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Potentising and application of an extract of *Melianthus comosus* against plant fungal pathogens

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A thesis submitted in fulfilment of the requirements for the degree of Master of Science

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June 2006

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Declaration

This research was carried out in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, under the supervision of Prof JN Eloff and Dr LJ McGaw.

This thesis represents work done by Irene Esah Angeh, except where the work of others is acknowledged and the results have not been submitted anywhere else before.

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Acknowledgements

I am very grateful to my promoter Prof J. N. Eloff whose ideas contributed greatly to this work, thank you for your guidance, financial support and for being like a father to me and my family. Special thanks to my co-promoter Dr L.J. McGaw for timely advice and for helping me carry out certain assays.

My deep appreciation goes to the Healthtech Laboratories for their financial support of this project.

My colleagues of the Phytomedicine group have been very supportive and I appreciate all the things you did for me. I am grateful to Masoko Peter for help with the antifungal techniques and Dr L.K. Mdee for help in structural elucidation of the isolated compound.

My supervisor's wife, Christna Eloff made my life very comfortable and filled with so much love; there are no words that can describe how grateful I am for what you have done for me and my family. Thanks a million!

Naomi Nkealah, Irene Ifughe, Angie Pasetti, Estelle Boshoff and Audrey Cathrow have been the most wonderful of friends, thank you very much.

I am very grateful to my family back at home especially my parents Lucy and Lucas Abia for looking after my daughter, Sandy, while I was doing this work.

Deep appreciation goes to my husband Dr JE Angeh for all the encouragement and help in structural elucidation of compound.

My most profound gratitude goes to Jehovah God who gave me the strength and insight to carry out this research.

Table of contents

Declaration ii

Acknowledgements iii

Table of Figures viii

List of Tables xii

List of Abbreviations used xiv

Abstract xvi

1. Chapter 1: Literature Review.. 1

1.1 Introduction 1
1.2 Fungi as plant pathogens..2
1.3 Effects of fungi on plant health..3
1.4 Diagnosis of plant disease 5
1.5 Control of plant disease 6
1.5.1 Evasion of disease 7
1.5.2 Direct chemical control 8
1.5.3 Non-systemic organic fungicides...10
1.5.4 Systemic fungicides 12
1.5.5 Other systemic fungicides 14
1.6 Fungicide resistance 15
1.7 Use of plant extracts to combat fungal pathogens 16
1.7.1 Why choose plant extracts? 17
1.8 Sources of antimicrobial activity in plants..18
1.8.1 Secondary metabolites...18
1.9 Contract research for Healthtech Laboratories..20
1.10 The Melianthaceae family 20
1.10.1 Phytochemistry of Melianthaceae 20
1.10.2 *Melianthus comosus* Vahl 20
1.10.3 Ethnobotanical uses of *Melianthus comosus* 22

1.11 Aim and objectives 23

2. Chapter 2: Plant collection, extraction and analysis 24

2.1 Introduction 24
2.2 Materials and Methods 24
2.2.1 Plant collection 24
2.2.2 Plant extraction 25
2.2.3 Phytochemical analysis of extracts 25
2.2.4 Retardation factor (R_f) values of compounds 26
2.3 Results and Discussion 26
2.3.1 Extraction 26
2.3.2 Phytochemical analysis 29
2.3.3 R_f values 30
2.4 Conclusion 31

3. Chapter 3: Biological assays 33

3.1 Introduction 33
3.2 Materials and Methods 34
3.2.1 Fungal organisms 34
3.2.2 Antifungal assays 36
3.3 Results and Discussion 37
3.3.1 Quantification of fungal inoculum 37
3.3.2 Bioautography 37
3.3.3 Microplate dilution assay 41
3.4 Conclusion 42

4. Chapter 4: Enrichment of Acetone Extract 44

4.1 Introduction 44
4.2 Materials and Methods 44
4.2.1 Pathway 1: “HT01” 44

4.2.2 Pathway 2: "HT02" 46
4.2.3 Comparison of HT02 with commercial fungicides....48
4.3 Results and Discussion 48
4.3.1 HT01 48
4.3.2 HT02 49
4.3.3 Comparison of HT02 with commercial fungicides....51
4.4 Conclusion 52

5. Chapter 5: Cytotoxicity, Solubility and Stability of HT02....53

5.1 Introduction 53
5.2 Materials and Methods 53
5.2.1 Cytotoxicity....53
5.2.2 Solubility of HT02 55
5.2.3 Stability of HT02 55
5.3 Results and Discussion 56
5.3.1 Brine shrimp assay....56
5.3.2 MTT cell line assay 56
5.3.3 Solubility of HT02 57
5.3.4 Stability of HT02....58
5.4 Conclusion 59

6. Chapter 6: Field trial of HT02 60

6.1 Introduction 60
6.2 Materials and Methods 60
6.3 Results and Discussion 61
6.4 Conclusion 62

7. Chapter 7: Selective removal of cytotoxic constituents....63

7.1 Introduction 63
7.2 Materials and Methods 63

7.2.1 Extraction and detection of cardiac glycosides....63
7.2.2 Removal of cardiac glycosides....64
7.3 Results and Discussion....65
7.3.1 Extraction and detection of cardiac glycosides....65
7.3.2 Removal of cardiac glycosides....68
7.4 Conclusion....74

8. Chapter 8: Isolation of Bioactive Compound(s)....75

8.1 Introduction....75
8.2 Materials and Methods....75
8.2.1 Extraction....75
8.2.2 TLC analysis of extracts....76
8.2.3 Biological assays of extracts....76
8.2.4 Vacuum liquid column chromatography (VLC)....76
8.2.5 TLC analysis and bioassay of column fractions....78
8.2.6 TLC analysis, bioassay and cytotoxicity of isolated compound....79
8.2.7 Spectroscopic analysis of isolated compound....79
8.3 Results and Discussion....80
8.3.1 Extraction results....80
8.3.2 TLC analysis of extracts....80
8.3.3 Biological assays of extracts....81
8.3.4 TLC analysis of column fractions....83
8.3.5 Bioassay of column fractions....86
8.6 TLC analysis and bioassay of isolated compound....88
8.7 Spectroscopic analysis of isolated compound....90
8.8 Conclusion....96

9. Chapter 9: General conclusion....97

10. Chapter 10: References....100

Table of Figures

Figure 1-1: Chemical structures of some non-systemic fungicides (Isaac, 1992) 12

Figure 1-2: Chemical structures of some systemic fungicides (Isaac, 1992). 15

Figure 1-3: Melianthugenin (left) and oleanolic acid (right), (Van Wyk et al., 2002) 21

Figure 1-4: *Melianthus comosus* shrub (above), its flowers and fruits (below) (Van Wyk et al., 2002) 22

Figure 2-1: Percentage yield of extractables of *Melianthus comosus* after three extractions with hexane (Hex), carbon tetrachloride (Ctc), diethyl ether (Dee), dichloromethane (Dcm), chloroform (Chl), acetone (Act), ethanol (EtOH), ethyl acetate (EtAc), methanol (MeOH) and water (Wat) from left to right. 27

Figure 2-2: Percentage yield of extractables of *Melianthus comosus* after three extractions with hexane (Hex), carbon tetrachloride (Ctc), diethyl ether (Dee), dichloromethane (Dcm), chloroform (Chl), ethanol (EtOH), ethyl acetate (EtAc), acetone (Act), methanol (MeOH) and water (Wat) from left to right. 28

Figure 2-3: TLC chromatograms of *Melianthus comosus* extracted by 10 different solvents: developed in BEA (top), CEF (centre) and EMW (bottom) sprayed with vanillin-phosphoric acid (left) and anisaldehyde-sulphuric acid (right). Lanes from left to right: hexane (1), carbon tetrachloride (2), diethyl ether (3), dichloromethane (4), chloroform (5), acetone (6), ethanol (7), ethyl acetate (8), methanol (9) and water (10). 30

Figure 3-1: Bioautogram of extracts of *Melianthus comosus*. TLC chromatogram was developed in BEA sprayed with *Coletotrichum gloeosporioides* (top), *Penicillium janthinellum* (centre), *Fusarium oxysporum* (bottom); incubated and sprayed with INT. Colourless areas denote inhibition of fungal growth. Lanes from left to right: hexane (1), carbon tetrachloride (2), diethyl ether (3), dichloromethane (4), chloroform (5), acetone (6), ethanol (7), ethyl acetate (8), methanol (9) and water (10). 38

Figure 3-2: Left: TLC chromatogram of acetone (Acet) and ethanol (EtOH) extracts developed in BEA, not sprayed. Middle: TLC chromatogram of acetone (6) and ethanol (7) extracts developed in BEA and sprayed with vanillin-phosphoric acid spray reagent. Right: Bioautogram of acetone (Acet) and ethanol (EtOH) extracts. TLC chromatograms developed in BEA sprayed with *Fusarium oxysporum*, incubated and sprayed with INT. Colourless areas denote inhibition of fungal growth. 39

Figure 3-3: Left: TLC chromatogram of acetone (Acet) and ethanol (EtOH) extracts developed in EMW but not sprayed. Middle: TLC chromatogram of acetone (6) and ethanol (7) extracts developed in EMW and sprayed with vanillin-phosphoric acid spray reagent. Right: Bioautograms of acetone (Acet) and ethanol (EtOH) extracts. TLC chromatograms developed

in EMW sprayed with *Trichoderma harzianum,* incubated and sprayed with INT. Colourless areas denote inhibition of fungal growth. 40

Figure 4-1: Flow chart for the development of HT01 46

Figure 4-2: Flow chart for the development of HT02. 47

Figure 4-3: TLC profiles of extracts developed in BEA and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane after water “wash” (P-W-H, a), acetone after water and hexane “washes” (P-W-H+A, b), DCM following water “wash (P-W-D, c), acetone following water and DCM “washes” (P-W-D+A, d) “HT01”, ethyl acetate following water “wash” (P-W-E, e), acetone following ethyl acetate and water “washes”(P-W-E+A, f), “HT02” (g). 49

Figure 4-4: Bioautogram of extracts developed in BEA and sprayed with *Fusarium oxysporum* (top) and *Phytophthora nicotiana* (bottom). Lanes from left to right: hexane after water “wash” (P-W-H, a), acetone after water and hexane “washes” (P-W-H+A, b), DCM following water “wash (P-W-D, c), acetone following water and DCM “washes” (P-W-D+A, d) “HT01”, ethyl acetate following water “wash” (P-W-E, e), acetone following ethyl acetate and water “washes”(P-W-E+A, f), “HT02” (g). Arrows showing active compounds. 50

Figure 5-1: Influence of concentration of HT02 on brine shrimp mortality. 56

Figure 5-2: MTT assay results; wells from left to right: no cells (1), control (2), HT02 with cells (3&4), acetone and cells (5&6), Berberine (7&8), HT02 with cells (9&10), control (11) and no cells (12). 57

Figure 6-1: Bed A treated with HT02. 61

Figure 6-2: Bed B treated with Bravo 500. 61

Figure 6-3: Bed C untreated, negative control. 62

Figure 7-1: Fluorescence of extracts under UV 254 nm (left) and UV 365 nm (right). Lanes from A to D: CG1 (cardenolides), Digitoxin, CG2 (lead precipitate or cardiac glycosides) and Pb-washed acetone extract (one wash) developed in EMW solvent system. 66

Figure 7-2: Chromatograms of extracts sprayed with antimony viewed under visible light (left) and under UV 365 nm (right). Lanes from A to D: CG1 (cardenolides), Digitoxin, CG2 (lead precipitate or cardiac glycosides) and Pb-washed acetone extract (one wash) developed in EMW solvent system. 67

Figure 7-3: Chromatogram of extracts sprayed with Kedde reagent. Lanes from left to right: CG1 (cardenolides), digitoxin, CG2 (cardiac glycosides) and Pb-washed acetone extract (one wash) developed in EMW solvent system. 67

Figure 7-4: Fluorescence of extracts under UV 254 nm (left) and UV 365 nm (right) without chemical treatment. Lanes from A to E: First Pb extract (A), second Pb extract (B), third Pb extract (C), digitoxin (D) and the Pb-washed acetone extract (exhaustive) (E). 69

Figure 7-5: Chromatogram of extracts sprayed with antimony (left) and fluorescence under UV 365 nm (right). Lanes from A to E: First Pb extract, second Pb extract, third Pb extract, digitoxin and the Pb-washed acetone extract (exhaustive). 69

Figure 7-6: Chromatograms of fractions (F1 to F8) of HT02 developed in BEA, EMW and CEF and sprayed with vanillin sulphuric acid. 71

Figure 7-7: Bioautogram of fractions of HT02 developed in BEA (left) and EMW (right) and sprayed with *Penicillium janthinellum*.. 72

Figure 7-8: Bioautogram of fractions of HT02 developed in EMW (left) and BEA (right) and sprayed with *Fusarium oxysporum*. 73

Figure 7-9: Cytotoxicity of inactive fractions. 73

Figure 8-1: Quantity of material extracted from the first (1st), second (2nd), third (3rd), fourth (4th) and fifth extractions from 500 g of *M. comosus* leaves by hexane, dichloromethane (DCM), acetone and methanol in serial exhaustive extraction. 80

Figure 8-2: TLC profiles of extracts from serial exhaustive extraction (SEE) developed in EMW (left), CEF (middle) and BEA (right) solvent systems and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane (Hex), dichloromethane (DCM), acetone (Act) and methanol (Met). 81

Figure 8-3: Bioautogram of acetone extract of *Melianthus comosus* developed in BEA sprayed with *Penicillium expansum* (left), *Fusarium oxysporum* (middle) and *Phytophthora nicotiana* (right). Plates were incubated at 37°C for 24 hours and sprayed with INT. 82

Figure 8-4: Bioautogram of extracts of *Melianthus comosus* developed in BEA, sprayed with *Phytophthora nicotiana* (left) and sprayed with *Penicillium expansum* (right). Lanes from left to right: hexane (Hex), dichloromethane (DCM), acetone (Act) and methanol (MeOH)........ 83

Figure 8-5: TLC profiles of acetone extract developed in hexane:ethyl acetate 9:1, 8:2, 7:3, 6:4, 5:5 solvent systems from left to right... 83

Figure 8-6: TLC profiles of fractions of acetone by VLC. TLC plates developed in BEA (top), CEF (middle), and EMW (bottom) and sprayed with vanillin-sulphuric acid. Lanes from left to right: crude extract, F1, F2, F3, F4, F5, F6, F7, F8 and F9. 84

Figure 8-7: TLC profiles of the tubes (10-32) obtained from column separation of F1. TLC plate developed in hexane:ethyl acetate (85:15) and sprayed with vanillin-sulphuric acid. 85

Figure 8-8: TLC profiles of the tubes (41-61) obtained from column separation of F1. TLC plate developed in BEA and sprayed with vanillin-sulphuric acid. 85

Figure 8-9: Bioautogram of fractions of crude acetone extract of M. comosus. TLC plate developed in EMW (below) and BEA (above) and sprayed with *Penicillium expansum*. Lanes from left to right: crude, F1, F2, F3, F4, F5, F6, F7, F8, and F9. 86

Figure 8-10: TLC profiles (left) and bioautogram (right) of sub-fractions of F1. TLC plate developed in EMW and sprayed with vanillin-sulphuric acid and *Penicillium expansum* respectively. Lanes from left to right: f1, f2, f3, f4, f5 and f6. 87

Figure 8-11: TLC profiles of tubes of SF1 (560-598). TLC plate developed in hexane:ethyl acetate (7:3) and sprayed with vanillin-sulphuric acid. 87

Figure 8-12: TLC profiles of tubes of SF1 (tubes 756-792 above; tubes 890-924 and crude fraction below). TLC plate developed in hexane:ethyl acetate (7:3) and sprayed with anisaldehyde. 88

Figure 8-13: TLC profiles of the isolated compound developed in CEF (left), hexane:ethyl acetate [(7:3), middle], EMW (right) and sprayed with vanillin-sulphuric acid. 89

Figure 8-14: Bioautogram of acetone crude and compound against from left to right: *Colletotrichum gloeosporioides, Phytophthora nicotiana* and *Aspergillus parasiticus.* 89

Figure 8-15: Skeletal unit of olean-12-ene type of pentacyclic triterpenoid isolated from *M. comosus.* 90

Figure 8-16: Structure of compound 1: 3-hydroxy-12-oleanen-28-oic acid (oleanolic acid). 92

Figure 8-17: ^{1}H-NMR spectrum of compound 1 92

Figure 8-18: ^{13}C-NMR spectrum of compound 1 93

Figure 8-19: HSQC spectrum of compound 1 93

Figure 8-20: HMBC spectrum of compound 1 94

Figure 8-21: Mass spectrum of compound 1 94

List of Tables

Table 1-1: Non-systemic fungicides (Isaac, 1992) 11

Table 1-2: Systemic fungicides (Isaac, 1992) 14

Table 2-1: Quantity in mg extracted from 3 g of leaves using hexane (Hex), carbon tetrachloride (Ctc), diethyl ether (Dee), dichloromethane (Dcm), chloroform (Chl), acetone (Act), ethanol (EtOH), ethyl acetate (EtAc), methanol (MeOH) and water (Wat), with extraction repeated twice on the same marc. 27

Table 2-2: R_f value of compounds (√ = present, ×= not present) separated in BEA extracted by hexane (Hex), carbon tetrachloride (Ctc), diethyl ether (Dee), dichloromethane (Dcm), chloroform (Chl), ethanol (EtOH), ethyl acetate (EtAc), acetone (Act), methanol (MeOH) and water (Wat) 31

Table 3-1: Origin of fungal pathogens used in this study obtained from the University of Pretoria Fungal Collection (UPFC) 34

Table 3-2: MIC values (mg/ml) of extracts tested against 8 moulds. A (hexane), B (carbon tetrachloride), C (diethyl ether), D (dichloromethane), E (chloroform), F (acetone), G (ethanol), H (ethyl acetate), I (methanol), J (water). 41

Table 3-3: MIC values in mg/ml for acetone and ethanol extracts stored for one month at 5°C (2nd MIC) compared to the MIC values previously determined (1st MIC). All values were recorded after 24 hours of incubation of extract with fungus. 42

Table 4-1: Enrichment procedures of the acetone extract 45

Table 4-2: Commercial fungicides and their active compounds 48

Table 4-3: MIC values (mg/ml) of hexane after water “wash” (P-W-H, a), acetone after water and hexane “washes” (P-W-H+A, b), DCM following water “wash (P-W-D, c), acetone following water and DCM “washes” (P-W-D+A, d), ethyl acetate following water “wash” (P-W-E, e), acetone following ethyl acetate and water “washes”(P-W-E+A, f), “HT02” (g) 51

Table 4-4: MIC values of HT02 and other commercial fungicides: triforine, propiconazole, dicarboximide, chlorothalonil, tebuconazole, and copper oxychloride against *Fusarium oxysporum* and *Penicillium expansum* 52

Table 5-1: Solubility of HT02 in water, ethanol, ethyl acetate and acetone 58

Table 5-2: Average MIC values of HT02 extracts to determine stability at room temperature and in the fridge. 58

Table 7-1: R_f values of cardiac glycosides present (+) or absent (-) in CG1, CG2 and lead-washed acetone extract (one wash) in EMW solvent system. 68

Table 7-2: MIC values (mg/ml) of acetone, exhaustive lead precipitate and lead-washed acetone (exhaustive wash) extracts against *Penicillium janthinellum.* 70

Table 7-3: MIC values (mg/ml) of fractions of HT02. 72

Table 8-1: Gradients of solvent used in VLC for the separation of the acetone extract of *M. comosus.* 77

Table 8-2: Gradients of solvent used in VLC for the separation of F1. 78

Table 8-3: MIC values of acetone extract from direct extraction (DE) and hexane, DCM, acetone and methanol extracts from serial exhaustive extraction (SEE); against *Phythophthora nicotiana* and *Penicillium expansum.* 82

Table 8-4: Mass of fractions F1-F9 of acetone extract. 84

Table 8-5: Mass of fractions f1-f6 of F1. 86

Table 8-6: MIC values (µg/ml) of isolated compound in triplicate (1, 2, 3) against *Colletotrichum gloeosporioides* (C.g.), *Phytophthora nicotiana* (P.n.), *Penicillium expansum* (P.e.), *Aspergillus parasiticus* (A.p.), *Staphyloccocus aureus* (S.a.), *Enteroccocus faecalis* (E.f.), *Escherichia coli* (E.c.) and *Pseudomonas aeruginosa* (P.a.). 90

Table 8-7: NMR data of compound 1 95

List of Abbreviations used

1D	1-dimensional
2D	2-dimensional
Act	Acetone
ATCC	American Type Culture Collection
BEA	Benzene:Ethanol:Ammonium hydroxide (90:10:1 v/v/v)
CEF	Chloroform:Ethyl acetate:Formic acid (5:4:1 v/v/v)
CF	Commercial fungicide
Chl	Chloroform
COSY	Correlation spectroscopy
Ctc	Carbon tetrachloride
Dcm	Dichloromethane
DE	Direct extraction
Dee	Diethyl ether
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl sulfoxide
E.c.	*Escherichia coli* (ATCC 25922)
E.f.	*Enteroccocus faecalis* (ATCC 29212)
EI	Electron impact
EMW	Ethyl acetate:methanol:water (40:5.4:4 v/v/v)
EtOH	Ethanol
EtAc	Ethyl acetate
HCl	Hydrochloric acid
Hex	Hexane
HMBC	Heteronuclear multiple bond correlation
HPLC	High performance liquid chromatography
HSQC	Heteronuclear single quantum coherence
HT	Healthtech
INT	p-iodonitrotetrazolium violet
LC_{50}	Lethal concentration for 50% of the cells

m/z	Mass to charge ratio
MEM	Minimal essential medium
MeOH	Methanol
MIC	Minimum inhibitory concentration
MS	Mass spectrometer
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye
NMR	Nuclear magnetic resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
P.a.	*Pseudomonas aeruginosa* (ATCC 27853)
PBS	Phosphate buffer solution
PDA	Potato dextrose agar
PDB	Potato dextrose broth
R_f	Retardation factor
S.a.	*Staphyloccocus aureus* (ATCC 29213)
SD	Standard deviation
SEE	Serial exhaustive extraction
SF	Sub fraction
ssp	Subspecies
TLC	Thin layer chromatography
TMS	Tetramethylsilane
UV	Ultraviolet radiation
v/v	Volume per volume
VLC	Vacuum liquid chromatography
Wat	Water

Abstract

Due to consumer resistance to the use of synthetic chemicals in agriculture, the aim of this research was to develop an antifungal extract from the leaves of *Melianthus comosus* that could be marketed as an organic fungicide by Healthtech Laboratories (Pty) Ltd.
Ten solvents of varying polarities (hexane, carbon tetrachloride, diethyl ether, dichloromethane, chloroform, acetone, ethanol, ethyl acetate, methanol and water) were used to extract the dried and powdered leaves of *Melianthus comosus*. The antifungal activity of each extract was tested against 10 plant pathogenic fungi (*Rhizoctonia solani, Fusarium oxysporum, Penicillium janthinelum, Penicillium expansum, Colletotrichum gloeosporioides, Trichoderma harzianum, Pythium ultimum, Phytophthora nicotiana, Aspergillus niger,* and *Aspergillus parasiticus*). The acetone extract had the highest antifungal activity (average MIC of 0.10 mg/ml against 8 pathogens), followed by the ethanol extract with an average MIC of 0.22 mg/ml against 8 pathogens.

Bioautography was used to determine the number of antifungal compounds in the extracts and to locate the active compounds on bioautograms, to facilitate bioassay guided isolation. The main active compound against all organisms was present in the acetone extract and was of intermediate polarity with an R_f value of 0.33 in BEA. There was another compound that was non-polar with an R_f value of 0.86 that had moderate antifungal activity against *Fusarium oxysporum*. The inactive compounds were generally of high polarity (4) and some were also non-polar (5). Enrichment procedures removing inactive compounds were developed to increase the concentration of the active compounds.

Acetone was the best primary extractant for enrichment based on antifungal activity. Two pathways were used to enrich the acetone extract. Pathway 1 included the use of water to extract the highly polar inactive compounds from the plant material. The water-“washed” marc was dried and “washed” (extracted once) separately with hexane or dichloromethane or ethyl acetate. The respective marcs were again dried and extracted with acetone. All extracts were tested for antifungal activity. The acetone extract following the water and dichloromethane “washes” was the most active (average MIC of 0.088 mg/ml against all 10 fungal organisms) and was termed “HT01”.

In pathway 2, a solvent-solvent extraction method was used. The plant material was first extracted with acetone and the acetone extract dried. The dried extract was dissolved in a 1:1 mixture of ethyl acetate and water in a separating funnel. The ethyl acetate fraction was extracted with water several times to remove all the very polar inactive compounds. The water and the ethyl acetate fractions were both dried and used for phytochemical analysis and bioassays. The water fraction was relatively inactive against all organisms. The ethyl acetate fraction was termed "HT02" and had an average MIC of 0.066 mg/ml against all 10 fungal organisms.

Work was continued on HT02 including a field trial and cytotoxicity assays as it was the most active extract. The HT02 extract was used for a field trial (done in collaboration with the Healthtech Laboratories) on the plant *Symphytum officinale* (Comfrey) infected by rust. When the *in vitro* activity of HT02 was compared with six commonly used fungicides, it had the most activity against *Penicillium expansum* and the second highest activity against *Fusarium oxysporum*. After nearly two months plants treated with HT02 (0.2 mg/ml) had c. 50 infected leaves. Plants treated with a commercial fungicide "Bravo 500" (containing 1.5 mg/ml chlorothalonil) had c. 250 infected leaves and untreated plants had extensive infection. Moreover, plants treated with HT02 had flourished when compared to the other treatments. The HT02 extract was more effective against the particular fungus at a six times lower concentration than "Bravo 500".

The cytotoxicity of HT02 was determined using the brine shrimp and cell line toxicity assays. The LC_{50} of HT02 was 4.5 mg/ml against brine shrimp larvae and 0.0445 mg/ml against Vero cell lines. The toxic principles present in the extract were tentatively identified on TLC using a 20% solution of antimony-III-chloride in ethanol and Kedde's reagent as cardiac glycosides of R_f values: 0, 0.08, 0.23, 0.34 and 0.58 in EMW solvent system. Lead precipitation and vacuum column chromatography were used to remove the cardiac glycosides from the extract, hence reducing the cytotoxicity of the extract. Lead precipitation removed all five cardiac glycosides but the antifungal activity of the extract was reduced four-fold. Fractionation of HT02 by means of column chromatography gave nine fractions. The antifungal fractions (F3 with MIC values of 0.04 and 0.08 mg/ml against *Fusarium oxysporum* and *Penicillium janthinellum* respectively, and F2 with MIC values of 0.08 and 0.16 mg/ml against *Fusarium oxysporum* and *Penicillium janthinellum*) were

combined and showed no apparent cytotoxicity against the Vero cell lines. The inactive fractions were combined and had an LC_{50} of 0.0861 mg/ml against the Vero cell lines.

To isolate the main antifungal compound, gradient vacuum liquid chromatography and gravity assisted column chromatography was used in the bioassay-guided fractionation of the acetone extract. The major active compound of R_f 0.33 in BEA, 0.71 in CEF and 0.44 in hexane: ethyl acetate (7:3, v/v) was isolated and identified as the triterpene 3-hydroxy-12-oleanen-28-oic acid (oleanolic acid). The compound had high antifungal activity with MIC values ranging from 7.8 to 15.6 µg/ml against fungal pathogens used. This compound has been isolated from the root bark of *Melianthus comosus* and other plants but this is the first description of its antifungal activity against plant pathogenic fungi. The compound had no apparent cytotoxicity on Vero cell lines.

1. Chapter 1: Literature Review

1.1 Introduction

Humans depend directly or indirectly on plants for food. Spoilage and plant pathogenic fungi are responsible for some 20% loss of the potential global plant production (BioMatNet, 2004). Considerable foliage and postharvest losses of fruits and vegetables are brought about by decay caused by fungal plant pathogens. Fruits, due to their low pH, high moisture content and nutrient composition are very susceptible to attack by pathogenic fungi, which, in addition to causing rots, may also make them unfit for consumption either aesthetically or for example by producing mycotoxins (Phillips, 1984; Moss, 2002). About 23 million kg of fungicides are applied to fruits and vegetables annually, and it is generally accepted that production and marketing of these perishable products would not be possible without their use (Ragsdale and Sisler, 1994).

Because fruits and vegetables are commonly treated with fungicides to prevent postharvest diseases, there is a greater likelihood of direct human exposure to these compounds than to chemicals that are applied to increase harvests by treating foliage. The use of synthetic chemicals to control postharvest deterioration has been restricted due to their potential carcinogenicity, teratogenicity, high and acute residual toxicity, long degradation period, environmental pollution and their effects on food and other side-effects on humans (Lingk, 1991; Unnikrishnan and Nath, 2002). In addition, phytotoxic and off-odour effects of some prevalent fungicides have limited their use.

