

**MEDICINAL PROPERTIES AND
GROWTH OF
*MERWILLA NATALENSIS***

By

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DECLARATION

I hereby declare that the work described in this thesis, unless otherwise acknowledged to the contrary in the text, is the result of my own investigation, under the supervision of Professor J. van Staden and Professor A.K. Jäger, in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal, Pietermaitzburg.

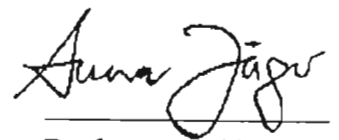


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We that declare the above statement is correct.



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ABSTRACT

Merwillia natalensis (Planchon) Speta is ranked as one of the most commonly sold medicinal plants at most of the informal medicinal plant markets found throughout South Africa. The increasing demand for medicinal plants has resulted in over-exploitation of many of the wild populations. Overharvesting has resulted in *M. natalensis* being declared vulnerable. Although this species is so popular, and reports state that the bulbs are used for a variety of ailments, very little is known about its pharmacological activity or phytochemical composition.

Extracts were made from mature *M. natalensis* bulbs using hexane, dichloromethane, methanol and water. These extracts were screened for antibacterial, anticancer, anti-inflammatory, antischistosomal and anthelmintic activity. Antibacterial activity was evaluated using the minimal inhibitory concentration (MIC) assay. Methanol extracts displayed good antibacterial activity against both Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria. Anti-inflammatory activity was evaluated using the COX-1 and COX-2 bioassays. Dichloromethane extracts displayed the highest inhibitory activity against both COX-1 and -2 enzymes. (80% and 91% inhibition respectively) Very good activity was displayed against the free-living nematode *Caenorhabditis elegans* and the schistosome worms of *Schistosoma haematobium* using microdilution techniques. Anticancer activity was evaluated using the biochemical induction assay (BIA) in which DNA-damaging properties are tested for. No activity was found using this assay, however, these results do not prove that *M. natalensis* does not have other anticancer properties.

The phytochemical investigation of mature *M. natalensis* plants showed the bulbs to contain both saponins and bufadienolides. One of the bufadienolides had the same R_f value as proscillaridin A. Cytotoxicity tests reveal *M. natalensis* to be extremely cytotoxic, yet the bulbs are commonly sold at traditional medicine markets around South Africa. This cytotoxicity may be accredited to the presence of saponins within the bulbs. No alkaloids or tannins were detected in the bulbs.

With the growing population in South Africa, there is an increasing demand for traditional medicines. This increasing demand is placing tremendous strain on natural populations growing in the wild. However, as the demand cannot continue to be met other sources are needed. Tissue cultured plants have been grown at two different regions of South Africa. These plants have been grown under different conditions to determine the optimal ones needed to grow *M. natalensis* as a commercial crop on small-scale farms.

Plantlets taken directly from tissue culture were acclimatized successfully for cultivation by means of simple and cost effective methods. Cultivated plants were harvested on a six-monthly basis for a period of two years. Field cultivation produced bulbs of almost marketable size ($\pm 300\text{g}$ fresh weight) after 24 months. Bulb size was not dependent on additional fertilizer or irrigation. No significant differences ($p \leq 0.05$) were shown in the average dry weights of bulbs grown under different treatments (control, fertilizer without irrigation, fertilizer with irrigation). Leaf senescence and dormancy of young plants were prevented with irrigation. Flowering occurred after 24 months, with the irrigation and fertilizer plot having the most flowering plants. TLC fingerprinting revealed differences in the chemical composition of the bulbs harvested at different stages of growth. Noticeable differences were found between bulbs cultivated at the different growing sites.

Pharmacological screenings were done of the harvested bulbs to investigate the effect of age (time of harvest) and growing conditions on antibacterial, anti-inflammatory and anthelmintic activity. Methanol extracts were screened against Gram-positive (*Bacillus subtilis* and *Staphylococcus aureus*) and Gram-negative (*Escherichia coli* and *Klebsiella pneumoniae*) bacteria. Variations in activity were found. The time of harvest had a significant effect ($p \leq 0.05$) on biological activity, with the younger plants being more active. Antibacterial activity decreased with an increase in plants age.

Methanol extracts were also screened for anthelmintic activity against *Caenorhabditis elegans*. Activity was found to increase with plant maturity. Irrigation was found to increase activity at the low rainfall (Fort Hare) site. Bulbs harvested from the irrigation treatment had significantly higher anthelmintic activity ($p \leq 0.05$)

than bulbs harvested from treatments without irrigation. Dichloromethane extracts from bulbs grown at both sites had high anti-inflammatory activity. There were no significant differences ($p \leq 0.05$) in the activity of bulbs harvested from the different treatment plots. The time of harvest had an effect on the inhibition of prostaglandin synthesis by COX-1 enzymes.

This study provides not only scientific verification for the use of *M. natalensis* to some extent as a medicinal plant, but also important data needed to successfully cultivate this species as a crop for small-scale farming.

PUBLICATIONS FROM THIS STUDY

SG SPARG, J VAN STADEN and AK JÄGER (2001). Pharmacological and phytochemical screening of two Hyacinthaceae species: *Scilla natalensis* and *Ledebouria ovatifolia*. *Journal of Ethnopharmacology*. 80, 1: 95-101.

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CONFERENCE CONTRIBUTIONS

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SG SPARG, J VAN STADEN and AK JÄGER (2001). Why is *Scilla natalensis* such a popular medicinal plant? World Conference on Medicinal and Aromatic Plants. Budapest, Hungary.

SG SPARG, J VAN STADEN and AK JÄGER (2002). Isolation of Biologically Active Phytochemicals from *Scilla natalensis*. 28th Annual Conference of South African Association of Botanists (SAAB). Grahamstown, South Africa.

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TABLE OF CONTENTS

DECLARATION	i
ACKNOWLEDGEMENTS	ii
ABSTRACT	iii
PUBLICATIONS FROM THIS STUDY	vi
CONFERENCE CONTRIBUTIONS	vi
TABLE OF CONTENTS	vii
LIST OF FIGURES	xiv
LIST OF TABLES	xxii

CHAPTER 1 INTRODUCTION

1.1. Plants in Medicine	1
1.2. Traditional Medicine	1
1.3. Traditional Medicine in South Africa.....	2
1.4. Conservation of Medicinal Plants in South Africa	4
1.5. The Genus <i>Merwillia</i>	5
1.6. <i>Merwillia natalensis</i> (Planchon) Speta.....	6
1.6.1. <i>Distribution and Morphology</i>	6
1.6.2. <i>Ethnobotany</i>	7
1.6.3. <i>Conservation</i>	8
1.6.4. <i>Medicinal Uses</i>	8
1.6.5. <i>Screenings Previously Performed</i>	9
1.7. Aims.....	12

**CHAPTER 2 PHARMACOLOGICAL INVESTIGATION OF
 MERWILLA NATALENSIS**

2.1. Introduction	13
2.2. Collection and Preparation of <i>Merwilla natalensis</i> Bulb Material	13
2.3. Antibacterial Screening	14
2.3.1. <i>Introduction</i>	14
2.3.2. <i>Method of Screening</i>	15
2.3.2.1. <i>Preparation of Extracts</i>	15
2.3.2.2. <i>Bacterial Strains Screened for Antibacterial Activity</i>	16
2.3.2.3. <i>MIC bioassay</i>	16
2.3.3. <i>Results and Discussion</i>	16
2.4. Anti-inflammatory Screening	18
2.4.1. <i>Introduction</i>	18
2.4.2. <i>Method of Screening</i>	20
2.4.2.1. <i>Preparation of Extracts</i>	20
2.4.2.2. <i>COX-1 Enzyme Preparation</i>	20
2.4.2.3. <i>COX-1 Bioassay</i>	20
2.4.2.4. <i>COX-2 Bioassay</i>	21
2.4.3. <i>Results and Discussion</i>	22
2.5. Antischistosomal Screening	24
2.5.1. <i>Introduction</i>	24
2.5.2. <i>Method of Screening</i>	26
2.5.2.1. <i>Preparation of Extracts</i>	26
2.5.2.2. <i>Collection and Storage of Snails</i>	26
2.5.2.3. <i>In Vitro Bioassay</i>	26

2.5.2.4. <i>Culture Medium</i>	27
2.5.3. <i>Results and Discussion</i>	27
2.6. Anthelmintic Screening	28
2.6.1. <i>Introduction</i>	28
2.6.2. <i>Method of Screening</i>	29
2.6.2.1. <i>Preparation of Extracts</i>	29
2.6.2.2. <i>In Vitro Bioassay</i>	29
2.6.3. <i>Results and Discussion</i>	29
2.7. Anticancer (DNA-damaging) Screening	31
2.7.1. <i>Introduction</i>	31
2.7.2. <i>Method of Screening</i>	32
2.7.2.1. <i>Preparation of Extracts</i>	32
2.7.2.2. <i>Preparation of Base Layer</i>	32
2.7.2.3. <i>Preparation of Inoculum</i>	32
2.7.2.4. <i>Preparation of Assay Plates</i>	33
2.7.2.5. <i>Biochemical Induction Assay (BIA)</i>	33
2.7.3. <i>Results and Discussion</i>	33
2.8. Cytotoxicity Testing	34
2.8.1. <i>Introduction</i>	34
2.8.2. <i>Method of Testing</i>	34
2.8.3. <i>Results and Discussion</i>	35
2.9. Summary	36

CHAPTER 3 PHYTOCHEMICAL SCREENING

3.1. Saponins	37
3.1.1. <i>Introduction</i>	37
3.1.2. <i>Method of Detection</i>	40
3.1.2.1. <i>Froth Test</i>	40
3.1.2.2. <i>Haemolysis Test</i>	40
3.1.3. <i>Results and Discussion</i>	41
3.2. Cardiac Glycosides	41
3.2.1. <i>Introduction</i>	41
3.2.2. <i>Method of Detection</i>	42
3.2.3. <i>Results and Discussion</i>	43
3.3. Alkaloids	45
3.3.1. <i>Introduction</i>	45
3.3.2. <i>Method of Detection</i>	46
3.3.2.1. <i>Method A</i>	46
3.3.2.2. <i>Method B</i>	47
3.3.2.3. <i>Method C</i>	47
3.3.3. <i>Results and Discussion</i>	47
3.4. Tannins	48
3.4.1. <i>Introduction</i>	48
3.4.2. <i>Method of Detection</i>	49
3.4.3. <i>Results and Discussion</i>	49
3.5. Summary	50

**CHAPTER 4 GROWTH ANALYSIS OF MATERIAL COLLECTED
FROM FIELD TRIALS**

4.1. Introduction	51
4.2. Methodology	53
4.2.1. <i>Cultivation Sites</i>	53
4.2.1.1. <i>H₂A Botanicals</i>	53
4.2.1.2. <i>Fort Hare</i>	56
4.2.2. <i>Cultivation Methods</i>	56
4.2.3. <i>Harvesting</i>	56
4.2.4. <i>Drying and Storage</i>	57
4.3. Results and Discussion	57
4.3.1. <i>H₂A Botanicals</i>	57
4.3.2. <i>Fort Hare</i>	63
4.4. Summary	66

CHAPTER 5 CHEMICAL FINGERPRINTING BY TLC

5.1. Introduction	67
5.2. Methodology	69
5.2.1. <i>Extract Preparations</i>	69
5.2.2. <i>Sample Preparation</i>	69
5.2.3. <i>TLC Separation</i>	69
5.2.3.1. <i>Methanol Extracts</i>	70
5.2.3.2. <i>Dichloromethane Extracts</i>	70
5.2.3.3. <i>Water Extracts</i>	70

5.3. Results and Discussion	71
5.3.1. <i>Methanol Extracts</i>	71
5.3.2. <i>Dichloromethane Extracts</i>	74
5.3.3. <i>Water Extracts</i>	74
5.4. Summary	77
CHAPTER 6	PHARMACOLOGICAL ANALYSIS OF MATERIAL COLLECTED FROM FIELD TRIALS
6.1. Introduction	78
6.2. Extract Preparations	78
6.3. Antibacterial Analysis of Cultivated Bulbs	79
6.3.1. <i>Methodology</i>	79
6.3.1.1. <i>Extracts</i>	79
6.3.1.2. <i>Antibacterial Bioassay</i>	79
6.3.1.3. <i>Bioautographic Assay</i>	79
6.3.2. <i>Results and Discussion</i>	80
6.4. Anti-inflammatory Analysis of Cultivated Bulbs	91
6.4.1. <i>Methodology</i>	91
6.4.1.1. <i>Extracts</i>	91
6.4.1.2. <i>Anti-inflammatory Bioassay</i>	93
6.4.2. <i>Results and Discussion</i>	93
6.5. Anthelmintic Analysis of Cultivated Bulbs	96
6.5.1. <i>Methodology</i>	96
6.5.1.1. <i>Extracts</i>	96
6.5.1.2. <i>Anthelmintic Bioassay</i>	96

6.5.2. <i>Results and Discussion</i>	97
6.6. Summary	101
 CHAPTER 7 CONCLUSIONS	
7.1. Pharmacological Investigation of <i>Merwillia natalensis</i>	104
7.2. Phytochemical Screening	104
7.3. Growth Analysis of Material Collected From Field Trials.....	104
7.4. Chemical Fingerprinting By TLC.....	105
7.5. Pharmacological Analysis of Material Collected From Field Trials.....	105
7.6. Conclusion	106
 REFERENCES	 107

LIST OF FIGURES

- Figure 1.1. (A) *Merwilla natalensis* plant in flower. (B) Inflorescence with a bee collecting pollen. (C) Bagged plant material being sold at a Pietermaritzburg traditional market. *M. natalensis* bulbs are one of the more commonly sold medicinal plants. (D) Distribution of *M. natalensis* in South Africa. (E) *M. natalensis* bulbs found in the Drakensberg. An antelope possibly ate the leaves, as spoor was found around the bulbs. 10
- Figure 2.1. Percentage inhibition of *M. natalensis* extracts (250 µg/ml) against prostaglandin synthesis by (A) COX-1 and (B) COX-2 enzymes. Values within the same graph with the same letter are not significantly different (at 5% level of significance) from each other. DCM = dichloromethane. 23
- Figure 2.2. Mean percentage of *Caenorhabditis elegans* nematodes alive after being exposed to *M. natalensis* extracts for two hours. Values within the same graph with the same letter are not significantly different (at 5% level of significance) from each other. DCM = dichloromethane. 30
- Figure 3.1. (A) Pill vial containing dried *M. natalensis* material in distilled water. The arrow points to the froth layer that resulted from shaking, indicating the possible presence of saponins. (B) Blood agar plate. The arrow points to the zones of ruptured erythrocytes (haemolysis test), which confirms the presence of saponins within the bulbs of *M. natalensis*. SC = saponin control, H₂O = water control and E1-3 = *M. natalensis* extracts 1, 2 and 3. 44

