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***In vitro* efficacy tests against
Mycobacterium species of South
African traditional medicinal plants**

Siyabulela Calvin Sibusiso Ntutela

2002

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Submitted in fulfilment of the requirements for the degree,

Doctor of Philosophy

In the

Division of Pharmacology,

Department of Medicine,

Faculty of Health Sciences,

At the

University of Cape Town

DECLARATION

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A Dedication

To the four most important women in my life at present:

My mother – Lulu: A silent worship of your motherhood

My Granny – Ntsiki: Will always love you

My daughter (0-yrs) – Malaika (SJ): You are my angel!

And to my wife - Thembi: All the way, with me

A Tribute

To my grandfather, GBN who passed away a month before the submission of this thesis

I thought you'd wait a little longer to witness this, but I was just too slow!

Lena yeyakho, Hlubi!

Storms make oak take deeper root – George Herbert (1593 – 1633)

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ACKNOWLEDGEMENTS

Firstly, I thank God, the Almighty, for giving me the strength and courage to perform this work

Large portions of this work were performed at the following laboratories: Departments of Pharmacology and Medical Microbiology, University of Cape Town, South Africa; Departments of Respiratory Medicine, Medical Microbiology and Chemistry, University of Sheffield, United Kingdom, with the generous donations from Lord John Sainbury/Linbury Trust Scholarship, South African Medical Research Council, and Paul and Stella Lowenstein Charitable Trust Travel Grant. I am very thankful to these institutions and donors for their kindness.

I would like to thank Professors Peter I Folb and Peter J Smith for supervising this work and allowing me to be registered in their department.

Great thanks to the following Professors/Doctors for allowing me to work in their departments: Prof. Lafras Steyn (Medical Microbiology - University of Cape Town), Prof. Tim Higgenbottam (Respiratory Medicine – University of Sheffield, Dr Robert Read (Medical Microbiology – University of Sheffield), Dr Joe Harrity (Chemistry – University of Sheffield) and to Prof. Sir Gareth Roberts (Vice-Chancellor – University of Sheffield)

A special thanks goes to Dr. William Campbell for assistance with the analysis and interpretation of the chemical data

I am very thankful to all the traditional healers in Southern Africa that have shared their information with me in order to perform this work

A million words of gratitude to the personnel at the Division of Pharmacology, UCT for their constant encouragement in all the years I've spent with them as a student. Colleagues at the Plant Research lab, you were the main pillar!

To every member of the Ntutela family – without you there would be darkness all over. You brought the light on my journey. Keep the spirits high!

Friends I met or made both in South Africa, in Cape Town and in Sheffield – your contribution was unique. Thank You All! A special thanks to Chief Bheki Buthelezi and Bonginkosi Gumede for being there all the time

Lastly, and by no means least, my cousins in Cape Town: Litha, Zola, Buhle, and Olona. Your company kept the house warm.

ABBREVIATIONS

A

ADC	Albumin-dextrose-catalase
AIDS	Acquired immune deficiency syndrome
AMP	Adenosine monophosphate
ATP	Adenosine triphosphate

B, C

CFU	Colony-forming units
CHOs	Chinese hamster ovarian cell lines
CMC	Complementary Medicines Committee, South Africa
CMI	Cell-mediated immunity
CoA	Co-enzyme A

D

DCM	Dichloromethane
DHS	District Health Systems
DMEM	Dulbecco's modified enrichment media
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOTS	Directly observed treatment, short course
DTH	Delayed-type hypersensitivity

E

EDTA	Ethylene diamine tetra acetic acid
EMB	Ethambutol

F, G

g	Gram
---	------

H

HIV	Human immunodeficiency virus
HPLC	High-pressure liquid chromatography

I

INH	Isoniazid
IOCD	International Organisation for Chemical Sciences in Development

J, K, L, M

LC	Liquid Chromatography
MAC	<i>Mycobacterium avium-intracellulare</i> complex
MCC	Medicines Control Council, South Africa
MDR	Multi-drug resistant
MeOH	Methanol
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide)

N

NADP	Nicotinamide adenine dinucleotide phosphate
NHP	National Health Plan, South Africa
NRCTM	National Reference Centre for Traditional Medicines, South Africa
NTCC	National Tissue Culture collection, United Kingdom
NTM	Non-tuberculous mycobacteria
NMR	Nuclear magnetic resonance spectroscopy

O

OD	Optical density
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P

PBS	Phosphate-buffered saline
PZA	Pyrazinamide
PZOA	Pyrazinoic acid

Q, R

RIF	Rifampicin
RNA	Ribonucleic acid
rpm	Revolutions per minute
RRDR	rifampicin resistance-determining region

S

SA	South Africa
SLs	Sesquiterpene lactones
SM	Streptomycin

T

TB	Tuberculosis
TCA	Trichloroacetic acid
TMS	Tetramethylsilane
Thia	Thionamide
TLC	Thin-layer chromatography

U

USA	United States of America
UV	Ultraviolet

V, W

WHO	World Health Organisation
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X, Y, Z

ZN	Ziehl-Neelsen stain
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OVERVIEW OF THE PROJECT

Chapter 1.

This chapter focuses on the literature review of the various aspects of tuberculosis. These aspects include the epidemiology of tuberculosis, bacteriology of tuberculosis, treatment and resistance to therapy, and also the public health programs in the prevention and control of tuberculosis. An introduction on the role of ethnopharmacology in the treatment of tuberculosis by indigenous health systems practitioners is visited.

Chapter 2.

Methods used in the projects are outlined in this chapter. These are tabulated in detail together with their principles.

Chapter 3

This chapter discusses, in depth, the role of ethnopharmacology in the treatment and control of tuberculosis. This chapter further discusses in context, the views and perceptions of traditional healers about tuberculosis and their understanding of the disease. These include their diagnosis modes, treatment systems, and the differences from one area to another.

Chapter 4

The results of the tests against *Mycobacterium aurum* A⁺ and Chinese hamster ovarian cells of the various plants collected during the ethnopharmacological survey into the communities of Southern Africa are tabulated and briefly summarised in this chapter. A special focus is attended to the plant *Artemisia afra* Jacq and its activity against *M. tuberculosis*. Various biological assays done on this plant and its extracts to validate its activity are shown in this chapter. The studies on the isolated compounds from this plant are, like-wise, reported in this chapter.

Chapter 5

Chemical analysis of the isolated sesquiterpene lactones is the main focus of this chapter. This involves the extraction procedures, the isolation of the fractions, the purification of the isolate and its structural elucidation. The various techniques used to elucidate the structures are reported in detail in this chapter.

Chapter 6

Discussion of the overall project is done in this chapter. This concludes the project based on the set objectives to see which have been met and what obstacles were encountered. This chapter further addresses the relevance of this study in the medical field.

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ABSTRACT

Problem statement: Tuberculosis is the leading cause of death due to a single organism; with a mortality of more than 3million people each year, worldwide. The emergence of multi-drug resistance and HIV/AIDS are the major causes of this problem. New therapeutic agents with a different mode of action, and thereby of resistance to *Mycobacterium tuberculosis*, the causative agent, are needed urgently. Amongst the methods used, the area of ethnopharmacology is explored in this study.

Methods: Visits were performed to collect the plants used by traditional healers in 7 provinces of South Africa for the treatment of tuberculosis. Extracts from the plants were tested against *M. aurum* A⁺, *in vitro*, using firefly luciferase bioluminescence assay at 200 µg/ml concentrations. Active extracts were further tested against *M. tuberculosis* H₃₇Rv, *in vitro*. Isolated compounds were tested against 5 clinical strains of *M. tuberculosis* using the BACTEC 460 TB system. Cytotoxicity testing was performed using MTT assay against Chinese hamster ovarian cell lines. **Results:** A total of 24 plants were collected. The dichloromethane extracts of *Artemisia afra* Jacq (**Asteraceae**) showed the highest activity against *M. aurum* A⁺ and *M. tuberculosis* H₃₇Rv with IC₅₀ of 270 µg/ml and 290 µg/ml, respectively. Further fractionation of the plant yielded two sesquiterpene lactones of eudesmanolide type, **tanacetin** and **artemin/arsubin**, with molecular weights of 264 g/mol and 266 g/mol, respectively. The biological assay of a mixture of the two sesquiterpenes showed an *in vitro* activity against *M. aurum* A⁺ and *M. tuberculosis* H₃₇Rv, with IC₅₀ of 1.9 µg/ml and 2.0 µg/ml, respectively. The sesquiterpenes had an IC₅₀ of 20.5 µg/ml against Chinese hamster ovarian cells, compared to daunomycin, with an IC₅₀ of 3.5 µg/ml. **Conclusions:** The crude dichloromethane extract of *A. afra* was active against *M. aurum* A⁺, *in vitro*. The isolated sesquiterpene lactones were also active against *M. tuberculosis* H₃₇Rv and some clinical strains of mycobacteria. The sesquiterpenes were not cytotoxic against Chinese hamster ovarian cells. The study reports on the finding of these visits to the traditional healers and on the biological assays performed to validate the activity of the two sesquiterpenes.

CHAPTER ONE

Introduction and Literature Review

*In April 1993 the World Health Organisation declared tuberculosis to be a
GLOBAL EMERGENCY' - the first declaration of its kind in the WHO
history.*

1.1 TUBERCULOSIS

1.1.1 Epidemiology of tuberculosis

Tuberculosis (TB) is a leading contender for the dubious distinction of being the most important plague of mankind. It is estimated to be the cause of death to more than 3 million people in the world, each year [1, 2]. TB is the most common cause of death due to a single infectious agent and is responsible for more than 26% of all avoidable deaths [1] and 80% of deaths due to TB occur in young to middle-age women and men [3]. Accentuating the impact of TB on the world's well being is its concentration among young adults throughout most of the developing world and its airborne spread from person to person, especially to household members. Because TB is a communicable disease, it is not surprising that the risk of an uninfected person becoming infected is strongly associated with the probability of coming in contact with someone with infectious TB, the duration of that contact, and the degree of infectiousness of the case. The transmission of TB among household members has introduced another obstacle in the control of TB: - *transmission to children*. The occurrence of TB in children is a marker for ongoing transmission of infection among all age groups in a society [4]. Infected children represent a large pool from which future TB cases will arise. The reason for this is that diagnosis of tuberculosis in children is usually limited to characteristics of the disease [5, 6, 7, 8]. Smear and culture tests are usually difficult in children since sputum cannot be easily obtained from children [9, 10]. This results in undiagnosed TB cases and the children then either become the source of infection or die from the disease [6]. The World Health Organisation (WHO) has recently estimated that in developing countries there are 1.3 million cases and 450 000 deaths, annually, of TB in children between 5-15 years of age [11]. The mortality rates of children from newborn to 4 years of age have been about twice that for children between 5-15 years, mostly because of the higher incidence of TB meningitis in the younger population [1].

Figure 1.1 on page 3 shows the incidence rates of TB in various parts of the world. A large proportion of the world has incidences higher than 100 cases per 100 000 people. It is estimated that 1700 million people have been infected with *Mycobacterium tuberculosis*, and 8.4 million of these people develop tuberculosis in a recognisable form, each year [11].



Figure 1.1: Estimated tuberculosis incidence rates during the year 1999 as supplied by the WHO. The increase in the HIV status might increase these rates by 5-10%.

Source: WHO TB report, 2001

In infected but healthy individuals, the TB bacteria are kept dormant by the host's immune system. The use of immunosuppressive therapies and the immunosuppressive diseases increase the risk of developing tuberculosis in infected persons. However, tuberculosis occurring among such immunocompromised persons contributes very little to the incidence of tuberculosis. The epidemic of infection with human immunodeficiency virus (HIV) is not only an exception to this case, but proves the opposite to be correct. The relative effect of immunosuppression on the incidence of TB has changed due to the HIV epidemic. It is now well established that infection with HIV is the most potent factor promoting the development of TB in individuals infected with *M. tuberculosis* [12, 13].

1.1.2 Tuberculosis and human immunodeficiency virus

M. tuberculosis exists in the lungs of a number of infected but healthy people for years. This is kept dormant in the macrophages that are immediately activated when the initial infection occurs [14]. When the immunity of the person is compromised due to a certain reason the macrophage activation no longer contains the mycobacteria and the bacilli

start to multiply and are released into the alveoli. Among many factors that lead to the inadequacy of the immune system, human immunodeficiency virus (HIV) constitutes the strongest risk factor for development of tuberculosis in individuals with dormant *M. tuberculosis* infection [15]. In fact, due to the high incidence of both HIV and *M. tuberculosis* infection in the developing countries, TB has emerged as the most common opportunistic infection in HIV infected patients, worldwide [15]. The WHO estimates 8.4 million new tuberculosis cases in 1999, up from 8.0 million in 1997 [11]. It is suggested that this rise is due largely to a 20% increase in incidence in African countries most affected by the HIV/Acquired immunodeficiency syndrome (AIDS) epidemic. A further report from WHO suggests that if current trends continue, 10.2 million new cases are expected in 2005, and Africa will have more cases than any other WHO region [11]. Figure 1.2 on page 5 shows the prevalence of HIV positive tuberculosis cases reported in 1999. As the figure shows, most of the incidences of co-infection by HIV and TB above 10 per 100 000 cases occur in the sub-Saharan region of Africa and parts of the Caribbean islands. This number is subject to increase due to the fact that most people are not yet tested for HIV and/or TB.

The WHO had suggested that tuberculosis patients who are at risk of HIV be offered HIV testing [16]. One of the reasons for this is the fact that HIV co-infection may complicate the management of TB through, for example, atypical case presentation, decreased sensitivity of diagnosis by sputum smear examination, increased frequency of adverse drug reactions, and increased mortality from other infections during treatment [16].



Figure 1.2: The estimated rate of tuberculosis and HIV co-infection in 1999 as estimated by the WHO. Source: WHO TB report, 2001

Active tuberculosis in HIV-infected subjects is associated with a significant accentuation in HIV-related immunodeficiency and mortality [12, 13, 15, 17, 18]. This further poses another threat to human health, as the two pathogens seem to be synergistic to each other [15]. Although the mechanism of this synergy is not clearly understood, in clinical terms the effects of this synergy could soon be the major killer-duo in all human life. Figure 1.3 on page 6 summarises the incidences of HIV cases, tuberculosis cases, and those of HIV/tuberculosis co-infection, as outlined by the WHO in the 1999 report [19]. In most developing countries the incidences are on the rise due to either socio-political matters or due to inadequate health facilities.

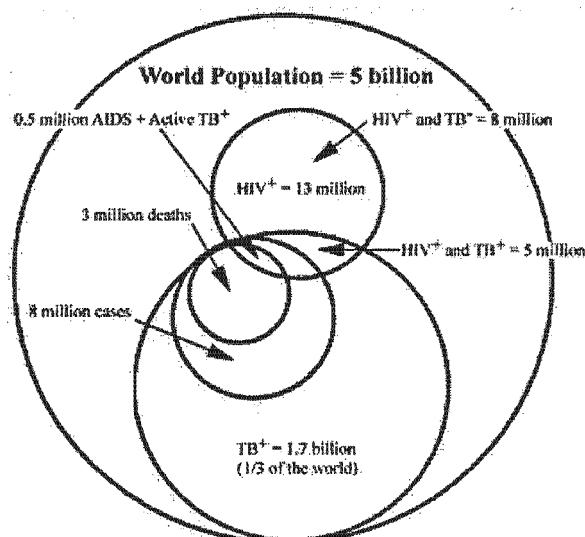


Figure 1.3: Venn diagram of tuberculosis (TB) and HIV infection and co-infection.

Source: WHO TB report, 1999

Apart from the co-activation of these two pathogens, one major threat with HIV/TB co-infection is the introduction of new mycobacterial pathogens that were normally less important due to their low pathogenicity [20]. This poses a threat in various forms such as diagnosis, treatment and cost of management [20, 21, 22]. The *M. avium-intracellulare* complex (MAC) has been mostly associated with non-*M. tuberculosis* cases of tuberculosis in HIV positive patients [23, 24]. These species are not at all pathogenic to healthy individuals but have been shown to be of clinical importance in immune-deficient patients. However, many other species have been identified that do not belong to the MAC that cause disease in HIV/AIDS patients [20, 24]. These include the *M. kansasii*, *M. marinum*, *M. fortuitum*/*M. chelonae* complex, and *M. scrofulaceum* [21]. These present clinically in disseminated forms such as lymphadenopathy, skin and soft-tissue involvement, skeletal system infections and the gastric mucosa [20, 21, 25, 26]. With these new pathogens requiring special attention, the mycobacterial genus is now posing a great challenge to all clinicians and scientists for new forms of management. As most developing countries still lack high-tech screening and identification facilities for mycobacterial infections, many of these infections will go undiagnosed until fatality.

1.1.3 Bacteriology of tuberculosis

There are more than 70 species of mycobacteria. Of these, two are major pathogens: *M. leprae* and *M. tuberculosis*. The remaining mycobacteria are environmental organisms collectively known as **Non-Tuberculous Mycobacteria** (NTM). Of the tuberculosis complex (*M. tuberculosis*; *M. bovis*; *M. africanum*), *M. tuberculosis* is considered to be the sole species of tubercle bacilli because of its clinical importance [27].

M. tuberculosis is a thin, non-motile rod without capsule. It does not form spores, but is resistant to drying. It is an acid-fast bacillus, as it does not decolour with acid and alcohol once stained with aryl methane dyes. The species grows under aerobic conditions and the cell wall has mycolic acids [28, 29]. Because of its thickness and also the high content in lipids, the cell wall is much more impermeable to hydrophilic molecules than most bacilli [30].

1.1.4 Pathogenesis of tuberculosis

TB is an infectious airborne disease that is caused mainly by *M. tuberculosis*. It occurs in almost any part of the body but the lungs are the most common sites. Although there are other modes of infection, inhalation is by far the most important mode of transmission. To begin an infection, virulent tubercle bacilli must reach the alveolar surface. The first host defence against infection is the alveolar macrophages. This initial contest between host and parasite is in many ways crucial to all subsequent events, determining whether the infection will occur at all. It is believed that in some cases viable bacilli do not reach the alveolar spaces and probably die [31], and in a majority of cases the bacilli are killed, probably by alveolar macrophages, before they can invade the alveolar basement membrane [31]. In cases where infection happens, it initiates recruitment to the focus of both cytotoxic cells (delayed-type hypersensitivity)[DTH] and activated macrophages (cell-mediated immunity)[CMI] [2]. In a study performed by Bodnar *et al* [32], they suggest that the activation of dendritic cells is playing a role in the phagocytosis of *M. tuberculosis*, but this is not responsible for bactericidal effect. A study done in mice by Jiao *et al* [33] has shown that dendritic cells are involved in triggering both the innate and acquired immune responses to *M. tuberculosis*, *in vivo*. Acquired immunity is expressed relatively slowly in the lungs [34]. The reason for this is

unclear, as it is the site where the protective T cells become sensitized. The lymphoid tissues surrounding the bronchi are potential sites, but for these to be the sites, antigen has to be physically carried to these tissues. It has been shown that one type of myeloid cells capable of such an action are the dendritic cells [35]. This study has shown that CD11c-positive dendritic cells are well distributed throughout the alveolar region. The study further demonstrates that these cells are capable of phagocytosing live *M. tuberculosis* bacteria, leading to the secretion of interleukin-12 (IL-12) and stimulation of CD4 T cells to produce gamma interferon (IFN- γ). Given the knowledge that dendritic cells are motile and capable of homing from the peripheral tissues to lymphoid organs [36], their data support the hypothesis that dendritic cells that engulf *M. tuberculosis* play an important role in the transition from the initial innate response in the lungs to a state of acquired specific immunity [35].

Some infected individuals develop a cascade of processes that lead to the active disease, whereas others will harbour these bacilli with the risk of developing the disease at a later stage. The *M. tuberculosis* is assumed to survive inside the non-degradative phagocytic vacuole in alveolar macrophages. By residing inside these vacuoles, the bacilli evade destruction within lysosomes and block the generation of antigens in the phagosome [37]. The nature of the bacilli at this state is highly debated. Some authors believe that the bacilli enter a stage of latency or dormancy, from which they re-emerge when the conditions in the lung are favourable for them [38].

In a counterpoint article Orme *et al* [31] argue against the existence of latency of mycobacteria using various examples of studies. In the first example, they argue the assumption by McKinney *et al* that latent bacilli utilize the glyoxylate shunt off the Krebs cycle to continue to metabolise two carbon sugars to generate energy in the form of NAD [39]. Orme *et al* argue that this is a form of active metabolism and is inconsistent with latency [31]. This argument further assumes the need for terminology change in recognition of the state of the mycobacteria during this stage. In Orme's argument, the term "non-replicating persistence" is recommended, as latency does not agree with the state of the bacilli. This is supported by a study conducted by Wayne & Hayes [40], in which bacteria starved of oxygen go into a state of non-replicating persistence in which there appear to be two definable stages. This oxygen starvation was based on the assumption that it is what happens when the activated monocytes surround the site of infection. In this process Wayne *et al* recognised that there was cessation of DNA replication but other metabolic processes continued [40]. Although there is no clear evidence of what happens when the bacteria are inside the lungs in the absence of

clinical disease, there is a cascade of processes that happen when the immunity of the person wanes and the conditions are favourable for the mycobacteria to replicate.

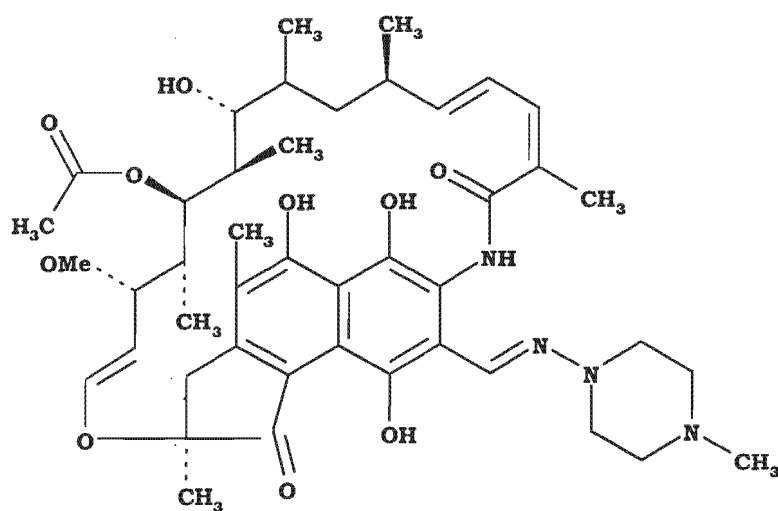
When innate macrophage microbicidal capacity is inadequate to destroy the initial few tubercle bacilli of the droplet nucleus, they replicate within the macrophage, causing it to rupture. Monocytes are attracted to the focus by various chemotactic factors, initiating granuloma formation. The monocytes acquire microbicidal activity upon activation by T-lymphocytes as CMI develops. Bacilli released from immature macrophages may be taken up and controlled by T cell-activated macrophages. After control is established, mature macrophages accumulate around the periphery of the caseous lesion (epithelioid), preventing further extension [41, 42]. Cytotoxic cells (DTH) also destroy the immature macrophages, which may provide an environment conducive to bacillary growth. Later in the course of tuberculosis, DTH may be more harmful to the host, causing the tissue damage characteristic of TB, especially lung cavitations. Lung cavities are largely responsible for both transmission of tuberculosis and for the development of resistance. However, in normal host, the bacillary population is stable during this stage as growth is counterbalanced by bacillary destruction and inhibition [43].

Although immuno-compromised persons in whom CMI fails to control primary TB may progress to cavitary TB (progressive primary disease), this final stage of pathogenesis usually results from a second (post primary) phase of disease activity, occurring months to decades after apparent recovery from initial process. There is an ongoing debate on the cause of this reactivation, some authors arguing that latent mycobacteria are the cause of this reactivation whilst there is equally evidence of the role played by re-infection. In a review by Manabe and Bishai [2], a series of studies to prove both the endogenous reactivation and exogenous re-infection were debated. There is a strong argument that reactivation mainly results from exogenous re-infection [44]. Reactivation tuberculosis is characterised by chronic tissue lesions, formation of tubercles, caseation, and fibrosis [28, 42]. The reactivation type almost always begins at the apex of the lung, where the oxygen tension is highest [45]. In most cases of tuberculosis the tubercle bacilli are disseminated to a site distant from the primary complex. This lymphohematogenous dissemination depends on the quality of organism released and the host immune response. The occult dissemination usually produces no symptoms, but it is the event that causes extra-pulmonary foci that can become the site of disease months to years after the final infection. Children may experience a protracted haematogenous infection caused by intermittent release of tubercle bacilli when a

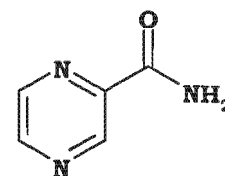
caseous focus erodes through the wall of a blood vessel. Multiple organ involvement is frequent, with the central nervous system being the most problematic [28].

1.1.5 Treatment of tuberculosis

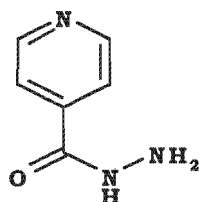
Chemotherapy for tuberculosis starts immediately when a positive diagnosis is made, usually beginning with radiology results. In the case of a drug sensitive strain infection a 6 months therapy course is followed with the first-line drugs. In a multi-drug resistant strain infection a longer course with an intense combination of first-line and second-line drugs is used. The most widely used chemotherapy regimen consists of 2 months of an intensive phase of treatment with isoniazid, rifampicin, pyrazinamide, and ethambutol, (see chemical structures in figure 1.4 on page 11). This is followed by 4 months' treatment with isoniazid and rifampicin. In the case of resistance to isoniazid and any of the first-line drugs, multi-drug resistance is assumed. The second line drugs are then implemented. These include capreomycin, cycloserine, streptomycin, clarithromycin, and ciprofloxacin. The first-line drugs may present with unwanted reactions and thereby the second-line drugs are used [46]. The major problem with tuberculosis treatment is the length of treatment. Conventional treatment lasts a minimum of 6 months. This has become the source of most of the problems associated with the eradication of tuberculosis. This is mainly due to lack of adherence to the treatment course, irregular taking of drugs, non-compliance and toxicity. The WHO has introduced the concept of directly observed treatment strategy, commonly known as DOTS. This involves the practice of giving patients their pills and observing that they are swallowed until the full treatment is completed [47].



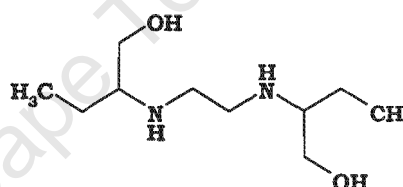
Rifampicin



Pyrazinamide



Isoniazid



Ethambutol

Figure 1.4: Chemical structures of the first-line anti-tuberculosis drugs.

Source: The Merck Index

1.1.6 The role of individual drugs in TB treatment

Tuberculosis treatment is given as a combination of drugs mainly to prevent resistance from emerging. Isoniazid (INH) has been shown to be responsible for the initial kill of bacilli during the early stages of treatment [48, 49]. It is shown that isoniazid reduces the bacterial colony counts by approximately 95% during the first 2 days of therapy [48, 49]. This is due to its ability to inhibit growth on actively multiplying bacilli compared to resting organisms. INH is usually dosed in humans at 5mg/kg/day, which is up to 300mg/day. This leads to peak serum levels of 3 to 5µg/ml [48]. *In vitro* studies show that the minimum inhibitory concentration of INH is approximately 0.05µg/ml. Theoretically; the C_{max} values would be sufficient in having a sterilising effect. However, the bactericidal activity of INH decreases over the course of treatment. It is assumed that isoniazid has limited activity at the later stages of therapy as all the organisms are

metabolising in spurts [46]. This explains the need for addition of other drugs in therapy against active tuberculosis. The dormant mycobacteria are killed by pyrazinamide (PZA) and rifampicin. Pyrazinamide is inactive at neutral pH and is tuberculostatic at acid pH of 5.5 or lower. However, the role of PZA inside human macrophages is still unclear. Some authors believe that it is bacteriostatic [50] while others believe that it is not active at lower pH values [51]. A similar situation is noticeable with rifampicin, which is also effective against organisms growing intracellularly. Rifampicin is believed to inhibit mycobacterial RNA-polymerase [46].

Chemotherapy for treating multi-drug resistant tuberculosis and relapse requires assessment of the history of treatment as well as meticulous laboratory studies to characterise the susceptibility of the specific strain. As part of the treatment, second-line drugs are administered to overcome resistant organisms. Among the drugs used are fluoroquinolones, aminosalicylic acid and thionamide, capreomycin. These drugs are given over a longer period of treatment that spans from 12 to 24 months depending on the extent of resistance [49].

1.1.7 Drug resistance in tuberculosis

1.1.7.1 Incidence of resistance

Like all bacterial genomes, the mycobacterial genome is subject to a wide range of mutations. Mutants affecting colony morphology, biochemical and virulence characteristics, and sensitivity are of major interest in view of their importance for the diagnosis, treatment, and epidemiology surveillance of tuberculosis [1]. The outbreak of MDR-TB during the past 10 - 15 years has been a major threat to developed countries. With the occurrence of HIV/TB co-infection, MDR-TB has increased with alarming rates [15, 52]. Tubercle bacilli that are resistant to two of the commonly used antituberculosis drugs, INH and rifampicin or pyrazinamide, are considered to be multi-drug resistant. Many factors have been associated with the development of resistance of *M. tuberculosis* to therapeutic agents. These factors include non-compliance to treatment, inappropriate regimens, and irregular drug supply [53]. In most cases patients tend to stop treatment once the symptoms start to subside, whereas the bacteria still remain latent and the immunity of the patients is still waning. This gives the tubercle bacilli the chance to mutate in the presence of sub-therapeutic levels of the anti-tuberculosis drugs leading to resistance emergence. The problem of drug-resistant tuberculosis seems to threaten

all efforts to control the disease [53]. When the WHO introduced the DOTS program one of the objectives was to avoid the development of resistant strains due to non-compliance to therapy [47]. Also, the extent of this problem is yet unclear as various recommendations on its surveillance are still being debated [54].

1.1.7.2 Mechanisms of resistance

Resistance to the drugs occurs due to different mutations as they have different mechanisms of action.

1.1.7.2.1 Resistance to isoniazid

As *M. tuberculosis* resides within macrophages, it has evolved to resist toxic oxygen species. However, INH interacts with components of the mycobacterial defence against oxidative stress in complex ways. INH is a prodrug that requires activation to an unstable electrophilic species by the mycobacterial catalase-peroxidase KatG, with hydrogen peroxide acting as an electron sink for the reaction. The activated drug subsequently interacts with one or more targets. These are the InhA, an NADH-dependent enoyl reductase, and/or KsA, a β -keto acyl carrier protein synthase [55]. Once activated, INH inhibits the biosynthesis of cell mycolic acids. This renders the mycobacterial cell wall defective, thereby penetratable to toxic oxygen species. As KatG is the only enzyme in *M. tuberculosis* capable of activating INH, KatG-mutant strains are resistant to INH [56, 57]. The KatG is considered an important factor in the survival of the organism. Expression of either KatG or an alkyl hydroperoxidase AhpC is considered sufficient to protect the bacilli against toxic peroxides. It has been shown that INH-resistant organisms compensate the loss of KatG catalase activity by a second mutation resulting in hyperexpression of AhpC [57, 58]. Other sites whose mutations are associated with resistance of *M. tuberculosis* to isoniazid are the two-gene operon (*inhA* locus) encoding the enoyl – acyl carrier protein reductase [59, 60], the 3-keto-acyl-acyl carrier protein reductase involved in mycolic acid biosynthesis [58], the *ahpC*, which is involved in the cellular responses to oxidative stress, and the β -keto acyl carrier protein synthase, which is important in fatty acid elongation [61].

1.1.7.2.2 Resistance to rifampicin

Mutation in RNA polymerase beta subunit gene (*rpoB*) is the major mechanism of resistance to rifampicin (RFP) with high frequencies of 90% or more [62]. Evaluation of the relationship between RFP susceptibility and genetic alteration in *rpoB* gene also showed that 95% of RFP-resistant *M. tuberculosis* isolates involved genetic alterations in an 81- base pair core region of *rpoB* gene. This region is called the rifampicin resistance-determining region (RRDR) [63]. Moreover, these genetic alterations in *rpoB* gene are suspected as the resistance mechanism to rifamycins [62].

