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The antimicrobial and antimycobacterial activity of plants used for the treatment of respiratory ailments in Southern Africa and the isolation of anacardic acid from ozoroa paniculosa.

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Title page, illustration from Van Wyk, 2001
Declaration……..II
Acknowledgements……III
Abstract……..IV
Table of Contents……..VII
List of Figures……..IX
List of Tables……XII
List of Abbreviations……...XIV

1 Introduction
1.1 Tuberculosis 1
1.2 The organism..... 2
1.3 Symptoms and diagnosis 3
1.4 Treatment of tuberculosis 4
1.5 Anti-tuberculosis drugs 4
1.6 Drug-resistant tuberculosis..... 5
1.7 Traditional medicine in South Africa 7
1.8 Drugs derived from natural products 8
1.9 Plants with antimycobacterial activity 9
1.10 Tuberculosis drug development 14
1.11 Project objectives 17
2 Plant selection and crude extract preparation
2.1 Introduction..... 18
2.2 Materials and methods 18
2.3 Results 20
2.4 Discussion 29
3 Antimicrobial testing of crude extracts
3.1 Introduction..... 32
3.2 Materials and methods 32
3.3 Results 34
3.4 Discussion 40
4 Antimycobacterial testing of crude extracts
4.1 Introduction..... 46
4.2 Materials and methods 47
4.3 Results 49
4.4 Discussion 51
5 The isolation and characterization of anacardic acids from *Ozoroa paniculosa*
5.1 Introduction..... 53
5.2 Materials and methods 55
5.3 Results 59
5.4 Discussion 69
6 The antimicrobial and antimycobacterial activity of anacardic acids
6.1 Introduction..... 72
6.2 Materials and methods 72
6.3 Results: Compound 1 72
6.4 Results: HPLC Fractions 85
6.5 Discussion 90
7 The cytotoxicity testing of anacardic acids
7.1 Introduction..... 93
7.2 Materials and methods 93

7.3 Results 95
7.4 Discussion 96
8 Conclusion……..97
9 Future work……...100
10 Presentations……101
11 References……102
12 Appendix: Nuclear Magnetic Resonance: 2-Dimensional Data ..…….……...114

Figure 1.1: The estimated geographical distribution of tuberculosis cases in 2003 (WHO, 2005) 1

Figure 1.2: A model of the mycobacterial cell wall, proposed by Minnikin, showing the primary constituents of lipids, mycolates, arabinogalactan and peptidoglycan. The funnel-shaped structure in the centre represents a porin, responsible for the movement of molecules across the cell wall (Hong and Hopfinger, 2004). 2

Figure 1.3: The structures of RIF, INH, PYR and EMB..... 5

Figure 1.4: Prevalence of MDR-TB among new TB cases between 1994 and 2002 (WHO, 2004a)…….6

Figure 1.5: The chemical structures of some antimycobacterial natural products…….11

Figure 2.1: A diagramatic representation of the experimental processes followed in this project……..19

Figure 2.2: Pictures of selected medicinal plants used in this study. (A)

Siphonochilus aethiopicus; (B) *Mentha longifolia*; (C) *Salvia africana-lutea*; (D) *Tetradenia riparia*; (E) *Helichrysum odoratissimum*; (F) and (G) *Eriocephalus africanus*; (H) *Agathosma betulina*; (I) *Ozoroa paniculosa* 30

Figure 3.1: Examples of the disc diffusion method. Plate 'A' shows the activity of the acetone extract of *H. odoratissimum* (45) and *C. scabrida* acetone and methanol extracts (47 and 63 respectively) against *B. cereus*. Plate 'B' shows the activity of the acetone extracts of *T. riparia* (35), *S. cordatum* bark (42) and *H. odoratissimum* (45) against *S. aureus*…….35

Figure 3.2: An example of the broth micro-dilution method for the determination of MIC's of extracts against *E. faecalis*. The wells from left to right contain the negative control, culture control, solvent control, antibiotic control and in duplicate, the acetone extracts of *A. robusta, C. scabrida, D. stramonium* and *O. paniculosa*……..….35

Figure 4.1: The BACTEC 460 apparatus (A) with racks containing the inoculated vials ready to be tested and the individual 12B vials containing the radiolabelled carbon substrate (B). 47

Figure 5.1: Selected plant extracts exhibiting the greatest antimicrobial and antimycobacterial activity……...55

Figure 5.2: The zones containing the active principles on TLC viewed under UV 365nm (A) and agar overlay bio-autography plates of fractions 2 (B) and 3 (C) after column chromatography of the crude extract of *O. paniculosa* showing the zones of inhibition of *M. aurum* growth……...60

Figure 5.3: The HPLC profile of the eluted fractions HPLC1, 2 and 3 using a semi-preparative C-18 column and visualizing at 300nm on a Shimadzu LC10AS at the University of Cape Town…….…….61

Figure 5.4: LC-MS results of active fraction 3 of *O. paniculosa* performed at the University of Stellenbosch……..61

Figure 5.5: High resolution mass spectrum of compound 1 62

Figure 5.6: The ^{13}C spectrum of compound 1 62

Figure 5.7: The ^{1}H spectrum of compound 1 63

Figure 5.8: The structure of 6-[8(*z*)-pentadecenyl]salicylic acid isolated from *Ozoroa paniculosa*……65

Figure 5.9: Mass spectrometry indicating the isotopic distribution of the tailing edge of fraction HPLC3……..66

Figure 5.10: The ^{13}C spectrum of HPLC3..... 67

Figure 5.11: The ^{1}H spectrum of HPLC3..... 67

Figure 5.12: The structure of 6-pentadecylsalicyclic acid with the saturated side chain isolated from fraction HPLC3 of *O. paniculosa*……..…….69

Figure 5.13: The structures of the saturated (structure A) and unsaturated (B) versions of anacardic acid, previously isolated from *Ozoroa* species ……..69

Figure 6.1: The dose-response curve representing the effect of anacardic acid on *Staphylococcus aureus* ……73

Figure 6.2: The dose-response curve representing the effect of anacardic acid on drug-resistant *Staphylococcus aureus* strain 1..…….74

Figure 6.3: The dose-response curve representing the effect of anacardic acid on drug-resistant *Staphylococcus aureus* strain 2……..75

Figure 6.4: The dose-response curve representing the effect of anacardic acid on *Enterococcus faecalis*…….……76

Figure 6.5: The dose-response curve representing the effect of anacardic acid on *Bacillus cereus*……77

Figure 6.6: The dose-response curve representing the effect of anacardic acid on *Pseudomonas aeruginosa*……..……78

Figure 6.7: The dose-response curve representing the effect of anacardic acid on *Klebsiella pneumoniae*……79

Figure 6.8: The dose-response curve representing the effect of anacardic acid on *Serratia odorifera*……...……80

Figure 6.9: The dose-response curve representing the effect of anacardic acid on *Candida albicans*……...……81

Figure 6.10: The dose-response curve representing the effect of anacardic acid on *Mycobacterium smegmatis*……82

Figure 6.11: The dose-response curve representing the effect of anacardic acid on *Mycobacterium aurum*…….…….....83

Figure 6.12: The dose-response curves representing the effects of fractions HPLC2 (A) and HPLC3 (B) on *Mycobacterium smegmatis*……..…….....85

Figure 6.13: The dose-response curves representing the effects of fractions HPLC2 (A) and HPLC3 (B) on *M. aurum*.…….....86

Figure 6.14: The dose-response curves representing the combined effect of compound 1 ($C_{15:1}$ anacardic acid) and fractions HPLC2 and HPLC3 on *Mycobacterium aurum* (A) and *Mycobacterium smegmatis* (B)…….....86

Figure 6.15: The antimicrobial and antimycobacterial activities of anacardic acids.…….89

Figure 7.1: The dose response curves representing the cytotoxicity of $C_{15:1}$ anacardic acid compound 1 (A) and fractions HPLC2 (B) and HPLC3 (C) isolated from *Ozoroa paniculosa*, together with the crude extract (D) on CHO cells. The emitine and methanol controls are illustrated in graphs E and D respectively…….95

Figure 12.1: Compound 1- GHMQC Plot……...…….114

Figure 12.2: Compound 1 - GHSQC Plot.……..……..115

Figure 12.3: Compound 1 - RELAYH Plot……..116

Figure 12.4: Compound 1 - DEPT Plot.……..……..117

Figure 12.5: HPLC3- GHMQC Plot.……..…..118

Figure 12.6: HPLC3 - GHSQC Plot.……..…...119

Figure 12.7: HPLC3 - RELAYH Plot…….……..120

Figure 12.8: HPLC3 - DEPT Plot……..……...121

LIST OF TABLES

Table 1.1: Examples of plant extracts exhibiting antimycobacterial activity……10

Table 1.2: Antimycobacterial activities of compounds isolated from plants……12

Table 2.1: Plant extract yield using acetone and methanol as extractants……31

Table 3.1: Antimicrobial activity of the crude extracts against three Gram-positive organisms using the disc diffusion and broth micro-dilution method. Zones were measured in millimetres (mm) from the edge of the disc to the end of the zone of inhibition, and MIC's in mg/ml. Each sample was tested in duplicate. Neomycin discs and ciprofloxacin served as positive controls for the agar and broth-based methods respectively……...36

Table 3.2: Antimicrobial activity of the crude extracts against four Gram-negative organisms using the disc diffusion and broth micro-dilution method. Zones were measured in millimetres (mm) from the edge of the disc to the end of the zone of inhibition and MIC's in mg/ml. Each sample was tested in duplicate. Neomycin discs and ciprofloxacin served as positive controls for the agar and broth-based methods respectively…….....38

Table 3.3: Antimicrobial activity of the crude extracts against *Candida albicans* using the disc diffusion and broth micro-dilution method. Zones were measured in millimetres (mm) from the edge of the disc to the end of the zone of inhibition and MIC's in mg/ml. Each sample was tested in duplicate. Nystatin served as the positive control in both methods...40

Table 4.1: Antimycobacterial activity of the crude extracts against three mycobacterial species as determined by the broth micro-dilution method for *M. smegmatis* and *M. aurum* A+, and the BACTEC 460 method for *M. tuberculosis* H37Ra. The results are expressed in mg/ml as MIC's. Rifampicin was used as the positive control for *M. aurum* and *M. tuberculosis* and ciprafloxacin for *M. smegmatis*……..50

Table 5.1: Extracts with activity against two or more test organisms. Activities are indicated as MIC's in mg/ml. An extract is regarded as having good activity if its MIC is less than 1mg/ml……54

Table 5.2: The ^{1}H, ^{13}C, HMQC and COSY spectral data of compound 1……64

Table 5.3: The ^{1}H, ^{13}C, HMQC and COSY spectral data of HPLC3……68

Table 6.1: The effect of anacardic acid on the viability of *Staphylococcus aureus*……...73

Table 6.2: The effect of anacardic acid on the viability of drug-resistant *Staphylococcus aureus* strain 1……74

Table 6.3: The effect of anacardic acid on the viability of drug-resistant *Staphylococcus aureus* strain 2……75

Table 6.4: The effect of anacardic acid on the viability of *Enterococcus faecalis*……....76

Table 6.5: The effect of anacardic acid on the viability of *Bacillus cereus*……...77

Table 6.6: The effect of anacardic acid on the viability of *Pseudomonas aeruginosa*……...78

Table 6.7: The effect of anacardic acid on the viability of *Klebsiella pneumoniae*…….....79

Table 6.8: The effect of anacardic acid on the viability of *Serratia odorifera*……..80

Table 6.9: The effect of anacardic acid on the viability of *Candida albicans*……...81

LIST OF TABLES

Table 6.10: The effect of anacardic acid on the viability of *Mycobacterium smegmatis*……...82

Table 6.11: The effect of anacardic acid on the viability of *Mycobacterium aurum*…….83

Table 6.12: The effect of anacardic acid on the viability of *Mycobacterium tuberculosis* as determined using the BACTEC 460 method…….……..……84

Table 6.13: The control drugs and MIC's used for each organism…….……...84

Table 6.14: The effect of HPLC2 and HPLC3 on *Mycobacterium tuberculosis* H37Ra ATCC 25177 using the BACTEC460 system. The organism is sensitive to the test substance if the ? GI of the sample is less than the ? GI of the control vial…….……86

Table 6.15: A summary of the effect of compound 1, as well as the effects of fractions HPLC2 and HPLC3 individually and in combination with the $C_{15:1}$ anacardic acid in equal proportions, on a range of organisms. All values are reported in µg/ml. Ciprofloxacin was used as the positive control for the Gram-positives, Gram-negatives, *M. smegmatis* and *M. aurum*, nystatin for *C. albicans*, and rifampicin for *M. tuberculosis*. All experiments, excepting those involving *M. tuberculosis*, were performed in duplicate.....88

ABBREVIATIONS

ACN – acetonitrile

AFB – acid fast bacilli

AIDS – Acquired Immunodeficiency Syndrome

AG - arabinogalactan

BCG – Bacillus Calmette-Guérin

CHS - chalcone-synthase

CM – complete medium

DMEM - Dulbecos Modified Eagles Medium

DMSO – dimethylsulfoxide

DOTS – Directly Observed Therapy Short-course

EMB – ethambutol

GATB - The Global Alliance for TB Drug Development

GI – growth index

HIV – Human Immunodeficiency Virus

HPLC – high pressure liquid chromatography

HR-MS – high resolution mass spectrometry

INH – isoniazid

INT – p-iodonitrotetrazolium violet

LC-MS – liquid chromatography - mass spectrometry

MDR-TB – multi-drug resistant tuberculosis

MIC – minimum inhibitory concentration

MRSA – methicillin-resistant *Staphylococcus aureus*

MS – mass spectrometry

MTB – *Mycobacterium tuberculosis*

MTT - 3-[4,5-Dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide

ABBREVIATIONS

NMR – Nuclear magnetic resonance

OADC – oleic acid, albumin, dextrose, catalase supplement

OD – optical density

PBS – phosphate buffered saline

PKS - polyketide synthases

PYR – pyrazinamide

RIF – rifampicin

RNA – ribonucleic acid

TAACF - Tuberculosis Antimicrobial Acquisition and Coordinating Facility

TB – tuberculosis

TDR – The Special Programme for Research and Training in Tropical Diseases

TLC – thin layer chromatography

TSA – tryptone soya agar

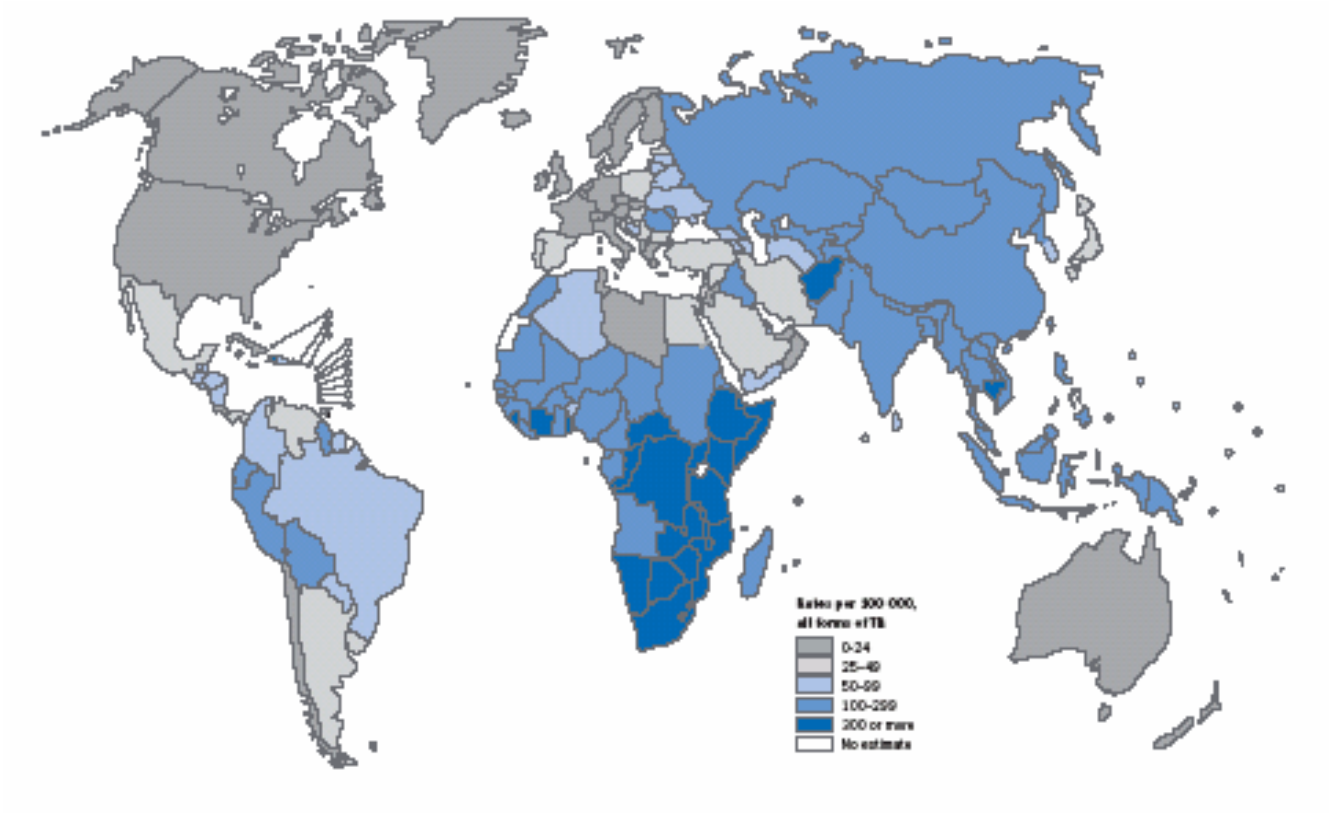
TSB – tryptone soya broth

WHO – World Health Organization

1.1 Tuberculosis

More than a hundred years after Robert Koch's discovery of the tubercle bacillus, *Mycobacterium tuberculosis* (MTB), the organism causing tuberculosis (TB) remains a major cause of morbidity and mortality. A third of the world's population is now estimated to be infected with this organism, the worldwide distribution in 2003 being represented in Figure 1.1. The World Health Organization (WHO) reported that 1.7 million people died of TB worldwide in 2003 (WHO, 2005). A total of 8.8 million new cases and 15.4 million prevalent cases of TB were estimated in 2003. The TB incidence rate appears to be increasing globally by 1% per annum, although the incidence in African countries has been rising far more quickly than the global trend as a result of high Human Immunodeficiency Virus (HIV) prevalence rates (WHO, 2005).

Figure 1.1: The estimated geographical distribution of tuberculosis cases in 2003 (WHO, 2005)



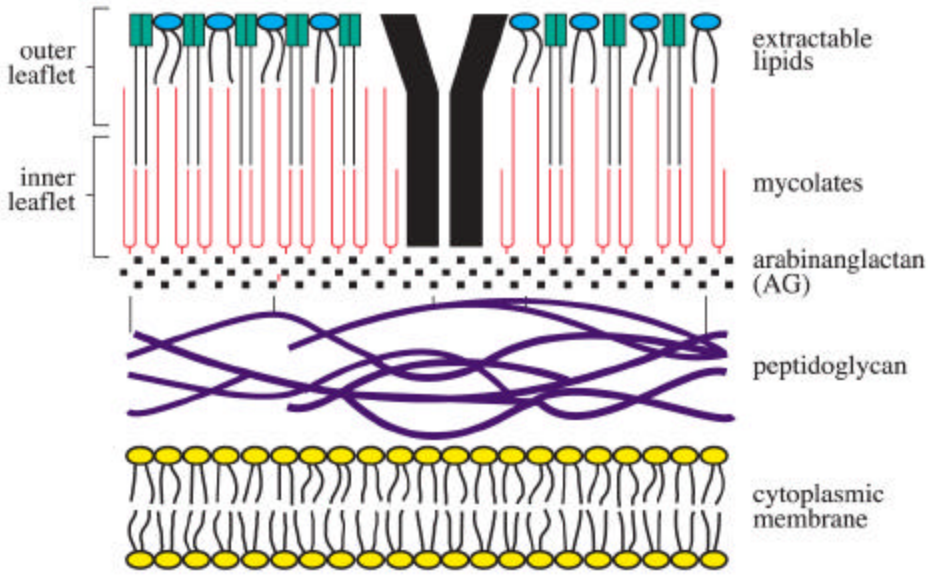
TB and HIV co-infection form a lethal combination, each accelerating the other's progress (Badri *et al.*, 2001). TB accounts for 13% of Acquired Immunodeficiency Syndrome (AIDS) deaths worldwide (WHO/TDR, 2004). In southern Africa, HIV infection is seen as the highest risk factor for TB. A person already infected with MTB has a 10% chance of developing active disease in a lifetime;

concurrent HIV infection increases this chance to 10% a year (WHO/TDR, 2004). In the WHO Africa region 31% of new TB cases are co-infected with HIV, which partly explains the fact that nine of the ten countries with the greatest burden of TB are in Africa (WHO, 2005). South Africa has the eighth highest rate of TB in the world with an estimated 61% of adult TB patients co-infected with HIV (WHO, 2005).

1.2 The organism

MTB is a rod-shaped bacillus. Many other species of saprophytic mycobacteria exist in addition to the TB-causing organism and rarely cause disease (Falkinham, 1996). The mycobacterial protective outer lipid bilayer is the thickest biological membrane known, rendering mycobacteria naturally resistant to many antibiotics. The cell wall can be divided into two portions. As seen in Figure 1.2, just above the cell membrane resides the peptigoglycan, covalently attached to arabinogalactan (AG), which is in turn attached to mycolic acids. This insoluble section is known as the cell wall core (Brennan, 2003). The outer leaflet consists of free lipids, interspersed with cell-wall proteins, phosphatidylinositol mannosides, the phthiocerol-containing lipids, lipomannan and lipoarabinomannan. These are the signaling and effector molecules in the disease process (Brennan, 2003).

Figure 1.2: A model of the mycobacterial cell wall, proposed by Minnikin, showing the primary constituents of lipids, mycolates, arabinogalactan and peptidoglycan. The funnel-shaped structure in the centre represents a porin, responsible for the movement of molecules across the cell wall (Hong and Hopfinger, 2004).



The tight packing of the mycolic acid residues of the AG decreases the permeability of the cell wall, thereby protecting the organism from passive transport of antibiotics as well as the host's immune system (Lowary, 2003). Pathogenic mycobacteria multiply in macrophages, which ordinarily destroy most microorganisms. The presence of a mycobacterial capsule, viewed under an electron microscope, can prevent the diffusion of harmful host-derived macromolecules into the organism. Furthermore, the organism contains superoxide dismutase and catalase/peroxidase enzymes capable of neutralizing host reactive oxygen species bombarding the mycobacterium (Daffé and Etienne, 1999).

The high lipid content of the cell wall binds fuchsin dye so that it is not destained by acid alcohol, which is why mycobacteria are referred to as "acid-fast bacilli" (AFB's) (Brennan and Nikaido, 1995). Bardou *et al*. (1996) have suggested that the function of mycolic acids may be involved in the export of proteins secreted by the organism. Pore proteins are thought to mediate the diffusion of particles across the cell wall and Engelhardt *et al.* (2002) found that *M. smegmatis* has significantly fewer protein pores than Gram-negative bacteria, and these pores are 'drastically different' from the channel proteins found in Gram-negatives.

The cell wall of mycobacteria represents numerous potential drug targets. The insoluble cell wall core needs to be maintained for organism viability, for instance, representing a very attractive target (Brennan, 2003). Enzymes responsible for the incorporation of mycolic acids in the cell wall are also expected to be potent targets.

1.3 Symptoms and diagnosis

TB bacilli are spread in droplet aerosols arising from infected individuals breathing, coughing, sneezing or spitting. The immune system isolates the TB bacilli which can remain dormant for years until the person's immune system deteriorates. If left untreated, a person with active disease can infect ten to fifteen people every year (WHO, 2004b). Symptoms associated with TB include a productive cough for longer than three weeks, loss of appetite with resultant weight loss, night sweats, fatigue, chest pains and haemoptysis (blood in sputum). A diagnosis is made by means of smear microscopy with the visualization of AFB's in sputum, culture of organisms on agar or in liquid broth and in some cases, with chest X-rays indicating lung damage. The progression of a TB granuloma is variable, and in some patients the disease remains localized and may even resolve. Lesions may spread in aggressive cases causing massive lung tissue destruction which may be fatal (WHO, 2004b).

1.4 Treatment of tuberculosis

Although an effective vaccine would be the first choice for the prevention of TB, chemotherapy to treat infection is still currently the key weapon to controlling this scourge as the efficacy of the BCG (bacilli Calmette-Guérin) vaccine currently in use is debatable (Fine, 1995; Maes, 1999). Many efforts are under way to either improve the protection afforded by this vaccine, or to develop new vaccines (Orme, 2005).

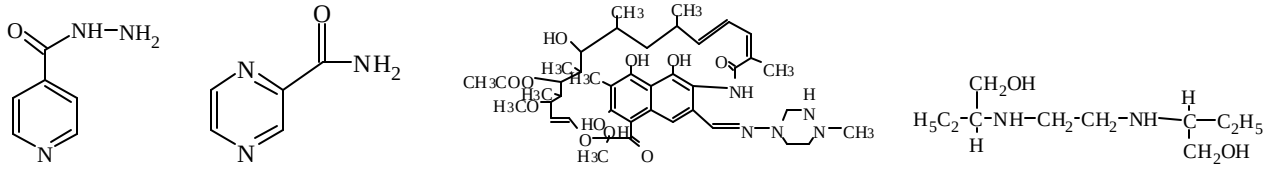
The WHO Directly Observed Therapy short-course (DOTS) strategy has been implemented worldwide and aims at curing 85% of new smear positive cases in 2005 and to have halted and begun to reverse incidence by 2015 (WHO, 2005). The purpose of this internationally recommended TB control strategy is to provide standardized regimens and proper case management to ensure completion of treatment and cure (WHO, 2003). Treatment regimens for new patients consist of an initial phase where a fixed-dose combination of isoniazid (INH), rifampicin (RIF), pyrazinamide (PYR) and ethambutol (EMB) is given for two months. Patients become non-infectious within two weeks. This is followed by a continuation phase of sterilizing drugs such as INH and RIF, given for four to six months (WHO, 2003). Re-treatment patients are given five drugs in the initial and three drugs in the continuation phase (WHO, 2003). Patients are monitored for the entire duration of treatment and take their medication under the supervision of a healthcare worker. Such a lengthy treatment period is necessary as a proportion of the infecting organisms are not effectively eliminated by the current TB drugs (Zhang and Amzel, 2002). The major factor determining the outcome of treatment is adherence to the treatment regimen (American Thoracic Society, 1994). National TB Programmes will help to minimise delays in the diagnosis and treatment of TB which is necessary to prevent uninfected individuals in the community being exposed for prolonged periods and progression of disease in the infected persons, which could lead to increased morbidity and mortality (Lawn *et al.*, 1997).

1.5 Anti-tuberculosis drugs

Anti-TB drugs must have three properties: bactericidal activity, sterilizing activity and the ability to prevent resistance. INH and RIF are the most effective drugs with activity against all populations of MTB (WHO, 2003). RIF, a first-line drug, is a semi-synthetic derivative of rifamycin and is the most potent sterilizing drug available. RIF binds to the ß-subunit of MTB ribonucleic acid (RNA) polymerase, thereby inhibiting transcription initiation. Mutations in the MTB *rpoB* gene are

responsible for resistance to rifampicins (Zhang and Amzel, 2002; Ramaswamy and Musser, 1998). INH is a synthetic agent used as a first-line drug in TB therapy active against susceptible organisms at 0.05µg/ml, although almost all other mycobacteria are naturally resistant. Mutations in the *inhA* and *katG* genes have been associated with resistance to INH, and data from countries surveyed between 1994 and 2002 indicated that more isolates are resistant to INH than any other drug (WHO, 2004a). INH affects the biosynthesis of mycolic acids with multiple drug targets, including the acyl carrier protein reductase and ß-ketoacyl synthase (Zhang and Amzel, 2002). It has been shown to reduce the weight of mycolic acids by 20-35% (Bardou *et al.*, 1996; Chatterjee, 1997). PZA is a structural analogue of nicotinamide and kills semi-dormant tubercle bacilli under acidic conditions. The bacteria convert PZA to pyrazinoic acid, the active derivative. This drug disrupts the membrane function and energy metabolism and possibly also inhibits fatty acid synthesis. Mutations in the *pncA* gene are responsible for resistance to this drug (Zhang and Amzel, 2002; Ramaswamy and Musser, 1998). EMB targets an arabinosyl transferase responsible for incorporation of mycolic acids into the mycobacterial cell wall and needs to be used in combination with more powerful drugs to prevent the emergence of resistant MTB (WHO, 2003). Mutations in the genes *embCAB* have been associated with resistance to EMB (Zhang and Amzel, 2002; Chatterjee, 1997). Of the second-line drugs, ethionamide is a structural analogue of INH and has a similar mode of action. Isoxyl, a thiourea, inhibits mycolic acid synthesis, but also inhibits the synthesis of shorter-chain fatty acids (Phetsuksiri *et al.*, 1999). Streptomycin inhibits protein synthesis in rapidly multiplying MTB, with the target being ribosomal S12 protein and 16S rRNA (Zhang and Amzel, 2002).

Figure 1.3: The structures of INH (A), PYR (B), RIF (C) and EMB (D)

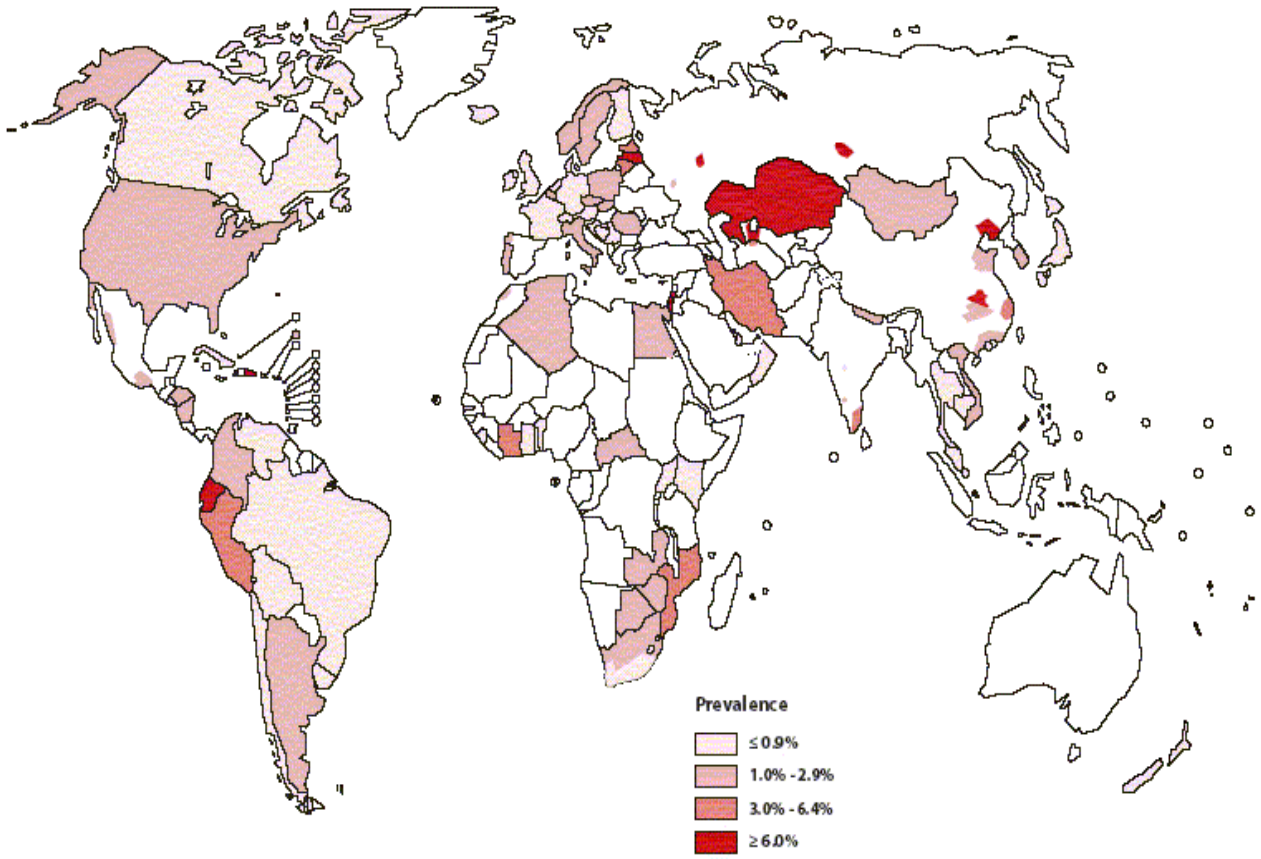


1.6 Drug-resistant tuberculosis

Anti-TB drugs have been in use for approximately 50 years, and yet, in this time, resistant strains have been reported from every country in the world. More concerning is the emergence of strains resistant to every major class of TB drug available, referred to as multi-drug resistant TB (MDR-TB), and defined as a strain resistant to at least INH and RIF (WHO, 2004a). Drug resistance arises as a result of spontaneous genetic mutations in MTB due to inconsistent or partial treatment, such as patients not

completing the prescribed course of treatment, medical staff not prescribing the correct treatments, or unreliable drugs (WHO, 2004a).

Figure 1.4: Prevalence of MDR-TB among new TB cases between 1994 and 2002 (WHO, 2004a)



The 'Third Global Report of Anti-tuberculosis Drug Resistance in the World' (WHO, 2004a) surveyed 75 settings and found that drug-resistant MTB was present, and increasing, in all regions of the world with a median prevalence of any resistance and MDR-TB among new TB cases of 10.2% and 1.1% respectively, as illustrated in Figure 1.4. It also highlighted a greater increase in the prevalence of drug resistance in patients who had previously received antituberculosis treatment than new cases. As indicated in previous reports, MDR-TB is of an especially high magnitude in most countries of the Russian Federation (WHO, 2004a; Morozova *et al.*, 2003). Kazakhstan and South Africa have the highest number of MDR-TB cases in the world (WHO, 2004a), although Africa in general has relatively low rates (WHO, 2004a; Aziz *et al.*, 2004; Mac-Arthur *et al.*, 2001). The emergence of MDR-TB in many cities of developing countries is a cause for concern as a lack of policies and facilities for the treatment and management of MDR-TB could lead to its uncontrolled spread across densely populated areas (Githui *et al.*, 2004). In areas where HIV-infection is of

epidemic proportions, such as Sub-Saharan Africa in general, even small increases in resistance could have enormous implications if HIV positive patients become co-infected with drug resistant strains of MTB.

Patients diagnosed with drug-resistant TB are given specially designed standardized or individualized regimens starting with at least four drugs in an initial phase lasting six months, followed by at least three drugs in the continuation phase of 12 – 18 months (WHO, 2003). The second-line drugs (such as amikacin, ciprofloxacin, ethionamide, kanamycin and ofloxacin) used to treat MDR-TB are less effective, need to be taken for longer periods of time (Nolan and Goldberg, 2002) and often result in adverse reactions, resulting in lower cure rates than for non-MDR-TB (WHO, 2003). The WHO estimated that the standardized treatment regimen for MDR-TB in South Africa cost about US$3400 per patient for drugs only (WHO, 2004).

1.7 Traditional medicine in South Africa

Approximately 4000 species of plants are used as medicines in southern Africa (van Wyk and Gericke, 2000). In KwaZulu-Natal alone, approximately 1020 plant and 150 animal species are utilized in traditional medicines. The total volume of plant material traded on an annual basis is estimated to be around 500 tonnes (www.kznwildlife/muthi_trade). 200 000 to 350 000 traditional healers practise in South Africa, with a statutory council elected by parliament to regulate their practises (van Wyk *et al.*, 2002; Baleta, 1998). Traditional healers in South Africa are referred to as 'inyanga' and 'sangoma', meaning herbalist and diviner respectively (van Wyk *et al.*, 2002). Each culture in this country has medicinal solutions for the prevention and curing of disease, as well as the maintenance of health, relating to physical health and spiritual well-being (Cocks and Møller, 2002). About 80% of the black population utilise the services of traditional healers, often as a primary source of affordable and accessible healthcare. The healers command a great deal of respect from the community (Kale, 1995). Traditional remedies given by healers can be consumed orally, smoked, inhaled, applied as washings, smeared on the body or administered as enemas (van Wyk *et al.*, 2002). However, adverse effects of these treatments have been reported and in some cases, delays in consulting professionally qualified physicians as a result of first visiting local healers have had fatal consequences (Smyth *et al.* 1995).

Edginton *et al*. (2002) found that many people in this country still believe that one acquires TB as a result of breaking cultural rules and that the resulting disease can only be treated by traditional healers, which leads to consequent delays in presentation to local healthcare services. Recognising the potential role of traditional healers in healthcare in South Africa, an effort was made to incorporate traditional healers into TB programmes as DOTS supervisors. This pilot scheme initiated in Hlabisa, KwaZulu-Natal (South Africa) was reported by Colvin *et al.* (2003). Treatment completion was not significantly higher among patients supervised by traditional healers than among patients supervised by other categories of DOTS supervisors, but patients who had completed treatment revealed high levels of satisfaction with the personalized care received.

In the past, conservation of medicinal plants was achieved by social, religious and seasonal restrictions, but with the increasing demand to supply a trade estimated to be around R63 million a year, over-harvesting of medicinal plants has led to some species being listed as endangered (ww.kznwildlife/muthi_trade). Efforts are being made by the South African government to control the illicit harvesting of endangered medicinal plants, while making indigenous knowledge available for research and development. In the process, communities can create wealth from their indigenous knowledge and the natural habitat will be protected (Coetzee *et al*., 1999).