- Figure 3.2. TLC plate developed with ethyl acetate: methanol: water (81:11:8 v:v:v) solvent system. The plate was stained with antimony trichloride reagent and viewed under UV₃₆₆ nm. MN = *M. natalensis*. PA = Proscillaridin A (reference bufadienolide). Florescent bands indicate bufadienolides. Arrow indicates bufadienolide from *M. natalensis* with same R_f as the reference compound.....44
- Figure 4.1. Provincial map of South Africa showing the distribution of wild populations of *M. natalensis* and the two cultivation sites, H₂A Botanicals and the University of Fort Hare where field trials were conducted. 54
- Figure 4.2. (A) *M. natalensis* bulblets being acclimatized in seedling trays under 80% shade cloth at H₂A Botanicals. (B) Young plants were put into bags after acclimatized to allow the plants to establish and grow before planting out into the field. (C) Overview of the terraced site at H₂A Botanicals where plants were planted out. (D) Rows of *M. natalensis* plants growing in the irrigation and fertilizer plot. (E) Bulbs harvested after two years of growing in the field..... 55
- Figure 4.3. Average dry weight and relative growth rate of *M. natalensis* bulbs harvested from the control treatment at the H₂A Botanicals site. .60
- Figure 4.4. Average dry weight and relative growth rate of *M. natalensis* bulbs harvested from fertilizer treatment at the H₂A Botanicals site.....61
- Figure 4.5. Average dry weight and relative growth rate of *M. natalensis* bulbs harvested from irrigation treatment at the H₂A Botanicals site.62

- Figure 4.6. Average dry weights and relative growth rates of harvested *M. natalensis* bulbs from the Fort Hare site. Measurements were taken from bulbs harvested from (A) Control treatment (B) Fertilizer treatment, and (C) Irrigation treatment.....65
- Figure 5.1. TLC Fingerprinting profiles of methanol extracts of *M. natalensis* bulbs harvested from the H₂A Botanicals site. TLC plates were developed in chloroform: glacial acetic acid: methanol: water (64:32:12:8 v:v:v:v) solvent system. (A) Anisaldehyde-sulphuric acid stained TLC plate viewed under visible light. (B) Antimony trichloride stained TLC plate viewed under visible light. (C) Antimony trichloride stained TLC plate viewed under UV₃₆₆ nm. M = mature *M. natalensis* plant, C = control treatment, F = fertilizer treatment, IF = irrigation treatment and 6-24 = month of harvest.72
- Figure 5.2. TLC Fingerprinting profiles of methanol extracts of *M. natalensis* bulbs harvested from the Fort Hare site. TLC plates were developed in chloroform: glacial acetic acid: methanol: water (64:32:12:8 v:v:v:v) solvent system. (A) Anisaldehyde-sulphuric acid stained TLC plate viewed under visible light. (B) Antimony trichloride stained TLC plate viewed under visible light. (C) Antimony trichloride stained TLC plate viewed under UV₃₆₆ nm. M = mature *M. natalensis* plant, C = control treatment, F = fertilizer treatment, IF = irrigation treatment and 6-24 = month of harvest.....73
- Figure 5.3. TLC Fingerprinting profiles of dichloromethane extracts of *M. natalensis* bulbs harvested from the (A) H₂A Botanicals site and (B) Fort Hare site. TLC plates were developed in dichloromethane: chloroform: methanol (10:1:1 v:v:v) solvent system. TLC plates stained with antimony trichloride reagent and viewed under UV₃₆₆ nm. M = mature *M. natalensis* plant, C = control treatment, F = fertilizer treatment, IF = irrigation treatment and 6-24 = month of harvest.75

- Figure 5.4. TLC Fingerprinting profiles of water extracts of *M. natalensis* bulbs harvested from the (A) H₂A Botanicals site and (B) Fort Hare site. TLC plates were developed in dichloromethane: methanol: water (1:3:1 v:v:v) solvent system. TLC plates stained with anisaldehyde-sulphuric acid reagent and viewed under visible light. M = mature *M. natalensis* plant, C = control treatment, F = fertilizer treatment, IF = irrigation treatment and 6-24 = month of harvest..... 76
- Figure 6.1. Mean MIC values of *M. natalensis* bulb extracts harvested from the H₂A Botanicals site over a period of two years against *B. subtilis*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.137$). The treatments and time of harvest had no significant effect on the antibacterial activity of the bulbs ($p=0.221$ and $p=0.184$ respectively)..... 82
- Figure 6.2. Mean MIC values of *M. natalensis* bulb extracts harvested from the H₂A Botanicals site over a period of two years against *S. aureus*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.278$). The treatments had no significant effect on the antibacterial activity ($p=0.076$). Time of harvest had a significant effect on the antibacterial activity of the bulbs ($p=0.002$). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other..... 83

Figure 6.3. Mean MIC values of *M. natalensis* bulb extracts harvested from the H₂A Botanicals site over a period of two years against *E. coli*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant (p=0.887). Both the treatments and time of harvest had a significant effect on the antibacterial activity of the bulbs (p=0.008 and p=0.001 respectively). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other....
 84

Figure 6.4. Mean MIC values of *M. natalensis* bulb extracts harvested from the H₂A Botanicals site over a period of two years against *K. pneumoniae*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was highly significant (p<0.001). Both the treatments and time of harvest had no significant effect on the antibacterial activity of the bulbs (p=0.193 and p=0.221 respectively). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other..... 85

Figure 6.5. Mean MIC values of *M. natalensis* bulb extracts harvested from the Fort Hare site over a period of two years against *B. subtilis*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant (p=0.847). The treatments had no significant effect on the antibacterial activity of the bulbs (p=0.579). Time of harvest had a significant effect on the antibacterial activity of the bulbs (p=0.002). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other..... 87

- Figure 6.6.** Mean MIC values of *M. natalensis* bulb extracts harvested from the Fort Hare site over a period of two years against *S. aureus*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.866$). The treatments had no significant effect on the antibacterial activity of the bulbs ($p=0.484$). Time of harvest had a significant effect on the antibacterial activity of the bulbs ($p<0.001$). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other. 88
- Figure 6.7.** Mean MIC values of *M. natalensis* bulb extracts harvested from the Fort Hare site over a period of two years against *E. coli*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.983$). The treatments had no significant effect on the antibacterial activity of the bulbs ($p=0.583$). Time of harvest had a significant effect on the antibacterial activity of the bulbs ($p<0.001$). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other. 89
- Figure 6.8.** Mean MIC values of *M. natalensis* bulb extracts harvested from the Fort Hare site over a period of two years against *K. pneumoniae*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.847$). The treatments and time of harvest had no significant effect on the antibacterial activity of the bulbs ($p=0.071$ and $p=0.242$ respectively)..... 90

- Figure 6.9. Bioautographic assay - TLC plates of *M. natalensis* methanol extracts from bulbs harvested from the control treatment from both the H₂A Botanicals and the Fort Hare sites. TLC plates were developed with a chloroform: methanol: water (8:5:1 v:v:v) solvent system. (A) Reference TLC plate stained with anisaldehyde-sulphuric acid reagent. (B) Corresponding TLC plate with *S. aureus* bacterial overlay. Clear zones indicate zones of bacterial inhibition. H = H₂A Botanicals. F = Fort Hare. 1 – 4 = Harvests (6, 12, 18 and 24 months).....92
- Figure 6.10. Mean percentage inhibition of COX-1 enzyme by *M. natalensis* bulb extracts. Bulbs harvested from the H₂A Botanicals site. (A) Mean inhibition for each extract. (B) Mean inhibition of each treatment. (C) Mean inhibition of each harvest. There was no significant ($p \leq 0.05$) interaction between the treatments and the time of harvest ($p = 0.985$). The treatments had no significant effect on COX-1 inhibition ($p = 0.613$). Time of harvest had no significant effect on COX-1 inhibition ($p = 0.128$)..... 94
- Figure 6.11. Mean percentage inhibition of COX-1 enzyme by *M. natalensis* bulb extracts. Bulbs harvested from the Fort Hare site. (A) Mean inhibition for each extract. (B) Mean inhibition of each treatment. (C) Mean inhibition of each harvest. There was no significant ($p \leq 0.05$) interaction between the treatments and the time of harvest ($p = 0.550$). The treatments had no significant effect on COX-1 inhibition ($p = 0.881$). Time of harvest had a significant effect on COX-1 inhibition ($p = 0.011$). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other. 95

Figure 6.12. Percentage of *Caenorhabditis elegans* nematodes alive after being exposed to methanol extracts of *M. natalensis* bulbs harvested from the H₂A Botanicals site. (A) Mean percentage of nematodes surviving for each extract. (B) Mean percentage of nematodes surviving for each plot treatment. (C) Mean percentage of nematodes surviving for each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.510$). The treatments had no significant effect ($p\leq 0.05$) on the anthelmintic activity ($p=0.092$). The time of harvest had a significant effect on the anthelmintic activity ($p=0.020$). 99

Figure 6.13. Percentage of *Caenorhabditis elegans* nematodes alive after being exposed to methanol extracts of *M. natalensis* bulbs harvested from the Fort Hare site. (A) Mean percentage of nematodes surviving for each extract. (B) Mean percentage of nematodes surviving for each plot treatment. (C) Mean percentage of nematodes surviving for each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.998$). The treatments and time of harvest had a significant effect on the anthelmintic activity ($p=0.024$ and $p<0.001$ respectively). 100

LIST OF TABLES

- Table 2.1. Minimum inhibitory concentrations (MIC) of *M. natalensis* bulb extracts against four common bacterial types. Values with the same letter are not significantly different (at 5% level of significance) from each other.17
- Table 2.2. Lethal concentrations (LC) of aqueous *M. natalensis* extracts against schistosomula of the species *Schistosoma haematobium*.....27
- Table 2.3. Survival of secondary vervet monkey kidney (VK) cells in the presence of varying concentrations of aqueous extracts of *M. natalensis*.....35

CHAPTER 1 INTRODUCTION

1.1. Plants in Medicine

Selection of plants from the wild to be used as medicines has occurred for centuries. The knowledge and administrative techniques of these traditional medicines have been passed from generation to generation (COUZINIER and MAMATAS 1986; LEWIS 1992). Plants were once the primary source of all the medicines in the world. More than 50 % of clinical drugs used today are made up of plant products or their derivatives (KINGHORN and BALANDRIN 1993). Although this number is high, about 90% of the 119 plant derived pharmaceutical products currently marketed are derived from approximately 90 different plant species (FARNSWORTH 1994). Well-known examples of plant-derived medicines that are used as clinical drugs include quinine, morphine, codeine, aspirin, atropine, reserpine and cocaine (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). If one considers that there are approximately 500 000 species of higher plants, there remains an abundance of plants that have not been examined and these species may have the potential to be pharmacologically active and the source of new pharmaceutical drugs (FARNSWORTH 1994). It was not until the 1990's that the developed world started to pay attention towards indigenous traditional knowledge and cultures of under-developed Countries. Increasing interest in natural products led to increasing respect for the indigenous peoples and their knowledge (CORDELL 1995).

Medicinal plant drugs can be divided into two broad categories. The first consists of complex mixtures containing a wide variety of compounds (concoctions) such as infusions, essential oils, tinctures or extracts. The second consists of pure, chemically active principles. These are stronger and more specific (HAMBURGER and HOSTETTMANN 1991).

1.2. Traditional Medicine

In most developing countries a large proportion of the population use traditional medicines either due to the high cost and unavailability of western medicines and

health care, or because the traditional medicines are more acceptable from a cultural and spiritual perspective (CUNNINGHAM 1988). Many traditional African medicines are regarded as food plants that are not poisonous and are eaten for therapeutic purposes in contrast to orthodox western medicines, which are in essence, poisons taken at low doses (IWU 1993). African traditional medicine is not as well documented as Indian and Chinese traditional medicine. Some of the African medicinal plants investigated for their chemical components contain compounds with interesting biological activity, which have proved to be therapeutically active (IWU 1993).

The World Health Organisation's (WHO) resolution on a strategy to promote "Health for All" recognises that health is a fundamental right (PATEL 1983). The current modern health care cannot meet the needs of the entire world's population. LE GRAND and WONDERGEM (1989) proposed that there is a need to promote traditional medicine as a means to improve the health in many developing countries.

Modern drugs available to remote villages often lack adequate information on their use, which can result in over-consumption and even drug abuse. Generally synthetic drugs have very high pharmacodynamic effects, but this activity is in most cases accompanied by strong and even dangerous side effects. Medicinal plants do not have similarly high therapeutic potencies and show no serious side effects except for a few exceptions (NARANJO, 1995).

1.3. Traditional Medicine in South Africa

Medicinal plants form an important aspect of the daily lives and cultural heritage of many South Africans. Southern Africa has over 30 000 species of higher plants (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). The Cape Floral Kingdom alone has nearly 9 000 species and is regarded as the most diverse temperate flora on earth (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). With this kind of biodiversity it is not surprising that there are approximately 3 000 plant species that are used in traditional medicines. Three hundred and fifty of these are commonly used and traded medicinal plants (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). Even today, a large part of day-to-day medicines are still derived from plants. South Africa has contributed to world medicine with the discovery of Cape aloes (*Aloe ferox*), buchu

(*Agathosma betulina*) and Devil's claw (*Harpagophytum procumbens*). However, there is still a lack of detailed documentation on the use of medicinal plants in South Africa. This is an important issue since most of the traditional knowledge is passed down through the generations from one person to the next. With the increase in urbanisation and the loss of many traditional customs, traditional knowledge is gradually being lost (VAN WYK, VAN OUDTSHOORN and GERICKE 1997).

Traditional medicine is still widely used by the majority of South Africans, especially as western medicines are not readily available to the majority of rural people. It is estimated that up to 80 % of the Zulu people use traditional medicines. For this reason, medicinal plants have become the focus of intense study in recent years in terms of conservation and as to whether their traditional uses are supported by pharmacological effects or merely based on folklore (CUNNINGHAM 1988; JÄGER, HUTCHINGS and VAN STADEN 1996; MANDER 1997). Up to 60% of the South African population consult one of the estimated 200 000 traditional healers (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). This is especially true in rural areas where traditional healers are more numerous and accessible than western doctors (CUNNINGHAM 1988). Traditional medicines are most frequently used by low-income groups, as it is considerably cheaper than Western medicines. This is prevalent in the KwaZulu-Natal region, which is known to have one of the highest incidences of poverty in South Africa (MANDER, MANDER and BREEN 1996).

A wide gap exists between modern and traditional medical practices. Attempts are being made to try and narrow this gap through the establishment of Healer's associations. There is a need for more professionalism and regulation (SINDIGA, KANUNHA, NYAIGOTTI-CHACHA and MWANGOLA 1995). In South Africa, the recognition of traditional medicine aims to improve the safety, availability and allow for wider application at low cost. With the increasing acceptance of traditional medicine as an alternative form of health care, there is an urgent need for an evaluation of traditional methods of treatment. A lot of importance has been placed on the screening of medicinal plants for active compounds.

1.4. Conservation of Medicinal Plants in South Africa

The increase in demand for novel phytochemical drugs has coincided with the accelerated destruction of tropical rain forests resulting in an estimated loss of 20 000 species over and above the normal rates of extinction (LEWIS 1992).