1.1.7.2.3 Resistance to pyrazinamide

The mechanism of action, and thereby of resistance of *M. tuberculosis* to pyrazinamide (PZA) has also been partially identified. PZA is crucial for achieving sterilisation by killing persisting semi-dormant bacilli in the lungs. Its activity depends on the presence of a bacterial amidase, which converts PZA to pyrazinoic acid (PZOA), which is the active antibacterial molecule [64]. PZA-resistant bacilli lack this amidase activity. The gene encoding for this has been identified and the mutation to this *pnca* gene has been associated with resistance to PZA [65].

1.1.7.2.4 Resistance to ethambutol

Ethambutol ([S,S]-2,2-[ethylenediimino])di-1-butanol is a bactericidal first line drug in the treatment of TB. It has been shown to inhibit the incorporation of mycolic acids into the cell wall. It has also been shown to inhibit the transfer of arabinogalactan into the cell wall of mycobacteria [63]. Only the dextro isomer of ethambutol is biologically active, an observation consistent with the idea that the drug binds to a specific drug target, which is assumed to be an arabinosyl transferase [63]. Ethambutol resistant strains have a mutation on the *embB* gene encoding an arabinosyl transferase which catalyses cell wall synthesis [62].

1.1.7.2.5 Resistance to streptomycin

Streptomycin is an aminocyclitol glycoside antibiotic that is one of the first line drugs used to treat TB. Most streptomycin resistant strains have a mutation on the *rrs* and *rpsL* genes encoding a 16S rRNA and a S12 ribosomal subunit protein, respectively [62]. In contrast to other bacteria, which have multiple copies of rRNA genes, *M. tuberculosis* complex members have only one copy. Hence, single nucleotide changes can potentially produce antibiotic resistance [63].

1.1.8 Trends in tuberculosis

The pandemic of HIV infection and acquired immunodeficiency syndrome (AIDS) has caused marked increases in TB notifications throughout the world. By virtue of its ability to destroy the immune system, HIV has emerged as the most important risk factor for progression of dormant TB infection to clinical disease. Recent studies estimate that, if present trends continue, the incidence of TB will rise by an additional one third over the next decade [11]. Further complications associated with TB/HIV complex are the emergence of new pathogens from the mycobacteria genus. These include *M. avium-intracellulare* complex (MAC) [66, 67]; and *M. africanum* [25]. Most TB infections are transmitted among household members, with children usually becoming victims of this cross-transmission. This is mainly due to the fact that many cases of TB remain undiagnosed until the later stages of disease manifestation. One of the major causes of this is the difficulty of confirming the diagnosis of childhood tuberculosis [10]. In countries with poor socio-economic status and inadequate medical facilities, where many children present with conditions similar to TB (but not TB), TB therapy may be initiated. As the children fail to respond to the treatment, further tests may reveal false initial diagnosis and therapy will be stopped. The latent bacilli in the lungs will then develop resistance, and they will further be transmitted to some individuals (primary resistance). A similar case usually occurs in patients who fail to complete their regimens, leading to bacilli being resistant to the drugs and the relapse will then be a resistant TB (acquired resistance) [68]. Outbreaks of multi-drug resistant TB and the rate at which they are transmitted is becoming a major threat in the control of TB [69]. An accurate account of drug-resistant TB throughout the world is not available because only a few countries have reliable drug resistance surveillance systems. Some molecular

epidemiological studies have been attempted to track transcontinental transmission of multi-drug resistant TB [70]. Disease with drug resistant *M. tuberculosis* has been recognised as an important global threat with a median of 9.9% of *M. tuberculosis* strains now resistant to at least one drug in 35 countries or regions [71]. It has also been shown that transmission of MDR-TB occurs in long distance airplane flights, which adds to the hypothesis of transcontinental transmission of MDR-TB [72, 73]. With the parallel increase in immunodeficiency diseases, the MDR-TB will eventually lead into recognisable tuberculosis; increasing the proportion of resistant TB thereby adding to the problem of the TB pandemic. With these immediate threats, a counter response from scientists and clinicians for combating the disease is rather urgent. In dealing with the MDR-TB, strategies to curb this problem have been proposed. Dye *et al* [69] recommend a three-part response: widespread implementation of short-course chemotherapy as the cornerstone of good tuberculosis control, improved resistance testing and surveillance, and the careful introduction of second-line drugs after a sound evaluation of cost, effectiveness, and feasibility.

1.1.9 Tuberculosis: the South African scenario

1.1.9.1 Epidemiology of tuberculosis in South Africa

TB is a true example of a disease directly influenced by unfavourable socio-economic conditions. Poor housing, overcrowding, malnutrition, lack of hygiene, emotional and physical stress, long hours of work, loss of sleep, lowered resistance, and unpasteurised milk are a few of the many factors that favour the development and spread of TB [74]. South Africa, having a high proportion of its population living under these conditions, is facing a burden of the disease becoming uncontrollable. The WHO has classified South Africa as one of the high burden countries, ranked position 8 globally and with estimated TB incidences of 495 per 100 000 [11]. Figure 1.5 on page 17 shows the geographical distribution of all TB incidences among the 9 provinces of South Africa [75].

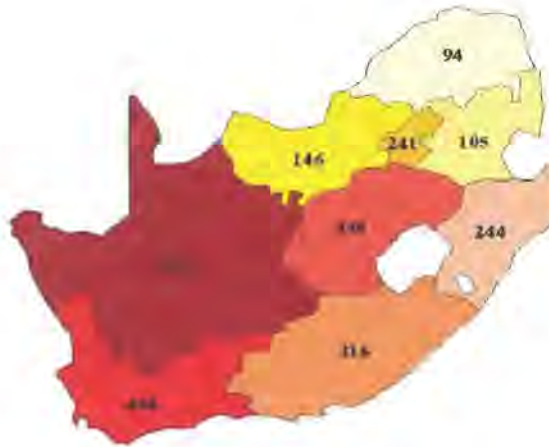


Figure 1.5:Incidence rates per 100 000 population in 1997 in South Africa

Source: National Health Office in Pretoria

Although the African population has the highest overall incidence, the Western Cape has a high and increasing risk of TB among the coloured community [74]. Among the age groups, the high incidences of active TB are seen in young adults up to old age (>15years) [11]. These statistics suggest a high notification rate among females below the age of 24 years. The notification rates of males are higher than those of females from the age of 25 upward, as shown in Figure 1.6 below. It should be noted that this population of males constitutes most of the labour force of the country, and its economic impact is therefore high.

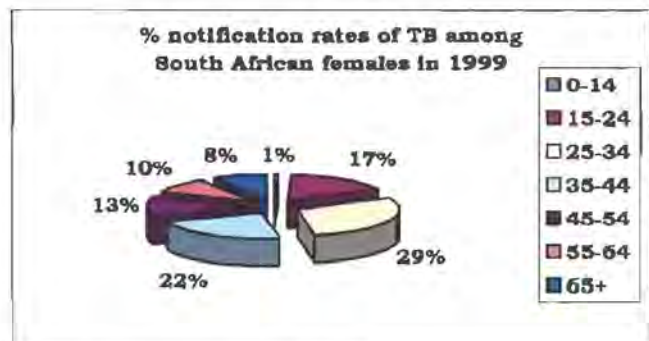
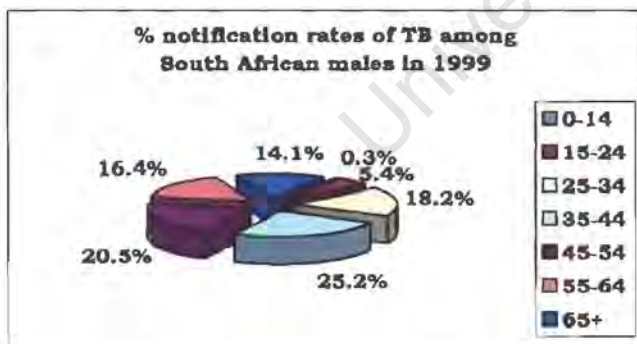


Figure 1.6: Percentage incidences of smear-positive rates of tuberculosis in South Africa by age and gender during the year 1999.

Source: WHO 2001[19]

Another high-risk group of TB infection in South Africa are the miners [76, 77]. The gold mining industry is one of South Africa's largest employers. An average of 300 000 men are employed by this industry [76]. TB has long been recognised as an important health hazard in the mining industry since the early 1900s. The incidence of pulmonary

tuberculosis in this group has increased from 686 per 100 000 workers in 1989 to more than 1800 per 100 000 in 1995 [77]. This increase has been associated with the long underground working hours, previous incomplete treatment of tuberculosis and coincidental silicosis. The exposure of the gold-miners to silica-containing dust has been shown to be a strong risk factor for TB and has been associated with non-tuberculous mycobacterial disease [76, 78]. Another accelerating factor to TB is the increase of HIV infection among the mineworkers and the general public as a whole. Prior to 1992, the incidence of TB in the gold mines was stable but high. However since the rise in HIV/AIDS epidemic, TB has ever been on the rise [77]. Since the mineworkers constitute a large proportion of the South African workforce, originally from the rural areas, these men act as potential traffickers of the epidemic to the rural population of their wives and young women, which leads to a further spread of the disease. In a study conducted by Kleinschmidt *et al* [79] between 1994 and 1995 in South Africa, they published that for the country as a whole TB mortality increased from 38 to 53 per 100,000 for males and from 15 to 23 per 100,000 for females. In the Eastern Cape, TB deaths accounted for 10% of registered male deaths and 7% of registered female deaths. The two provinces that had the highest HIV prevalence among women attending antenatal clinics, namely KwaZulu-Natal and North West, recorded increases in registered TB deaths of 100% and 75% respectively for males, and 130% and 74% respectively for females. The WHO in their latest report has estimated approximately 80% of all South African TB cases to be HIV positive [11]. This means that the HIV/TB duo is likely (if not already) to be the highest killer in the South African population.

1.1.9.2 Drug resistance in South Africa

The incidence of multi-drug resistance tuberculosis in the 3 provinces of Mpumalanga, KwaZulu-Natal and Eastern Cape is about 1% of new tuberculosis cases and about 4% of previously treated cases, with Mpumalanga having 1.5% of new cases [11]. This amounts to about 2000 new cases of MDR-TB each year [75]. Another problem associated with MDR TB is the cost of its treatment. In South Africa it is estimated to amount to R30 000 to treat one MDR TB patient, while the cure rates range from 30% - 50%. This cost is 10 to 20 times higher than in the treatment of uncomplicated drug-sensitive cases [75]. In the Western Cape province, which has the highest incidence of tuberculosis in South Africa, it is estimated that the almost 7% of all TB cases are isoniazid resistant and in children up to 5.6% are INH resistant [80]. This study also

estimates that approximately 1% of children under 5 years with tuberculosis are multi-drug resistant. Although the study suggests a low incidence of resistance, this is likely to escalate due to other factors such as increase in MDR in adults, HIV-TB co-infection, and acquired and natural resistance due to poor treatment management and drug supply [80]. With this increase in HIV cases in South Africa, the need to take serious action against tuberculosis is a priority as this is a leading cause of death in most HIV cases. The WHO has estimated that the treatment success of tuberculosis in South Africa is approximately 72%, with DOTS coverage of 66% of the population [11]. The increase in the DOTS coverage would yield a higher proportion of the treatment success and thereby would prevent the escalation of MDR-TB cases [54].

1.2 TB and ETHNOPHARMACOLOGY

Ethnopharmacology is the study of naturally occurring substances that have medicinal action. Although it focuses on medicinal properties, it is not entirely limited to these substances. It is closely related to botany, chemistry and pharmacology (and toxicology) [81].

Over the years, the World Health Assembly has adopted a number of resolutions drawing attention to the fact that most of the populations in various developing countries around the world depend on traditional medicine for primary health care, that the work force represented by practitioners of traditional medicine is essentially an important resource for the delivery of health care and that medicinal plants are of great importance to the health of individuals and communities [82]. This has been driven by initiatives taken by most countries in recognizing the role played by this health system in bettering the lives of many people. The South African government also adopted a National Health Plan (NHP) that included traditional healing as part of the broader health care service [83]. This new health care system is based mainly on primary health care approach. The District Health System (DHS) is the essence of this primary health care approach. The NHP proposes that the roles of traditional health systems and their interface with modern health care systems are best achieved through the primary health care and district health systems. In practice, however, the role of traditional medicines practitioners extends far more than primary health care. Direct intervention in disease progression and health promotion are mostly practised in many parts of South Africa, and Africa in general. In this system, the traditional healers are involved in the treatment of diseases and serious illnesses and in the performance of surgical

procedures such as midwifery, circumcisions and so on [84]. Traditional diagnosis is a system that is both an art and a method of seeking to discover the origins of the disease and determining what it is. The diagnostic process not only seeks to answer the question of how the disease originated, but who or what caused the disease and why it has affected this particular person at this time [85, 86]. Diagnosis involves a combination of information such as observation, patient self-diagnosis, family information and divination.

A traditional healer is defined as someone who is recognized by the community in which they live as competent to provide health care, with special care to vegetable, animal and religious backgrounds as well as the prevailing knowledge, attitudes and beliefs regarding diseases [87]. This includes the various types of traditional healers; as they do not all perform the same function. Each type of traditional medicinal practitioner has their own field of expertise, utilizing different techniques.

The traditional healers dominant in South Africa include diviners (*amagqirha*), herbalists (*amaxhwele*), prophets or faith healers (*abathandazeli*), general members of communities such as the elderly, traditional surgeons (*iingcibi*) and traditional birth attendants.

Diviners mainly diagnose through supernatural powers. Traditional healers argue that no one becomes a diviner by choice. There has to be a calling from the ancestors and the individual has to contact another diviner of superior power (*isanuse*) who will then give training for a period ranging from 5 years to 15 years. In many cultures in South Africa, the diviners use bones and other similar items as a way of communicating with ancestors. Certain diviners also dispense medicines while others refer patients to herbalists. Herbalists become either by choice or by learning from other herbalists. Most do not possess supernatural powers other than their special knowledge and experience in medicinal plants. Prophets or faith healers are new within the traditional medicinal health systems. They are often linked with a religious denomination, and are well accepted in communities in South Africa. Their diagnosis and treatment systems include prayers and constant visits by the patient to that church from which the healer comes.

The role of the traditional health system in communities is not mainly about the curative effects of medicines but it also includes disease prevention and other health service delivery. Services offered by traditional birth attendants, elderly people and traditional surgeons are the basis of primary health care services in communities. These services include advice, health monitoring and health communication to community or individual [88]. The one important aspect used by the traditional healers in providing their services is the element of culture and its role in health communication and disease

interpretation. Culture influences the way people understand and interpret diseases [89]. This influence of culture to the way of understanding of diseases by traditional healers has played a role in getting traditional healers accepted by communities. The respect that is still paid by many communities on their cultures need to be taken into serious consideration. In order for western-trained scientists to be effective in communicating disease prevention and control strategies in indigenous communities, the role of culture and cultural communication and perspective of diseases need to be well understood. This understanding needs to go to an extent of coupling research activities with cultural norms of that community being studied. In that way the communities would be persuaded by the changes that are then brought about by such researchers. A typical example for this is the concept of isolating a tuberculosis patient who is at the infectious stage from other family members. In the western context of health, this practice is highly acceptable. In the cultural context, a sick person needs constant visits by his family members and neighbours. In the cultural context, the isolation becomes more of a pressure both to the patient and to the family of the patient. When the patient recovers, it becomes difficult to face the community again if the patient was in isolation in the knowledge of the community. They become a social outcast and that contributes negatively to the recovery process. Another problem that arises from such practices is that patients fear to be seen going to clinics to pick up their medicines as they want to be seen as having recovered. This stigmatisation of tuberculosis and tuberculosis patients in general is mostly associated with health practices that are new and outside communities' cultural circles. It is for this reason that one would suggest the merging of traditional practices and cultural norms with disease prevention and health promotion strategies in indigenous communities. Allowing traditional healers as key and pivotal players in such programs could further enhance their role in communities. With both systems of health care given equal opportunity to play a role in community health awareness and promotion processes, diseases such as tuberculosis would be more easily controlled.

With the urgent need for new antimycobacterials, researchers are now beginning to find safer, rapid and effective ways of assaying plants against *M. tuberculosis*. Assay methods have been developed using simple means and different mycobacteria species that have been shown to be representative of the *M. tuberculosis* antibiotic profile [90]. Other means include modification of the previously used methods in order to meet the demands of the pace of research. Use of these modified and simplified methods and the bioassay-guided search for antimycobacterials from higher plants is showing signs of

success. Although no marketable products for the treatment of tuberculosis have been isolated from plants, some lead molecules have been identified. [91, 92, 93, 94, 95]. In structure-activity related studies against *M. tuberculosis* different research groups have found activity in a variety of natural products with no definite trend towards a specific group of compounds. The Houghton group [95] has found activity in a group of alkaloids from *Galipea officinalis* while cycloartanes have also been found by Cantrell *et al* to possess some activity [91]. They suggested that the presence of an epoxide in a triterpene isolated from *Borrchia frutescens* has an enhancement in activity against *M. tuberculosis* [91]. However, in a study on a series of natural and semi-synthetic guaianolide-type sesquiterpene lactones, epoxidation resulted in loss of activity. They then suggested that the polarity of the compound, instead, was the enhancer of activity, which then indicated that within this structurally related group of sesquiterpene lactones the activity was mostly likely controlled by the transport through the outer layer of mycobacteria [92]. In a similar study performed by Fischer *et al*, [96], on germacranolide-type sesquiterpene lactones against *M. tuberculosis* and *M. avium* they concluded that the alpha-methylene-gamma-lactone moiety is an essential, but not sufficient, structural requirement for significant *in vitro* activity. Attention has also been focussed on marine-derived natural products [97]. Although these are not necessarily of the phytoplankton family, the value of searching for antimycobacterial compounds from marine-derived natural products was further strengthened. One study mainly focused on the enhancement of activity from the existing antimycobacterial drugs using the resinous material from bee wax, propolis, and showed potentiation of activity when combined with isoniazid, rifampicin and streptomycin [98].

The sudden emergence of resistant *M. tuberculosis* to the commonly used drugs like isoniazid, rifampicin, and pyrazinamide has focused attention on the urgent need for development of new antimycobacterial agents [99]. Improving pharmacokinetic properties may alleviate compliance problems, thus permitting reduced frequency and/or duration of therapy.

With emergence of new mycobacterial pathogens such as *M. avium-intracellulare*, the new antimycobacterials will have to possess a broader spectrum of activity [25, 100].

1.3 HYPOTHESIS OF THE STUDY

The study examined the hypothesis that traditional healers play an active role in the treatment of tuberculosis. An ethnobotanical survey of antimycobacterial plants a search was conducted. The plants collected were considered as not only having direct antimycobacterial activity, but also an attempt was made to establish ways of testing their toxic effects.

1.4 MOTIVATION FOR THE STUDY

It is estimated that more than 80% of the Black South African population use traditional preparations from the healers [101]. With the epidemiological status of TB in South Africa showing that Africans are the most susceptible population [75], and considering that a high percentage of the South African population live in underdeveloped, overcrowded areas and in industrialised areas, it follows that some of these people use traditional medicines for the treatment of TB. At the moment, South African health care systems are still inaccessible to most people, considering the fact that some areas do not have a health facility within a 20-kilometre radius. As TB is a 'poor man's disease' and TB therapy runs over a long period of time, people tend to believe that traditional medicine, which is usually accessible and usually short-term, is the better way. Most TB patients tend to ignore or postpone the idea of suffering from TB. This is mainly the fear of social consequences, which makes the patients postpone their TB diagnosis. As they are considered contagious to the community they then fear social deprivation. Also, with TB being associated with HIV infection, most people tend to avoid being seen in TB clinics, as the communities will assume them to be suffering from HIV. In response to this, people tend to secretly visit traditional healers with the idea that no information will leak out to the community that they are TB sufferers. With this knowledge, there is then a need to look into those medicines that the traditional healers use, scientifically validating their use and possibly evaluating their toxicity.

It is the interest of this study to investigate the use of these plants from certain areas of Southern Africa, capturing the methods by which they are used and, using scientific knowledge, explaining the basis of the methodology in which they are used, and lastly, isolating and characterising the active principles of the plants for further scientific analysis.


1.5 AIM AND OBJECTIVES

1.5.1 Aim

The aim of this study is to test the efficacy and safety of plants used in the traditional treatment of tuberculosis in Southern Africa.

1.5.2 Objectives

The following were the objectives of the study:

1. To study the use of traditional medicines in the treatment of tuberculosis in South Africa by traditional healers and to investigate how illness associated with TB infection is treated in traditional medical practice.
 2. To test extracts from plants used for traditional treatment of tuberculosis against *M. aurum*, *in vitro*.
 3. To perform bioassay-guided fractionation and testing the plant species with the best activity against *M. tuberculosis*, *in vitro*.
 4. To test the cytotoxic effect of the plant extracts and isolates against mammalian cell lines.
 5. To isolate and characterise active plant isolates using analytical chemistry techniques.
- 

CHAPTER TWO

Methodology

University of Cape Town

2.1 ETHNOBOTANY

Various parts of South Africa were visited. These were the Eastern Cape, KwaZulu-Natal, Limpopo, Western Cape, Northern Cape, Free State and Mpumalanga. The southern parts of Lesotho were also visited. The areas were selected on the basis of the prevalence of tuberculosis. Various types of traditional healers were visited and interviewed. The community, in most cases, recommended the healer/s who had knowledge of the disease. The traditional healers were asked several questions in order to investigate their knowledge and understanding of tuberculosis (TB), including their method of diagnosis of TB, symptoms of TB, the knowledge of the causative agent(s), and the difference between TB and other pulmonary disorders. Once the traditional healers had elaborated fully on their understanding of the disease, they were asked about the mode of treatment that they use to cure the disease. The main interest was on the plants they use. Traditional healers who were willing to provide the information on plants would either give the name of the plants or take the researcher to the area where the plant was picked for treatment purposes. Where only the name of the plant was provided, the plant would be collected either at the botanical garden or in a local herb market. A specimen sample of the plant would be taken for positive identification at the Bolus Herbarium in Cape Town. The particular plant part that was used was recorded for collection purposes. The ways traditional healers combine plants was noted as this would assist in knowing which plants to extract in combinations. The collected specimens were put into containers and sent to the laboratory for air-drying. The three methods of approach used in the collection process were the ethnobotanical approach, phylogenetic-chemotaxonomic approach, and ecological survey. This involved obtaining information from traditional healers and community members on plants that they use to treat TB or other diseases with similar symptoms to TB. Recorded information from botanical gardens was used in the search. The chemotaxonomic approach assumes that plants that have close genetic similarity may produce similar compounds. In this survey, plants that belong to a family with known or recorded traditional use against TB were investigated. The approach takes into account the fact that certain known compounds are active against a broad spectrum of microorganisms. Plants traditionally used to treat certain bacterial infections were investigated for activity against TB. The healers in the latter case were asked about the plants that they used to treat symptoms such as cough. As TB can present in many forms such as pulmonary TB and bone TB,

healers might treat symptoms without any idea that they are treating TB. This further broadened the search for plants used for similar symptoms caused by diseases other than pulmonary TB. Although the ecological survey approach had a low success rate, it was also based on investigating plants that were constantly used by communities with a very low TB incidence. Such plants might enhance immunity to TB or they might be directly active against *M. tuberculosis*. The low success of this collection system was due to the fact that very few communities used a certain group of plants either as a food or as a tonic. Most plants used by such communities were for medicinal purposes.

2.2 PHYTOCHEMICAL STUDIES

Phytochemical analysis was used to test for the compounds of known pharmacological activity. The test is based on colorimetric phytochemistry. Colour changes due to chemical reactions exhibited by these compounds are used. The tests are based either on test tube reactions or thin-layer chromatography. Compounds tested in this way were saponins and tannins [102].

2.2.1 Test for saponins

5 mg of each plant were put into a 500-ml test tube and 200 ml of water were added. This was brought to boil. The sample was allowed to cool to 60°C and shaken to form foam. The presence of persistent foam suggested the presence of saponins.

2.2.2 Test for tannins

200 ml of water were added to 5 mg of the plant in a 500-ml test tube. This was set to boil in a water bath for 5 minutes. The plant mixture was allowed to cool and filtered into a clean test tube. Two drops of 5% ferric chloride solution were added to the filtrate using a Pasteur pipette. The presence of a bluish-black precipitate indicated a positive test for tannins.

2.3 CHEMICAL EXTRACTION

2.3.1 Removal of tannins

Tannins were removed because of their interference with many biological assays. Studies have shown that they may inhibit reverse transcriptase [103, 104] and DNA polymerase [105]. Rapid removal of tannins from samples prior to screening for activity in various mechanistic and biochemical assays and high throughput screening assays is recommended. The collected plant samples were air-dried at room temperature away from direct sunlight. Each sample was ground into finer material to increase surface area during extraction. Tannins were removed from plant extracts using the modified Collins method [106]. Two grams of the crude extract were dissolved in 200 ml of methanol (Saarchem, UniLab). 5 Grams of Polyamide-6-S (Riedel-de Haen, Germany) were added to the solution of the extract. This was allowed to stand for 10 minutes in a shaker at room temperature. The solution was filtered with filter paper (Macherey-Nagel GmbH, Germany) and the filtrate concentrated on the rotary evaporator (Buchi). The extract that was formed was tannin-free and the removed tannins were bound to the Polyamide-6-S residue. The tannin-free extract was concentrated by rotary evaporation and used to test in biological assays.

2.3.2 Solvent extraction

The material was divided into two samples. One sample was extracted with distilled deionised water (Millipore) to extract the water-soluble material from the plants. Furthermore, water is used to simulate the extraction procedure by the traditional healers as they use water to prepare their medicines. The other sample was extracted with dichloromethane (Saarchem, UniLab) and, sequentially, methanol (Saarchem, UniLab). The residue was air-dried to ensure total evaporation of dichloromethane and extracted with methanol. Methanol is a polar organic solvent that extracts most of the polar compounds that dichloromethane did not extract. The extraction procedure was performed at room temperature in a shaker for 24 hours. The solvent was changed and new solvent added until extraction was completed.

2.3.3 Solvent removal

Organic solvents were removed from the extracted plant material by rotary evaporation (Buchi). The aqueous extracts were concentrated by free-drier.. This system of sublimation converts the solid ice extract into a vapour and the vapour is converted directly back to ice, bypassing the liquid state.

2.3.4 Plant preparation for assay

4 mg of each extract were weighed into a micro tube (Eppendorf), dissolved in 100 µl of methanol (Merck, SA), and sonicated for 2 minutes until the extract was completely dissolved. The extract was made up to a concentration of 4 mg/ml with phosphate-buffered saline (PBS) pH 7.0. This was filter-sterilised with 0.2 µm-filter cartridge (Sterilin) into sterile micro tubes. Aqueous extracts were weighed as with organic extracts. They were directly dissolved in PBS to make the required concentration. The extracts were freshly prepared before each assay. Isoniazid was used as a positive control and was prepared in a similar manner. Methanol was prepared as a solvent control by adding 200 µl of methanol to 800 µl of PBS. This was performed to test the effect of methanol.

2.4 BIOLOGICAL ASSAYS

2.4.1 Firefly luciferase bioluminescence assay (ATP assay)

2.4.1.1 Principle

The firefly luciferase bioluminescence assay has been shown to be in full agreement with the results of other biological assays testing anti-mycobacterial activity, including the radiometric system (Bactec system) and the resistance ratio methods [107]. The main advantages of the ATP assay is its rapidity, the lower initial costs of the analytical equipment, the lower reagent cost per analysis, and the use of non-radioactive culture medium. This assay was used to measure the growth of *M. aurum* and *M. tuberculosis*

cultures in test cultures. The assay measures the amount of 5,6-adenosine triphosphate (ATP) extracted from living cells. It is based on a principle of light production, catalysed by the enzyme luciferase as follows:



The firefly luciferase catalyses activation of the substrate D-luciferin by ATP and its subsequent oxidation to excited oxyluciferin. Transition of excited oxyluciferin to its ground state results in light emission. Light output is directly proportional to the amount of ATP and the light emitted is measured by the luminometer [107, 108].

2.4.1.2 Process

The process behind the assay involves extraction of ATP from culture and comparing it with ATP from control cultures. Extracting ATP from non-pathogenic cultures is different from extracting it from pathogenic cultures. This assay was only used to test compounds against mycobacteria species.

2.4.1.3 Extraction of ATP from *M. aurum*

A solution of 15% trichloroacetic (TCA) acid (Merck, Germany) in ultra-pure ATP-free water was prepared and stored at 4°C. It was used to extract the ATP from the *M. aurum* A⁺ cultures. 10 µl of 15% TCA were added to micro tubes and placed on ice. Each assay was done in duplicate. 50 µl of well-vortexed culture were added to each tube and incubated on ice for 30 minutes. The tubes were removed from ice and 50 µl of 100 mM tris-acetate/2 mM ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.75) was added to each tube, followed by 622 µl ultra-pure water. The extract was mixed well and was then ready for ATP measurement. 100 µl of each extract was used to assay ATP using luciferin/luciferase reagent. When cultures were heavily turbid the ATP extract was diluted in 100 mM tris-acetate/2 mM ethylenediaminetetraacetic acid (EDTA) buffer (pH 7.75) before assaying.

2.4.1.4 Extraction of ATP from *M. tuberculosis*

In assaying against pathogenic strains of mycobacteria, stringent safety measures were taken. The assay system for testing *M. tuberculosis* is different from that of *M. aurum* due to the safety concern. A process of boiling of the cultures for 5 minutes is involved, in which it is necessary to heat-kill the mycobacteria [109]. This process is not necessary for other non-pathogenic mycobacteria as the heating puts a stress on the ATP being extracted. 500 µl of preheated (at 95°C) buffer were added to 50 µl of culture in a micro tube. The tubes were sealed securely with parafilm. The cultures were then heat-killed by placing the tubes in boiling water for 5 minutes. 100 µl of the heat-killed cultures were used to assay the ATP using luciferin/luciferase reagent. Six (6) samples of ATP controls were prepared for ATP hydrolysis by adding 2.5 µl of 10⁻⁷M ATP standard solution (Boehringer Mannheim GmbH, Germany) to 47.5 µl of ultra-pure water. Three were placed in hot buffer in the water bath at 100°C as for *M. tuberculosis* samples and three for treatment with room-temperature buffer (unhydrolysed ATP controls).

2.4.1.5 ATP assaying with luciferin/luciferase reagents

An amount of 347.5 µl of 100 mM tris/2 mM EDTA buffer (pH 7.75) was transferred into luminometer cuvettes and 50 µl of thawed luciferin/luciferase reagent added to each cuvette and slightly mixed by vortexing and the light output read on a luminometer (Bio-Orbit 1253) for background (B). 100 µl of the ATP extract were added to the cuvette, mixed and light output read on luminometer (C). 2.5 µl of 10⁻⁷M ATP solution (as internal standard) were added, mixed and the light output read (T).

2.4.1.6 Data analysis

The results from this test were expressed as percentage survival measured against the various test concentrations. The IC₅₀ was used as a measure of potency of the test compound. To determine the amount of ATP the following equation was used:

$$\text{ATP} = \{[C - B]/[T - C]\} \times \text{number of pmoles ATP in the standard solution}$$

For *M. tuberculosis* the amount of hydrolysed ATP was subtracted from unhydrolysed

ATP to calculate the amount of ATP in all cultures. To calculate the growth of the culture the following calculation was made:

$$\text{Percentage growth} = (\text{ATP of Control} / \text{ATP of test}) \times 100$$

A line graph was constructed with the percentage growth plotted against the concentration on a logarithmic scale. As each experiment was repeated three or four times, the average of the readings was calculated and regarded as the overall reading, with standard deviation as error bars.