One problem with these synthetic chemicals is that as their potency has been enhanced, so have their side-effects, and also their cost (Tyler, 1992; Castro et al., 1999; Falandysz, 2000; Kast-Hutcheson et al., 2001; Sorour and Larink, 2001). Synthetic fungicides can leave significant residues in treated commodities (Parmar and Devkumar, 1993; Fernandez et al., 2001; Dogheim et al., 2002; Zahida and Masud, 2002). Due to consumer resistance to the use of synthetic chemicals in agriculture, the search for natural products to be applied in organic farming systems either as herbicides, fungicides, or pesticides has become a priority (Duke et al., 1995; Ushiki et al., 1996; Lovag & Wildt-Persson, 1998). Compounds of plant origin are generally preferred since they are regarded as environmentally safer (Ganesan and Krishnaraju, 1995). This has led to renewed

interest in the screening of extracts of plants for their potential application in agriculture (Poswal *et al*., 1993). After discussing a fresh impetus in search for novel antifungal compounds, Hostettmann et al. (2000) made the following statement "Another area which is badly in need of lead compounds is agrochemicals. Consequently, the investigation of higher plants for antifungal properties is of great importance at the moment."

1.2 Fungi as plant pathogens

Some plant growth problems are caused by disturbed metabolism produced, for example, by a deficiency of essential mineral elements in the soil. However, most plant diseases are caused by fungal, viral or bacterial pathogens. Fungi, rather than bacteria, are the most widespread and destructive plant pathogens. This is the reverse of the situation in man, where bacterial diseases are much more important and serious (Read, 1970).

In adopting parasitism as a mode of life, fungi derive their organic requirements from living plants or animals. A system of living together is established but only the fungus benefits and the host is harmed. Such a relationship is called a parasitic symbiosis (Isaac, 1992). Parasitic fungi can be classified in a number of ways. Obligate parasites are very specialized and can only grow in association with a living host. They must be associated with living cells of the host in order to grow and reproduce. With facultative parasites this association is not absolutely necessary. They are less specialized and able to grow away from their hosts as competitive saprotrophs.

Parasitic fungi can also be classified by their modes of their nutrition. Two extreme modes can be recognized – nectrotrophs and biotrophs (Wheeler, 1984). Nectrotrophic fungi derive their organic nutrients from dead cells which have been killed. They are 'dead feeders'. They are destructive parasites, differing from saprotrophs only in that they kill host cells. They often kill their host quickly (Wheeler, 1984). Many nectrotrophs are facultative parasites which can live as competitive saprotrophs on the dead organic matter in their environment. The mode of nutrition of such fungi depends very much on the conditions in which they find themselves (Read, 1970). By contrast, biotrophs are 'living feeders'. They are only capable of deriving their nutrition from living host cells. The fungus either dies or goes into the survival state should the host, or that part of it infected by the fungus, die. Away from their hosts, biotrophs are only found as dispersal or dormant spores. A

very balanced or long-lasting relationship may be established with the host. Nectrotrophy and biotrophy are two nutritional extremes. Some parasites, such as *Phytophtora infestans*, which causes potato blight, are truly intermediate, being at first biotrophic and then nectrotrophic (Georgopoulos, 1987).

1.3 Effects of fungi on plant health

Fungi affect the growth of plants in a range of different ways. As the result of the symbiotic association with fungi, for example the formation of mycorrhizae, the effects on plants are positive. Plants may have increased survival rates, greater biomass production, improved vigour and general health, and even better resistance to attack by pathogens. Conversely, infections by fungal pathogens cause reduction in the growth rate and the development of plants, either rapidly or in the long term, which may be so severe as to be fatal (Ayres, 1984).

Fungal pathogens arrive on the surface of the leaves or shoots of susceptible hosts as spores. Once deposited, free water is normally necessary for spores to germinate, although the conidia of some powdery mildews provide a conspicuous exception to the rule (Read, 1970). Droplets of water on the epidermis of the host resulting from dew or rain provide potential infection drops. If infection is to occur, the infected drops must persist long enough to allow penetration to take place. Apart from their duration, other factors may operate in the infection drops, for example substances may diffuse into them from the living cells of the host, which stimulate or retard the germination of the spores (Burge, 1988). Penetration can occur by two distinct methods in the leaves and soft shoots. They may penetrate either directly through the cuticle and cell-wall or via natural openings such as stomatal pores (Dixon, 1981). The germ tubes of the conidia of powdery mildews, such as *Erysiphe graminis*, make contact with the cuticle and swell to form an ovoid, limpet-like appressorium. From the underside of the appressorium a very narrow infection peg penetrates the cuticle and cell-wall. Penetration is partly enzymatic and partly mechanical. In many pathogens a cutinase is liberated in response to the presence of a cuticle. Basal levels of spores break down the cuticle and release monomers on contact. This stimulates the switching on of the fungal gene for cutinase production. On penetration and the utilization of the monomers, the gene is switched off again. Mutants lacking the gene are non-pathogenic (Isaac, 1992). The formation of appressoria further illustrates the versatility of fungal hyphae in their ability to penetrate and permeate tissues.

The firmly attached appressorium prevents any lift-off from the surface as the infection peg applies mechanical pressure, coupled with the production of lytic enzymes, as it bores through the cuticle and wall. Many nectrotrophic pathogens and mycorrhizal fungi penetrate their host in a very similar manner (Ayres, 1984).

After penetration, a swelling or vesicle usually arises from the tip of the infection peg and in the majority of biotrophs hyphal branches arise from this and grow between the host cells. They remain intercellular and do not penetrate the host cells. The cells are penetrated by very specialized branches of determinate growth and multiplicity of shapes called haustoria. These penetrate the wall but only invaginate the cell membrane. Haustoria are nucleated and rich in organelles such as mitochondria, ribosomes and vesicles, indicating that they are sites of high metabolic activity. They are presumed to be the structures responsible for the absorption of major nutrients from the host cells.

Infection of a leaf or shoot creates a sink into which a variety of host-produced nutrients, especially sucrose are rapidly removed. This induces the translocation of host metabolites into infected regions. These changed patterns of translocation are probably brought about by changes in the balance of growth substances within the host by the pathogen (Dixon, 1981). Overall, there is a tendency to increase the biosynthetic processes of the host to provide materials which the fungus requires, but which are also useful to the host in its attempt to resist or ward off the pathogen. Just as the rate of respiration increases on infection, very severe depressions in the rate of photosynthesis occur in green tissues often within a few hours of infection (Burge, 1988). As the rate of infection progresses the rate of photosynthesis continues to decline until the amount of carbon dioxide given off by the combination of host and pathogen exceeds the amount fixed. The increased respiration of infected tissues leads to losses of carbohydrates (Harley and Smith, 1983). This loss is compounded by the effects of reduced rates of photosynthesis. Invasion of vascular tissues results in a range of visible or measurable changes, which are considered to be symptoms of the interaction. Many plant diseases are manifested by a characteristic pattern of symptom development which is often referred to as the disease syndrome (Georgopoulos, 1987). Diagnosis may be difficult, since plants do not exhibit many different symptoms. Different causes may give

rise to similar symptoms resulting in confusion. A combination of biotic, or biotic and abiotic factors operating simultaneously will also complicate the situation (Harley and Smith, 1983).

1.4 Diagnosis of plant disease

In the diagnosis of plant disease, it is important to distinguish between environmental effects and those of disease. Where microbial pathogens are involved, it is usually possible to detect the organism on the surface of the plant or within the tissues. Detailed examination, with a microscope, may reveal the causal agent, particularly in the case of a fungus which is sporulating. However, if identification within the tissues is not possible, isolation procedures can be employed. In some cases it may be possible to encourage the sporulation of a fungus by incubation of infected tissues under more favourable conditions, or alternatively the organism may be isolated into axenic culture (Georgopoulos, 1987).

Koch's postulates

In 1882, Robert Koch laid down a series of rules to be followed in the demonstration of the pathogenicity of microbes in medicine. These have been subsequently modified for application to plant pathogens (Wheeler, 1984).

1. The microorganism (pathogen) must always be associated with the disease.
2. The pathogen must be isolated from diseased tissue, grown in pure culture and its characteristics described. Alternatively (for obligate biotrophs), it must be grown on a susceptible host plant and all characteristics of the plant and pathogen recorded.
3. The specific disease must be reproduced when the pathogen, isolated into pure culture, is re-inoculated into healthy host plants.
4. The microorganism must be re-isolated from an inoculated host which has developed the disease and the characteristics must match, exactly, with the previous isolation.

These rules are still very widely used in the determination of causal agents although it is now recognized that they are not equally applicable to all pathogens. In the case of obligate biotrophs, which cannot be grown in pure culture, it may be necessary to isolate the unknown organism into

healthy plants, rather than onto laboratory medium, and then apply rigorous controls to be certain that a particular organism is the causal agent of a disease. The direct transfer of isolated fungal spores on to a potential host can also be carried out. The sporulation of a fungus is important with regard to the identification of an isolate since many of the taxonomic criteria applied to the fungi depend on spore production and morphology.

1.5 Control of plant disease

Information presented in this section was compiled mainly from the book: Fungal-Plant Interactions by Susan Isaac, 1992.

The development of plant disease depends upon a series of dynamic interactions between host plants and potential pathogens at cell, tissue and whole plant level. The outcome of such encounters will be affected by the initial health and status of the plants concerned and will be moderated by the environment. Owing to the wide range of variables involved, the course of disease development is therefore erratic, highly variable and unpredictable. The occurrence of epidemics has caused very substantial economic losses and imposed serious food shortages throughout the world. For example, potato blight disease (caused by the fungus *Phytophthora infestans*) spread through southern Ireland in the mid 1840's and devastated the potato crop which was, at that time, the staple food of the predominantly poor population. As a result, that population was reduced by 25% due to immigration, or starvation and death, in just a few years. Such an epidermic that changed the course of history is rare. Less catastrophic losses occur annually, with major impacts on agriculture and environment.

Such economic and human considerations, together with the application of modern agricultural practices, including the large-scale cultivation of plants in close proximity, have necessitated the development of both preventative and direct control methods. Serious diseases are often persistent in an area and may spread very rapidly. A good understanding of the infection cycles and life-styles of the pathogens concerned in interaction with their hosts is important in the identification of suitable combative measures. The recognition of the mode of infection and the mechanism by which disease becomes established help to pin-point the possible sites and times of pathogen vulnerability leading to the development of strategies by which the disease cycle may be

interrupted. In general terms the steps which are taken include avoidance of disease, direct control measures, usually involving chemical treatments, and biological control. In many instances a combination of methods is most effective, and integrated control systems are most useful.

1.5.1 Evasion of disease

Disease will not develop if it is possible to prevent host and pathogen coming into contact. In most instances this is not possible but some measures can be used to limit the potential contact or to protect seedlings at the most vulnerable stages of development. In some cases simple means can be used most effectively to avoid disease or at least to aid in the limitation of disease establishment.

1.5.1.1 Quarantine

Strictly enforced Government Legislation now prevents movements of diseased plant materials between countries in efforts to exclude non-indigenous pathogens and highly infective strains from previously uninvaded regions. Such controls operate on a global scale. The importation of plant materials, plant products and soils is restricted and as a result the spread of some diseases has been reduced. The introduction of a novel pathogen to an area may result in the massive development of the infection, if it occurs, because the indigenous and hitherto unchallenged plant population is likely to have little inherent resistance.

1.5.1.2 Crop rotation

If a crop can grow in an isolated area clean from potential pathogens then disease is unlikely to develop. In practice this would be considered a very unusual situation, particularly in agriculturally active regions. However, the rotation planting of immune and susceptible crops in a particular area is an extremely successful, easy and cheap way to limit many plant diseases. This is a widely used crop protection measure. The technique is particularly suitable for soil borne fungi but in instances where resting spores are very long-lived, little success will be achieved.

1.5.1.3 Crop husbandry

An understanding of plant crop management together with the physiological mechanisms of disease production is essential to ensure successful husbandry. Appropriate treatment of crops will ensure good plant health and minimize the influx of disease. The optimization of various parameters including, for example, planting distances between rows and individual rows and the provision of suitable fertilizer levels, will maximize plant health and the rate of development. A healthy crop is most likely to result if it is possible to protect the seedlings through stages when they are most vulnerable to pathogen invasion. In some instances it is possible to control some environmental factors relatively easily, such as drainage and soil pH, and to great effect in counteracting the activities of selected fungal pathogens.

1.5.1.4 Hygiene

Avoidance of disease is achieved in the absence of the pathogen and therefore a useful protection measure is to maintain plants in conditions as free from infected material as possible. At planting, disease free seeds should preferably be used. It is an interesting and important observation that even a very low proportion of infected seed material may rapidly give rise to disease outbreaks and therefore it is clear that this is an important step in disease prevention. Parent plants should be grown in clean conditions for seed production. Good hygiene in fields has proved to be essential for crop protection. The removal of potential sources of infection such as dead plant remains or previously infected material, which may act as sources of infection, is essential. The careful pruning (roughing) of infected plants is an especially useful treatment for long-lived plants, trees and shrubs, to remove sources of infection. This is a labour-intensive means of control but it is a useful system providing that the rate of disease spread is not too rapid. If the spread is particularly fast then such treatment may not be cost-effective.

1.5.2 Direct chemical control

Using chemicals to control plant diseases is one of the oldest and still most widely practiced methods of disease control. Chemical treatments have been very successful in the control and eradication of potential fungal pathogens, particularly with fruit and vegetable crops. Essentially

there are three main classes of fungicides: the inorganic, the non-systemic organic and the systemic fungicides.

1.5.2.1 Inorganic fungicides

1.5.2.1.1 Copper

Copper treatment is one of the oldest and most famous treatments for the protection of plants. However, copper ions in solution are toxic to plants and more or less soluble copper salts are now used. Some plants are particularly sensitive, especially in wet weather. For example, the leaves of some varieties become scorched and photosynthesis is then impaired. Cuprous oxide (Cu_2O) is employed as a seed treatment and cuprous oxychloride ($CuCl_2.3Cu(OH)_2$) as a foliar spray or dip treatment. Some copper derivatives are only used as timber treatments because of their extreme phytotoxicity. Copper-based treatments are effective against a wide spectrum of fungal pathogens. Their action is fungistatic and prevents spore germination. However, if the spores are well washed after treatment the copper is removed and the spores may subsequently germinate. It is thought that the effects on mycelium are as a result of reaction with sulphydryl groups of amino acids, which denature proteins and enzymes.

1.5.2.1.2 Sulphur

Elemental sulphur is useful for the control of a wide range of fungal diseases, particularly powdery mildews (Erysiphales), leaf blights, rusts and apple scab. It is mostly used as a fine powder or as a paste. Some plants, such as tomatoes, and apple varieties, are sensitive to sulphur under dry conditions when leaf burn occurs, with loss of chlorophyll (chlorosis) and premature senescence of leaves and fruit drop. The action is thought to be fungitoxic and sulphur is suggested to interfere with electron transport through the cytochromes. Hydrogen sulphide is formed which denatures essential proteins.

1.5.2.1.3 Mercury

Compounds containing mercury are effective against fungal pathogens. Mercurous chloride (calomel; Hg_2Cl_2) has been used as a grass treatment and is especially suitable for the eradication

of clubroot disease (*Plasmodiophora*). Mercuric chloride ($HgCl_2$) has also been used in aqueous solution as a dip or seed treatment. Phenyl mercury chloride is an effective foliar spray for apple canker (*Nectria galligena*). However, these compounds are extremely toxic to mammals and are therefore not generally in use today for such environmental reasons.

1.5.3 Non-systemic organic fungicides

1.5.3.1 Dithiocarbamates

The dithiocarbamates, derivatives of dithiocarbamic acid, are the most widely used of the fungicidal preparations which are commercially available today (Table 1-1). The fungitoxic action is derived from the inactivation of sulphydryl groups in amino acids, which inhibits the production of proteins and important enzymes in the pathogen.

Thiram (tetramethylthiuram disulphate) (Figure 1-1) is used mainly as a seed dressing against vascular wilts and *Botrytis*. It is used as a very successful seed soak (24 h at 30°C) to protect against deep-seated, seed-borne pathogens, such as leaf spot of celery crops and as a control for *Alternaria* diseases of carrots. Maneb and zineb (Figure 1-1), which are manganese and zinc salts of dithiocarbamate, are used widely for the treatment of vegetable crops, either as seed treatments or often as post-harvest dips. These are particularly useful for the control of some rust diseases and powdery mildews. However, the possible effects of dithiocarbamates on mammals currently gives rise to concern.

1.5.3.2 Phthalimides

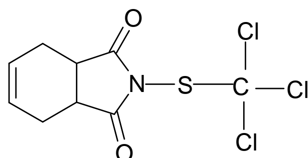
This group of fungicides is very widely used and is agriculturally important. Captan (Figure 1-1) is a very effective, broad spectrum, insoluble fungicide. It is used for the treatment of apple scab, tomato blight and *Botrytis* diseases. It is taken up into fungi very readily and the metabolism of these compounds results in interference with the production of amino acids and proteins in the cells. Captafol (Figure 1-1) is a chemically similar compound which is used widely for the control of potato blight and peach leaf curl (*Taphrina deformans*). Folpet is another similar compound but has a more phytotoxic action.

1.5.3.3 Dinitrophenols

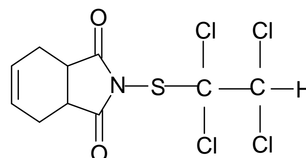
The dinitrophenols are used as fungicides and also as insecticides and herbicides. These compounds uncouple oxidation from phosphorylation in mitochondria and are therefore highly efficient fungicides. Dinocap is used particularly to control powdery mildew diseases (Erysiphales), especially on apples and soft fruit. It is often used, in wettable powder form, as a spray in fruit growing areas.

Table 1-1: Non-systemic fungicides (Isaac, 1992)

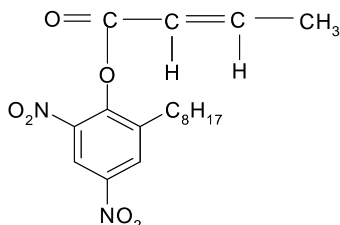
Fungicide	Usage
Thiram	Seed treatment for vascular wilt disease; celery leaf spot.
Maneb	Treatment against *Botrytis*, rust diseases and as a turf treatment.
	Potato blight; rusts of cereals; black spot of roses; also used as post-harvest treatment for soft fruits.
Zineb	Downy mildews, tomato blight and root rot; also used to protect mushroom crops
Captan	*Botrytis* on lettuce and soft fruit; apple scab; tomato stem rot.
Captafol	Potato blight (*Phytophthora infestans*).
Dinocab	Powdery mildew diseases.



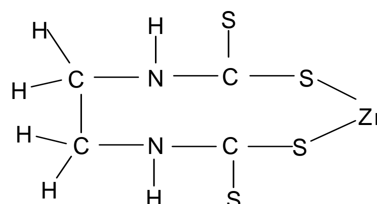
Captan



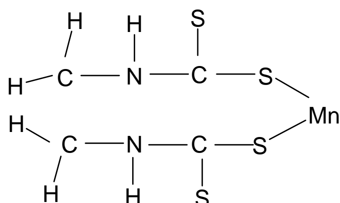
Captafol



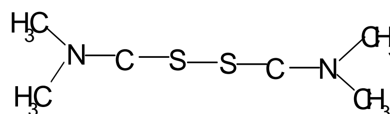
Dinocap



Zineb



Maneb



Thiram

Figure 1-1: Chemical structures of some non-systemic fungicides (Isaac, 1992)

1.5.4 Systemic fungicides

1.5.4.1 Antibiotics

Streptomycin which is produced by the actinomycete *Streptomyces griseus* is soluble in water and is readily absorbed by plant roots (Table 1-2). The main fungicidal action is interference with mitochondrial protein synthesis. It is used for the treatment of downy mildew disease of hops, although it lowers the rate of chlorophyll synthesis and may therefore be slightly phytotoxic under some conditions.

Other antibiotics produced by species of Streptomycetes are effective as fungicides but are also phytotoxic and are, therefore, used only in limited applications. Cycloheximide, which inhibits protein synthesis, is used to control stem rust on wheat. Kasugamycin is used against rice blast disease, very effectively, since this has lower phytotoxicity than other antibiotics.

1.5.4.2 Benzimidazoles

Benomyl (Figure 1-2) is a very useful, broad spectrum, antifungal agent which has been used for the successful control of many pathogenic fungi. It is used as a foliar spray as a protectant from powdery mildews, as a seed treatment for wheat smut, as a dip for bulbs to protect against *Fusarium spp*., and *Sclerotinia*, and sometimes as a soil treatment against vascular wilts. The toxic action is by interference with nuclear division and blockage of further mycelial growth. The formation of β-tubulin is prevented so that the microtubules, normally present when chromosomes separate, do not form and mitosis is therefore blocked. Thiabendazole, thiophanate and thiophanate-methyl are also used as very effective protectants and treatments for fungal infections. The benzimidazoles have proved commercially effective in disease control but resistance has been identified in *Botrytis cinerea, Venturia inaequalis and Pseudocercosporella herpotrichoides* in field situations. Benzimidazole resistance is very stable and is probably a major gene resistance so that the effectiveness of treatments can be easily lost in agricultural situations.

1.5.4.3 Pyrimidines

This group of fungicides is most widely used for the control of powdery mildews. Ethirimol is highly effective against *Erysiphe graminis* infections on barley. It is absorbed readily through plant roots and is translocated through to leaf margins. The toxic action probably occurs by interference with nucleotide synthesis. This group also includes the fungicides triarimol and dimethimirol.

1.5.4.5 Morpholines

The most important fungicides in this group are tridemorph and dodemorph and these are used against powdery mildew infections and leaf spot diseases. These compounds interfere with sterol biosynthesis (ergosterol) in fungi and as a result membrane function is disrupted.

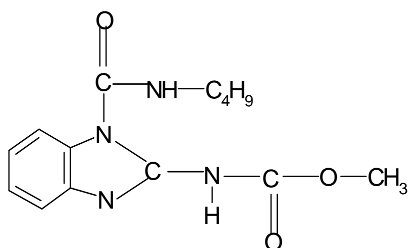
1.5.5 Other systemic fungicides

Metalaxyl (Figure 1-2) is a useful fungicide (an alanine derivative) which is effective against Oomycete fungi and can be used in the treatment of potato blight (*Phytophthora infestans*). It is also used as a protective treatment against a number of fruit diseases.

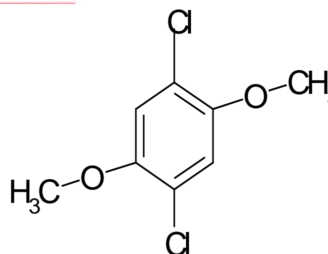
Chloroneb is also efficient for the treatment of soil-borne Oomycete fungi. The toxic action is limited to *Rhizoctonia* and *Pythium* but it is effective as a seed treatment in preventing seedling blights. It is persistent in soil and protection to the root system is therefore long lasting.

Table 1-2: Systemic fungicides (Isaac, 1992)

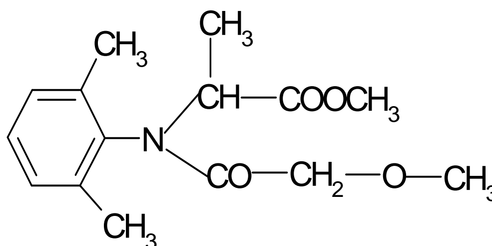
Fungicide	Usage
Antibiotics	
Streptomycin	Downy mildew on hops.
Cycloheximide	Wheat stem rust.
Kasugamycin	Rice blast disease (*Pyricularia*).
Benzimidazoles	
Benomyl	Very wide usage. Powdery mildews, apple scab, leaf spots, eyespot of cereals.
Thiabendazole	Post-harvest treatment.
Pyrimidines	
Ethirimol	Powdery mildews, especially *Erysiphe graminis* on barley.
Morpholines	
Tridemorph	Cereal mildews; leaf spots.
Others	
Metalaxyl	Treatment of soil-borne Oomycetes, often as seed treatment.
Chloroneb	Treatment of Oomycetes.



Benomyl



Chloroneb



Metalaxyl

Figure 1-2: Chemical structures of some systemic fungicides (Isaac, 1992).

1.6 Fungicide resistance

The use of systemic fungicides has increased the occurrence of resistance in pathogen populations. This has occurred because the toxic action is very site-specific in the pathogen and resistant strains have arisen by the selection of resistant individuals or as the result of single mutations. Resistance is less likely to arise if compounds which are less site-specific, or multisite fungicides, are used. Some of the more important pathogens, which are difficult to control, have developed resistance to some systemic fungicides, e.g. *Fusarium, Colletotrichum* and *Botrytis* (BioMatNet, 2004).

In physiological terms, resistance to fungicides may develop in a variety of ways, and it is obviously important to understand the mechanism of the toxic action in order to understand resistance. The pathogen may become less permeable to the compound concerned. The compound may become bound to cell wall materials, and therefore may not exhibit toxic activity in the fungus. There may be changes in the metabolism of the compound in the fungus so that toxic intermediates are not formed and the fungicidal action is lost. If the action is by virtue of a metabolic block in a biochemical pathway, then the fungus may become able to bypass that block and circumvent the

action of the fungicide. Alternatively, if the action is to block the activity of a particular enzyme then the fungus may produce more of the enzyme to compensate for the fungicidal action (Tyler, 1992).

Resistance to some fungicides can be acquired by changes in major genes within a pathogen. This type of resistance can develop very rapidly and be very stable and may therefore seriously affect the future use of these chemicals. Resistance to other compounds may be multi- or polygenic and may develop only over a long period of exposure to those chemicals (Georgopoulos, 1987).

Knowledge of resistance to systemic fungicides and its development is important. It is now realized that fungicides should be used in combination and that chemical treatment can be alternated, effectively minimizing the possibilities of resistance of a pathogen developing in a particular area. Repeated applications of the same fungicides, especially in relatively quick succession at suboptimal concentrations, provide the fungus with a perfect opportunity to develop resistance. A more promising approach to plant disease control in the future will be through programmes of Integrated Disease Management (IDM), the use of a number of fungicides in conjunction with resistant cultivars (Baldwin and Rathmell, 1988). The diversification of the control systems employed is obviously beneficial and the use of limited quantities of fungicides is important in both economic and environmental terms. In this search for a solution to the problems associated with the use of synthetic crop protectants in agriculture, plant extracts are being used (Poswal et al., 1993).

1.7 Use of plant extracts to combat fungal pathogens

Plants are good candidates in the search for fungicidal compounds because they have to exist under difficult conditions and attack by all manner of parasites (Hostettmann et al., 2000). Without an immune system to combat pathogenic microorganisms, plants rely primarily on chemical protection with secondary compounds. Phytoalexins are among the compounds that inhibit the establishment and growth of plant pathogens. Many other plant secondary compounds have been chemically characterized and evidence is developing that these compounds are involved in plant disease prevention and control. There is even some evidence that certain synthetic fungicides used in plant protection act by inducing the production of phytoalexins in plants (Duke et al., 1995).

Plants have many ways of generating antifungal compounds to protect themselves against pathogens. External plant tissue can be rich in phenolic compounds, alkaloids, terpenoids and other compounds that inhibit the development of fungi and bacteria (Kuc, 1985).

1.7.1 Why choose plant extracts?

(1) Each plant is a unique chemical factory capable of synthesizing large numbers of highly complex and new chemical substances (Farnsworth, 1984). The abundance of plant species therefore potentially provides us with a very broad spectrum of natural and new chemical substances for our scientific inquiries.
(2) Synergism occurs when the effect of two or more compounds applied together to a biological system is greater than the sum of the effects when identical amounts of each constituent are used. Since plant extracts contain more than one biologically active compound, synergism may explain why the activity of an extract is frequently greater than that of pure isolated compounds.
(3) The biologically active substances derived from plants have served as templates for synthesis of pharmaceuticals. In the United States of America, about 25% of human prescription drugs contain active principles that were derived from higher plants and there is increasing popularity in the use of herbal medicines (Farnsworth and Morris, 1976). It has also been estimated by the World Health Organization that about 80% of the population of the developing countries rely exclusively on plants to meet their health care needs (Farnsworth et al., 1985).
(4) Less time and resources are used in the development of an extract; it is therefore more economic to come up with biologically active extracts than to isolate pure compounds and research has shown fewer cases of undesirable side effects when dealing with extracts (Zishiri, 2004).
(5) The main sources of the biological activity of plants are their secondary metabolites (Farnsworth, 1984). Plant extracts may contain more than one antifungal compound and if resistance should develop for one compound, the other compounds present in the extract may still be active if they work using different mechanisms. There may also be increased stability of compounds present in an extract since some constituents may prevent the inactivation of others, e.g. antioxidant compounds like flavonoids may preserve molecules susceptible to oxidation.