South Africa has a rapidly increasing population that needs to be fed and housed. The increase in agriculture, forestry, industrial and urban housing encroachment has caused many wild medicinal plant resources to become threatened. Development of private and communal land has resulted in localised extinctions of many popular medicinal plants in KwaZulu-Natal (MANDER, MANDER and BREEN 1996).

Another threat to wild resources is the need for more medicinal plants. A large and active trade in medicinal plants exists in KwaZulu-Natal and other parts of South Africa. The increasing demand for medicinal plants has resulted in over-exploitation of many of the wild populations (MANDER, MANDER and BREEN 1996). There has also been an increase in non-specialist gatherers and commercial traders. These gatherers have little knowledge of traditional healing and sustainability (VAN STADEN 1999). This has been brought about due to the increase in unemployment and increasing demand for medicinal plants. In the past, professional herb gatherers and traditional healers used to collect and store plants according to their traditions. In this way they prevented overharvesting. Today, the urbanised healers purchase their ingredients from the street markets and stores which provides an income to many otherwise unemployed people. However, this has a negative effect on the environment, as this provides an incentive for the destructive harvesting of vulnerable medicinal plants (VAN WYK, VAN OUDTSHOORN and GERICKE 1997).

The ever increasing demand for medicinal plants has resulted in a greater harvesting intensity being placed on the remaining natural resources (MANDER, MANDER and BREEN 1996). There is a growing need for sustainable use of natural resources and for enhanced supply by means of cultivation. VAN STADEN (1999) suggested that if the concept of sustainable use is to be effective, the population needs to be in equilibrium with the landmass it occupies. However, with South Africa's

growing population this equilibrium is seriously out of phase. Most of the natural populations have already been overexploited, and those that remain are largely within conservation areas. It is these conservation areas that are targeted for so-called sustainable harvesting. It is therefore believed that the demand for medicinal plants is already too large and that meeting the demand by sustainable use would be extremely difficult (VAN STADEN 1999). VAN STADEN (1999) suggested that the only real solution would be to develop the valuable medicinal plants as crops through small-scale farming. The outcomes of this would be very positive, as not only would there be sustainable development and protection of biodiversity, but this would generate income for many through entrepreneurial farmers.

Although the cultivation of medicinal plants has been suggested, there remains a lack of understanding with respect to the cultivation and economics of producing medicinal plants. This lack of understanding can be considered as one of the most limiting factors in the commercialisation of indigenous medicinal plants (MANDER, MANDER and BREEN 1996). There is an urgent need for field trial analysis on commonly used medicinal plants. Basic information on how to grow the plants, planting to harvest periods, yield, expected financial returns, storage and shelf-life is needed before small-scale farmers can be expected to venture into growing medicinal plants (JÄGER and VAN STADEN 2000). JÄGER and VAN STADEN (2000) suggested that if this information were available and the cultivation of medicinal plants was phased in properly, the financial rewards for small-scale farmers could be higher than the returns obtained from maize or sugarcane.

1.5. The Genus *Merwillia*

The genus *Merwillia* (SPETA 1998) is a relatively new genus that is comprised of species that were initially members of the genus *Scilla*. The genus *Scilla* has been under investigation many times and still remains a very controversial one. Initially, the genus *Scilla* was placed under the family Liliaceae, but now it has been placed into a new family known as the Hyacinthaceae that comprises of the four sub-families Hyacinthoideae, Ornithogaloideae, Urineoideae and Oziroeoideae (PFOSSER and SPETA 1999).

In 1970, JESSOP reviewed the genus *Scilla* sensu lato. The genus *Ledebouria* was initially placed under the title *Scilla* sensu lato, but JESSOP (1970) showed that cytological as well as anatomical evidence suggested otherwise. JESSOP (1970) recognised six *Scilla* species found in South Africa. These being *Scilla dracomontana* HILLARD & BURTT, *S. firmifolia* BAKER, *S. kraussii* BAKER, *S. natalensis* PLANCH., *S. nervosa* (BURCH.) JESSOP and *S. plumbea* LINDL. In SPETA's (1998) review of the family Hyacinthaceae, the family was found to accommodate approximately 1000 species distributed into approximately 70 genera. This review showed there to be no true *Scilla* species existing in Africa south of the Sahara (SPETA 1998). Instead SPETA (1998) recognises there to be a new genus *Merwillia*, which consists of five species *Merwillia dracomontana* (HILLIARD & BURTT) SPETA (= *Scilla dracomontana*), *Merwillia kraussii* (BAKER) SPETA (= *Scilla kraussii*), *Merwillia lazulina* (WILD) SPETA (= *Scilla lazulina*), *Merwillia natalensis* (PLANCHON) SPETA (= *Scilla natalensis*) and *Merwillia plumbea* (LINDL.) SPETA (= *Scilla plumbea*). Four of these species are found in South Africa, *Merwillia lazulina* (WILD) SPETA is found in Zimbabwe. The other two *Scilla* species that were recognised by JESSOP (1970) to be found in South Africa, *Scilla firmifolia* BAKER and *Scilla nervosa* (BURCH.) JESSOP were recognised by SPETA (1998) to be *Pseudoprospero firmifolium* (Baker) Speta and *Schizocarphus nervosus* (BURCH.) MERWE respectively.

1.6. *Merwillia natalensis* (PLANCHON) SPETA (= *Scilla natalensis*)

Many of the species used in traditional medicines have bulbs as their main storage organ. South Africa has been regarded as having a treasure trove of bulbous plants. Many of these species have the potential to be exploited commercially both horticulturally and medicinally. One of the more commonly traded and used medicinal plants is *Merwillia natalensis* (= *Scilla natalensis*).

1.6.1. *Distribution and Morphology*

Merwillia natalensis is a summer-growing plant, restricted to the eastern parts of South Africa (Figure 1.1D), which experience summer rainfall, from the Eastern Cape

Province through Lesotho, KwaZulu-Natal, Free State and Swaziland to the Northern Province (JESSOP 1970). In KwaZulu-Natal it is found from the coastal plateaux to the Drakensberg escarpment where it is most common. The bulbs of this species are frost resistant and are usually associated with well-drained steep slopes and rocky sites, in Mistbelts and Montane Grasslands. However, this species has been found to be growing in soils with a high clay content in parts of the Drakensberg.

Merwillia natalensis (Figure 1.1A) is a bulbous plant with broad, sharply tapering leaves arising from medium to large-sized bulbs of about 100 mm - 150 mm in diameter. The bulbs often grow in large groups and are mostly exposed above the soil surface. Brown, papery "scales", the remains of old leaves are found on the exterior of the bulb. The flowers are small, blue or purplish-blue in colour (Figure 1.1B) and are borne on long slender stalks of about 1 m in height. The blue flowers and broad leaves are what make *S. natalensis* distinguishable from the other South African *Scilla* species (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). However, even with these rather distinctive characteristics, certain authors (JESSOP 1970) placed *S. natalensis* Planch together with *S. dracomantana* (now known as *M. dracomantana*) and *S. kraussii* (now known as *M. kraussii*) under the same species name *Scilla natalensis*. These three species have since been shown to be different species using morphological characteristics (HILLIARD and BURTT 1982) and phytochemical evidence (CROUCH, BANGANI and MULHOLLAND 1999). VAN STADEN and PAN (2001) confirmed the differences between the species using random amplified polymorphic DNA (RAPD) makers. In the review by SPETA (1998) all three species were recognised as three separate *Merwillia* species. In this study the *Merwillia natalensis* species that will be investigated will not be its broad description, i.e. not including *M. dracomantana* and *M. kraussii*.

1.6.2. Ethnobotany

According to the case studies on the indigenous medicinal market by CUNNINGHAM (1988), and MANDER (1997), *Scilla natalensis* is ranked as the most popular plant species sold at the markets (Figure 1.1C). MANDER (1997) reported that 95.5 tonnes of *S. natalensis* bulbs are sold annually in the Durban medicinal plant

trade. In the Witwatersrand informal medicinal plant trade, *S. natalensis* is the second most commonly stocked medicinal plant after *Artemisia afra* (WILLIAMS 1996).

1.6.3. Conservation

Although *S. natalensis* is a popular bulbous plant, there are little supply constraints yet (MANDER 1997). Large populations still exist within KwaZulu-Natal. For this reason *S. natalensis* is still sold as the cheapest popular product per kg, in both the shop and street trade. They fetch a price of approximately R6.46/kg (MANDER 1997). However, this exploitation can only continue for so long. The demand is too high to be sustainable. SCOTT-SHAW (1999) suggested that the conservation status of *S. natalensis* is vulnerable (Alacd, A2cd), as it has been over-exploited over most of its distribution range. HILTON-TAYLOR (1996) rated *S. natalensis*' conservation status to be higher (K), stating that its exact population size in the wild was "insufficiently known". To alleviate pressures on the natural resources, some form of conservation needs to be implemented. McCARTAN and VAN STADEN (1998) successfully micropropagated *S. natalensis* plantlets *in vitro*. CUNNINGHAM (1988) suggested that if we assume that *S. natalensis* grows at the same rate and density as *Urginea maritima* (harvesting after 6 years, 10 000 bulbs per acre), which were cultivated by GENTRY, VERBISCAR and BANIGAN (1987), then 3000 bags could be sold from 70 ha of land. This would alleviate over-exploitation from the wild.

1.6.4. Medicinal Uses

Merwillia natalensis (= *Scilla natalensis*) is known to the Zulu-speaking people of South Africa as *inguduza*, which means "searching the body for the cause of the ailment" (WILLIAMS 1996). The bulbs of *M. natalensis* are sold for the treatment of various ailments. These include gastro-intestinal ailments, which includes stomach aches, constipation, intestinal worms, diarrhoea, dysentery, nausea and indigestion (HUTCHINGS 1989). *M. natalensis* is also taken in the treatment of sprains and fractures, sores, boils and growths such as tumours, lumps or other swellings that may or may not be cancerous (PHILLIPS 1917; WATT and BREYER-BRANDWIJK 1962; HUTCHINGS 1989). There have also been reports that *M. natalensis* is used gynaecologically in the treatment of menstrual pains and to facilitate delivery during

pregnancy (GERSTNER 1941; HUTCHINGS 1989; HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996). Decoctions are also taken as enemas for female fertilization and to enhance male potency and libido. *M. natalensis* has also been included in the treatment of chestpains and kidney troubles (ROTHWELL 1999). In most cases the bulbs are prepared as enemas and purgatives (GERSTNER 1938; 1939).

1.6.5. Screenings Previously Performed

JÄGER, HUTCHINGS and VAN STADEN (1996) screened aqueous and ethanol extracts of *M. natalensis* for prostaglandin-synthesis inhibitors (prostaglandins are involved in the complex process of inflammation and are responsible for the sensation of pain). The result showed that aqueous extracts from *M. natalensis* bulbs only had low inhibition of prostaglandin-synthesis. Higher activity was displayed with the ethanol extracts. However, the activity was not as high as many of the other species that were also tested.

Preliminary studies done by MATTHE (1988, 1989) indicated that extracts from *S. natalensis* have antimicrobial activity against a range of pus-forming organisms *in vitro*. RABE and VAN STADEN (1997) screened *M. natalensis* for *in vitro* antibacterial activity using the agar disc-diffusion assay (RASOANAIVO and RATSIMAMANGA-URVERG 1993). No activity was reported against any of the five bacteria tested.

Two homoisoflavanones have been isolated from *M. natalensis*. These are 5,7-dihydroxy-6-methoxy-3-(4-hydroxybenzyl) chroman-4-one and 5,7-dihydroxy-3-(3-hydroxy-4-methoxybenzyl) chroman-4-one (CROUCH, BANGANI and MULHOLLAND 1999). Homoisoflavanones have been reported in other *Scilla* species as well. DELLA LOGGIA, DEL NEGRO, TUBARO, BARONE and PARRILLI (1989) showed the homoisoflavanones of *Muscari comosum* to have anti-inflammatory activity. Homoisoflavanones have also been shown to have anti-allergenic, anti-histaminic, antimutagenic and angioprotective properties as well as being potent phosphodiesterase inhibitors (AMSCHLER, FRAHM, HATZELMANN, KILIAN, MULLER-DOBLIES and MULLER-DOBLIES 1996).

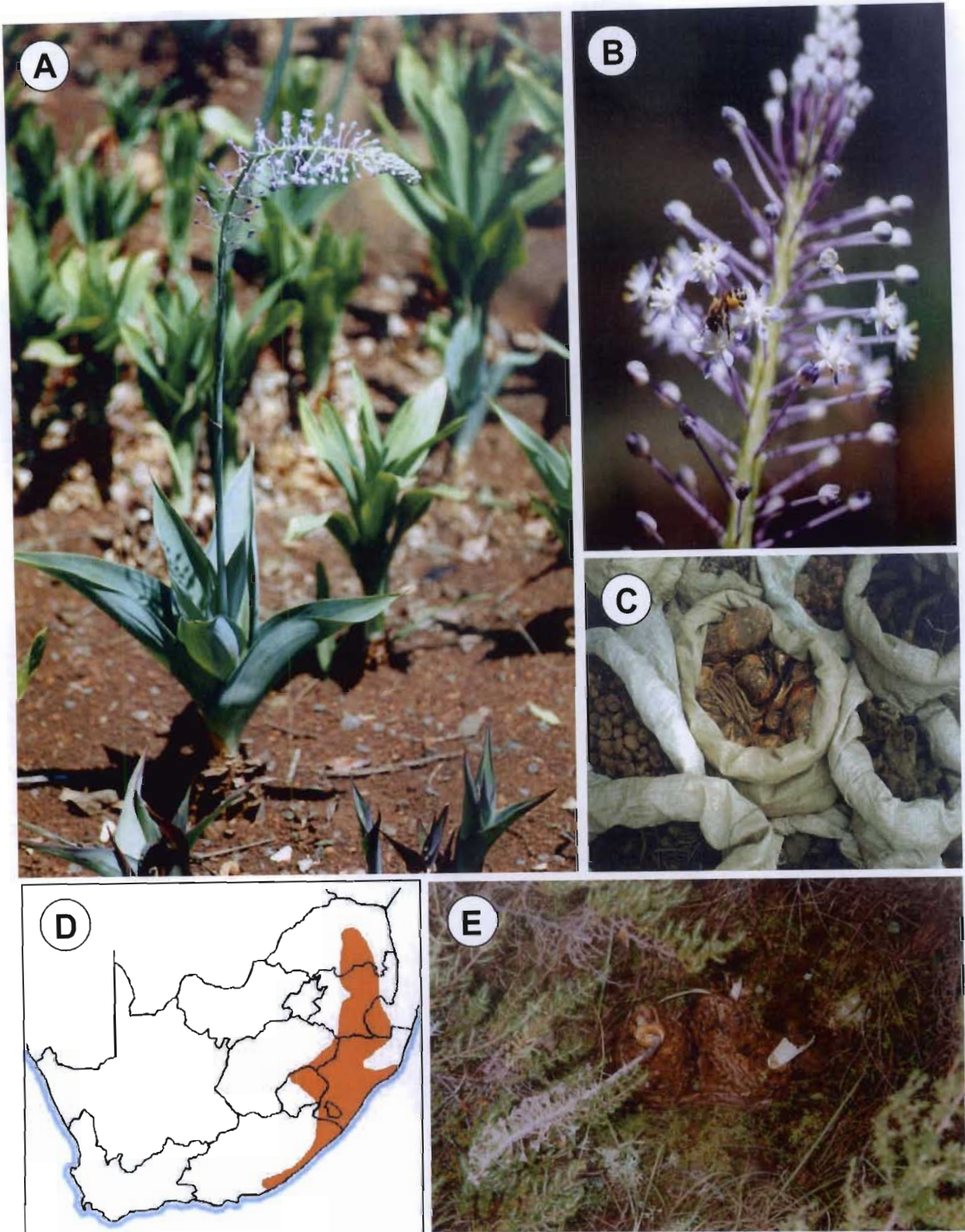


Figure 1.1. (A) *Merwillia natalensis* plant in flower. (B) Inflorescence with a bee collecting pollen. (C) Bagged plant material being sold at a Pietermaritzburg traditional market. *M. natalensis* bulbs are one of the more commonly sold medicinal plants. (D) Distribution of *M. natalensis* in South Africa. (E) *M. natalensis* bulbs found in the Drakensberg. An antelope possibly ate the leaves, as spoor was found around the bulbs.