2.4.2 Optical density determination

The optical density determination was used as a test assay for growth of Gram-positive and Gram-negative bacteria. This system measures spectrophotometrically the turbidity of a test culture compared with that of the control culture. The absorbance is measured at a wavelength of 600 nm using a spectrophotometer. The bacterial culture was vortexed thoroughly until it formed a homogenous culture. The optical density of the culture was then measured at 600 nm. Using sterile media, the culture was diluted to get an OD₆₀₀ of 0.001 units. 1.89 ml of media and 100 µl of test sample were transferred to the culture bottle. 10 µl of the 0.001 OD₆₀₀ test culture were added into the culture bottle, the bottle was tightly sealed and the culture slightly vortexed. Incubation was for the specified period, depending on the culture. *E. coli* cultures were incubated for 12 hours. Other Gram-positive and Gram-negative cultures were incubated for 16 hours. *M. aurum* was incubated for 24 – 30 hours.

After the incubation period, the cultures were prepared for measuring their OD₆₀₀. They were diluted with media 10-fold and 100-fold. The OD₆₀₀ was measured and the growth calculated as the optical density multiplied by the dilution factor. These readings were compared to those of the control culture where no drug or agent was added.

2.4.3 BACTEC 460 TB system

2.4.3.1 Principle

The BACTEC 460 TB system was used to test the effect of isolates on a number of clinical strains of *M. tuberculosis*. This method was chosen because of its safety. This work was done by Centre for Molecular Medicine at the University of Stellenbosch in Tygerberg Hospital.

The Bactec system measures the growth of *M. tuberculosis* using the uptake of radioactive ^{14}C from palmitic acid and the subsequent release of $^{14}\text{CO}_2$ from the actively growing bacteria.

2.4.3.2 Culture typing

Patient samples of *M. tuberculosis* were isolated and DNA-typed using restriction fragment length polymorphism. The Centre for Molecular Medicine at the University of Stellenbosch performed this work. This was to confirm that the strains were different in genetic make-up.

2.4.3.3 Preparing the test material

Test material was prepared in PBS pH 7.0 at maximum concentrations of 12 mg/ml. The dilution factor in the Bactec was 1 in 40 ml. The final maximum concentration in the test tube was 30 $\mu\text{g}/\text{ml}$. Serial dilutions of stock were prepared to a range suitable for the test.

2.4.3.4 Setting the assay

The compound solution was filter-sterilised with a 0.2 μm filter unit. Cultures of *M. tuberculosis* were incubated at 37°C and allowed to reach a concentration of 1 McFarland unit, equivalent to approximately 500-growth index reading on the Bactec.

100 µl of that culture were transferred to the Bactec tube and 100 µl of the test isolate were added. An additional tube containing 1% of the control culture was prepared and incubated under similar conditions. The culture was incubated at 37°C and readings were taken on daily basis to monitor growth of the cultures.

2.4.3.5 Data analysis

The growth index was used as a monitor of the growth of the cultures. The 1% control culture was used as a measure of the minimum inhibitory concentration (MIC). After a seven-day period, culture that maintained a similar growth index with the 1% control culture was regarded as the MIC.

2.4.4 Cytotoxicity assay

2.4.4.1 The MTT assay

The cytotoxicity of the plant extracts and of isolates was tested using the MTT assay. The assay is based on the reduction of soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) (Sigma, SA) to an insoluble formazan product. The purple colour formed is a measure of cell viability, the reducing enzymes being present only in metabolically active live cells. Dead cells do not reduce MTT. The purple-coloured formazan crystals dissolve in a variety of organic solvents and the optical density of the resulting solution is measured [110].

2.4.4.2 Culturing the Chinese hamster ovarian cell lines

Chinese Hamster Ovarian cell lines were maintained in 45% Dulbecco's Modified Eagle's medium (DMEM) (Gibco), 45% Nutrient Mixture F-12 Ham (Sigma, SA) and 10% heat-inactivated fetal calf serum (Gibco). They were subcultured every 48 hours at 37°C in 5% CO₂ in a tissue culture-treated flask.

2.4.4.3 Plating the cells

When the cells became confluent they were regarded as ready for plating. The cells were detached from the flask with 5 ml of 1% of trypsin (Sigma) in PBS (pH 7.0) after aspirating the media. The medium was incubated at 37°C for five minutes and 5 ml of medium were added to make a 10-ml solution and centrifuged at 1700rpm for 5 minutes. The cells were stained with crystal violet and counted in a haemocytometer. The cells were adjusted to a concentration of 10^4 cells/ml with media. 100 μ l were added to each well of the tissue culture-treated 96-well microtiter plate and incubated for 24 hours to allow for attachment to the plate.

2.4.4.4 Preparing the test material

4 mg of each extract were weighed into a micro tube, dissolved in 100 μ l of dimethyl sulfoxide and sonicated for 2 minutes until the extracts were totally dissolved. The extract was made to a concentration of 4 mg/ml with phosphate-buffered saline pH 7.0 and filter-sterilised with 0.2 μ m-filter cartridge (Sterilin) into a sterile micro tube. Aqueous extracts were weighed as with organic extracts, directly dissolved into PBS, and made to the required concentration. The extracts were tested immediately after preparation.

2.4.4.5 Setting the assay

After the 24-hour incubation period, the media was aspirated from each well. 25 μ l of the test and control compounds were added into their respective wells. Daunomycin (Sigma) was the control compound. An aliquot of 175 μ l of medium was added to each well and incubated for 24 to 48 hours, depending on the requirements of the experiment.

2.4.4.6 Harvesting the cultures

After incubation 25 μ l of 25 mg/ml concentration of MTT were added to each well and incubated for 4 hours away from direct sunlight. The plate was then spun at 30 000 rpm for 15 minutes, the medium aspirated, taking care not to remove the crystals of formazan, and 100 μ l of dimethyl sulfoxide were added to each well to dissolve the crystals. The mixture was carefully vortexed away from direct sunlight and the absorbance read at 600 nm.

2.4.4.7 Data analysis

The percentage survival of the cells was plotted against concentration of the test material in a line graph or histogram. The IC₅₀ value gave an indication of the toxicity of the test material.

2.5 BACTERIOLOGY

2.5.1 Tissue culture

7H9 Middlebrook liquid broth was prepared by dissolving 4.7 g of Middlebrook broth (Difco Laboratories, USA) in 900 ml of distilled water. An aliquot of 90 ml was added to culture bottles and autoclaved at 121°C for 10 minutes. 10 ml of bovine serum albumin Fraction V-dextrose-catalase (Boehringer Mannheim GmbH, Germany) supplement was added. In preparing the 7H11 Middlebrook solid agar, 2.0 g of bacteriological agar (Difco Laboratories, USA) was added to the Middlebrook broth and processed as in the liquid broth. In the solid agar, oleic acid-albumin-dextrose-catalase supplement was used. The agar was prepared in Petri dishes (Sterilin, SA), 10 ml being added to each plate.

For growing the non-mycobacterial cultures, 2YT agar was used. This was prepared by adding 16 g of tryptone, 10 g of yeast extract, 5 g of NaCl, and 15 g of bacteriological agar and made to 1 litre with distilled deionised water in a flask. The mixture was boiled in an oven for 1-2 minutes to dissolve the agar and transferred to Petri dishes in 10 ml

aliquots in each plate.

All bacterial cultures, with the exception of *M. tuberculosis*, were transferred from the solid medium to the liquid medium 24 hours before the assay was conducted and incubated at 37°C in a shaker. *M. tuberculosis* cultures were incubated for 7 days in 7H9 Middlebrook broth before the assay was conducted. An inoculum of each culture was stained with the appropriate stain and viewed under the microscope for morphological identification.

2.5.2 Maintenance of bacterial cultures

M. tuberculosis H₃₇Rv was used as a slow-growing pathogenic strain of tuberculosis. This is a sensitive mycobacterial. The cultures were maintained in Lowenstein-Jensen solid slopes at 37°C and were subcultured every two weeks. In preparing for an assay an inoculum was transferred to liquid 7H9 Middlebrook broth with 10% of albumin-dextrose-catalase (ADC) supplement. The cultures were vortexed to disperse any bacterial clumps in the culture. They were incubated in a shaker at 37°C. To count colonies, the cultures were spread-plated on 7H11 Middlebrook agar. The plates were sealed with parafilm to prevent loss of moisture and incubated for 14 days. *M. aurum* A⁺ was used as a fast-growing non-pathogenic strain of mycobacteria to test crude plant extracts. *M. aurum* A⁺ is a non-pathogenic strain with a similar antibiotic sensitivity profile to that of *M. tuberculosis* [90]. The cultures were subcultured once every 24 hours in 7H9 Middlebrook broth with 10% of albumin-dextrose-catalase (ADC) supplement in culture bottles at 37°C in a shaker incubator. *M. aurum* was also subcultured in 2YT broth under similar conditions to the 7H9 Middlebrook broth. For counting colony-forming units (CFU), the cultures were plated in 2YT agar plates.

Gram-positive and Gram-negative bacteria were used to test for specificity of the extracts. The species used were *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* (Gram-negative) bacteria and *Micrococcus* sp, *Staphylococcus aureus*, and *Bacillus subtilis* as Gram-positive bacteria. The cultures were maintained in 2YT agar plate, with subculturing every 7 days. All cultures were viewed under the simple microscope oil immersion lens. All mycobacterial cultures were stained with Ziehl-Neelsen stain and other bacteria were stained with Gram stain.

2.5.3 Setting the assays

In setting the test tubes for biological assays, the medium was pre-warmed to 37°C in a water bath. The assays were conducted in 20 ml culture bottles. 1.8 ml of media were transferred to the test bottle. 100 µl of well-vortexed culture were transferred to the culture bottle and 100 µl of the test material were transferred into the culture bottle. This was tightly sealed and incubated in a shaker at 37°C for the required time.

The growth curve was done to determine the various growth phases of the cultures. This made it possible to determine the stage when the culture was ready for use in the biological assays. The various growth phases included the lag phase, the early and late log phases, and the stationary phase. In determining the growth phase, the culture was incubated in liquid media at 37°C. At a predetermined time interval an aliquot of culture was used to measure the amount of bacteria present. The various points were plotted until the culture reached a constant growth stage, when there was no longer active bacterial division. For the *M. aurum* the growth was measured at 4 hourly intervals over 2 days. For *M. tuberculosis* the growth was measured every 24 hours for 4 days. The cultures were measured using the firefly luciferase bioluminescence assay. Growth of the other bacteria was measured every ½ hour for 24 hours. Optical density was used to measure culture growth.

2.6 BIOASSAY-GUIDED FRACTIONATION

The dichloromethane extracts of *Artemisia afra* were fractionated to isolate the active compounds.

2.6.1 Vacuum liquid chromatography and flash column chromatography

2.6.1.1 Principle

Vacuum liquid chromatography involves separation of compounds bound to silica, based on their polarity. A variety of solvents with increasing polarity are used to isolate

the various compounds. A vacuum is created at the bottom of the flask to pull the liquid into a collection vessel. In flash chromatography pressure is applied from above the solvent to push the solvent down [111].

2.6.1.2 Process

The initial fractionation of *A. afra* dichloromethane extract was by column chromatography. Two types of columns were used. To isolate the various fractions of the extract a column of 3 cm diameter and 50 cm height was used. The silica gel was 60 – 120 μm (E. Merck, Germany). Hexane was used as the initial solvent to pack the column. Thin-layer chromatography was used to run the fractions obtained from flash chromatography. The process of fractionation was done in a four-step process based on bioassay of the fractions. The first fractionation step yielded 12 fractions, the second step yielded 9 fractions, and the third yielded 2 compounds.

2.6.1.3 Fractionation of *A. afra* crude extract

The active crude dichloromethane extract of *A. afra* was chromatographed over a silica gel (96 g) (60-120 μm)(Merck, Germany) column (6 cm x 45 cm), eluted with a gradient of ethyl acetate in *n*-hexane (v/v) (0, 10, 50, 75, 100) and methanol in ethyl acetate (v/v) (10, 50, 100). Fractions were pooled or separated based on thin-layer chromatography (TLC) analysis results. This generated 12 fractions, designated Shef1 through Shef12, as shown in table 2.1 on page 40. Following biological activity, fractions Shef5 (1:1), Shef6 (1:3) and Shef7 (0:1) were pooled and further chromatographed over a silica gel (50 g) column (4 cm x 30 cm), eluting with a gradient of ethyl acetate in *n*-hexane (v/v) (25, 30, 35, 40, 50, 100), as shown in table 2.2 on page 40. TLC analysis results were used to separate or pool isolates, obtaining a further 12 isolates, designated C1 through to C12. Isolate C8, the most biologically active isolate, was isolated with a hexane: ethyl acetate ratio of 65:35 (v/v) as a white crystalline isolate, showing one spot on TLC, visualised with anisaldehyde.

Table 2.1: The first fractionation process of the dichloromethane extract of *A. afra* on flash column chromatography

Fraction	Eluting solvent system (v/v)		
	Hexane	Ethyl Acetate	Methanol
Shef1	100	0	0
Shef2	90	10	0
Shef3	90	10	0
Shef3B	90	10	0
Shef4	50	50	0
Shef5	50	50	0
Shef6	25	75	0
Shef7	0	100	0
Shef8	0	90	10
Shef9	0	50	50
Shef9B	0	50	50
Shef10	0	0	100

Table 2.2: The second fractionation process of the dichloromethane extract of *A. afra* on column chromatography.

Fraction	Eluting solvent system	
	Hexane:	Ethyl Acetate
C1	75	25
C2	75	25
C3	75	25
C4	75	25
C5	75	25
C6	75	25
C7	70	30
C8	65	35
C9	60	40
C10	50	50
C11	50	50
C12	100	0

Table 2.1 and Table 2.2 show the gradient of the solvent systems used in the 1st and 2nd fractionation processes of the crude dichloromethane extract of *A. afra*.

2.6.2 Thin-layer chromatography

2.6.2.1 Principle

Thin-layer chromatography is based on separation of compounds as they migrate on a film of silica gel - an amorphous, porous substance. The properties of the compound and of the mobile phase determine the distance covered by the compound along the thin layer chromatography plate. An important factor in quantifying the migration of a compound on a particular sorbent and solvent system is the R_f value. R_f value is the ratio of the compound distance from origin to that of solvent front, as shown below:

$$R_f = \frac{\text{Compound distance from origin}}{\text{Solvent front distance from origin}}$$

In the case of adsorption chromatography, where the sorbent is silica, polar compounds have a higher affinity for the sorbent and migrate slowly up the plate as the mobile phase migrates. These compounds will have a relatively small R_f values. Non-polar compounds have less affinity for the stationary phase and move comparatively quickly up the plate and have larger R_f values. Compounds of a mixture will separate according to their relative polarities.

2.6.2.2 Process

Thin-layer chromatography was used in the fractionation process as a marker of the number of compounds present in a fraction. The material was dissolved in the mobile phase and spotted using a 3- μ l capillary tube. Depending on the concentration of the material, the application process was repeated to increase the concentrations of the material on the plate. The chromatography was performed in a closed glass chamber to prevent evaporation of the mobile phase. The stationary phase used was the aluminium backed silica gel 60 F₂₅₄ plate. The mobile phase was prepared from the same solvent

that was used to elute the compound/s from the flash chromatography. Isolate C8 was eluted with a mobile phase of 35% ethyl acetate in hexane. The R_f value for C8 was 0.6. Following the elution process, visualising agents were used. These included UV₂₅₄, UV₃₆₆ and anisaldehyde.

2.6.3 High-pressure liquid chromatography

High-pressure liquid chromatography was used to achieve purification of the C8 isolate and to determine its UV absorbance. Isolate C8 was run on a high-pressure liquid chromatograph (Shimadzu LC10S) connected to a Diode Array detector (Shimadzu, LC10D) with variable wavelength. The gradient mobile phase run of the isolate was performed with a 5 micron 150 x 4.50 mm analytical column (Phenomenox, Prodigy 5 ODS-2) linked to a guard column (Phenomenox) at a flow rate of 1 ml/min, with a mobile phase starting with 20% methanol in water for duration of 60 minutes. An isocratic run of the isolate was performed using a mobile phase of 50% methanol (v/v) in water for 10 minutes. This was run on a high-pressure liquid chromatograph (Shimadzu LC10S) connected to a Diode Array detector (Shimadzu, LC10D) with variable wavelength through a Supelco analytical Dimension 5u 150 x 4.60 mm column linked to a Phenomenox guard column. Absorbance was measured at 254 nm.

2.7 CHEMICAL ANALYSIS

Following the purification process analytical chemistry techniques were used to analyse isolate C8.

2.7.1 Nuclear magnetic resonance

2.7.1.1 Principle

Nuclear magnetic resonance (NMR) provides information about the types, numbers and connectivity of particular atoms [112]. It is usually the first step towards studying the

structures of many biological and organic and inorganic molecules. Proton NMR (^1H -NMR) spectroscopy provides a means of determining the structure by measuring the magnetic moments of its hydrogen atoms. In most compounds, hydrogen atoms are attached to different functional groups and the proton NMR spectrum provides a record of the number of hydrogen atoms in these different situations. Carbon-13 NMR (^{13}C -NMR) spectroscopy, on the other hand, gives direct information on the nature of the carbon skeleton on the molecule.

In practice, the sample is placed in solution, in an inert solvent, between the poles of a powerful magnet and the protons undergo different chemical shifts according to their molecular environments within the molecule. These are measured in the NMR relative to a standard, usually tetramethylsilane (TMS), which is an inert compound, which is added to the sample without the likelihood of chemical reaction occurring.

^1H -NMR and ^{13}C -NMR complement each other and the combination of the two techniques provides a means of structural elucidation [112].

2.7.1.2 Process

The NMR spectra were measured in CDCl_3 with TMS as an internal reference. The ^1H -, ^{13}C -NMR, HSQC, HMBC, and ^1H - ^1H COSY spectroscopic data for the isolate were recorded on a Varian 300 MHz (VXR 300) instrument.

2.7.2 Mass spectrometry

2.7.2.1 Principle

Mass spectroscopy (MS) consists of degrading trace amounts of organic compounds and recording the fragmentation pattern according to mass. The sample vapour diffuses into a low-pressure system of the mass spectrometer where it is ionised with sufficient energy to cause fragmentation of chemical bonds forming a molecular ion and fragment ions. The resulting positively charged ions are accelerated in a magnetic field, which disperses and permits relative abundance measurements of ions of given mass-to-charge ratio. The resulting record of ion abundance versus mass constitutes the mass

spectral graph, which thus consists of a series of lines of varying intensity at different mass units. In some instances the parent compound does not give a stable molecular ion. The measurement of mass on this particular ion is then performed with the introduction of soft ionisation technique [112].

2.7.2.2 Process

High-resolution electron impact mass spectrometry of isolate C8 was performed on a VG-70-SEQ mass spectrometer operating at 70 eV.

CHAPTER THREE

The role of traditional healers in the control and treatment of tuberculosis

*"It is hard indeed to accept that one single model could be used
equally well to understand the process of innovation and
technological change of societies that differ substantially."*

Jorge Katz

3.1 INTRODUCTION

The impact of the traditional health system on control and prevention of tuberculosis in South Africa is one aspect of assessing the efficacy of the traditional approach. Tuberculosis is an old disease that has been aggravated by the social and political systems of South Africa [113].

The aim of the study was to research and report the different ways in which traditional healers around South Africa respond to tuberculosis. The study concentrated mainly on the treatment of the disease by traditional healers. However, certain trends were observed, and recorded, on their understanding of the cause of tuberculosis and their criteria for diagnosis. Traditional healers were interviewed in an open discussion rather than by standard questionnaire in order not to curtail the traditional healer's explanation. The study was carried out by visits to traditional healers in 7 South African provinces, namely, the Western Cape, Limpopo, Eastern Cape, Free State, Northern Cape, Mpumalanga, and KwaZulu-Natal. For purpose of the study, the definition of traditional healers included diviners, faith healers, herbalists, and Rastafarians. Selection of traditional healers in each village was either by random selection process or by recommendation by community members. The findings from these visits on the plants used by traditional healers to treat tuberculosis are reported. Because of the lack of robust anthropological methodology in conducting this study, observations on the interaction of traditional healers with their patients have simply been noted.

3.2 RESULTS

3.2.1 Tuberculosis: traditional healer's perspective

3.2.1.1 Methods of diagnosis

Traditional healers mostly employed visual means of diagnosis for tuberculosis. This was supplemented by the spiritual systems such as bone throwing for diviners, prophecy for spiritual healers or information from family members. In most instances the traditional healers did not necessarily need to know most of the symptoms that are used to distinguish tuberculosis from other forms of chest complaints. Most diviners preferred to perform the divining before the patient reveals his/her complaint. In such cases this showed that the traditional healer had some knowledge of the disease that they were about to cure. This was shown to be similar with the spiritual healers who would perform prophesying rituals to determine what the patient was suffering from. In this case the patient, together with his family members, would be given the chance to agree or disagree with the healer's prophecies. The symptoms that were mostly mentioned were coughing persistently, loss of weight, night sweats and bloody sputum. These seem to correlate with the conventional ways in which TB is diagnosed in modern medicine. Differentiation between TB and other forms of respiratory diseases was usually less clear as most healers had difficulty in understanding what tuberculosis is in comparison with other chest complaints.

3.2.1.2 Understanding the cause

Different schools of thought were observed on what causes tuberculosis amongst adults. Most traditional healers were of the idea that tuberculosis was an ancestral way of demanding a ritual to be performed by the unhealthy being. In this way, the ancestors would induce the disease in the person in order for the individual to respond by performing the required custom. The healers' role would be firstly to find the type of ritual custom that the sufferer had to perform. The custom would then be performed as the initial treatment step. Following the performance the person would either recover automatically or the healer would then proceed with the treatment.

This form of belief was mainly found among *amagqirha*, the elders and *amaxhwele*. Some traditional healers believed that tuberculosis is a form of poisoning or *idliso*, as it is termed in isiXhosa. This form of poisoning could either be through food or water or beer. Their belief was based on the idea that the poisoning would rise to other organs such as the lungs, when it matures. Similar cases were directed to a form of a bad spirit or witchcraft, known in many parts as *impundulu*. These two beliefs were predominant amongst the *izangoma* and *abathandazeli*.

Others believe that tuberculosis is either transmitted through coughs from those who are sufferers or by inhaling it in the air. These views were mostly prevalent amongst herbalists and Rastafarians, as their practice involved treatment aimed at eradication of the bacteria.

3.2.1.3 Treatment of tuberculosis

The treatment of tuberculosis with plants varied from one region to another and from one type of traditional healer to another. The system of diagnosis used, the understanding of the cause of tuberculosis and the type of traditional healer mainly influenced the different approaches to treatment. From the various reports obtained from the field trips the treatment approaches could be grouped into 5 categories. These are outlined as symptomatic mode, causative mode, diagnostic mode, primary health care mode, and holistic approach.

3.2.1.3.1 Symptomatic mode

In this approach the symptoms observed from the patient influence the type of treatment that a traditional healer would give to a patient. The treatment was mainly medication using plant medication. For example, when loss of weight was the main symptom, the traditional healer would prescribe medication to enhance appetite and thereby lead to weight gain.

3.2.1.3.2 Causative mode

In this case, the traditional healers would first treat or address the cause of the disease as they understand it. An example is that when the traditional healer believes that the reason for the patient to be ill is the need for a ritual to be

performed, the healer would request the family of the ill person to perform the ritual. As part of the treatment, the healer would also recommend to the family what type of ritual the person had not performed or needs to perform. This form of healing is sometimes accompanied by medication to fight the invading disease. However, in some instances, ritual cleansing takes place first and then the patient is referred to another healer who deals with herbal remedies.

Another example that is prevalent is when a patient is given medication to induce vomiting when *idliso* (poison or evil spirit) is suspected to be the cause of illness. In this case, the assumption is based on the argument that once the bad spirit or poison is released the patient will recover.

3.2.1.3.3 Diagnostic mode

In the diagnostic mode, the diagnosis of the disease and what the disease does to the body are taken into consideration. In such an approach, the traditional healers would treat the cause while trying to repair the body to its original form. An example is where a bacterial cause is diagnosed to have caused a scar in the lungs, leading to the bleeding when coughing. In such a case the traditional healer would give medication to treat the scar while he is in the process of eradicating the bacterium with another medication or to “cleanse the blood”, as most traditional healers would term it. Sometimes these processes follow each other.

3.2.1.3.4 Primary health approach

This is the most prevalent form of healing in most rural areas. The traditional healer would be required to look at engaging the whole family in the healing process. This sometimes becomes a lengthy and expensive process, as it requires certain practices such as slaughter of sheep or cattle. This is termed as the primary health approach in the sense that the healer looks at the history of the patient, his surroundings and family. In some instances the healer would involve other members of the community. Another example of this is the understanding of the cause, such as cross-infection amongst family members and genetic causes (*ufuzo*) of low immunity.

3.2.1.3.5 Holistic approach

This is another prevalent approach where an intensive treatment system is used to treat the symptoms, the causes, and the anatomical deformity that has resulted therefrom.

3.2.2 Ethnopharmacological survey

Part of the study involved collection and recording of the various medicines used by the traditional healers to treat tuberculosis. The preparation of these remedies, the treatment regimens and the doses were recorded. The observation from this was that most of the plants were used in combinations. However, this varied amongst traditional healers in the different areas. Some of the medicinal preparations and plants used were recorded as follows:

Combination 1: *Alepidea amatymbica* (roots) + *Artemisia afra* (leaves) + *Warburgia salutaris* (bark). The bark is ground into powder and mixed with the leaves. These are then boiled into a tea. The tea is filtered and allowed to cool. This is drunk as “half-cup” in once a day regimens until the patient is cured. The plants for this mixture were obtained from Ngqeleni in the Eastern Cape. *A. amatymbica* is available mostly in the Eastern Cape and KwaZulu-Natal but is also found in the other parts of South Africa and Lesotho. *A. afra* is widely distributed in Southern Africa. *Warburgia salutaris* is facing extinction in South Africa. It is scarcely available in the Northern Province of South Africa. Sample for the study was bought in KwaZulu-Natal.

Combination 2: *Alepidea amatymbica* (roots) + *Strychnos henningsii* (bark) + *Afzelia quanzensis* (bark) + *Schotia brachypetalia* (bark) + *Ledebouria revoluta*.

L. revoluta is distributed mainly in the Eastern Cape but is also available in the Western Cape and KwaZulu-Natal. *S. brachypetalia* grows in upper areas of KwaZulu-Natal and Mpumalanga. People from the Eastern Cape usually pick it up in KwaZulu-Natal and it is therefore sold in herb markets for medicinal uses.

Combination 3: *Agathosma betulina* (leaves) + *Salvia africana-caeruleae* (leaves) + *Coryza scabrida* + *Plantago major* + *Rorippa nasturtium-aquaticum*. An informant said that this mixture was used by the Khoisan people in the treatment of chest

complaints in the Cape Province. A tea is prepared from this mixture of plants. The tea is taken daily for five to six months, one cupful per day.

Agathosma betulina grows as a shrub in the Western Cape. Its medicinal use dates back to the times of the early settlers in the Cape Colony. It is also used in the treatment of a variety of other ailments. It is used as a tea in most families. *Salvia africana-caeruleae*, *Plantago major*, *Rorippa nasturtium-aquaticum* and *Coryza scabrida* are distributed in parts of the Western Cape and Northern Cape provinces.

Combination 4: *Euphorbia natalensis* (whole plant) + *Tshetshafike* (unidentified powder) + *Pittisporum viridiflorum* (bark). Used for the treatment of asthma and “other types of coughs”. The three plants are mixed in specific proportions. A sample of the mixture is boiled and the tea is drunk. The recipe was obtained from Umtata and the plants were picked at the same place.

E. natalensis is scarcely available in the Eastern Cape and certain parts of the Western Cape and KwaZulu Natal.

Other plants were collected singly as specified by the healers. These were either used in combination with other plants or commercial products that are not of plant origin. These plants are as follows:

Olea europea subsp. africana. The leaves of this plant are used for treatment of TB in Lesotho. The leaves are boiled in water and the tea is drunk until the patient is healed. The plant was picked in Lesotho. This plant also grows wild in many parts of South Africa.

Solanum aculeastrum. The leaves and fruits are used in Lesotho and certain parts of Mpumalanga for the treatment of cough and other chest complaints. In the Eastern Cape the plant is dried and the leaves are smoked for “strong TB”. The fruits are used in other parts of the country for other reasons. The plant is known to be toxic.

Pentanasia prunelloides. The bark of the plant is used in TB treatment. The bark from this plant is boiled and the tea is drunk either alone or in combination with the leaves of *O. europeae*.

Leonotis ocymifolius. The leaves are used for treatment of TB and other chest complaints. The plant is widely available in the Southern African region.

Syzygium cordatum. The bark of this plant has been mentioned in literature as being used to treat tuberculosis and other chest complaints. A decoction of this plant is prepared in water and administered to TB patients until symptoms subside. The bark sample was obtained in KwaZulu Natal.

Cassine transvaalensis. The bark from this plant is used to treat chest complaints such as coughs, asthma. The plant was picked on the basis of its use for such complaints as that may include antibacterial activities. The red colouration of the plant tea has made it to be used for "*cleansing blood*". This means it is used when one suffers from an infection in the blood.

Ekebergia capensis. The bark of this plant is ground and boiled for a long unspecified time. The tea is cooled and drunk as medicine for coughs and chest pains.

Kedrotis africana. The tuber is used to treat tuberculosis and other chest complaints. Traditional healers use this plant as an emetic to "*get rid of the infection*" during their treatment for tuberculosis. Although this plant is not claimed to have direct treatment of tuberculosis, it is usually used sequentially with other plants that are antibacterial.

Siphonochilus aethiopicus. This almost extinct plant is used in KwaZulu-Natal as a TB treatment by boiling it in water and drinking the tea while still hot. The plant is also used as a cold tea by placing in cold water for three to four hours. Filter and add honey and drink two spoons three times a day. The plant is mainly found in herb markets and is sold illegally due to its extinction rate.

Croton gratissimus. The bark and the roots of the tree are assumed to have the same medicinal value. The part is dried and ground to powder. The powder is either boiled to make tea, or is snuffed to relieve tension headache.

Cannabis sativa. This plant is traditionally used in the treatment of chest complaints. This is prepared as a tea of fresh leaves. It is drunk as a cold tea. Certain traditional healers recommend the use of steam from boiling this plant.

Asclepias fruticosa. The leaves of this plant have been quoted in literature to be used to treat tuberculosis. A sample of this was collected in KwaZulu-Natal as being used to treat chest complaints and other bacterial infections. The leaves of the plant are also boiled and a teaspoon of the tea is taken orally to treat internal infections and chest complaints.

Allium sativum. The plant is usually drunk as a tea or used as a spice. Most traditional healers use the plant together with other plants to avoid the bad smell that comes after ingestion of the tea made therefrom. The plant has been quoted in literature as having antituberculosis effects.

A summary of the medicinal uses of the plants collected and their traditional use in the treatment of other ailments is presented in table 3.1 on page 54 - 55.

The Role of Traditional Healers in the Control and Treatment of Tuberculosis

Table 3.1: Plants used in the treatment of tuberculosis in South Africa.