1.8 Sources of antimicrobial activity in plants

Plants are highly vulnerable to attack by pathogenic organisms. Secondary metabolites are vital defensive compounds protecting plants from pathogens and herbivores. Since plants are more susceptible to fungal than bacterial attack, plants have probably evolved more potent antifungal than antibacterial compounds. Many different secondary compounds may have developed to protect plants against pathogens. Such compounds would be suitable for application as pest control agents in agriculture.

1.8.1 Secondary metabolites

Secondary metabolites are molecules that are not necessary for the growth and development of a plant, but may play a role in herbivore deterrence due to astringency or they may act as phytoalexins, killing bacteria that the plant recognizes as a threat. Secondary compounds are often involved in key interactions between plants and their abiotic and biotic environments that influence them (Facchini et al., 2000). There are three large classes of secondary metabolites: phenolic derivatives, terpenoids and alkaloids. The phenolic derivatives include the tannins and the flavonoids.

1.8.1.1 Phenolics

Phenolics are widespread in nature and are found in many natural compounds with aromatic rings. They include tannins, flavonoids, anthocyanins, anthraquinones, coumarins and simple phenolic compounds.

1.8.1.1.1 Tannins

Tannins are water soluble polyphenols which differ from other phenolics in their ability to precipitate proteins such as gelatine from solution (Bruneton, 1995). They are classified into two groups according to their structures: proanthocyanins (condensed tannins) and hydrolysable tannins. Hydrolysable tannins are hydrolysed by mild acid or base to yield carbohydrate and phenolic acid, whereas under the same conditions, condensed tannins are not hydrolysed. The phenolic acids are either gallic acid in gallotannins or other phenolic acids derived from oxidation of gallic acid in ellagitannins. One of their molecular actions is to complex with polymers such as proteins and

polysaccharides through non-specific forces such as hydrogen bonding and hydrophobic effects, as well as covalent bonding (Haslam, 1999). Thus their mode of antimicrobial action may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport protein and to complex with cell walls (Cowan, 1999).

1.8.1.1.2 Flavonoids

Flavonoids include: chalcones, flavononones, flavones and flavonols. Flavonoids are generally present in plants as glycosylated conjugates in fruits, vegetables, flowers and other plant products consumed in diet. Their activity is probably due to their ability to complex with extra-cellular and soluble proteins and to complex with bacterial cell walls (Tsuchiya et al., 1996).

1.8.1.2 Terpenoids

The linking together of isoprene units form the building blocks of terpenoids and they occur as sesquiterpenoids (C_{15}), diterpenoids (C_{20}), triterpenoids (C_{30}) and tetraterpenoids (C_{40}). Triterpenoids are present in plants in the form of saponins; monoterpenoids and sesquiterpenoids are common constituents of essential oils. Terpenoids exhibit antifungal and antibacterial properties (Taylor et al., 1996). The mechanism of action of terpenes is not fully understood but it is speculated to involve membrane disruption by lipophilic compounds.

1.8.1.3 Alkaloids

Heterocyclic nitrogen-containing compounds are called alkaloids. Many alkaloids are toxic and have a wide range of different pharmacological effects. The first medically useful example of an alkaloid was morphine, isolated in 1805 from the opium poppy *Papaver somniferum* (Fessenden and Fessenden, 1982); the name morphine comes from the Greek “Morpheus”, god of dreams. Codeine and heroin are both derivatives of morphine. Berberine is also an important representative of the alkaloid group. Indoquinolidizine alkaloids and glycoalkaloids and saponins, the essential constituents of *Naclea latifolia,* have antibacterial activity against Gram-negative and Gram-positive bacteria, as well as antifungal activity (Iwu, 1993).

1.9 Contract research for Healthtech Laboratories

As part of a service provided to the herbal industry, the Phytomedicine Laboratory, University of Pretoria, evaluated the antibacterial activity of extracts of *Melianthus comosus* (belongs to the Family Melianthaceae) supplied to us for analysis by Healthtech Laboratories Pty (Ltd), South Africa. The acetone and ethanol extracts of the leaves of this species were tested for antibacterial activity. These extracts had very low antibacterial activity.

Because we have developed an antifungal assay (Masoko et al., 2005) we determined the antifungal activity of extracts against animal pathogens. The antifungal activity against human fungal pathogens was high but *Melianthus comosus* contains toxic principles (Watt and Breyer-Brandwijk, 1962) and this would be a barrier to the use in animals or humans.

Hostettman et al. (2000) stressed the importance of agrochemical antifungals. Therefore the plant extracts were tested for antifungal activity against plant pathogenic fungi. The acetone and ethanol extracts had minimum inhibitory concentration (MIC) values of 0.04 to 0.63 mg/ml against the following plant pathogenic fungi: *Rhizoctonia solani, Fusarium oxysporum, Penicillium janthinellum, Penicillium expansum, Colletotrichum gloesporioides, Trichoderma horzianum, Pythium ultimum* and *Phytophthora nicotiana.* Later we found that leaf decoctions of *Melianthus comosus* are used traditionally to treat ringworm infection (Van Wyk et al., 1997). Healthtech Laboratories decided to sponsor further research on this topic.

1.10 The Melianthaceae family

1.10.1 Phytochemistry of Melianthaceae

Members of this family are tanniferous, producing ellagic acid but not proanthocyanins, and are not saponiferous, cyanogenic or mucilaginous (Cronquist, 1981). Styloids (elongate, slender prismatic crystals) are commonly present in some cells of the parenchymatous tissues.

1.10.2 *Melianthus comosus* Vahl

Melianthus comosus is a sturdy, woody shrub standing up to three metres in height, with leaves grouped at the ends of branches (Van Wyk et al., 2002). The yellowish-green leaves are pinnately

compound with approximately five pairs of toothed leaflets. A distinctive feature of the leaves is that the leaf stalks and midribs are winged. Brownish-red flowers, heavy with nectar, are borne in clusters below the leaves (Figure 1-4). The fruit is a membranous, inflated, four-chambered capsule, each chamber of which contains a round glossy seed. *Melianthus comosus* is found throughout the North West and Eastern Cape Provinces (Kellerman et al., 2005). Plants produce a pungent, unpleasant smell.

The six main toxic principles, extracted from the root bark of *Melianthus comosus* are helibrigenin 3-acetate and five new bufadenolides, melianthugenin, melianthusigenin, 6β-acetoxymelianthugenin, 6β-acetoxy-14-deoxy-15β-epoxymelianthugenin and 14-deoxy-15β-16β-epoxymelianthugenin (Anderson and Koekemoer, 1968). A novel feature of these compounds is the presence of an orthoacetate group on the ring A (Figure 1-3). Hydrolysis of the methanolic extract of the seeds with acetone containing 1% hydrochloric acid (gas) yielded scillaridine A, anhydroscillarine A and 3-anhydroscilliglaucosidine (Anderson and Koekemoer, 1969b). Anderson and Koekemoer (1969a) also isolated a triterpenoid acid (oleanolic acid) from the root bark of *Melianthus comosus.*

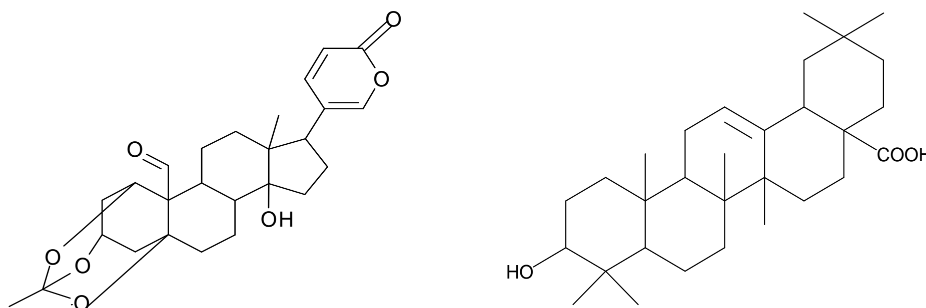


Figure 1-3: Melianthugenin (left) and oleanolic acid (right), (Van Wyk et al., 2002)

The plants seldom cause poisoning, although some mortality has been reported in equines and ruminants when grazing is scarce. Marloth (1913) stated that although stock avoids *M. comosus*, they can be poisoned by the palatable mistletoe or voëlent, *Tapinanthus* sp. growing parasitically on it. This indicated to him that the poisonous principle could pass from the host to the parasite. Some fifty years later, Adelaar and Terblanche (OVI, unpublished data, 1964; reported by Kellerman et al., 2005) fatally poisoned a sheep at the laboratory with a *Tapinanthus* sp., removed

from *Melianthus comosus* associated with the death of cattle. Similar specimens of Loranthaceae collected from *Acacia* spp. were non-toxic (Kellerman et al., 2005).

This transfer of toxicity from the host to the parasite has been confirmed later with the aid of fluorescence polarization immunoassay (FPIA). Cardiac glycoside activity of the same order as that of the host plant was registered in the hemiparasitic bird limes, *Tapinanthus leendertzii* (Loranthaceae) and *Viscum rotundifolium* (Viscaceae), growing on the very toxic *Nerium oleander* (Schultz & Naudé, OVI, unpublished data, 1995; reported by Kellerman et al., 2005).

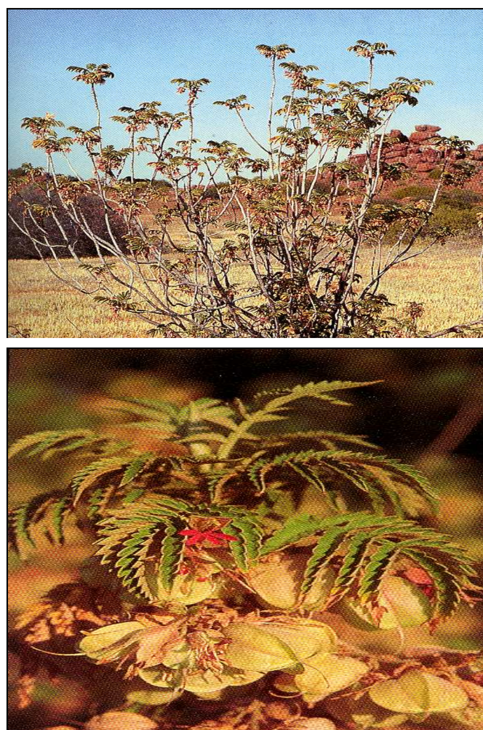


Figure 1-4: *Melianthus comosus* shrub (above), its flowers and fruits (below) (Van Wyk et al., 2002)

1.10.3 Ethnobotanical uses of *Melianthus comosus*

Leaf poultices and leaf decoctions are used to treat septic wounds, sores, bruises, backache and rheumatic joints (Van Wyk et al., 2002). *Melianthus comosus* is a traditional remedy for snake bites, root infusions are used for treating cancer and strong leaf decoctions are applied topically for

ringworm (Van Wyk et al., 2002). The root bark is thought to be poisonous and deaths are said to have been caused by it (Watt and Breyer-Brandwijk, 1962).

1.11 Aim and objectives

This project was aimed at developing an antifungal extract from the leaves of *Melianthus comosus* for the protection of plants and plant products from pathogenic fungi that could be licensed to Healthtech Laboratories for marketing as an organic fungicide.

The objectives include the following:

- To extract plant material with different solvents: hexane, acetone, dichloromethane, diethyl ether, ethyl acetate, chloroform, ethanol, methanol, water and carbon tetrachloride to select the best extractant for antifungal activity.
- To develop a product that can compete with other antifungals already in the market by increasing the antifungal activity of extract(s).
- To evaluate the toxicity of the extract and investigate the possibility of separating the toxic compounds from the active antifungal compounds.
- To determine the stability of the extract and test the efficacy in a field trial.
- To isolate the antifungal compound(s) from the most active extract using bioassay-guided fractionation and determine their structure(s).
- To evaluate the *in vitro* efficacy of the isolated compound(s).

2. Chapter 2: Plant collection, extraction and analysis

2.1 Introduction

Fresh or dried plant material can be used for phytochemical analysis or biological assays; most phytochemists have used dried material for several reasons:

- Traditional healers frequently use dried plant material.
- The time delay between collecting plant material and processing it makes it difficult to work with fresh material.
- There are fewer problems associated with large-scale extraction of dried plant material. Freshly harvested and dried material is more commonly used since old dried material stored for a long period of time may undergo some qualitative changes such as oxidative reactions resulting in a break-down of active compounds.

The choice of solvents used in an extraction is based on polarity, efficacy, ease of removal, safety and toxicity to assay organisms in the case of bioassay guided work (Eloff, 1998b). Secondary metabolites from plants can be extracted using solvents of different polarities. In order to find an extractant that would be optimally useful in screening and isolation of antifungal compounds from plants, the following extractants were compared: hexane, carbon tetrachloride, diethyl ether, dichloromethane, chloroform, acetone, ethanol, ethyl acetate, methanol and water (Kotze and Eloff, 2002). Kotze and Eloff (2002) used these solvents to extract from plant leaves and found that acetone was the best extractant. The quantity extracted and the diversity of compounds extracted will be investigated by thin layer chromatography of the crude extracts of *Melianthus comosus*. Qualitative antifungal activity will be determined by bioautography.

2.2 Materials and Methods

2.2.1 Plant collection

The leaves of *Melianthus comosus* were collected from a farm in Pretoria during summer 2004. A voucher specimen is deposited in the medicinal plant herbarium of the Division of Pharmacology and Toxicology (Department of Paraclinical Sciences) of the University of Pretoria. The leaves were air dried in the shade at room temperature (24°C) and ground into fine powder using a Jankel

and Kunkel Model A10 mill. The ground leaf material was stored in sealed plastic bags in a dark cupboard at room temperature.

2.2.2 Plant extraction

Ten solvents of varying polarities were used for extracting separate aliquots of powdered plant material: hexane, carbon tetrachloride, diethyl ether, dichloromethane, chloroform, acetone, ethanol, ethyl acetate, methanol and water (Kotze and Eloff, 2002). Three grams (3 g) of powdered material were extracted with 30 ml of each solvent by shaking vigorously for 10 minutes, then centrifuged at 3600 rpm for 5 minutes and filtered into pre-weighed glass vials. The process was repeated twice on the same marc and extracts were combined and dried under a stream of cold air at room temperature.

2.2.3 Phytochemical analysis of extracts

To get an indication of the chemical complexity, plant extracts were analysed by thin layer chromatography (TLC) on Merck (Kieselgel 60 F_{254}) plates in one of the following three solvent systems (Kotze and Eloff, 2002):

1. Polar/neutral = ethyl acetate: methanol: water (EMW) (10:1.35:1, v/v/v)
2. Intermediate polarity/acidic = chloroform: ethyl acetate: formic acid (CEF) (10:8:2, v/v/v)
3. Non-polar/basic = benzene: ethanol: ammonium hydroxide (BEA) (18:2:0.2, v/v/v)

All extracts were dissolved in acetone to give a 10 mg/ml concentration. Aliquots of 10 µl of extracts [containing 100 µg of extract] were loaded with a micropipette 1 cm from the bottom of a labeled TLC plate. The plates were air-dried and immediately placed in the tanks saturated with the eluent. The plates were developed until solvent was approximately 1 cm from the top and the plates were removed from the tanks and air-dried. Separated compounds were viewed under visible light and ultraviolet (UV) light (254 and 365 nm, Camac universal UV lamp TL-600). For further detection of compounds, the TLC chromatograms were sprayed with anisaldehyde-sulphuric acid (1 ml anisaldehyde + 18 ml ethanol + 1 ml sulphuric acid) or vanillin-phosphoric acid (0.4 g of vanillin + 40 ml of 85% phosphoric acid) (Stahl, 1969). The plates were heated for *c.* 5 minutes at 105°C for optimal colour development.

2.2.4 Retardation factor (R_f) values of compounds

The R_f value is the distance from the baseline (point of application) to the center of the zone of a visible compound divided by the distance from the baseline to the solvent front. Qualitative analysis is done by comparing the retardation factor (R_f value) on the TLC against a reference value of a standard because R_f values are constant under defined conditions.

$$R_f = \frac{\text{distance moved by analyte}}{\text{distance moved by solvent front}}$$

The R_f values of compounds in the *Melianthus comosus* extracts were calculated using the formula above.

2.3 Results and Discussion

2.3.1 Extraction

Methanol (MeOH) extracted the most material from the plant c. 20.7%, followed by water (Wat) c. 11%, ethanol (EtOH) c. 6.3%, dichloromethane (Dcm) c. 4.9%, acetone (Act) c. 4.7%, ethyl acetate (EtAc*)* c. 3.8%, chloroform (Chl) c. 3.3%, diethyl ether (Dee) and hexane (Hex) c. 2.9%, while carbon tetrachloride (Ctc) extracted the least, c. 1.8% (Table 2-1 and Figure 2-1). Figure 2-2 shows the extraction yields of the first, second and third extractions of different solvents on the plant material.

Table 2-1: Quantity in mg extracted from 3 g of leaves using hexane (Hex), carbon tetrachloride (Ctc), diethyl ether (Dee), dichloromethane (Dcm), chloroform (Chl), acetone (Act), ethanol (EtOH), ethyl acetate (EtAc), methanol (MeOH) and water (Wat), with extraction repeated twice on the same marc.

Solvents	Polarity Index	1st Extraction (mg)	2nd Extraction (mg)	3rd Extraction (mg)	Total yield (mg)	% Yield
Hex	0.1	40	37	10	87	2.9
Ctc	1.6	27	14	13	54	1.8
Dee	2.8	53	23	11	87	2.9
Dcm	3.1	86	48	12	146	4.9
Chl	4.1	54	31	13	98	3.3
EtOH	4.3	98	60	32	190	6.3
EtAc	4.4	76	24	15	115	3.8
Act	5.1	99	30	13	142	4.7
MeOH	5.1	400	131	90	621	20.7
Wat	10.2	213	88	31	330	11

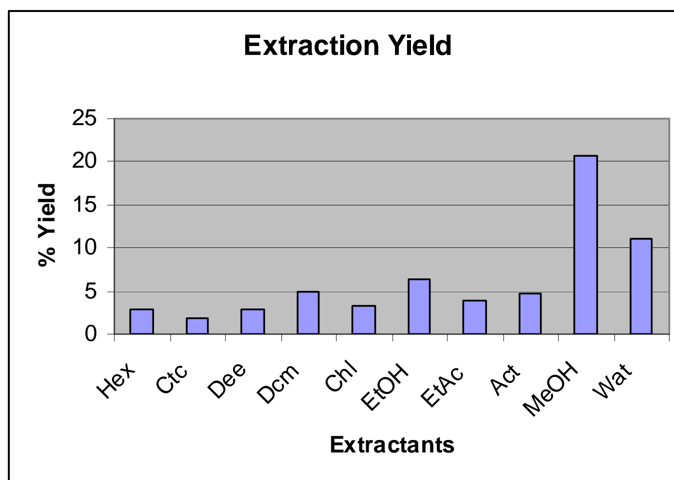


Figure 2-1: Percentage yield of extractables of *Melianthus comosus* after three extractions with hexane (Hex), carbon tetrachloride (Ctc), diethyl ether (Dee), dichloromethane (Dcm), chloroform (Chl), acetone (Act), ethanol (EtOH), ethyl acetate (EtAc), methanol (MeOH) and water (Wat) from left to right.

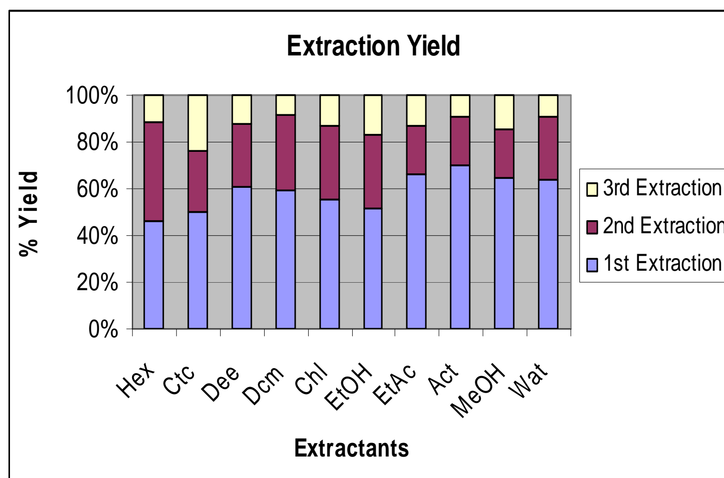


Figure 2-2: Percentage yield of extractables of *Melianthus comosus* after three extractions with hexane (Hex), carbon tetrachloride (Ctc), diethyl ether (Dee), dichloromethane (Dcm), chloroform (Chl), ethanol (EtOH), ethyl acetate (EtAc), acetone (Act), methanol (MeOH) and water (Wat) from left to right.

The choice of solvent depends on what it is intended with the extract. In this case, the aim was to extract antifungal compounds. The main reason for using solvents of different polarities was to determine if any of these extractants could selectively extract antifungal compounds. In general, the larger the variety of compounds that can be extracted by extractants, the better the chance that biologically active components will also be extracted (Eloff, 1998a). Methanol and water extracted a larger mass of material than the other solvents (20.7% and 11% respectively). This might indicate that *Melianthus comosus* contains high concentrations of polar compounds such as sugars, amino acids and glycosides.

To analyze extracts, the concentration has to be known. It is therefore important to dry the extracts or at least an aliquot (Eloff, 2004). A water extract takes a long time to dry in a stream of air at room temperature, so it was dried in a rotavaporator (Kotze and Eloff, 2002). The volatility of the extractant is therefore important. Another factor influencing the choice of solvent is toxicity to the test organisms and to the operator. For example, chloroform and diethyl ether are toxic to test organisms. Inhalation of large doses of chloroform or diethyl ether can cause respiratory depression and central anaesthesia. Methanol is considered to be highly poisonous. Chronic exposure to small quantities of carbon tetrachloride may cause more insidious effects such as

hepatoxicity (Houghton and Raman, 1998). Water-miscibility of extracts is also important for any further biological assays. Eloff (1998b) found that acetone is a very useful extractant because it has low toxicity to the test organism, dissolves many hydrophilic and lipophilic components and is miscible with water. Thus, acetone was chosen as the best extractant of *Melianthus comosus* leaf extract and ethanol as an alternate extractant if these extracts had high antifungal activity. Both solvents also had reasonably good extraction yields (4.7% and 6.3% respectively).

2.3.2 Phytochemical analysis

The chromatographic profiles of acetone (lane 5) and chloroform (lane 6) were similar (Figure 2-3). They both had the same number of compounds visible on the chromatogram, similar size of bands and the same colour intensities when the plates were sprayed with vanillin-phosphoric acid spray reagent. This suggests a similar composition and occurrence of the compounds in approximately the same quantities in the two extracts (Figure 2-3). Comparing the chemical profiles of the extracts, chloroform (lane 5) and acetone (lane 6) extracted more compounds separated by the TLC solvent systems used than the other extractants. The water extract had only one visible compound, probably because the solvent systems used could not resolve the very highly polar components. The methanol extract had fewer compounds but in high concentration based on the quantity extracted. There was little difference in the composition of the other extracts. The BEA solvent system gave a better resolution of the compounds in the crude extracts than either EMW or CEF (Figure 2-3). Hence BEA was chosen as the best solvent system to determine the complexities of the extracts and the R_f values of compounds. The vanillin-phosphoric acid reagent was a better chromogenic spray reagent than anisaldehyde-sulphuric acid. More compounds were visible when sprayed with the former than the latter (Figure 2-3).

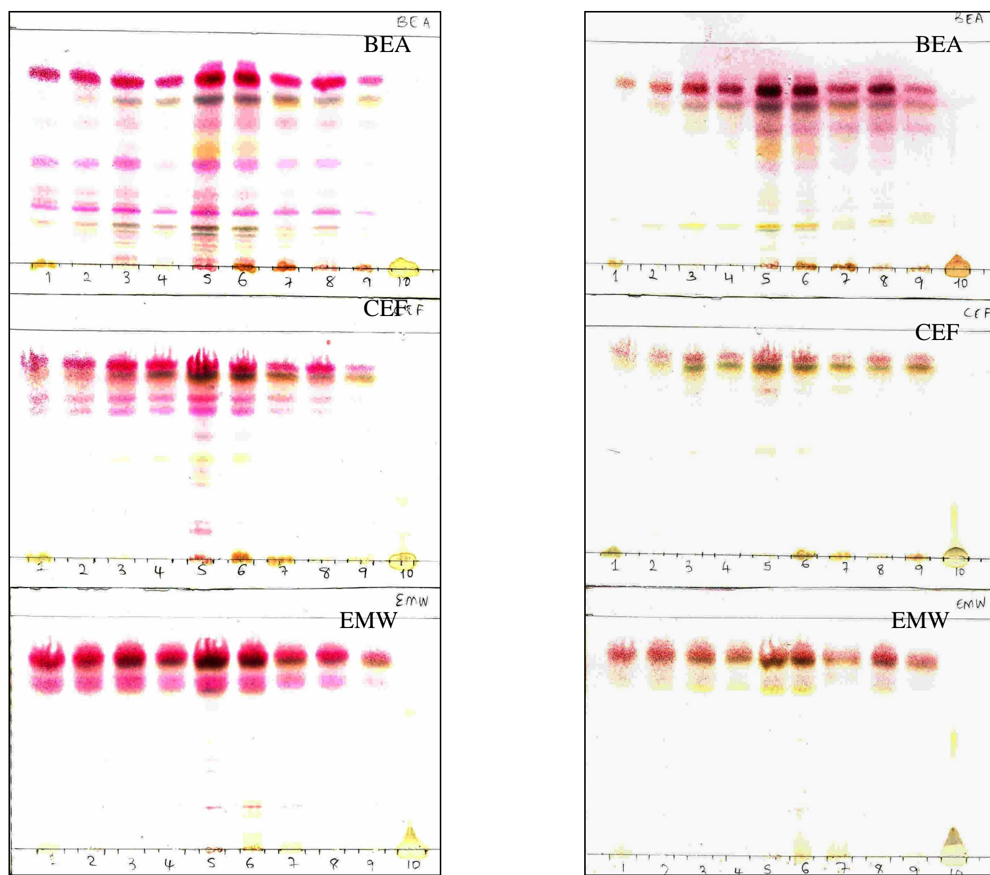


Figure 2-3: TLC chromatograms of *Melianthus comosus* extracted by 10 different solvents: developed in BEA (top), CEF (centre) and EMW (bottom) sprayed with vanillin-phosphoric acid (left) and anisaldehyde-sulphuric acid (right). **Lanes from left to right:** hexane (1), carbon tetrachloride (2), diethyl ether (3), dichloromethane (4), chloroform (5), acetone (6), ethanol (7), ethyl acetate (8), methanol (9) and water (10).

2.3.3 R_f values

Slight variations in the R_f values of compounds may occur due to changes in temperature, stationary phase or mobile phase composition and saturation status of the TLC tank (Houghton and Raman, 1998). Acetone and chloroform extracts had thirteen compounds ranging from R_f 0.01 to 0.86. All the extracts except water contained a compound with R_f 0.23 (Table 2-2). All extracts except methanol and water contained the compounds with R_f 0.16 and 0.43. The water extract had only one compound or several co-eluting compounds unable to be separated in the solvent systems with R_f 0.01.

Table 2-2: R_f value of compounds (√ = present, ×= not present) separated in BEA extracted by hexane (Hex), carbon tetrachloride (Ctc), diethyl ether (Dee), dichloromethane (Dcm), chloroform (Chl), ethanol (EtOH), ethyl acetate (EtAc), acetone (Act), methanol (MeOH) and water (Wat).

Solvents	**Rf values of compounds**												
	0.01	**0.02**	**0.09**	**0.13**	**0.16**	**0.23**	**0.3**	**0.43**	**0.49**	**0.61**	**0.72**	**0.8**	**0.86**
Hex	√	×	×	×	√	√	√	√	×	√	×	√	×
Ctc	×	×	×	×	√	√	√	√	×	√	√	√	×
Dee	√	√	√	√	√	√	√	√	√	√	√	√	×
Dcm	×	×	×	×	√	√	×	√	×	×	√	√	×
Chl	√	√	√	√	√	√	√	√	√	√	√	√	√
EtOH	√	√	×	×	√	√	×	√	×	√	√	√	×
EtAc	√	√	×	×	√	√	×	√	×	√	√	√	×
Act	√	√	√	√	√	√	√	√	√	√	√	√	√
MeOH	√	×	×	×	×	√	×	×	×	√	√	×	×
Wat	√	x	x	x	x	x	x	x	x	x	x	x	x

2.4 Conclusion

The direct extraction method used has the following advantages: it is quick, easy to use and compounds that are heat sensitive are extracted from the leaf material without being destroyed. The short time frame in which the procedure is performed limits the possible degradation of compounds due to prolonged exposure to solvent.