M. natalensis is also reported to possibly contain saponins (HUTCHINGS, SCOTT, LEWIS and CUNNINGHAM 1996). LOWER (1985) showed that saponins have potential antimicrobial, cytostatic and anti-inflammatory activity.

KAMANO and PETIT (1974) reported the presence of cardiac glycosides in different *Scilla* species, but the presence of these glycosides in *M. natalensis* still needs to be demonstrated (VAN WYK, VAN OUDTSHOORN and GERICKE 1997). KELLERMAN, COETZER and NAUDE (1988) stated that electrocardiographic changes likened to those of cardiac glycoside poisoning were recorded in their work on *M. natalensis*. It is thought that *M. natalensis* may contain heart glycosides of the bufadienolide type, such as scillaren A, but no proof of this has been reported. Although the bulbs are thought to contain cardiac glycosides and are toxic to livestock (KELLERMAN, COETZER and NAUDE 1988), evidence found in the Drakensberg (Figure 1.1E) indicates that the wild animals browse the leaves.

1.7. Aims

The aims of this study were to firstly evaluate the rational usage of *Merwillia natalensis* as a medicinal plant and screen the bulbs for any phytochemical compounds that may have pharmacological activity. Secondly, analyse cultivated field trials of *M. natalensis* and develop a chemical fingerprinting system to use to analyse field-grown material.

Due to the broad usage of *M. natalensis* as a medicinal plant, the first part of this study was to determine the pharmacological activity of this species. This involved *in vitro* testing of bulb extracts for antibacterial, anti-inflammatory, anti-cancer, anti-viral, antischistosomal and anthelmintic activity. Once activity had been assessed, phytochemical screenings were performed to investigate for potentially active compounds.

The second part of the study was aimed at analyzing material grown in field trials from two different climatic and geographic regions within South Africa. Analysis was to be conducted on growth, and pharmacological activity. A chemical fingerprinting system was to be developed and chemical fingerprinting analysis done on the harvested material.

CHAPTER 2 PHARMACOLOGICAL INVESTIGATION OF *MERWILLA NATALENSIS*

2.1. Introduction

Over the last few years pharmacological investigations have been carried out on a number of commonly used medicinal plants to establish a scientific rationale for their usage in traditional medicine. This is very important not only to determine new possible drugs that are plant based but also to show that many of the medicinal plants used in traditional medicine do in fact have pharmacologically active properties. In many instances, traditional usage has been backed up with scientific data.

There is also the need to determine whether certain plants that are being used are not toxic to humans. This is very important as the number of non-specialist gatherers and traditional healers increase. The potential for poisonings also increases as more people make use of traditional medicines.

2.2. Collection and Preparation of *Merwillia natalensis* Bulb Material

Mature (5-10 years old) healthy looking bulbs of *Merwillia natalensis* were collected from stock plants grown at the University of Natal Botanical Gardens. The bulbs were approximately 8 cm in diameter and on average had a fresh weight of ± 400 g (70 g dry weight). The bulbs were rinsed under tap water followed by distilled water before being cut into small pieces. The cut pieces were placed into paper bags and dried at 50°C for approximately 72 h. Once dry, the pieces of bulbs were ground into powders using a mill (Greiner Labortechnik). The powdered bulbs were placed into air-tight containers and stored in the dark until extraction.

Extracts were made using four solvents, ranging in polarity (water, methanol, dichloromethane and hexane). The extraction procedure consisted of sonicating 500 mg of powdered *M. natalensis* bulb material in 5 ml extraction solvent for 30 min. The plant material was removed by filtering the extracts under vacuum through

Whatman No. 1 filter paper discs using a Büchner funnel. The filtered extracts were taken to dryness before being stored at 5°C.

2.3. Antibacterial Screening

2.3.1. Introduction

Bacteria are amongst the most abundant organisms on earth and are able to adapt to almost any living condition. They are both useful and harmful to humans. Infections caused by bacteria are very common and frequently cause death. For this reason antibacterial drugs are among the most important therapeutic discoveries of the twentieth century (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN 1997).

An increasing problem with antibiotics is the development of resistance by a variety of bacteria. Over-prescription and use of antibiotics have contributed significantly to antibiotic resistance (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN 1997). LAURENCE and BENNETT (1980) recognized two types of drug resistance. The first type, and the most common, is drug tolerance. This occurs when the organism is capable of growing in the presence of the drug. The second is drug destroying, which is when the organisms inactivate the drug. PAGE, CURTIS, SUTTER, WALKER and HOFFMAN (1997) classified antibiotic resistance as being either innate or acquired. Innate resistance is based on the mechanism of the drug, while acquired resistance refers to the genetic resistance of the bacteria. According to PAGE, CURTIS, SUTTER, WALKER and HOFFMAN (1997) there is no antibiotic that does not experience acquired resistance. For this reason it is clear that new antibiotic drugs need to be developed on a continuous basis.

Antibacterial activity of medicinal plant extracts has previously been evaluated using various methods, which are classified into three groups, i.e. disc-diffusion, dilution and bioautographic assays. There is no standardised method for expressing the results of antibacterial screening. Results can also be influenced by many factors, including the extraction method, inocula volume, culture medium composition, pH and incubation temperature (RIOS, RECIO and VILLAR 1988). The disc-diffusion assays allow several plant extracts to be screened against various

bacteria at the same time. This is suitable for preliminary screenings. The dilution assays are used to determine more precisely the antibacterial activity of extracts, giving the minimum inhibitory concentration (MIC) or minimum bactericidal concentration (MBC) values of test extracts for a given microorganism. The MIC value is the concentration that inhibits bacterial growth, while the MBC is the lowest concentration required to kill the bacterium. The MBC is often two to eight times that of the MIC (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN 1997). The bioautographic assays allows for rapid detection of bioactive constituents of plant extracts during bioassay-guided fractionation (RASOANAIVO and RATSIMAMANGA-URVERG 1993).

Many South African plants have potential antibiotic activity. RABE and VAN STADEN (1997), ELOFF (1998), KELMANSON, JÄGER and VAN STADEN (2000), and McGAW, JÄGER and VAN STADEN (2000) have examined many South African plants that have potential antibacterial activity. RABE and VAN STADEN (1997) screened 21 different plant species at a concentration of 1 mg/ml against five of the more common bacteria that plague our lives. The plants selected were based on reported traditional treatment of symptoms such as wounds, boils and purulent sores. One of the species screened for *in vitro* antibacterial activity was *M. natalensis* (*Scilla natalensis*). Methanol and aqueous extracts were screened using the disc-diffusion assay of RASOANAIVO and RATSIMAMANGA-URVERG (1993). *S. natalensis* bulb extracts showed no activity at concentrations of 1 mg/ml (RABE and VAN STADEN, 1997). Preliminary studies done by MATTHE (1988; 1989) indicated that extracts from *S. natalensis* have antimicrobial activity against a range of pus-forming organisms *in vitro*.

2.3.2. Method of Screening

2.3.2.1. Preparation of Extracts

Fifty mg/ml of the plant extract residues were dissolved in 1 ml of water (aqueous extract), methanol (methanol extract) and acetone (dichloromethane and hexane extracts). Dichloromethane and hexane were toxic to the test bacteria.

2.3.2.2. *Bacterial Strains Screened for Antibacterial Activity*

Each extract was bioassayed against two Gram-positive bacteria, *Bacillus subtilis* (ATCC No. 6051) and *Staphylococcus aureus* (ATCC No. 12600) and two Gram-negative bacteria *Escherichia coli* (ATCC No. 11775) and *Klebsiella pneumoniae* (ATCC No. 13883). The bacterial strains were obtained from the American Type Culture Collection (ATCC). The bacterial strains were maintained on Mueller-Hinton nutrient agar (Biolab) at 4°C.

2.3.2.3. *MIC Bioassay*

The bioassay used for antibacterial screening was the microdilution method for MIC determination as described by ELOFF (1998). Prior to use in the assay, suspension cultures were inoculated in Mueller-Hinton (MH) broth (Oxoid) from bacterial stock cultures and incubated overnight at 37°C in a waterbath with an orbital shaker. For each of the four bacteria, 100 µl of redissolved extract (50 mg/ml) were two-fold serially diluted with 100 µl sterile distilled water in a sterile 96-well microtitre plate (Greiner Labortechnik). A similar two-fold serial dilution of neomycin (Sigma) (100 µg/ml) was used as a positive control against each bacterium. Extraction solvents, extracts and bacteria-free controls were included as negative controls. The bacterial-saturated suspension cultures were diluted 1:100 with sterile MH broth, with 100 µl being added to each of the wells containing the test and control solutions. The plates were covered and incubated overnight at 37°C. Bacterial growth was indicated by adding 40 µl of 0.2 mg/ml p-iodonitrotetrazolium chloride (Sigma) to each of the wells. The plates were incubated at 37°C for a further 30 min. Lack of antibacterial activity is indicated by a change in colour. The MIC was taken as the lowest concentration of plant extract to elicit an inhibitory effect on the growth (last well not to exhibit a colour change) of the test bacterium.

2.3.3. *Results and Discussion*

One of the uncertainties with this bioassay is that the recorded MIC values are possibly MBC values. It is uncertain whether the bacteria are actually killed by the extract at the given value, or at the low concentrations, do not cause a colour change

with the addition of the p -iodonitrotetrazolium chloride. Those wells that do exhibit a colour change may represent concentrations that do not inhibit growth, or their growth is inhibited, but the concentrations are not lethal to the test bacterium. The values taken are therefore recorded as MIC values (last well not to exhibit a colour change) as we know growth has been inhibited, but there is no certainty of bactericidal activity.

Results for the general screening for antibacterial activity are shown in Table 1. No inhibitory activity was detected against the four test bacteria with the *M. natalensis* hexane extracts. Poor inhibitory activity was detected for both the water and dichloromethane extracts. The methanol extracts gave the highest inhibitory activity. The results suggest that the concentrations used by RABE and VAN STADEN (1997) were too low (1 mg/ml) to detect any antibacterial activity in the methanol extracts of *M. natalensis* (*S. natalensis*) bulbs. In the disc diffusion assay as used by RABE and VAN STADEN (1997) active compounds present in the extract diffuse through the bacteria inoculated agar, killing or inhibiting multiplication of the bacteria. If the compounds cannot diffuse readily through the agar, the extract is seen as being inactive. Therefore it is important to test extracts for antibacterial activity using the MIC assay as well.

Table 2.1. Minimum inhibitory concentrations (MIC) of *M. natalensis* bulb extracts against four common bacterial types. Values with the same letter are not significantly different (at 5% level of significance) from each other.

Extraction Solvent	Bacteria ¹ used (MIC (mg/ml))			
	<i>E. c.</i>	<i>K. p.</i>	<i>B. s.</i>	<i>S. a.</i>
Water	6.3 c	6.3 c	12.5 d	6.3 c
Methanol	1.6 a	1.6 a	1.6 a	1.6 a
Dichloromethane ²	3.1 b	6.3 c	6.3 c	6.3 c
Hexane ²	>12.5 e	>12.5 e	>12.5 e	>12.5 e
Neomycin	6.3×10^{-2}	1.6×10^{-2}	1.6×10^{-2}	3.1×10^{-2}

¹Bacteria: *E.c.* = *Escherichia coli*, *K.p.* = *Klebsiella pneumoniae*, *B.s.* = *Bacillus subtilis*, *S.a.* = *Staphylococcus aureus*

²Extract: dissolved in acetone

Extract concentration is important in terms of its traditional usage. At the wrong dosage, the plant extracts may not have any antibacterial activity. RABE and VAN STADEN (1997) reported that the methanol extracts of those plant species that exhibited antibacterial activity, had higher activity than the aqueous extracts.

Generally no significant differences ($p \leq 0.05$) were noted between the activity obtained against the Gram-positive or Gram-negative bacteria. The only extracts that showed any significant differences ($p \leq 0.05$) in activity between the test bacteria were the aqueous and dichloromethane extracts which yielded significant differences for *Bacillus subtilis* and *Escherichia coli* respectively. Due to the outer membrane structure of Gram-negative bacteria, the effectiveness of a drug is somewhat challenging (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN 1997). This is often the case with plant extracts as well. Plant extracts are usually more active against Gram-positive bacteria (McGAW, JÄGER and VAN STADEN 2000).

2.4. Anti-inflammatory Screening

2.4.1. Introduction

Inflammation is said to be a mechanism by which protective immune processes can be localized at a region where the infection occurs (VILLEE, SOLOMON, MARTIN, MARTIN, BERG and DAVIS 1989). Inflammation is a complex process with a number of different mediators, including prostaglandins (CAMPBELL 1990). Prostaglandins are responsible for the sensation of pain and inflammation. Prostaglandins are not stored in tissues for later release, but are biosynthesized within seconds of the activation of the appropriate enzymes and are rapidly metabolized (SMITH 1990). There are at least 14 naturally occurring prostaglandins. Six are "primary" prostaglandins (PGE_1 , PGE_2 , PGE_3 , $\text{PGF}_{1\alpha}$, $\text{PGF}_{2\alpha}$ and $\text{PGF}_{3\alpha}$) and give rise metabolically to another eight prostaglandins (LAURENCE and BENNETT 1980). The prostaglandin biosynthesis pathway begins with the conversion of arachidonic acid firstly, to prostaglandin G_2 and then to prostaglandin GH_2 (both are endoperoxide intermediates). These reactions are catalysed by the same enzyme, prostaglandin endoperoxide synthase. This enzyme consists of two components, cyclooxygenase and peroxidase.