1.8 Drugs derived from natural products

For centuries, natural products have been used to treat varying ailments. Well-known drugs derived from natural sources include aspirin, morphine, codeine, digoxin, atropine, quinine and artimisinin (Balick and Cox, 1997; Clark, 1996; Mueller *et al.*, 2000). Natural products have had an important impact on the longevity and quality of life for cancer patients. Most (62%) approved anticancer drugs are of natural origin or models thereof (Cragg *et al*., 1997). Many anticancer agents were developed as a result of large-scale screening of extracts of plants, microorganisms and marine organisms (Clarke, 1996). Similarly, the discovery of antibiotics had an enormous impact on life expectancy and quality of life. The antibiotic era was launched with the discovery of penicillin from the fungus *Penicillium*. Since then, hundreds of antibiotics have been isolated from many microorganisms (Clarke, 1996). Cragg *et al.* (1997) estimated that between 1983 and 1994, 78% of new antibacterial drugs were of natural origin. These agents paved the way for research into structural analogues, the pathogenicity of organisms, as well as human pharmacology. However, new and re-emerging infectious diseases and resistance to currently available therapeutics (Bouchillon *et al*., 2004; Ehrhardt and Russo, 2001;

Walsh and Amyes., 2004) has made it apparent that the life span of any antibiotic is limited, and that there is a desperate need for the development of new drugs (Cragg, *et al.*1997).

1.9 Plants with antimycobacterial activity

Various reviews have been published on antimycobacterial extracts and compounds derived from natural products (Copp, 2003; Cantrell *et al.*, 2001; Okunade *et al.*, 2004; Newton *et al.*, 2000), examples of which are shown in Tables 1.1 and 1.2 respectively, while some of the chemical structures are shown in Figure 1.5. The compound with the greatest activity reviewed by Okunade *et al.* (2004) was a peptide with an MIC of 0.1µg/ml, whereas Cantrell *et al.* (2001) found that the most active compound in that review was a norditerpenoid with an MIC of 0.46µg/ml. To put these findings into perspective, the front-line TB drugs, namely INH, RIF, PYR and EMB, have MIC's of 0.05, 0.25, 100 and 3.8µg/ml respectively. It has been observed that more lipophilic components were associated with increased activities as opposed to more polar substituents, findings verified by numerous authors (Cantrell *et al.*, 2001; Rajab *et al.*, 1998; Lu *et al.*, 1998; Appendino *et al.*, 2004).

One such hydrophobic compound with antimycobacterial activity is *(E)*-phytol (Figure 1.5, structure B), with an MIC of 2µg/ml (Rajab *et al.*, 1998). The authors found that a free hydroxy group and overall lipophylicity were the two most important structural characteristics responsible for *in vitro* antituberculosis activity of derivatives of this compound. Similarly, the activity of compounds from *Ferula communis* and synthesized derivatives was dependent on the degree of lipophilicity (Appendino *et al.*, 2004), but the authors concluded that a hydroxyl or acetoxyl group caused a reduction in inhibitory activity, while large lipophilic groups at the same position were well tolerated. Similarly, Mata *et al.* (2004) suggested that a free hydroxyl group was not important for activity and that an extra methoxy group decreased the antimycobacterial activity. Ma *et al.* (2005), however, found that the presence of a methoxy group increased activity.

Another example of the activity of lipophilic compounds against mycobacteria was illustrated by compounds isolated from members of the Asteraceae family, specifically members of the tribe Astereae (Lu *et al.*, 1998). Three of these compounds exhibited activity against *M. tuberculosis* with MIC's of 25, 25 and 12.5 µg/ml respectively. The stereochemistry in the ester moiety at C-10 in derivatives of all three compounds resulted in significant differences in activity. The authors projected that these compounds inhibit thiol groups and other essential nucleophilic centers of

Table 1.1: Examples of plant extracts exhibiting antimycobacterial activity

Plant	Traditional use	Extractant	Organism	MIC	Remarks	References
Amborella trichopoda	Vanuatu medicine to treat TB-like symptoms	methanol	*M. bovis* BCG	1-2.5µg/ml	-	Billo *et al*., 2005
Chelidonium majus	Treatment of TB	ethanol	MTB	50µg/ml	aerial parts, Turkey	Tosun *et al*., 2004
Chenopodium ambrosoides	Treatment of pulmonary diseases	acetone	MDR-MTB	100µg/ml	aerial parts, South Africa	Lall and Meyer, 1999
Commiphora mukul	Treatment of TB and leprosy	methanol	*M. aurum*	62.5µg/ml	resin	Newton *et al.*, 2002
Croton pseudopulchellus	Treatment of pulmonary diseases	acetone	MTB	100µg/ml	aerial parts, South Africa	Lall and Meyer, 1999
Ekebergia capensis	Treatment of pulmonary diseases	acetone	MTB MDR-MTB	100µg/ml 100µg/ml	bark, South Africa	Lall and Meyer, 1999
Euclea natalensis	Treatment of pulmonary diseases	acetone	MTB MDR-MTB	100µg/ml 100µg/ml	roots, South Africa	Lall and Meyer, 1999
Helichrysum melanacme	Treatment of pulmonary diseases	acetone	MDR-MTB	100µg/ml	whole plant, South Africa	Lall and Meyer, 1999
Myristica fatua	Vanuatu medicine to treat TB-like symptoms	methanol dichloromethane	*M. bovis* BCG	≤100µg/ml	100µg/ml lowest concentration tested	Billo *et al*., 2005
Nidorella anomala	Treatment of pulmonary diseases	acetone	MTB MDR-MTB	100µg/ml 100µg/ml	whole plant, South Africa	Lall and Meyer, 1999
Polygala myrtifolia	Treatment of pulmonary diseases	acetone	MTB MDR-MTB	100µg/ml 100µg/ml	aerial parts, South Africa	Lall and Meyer, 1999
Psoralea corylifolia	Treatment of TB and leprosy	methanol	*M. aurum*	62.5µg/ml	leaves	Newton *et al.*, 2002
Salvia aethiopis	Treatment of tuberculosis	ethanol	MTB	50µg/ml	aerial parts, Turkey	Tosun *et al*., 2004
Sanguinaria canadensis	Treatment of TB and leprosy	methanol	*M. aurum*	62.5µg/ml	root	Newton *et al.*, 2002
Stachys sylvatica	Treatment of TB	ethanol	MTB	50µg/ml	aerial parts, Turkey	Tosun *et al*., 2004
Ulmus glabra	Treatment of TB	ethanol	MTB	50µg/ml	leaves, Turkey	Tosun *et al*., 2004
Urtica dioica	Treatment of TB	ethanol	MTB	50µg/ml	aerial parts, Turkey	Tosun *et al*., 2004

Figure 1.5: The chemical structures of some antimycobacterial natural products

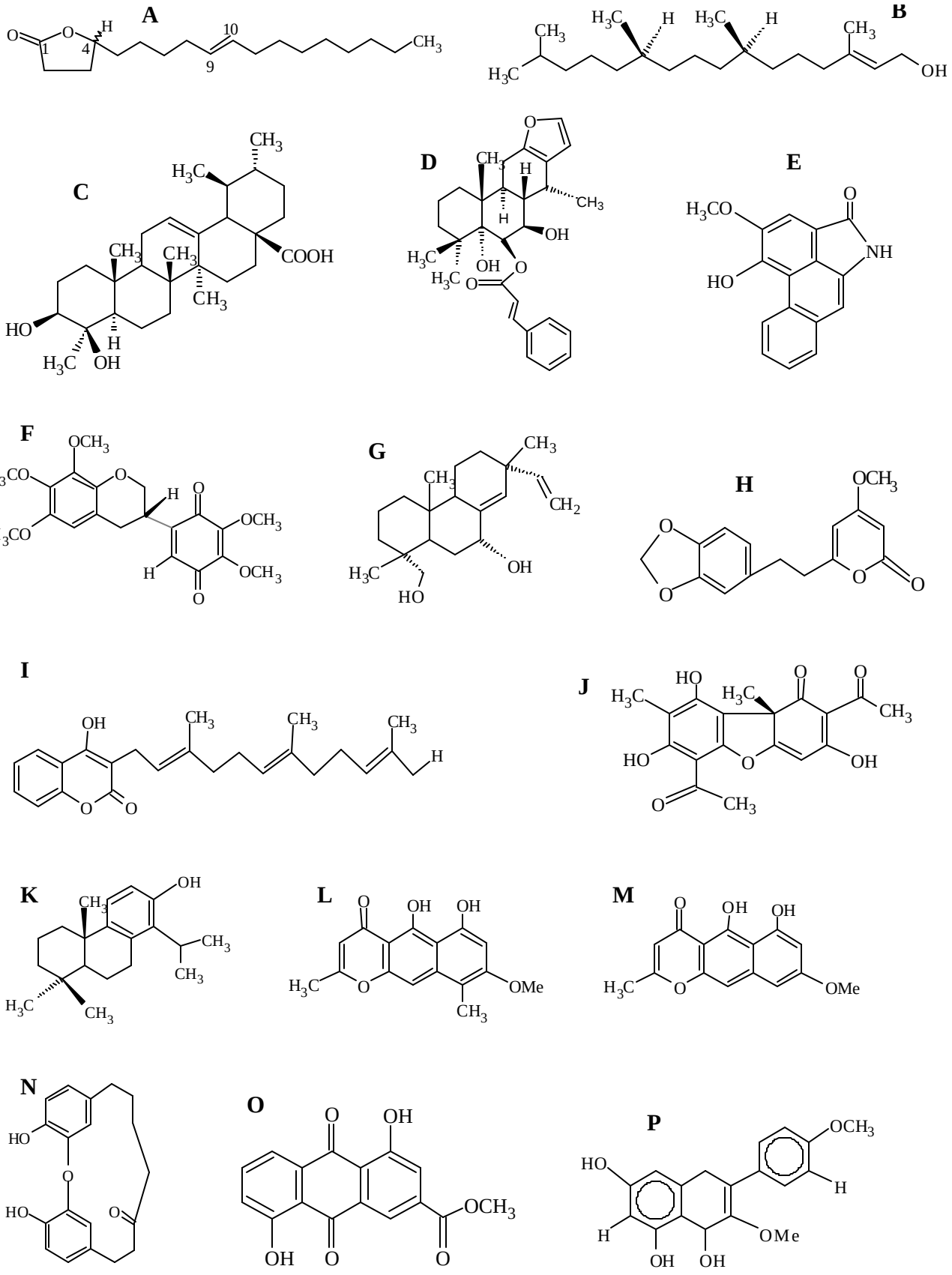


Table 1.2: Antimycobacterial activities of compounds isolated from plants

Plant	**Compound**	**Organism**	**MIC**	**References**
Abrus precatorius	abruquinone B, (Figure 1.5 F)	MTB	12.5µg/ml	Limmatvapirat *et al*., 2004
Ajuga remota	ergosterol-5,8-endoperoxide	MTB	1µg/ml	Cantrell *et al*., 2001
Caesalpinia pulcherrima	6β-cinnamoyl-7β-hydroxyvouacapen-5α-ol (Figure 1.5D)	MTB	6.25µg/ml	Promsawan *et al.*, 2003
Cetraria islandica	protolichesterinic acid	*M. aurum*	=125µg/ml	Ingólfsdóttir *et al.*, 1998
Chamaecyparis nootkatensis	totarol (Figure 1.5K)	MTB	16µg/ml	Constantine *et al.*, 2001
Chromolaena odorata	isosakuranetin	MTB	174.8µM	Suksamram *et al.*, 2000
Cladonia arbuscula	ursnic acid (Figure 1.5J)	*M. aurum*	32µg/ml	Ingólfsdóttir *et al.*, 1998
Engelhardia roxburghiana	engelhardione (Figure 1.5N)	clinical MTB MTB	3.125µg/ml 0.2µg/ml	Lin *et al*., 2005
Engelhardia roxburghiana	(-)-5-hydroxy-4-methoxy-1-tetralone	clinical MTB MTB	3.125µg/ml 0.2µg/ml	Lin *et al*., 2005
Engelhardia roxburghiana	3-methoxycarbonyl-1,5-dihydroxyanthraquinone (Figure 1.5O)	Clinical MTB MTB	6.25µg/ml 4µg/ml	Lin *et al*., 2005
Evodia rutaecarpa	evocarpine	*M. fortuitum* *M. smegmatis* *M. phlei*	2µg/ml 2µg/ml 2µg/ml	Adams *et al.*, 2005
Ferula communis	ferulenol (Figure 1.5I)	*M. fortuitum* *M. phlei* *M. aurum* *M. smegmatis*	2µg/ml 2µg/ml 2µg/ml 0.5µg/ml	Appendino *et al.*, 2004
Haplopappus sonorensis	ermanin, or 5,7-dihydroxy-3,4'-dimethoxyflavone (Figure 1.5 P)	MTB	Inhibited 98% growth at 100µg/ml	Murillo *et al.*, 2003
Lucas volkensii	(E)-phytol (Figure 1.5B)	MTB	2µg/ml	Rajab *et al.*, 1998
Micromelum hirsutum	micromolide (Figure 1.5A)	MTB Rv EC_{90} of 5.6µg/ml in a mouse macrophage model	1.5µg/ml	Ma *et al*., 2005

Parmelia saxatilis	salazinic acid	*M. aurum*	=125µg/ml	Ingólfsdóttir *et al.*, 1998
Piper sanctum	2-oxo-14-(3`,4`-methylenedioxyphenyl)dodecane	MTB	6.25µg/ml	Mata *et al.*, 2004
Piper sanctum	2-oxo-16-(3`,4`-methylenedioxyphenyl)hexadecane	MTB	6.25µg/ml	Mata *et al.*, 2004
Piper sanctum	5,6-dehydro-7,8,dihydromethysticin (Figure 1.5H)	MTB	4µg/ml	Mata *et al.*, 2004
Piper sanctum	piperolactam A (Figure 1.5E)	MTB	8µg/ml	Mata *et al.*, 2004
Psoralea corylifolia	bakauchiol	*M. aurum* *M. smegmatis* *M. bovis BCG*	IC_{50} = 15.8µg/ml IC_{50} = >500µg/ml IC_{50} = 21.4µg/ml	Newton *et al.*, 2002
Salvia multicaulis	multiorthoquinone	MTB	2µg/ml	Cantrell *et al.*, 2001
Sanguinaria canadensis	chelerythrine sanguinarine	*M. aurum* *M. smegmatis* *M. bovis BCG* *M. aurum* *M. smegmatis* *M. bovis BCG*	IC_{50} = 7.3µg/ml IC_{50} = 29µg/ml IC_{50} = 14.3µg/ml IC_{50} = 9.61µg/ml IC_{50} = 41.2µg/ml IC_{50} = 24.5µg/ml	Newton *et al.*, 2002
Sanguinaria canadensis	chelerythrine	*M. aurum* *M. smegmatis*	7.3µg/ml 29µg/ml	Newton *et al.*, 2002
Saussurea lappa	dehydrocostuslactone	MTB	2µg/ml	Cantrell *et al.*, 2001
Senna obliqua	quinquangulin (structure 1.5 L) rubrofusarin (structure 1.5M)	MTB	12.0µg/ml	Graham *et al.*, 2004
Stereocaulon alpinum	atranorin lobaric acid	*M. aurum*	=125µg/ml	Ingólfsdóttir *et al.*, 1998
Tetradenia riparia *Valeriana laxiflora*	8(14),15-sandaracopimaradiene-7α,18-diol (structure 1.5G) 23-hydroxyursolic acid (Figure 1.5C)	*M. smegmatis* MTB	12.5µg/ml 15.5µg/ml	Van Puyvelde *et al.*, 1986 Gu *et al.*, 2004

MTB = *M. tuberculosis*

mycobacterial enzymes. Promsawan *et al.*, (2003) also found that the benzoyl analogue of the cassane diterpene, 6β-cinnamoyl-7β-hydroxyvouacapen-5α-ol shown as structure D in Figure 1.5, had less activity against MTB.

Tables 1.1 and 1.2 indicate that plants have the ability to provide researchers with a largely untapped resource of chemically diverse compounds, of which many have been shown to have potent antimycobacterial properties. These lead compounds with *in vitro* activities need to be further investigated for their potential to be developed into viable drugs

1.10 Tuberculosis drug development

1.10.1 Research Groups

Very little TB drug development has occurred over the last 30 years. Pharmaceutical companies lack profit motive and the cost of development and clinical trials is prohibitive (O'Brian and Nunn, 2001). The Global Alliance for TB Drug Development (GATB, www.tballiance.org) has been established to encourage collaborative research between academic, commercial and non-profit organizations (Duncan and Barry, 2004; Zhang and Amzel, 2002) and to facilitate the the progression of promising drugs through the development pipeline (Duncan, 2003). The National Institutes of Health have also established a Tuberculosis Antimicrobial Acquisition and Coordinating Facility (TAACF) which facilitates high-throughput and structured screening of compounds against *M. tuberculosis* (www.taacf.org; Orme, 2001). At the time of publication, 5251 lead compounds had been screened, of which 11% had exhibited *in vitro* activity against *M. tuberculosis* H37Rv. The cytotoxicity of these lead compounds is then determined after which a Selectivity Index (SI) value is assigned, designated as the quotient of the minimum cytotoxic and antimycobacterial concentrations. A value of greater than ten initiates further testing procedures. The first of these involves a macrophage infection model followed by testing in a mouse model where mice are exposed to a low-dose aerosol infection with *M. tuberculosis*. Nine of the initial 5251 compounds (0.17%) were shown to significantly reduce the bacterial load in infected mice (Orme, 2001) and these lead compounds are being further pursued as potential antimycobacterial drugs.

1.10.2 Objectives for tuberculosis drug development

According to the 'Scientific Blueprint for Tuberculosis Drug Development' laid out by GATB, a new anti-TB drug should aim to achieve three objectives: firstly, to shorten the

duration of therapy or decrease the number of doses required under DOTS supervision to encourage patient and healthcare provider compliance; secondly, the new treatment must be able to treat MDR-TB; and it should be effective against latent infection (The Global Alliance for TB Drug Development, 2001). However, a novel drug released into poorly controlled programmes will only accelerate the emergence of resistance to that compound (O'Brian and Nunn, 2001). The focus of antituberculosis drug development should be on targeting dormancy or persistence, transcription, virulence factors, two-component systems, cell wall synthesis, weaknesses of the tubercle bacillus and the use of TB genomics for the identification of drug targets (Zhang and Amzel, 2002).

1.10.3 Prospective drugs

Encouraging work has been performed on fluoroquinolone compounds, oxazolidinone compounds, clofazimine, phenazine, phenathiazines, azoles and peptides, nitroimidazopyrans and long-acting rifamycins (rifapentine, rifabutin, rifalazil) (O'Brian 2003; O'Brian and Nunn, 2001; Tomiaka, 2000; Hudson *et al*., 2003; Duncan, 2003; Zhang and Amzel, 2002). Rifapentine, which was approved for the treatment of TB in the USA in 1998, is a rifamycin antibiotic that inhibits mycobacterial RNA synthesis. Rifapentine has a half-life of 16 to 20 hours, compared to that of rifampicin which is 2 to 3 hours, which makes it possible to reduce the doses to once-weekly administration during the continuation phase of treatment. Although it is more active against MTB than rifampicin, it is not effective against drug-resistant MTB as rifampicin resistant strains are also cross-resistant to rifapentine (Jarvis and Lamb, 1998; Tam *et al*., 1998; Tam, 1998; Chaulet; 1998). This emphasizes the need for new drugs with novel targets (Zhang and Amsel, 2002).

An application to the Food and Drug Administration (FDA) has been filed by GATB for the new investigational drug, the nitroimidazole PA-824 (Chiron Corporation, Emeryville, California) (Elias, 2005). Sequella Incorporated (Rockville, Md) are also currently performing preclinical work on the ethambutol analogue, SQ109. A few drug candidates are in the clinical trial stage, including pyrrole LL-3858 (Lupin Limited, Mumbai, India), moxifloxacin and gatifloxacin, which are already approved for the treatment of other ailments, and a diarylquinoline (Johnson & Johnson Pharmaceutical Research and Development Division of Jannssen Pharmaceutical N.V), R207910, under development as TMC207 (Tibotec Pharmaceuticals Limited, Mechelen, Belgium) (Hampton, 2005). The

latter, chemically known as 1-(6-bromo-2-methoxy-quinolin-3-yl)-4-dimethylamino-2-naphthalen-1-yl-1-phenyl-butan-2-ol, has MIC's of 0.030 to 0.120µg/ml against reference and antibiotic-susceptible strains of MTB. Furthermore, it has also displayed very potent activity against MDR-TB isolates, as well as a broad range of non-tuberculous mycobacterial strains. This compound also has a novel drug target, namely mycobacterial ATP synthase, and this means that it has no cross-resistance with existing anti-TB drugs (Andries *et al*., 2005).

1.10.4 Strategies to tuberculosis drug discovery

Varying approaches can be used for the discovery of new medicines. These include biodiversity screening, based on either random screening or on plant therapies used by cultural groups over many years, known as 'ethnopharmacology', to find lead compounds with a pre-defined set of biological activities which can then be synthesized or modified to elicit the desired activities (Clark, 1996; Harvey, 1999). The use of natural products for drug discovery provides a large, structurally diverse pool of potential precursors of new drugs. A survey with the aim of assessing the usefulness of ethnobotany in drug discovery found 122 compounds, 80% of which were used for the same or similar ethnomedical purposes, which were derived from only 94 species of plants (Fabricant and Farnsworth, 2001). These approaches should always be observant of the preservation and diversity of the environment (Rates, 2001). With the rate of rapid industrialization worldwide, there is a real fear that ethnomedical knowledge held by ethnic cultures and customs will be lost (Fabricant and Farnsworth, 2001).

Another approach is based on the characterisation of a molecular target for the medicinal agent by rational drug design using computer-aided techniques or by the manipulation of genetic targets (Harvey, 1999; Barry *et al*., 2000). The determination of the complete genome sequence of MTB (Cole *et al*., 1998) has enabled researchers to develop new drugs by means of comparative genomics (Cole, 2002), proteonomics, transcritomics and structural genomics (Duncan, 2003; Warner and Mizrahi, 2004). This project will follow a structured approach using ethnomedical claims to identify possible lead compounds with promising activity against MTB.

1.11 Project objectives

The intention of this study is to use an ethnopharmacological approach to determine a scientific basis for the use of selected medicinal plants in the search for compounds active against *M. tuberculosis* and other selected organisms pathogenic to humans.

This study aims to:

- Investigate the *in vitro* activities of 19 medicinal plants used in southern Africa against a range of organisms encompassing mycobacteria, Gram-positive bacteria, Gram-negative bacteria and fungi
- Identify, isolate and characterise a compound from a plant extract exhibiting appreciable antimycobacterial activity using a structured approach
- Determine the antimicrobial and antimycobacterial activity of this isolated compound
- Demonstrate the cytotoxicity of the isolated compound in the Chinese Hamster Ovarian cell line

2.1 Introduction

Figure 2.1 describes the entire experimental process followed through the course of this project. This section describes the literature search for medicinal plants fitting the specified criteria and the preparation of methanol and acetone extracts of each plant part.

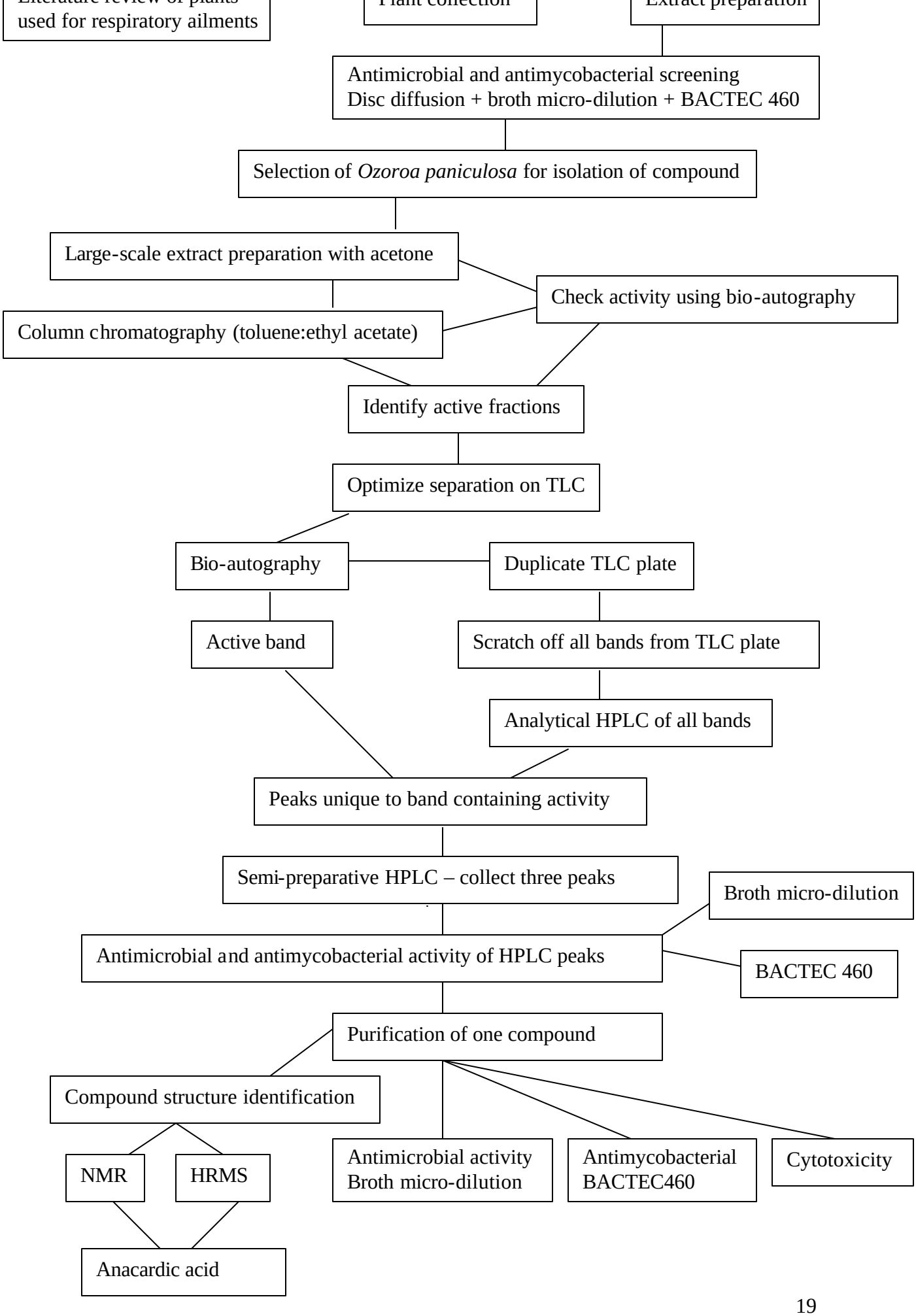
2.2 Materials and methods

2.2.1 Plant selection

For the compilation of a list of twenty-three plants from nineteen species, three primary literature sources were consulted: 'Zulu Medicinal Plants: An Inventory' by Hutchings (1996), 'Medicinal Plants of South Africa' by van Wyk *et al*. (2002) and 'The Medicinal and Poisonous Plants of Southern and Eastern Africa' by Watt and Breyer-Brandwijk (1962). Plants were selected based on their traditional use in southern Africa to treat symptoms associated with respiratory ailments that could be indicative of TB. The keywords used in this search were 'respiratory', 'cough', 'chest', 'asthma', 'catarrh' and 'haemoptysis'. Methods of administration of the various treatments included the oral consumption of decoctions, enemas, as well as the inhalation of smoke from burning plant material, described in section 2.3.1.

2.2.2 Extract preparation

Plants were collected and plant identities were confirmed. Voucher specimens are housed at the National Herbarium in Pretoria and / or at the Department of Pharmacy and Pharmacology, WITS University. Plant material was dried at room temperature or 50ºC. The dried plant material was ground with a coffee grinder until a minimum of 10g of finely ground material was obtained. The plant material was divided into 2 x 5g in Erlenmeyer Flasks to which 40ml of methanol or acetone was added. The flasks were placed in a 40ºC water bath for 1 hour after which the insoluble material was filtered off. The samples were washed in this manner three times. The extract was taken to dryness using a rotary evaporator under reduced pressure after which it was resuspended in a small volume of the same solvent. The extract was then transferred into pre-labelled and pre-weighed glass vials and allowed to dry. The total extract mass was measured and recorded, as shown in Table 2.1.


used for respiratory ailments
Plant collection
Extract preparation
Antimicrobial and antimycobacterial screening
Disc diffusion + broth micro-dilution + BACTEC 460
Selection of *Ozoroa paniculosa* for isolation of compound
Large-scale extract preparation with acetone
Check activity using bio-autography
Column chromatography (toluene:ethyl acetate)
Identify active fractions
Optimize separation on TLC
Bio-autography
Duplicate TLC plate
Active band
Scratch off all bands from TLC plate
Analytical HPLC of all bands
Peaks unique to band containing activity
Semi-preparative HPLC – collect three peaks
Broth micro-dilution
Antimicrobial and antimycobacterial activity of HPLC peaks
BACTEC 460
Purification of one compound
Compound structure identification
NMR
HRMS
Antimicrobial activity
Broth micro-dilution
Antimycobacterial
BACTEC460
Cytotoxicity
Anacardic acid

2.3 Results

2.3.1 Recorded usage of selected plants

2.3.1.1 *Acacia robusta* Burch. subsp. *robusta*

FAMILY**:** Mimosaceae

Steam from the crushed boiled bark of subspecies *clavigera* is inhaled for chest complaints by the Zulu people. The roots are reported to be poisonous and there is a possibility that cyanogenic glycosides and hydrocyanic acid are present in immature pods and fresh dry leaves (Watt and Breyer-Brandwijk, 1962; Hutchings 1996). The bark of this plant was collected from the Nylstroom district in Limpopo Province by P.M. Burgoyne.

2.3.1.2 *Adiantum capillus-veneris* L.

FAMILY: Adiantaceae

Common names: maiden-hair fern, 'vrouehaar'

The plant is used by the Zulu people to make tea that is taken for chest colds (Hutchings, 1996). The Southern Sotho smoke the dried leaves for head and chest colds and it has been used in Europe to treat chronic pulmonary catarrh and other respiratory ailments and is reported to suppress coughing. Furthermore, it has been used as an expectorant (Watt and Breyer-Brandwijk, 1962). The fern was used in tea as an early Cape remedy for colds and chest ailments. The plant is known to contain gallic, tannic and quinnic acids, a bitter substance, essential oil and gum. Many terpenoids have been isolated from this plant around the world (Nakane *et al.*, 2002) including adiantone, while glycosides include an acylated glycoside, quercetin, seven flavonol glycoides, nicotiflorin, isoquercitrin, rutin and kaempferol 3-*O*-rutinoside sulphate and four hydroxycinnamic acid-sugar derivatives. An extract from this plant has been reported to produce glycaemic and diuretic activity in animals (Hutchings, 1996; van Wyk *et al.*, 2002). Plants used in this study were collected near Nelspruit in the Mpumalanga Province.

2.3.1.3 *Agathosma betulina (Barosma betulina* Bartl. & Wendl.*)*

FAMILY: Rutaceae

Also known as *Barosma betulina* (Watt and Breyer-Brandwijk, 1962)

Common names: 'buchu', 'boegoe', 'ibuchu'

This plant is a shrub that grows up to two metres in height with broad leaves about 20mm long with a rounded apex that curves backwards. Oil glands are present along the margins and lower surfaces of the leaves and the white/pale purple flowers are small and star-shaped, illustrated in Figure 2.2. The plant naturally occurs in the mountains of the Western Cape (van Wyk *et al*., 2002). The powdered, dried leaves were mixed with sheep fat by the San and Khoi people and used to anoint the body, which reportedly gives protection against microorganisms. A brandy tincture of the leaves was also used by Dutch colonists in the Cape to treat stomach ailments while buchu vinegar was used to clean wounds. Buchu is widely used in South Africa to this day to treat kidney and urinary tract infections, for symptomatic relief of rheumatism and for application on external wounds. The major compounds isolated from the essential oil are isomenthone and diosphenol (van Wyk *et al.*, 2002). A commercial sample was obtained from Afriplex for use in this study.

2.3.1.4 *Alipedia amatymbica* Eckl. & Zeyh. var *amatymbica*

FAMILY: Apiaceae

Common names: 'kalmoes', 'ikhathazo', 'lesoko', 'iqwili'

This plant grows in grassland areas from the Eastern Cape northwards to Mpumalanga, the Northern Province and eastern Zimbabwe (van Wyk *et al*., 2002). This robust perennial has dark green rosettes of long, toothed leaves arising from a rhizome. The flowering stalk can reach up to two metres in height with numerous small flowers arranged at the end. Zulu adults and children eat a table- or teaspoonful, respectively, of the raw or cooked roots to treat colds, influenza and coughs. Children with coughs and colds are also given root infusions (Watt and Breyer-Brandwijk, 1962), snuff from the powdered root, or made to inhale smoke from the burning roots. The steam arising from the burnt end of the stem is inhaled through the hollow stem. Similarly, the Sotho, Swati and Xhosa people utilize rhizomes to treat respiratory ailments. It has been reported that small doses of powdered root act as a tonic, but large doses have purgative effects. The roots are very aromatic, resinous and taste of turpentine. Terpenoid kaurene derivatives have been isolated from the roots and aerial parts (Hutchings, 1996). Leaves were chosen for testing in this study as their collection is less harmful to the plant. Plant material used in this study was collected southwest of Sabie near the Long Tom Pass in the Mpumalanga Province by P.M. Burgoyne.

2.3.1.5 *Conyza scabrida* DC.

FAMILY: Asteraceae

Common names: 'bakbossie', 'galsiektebossie', 'kouebos', oven bush, 'uhlabo', 'umanzimnyama'

The Zulu people take leaf infusions, or administer them as enemas, for colds and coughs (Watt and Breyer-Brandwijk, 1962). Children suffering from pleuritic pain have powder from charred roots rubbed into incisions on their chests. During the 1918 influenza epidemic, white South Africans were reported to use the plant extensively for fevers. Plants are aromatic and hautriwaic acid, 12 clerodane derivatives and diterpenoids have been isolated from aerial parts (Hutchings, 1996). Plant material for this study was collected about 14km south of the town of Warmbaths in Limpopo Province by P.M. Burgoyne.

2.3.1.6 *Datura stramonium* L.

FAMILY: Solanaceae

Common names: apple of Peru, 'bloustinkblaar', 'Jimson weed', 'makolieboom', 'pietjelaporte', prickly apple, thorn apple, 'iloyi', 'iyoli', 'iyoye', 'iyoyi'

This annual is probably indigenous to tropical America and is regarded as a weed in South Africa. It grows to about one and a half metres in height. The green, irregularly toothed leaves smell unpleasant when crushed. The plant produces a white or purplish tubular flower (van Wyk *et al*., 2002). The leaves are widely smoked in southern Africa, Israel and Madagascar for relief of asthma and coughs, but the plant is also used as a treatment for other ailments (Hutchings, 1996; van Wyk *et al*., 2002). Incidences of intoxication and poisoning, sometimes fatal, from the seed have been reported, but less frequently as a result of the leaf (Watt and Breyer-Brandwijk, 1962). Leaves are reportedly added to beer in Zimbabwe to make it more intoxicating (van Wyk and Gericke, 2000). Numerous compounds have been isolated from this plant. The two major alkaloids of *D. stramonium* are used commercially – atropine is an ingredient in eye drops, while hyoscine is used to treat motion sickness, Parkinsonism and visceral spasms (van Wyk *et al*., 2002). Plant material for this study was collected 5km south of Warmbaths in the Limpopo Province by P.M. Burgoyne.

2.3.1.7 *Dioscorea sylvatica* (Kunth) Eckl.

FAMILY: Dioscoreaceae

Common names: 'ingwevu', 'ufudu'

The Xhosa people inhale steam from root decoctions for chest complaints. A lotion made from the tuber is used to treat udder and uterine problems of cows by the Zulu people. Known constituents include diosgenin, a pre-cursor of cortisone, from the tuber, which was used in the 1950s to produce steroidal contraceptive compounds. The roots have tested positive for haemolytic properties (Watt and Breyer-Brandwijk, 1962; Hutchings, 1996). People in Zimbabwe also use the rhizomes for the treatment of skin problems and rheumatism. The discovery of raphides of calcium oxalate in the plant is thought to be partly responsible for this activity (Cogney *et al.*, 2001). Plant material for this study was collected by P.M. Burgoyne about 16km from the town of Kaapmuiden on the road to Barberton in the Mpumalanga Province.

2.3.1.8 *Eriocephalus africanus* L.

FAMILY: Asteraceae

Common names: 'kapokbos', wild rosemary

This shrub grows up to a metre in height and has clusters of small silver, hairy leaves along the branches. White to light purple flowers are followed by tufts of seed hairs, shown in Figure 2.2. The plant is found in the Western and Eastern Cape, as well as the Namaqualand. The plant has been used in the Cape as a diaphoretic and a diuretic (Watt and Breyer-Brandwijk, 1962). Plant material used in this study was collected by A.M. Viljoen in Melkbosstrand in the Western Cape of South Africa.

2.3.1.9 *Helichrysum nudifolium* (L.) Less.

FAMILY: Asteraceae

Common names: 'icholocholo', 'imphepho', 'isidwaba-somkhovu', everlastings, 'kooigoed'

Approximately 254 *Helichrysum* species occur in South Africa. All are aromatic perennial herbs or shrublets with hairy or woolly leaves and persistent flower heads. *Helichrysum nudifolium* is distributed along the southern and eastern coast of South Africa, Mpumalanga and Northern Province (van Wyk *et al.*, 2002). The Zulu people sometimes prepare root decoctions as emetics for chest complaints, and the roots are widely used throughout southern Africa to treat colds. The Xhosa also use the leaf as a

remedy for colds (Watt and Breyer-Brandwijk, 1962). Isocomene, ß-isocomene, siliphinene, modhephene, d-cadinene, isoabienol, isocomene-5,5-epoxide, and isobienol derivative and a sesquiterpenoid have been isolated from roots. Isocomene, ß-isocomene, modhephene, cadinene, cryophyllene, squalene and isobienol, two phloroglucinol derivatives and two new diterpenoids were isolated from aerial parts (Hutchings, 1996). Plant material for this study was collected by P.M. Burgoyne in the Cullinan District close to Mooiplaats in the Gauteng Province of South Africa.