The extraction results showed that acetone and chloroform extracted more compounds (13) which separated in BEA than any of the other solvents. Methanol and water possibly extracted more sugars, amino acids and glycosides from the plant material (20.7 % and 11% yields respectively) than the other solvents. The fact that more polar solvents like ethanol, ethyl acetate, dichloromethane, chloroform and acetone extracted similar concentrations of non-polar compounds as did hexane suggested the presence of saponin-like compounds with polar and non-polar ends, which solubilize in either polar or non-polar solvents (Bruneton, 1995).

Because small particles of powdered plant material are used, the extraction equilibrium establishes very quickly. From the data presented in Table 2-1, it can be calculated that 91% of the quantity that would be extracted in 6 cycles is extracted after 3 cycles. The yield after the sixth extraction is same as that after the third extraction. Hence there was no need to further extract from plant material after three cycles of extraction.

3. Chapter 3: Biological assays

3.1 Introduction

Four major roles of bioassays can be distinguished: prescreening, screening, monitoring and secondary testing (Suffness and Pezzuto, 1991). In a prescreen, a bioassay is applied to large numbers of initial samples to determine whether or not they have any bioactivity of the desired type. Such bioassays must have high capacity, low cost, and must give rapid answers. A bioassay in a screen is used to select materials for secondary testing, whereas in a monitor, a bioassay is used to guide fractionation of a crude material towards isolation of the pure bioactive substances. It must therefore be fast and cheap, have high capacity, and be readily available to the phytochemist. In secondary testing, lead compounds are evaluated in multiple models and test conditions to select candidates for development towards clinical trials. Secondary testing is consequently characterized by a low capacity and expensive and slow bioassays.

The methods for the detection of natural product mixtures can also be divided into two groups: general screening bioassays and specialized screening bioassays. Depending on the aims of the screening programme, either a general screening which can pick up many different effects, or a specific assay which is directed at finding some effect against a specific disease, has to be performed (Tringali, 2001). The specific assay approach (antifungal assay) was used in this research. Plant-pathogenic fungi were used in the bioassay with the aim of selecting the best extract to protect plant and plant products from these organisms.

There are different methods for antifungal assay: bioautographic method (Begue and Kline, 1972), microtitre plate method (Masoko et al., 2005) and the disc diffusion method (Barry et al., 1979). The disc diffusion assay does not provide good comparative data (personal communication, J.N. Eloff). The bioautographic method is quantitative and gives the number of active compounds present in an extract. The microtitre plate method is qualitative, giving the concentration of active compounds in the extract. The bioautographic method (Begue and Kline, 1972) and microtitre plate method (Masoko et al., 2005) were used in this study. The p-iodonitrotetrazolium salt solution was used as an indicator of fungal growth. The colourless tetrazolium salt solution acts as an electron acceptor and is reduced to a red coloured formazan product by biologically active organisms.

3.2 Materials and Methods

3.2.1 Fungal organisms

Ten plant-pathogenic fungi (moulds) obtained from the Department of Plant Pathology, University of Pretoria (Table 3-1) were used as test organisms: *Rhizoctonia solani, Fusarium oxysporum, Penicillium janthinelum, Penicillium expansum, Colletotrichum gloeosporioides, Trichoderma horzanum, Pythium ultimum, Phytophthora nicotiana, Aspergillus niger,* and *Aspergillus parasiticus.* The fungi were chosen because they are the most important plant pathogenic fungi of economic significance.

Table 3-1: Origin of fungal pathogens used in this study obtained from the University of Pretoria Fungal Collection (UPFC)

UPFC no	Isolate name	Origin/Area	Person isolated	Substrate/host	Isolation date	Other collections codes
21	*Fusarium oxysporum*	Delmas, Gauteng	C Cronje	Maize roots	Jun-99	none
40	*Penicillium janthinellum*	Letaba Estate, Tzaneen	C Cronje	Citrus soil	Jan-99	2F205
43	*Colletotrichum gloeosporioides*	Pretoria	M Truter	*Phormium* leaves	Jun-00	As157
72	*Penicillium expansum*	Unrecorded	E de Jager	Litchi fruit	Unrecorded	none
89	*Trichoderma harzianum*	Pretoria	M Truter	*Acacia* roots	Apr-00	As101
411	*Aspergillus niger*	Letaba Estate, Tzaneen	C Cronje	Citrus soil	Jan-99	2F81
454	*Aspergillus parasiticus*	Letaba Estate, Tzaneen	C Cronje	Citrus soil	Jan-99	2F240
Other collections						
RS2366	*Rhizoctonia solani*	KwaZulu-Natal	M Truter	Soil, potato	May-01	none
--	*Pythium ultimum*	Johannesburg	M Truter	*Lisianthus* roots	Mar-05	none
	Phytophthora nicotiana	Johannesburg	M. Truter	Unrecorded	Mar-05	none

3.2.1.1 Culture preservation techniques

All organisms were maintained on Potato dextrose agar (Merck). The agar was prepared by dissolving 25 g of agar into one litre of distilled water. The mixture was boiled while stirring with a

magnetic stirrer for 15 minutes to dissolve the agar. The agar was autoclaved for 15 minutes, allowed to cool slightly and poured into sterile petri dishes. The agar was allowed to cool completely before being used for maintaining fungal cultures. The fungal organisms were subcultured from the mother cultures obtained from the University of Pretoria using surgical blades. The agar on which the fungus was growing was cut into small squares with the surgical blade and transferred to newly prepared agar in petri dishes. The new cultures were incubated at room temperature for 3-7 days in the dark after which they were sealed with parafilm and stored at 8°C. These cultures could now be used for preparing the fungal inoculum for use in bioassays.

3.2.1.2 Preparation of fungal inoculum

Potato dextrose broth (Merck) was used as the liquid medium for fungal culture. The broth was prepared by dissolving 30 g of broth powder into one litre of distilled water. The mixture was boiled while being stirred by a magnetic stirrer for 15 minutes and then autoclaved for 15 minutes. The conidia of fungi were harvested from the agar plate using a sterile cotton swab and suspended uniformly in the broth. The inoculum was quantified immediately before being used for bioassay.

3.2.1.3 Quantification of fungal inoculum

The conidia of each fungal species from one agar plate were harvested with cotton swabs and suspended uniformly in 25 ml of Potato dextrose broth each. The inoculum of each fungal species was quantified by counting the number of conidia using a haemocytometer and their number was adjusted to 1 x 10^6 ml^{-1} of the suspension.

The haemocytometer (Merck) was cleaned with 70% ethanol. The glass cover slip was cleaned with 70% ethanol and placed over the grooves and semi-silvered counting area. A micropipette was used to transfer approximately 20 µl of conidia suspension to the edge of the haemocytometer and allowed to spread evenly by capillary action. A compound microscope (Zeiss) was used at 100x magnification to focus on the grid lines in the chamber. The slide was adjusted to view one corner (16 square grid area), which is a 1 mm^2 area. The conidia in the two corners diagonally opposite to each other were counted and averaged to obtain the number of conidia (n). For standard subculture, counts between 100 and 300 conidia (cells) per mm^2 are ideal. The accuracy of the results increases when more cells are counted.

The concentration of the sample was calculated as follows:

c = n/v

c = cell concentration (conidia/ml)

n = number of cells counted

v = volume counted

For a haemocytometer, the depth of the chamber is 0.1 mm and the area counted is 1 mm^2.

Therefore, the volume is 0.1 mm x 1.0 mm^2 = 0.1 mm^3 or 10^{-4} ml. Hence,

c= n/v = n/10^{-4} ml = n x 10^4/ml

The cell concentration is equal to this number multiplied by the total volume of the cell suspension. After quantification, the inoculum was used for antifungal assay.

3.2.2 Antifungal assays

3.2.2.1 Bioautography

TLC plates (10 x 10 cm) were loaded with 100 µg of each of the acetone and ethanol extracts of *Melianthus comosus*. The prepared plates were developed in three different mobile systems: CEF, BEA and EMW. The chromatograms were dried overnight at room temperature under a stream of air to remove the remaining solvent. The TLC plates developed were inoculated with a fine spray of the quantified suspension of actively growing fungi (conidia) in a Biosafety Class II cabinet (Labotec, SA). The plates were sprayed until they were just wet and incubated at 37°C in a clean chamber at 100% relative humidity in the dark for 24 hours. Plates were then sprayed with a 2-mg/ml solution of *p*-iodonitrotetrazolium violet (3-{4-iodo-phenyl}-2-{4-nitrophenyl}-5-phenyl-2H-tetrazolium chloride, INT) (SIGMA) and incubated at 37°C for 30 minutes. White spots on the plate against a purple background indicate active compounds. INT is reduced to a red formazan salt in the presence of actively dividing cells.

3.2.2.2 Microplate dilution assay

A serial microdilution assay method (Masoko et al., 2005) was used to determine the minimum inhibitory concentration (MIC) values for plant extracts. This method had previously been used only for antibacterial activities (Eloff, 1998c; McGaw et al., 2001). To apply it to measuring antifungal

activities, a slight modification was made to suit fungal growth conditions. Residues of the different extracts were dissolved in acetone to a concentration of 10 mg/ml. The plant extracts (100 µl) were serially diluted 50 % with water in 96 well microtitre plates (Eloff, 1998c). Fungal cultures (*Rhizoctonia solani, Fusarium oxysporum, Penicillium janthinelum, Penicillium expansum, Colletotrichum gloeosporioides, Trichoderma harzianum, Pythium ultimum* and *Phytophthora nicotiana)* were transferred into fresh Potato dextrose broth, quantified and 100 µl of this was added to each well. As an indicator of growth, 40 µl of 0.2 mg/ml of INT dissolved in water was added to each of the microplate wells. The covered microplates were sealed in plastic sleeves to minimize cross contamination and incubated for two to three days at 37°C and 100% relative humidity. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth after 24 hours as shown by the red colored formazan. The assays were done in triplicate for each extract against each organism.

To confirm the results obtained, the microdilution assay was repeated against 10 organisms for acetone and ethanol extracts that had been stored for one month in a refrigerator at 5°C. *Aspergillus niger* and *Aspergillus parasiticus* were incubated with extracts for 16 hours instead of 24 hours as for the other organisms because they sporulate faster than the others.

3.3 Results and Discussion

3.3.1 Quantification of fungal inoculum

The number of cells of *Penicillium expansum* in the two diagonally opposite corner grids of the haemocytometer were counted and averaged. The average was 166 cells; hence the concentration of the sample was 166 x 10^4cells/ml. This concentration is higher than the required concentration; hence the inoculum was diluted with more broth to obtain an average of 100 cells. Hence the cell concentration was 100 x 10^4 cells /ml = 1.0 x 10^6 cells/ml. The same procedure was followed for the other fungal species to obtain a concentration of 1.0 x 10^6 cells/ml.

3.3.2 Bioautography

Bioautography was done to determine the number of active compounds and their retardation factors (R_f) values. The bioautograms (Figure 3-1) with *Colletotrichum gloeosporioides* (top),

Penicillium janthinellum (centre), *Fusarium oxysporum* (bottom); did not produce clear results. The cultures did not grow evenly on TLC plates. Zones of inhibition were present but not very distinct.

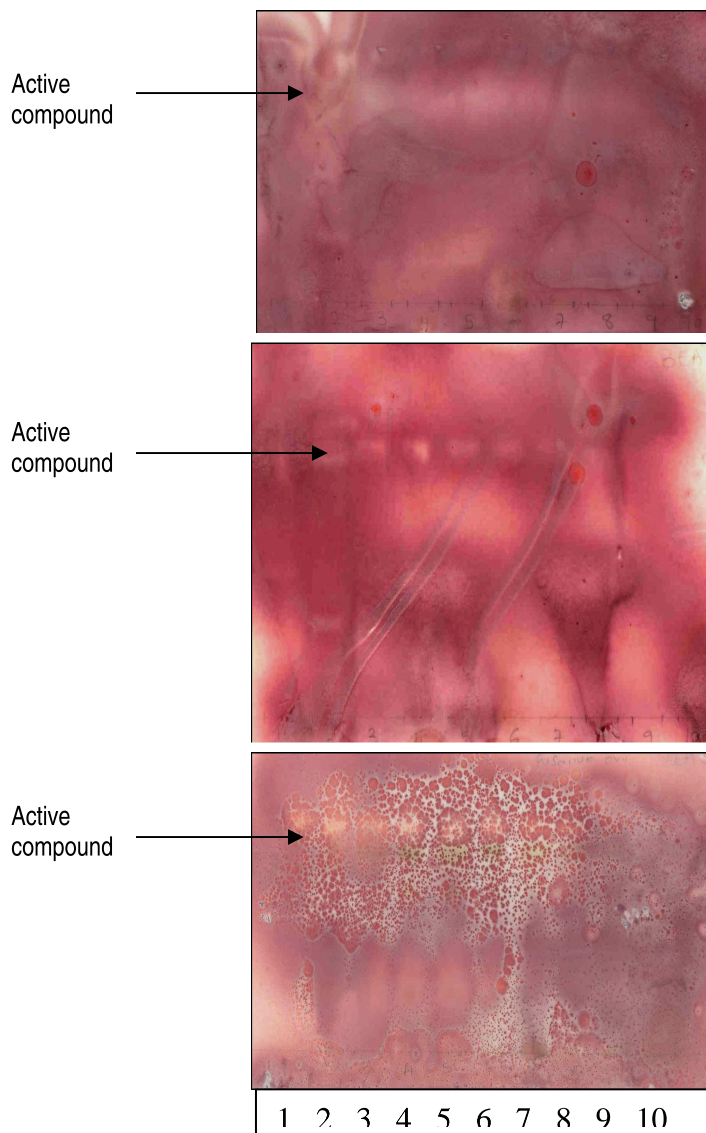


Figure 3-1: Bioautogram of extracts of *Melianthus comosus*. TLC chromatogram was developed in BEA sprayed with *Colletotrichum gloeosporioides* (top), *Penicillium janthinellum* (centre), *Fusarium oxysporum* (bottom); incubated and sprayed with INT. Colourless areas denote inhibition of fungal growth. **Lanes from left to right:** hexane (1), carbon tetrachloride (2), diethyl ether (3), dichloromethane (4), chloroform (5), acetone (6), ethanol (7), ethyl acetate (8), methanol (9) and water (10).

Most compounds separated by TLC did not have antifungal activity against *Colletotrichum gloesporioides, Penicillium janthinellum* and *Fusarium oxysporum*. There was a compound of R_f value 0.86 in BEA that was active against *Colletotrichum gloeosporioides, Penicillium janthinellum* and *Fusarium oxysporum*. The bioautography results were not very clear possibly because generally moulds are difficult to grow on TLC plates. Since the solvents of interest were acetone and ethanol, bioautography was repeated for these two extracts. This time the plates were incubated for 24 hours instead of 18 hours allowing the organisms to grow for a longer period of time before being sprayed with INT.

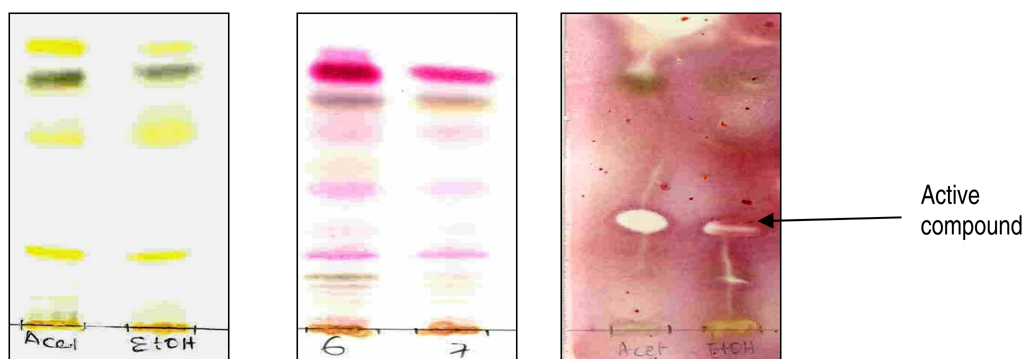


Figure 3-2: Left: TLC chromatogram of acetone (Acet) and ethanol (EtOH) extracts developed in BEA, not sprayed. **Middle:** TLC chromatogram of acetone (6) and ethanol (7) extracts developed in BEA and sprayed with vanillin-phosphoric acid spray reagent. **Right:** Bioautogram of acetone (Acet) and ethanol (EtOH) extracts. TLC chromatograms developed in BEA sprayed with *Fusarium oxysporum*, incubated and sprayed with INT. Colourless areas denote inhibition of fungal growth.

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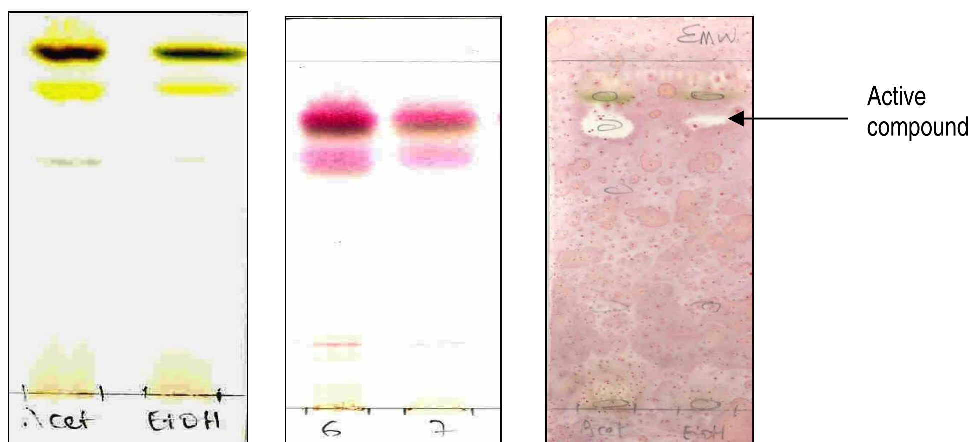

Figure 3-3: Left: TLC chromatogram of acetone (Acet) and ethanol (EtOH) extracts developed in EMW but not sprayed. **Middle:** TLC chromatogram of acetone (6) and ethanol (7) extracts developed in EMW and sprayed with vanillin-phosphoric acid spray reagent. **Right:** Bioautograms of acetone (Acet) and ethanol (EtOH) extracts. TLC chromatograms developed in EMW sprayed with *Trichoderma harzianum,* incubated and sprayed with INT. Colourless areas denote inhibition of fungal growth.

There was one major compound in the acetone extract that was very active with R_f value of 0.33 in BEA solvent system and an R_f value of 0.81 in EMW solvent system (indicated by arrow in Figure 3-3). The compound was invisible when viewed under visible light and UV light but was purple in colour when sprayed with vanillin-phosphoric acid. This compound inhibited the growth of *Fusarium oxysporum* and *Trichoderma harzianum* (Figures 3-2 and 3-3 as indicated by the arrows). There was another compound of R_f value of 0.86 in BEA that had moderate activity against *Fusarium oxysporum*. This compound could not be seen in the EMW system possibly because it was masked by other compounds. There were two compounds in the ethanol extract developed in BEA (R_f 0.23 and 0.33) that inhibited the growth of *Fusarium oxysporum* but they were in small quantities as seen by the relatively small size of the white spot against the purple background (Figure 3-3).

The fact that the major antifungal compound did not have a very high R_f value in BEA (non-polar solvent system) indicated that it was of intermediate polarity.

3.3.3 Microplate dilution assay

The antifungal activities of extracts against 8 out of the 10 organisms were determined and recorded because *Aspergillus niger* and *Aspergillus parasiticus* were too overgrown for results to be read. The methanol extract had high MIC values (average of 1.17 mg/ml for 8 organisms) [Table 3-2] thus low antifungal activity although it extracted a large quantity of material from the ground plant material. The acetone extract had the lowest MIC (average of 0.12 mg/ml for 8 organisms) thus highest antifungal activity, followed by ethanol. The water extract was inactive. This may be because any inhibitory compounds present may have been insoluble in water or contained within lipid soluble membranes in the cell (Martini and Eloff, 1998). The acetone extract was chosen as the extract to be developed further based on its high antifungal activity. With the exception of *Fusarium oxysporum*, the acetone extract had the highest activity against all pathogens tested.

Table 3-2: MIC values (mg/ml) of extracts tested against 8 moulds. A (hexane), B (carbon tetrachloride), C (diethyl ether), D (dichloromethane), E (chloroform), F (acetone), G (ethanol), H (ethyl acetate), I (methanol), J (water).

Fungi	A	B	C	D	E	F	G	H	I	J
Rhizoctonia solani	2.5	2.5	1.25	1.25	1.25	**0.02**	0.16	0.63	0.31	2.5
Fusarium oxysporum	2.5	0.63	0.63	0.63	0.16	0.16	**0.04**	0.08	2.5	2.5
Penicillium janthinellum	2.5	2.5	1.25	0.31	1.25	**0.04**	0.31	0.31	1.25	2.5
Penicillium expansum	2.5	1.25	0.31	0.63	0.16	**0.04**	0.31	0.16	0.31	2.5
Colletotrichum gloeosporiodes	2.5	1.25	2.5	2.5	2.5	**0.04**	0.16	0.08	0.63	2.5
Trichoderma harzianum	1.25	0.63	0.63	0.63	0.31	**0.31**	0.31	0.63	0.63	2.5
Pythium ultimum	2.5	0.63	1.25	0.31	0.16	**0.16**	0.31	0.16	1.25	2.5
Phytophthora nicotiana	1.25	0.31	2.5	0.63	0.31	**0.04**	0.16	0.16	2.5	2.5

The repeated average MIC values of acetone and ethanol extracts after storage were slightly higher (Table 3-3) than values determined previously [0.20 vs 0.12 mg/ml] for ten organisms, indicating lower activity. The average MIC values differ only by less than one well in the microplate assay which is not such a big difference in activity. The acetone extract was still more active than the ethanol extract (Table 3-3).

Table 3-3: MIC values in mg/ml for acetone and ethanol extracts stored for one month at 5°C (2nd MIC) compared to the MIC values previously determined (1st MIC). All values were recorded after 24 hours of incubation of extract with fungus.

Fungi	Acetone				Ethanol			
	1st MIC	2nd MIC	Average	SD	1st MIC	2nd MIC	Average	SD
Rhizoctonia solani	0.02	0.08	0.05	0.042	0.16	0.16	0.16	0
Fusarium oxysporum	0.16	0.16	0.16	0	0.04	0.04	0.04	0
Penicillium janthinellum	0.04	0.16	0.1	0.085	0.31	0.31	0.31	0
Penicillium expansum	0.04	0.31	0.175	0.191	0.31	0.31	0.31	0
Colletotrichum gloeosporiodes	0.04	0.08	0.06	0.028	0.16	0.63	0.395	0.332
Trichoderma harzianum	0.31	0.63	0.47	0.226	0.31	0.31	0.31	0
Pythium ultimum	0.16	0.16	0.16	0	0.31	0.31	0.31	0
Phytophthora nicotiana	0.04	0.04	0.04	0	0.16	0.08	0.12	0.057
Aspergillus niger	0.16	0.16	0.16	0	0.31	0.31	0.31	0
Aspergillus parasiticus	0.16	0.16	0.16	0	0.31	0.63	0.47	0.226

In most cases with regard to the acetone extracts there was an increase in the MIC values, indicating lower activity of the extracts. This difference may be attributed to experimental error or probably to the extracts losing some of their activity during storage in the refrigerator for a month at 5°C. In most cases the ethanol extracts did not lose any activity. Because ethanol is a much more efficient antiseptic compound than acetone, microbial decomposition of acetone extracts and not experimental error may explain the loss of activity.

The major difference in antifungal activity over time for different test organisms (e.g. *P. expansum* vs *F. oxysporum*), indicated that different compounds may be responsible for antifungal activity in different extracts.

3.4 Conclusion

The bioautography assay results were not very clear as some cultures grew unevenly on the plates probably because moulds are difficult to grow. Two active compounds were present in the acetone extract against *Fusarium oxysporum* and *Trichoderma harzianum*; the compound of R_f value 0.33 in BEA was a major active compound and that of R_f value 0.86 in BEA was a minor active compound with respect to their band sizes on TLC. Acetone was the most active extract followed by ethanol against all ten organisms. The ethanol extract had two active compounds against *Fusarium oxysporum* of R_f 0.23 and 0.33 in BEA. Acetone and ethanol extracts had a similar active

compound of R_f 0.33 in BEA. The compound is present in a smaller quantity in ethanol extracts than in acetone as seen by the relatively small size of the compound on the bioautogram. The water extract was inactive against all organisms. This indicates that plant material could be extracted with water first to remove inactive polar compounds before extracting with a relevant solvent without loss of antifungal activity. Based on antifungal activity, the acetone extract was chosen as the extract to be further developed.

Results in the previous chapter indicate that acetone and ethanol were the best extractants. Although the ethanol extract appear to be more stable than the acetone extracts, the same compounds are extracted. The ease of handling acetone extracts and the low toxicity of acetone to the test organisms led to selecting acetone as the primary extract. In the next chapter ways of increasing the activity of the extract will be investigated.

4. Chapter 4: Enrichment of Acetone Extract

4.1 Introduction

The acetone extract was more active against many fungal species than the ethanol extract. Hence the acetone extract was chosen to be potentized. The aim was to remove the inactive compounds by means of selective extraction and thereby to increase the concentration of the active compounds. Chikoto (2003) used selective extraction to produce a grape seed extract with high antioxidant activity. Zishiri (2004) successfully used selective extraction to develop a *Combretum woodii* leaf extract with high antibacterial and antioxidant activity. Selective extraction has advantages over isolation of active compounds. It allows for synergism between compounds hence increasing the activity of the extract; it is less time consuming, less labour intensive and less expensive than isolating the bioactive compound.

The main active compound in the acetone extract against *Fusarium oxysporum* was of intermediate polarity with an R_f value of 0.33 when separated by TLC using the BEA solvent system. There was another compound that was non-polar with an R_f value of 0.86 in BEA and had moderate activity against *Fusarium oxysporum* (Figure 3-2). The inactive compounds are of high polarity and some are also non-polar. Two different approaches were used for the enrichment of the acetone extract: pathway 1 which led to the development of “HT01” and pathway 2 which led to the development of “HT02”.

4.2 Materials and Methods

4.2.1 Pathway 1: “HT01”

Pathway 1 involved the use of water followed by hexane, dichloromethane or ethyl acetate to “wash” the dried and powdered plant material before extracting the dried marc with acetone. The term “wash” is defined as removing and discarding compounds soluble in a particular extractant. To remove the highly polar inactive compounds, water was therefore used to “wash” the plant material and the water extract was discarded. The marc (P-W) was dried at room temperature. Three samples (P-W) were prepared. Hexane was used to extract the dried marc (P-W). The hexane extract and the resulting marc (P-W-H) were dried at room temperature. Dichloromethane

was also used to extract from another sample of P-W. The dichloromethane extract and the resulting marc (P-W-D) were dried at room temperature. Ethyl acetate was used to extract from the last sample of P-W. The ethyl acetate extract and the resulting marc (P-W-E) were dried at room temperature. Three grams (3 g) of plant material was used in each case and extracted with 30 ml of solvent once. Following these "washes", the dried marcs (P-W-H, P-W-D and P-W-E) were each extracted with 30 ml of acetone three times resulting in extracts P-W-H+A, P-W-D+A and P-W-E+A respectively.
Below is a table (Table 4-1) of the different "washes", the desired effects and the shortcomings.

Table 4-1: Enrichment procedures of the acetone extract.

Pretreatments	Desired effects	Possible shortcomings
Water "wash" (P-W)	Removal of very polar inactive compounds	None
Hexane "wash" following water "wash"(P-W-H)	Removal of non-polar inactive compounds	Removal of the minor active compound
Ethyl acetate "wash" following water "wash" (P-W-E)	Removal of inactive compounds of intermediate polarity	Removal of major active compound
DCM "wash" following water "wash" (P-W-D)	Removal of inactive compounds of intermediate polarity	Trace of residue of solvent in the dried extract can be toxic and removal of major active compound

The pretreated extracts and the acetone ('washed" acetone) extracts were dried in pre-weighed glass vials under a cold stream of air. The dried extracts were reconstituted in acetone to obtain a stock solution of 10 mg/ml. These extracts (10 µl) were each spotted on TLC plates and developed in the BEA solvent system. The plate was sprayed with vanillin-sulphuric acid. Some plates were dried overnight and used for bioautography with *Fusarium oxysporum* and *Phytophthora nicotiana* following the method described in section 3.2.2.1. These organisms were chosen because they were easy to subculture and are more sensitive than the other organisms.

The antifungal activities of the extracts were also determined using the serial dilution microtitre plate technique (Masoko et al., 2005) as described in section 3.2.2.2. Ten mg/ml solution of each

extract was tested against all ten organisms. The extract with the highest antifungal activity was termed "HT01". The process is summarized in Figure 4-1 below.