Drugs that are developed for the treatment of pain and inflammation are targeted at inhibiting the cyclooxygenase enzyme and so prevent the formation of prostaglandins (NASIRI, HOLTH and BJORK 1993). Cyclooxygenase can be inhibited by three different mechanisms. These are: competitive inhibition, where there is reversible competition with arachidonate for binding to the cyclooxygenase site; mechanism based or time-dependent inactivation, where there is the formation of an enzyme-inhibitor complex; and non-competitive inhibition, where there is irreversible binding to the cyclooxygenase active site (SMITH and DEWITT 1995). Drugs that have this pharmacological action are known as non-steroidal anti-inflammatory drugs (NSAID) (RANG and DALE 1987). Aspirin, an anti-inflammatory drug, was one of the first drugs discovered from medicinal plants (COX and BALCK 1994). Other NSAIDs that are effective in the treatment of pain and inflammation are indomethacin, ibuprofen and paracetamol (RANG and DALE 1987). Recent investigations into the cyclooxygenase enzyme have found that this enzyme actually consists of two isozymes, COX-1 and COX-2 (VANE 1994; HERSCHMANN 1996; VANE and BOTTING 1996). The COX-1 isozyme is constitutively expressed while the COX-2 isozyme seems to be induced during inflammation. According to VANE and BOTTING (1996) little or no COX-2 is found in resting cells. Nearly all NSAIDs that have been discovered are COX-1 inhibitors, however all cyclooxygenase inhibitors seem to inhibit both the COX-1 and the COX-2 isozymes, but with different selectivity. The problem that exists is that many of the currently used anti-inflammatory drugs that are on the market have certain side effects which is why there is a growing need to discover new and improved drugs.

HUTCHINGS (1989) listed many of the plants used in South Africa for the treatment of pain and inflammation. HUTCHINGS and VAN STADEN (1994) revised this list. However, no scientific evidence is given of the efficacy of these plants in treating inflammation. JÄGER, HUTCHINGS and VAN STADEN (1996) screened 39 Zulu medicinal plants for prostaglandin-synthesis inhibitors. Aqueous and ethanolic extracts of *Scilla natalensis* were included in the screening. The inhibition was low compared to many of the other species screened. McGAW, JÄGER and VAN STADEN (1997) screened 26 plant used by Zulu healers for pain and inflammation for COX-1 activity at concentrations of 50, 500 and 1000 µg/ml. SHALE, STIRK and VAN STADEN (1999) screened 12 Lesotho medicinal plants for prostaglandin-synthesis inhibitors. Six species were found to have above 90% inhibition at 200 µg/ml.

2.4.2. Method of Screening

2.4.2.1. Preparation of Extracts

The dried extract residues were dissolved in water (aqueous extract) and ethanol (methanol, dichloromethane and hexane extracts). The methanol, dichloromethane and hexane residues were dissolved to 10 mg/ml ethanol, and the water residues to 2.5 mg/ml.

2.4.2.2. COX-1 Enzyme Preparation

Cyclooxygenase was obtained from sheep seminal vesicle microsomal fractions. Sheep seminal vesicles were homogenized at 4°C in potassium phosphate buffer with 1 mM EDTA. The homogenate was centrifuged at 4 000 g for 15 min (Avanti J-251 Centrifuge) to remove cell debris. The supernatant was recentrifuged at 17 000 g for 10 min to remove the mitochondria. Microsomes were isolated from the supernatant by centrifuging at 100 000 g for 1 h (Beckman L7-5S Centrifuge). The microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4). The concentration of the enzyme was determined using the Bio-Rad Protein Assay Kit. Aliquots of 10 µl microsome suspensions (0.3 µg protein) were pipetted into Eppendorf tubes and stored at -70°C.

2.4.2.3. COX-1 Bioassay

The assay was performed as described by JÄGER, HUTCHINGS and VAN STADEN (1996). The COX-1 enzyme (10 µl) was activated with 50 µl of co-factor solution on ice for 15 min. The co-factor solution consisted of 0.3 mg/ml L-epinephrine and 0.3 mg/ml reduced glutathione in 0.1 M tris buffer, pH 8.2. Sixty µl of the enzyme/co-factor solution were added to 20 µl of sample (20 µl of aqueous plant extracts, 2.5 µl ethanolic plant extract + 17.5 µl water or 2.5 µl indomethacin standard (20 mM) + 17.5 µl water). Extracts were tested at a final concentration of 250 µg/ml. Twenty µl of [¹⁴C] arachidonic acid (16 Ci/mol, 30 µM) were added to each sample and the assay mixture was incubated at 37°C for 10 min. The reaction was terminated by adding 10 µl of 2N HCl. A background (2.5 µl ethanol + 17.5 µl water) was kept in the ice bucket. After

incubation 4 μ l of a 0.2 mg/ml carrier solution of unlabelled prostaglandins (PGE₂:PGF₂ 1:1 v/v) were added.

The ¹⁴C-labelled prostaglandins synthesised during the assay were separated from the unmetabolized [¹⁴C] arachidonic acid by column chromatography. Silica gel in eluent 1 (hexane:dioxane:acetic acid 350:50:1 v/v/v) was packed to a height of 3 cm in Pasteur pipettes. One ml of eluent 1 was added to each of the assay mixtures and this mixture was applied to the separate columns. The arachidonic acid was eluted from the column with 4 ml of eluent 1 and discarded. The labelled prostaglandins were subsequently eluted with 3 ml of eluent 2 (ethyl acetate: methanol 85:15 v:v) into scintillation vials. Four ml of scintillation fluid were added to each vial and the radioactivity measured using a Beckman LS 6000LL scintillation counter. The percentage inhibition of the test solutions was calculated by comparing the amount of radioactivity present in the samples to that in the solvent blank, using the following formula:

$$\text{Inhibition (\%)} = \left[1 - \frac{\text{radioactivity}_{\text{sample}} - \text{radioactivity}_{\text{background}}}{\text{radioactivity}_{\text{blank}} - \text{radioactivity}_{\text{background}}} \right] \times 100$$

The same formula was applied to the indomethacin standard to determine the validity of the assay-system. Extracts were assayed in duplicate.

2.4.2.4. COX-2 Bioassay

The cyclooxygenase-2 (COX-2) assay was performed as described by NOREEN, RINGBOM, PERERA, DANIELSON and BOHLIN (1998) with slight modifications (ZSCHOCKE and VAN STADEN 2000). The assay follows the same protocol as the COX-1 assay. The purified COX-2 enzyme however, was obtained from sheep placental cotyledons, purchased from Cayman Chemicals. The enzyme (10 μ l) was activated with 50 μ l cofactor solution (0.9 mM L-epinephrine, 0.49 mM glutathione and 1 μ M hematin in 0.1 M Tris buffer, at pH of 8). This was done on ice for 5 min. The enzyme solution (60 μ l) and the sample solutions (2.5 μ l dissolved plant extract applied

to 17.5 μl distilled water) were incubated for 5 min at room temperature. Extracts were tested at a final concentration of 250 $\mu\text{g}/\text{ml}$. The reaction was started with the addition of 20 μl [^{14}C] arachidonic acid (16 Ci/mol, 30 μM) to each of the samples. The samples were incubated for 10 min at 37°C before the reaction was terminated by adding 10 μl of 2N HCl. Four μl of a 0.2 mg/ml carrier solution of unlabelled prostaglandins (PGE₂:PGF₂ 1:1 v/v) were added. COX-2 inhibition was determined as described for the COX-1 assay.

2.4.3. Results and Discussion

Both the COX-1 and the COX-2 assays follow the same protocol, which facilitates comparisons between activities of the extracts on the two enzymes (ZSCHOCKE and VAN STADEN 2000). The results were recorded as the mean percentage inhibition by the extracts of the enzymes cyclooxygenase-1 and cyclooxygenase-2 (Figure 2.1). Results for the COX-1 inhibition assay showed the aqueous and methanol extracts to have significantly lower activity. JÄGER, HUTCHINGS and VAN STADEN (1996) reported 81% inhibition by the ethanolic *S. natalensis* extracts against COX-1. However, the extracts were screened at a final concentration of 500 g/ml which is double the concentration used in this study. This would explain the difference in the results of these screenings. Only the lipophilic extracts showed significantly high levels of anti-inflammatory activity, with 80 and 71% inhibition for the dichlorormethane and hexane extracts respectively. Similar results were obtained with the COX-2 inhibition. Dichloromethane gave 91% inhibition, which was significantly higher ($p \leq 0.05$) than the other extracts. Hexane gave 82% inhibition to the COX-2 enzyme. No significant differences ($p \leq 0.05$) were found between the more polar extracts (water and methanol) which both have very low inhibition. The indomethacin controls were 54% and 60% inhibition for COX-1 and COX-2 respectively. Polar solvents are known to extract compounds that may interfere in the enzyme-based bioassays. Lipophilic compounds, which are extracted with the non-polar solvents are most likely present in the polar solvent extracts as well, though at very low concentrations. If one considers that lipophilic compounds have better absorption through the cell membrane, having good inhibition of both COX-1 and COX-2 by the dichloromethane extracts is highly desirable.

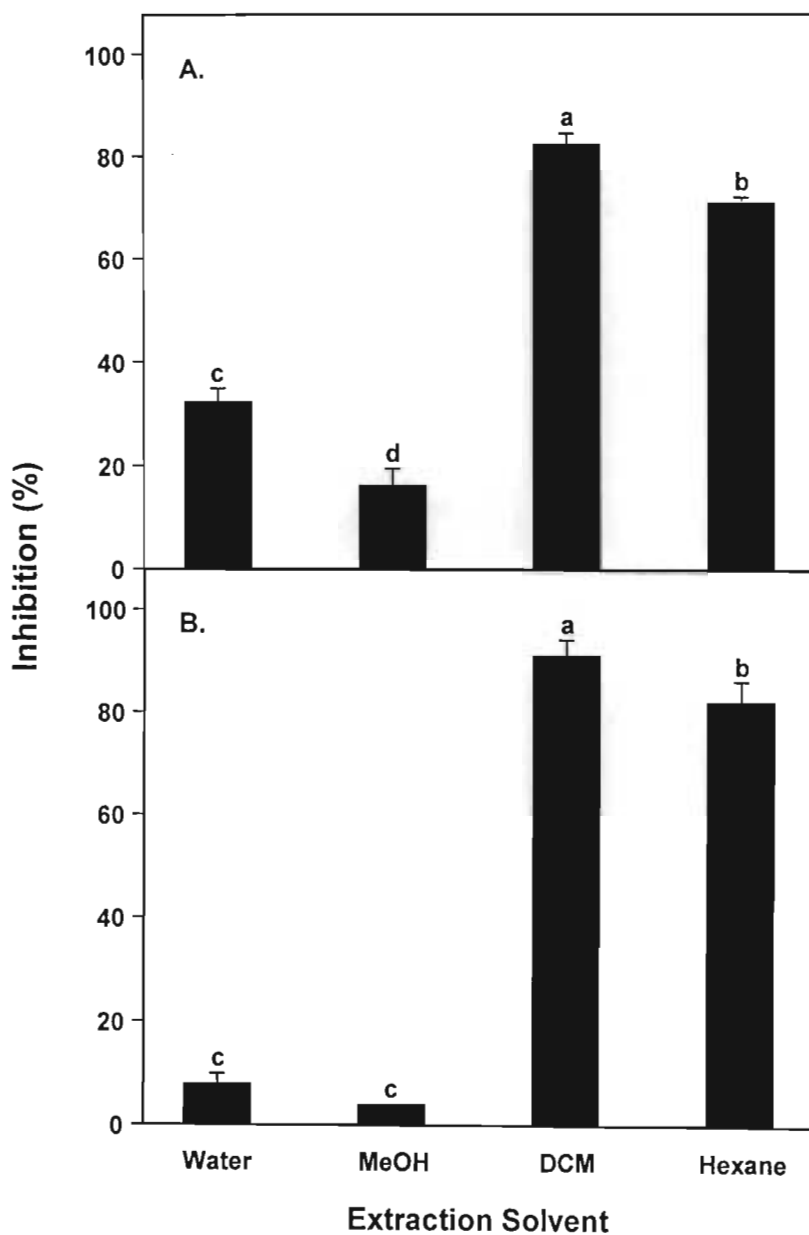


Figure 2.1. Percentage inhibition of *M. natalensis* extracts (250 µg/ml) against prostaglandin synthesis by (A) COX-1 and (B) COX-2 enzymes. Values within the same graph with the same letter are not significantly different (at 5% level of significance) from each other. DCM = dichloromethane.

In general plant material extracted with ethanol shows higher inhibitory activity than plant material extracted with water (McGAW, JÄGER and VAN STADEN 1997, LINDSEY, JÄGER, RAIDOO and VAN STADEN 1999). Although water is the most commonly used solvent, ethanol is also available to many traditional healers. Solvents like hexane and dichloromethane, which showed the highest percentage inhibition, are not easily available to the majority of traditional healers. As these solvents are toxic, if they were made available to the healers, extracts made with them would have to be dried down to remove the dichloromethane and hexane. The dried residues could be administered in the form of a powder or redissolved in an ethanol solution. However, these solvents may also extract other compounds in higher concentrations, causing the crude extracts to be toxic. Dosage is important with regard to which solvent is being used. With aqueous solvents, the dosage would have to be higher, however, this may then increase the toxicity as the concentration of possible toxic polar compounds increases. The same dosage using a dichloromethane extraction may be potentially dangerous.

2.5. Antischistosomal Screening

2.5.1. Introduction

Schistosomiasis, commonly known as "bilharziasis", is an important health issue in Third World countries found in the tropics and subtropics. It is estimated that over 200 million people in 73 countries are infected with the disease (McCULLOUGH and MOTT 1983), while a further 4-5% of the world's population are at risk of being infected (BASCH 1991; BROWN 1994). Most of these people live in rural communities that are situated near or around slow-moving water sources. The most extensively infected areas are found in Africa, China and the neotropics. According to BROWN (1994) the spreading of schistosomiasis is aided by the creation of suitable habitats for the host snails and the travelling of infected people.

Schistosomiasis is a parasitic infection that is caused by blood-flukes which are a type of trematode of the genus *Schistosoma*. There are 11 different *Schistosoma* species worldwide (BASCH 1991), with three of these species residing in South Africa

(PITCHFORD 1980; VAN WYK 1983). These are *Schistosoma haematobium* (causes urinary bilharzia), *Schistosoma mansoni* (causes intestinal bilharzia), and *Schistosoma mattheei* (primarily found in domestic livestock).

The adult worms live in the abdominal blood system of their mammalian hosts, situated in the veins associated with the intestine or those around the bladder. The adult worms are dioecious, and produce non-operate eggs, which are laid in the blood vessels of their host. Each egg contains a miracidium larva, which hatches only when the egg comes in contact with water. The miracidia are ciliated and capable of swimming. They can only survive for 48 h if an intermediate host snail is not found. Many of the *Schistosoma* species are intermediate host specific. This means that they are only compatible with a particular snail species. This compatibility is thought to be associated with genetically-based variations found in the snails and the *Schistosoma* species. Once a compatible host is located, the miracidia penetrate the snail's epithelium with the aid of histolytic secretions (BROWN 1994). Through a series of transformations, cercariae emerge from the snail's mantle collar and pseudobranch (PAN 1965). If the compatibility between the host and parasite is good, then there is a high rate of infection, with many cercariae being shed soon after penetration (BROWN 1994). The cercariae are also motile and have to find a compatible mammalian host within 48 h. Once a host has been located, the cercariae penetrates the skin and in so doing loses its tail and transforms into a schistosomula worm before entering into the blood stream of the host (STURROCK 1993).