2.3.1.10 *Helichrysum odoratissimum* (L.) Sweet

FAMILY: Asteraceae

Common name: 'imphepho'

A description of this plant is illustrated photographically in Figure 2.2. Plants are widely used in the Transkei as medicines for coughs and colds (Hutchings, 1996), as well as in East Africa (Watt and Breyer-Brandwijk, 1962). a-pyrone substituted phloroglucinols have been isolated. An antimicrobial flavonoid helichrysetin has been isolated from this species (van Puyvelde *et al*., 1989). Plant material for this study was collected by J.E. Victor in the Amatolas of the Eastern Cape Province of South Africa.

2.3.1.11 *Mentha longifolia* (L.) L.

FAMILY: Lamiaceae

Common names: 'kruisement', mint, pennyroyal, wild mint, 'ufuthane lomhlange'

This aromatic herb that smells like mint is perennial with rhizomes below the ground and flowering stems. Small white or pale purple flowers form at the end of stems, depicted in Figure 2.2. This plant is widely distributed throughout South Africa and grows in wet places (van Wyk *et al*., 2002).

The Zulu people take infusions from roots, stems and leaves against incipient colds (Watt and Breyer-Brandwijk, 1962). The Xhosa use milk or water decoctions for coughs, colds, asthma and other bronchial ailments. The Sotho use the crushed leaves to plug the nose for the relief of colds. Plants are also placed under the bed to facilitate breathing in patients with respiratory ailments. The plant has popularly been used in the Western Province for inflammatory conditions of the chest, croup, diphtheria, whooping cough, tuberculosis, typhoid fever, scarlet fever, gynaecological conditions and oedema (Watt and Breyer-Brandwijk, 1962). Compounds isolated include luteolin 7-glucoside,

luteolin 7-rutinoside 7-glucuronide, apigenin 7-glucuronide, acacetin 7-rutinoside, dismetin 7-rutinoside, hesperetin 7-rutinoside, eriodictyl 7-rutinoside and the aglycones acacetin and eriodictyol. Volatile oils are known to have decongestant and antiseptic effects (van Wyk *et al*., 2002). Plant material for this study was collected from the garden of the WITS Faculty of Health Sciences in Johannesburg, Gauteng Province.

2.3.1.12 *Ozoroa paniculosa* (Sond.) R. & A. Fernandes var. *paniculosa*

FAMILY: Anacardiaceae

Common names: common resin tree, 'gewone harpuisboom', 'isifica', 'isifice', 'isifico sehlanze'

Watt and Breyer-Brandwijk (1962) report that the Zulu people use the powdered bark for acute inflammatory conditions of the chest, administered as an enema or by mouth to adults, preferably mixed with *Berchemia zeyheri*. It is suspected to be the cause of at least one human death. The pericarp and crushed fruit yield a volatile oil, as well as a fixed oil for the latter. A coagulating liquid is present in the bark and roots, responsible for colouring matter and tannin (Hutchings, 1996). A picture of this tree is available in Figure 2.2, obtained from van Wyk (2001). The bark used in this study was collected from trees in the Verena District along the Doornfontein road in the Gauteng Province.

2.3.1.13 *Pellaea calomelanos* (Swartz) Link

FAMILY: Adiantaceae

Common names: hard fern, 'phaladza', 'inkomankomo'

This common fern is covered with small brown scales and has an underground rootstock of about six millimetres in diameter. The leaves have a firm texture, blue-green coloured leaflets aligned in a triangular shape with spore-producing bodies along the edges. The plant is found over large parts of southern Africa (van Wyk *et al*., 2002). The Xhosa and Sotho smoke burnt leaves for relief from asthma, head and chest colds (Watt and Breyer-Brandwijk, 1962), while the Zulu people inhale smoke from burnt green leaves for headaches, and the Tswana people take decoctions for coughs and colds (Hutchings, 1996; van Wyk *et al*., 2002). The Lobedu, Kwena and Kgatla make a decoction from the rhizome to treat boils, 'internal pimples', 'internal sores' and for external application (Watt and Breyer-Brandwijk, 1962). The active ingredients and pharmacological action are as yet unknown. Plant material for this study was collected

by P.M. Burgoyne about 11km south-west of Bronkhorstspruit on the farm of Cloverhill in the Gauteng Province.

2.3.1.14 *Pollichia campestris* Ait.

FAMILY: Illecebraceae

Common names: 'teesuikerkarroo'

This plant is a herb or small shrub with small leaves and small white berries, eaten by rural children (van Wyk and Gericke, 2000). Cooked roots are used by the Zulu people to treat bronchitis (Hutchings, 1996). The Sotho people inhale steam from a decoction of unspecified parts to treat asthma followed by massage with the lukewarm water. Watt and Breyer-Brandwijk (1962) report the presence of a volatile oil, the vapour of which is used to treat rheumatism. The plant is also used as a liniment applied to swellings and bruises. Plant material for this study was collected by P.M. Burgoyne in the Rustenburg district near Breeds Nek in the North West Province of South Africa.

2.3.1.15 *Salvia africana-lutea* L.

FAMILY: Lamiaceae

Common names: beach salvia, dune salvia, golden salvia, 'geelblomsalie', 'sandsalie'

This is a hardy aromatic shrub which grows rapidly up to two metres high. Flowers are initially yellow and turn orange and finally reddish-brown, a picture of which is available in Figure 2.2. The calyx remains for a fair amount of time after the petals have fallen. The foliage is greyish-green. This shrub is distributed from the coast of Namaqualand to the Cape Peninsula, and east up to Port Alfred. It is a common constituent of vegetation on coastal sand dunes (www.ncbi.co.za). Infusions and decoctions are taken by the European and Nama respectively to treat colds and coughs (Watt and Breyer-Brandwijk, 1962). Plant material for this study was collected at Dynefontein along the West Coast of South Africa.

2.3.1.16 *Siphonochilus aethiopicus* (Schweinf.) B.L. Burtt

FAMILY: Zingiberaceae

Common names: Natal ginger, wild ginger, 'indungulo', 'isiphephetho'

This deciduous plant develops annually from a small rhizome to form large, hairless leaves with predominantly pink flowers forming at ground level in early summer, as shown in Figure 2.2. The leaves and rhizomes smell similar to real ginger, *Zingiber*

officinale. This plant is extinct in KwaZulu-Natal due to its demand in traditional medicines, and is very scarcely distributed in Mpumalanga and the Northern Province. The Zulu people chew the fresh roots and rhizomes to treat coughs, colds and influenza. The tuber has volatile oil containing a-terpineol, a natural antiseptic, various monoterpenoids, and a sesquiterpenoid (van Wyk *et al*., 2002). Furantoterpenoid derivatives were also isolated by Holzapfel *et al*. (2002). Plant material for this study was a commercial sample obtained from P. Soundy of the University of Pretoria.

2.3.1.17 *Syzigium cordatum* Hochst.

FAMILY: Myrtaceae

Common names: 'umdoni', 'waterbessie', water berry, 'montlho'

A medium-sized evergreen tree up to 15 metres in height, with a rough dark brown bark, the leaves are blue-green, broad and almost circular. Fluffy cream to pink flowers with many stamens form at the tips of branches in clusters. The tree produces edible red to dark purple berries. The tree is widely-distributed in the eastern and north-eastern parts of South Africa (van Wyk *et al.*, 2002; van Wyk and Gericke, 2000), and is eaten for food by the Bemba and Luvale (Watt and Breyer-Brandwijk, 1962). Decoctions of the bark, and occasionally the leaves and roots, are used to treat respiratory ailments, including symptoms associated with tuberculosis, by the Zulu people (Watt and Breyer-Brandwijk, 1962). The wood and bark contain proanthocyanidins, pentacyclic triterpenoids, steroidal triterpenoids as well as gallic acid, ellagic acid and various gallic acid derivatives. Hydrolysis of the bark has yielded delphinidin (Hutchings, 1996 and van Wyk *et al.*, 2002). The pharmacological action of this plant is unknown, although the extract has been shown to have anti-mutagenic effects (Verschaeve *et al.*, 2004). Plant material for this study was collected by P.M. Burgoyne near Schoemanskloof south of Lydenburg on the road to Nelspruit in the Mpumalanga Province of South Africa.

2.3.1.18 *Tetradenia riparia* (Hochst.) Codd

FAMILY: Lamiaceae

Common names: ginger bush, 'watersalie', 'iboza', 'ibozane'

The plant is a shrub or small tree up to five metres in height. The large rounded, aromatic leaves are somewhat succulent, with glandular hairs and the flower colour varies from white to mauve. Figure 2.2 contains a picture of this plant. The plant is

common in the north-eastern and eastern parts of South African and distribution extends to Namibia and Angola through east tropical Africa and to Ethiopia. Plants may be found on dry rocky slopes on stream banks (van Wyk *et al*., 2002). The Zulu people use leaf decoctions and infusions for coughs and sore throats. For chronic coughs, cold water infusions of pounded leaves are administered, followed by warm water to induce vomiting. The Xhosa use decoctions, together with umhlonyane (*Artemesia afra*) and salt for coughs. Watt and Breyer-Brandwijk (1962) have also reported the use of leaves in unspecified parts of southern Africa for haemoptysis. Cases of poisoning have been reported as a result of overdosing on hot water extracts of the leaves.

Compounds from the leaves include 8(14),15-sandaracopimaradeine-7a, 18-diol, ibozol, 7^{a}-hydroxyroyleanone, umavumbolide, deacetylumuravumbolide, deacetylboronolide, 1'2'-dideacetylboronolide (van Puyvelde *et al.*, 1986; Hutchings, 1996), tetradenolide (van Puyvelde and De Kimpe, 1998) and umuravumbolide (Davies-Coleman and Rivett, 1995). 8(14),15-Sandaracopimaradiene-7^{a}, 18-diol from leaves has shown significant antimicrobial activity against several bacteria and fungi (van Puyvelde *et al*., 1986). Extracts from the leaves have shown *in vitro* activity against *M. tuberculosis* at 1mg/ml (van Puyvelde *et al*., 1994). 8(14),15-Sandaracopimaradiene-7^{a}, 18-diol inhibited *M. tuberculosis* at concentrations varying between 25µg/ml and 100µg/ml. Campbell *et al*. (1997) found that the essential oil had moderate activity against two strains of *Plasmodium falciparum in vitro*. Plants used in this study were collected by P.M. Burgoyne west of Nelspruit in the Mpumalanga Province of South Africa.

2.3.1.19 *Xerophyta retinervis* Bak.

FAMILY: Velloziaceae

Common names: 'isiphemba', 'isiquumama', monkey's tail, 'bobbejaanstert'

This plant has a thick stem covered with the charred remains of leaf bases as a result of annual veldfires. Long leaves are found at the tip of the stem and mauve or pale blue, sometimes white, flowers are found at the end of long stems. *X. retinervis* grows in rocky areas in the grassland areas of South Africa (van Wyk *et al*., 2002). The dried roots are smoked for asthma relief while the stem bark of the related *X. spekei* is used for general aches, as an anti-inflammatory and for post-partum haemorrhage. Biflavonoids have been reported, while the main compound is amentoflavone (found in *Ginkgo biloba*). Diterpenoids called cleistanthatetraenes and cleistanthatetranols have

been isolated from the closely related *Vellozia* genus. It has been surmised that flavonoids may be responsible for the medicinal properties of this plant (van Wyk *et al.*, 2002). Plant material for this study was collected about 15km south-west of Bronkhorstspruit on the road to Bapsfontein in the Gauteng Province of South Africa.

2.3.2 Extract Yields

For most of the collected plants, 10g of ground plant material was obtained from which the respective extracts were prepared, except for *A. betulina* leaves and *H. nudifolium* roots, indicated in Table 2.1. The percentage yield for the methanol extracts was far greater in general than for the acetone extracts.

2.4 Discussion

Methanol extracted a significantly larger percentage of the dry mass of the plant material than acetone. It should be noted, however, that methanol extracts were more likely to remain moist when compared to acetone extracts which dried completely in a shorter period of time. It is possible that residual methanol present in the samples, together with extended periods of storage before testing could lead to the degradation of compounds within the extract, as has been shown by Stafford *et al.* (2005), using thin layer chromatography (TLC) as a means of monitoring the effect of storage on dried and non-dried plant extracts. The authors speculated that extracts from bark and underground storage organs (roots and tubers) were less susceptible to chemical breakdown than those of leaves, a belief also held by traditional healers who prefer to buy only fresh leaf material, but are not as stringent when choosing bark, roots and tubers.

Figure 2.2: Pictures of selected medicinal plants used in this study. (A) *Siphonochilus aethiopicus*; (B) *Mentha longifolia*; (C) *Salvia africana-lutea*; (D) *Tetradenia riparia*; (E) *Helichrysum odoratissimum*; (F) and (G) *Eriocephalus africanus*; (H) *Agathosma betulina*; (I) *Ozoroa paniculosa*

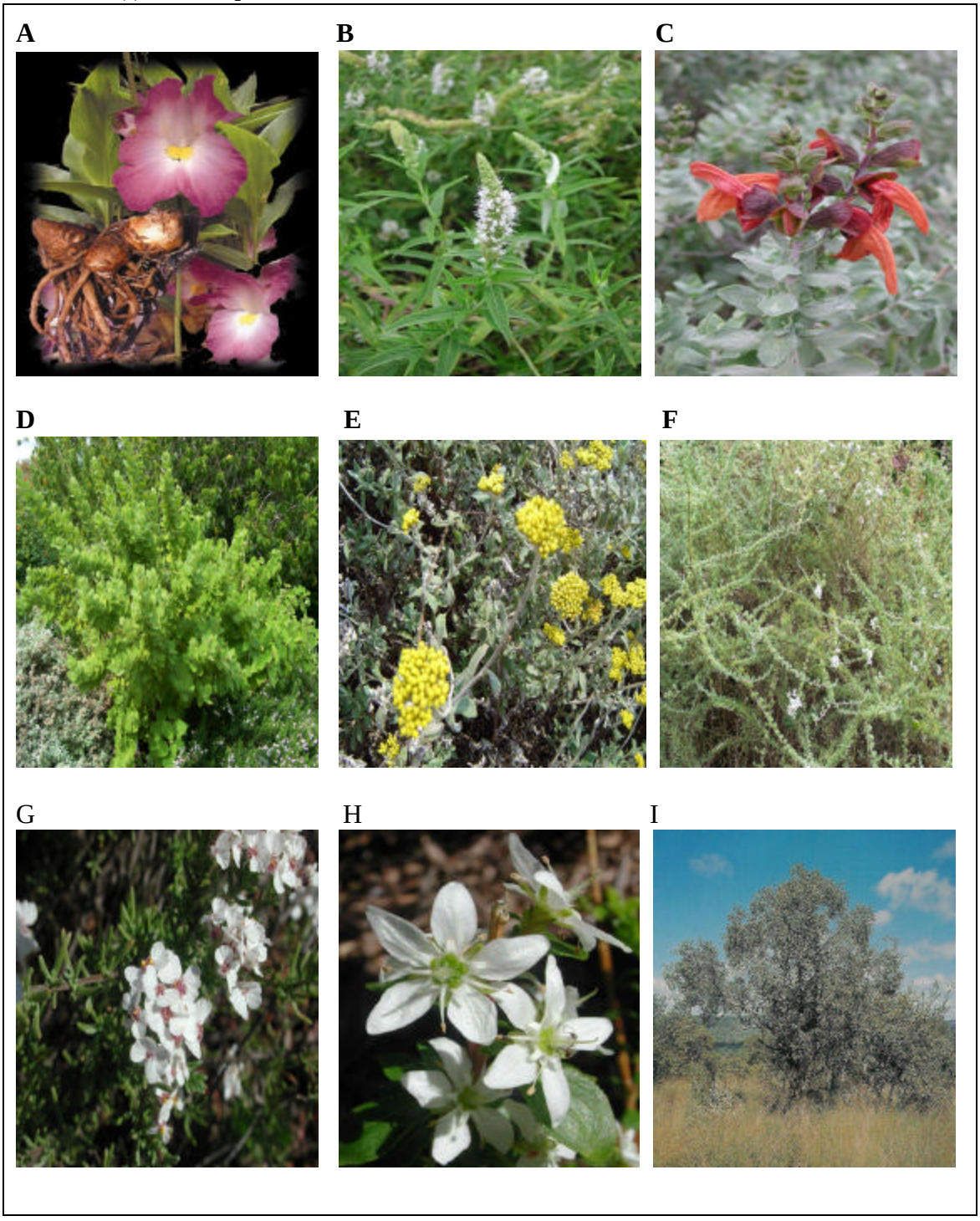


Table 2.1: Plant extract yield using acetone and methanol as extractants

	ACETONE			**METHANOL**		
PLANTS	Plant mass (g)	Extract mass (g)	% yield	Plant mass (g)	Extract mass (g)	% yield
Acacia robusta bark	5	0.46	9.12	5	0.63	12.69
Adiantum capillus-veneris leaves	5	0.18	3.66	5	0.51	10.13
Agathosma betulina leaves	1	0.11	10.78	1	0.32	31.80
Alipedia amatymbica leaves	2	0.13	6.71	2	0.38	18.75
Conyza scabrida leaves	5	0.37	7.43	5	0.97	19.39
Datura stramonium leaves	5	0.15	2.92	5	0.73	14.51
Dioscorea sylvatica tubers	5	0.10	1.95	5	0.81	16.29
Eriocephalus africanus leaves	5	0.08	1.54	5	0.40	8.10
Helichrysum nudifolium leaves	5	0.19	3.79	5	0.51	10.22
Helichrysum nudifolium roots	4.6	0.10	2.10	4.6	0.15	3.22
Helichrysum odoratissimum leaves	5	0.26	5.19	5	0.41	8.12
Mentha longifolia leaves	5	0.22	4.38	5	0.65	12.94
Ozoroa paniculosa bark	5	0.32	6.45	5	1.20	24.00
Pellaea calomelanos leaves	5	0.13	2.59	5	0.90	17.92
Pollichia campestris leaves	5	0.19	3.71	5	0.81	16.30
Pollichia campestris roots	5	0.03	0.59	5	0.25	5.00
Salvia africana-lutea leaves	5	0.23	4.69	5	0.64	12.71
Siphonochilus aethiopicus roots	5	0.25	5.00	5	0.33	6.54
Syzigium cordatum bark	5	0.19	3.86	5	0.63	12.49
Syzigium cordatum leaves	5	0.35	6.91	5	0.67	13.42
Tetradenia riparia leaves	5	0.49	9.76	5	0.61	12.36
Xerophyta retinervis bark	5	0.08	1.67	5	0.10	1.94
Xerophyta retinervis roots	5	0.02	0.49	5	0.10	2.05

3.1 Introduction

In this section, the antimicrobial activities of the acetone and methanol extracts are reported. The extracts were first tested using the agar-based disc-diffusion method with the intention of only testing active extracts further with the broth-micro dilution method. Broth dilution methods can be used for determining the lowest concentration at which the test sample inhibits microbial growth, known as the minimum inhibitory concentration (MIC). However, the protocol was adapted as described in Section 3.4 and the MIC's of all extracts against each organism were determined. Bio-autography was then performed on promising extracts to determine the location on TLC of the active principles.

3.2 Materials and methods

3.2.1 Disc diffusion

Disc diffusion methodology was adapted from Rabe and van Staden (1997) and Salie *et al*. (1996). A single colony of *Staphylococcus aureus* ATCC 12600, *Enterococcus faecalis* ATCC 29212, *Bacillus cereus* ATCC 11778, *Pseudomonas aeruginosa* ATCC 9027, *Klebsiella pneumoniae* ATCC 13883, *Serratia odorifera* ATCC 33132, *Candida albicans* ATCC10231 and a clinical strain of and *Moraxella catarrhalis* was inoculated from Tryptone Soya Agar (TSA) (Oxoid Ltd., Basingstoke, Hampshire, England) plates into 5ml tryptone soya broth (TSB) (Oxoid Ltd., Basingstoke, Hampshire, England) and incubated overnight at 37ºC.

Extracts were prepared to a concentration of 64mg/ml in the appropriate solvent (methanol or acetone). A 1/100 dilution of organism was prepared in TSB and added to sterile TSA, in a test tube, mixed thoroughly, and poured into sterile Petri dishes, avoiding bubble formation. Once set, the plates were stored at 4ºC while preparing the discs. Sterile filter paper discs were transferred to sterile Petri dishes and saturated with extract (approximately 15µl or 1mg of extract per disc). The discs were transferred to designated positions on the agar surface using a sterile needle. Neomycin (10µg) and nystatin (100IU) discs (Oxoid Ltd., Basingstoke, Hampshire, England) were used as positive controls for the bacteria and fungi respectively, a sufficient zone of inhibition required for experiment success and subsequent interpretation of test sample results. Sterile filter paper discs, together with solvent-saturated discs, were used as negative controls. The plates were refrigerated at 4ºC for 1 hour to allow diffusion of extract into the agar, and then incubated

inverted at 37°C overnight. All extracts were tested in duplicates, and those exhibiting activity were confirmed. Zones are reported as an average.

3.2.2 Broth micro-dilution MIC determination

The microplate method for MIC determination as described by Eloff (1998) was used as the basis for these experiments. Sterile 96-well microtitre plates with flat-bottomed wells were used. 100µl sterile distilled water was added to each well and the same volume of TSB was added to the first column as a negative control. 100µl of 62.5µl/ml ciprofloxacin (Sigma, St Louis, MO, USA) and 1mg/ml nystatin (Sigma) was used as a positive control for the bacteria and fungi respectively. The inhibitory action of these two antimicrobials had to be within acceptable ranges for experiment success and subsequent interpretation of MIC's obtained from test samples. 100µl of each re-dissolved extract (64mg/ml unless otherwise specified), and solvent controls were added to the first well of respective rows, the latter to ensure that the solvent had no negative effect on organism growth. A serial dilution was then prepared by transferring 100µl aliquots from the top to the bottom of the plate using a multi-channel pipette. The remaining 100µl from the last row was discarded. Thereafter, 100µl of organism, diluted a hundred-fold in TSB from an overnight culture, was added to each well of the 96-well plate, excluding the medium control, which remained uninoculated to test sterility of the medium. Extract concentrations therefore ranged from 16mg/ml to 125µg/ml, unless otherwise specified. The plates were sealed in plastic bags and incubated for 24 - 48 hours at 37ºC. Each extract was tested in duplicate against each organism.

40µl of 0.4mg/ml p-iodonitrotetrazolium salt (INT) (Sigma, St Louis, MO, USA) was added to each well and the plates were left at room temperature for 6 hours. This tetrazolium salt is used as an indicator as it is reduced from a colourless compound to red in the presence of actively metabolizing organisms (Eloff, 1998). The author recommends that the same volume of a 0.2mg/ml solution be added to each well, but it was found that increasing the concentration yielded results in a shorter period of time, as well as producing a more intense colour that increased the probability of visualization of the red produced by INT in the higher concentrations of strongly coloured extracts. The results were determined by visual inspection of the wells with the MIC being reported as the lowest concentration of extract containing no indication of red.

3.2.3 Direct and agar-overlay bio-autography assays

The direct and agar-overlay bio-autography methods were adapted from Chaaib *et al*. (2003). For the agar-overlay bio-autographic assay, a base layer of the appropriate agar was poured into a sterile Petri dish. Once solidified, the sterile, trimmed TLC plate spotted with extract was firmly placed onto the agar surface, pressing it gently to avoid air bubbles. An aliquot of the liquid culture, containing the appropriate organism, was then mixed into agar to dilute 100-fold and then poured over the surface of the plate. The agar was allowed to set and the plate was incubated, inverted, at 37ºC for 24 hours. For the direct bio-autography, organism diluted 100-fold in broth was dabbed onto the sterile TLC plate with sterile cotton wool, sealed in a sterile Petri dish containing wet sterile paper towel, sealed in a bag and incubated overnight. For both methods, the plate was then sprayed with 0.4mg/ml INT and left at room temperature for 6 hours. Zones of clearing indicate the bands responsible for antimicrobial activity within the extracts.

3.3 Results

Each extract was tested in duplicate using the disc diffusion and broth micro-dilution techniques. Zones of inhibition in the disc-diffusion method are reported as an average value. Concentrations indicated with 'greater than or equal to' indicate strongly coloured extracts where it was not possible to distinguish the red colour of INT through the colour of the diluted sample. For an experiment to be regarded as successful all the wells of the culture-control had to be positive for growth, the organism-free control growth-free and the drug-control had to indicate sufficient inhibition of growth. The highest concentration of solvents (25%) in the first wells indicated that methanol and acetone at 25% had no negative effect on *S. aureus, E. faecalis, B.cereus, K. pneumoniae* and *M. catarrhalis* growth. Methanol at 25%, however, was detrimental to *P. aeruginosa* while acetone at this concentration had no effect on the growth of the organism. If insufficient quantities of extracts prevented the determination of the MIC, the results were recorded as ND, representative of 'Not determined'. Examples of the disc diffusion and broth micro-dilution methods are illustrated in Figures 3.1 and 3.2 respectively. The results of the antimicrobial activities of the crude extracts against Gram-positive and –negative bacteria and yeast are reported in Tables 3.1, 3.2 and 3.3 respectively.

Figure 3.1: Examples of the disc diffusion method. Plate 'A' shows the activity of the acetone extract of *H. odoratissimum* (45) and *C. scabrida* acetone and methanol extracts (47 and 63 respectively) against *B. cereus*. Plate 'B' shows the activity of the acetone extracts of *T. riparia* (35), *S. cordatum* bark (42) and *H. odoratissimum* (45) against *S. aureus*.

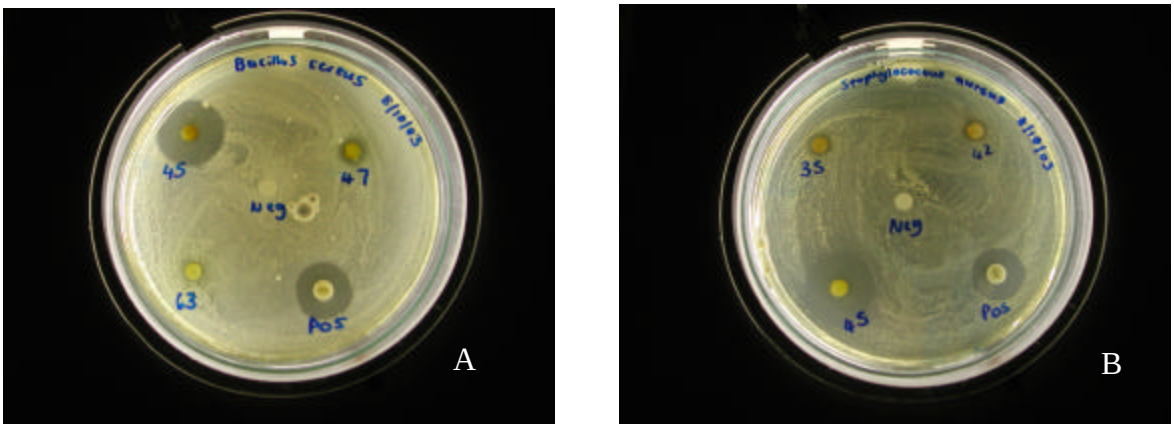


Figure 3.2: An example of the broth micro-dilution method for the determination of MIC's of extracts against *E. faecalis.* The wells from left to right contain the negative control, culture control, solvent control, antibiotic control and in duplicate, the acetone extracts of *A. robusta, C. scabrida*, *D. stramonium* and *O. paniculosa*.

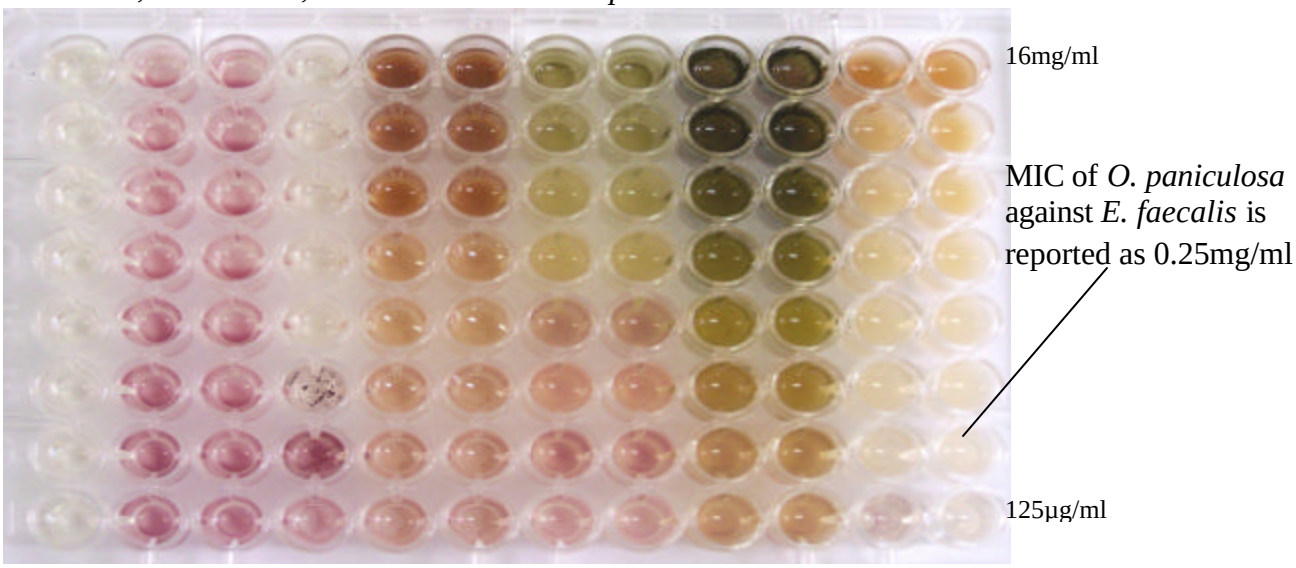


Table 3.1: Antimicrobial activity of the crude extracts against three Gram-positive organisms using the disc diffusion and broth micro-dilution method. Zones were measured in millimetres (mm) from the edge of the disc to the end of the zone of inhibition, and MIC's in mg/ml. Each sample was tested in duplicate. Neomycin discs and ciprofloxacin served as positive controls for the agar and broth-based methods respectively.

			S. aureus		***E. faecalis***		***B. cereus***	
Plant	Part	Solv	Disc Diffusion	MIC	Disc Diffusion	MIC	Disc Diffusion	MIC
Acacia robusta	B	A	0	≥1	0	≥1	0	2
Acacia robusta	B	M	0	≥2	0	≥1	0	4
Adiantum capillus veneris	L	A	0	1	0	2	0	2
Adiantum capillus veneris	L	M	0	8	0	8	0	≥16
Agathosma betulina	L	A	0	4	0	2	0	8
Agathosma betulina	L	M	0	8	0	8	0	8
Alipedia amatymbica	L	A	0	6	0	3	0	ND
Alipedia amatymbica	L	M	0	4	0	4	0	2.125
Conyza scabrida	L	A	0	0.5	0	2	1.4	0.5
Conyza scabrida	L	M	0	2	0	4	1.1, 0	1
Datura stramonium	L	A	0	≥2	0	4	0	4
Datura stramonium	L	M	0	4	0	8	0	8
Dioscorea sylvatica	T	A	0	0.5	0	2	0	2
Dioscorea sylvatica	T	M	0	0.5	0	1	0	2
Eriocephalus africanus	L	A	0	1	0	0.5-1	0	ND
Eriocephalus africanus	L	M	0	4	0	4-8	0	4
Helichrysum nudifolium	L	A	0	1	0	2	0	4
Helichrysum nudifolium	L	M	0	4	0	4	0	ND
Helichrysum nudifolium	R	A	0	4	0	8	0	>8
Helichrysum nudifolium	R	M	0	8	0	8	0	>8
Helichrysum odoratissimum	L	A	7.7	0.125-0.25	4.1	0.25-0.5	9.2	<0.125
Helichrysum odoratissimum	L	M	7.6	2	5.2	0.5	9.4	0.25-1
Mentha longifolia	L	A	0	≥1	0	≥4	0	16
Mentha longifolia	L	M	0	4	0	8	0	16

			S. aureus		***E. faecalis***		***B. cereus***	
Plant	Part	Solv	Disc Diffusion	MIC	Disc Diffusion	MIC	Disc Diffusion	MIC
Ozoroa paniculosa	B	A	0	1	0	≤0.125	0	2
Ozoroa paniculosa	B	M	0	1	0	0.5	0	2
Pellaea calomelanos	L	A	0	4	0	≥4	0	>8
Pellaea calomelanos	L	M	0	8	0	8	0	8
Pollichia campestris	L	A	0	>16	0	>16	0	8
Pollichia campestris	L	M	0	8	0	8	0	8
Pollichia campestris	R	A	0	ND	0	ND	0	ND
Pollichia campestris	R	M	0	8	0	8	0	ND
Salvia africana-lutea	L	A	0	2	0	≥8	0	4-8
Salvia africana-lutea	L	M	0	4	0	2	0	4
Siphonochilus aethiopicus	R	A	0	0.25	0	4-8	0	0.25
Siphonochilus aethiopicus	R	M	0	2	0	4	0	1
Syzigium cordatum	B	A	1.3	0.25-2	1.6	1	0	2.5
Syzigium cordatum	B	M	0	≥1	1.7	1-2	0	1
Syzigium cordatum	L	A	0	2	0.9	1	0	4
Syzigium cordatum	L	M	0	4	0	1	0	4
Tetradenia riparia	L	A	1.3	<0.125	0	8	0	2
Tetradenia riparia	L	M	0	0.25	0	8	0	4
Xerophyta retinervis	B	A	0	1	0	C	0	C
Xerophyta retinervis	B	M	0	0.5	0	0.25	0	5.25
Xerophyta retinervis	R	A	0	ND	0	ND	0	ND
Xerophyta retinervis	R	M	0	ND	0	ND	0	ND
Positive control (neomycin and ciprofloxacin)			4.7	0.0005	1.3	0.001	5.7	0.0016

L = leaves; R = roots; T = tubers; B = bark; A = acetone; M = methanol; MIC = minimum inhibitory concentration; ND = Not determined due to insufficient extract quantities; C = contamination

Table 3.2: Antimicrobial activity of the crude extracts against four Gram-negative organisms using the disc diffusion and broth micro-dilution method. Zones were measured in millimetres (mm) from the edge of the disc to the end of the zone of inhibition and MIC's in mg/ml. Each sample was tested in duplicate. Neomycin discs and ciprofloxacin served as positive controls for the agar and broth-based methods respectively

			K. pneumoniae		***P. aeruginosa***		***M. catarrhalis***		***S. odorifera***	
Plant	Part	Solv	DD	MIC	DD	MIC	DD	MIC	DD	MIC
Acacia robusta	B	A	0	≥4	0	0.25	1.2	<0.125	0	2
Acacia robusta	B	M	0	4	0	1-2	0	4	0	8
Adiantum capillus veneris	L	A	0	1	0	2	0	>16	0	2
Adiantum capillus veneris	L	M	0	4	0	4	0	>16	0	4
Agathosma betulina	L	A	0	2	0	8	0	>8	0	>8
Agathosma betulina	L	M	0	4	0	4	0	8	0	>16
Alipedia amatymbica	L	A	0	12	0	3	0	ND	0	ND
Alipedia amatymbica	L	M	0	4-8	0	2-4	0	10.5	0	2.5
Conyza scabrida	L	A	0	2	0	4	0	8	0	>8
Conyza scabrida	L	M	0	2	0	4	0	16	0	16
Datura stramonium	L	A	0	≥1	0	≥4	0	>8	0	>8
Datura stramonium	L	M	0	4	0	4	0	16	0	16
Dioscorea sylvatica	T	A	0	4	0	1	0	4	0	1
Dioscorea sylvatica	T	M	0	4	0	8	0	4	0	2
Eriocephalus africanus	L	A	0	0.5	0	1-2	0	ND	0	ND
Eriocephalus africanus	L	M	0	4-8	0	8	0	>16	0	>16
Helichrysum nudifolium	L	A	0	4	0	0.5-1	2.4	4	0	2
Helichrysum nudifolium	L	M	0	4	0	2	0	ND	0	2
Helichrysum nudifolium	R	A	0	>8	0	2	0	>8	0	>8
Helichrysum nudifolium	R	M	0	8	0	4	0	>8	0	>8
Helichrysum odoratissimum	L	A	0	2	0	0.5	1.4	0.25	0	ND
Helichrysum odoratissimum	L	M	0	4	0	1	0	8	0	>16
Mentha longifolia	L	A	0	≥1	0	≥2	0	4	0	>16
Mentha longifolia	L	M	0	4	0	4	0	16	0	≥16

			K. pneumoniae		***P. aeruginosa***		***M. catarrhalis***		***S. odorifera***	
Plant	Part	Solv	DD	MIC	DD	MIC	DD	MIC	DD	MIC
Ozoroa paniculosa	B	A	0	1	0	0.5	0	8	0	16
Ozoroa paniculosa	B	M	0	4	0	0.5	0	1	0	8
Pellaea calomelanos	L	A	0	≥1	0	4	0	>8	0	1
Pellaea calomelanos	L	M	0	4	0	2	0	16	0	16
Pollichia campestris	L	A	0	4	0	4	0	>16	0	1
Pollichia campestris	L	M	0	4	0	2	0	16	0	8
Pollichia campestris	R	A	0	ND	0	ND	0	ND	0	ND
Pollichia campestris	R	M	0	4	0	4	0	ND	0	ND
Salvia africana-lutea	L	A	0	4	0	4	0	>8	0	1
Salvia africana-lutea	L	M	0	4	0	4	0	≥16	0	≥16
Siphonochilus aethiopicus	R	A	0	4	0	4	2.55	13.4	0	>8
Siphonochilus aethiopicus	R	M	0	4	0	4	0	>16	0	>16
Syzigium cordatum	B	A	0	2	1.5	0.25	2.1	>5	0	>5
Syzigium cordatum	B	M	0	2	0	0.25-0.5	0	4-8	0	8
Syzigium cordatum	L	A	0	1	0	0.25-0.5	0	4	0	4
Syzigium cordatum	L	M	0	1	0	0.5	0	8	0	16
Tetradenia riparia	L	A	0	8	0	4	1.1	4	0	2
Tetradenia riparia	L	M	0	8	0	2-4	0	16	0	≥16
Xerophyta retinervis	B	A	0	C	0	C	0	C	0	C
Xerophyta retinervis	B	M	0	0.5	0	0.25	0	5.25	0	5.25
Xerophyta retinervis	R	A	0	ND	0	ND	0	ND	0	ND
Xerophyta retinervis	R	M	0	ND	0	ND	0	>10.5	0	>10.5
Positive control (neomycin and ciprofloxacin)			1.6	0.0003-0.003	1.2	0.001	9.7	0.0005	1.125	0.002-0.004

L = leaves; R = roots; T = tubers; B = bark; A = acetone; M = methanol; MIC = minimum inhibitory concentration; ND = Not determined due to insufficient extract quantities; C = contamination; DD = disc diffusion; solv = solvent

Table 3.3: Antimicrobial activity of the crude extracts against *Candida albicans* using the disc diffusion and broth micro-dilution method. Zones were measured in millimetres (mm) from the edge of the disc to the end of the zone of inhibition and MIC's in mg/ml. Each sample was tested in duplicate. Nystatin served as the positive control in both methods.