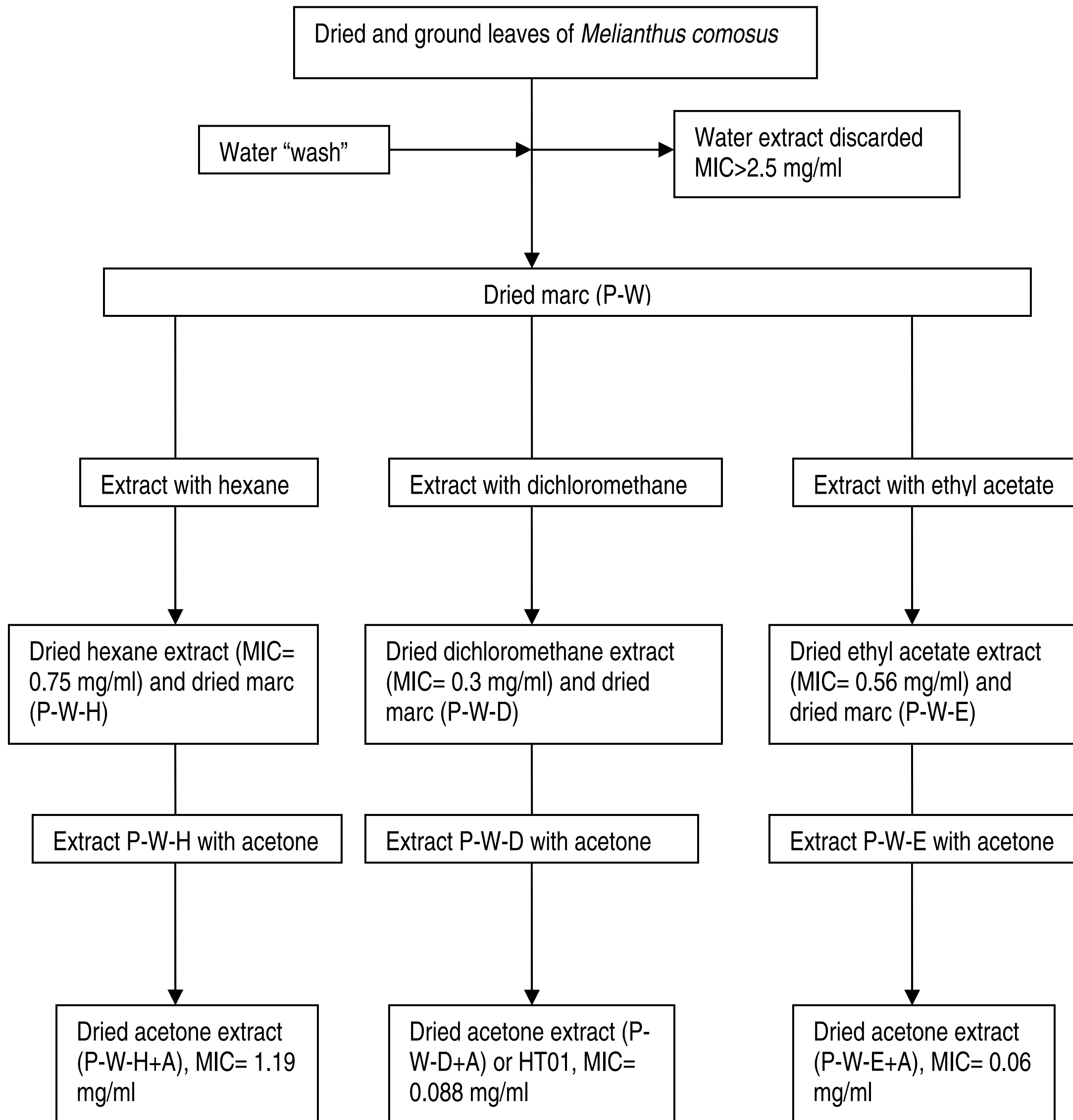

Figure 4-1: Flow chart for the development of HT01

4.2.2 Pathway 2: "HT02"

Pathway 2 involved the use of water to "wash" the plant material; extraction of the dried marc with acetone and lastly water: ethyl acetate (solvent: solvent) extraction of the dried acetone extract. Thirty grams (30 g) powdered plant was first extracted with 300 ml of acetone and the extract was

dried. The marc was discarded. The first acetone extraction would have broken cell membranes of the plant material and then made it easier to remove polar inactive compounds. The dried acetone extract was dissolved in a mixture of ethyl acetate (300 ml) and distilled water (300 ml) in a 1000 ml separating funnel. The water fraction was removed from the separating funnel. More distilled water (300 ml) was added to the ethyl acetate fraction to remove all the very polar inactive compounds. This process was repeated three times. The water and the ethyl acetate fractions were dried separately under a stream of cold air at room temperature and used for phytochemical analysis and bioassays. The ethyl acetate fraction was termed “HT02”. The process is summarized in Figure 4-2 below.

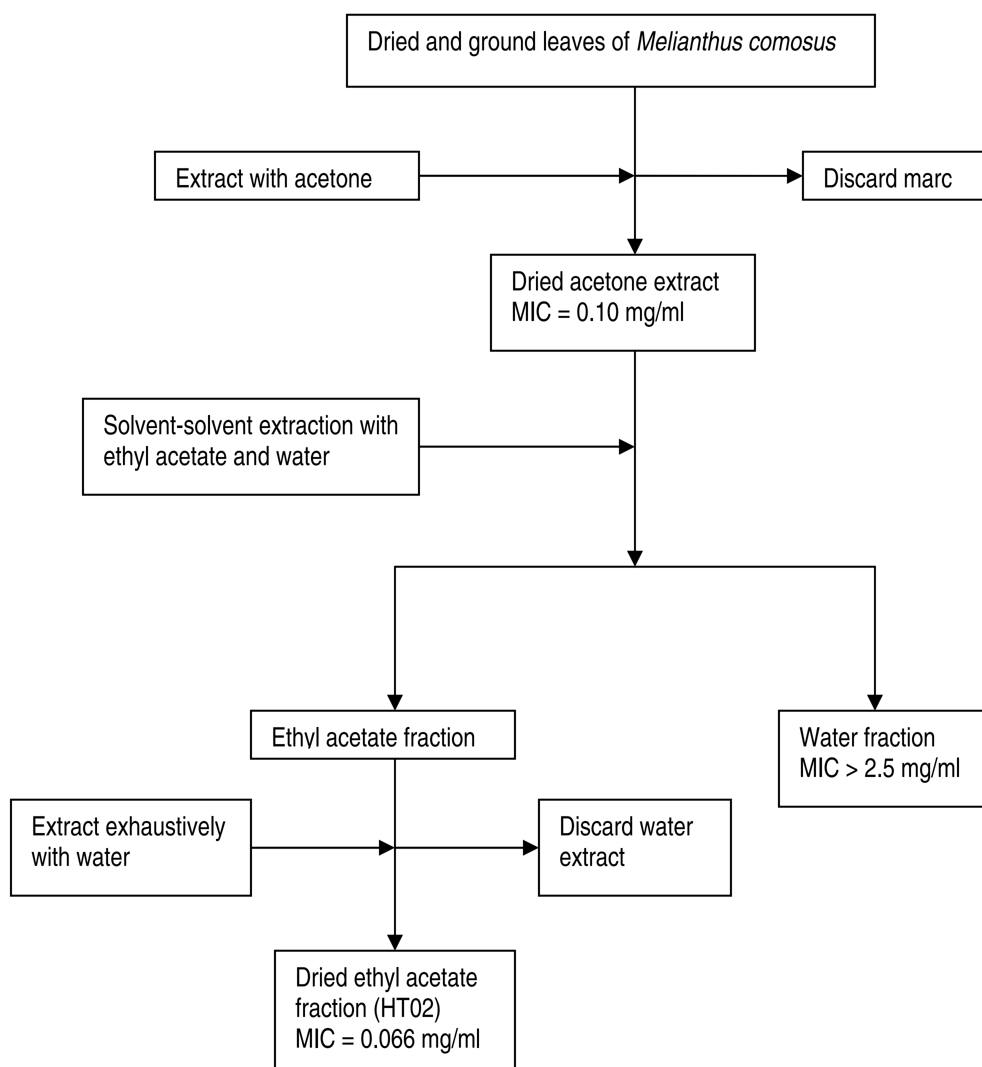

Figure 4-2: Flow chart for the development of HT02.

4.2.3 Comparison of HT02 with commercial fungicides

The antifungal activity of HT02 was compared to six different commercial fungicides. The microdilution assay method described in section 3.2.2.2 was used with 2 fungal organisms (*Fusarium oxysporum* and *Penicillium expansum*). These organisms were chosen because they were easy to subculture. The six commercial fungicides (Table 4-2) had active compounds: tebuconazole, propiconazole, chlorothalonil, triforine, dicarboximide and copper oxychloride. These fungicides were obtained from Healthtech Laboratories. The dilution used was 10 mg/ml for all samples.

Table 4-2: Commercial fungicides and their active compounds

Commercial name	Active compound
Bayer Folicur	Tebuconazole
Kombat Fungi Rid	Propiconazole
Effecto Bravo 500	Chlorothalonil
Effecto Funginex	Triforine
Kirchhoffs Rot and Spot	Dicarboximide
Pestex Copperchlor 50	Copper oxychloride

4.3 Results and Discussion

4.3.1 HT01

Water extracted 180 mg of material from 3 g of plant material. The water extract had little antifungal activity (MIC >2.5 mg/ml against all 10 organisms) as it contained highly polar inactive compounds as established before. The extraction yield of the solvents following the water "wash" were: hexane (P-W-H, 27 mg), dichloromethane (P-W-D, 76 mg) and ethyl acetate (P-W-E, 44 mg). The three acetone extracts following the hexane, dichloromethane and ethyl acetate "washes" (P-W-H+A, 22 mg; P-W-D+A, 34 mg; and P-W-E+A, 27 mg respectively) were pale green in colour. The extracts had similar chemical profiles when sprayed with vanillin-sulphuric acid (Figure 4-3). Hexane following water "wash" extract (P-W-H, a) removed most of the compounds from the plant material, hence acetone extract (P-W-H+A, b) after this "wash" contained very few compounds.
All the extracts except for (P-W-H+A, b) had two antifungal compounds (Figure 4-4).

4.3.2 HT02

The extraction yield of HT02 was 3.1%. The HT02 extract had more compounds (14) than all the extracts developed in pathway 1(Figure, 4-3). The HT02 extract (g) had a bigger band of the two active compounds R_f 0.29 and 0.86 in BEA against *Phytophthora nicotiana* and *Fusarium oxysporum* (Figure, 4-4). The compound of R_f 0.29 in BEA may be the same as the major active compound of R_f 0.33 in BEA since they both appeared purple upon spraying with vanillin-sulphuric acid. The difference in R_f may be due to changes in composition of solvent system caused by some degree of evaporation of one of the solvents.

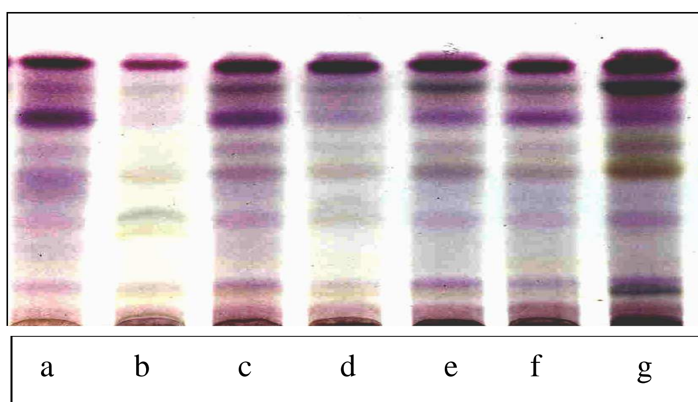

Figure 4-3: TLC profiles of extracts developed in BEA and sprayed with vanillin-sulphuric acid. **Lanes from left to right:** hexane after water “wash” (P-W-H, a), acetone after water and hexane “washes” (P-W-H+A, b), DCM following water “wash (P-W-D, c), acetone following water and DCM “washes” (P-W-D+A, d) “HT01”, ethyl acetate following water “wash” (P-W-E, e), acetone following ethyl acetate and water “washes”(P-W-E+A, f), “HT02” (g).

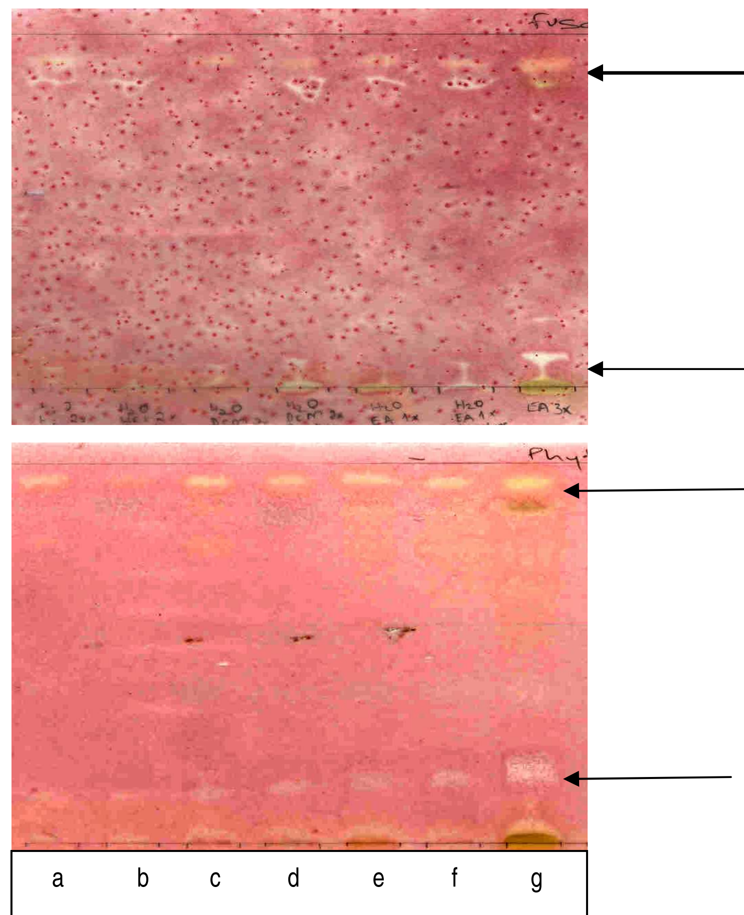

Figure 4-4: Bioautogram of extracts developed in BEA and sprayed with *Fusarium oxysporum* (top) and *Phytophthora nicotiana* (bottom). **Lanes from left to right:** hexane after water "wash" (P-W-H, a), acetone after water and hexane "washes" (P-W-H+A, b), DCM following water "wash (P-W-D, c), acetone following water and DCM "washes" (P-W-D+A, d) "HT01", ethyl acetate following water "wash" (P-W-E, e), acetone following ethyl acetate and water "washes"(P-W-E+A, f), "HT02" (g). Arrows showing active compounds.

The antifungal activity of the acetone extract following water and DCM "washes" (P-W-D+A, d) was higher than that of the direct acetone extract (an average of 0.088 mg/ml and 0.113 mg/ml against all 10 organisms respectively) (Table 4-3). This extract (d) was the most active extract amongst the extracts obtained using pathway 1 and was therefore called HT01. The acetone extract following the water and ethyl acetate "washes" (e) was more active against *Pythium ultimum* and *Penicillium janthinellum* than against the rest of the organisms. The acetone extract (b) following the water and hexane washes was the least active extract with an average MIC of 1.19 mg/ml against 10 organisms. This is probably because hexane, being non-polar, extracted some of the non-polar

active compound of R_f 0.81 in BEA. The ethyl acetate "wash" removed the inactive compounds alongside some of the active compounds.

The ethyl acetate fraction obtained from pathway 2 or HT02 (g) was the most active extract with an average MIC of 0.066 mg/ml against all 10 organisms followed by the acetone extract following water and DCM washes (d) with an average MIC of 0.088 mg/ml against all 10 organisms. The development of HT02 increased the activity of the acetone extract from an average MIC of 0.154 to 0.066 mg/ml; that is by 133%.

Table 4-3: MIC values (mg/ml) of hexane after water "wash" (P-W-H, a), acetone after water and hexane "washes" (P-W-H+A, b), DCM following water "wash (P-W-D, c), acetone following water and DCM "washes" (P-W-D+A, d), ethyl acetate following water "wash" (P-W-E, e), acetone following ethyl acetate and water "washes"(P-W-E+A, f), "HT02" (g).

	MIC values (mg/ml)						
Fungi	**a**	**b**	**c**	**d**	**e**	**f**	**g**
Rhizoctonia solani	1.25	1.25	1.25	0.08	0.63	0.31	0.04
Fusarium oxysporum	1.25	0.63	0.16	0.04	0.63	0.16	0.04
Penicillium janthinellum	0.63	0.63	0.08	0.08	0.16	0.16	0.02
Penicillium expansum	0.31	1.25	0.31	0.16	0.63	1.25	0.16
Colletotrichum gloeosporioides	0.31	1.25	0.16	0.04	1.25	1.25	0.04
Trichoderma harzianum	0.31	0.63	0.08	0.08	0.63	0.31	0.08
Pythium ultimum	0.63	0.63	0.31	0.04	0.16	1.25	0.04
Phytophthora nicotiana	0.31	0.63	0.16	0.04	0.31	1.25	0.04
Aspergillus niger	1.25	2.5	0.31	0.16	0.63	0.16	0.16
Aspergillus parasiticus	1.25	2.5	0.16	0.16	0.63	0.16	0.04
Average MIC	0.75	1.19	0.298	0.088	0.566	0.626	0.066

4.3.3 Comparison of HT02 with commercial fungicides

The MIC values were determined against *Fusarium oxysporum* and *Penicillium expansum.* Copper oxychloride was the most active fungicide with an MIC value as low as 0.02 mg/ml against *Fusarium oxysporum* (Table 4-4). This is probably because copper-based fungicides have fungistatic action and prevent spore germination. HT02 was the second most active extract with MIC value of 0.16 mg/ml against *Fusarium oxysporum* but it was as active as copper oxychloride against *Penicillium expansum* (MIC value of 0.04 mg/ml). Tebuconazole was the least active with

MIC values of 2.5 and 1.25 mg/ml against *Fusarium oxysporum* and *Penicillium expansum* respectively.

Table 4-4: MIC values of HT02 and other commercial fungicides: triforine, propiconazole, dicarboximide, chlorothalonil, tebuconazole, and copper oxychloride against *Fusarium oxysporum* and *Penicillium expansum.*

	Fusarium oxysporum		***Penicillium expansum***	
Samples	**Average MIC**	**SD**	**Average MIC**	**SD**
HT02	0.16	0	0.04	0
Triforine	0.63	0	1.25	0
Propiconazole	0.84	0.36	0.63	0
Dicarboximide	0.63	0	1.25	0
Chlorothalonil	1.67	0.72	2.08	0.72
Tebuconazole	2.5	0	1.25	0
Copper oxychloride	0.02	0	0.04	0

4.4 Conclusion

Two pathways were used for the enrichment of the acetone extract of *Melianthus comosus* leaves. Pathway 1 involved the use of selective extraction using various solvents while pathway 2 involved the use of solvent-solvent fractionation. Pathway 1 led to the development of HT01 while pathway 2 led to the development of HT02. HT01 was obtained by first extracting the plant material with water three times, and then extracting the dried marc with dichloromethane once and finally extracting the dried marc with acetone three times. HT01 had an average MIC of 0.088 mg/ml against all 10 fungal organisms.

HT02 was obtained by extracting the plant material with acetone three times; this acetone extract was dried and used in solvent-solvent fractionation with water and ethyl acetate. HT02 was more active than HT01 with an average MIC of 0.066 mg/ml against all 10 organisms. Hence HT02 was chosen as the new product for application on plants and plant products. This product may possibly be considered as an 'organic' or 'natural' fungicide. The antifungal activity of HT02 was compared to that of 6 commercial fungicides (triforine, propiconazole, dicarboximide, chlorothalonil, tebuconazole and copper oxychloride) against two fungal pathogens. Copper oxychloride was the most active followed by HT02; tebuconazole was the least active. The cytotoxicity, solubility and stability of HT02 have to be determined before use in field trials.

5. Chapter 5: Cytotoxicity, Solubility and Stability of HT02

5.1 Introduction

HT02 is to be used commercially to protect plants and plant products; hence it was necessary to determine the cytotoxicity if is to be used on food plants or contact toxicity for use on ornamental plants. The *in vitro* toxicity of HT02 was assessed in order to determine the concentrations at which HT02 was cytotoxic. The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide, Sigma) microculture tetrazolium assay described by Mosmann (1983) was used in order to evaluate the cellular viability in the presence of HT02. MTT is a tetrazolium dye; the conversion of the tetrazolium compound into a coloured formazan product by viable cells is monitored by a shift in absorbance. A linear relationship exists between cell numbers and the amount of formazan produced. This method is used in order to determine the number of living cells after incubation with a test compound relative to untreated control cells.

The developed extract (HT02) was also tested for *in vitro* toxicity using *Artemia salina* (brine shrimp) (Desmarchelier *et al*, 1996). The survival rate of the brine shrimp larvae after inhibition with plant extract determines the toxicity of the extract. Brine shrimp assay has been correlated with cytotoxicity in various cell lines. The brine shrimp assay shows a good correlation with cytotoxicity in cell lines such as 9KB, P388, L5178Y and L1210 (Meyer et al., 1982; McLaughlin, 1991; De Rosa et al., 1994; McLaughlin et al., 1998).

Most farmers or gardeners dissolve their fungicides in water, so it was necessary to determine the solubility of HT02 in different solvents and its stability in these solvents.

5.2 Materials and Methods

5.2.1 Cytotoxicity

Two methods were used: the brine shrimp assay and the MTT cell-line assay.

5.2.1.1 Brine shrimp assay

HT02 extract was tested at different concentrations: 0.1, 0.2, 0.5, 1 and 2 mg/ml in triplicate. The brine shrimp eggs obtained from a local pet shop were hatched in artificial sea water (3.8 g NaCl + 100 ml distilled H_2O) and left for 48 hours at room temperature. Extracts of different concentrations and the brine shrimp larvae (approximately 20 in each well) were incubated in microtitre plates at room temperature overnight. The survival rate of brine shrimp was recorded by counting the number of dead and live nauplii in each well using a dissecting microscope. LC_{50}, that is, the lethal concentration that would kill 50% of the organisms, was calculated. Podophyllotoxin (Sigma) was used for positive control and negative solvent controls were included.

5.2.1.2 Cell-line Cytotoxicity (MTT) assay

Logarithmically growing monkey kidney cells (Vero) were seeded into 96-well microtitre plates (200 ul/well at a density of 0.5-10 x 10^3 cells/ml) and exposed to various concentrations (0.001-1 mg/ml) of the HT02 extract for 120 hours. The growth medium used was Minimal Essential Medium (MEM, Highveld Biological) supplemented with 0.1% gentamicin and 5% foetal calf serum. A total of 200 µl of the cell suspension was pipetted into each well of columns 2 to 11 of a sterile 96-well microtitre plate. Growth medium (200 µl) was added to wells of columns 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated for 24 h at 37ºC in a 5% CO_2 incubator, until the cells were in the exponential phase of growth. The MEM was aspirated from the cells using a fine tube attached to a hypodermic needle, and replaced with 200 µl of HT02 at differing concentrations (serial dilution prepared in growth medium). The cells were disturbed as little as possible during the aspiration of medium and addition of extract. Each dilution was tested in quadruplicate. The microtitre plates were incubated at 37ºC in a 5% CO_2 incubator for 5 days or 120 hours. Untreated cells and positive control (berberine chloride, Sigma) were included.
After incubation, 30 µl MTT (Sigma, stock solution of 5 mg/ml in PBS) was added to each well and the plates were incubated for a further 4 h at 37ºC. After incubation with MTT the plates were centrifuged for 10 min at 1500 rpm. The medium in each well was carefully removed as before, without disturbing the MTT crystals in the wells, before adding 150 µl fresh PBS to each well. The microtitre plates were again centrifuged for 10 min at 1500 rpm and the PBS removed from the wells. After washing with PBS, the MTT formazan crystals were dissolved by adding 50 µl DMSO

to each well. The plates were shaken gently until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (Titertek Multiscan MCC/340) at a test wavelength of 540 nm and a reference wavelength of 690 nm. The wells in column 1, containing medium and MTT but no cells were used to blank the plate reader. The LC_{50} values were calculated as the concentration of extract resulting in a 50% reduction of absorbance compared to untreated cells. Alternatively, viability of cells after exposure to test substance was expressed as a percentage of viability of untreated control cells.

5.2.2 Solubility of HT02

The HT02 extract was tested for its solubility using four solvents: water, ethanol, ethyl acetate and acetone. Different quantities of the extract: 0.5, 1, 2, 5, 10, 50, 100, 200, 400 and 500 mg were weighed into pre-weighed glass vials. In the first vial with 0.5 mg of HT02, 0.5 ml of water was added. The vial was closed and shaken vigorously, and sonicated for 10 to 15 minutes for the extract to dissolve. The solvent was then poured out of the vial and the residue dried and weighed. This was done for each vial and each solvent and the results recorded. A drop of concentrated hydrochloric acid (12 molar) was added to water (0.5 ml) to make it acidic and the HT02 was added to it. Also, a drop of sodium hydroxide (2M NaOH) was added to water (0.5 ml) to make it alkaline and the HT02 was added to it. In the cases where the extract was very soluble, concentrations were increased up to 1100 mg/ml to test the solubility limit.

5.2.3 Stability of HT02

The HT02 extract was concentrated to 1 g/ml and put into two glass vials; one was stored at room temperature (25°C) and the other in the fridge at 5°C. Extracts were diluted after a month to make a stock solution of 10 mg/ml and tested against *Trichoderma harzianum and Fusarium oxysporum.* These organisms were chosen because they grow faster and give better results than the other fungal organisms. On the day of the test, a new HT02 extract was prepared, tested and the MIC value compared with the others.

5.3 Results and Discussion

5.3.1 Brine shrimp assay

There were deaths encountered in the wells with no HT02 extract (untreated wells) hence the % mortality of brine shrimp larvae in the treated wells was corrected using Abbott's formula:

Corrected mortality percentage: (m-M)/S x 100

m = mean of dead larvae in treated tubes %

M = mean of dead larvae in controls %

S = mean of living larvae in controls %

The extract was not toxic at low concentrations of 0.1, 0.2 and 0.5 mg/ml. At a concentration of 1 mg/ml, 9.76 % of the brine shrimp larvae were dead and at 2 mg/ml of HT02 21.65 % of the larvae were dead. The LC_{50} could not be calculated since a higher concentration was not used (Figure 5-1). The LC_{50} value is therefore substantially higher than 2 mg/ml. By extrapolating from Figure 5-1, 50% mortality would probably have been attained at 4.5 mg/ml. The extract is active at 0.04 mg/ml, which provides a safety margin greater than 100-fold.

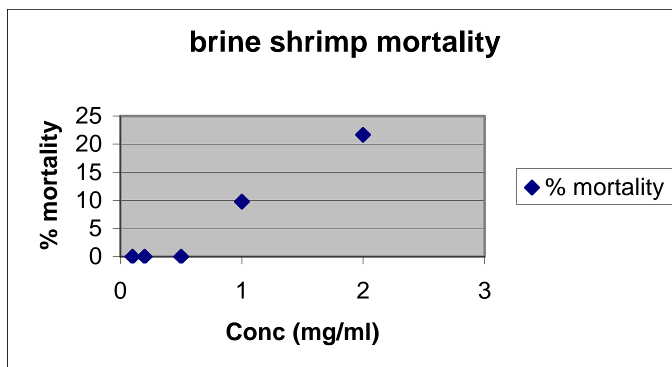

Figure 5-1: Influence of concentration of HT02 on brine shrimp mortality.

5.3.2 MTT cell line assay

The LC_{50} values of the HT02 determined in two separate experiments (sample 1 and 2) were 0.0442 and 0.0448 mg/ml which was higher than that of berberine (LC_{50}= 0.0076 mg/ml). This

implies that a much higher concentration of HT02 extract than of berberine is required to kill 50% of the cells, which means it is less toxic than berberine, a toxic alkaloid.

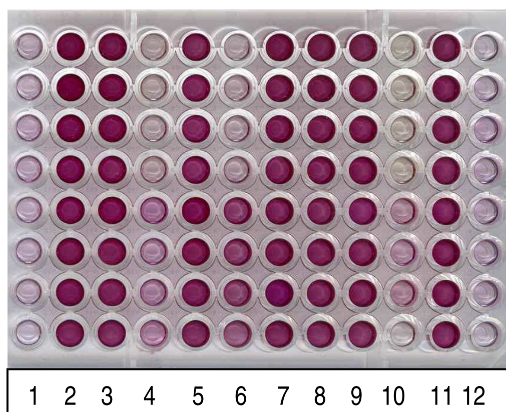

Figure 5-2: MTT assay results; wells from left to right: no cells (1), control (2), HT02 with cells (3&4), acetone and cells (5&6), Berberine (7&8), HT02 with cells (9&10), control (11) and no cells (12).