Most research into controlling schistosomiasis has been done on developing molluscicides from South African plants (BRACKENBURY, APPLETON and THURMAN 1996; CLARK and APPLETON 1996; CLARK, APPLETON and DREWES 1996; BRACKENBURY and APPLETON 1997; BRACKENBURY, APPLETON and KAYONGA 1997; CLARK, APPLETON and KVALSVIG 1997). More recent studies have focussed on possible use of medicinal plants to treat the disease at the human stage of the cycle. SPARG, VAN STADEN and JÄGER (2000) and MØLGAARD, NIELSEN, RASMUSSEN, DRUMMOND, MAKAZA and ANDREASSEN (2001) investigated the use of southern African plant species to treat schistosomiasis *in vitro*.

2.5.2. Method of Screening

2.5.2.1. Preparation of Extracts

The aqueous residues were dissolved to 50 mg/ml with distilled water. Fresh *M. natalensis* bulbs were also macerated and extracted with distilled water. The residues were dissolved to 50 mg/ml.

2.5.2.2. Collection and Storage of Snails

Bulinus africanus (Krauss) snails were collected from the Slangspruit at Campsdrift, Pietermaritzburg. Snails were identified and confirmed with specimens housed at the Pietermaritzburg Museum. The snails were housed in tanks, which were kept under controlled conditions of aeration, 12 h light and $25 \pm 2^\circ\text{C}$. The snails were fed on freeze-dried lettuce. The snails were kept for up to 4 weeks.

2.5.2.3. In vitro Bioassay

The antischistosomiasis bioassay as described by SPARG, VAN STADEN and JÄGER (2000) was used in the screening. Infected *Bulinus africanus* snails were placed into test tubes under a 60 watt electric lamp to promote the shedding of *Schistosoma haematobium* cercariae. The cercariae were collected and subjected to a sheering stress using a syringe with a 0.8 ml needle attached. The sheering procedure was repeated approximately five times, causing the cercariae to lose their tails and thereby transforming them into schistosomula worms. The bioassay was run in microtiter plates. The culture medium (100 μl) was placed into each well. The extracts (100 μl) were placed into the first well from which two-fold serial dilutions were made. Four schistosomula worms along with 100 μl culture medium were pipetted into each of the wells containing the extract dilutions. The bioassay was incubated at 25°C for 1 h after which each well was examined to see how many of the schistosomula had survived and the fatal concentration calculated. The assay was repeated three times for each extract. Praziquantel (Sigma) and a culture medium blank were used as controls.

2.5.2.4. Culture Medium

The culture medium used was the mineral salt component of Roswell Park Memorial Institute (RPMI) 1640 media (NaCl 6 g/l; KCl 400 mg/l; Ca(NO₃)₂·4H₂O 100 mg/l; MgSO₄·7H₂O 100 mg/l; Na₂HPO₄·12H₂O 2.02 g/l; and NaHCO₃ 2 g/l) (BUTLER 1992), supplemented with 5% bovine calf serum.

2.5.3. Results and Discussion

The results of the screening for antischistosomal activity are shown in Table 2. The praziquantel (Sigma) control was lethal at a concentration of 0.001 mg/ml. Aqueous extracts from both fresh and dry bulb material of *M. natalensis* had high activity. The results show the activity to be higher than any of the plants screened previously for antischistosomal activity using this assay (SPARG, VAN STADEN and JÄGER 2000; MØLGAARD, NIELSEN, RASMUSSEN, DRUMMOND, MAKAZA and ANDREASSEN 2001). *Abrus precatorius* root extracts had the highest activity with a lethal concentration of 0.6 mg/ml (MØLGAARD, NIELSEN, RASMUSSEN, DRUMMOND, MAKAZA and ANDREASSEN 2001). *M. natalensis* bulb extracts were lethal to *S. haematobium* at 0.4 mg/ml. However, there is no report of *M. natalensis* being used traditionally for the treatment of schistosomiasis. The presence of saponins, which have potential molluscicidal activity combined with antischistosomal activity, would make *M. natalensis* very useful in controlling schistosomiasis in many of the rural communities where this disease has its biggest threat.

Table 2.2. Lethal concentrations (LC) of aqueous *M. natalensis* extracts against schistosomula of the species *Schistosoma haematobium*

Type of material	LC against schistosomula (mg/ml)
Fresh bulb	0.4
Dry bulb	0.4

2.6. Anthelmintic Screening

2.6.1. Introduction

Helminthiasis is a disease that is characterized by the presence of parasitic worms in the body. The intestines of humans and their domestic animals are infected with several types of worms. The most important types are nematodes or round worms. Most nematodes are free-living, but many species are parasitic on insects, fish, birds, fungi, plants or animals including man. Basically, they have been found to occur in almost every type of environment found on the planet (LEE 1965). Most of the nematodes and other worms that live in the intestines of man do little to impair the health of their hosts. Symptoms of their presence usually only appear in cases of heavy infestation (LEWIS and ELVIN-LEWIS 1977).

Helminthiasis is controlled by chemotherapy using broad spectrum anthelmintics (NJOKU, OKEYODE and ASUZU 1996). The more commonly used anthelmintics include the benzimidazoles (albendazole and thiabendazole) and the non-benzimidazoles (levamisole, pyrantel and ivermectin) (SIMPKIM and COLES 1981). However, problems do exist with these drugs. Continued usage can result in the development of resistance, while some of the synthetic drugs used are expensive and not always readily available (ASUZU and NJOKU 1996). There is an urgent need for new, inexpensive drugs that will not allow for easy resistance to develop. Much effort is being made to discover new anthelmintic drugs that are of plant origin (NJOKU, OKEYODE and ASUZU 1996). Anthelmintic drugs must have a wide margin of safety between its toxicity to the worms and its toxicity to the host. LEWIS and ELVIN-LEWIS (1977) stated that the anthelmintic properties of many naturally occurring products have been known since the dawn of civilizations and are still in use today. ASUZU and NJOKU (1996) believe that medicinal plants may be the most important source of new anthelmintic drugs.

SIMPKIN and COLES (1981) reviewed a number of *in vitro* test systems that have been used to test against different nematode species. *Caenorhabditis elegans* has unofficially been adopted as a convenient model to study important problems in the developmental genetics and biology of metazoans (BRENNER 1974). SIMPKIN and

COLE (1981) recommend using free-living nematodes for screening anthelmintic compounds. Screenings could be quite simple and would not require sophisticated equipment. SIMPKIN and COLES (1981) suggested that *C. elegans* be used in *in vitro* assays to screen for potential anthelmintics. McGAW, JÄGER and VAN STADEN (2000) successfully screened 24 South African medicinal plants using an *in vitro* bioassay involving the free-living nematode *C. elegans*.

2.6.2. Method of Screening

2.6.2.1. Preparation of Extracts

The aqueous, methanol, dichloromethane and hexane extract residues were dissolved to 100 mg/ml. The dichloromethane and hexane extracts were dissolved in acetone.

2.6.2.2. In Vitro Bioassay

The nematode *Caenorhabditis elegans* var. Bristol (N2) was cultured on nematode growth agar seeded with *E. coli* according to the method of BRENNER (1974). The anthelmintic assay used was the method of RASOANAIVO and RATSIMAMANGA-URVERG (1993) as described by McGAW, JÄGER and VAN STADEN (2000). Extract solutions were prepared (5 μ l dissolved extract + 495 μ l distilled water) to which 10 μ l M9 buffer (BRENNER 1974) containing approximately 100 nematodes from 7-10 day old cultures were added. The samples were incubated for 2 h at 25°C in the dark. A standard concentration of levamisole (Sigma) (5 μ g/ml = 2.5 μ l of 1 mg/ml levamisole solution + 497.5 μ l distilled water) was used as a positive control. Solvent blanks and a buffer only solution were included as negative controls. Using a dissecting microscope, the percentage of nematodes still alive was estimated, and their movement noted and compared to the controls.

2.6.3. Results and Discussion

The anthelmintic assay used is a cheap and simple assay that can be used to test for anthelmintic activity. Methanol extracts of *M. natalensis* showed good activity,

killing approximately 47% of the nematodes in the anthelmintic assay (Figure 2.2), with the remaining 53% moving slower than those of the negative control. Both the dichloromethane and the hexane extracts were significantly less active ($p \leq 0.05$) killing approximately 28% of the nematodes while the water extracts killed only 22% of the nematodes. The levamisole control (5 $\mu\text{g/ml}$) killed approximately 40% of the nematodes.

McGAW, JÄGER and VAN STADEN (2000) found that the aqueous extracts were generally more active than the ethanolic extracts. Hexane extracts were found to have low activity as well. The results from this study with *M. natalensis* extracts showed that both the methanol and the hexane extracts had higher activity against *C. elegans* than the aqueous extracts. From the results it would seem that there are both polar and non-polar compounds with anthelmintic activity in the bulbs of *M. natalensis*.

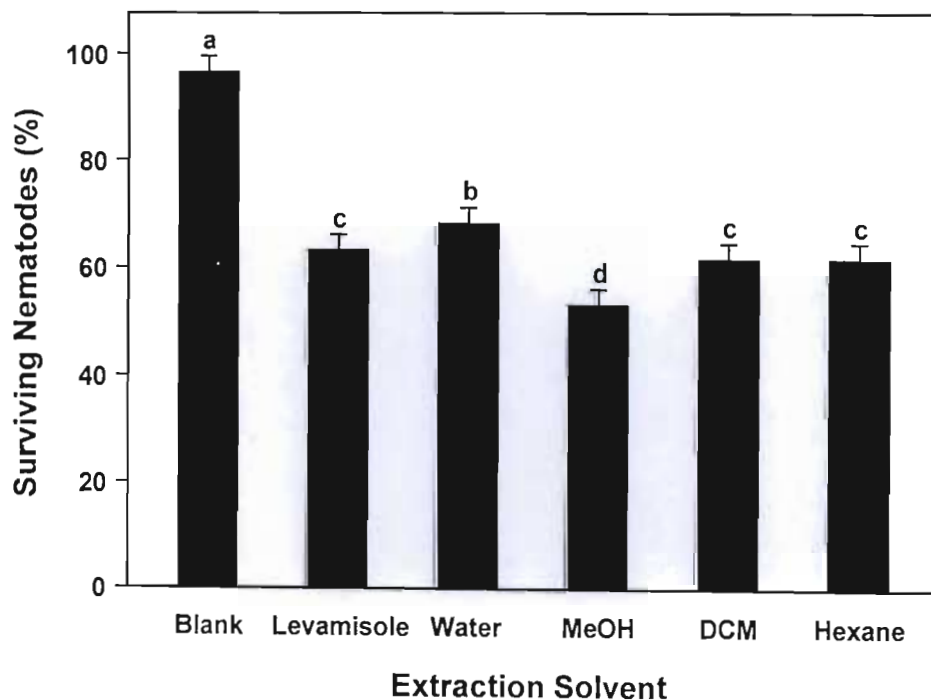


Figure 2.2. Mean percentage of *Caenorhabditis elegans* nematodes alive after being exposed to *M. natalensis* extracts for two hours. Values within the same graph with the same letter are not significantly different (at 5% level of significance) from each other. DCM = dichloromethane.

Merwillia natalensis is used traditionally to treat intestinal worms (HUTCHINGS 1989). Ethanol is used sometimes by traditional healers for extraction therefore the healers could obtain similar anthelmintic activity. However, possible oral application of such ethanol extracts would be highly dangerous if not fatal if administered to children, who often have the highest worm-load. Residues of ethanol or methanol extracts should be dissolved in water before administration or possibly taken in powder form.

2.7. Anticancer (DNA-damaging) Screening

2.7.1. Introduction

One of the dreaded illnesses that plague humans and animals is cancer. There are many forms, affecting different organs and parts of the body. In most cases, cancers are treated with anticancer drugs and chemotherapy. Although Western drugs are still used, research has focussed on discovering new potential anticancer compounds from indigenous medicinal sources. To date, large numbers of higher plants have been tested for anticancer properties and have yielded some positive results. The National Cancer Institute (NCI) have screened over 120 000 extracts from 35 000 plant species for novel anticancer agents over a period of 25 years and have made some promising discoveries (SPJUT 1985). Two successes are: taxol (*Taxus brevifolia* Nutt.) which is used to treat breast, lung and neck cancer (CRAGG 1998) and the drug alkaloids vinblastine and vincristine which are both from the Madagascan rosy periwinkle (*Catharanthus roseus*) and are used in treating leukaemia.

Many of the natural products that have demonstrated clinical utility as cancer chemotherapeutic agents, function by causing DNA damage in tumor cells (DEMAIN and SOLOMON 1986). Most of the detection systems have been developed specifically to find compounds with this mechanism of action. One such system is the biochemical induction assay (BIA) that was developed for the screening of anticancer drugs (ELESPURU and WHITE 1983). This assay is a simple, sensitive and rapid method, which has been successfully employed in the screening of microbial culture broths for anticancer agents (DEMAIN and SOLOMON 1986). The BIA is a modification on the lambda (λ) prophage induction assay, where DNA damage

releases λ prophage from its latent, repressed state in an *Escherichia coli* lysogen. Any complete virus particles that are formed when DNA damage occurs can be assessed in an overnight plaque assay (HEILEMANN and HOWARD 1964; PRICE, BUCK and LEIN 1964). Instead of virus particle, DNA damage is assessed in the BIA by the formation of the enzyme β -galactosidase. In the BIA, the λ lysogenic indicator is a special *E. coli* BR 513 strain (ATCC 33312) (WHITE, MAIESE and GREENSTEIN 1986).

2.7.2. Method of Screening

2.7.2.1. Preparation of Extracts

The dried *M. natalensis* extracts were re-dissolved to 100 mg/ml in their appropriate solvents. The dissolved extracts (10 μ l) were applied to sterile filter paper discs, which were placed onto agar assay plates.

2.7.2.2. Preparation of the Base Layer

Agar base layers were prepared using Nunc bioassay plates (243 mm x 243 mm x 5 mm) dispensed with 100 ml LBE-amp agar and allowed to set. The LBE-amp agar was made up of 15 g LBE-amp agar in 1000 ml LBE medium (Tryptone 10g; yeast extract 5 g; NaCl 10 g; 1M Tris 5 ml; distilled water 995 ml), autoclaved and allowed to cool to 50°C before adding 4 ml salt medium (distilled water 670 ml; MgSO₄·7H₂O 10 g; K₂HPO₄ 500 g; Na(NH₄)HPO₄·4H₂O 175 g; citric acid·H₂O 100 g), 10 ml 20% glucose and 1 ml of 10 mg/ml sodium ampicillin solution.