		Acetone		**Methanol**	
Plant	Part	Disc Diffusion	MIC	Disc Diffusion	MIC
Acacia robusta	B	0	≥2	0	≥1
Adiantum capillus veneris	L	0	≥0.5	0	4
Agathosma betulina	L	0	8	0	4
Alipedia amatymbica	L	0	ND	0	ND
Conyza scabrida	L	0	8	0	4
Datura stramonium	L	0	≥0.5	0	0.25
Dioscorea sylvatica	T	0	≥4	0	≥4
Eriocephalus africanus	L	0	>2	0	4
Helichrysum nudifolium	L	0	≥2	0	4
Helichrysum nudifolium	R	0	8	0	4
Helichrysum odoratissimum	L	0	2	0	8
Mentha longifolia	L	0	≥0.25	0	≥2
Ozoroa paniculosa	B	0	4	0	≥2
Pellaea calomelanos	L	0	≥1	0	4
Pollichia campestris	L	0	4-8	0	4
Pollichia campestris	R	0	ND	0	4
Salvia africana-lutea	L	0	≥8	0	4
Siphonochilus aethiopicus	R	0	2	0	2
Syzigium cordatum	B	2.2	≥4	0	2
Syzigium cordatum	L	0	≥4	0	≥2
Tetradenia riparia	L	0	8-16	0	4
Xerophyta retinervis	B	0	C	0	0.5
Xerophyta retinervis	R	0	ND	0	ND
Positive control (nystatin)		6.25	0.0078-0.0156	6.25	0.0078-0.0156

L = leaves; R = roots; T = tubers; B = bark; A = acetone; M = methanol; MIC = minimum inhibitory concentration; ND = Not determined due to insufficient extract quantities; C = contamination

3.4 Discussion

Initially, only extracts exhibiting activity in the disc-diffusion method were to be further tested for the determination of an MIC. To confirm results of the agar-based method, several extracts not exhibiting zones of inhibition in this method were also tested using the broth micro-dilution method and did, in fact, exhibit potent activity. This study has confirmed findings by other authors (Eloff, 1998; Ríos and Recio, 2005) that agar based methods are not suitable for the high-throughput screening of plant-derived extracts for antimicrobial activity as non-polar compounds do not dissolve easily into the agar and therefore are often not detected. The

protocol was therefore revised and all extracts were subsequently tested in the broth micro-dilution method. An extract with an MIC of less than 1mg/ml was considered to have good antimicrobial activity.

Of the nine extracts which exhibited MIC's of less than 1mg/ml against *S. aureus*, only three exhibited activity by means of the disc diffusion method, together with one other extract which had an MIC of 2mg/ml (methanol extract of *H. odoratissimum*). The acetone extracts of *T. riparia*, *S. cordatum* bark, and *H. odoratissimum* exhibited good activity by both methods. Six of the nine extracts with good broth micro-dilution results were acetone extracts. The most active samples against *S. aureus* were the acetone extracts of *T. riparia* and *H. odoratissimum*. The acetone extract of *S. cordatum* leaves and both extracts of *S. cordatum* bark were active against *E. faecalis* by means of the disc diffusion method, and had MIC's of greater than or equal to 1mg/ml. Both extracts of *H. odoratissimum* exhibited potent activity with both methods. Furthermore, *X. retinervis* bark (methanol), *E. africanus* leaves (acetone) and both extracts of *O. paniculosa* bark had MIC's against this organism of less than 1mg/ml. The acetone extract of the latter exhibited the lowest MIC (=0.125mg/ml). Equal numbers (four each) of acetone and methanol extracts had activity against this organism. Four extracts exhibited activity against *B. cereus* as indicated by the disc diffusion method. Both the acetone and methanol extracts of *C. scabrida* had zones of inhibition that corresponded with MIC's of 0.5 and 1mg/ml respectively. Similarly, the acetone and methanol extracts of *H. odoratissimum* had substantial zones of inhibition with MIC's of <0.25 and 0.25-1mg/ml respectively. Apart from the acetone extract of *S. aethiopicus* that had an MIC of 0.25mg/ml, no other extracts exhibited pronounced activity against this organism. Three plants exhibited significant activity against all three Gram-positive organisms from these experiments: *X. retinervis* bark, *S. cordatum* bark and *H. odoratissimum* leaves, with *C. scabrida* exhibiting activity against *B. cereus* and *S. aureus.*

Four methanol and six acetone extracts exhibited activity against *P. aeruginosa*, with *X. retinervis* bark (methanol), *S. cordatum* leaves (acetone) and bark (both extractants) and *A. robusta* bark (acetone) having the lowest MIC's (0.25mg/ml). The activity of one extract was determined using both the agar and broth methods, and one (*P. calomelanos* leaves) with only the former method, although the zone of inhibition was very small. The remaining extracts with pronounced activity were *H. nudifolium* leaves (acetone), *H. odoratissimum* leaves (acetone) and *O. paniculosa* bark (both extractants). Two extracts exhibited inhibition of growth of *K. pneumoniae* with the broth micro-dilution method, namely the methanol and acetone extracts of *X. retinervis* bark and *E. africanus* leaves respectively. Six extracts resulted in zones of

inhibition with the agar method against a clinical strain of *M. catarrhalis*, all actone extracts. The resultant MIC's indicated that *H. odoratissimum* leaves and *A. robusta* bark (both acetone extracts) had activities of 0.25µg/ml and <0.125µg/ml respectively. No extracts indicated activity against *S. odorifera* by means of the disc diffusion method, corresponding with no MIC's of less than 1mg/ml. Those extracts with the lowest MIC's were the acetone extracts of *D. sylvatica*, *P. calomelanos*, *P. campestris* leaves and *S. africana-lutea*. Extracts which exhibited activity against any two Gram-negative organisms tested were *X. retinervis* bark, *P. calomelanos*, *H. odoratissimum*, *A. robusta* and *S. cordatum* bark.

Only one extract exhibited zones of inhibition on agar seeded with *C. albicans*, namely the acetone extract of *S. cordatum* bark. The three extracts with MIC's less than 1mg/ml were both extracts of *D. stramonium* and the acetone extract of *M. longifolia*.

Activity of *Helichrysum* species has been reported previously. Lourens *et al.* (2004) and Mathekga and Meyer (1998) found that *H. odoratissimum* (formerly known as *H. hochstetteri*) inhibited the growth of all Gram-positive bacteria tested, results in agreement with this study. However, the authors obtained no activity against the Gram-negative organisms, unlike the current study where the broth-based system indicated activity (MIC=0.5mg/ml) against *P. aeruginosa* of the acetone extracts of this plant species. *H. odoratissimum* also showed good activity by the agar and broth susceptibility methods in the current study against *M. catarrhalis*, with MIC's of 1-2mg/ml for the methanol extract and 0.25mg/ml for the acetone extract. Similarly, Vlietinck *et al*. (1995) also found activity of this plant against *S. aureus*, *E. coli* and *P. aeruginosa* when screening 100 Rwandese medicinal plants. Unlike the current study, the root and stem extracts exhibited activity, but not the leaves. Van Puyvelde *et al*. (1989) isolated a flavonoid with antimicrobial activity from the chloroform/ethanol (35:65) extract of *H. odoratissimum* flowers collected in Rwanda. The compound, 3-*O*-methylquercetin, exhibited MIC's of 50µg/ml against *Bacillus subtilis*, 6.25µg/ml against *S. aureus*, and 100µg/ml against *K. pneumoniae*, *P. aeruginosa* and *Serratia marcescens*. Galangin has been isolated from *H. aureonitens* and has potent activity against Gram-positive but not Gram-negative bacteria (Afolayan and Meyer, 1997), and has been shown to cause cytoplasmic membrane damage in *S. aureus* (Cushnie and Lamb, 2005). Furthermore, it has activity against herpes simplex type 1 and coxsackie B virus type 1 (Meyer *et al*., 1997). An extract of *H. italicum* has also shown inhibition of *S. aureus* affecting growth and enzyme activity (Nostro *et al*., 2001).

Anti-bacterial testing of *S. aethiopicus* plant parts by Light *et al*. (2002) indicated that ethanol and ethyl acetate extracts of the roots yielded MIC's of 0.78mg/ml and 1.56mg/ml against *S. aureus*, and 6.25mg/ml and 12.5mg/ml against *K. pneumoniae*. The roots resulted in MIC's of 2mg/ml and 0.25mg/ml for the methanol and acetone extracts against *S. aureus* and 4mg/ml against *K. pneumoniae* respectively in this study. Against *B. subtilis*, the authors obtained MIC's of 1.56mg/ml and 0.78mg/ml for the ethanol and ethyl acetate extract respectively, while the current study obtained MIC's of 1mg/ml and 0.25-0.8mg/ml for the methanol and acetone extracts respectively. The authors found that seasonal changes affected the antimicrobial activity of the plant parts, and that the roots harvested after senescence had increased activity against *S. aureus*. The composition of the essential oils obtained from the roots and rhizomes of *S. aethiopicus* has been previously determined, with the major components being 1,8-cineole, (E)-ß-ocimene, *cis*-allocimene and a furanoterpenoid (4aa,5 ß,8a)-3,5,8a-trimethyl-4,4a,9-tetrahydo-naptho[2,3-*b*]-furan-8(5H)-one (Viljoen *et al*., 2002).

Salie *et al*. (1996) found that petroleum ether extracts of the stems and methanol extracts of the roots of *E. africanus* had activity against *S. aureus* while only the former had activity against *C. albicans* using the disc diffusion assay. In the current study, the leaves of this plant showed activity against *E. faecalis*, but not *S. aureus* or *C. albicans*. Compounds have been isolated from *Salvia* species (Ulubelen 2003), not including the species used in this study. Four of the seven compounds tested had activity against *S. aureus*, one of the seven had activity against *E. faecalis*, four of the seven activity against *B. subtilis*, and none exhibited activity against *K. pneumoniae*, *P. aeruginosa*, nor *C. albicans*. In this study, *S. africana-lutea* had no noticeable activity against any of the organisms tested.

Vlietinck *et al*. (1995) found that the leaves and stems of *T. riparia* and the leaves of *Acacia sieberiana* exhibited activity against *S. aureus*, the former confirming findings from this study whereas *A. robusta* screened in this study had MIC's of 1-≥2mg/ml for the acetone and methanol extracts. The authors also found that *T. riparia* had activity against *C. albicans*. 8(14),15-sandaracopimaradiene-7α,18-diol isolated from *T. riparia* has been shown to have activity against Gram-positive, but not Gram-negative, bacteria with MIC's of 6.25 and 12.5µg/ml against *B. subtilis* and *S. aureus* respectively, the latter in agreement with this study (van Puyvelde *et al*., 1986). *Acacia sieberiana* also exhibited activity against Gram-positives, but had no activity against the Gram-negative organisms tested (Rabe and Van Staden, 1997). Geyid *et al*. (2005) screened Ethiopian medicinal plants against a range of organisms and found

that *Syzigium guineense* leaves and bark stem (methanol extracts) exhibited activity against *S. aureus* at 250µg/ml, but was inactive against *C. albicans* at 4mg/ml.

D. stramonium has previously been tested for antimicrobial activity against a range of Gram-positive and –negative organisms, but only displayed limited activity against *B. subtilis* (Rabe and van Staden, 1997). Similarly, Eftekhar *et al*. (2005) obtained a dose-dependent activity against all three Gram-positives tested, but little to no activity against the three Gram-negatives tested with the methanol extracts. In this study, both extracts of *D. stramonium* only had activity against *C. albicans* with MIC's of =0.5 and 0.25mg/ml for the acetone and methanol extracts respectively. Various activities have also been reported for *M. longifolia*. The essential oils have demonstrated activity against *E. coli*, *S. aureus* and *B. subtilis*, but had no activity against *P. aeruginosa* (Mimica-Dukic *et al*., 2003). Tassou *et al*. (2000) showed that the oil decreased the growth of *S. aureus* by six to seven logs. The oils were also shown to be effective against fungi, not including *C. albicans* (Abou-Jawdah *et al*., 2002). The strong green colour of the extracts made visualisation of the INT colour difficult.

Several studies have been performed on the activities of *D. sylvatica*. Rabe and Van Staden (1997) found no activity against *S. aureus*, *K. pneumoniae* or *B. subtilis* from water and methanol extracts of the leaves and tubers using a disc diffusion assay. A later study by Kelmanson *et al*. (2000) found activity of the methanol extract of the roots against *B. subtilis* and nothing else, but the methanol and ethyl acetate extracts of the tuber bark had activity against all the Gram-positive organisms tested, but very little activity against the Gram-negatives. These findings partially confirm results from the current study where both extracts had good activity against *S. aureus* and moderate activity (1mg/ml) against *P. aeruginosa* and *S. odorifera*. The antimicrobial testing of *A. betulina* extracts in this study indicated poor activity against all organisms tested (MIC's of 2mg/ml or greater). Similar results have been found with the essential oils of this species, with no activity against Gram-negatives, and very little activity against *S. aureus* (Lis-Balchin *et al*., 2001).

The results from this study indicate that plants are a viable potential source of products active against pathogenic microorganisms. 19.6%, 15.2% and 8.7% of the extracts tested had activity against *S. aureus*, *E. faecalis* and *B. cereus* respectively. Furthermore, 4.3%, 19.6%, 4.3% and 6.5% of the 46 extracts tested had activity against *K. pneumoniae*, *P. aeruginosa*, *M. catarrhalis* and *C. albicans* respectively. The observation that fewer extracts were active against Gram-negative than Gram-positive bacteria can be attributed to the fact that the former are known to

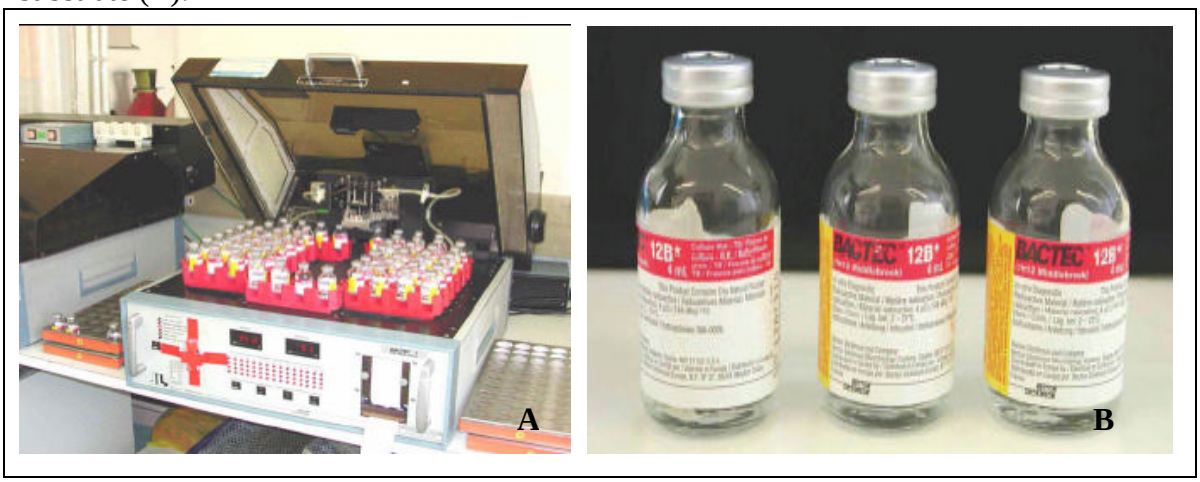
have greater intrinsic resistance to antimicrobial agents as a result of their two cell-envelope membranes. The outer membrane functions as a selective permeability barrier and, in combination with efflux, is responsible for the high resistance to external detergents and dyes (Hancock, 1997). *P. aeruginosa* is an example of a Gram-negative bacterium that has high intrinsic resistance to all classes of antibiotics. The outer membrane has very low permeability to β-lactams and the bacterium possesses inducible β-lactamase and active efflux which together form effective resistance mechanisms (Hancock, 1997).

4.1 Introduction

Due to the dangerous and slow-growing nature of MTB, various techniques have been developed for the rapid and safe screening of potential anti-TB agents. Basic broth dilution methods have been adapted for the high-throughput screening of antimycobacterial agents using fast growing, non-pathogenic species of mycobacteria, such as *M. aurum*, which has similar susceptibility patterns to *M. tuberculosis*. These methods have been adapted to measure radiolabelled substrate uptake (Chung *et al.*, 1995), bioluminescence from firefly luciferase (Cooksey *et al*., 1993; Arain *et al*., 1996; Deb *et al*., 2000), L-rhamnosyl produced by enzymes as an indicator of inhibition of cell wall synthesis (Ma *et al.*, 2001), and colour changes as a result of tetrazolium salts (Eloff, 1998; Palomino *et al.*, 2002) to indicate metabolizing organisms. High-throughput screening in the above-mentioned manner does, however, have a downfall. PZA, for example, has a relatively high MIC against MTB of 50µg/ml at a pH of 5.5 and would almost certainly have been missed in such *in vitro* screening methods. In *in vitro* conditions with low oxygen and acidic pH, the activity of PZA against *Mycobacterium tuberculosis* increases significantly (Zhang and Amzel, 2002).

Many researchers have used agar based techniques for the susceptibility testing of this nature (Newton *et al*., 2000; Eloff, 1998). Lall and Meyer (1999) found the radiometric BACTEC 460 method, illustrated in Figure 4.1, comparable to the conventional agar plate method. Furthermore, results were obtained within 6-7 days using the radiometric method as opposed to 5-6 weeks using the plate method, decreasing the chance of the test compounds decomposing. The 12B vials, illustrated in Figure 4.1B, contain a ^{14}Carbon-labelled substrate and in the presence of metabolising organism, radiolabelled CO_2 is released into the atmosphere above the broth, which is aspirated and measured with every reading. The radiometric method also allows greater cell-to-drug interaction in the liquid medium. The principles of the radiometric method of susceptibility testing (Siddiqi *et al.*, 1981) indicate that the concentration of the extract resulting in a daily GI increase and final GI reading lower than in the 1:100 control can be considered to be the concentration at which more than 99% of the bacterial population is inhibited. The MIC is defined as the lowest concentration of drug inhibiting more than 99% of the bacterial population. Drawbacks of this method include cost, working with radioactivity and the need to handle needles.

Figure 4.1: The BACTEC 460 apparatus (A) with racks containing the inoculated vials ready to be tested and the individual 12B vials containing the radiolabelled carbon substrate (B).



This chapter reports the effect of the methanol and acetone plant extracts on three mycobacterial species. Initially, only extracts with MIC's of less than 1mg/ml against *M. smegmatis* were to be tested against MTB due to the cost of the BACTEC 460 medium. Subsequently, *M. aurum* was obtained with numerous literature reports advocating its use for the high-throughput screening of plant extracts or compounds, as its drug susceptibility profiles are similar to that of MTB, unlike *M. smegmatis*. Therefore, *M. aurum* was also used for the screening of all the plant extracts. The results described in section 4.3 encouraged the testing of all the extracts against MTB in the radiometric method as conclusive proof of antitubercular activity.

4.2 Materials and methods

4.2.1 Broth micro-dilution MIC determination

Middlebrook 7H11 agar (Becton Dickinson, Sparks, MD, USA) supplemented with 10% OADC (oleic acid, albumin, dextrose, catalase) (Remel, Lenexa, KS, USA) plates were used for the routine maintenance and growth of *Mycobacterium smegmatis* and *Mycobacterium aurum* A+ (Pasteur Institute, France) from glycerol stocks stored at -70ºC. For experimentation purposes, a single colony of growth was then used to inoculate supplemented Middlebrook 7H9 broth (Becton Dickinson, Sparks, MD, USA) and incubated at 37ºC for 72 hours.

The basis of the microplate method described by Chung *et al.* (1995) for the high-throughput screening of antimycobacterial agents, combined with the use of tetrazolium

salts (Eloff, 1998) was utilized for MIC determination of test samples against *M. aurum A+* and *M. smegmatis*. Sterile 96-well microtitre plates with flat-bottomed wells were used for these experiments. 100µl supplemented 7H9 broth was added to each well. 100µl 1mg/ml ciprofloxacin (Sigma, St Louis, MO, USA) was used as a positive control for *M. smegmatis*, while 100µl 1 mg/ml rifampicin (Duchefa Biochemie, Haarlem, Netherlands) was used for *M. aurum*, the MIC's of both positive controls required to be within acceptable limits for experiment success and subsequent interpretation of results. 100µl of broth was added to all the wells of the first row which served as a negative culture-free control. 100µl of each extract (64mg/ml unless otherwise specified) dissolved in the same solvent used for the initial extraction, and solvent controls were added to the first well of respective rows. A dilution series was then prepared by transferring 100µl aliquots from the top to the bottom of the plate using a multi-channel pipette. The remaining 100µl from the last row was discarded. Thereafter, 100µl of organism, diluted twenty-fold in supplemented broth for *M. smegmatis* and to an optical density of 0.125 at 550nm for *M. aurum* (Chung *et al*., 1995) to yield approximately $1x10^5$ cfu/ml for both organisms, was added to each well of the 96-well plate. Extract concentrations therefore ranged from 16mg/ml to 125µg/ml, unless insufficient extract was available. The plates were sealed in plastic bags and incubated for 48 hours for *M. smegmatis* and 72 hours for *M. aurum* at 37ºC. Each extract was tested in duplicate against each organism.

40µl of 0.4mg/ml INT (Sigma, St Louis, MO, USA) was added to each well and the plates were left at room temperature for eight hours for *M. smegmatis* and overnight for *M. aurum*. The results were determined by visual inspection of the wells with the MIC being reported as the lowest concentration of extract containing no red.

4.2.2 BACTEC susceptibility testing

All procedures involving the use of MTB were performed in biological safety cabinets.
The BACTEC™ S.I.R.E Drug Kit protocol (Becton Dickinson, Sparks, MD, USA) for the drug susceptibility testing of *M. tuberculosis* was used for these experiments. A 12B vial was inoculated with *M. tuberculosis* H37R*a* ATCC 25177 from a glycerol stock stored at -70ºC. The vial was incubated at 37ºC and read on a daily basis until a GI reading of 999 was obtained. A 12B vial was then inoculated with 100µl of this suspension of H37Ra ATCC25177 and incubated at 37ºC until a GI reading of 400-500 was obtained. Test vials were inoculated with 0.1ml of this growth. 100µl of extract was added to the vial to obtain

final concentrations of 1, 0.5, and 0.1mg/ml initially. The extracts were checked for sterility before inoculation by streaking out on TSA plates, and the 12B medium was checked on a daily basis for murkiness as an indicator of contamination. Any suspected contaminants were confirmed by culture on 2% blood agar plates. Control vials were prepared at the same time – one inoculated with the same volume of the MTB suspension and the other with a 1:100 dilution of the MTB suspension prepared in TB diluting fluid (NHLS Media Department, Greenpoint, Cape Town), to represent 1% of the bacterial population present in the test vials.

Vials were incubated at 37ºC and readings were taken every day. The GI readings of the test vials were compared with the control vial containing a 1:100 dilution of the inoculum. Readings were taken until this control vial had reached a GI greater than 30. The difference in the GI values of the last two days is designated as ?GI. If the ?GI value of the vial containing extract was less than the control, the population was reported to be susceptible to the extract at that concentration. Due to the high cost of the 12B medium, each extract at each concentration was only tested once. If no inhibition was exhibited at 1mg/ml, the MIC is designated as >1mg/ml. If inhibition was exhibited at 1mg/ml, but not at 0.5mg/ml, then the MIC is reported as 1mg/ml. If the extract exhibited inhibition of growth at 0.5mg/ml, but not at 0.1mg/ml, then the extract was further tested at 0.3mg/ml. If active at this concentration, the MIC was reported as 0.3mg/ml. An MIC reported as 'Not determined' implies that insufficient extract was available to perform testing.

4.3 Results

The antimycobacterial effects of the crude extracts are reported in Table 4.1. Acetone and methanol controls at 2.38%, equivalent to the maximum solvent concentration in test 12B samples, exhibited no inhibitory effect on mycobacterial growth. As mentioned previously for the broth micro-dilution experiments, some strongly coloured extracts prevented visualization of the INT and MIC's are reported with a '=' symbol.

Table 4.1: Antimycobacterial activity of the crude extracts against three mycobacterial species as determined by the broth micro-dilution method for *M. smegmatis* and *M. aurum* A+, and the BACTEC 460 method for *M. tuberculosis* H37R*a*. The results are expressed in mg/ml as MIC's. Rifampicin was used as the positive control for *M. aurum* and *M. tuberculosis* and ciprofloxacin for *M. smegmatis*.

		M. smegmatis		***M. aurum***		***M. tuberculosis***	
Extract	**Plant part**	**Methanol**	**Acetone**	**Methanol**	**Acetone**	**Methanol**	**Acetone**
Acacia robusta	B	2	2	4	2l	1	1
Adiantum capillus veneris	L	8	0.5-4	8	1	>1	1
Agathosma betulina	L	4	8	=16	16	>1	>1
Alipedia amatymbica	L	2	0.5-2	4	4	0.3	1
Conyza scabrida	L	4	1-2	2	0.5	1	0.3
Datura stramonium	L	16	0.5	2	2	0.3	0.5
Dioscorea sylvatica	T	2-4	2- 8	1-2	0.25-0.5	<0.5	>1
Eriocephalus africanus	L	4	0.5-1	4	>2.3	0.5	0.3 or 0.5
Helichrysum nudifolium	L	4-8	2	8	1	1	1
Helichrysum nudifolium	R	8	8	8	16	1	>1
Helichrysum odoratissimum	L	1-2	0.5-1	1-2	0.68	>1	0.3
Mentha longifolia	L	2	0.5	8	2	>1	1
Ozoroa paniculosa	B	2	1	1	0.25	1	0.3
Pellaea calomelanos	L	8	1	16	2	>1	1
Pollichia campestris	L	2	1	2-4	8	>1	1
Pollichia campestris	R	16	2	>16	>5.2	1	>1
Salvia africana-lutea	L	8	4	4-8	2	1	>1
Siphonochilus aethiopicus	R	1-4	1-3.3	2-4	0.25-1	≤0.1	0.3
Syzigium cordatum	B	2	4	1	2	0.3	1
Syzigium cordatum	L	8	8	4	4	1	>1
Tetradenia riparia	L	0.5	4	4	2	0.3	1
Xerophyta retinervis	B	0.5	2	4	>16	0.3	≥1
Xerophyta retinervis	R	8	>16	4	>2.7	0.3	Not done
Positive controls (Rifampicin/ciprofloxacin)		0.0082 – 0.0163	0.0082 – 0.0163	0.0078	0.0078	0.002	0.002

B = bark, L = leaves; R = roots; T = tubers; ND = not determined due to insufficient quantities of extract

4.4 Discussion

The eight most active extracts against *M. smegmatis*, with MIC's of 0.5mg/ml were the acetone extracts of *A. capillus-veneris* leaves, *A. amatymbica* leaves, *E. africanus* leaves, *H. odoratissimum* leaves, *D. stramonium* leaves and *M. longifolia* leaves. Only two methanolic extracts exhibited the equivalent activity, namely *T. riparia* leaves and *X. retinervis* scales. *M. aurum* has in recent times become more frequently used as a substitute for *M. tuberculosis* for the high-throughput screening of plant derived products due to its similar drug susceptibility profile. In this study using the broth micro-dilution method, 5 acetone extracts resulted in MIC's of 0.25-0.68mg/ml, namely, *D. sylvatica* tubers, *H. odoratissimum* leaves, *C. scabrida* leaves, *O. paniculosa* bark and *S. aethiopicus* roots. Extracts which exhibited the greatest activity against *M. tuberculosis* in the BACTEC 460 method with MIC's of 0.3mg/ml or less, were the methanol extracts of *A. amatymbica, S. aethiopicus, T. riparia* leaves, *X. retinervis* roots and bark, *S. cordatum* bark, *D. stramonium* leaves and the acetone extracts of *H. odoratissimum* leaves, *C. scabrida* leaves, *E. africanus* leaves, *O. paniculosa* bark and *S. aethiopicus* roots. The methanol extract of *S. aethiopicus* roots was the most active extract tested, with activity at 0.1mg/ml, the lowest concentration tested.

The only extract with good activity against all three mycobacterial species investigated was *H. odoratissimum*. Lall and Meyer (1999) investigated its activity using the agar plate method and found that the acetone extract of the whole plant had an activity of 0.5mg/ml against *M. tuberculosis* (H37Rv). Using the BACTEC 460 method, the same extract exhibited an MIC of 0.5mg/ml against *M. tuberculosis* (H37R*v*) and an MDR-TB strain, compared to the MIC of 0.3mg/ml obtained from an acetone extract of only the leaves against *M. tuberculosis* (H37R*a*) used in the current study. The acetone extract of *O. paniculosa* bark was very effective at inhibiting the growth of *M. tuberculosis* and *M. aurum*, resulting in low MIC's of 0.3 and 0.25mg/ml respectively, and moderate activity (1mg/ml) against *M. smegmatis*.

The methanol extract of the leaves of *T. riparia* exhibited good activity against *M. smegmatis* and *M. tuberculosis* in this study. Van Puyvelde *et al*. (1994) found that extracts from the leaves had *in vitro* activity against *M. tuberculosis* at 1mg/ml, slightly higher than experienced in the current study (0.3mg/ml). Compounds isolated previously from the

leaves include 8(14),15-sandaracopimaradeine-7a,18-diol, which had activity against *M. smegmatis* with an MIC of 12.5µg/ml (van Puyvelde *et al*., 1986) and *M. tuberculosis* at concentrations varying between 25µg/ml and 100µg/ml (van Puyvelde *et al*., 1994).

The methanol extract of *X. retinervis* scales exhibited good activity against *M. tuberculosis* and *M. smegmatis* in this study. Several flavonoids, including 6-C-methyl quercetin 3-methyl ether, have been isolated from the leaves of this plant (Williams *et al*., 1994). A dimer also found in *Ginkgo biloba*, amentoflavone, is also found in *X. retinervis* (van Wyk *et al*., 2002), but as yet, no antimycobacterial data is available. Acetone extracts of the leaves of *E. africanus* had moderate activity against *M. smegmatis* (0.5 – 1mg/ml) and *M. tuberculosis* (MIC of 0.5mg/ml). Salie *et al.* (1996), however, found that varying solvent extracts of *E. africanus* plant parts (leaves, stems and roots) had no activity against *M. smegmatis*.

Fifteen of the 46 extracts tested (32.6%) in this study had activity against *M. tuberculosis* H37R*a*, five (10.9%) against *M. aurum* and eight (17.4%) against *M. smegmatis*. The fact that more extracts exhibited activity against *M. tuberculosis* in the BACTEC 460 method than in the broth dilution methods with non-pathogenic mycobacteria can be attributed to this being a well established and controlled method of susceptibility testing, together with the fact that fairly high concentrations of organism are required for visual detection by INT in the microplate method at the cost of sensitivity (Eloff, 1998). These findings emphasize the need to confirm activity from plant products against non-pathogenic mycobacteria with the radiometric method involving *M. tuberculosis.*

5.1 Introduction

In summary of the results obtained from the antimicrobial and antimycobacterial testing of the crude extracts, twelve extracts exhibited activity against two or more organisms as summarized in Table 5.1. A bar graph representing the average MIC values (less than 1mg/ml) of some of these active extracts against selected organisms is illustrated in Figure 5.1. The extract exhibiting activity at less than 1mg/ml across the broadest range of organisms was the methanol extract of *X. retinervis* bark/scales, active against seven of the eleven organisms tested, followed by the acetone extract of *H. odoratissimum* leaves which inhibited the growth of all three Gram-positive bacteria, all mycobacteria and two Gram-negatives. The acetone extract of *O. paniculosa* inhibited the growth of four of the eleven test organisms below 1mg/ml, as did *E. africanus*. It is interesting to note that despite being a small percentage of the types of plant parts collected, bark and tubers accounted for half of the active plant parts in Table 5.1. It is possible that the protective effect on chemicals within storage organisms and bark may account for their antimicrobial activity (Stafford *et al*., 2005).

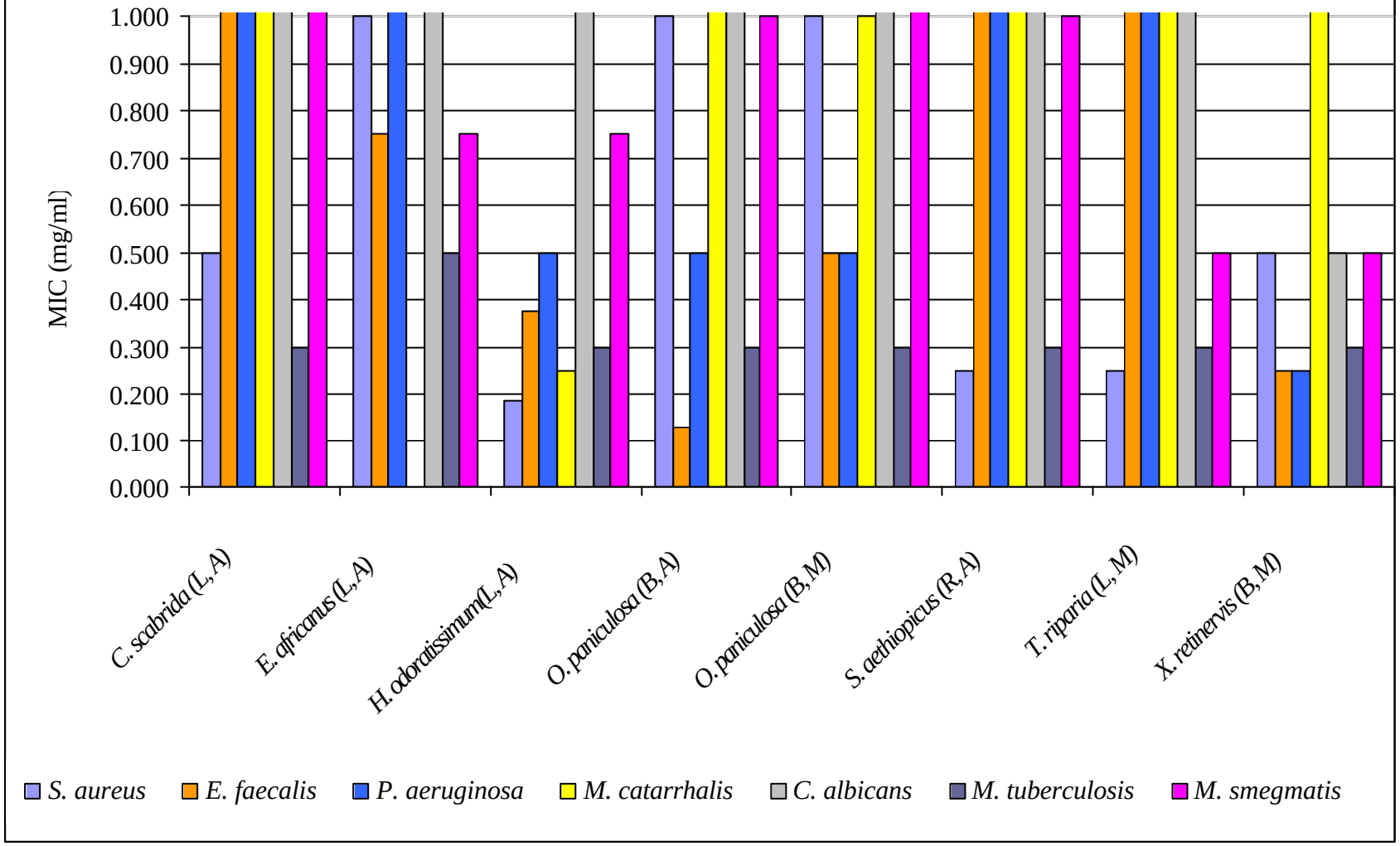
Ozoroa paniculosa was selected for the isolation of its active constituents as very little literature is available on antimicrobial and more specifically, antimycobacterial compounds derived from this genus in general. The Zulu people use the powdered bark for acute inflammatory conditions of the chest, often mixed with *Berchemia zeyheri* (Hutchings, 1996). The Midzichenda tribes of Kenya use the roots of related species, *O. insignis* and *O. obovata*, to treat complications in menstruation, venereal diseases and as protective charms (Pakia and Cooke, 2003). Mølgaard *et al*. (2001) found that extracts of the stem bark, leaves and root bark of *O. insignis* had good anti-helminthic activity, in order of increasing activity. Asase *et al*. (2005) have reported the use of *O. insignis* leaves and twigs boiled in water and consumed as a treatment for malaria in Ghana. This species has also been used as a treatment of schistosomiasis in Zimbabwe and was lethal to adult schistosomes, reducing both the egg count and worm load in hamsters (Ndamba *et al*., 1994). Moronic acid ($C_{30}H_{46}O_3$, MW = 454) has previously been isolated from another species, *O. mucronata* (Hostettman-Kaldas and Nakanishi, 1979). The bark of this plant, known as 'msimbwi' in Swahili, is used to treat intestinal parasites, dysentery, diarrhoea, gonorrhoea, bilharzia and abortion. This compound was active against *S. aureus* and *Bacillus subtilis* with MIC's of 6.25 and 12.5µg/ml respectively, but was devoid of activity

Table 5.1: Extracts with activity against two or more test organisms. Activities are indicated as MIC's in mg/ml. An extract is regarded as having good activity if its MIC is less than 1mg/ml.