Botanical	Family	Vernacular names	Part used	Other uses of the plant	Reference:
<i>Afzelia quanzensis</i>	Celastraceae	Umdlavuz	Bark	Uterine pain, eye complaint, bilharzia, eczema, depressed fontanelle, snake bite	114
<i>Agathosma betulina</i>	Rutaceae	Buchu	Leaves	Antibiotic, antispasmodic, backache, bladder complaint, bruise, flatulence, cold, cough, colic, cystitis, fractures, gout, hangover, indigestion, rheumatism, kidney ailment, dysmenorrhea, nausea, stomach ailment, urinary infection, vomiting, wound/s	115
<i>Alepiidea amatymbica</i>	Apiaceae	Ikthathazo, Iqwili, kalmoes	Tuber	Abdominal pain, asthma, chest complaint, cold, cough, influenza, diarrhea, headache, respiratory ailment, rheumatism, sedative, stomach ailment, sore throat, wound/s	115
<i>Allium sativum</i>	Alliaceae	Garlic	Tuber	Acne, athlete's foot, boils, bronchitis, cold, digestive infection, earache, fungal infection, high blood pressure, herpes simplex, influenza, old age tonic, tonsillitis, urinary infection	114
<i>Artemisia afra</i>	Asteraceae	Umdlonyane, wildeals, African wormwood	Leaves, Flowers	Abdominal pain, acne, inflammation, fever, asthma, boil, bronchitis, cold, cough, influenza, colic, nasal congestion, constipation, diabetes, dyspepsia, earache, emphysema, gastritis, gout, haemorrhoids, hay fever, headache, indigestion, malaria, measles, dysmenorrhea, mumps	115
<i>Asclepias fruticosa</i>	Asclepiadaceae	Umsinga-lwesalukazi	Leaves	Abdominal pain, asthma, bile, cough, diabetes, diarrhea, gonorrhoea, headache, hepatitis, female infertility, nasal catarrh, pain, purgative, stomach ailment, wart	115
<i>Bulbine latifolia</i>	Asphodelaceae	Ibuchu, Rooiwortel	Leaves	Blood disorder, convulsion, diabetes, diarrhea, dysentery, eczema, pruritus, lumbago, rheumatism, syphilis, urinary ailment, venereal disease, vomiting	114
<i>Cannabis sativa indica</i>	Cannabaceae	Umya, dagga, marijuana	Leaves	Appetite loss, asthma, bronchitis, cold, cough, influenza, depression, diabetes, dysentery, epilepsy, glaucoma, headache, hypertension, insomnia, labour pain, malaria, migraine, nausea, pain, snake bite, septicemia	115
<i>Capparis tomentosa</i>	Capparaceae	Iqwaningi/Iqwaningi	Bark	Aphrodisiac, apoplexy, bronchitis, chest complaint, constipation, cough, diarrhea, eye complaint, gonorrhoea, headache, impotence, infertility, insanity, jaundice, leprosy, lung congestion, malaria, ophthalmic, pruritus, piles, pleurisy, pneumonia, prevent abortion, purgative, purify blood, rheumatism, rubefacient, scrofula, sepsis, snake bite, sore/s, stomach ailment, wound/s	115
<i>Cassine transvaalensis</i>	Celastraceae	Ingwavuma	Bark	Anaemia, angina, blood disorder, cold sore, cough, diarrhea, fever, gout, haemorrhoids, infection, intestinal cramp, lice infestation, menorrhagia, pain, psoriasis, rash, renal ailment, scabies, shingles, stomach ailment, urticaria	115
<i>Conyza scabrida</i>	Asteraceae	Bakbossie	Leaves	Cold, convulsion, cough, delivery of placenta, fever, gall bladder disorders, headache, heart & chest complaint, inflammation, pain,	115
<i>Croton gratissimus</i>	Euphorbiaceae	Umahlabekeufeni	Bark	Abdominal complaint, aphrodisiac, bleeding gums, chest complaint, cough, dropsy, eye complaint, fever, indigestion, inflammation, insomnia, pain, purgative, rheumatism, uterine complaint	120
<i>Ekebergia capensis</i>	Meliaceae	Umnyamathi, Cape ash, essenhout	Bark	Abscess, acne, backache, blood purifier, boil, chest complaint, cough, diarrhea, disinfectant, dysentery, emetic, fatigue, gastritis, headache, heart ailment, heartburn, infertility, internal parasites, purgative, scabies, skin complaint, vermifuge	120
<i>Euphorbia natalensis</i>	Euphorbiaceae	Inkalimasana, inkalamazane	Whole	Variety of medicinal uses	120
<i>Kedrostis africana</i>	Cucurbitaceae	Uthuvana	Tuber	Purgative, emetic	120
<i>Ledebouria revoluta</i>	Hyacinthaceae	Inqwebaba	Bulb	Enema, gall bladder disorders, lumbago, skin irritation, sore/s, wound/s	120
<i>Leonotis ocymifolius</i>	Lamiaceae	Klipdagga	Leaves	Cold, gall bladder disorders, hypertension, purgative	120
<i>Olea europea subsp. africana</i>	Oleaceae	Umquma, wild olive	Leaves	Bladder infection, blood cleansing, blood pressure (high), colic, constipation, corn/s, diarrhea, diphtheria, eye complaint, fatigue, fever headache, renal ailment, rheumatism, scrofula, sore throat, urinary ailment/infection	114

Table 3.1: Plants used in the treatment of tuberculosis in South Africa. (Continued)

Botanical names	Family	Vernacular names	Part used	Other uses of the plant	Reference:
<i>Pentanisia prunelloides</i>	Rubiaceae	Isicimamlilo, setima-mollo, wild verbena	Leaves	Blister/s, blood purifier, boil, burn/scald, cut/laceration, chest pain, eczema, expel retained placenta, facilitate birth, fever, graze, haemorrhoid, heartburn, insect bite/sting, palpitations, rheumatism, snake bite, sore joints, swelling, toothache, vomiting	120
<i>Pittosporum viridiflorum</i>	Pittosporaceae	Umkwenkwe	Bark	Abdominal complaint, aphrodisiac, back pain, biliousness, boil, chest complaint, dizziness, dysentery, fever, gall bladder disorders, malaria, pain, respiratory disorder, sedative	115
<i>Plantago major</i>	Plantaginaceae	Weeblare	Leaves	Anthelmintic, bleeding, blepharitis, conjunctivitis, cough, diarrhea, diuretic, dysentery, ear complaint, enuresis, eye infection, fatigue, hepatitis, insect bite/sting, malaria, mouth ailment, otitis media, praecordial pain, purgative, rheumatism, skin complaints, snake bite, sore/s, tachycardia, toothache, tubercular ulcer, urinary tract disease, uterine complaints, vermifuge, whooping cough, wound/s	120
<i>Rorippa nasturtium-aquaticum</i>	Brassicaceae	Bronkoslaai	Leaves	None	
<i>Salvia africana-caerulea</i>	Lamiaceae	Bloublomsalie	Leaves	Abdominal complaint, bronchitis, cold, indigestion, influenza	115
<i>Schotia brachypetalia</i>	Fabaceae	Ihluze, Umgxam, weeping boer-bean	Bark	Alcohol abuse, acne, colic, diarrhea, dysentery, hangover, heartburn, inflammation, nervous heart condition, nausea, nervous symptom, nosebleed, swelling, tropical ulcer	115
<i>Siphonochilus aethiopicus</i>	Zingiberaceae	Isiphephetho, indungulo, serololo	Bulb	Abdominal complaint, asthma, catarrh, cold, colic, cough, dysmenorrhoea, epilepsy, hysteria, influenza, malaria, pain	115
<i>Solanum aculeastrum</i>	Solanaceae	Umthuma	Laves	Anthrax, anuria, back and leg pain, bowel cleanser, bronchitis, dysentery, gonorrhoea, haemorrhoids, rheumatic pain, ringworm, toothache, vomiting, wound/s	115
<i>Strychnos henningsii</i>	Loganiaceae	Mqalothi, umnonono	Bark	Appetite stimulant, chest pain, colic, dysmenorrhoea, fatigue, nausea, purgative, snake bite, stomach ailment, tapeworm	120
<i>Syzygium cordatum</i>	Myrtaceae	Umdoni, waterberry	Bark	Amenorrhoea, cold, diarrhea, emetic, fever, headache, indigestion, increase lactation, respiratory ailment, stomach ailment, wound	120
<i>Tetradenia riparia</i>	Lamiaceae	Iboza	Leaves	Boil, bronchitis, chest complaint, cold, colic, cough, dengue fever, diarrhea, dropsy, fever, flatulence, gall bladder disorders, gingivitis, haemoptysis, headache, influenza, malaria, mouth ulcer, mumps, nasal congestion, nausea, respiratory ailment, rheumatism, stomach ailment, swelling of legs, sore throat	115
<i>Warburgia salutaris</i>	Canellaceae	Isibhara, isibaha	Bark	Angina, aphrodisiac, arthritis, backache, blisters, bronchitis, burn/scald, cancer, chest complaint, cold, constipation, cough, cut/laceration, diarrhea, emphysema, fatigue, fever, gastric ulcer, gastro-intestinal problem, graze, headache, herpes simplex, herpes zoster, influenza, inflamed urethra, intercostal neuralgia, malaria, pain, penial irritation, pneumonia, purgative, rheumatism, scabies, skin complaint, sore/s, stomach ailment, toothache, venereal disease	120

Table 3.1 shows the various plants collected in the ethnopharmacological survey for the plants used in the traditional treatment of tuberculosis. The plants were mostly presented in vernacular names. Identification of plants was performed at the Bolus Herbarium at the University of Cape Town or through literature by using the vernacular name given by the traditional healer. Voucher specimen of *Artemisia afra* **Jacq** was deposited at Bolus Herbarium (BOL) with specimen number 11097. Most plants have been quoted in literature to have a variety of other uses, as presented in the table. No other medicinal use of *R. nasturtium-aquaticum* was reported, except the anti-tuberculosis use. *E. natalensis* was reported as having a variety of medicinal uses; however, these were not specified.

3.2.3 Chemical extraction

Plants were extracted with three different solvents. The various extraction procedures yielded different extracts of each plant or plant mixture. The dichloromethane extracts are marked as “D”, the methanol extracts with an “M” and the water extracts with a “W”. Various extract yields were calculated to show how much content of each plant the solvents extracted. Table 3.2 on page 57 gives the percentage extract yield of each plant and the solvent used.

Table 3.2: Extract yields of plants using different solvent systems.

Botanical Name	Extract	Yield (g)	Yield (%)
	S1D	0.2	3.3
<i>O. europea</i>	S1M	0.6	11.4
	S1W	0.9	49.5
	S2D	0.1	2.5
<i>S. aculeastrum</i>	S2M	0.1	0.9
	S2W	0.2	8.9
	S3D	0.1	0.7
<i>P. prunelloides</i>	S3M	0.2	4.3
	S3W	0.1	4.5
	S4D	0.2	3.9
<i>L. ocymifolius</i>	S4M	0.7	14.4
	S4W	0.3	15.7
	S5D	0.4	7.5
<i>C. gratissimus</i>	S5M	0.5	10.9
	S5W	0.9	5.9
	S6D	0.2	3.6
Combination 3	S6M	0.5	9.3
	S6W	0.2	10.7
	S7D	0.6	12.2
<i>A. amatymbica</i>	S7M	0.6	12.3
	S7W	0.2	11.9
	S8D	0.6	11.7
<i>S. henningsii</i>	S8M	0.5	8.9
	S8W	0.7	35.0
	S9D	1.5	30.8
<i>A. quanzensis</i>	S9M	2.8	55.2
	S9W	0.9	5.9
	S10D	0.6	11.3
<i>A. afra</i>	S10M	1.1	21.5
	S10W	0.5	22.4
	S11D	0.3	6.8
<i>T. riparia</i>	S11M	0.3	6.3
	S11W	1.1	55.1

Botanical Name	Extract	Yield (g)	Yield (%)
	S12D	0.3	4.6
<i>E. natalensis</i>	S12M	0.3	6.4
	S12W	0.4	19.7
	S13D	0.2	3.4
Plant from Home	S13M	0.2	4.6
	S13W	0.3	14.8
	S14D	0.1	2.0
<i>L. revoluta</i>	S14M	2.1	42.1
	S14W	0.5	25.0
	S15D	0.1	1.6
<i>C. transvaalensis</i>	S15M	2.0	39.2
	S15W	0.3	15.0
	S16D	1.1	22.0
<i>C. tomentosa</i>	S16M	2.2	45.2
	S16W	0.2	10.0
	S17D	0.1	0.2
<i>E. capensis</i>	S17M	0.2	3.3
	S17W	0.1	3.6
	S18D	0.1	1.9
<i>S. cordatum</i>	S18M	1.2	24.2
	S18W	0.3	12.3
	S19D	0.1	0.4
<i>K. africana</i>	S19M	1.9	39.3
	S19W	0.1	3.6
	S20D	2.9	58.2
<i>S. aethiopicus</i>	S20M	0.3	6.4
	S20W	0.8	40.1

Botanical Name	Extract	Yield (g)	Yield (%)
	S21D	0.1	0.2
<i>A. fruticosa</i>	S21M	1.9	38.4
	S21W	0.8	40.0
	S22D	0.1	1.9
<i>A. sativum</i>	S22M	0.2	2.6
	S22W	0.3	12.3
<i>S. brachypetalia</i>	S23D	0.1	0.4
	S23M	3.1	60.1
	S23W	0.1	3.6
	S24D	1.9	38.1
<i>P. viridiflorum</i>	S24M	0.6	12.0
	S24W	0.2	10.3
	S25D	0.1	0.4
<i>C. sativa</i>	S25M	2.9	58.2
	S25W	0.1	3.6
	S26D	2.0	39.2
<i>Tshetsha</i> (Vernacular name)	S26M	1.2	24.2
	S26W	0.4	19.7

3.2.4 Phytochemical analysis

Using colorimetric phytochemistry, the plants were screened for compounds with known pharmacological activity [103]. These included saponins and tannins. Table 3.3 below shows the compounds that were detected in some of the plants, presented qualitatively as no quantity of the compounds was measured.

Table 3.3: Phytochemical analysis of plants.

Botanical Name	Saponins	Tannins	Botanical name	Saponins	Tannins
<i>A. quanzensis</i>	+++	+++	<i>S. brachypetalia</i>	-	-
<i>A. amatymbica</i>	-	++	<i>S. aculestrum</i>	-	+
<i>C. tomentosa</i>	+	-	<i>S. henningsii</i>	-	+
<i>E. capensis</i>	++	++	<i>S. cordatum</i>	+	++
<i>K. africana</i>	-	-	<i>Combination 3</i>	+	++
<i>L. ocymifolius</i>	-	++	<i>T. riparia</i>	-	+
<i>O. europea</i>	++	++	<i>P. prunelloides</i>	-	-
<i>P. viridiflorum</i>	+++	+			

(+) Indicates the presence of the compounds, (-) indicates absence

The table gives the results of the plants tested for saponins and tannins. Other plants were not tested because data on the two compounds were readily available in literature [114]. Multiplicity of each positive sign (+) shows that the plant had an abundance of the tested compound. *A. quanzensis* had the highest content of both the saponins and tannins, whereas *K. africana*, *P. prunelloides* and *S. brachypetalia* were both tannin- and saponin-free.

3.3 SUMMARY OF RESULTS

The toxicity of other plants in the traditional preparations could not be measured since the concentration of the plant in the prepared mixtures was *not* determined. However, most of these plants used by traditional healers tested positive for tannins. Six plants tested positive for saponins. Saponins are toxic to red blood cells. This observation raises concern regarding prolonged use of these plants in the traditional treatment of tuberculosis, as most healers would treat patients for a long period until they are “feeling better”. No report on the toxic effects of these plants in patients was obtained from the traditional healers. The plants reported to have high saponin content were *A. quanzensis*, *E. capensis*, *P. viridiflorum* and *O. europea* subspecies *africana*. The bark of *E. capensis* contains about 7.3% tannins and has been reported to be poisonous [117]. The toxicity of this plant as used in treatment *Combination 2* may be minimized by the presence of the other plants. The other plant that showed a high content of saponins was the *P. viridiflorum*. This plant has been reported to yield a high level of saponins. *Barrigenol A1* and other structurally related triterpenoids, as well as sapogenins, have been isolated from this plant [118]. It is assumed that these are the major agents accounting for of the biological activity. No report on the toxicity effect of this plant exists, despite its high content of saponins. Phytochemical studies on these plants have revealed a high number being rich in tannins. Tannins are known to interfere with a number of biological assays [104, 105, 106]. Certain studies have shown that they inhibit reverse transcriptase [101, 103] and DNA polymerase [105]. There seems to be no correlation between the tannin-content and the cytotoxicity of these plants. For the other biological assays, the tannins were removed prior to the assays.

CHAPTER FOUR

Comparative antimycobacterial and cytotoxicity activity of *Artemisia afra*

4.1 INTRODUCTION

4.1.1 Aim of this chapter

The activity tests against *Mycobacterium aurum* A⁺ and toxicity tests against Chinese hamster ovarian cells of the crude extracts that were obtained from the plants collected from traditional healers are reported in this chapter. *M. aurum* A⁺ was used for preliminary screening. The dichloromethane extracts of *A. afra* Jacq have shown the highest activity against *M. aurum*. Tests on the plant have shown it to be active against *M. tuberculosis*, using *in vitro* biological assays. It was the aim of this study to show the results of the biological tests of the various extracts against a selection of assays. In addition, the results of various tests performed on *A. afra*, are reported.

4.1.2 *Artemisia afra* and its traditional use

Artemisia afra [Jacq] (**Asteraceae**); *umhlonyane*; *lengana*; *African wormwood* is a strongly aromatic, much-branched greyish-green shrublet common on cool slopes, river terraces and in narrow valleys in many parts of Southern Africa [117, 118, 119]. It is one of the most widely used plants in traditional medicinal practices in South Africa. The leaves are boiled in water and the 'tea' is drunk at night for a variety of fevers, colds, and chest complaints, including coughs, malaria, intestinal worms, earache and influenza, amongst others. African women also use the plant as a fragrance because of its aromatic smell [85]. Alternatively, the leaves may be inserted in the nostrils for treatment of fever [120, 121]. In other parts of Southern Africa the plant is used for a variety of reasons. In Zimbabwe, the dried aerial parts are used to treat diabetes if taken regularly [117] and in Ethiopia the stems and fresh leaves are used for stomach disorders [122, 123].

4.1.3 Literature review on the chemical constituents of *A. afra*

A. afra is one of the most studied plants in the South African fauna. It is rich in a number of compounds including carbohydrates, phenolics, steroids, alkaloids and

terpenoids, of which the essential oil is the most studied. The latter includes 1,8-cineole, thujone, camphor and borneol [124], which have been found to possess biological activities such as antimicrobial [124, 125] and antifungal activity [126]. Specifically, the essential oil of *A. afra* Jacq has shown inhibition of growth of *Aspergillus ochraceus*, *A. niger*, *A. parasiticus*, *Candida albicans*, *Alternaria alternata*, and *Geotichum candidum* [126]. Another study showed the essential oil of *A. afra* also has antioxidant activity [127].

Another class of compounds that has biological activity in this plant species are the sesquiterpene lactones. Amongst the isolated sesquiterpene lactones of *A. afra* are artemisia glaucolide, hydroxyafra-glaucolide, hydroxyisoafra-glaucolide, eudesmaafra-glaucolide and 12-hydroxy- α -cyperone [128]. The lactone moiety and the epoxides are assumed to be the reactive sites for biological nucleophiles, mainly thiols and amines of the reactive sites of various enzymes such as glycogen synthase, DNA polymerase, and thymidylate synthase [128]. The resultant effect of these compounds on these enzymes is irreversible alkylation. The sesquiterpene lactones have also been associated with antibacterial activity against Gram-positive bacteria, antifungal activity, antiparasitic and anthelmintic activity [124]. *A. afra* has been shown to possess activity against *Plasmodium falciparum* [129].

Other groups of compounds that have been isolated and characterised from *A. afra* include phenolics, which contain mainly coumarins [130, 131] and flavonoids [131, 132] and polyalkenes (ceryl cerotate) [131]. These studies showed no data on biological activity of the compounds.

4.1.4 Cytotoxicity testing

Testing of natural products against biological systems provides an indication of the reaction that happens between various compounds in the product and the biological system tested against. Various natural products have been known to possess particular reactions against certain biological systems [104, 105]. Cytotoxicity tests are part of developing a product into a clinically accepted drug. This provides a screening system to ascertain that the compounds being tested are not harmful to the normal biological processes more than they are for the effect they are tested for. A variety of methods are available to assess cytotoxicity of compounds, including dye uptake, leakage of lactate dehydrogenase and reduction of MTT [132b]. In this study, the MTT assay is performed, testing Chinese hamster ovarian cells against various crude extracts and isolates.

4.2 RESULTS

4.2.1 Antimycobacterial testing of crude extracts

The firefly luciferase bioluminescence assay was used to test the extracts against *M. aurum* A⁺ and *M. tuberculosis* H₃₇Rv, *in vitro*. To standardize the assay system and validate its use, isoniazid and rifampicin were tested against *M. aurum* A⁺. This was to reproduce the minimum inhibition concentrations of the two antimycobacterial drugs, as published in literature. Figure 4.1 below shows the dose-response curve of isoniazid and rifampicin against *M. aurum* A⁺.

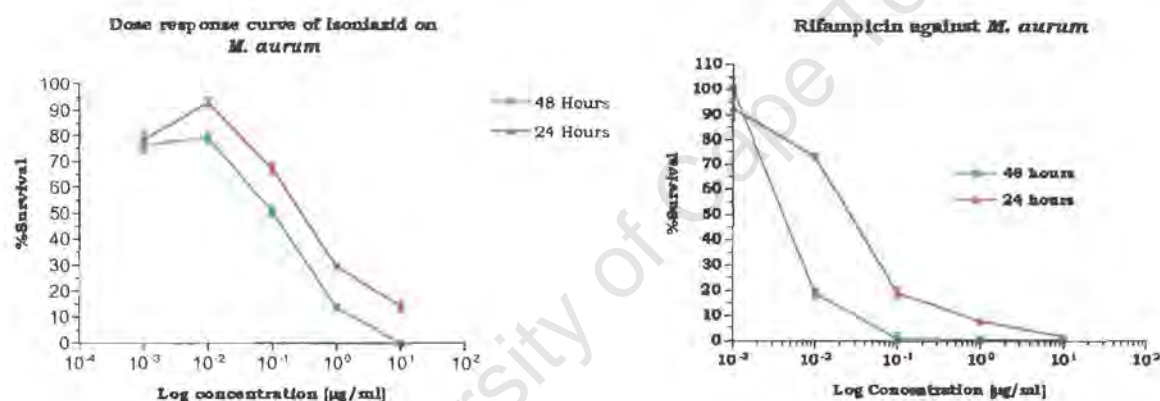


Figure 4.1: Dose-response effect of isoniazid and rifampicin against *M. aurum* A⁺ over 24 and 48 hours, respectively. Results are presented as a mean of two readings with the standard deviation as the error bars.

The minimum inhibitory concentration (MIC) value of isoniazid (INH) against *M. aurum* A⁺ was 0.02 μg/ml and that of rifampicin was 0.15 μg/ml when tested at 48 hours. Higher values in each case were obtained when the test was done over 24 hours. The graphs in Figure 4.1 show that the firefly luciferase bioluminescence assay produces the known minimum inhibitory concentration (MIC) values of the two drugs. Based on these results, the assay system was used to test the plant extracts. The plant extracts were assayed against *M. aurum* A⁺, *in vitro*, at 200 μg/ml concentrations. This was to identify those extracts that were antimycobacterial at this concentration. Figures 4.2 to 4.5 (pages 64 – 66) show the graphs of the various extracts tested against *M. aurum* A⁺.

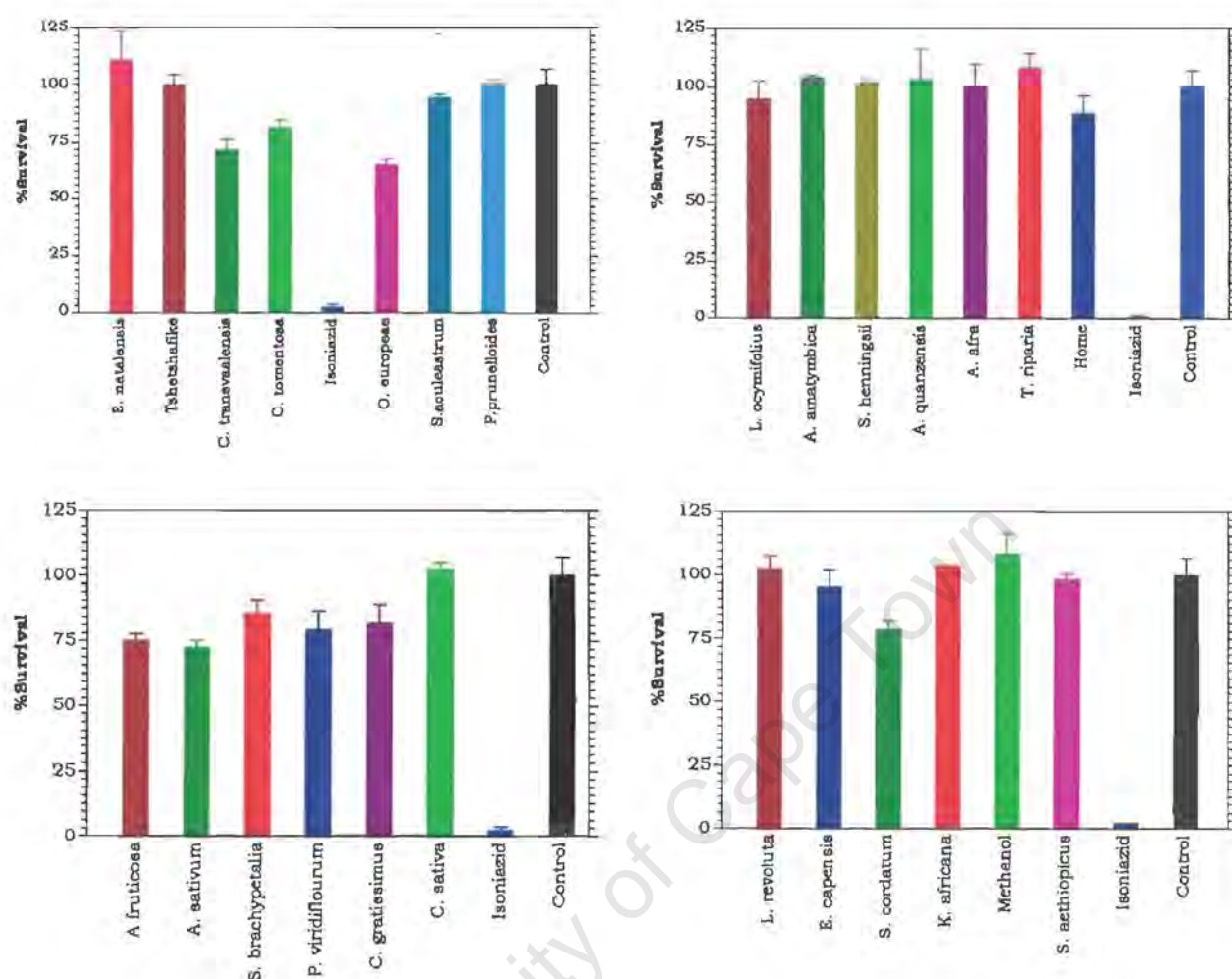


Figure 4.2: Methanol extracts tested against *M. aurum* A⁺ at 200 µg/ml for 48 hours. The extracts were dissolved in 100 µl of methanol and made up to 1.0 ml in medium. The effect of methanol on *M. aurum* was tested at concentrations equal to those of the solutions with extracts. Results are presented as a mean of two readings with the standard deviation as the error bars.

Each of the extracts was considered arbitrarily to be active when the percentage survival of 75% or less was demonstrated against *M. aurum* A⁺.

The methanol extract of *O. europaea* tested against *M. aurum* A⁺ had the highest activity, with percentage survival of 65.0 ± 2.8%. Other active extracts were *C. transvaalensis* (71.5 ± 4.7%), *A. sativum* (72.3 ± 2.9%) and *A. fruticosa* (75.0 ± 2.7%).

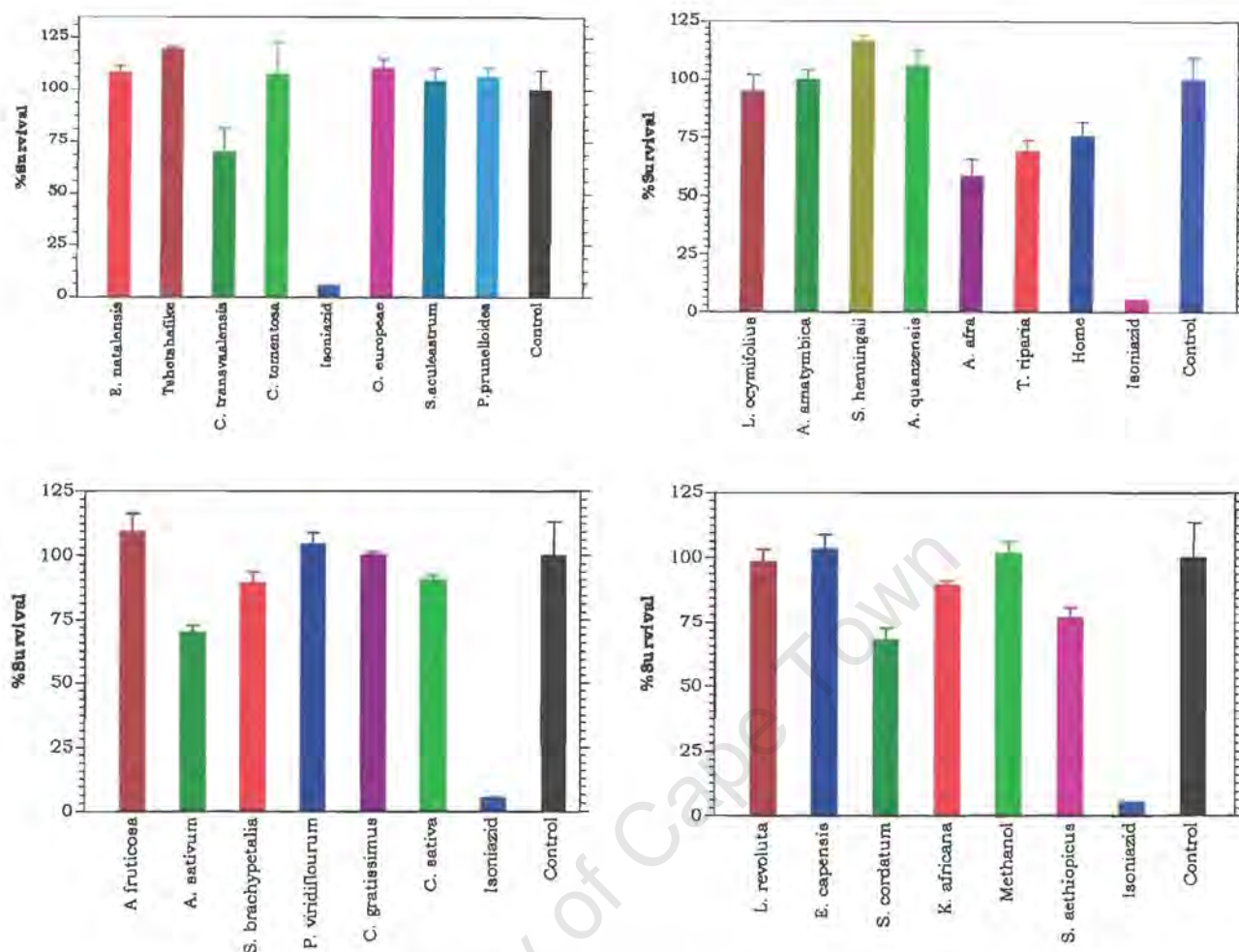


Figure 4.3: Dichloromethane extracts tested against *M. aurum* A⁺ at 200 µg/ml for 48 hours. The extracts were dissolved in 100 µl of methanol and made up to 1.0 ml in medium. The effect of methanol on *M. aurum* A⁺ was tested at concentrations equal to those of the solutions with extracts. Results are presented as a mean of two readings with the standard deviation as the error bars.

Each of the extracts was considered arbitrarily to be active when the percentage survival of 75% or less was demonstrated against *M. aurum* A⁺.

Of all the dichloromethane extracts tested, *A. afra* exhibited the highest activity against *M. aurum* A⁺ with percentage survival of $58.6 \pm 7.6\%$. Other active dichloromethane extracts were *S. cordatum* ($67.8 \pm 5.5\%$), *T. riparia* ($69.6 \pm 4.5\%$), *C. transvaalensis* ($69.8 \pm 7.5\%$) and *A. sativum* ($69.9 \pm 2.7\%$).