2.7.2.3. Preparation of Inoculum

The test bacteria *E. coli* BR 513 (ATCC 33312) were grown overnight in 5 ml LBE broth at 37°C under constant agitation. The culture (0.7 ml) was diluted into 35 ml LBE broth supplemented with 0.5 ml 20% glucose (filter sterilized through a 0.22 μ m filter) and 0.2 ml of a salt medium (MgSO₄·7H₂O 14.9 g/l; K₂HPO₄ 746.3 g/l; Na(NH₄)HPO₄·4H₂O 261.2 g/l; citric acid·H₂O 149.3 g/l). The diluted solution was

agitated at 37°C until an OD₆₀₀ of 0.4 was reached. The solution was then centrifuged for 10 min at 5000 rpm (3000 x g). The supernatant was discarded and the pellet resuspended in 2 ml cold, sterile distilled water to give the inoculum.

2.7.2.4. Preparation of the Assay Plates

The inoculum was added to 75 ml 1% soft agar ($\pm 50^\circ\text{C}$) and poured evenly over a prewarmed (37°C) base layer before being allowed to solidify for 10 min at room temperature.

2.7.2.5. Biochemical Induction Assay (BIA)

The dissolved extracts (10 μl) were loaded onto sterile Whatman No. 1 filter-paper discs (6 mm) before being placed onto the assay plates. 4-Nitroquinoline-1-oxide (100, 50 and 5 $\mu\text{g/ml}$) was used as a positive control. Solvent controls were also included. The assay plates were incubated at 37°C for 5 h before pouring over a 1% soft agar substrate containing 120 mg fast blue RR and 20 mg 6-bromo-2-naphthyl- β -galactosidase dissolved in 2 ml dimethyl sulfoxide. The plates were examined after 15 min for the development of red-violet zones at the sites of sample application. The presence of a red-violet zone indicates positive anticancer activity.

2.7.3. Results and Discussion

Clear red-violet zones of activity were observed around the positive controls, but no colour changes were observed for the *M. natalensis* extracts. This however, does not indicate that there is no potential anticancer activity found in *M. natalensis*. One of the problems with this assay is that there is no guarantee that active compounds found in the extracts can diffuse through the agar. If this was the case, then no colour zone would be detected. Crude extracts of *M. natalensis* may also contain other compounds that either mask the active compounds by binding to them, so rendering them inactive. According to ELESURU and WHITE (1983) the anticancer drug vinblastine, which is known to interfere with DNA, also showed no activity with this assay. Therefore, this assay may not be ideally suited for anticancer screening.

2.8. Cytotoxicity Testing

2.8.1. Introduction

Though many plants are used medicinally in the treatment of almost every ailment that affects humans and animals, many are highly cytotoxic to human and animal cells. However, this toxicity is not always a negative one. Plants that are cytotoxic have been used and are still investigated to treat tumor cells.

Hundreds, if not thousands, of plant constituents have been found to be cytotoxic against one or more tumor cells in culture. However, not all classes of cytotoxic compounds have useful antitumor activity (CORDELL, FARNSWORTH, BEECHER, SOEJARTO, KINGHORN, PEZZUTO, WALL, WANI, BROWN, ONEILL, LEWIS, TAIT and HARRIS 1993). Cytotoxicity is extremely important when novel plant drugs are being investigated, especially if these drugs are to be taken orally for internal ailments.

2.8.2. Method of Testing

The cytotoxicity testing was performed by the Department of Medical Virology, Institute of Pathology, University of Pretoria, South Africa. Extracts of *M. natalensis* were tested for cytotoxicity by exposing monolayers of secondary vervet monkey kidney (VK) cells to dilutions of the filter sterilised (0.45 µm membrane) plant extracts. Serial two-fold dilutions of the plant extracts, in serum-free Eagle's minimum essential medium (MEM) (National Institute for Virology, Johannesburg, South Africa), from a concentration of 3.9 µg/ml to 1000 µg/ml, were used for testing on 24-hour-old monolayers of VK cells. The cells were monitored visually, by light microscopy, over a period of seven days and on the seventh day tested for cytotoxicity using a tetrazolium salt reduction (MTT) assay (VAN RENSBURG, ANDERSON, MYER JOONÉ and O'SULLIVAN 1994), based on the method of HUSSAIN, NOURI and OLIVER (1993). Monolayers of cells exposed to serum-free MEM alone were used as a control.

2.8.3. Results and Discussion

Visual examination of the monolayers of VK cells treated with the *M. natalensis* extract, from 3.9 µg/ml up to 1000 µg/ml, showed gross morphological changes and cell death, suggestive of severe cytotoxicity, at all concentrations tested (Table 2.3).

Table 2.3. Survival of secondary vervet monkey kidney (VK) cells in the presence of varying concentrations of aqueous extracts of *M. natalensis*.

Extract Concentration (µg/ml)	Percentage survival of VK cells (%)
1000.0	15
500.0	16
250.0	16
125.0	17
62.5	18
31.3	42
15.6	59
7.8	62
3.9	66

These observations were confirmed by the MTT assay. This high level of cytotoxicity could possibly account for the use of this species in the treatment of tumors by traditional healers. However, such a high level of cytotoxicity could be dangerous and may not even have any antitumor activity. These levels of toxicity are cause for concern since *M. natalensis* is one of most commonly sold medicinal plants at traditional medicine markets. The toxicity is most likely attributed to the presence of saponins.

2.9. Summary

- Four solvent extracts were made from *Merwillia natalensis* bulbs. Solvents ranged in polarity (water, methanol, dichloromethane, hexane).
- All four extracts were screened for antibacterial, anti-inflammatory, anthelmintic and DNA-damaging activity. Aqueous extracts were also screened for antischistosomal activity and cytotoxicity.
- Methanol extracts had both the highest antibacterial and anthelmintic activity.
- Methanol extracts were significantly more effective ($p \leq 0.05$) than other solvent extracts against both Gram-positive and Gram-negative test bacteria (MIC = 1.6 mg/ml).
- Approximately 47% of the *Caenorhabditis elegans* nematodes were killed after being exposed for 2 h to the methanol extracts (concentration of 1 mg/ml).
- Non-polar extracts had higher anti-inflammatory activity. Dichloromethane extracts gave the highest inhibitory effect on prostaglandin synthesis by both COX-1 and COX-2 enzymes (80 and 91% inhibition respectively at a concentration of 250 $\mu\text{g/ml}$).
- Aqueous extracts of both fresh and dry material tested against *Schistosoma haematobium* gave very high activity. The crude extracts were lethal at a concentration of 0.4 mg/ml.
- All the extracts showed no DNA-damaging activity at 100 mg/ml.
- Aqueous extracts were highly cytotoxic, even at very low concentrations, which suggests that extracts could possibly have antitumor properties.
- Results show that the bulbs of *M. natalensis* have a range of pharmacological activities, which might rationalise its popularity and use as a medicinal plant.

CHAPTER 3 PHYTOCHEMICAL SCREENING

3.1. Saponins

3.1.1. Introduction

Saponins are a large group of glycosides, which are ubiquitous in plants. Their surface-active properties are what distinguish these compounds from others. They dissolve in water to form foamy solutions. These saponin-containing plants are sought after for use in household detergents. One such example is the soapwort (*Saponaria officinalis* L.), which has been widely used for centuries. Most saponins have haemolytic properties and are toxic to most cold-blooded animals. However, since these properties are not common to all saponins, they cannot be identified from other compounds on the basis of these properties (BRUNETON 1995). Saponins have been sought after in the pharmaceutical industry because some form the starting materials for the semi-synthesis of steroidal drugs. They also have pharmacological properties and are used in phytotherapy and in the cosmetic industry.

Saponins can be classified into two groups based on the nature of their aglycone. The first group consists of the steroidal saponins, which are almost exclusively present in the monocotyledonous angiosperms. The second group consists of the triterpenoid saponins, which are the most common and occur mainly in the dicotyledonous angiosperms (BRUNETON 1995). Some authors distinguish a third group called steroidal amines, which are classified by others as steroidal alkaloids (BRUNETON 1995).

Saponins have the ability to rupture erythrocytes. This has led to the development of haemolytic assays for detecting the presence of saponins in drugs or plant extracts. The haemolytic properties are generally attributed to the interaction between the saponins and the sterols of the erythrocyte membrane. As a result, there is an increase in permeability and a loss of haemoglobin. Although toxic to cold-blooded species, if taken orally by warm-blooded species, saponins have only weak toxicity (BRUNETON 1995), which is probably attributed to low absorption

rates. The toxicity towards cold-blooded species has led to the use of saponin-containing drugs to catch fish. Saponins are also highly toxic to molluscs and have been investigated as molluscicides in the control of schistosomiasis (MARSTON and HOSTETTMANN 1985; MOTT 1987; ABDEL-GAWAD, EL-SAYED and ABDEL-HAMEED 1999). More than 1000 plant species have been tested for molluscicidal activity (KLOOS and McCULLOUGH 1982). Molluscs, in particular *Bulinus* and *Biomphalaria* species, act as intermediate hosts in the life cycle of schistosomes. Many trials have been run in African countries, where the occurrence of schistosomiasis is very prevalent. *Phytolacca dodecandra* berries contain saponins with highly potent molluscicidal activity (LEMMA, HEYNEMAN and SILANGWA 1984). Another species that has potent saponins is *Swartzia madagascariensis* (BOREL and HOSTETTMANN 1987). Field trial performed in Tanzania with *S. madagascariensis*, was very successful in reducing the densities of aquatic snails (SUTER, TANNER, BOREL, HOSTETTMANN and FREYVOGEL 1986).

Saponins also have anti-inflammatory activity (HILLER 1987; LI, LEE, KANG, HYUN and WHANG 2002; KWAK, HAN, CHANG, KIM, KANG and SON 2003). Many saponins isolated from plant sources produce an inhibition of inflammation of carrageenin-induced edema. WOO, SHIN and KANG (1976) and WOO, KANG, WAGNER, SELIGMANN and CHARI (1978) showed that the saponins from different *Phytolacca* species had anti-inflammatory activity. Many different species of the genus *Phytolacca* contain saponins that have antifungal activity (MORENO and RODRIGUEZ 1981; KOBAYASHI, KOHTATSU and KAJIYAMA 1995; QUIROGA, SAMPIETRO and VATTUONE 2001; ESCALANTE, SANTECCHIA, LÓPEZ, GATTUSO, GUTIÉRREZ RAVELO, DELLE MONACHE, GONZALEZ SIERRA and ZACCHINO 2001). Triterpenoid saponins from *Chenopodium quinoa* have been reported to have antifungal activity (WOLDEMICHAEL and WINK 2001). Saponins isolated from *Panax notoginseng* exhibited an inhibitory effect on *Aphanomyces cochlioides* zoospore motility (MA, MIZUTANI, MALTERUD, LU, DUCREY and TAHARA 1999). Saponins also show antimicrobial activity (KILLEEN, MADIGAN, CONNOLLY, WALSH, CLARK, HYNES, TIMMINS, JAMES, HEADON and POWER 1998; IORIZZI, LANZOTTI, RANALLI, DEMARINO and ZOLLO 2002).

QIU, LI, XIONG, DONG, CHAI, FARNSWORTH, PEZZUTO and FONG (2000) isolated the saponin chloromaloside A from *Chlorophytum malayense*, which is highly cytotoxic. *In vitro* studies showed the steroidal saponin to have cytotoxicity against human cancer cell lines. Saponins isolated from *Camassia leichtlinii* show cytotoxic activity against human oral squamous cell carcinoma (HSC-2) cells and normal human gingival fibroblasts (KURODA, MIMAKI, HASEGAWA, YOKOSUKA, SASHIDA and SAKAGAMI 2001). Saponins isolated from *Allium chinense* have antitumor-promoting activity (BABA, OHMURA, KISHI, OKADA, SHIBATA, PENG, YAO, NISHINO and OKUYAMA 2000). *In vivo* studies on antitumor activity by *Nigella sativa* seed extracts yielded the principle bioactive compound α -hederin, a monodesmosidic triterpene saponin (KUMARA and HUAT 2001). TRUTE, GROSS, MUTSCHLER and NAHRSTEDT (1996) showed the saponin α -hederin, isolated from *Hedera helix*, to have significant antispasmodic activity.

Saponins also have antiviral activity. Triterpenoid saponins from the Fabaceae showed anti-herpes virus activity (KINJO, YOKOMIZO, HIRAKAWA, SHII, NOHARA and UYEDA 2000). Saponins from *Tieghemella heckelii* yielded antiviral activity (GOSSE, GNABRE, BATES, DISUS, NAKKIEW and HUANG 2002). A mixture of tea-seed saponins from *Camellia sinensis* var *sinensis*, inactivate human type A and B influenza viruses. However, these saponins are also toxic to the host cells and further studies need to be conducted (HAYASHI, SAGESAKA, SUZUKI and SUZUKI 1999). Saponins from soybean exhibit strong anti-HIV activity *in vitro* (NAKASHIMA, OHUBO, HONDA, TAMURA, MATSUDA and YAMAMOTO 1989).

Korean red ginseng saponins have been shown to have various effects on the spatial memory (BURESOVA and BURES 1982; JAENICKE, KIM, AHN and LEE 1991; PARK, NAM, HYAN, JIN, CHEPURNOV and CHEPUNORA 1994). KIM, JANG, SEONG, RHEN, CHO and KANG (1996) demonstrated the effects of Korean red ginseng saponins on cocaine-induced intoxication. Korean red ginseng saponins were also found to have an effect on ethanol-induced amnesia (LEE, MOON and YOU 1999).

Saponins are present in many bulbous South African plants. When working with *M. natalensis*, especially aqueous extracts, there develops persistent foam,

which is characteristic of saponins. HUTCHINGS (1989) suggested that *M. natalensis* contains saponins, however, there is no definite evidence.

3.1.2. Method of Detection

3.1.2.1. Froth Test

One hundred mg of dried *M. natalensis* bulb material were placed into a test-tube containing 10 ml distilled water. The test-tube was sealed with a rubber stopper before being vigorously shaken for 30 sec. The test-tube was left to stand vertically for 30 min, before being examined. If a froth layer greater than 1 cm existed on top of the water surface, then it could be presumed that there are saponins in the plant. However, this is just a simple test that does not prove that there are definitely saponins in the material. A further test exists, which does prove that there are definitely saponins within the bulbs of *M. natalensis*. This is the haemolysis test (LUYT, JÄGER and VAN STADEN 1999).

3.1.2.2. Haemolysis Test

This test makes use of blood agar plates to which the plant extracts are added. The blood agar plates were made up as follows: Columbia blood agar base (OXOID) (39 g) was suspended in 1 l distilled water. The agar solution was autoclaved at 121°C for 15 min, and then cooled to 50°C in a waterbath. Under aseptic conditions, 50-70 ml sterile citrated blood (100 g tri-sodium citrate in 500 ml distilled water; 15 ml per 500 ml blood) from either a sheep or cow was added. The blood agar was dispensed into sterile Petri dishes.