Plant	**SA**	**EF**	**BC**	**PA**	**KP**	**SO**	**MC**	**CA**	**MTB**	**MS**	**MA**
C. scabrida (L, A)	0.5	2	0.5	4	2	>8	8	8	0.3	1-2	0.5
D. stramonium (L, M)	4	8	8	4	4	16	16	0.25	0.3	16	2
D. sylvatica (T, M)	0.5	1	2	8	4	2	4	=4	>1	2-4	0.25-0.5
E. africanus (L, A)	1	0.5-1	ND	1-2	0.5	ND	ND	>2	0.5	0.5-1	>2.3
H. odoratissimum (L, A)	0.125-0.25	0. 25-0.5	<0.125	0.5	2	>16	0.25	2	0.3	0.5-1	0.68
O. paniculosa (B, A)	1	=0.125	2	0.5	1	16	8	4	0.3	1	0.25
O. paniculosa (B, M)	1	0.5	2	0.5	4	8	1	=2	0.3	2	1
S. aethiopicus (R, A)	0.25	4	4	4	4	>8	13.4	2	0.3	>1	0.25-1
S. cordatum (B, A)	0.25-2	1	2.5	0.25	2	>5	>5	=4	1	4	2
S. cordatum (B, M)	=1	1	1	0.25-0.5	2	8	4-8	2	0.3	2	1
T. riparia (L, M)	0.25	8	2	2-4	8	≥16	16	4	0.3	0.5	4
X. retinervis (B, M)	0.5	0.25	5.25	0.25	0.5	5.25	5.25	0.5	0.3	0.5	4

M = methanol; A = acetone; B = bark; L = leaves; T = tubers; SA = *S. aureus*; EF = *E. faecalis*; BC = *B. cereus*; KP = *K. pneumoniae*; PA = *P. aeruginosa*; SO = *S. odorifera*; MC = *M. catarrhalis*; MTB = *M. tuberculosis*; MS = *M. smegmatis*; MA = *M. aurum;* ND = not determined due to insufficient extract quantities

Figure 5.1: Selected plant extracts exhibiting the greatest antimicrobial and antimycobacterial activity



against Gram-negative bacteria. Anacardic acid and ginkgolic acid have also previously been isolated from *O. insignis* (Rea *et al*., 2003; Kubo *et al*., 1987).

5.2 Materials and methods

5.2.1 Column chromatography

All solvents were obtained from Merck (Darmstadt, Germany) unless otherwise specified. *Ozoroa paniculosa* bark was dried at 50ºC and finely ground to obtain a total mass of 903.6g. Four acetone extractions were performed of 5 hours each by exposing finely ground bark to the solvent in a cylinder at room temperature. Solvent was evaporated with the aid of a Rotary evaporator under reduced pressure. Column chromatography was performed by packing a column (approximately 90cm long with a diameter of 15cm) with silica gel 60 (Sigma, St Louis, MO, USA) and eluting with a mobile phase of toluene 93: ethyl acetate 7. The extract remained 'sticky' and small amounts of silica 60 were used to dry the extract completely before gradual addition to the silica column. A total of 87,3g of extract was added to the column. Fractions were collected on the basis of colour changes. Four fractions were collected from the first mobile phase. Once the fractions became colourless, methanol was used to wash the remnants of extract from the column. Bio-autography was performed on each fraction to determine which contained the components responsible for antimycobacterial activity against *M. aurum* using toluene 93: ethyl acetate 7 as mobile phase (see Section 3.2.3). Two active fractions were allowed to air dry in pre-weighed glass vials initially, and were later frozen at -80ºC and freeze-dried to remove excess toluene. The fractions remained very 'sticky' and somewhat oily and were soluble in DMSO and ethyl acetate.

5.2.2 Thin Layer Chromatography (TLC)

5.2.2.1 Conventional TLC

Conventional TLC was performed on silica gel 60 F254 coated aluminium plates (20 x 20cm x 0.55mm) (Machery-Nagel, Germany). Several mobile phases were evaluated until a combination of hexane 70: ethyl acetate 10: methanol 5 yielded the best separation of bands from the active fractions. The active fractions of *O. paniculosa* were prepared in ethyl acetate to a concentration of 50mg/ml. 15µl bands were spotted out onto TLC plates (3 x 5µl bands thick) in duplicate (approximately 750µg of material per band) and allowed to run in the mobile phase for approximately 10 minutes at room temperature. The plates

were allowed to air dry and viewed at 254 and 365nm. Bands were clearly marked with pencil. One set of plates was used to perform bio-autography, as described in Section 3.1.3, and the other set kept for reference purposes. The bands were scratched off individually from the TLC plates, placed in separate eppendorfs and 1ml acetonitrile (ACN) added to each sample. The samples were sonicated for 30 minutes and left at room temperature overnight. The HPLC profiles of all the bands were evaluated to determine peaks unique to the active bands from TLC as compared to the plates resulting from bio-autography using *M. aurum*.

5.2.2.2 Preparative TLC

The Chromatotron is a preparative, centrifugally accelerated, radial, thin layer chromatograph (Harrison Research, Palo Alto, California, USA). The TLC area of the two fractions responsible for antimycobacterial activity fluoresced purple/blue at 254 and 365nm, making it suitable for concentration using the Chromatotron. 2mm silica gel 60 PF254 plates containing gypsum (Merck, Darmstadt, Germany) were prepared as per manufacturer's instructions. A 100mg/ml solution of the active fraction was prepared in ethyl acetate. The plate was equilibrated with hexane at a flow rate of 6-8ml/min. 2ml of the active fraction was loaded onto the plate at a time. The polarity of the mobile phase was increased once it was evident that no bands were being removed from the plate. The active band was removed using a mobile phase of hexane: ethyl acetate: methanol at a ratio of 70:20:10. The plate was rinsed with 100% methanol to remove remaining extract. The solvent was allowed to air-dry from the collected fraction before being dissolved in DMSO for HPLC analysis. Antimycobacterial activity of all bands collected from the Chromatotron was ascertained by means of the broth micro-dilution method.

5.2.3 High Pressure Liquid Chromatography (HPLC)

HPLC analysis was performed using a Shimadzu LC10AS high pressure gradient system controlled via a desktop computer with Shimadzu control software and a Shimadzu CBM10A communication bus module. The equipment consisted of a diode array detector (Shimadzu SPDM10A), an automatic sample injector and two solvent delivery systems (LC10AS pumps). The diode array detector was set to acquire spectra at wavelengths of 210 and 300nm. HPLC grade acetonitrile (BDH, Poole, England) was used with purified deionised water (Millipore, Milli-Q Water System). HPLC was used to isolate and purify compounds from the active fractions.

5.2.3.1 Analytical HPLC conditions

All of the analytical HPLC separations were made using a pre-packed Supelco Discovery® C18 504955 5µm (150 x 4.6mm) column with C18 40 (Bondesil) guard column (Anatech). The initial TLC fractions were centrifuged for 10 minutes at 10000rpm to pellet the silica from the samples. The column flow rate was set to 1ml/min and the injection volumes were 100µl. The samples were all run in ACN/water mobile phase. Samples were separated using a gradient of 10% to 90% ACN over 40 minutes, followed by a wash with 100% ACN for 2 minutes. The equilibration time between analyses was 5 minutes.

The identified active peaks were separated using an ACN/water gradient, starting with 60% ACN to 66% ACN over 10 minutes, followed by 100% ACN for 2 minutes. The equilibration time between analyses was 5 minutes. A maximum of 1mg of the separated Chromatotron fraction, dissolved in 50%DMSO/ACN was loaded onto the analytical column at a time. Compound 1 was further purified under these analytical conditions.

5.2.3.2 Preparative HPLC conditions

The TLC region containing the active components of the active fraction of *O. paniculosa* was used for the collection of the active principles by means of HPLC. This region was concentrated with the use of a Chromatotron (model 8924, Harrison Research, Palo Alto, California, USA), as described in section 5.2.2.2. The sample was dissolved in DMSO/ACN.

The collection of the active peaks was achieved by up-scaling the analytical method using a pre-packed Supelco Discovery® C18 504955 5µm (150 x 4.6mm) column with C18 40 (Bondesil) guard column (Anatech). The gradient was adjusted from that described above for the collection of the maximum volume of the separated peaks. An ACN/water gradient and a flow rate of 3ml/min were used. The gradient started at 72% ACN to 78% ACN over 20 minutes, followed by a wash with 100% ACN for 2 minutes. The equilibration time between analyses was 5 minutes. 200µl sample injections at a concentration of 50mg/ml were loaded onto the column at a time. The ACN was evaporated under reduced pressure and the water displaced by freeze drying. Dried material was stored in pre-weighed glass vials. The percentage purity was determined by integrating the area under the peak and dividing that by the total area under the spectra curve.

5.2.4 Liquid Chromatography – Mass Spectrometry

Liquid chromatography – mass spectrometry was performed at the University of Stellenbosch on a Waters API Q-TOF Ultima LCMS 0507. 1mg of the active fraction obtained from the column chromatography was dissolved in DMSO and diluted ten-fold in ACN. 10µl of sample was loaded at a time with a 1ml/min flow rate split 20/80 to the mass spectrometer and diode array detector. The mass spectrometry conditions were as follows: capilliary voltage of 3.5kV; cone voltage of 35; RFI of 40; source temperature of 120°C; desolvation temperature of 380°C; desolvation gas of 400L/h; and cone gas of 50L/h. The solvents used were ACN and water, run over a gradient of 2% to 100% ACN over 30 minutes. A Phenomenex Gemini C18, 30x2mm, 5µm column was used.

5.2.5 High Resolution Mass Spectrometry

High-resolution electron impact mass spectra (HREIMS) were obtained on a VG-70-SEQ mass spectrometer operating at 70eV at the Department of Chemistry, University of the Witwatersrand, Johannesburg.

5.2.6 Nuclear Magnetic Resonance

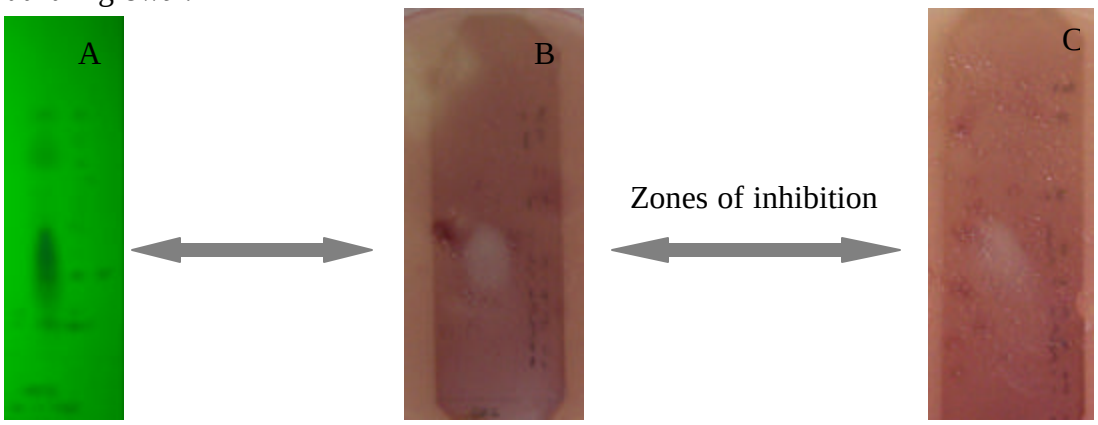
The ^{1}H, ^{13}C, HSQC, HMBC, DEPT and COSY NMR data were obtained on a Varian 600MHz (VXR 600) machine at the University of Stellenbosch, South Africa.

5.3 Results

5.3.1 TLC results

After column chromatography of the crude extract, fractions 2 and 3, which were yellow-green in colour, were identified as containing the active principles by means of bio-autography using *M. aurum*, shown in Figure 5.2 below. When viewed at 254nm and 365nm, the common band fluoresced purple/blue over a large area (Rf = 0.13 – 0.28), suggesting large quantities of this active component/s in the fractions. This property made it possible to concentrate the active band on a Chromatotron before applying to HPLC.

Figure 5.2: The zones containing the active principles on TLC viewed under UV 365nm (A) and agar overlay bio-autography plates of fractions 2 (B) and 3 (C) after column chromatography of the crude extract of *O. paniculosa* showing the zones of inhibition of *M. aurum* growth.



Three major bands were eluted from the Chromatotron using the mobile phases as described above. These bands were tested for activity against *M. aurum* using the broth micro-dilution method. The first band was visible at 254nm, the second at 254nm and 365nm and the third only at 365nm. All three bands produced cream crystals with MIC's of 1mg/ml against *M. aurum*. The second band was identified as having the unique peaks and was further used for the isolation of a compound on HPLC.

5.3.2 HPLC results

Three fractions containing three primary peaks were collected using semi-preparative HPLC. The first peak eluted at approximately 73.5% ACN (HPLC1), the second at 75% ACN (HPLC2) and the third at 76.5% ACN (HPLC3) as illustrated in Figures 5.3 and 5.4. The crude, collected peaks were screened against a range of organisms and the results are shown in Chapter 6.

Figure 5.3: The HPLC profile of the eluted fractions HPLC1, 2 and 3 using a semi-preparative C-18 column and visualizing at 300nm on a Shimadzu LC10AS at the University of Cape Town.

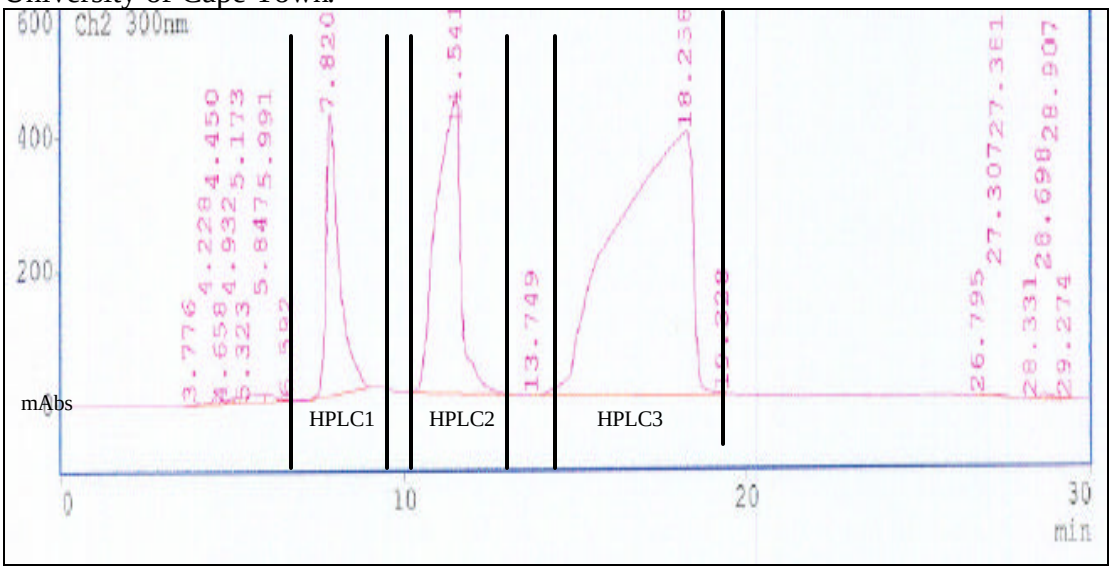
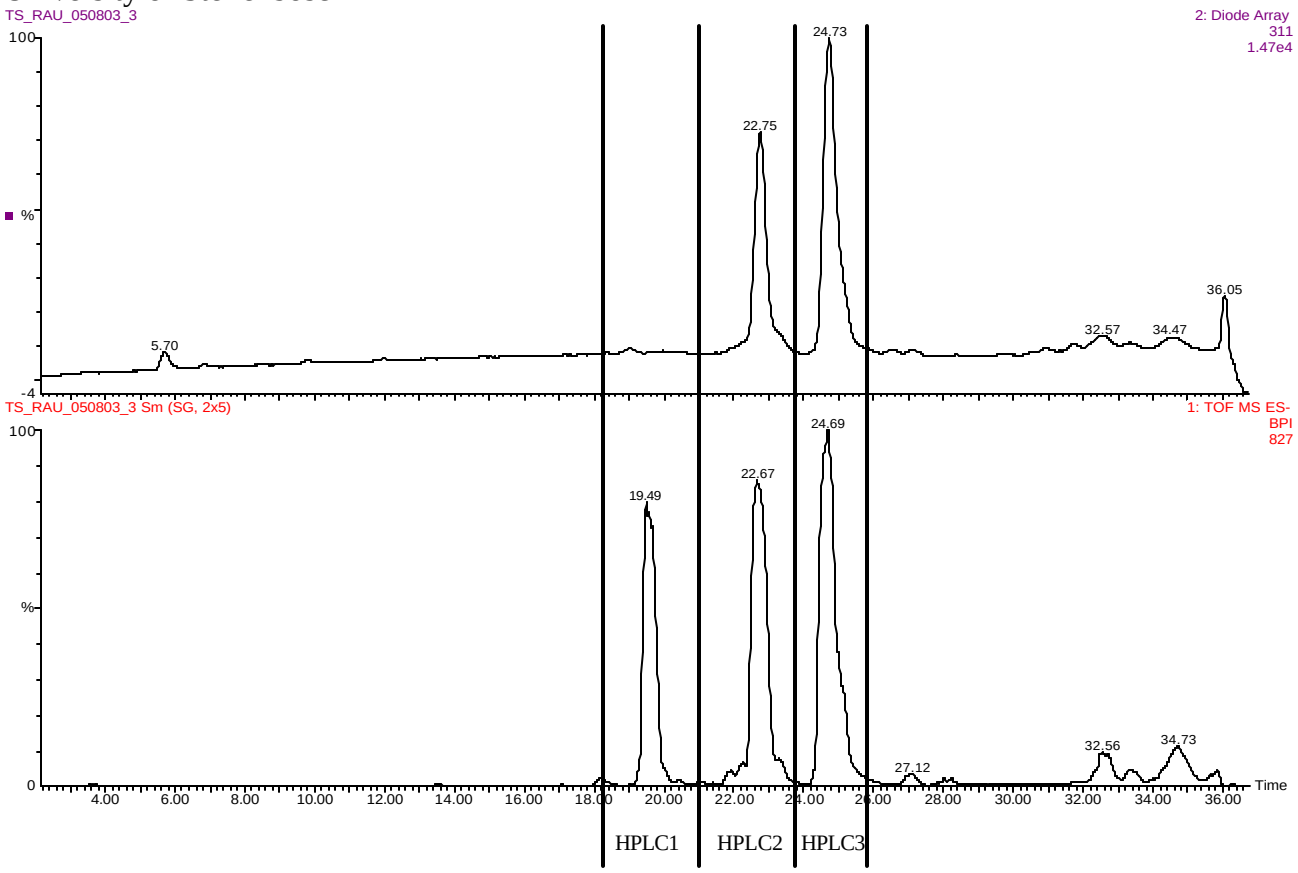
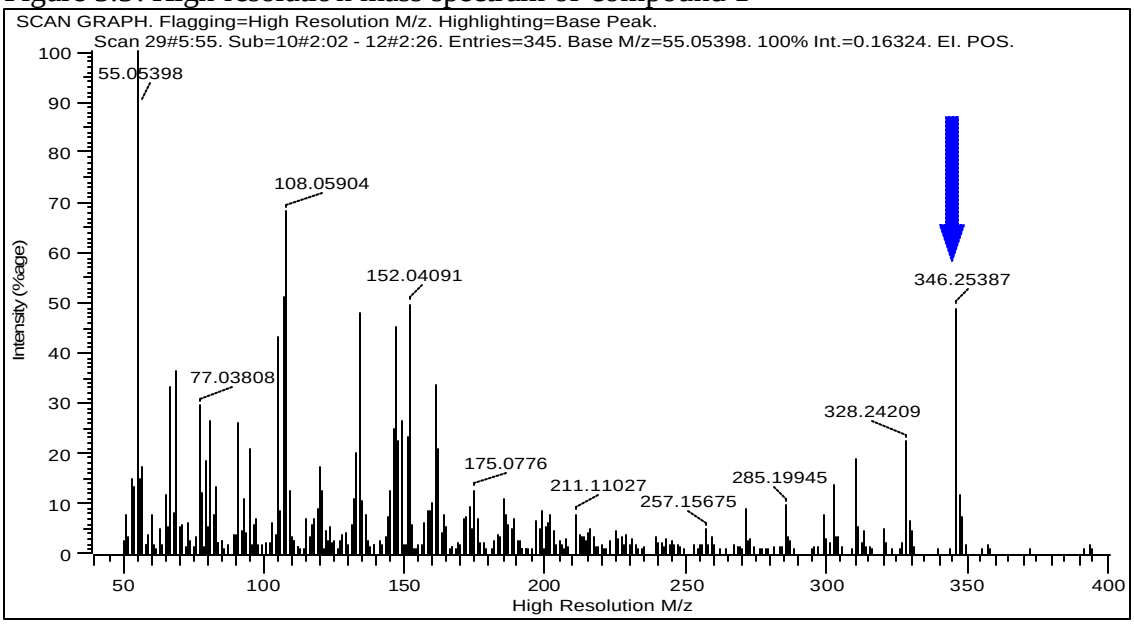


Figure 5.4: LC-MS results of active fraction 3 of *O. paniculosa* performed at the University of Stellenbosch



5.3.3 High resolution mass spectrometry of Compound 1 from HPLC1

Figure 5.5: High resolution mass spectrum of compound 1



Allowable error = minimum of 25.0 ppm, 10.0 mmu

5.3.4 Nuclear magnetic resonance of compound 1

Figure 5.6: The ^{13}C spectrum of compound 1

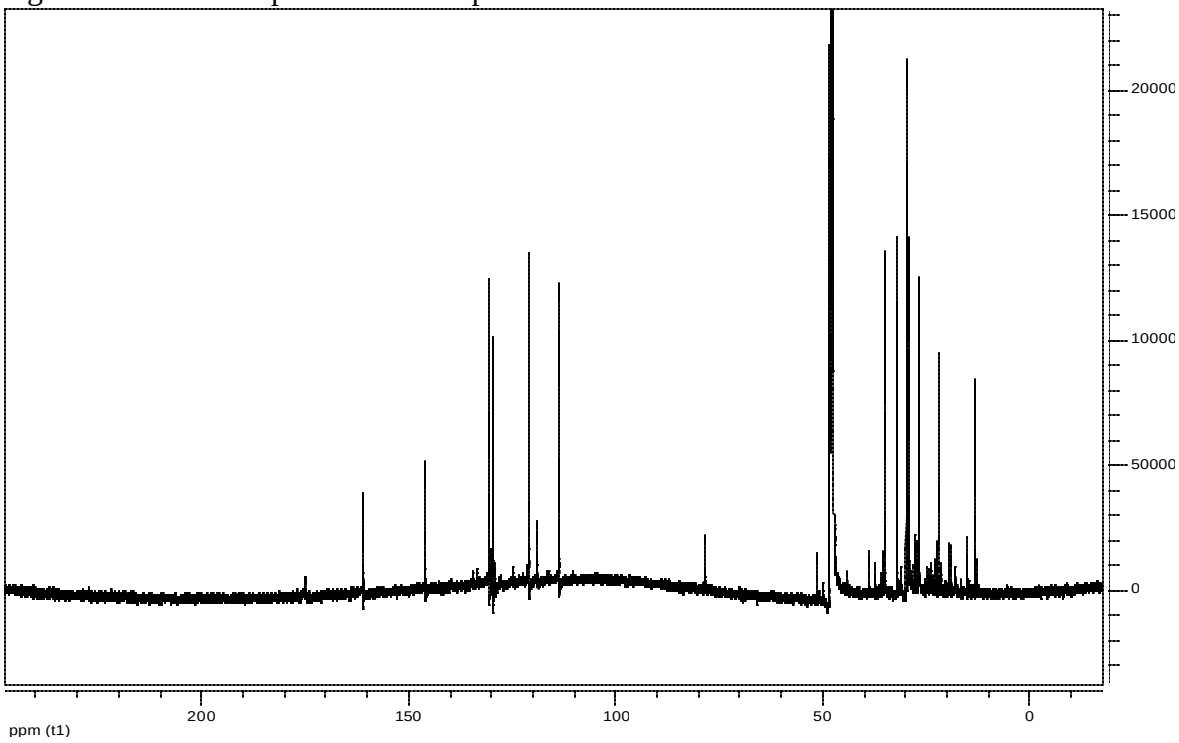


Figure 5.7: The ^{1}H spectrum of compound 1

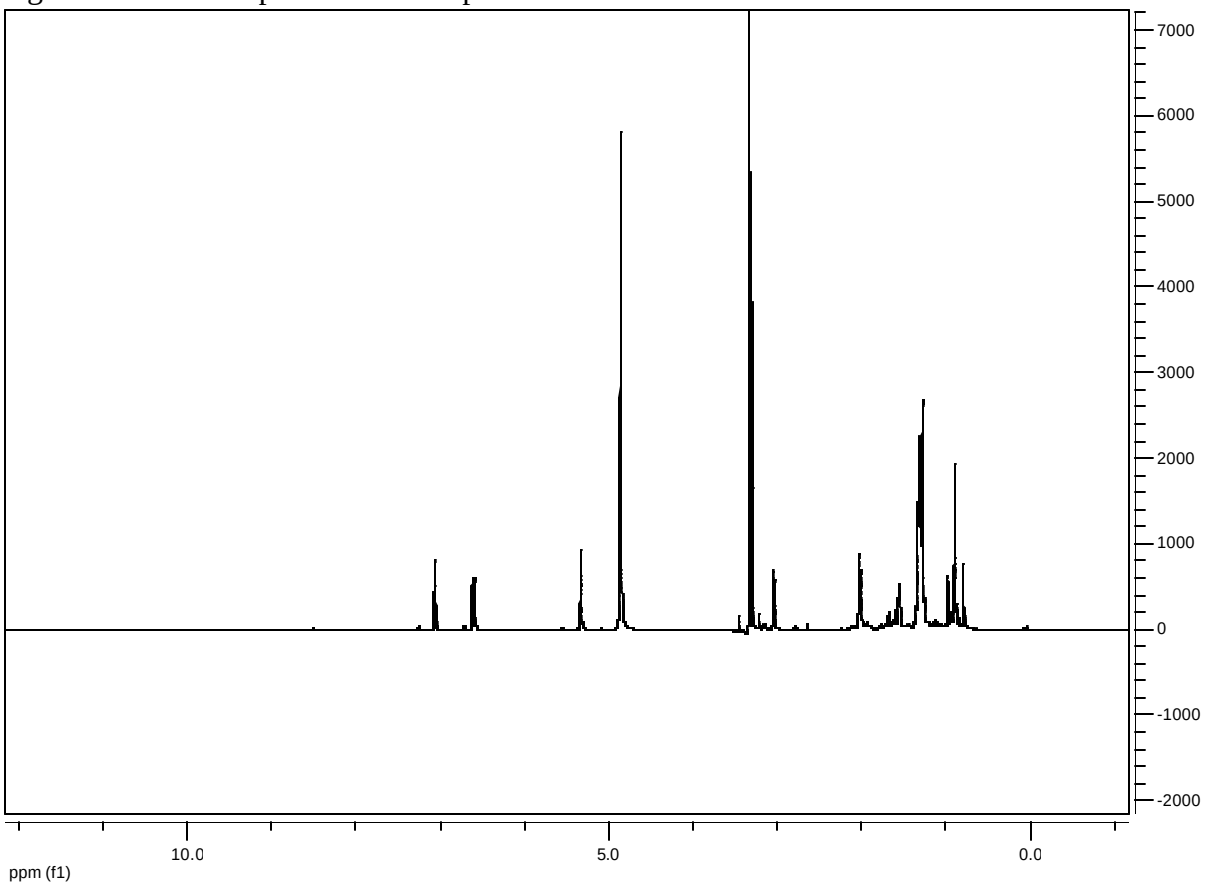


Table 5.2: The ^{1}H, ^{13}C, HMQC and COSY spectral data of compound 1

Position	d ^{1}H	J (Hz)	d ^{13}C	HMQC	COSY
1			118.9		
2			161.3		
3	6.61	*d* (7.8)	113.7, *d*	C-1, C-5	H-4
4	7.06	*t* (7.8)	130.4, *d*	C-2, C-6	H-3, H-5
5	6.59	*d* (7.8)	121.0, *d*	C-1, C-3	H-4
6			146.1, *s*		
1`	2H 3.04	*t* (7.8)	35.2, *t*	C-1, C-5, C-6	H-2`
2`	2H 1.57	*quintet*	32.2, *t*		H-3`
3`	2H	*m*	32.0, *t*	29.146, 29.277,	
4`	2H	*m*		29.454, 29.584,	
5`	2H	*		29.627, 29.663,	
6`	2H	*q*		29.875	
7`	2H 2.02	*m*	26.9, *t*		H-8`
8`	5.33	4.8 (*t)*	129.7, *d*		H-7`, H-9`
9`	5.33	4.8 (*t)*	129.6, *d*		H-8`, H-10`
10`	2H 2.02	*m*	26.7, *t*		H-9`
11`	2H		31.9, *t*		
12`	2H			29.2, 29.3,	
13`	2H	*		29.5, 29.6,	
14`	2H		22.2, *t*	29.6, 29.7, 29.9	H-15`
15`	3H 0.90		13.1, *q*		H-14`
C=O			175.0		

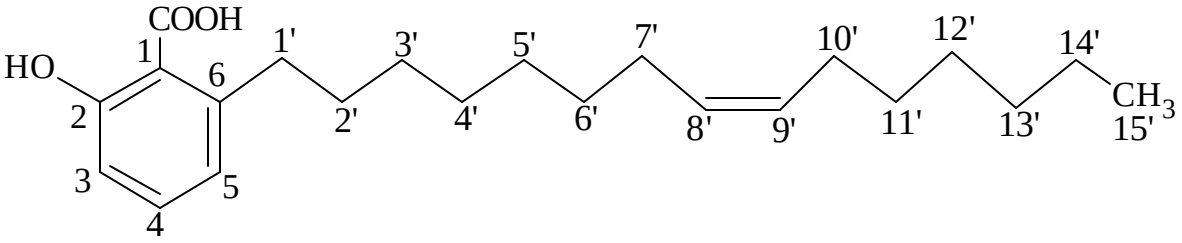
* 1.28, 1.3, 1.3, 1.31, 1.32, 1.33, 1.33

5.3.5 Structure determination of compound 1

Table 5.2 summarises the NMR elucidation of the structure of compound 1. The molecular formula of compound 1, the chemical structure of which is illustrated in Figure 5.8 below, was established as $C_{22}H_{34}O_3$ from the molecular ion 346,25387 (calculated value = 346,25080), as shown in Figure 5.5. The ^{1}H-nmr spectrum (Figure 5.7) showed signals characteristic of a 1,2,3-trisubstituted aromatic moiety as well as 27 aliphatic and 2 olefinic

protons. Confirmation of the existence of 22 carbons in the molecule was demonstrated by the ^{13}C-nmr spectrum in Figure 5.6. The DEPT spectrum indicated 4 non-protonated, 1 methyl, 12 methylene and 5 methine resonances. Of the four non-protonated carbon atoms, three were associated with the aromatic ring while the remaining one (d175) was assigned as a carboxyl carbonyl group. The aromatic ring and carbonyl group accounted for five of the 6 degrees of unsaturation, while the remaining double bond was assigned to the aliphatic chain (8`-9` or 10'-11') with its associated two methine protons resonating at d5,33 associated with carbon resonances at d129.6 and 129.7. The proton and carbon chemical shifts identified two of the methines as olefinic and the remaining three as aromatic. The resonance effects of the –COOH and –OH substituents would allow us to predict that the C-2, C-6 and C-4 in the aromatic ring are the most deshielded carbons and the C-1, C-3 and C-5 would be relatively shielded. The two doublets at 6.69 and 6.59 and the triplet at 7.06 could be ascribed to H-3, H-5 and H-4 respectively attached to C-3, C-5 and C-4 at d113.7, 121.0 and 130.4 (HSQC). The benzyllic protons 2H-1` could be identified from the ^{1}H and ^{13}C chemical shifts at d3.04 and d 35.2 respectively and by the presence of a two-bond coupling to the aromatic 6-carbon and three-bond couplings to the aromatic 1-and 5-carbons (HMQC). The COSY spectrum allowed us to distinguish some adjacent protons as described in Table 6.1, but it was not possible to differentiate between the individual methylene protons (2H – 3` - 2H – 6` and 2H-11` - 2H – 14`) as these resonated as a complex unresolved multiplet (d1.33 – 1.28). Similarly, only C-3` and C-15` could be positively identified. All two dimensional spectra are illustrated in the Appendix.

Figure 5.8: The structure of 6-[8(*z*)-pentadecenyl]salicylic acid isolated from *O. paniculosa*

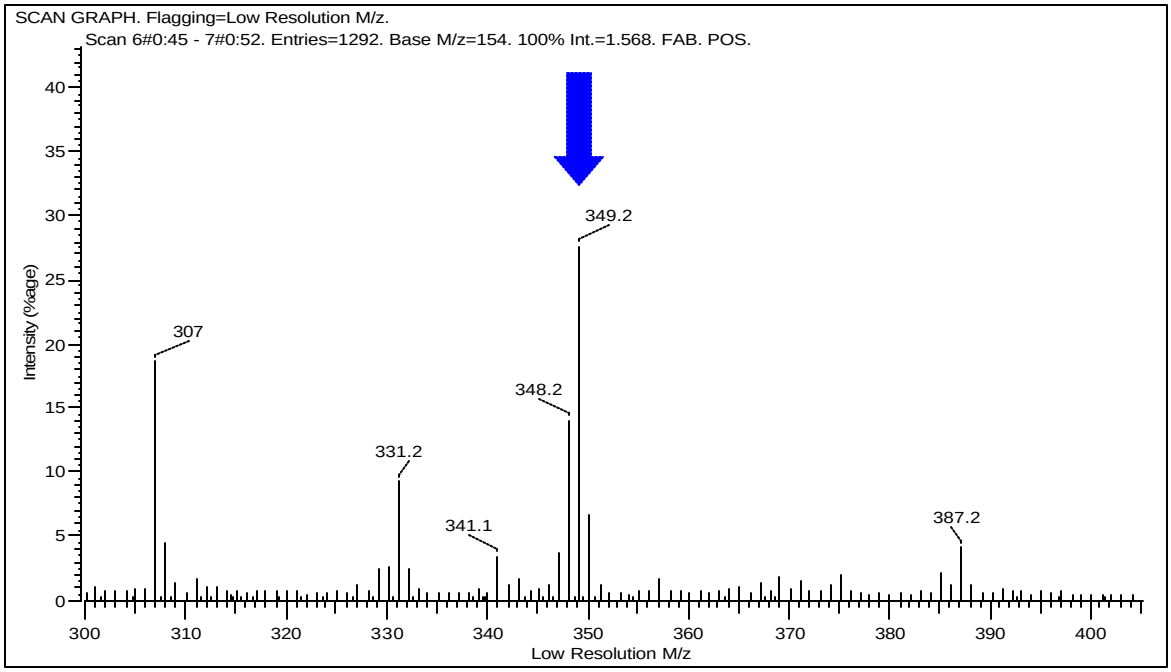


The stereochemistry of the double bond was determined to be *cis* as has previously been reported from anacardic acids (Kubo *et al*., 1987; Tyman, 1979; Green and Tocoli, 2002; Spencer *et al*., 1980; Chen *et al*., 1998) and due to the two bisallylic methylene carbons resonating at dC 26.9 and 26.7. The *trans* configuration would have been associated with

approximately a 10 ppm shift downfield (Coates *et al.*, 1994). Two compounds with the same molecular formulae have been isolated from two different species of *Ozoroa*, *O. mucronata* (Kubo *et* al., 1987) and *O. insignis* (Rea *et al*., 2003). The first compound was shown conclusively by means of ozonization to have a double bond between the 10` and 11`, while the second reported without detail that the double bond existed between the 8` and 9` carbons of the aliphatic chain. Without further experimentation, it is not possible to determine to which carbons the double bond of compound 1 can be assigned as the melting points for both compounds are in the same range (45.5-48°C; Budavari *et al*., 1996). These NMR spectra correspond well with those published by Coates *et al*. (1994), who also used methanol as solvent for the analysis of an anacardic acid with a C_{17} aliphatic chain containing three double bonds, as well as Gonzalez *et al*. (1996) and Green and Tocoli (2002).