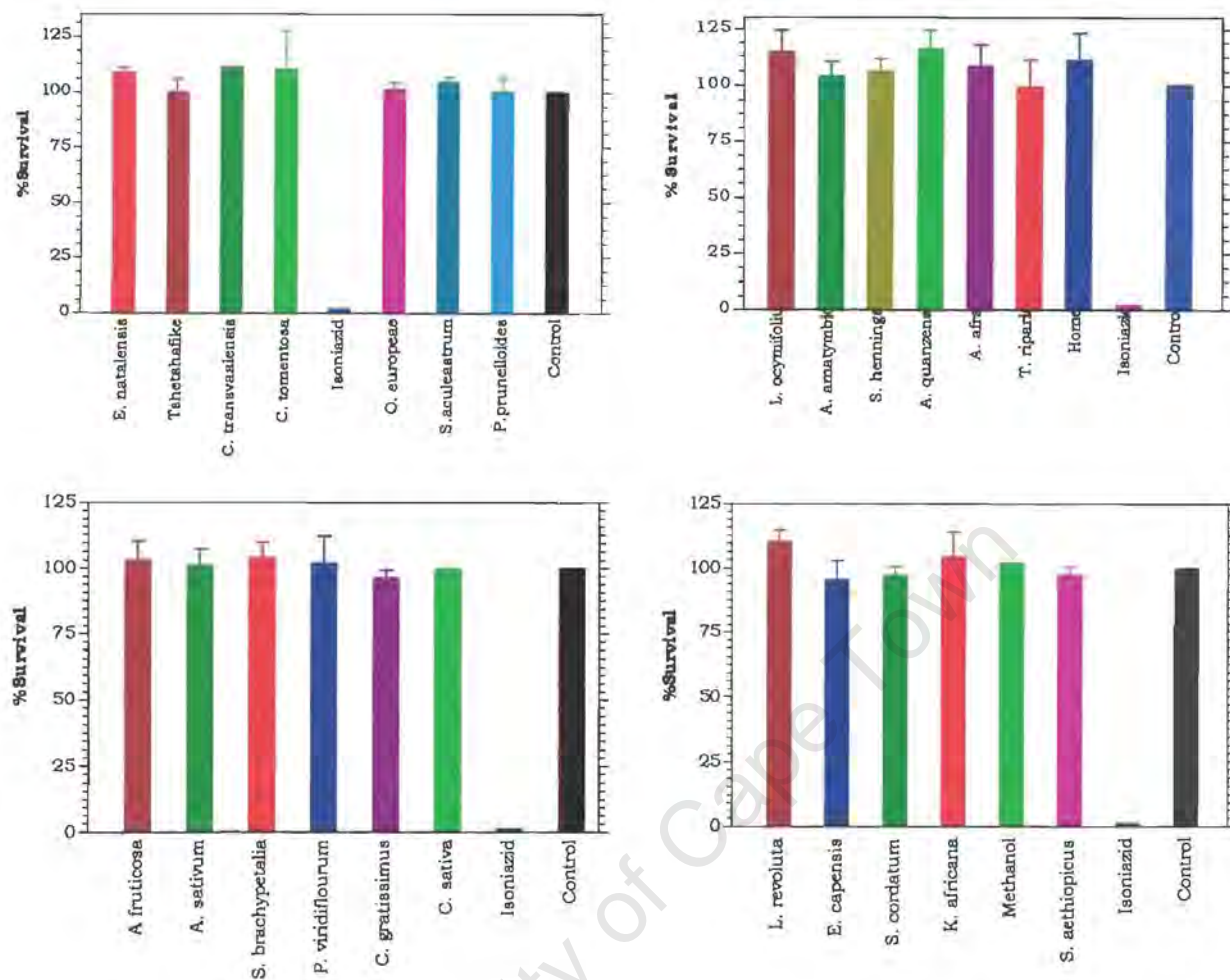


Figure 4.4: Water extracts tested against *M. aurum* A⁺ at concentrations of 200 µg/ml. Dissolving in media and using the firefly luciferase bioluminescence assay over a 48-hour incubation period tested the extracts.

Each of the extracts was considered arbitrarily to be active when the percentage survival of 75% or less was demonstrated against *M. aurum* A⁺. All the water extracts were not active against *M. aurum* A⁺.

The combinations of plants as they are prescribed by traditional healers were tested against *M. aurum* A⁺ at 200 µg/ml. The extracts were tested to investigate any possibility of synergistic effect between the plants when they are combined. The findings of the tests are presented in Figure 4.5 on page 67.

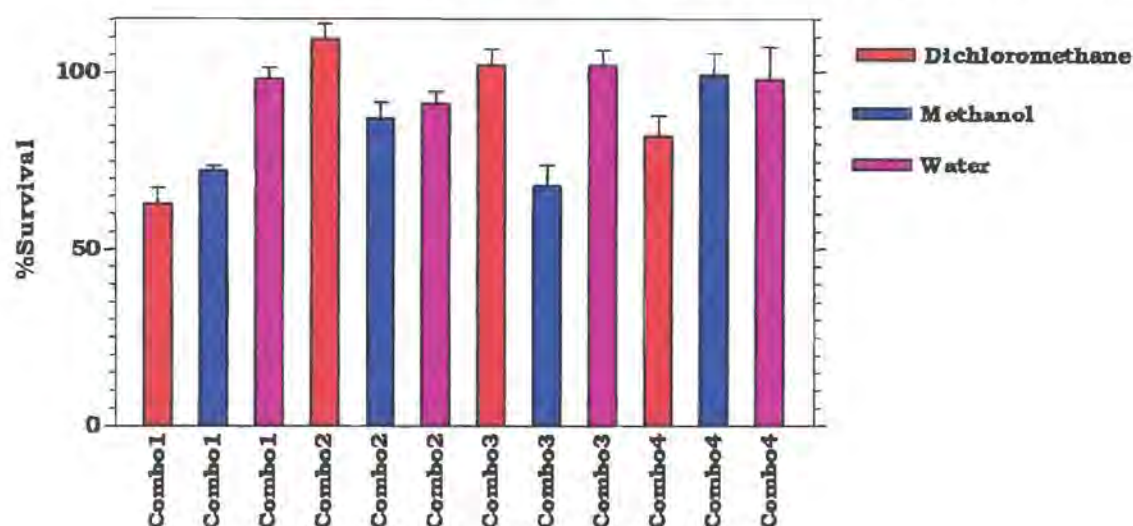


Figure 4.5: Extracts of the combination of plants tested against *M. aurum* A⁺ at 200 µg/ml using the firefly luciferase bioluminescence assay. The red bars represent the dichloromethane extracts, the blue bars represent methanol and the purple bars represent water extracts. Results are presented as a mean of two readings with the standard deviation as the error bars.

Each of the extracts was considered arbitrarily to be active when the percentage survival of 75% or less was demonstrated against *M. aurum* A⁺. The activity against *M. aurum* A⁺ of combinations of extracts of certain plants showed that the dichloromethane extract of Combination 1 had the highest activity (percentage survival of 62.8 ± 4.5%).

Table 4.1: % Survival of *M. aurum* A⁺ against active plant extracts. The extracts were tested at 200 µg/ml. No water extracts showed any activity at the tested concentration.

Dichloromethane Extracts		Methanol extracts	
Plant	% Survival	Plant	% Survival
<i>A. sativum</i>	69.9 ± 2.7	<i>A. sativum</i>	72.3 ± 2.9
<i>A. afra</i>	58.6 ± 7.6	<i>A. fruticosa</i>	75.0 ± 2.7
<i>C. transvaalensis</i>	69.9 ± 7.5	<i>C. transvaalensis</i>	71.5 ± 4.7
Combination 1	62.9 ± 4.5	Combination 1	72.5 ± 1.3
<i>S. cordatum</i>	67.9 ± 4.6	Combination 3	68.0 ± 5.9
<i>T. riparia</i>	69.7 ± 4.5	<i>O. europeae</i>	65.0 ± 2.8

There was no activity observed from the water extracts. There were a number of active extracts from the methanol and dichloromethane extracts. The dichloromethane extract of *A. afra* was the most active, with percentage survival of 58.6%. Combination 1, which had *A. afra* as one of the plants, had a 62% survival of *M. aurum* A⁺.

4.2.2 Cytotoxicity testing of crude extracts

The plants were tested for cytotoxicity against the Chinese hamster ovarian cell lines. Their ability to kill these cells is an indication of their ability to cause damage to human cells. Figure 4.6 below shows the activities of the crude extracts against Chinese hamster ovarian cell lines.

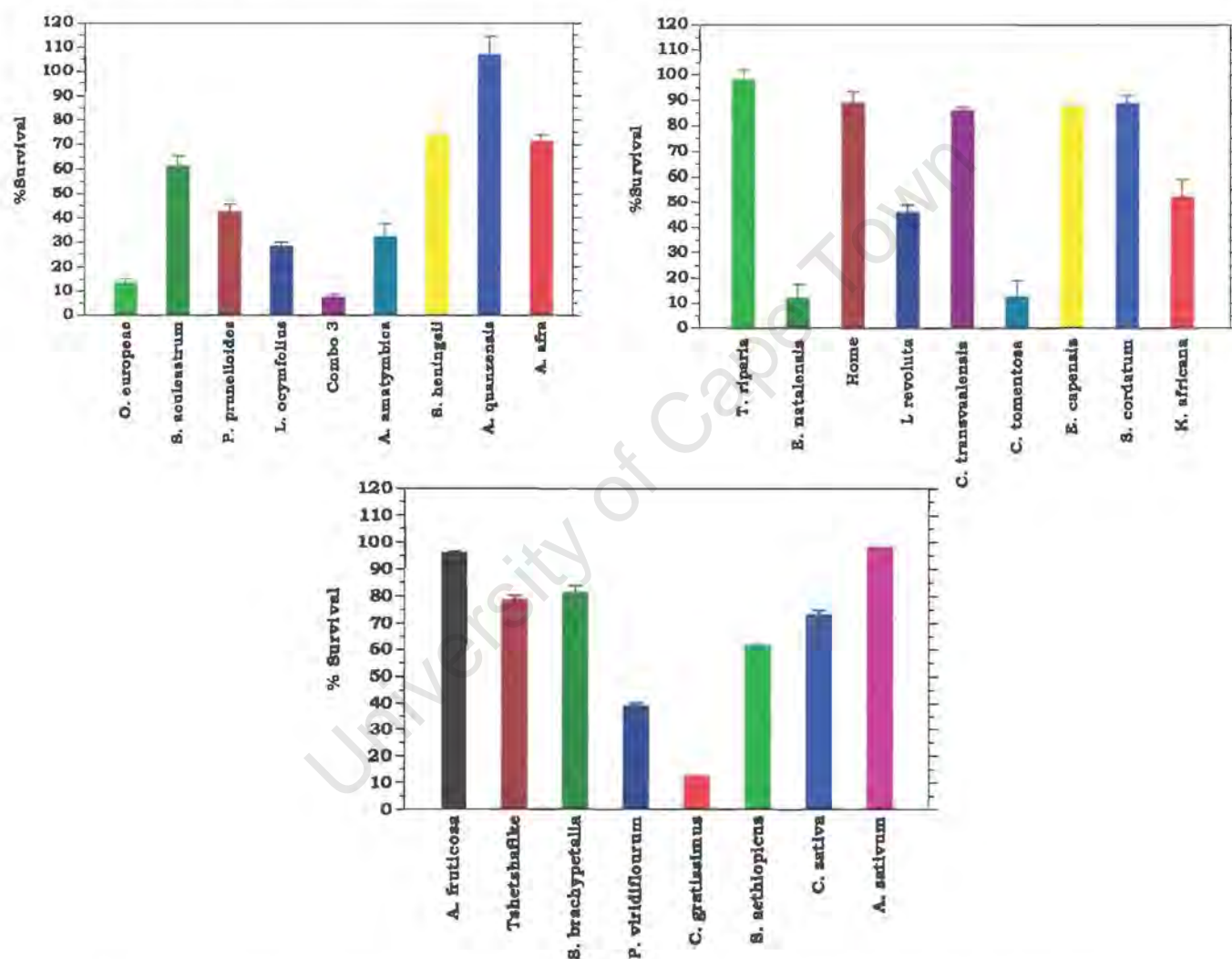


Figure 4.6: Crude dichloromethane extracts of plants tested against Chinese hamster ovarian cell lines over 48-hour incubation period. Extracts were tested at concentrations of 200 µg/ml. Cytotoxicity was rated on percentage survival of 50 or more. The cytotoxicity was tested using the MTT assay. Results are presented as a mean of four readings with the standard deviation as the error bars.

The activity of these extracts was assumed arbitrarily to be significant if there was a percentage survival of 75% of the cells or less. Figure 4.6 shows most of the

dichloromethane extracts to be cytotoxic to the Chinese hamster ovarian cell lines, with Combination 3 having the highest activity (percentage inhibition of $91.5 \pm 1.1\%$). Table 4.2 below summarises the results presented in Figure 4.6 showing the cytotoxic extracts.

Table 4.2: Summary of the results of Figure 4.6. The table shows the activity of dichloromethane extracts against Chinese hamster ovarian cell lines. Percentage survival was determined according to the formula: Percentage survival = (ATP of control/ATP of test) 100. Results are presented as a mean of four readings with the standard deviation

Extract	Percentage survival	
Combination 3	7.5	: 1.1
<i>E. natalensis</i>	12.0	: 5.4%
<i>C. tomentosa</i>	12.4	: 6.5%
<i>C. gratissimus</i>	12.5	: 0
<i>O. europeae</i>	13.4	: 1.8%
<i>L. ocymifolius</i>	28.1	: 1.9%
<i>A. amatymbica</i>	32.0	: 5.6%
<i>P. viridiflorum</i>	39.0	: 1.0%
<i>P. prunelloides</i>	42.6	: 2.9%
<i>L. revolute</i>	45.8	: 2.9%
<i>K. africana</i>	52.0	: 7.2%
<i>S. aculestrum</i>	61.1	: 4.3%
<i>S. aethiopicus</i>	61.5	: 0.5%
<i>A. afra</i>	71.5	: 2.6%
<i>S. henningsii</i>	73.2	: 7.0%
<i>C. sativa</i>	73.2	: 1.6%

Methanol extracts were also tested against Chinese hamster ovarian cell lines. The results are presented in Figure 4.7.

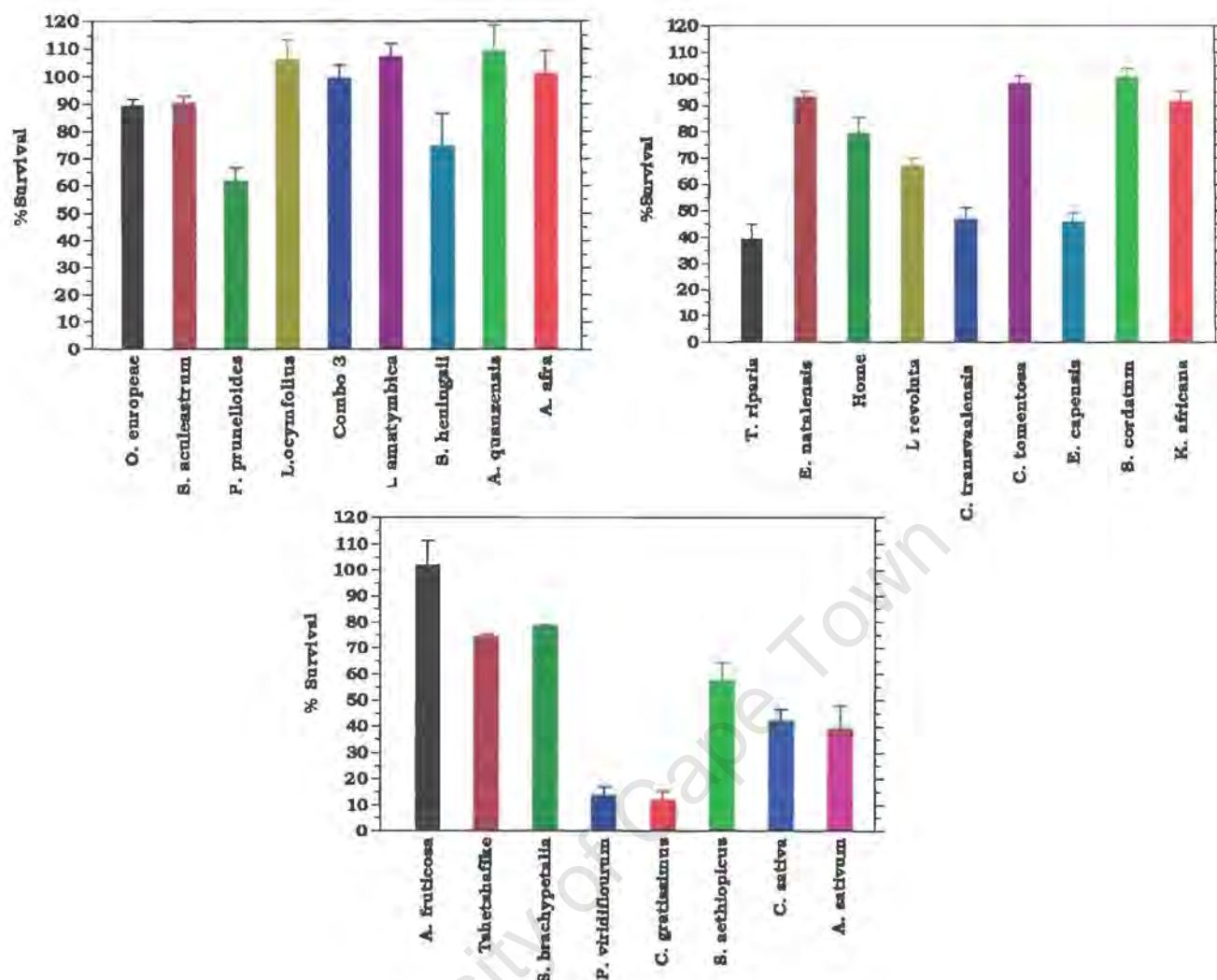


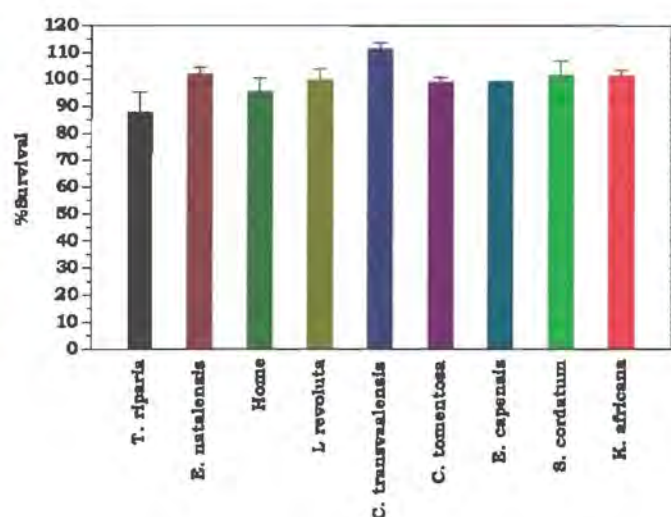
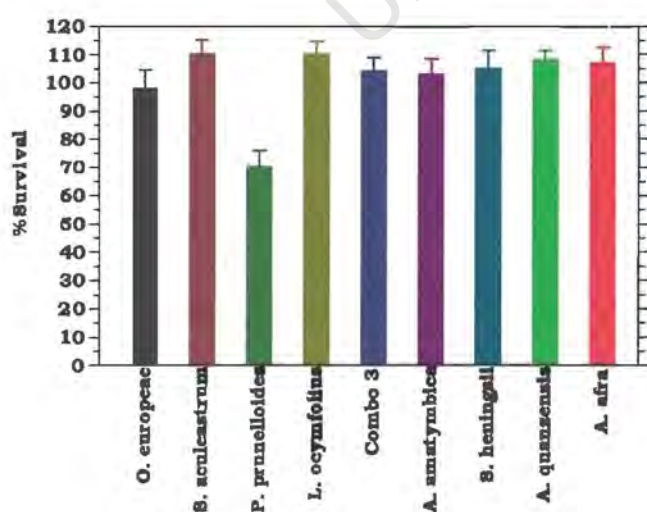
Figure 4.7: Crude methanol extracts of plants tested against Chinese hamster ovarian cell lines over 48-hour incubation period. Extracts were tested at concentration of 200 µg/ml. Cytotoxicity was determined as % survival of 50 or more, using the MTT assay. Results are presented as a mean of four readings with the standard deviation as the error bars.

Similarly to the dichloromethane extracts, the activity of the methanol extracts was assumed arbitrarily to be significant if there was a percentage survival of 75% of the cells or less. Most extracts showed low toxicity levels to the Chinese hamster ovarian cells. The extracts of *C. gratissimus* and *P. viridiflorum* had the highest cytotoxicity, with percentage survivals of $11.9 \pm 3.4\%$ and $13.6 \pm 3.3\%$, respectively. The values for the inhibition levels for the methanol extracts are shown in table 4.3 on page 71.

Table 4.3: Summary of the results of figure 4.7. The table shows the active methanol extracts against Chinese hamster ovarian cell lines. Percentage survival was obtained with the formula: Percentage survival = (ATP of control/ATP of test) 100. Results are presented as a mean of four readings with the standard deviation as the error bars.

Extract	Percentage survival
<i>C. gratissimus</i>	11.9 : 3.4%
<i>P. viridiflorum</i>	13.6 : 3.3%
<i>A. sativum</i>	39.3 : 8.9%
<i>T. riparia</i>	39.4 : 5.7%
<i>C. sativa</i>	42.7 : 4.3%
<i>E. capensis</i>	45.8 : 3.2%
<i>C. transvaalensis</i>	46.9 : 4.1%
<i>S. aethiopicus</i>	57.5 : 6.8%
<i>P. prunelloides</i>	61.6 : 4.9%
<i>L. revolute</i>	67.1 : 2.6%
<i>Tshetsha</i>	74.3 : 1.3%
<i>S. henningsii</i>	74.3 : 11.9%

The methanol extracts were tested against Chinese hamster ovarian cells in a similar manner to the dichloromethane and methanol extracts. The water extracts results are shown in figure 4.8 below. As shown in the figure, all the water extracts did not exhibit any cytotoxic activity to the cell lines.



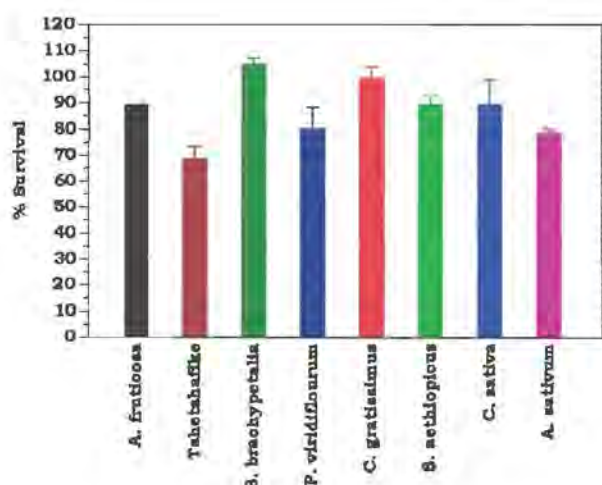


Figure 4.8: Crude water extracts tested against Chinese hamster ovarian cell lines over 48-hour incubation period, in a concentration of 200 µg/ml. Cytotoxicity was rated as percentage survival of 50 or more, using the MTT assay. Results are presented as the mean of four readings with standard deviation as the error bars.

This figure shows the water extracts of plants tested for cytotoxicity against Chinese hamster ovarian cell lines. The activity of the water extracts was assumed arbitrarily to be significant if there was a percentage survival of 75% of the cells, or less. The water extracts did not exhibit meaningful activity against the cell lines. The most active extract was *P. prunelloides* with percentage survival of $70.1 \pm 10.8\%$.

4.2.3 Antimycobacterial testing of isolates of *A. afra*

Crude dichloromethane extracts of *A. afra* showed the most potent activity against *M. aurum* A⁺ when tested at a fixed dose of 200 µg/ml, as shown in Figure 4.3 on page 65. Further antimycobacterial investigations of the extract were conducted in an attempt to isolate the active entity. To find the dose response effect of the extract, it was tested at varying concentrations from 62.5 µg/ml to 1mg/ml against *M. aurum* A⁺ and *M. tuberculosis* H₃₇Rv. Figure 4.9 on page 73 shows the dose response curve of the extract when tested against both the *M. aurum* A⁺ and *M. tuberculosis* H₃₇Rv.

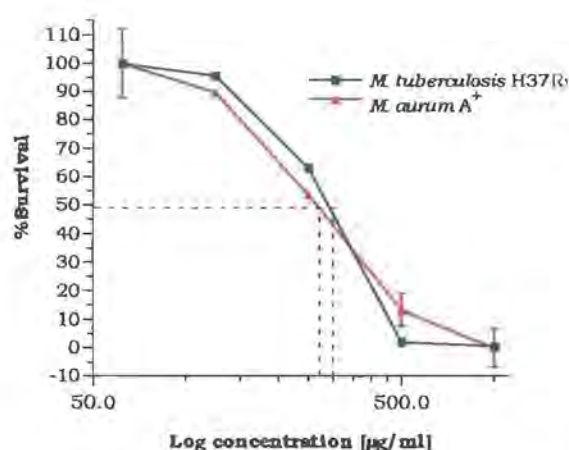


Figure 4.9: Dose-response effect of dichloromethane extract of *A. afra* against *M. tuberculosis* H₃₇Rv and *M. aurum* A⁺. *M. tuberculosis* H₃₇Rv was tested over 7 days and *M. aurum* was tested over 48 hours using the firefly luciferase bioluminescence assay. Results are presented as a mean of three readings with the standard deviation as the error bars.

The dichloromethane extract of *A. afra* had similar activity against *M. tuberculosis* H₃₇Rv and *M. aurum* A⁺, when tested *in vitro* using the firefly luciferase bioluminescence assay. The extract showed significant similarities in activity between *M. aurum* A⁺ and *M. tuberculosis* H₃₇Rv, with IC₅₀ of 270 µg/ml and 290 µg/ml, respectively.

The activity of the crude dichloromethane extract of *A. afra* against mycobacteria gave rise to the further question of selectivity. The extract was tested against a number of Gram-positive and Gram-negative bacterial species; namely, *Klebsiella pneumoniae*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Micrococcus* species. The doses varied from 62.5 µg/ml to 1mg/ml using the optical density determination assay. Figure 4.10 on page 74 shows the dose-response effect of the extract against the bacteria. The incubation times were different depending on the doubling time of each of the bacteria. From the Figure it could be seen that the extract had no activity against the Gram-negative bacteria tested. Activity was observed against Gram-positive bacteria, especially against *Micrococcus* species. However, the activity was significantly lower against *Micrococcus* species (IC₅₀ ≅ 530 µg/ml), compared with *M. aurum* A⁺ (IC₅₀ ≅ 270 µg/ml).

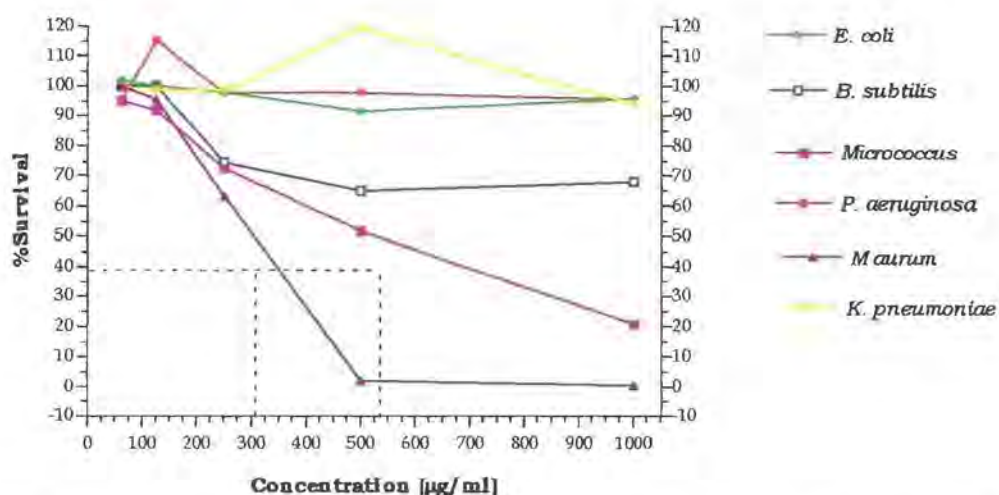


Figure 4.10: Dose-response curves of dichloromethane extract of *A. afra* against Gram-positive and Gram-negative bacteria. The extract was tested over a period of 16 to 24 hours, depending on the doubling time of the bacteria. The results are presented as a direct reading of growth from an assay performed once.

The activity against *Micrococcus* suggested activity against Gram-positive bacteria, but the finding was inconclusive since there was no response from other Gram-positive bacteria. Further fractionation of the crude extract of *A. afra* generated ten crude fractions, designated Shef1 through Shef10. These crude fractions were tested against *M. aurum* NTCC23366 using the optical density determination on a spectrophotometer at 600nm visible light. This strain was used because of the unavailability of *M. aurum* A⁺ strain as this part of the study was performed in The University of Sheffield, United Kingdom.

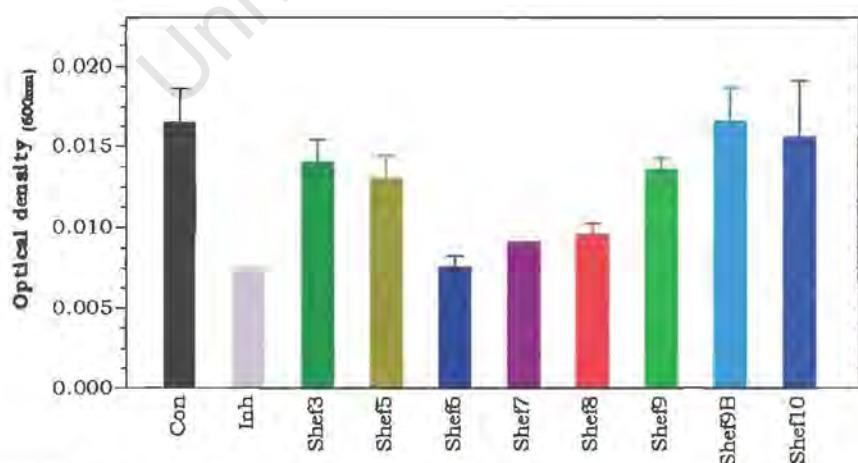


Figure 4.11: The activities of crude fractions of the dichloromethane extract of *A. afra* tested against *M. aurum* NTCC23366 over 48 hours. The crude fractions were tested at concentrations of 50 µg/ml using the optical density determination assay system. Results are presented as a mean of two readings with the standard deviation as the error bars.

Fractions Shef6, Shef7 and Shef8 showed the highest activity with optical densities of $0.0075 \pm 0.707 \times 10^{-3}$; 0.009; and 0.0095 ± 0.0071 , respectively, with control cultures having an optical density of 0.0165 ± 0.0021 . These optical density values corresponded with a total inhibition of 45.5%, 54.6% and 57.6% for Shef6, Shef7 and Shef8, respectively.

After measuring the OD, the test culture of *M. aurum* with 10 µg/ml of isoniazid was immediately cultured on an agar plate in order to count colony-forming units. After a 5-day incubation period there was no growth on the plates, suggesting that the concentrations of 10 µg/ml of isoniazid gave a 100% sterilising effect. This was regarded as the control for the study. From the Figure, extrapolation assumed that the fraction Shef6 had a 100% inhibition of the culture at 50 µg/ml. Fractions Shef6, Shef7 and Shef8 were pooled and further purification was conducted.

Isolates C1 through C10 were isolated from the pooled fractions Shef6, Shef7 and Shef8. The isolate C2 was tested against *M. aurum* NTCC23366 using the optical density determination test. The results of the test are shown in Figure 4.12 below.