5.3.3 Solubility of HT02

The HT02 extract was not well soluble in water; this may be because all the water-soluble compounds had been extracted during the water-ethyl acetate solvent-solvent fractionation. The HT02 did not dissolve well in the acidic or alkaline water either (Table 5-1). The extract was reasonably soluble in ethanol. The extract was much more soluble in ethyl acetate (400 mg/ml) and highly soluble in acetone (>1000 mg/ml). This again indicates that the extract contains many compounds of intermediate polarity because ethyl acetate (polarity of 4.4) dissolves compounds of intermediate polarity. In the enrichment process of the acetone extract to obtain HT02, the dried acetone extract was partitioned between water and ethyl acetate. All the water soluble compounds had been removed by extraction with water in the solvent-solvent fractionation.

Table 5-1: Solubility of HT02 in water, ethanol, ethyl acetate and acetone

Solvents	Solubility
Water	< 1 mg/ml
Water + HCl	< 1 mg/ml
Water + NaOH	< 1 mg/ml
Ethanol	1 mg/ml
Ethyl acetate	400 mg/ml
Acetone	> 1000 mg/ml

5.3.4 Stability of HT02

The HT02 extract did lose some activity when stored over some time (MIC values of 0.31 mg/ml against *Trichoderma harzanium* and 0.16 mg/ml against *Fusarium oxysporum* for the stored and freshly prepared extracts). The dried extracts would probably be stable, but if product is to be marketed, it would probably be sold as an extract that can be diluted. The stability of the product developed is therefore important. The HT02 extract was stable in the fridge at 5°C and also at room temperature after a month as the antifungal activity did not change against the 2 fungal organisms selected for testing (Table 5-2). The MIC values are the same as those of the freshly prepared acetone extract determined earlier in section 3.3.3 (Table 3-2). This showed that the active ingredient of the plant does not lose activity in the fridge or at room temperature. However this does not prove whether or not the extract will lose activity if kept for longer periods and in different containers. Exposure of extracts to other factors apart from temperature, for example light, may influence stability.

Table 5-2: Average MIC values of HT02 extracts to determine stability at room temperature and in the fridge.

	Average MIC values (mg/ml)	
Extracts	***Trichoderma harzianum***	***Fusarium oxysporum***
HT02 (freshly prepared)	0.31	0.16
HT02 stored at room temperature	0.31	0.16
HT02 stored in the fridge	0.31	0.16

5.4 Conclusion

The LC_{50} of HT02 was c. 4.5 mg/ml based on the brine shrimp assay. HT02 was an antifungal extract with an average MIC of 0.066 mg/ml against all 10 fungal organisms, which provides a safety margin greater than 60-fold. An average LC_{50} of HT02 was 0.0445 mg/ml using the MTT cytotoxicity assay, providing practically no safety margin. There is quite a big difference between the LC_{50} values obtained from the brine shrimp assay and MTT assay (4.45 mg/ml and 0.045 mg/ml). A lower concentration of extract (0.0445 mg/ml) is required to kill half of the population of cell lines than to kill half of the population of the brine shrimp larvae (4.5 mg/ml). This might be because the cell lines are more susceptible to toxic test samples than are the brine shrimp larvae. In cytotoxicity assays, cell cultures are much more reliable than brine shrimp larvae. Cell lines from animals are more closely related to human cells than brine shrimps which are crustaceans.

HT02 was far more soluble in acetone (>1000 mg/ml) than in other solvents; it was fairly soluble in ethyl acetate (400 mg/ml) and insoluble in water. HT02 was stable in the fridge at $5^{o}C$ and also at room temperature at $25^{o}C$ up to a month; hence the extract could be stored either at room temperature or in a fridge at $5^{o}C$ during field trials.

6. Chapter 6: Field trial of HT02

6.1 Introduction

This part of the project was done in collaboration with Mr. William Quin of Healthtech Laboratories. The developed HT02 extract had a high *in vitro* antifungal activity (an average MIC of 0.066 mg/ml against all 10 fungal species). To determine *in vivo* antifungal activity, HT02 was used as a natural fungicide on plants in the field. An area on an agricultural holding used for experimental organic growing was selected. The area is on a south westerly slope, with weather patterns roughly between those of Johannesburg and Pretoria. The area is fertilized with compost made on the property from horse manure and bedding from the stables. The bedding usually contains pine shavings and this necessitates the occasional pH adjustment by the addition of lime. No fungicides are normally used in the area.

6.2 Materials and Methods

The plant *Symphytum officinale* was selected as:

- There were approximately 20 beds of the plant on the site
- The plants were presumably genetically identical, grown from cuttings of a single plant developed as a low alkaloid version of the plant
- The plant is susceptible to “rust” (unidentified species) that is controlled by bi-weekly removal of the infected leaves

Three adjoining beds (A, B and C) were selected. All infected leaves were removed. Plants were sprayed at the commencement of the trial and again at 17 days into the trial.

Bed A was thoroughly sprayed with a 1 in 5000 mixture (0.2 g/l or 0.2 mg/ml) of HT02. Bed B was similarly treated with a widely used commercial fungicide commonly known as “Bravo 500”. This material was used at an effective dilution of 7.5 g of active (Chlorothalonil) in 5000 ml of water (1.2 g/l or 1.2 mg/ml).

Bed C was untreated and served as the negative control.

The plants were not watered during the eight week test period but received some rain. Due to the water insolubility of the extract, this may not be too critical. The tests were concluded 56 days after commencement.

6.3 Results and Discussion

All three beds had infected leaves at the close of the trial (Figures. 6-1, 6-2, 6-3). An approximate count gave about 50 infected leaves for bed A, 250 for bed B and an extensive infection for Bed C. A further observation indicated that bed A had flourished when compared to beds B and C. It is not clear whether this was due to the low level of infection, or growth stimulation caused by the HT02 extract or another activity. We are in the process of isolating pathogens that were present on the leaves.

Figure 6-1: Bed A treated with HT02.

Figure 6-2: Bed B treated with Bravo 500.

Figure 6-3: Bed C untreated, negative control.

6.4 Conclusion

Whilst the trial was extremely limited, the results indicate that the HT02 extract at a six times lower concentration than a commercial fungicide is effective on the particular fungus. The HT02 will be used for a field trial on a larger scale on ornamental plants to confirm the results; this is outside the scope of the present study.

It is clear that rainfall during the experimental period appeared not to have removed HT02 from the leaves of the infected plant due to the limited solubility of HT02 in water.

7. Chapter 7: Selective removal of cytotoxic constituents

7.1 Introduction

Melianthus comosus is known to be a toxic plant because it contains cardiac glycosides (Van Wyk et al., 2002). Cardiac glycoside drugs contain steroid glycosides which specifically affect the dynamics and rhythm of the heart muscles (Wagner and Bladt, 1996). The steroids are structurally derived from the tetracyclic 10,13-dimethylcyclopentanoperhydrophenanthrene. They possess a γ–lactone ring (cardenolides) and a δ–lactone ring (bufadenolides) attached to the β–position at C-17 (Wagner and Bladt, 1996). Glycosides vary widely in their chemical type, particularly in the nature of the aglycone. Their solubility properties correspondingly exhibit a wide range and it is therefore necessary to use different extraction methods to test for the different types of glycosides. In order to reduce the cytotoxicity of HT02 extract, the cardiac glycosides were identified by their colour and R_f values on TLC separations, and attempts were made to selectively remove these compounds from HT02. Two methods were used in attempts to remove the cardiac glycosides: lead precipitation and column fractionation.

7.2 Materials and Methods

7.2.1 Extraction and detection of cardiac glycosides

To extract cardenolides, 1 g powdered leaf material of *Melianthus comosus* was heated with 10 ml of ethanol in a water bath (at 50^oC) for 30 minutes (modified from Wagner and Bladt, 1996). The extract was cooled and filtered. This extract was named CG1.

To precipitate cardiac glycosides, 1 g leaf material of *Melianthus comosus* was extracted by boiling for 15 minutes with 30 ml 53% ethanol containing 3% lead acetate (Jäger and van Staden, 1995). The extract was filtered and cooled. The filtrate was acidified with a few drops of glacial acetic acid and partitioned three times in succession with 15 ml dichloromethane (DCM). The DCM phases were combined, dried with anhydrous sodium sulphate and solvent was removed under vacuum. The dried extract was redissolved in 1 ml dichloromethane: ethanol (1:1, v/v). This extract was named CG2.

Extracts (CG1 and CG2) were spotted on TLC plates. The TLC plates were developed in ethyl acetate:methanol:water (81:11:8, v/v). Ten µl of digitoxin (0.4 mg/ml in methanol), a standard cardiac glycoside was used as reference.

Cardiac glycosides were detected in two ways. Firstly, developed TLC plates of the cardiac glycosides were viewed under an ultraviolet lamp at UV-254 nm and UV 365 nm. All cardiac glycosides exhibit a very weak fluorescence quenching at 254 nm and do not fluoresce at all at 365 nm. Secondly, 15 ml of a 20% solution of antimony-III-chloride in ethanol was sprayed on developed TLC plates. The plates were then heated for 5-6 min at 110°C. Evaluation was done in visible light and at UV-365 nm. Cardiac glycosides generally appear yellow to yellow-brown under UV-365 nm (Wagner and Bladt, 1996). In visible light the zones appear mainly grey, violet or brown.

To detect cardenolides only, the developed TLC plates were sprayed with 8 ml of Kedde reagent (alkaline 3,5-dinitrobenzoic acid) to detect cardenolides. Kedde reagent was prepared by mixing 5 ml freshly prepared 3% ethanolic 3,5-dinitrobenzoic acid with 5 ml 2M NaOH. Evaluation was done in visible light. Immediately on spraying, cardenolides generate a pink or blue-violet (visible) colour. The colour fades after a few minutes but can be regained by repeated spraying. Some reagents also give red, red-orange or violet cardenolide specific colours. Bufadienolides do not react.

7.2.2 Removal of cardiac glycosides

7.2.2.1 Lead Precipitation

Lead precipitation of cardiac glycosides from the leaf material of *Melianthus comosus* was carried out as described in section 7.2.1. The marc was not discarded but it was dried under a stream of cold air at room temperature. The dried marc was then extracted with 10 ml of acetone three times. The acetone extract (called lead-washed acetone extract) was dried and redissolved to form a 10-mg/ml stock solution for phytochemical analysis. Thirty µl (30 µl) of lead extract and 10 µl of the acetone extract were spotted on a TLC plate. The TLC plate was developed in ethyl acetate:methanol:water (EMW, 81:11:8, v/v/v). Cardiac glycosides were detected as described in section 7.2.1.

The lead precipitate and the lead-washed acetone extracts were tested for antifungal activity by microdilution assay (as described in section 3.2.2.2) against *Penicillium janthinellum* to find out whether this process of lead precipitation reduced the activity of the extract.

7.2.2.2 Column fractionation and bioassay of fractions

Ten grams (10 g) of HT02 extract was fractionated by means of silica gel 60 column chromatography using 6 litres of chloroform and 2 litres of chloroform: methanol (50:50, v/v). The extract was loaded on 500 g of silica gel 60 (Merck) in a 10 x 30 cm column. Small fractions (25 ml) were collected in test tubes. Tubes with similar contents as determined by TLC analysis were combined to form 8 major fractions.

The MIC values of the fractions were determined as described in section 3.2.2.2. Bioautography was carried out as described in section 3.2.2.1 and the MTT cell-line assay (as described in section 5.2.1.2) was used to determine the cytotoxicity of the fractions. The active and non-toxic fractions were combined whereas the inactive and toxic fractions were also combined.

7.3 Results and Discussion

7.3.1 Extraction and detection of cardiac glycosides

7.3.1.1 Detection without chemical treatment

The CG1 and CG2 extracts were bright yellow in visible light. There was a weak fluorescence by compounds of CG1, CG2 and the Pb-washed acetone extracts at UV 254 nm. Most of the compounds fluoresced under UV 365 nm as shown in Figure 7-1 below. Digitoxin did not fluoresce at either of the two wavelengths.

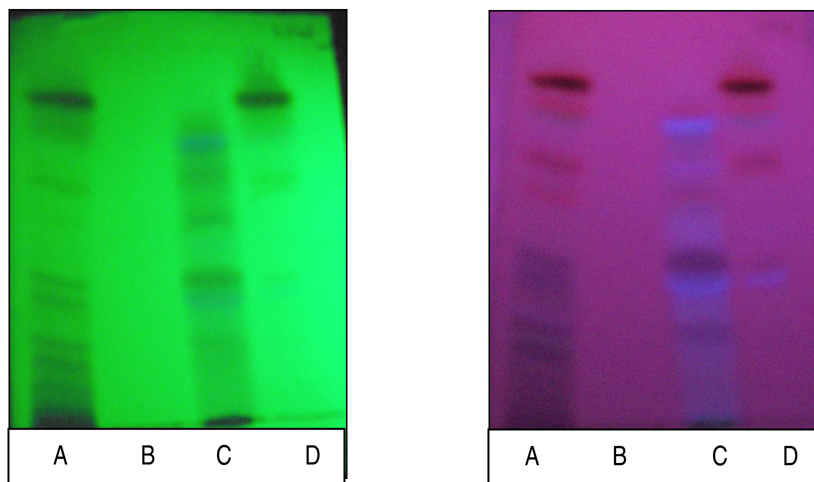

Figure 7-1: Fluorescence of extracts under UV 254 nm (left) and UV 365 nm (right). **Lanes from A to D:** CG1 (cardenolides), Digitoxin, CG2 (lead precipitate or cardiac glycosides) and Pb-washed acetone extract (one wash) developed in EMW solvent system.

7.3.1.1 Detection with chemical treatment

7.3.1.1.1 Antimony chloride reagent

Digitoxin appeared grey upon heating and fluoresced as a yellow-brown compound at UV 365 nm. Digitoxin had an R_f value of 0.44 in EMW. There was a blue-violet compound present in all three extracts (CG1, CG2 and lead-washed acetone extract) of R_f value 0.34 in EMW. This same compound appeared yellow-brown under UV 365 nm.

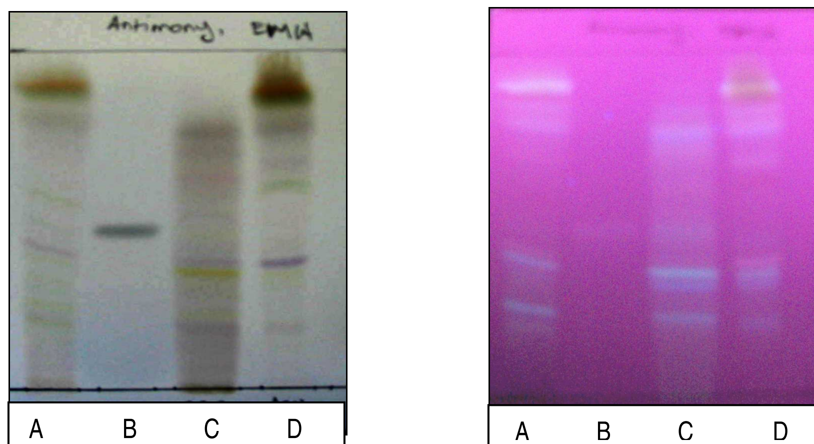

Figure 7-2: Chromatograms of extracts sprayed with antimony viewed under visible light (left) and under UV 365 nm (right). **Lanes from A to D:** CG1 (cardenolides), Digitoxin, CG2 (lead precipitate or cardiac glycosides) and Pb-washed acetone extract (one wash) developed in EMW solvent system.

7.3.1.1.2 Kedde Reagent

The CG1 extract contained very polar cardenolides that appeared red-orange upon spraying. Digitoxin appeared pink upon spraying. The colours quickly faded after 2 minutes which could give very misleading results if viewed after 2 minutes.

Figure 7-3: Chromatogram of extracts sprayed with Kedde reagent. **Lanes from left to right:** CG1 (cardenolides), digitoxin, CG2 (cardiac glycosides) and Pb-washed acetone extract (one wash) developed in EMW solvent system.

The *Melianthus comosus* leaf extracts contained five cardiac glycosides with R_f values of 0, 0.08, 0.23, 0.34 and 0.58 in EMW as shown below in Table 7-1. The CG1 contained three cardenolides of R_f 0, 0.08 and 0.23 whereas CG2 contained 2 cardiac glycosides of R_f 0 and 0.34 in EMW. The Pb-washed acetone extract contained 3 of the 5 cardiac glycosides.

Table 7-1: R_f values of cardiac glycosides present (+) or absent (-) in CG1, CG2 and lead-washed acetone extract (one wash) in EMW solvent system.

	Extracts		
R_f values	**CG1**	**CG2**	**Pb-washed Acetone extract**
0	+	+	-
0.08	+	-	+
0.23	+	-	-
0.34	-	+	+
0.58	-	-	+

7.3.2 Removal of cardiac glycosides

7.3.2.1 Lead precipitation

Exhaustive lead precipitation of leaf material of *Melianthus comosus* resulted in all five of the detected cardiac glycosides being removed from the extract. However the lead precipitates did not only contain the cardiac glycosides but also contained other important compounds from the plant. Two cardiac glycosides were removed when the leaf material was washed only once with the lead-acetate solution, whereas all five were removed alongside most of the other compounds with exhaustive precipitation. Hence, this is a disadvantage in using this method to remove the cardiac glycosides from the plant. The remaining lead in the acetone extract will have to be removed which can be very costly and time consuming.

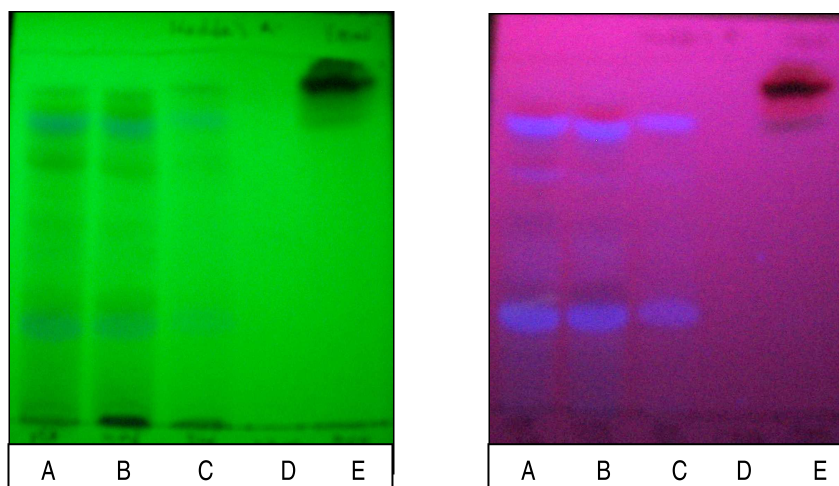

Figure 7-4: Fluorescence of extracts under UV 254 nm (left) and UV 365 nm (right) without chemical treatment. **Lanes from A to E:** First Pb extract (A), second Pb extract (B), third Pb extract (C), digitoxin (D) and the Pb-washed acetone extract (exhaustive) (E).

The plates sprayed with antimony chloride (Figure 7-5) showed that the lead precipitates contained 5 cardiac glycosides and many other compounds. The Pb-washed acetone extract contained fewer compounds in very small concentrations.

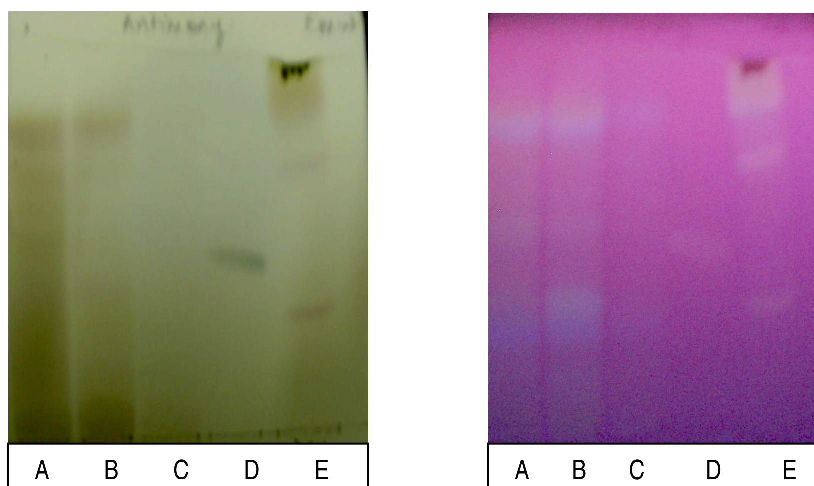

Figure 7-5: Chromatogram of extracts sprayed with antimony (left) and fluorescence under UV 365 nm (right). **Lanes from A to E:** First Pb extract, second Pb extract, third Pb extract, digitoxin and the Pb-washed acetone extract (exhaustive).

Substantial antifungal activity of *Melianthus comosus* was lost to the cardiac glycoside extraction process. The activity was reduced from MIC 0.08 mg/ml to 0.31 mg/ml against *Penicillium janthinellum* (Table 7-2), that is, a loss of c. 75%.

Table 7-2: MIC values (mg/ml) of acetone, exhaustive lead precipitate and lead-washed acetone (exhaustive wash) extracts against *Penicillium janthinellum.*

Extracts	1st MIC	2nd MIC	3rd MIC	Average	SD
Acetone extract	0.08	0.08	0.08	0.08	0
Exhaustive lead precipitate	0.63	0.63	0.63	0.63	0
Lead-washed acetone extract	0.31	0.31	0.31	0.31	0

7.3.2.2 Column fractionation

Fractionation of HT02 with chloroform and chloroform: methanol (50:50, v/v) led to the collection of 8 fractions (F1-F8). The chemical profiles obtained using TLC analysis (Figure 7-6) of the fractions showed the presence of non-polar compounds (F1, F2), compounds of intermediate polarity (F3, F4) and highly polar compounds (F7, F8).

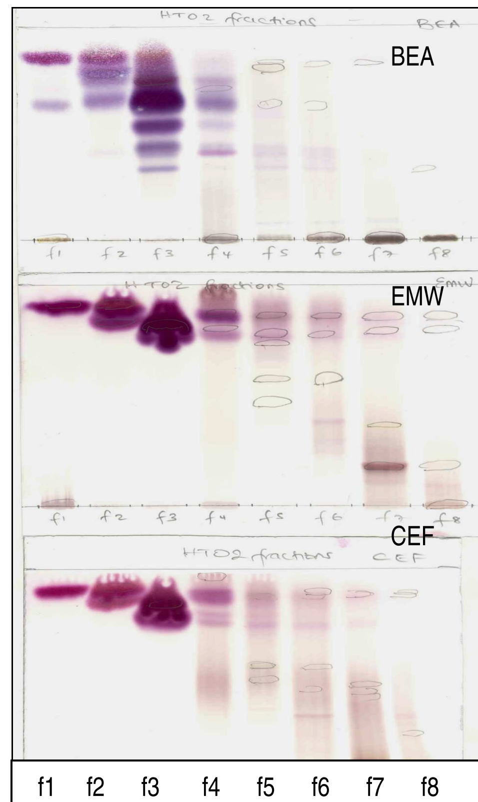

Figure 7-6: Chromatograms of fractions (F1 to F8) of HT02 developed in BEA, EMW and CEF and sprayed with vanillin sulphuric acid.

The microdilution assay of the fractions revealed that F3 was the most active fraction with MIC values of 0.04 and 0.08 mg/ml against *Fusarium oxysporum* and *Penicillium janthinellum* respectively followed by F2 with MIC values of 0.08 and 0.16 mg/ml against *Fusarium oxysporum* and *Penicillium janthinellum* respectively (Table 7.3). The least active fraction was F6 with MIC values of 0.63 and 1.25 mg/ml against *Fusarium oxysporum* and *Penicillium janthinellum* respectively. The same compounds appear to be bioactive against these two fungi.

Table 7-3: MIC values (mg/ml) of fractions of HT02.

Fractions	*Penicillium janthinellum*	*Fusarium oxysporum*
F1	0.31	0.31
F2	0.08	0.16
F3	0.04	0.08
F4	0.63	1.25
F5	1.25	1.25
F6	0.63	2.5
F7	0.31	0.31
F8	0.16	0.63

There were two active compounds present in F3 and one active compound present in F2 as determined using bioautography. The fractions F7 and F8 contained very highly polar compounds that had little antifungal activity (Figures 7-7 and 7-8).

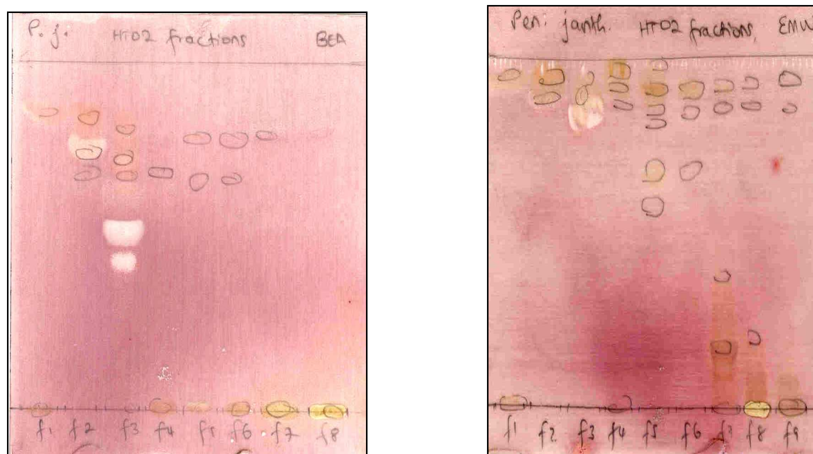

Figure 7-7: Bioautogram of fractions of HT02 developed in BEA (left) and EMW (right) and sprayed with *Penicillium janthinellum*.

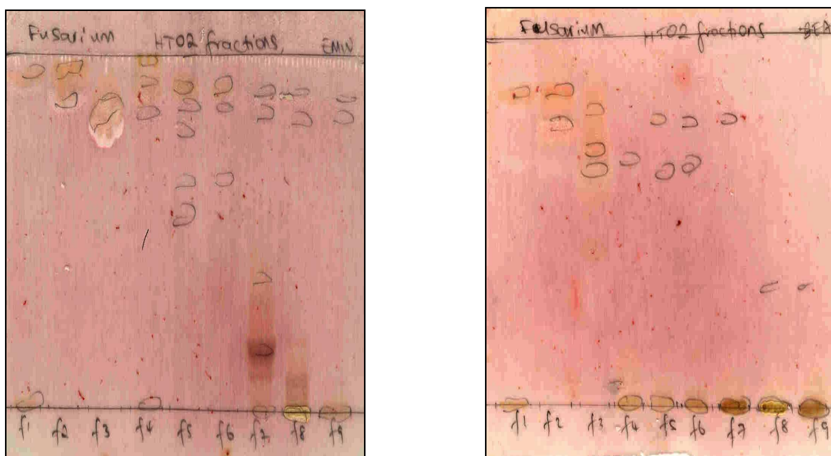

Figure 7-8: Bioautogram of fractions of HT02 developed in EMW (left) and BEA (right) and sprayed with *Fusarium oxysporum*.

The most active fractions F2 (average MIC of 0.12 mg/ml) and F3 (average MIC of 0.06 mg/ml) were combined, and the inactive fractions F1, F4, F5, F6, F7 and F8 were combined. The average MIC value of the combined active fractions was 0.04 mg/ml against *Fusarium oxysporum* and that of the combined inactive fractions was 0.63 mg/ml. The antifungal fractions had no apparent cytotoxicity on the Vero cell lines. The antifungal inactive fractions had very high cytotoxicity on the cells with LC_{50} of 0.0261 mg/ml (Figure 7-9). The inactive fractions had more cytotoxic effect than the HT02 (average LC_{50} of 0.0445 mg/ml as determined earlier in section 5.3.2) extract probably because the concentration of the cardiac glycosides was higher in the inactive fractions than in HT02.

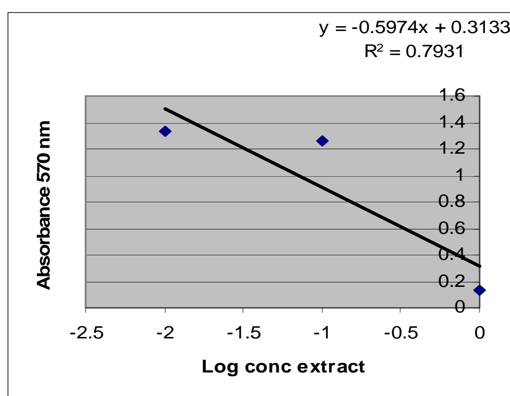

Figure 7-9: Cytotoxicity of inactive fractions.