5.3.6 Mass spectrometry of HPLC3

Figure 5.9: Mass spectrometry indicating the isotopic distribution of the tailing edge of fraction HPLC3



5.3.7 Nuclear magnetic resonance of HPLC3

Figure 5.10: The ^{13}C spectrum of HPLC3

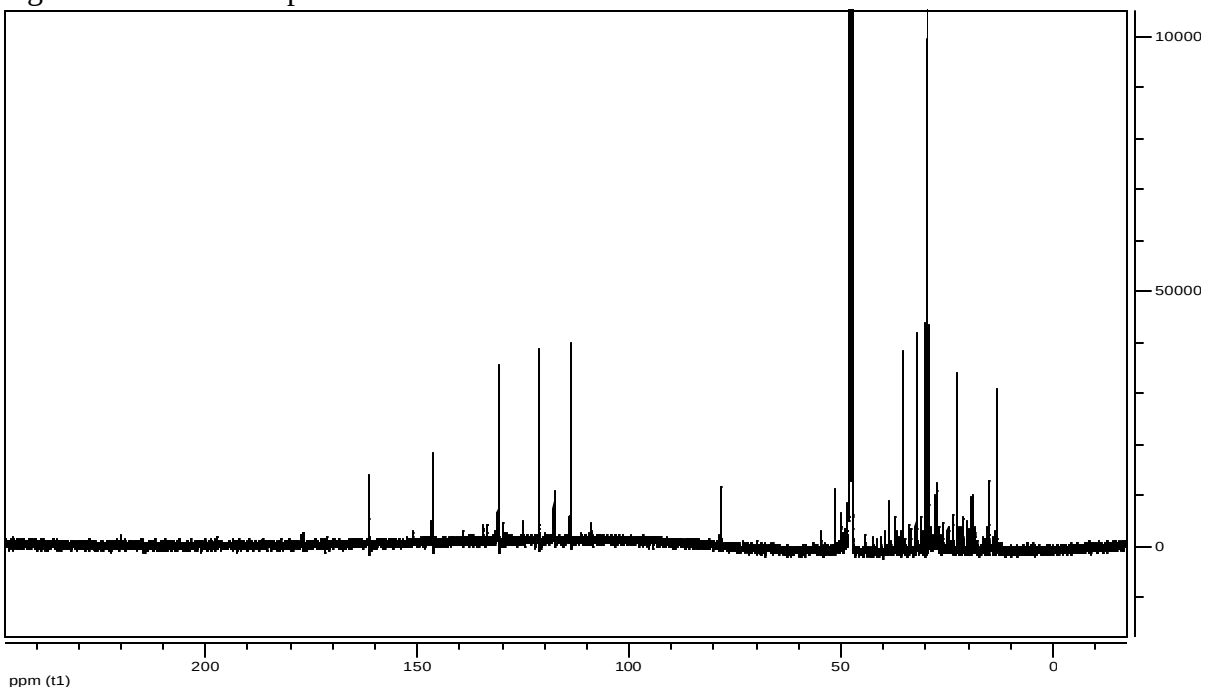


Figure 5.11: The ^{1}H spectrum of HPLC3

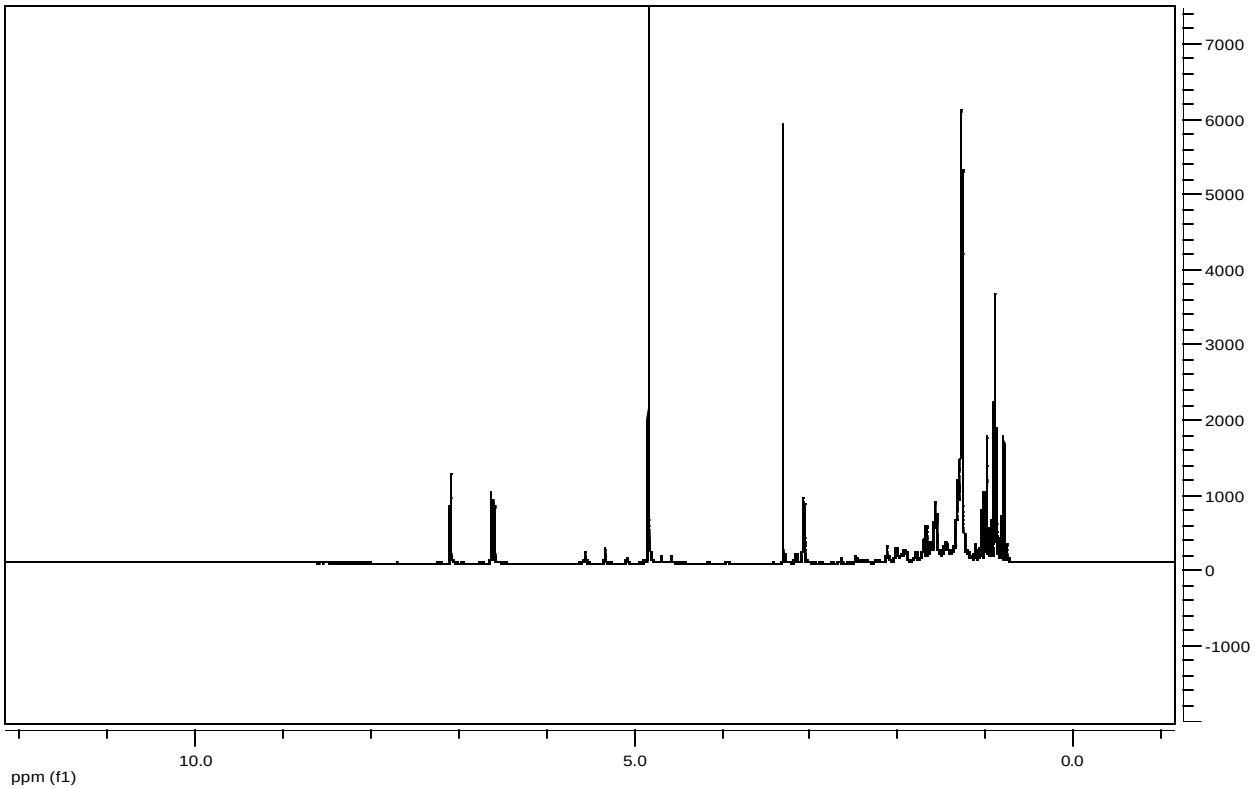


Table 5.3: The ^{1}H, ^{13}C, HMQC and COSY spectral data of HPLC3

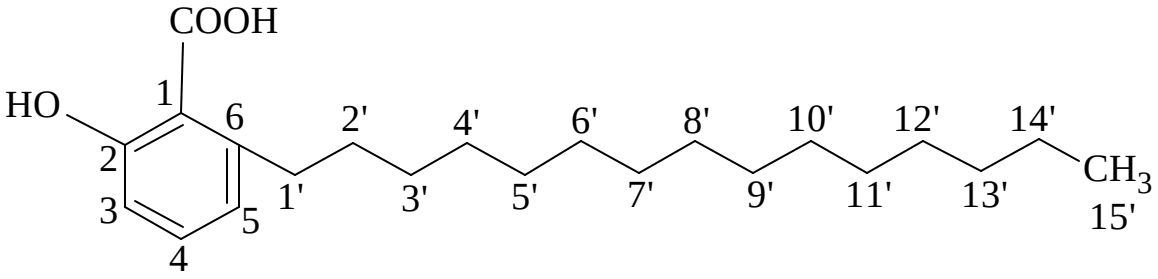
Position	**d ^{1}H**	**J (Hz)**	**d ^{13}C**	**HMQC**	COSY
1			117.75 (*s*)	C-3, C-5, COOH	
2			161.5 (*s*)	C-4, C-6, OH	
3	6.63	7.8 (*d*)	113.9 (*d*)	C-1, C-5	H-4
4	7.09	7.8 (*t*)	130.9 (*d*)	C-2, C-6	H-3, H-5
5	6.60	7.8 (*d*)	121.2 (*d*)	C-1, C-3	H-4
6			146.5 (*s*)	C-2, C-4	
1`	2H 3.07	7.8 (*t*)	35.2 (*t*)	C-1, C-5, C-6	H-2`
2`	2H 1.57	*quintet*	32.3 (*t*)		H-3`
3`	2H		31.9 (*t*)		
4`	2H				
5`	2H				
6`	2H				
7`	2H				
8`	2H	1.27 -		29.9, 29.7, 29.7,	
9`	2H	1.26 (*m*)		29.6, 29.6, 29.3	
10`	2H				
11`	2H				
12`	2H				
13`	2H				
14`	2H				H-15`
15`	3H 0.89	6.9 (*t*)	13.3 (*q*)		H-14`
C=O			177.2		

Structure determination of HPLC3

Table 5.3 summarises the NMR elucidation of the structure of HPLC3. Although not completely pure, it is evident that the primary component of the tailing edge of HPLC3 appears to be the saturated anacardic acid (Figure 5.12), almost identical to compound 1 described previously, except for the two extra hydrogen atoms arising from the hydrogenation of the olefinic bond. The molecular formula of HPLC3 was established as $C_{22}H_{36}O_3$ from the molecular ion 348.2 in the HRFAB spectrum (calculated value =

348,51944) (Figure 5.9). The ^{13}C and ^{1}H spectra (Figures 5.10 and 5.11 rspectively) for this compound are very similar to compound 1, with the absence of the double bond and its associated two methine protons resonating as a triplet at d533 associated with carbon resonances at d129.6 and 129.7. The melting point was found to be high (approximately 100°C) relative to the unsaturated derivative previously discussed (Paul and Yeddanapalli, 1954).

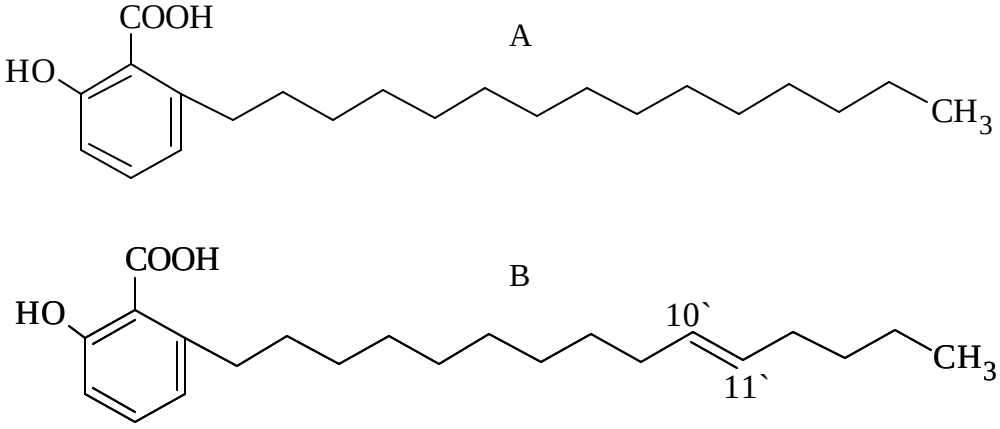
Figure 5.12: The structure of 6-pentadecylsalicyclic acid with the saturated side chain isolated from fraction HPLC3 of *Ozoroa paniculosa*



5.4 Discussion

The isolation of two anacardic acids, including the unsaturated analogue isolated in this study, has been reported from *Ozoroa insignis* in Zimbabwe (Rea *et al*., 2003).

Figure 5.13: The structures of the saturated (structure A) and unsaturated (B) versions of anacardic acid, previously isolated from *Ozoroa* species



Furthermore, the saturated anacardic acid was also isolated from *O. mucronata* (Kubo *et* al., 1987) together with an anacardic acid of the same aliphatic chain length, but with a double bond between the C10` and C11` determined by the products of ozonization. The structures are shown in Figure 5.13.

Anacardic acids (2-hydroxy-6-pentadecylbenzoic acid) are derivatives of salicylic acid with linear saturated or unsaturated 15-carbon alkyl side chains. The double bonds occur in the *cis* configuration and the saturated, monoene, diene and triene versions do not have separate trivial names (Tyman, 1979). The various forms of anacardic acids are often isolated together of which the saturated version is very little compared to the mono-, di- and tri-olefin components (Ha and Kubo, 2005; Paul and Yeddanapalli, 1954), although in this study the saturated version represented the major component. It is suspected that the major peak of fraction HPLC2 is also an anacardic acid eluting at a different ACN concentration, with preliminary NMR data showing similar structural characteristics to compound 1 and HPLC3. At this stage, it appears as though HPLC2 is also an anacardic acid with a single double bond in the aliphatic chain of unknown location. These compounds potentially originate from the polyacetate pathway and are extremely hydrophobic as a result of the long aliphatic chain. Plants from which anacardic acids are frequently isolated include the families Anacardiaceae (Cashew, *Ozoroa*) and Ginkgoaceae (*Ginkgo biloba*), although these compounds have also been isolated from other families, including Myristicaceae (*Knema elegans*) (Kozubek *et al*., 2001; Spencer *et al*., 1980). Those isolated from Anacardiaceae have aliphatic chains of variable lengths and degrees of saturation, and are hence known as anacardic acids. The biological synthesis of these compounds is similar to that of fatty acids, and is enhanced with decreased temperature and access to light (Kozubek *et al*., 2001). Similar resorcinolic lipids have been found in various organisms, including *Mycobacterium* and *Pseudomonas*. It has been suggested that the chalcone-synthase (CHS) superfamily of type III polyketide synthases (PKSs) enzymes are involved in the biosynthesis of anacardic acid (Abe *et al*., 2004). Interestingly, mycobacterial type III PKS isolated from *M. tuberculosis* shares 25-45% amino acid sequence similarity to plant CHS's (Saxena *et al*., 2003).

The affinity of anacardic acids, particularly those with the saturated alkyl chain, for certain metal ions has been reported, with a preference for Fe^{2+}, Cu^{2+} and less so, Zn^{2+} (Nagabhushana *et al*., 1995). These compounds can form water soluble monosodium and disodium salts (anacardate salts) and their transportation across an organic layer representative of a biological membrane has been reported under experimental conditions. These ion chelating properties could make them good secondary antioxidants as they reduce the redox potential, stabilizing the oxidized form of the metal ion (Ha and Kubo, 2005).

Various biological activities have been reported for anacardic acids. They exhibited inhibitory properties against various enzymes, including DNA polymerase β ($C_{17:1}$) (Chen *et al*., 1998) and β-lactamase inhibitory activity (Coates *et al*., 1994), capable of protecting β-lactam antibiotics from inactivation by bacterial β-lactamase. The $C_{15:1}$ anacardic acid was also shown to have lipoxygenase inhibitory activity (Ha and Kubo, 2005). This enzyme is thought to be involved in artherosclerosis as well as the deterioration during processing and storage of food. Kubo *et al*. (1999) also showed that an anacardic acid inhibited urease activity. The activity of anacardic acids against molluscs (snails), vectors for the parasitic disease schistosomiasis, has also been shown experimentally (Kubo *et al*., 1986; Sullivan *et al*., 1982), where it was found that the unsaturated forms were more toxic than the saturated form.

Anacardic acids have been reported to have anti-cancer activities. The $C_{17:1}$ version inhibits an enzyme responsible for DNA repair, which could be useful after treatment with anti-tumour agents which purposefully damage DNA (Chen *et al*., 1998). Rea *et al*. (2003) reported the cytotoxic properties of the saturated and monoene versions against six cancer cell lines. The saturated and unsaturated C_{15} anacardic acids have also been shown to inhibit breast and cervical carcinoma cell lines (Kubo *et al*., 1993b). Furthermore, anacardic acid from *Rhus semialata* has shown antithrombin activity (Kuo *et al*., 1991).

6.1 Introduction

In this section, the effect of the isolated anacardic acid and fractions HPLC2 and HPLC3 on a range of pathogens is reported.

6.2 Materials and methods

6.2.1 Broth micro-dilution for IC_{50} determination

The MIC's and IC_{50}'s of compound 1, as well as HPLC2 and HPLC3 were determined using the broth micro-dilution method in 96-well plates, as described previously. The test samples were prepared in methanol to a concentration of 1mg/ml. 100µl, in duplicate, was added to the first well and a serial dilution was performed. After addition of organism at a concentration of approximately $1x10^5$ to $1x10^6$ cfu/ml, the final concentration of the test compounds ranged between 1.95µg/ml and 250µg/ml. The controls described previously were included in each test plate, except for *M. aurum* A+, where ciprofloxacin was used instead of rifampicin, the colour of which would have interfered with the microplate reader (see Table 6.13 at the end of section 6.3). After addition of the INT, the plates were left at room temperature for 6 hours before being read at 620nm using a microplate reader, as described for the cytotoxicity determination and adapted from Newton *et al*. (2002). The percentage viability of organisms at the Log concentration of compound was determined, calculating the IC_{50} concentration at which 50% of the organism is killed. The activity of each test sample was determined in duplicate.

6.2.2 BACTEC 460

The BACTEC 460 radiometric method described earlier was used to determine the minimum inhibitory concentration of the isolated anacardic acid and HPLC2 and HPLC3 against *M. tuberculosis*. The compound/fractions were prepared in methanol and 100µl of each sample concentration was tested until the control vial reached a reading of greater than 30. A concentration range of between 32 and 250µg/ml was tested. Rifampicin was used as the positive control as described previously.

6.3 Results: Compound 1

Due to the hydrophobic nature of anacardic acids, microplate readings were affected by the turbidity of wells, as has been experienced by previous investigators (Kubo *et al*., 1999; Nagabuhshana *et al*., 1995). The MIC, indicated by the presence of red from INT was

used to help estimate an IC_{50} value against each organism. At concentrations of 62.5µg/ml to 250µg/ml the wells were visibly turbid and this was reflected in the optical density readings despite the absence of growth.

6.3.1 Gram-positive organisms

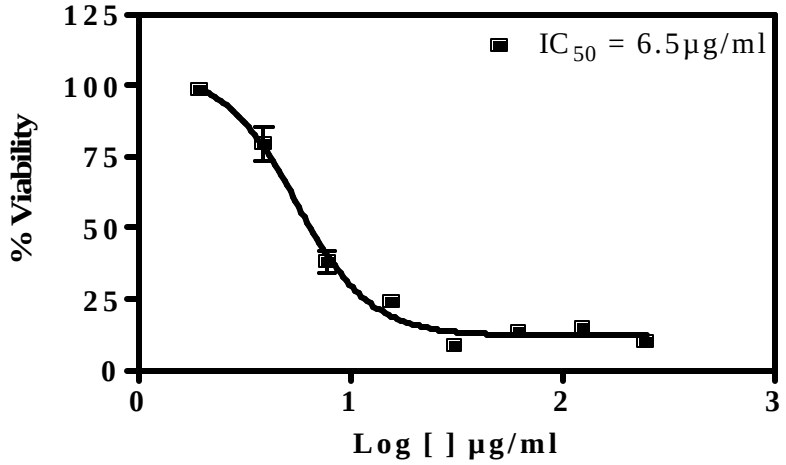
6.3.1.1 Staphylococcus aureus ATCC 12600

The MIC of compound 1 against *S. aureus*, tested in duplicate, was 31.3µg/ml. The actual and adjusted percentage viabilities are reported in Table 6.1 and the dose-response curve is illustrated in Figure 6.1 below. Anacardic acid exhibited good activity against *S. aureus* with an IC_{50} value of 6.5µg/ml.

Table 6.1: The effect of anacardic acid on the viability of *Staphylococcus aureus*

Concentration (µg/ml)	**% Viability from OD**		**Growth as indicated by INT**		**Adjusted % viability**	
250	30.76	21.77	None	None	10	10
125	13.47	15.89	None	None	13.47	15.89
62.5	12.81	14.12	None	None	12.81	14.12
31.3	7.84	9.80	None	None	7.84	9.80
15.6	23.97	23.60	Growth with increasing intensity with decreasing concentration		23.97	23.60
7.8	34.36	41.65			34.36	41.65
3.9	85.59	73.31			85.59	73.31
2.0	98.35	98.35			98.35	98.35

Figure 6.1: The dose-response curve representing the effect of anacardic acid on *Staphylococcus aureus*



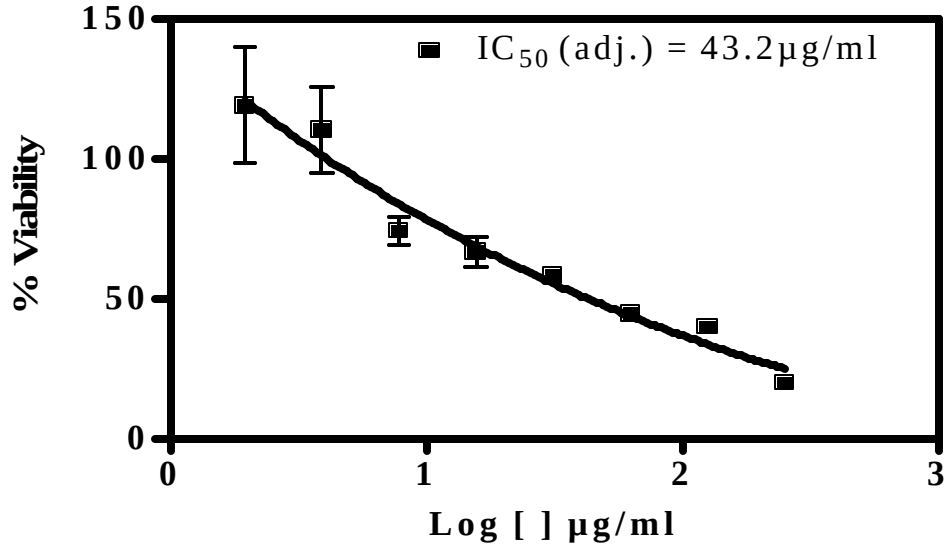
6.3.1.2 *Staphylococcus aureus clinical drug-resistant strain 1*

The encouraging activity shown by anacardic acid against a reference strain of *S. aureus*, prompted the investigation of this compound against two drug-resistant clinical strains of the organism, reported in sections 6.3.1.2 and 6.3.1.3. The MIC of compound 1 against ß-lactamase positive *S. aureus*, tested in duplicate, was 62.5µg/ml. The actual and adjusted percentage viabilities are reported in Table 6.2 and the dose-response curve is illustrated in Figure 6.2 below. Anacardic acid had moderate activity against this drug-resistant strain of *S. aureus* with an IC_{50} value of 43.2µg/ml.

Table 6.2: The effect of anacardic acid on the viability of drug-resistant *Staphylococcus aureus* strain 1

Concentration (µg/ml)	**% Viability from OD**		**Growth as indicated by INT**		**Adjusted % viability**	
250	91.99	93.49	None	None	20.00	20.00
125	81.58	86.47	None	None	40.00	40.00
62.5	65.43	92.29	None	None	45.00	45.00
31.3	58.71	58.27	Light	Light	58.71	58.27
15.6	61.69	72.15	Growth with increasing intensity with decreasing concentration		61.69	61.69
7.8	69.77	79.24			69.77	69.77
3.9	125.84	95.55			125.84	125.84
2.0	140.14	98.69			140.14	140.14

Figure 6.2: The dose-response curve representing the effect of anacardic acid on drug-resistant *Staphylococcus aureus* strain 1



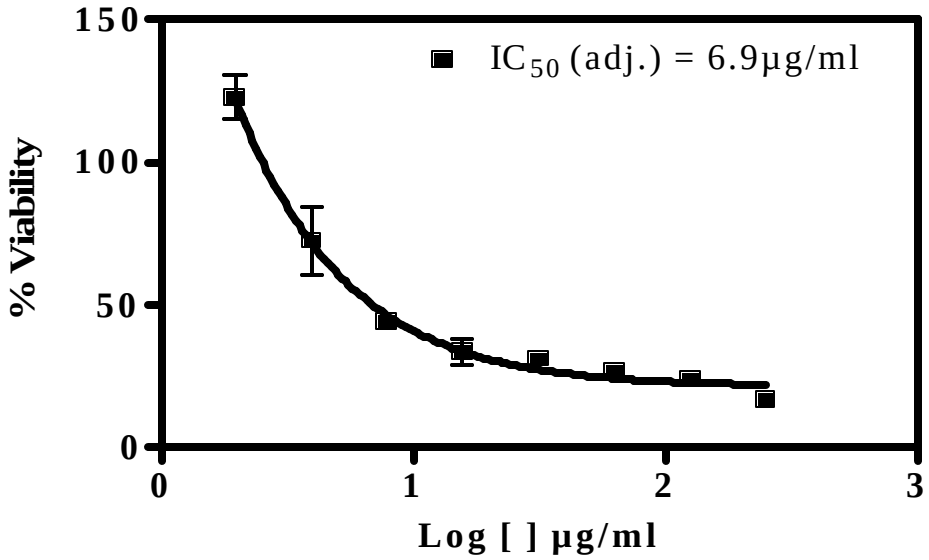
6.3.1.3 *Staphylococcus aureus clinical drug-resistant strain 2*

The MIC of compound 1 against a clinical strain of drug-resistant *S. aureus*, as tested in duplicate, was 62.5µg/ml. The actual and adjust percentage viabilities are reported in Table 6.3 and the dose-response curve is illustrated in Figure 6.3 below. Anacardic acid exhibited potent activity against this drug-resistant strain of *S. aureus* with an IC_{50} value of 6.9µg/ml.

Table 6.3: The effect of anacardic acid on the viability of drug-resistant *Staphylococcus aureus* strain 2

Concentration (µg/ml)	**% Viability from OD**		**Growth as indicated by INT**		**Adjusted % viability**	
250	69.11	68.08	None	None	19.00	15.00
125	39.95	23.31	None	None	25.00	23.31
62.5	29.67	31.67	None	None	28.00	25.00
31.3	32.85	29.45	Light	Light	32.85	29.45
15.6	38.34	28.83	Growth	Growth	38.34	28.83
7.8	45.95	42.07	Growth	Growth	45.95	42.07
3.9	84.14	60.75	Growth	Growth	84.14	60.75
2.0	115.26	130.26	Growth	Growth	115.26	130.26

Figure 6.3: The dose-response curve representing the effect of anacardic acid on drug-resistant *Staphylococcus aureus* strain 2



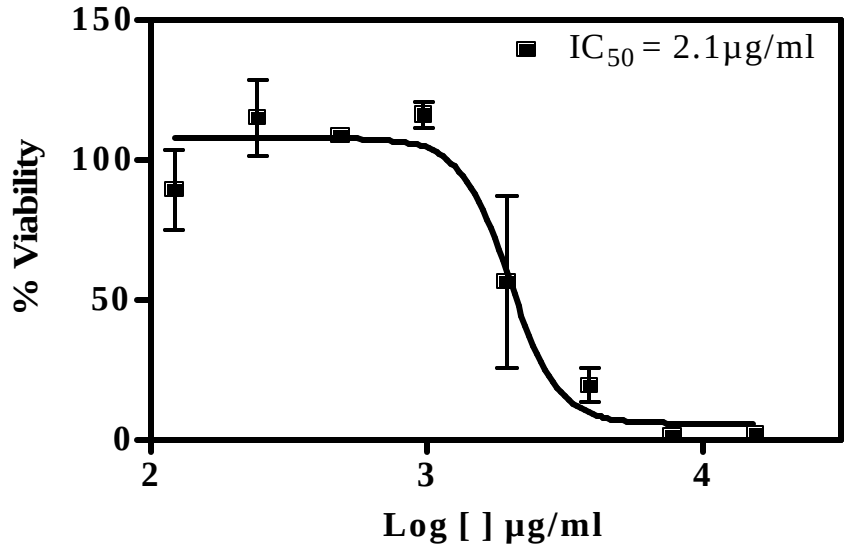
6.3.1.4 *Enterococcus faecalis ATCC 29212*

Compound 1 was tested against *E. faecalis* at concentrations from 250µg/ml to 2µg/ml and then from 15.6 to 0.1µg/ml as it appeared to exhibit potent activity. All concentrations were tested in duplicate. The MIC was determined to be 7.8µg/ml and the IC_{50} value was determined as 2.1µg/ml. The percentage viabilities are reported in Table 6.4 and the dose-response curve is illustrated in Figure 6.4 below.

Table 6.4: The effect of anacardic acid on the viability of *Enterococcus faecalis*

Concentration (µg/ml)	**% Viability from OD**		**Growth as indicated by INT**	
15.6	2.29	1.89	No growth	No growth
7.8	2.25	0.67	No growth	No growth
3.9	13.46	25.52	Growth	Growth
2.0	25.98	87.66	Growth	Growth
1.0	111.43	121.34	Growth	Growth
0.5	107.18	110.73	Growth	Growth
0.2	101.79	128.81	Growth	Growth
0.1	75.29	104.10	Growth	Growth

Figure 6.4: The dose-response curve representing the effect of anacardic acid on *Enterococcus faecalis*



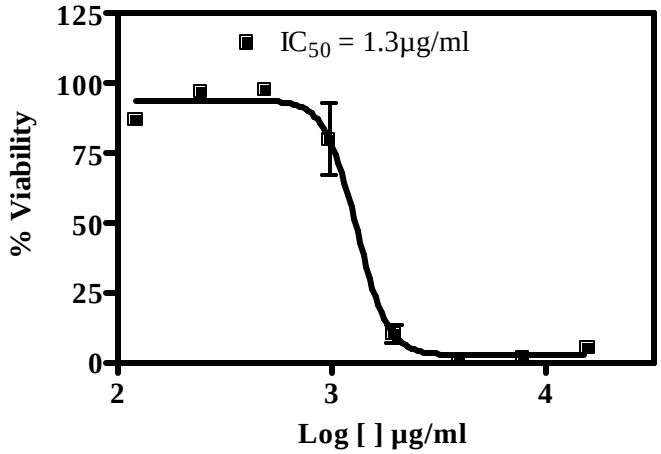
6.3.1.5 *Bacillus cereus ATCC 11778*

The all-round good activity against Gram-positives encouraged the inclusion of one more organism for testing of the antimicrobial activity of anacardic acid. The effect of compound 1 on *B. cereus* was tested at two concentration ranges, namely 250µg/ml to 2µg/ml and then from 15.6 to 0.1µg/ml, in duplicate for each concentration, as the compound appeared to have potent activity against *B. cereus*. The anacardic acid exhibited very good activity with an MIC and IC_{50} of 3.9µg/ml and 1.3µg/ml, respectively. This was the most potent antimicrobial activity observed in this study against any organism, and the range of activity is similar to currently available therapeutics. The viabilities are reported in Table 6.5 and the dose-response curve is illustrated in Figure 6.5 below.

Table 6.5: The effect of anacardic acid on the viability of *Bacillus cereus*

Concentration (µg/ml)	**% Viability from OD**		**Growth as indicated by INT**	
15.6	5.19	5.12	No growth	No growth
7.8	1.33	1.92	No growth	No growth
3.9	0.89	0.75	No growth	No growth
2.0	13.24	7.02	Growth	Growth
1.0	67.10	93.08	Growth	Growth
0.5	99.86	96.03	Growth	Growth
0.2	95.75	97.72	Growth	Growth
0.1	86.99	87.58	Growth	Growth

Figure 6.5: The dose-response curve representing the effect of anacardic acid on *Bacillus cereus*



6.3.2 Gram-negative organisms

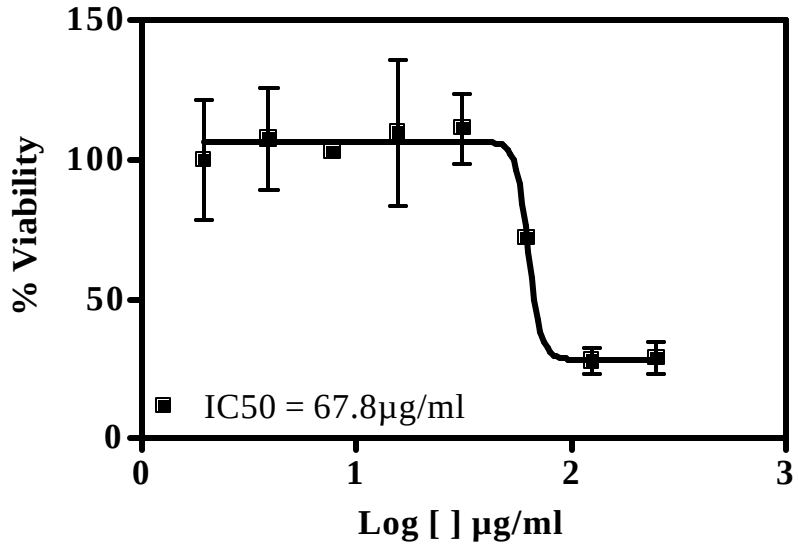
6.3.2.1 Pseudomonas aeruginosa ATCC 9027

The MIC of compound 1 against *P. aeruginosa*, as tested in duplicate at each concentration, was 250µg/ml, while an IC_{50} value of 67.8µg/ml was obtained. The growth in the first wells at 125 and 250µg/ml was very light and has therefore affected the latter value, indicating that in the first two rows less than 50% of the organism survived exposure to anacardic acid, as indicated by the percentage viabilities reported in Table 6.6 and the dose-response curve illustrated in Figure 6.6 below.

Table 6.6: The effect of anacardic acid on the viability of *Pseudomonas aeruginosa*

Concentration (µg/ml)	**% Viability from OD**		**Growth as indicated by INT**	
250	23.27	34.83	Light	Light
125	23.33	32.29	Growth	Growth
62.5	69.60	74.69	Growth	Growth
31.3	98.97	123.97	Growth	Growth
15.6	83.31	136.23	Growth	Growth
7.8	103.24	102.78	Growth	Growth
3.9	89.41	126.07	Growth	Growth
2.0	78.80	121.61	Growth	Growth

Figure 6.6: The dose-response curve representing the effect of anacardic acid on *Pseudomonas aeruginosa*



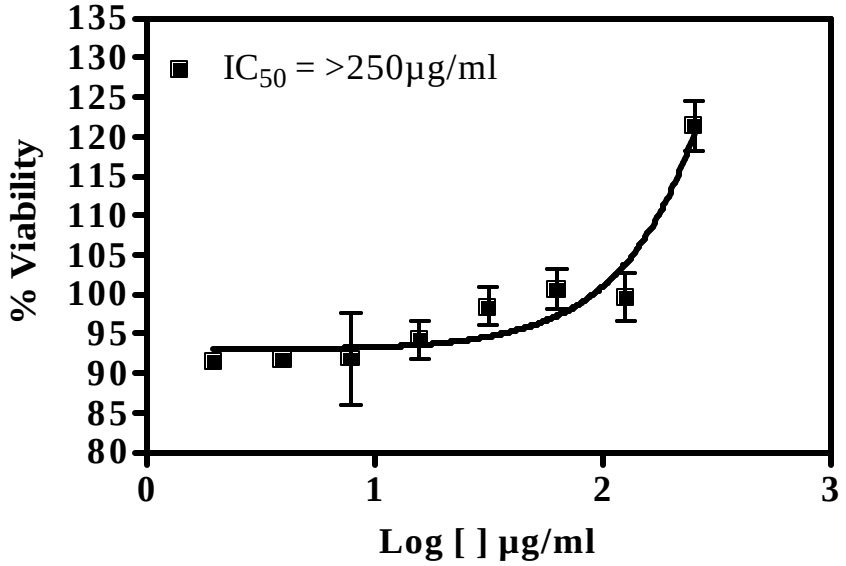
6.3.2.2 Klebsiella pneumoniae ATCC 13883

The MIC and IC_{50} values of compound 1 against *K. pneumoniae* were both >250µg/ml, as tested in duplicate at all concentrations. The percentage viabilities are reported in Table 6.7 and the dose-response curve is illustrated in Figure 6.7 below. The increasing viability at the higher concentrations could be attributed to the added effect of the turbidity resulting from the insoluble anacardic acids at high concentrations. This, however, does not affect the results, which indicate no inhibitory effect of this compound against. *K. pneumoniae*.

Table 6.7: The effect of anacardic acid on the viability of *Klebsiella pneumoniae*

Concentration (µg/ml)	**% Viability from OD**		**Growth as indicated by INT**	
250	124.45	118.20	Growth	Growth
125	96.71	102.66	Growth	Growth
62.5	98.07	103.27	Growth	Growth
31.3	96.01	100.81	Growth	Growth
15.6	91.72	96.61	Growth	Growth
7.8	97.76	85.95	Growth	Growth
3.9	90.93	92.30	Growth	Growth
2.0	90.80	92.09	Growth	Growth

Figure 6.7: The dose-response curve representing the effect of anacardic acid on *Klebsiella pneumoniae*



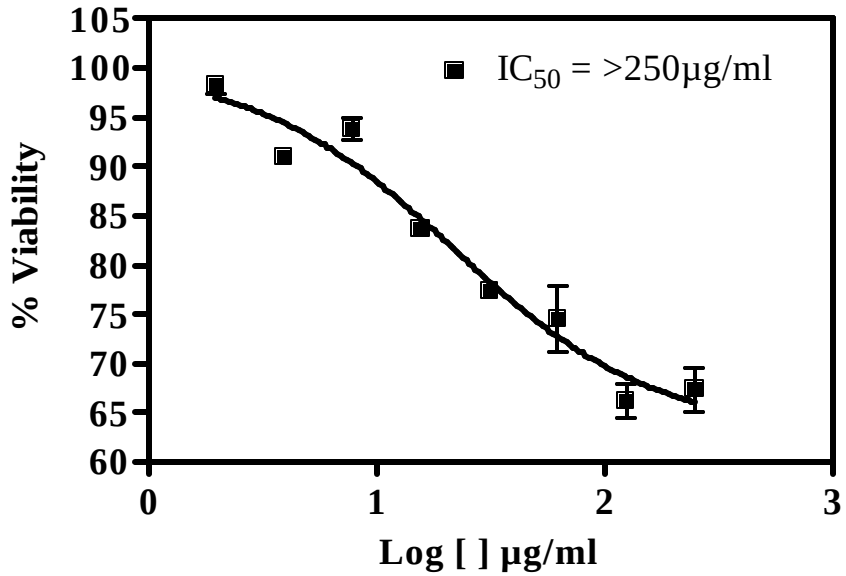
6.3.2.3 Serratia odorifera ATCC 33132

Once again, as seen with *K. pneumoniae*, anacardic acid also exhibited poor inhibition of growth of this Gram-negative organism, with only 30 – 35% of organism being killed at the highest concentration tested. The MIC and IC_{50} values of compound 1 against *S. odorifera* were both >250 µg/ml. Each concentration of compound was tested in duplicate. The percentage viabilities are reported in Table 6.8 and the dose-response curve is illustrated in Figure 6.8 below.