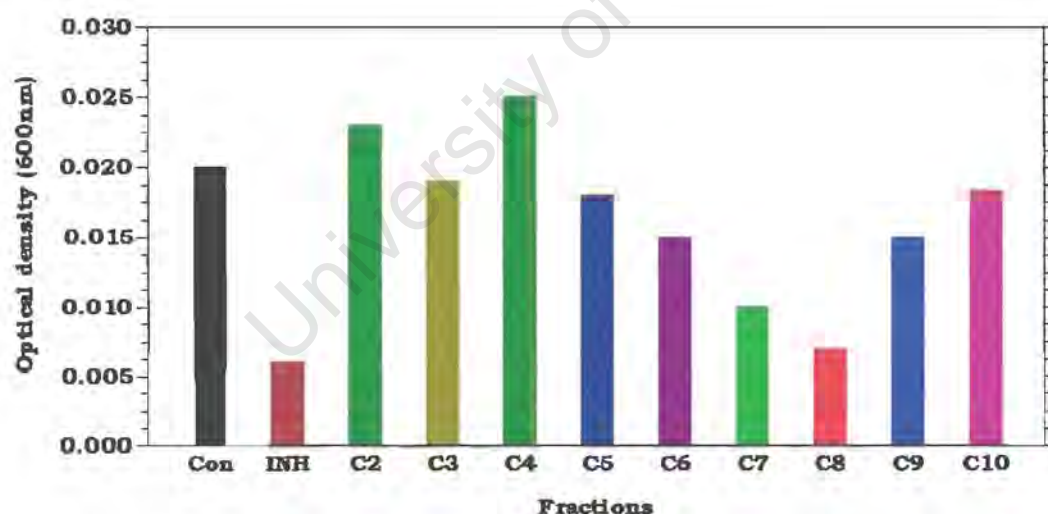


Figure 4.12: Graph of isolates C2 through C10 tested against *M. aurum* NTCC23366 at 5 µg/ml concentrations. The isolates were tested using the optical density determination assay using spectrophotometer at 600nm visible light. Isolate C1 was not tested due to small quantities of the isolate. The results are presented as a direct reading of growth from an assay performed once.

The readings in Figure 4.12 show that isolates C7 and C8 showed relative activity against *M. aurum* NTCC23366 using the optical density determination tests. Isolate C8

was more active than isolate C7. Since the initial tests of the crude extract on *M. aurum* A⁺ were done with the strain A⁺ and the bioassay-guided fractionation was performed with the NTCC23366 strain, it was necessary to test the isolates against *M. aurum* A⁺ to confirm the results and the activity shown against the NTCC23366 strain. The isolates were then tested against *M. aurum* A⁺ using the firefly luciferase bioluminescence assay. The results are shown in Figure 4.13 below.

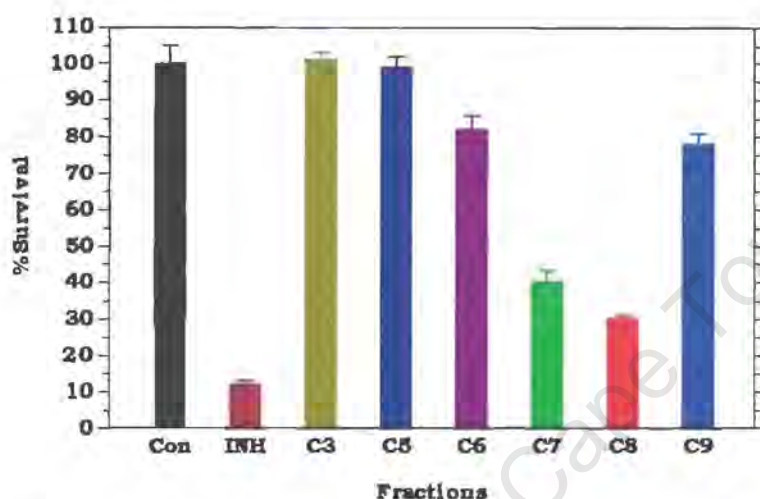


Figure 4.13: Graph of isolates C3 through C9 tested against *M. aurum* A⁺ at 5 µg/ml concentrations, using the bioluminescence firefly luciferase assay. Isolates C1, C2, C4 and C10 were not tested due to small quantities of the isolates available. Results are presented as a mean of two readings with the standard deviation as the error bars.

Figure 4.13 confirms a correlation with the results shown in Figure 4.12 on page 75. The results confirm that isolates C7 and C8 were exhibited inhibitory effect against *M. aurum*. Isolate C7 was later shown in Chapter 5 to be a mixture of traces of C8 and other compounds. It was concluded that the activity of C7 was due to traces of C8. Isolate C8 was further tested against *M. aurum* A⁺ in a dose-dependent fashion. The isolate was further tested against *M. tuberculosis* H₃₇Rv in a manner similar to *M. aurum* A⁺. The aim of these tests was to find the IC₅₀ value of the isolate against the two mycobacteria species. Figure 4.14 on page 77 shows the activity of the isolate against the two strains, *M. aurum* A⁺ and *M. tuberculosis* H₃₇Rv.

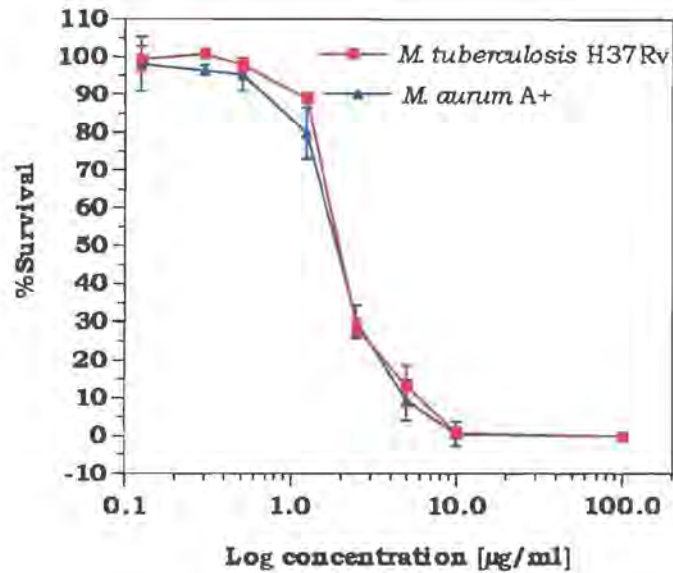


Figure 4.14: Dose-response curve of isolate C8 against *M. aurum* A⁺ and *M. tuberculosis* H₃₇Rv, using the firefly luciferase bioluminescence assay. Results are presented as a mean of three readings with the standard deviation as the error bars.

The responses of the two strains of mycobacteria to isolate C8 were similar. The IC₅₀ of *M. aurum* A⁺ was approximately 1.9 µg/ml while the IC₅₀ of *M. tuberculosis* H₃₇Rv was IC₅₀ of 2.0 µg/ml. The MIC value of C8 for each of the species was 10 µg/ml.

The effects of isolate C8 on the clinical strains of *M. tuberculosis* are shown in Figure 4.15 below. A total of 5 strains were selected for these tests.

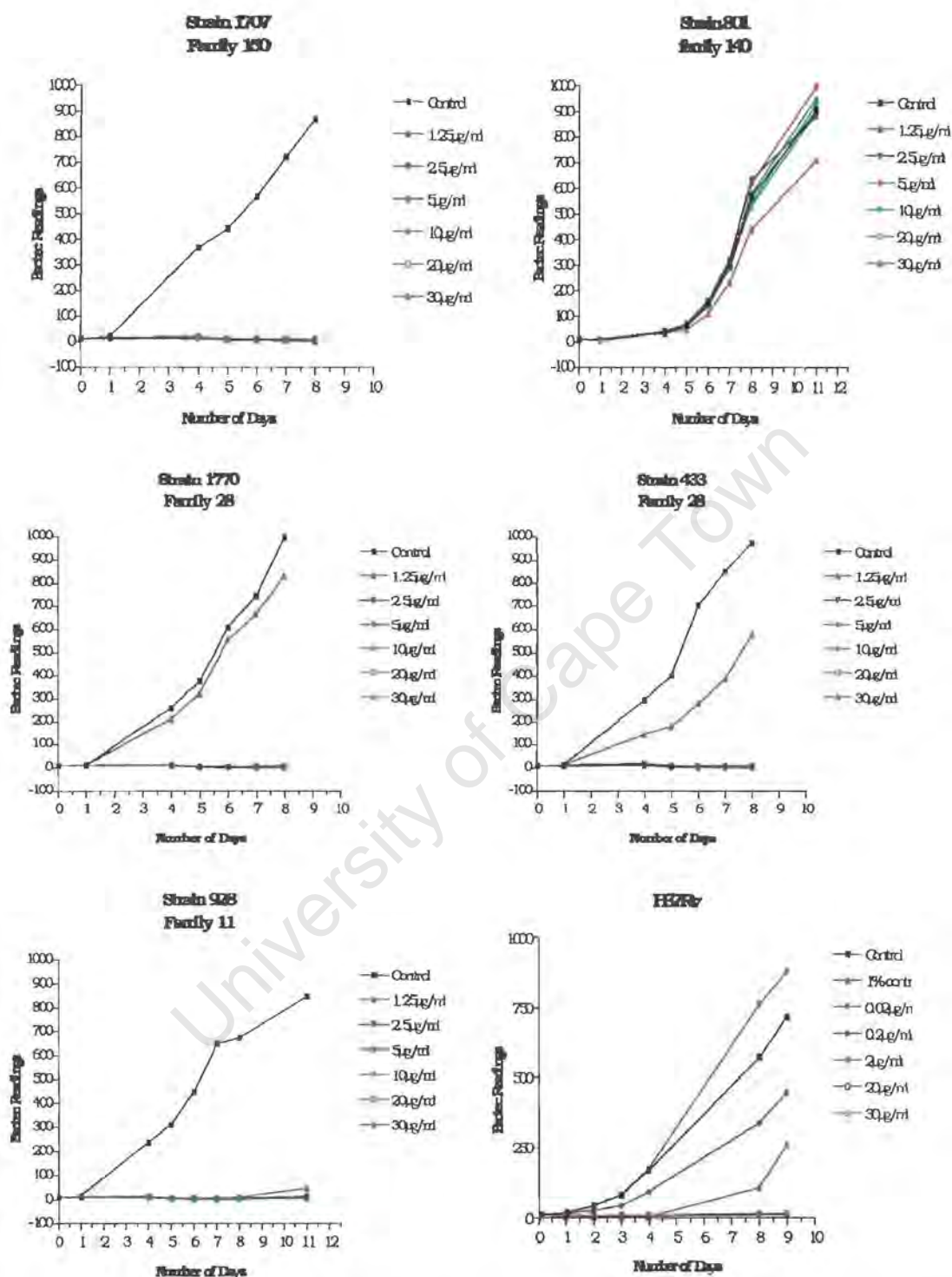


Figure 4.15: Dose-response curves of sesquiterpenes of C8 tested against various clinical strains of *M. tuberculosis*. Strain H₃₇Rv was used as a reference strain as it is a laboratory strain.

Figure 4.15 showed that isolate C8 showed no activity against strain 801. Strains 928 and 1707 were completely inhibited by concentrations of C8 of 1.25 $\mu\text{g/ml}$ and greater. Strains 433 and 1770 were completely inhibited by concentrations of C8 of 2.5 $\mu\text{g/ml}$ and greater. The response of the strains to anti-tuberculosis drugs in clinical use is tabulated in Appendix I.

4.2.4 Cytotoxicity studies of the sesquiterpenes of C8

Figures 4.16 and 4.17 on page 79 show the results of C8 sesquiterpene lactones and daunomycin, respectively, against Chinese hamster ovarian cell lines, tested at concentrations between 100 $\mu\text{g/ml}$ and 0.1958 $\mu\text{g/ml}$.

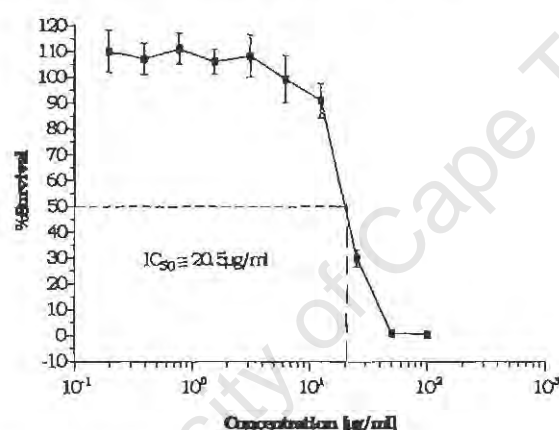


Figure 4.16: Dose-response curve of C8 sesquiterpene lactones tested against Chinese hamster ovarian cells to determine cytotoxicity. The IC_{50} of the isolate was 20.5 $\mu\text{g/ml}$. Results are presented as the mean of four readings with standard deviation as the error bars.

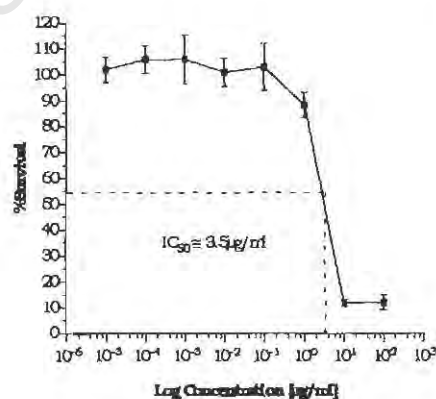


Figure 4.17: Dose-response curve of daunomycin tested against Chinese hamster ovarian cells to determine cytotoxicity. The IC_{50} of the isolate was 3.5 $\mu\text{g/ml}$. Daunomycin was used as the comparison drug. Results are presented as a mean of four readings with standard deviation as the error bars.

Figures 4.16 and 4.17 show that isolate C8 had an IC_{50} of 20.5 $\mu\text{g/ml}$ when tested against Chinese hamster ovarian cell lines, whereas daunomycin had an IC_{50} of 3.5 $\mu\text{g/ml}$. This indicated that isolate C8 was more selectively antimycobacterial than cytotoxic. This was reflected by the fact that the IC_{50} value against *M. tuberculosis* H₃₇Rv was 1.9 $\mu\text{g/ml}$ while it was 20.5 $\mu\text{g/ml}$ against Chinese hamster ovarian cells.

4.3 SUMMARY OF RESULTS

The cold-water extracts of all the plants did not show activity against *M. aurum* A⁺, with the highest concentration tested being 200 $\mu\text{g/ml}$. A similar lack of activity was observed with the cytotoxicity tests of these plant extracts against the Chinese hamster ovarian cells. *P. prunelloides* was the only water-extracted plant cytotoxic against the Chinese hamster ovarian cell lines, with 30% kill at 200 $\mu\text{g/ml}$. This overall lack of activity of the water extracts is attributed to the group of compounds that are extracted by water. The process extracts mainly highly polar hydrophilic compounds that are unlikely to have a major effect in either of these biological assays. A different result was observed with most dichloromethane extracts. The dichloromethane extracts exhibited the highest cytotoxicity levels against Chinese hamster ovarian cells. Cytotoxicity was observed in most dichloromethane extracts. Activity against *M. aurum* A⁺ was observed with a small number of extracts. Dichloromethane extracts of *A. afra* showed the highest antimycobacterial activity, with 58.64% survival. *A. afra* was also tested in a combination with other plants, in a manner comparable to that used by traditional healers. This combination (Combination 1) showed activity of 62% against *M. aurum* A⁺. The other plants that formed part of this combination did not show significant activity when tested alone. The assumption was made that *A. afra* was the active plant in this group.

The methanol extracts followed a similar trend to the dichloromethane extracts in their activity against the biological assays. Most of the methanol extracts exhibited cytotoxicity against Chinese hamster ovarian cell lines. These extracts, however, did not exhibit a significant activity against *M. aurum* A⁺.

Crude dichloromethane extracts of *A. afra* had similar activity when tested against *M. tuberculosis* and *M. aurum*, with IC_{50} values of 280 $\mu\text{g/ml}$ and 250 $\mu\text{g/ml}$, respectively. The combination that contained this plant, Combination 1, was tested and did not show increased activity compared with the activity of the single plant. The other plants were

also tested singly and did not show activity against *M. aurum* A⁺ suggesting that *A. afra* was the plant with the antimycobacterial activity.

Further fractionation of the *A. afra* dichloromethane extract yielded the fractions designated as Shef1 through Shef10. Shef6 had a 100% inhibition at 50 µg/ml against *M. aurum* NTCC23366. Fractions Shef7 and Shef8 had less activity, with 85% and 80% inhibition, respectively. The three fractions were pooled and purified further, yielding isolates C1 through C12. Isolate C8 exhibited the highest *in vitro* activity against *M. aurum* A⁺ and *M. tuberculosis* H₃₇Rv, with IC₅₀ of 1.9 µg/ml and 2.0 µg/ml. Isolate C8 was further tested against a variety of clinical strains of *M. tuberculosis*, *in vitro*. Strains 1707 and 928 were sensitive to the compounds at concentrations as low as 1.25 µg/ml. These strains were not related at all as strain 1707 belongs to family 150 while strain 928 belongs to family 11. The DNA fingerprinting confirmed that the two strains were different (see Appendix I). Strains 433 and 1770, which both belong to family 28, were sensitive to the compounds at concentrations of 2.5 µg/ml and greater. This concentration gave 100% inhibition for the duration of 10 days. As all the studies were halted at day 10, no follow up was performed to find the effect of the compound after a longer incubation period. Strain 801 had complete resistance to C8 at the highest concentration of 30 µg/ml. This strain, which belongs to family 140, was also markedly resistant to a series of antibiotics including isoniazid, rifampicin and streptomycin (see Appendix I). The cytotoxicity pattern of C8 showed that the compounds were not toxic to the Chinese hamster ovarian cell lines. The crude plant extract did not have any cytotoxic effect against these cell lines. This suggests that the plant is not toxic in its clinical use. The dichloromethane extract of *A. amatymbica*, the other component of the combination in which the plant is traditionally administered, had 30% inhibition of CHOs at 200 µg/ml. The effect of this toxicity in a clinical setting is not known as traditional healers commonly use the plant in the treatment of tuberculosis. The combination of these three plants was not tested for cytotoxicity.

In conclusion, the dichloromethane extract of *A. afra* contains the isolate C8 that exhibits the antimycobacterial activity against various strains of *M. tuberculosis*, *in vitro*. Isolate C8 does not have any cytotoxic effect against the Chinese hamster ovarian cells, an indication of its selective activity against the mycobacterium.

CHAPTER FIVE

Isolation, characterisation and structural elucidation of tanacetin and artemin/arsubin

5.1 INTRODUCTION

5.1.1 Aim of this chapter

This chapter is aimed at showing the processes involved in the structural isolation, characterization and structural elucidation of sesquiterpene lactones of eudesmanolide type from *Artemisia afra*. These sesquiterpenes were isolated as a mixture of biologically active isolate C8 shown in the previous chapters to be active

5.1.2 Chemical properties of sesquiterpenes

Sesquiterpenes are C-15 terpenoids that occur in nature as hydrocarbons or in oxygenated forms such as alcohols, ketones, aldehydes, acids or lactones.

5.1.3 Biogenesis of sesquiterpenes

Sesquiterpenes are biogenetically derived from farnesyl pyrophosphate. They may be linear, monocyclic or bicyclic in structure [81]. Figure 5.1 on page 84 shows the stages of biosynthesis of sesquiterpenes.

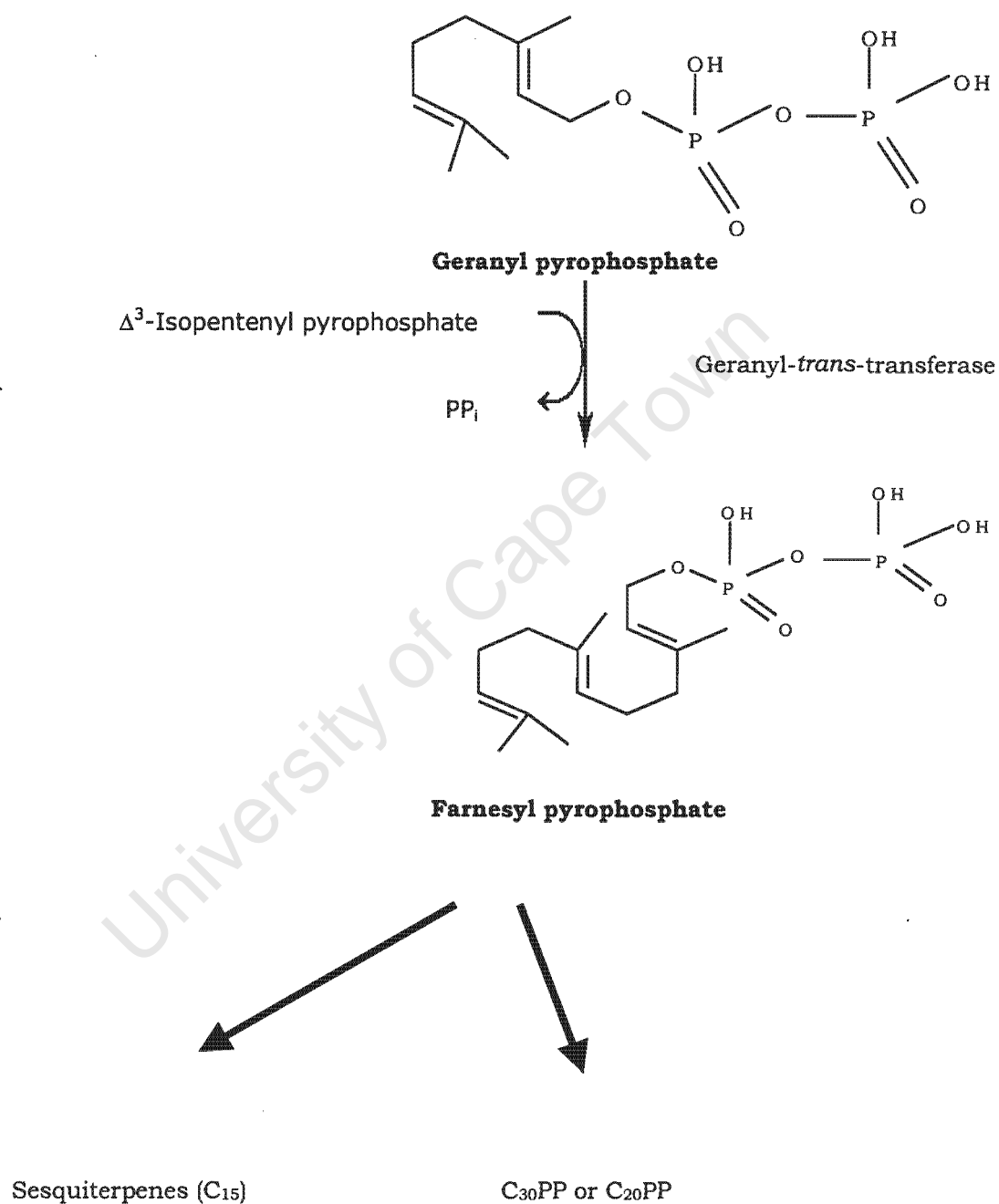


Figure 5.1: Stages in the biosynthesis of sesquiterpenes.

Source: Evans [81].

5.1.4 Biological uses of sesquiterpenes

Sesquiterpenes have been isolated from a number of plants and tested over a wide variety of biological assays. This group of compounds has been shown to be of biological importance in many studies, such as possessing activity against *Nipposstrongylus brasiliensis* [133], anti-*Plasmodium falciparum* [134, 135], trypanocidal activity [136], and against *Candida albicans* [137]. Sesquiterpenes have been shown to reverse resistance against multi-drug resistant *Leishmania* species. In a study done by Kennedy *et al* [138] and Perez-Victoria *et al* [139], a number of sesquiterpenes from *Maytenus magellanica* and *M. chubutensis* were tested on a multi-drug resistant *Leishmania tropica* line over-expressing a P-glycoprotein-like transporter. Some of these sesquiterpenes were shown to revert the resistance phenotype and modulate intracellular drug accumulation. Kim *et al* [140] performed a similar study on resistance reversing potential. Their studies were on sesquiterpene esters from *Celastrus orbiculatus* that were more active than verapamil in reversing vinblastine resistance in multi-drug resistant KB-V1 cells.

In a study performed by Burim *et al* [141], centratherin, a sesquiterpene lactone with trypanocidal activity [136], exhibited clastogenic and cytotoxic activity on *in vitro* and *in vivo* mammalian systems. A series of other experiments showed the cytotoxic activity of sesquiterpenes. Other biological activities of sesquiterpenes include nitric oxide production inhibition [142], analgesic activity [143], and hepatoprotective activity in mice [144].

5.1.5 Chemical analysis of sesquiterpene lactones

Sesquiterpene lactones (SLs) are classified according to their carbocyclic skeletons. The structural feature of these compounds is the α,β -unsaturated- γ -lactone [81]. This moiety has been associated with most of the biological activity of this group of compounds [96]. The SLs are known to be low in volatility and most of them are thermo-labile. Because of these properties, the SLs have been analysed from crude plant extracts by reversed phase high-pressure liquid chromatography [145]. In a study of 33 pseudoguaianolides and xanthanolides of the genus *Parthenium* [145] and of 21 pseudoguaianolides of *Arnica chamissonis* [146], HPLC was used successfully to analyse these compounds. This study

further proposed the use of methanol-water or acetonitrile-water gradients for elution of the SLs.

The use of UV detection in the analysis of SLs is sometimes unsuccessful as the SLs show weakly absorbing chromophores or no chromophores at all [147]. HPLC linked to other high sensitivity analytic chemistry techniques has been used in overcoming this problem. Vogel *et al* [148] have implemented the use of LC linked to Mass Spectrometry and LC linked to Nuclear Magnetic Resonance to elucidate the structures of 9 antibacterial sesquiterpene lactones from a partially purified extract of *Vernonia fastigiata*, without isolation of individual compounds. The rapid structural analysis of both major and minor components of this class of compounds demonstrates the power of structure-guided screening as a complementary method to assay-guided screening. Other systems that have been successfully used in the separation of SLs include supercritical fluid chromatography [147], gas chromatography [146] and thin layer chromatography.

5.2 RESULTS AND DISCUSSION

The sesquiterpene lactones that were isolated were fractionated through a series of bioassay guided processes. The various fractionation and purification processes included flash column chromatography, thin layer chromatography and high-pressure liquid chromatography. Structural elucidation was performed using various analytic chemistry techniques such as nuclear magnetic resonance and mass spectrometry.

5.2.1 Fractionation processes

5.2.1.1 Thin-layer chromatography

The plate showed one spot for the isolate, suggesting the presence of one compound or compounds with similar elution patterns. The R_f value of the isolate was 0.6.



Figure 5.2: Thin-layer chromatograph of C8. The isolate was run with 60% hexane in ethyl acetate. The plate was visualized using anisaldehyde.

Figure 5.2 above shows the TLC elution pattern of isolate C8. The pattern suggests a single compound.

5.2.1.2 High-pressure liquid chromatography

The isolate C8 was run on the high-pressure liquid chromatography to determine its elution pattern. The isolate was also compared with the elution pattern of the crude extract of *A. afra*, ran under the same conditions. Figures 5.3 below and 5.4 on page 89 show the HPLC profile of isolate C8.

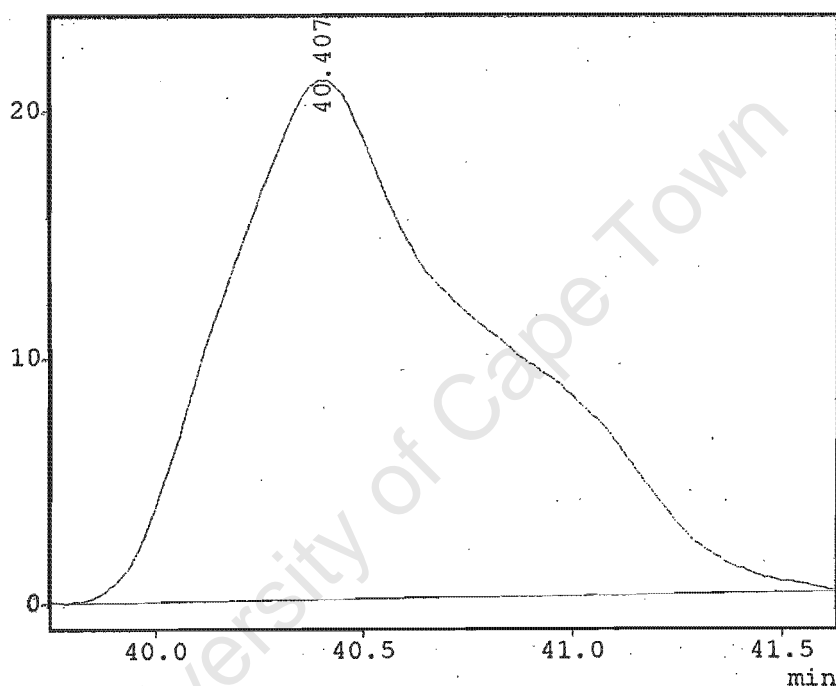


Figure 5.3: HPLC profile of C8. The isolate was run using a Phenomenox analytical Prodigy 5u ODS (2) 150 x 4.60mm column linked to a Phenomenox guard column. An amount of 100 μ l was injected and run at a flow rate of 1 ml/ml. The isolate was run on a gradient mobile phase starting with 30% methanol in water over 60 minutes to 100% water.

The elution time of isolate C8 was approximately 31 minutes. The peak, however, seemed to exhibit the presence of more than one compound due to the shoulder effect appearing before completion of the main peak. Further analysis of isolate C8 using an isocratic gradient of 50% methanol in water on a Supelco column gave significant separation of the two compounds, as shown in Figure 5.4 on page 89.

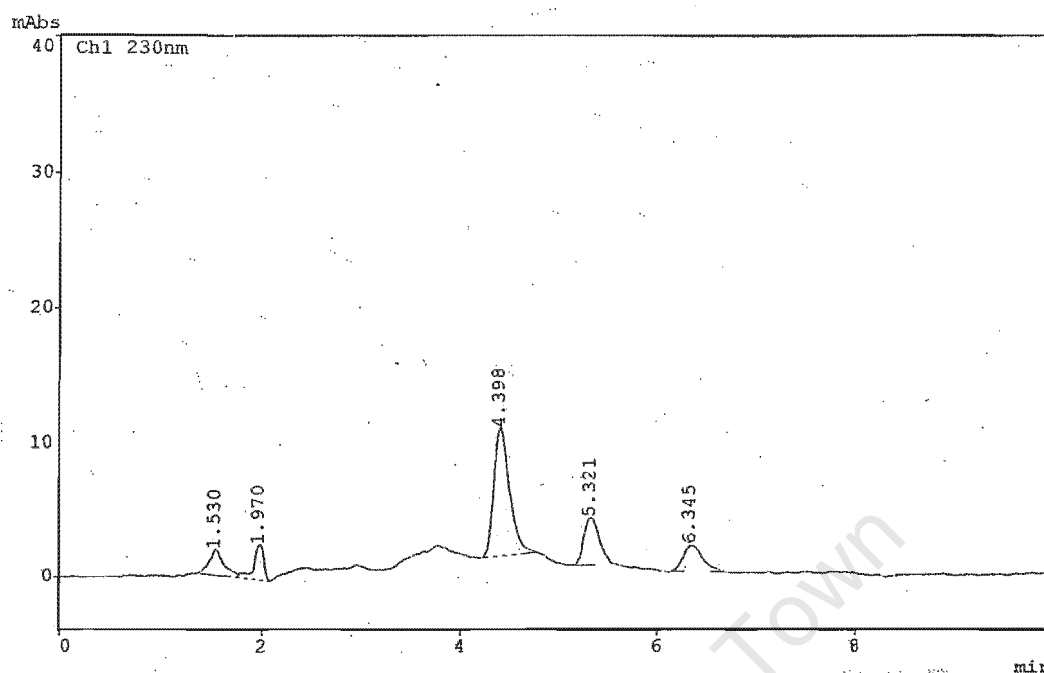


Figure 5.4: HPLC profile of C8. The isolate was run using a Supelco analytical Dimension 5u 150 x 4.60 mm column linked to a Phenomenox guard column. An amount of 30 μ l was injected and run at a flow rate of 1 ml/ml. The isolate was run isocratically with a mobile phase of 50% methanol in water over 10 minutes period.

This HPLC profile was used in a further attempt to fractionate isolate C8 into its minor and major components, the 266 amu and 264 amu components (explained in section 5.2.2) but was unsuccessful. The LC-MS spectrum showed that the minor compound eluted in all the three peaks of the major compound.