7.4 Conclusion

The leaf material of *Melianthus comosus* contained five cardiac glycosides separated by TLC in the EMW solvent system. The Pb-washed acetone extract still contained 3 of the 5 cardiac glycosides. However, exhaustive lead precipitation of the HT plant resulted in all the cardiac glycosides being removed. There was a major disadvantage in using this method to remove the cardiac glycosides because the antifungal activity of the plant was reduced. The activity was reduced from MIC 0.08 mg/ml to 0.31 mg/ml against *Penicillium janthinellum.*

By calculating the activity volume as 1/MIC (personal communication, J.N. Eloff), whereas 1 mg of HT02 before treatment could be added to 12.5 ml of solvent and still inhibit fungal growth, after treatment the treatment could only be diluted to 3.2 ml and retain activity.

Using column fractionation of HT02, 8 fractions were obtained. The two most active fractions were combined and the inactive fractions combined separately. The combined active fractions had an average MIC value of 0.04 mg/ml and the combined inactive fractions an average MIC value of 0.63 mg/ml against *Fusarium oxysporum*. The active fractions combined showed no apparent cytotoxicity whereas the inactive fractions showed more cytotoxicity than HT02.

Therefore the best method to use in the removal of cardiac glycosides from the HT plant would be column fractionation and combination of the most active fractions. The inactive fractions were made up of highly polar compounds, probably containing cardiac glycosides. Fractions F7 and F8 contained highly polar compounds with little antifungal activity. The procedure may probably be too expensive to apply industrially. It is fortunate that the antifungal compounds are released relatively early. It may therefore be possible to use as batch process after refining the eluents used.

The R_f values of the cardiac glycosides (Table 7-1) and the antifungal compounds differ indicating that the antifungal compounds are not cardiac glycosides. The isolation of the antifungal compound(s) is discussed in the next chapter.

8. Chapter 8: Isolation of Bioactive Compound(s)

8.1 Introduction

The primary aim of isolation work was to isolate, determine the structure and quantify the activity of the major antifungal compound. Separation techniques leading to the isolation of bioactive compounds are important in the natural product isolation process. The complexity of a plant extract can be simplified through different separation techniques. In this chapter the use of direct extraction, serial exhaustive extraction and vacuum liquid chromatography (VLC) processes as important techniques in the preliminary separation and isolation of natural product compounds were applied.

Chromatography relies on the differential distribution of compounds between two phases, one of which moves relative to the other. These phases are called the mobile and stationary phase, respectively. The mobile phase is a fluid which can either be a liquid, a gas or a supercritical fluid. In this isolation process, a liquid mobile phase was used. The stationary phase is commonly a solid consisting of fine particles. Conventionally, the stationary phase of column chromatography consists of adsorbents of high polarity, e.g. silica gel. However, recent years have seen the introduction of many other forms of stationary phase, e.g. reverse-phase silica, ion-exchange resins, exclusion chromatography stationary phases (Houghton and Raman, 1998).
Elution with the mobile phase can be isocratic, where mobile phase of only one composition is used, or gradient. Gradient elution consists of a sequence of different compositions of the mobile phase, most commonly as a gradient of increasing (or, in the case of reverse-phase systems, decreasing) polarity (Houghton and Raman, 1998). The fractions collected from the column are analysed either chemically (TLC) or biologically (using a bioassay).

8.2 Materials and Methods

8.2.1 Extraction

8.2.1.1 Direct extraction

The direct extraction method of Eloff (1998a) was used for the purpose of isolation. One kilogram (1 kg) of finely ground leaves of *M. comosus* was extracted with 10 litres of acetone solvent. The

process was repeated on the marc with fresh solvent until there was no noticeable colour change in the newly prepared extract. The extract was filtered and dried under vacuum using a Büchi vacuum rotary evaporator at 45°C and weighed.

8.2.1.2 Serial exhaustive extraction (SEE)

The purpose of serial exhaustive extraction was to simplify extracts by fractionating the chemical compounds into broad groups based on their solubility. In preliminary serial exhaustive extraction studies 500 g of *M. comosus* leaves (dried and ground) were extracted serially with 5 litres each of hexane, dichloromethane, acetone and methanol. Extraction with each solvent was carried out five times on the marc or until there was no noticeable colour change in the newly prepared extract. For quantitative determination, extracts were placed in pre-weighed flasks and the solvents removed in a Büchi vacuum rotary evaporator. The extracts were later re-dissolved in acetone for further analysis.

8.2.2 TLC analysis of extracts

One hundred µg of each of the hexane, DCM, acetone and methanol extracts were loaded on TLC plates and developed in three solvent systems described in section 2.2.3. The plates were sprayed with vanillin-sulphuric acid reagent.

8.2.3 Biological assays of extracts

The microdilution assay method of Masoko et al. (2005) described in section 3.2.2.2 was used to determine the minimum inhibitory concentration (MIC) of the direct acetone extract and the SEE extracts. Bioautography was used to determine the active compounds and their R_f values.The test organisms used were *Phytophthora nicotiana* and *Penicillium expansum*.

8.2.4 Vacuum liquid column chromatography (VLC)

Fifty grams (50 g) of dried acetone extract from direct extraction of *M. comosus* was loaded on 2000 g of silica gel 60 in a 10 x 50 cm VLC column. TLC plates were spotted with 100 µg of acetone extract and developed in 5 solvent systems [hexane:ethylacetate (9:1), (8:2), (7:3), (6:4) and (5:5), v/v]. The solvent system that gave the best resolution of compounds was chosen as the

starting eluent. Eluents (Table 8-1) were applied in a stepwise gradient and sucked through by gravity. The top of the column was sealed tightly with parafilm wax and the tap was opened. The solvent was allowed to run through the column; until the 1000 ml of each eluent system had been collected in test tubes of 50 ml each. Each tube content was concentrated overnight under a stream of cold air. TLC analysis was then carried out on fractions collected. The contents of the tubes were spotted on TLC plates and developed. Tubes with similar contents were combined to form major fractions (F1-F9, Table 8-1).

Table 8-1: Gradients of solvent used in VLC for the separation of the acetone extract of *M. comosus.*

Eluent system	Hexane (ml)	Ethyl acetate (ml)	Quantity used (l)
A	70	30	11
B	60	40	7
C	50	50	8
D	40	60	5
E	30	70	3
F	20	80	4
G	10	90	4
H	0	100	11
	Ethyl acetate (ml)	**Methanol (ml)**	
I	90	10	5
J	80	20	4
K	50	50	4
M	0	100	3

A second column chromatography procedure was undertaken to separate further the compounds in F1. Twenty grams (20 g) of the fraction containing the active compound (F1) was loaded on a 10 x 30 cm column. Silica gel 60 (1000 g) was used to pack the column. The gradient elution was performed with hexane, DCM, ethyl acetate and acetone in different ratios of increasing polarity as shown in Table 8-2.

Table 8-2: Gradients of solvent used in VLC for the separation of F1.

Eluent system	Hexane (ml)	DCM (ml)	Quantity used (l)
A	80	20	9
B	70	30	5
C	60	40	5
D	50	50	4
E	30	70	4
F	0	100	5
	DCM (ml)	**Ethyl acetate (ml)**	
G	90	10	4
H	80	20	3
I	60	40	5
J	50	50	5
K	30	70	3
M	0	100	3
	Ethyl acetate (ml)	**Acetone (ml)**	
N	90	10	3
O	70	30	3
P	50	50	2
Q	30	70	2
R	0	100	3

Small fractions (30 ml) were collected into test tubes. The tube contents were spotted on TLC plates, developed and sprayed with vanillin-sulphuric acid to determine their chemical compositions. Tubes with similar composition were combined to make up pooled sub-fractions.

8.2.5 TLC analysis and bioassay of column fractions

The pooled fractions were tested for antifungal activity by bioautography to identify the fraction in which the active compounds were present. The test organism used was *Penicillium expansum* because it was the most sensitive organism and grows fast. Sub-fractions f3, f4, f5 and f6 were combined (SF1) following these results.

A third column was packed (5 cm x 60 cm) with 40 g of silica gel 60 (Merck) and 4 g of SF1 was further separated by gravity assisted column chromatography. Hexane: ethyl acetate (9:1, v/v) gave a good resolution of compounds on TLC and was used as the eluent. Small fractions (about 20 ml) were collected into test tubes. About 1500 test tubes were collected and placed in a fume cupboard under a stream of air to concentrate the fractions. TLC analysis of column fractions was carried out with the intention to combine fractions with similar compounds based on colour and R_f values. One of the fractions crystallized to afford a clean compound as determined by TLC analysis.

8.2.6 TLC analysis, bioassay and cytotoxicity of isolated compound

The isolated compound was dissolved in acetone to give a stock solution of 2 mg/ml. Ten µg of solution was spotted on TLC plates and developed in EMW, CEF, and hexane:ethyl acetate (7:3, v/v). Some of the plates were sprayed with vanillin-sulphuric acid and the rest were dried overnight for bioassay against *Colletotrichum gloesporioides, Phytophthora nicotiana, Penicillium expansum* and *Aspergillus parasiticus.*

The isolated compound was dissolved in acetone to give a stock solution of 0.5 mg/ml. This was used in the microtitre assay method of Masoko et al. (2005) against *Colletotrichum gloeosporioides, Phytophthora nicotiana* and *Aspergillus parasiticus*. The antibacterial activity of compound was also tested against *Staphyloccocus aureus* (ATCC 29213), *Enteroccocus faecalis* (ATCC 29212), *Escherischia coli* (ATCC 25922) and *Pseudomonas aeruginosa* (ATCC 27853). The microplate method of Eloff (1998c) was used. The antibacterial assay was carried out to confirm the antifungal activity of the compound.

The isolated compound was dissolved in acetone to give a stock solution of 10 mg/ml and tested for cytotoxicity against monkey kidney (Vero) cells.

8.2.7 Spectroscopic analysis of isolated compound

Twenty milligrams (20 mg) of the isolated compound was dissolved in 2 ml of deuterated acetone. The nuclear magnetic resonance (NMR) experiments (^{13}C and ^{1}H) were carried out at the Regional Analytical Centre at the University of Johannesburg (RAU). This was done using a Varian Unity

Innova 300 MHz NMR system. The same sample was used for mass spectrometry, Heteronuclear Multiple Bond Connectivity (HMBC), and Heteronuclear Single Quantum Coherence (HSQC) experiments. The mass spectrum was recorded on an analytical VG 7070E mass spectrometer using electron impact at 70 eV as ionization technique.

8.3 Results and Discussion

8.3.1 Extraction results

One hundred and ninety one grams (191 g) of extract was obtained from the direct extraction of 1000 g of *M. comosus* with acetone. The results of the serial exhaustive extraction are presented in Figure 8-1 below. Hexane extracted the most compounds from the plant material (20.475 g) indicating a high concentration of non-polar components, followed by DCM (9.230 g), acetone (6.429 g) and methanol which extracted the least (5.050 g). All the solvents extracted more material in the first extraction than in the subsequent extractions.

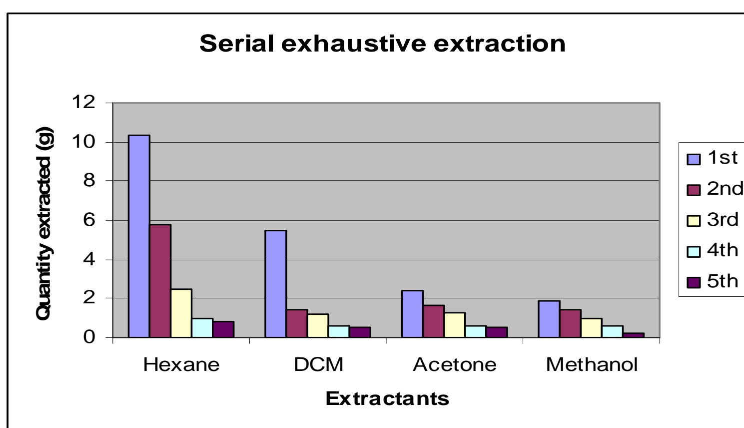

Figure 8-1: Quantity of material extracted from the first (1st), second (2nd), third (3rd), fourth (4th) and fifth extractions from 500 g of *M. comosus* leaves by hexane, dichloromethane (DCM), acetone and methanol in serial exhaustive extraction.

8.3.2 TLC analysis of extracts

The DCM extract had the highest number of compounds visible on TLC plates after spraying with vanillin spray reagent and had a relatively complex chemical composition as compared to the other

extracts. The hexane extract consisted of mainly non-polar compounds whereas the methanol extract consisted of highly polar compounds that could hardly move from the baseline in the solvent systems used.

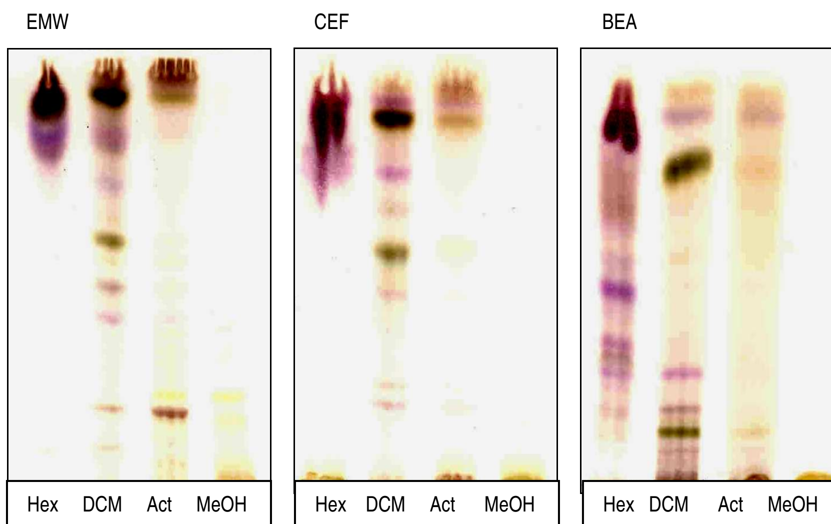

Figure 8-2: TLC profiles of extracts from serial exhaustive extraction (SEE) developed in EMW (left), CEF (middle) and BEA (right) solvent systems and sprayed with vanillin-sulphuric acid. **Lanes from left to right:** hexane (Hex), dichloromethane (DCM), acetone (Act) and methanol (Met).

8.3.3 Biological assays of extracts

The acetone extract from direct extraction was the most active extract with MIC values of 0.08 and 0.31 mg/ml against *Phythophthora nicotiana* and *Penicillium expansum* respectively (Table 8-3). This confirms earlier results and justifies the reason why the acetone extract was chosen for potentising. The DCM extract was less active than the acetone extract but more active than all the rest (MIC of 0.31 and 0.31 mg/ml against *Phythophthora nicotiana* and *Penicillium expansum* respectively).

Table 8-3: MIC values of acetone extract from direct extraction (DE) and hexane, DCM, acetone and methanol extracts from serial exhaustive extraction (SEE); against *Phythophthora nicotiana* and *Penicillium expansum*.

Extracts	MIC values (mg/ml)	
	Phythophthora nicotiana	***Penicillium expansum***
Acetone(DE)	0.08	0.31
Hexane	1.25	2.5
DCM	0.31	0.31
Acetone (SEE)	0.31	0.63
Methanol	1.25	0.63

As shown in bioautography, the direct acetone extract contained two active compounds of R_f 0.33 and 0.81 in BEA (Figure 8-3) comfirming the results obtained earlier in section 3.3.2.

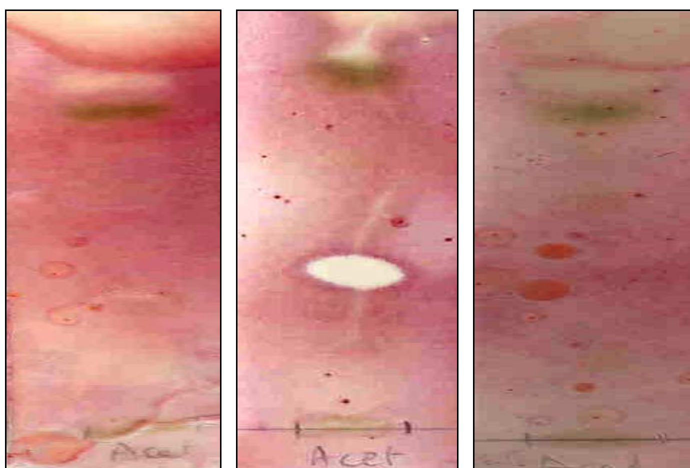

Figure 8-3: Bioautogram of acetone extract of *Melianthus comosus* developed in BEA sprayed with *Penicillium expansum* (left), *Fusarium oxysporum* (middle) and *Phytophthora nicotiana* (right). Plates were incubated at 37°C for 24 hours and sprayed with INT.

The SEE extracts were not as active as the acetone extract from DE with respect to the number of active compounds on the bioautogram.This is because in SEE, acetone was used to extract from the marc that had been exhaustively extracted with hexane and then dichloromethane. Acetone extracts few compounds from the marc unlike direct extraction with acetone on the powdered plant material. The hexane extract had one active compound as did the DCM extract (Figure 8-4) in the

EMW solvent sytem. The acetone and methanol extracts had no active compounds against *Phytophthora nicotiana* and *Penicillium expansum* (Figure 8-4), under the conditions used.

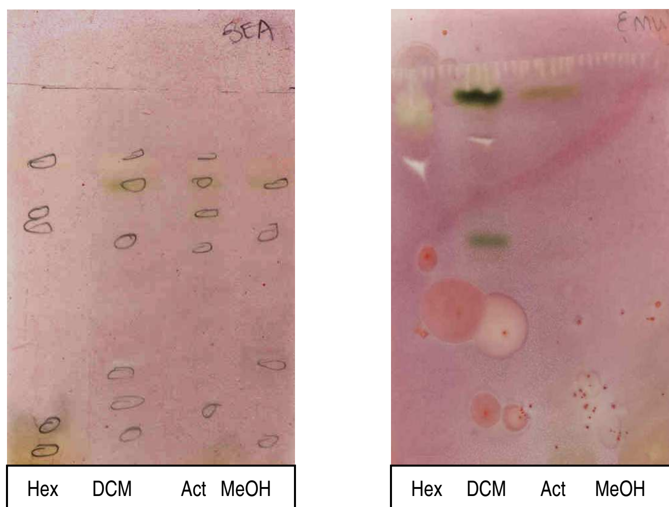

Figure 8-4: Bioautogram of extracts of *Melianthus comosus* developed in BEA, sprayed with *Phytophthora nicotiana* (left) and sprayed with *Penicillium expansum* (right). **Lanes from left to right:** hexane (Hex), dichloromethane (DCM), acetone (Act) and methanol (MeOH).

8.3.4 TLC analysis of column fractions

Hexane:ethyl acetate (7:3, v/v) indicated the best separation and was chosen to start the gradient elution (Figure 8-5) for the first column.

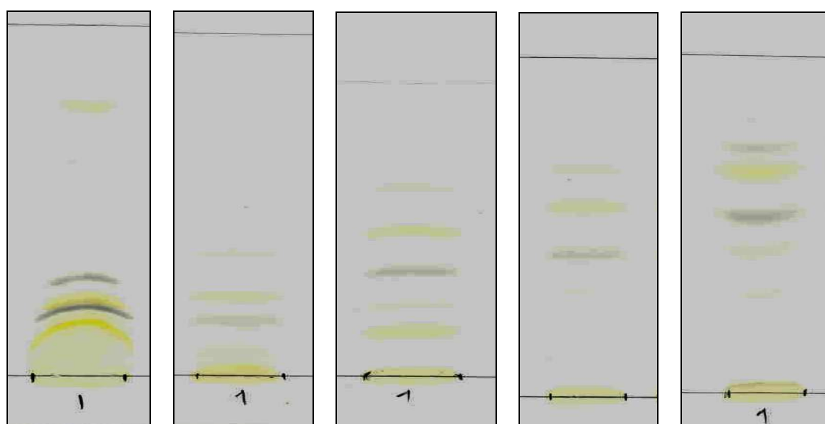

Figure 8-5: TLC profiles of acetone extract developed in hexane:ethyl acetate 9:1, 8:2, 7:3, 6:4, 5:5 solvent systems from left to right.

Nine fractions were obtained from the VLC using 50 g of acetone extract. The mass of the fractions are shown in Table 8-4 and their chemical compositions are shown in Figure 8-6.

Table 8-4: Mass of fractions F1-F9 of acetone extract.

Fractions	Mass of fractions (g)
F1	20.200
F2	2.414
F3	1.584
F4	0.531
F5	6.233
F6	8.902
F7	3.286
F8	2.186
F9	3.155

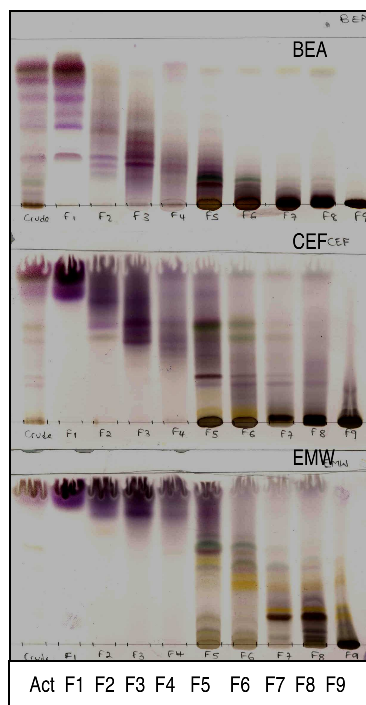

Figure 8-6: TLC profiles of fractions of acetone by VLC. TLC plates developed in BEA (top), CEF (middle), and EMW (bottom) and sprayed with vanillin-sulphuric acid. **Lanes from left to right:** crude extract, F1, F2, F3, F4, F5, F6, F7, F8 and F9.

The contents of the tubes collected from F1 contained many non-polar compounds and most of the compounds appeared purple when sprayed with vanillin-sulphuric acid suggesting the presence of terpenes (Wagner and Bladt, 1996).

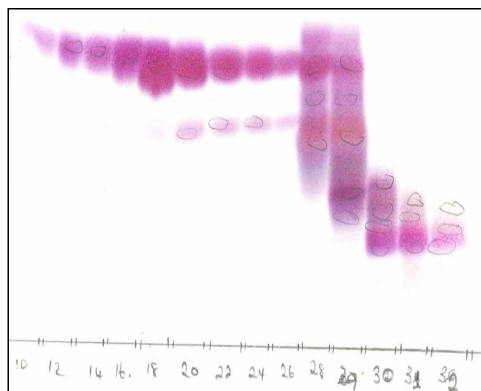

Figure 8-7: TLC profiles of the tubes (10-32) obtained from column separation of F1. TLC plate developed in hexane:ethyl acetate (85:15) and sprayed with vanillin-sulphuric acid.

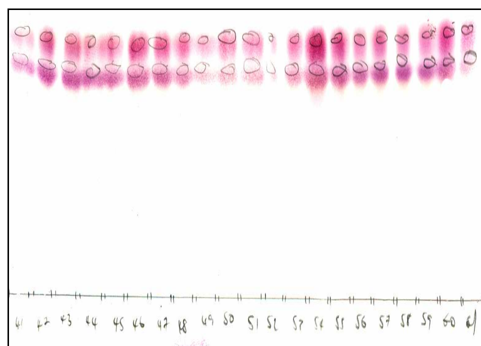

Figure 8-8: TLC profiles of the tubes (41-61) obtained from column separation of F1. TLC plate developed in BEA and sprayed with vanillin-sulphuric acid.

F1 was further fractionated and six sub-fractions were collected (f1-f6). Table 8-5 shows the mass of fractions.

Table 8-5: Mass of fractions f1-f6 of F1.

Fractions	Mass of fractions (g)
f1	3.212
f2	2.332
f3	3.431
f4	1.203
f5	4.318
f6	0.735

8.3.5 Bioassay of column fractions

The main active compound was found in fraction F1 (Figure 8-9).

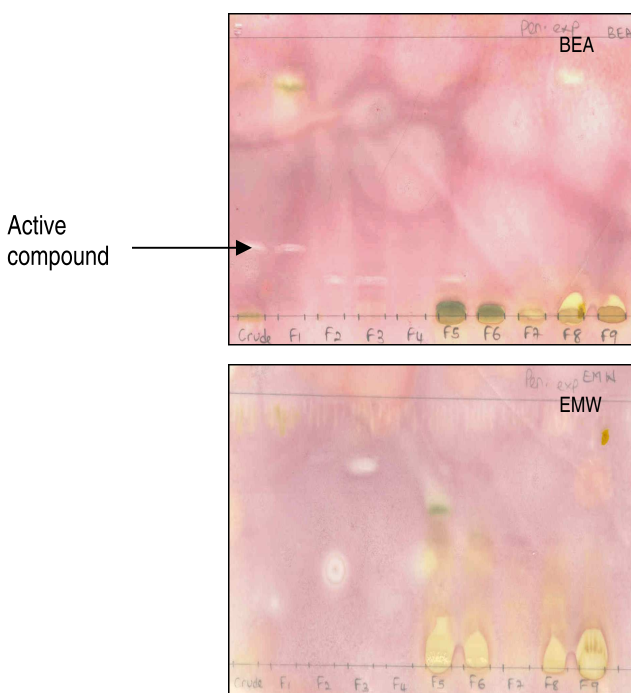

Figure 8-9: Bioautogram of fractions of crude acetone extract of M. comosus. TLC plate developed in EMW (below) and BEA (above) and sprayed with *Penicillium expansum*. **Lanes from left to right:** crude, F1, F2, F3, F4, F5, F6, F7, F8, and F9.

Six sub-fractions were obtained after combining contents of tubes. The chemical compositions of these sub-fractions (f1, f2, f3, f4, f5 and f6) are shown in Figure 8-10.

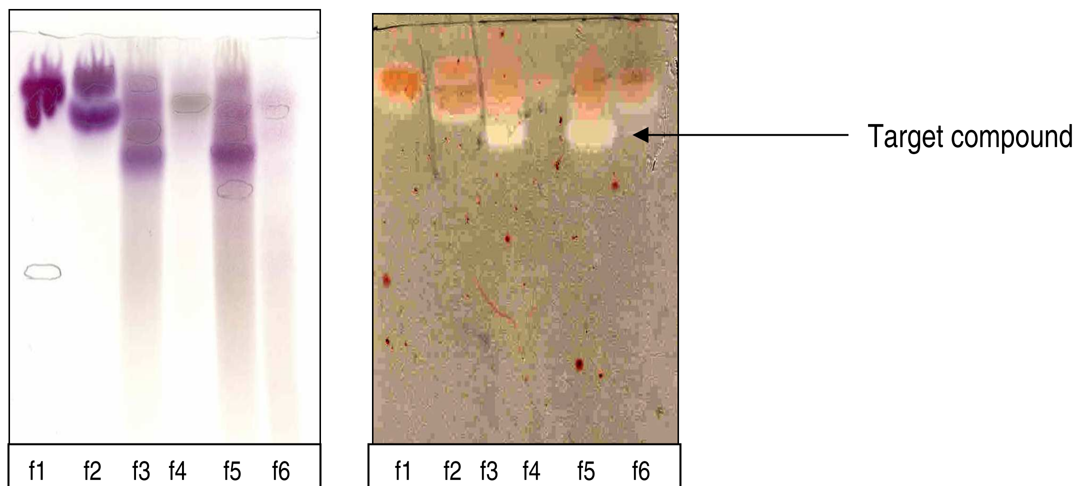

Figure 8-10: TLC profiles (left) and bioautogram (right) of sub-fractions of F1. TLC plate developed in EMW and sprayed with vanillin-sulphuric acid and *Penicillium expansum* respectively. Lanes from left to right: f1, f2, f3, f4, f5 and f6.

Sub-fractions f3 and f5 contained the target compound (indicated by arrow in Figure 8-10) and were combined to form SF1. Column chromatography of SF1 led to very simplified fractions (Figure 8-11) and eventually to the target compound (Figure 8-12).

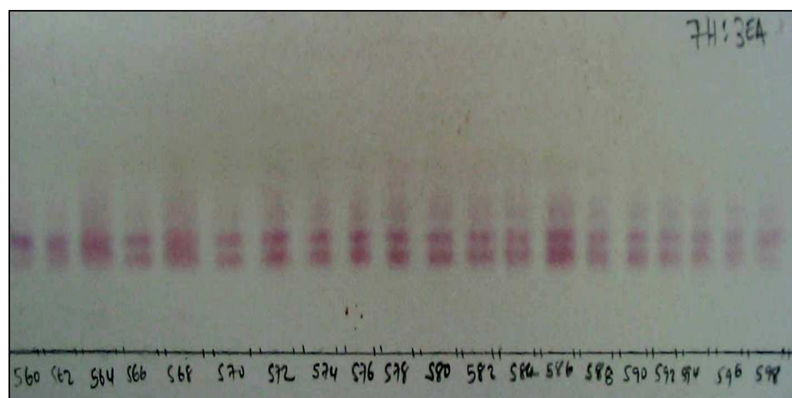

Figure 8-11: TLC profiles of tubes of SF1 (560-598). TLC plate developed in hexane:ethyl acetate (7:3) and sprayed with vanillin-sulphuric acid.