Table 6.8: The effect of anacardic acid on the viability of *Serratia odorifera*

Concentration (µg/ml)	**% Viability from OD**		**Growth as indicated by INT**	
250	69.64	65.18	Growth	Growth
125	67.94	64.42	Growth	Growth
62.5	77.86	71.18	Growth	Growth
31.3	77.19	77.64	Growth	Growth
15.6	83.72	83.78	Growth	Growth
7.8	94.88	92.59	Growth	Growth
3.9	91.47	90.57	Growth	Growth
2.0	97.30	99.06	Growth	Growth

Figure 6.8: The dose-response curve representing the effect of anacardic acid on *Serratia odorifera*



6.3.3 Fungi

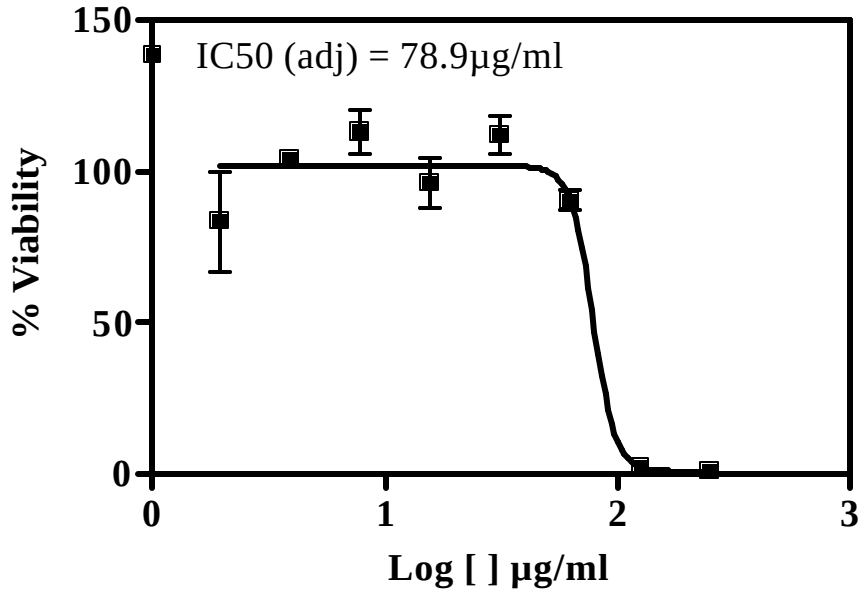
6.3.3.1 *Candida albicans* ATCC 10231

The MIC of compound 1 against *C. albicans* was 125µg/ml while the concentration at which 50% of growth was inhibited was calculated to be 78.9µg/ml. Each concentration of compound was tested in duplicate. The actual and adjusted percentage viabilities are reported in Table 6.9 and the dose-response curve is illustrated in Figure 6.9 below.

Table 6.9: The effect of anacardic acid on the viability of *Candida albicans*

Concentration (µg/ml)	**% Viability from OD**		**Growth as indicated by INT**		**Adjusted % viability**	
250	25.28	20.44	No growth	No growth	1.00	1.00
125	3.29	2.10	No growth	No growth	3.29	2.10
62.5	93.95	87.13	Growth	Growth	93.95	87.13
31.3	118.56	105.69	Growth	Growth	118.56	105.69
15.6	104.52	87.96	Growth	Growth	104.52	87.96
7.8	120.51	105.71	Growth	Growth	120.51	105.71
3.9	104.72	103.15	Growth	Growth	104.72	103.15
2.0	67.09	100.00	Growth	Growth	67.09	100.00

Figure 6.9: The dose-response curve representing the effect of anacardic acid on *Candida albicans*



6.3.4 Mycobacteria

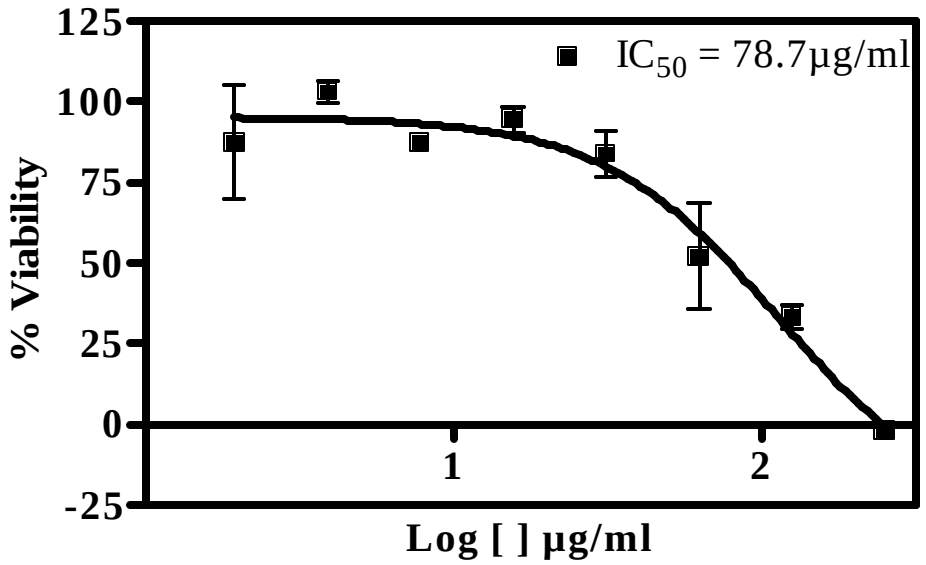
6.3.4.1 Mycobacterium smegmatis

The MIC and IC_{50} values of compound 1 against *M. smegmatis* are 125µg/ml and 78.7µg/ml, respectively, values almost identical to those obtained for the yeast, *C. albicans*. All concentrations were tested in duplicate. The percentage viabilities are reported in Table 6.10 and the dose-response curve is illustrated in Figure 6.10 below.

Table 6.10: The effect of anacardic acid on the viability of *Mycobacterium smegmatis*

Concentration (µg/ml)	**% Viability from OD**		**Growth as indicated by INT**	
250	-2.88	-1.19	No Growth	No Growth
125	36.80	29.84	Growth	Growth
62.5	68.39	35.64	Growth	Growth
31.3	77.05	90.85	Growth	Growth
15.6	98.38	90.73	Growth	Growth
7.8	89.80	84.74	Growth	Growth
3.9	99.84	106.57	Growth	Growth
2.0	105.22	69.65	Growth	Growth

Figure 6.10: The dose-response curve representing the effect of anacardic acid on *Mycobacterium smegmatis*



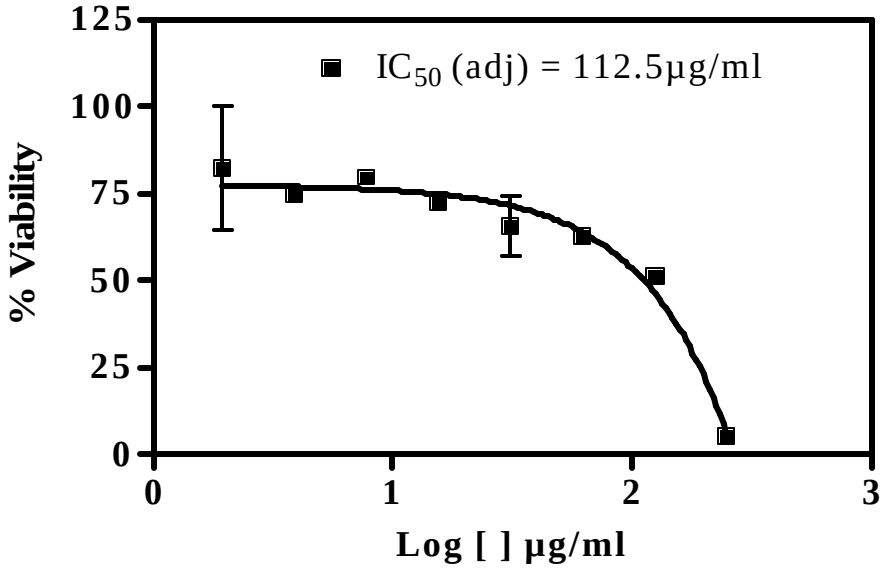
6.3.4.2 Mycobacterium aurum A+

The IC_{50} for *M. aurum* was calculated with and without adjustment, as shown in Table 6.11, to compensate for large error bars on two data points. Without adjustment, the IC_{50} was 106.7µg/ml, and with adjustment, slightly greater at 112.5µg/ml. These values are higher than those obtained for *M. smegmatis*. The dose-response curve is illustrated in Figure 6.11 below and each data point represents the average of duplicate testing. The MIC was determined as being 125µg/ml.

Table 6.11: The effect of anacardic acid on the viability of *Mycobacterium aurum*

Concentration (µg/ml)	**% Viability from OD**		**Growth as indicated by INT**		**Adjusted % viability**	
250	3.97	5.92	No Growth	No Growth	3.97	5.92
125	52.84	48.86	Growth	Growth	52.84	48.86
62.5	64.65	60.61	Growth	Growth	64.65	60.61
31.3	74.38	57.02	Growth	Growth	74.38	57.02
15.6	72.19	72.52	Growth	Growth	72.19	72.52
7.8	79.13	138.55	Growth	Growth	79.13	80.00
3.9	45.05	74.54	Growth	Growth	75.00	74.54
2.0	64.50	120.71	Growth	Growth	64.50	100.00

Figure 6.11: The dose-response curve representing the effect of anacardic acid on *Mycobacterium aurum*



6.3.4.3 Mycobacterium tuberculosis H37Ra ATCC 25177

The MIC of compound 1 against *M. tuberculosis* was 125µg/ml as determined by the BACTEC 460 GI readings reported in Table 6.12 below. Due to the high cost of 12B medium, the standardized nature of BACTEC susceptibility testing and the large amount of compound required for inoculation, each concentration was tested once. The organism is sensitive to the test substance if the ? GI of the sample is less than the ? GI of the control vial.

Table 6.12: The effect of anacardic acid on the viability of *Mycobacterium tuberculosis* as determined using the BACTEC 460 method.

Concentration	**? GI control**	**? GI sample**	**Sensitive/resistant**
250µg/ml	16	-1	Sensitive
125µg/ml	16	6	Sensitive
62.5µg/ml	16	24	Resistant

6.3.4.4 Antimicrobial positive controls utilised

Table 6.13 outlines the antimicrobial drugs used during these experiments as positive controls and the MIC's recorded. These results had to fall within an acceptable range for each experiment to be regarded as a success and for the subsequent test results to be interpretable.

Table 6.13: The control drugs and MIC's thereof for each organism

Organism	**Drug**	**MIC (µg/ml)**
S. aureus ATCC 12600	Ciprofloxacin	2.0
S. aureus drug resistant strain 1	Ciprofloxacin	0.5
S. aureus drug resistant strain 2	Ciprofloxacin	0.5
E. faecalis ATCC 29212	Ciprofloxacin	1.0
B. cereus ATCC 11778	Ciprofloxacin	0.2
K. pneumoniae ATCC 13883	Ciprofloxacin	0.5
P. aeruginosa ATCC 9027	Ciprofloxacin	<0.1
S. odorifera ATCC 33132	Ciprofloxacin	1.0
C. albicans clinical	Nystatin	3.9
M. smegmatis clinical	Ciprofloxacin	0.3
M. aurum A+	Ciprofloxacin	<0.1
M. tuberculosis H37Ra ATCC 25177	Rifampicin	2

6.4 Results: HPLC Fractions

The excellent activity of the $C_{15:1}$ anacardic acid against Gram-positive organisms and the indications that the principle components of fractions HPLC2 and HPLC3 were analogues of anacardic acid, prompted an investigation into their activities against a range of organisms. This activity was investigated by testing the fractions individually, and in combination with the identified unsaturated anacardic acid. This was done to determine potential enhanced individual activities compared to compound 1, particularly against mycobacteria, the focus of this project, as well as Gram-negative bacteria and yeast. Furthermore, possible synergy that could explain the overall effect of the compounds in the crude extract was also briefly explored. The results of these experiments are reported, together with a summary of the results of compound 1, in Table 6.15. Due to limited amounts of compound, it was not possible to perform experiments against every organism. As mentioned previously, the activity of these compounds against mycobacteria was first tested, the BACTEC radiometric method requiring large amounts of test sample (Table 6.14), followed by the Gram-negatives and fungi. All broth micro-dilution testing was performed in duplicate. Those not tested are reported as 'ND', or 'not determined'. The dose-response curves of fractions HPLC2 and HPLC3 individually, and in combination with compound 1, against *M. smegmatis* and *M. aurum* are illustrated in Figures 6.12, 6.13 and 6.14.

Figure 6.12: The dose-response curves representing the effects of fractions HPLC2 (A) and HPLC3 (B) on *Mycobacterium smegmatis*

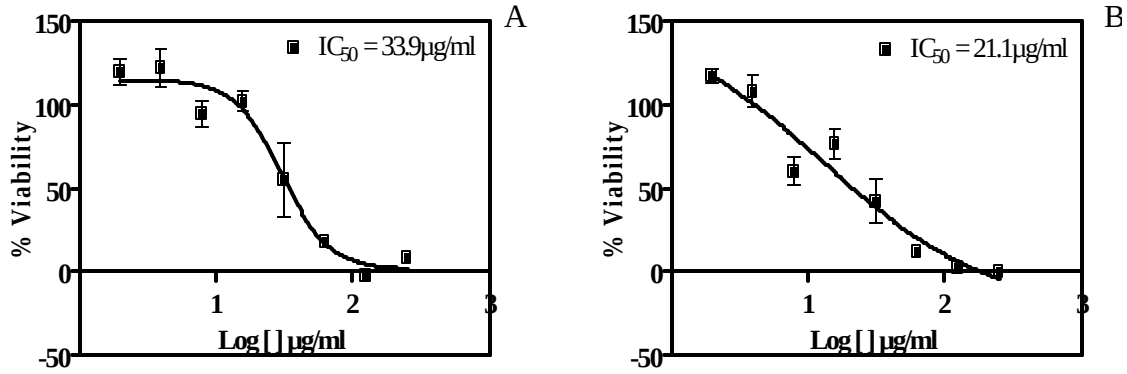


Figure 6.13: The dose-response curves representing the effects of fractions HPLC2 (A) and HPLC3 (B) on *Mycobacterium aurum*

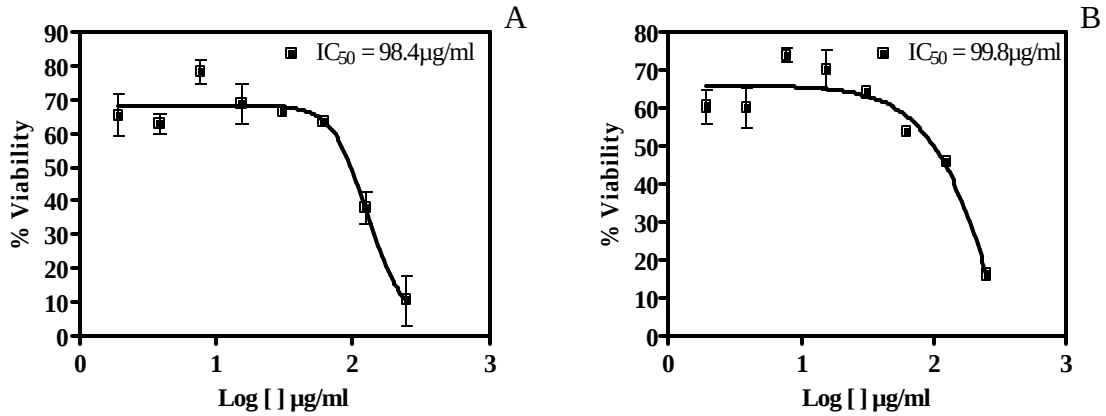


Figure 6.14: The dose-response curves representing the combined effect of compound 1 ($C_{15:1}$ anacardic acid) and fractions HPLC2 and HPLC3 on *Mycobacterium aurum* (A) and *Mycobacterium smegmatis* (B)

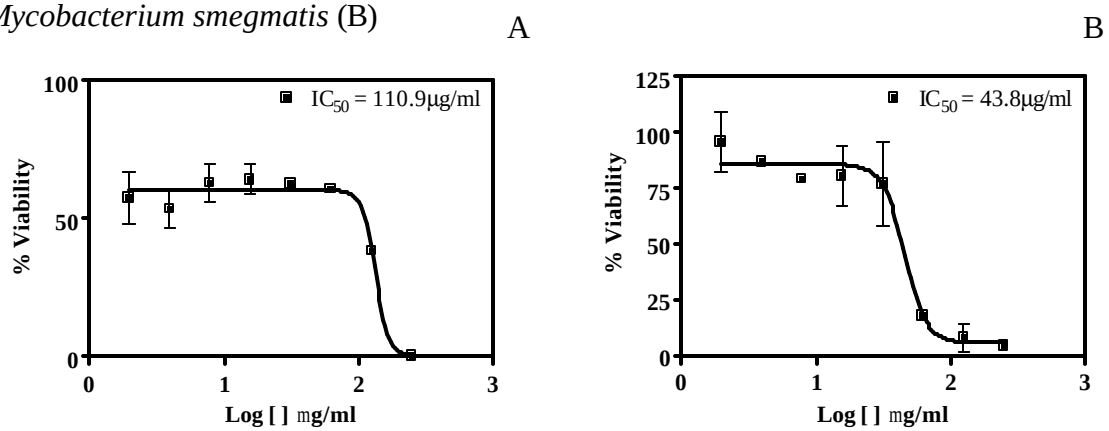


Table 6.14: The effect of HPLC2 and HPLC3 on *Mycobacterium tuberculosis* H37R*a* ATCC 25177 using the BACTEC460 system. The organism is sensitive to the test substance if the ? GI of the sample is less than the ? GI of the control vial.

Concentration of test substance	**? GI control**	Fraction HPLC2		Fraction HPLC3	
		? GI sample	**Sensitive/resistant**	**? GI sample**	**Sensitive/resistant**
250µg/ml	16	0	Sensitive	2	Sensitive
125µg/ml	16, 29	3, 1	Sensitive	3, 0	Sensitive
62.5µg/ml	16, 29	24,46	Resistant	7, -1	Sensitive
31.3µg/ml	29	134	Resistant	11	Sensitive
15.6µg/ml	18	ND	ND	68	Resistant

ND = not determined; ? GI = change in growth index

When analyzing Table 6.15 and Figure 6.15, it is apparent that compound 1, HPLC2 and the combination of 1, 2 and 3 had the same MIC's and very similar IC_{50} values against the reference strain of susceptible *S. aureus*. However, HPLC3 had remarkably decreased activity. Based on our observations that the primary component of HPLC3 is a saturated anacardic acid as well as finding from other research groups, the saturated anacardic acid is expected to have less activity against Gram-positives.

Previous authors have noted that anacardic acids in general have poor activity against Gram-negatives. Although the activity of 1, 2 and 3, alone and in combination, was less than against Gram-positives, the activity can be described as moderate to poor, ranging from IC_{50} values of 17.5µg/ml against *P. aeruginosa* by HPLC2, to >250µg/ml by compound 1 against *K. pneumoniae* and *S. odorifera*. For *K. pneumoniae*, HPLC2, HPLC3 and the combined fraction had considerably better activity (IC_{50}'s of 26.1 – 29.4) than compound 1 (>250µg/ml). Similarly for *S. odorifera*, the fractions and combination had IC_{50}'s of 19.0 – 20.6 µg/ml while compound 1 had values of >250µg/ml. However, for *P. aeruginosa*, compound 1 had decreased activity (IC_{50} of 67.8) in comparison to HPLC2 and HPLC3 (17.5 and 23.5µg/ml), but the combination exhibited an IC_{50} of >250µg/ml. The general trend that can be elucidated from the results of these compounds against Gram-negatives, therefore, is that the $C_{15:1}$ anacardic acid has less activity than HPLC2 and HPLC3, results that are consistent. Therefore, it can be surmised that increasing the lipophilicity of anacardic acid will increase activity against Gram-negative organisms. For *C. albicans*, a similar trend was noted, and in this case, the saturated anacardic acid also exhibited the best activity, while the combination and compound 1 had the poorest activity.

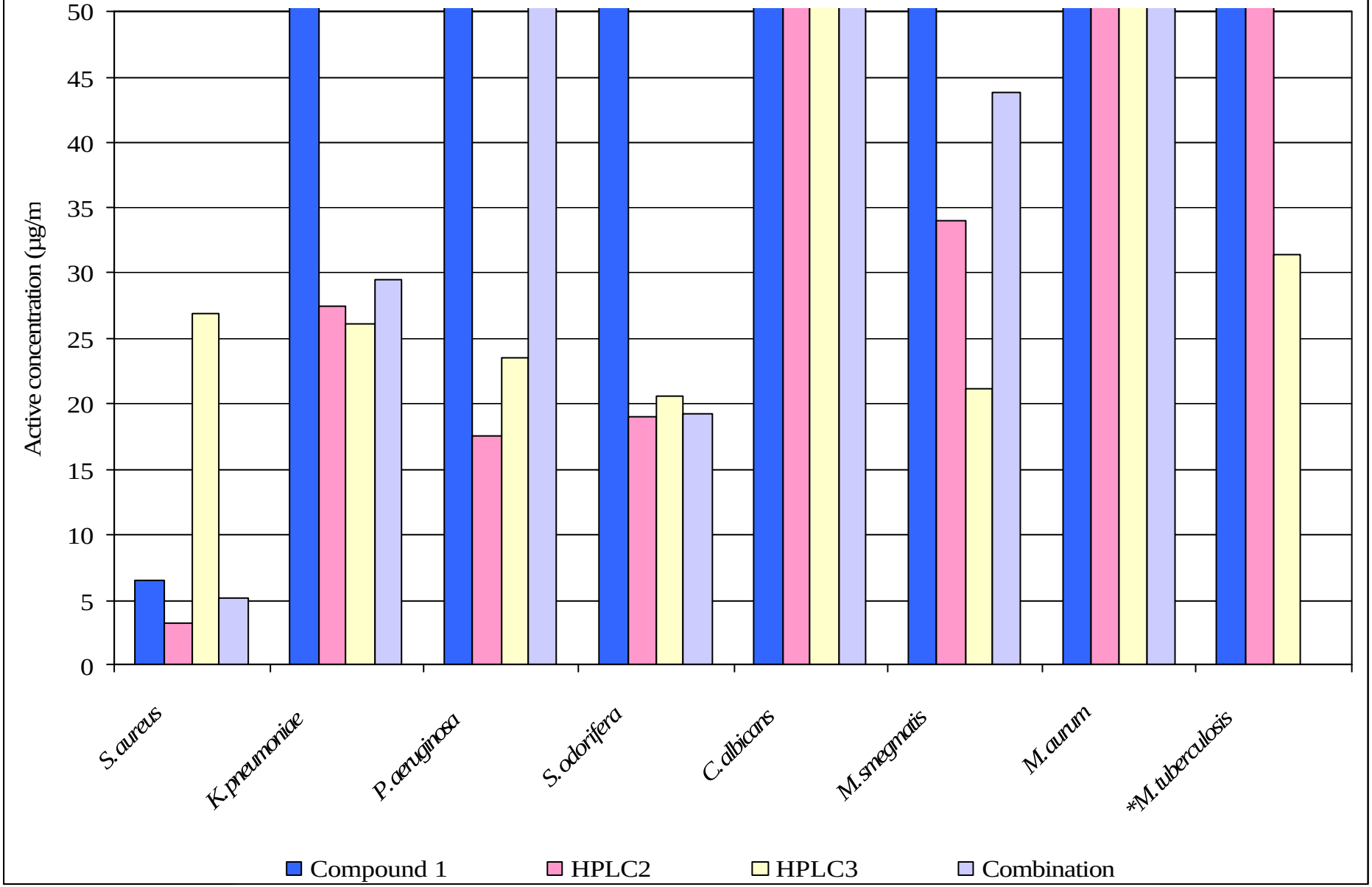
The mycobacteria excluding *M. aurum* A+, increasing lipophilicity resulted in increasing activity, with HPLC3, the saturated anacardic acid having an IC_{50} against *M. smegmatis* and an MIC against *M. tuberculosis* of 21.1 and 31.3µg/ml respectively. For *M. aurum*, the isolated compound, fractions and combination had similarly moderate activity against the organism.

Table 6.15: A summary of the effect of compound 1, as well as the effects of fractions HPLC2 and HPLC3 individually and in combination with the $C_{15:1}$ anacardic acid in equal proportions, on a range of organisms. All values are reported in µg/ml. Ciprofloxacin was used as the positive control for the Gram-positives, Gram-negatives, *M. smegmatis* and *M. aurum*, nystatin for *C. albicans*, and rifampicin for *M. tuberculosis*. All experiments, excepting those involving *M. tuberculosis*, were performed in duplicate.

	Compound 1 (µg/ml)		**HPLC2 (µg/ml)**		**HPLC3 (µg/ml)**		**Combination (µg/ml)**	
Organism	**MIC**	**IC_{50}**	**MIC**	**IC_{50}**	**MIC**	**IC_{50}**	**MIC**	**IC_{50}**
S. aureus ATCC 12600	31.3	6.5	31.3	3.3	62.5	26.8	31.3	5.2
S. aureus drug-resistant strain 1	62.5	43.2	ND	ND	ND	ND	ND	ND
S. aureus drug-resistant strain 2	62.5	6.9	ND	ND	ND	ND	ND	ND
E. faecalis ATCC 29212	7.8	2.1	<2.0	ND	15.6	ND	ND	ND
B. cereus ATCC 11778	3.9	1.3	ND	ND	ND	ND	ND	ND
K. pneumoniae ATCC 13883	>250	>250	62.5	27.4	62.5	26.1	62.5	29.4
P. aeruginosa ATCC 9027	250	67.8	250.0	17.5	250.0	23.5	250.0	>250.0
S. odorifera ATCC 33132	>250	>250	62.5	19.0	62.5	20.6	62.5	19.2
C. albicans ATCC 10231	125	78.9	31.3	82.0	31.3	79.4	125.0	80.9
M. smegmatis clinical	125	78.7	62.5	33.9	125.0	21.1	125.0	43.8
M. aurum A+	125	112.5	125.0	98.4	125.0	99.8	125.0	110.9
MTB	125	ND	125	ND	31.3	ND	ND	ND

ND = not determined; MTB = *M. tuberculosis*; MIC = minimum inhibitory concentration; IC_{50} = inhibitory concentration at which 50% of organisms is killed

Figure 6.15: The antimicrobial and antimycobacterial activities of anacardic acids. The activities are expressed as IC_{50} values for all organisms except **M. tuberculosis*, for which it is expressed as MIC values.



6.5 Discussion

The antimicrobial effects, particularly against Gram-positive organisms, of anacardic acids have been reported extensively. The greatest activity obtained by Kubo *et al*. (1995) against *S. aureus* was obtained from a $C_{15:3}$ anacardic acid with an MIC of 6.3µg/ml and increasing MIC's with decreasing double bonds. The authors found that the same compound had an MIC of 3.1µg/ml against *B. subtilis*, while the compounds with 2, 1 and no double bonds had MIC's of 6.3, 6.3 and 100µg/ml respectively. None of the anacardic acids had good activity against *Pseudomonas*, *Candida* and *Escherichia* species (MIC's >800µg/ml). However, activity of anacardic acids from cashew apples has been exhibited against another Gram-negative organism, *Helicobacter pylori*, considered to cause acute gastritis (Kubo *et al*., 1999), with MIC's of 200µg/ml to >800µg/ml. Murata *et al*. (1997) found that anacardic acids inhibited the growth of *Bacillus* and the yeast *Limomyces* and this activity was thought to be as a result of the inhibition of glycerol-3-phosphate dehydrogenase (GPDH), affecting lipid metabolism. It has also been shown that anacardic acids can be used synergistically to improve the activity of other antimicrobials, such as totarol activity against *S. aureus*, the concentration of which was decreased from 1.56 to 0.2µg/ml (Kubo *et al*., 1992). Furthermore, Muroi and Kubo (1996) showed that anacardic acid had a synergistic bactericidal activity with methicillin against MRSA, resulting in MIC's in almost the same range as for methcillin-susceptible *S. aureus* (1.6 and 6.3µg/ml, from 800µg/ml with methicillin alone). The authors obtained an MIC of 6.25µg/ml of anacardic acid ($C_{15:3}$) alone against MRSA. This compound showed rapid bactericidal activity at all stages of *S. aureus* growth. Only one report with discordant results has been found from Himejima and Kubo (1991) who reported that anacardic acids with C_{15} alkyl chains ranging from none to three double bonds all had poor activity against *S. aureus* with MIC's of >100µg/ml for all four compounds.

When the activity of salicylic acid has been tested, this compound, from which anacardic acid is derived, has shown poor antimicrobial activity despite the anacardic acids being relatively active against Gram-positive organisms (Kubo *et al*., 1993a). This suggests that the alkyl side chain is important for eliciting biological activity. Various authors have also observed that more double bonds in the aliphatic side chain correspond with greater biological activity (Kubo *et al*., 1999; Gellerman *et al*., 1969; Kubo *et al*., 1993a; Kubo *et al*., 1993b). The length of the alkyl chain also appears to play a role in activity (Kubo *et al*., 1993a; Kubo *et al*., 1999).

Kubo *et al*. (1995) have suggested a mechanism of action based on their findings. A double bond in the aliphatic chain is in the *cis* configuration, which results in a bend in the aliphatic chain, and shortens the length of the chain. The more double bonds, the more bends and the shorter the chain. These bends could lead to great disturbances of the fluid bilayer membrane, resulting in greater activity. The entire molecule would enter the molecular structure of the membrane with the hydrophilic hydroxyl group oriented into the aqueous phase and the aliphatic chain in the lipid phase. The end result would possibly be an alteration of the membrane form and function, which could explain the reported antimicrobial activities of these compounds (Kozubek *et al*., 2001).

In the current study, $C_{15:1}$ anacardic acid (compound 1) and fractions HPLC2 and HPLC3, all derivatives of anacardic acid, were active against Gram-positive organisms, concurring with previous reports. The lowest IC_{50} values were obtained from exposing *S. aureus*, *E. faecalis* and *B. cereus* to compound 1, with values less than 10µg/ml. Furthermore, the isolated anacardic acid was active against one strain of drug-resistant *S. aureus* with an IC_{50} of 6.87µg/ml. Compound 1 had relatively poor activity against Gram-negative organisms as has been shown previously, with moderate activity against *P. aeruginosa* and the fungus, *C. albicans*. HPLC3 had higher IC_{50} values in the Gram-positive organisms in comparison to compound 1 and HPLC2. However, the opposite effect was observed against *M. tuberculosis*, with HPLC3 exhibiting greater inhibitory activity (MIC = 32µg/ml) of mycobacterial growth than compound1 and fraction HPLC2 (MIC = 125µg/ml). This is an interesting result because previous biological activity screening has associated increasing numbers of double bonds in the aliphatic chain with increasing activity, and this holds true when screening Gram-positive organisms, but the converse appears to be true for *M. tuberculosis*. However, Adams *et al*. (2005) found that for quinolone alkaloids isolated from *Evodia rutaecarpa*, the increasing degree of unsaturation of the aliphatic chain containing ten to fourteen carbons resulted in increasing antimycobacterial activity. This is the first report of testing anacardic acids for antimycobacterial activity.

Fractions HPLC1, HPLC2 and HPLC3 eluted at increasing concentrations of ACN, indicating increasing hydrophobicity which would support the findings of decreasing numbers of double bonds in the aliphatic side chains. Previous observations suggest that compounds with more lipophilic constituents have greater activity against *M. tuberculosis*

than more polar analogs (Cantrell *et al*., 2001). Furthermore, the fact that type III polyketide synthases (PKS) isolated from *M. tuberculosis* share 25-45% amino acid sequence similarity to plant chalcone-synthase (CHS) (Saxena *et al*., 2003), thought to be involved in the biosynthesis of anacardic acid (Abe *et al*., 2004), suggests that these compounds may play a role in inhibiting these mycobacterial enzymes, therby inhibiting organism growth. The anacardic acids isolated from *O. paniculosa* have moderate activity against *M. tuberculosis* and appear to be potentially interesting lead compounds that could be further altered to illicit maximum antimycobacterial activity.

7.1 Introduction

This section reports the effect of the crude acetone extract of *O. paniculosa*, isolated anacardic acid, as well as fractions HPLC1 and HPLC2 on hamster cells. This is necessary to determine whether these substances are toxic and will be used to calculate a Selectivity Index (SI) suggested by Orme (2001) for the determination of the true potential of an isolated compound as an antimycobacterial agent for further investigation.

7.2 Materials and methods

7.2.1 Cell culture

The cytotoxicity of the isolated compounds was tested against Chinese hamster ovarian (CHO) cells. The cells were routinely maintained as adherent monolayers in 75cm^3 culture flasks in complete medium (CM) consisting of Dulbecos Modified Eagles Medium (DMEM) (Highveld Biologicials, Lyndhurst, South Africa): Hams F-12 medium (Sigma, St Louis, MO, USA) (1:1) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Highveld Biologicals, Lyndurst, South Africa). The cells were incubated in a 5% CO_2-air humidified atmosphere at 37°C. The culture medium was changed every 2-3 days and the cells sub-cultured once confluent, which involved digestion of the cellular matrix with a 1% trypsin solution.

7.2.2 Microtitre plate and compound preparation

Emitine (Sigma, St Louis, MO, USA) was used as the positive control for the cytotoxicity assays. An initial stock of 2mg/ml Emitine was prepared in Millipore water. Ten-fold dilutions of the positive control were prepared in CM on the day of experimentation. A 1mg/ml stock of each compound was prepared in 1% methanol/ 99% CM, from which six consecutive half dilutions were prepared in CM on the day of testing to give concentrations ranging between 1mg/ml – 31.25µg/ml. The crude extract was prepared to a concentration of 2mg/ml in 2% DMSO. Subsequent half-dilutions were prepared in CM and the concentration in the wells ranged between 1mg/ml – 0.49µg/ml. Each concentration was tested in quadruplicate.

100µl of a 10^5/ml cell concentration was added to each well, except those in row H (blank) in a 96 well microtitre plate. The plates were incubated at 37°C for 24 hours in a humidified atmosphere containing 5% CO_2. The medium was thereafter carefully aspirated from the adherent cells and 100µl of the control and test sample dilutions were

added, in quadruplicate, to the appropriate wells. 100µl of CM was then added to all the wells containing cells and drugs, and 200µl of CM was added to row H (blank) and column 2 (culture control). The microplate was incubated at 37°C for 48 hours.

7.2.3 Colorimetric MTT assay

The MTT (3-[4,5-Dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide) (Sigma, St Louis, MO, USA) assay described by Mosmann (1983) was used to determine the effect of the compounds on mammalian cell survival and proliferation. This colorimetric assay quantitates the degree of activation of the cells, thereby measuring the cytotoxicity, proliferation or activation of the cells. It is based on the ability of cells to metabolize the yellow water soluble tetrazolium salt into a water-insoluble purple formazan product. The intensity of the formazan product is proportional to the metabolic activity and number of cells in the microtitre wells and can be measured using a microplate reader.