5.2.2 Structural elucidation of the sesquiterpenes of C8

High-resolution electron impact mass spectrometry (HREIMS) (Appendix II) showed two molecular ions at m/z 266.15232 and m/z 264.13569. The calculated values of the ions were 266.15181 m/z and 264.13616 m/z , respectively. Mass spectrometry data suggested the molecular formulae of the two compounds to be $C_{15}H_{22}O_4$ and $C_{15}H_{20}O_4$, respectively. From the mass spectrometry data, there was a loss of H_2O ion from the parent compound, from 266 m/z to 248 m/z to 230 m/z , suggesting the presence of the two $-OH$ groups.

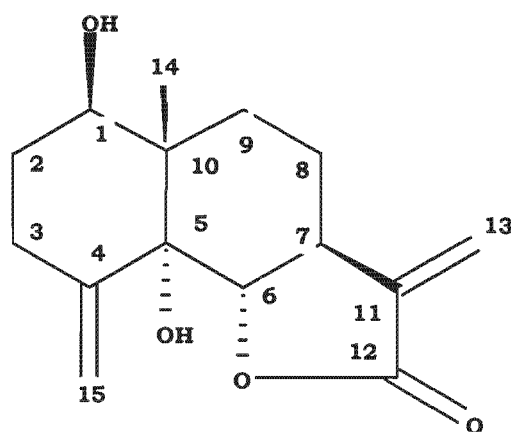
Features of the ^1H -NMR spectrum, as shown in table 5.3 and Appendix IV, showed protons at $\delta 4.23$, dd, $J = (11.5, 4.9)$ Hz linked to carbon at $\delta 71.7$ in the HSQC spectrum (see table 5.3 and Appendix VI) and at $\delta 4.25$, d, $J = 10.71$ Hz linked to carbon at $\delta 81.7$. These protons were assigned to H-1 and H-6, respectively. A methyl singlet at $\delta 0.86$ and a methyl doublet at $\delta 1.21$ were assigned to CH_3 -14 and CH_3 -13, respectively. ^1H - ^1H COSY (see table 5.3 and Appendix VIII) showed H-1 correlates to H-2 β ($\delta 1.49 - 1.67$ m) because the dihedral angle is 180° . There was small correlation to H-2 α ($\delta 1.71 - 1.88$ m) because of a dihedral angle of *ca.* 90° . H-2 β correlates to one of the H-3's, which is H-3 α because of the dihedral angle of 180° . H-6 β correlates to H-7 α ($\delta 2.32 - 2.45$ m), which in turn correlates with H-8 β ($\delta 1.30 - 1.34$ m). H-11 correlates to H-7 α ($\delta 2.32 - 2.45$ m) and to the protons of the methyl group at C-13 ($\delta 1.21$ d $J = 6.7$ Hz). H-8 β correlates to H-9 α ($\delta 1.71 - 1.88$ m). The protons of the methylene group (H-15a & H-15b) at $\delta 5.02$ s and $\delta 4.99$ s, respectively, correlate to H-3 α . ^1H - ^1H COSY spectrum showed a correlation between H-7 α and H-8 β and in the HSQC spectrum there was correlation with C-8 at $\delta 30.3$. Additionally, there was correlation between H-1 and H-2 and HSQC showed correlation at C-2 ($\delta 22.7$). These data differed from the data published by Gonzalez *et al* [149], who showed that the chemical shift of C-2 was $\delta 30.3$ and C-8 was $\delta 22.7$.

The carbon resonances of all the protonated carbons could be established from the HSQC spectrum (see table 4.3 and Appendix VI). Two and three bond C-H correlations from CH_3 -13 to C-12 at $\delta 179.2$, from H-15 (a, b), CH_3 -14 and H-3 β to C-5 at $\delta 76.9$, from CH_3 -14, H-8, H-9, to C-10 at $\delta 44.5$, and from H-2 α , H-3 α to C-4 at $\delta 144.9$ enabled us to assign the four quaternary carbons. From these data the structure of the major compound, **artemin** or **arsubin**, was assigned to be that shown in Figure 5.5 on page 92. No NOESY spectrum was run, so we could not distinguish between the spatial orientations of the methyl group, CH_3 -13, whether it is a β -methyl (**arsubin**) or α -methyl (**artemin**).

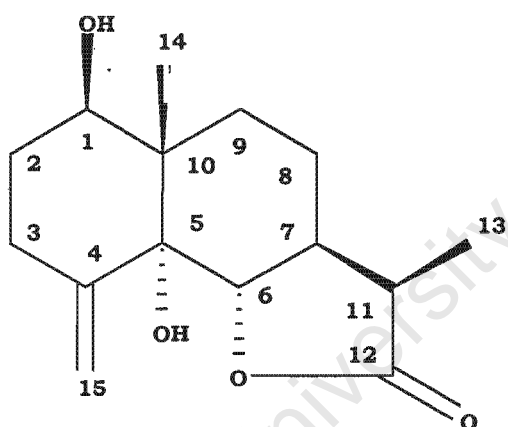
The ^1H -NMR showed that there was substitution of the CH_3 -13 with a $\text{C}=\text{CH}_2$, at H-13a ($\delta 6.1$, dd, $J = 3.2, 2.1$ Hz) and H-13b ($\delta 5.4$, dd, $J = 3.2, 2.1$ Hz) as evidence for the minor compound, **tanacetin**. H-13a is the most deshielded proton adjacent to the carbonyl group.

Table 5.3: ^1H -NMR, ^{13}C -NMR, ^1H - ^1H COSY, HSQC and HMBC spectral data of the major compound. To form the minor compound the methyl group of C-13 in major compound is substituted with a CH_2 group.

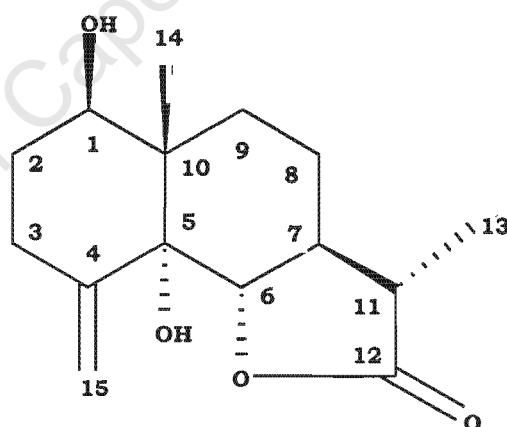
Position	δH	^1H - ^1H COSY	δC	HMBC
1	α 4.23 dd (11.5, 4.9)	2 α , 2 β	71.7d	C-14
2	α 1.71-1.88 m	1 α , 2 β , 3 α	22.7t	C-1, C-3, C-4, C-10
	β 1.49-1.67 m	1 α , 2 α , 3 α		C-1, C-3
3	α 2.14 ddd (13.9, 5.6, 1.9)	2 α , 3 β	29.8t	C-1, C-2, C-4, C-5, C-15,
	β 2.59-2.72 m	2 β , 3 α , 15(a, b)		C-2, C-14
4			144.9s	
5			76.9s	
6	β 4.25 d (10.7)	7 α	81.7d	C-11
7	α 2.32-2.45 m	8 β , 11 α	45.4d	
8	α 1.67-1.80 m	8 β , 9 β	30.3t	C-10
	β 1.30-1.34 m	7 α , 8 α , 9 α		C-7, C-9, C-11
9	α 1.71-1.88 m	8 β , 9 β	29.5t	C-1, C-5, C-8, C-10, C-14
	β 1.49-1.67 m	8 β , 9 α		C-1
10			44.5s	
11	α 2.32-2.45 m	7 α , 13	41.2d	C-7
12			179.2s	
13	1.21 d (6.7)	7 α , 11 α	12.4q	C-7, C-11, C-12
14	0.86 s		13.2q	C-5, C-1, C-10, C-9, C-2
15a	5.02 s	3 β	112.6t	C-3, C-5
15b	4.99 s	3 β		



Tanacetin



Artemin



Arsubin

Figure 5.5: Chemical structures of the two sesquiterpenes isolated from *A. afra*. Tanacetin is the minor compound and either artemin or arsubin is the major compound. The planar orientation of CH₃-13 was unresolved.

The structures depict the two sesquiterpenes isolated from *A. afra* Jacq. They differ by a molecular mass of two. The spatial orientation of the methyl group at carbon C-13 could not be decided, which would determine whether the major compound is artemin or arsubin. The compounds have been isolated in previous studies. Tanacetin has been

isolated from *Tanacetum vulgare* L. [150]. The plant has been associated with toxicity but no work has been done to link the toxicity with this sesquiterpene. No biological activity of tanacetin has been described. Artemin has been extensively worked on and isolated from a number of plants. Rybalko *et al* initially isolated artemin from *Artemisia taurica* [151]. They published a probable gross structure of the compound without definitely establishing the position of the secondary hydroxyl group, nor the stereochemistry of the asymmetric centres [152]. Gonzalez *et al* [149, 152] described intensive work on the structure and the stereochemistry of artemin isolated from *A. maritime* L ssp *Gallica* Willd [152]. The compound has been shown to possess antipyretic activity in a study by Martin *et al* in rats with induced fever [153]. Tarasov *et al* isolated arsubin; however, Gonzalez *et al* disproved the structure that they published as being that of artemin [152]. Feliciano *et al* performed further work on arsubin when they isolated arsubin and artemin, among other sesquiterpenes, from *A. cearulescens* ssp *Gallica* [154].

CHAPTER SIX

Discussion and Conclusions

Everyone who breathes air, from Wall Street to the Great Wall of China, needs to worry about this risk. Once MDR-TB is unleashed, we may never be able to stop it. We will face a deadly infectious disease that spreads through the air, yet is virtually as incurable as AIDS or Ebola. This frightening prospect must be avoided at any cost! – WHO

6.1 TRADITIONAL HEALERS and TB

Traditional healers throughout South Africa were interviewed about their understanding and treatment of tuberculosis. Most traditional healers used symptoms such as cough, loss of weight and night sweats in the diagnosis of TB. It is assumed that their focus was mainly on pulmonary tuberculosis. However, healers' understanding of the cause differs from the conventional knowledge of *M. tuberculosis*, and this influences the way they treat the disease. The various categories of approach to treatment have simplified understanding of their choice of treatment. A large proportion of plants collected were directed towards the treatment of symptoms, with the healers treating the patient until they returned to their normal condition. This does not provide evidence of full recovery, because in conventional medicine patients start looking normal within few months of treatment although not completely cured. Most traditional healers treat a patient for an unspecified time. They argue that they treat the patient until "they are cured". In scientific terms, this is vague, as it does not address the causative agent. However, it is in line with the traditional healers' understanding that there are other aspects involved in the disease than the microorganism. The level of understanding, and interpretation, of tuberculosis of most traditional healers differs considerably from the orthodox. Observations made in this study indicate that most traditional healers combine culture with health, in their practices. It is clear that the understanding of tuberculosis and its management by traditional healers is explained in the context of culture, to a degree not conventionally known by western-trained medical personnel. But there are also similarities in how most traditional healers understand tuberculosis. Symptoms are used to guide the form of treatment. The culture and background of the traditional healer and of the patient are the factors that determine treatment of the disease. As explained by Airhihenbuwa, culture influences the way people understand and interpret diseases [89]. In tuberculosis particularly, traditional healers believe that the individual's cultural background needs to be considered. A traditional healer gave one example when he argued:

"Tuberculosis is a form of communication by ancestors to an individual who has not performed some of his customs. Without that problem being addressed, the patient will constantly experience episodes of tuberculosis".

From the western-trained medical practitioner's point of view, this system of disease persistence could either be recognized as re-infection, relapse, or reactivation of latent tuberculosis. Thus, issues of culture and custom are relevant tools for understanding

tuberculosis and how traditional healers perceive it, as observed from constant emphasis by traditional healers.

Culture dictates the communication of diseases by traditional healers and by the communities they serve. A community that still believes in customs would understand the traditional healers' perspective of customary practice, more than they would understand concepts of relapse and reactivation after treatment. Some traditional healers even go to an extent of tracing the history of the family to understand the cause of the disease. The concept of *"call by ancestors to be a sangoma"* has been presented in many cases as the cause of someone to suffer from diseases such as tuberculosis. The direct role of this belief and understanding in actual treatment is not fully explained by this study. Whether the direct treatment that accompanies ritual performance plays a role in the treatment of the disease that needs further investigation. Another aspect of the recovery process is the role of medication usually taken by patients who attend to both paradigms of health care, the traditional and the western. One traditional healer mentioned that she:

"Does not stop patients from attending clinics while they are consulting with her".

In this case, the effect of the traditional healing cannot be measured, as there are concurrent systems of medication being used. This has the potential of being misinterpreted by most scientists if they do not include the cultural aspect of each community in their quest to investigate the use of traditional medicines. Traditional healers treat the cause of the disease, as they understand it. The use of terms such as "evil spirit" and "poison" as causes of tuberculosis makes it difficult to interpret the disease in scientific terms. Some of the plants used in the combinations were assumed to address such beliefs. In scientific language terms such as "combination therapy" and "synergy" would apply in understanding use of such mixtures of plants.

In this study, a series of plants that healers used was collected and evaluated using biological assays. Most plants did not exhibit activity against *M. aurum* A⁺, *in vitro*. Traditional healers, however, continue to use these plants in treatment of tuberculosis. This could pose a threat to communities if traditional healers regard their role in TB as primarily disease treatment. It needs to be examined and investigated thoroughly because traditional healers form a large workforce for health, on which many communities still rely. Furthermore, cytotoxicity tests suggest a level of toxicity among a number of the organic extracts. It should be noted, though, that almost all the water extracts were not cytotoxic to the Chinese hamster ovarian cells. Traditional healers mainly use water to prepare medicines, and most of their preparations are unlikely to be

toxic to humans. Some traditional preparations require rigorous boiling and this could have similar effect to organic extraction with similar toxicity effect.

Biological activity against the *in vitro* systems tested in this research serves as an indication of the level of anti-tuberculosis effect of plants in humans. With the inherent scientific limitations in validating the results in the human body, a conclusion based on *in vitro* tests that traditional medicines has no role in the control of tuberculosis may be wrong. There is no evidence that these medicines are either effective or ineffective in tuberculosis. It is the necessary that the scientific community should find common ground in working in collaboration with healers to combat tuberculosis. Amongst the potential roles that the traditional healers would play in tuberculosis include health promotion, health education and disease prevention strategies, and in implementation of the DOTS programme.

Traditional health systems can play a major role in the prevention of tuberculosis and in promotion of initiatives towards treatment of the disease. This includes participation of traditional healers in primary health care initiatives to educate people about the impact of tuberculosis, and how to prevent occurrence and recurrence of the disease. Promotion of the cross-referral system of patients between western and the traditional medical practitioners might be explored as a way of enhancing the primary health care role of traditional healers. Traditional healers involvement in control strategies of tuberculosis through implementation of the Directly Observed Treatment, short-course (DOTS) programme, should be promoted.

Traditional healers command considerable respect in their communities. They are regarded as advisors on health matters and family values. Through collaboration with local nurses and health service providers, traditional healers might play a role in motivating tuberculosis patients to continue taking their medicines until completion of regimen. DOTS can have maximum effect if it is coupled with cultural understanding of the disease. DOTS requires committed individuals to monitor patients and to ensure that they receive proper treatment. Traditional healers would play a role in implementation of DOTS through programme such as involvement in community education on prevention of tuberculosis and its treatment, monitoring of TB patients in adherence to their treatment, and through involvement in community health centres. The emergence of HIV/AIDS has created an even more damaging stigma to TB, as anyone suffering from TB is suspected of suffering from HIV/AIDS. This creates an additional problem of people refusing to accept that they suffer from TB. Traditional healers might play a role in destigmatisation of tuberculosis and HIV/AIDS as a strategy of disease control. In South Africa there are over 200 000 traditional healers, a figure

that is growing daily. This escalation is driven by factors such as the increase in genuine, trained healers, unemployment leading to charlatans, and registration of traditional healers with recognizable organisations. In many parts of the country traditional healers work as individuals and in other parts they form organisations. Nevertheless, there is still no overall controlling body. There is a lack of regulation as to who should become a traditional healer and what standards of training are necessary before one is regarded as a traditional healer. This haphazard approach creates a stigma, aggravated by an increase in deaths claimed to be due to poisoning by traditional medicines and by weak disease control strategies. Coordination of bodies of traditional healers would assist in implementation of education programs to equip traditional healers with the necessary skills for combating diseases such as TB and HIV/AIDS. It would also help to coordinate the collaborative efforts between the government and the other institutions with an interest in working with traditional healers.

6.2 ANTIMYCOBACTERIAL ACTIVITY of *ARTEMISIA AFRA*

This study has successfully tested a total of 90 extracts against *Mycobacterium aurum* A⁺, *in vitro*. The dichloromethane crude extract of *A. afra* had the highest activity of all the plants, with 58.6 percentage survival against *M. aurum* A⁺. The methanol and water extracts of the plant did not exhibit any activity, with percentage survivals of 100.13% and 108.6%, respectively. The dose-response curve of the extract against *M. tuberculosis* gave an IC₅₀ of 290 µg/ml, more than 1450-fold that of isoniazid. The crude plant extract also does not have cytotoxic activity against Chinese hamster ovarian cell lines, indicating that the plant is not toxic in the way it is used in practice. Selectivity against mycobacteria was examined by testing the dichloromethane extract against Gram-positive bacteria *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* and Gram-negative bacteria *Bacillus subtilis* and *Micrococcus*. The extract did not have any activity against gram-negative bacteria. The extract did have activity against *Micrococcus*, with an IC₅₀ of 540 µg/ml. This was 2-fold that of *M. aurum*, which had an IC₅₀ value of 250 µg/ml. Slight inhibition of *B. subtilis* was displayed but the inhibition did not go below 50%. Mycobacteria are known to have certain qualities of gram-positive bacteria in that they retain the gram stain in the thick cell wall. A general conclusion was drawn that the extract of *A. afra* has limited activity against gram-positive bacteria and high specificity against mycobacteria. The activity against *M. tuberculosis* of the

dichloromethane crude extract of *A. afra* led to the isolation of two sesquiterpene lactones of eudesmanolide type. The mixture of these sesquiterpenes showed similar activity, *in vitro*, against *M. aurum* A⁺ and *M. tuberculosis* H₃₇Rv, with IC₅₀s of 1.9 µg/ml and 2.0 µg/ml, respectively. The activity of these sesquiterpenes was 20-fold less than that of isoniazid; which has an IC₅₀ of approximately 0.1 µg/ml. It might be argued that these sesquiterpenes are less active compared with INH, and therefore that traditional use of the plant is of no advantage in comparison with proven therapy. However, sesquiterpene lactones with a methylene group bonded to the lactone ring have high activity against mycobacteria [92]. Due to the fact that the two compounds could not be separated, the individual activity of the two compounds isolated in this research programme cannot be determined. The compounds have been shown to have activity, and the plant is used in high concentrations so as to have an effect. Cytotoxicity testing of the isolated sesquiterpenes showed that the compounds are not toxic to Chinese hamster ovarian cell lines and they are 5-fold less toxic than daunomycin. The sesquiterpenes give mixed activity when tested against various clinical strains of *M. tuberculosis*. Strain 1707 and strain 928 are sensitive to the compounds at concentrations as low as 1.25 µg/ml. These strains are not related as strain 1707 belongs to family 150 while strain 928 belongs to family 11. The antibiotic resistance profile of the two strains was not performed but the DNA fingerprinting confirmed that the two strains were different. Strains 433 and 1770, which both belong to family 28, are sensitive to the compounds at concentrations of 2.5 µg/ml. This concentration gave a 100% inhibition for duration of 10 days. Strain 801 had a total resistance to the sesquiterpenes at the highest concentration of 30 µg/ml. This strain, which belonged to family 140, was also resistant to a series of antibiotics including isoniazid, rifampicin and streptomycin (see Appendix I).

6.3 ETHNOPHARMACOLOGICAL/PHARMACOTHERAPEUTIC IMPLICATIONS

6.3.1 Researching traditional medicines

The field of traditional medicines in South Africa is still shrouded in uncertainty due to lack of interest from other sectors of health and government. There is, however, some encouraging indication from the government that it intends to put traditional medicines into the limelight, through introduction of policies governing the incorporation into the national health agenda and giving it recognition. This comes with an increasing need to

research traditional medicines and to provide valid information for processes of recognition to proceed swiftly. Researching traditional medicines would then assist healers and communities who have limited opportunity of validating their claims. This project has provided information on a number of plants used by the people of this country for treatment of tuberculosis. The research has also provided information on how traditional healers understand TB, and how that understanding has caused them to have a different view of its treatment from conventional western ways.

6.3.2 Scientific relevance

In this thesis, two sesquiterpene lactones of eudesmanolide type with novel activity against *M. tuberculosis* have been isolated and partially characterised. This is the first time these sesquiterpene lactones have been isolated from *A. afra* and shown to be active. Novel methods of isolating the compounds from this plant have been determined, and their nuclear magnetic resonance spectral data have been compiled.

6.3.3 Relevance to policy issues

The area of traditional medicines in South Africa is deeply rooted in the culture of the people that use the medicines. It is practised in conjunction with the cultural beliefs of the society and in this manner. For this reason, it can be assumed that the expertise will continue to exist for a long period of time, irrespective of scientific, legal or any other disagreement from those who may have opposing beliefs. The challenge facing the country is to find effective means of incorporating this practice into the mainstream health system, as a parallel or an alternative health system. This would not only give recognition to traditional medicines and traditional healing, but also to ways of regulating the use of this system by the people. The South African government has introduced a number of initiatives to address the incorporation and regulation of traditional medicines. These include the formation of the Complementary Medicines Committee (CMC), a committee of the Medicines Control Council (MCC) and formation of the National Reference Centre for Traditional Medicines (NRCTM). The CMC deals with claims from traditional healers about medicines that they consider to be of clinical importance. The NRCTM aims at creating an infrastructure of experts and facilities around the country to perform bioassays and studies on these claims in an attempt to

confirm them. The data obtained from the research reported in this thesis would play an important role in the work of these committees, contributing to the scientific basis for claims that deal with tuberculosis, cytotoxicity and phytochemistry.

6.4 RECOMMENDATIONS

One of the objectives of this study was to understand how the traditional healers use their plants to treat patients. The study has shown that most traditional healers use the plants together with cultural rituals. However, traditions need to be changed in order to serve the people they are made for. Traditional healers need to adopt a practice of referring patients to clinics and health service centres for conventional tuberculosis therapy. On recovery, the patient may then undergo the traditional and cultural aspects as advocated by their traditional healers. On the other hand, western practitioners should recognise the role played by traditional healers in acting as primary health practitioners in their communities. They should encourage patients to return to their traditional healers to undergo cultural rituals, if they need to. In this way, proper collaboration will mutually benefit practitioners and communities. Depending on herbal remedies, or undermining the cultural aspects of a community, might act to the detriment of the patient and the communities.

In this study it has been confirmed that traditional healers have one main aim in their practice, to improve the health status of their communities. This can be achieved a number of ways; namely, by community wellness, disease prevention, disease control, and patient care. Traditional healers have voiced their eagerness to learn - traditional healing, as with other health systems, is dynamic. These aims and eagerness could be furthered if the National Department of Health and other health systems work together with traditional healers by providing the resources necessary to enable them to be involved in the education of communities about issues such as tuberculosis, HIV/AIDS, smoking, alcohol abuse, family violence and many others. Through this primary health approach, integration of the traditional health system into the main health policies of the country might be achieved. The findings from Chapter 3 of this study provide a basis for exploring such collaborative initiatives. Understanding the disease and traditional healers' of cultural interpretations of the disease might be used by western trained practitioners in understanding the traditional perspective of the disease.

6.5 SUMMARY AND CONCLUSIONS

1. Traditional healers have a special understanding of tuberculosis, based on their interpretation of the disease. This incorporates elements of culture and traditional rituals, and forms part of the process of traditional healing.
2. Biological assays of the plants that traditional healers use conducted in this research programme showed little evidence that the plants concerned have direct antimycobacterial activity when tested with *in vitro* biological systems.
3. The dichloromethane extract of *A. afra* Jacq clone AK6 had the highest antimycobacterial activity against *M. aurum* A⁺ and *M. tuberculosis* H₃₇Rv, *in vitro*.
4. Two sesquiterpene lactones, tanacetin and arsubin/artemin, isolated from dichloromethane extracts of *A. afra*, have been shown in this regard to have *in vitro* antimycobacterial activity against *M. aurum* A⁺ and *M. tuberculosis*.

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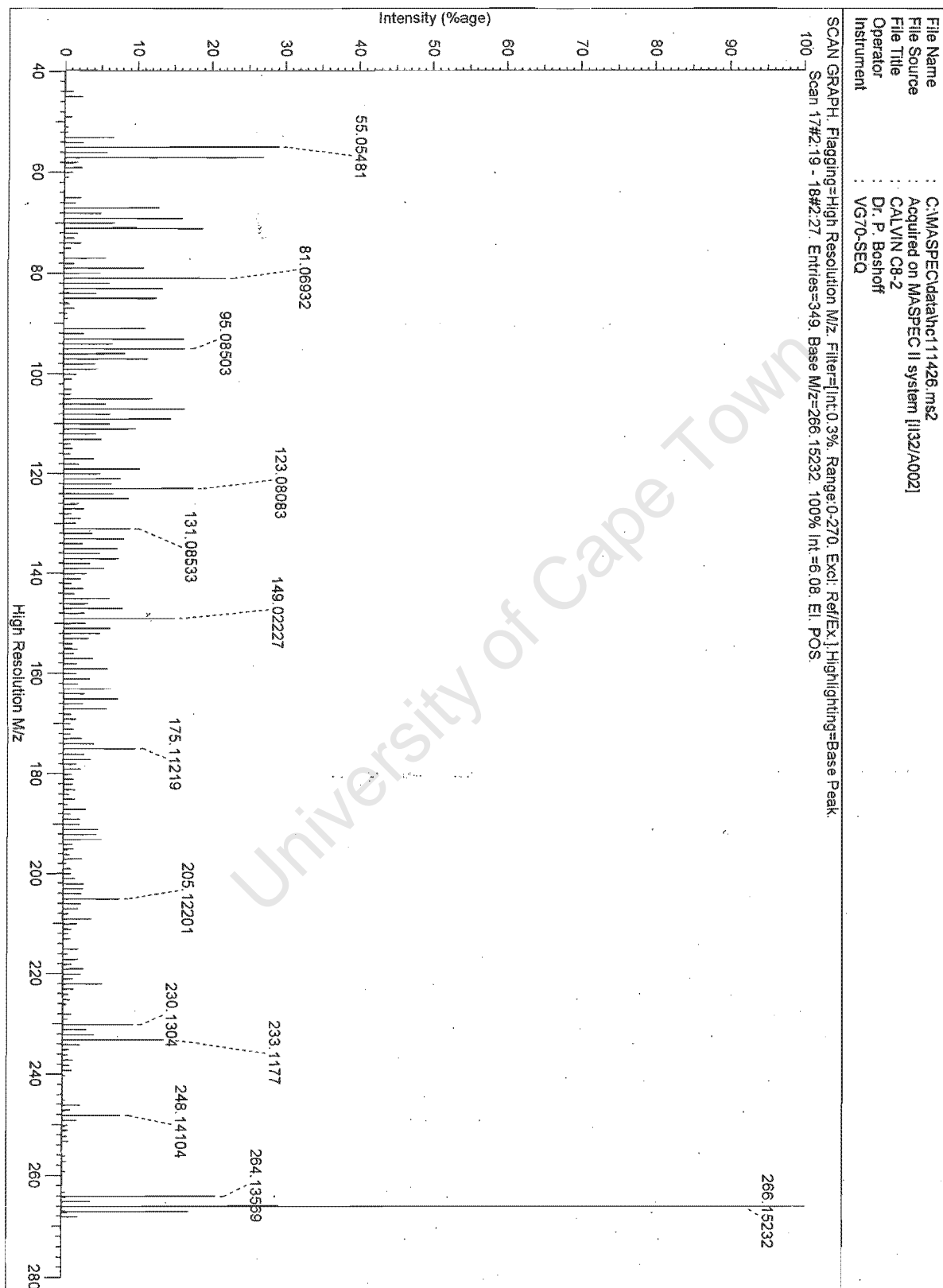
APPENDICES

Appendix I: Clinical strains of *Mycobacterium tuberculosis*

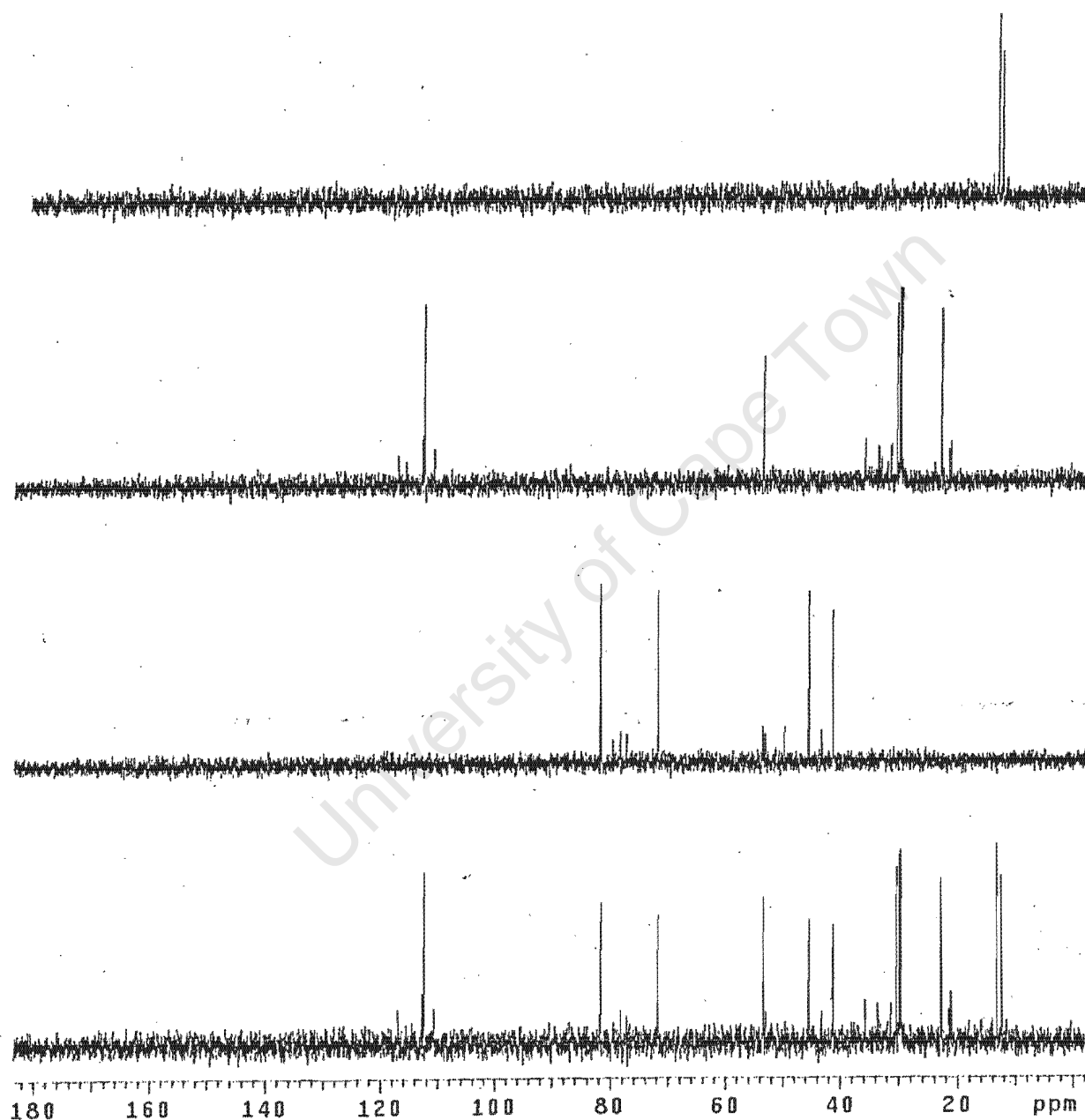
The antibiotic tests and DNA fingerprinting results of the various clinical strains of *M. tuberculosis* used in the Bactec 460 TB assay of isolate C8. This set of data was used to show that the strains were different from each other.