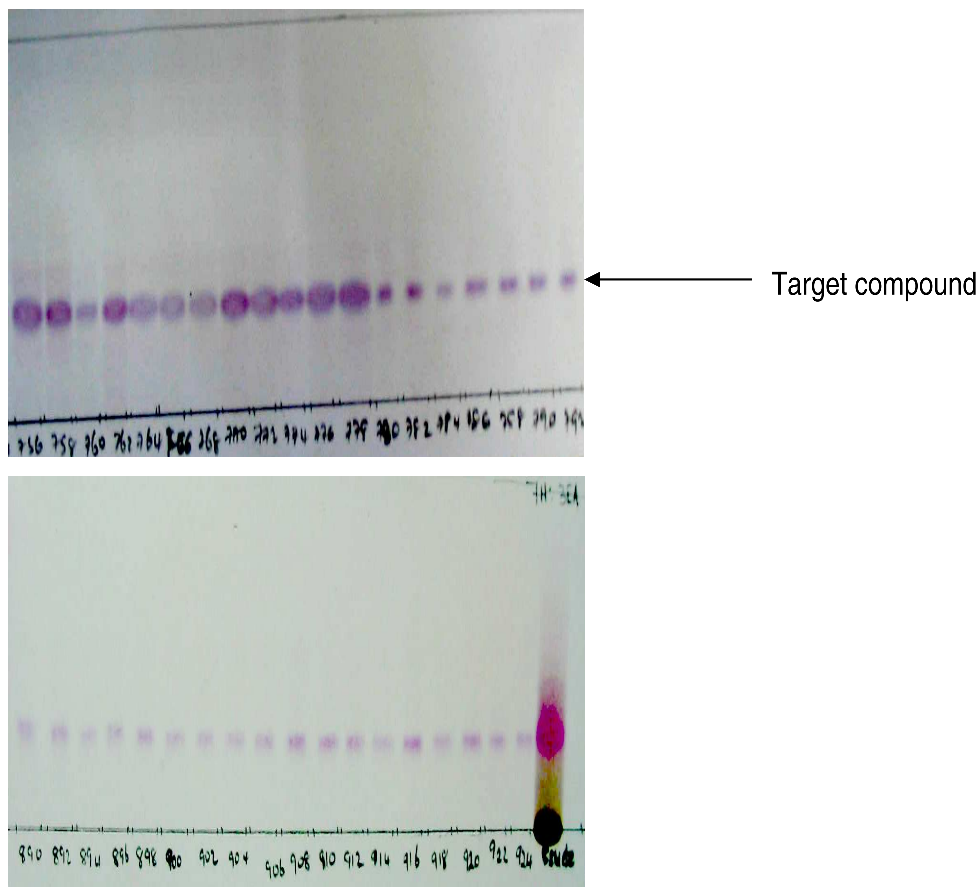

Figure 8-12: TLC profiles of tubes of SF1 (tubes 756-792 above; tubes 890-924 and crude fraction below). TLC plate developed in hexane:ethyl acetate (7:3) and sprayed with anisaldehyde.

The target compound was found from tube 756 to tube 792. The compound appeared clean from tube 780 to tube 850. Contents of these test tubes were combined and the volume of the solvent reduced with the rotary evaporator. Hot hexane (1 ml, 50°C) was added to the concentrated compound and allowed to cool down at room temperature. The target compound (76 mg, 0.2% yield) subsequently crystallized from hexane.

8.6 TLC analysis and bioassay of isolated compound

The compound appeared purple when sprayed with vanillin-sulphuric acid, hence possibly indicating a terpene. The compound appeared to be pure with no contaminants visible when

developed in different TLC systems. The R_f values of the compound were 0.71 in CEF, 0.44 in hexane: ethyl acetate (7:3, v/v) and 0.81 in EMW.

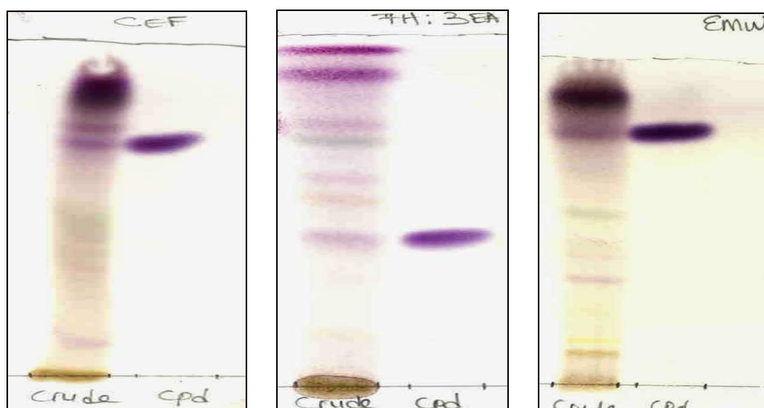

Figure 8-13: TLC profiles of the isolated compound developed in CEF (left), hexane:ethyl acetate [(7:3), middle], EMW (right) and sprayed with vanillin-sulphuric acid.

The compound was active against *Colletotrichum gloesporioides, Phytophthora nicotiana* and *Aspergillus parasiticus* (Figure 8-14) when bioautography was performed.

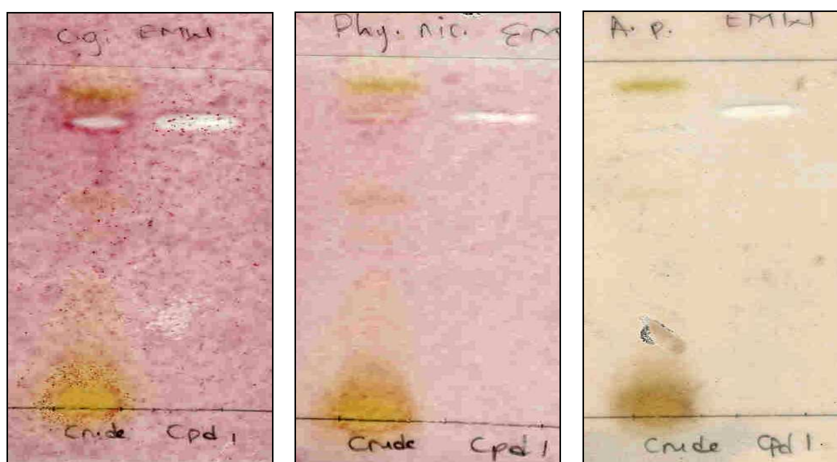

Figure 8-14: Bioautogram of acetone crude and compound against from left to right: *Colletotrichum gloeosporioides, Phytophthora nicotiana* and *Aspergillus parasiticus.*

The compound had high antifungal activity with MIC values ranging from 7.8 to 15.6 µg/ml (Table 8-6). The compound was less active against bacteria (*Staphyloccocus aureus*, *Enteroccocus*

faecalis, *Escherichia coli* and *Pseudomonas aeruginosa*) with MIC values ranging from 31.3 to 125 µg/ml (Table 8-6).

Table 8-6: MIC values (µg/ml) of isolated compound in triplicate (1, 2, 3) against *Colletotrichum gloeosporioides* (C.g.), *Phytophthora nicotiana* (P.n.), *Penicillium expansum* (P.e.), *Aspergillus parasiticus* (A.p.), *Staphyloccocus aureus* (S.a.), *Enteroccocus faecalis* (E.f.), *Escherichia coli* (E.c.) and *Pseudomonas aeruginosa* (P.a.).

	MIC values (µg/ml)				
Organisms	**1st MIC**	**2nd MIC**	**3rd MIC**	**SD**	**Average**
			Fungi		
C.g.	15.6	15.6	15.6	0	15.6
P.n.	7.8	7.8	7.8	0	7.8
P.e.	15.6	15.6	15.6	0	15.6
A.p.	7.8	7.8	7.8	0	7.8
			Bacteria		
S.a.	62.5	62.5	62.5	0	62.5
E.f.	125	125	125	0	125
E.c.	31.3	31.3	31.3	0	31.3
P.a.	31.3	31.3	31.3	0	31.3

The isolated compound had a low cytotoxicity on the Vero cell lines. At the highest concentration tested (1 mg/ml), no toxic effects on the cells were noted.

8.7 Spectroscopic analysis of isolated compound

The compound had a basic triterpenoid skeleton (Figure 8-15).

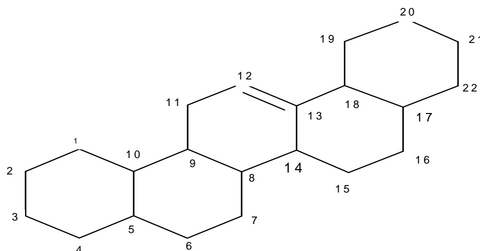

Figure 8-15: Skeletal unit of olean-12-ene type of pentacyclic triterpenoid isolated from *M. comosus.*

Compound **1** crystallized from hexane in most of the fractions from the third column and was isolated in large quantity and appeared to be the major constituent in the leaves of *M. comosus*. NMR spectra were suggestive of an oleanen-12 skeleton with a carboxylic function, a trisubstituted double bond, carbon bearing OH bond and an AB system, suggesting a similarity in the chemical structure in the main skeletal unit (Figure 8.15) modified by the various constituents.

MS analysis of compound **1** (Figure 8-21) gave a molecular formula of $C_{30}H_{48}O_3$ (m/z 456) and characteristic peak at m/z 248 $(M-C_{14}H_{24}O_2)^+$. This structural type was further supported by the indication of seven degrees of unsaturation and contained resonance for seven skeletal methyl groups and a broad triplet at δ_H 5.22 (Figure 8-17) for olefenic proton (H-12), carboxylic acid functionality (δ_c 183.4, Figure 8-18) and one hydroxyl group (δ_H 3.6, δ_c 79.1). The ^{13}C NMR data of compound **1** are given in Table 8-5 indicating the presence of a hydroxyl carbon at $\delta_{c,}$ 79.1 and total number of carbons to be 30. The assignment presented resulted from a combination of HSQC (Figure 8-19) and HMBC (Figure 8-20) experiments. In the HMBC, proton H-29 (δ_H 0.75) correlates with C-20 (δ_C 30.7) and also proton H-30 (δ_H 0.9) correlates with C-20 (δ 30.7) indicating that the carboxylic acid functionality which is not seen in the HMBC does not appear at C-30 and hence should be at C-28.

Using this information, along with the HSQC, HMBC data and literature comparison, the structure of compound **1** which was suggestive of a 3-hydroxy-12-oleanen-28-oic acid, corresponded to oleanolic acid previously isolated by Anderson and Koekemoer (1968) from the rootbark of *Melianthus comosus.* Oleanolic acid is widely spread in plants and has been reported to have antiallergic, antiatherosclerotic, antibacterial (MIC=625-1,250 µg/ml), anticarciogenic, anticomplement, antiedemic, antifertility, antigingivitic, antiHIV, anti-inflammatory, antimalarial, antiviral, antioxidant, antiseptic, antisarcomic, antiplaque, antiplasmodial, antitumor and antiulcer properties (Dr. Duke's Phytochemical and Ethnobotanical Databases)

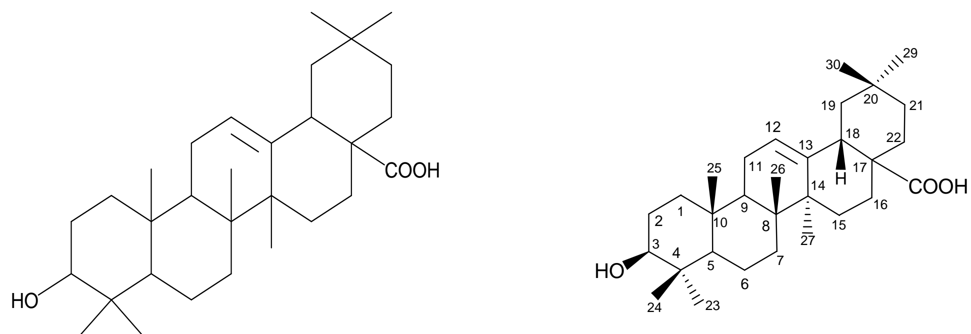

Figure 8-16: Structure of compound 1: 3-hydroxy-12-oleanen-28-oic acid (oleanolic acid).

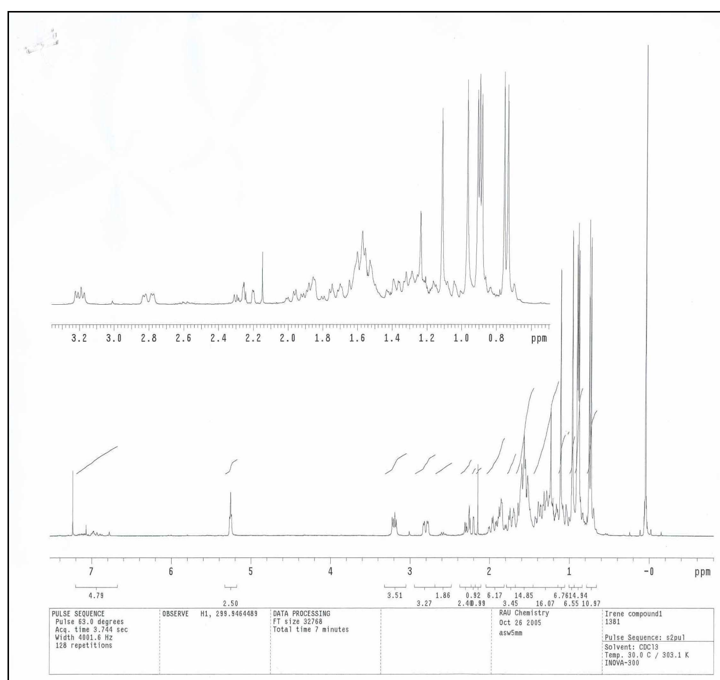

Figure 8-17: ¹H-NMR spectrum of compound 1

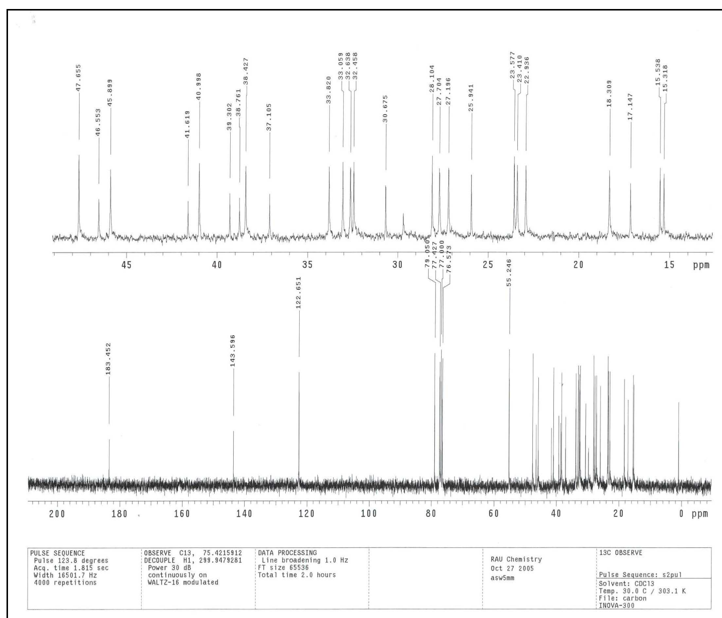

Figure 8-18: ^{13}C-NMR spectrum of compound 1

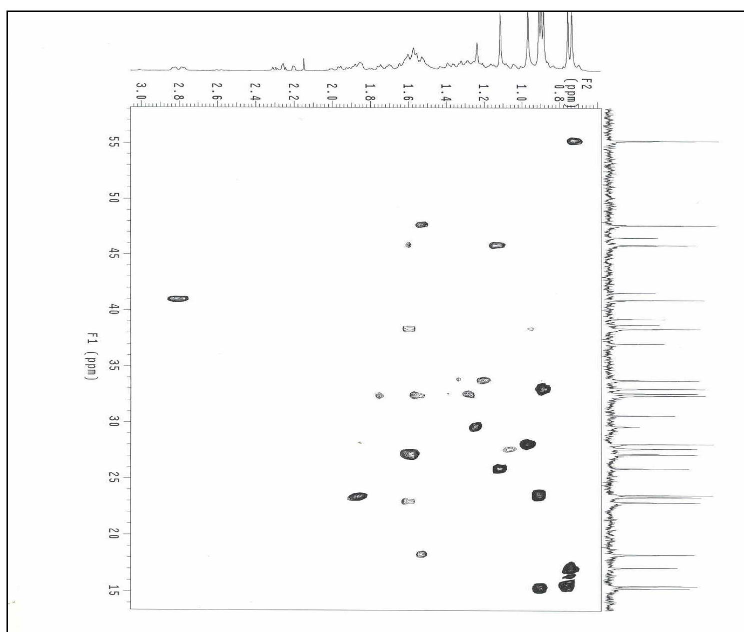

Figure 8-19: HSQC spectrum of compound 1

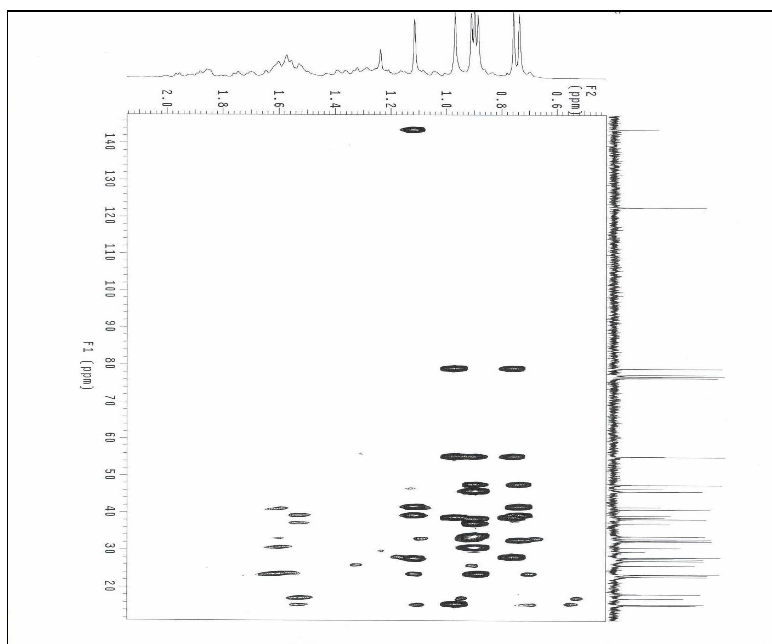

Figure 8-20: HMBC spectrum of compound 1

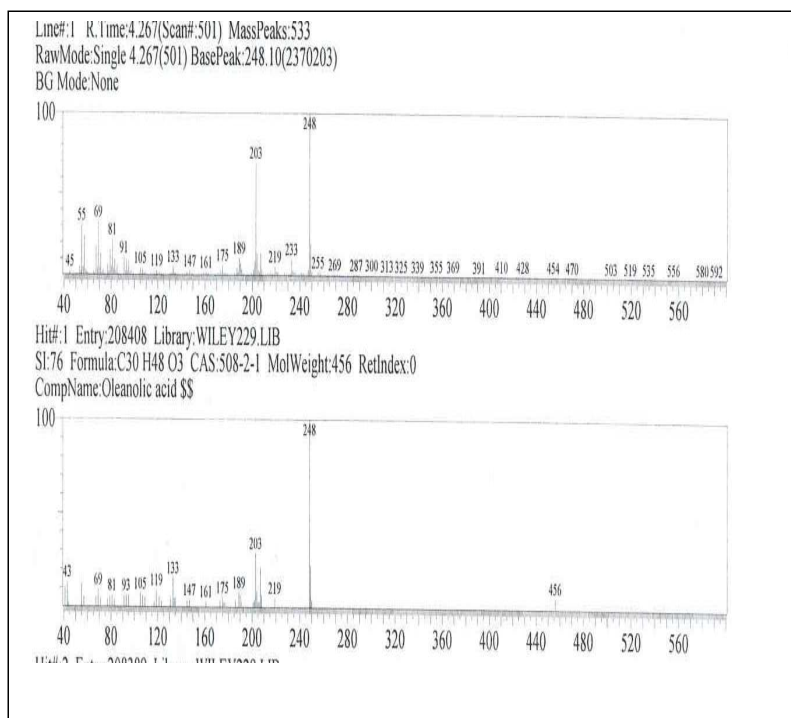

Figure 8-21: Mass spectrum of compound 1

Table 8-7: NMR data of compound 1

Carbon	Shift (ppm)
C-1	38.7
C-2	27.7
C-3	79.1
C-4	38.7
C-5	55.2
C-6	18.3
C-7	32.5
C-8	39.3
C-9	47.7
C-10	37.1
C-11	23.0
C-12	122.7
C-13	143.6
C-14	41.6
C-15	27.7
C-16	23.4
C-17	46.6
C-18	41.6
C-19	45.9
C-20	30.7
C-21	33.8
C-22	32.5
C-23	28.1
C-24	15.5
C-25	15.3
C-26	17.1
C-27	26.0
C-28	183.4
C-29	33.1
C-30	23.6

All measured in *d*-Acetone relative to TMS.

8.8 Conclusion

The major antifungal compound in *Melianthus comosus* leaves was found in the acetone extract from DE (direct extraction) with R_f values of 0.33 in BEA and 0.81 in EMW. Gradient vacuum liquid chromatography, as well as gravity assisted column chromatography was used in fractionating the acetone extract to obtain simplified fractions. Nine fractions were obtained. The active fractions with similar compounds were combined and further fractionated to obtain 6 smaller fractions. The major active compound was identified by TLC in two of these fractions. These fractions were combined and fractionated which led to the isolation of the major active compound (76 mg). The compound was identified as 3-hydroxy-oleanen-30-oic acid (oleanolic acid), a triterpene. The compound had high antifungal activity ranging from 7.8 µg/ml against *Phytophthora nicotiana* and *Aspergillus parasiticus* to 15.6 µg/ml against *Colletotrichum gloeosporioides* and *Penicillium expansum*. This compound showed no apparent cytotoxicity on Vero cell lines. Oleanolic acid was previously isolated by Anderson and Koekemoer (1968) from the rootbark of *Melianthus comosus.* It has not been isolated from the leaves of *Melianthus comosus* before. Oleanolic acid is widely spread in plants and its biological activities have been reported (Dr. Duke's Phytochemical and Ethnobotanical Databases). The antifungal activity of *Melianthus comosus* leaf extracts against plant fungal pathogens is reported here for the first time.

9. Chapter 9: General conclusion

The leaves of *Melianthus comosus* were brought to the Phytomedicine laboratory by Healthtech Laboratories Pty (Ltd) to test for antibacterial activity. The antibacterial activity was not impressive. We then tested the antifungal activity on animal fungal pathogens. Due to potential toxicity to humans and animals we decided to investigate the activity against plant fungal pathogens. Healthtech Laboratories sponsored further research to develop an antifungal-rich extract that could be marketed as an organic fungicide to protect plants and plant products. Ten plant pathogenic fungi were used in the biological assays: *Rhizoctonia solani, Fusarium oxysporum, Penicillium janthinellum, Penicillium expansum, Colletotrichum glocosporioides, Trichoderma harzianum, Pythium ultimum, Phytophthora nicotiana, Aspergillus niger,* and *Aspergillus parasiticu*s.

Extractants with different polarities were used to extract from the plant: hexane, carbon tetrachloride, diethyl ether, dichloromethane, chloroform, acetone, ethanol, ethyl acetate, methanol and water. These solvents have different polarities and fall under different selectivity groups hence will extract a wide range of compounds from the plant. The direct extraction method of Eloff (1998a) was used with a ratio of 1 g :10 ml (plant material:extractant). The extraction results showed that acetone and chloroform extracted more compounds (13) than the rest of the solvents. Methanol and water extracted more sugars, amino acids and glycosides from the plant material (20.7 % and 11% respectively) than the other solvents. The fact that more polar solvents like ethanol, ethyl acetate, dichloromethane, chloroform and acetone extracted similar concentrations of non-polar compounds as did hexane suggested the presence of saponin compounds with polar and non-polar ends, which solubilize in either polar or non-polar solvents.

By bioautography two active compounds were demonstrated in the acetone extract; the compound with an R_f value of 0.33 in BEA was a major active compound and that with an R_f value of 0.86 in BEA was a minor active compound based on their band sizes on TLC. Acetone and ethanol extracts had a similar active compound of R_f 0.33 in BEA. The compound was present in a smaller quantity in the ethanol extract than in the acetone extract. With its high antifungal activity, the acetone extract was chosen as the extract to be further developed.

Two pathways were used for the enrichment of the acetone extract. Pathway 1 involved the use of selective extraction while pathway 2 involved the use of solvent-solvent fractionation. Pathway 1 led to the development of HT01 while pathway 2 led to the development of HT02. HT01 was obtained by first extracting the plant material with water three times, and then extracting the dried marc with DCM once and finally extracting the dried marc with acetone three times. HT01 had an average MIC of 0.088 mg/ml against all 10 fungal organisms. HT02 was obtained by extracting the plant material with acetone three times; this acetone extract was dried and used in solvent-solvent fractionation with water and ethyl acetate. HT02 was more active than HT01 with an average MIC of 0.066 mg/ml against all 10 organisms. Hence HT02 was chosen as the new product for application on plants and plant products. The *in vitro* antifungal activity of HT02 was compared to that of 6 commercial fungicides (triforine, triazole, dicarboximide, chlorothalonil, tebuconazole and copper oxychloride). Copper oxychloride was the most active followed by HT02; tebuconazole was the least active.

The LC_{50} of HT02 was 4.5 mg/ml using the brine shrimp assay. HT02 was an antifungal extract with an average MIC of 0.066 mg/ml against all 10 organisms. An average LC_{50} of HT02 was 0.0445 mg/ml using the MTT cell-line cytotoxicity assay which was in all likelihood due to the presence of cardiac glycosides in the plant.

Based on the colour reaction with Kedde reagent five cardiac glycosides with R_f values of 0, 0.08, 0.23, 0.34 and 0.58 in EMW TLC separation were identified in the plant extract and an attempt was made to remove these to reduce the toxicity of HT02. Three-fold exhaustive lead precipitation of the extract resulted in all the cardiac glycosides being removed from the extract. There was a major disadvantage in using this method to remove the cardiac glycosides because the antifungal activity of the plant was reduced. The activity was reduced by nearly 75% from MIC 0.08 mg/ml to 0.31 mg/ml against *Penicillium janthinellum.* Using column fractionation of HT02, 8 fractions were obtained; the two most active fractions were combined. The antifungal fractions (average MIC of 0.09 mg/ml) combined showed no apparent cytotoxicity whereas the inactive fractions combined showed cytotoxicity. Therefore the best method to use in the removal of cardiac glycosides from the HT02 was column fractionation and combination of the most active fractions.

The major active compound was found in the acetone extract from direct extraction with R_f values of 0.33 in BEA and 0.81 in EMW. Gradient vacuum liquid chromatography, as well as gravity assisted column chromatography was used in fractionating the acetone extract to obtain simplified fractions. Bioassay-guided fractionation of the acetone extract led to the isolation of the major active compound (76 mg). The compound was identified as 3-hydroxy-13-oleanen-28-oic acid (oleanolic acid), a triterpenoid acid previously isolated by Anderson and Koekemoer (1968) from the rootbark of *Melianthus comosus*. The compound had high antifungal activity ranging from 7.8 µg/ml against *Phytophthora nicotiana* and *Aspergillus parasiticus* to 15.6 µg/ml against *Colletotrichum gloeosporiodes* and *Penicillium expansum*. This compound showed no apparent cytotoxicity on Vero cell lines at a concentration of 1 mg/ml. The antifungal activity of this compound against plant pathogenic fungi is reported here for the first time.

The aim of this research was to develop an antifungal extract with high activity which will be able to compete with products already in the market. This aim was achieved successfully as the HT02 extract was developed with an antifungal activity of 0.066 mg/ml and has been patented and licensed to the Healthtech Laboratories for marketing. The yield of HT02 from dried, powdered leaf material was 3.1%.

Whilst the field trial conducted was extremely limited, the results indicated that the HT02 extract was more effective than a widely used commercial product at a quarter of the dose. Larger scale field trials have to be carried out on other crops to confirm these findings. This is outside the scope of the present study.

Future tasks will also include investigating problems that may arise in the process of up-scaling the extraction procedures. For quality control, the HT02 extract could be characterized using TLC or HPLC t determine the levels of active and toxic compounds present. The stability of the extract on crops under natural conditions as well as the toxicity of the extract against animals will also have to be tested.

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