After the incubation period, 25µl of sterile MTT at a concentration of 5mg/ml in PBS was added to each well and the plate re-incubated for 4 hours at 37°C. The plates were centrifuged for 10 minutes at 2050rpm and the supernatant aspirated from the wells without disturbing the formazan crystals. 100µl of DMSO was added to each well and the plate was gently shaken for 5 minutes on a microplate shaker to dissolve the crystals. The microplate reader was set at a wavelength of 540nm and the plate blanked on the wells in row H. after which the absorbance of the formazan was measured. The cell viability was calculated in each well using the formula:

$$\% \text{ Cell Viability} = \frac{A_{?540} \text{ test well (cells + drug)}}{A_{?540} \text{ cell control well (cells + no drug)}} \times 100$$

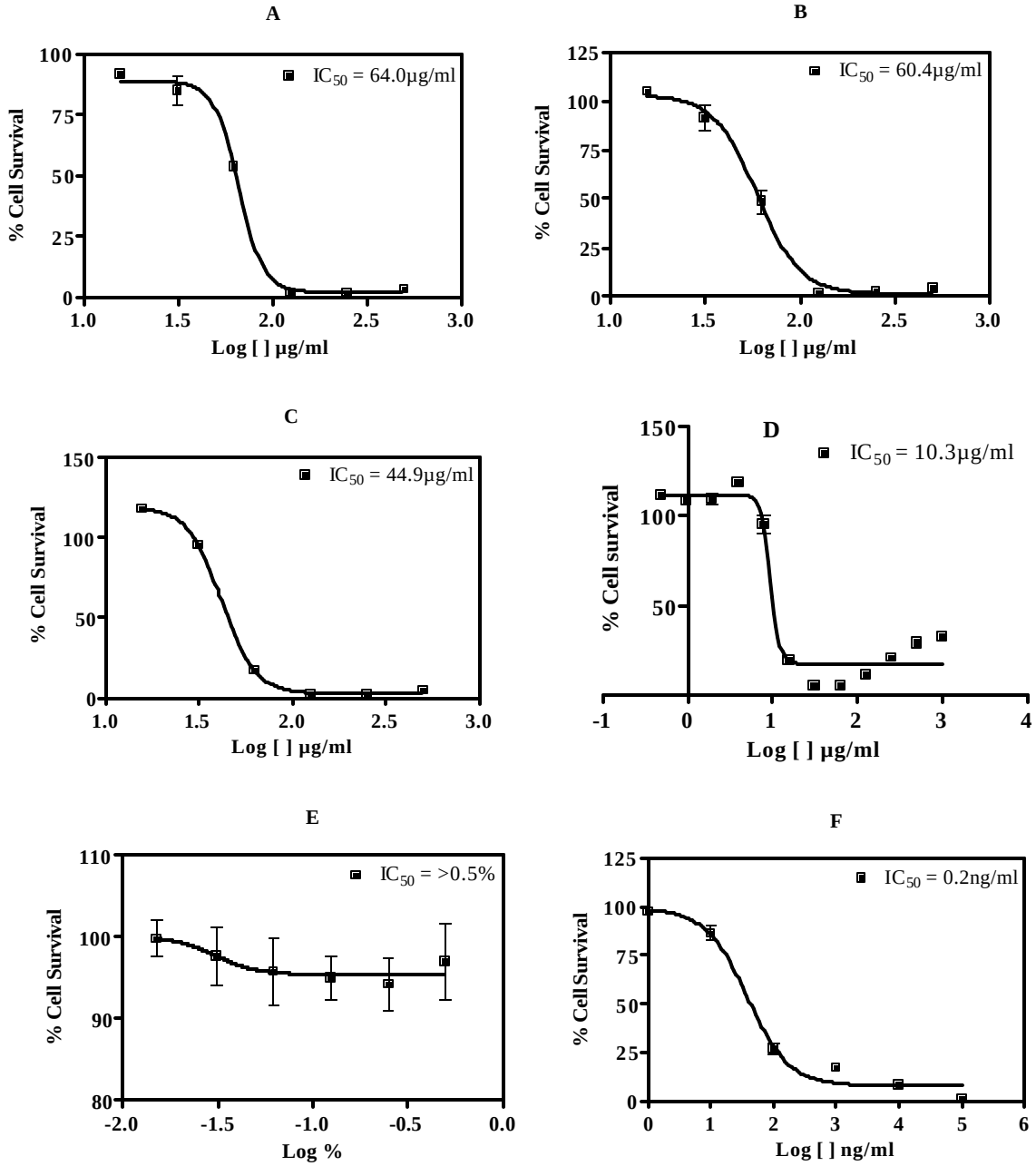
7.2.4 Data analysis

GraphPad Prism V.4.00 software was used to construct dose response curves using non-linear dose response curve fitting analyses from the percentage viability data (Microsoft Excel) of cells, shown in Figure7.1. The concentration of the compound at which 50% of the cell growth was inhibited (IC_{50} values) was determined from the dose response curves generated by GraphPad Prism. Each data point represents the average of four wells of a microtitre plate.

7.3 Results

Compound 1, HPLC2, HPLC3 and the crude extract of *O. pan*iculosa had IC_{50} values of 64, 60.4, 44.9 and 10.3µg/ml respectively, as shown in Figure 7.1 below. The crude extract is less cytotoxic as it contains smaller amounts of each compound, diluted with other unknown components. Methanol had no negative effect on cell growth.

Figure 7.1: The dose response curves representing the cytotoxicity of $C_{15:1}$ anacardic acid compound 1 (A) and fractions HPLC2 (B) and HPLC3 (C) isolated from *Ozoroa paniculosa*, together with the crude extract (D) on CHO cells. The emitine and methanol controls are illustrated in graphs E and D respectively.



7.4 Discussion

A related species, *Ozoroa insignis* from Zimbabwe, has shown *in vitro* cytotoxic activity against human hepatocellular carcinoma (Hep-G2), human mammary adenocarcinoma (MDA-MB-231) and human primary bladder carcinoma (5637) cells. The cytotoxic components were identified as anacardic acid (6-pentadecyldalicyclic acid) and ginkgolic acid with IC_{50} values of 229µM and 385µM respectively (Rea *et al*., 2003). It is interesting to note that the saturated anacardic acid is less toxic than its unsaturated analogues. The levels of cytotoxicity exhibited by the crude extract suggest that toxicity to humans would be experienced when consuming this extract in large quantities.

The level of activity of HPLC3 against MTB is lower than the concentration at which cytotoxicity is displayed. The selectivity index (Orme 2001), or quotient of the cytotoxicity and the antimicrobial activity, of compound 1, HPLC2 and HPLC3 was therefore determined as being 0.5, 0.4 and 1.3 respectively against *M. tuberculosis*. A value of greater than ten warrants further evaluation of compounds as promising antimycobacterial agents. When applying this calculation to the potent activity of compound 1 against Gram-positive organisms, SI values of 9.8, 30.5 and 49.2 are obtained for the reference strains of *S. aureus*, *E. faecalis* and *B. cereus* respectively. These results indicate that anacardic acid should be pursued as a viable drug source for the treatment of diseases resulting from infection with Gram-positive organisms.

A third of the world's population is infected with *M. tuberculosis.* The incidence rate appears to be increasing globally by 1% per annum, and HIV in Africa is having a great impact on these rates. Current treatment is based on multi-drug therapy consisting of the front line drugs INH, RIF, ETH and PYR over an extended period of time. Incomplete treatment leads to the development of MDR-TB, which is difficult to treat and leads to an increase in morbidity and mortality.

No new class of TB drug has been developed in more than thirty years. Various groups have been formed to fast-track the development of new anti-TB drugs, including The Global Alliance for TB Drug Development. Several promising new drugs are currently at various stages in the drug development pipeline, including a drug with a unique drug target in the MTB genome. A new TB drug should ideally shorten the duration of treatment or decrease the number of doses that need to be taken under observed therapy and should be active against drug resistant organisms and latent TB. Various compounds isolated from plants have promising activities against MTB in *in vitro* assays.

The purpose of this project was to evaluate plants traditionally used to treat respiratory ailments in southern Africa for antimicrobial and antimycobacterial activity, and to isolate the active principles responsible for activity from a single plant, followed by the determination of the cytotoxicity. The major findings of this project are listed below.

- Several plant extracts were active against two or more of the three Gram-positives, four Gram-negatives, one fungus and three mycobacterial organisms evaluated, with MIC's of less than 1mg/ml. These were *Xerophyta retinervis* bark, *Helichrysum odoratissimum* leaves, *Eriocephalus africanus* leaves, *Ozoroa paniculosa* bark, *Siphonochilus aethiopicus* roots, *Syzigium cordatum* bark, *Tetradenia riparia* leaves, *Datura stramonium* leaves *and Dioscorea sylvatica* tubers with MIC's of less than 1mg/ml.
- For the purposes of this study, *O. paniculosa* was further studied for the isolation of its active constituents as no literature on the antimicrobial and antimycobacterial properties of this tree have been published, although promising antihelminthic, antischistosomiasis, antimalarial and anticancer

activities have been reported from other species. Moronic, ginkgolic and anacardic acids have been isolated from related species, and anacardic acids are frequently isolated from members of the Anacardiaceae family.

- Using structured, bio-assay guided methodologies, a $C_{15:1}$ anacardic acid (compound 1) and its saturated analogue (HPLC3) were isolated from the active fraction of the acetone extract of *O. paniculosa* bark, identified using NMR and HR-MS. HPLC2 appears to have similar NMR spectra, also containing a single double bond, although the location is as yet uncertain.
- The isolated $C_{15:1}$ anacardic acid and the two HPLC fractions exhibited potent activity against Gram-positive organisms, with compound 1 exhibiting IC_{50} values of 6.5µg/ml, 2.1µg/ml and 1.3µg/ml against *S. aureus*, *E. faecalis* and *B. cereus*. These good activities warranted testing against two drug-resistant strains of *S. aureus*, with IC_{50} values of 43.2µg/ml and 6.9µg/ml.
- Against Gram-positives, the saturated anacardic acid (HPLC3) appeared to have less activity than the unsaturated analogues, results in agreement with other authors.
- Compound 1, HPLC2, HPLC3 and a combination of the three had IC_{50} values against *K. pneumoniae*, *P. aeruginosa* and *S. odorifera* ranging between 26.1 - >250µg/ml, 17.5 - >250µg/ml and 19.0 - >250µg/ml respectively. Previous authors have noted that anacardic acids in general have poor activity against Gram-negatives. It appears that the $C_{15:1}$ anacardic acid consistently had less activity than HPLC2 and HPLC3. It can be surmised that increasing the lipophilicity of anacardic acid will increase activity against Gram-negative organisms. Similar trends were noted for *C. albicans*.
- For mycobacteria excluding *M. aurum* A+, increasing lipophilicity resulted in increasing activity, with HPLC3, the saturated anacardic acid having an IC_{50} against *M. smegmatis* and an MIC against *M. tuberculosis* of 21.1 and 31.3µg/ml respectively. For *M. aurum*, the isolated compound, fractions and combination had similarly moderate activity against the organism.
- These results are in agreement with literature reports suggesting that greater antimycobacterial activity is associated with greater compound lipophilicity. This suggests a role of these anacardic acids in disrupting organism membrane function.

- The cytotoxicity of compound 1, HPLC2 and HPLC3 was determined to be 44.9 – 64.0µg/ml against Chinese Hamster Ovarian cells. The selectivity index (Orme 2001) of compound 1, HPLC2 and HPLC3 was therefore determined as being 0.5, 0.4 and 1.3 respectively. A value of greater than ten warrants further evaluation of compounds as promising antimycobacterial agents and it is evident that HPLC3 has the most activity.
- The selectivity index of compound 1 against Gram-positive organisms suggest that anacardic acids should be pursued as a source of drugs to treat infections caused by these organisms.

This study suggests that using ethnomedical claims in search of new anti-infectives from largely untapped natural resources is a useful way of maximizing the number of 'hits' that could yield potential actives. The anacardic acids isolated in this study have shown excellent activity against Gram-positive organisms. Despite not having as good activity against mycobacteria, these compounds have furthered our understanding of mechanisms of action of lipophilic compounds with long aliphatic chains in the inhibition of organisms.

Various plants have been highlighted in this project as having potent antimicrobial and antimycobacterial properties for which no information of previously isolated compounds is available. These plants include *Xerophyta retinervis*, *Eriocephalus africanus*, *Siphonochilus aethiopicus*, *Syzigium cordatum*, *Datura stramonium* and *Dioscorea sylvatica*. For the purpose of this project, these properties could not be further evaluated, but the preliminary results warrant further exploration and isolation of active principles.

With regard to the isolated anacardic acids from *Ozoroa paniculosa*, proton and carbon spectra have been obtained for fraction HPLC2, suggesting that it too has a single double bond in its aliphatic chain. This compound needs to be re-collected, purified and subjected to chemical procedures that can help elucidate the location of this double bond, together with that of compound 1. Although much work has been done on anacardic acids, this is the first reported case of antimycobacterial testing, with promising results. Analogues with variations in chain length, degrees of unsaturation, as well as lipophilicity must be subjected to antimycobacterial testing in the hope of enhancing the currently experienced activity of the saturated C_{15} anacardic acid. Futhermore, possible synergism of these compounds with current antituberculosis drugs should also be explored, as anacardic acids have shown synergistic effects against drug-resistant *S. aureus* when used in combination with existing therapeutics.

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Nuclear Magnetic Resonance: 2-Dimensional Data

Figure 12.1: Compound 1- GHMQC Plot

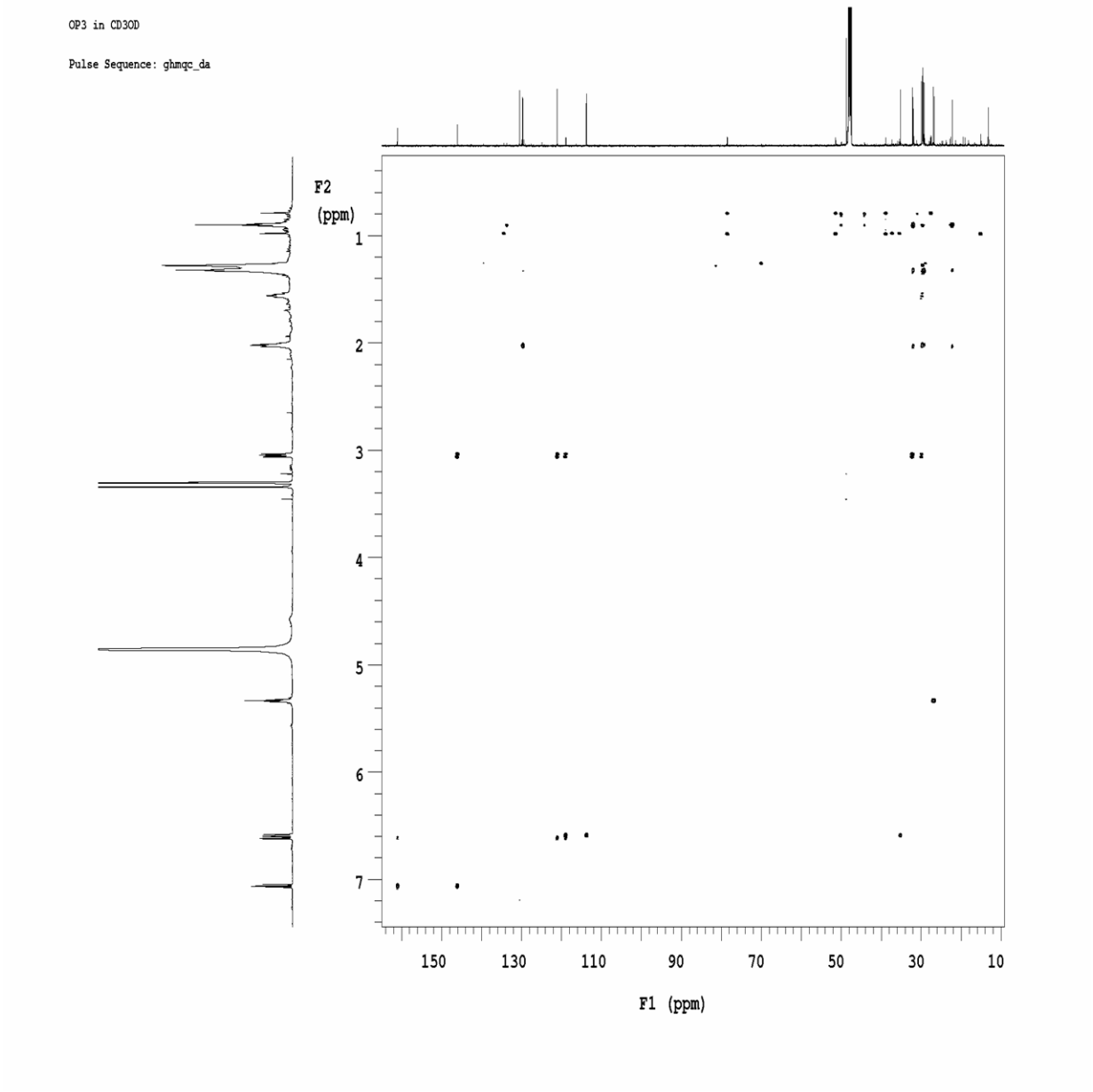


Figure 12.2: Compound 1 - GHSQC Plot

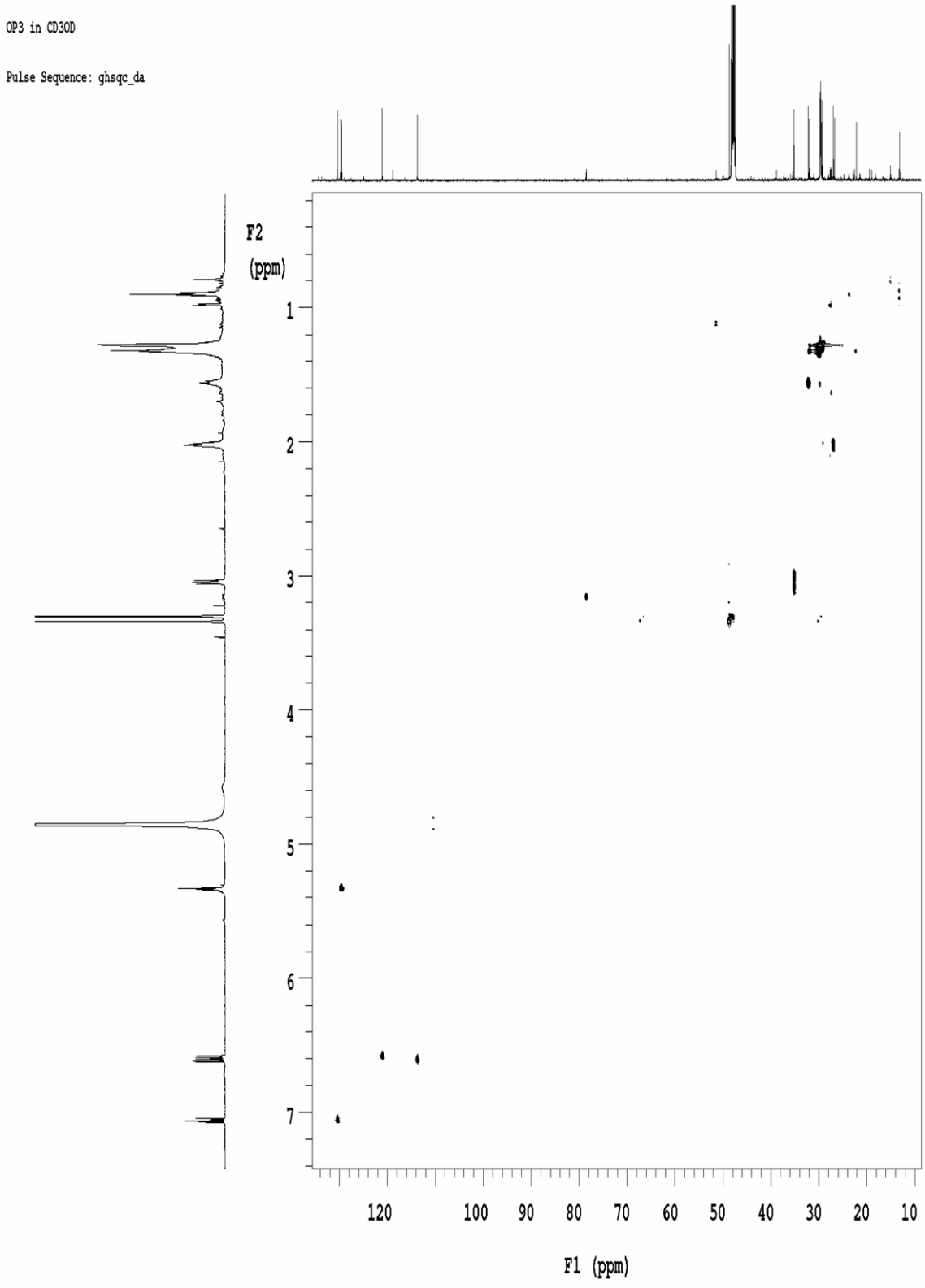


Figure 12.3: Compound 1 - RELAYH Plot

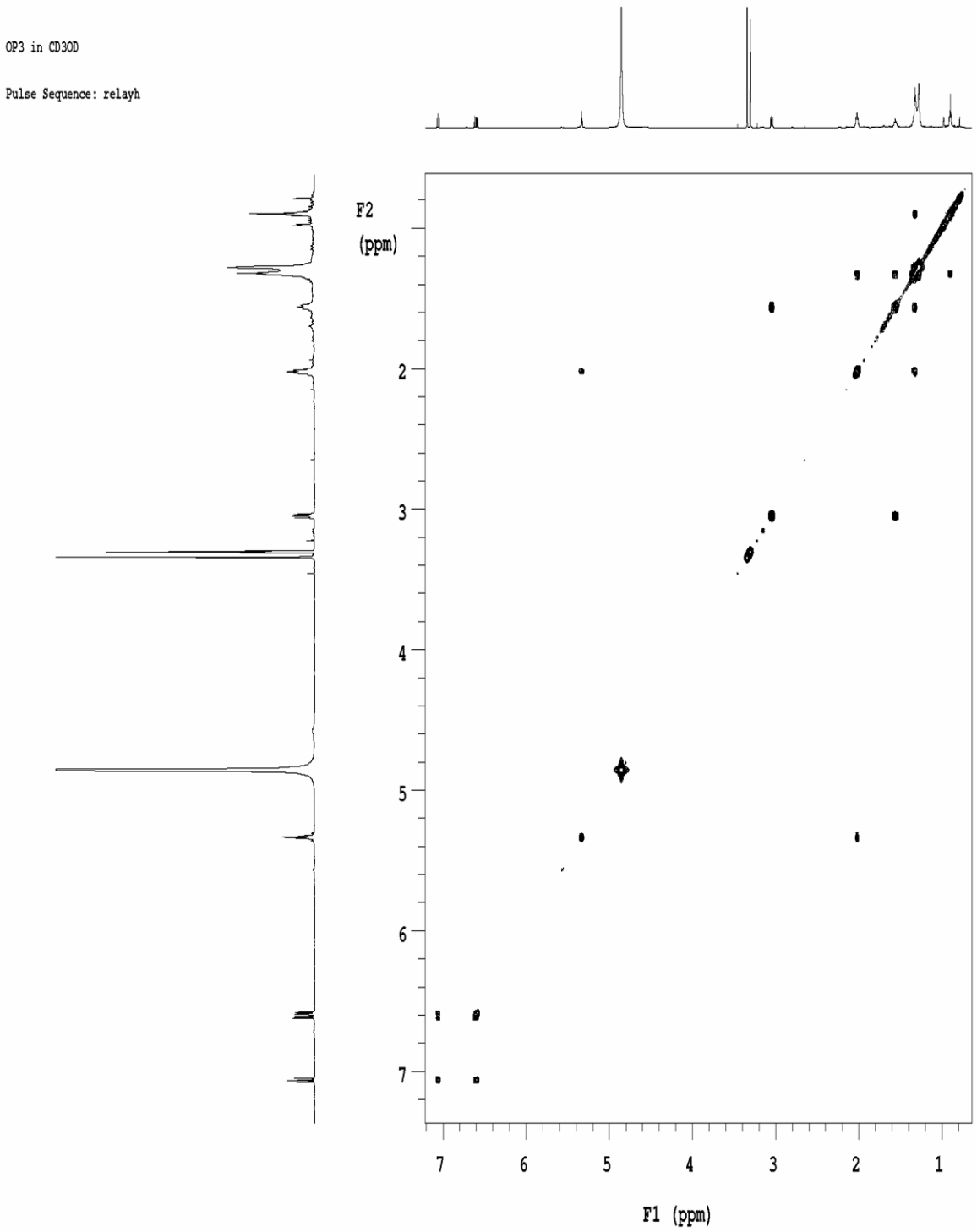


Figure 12.4: Compound 1 - DEPT Plot

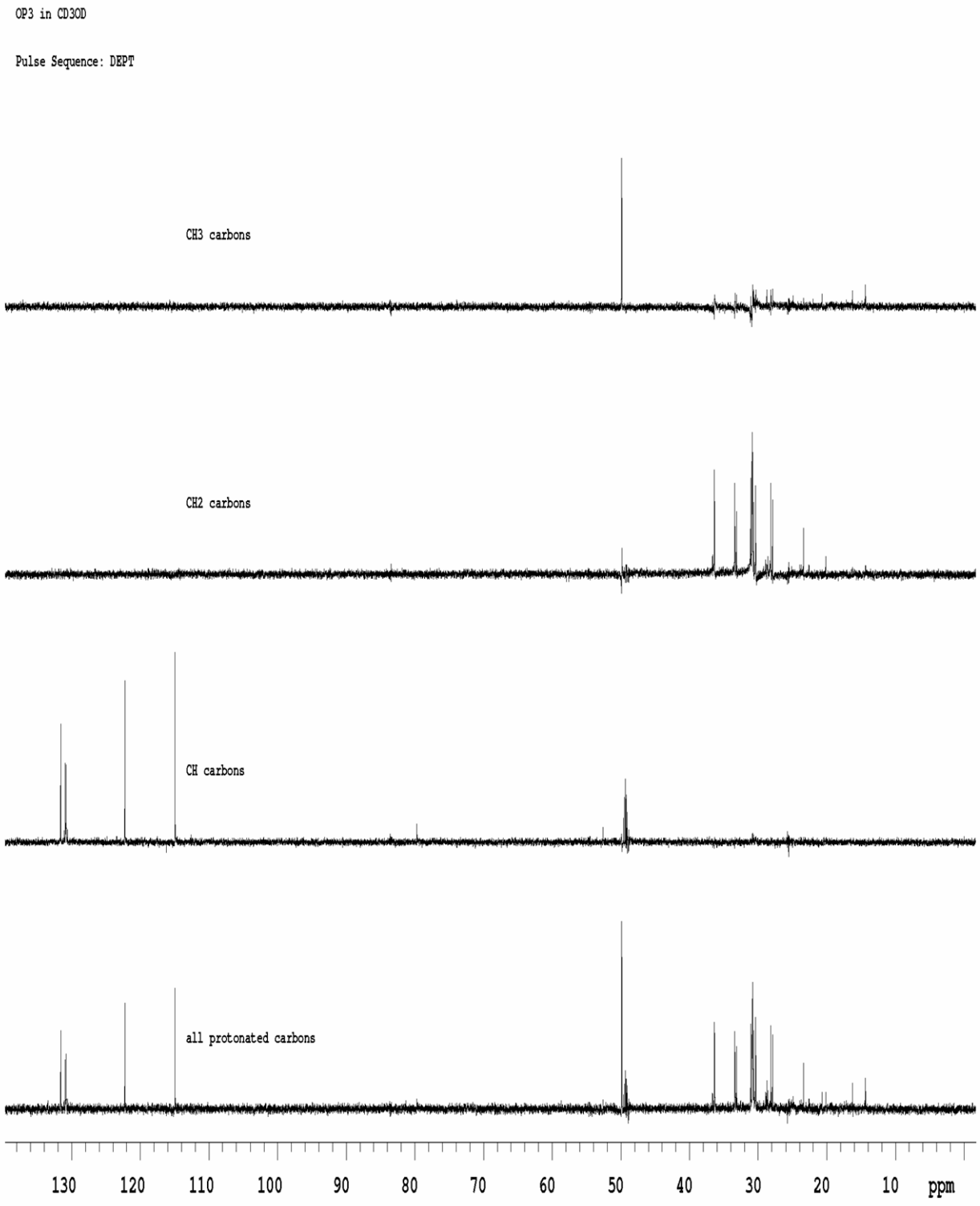


Figure 12.5: HPLC3 - GHMQC Plot

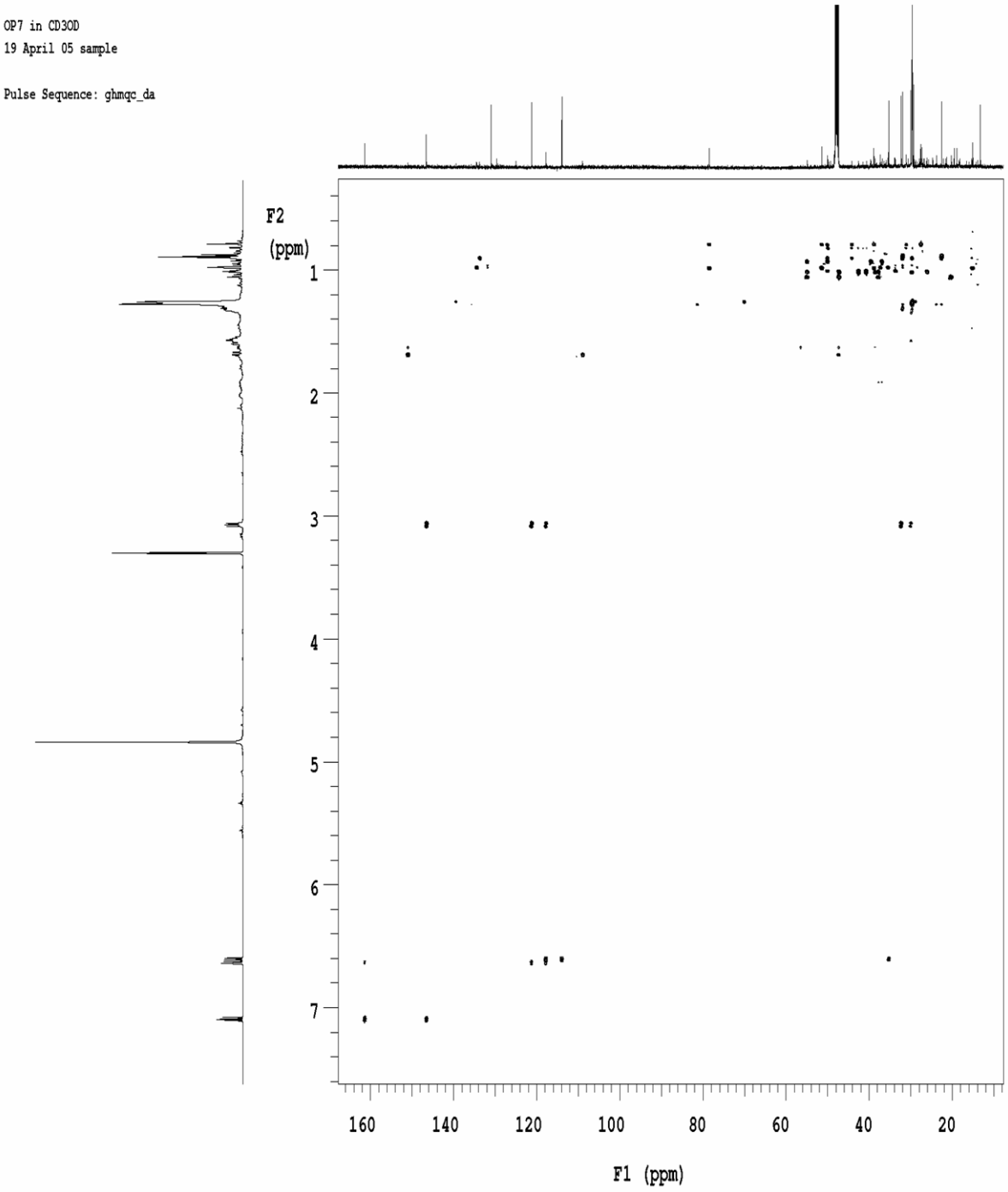


Figure 12.6: HPLC3 - GHSQC Plot

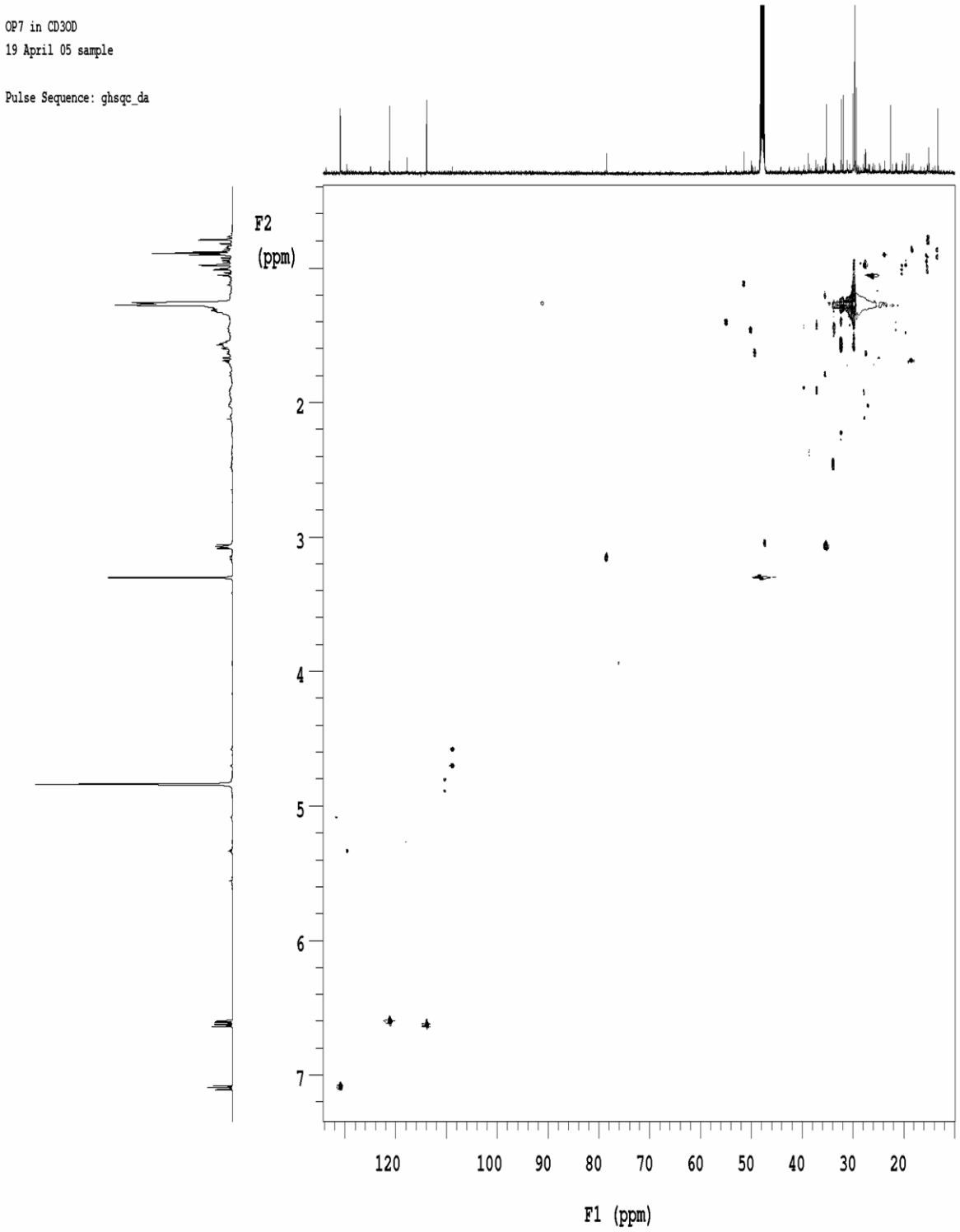


Figure 12.7: HPLC3 - RELAYH Plot

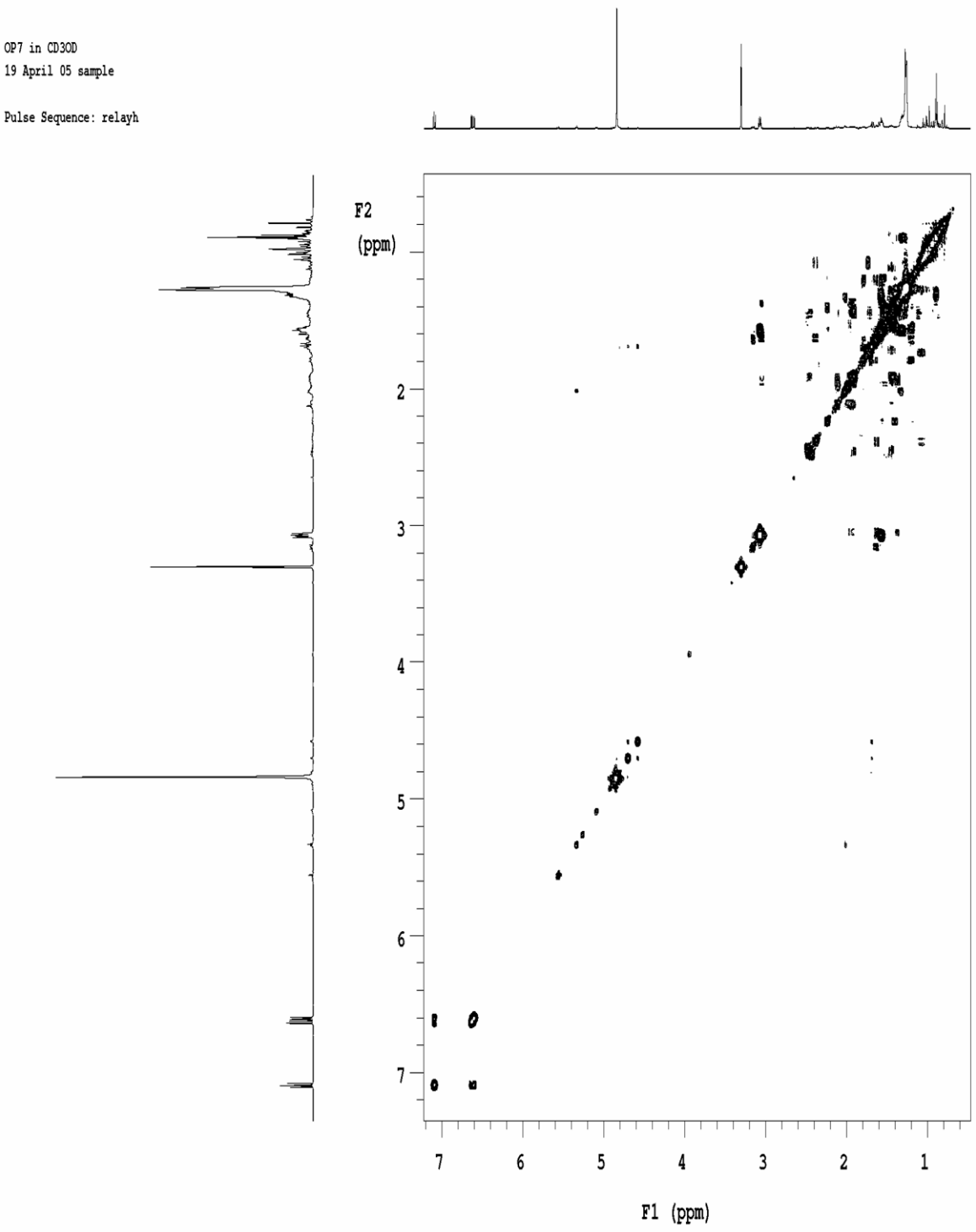


Figure 12.8: HPLC3 - DEPT Plot