PatientID	SAWC Strain No TB Strain	Date	Family	IS6110_3	IS6110_5	484	Bands	Resistance	Tested on
417	801	1992/10/10						Y	INH STREP RIF
417	801	1993/08/10						Y	INH RIF
417	801	1993/07/29						Y	INH RIF STREP KANA
417	801	268	1993/08/12	140	330	387	3 4	Y	INH RIF SM
417	801	250	1993/08/19	140	330	387	3 4	Y	INH RIF SREP
417	801	781	1995/01/05	140	330	387	3 4	Y	INH STREP RIF KANA CYCLO
417	801	801	1995/02/07	140	330	387	3 4	Y	INH STREP RIF KANA THIA CYCLO
417	801	824	1995/03/16	140	330	387	3 4	Y	INH RIF KANA STREP AMIKA
1234	928	928	1995/04/28	11	20	14	1 13	Y	NONE
1234	928		1995/09/21					N	
1234	928		1997/06/19					N	
1234	928		1998/03/26					N	
1540	433	433	1994/03/17	28	264	290	44 9	Y	NONE
1540	433		1994/08/04					N	
1540	433		1997/04/23					N	
2032	1707		1998/02/24					Y	None
2032	1707	1727	1998/03/30	150	338	388	1 5	N	
2032	1707		1998/08/04					N	
2124	1770	1771	1998/05/05	28	284	309	12	U	
2124	1770	1770	1998/05/06	28	284	309	12	U	
2124	1770	1770	1998/05/06	28	284	309	12	U	
2124	1770		1998/11/12					N	
2124	1770		1998/11/24					N	
2124	1770		1998/11/25					N	

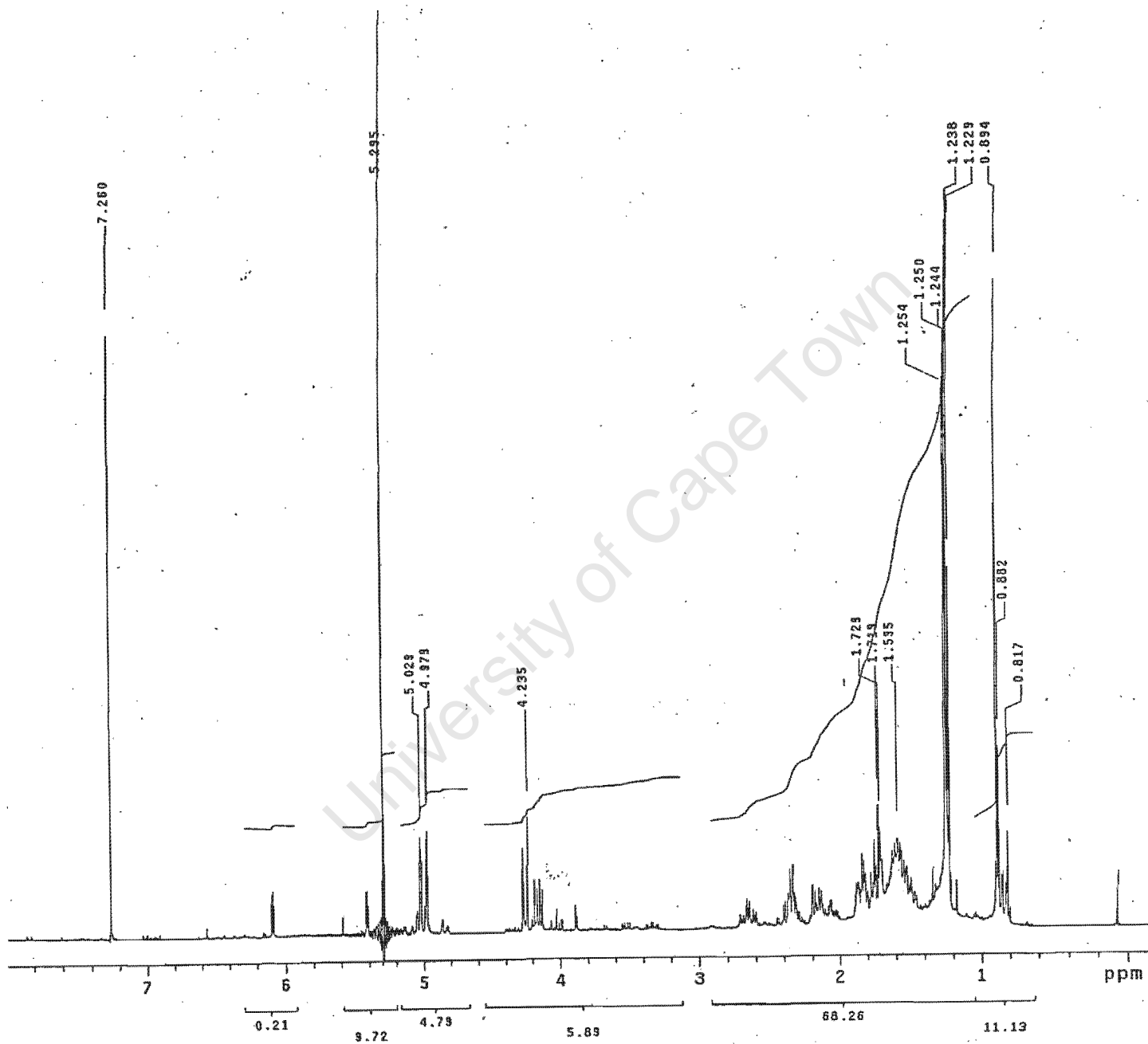
Appendix II: Mass spectrometry data of C8

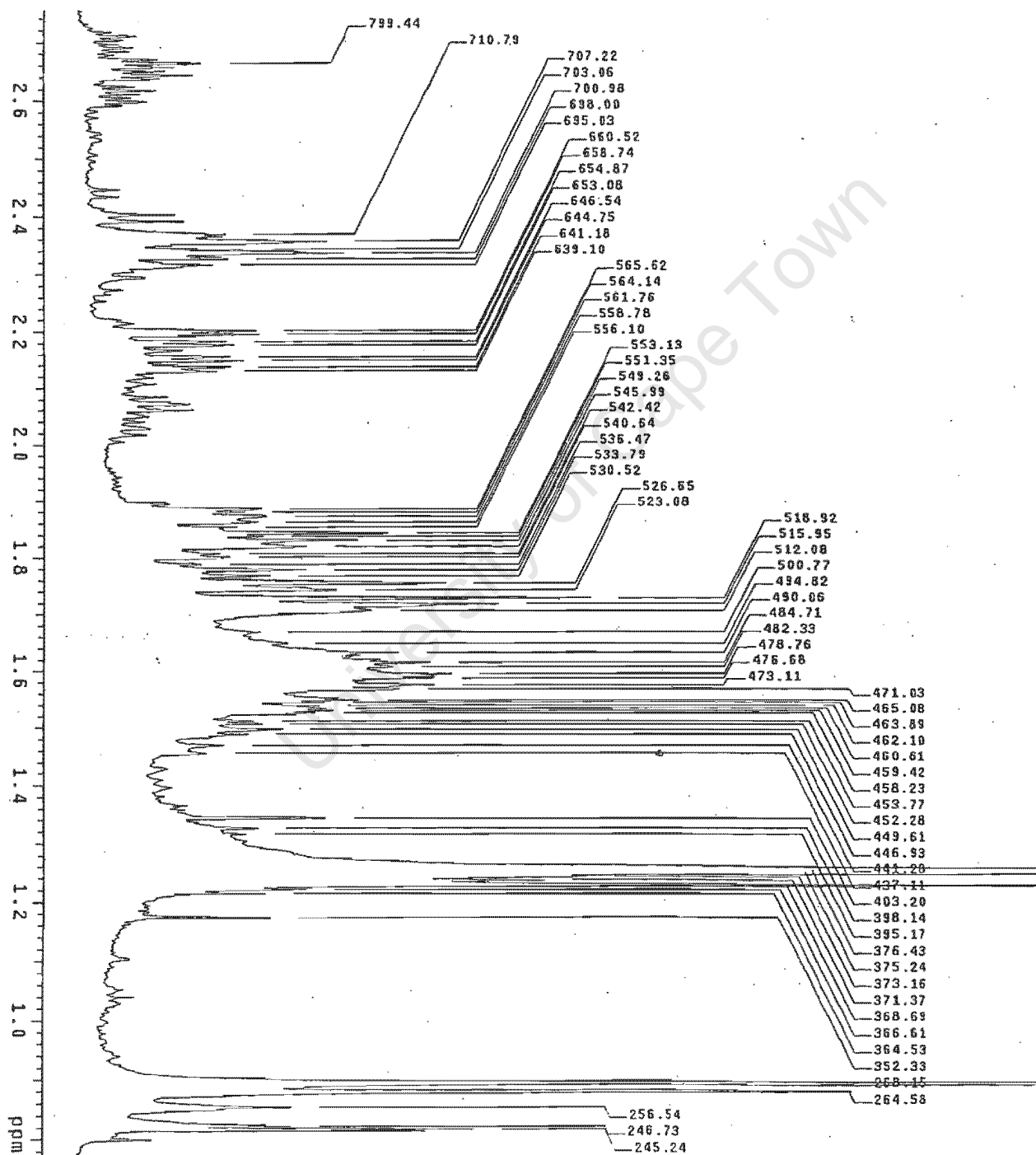


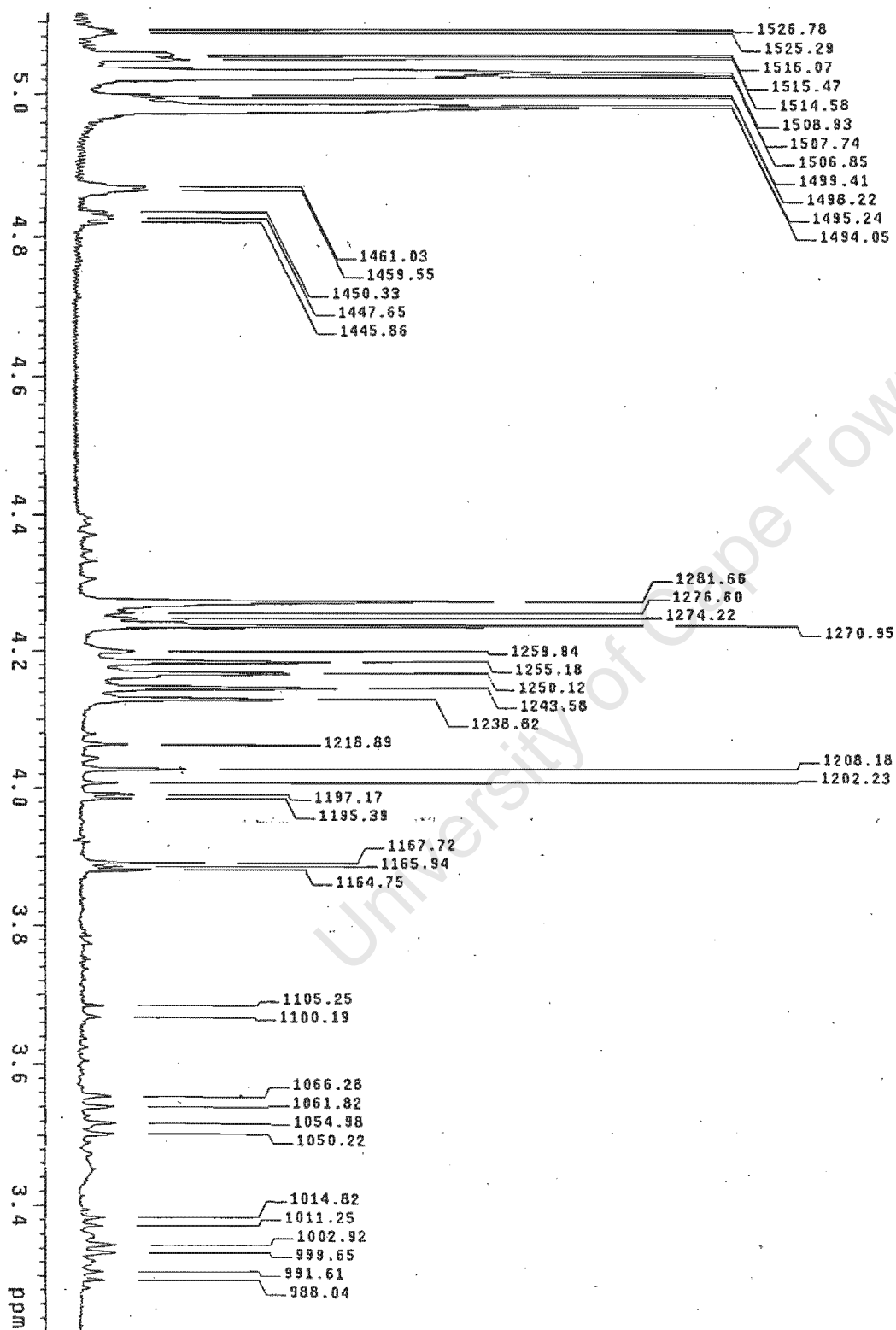
Appendix III: DEPT spectral data of C8

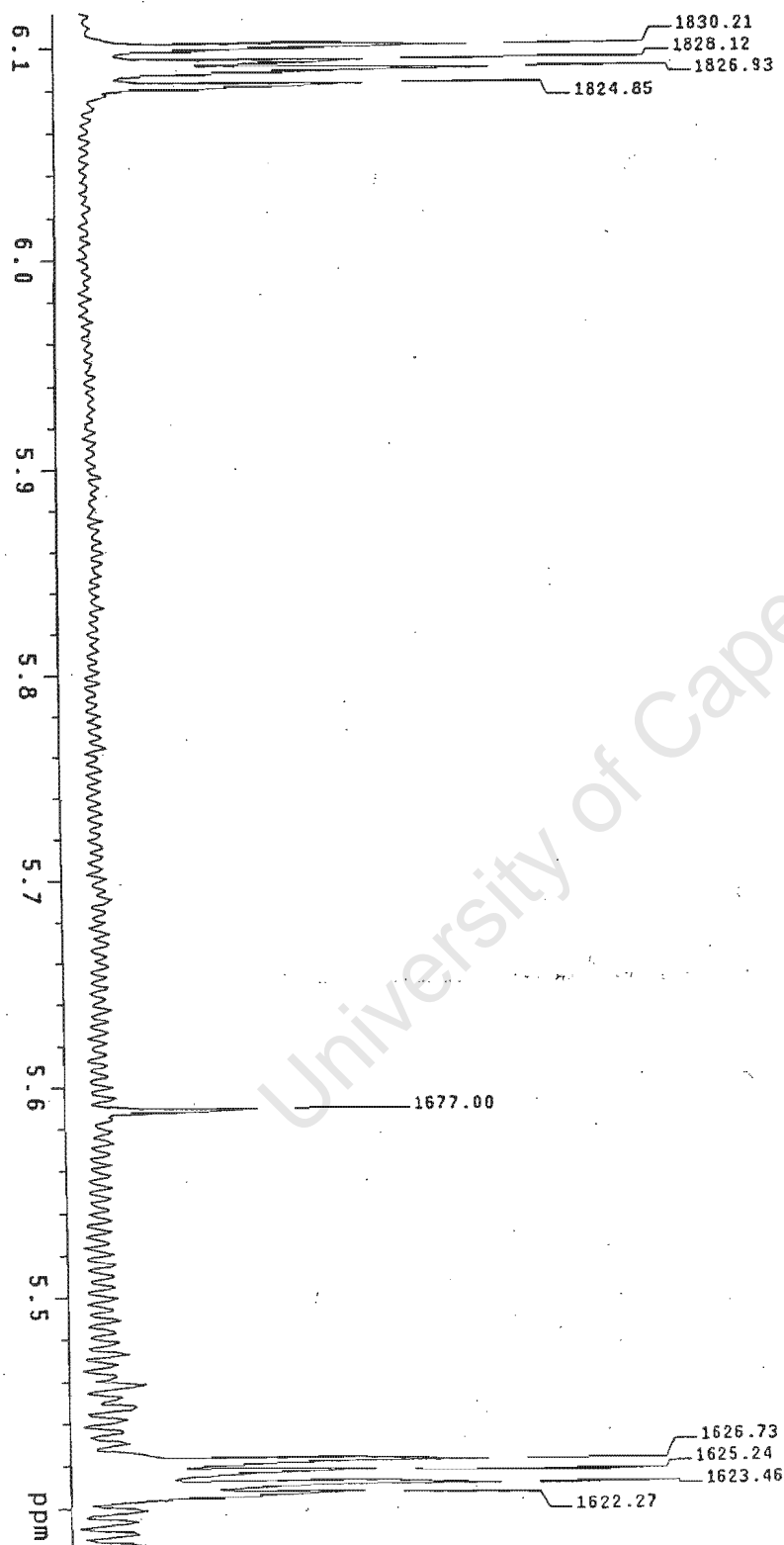


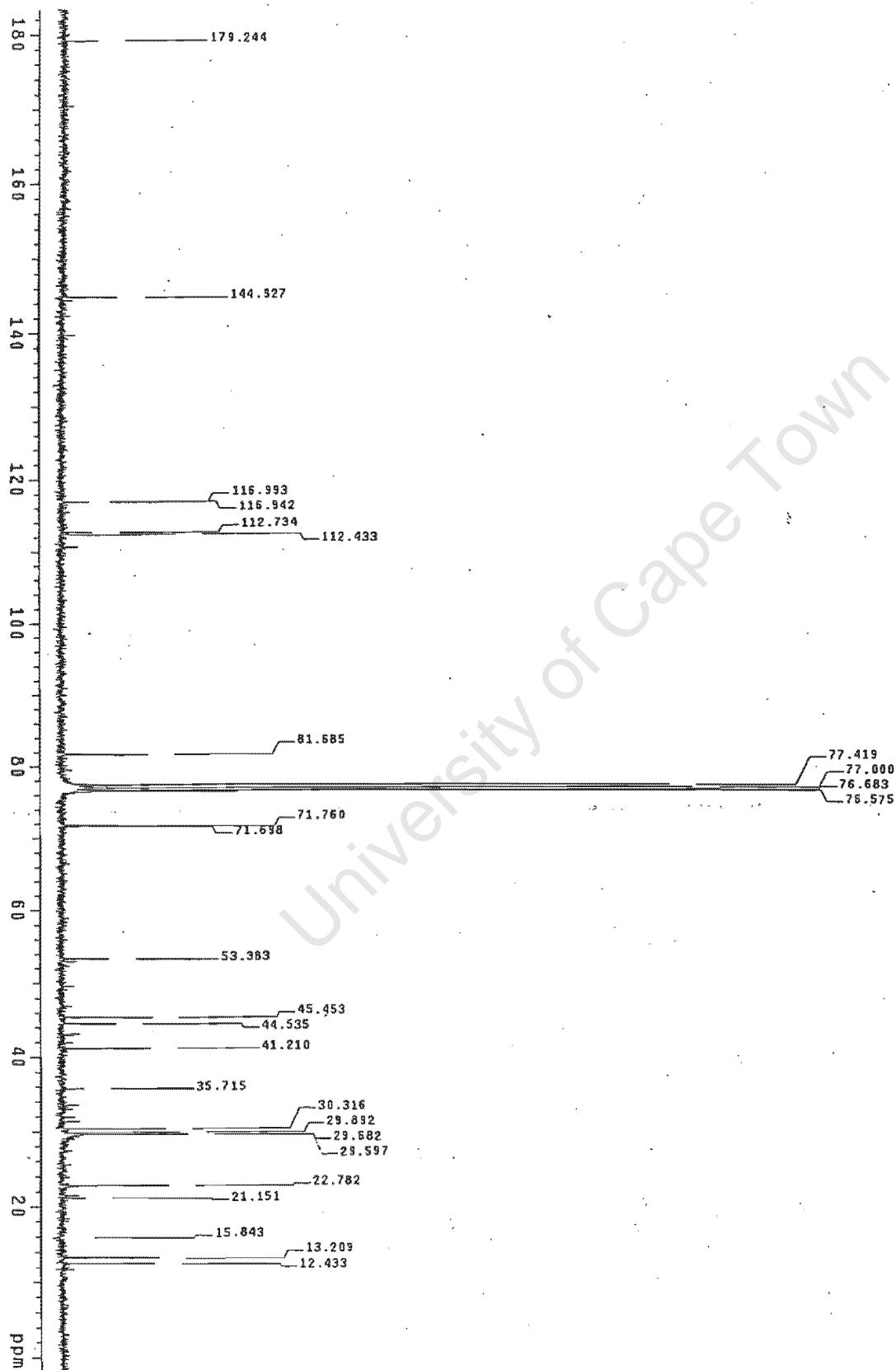
Appendix IV (a): ^1H -NMR data of C8

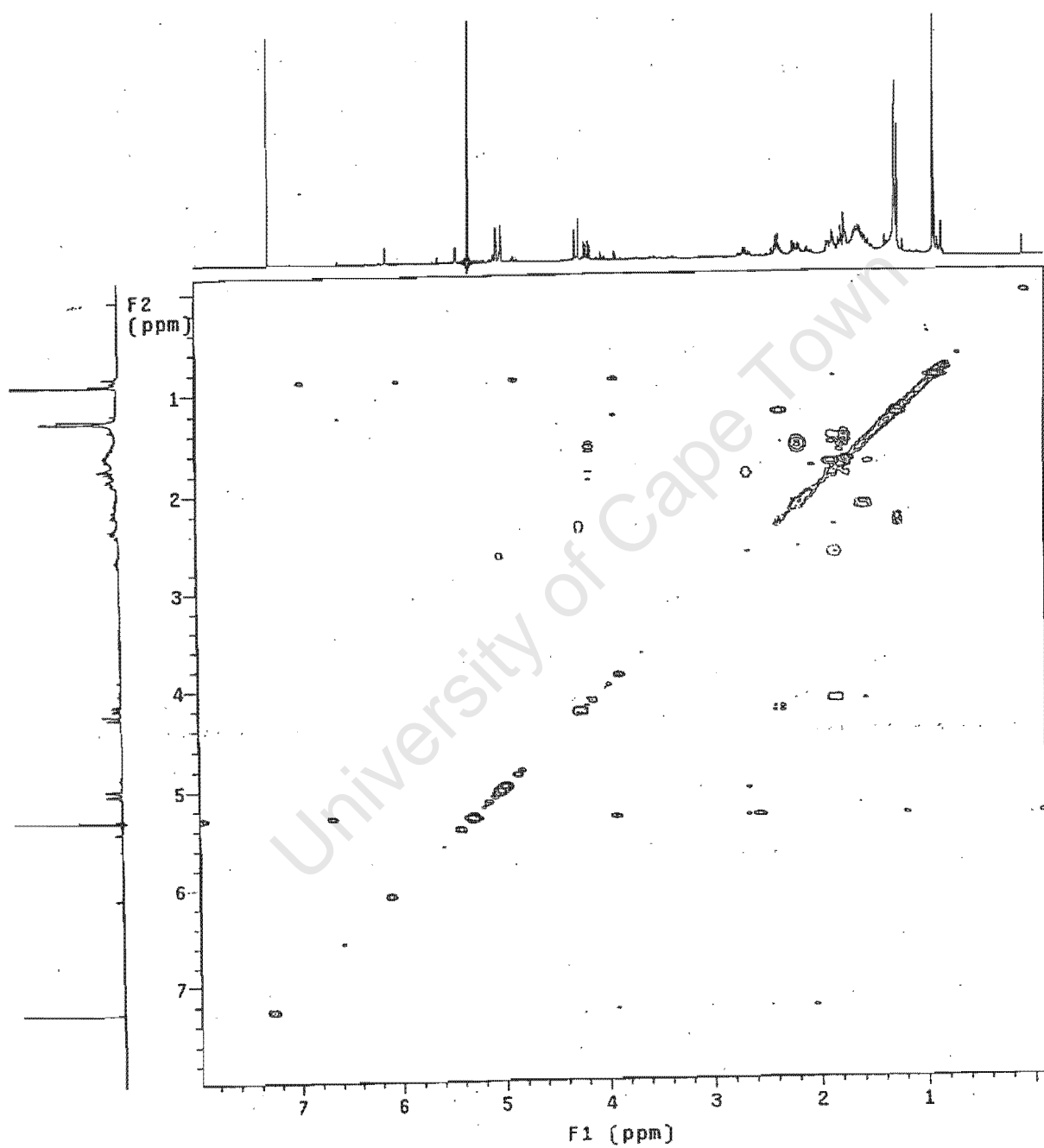


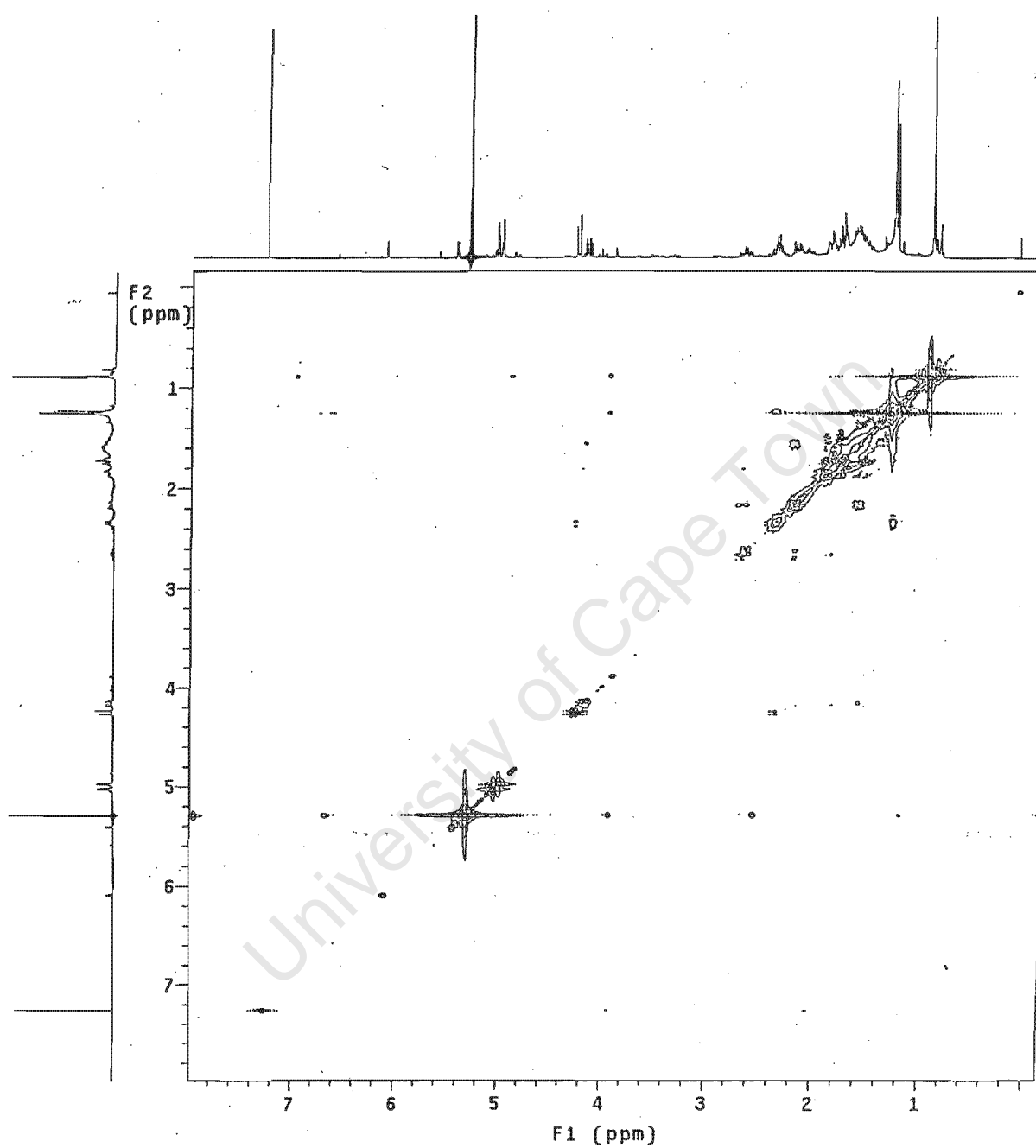
Appendix IV (b): ^1H -NMR data of C8

Appendix IV (c): ^1H -NMR data of C8

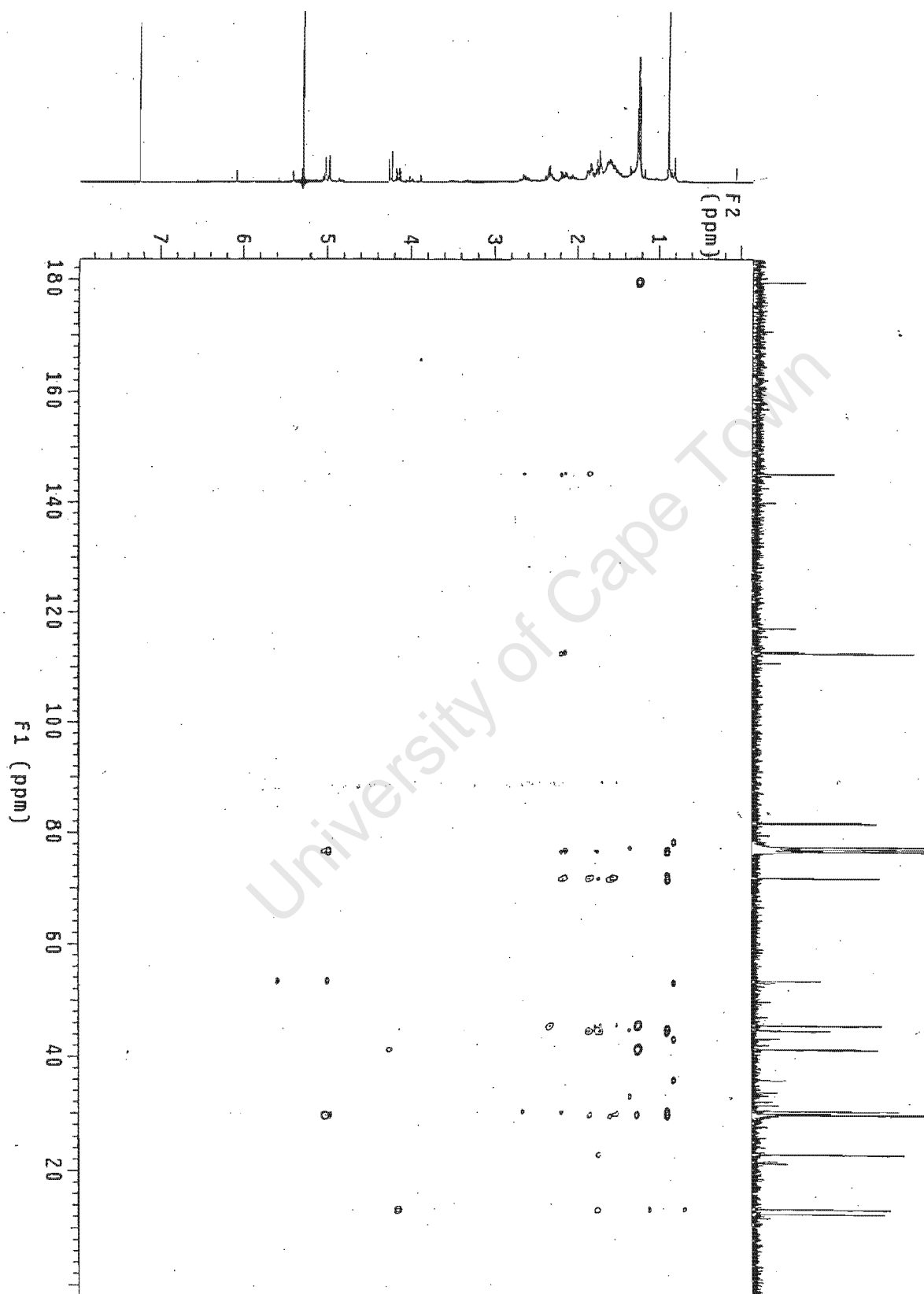
Appendix IV (d): ^1H -NMR data of C8

Appendix V: ^{13}C -NMR spectral data of C8

Appendix VI (a): ^1H - ^1H COSY spectral data of C8

Appendix VI (b): ^1H - ^1H COSY spectral data of C8

Appendix VII: HMBC spectral data of C8



Appendix VIII: HSQC spectral data of C8