Wells were cut into the blood agar equidistant apart. The bottom of each well was sealed by using a 1-2 mm diameter glass rod, heated over a flame. Sealing the wells prevented the extracts from spreading beneath the blood agar. Using a Pasteur pipette, the aqueous plant extract (10 mg/ml) was added. Each well was filled. Distilled water and saponin (Sigma) (10 mg/ml) were included as negative and positive controls respectively. The plates were allowed to stand at room temperature

for one h, after which the wells were examined for zones of haemolytic activity. These zones appear as clear rings around each well.

3.1.3. Results and Discussion

After 30 min, there was a very distinct froth layer on the surface of the water (Figure 3.1A). This froth layer remained for more than 12 h. Therefore, in order to confirm the results of the froth test a haemolysis test was also conducted. Saponins are known to have haemolytic activity (WAGNER, BLADT and ZGAINSKI 1984). The results of the haemolysis test confirmed the froth test findings as very clear zones of haemolysis were observed (Figure 3.1B). The presence of saponins could explain some of the pharmacological activities shown in Section 2.

3.2. Cardiac Glycosides

3.2.1. Introduction

Cardiac glycosides are naturally occurring steroids that have a powerful stimulating action on the cardiac muscle. Although new synthetic inotropic agents are appearing on the market, cardiac glycosides of plant origin still remain the chief drugs in treating cardiac insufficiency (BRUNETON 1995). Despite the early lack of awareness of their therapeutic potential, some of these drugs were known and used for their cardiac toxicity (BRUNETON 1995). These compounds may occur in any of the plant's organs, but in most cases, at very low concentrations (BRUNETON 1995). Their chemical structures are homologous, and comprise of a steroidal aglycone (two types) and a sugar moiety, which is most often an oligosaccharide. The aglycones all have a classic, tetracyclic steroidal nucleus. The four rings (A, B, C, and D) normally have a *cis-trans-cis* configuration. Sometimes the rings are bound in a *trans-trans-cis* configuration, but this is only so in a few cases (BRUNETON 1995). The aglycone has two hydroxyl groups and a β substituent (α , β -unsaturated lactone). The lactone ring is attached at C-17 and it is the size of the lactone ring, which distinguishes the two groups of cardiac glycosides (WAGNER,

BLADT and ZGAINSKI 1984; BRUNETON 1995). Cardenolides are C₂₃ steroids containing an α , β -unsaturated γ -lactone ring (butenolide), Bufadienolides are C₂₄ homologs of cardenolides which contain a di-unsaturated δ -lactone ring (pentadienolide). The lactone ring is essential for activity, with the substituted lactones remaining biologically active even when the steroid moiety is removed (RANG and DALE 1987).

Western medicines use cardenolides as cardio-active drugs, while bufadienolides are seldom used as cardiac drugs because of their low therapeutic index and adverse side effects (RANG and DALE 1987). Cardenolides are more commonly and abundantly found in plants than bufadienolides (HEGNAUER 1970). Cardiac glycosides are known to act on the heart at different levels. They affect the force and speed of contraction, rate and electrophysiological properties of the heart. Patients with cardiac insufficiency are treated with cardiac glycoside drugs resulting in an increase in the force and speed of contraction, but a decrease in heart rate. However, there is a notably small margin between their therapeutic effect and their toxicity, which means that dosage of cardiac glycoside drugs must be administered with great care.

Bufadienolides have antiviral activity. KAMANO, SATOH, NAKAYOSH, PETTIT and SMITH (1988) reported bufadienolides to have antirhinovirus properties. KAMANO, SATOH, NAKAYOSH, PETTIT and SMITH (1988) also reported that synthetic bufadienolides, including scillarenin, are active against poliovirus. Bufadienolides have also been reported to have antitumor properties. Bufadienolides isolated from *Kalanchoe pinnata* and *Kalanchoe daigrenontiana* were shown to be antitumor-promoting (SUPRATMAN, FUJITA, AKIYAMA, HAYASHI, MURAKAMI, SAKAI, KOSHIMIZU and OHIGASHI 2001). It was suggested that the bufadienolides are potential cancer chemopreventive agents.

3.2.2. Method of Detection

The method described by JÄGER and VAN STADEN (1995) was used to screen for cardiac glycosides. One g of dried powder of *M. natalensis* bulb material

was extracted with 30 ml 53% ethanol containing 3% lead acetate under reflux for 15 min. The extract was allowed to cool before being filtered through Whatman No. 1 filter paper using a Büchner funnel. The filtered extract was then acidified with acetic acid and partitioned three times in succession with 15 ml dichloromethane. The dichloromethane phases were filtered over sodium sulphate (anhydrous) and evaporated to dryness. The residue was dissolved in 1 ml dichloromethane:methanol (1:1 v/v). The dissolved extract (30-50 μ l) was strip-loaded onto a silica gel 60 F₂₅₄ TLC plate (Merck). The plates were run in an ethyl acetate: methanol: water (81:11:8 v:v:v) solvent system. Twenty μ l proscillaridin A (1 mg/ml) (Sigma) were used as a reference compound. The TLC plate was sprayed with antimony III chloride (SbCl₃) reagent (20% SbCl₃ in ethanol) to detect bufadienolides. After spraying the plate, it was incubated at 100°C for 6 min. Bufadienolides can be viewed under visible and UV₃₆₆ nm light. The same method was used to screen for cardenolides, except that Kedde's reagent (WAGNER, BLADT and ZGAINSKI 1984) was sprayed over the plates.

3.2.3. Results and Discussion

After spraying the TLC plates with antimony trichloride reagent, and examining under UV₃₆₆ nm it was clear that there are bufadienolide-type cardiac glycosides present in the bulbs of *M. natalensis*. This confirms the reports that these kinds of glycosides may be present in this species. It is clear that there are more than one kind of bufadienolide found in the bulbs, as there are a number of bands on the TLC plate. One of the bands matches the reference bufadienolide, proscillaridin A (Figure 3.2).

No cardiac glycoside bands were detected after spraying with Kedde's reagent. Kedde's reagent detects the presence of the cardenolide unsaturated lactone ring. The lack of bands indicates that there are no cardenolides present in the bulbs of *M. natalensis*. Since the bulbs were shown to contain cardiac glycosides, extreme care should be taken in using this species in traditional medicines. If administered incorrectly, it could have serious if not fatal consequences.

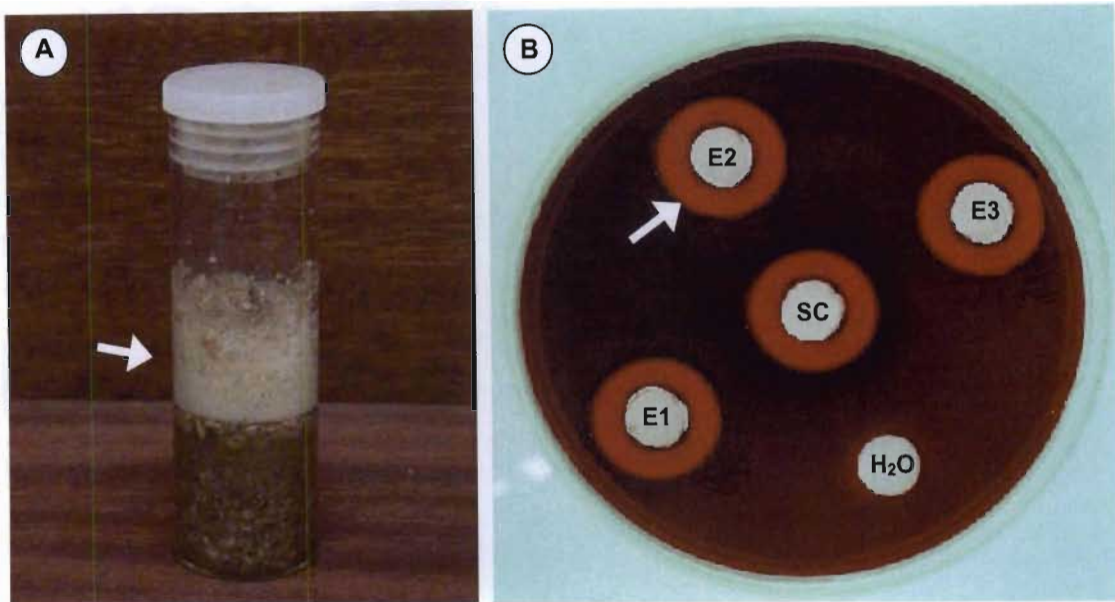


Figure 3.1. (A) Pill vial containing dried *M. natalensis* material in distilled water. The arrow points to the froth layer that resulted from shaking, indicating the possible presence of saponins. (B) Blood agar plate. The arrow points to the zones of ruptured erythrocytes (haemolysis test), which confirms the presence of saponins within the bulbs of *M. natalensis*. SC = saponin control, H₂O = water control and E1-3 = *M. natalensis* extracts 1, 2 and 3.

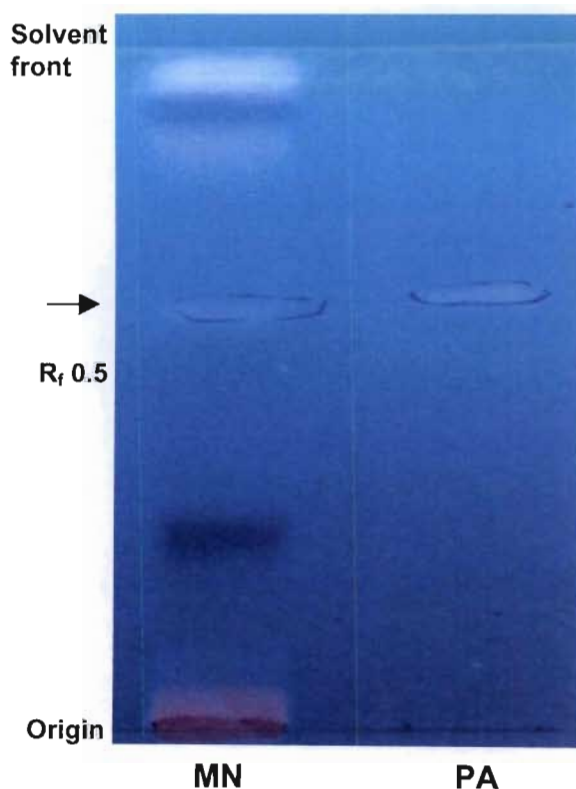


Figure 3.2. TLC plate developed with ethyl acetate: methanol: water (81:11:8 v:v:v) solvent system. The plate was stained with antimony trichloride reagent and viewed under UV₃₆₆ nm. MN = *M. natalensis* bulb extract. PA = Proscillaridin A (reference bufadienolide). Florescent bands indicate bufadienolides. Arrow indicates bufadienolide from *M. natalensis* with same R_f as the reference compound.

3.3. Alkaloids

3.3.1. Introduction

In plants, alkaloids occur as soluble salts such as citrates, malates, tartrates, meconates, isobutyrate and benzoates. They may also occur in combination with tannins (BRUNETON 1995). It has been estimated that 15-20% of vascular plants contain alkaloids. Although alkaloids are widely distributed in the plant kingdom, there are certain groups that are characteristically devoid of them (FARNSWORTH 1966). Since it is possible to obtain false-positive results when screening for alkaloids, it has been suggested that only 9-10% of vascular plant species contain alkaloids (FARNSWORTH 1966). False-positive reactions can most often be attributed to the presence of proteins found within the plant material.

In most cases, alkaloids are found in the peripheral tissues of plants, i.e. external layers of the bark of stems and roots. They are sometimes found in the integument of seeds. Most alkaloids have antimetabolite properties and therefore compartmentalized storage is necessary. They are mainly stored in cell vacuoles, which are sometimes specialized (laticiferous). Although they are stored in the vacuoles, alkaloid synthesis most often occurs in other specific sites such as growing roots, laticiferous cells or chloroplasts. The alkaloids are then transported to the storage sites (BRUNETON 1995). Most of the alkaloids found in plants are derivatives of tertiary amines, while others contain primary, secondary or quaternary nitrogens (WAGNER, BLADT and ZGAINSKI 1984). The basicity of alkaloids varies considerably, depending on which of the four types are being represented (WAGNER, BLADT and ZGAINSKI 1984; BRUNETON 1995).

Alkaloids have a wide range of pharmacological uses. Acetone extracts from leaves of *Murraya koenigii* revealed carbazole alkaloids, which have antimicrobial, anti-inflammatory, and antioxidant activity (RAMSEWAK, NAIR, STRASBURG, DEWITT and NITISS 1999). These alkaloids also have mosquitocidal properties. NAVARRO and DELGADO (1999) reported that alkaloids from *Bocconia arborea* have both antibacterial and antifungal activity. The isolated alkaloids were active against both Gram-positive and Gram-negative bacterial strains. The alkaloids were

also active against the fungus *Candida albicans*. Alkaloids have also been reported to have anti-inflammatory activity (WHITEHOUSE, FAIRLIE and THONG 1994). GUHA, DAWN, DUTTA, CHAKRABORTY and PAIN (1999) reported anti-inflammatory activity from alkaloid extracts of plants used in homeopathic medicines. Extracts were also reported to be extremely potent cardiotoxins. Naturally occurring bisbenzylisoquinoline alkaloids were reported to be cytotoxic. Bisbenzylisoquinoline alkaloids isolated from nine different plant species showed antiplasmodial activity and were cytotoxic to human KB cells (ANGERHOFER, GUINAUDEAU, WONGPANICH, PEZZUTO and CORDELL 1999).

Alkaloids were reported to be present in *M. natalensis* (= *Scilla natalensis*) (RAFFAUF 1996). However, the same reference reported that *S. natalensis* did not contain alkaloids. The reason for this contradiction may be attributed to the ongoing name changing and shuffling of the genus *Scilla*. As mentioned in Section 1.5 the species *S. kraussii* and *S. dracomontana* were once placed under the species *S. natalensis*. RAFFAUF (1996) also placed *S. natalensis* under the family Liliaceae instead of the family Hyacinthaceae, which indicates the confusion of the genus.

3.3.2. Method of Detection

Alkaloids are not always easily detected and therefore, three extraction methods were used to screen for their presence using Dragendorff Reagent.

3.3.2.1. Method A

Two g of dried *M. natalensis* bulb material were ground in a mortar with 2 ml 10% ammonia solution for 1-2 min. Seven g basic aluminum oxide were added and mixed thoroughly with the plant material. The mixture was packed loosely into a glass column (1.5 x 20 cm). Ten ml chloroform were added. The column was eluted with an additional 5 ml CHCl_3 and the eluate collected. The eluate was evaporated to 1 ml and 20 μl strip-loaded onto a silica gel plate (Merck). The plates were run in an ethyl acetate: methanol: water (100:13.5:10 v:v:v) solvent system and sprayed with Dragendorff reagent (WAGNER, BLADT and ZGAINSKI 1984). The plates were examined under visible light for brown or orange-brown zones.

3.3.2.2. Method B

Two g of dried *M. natalensis* bulb material were mixed thoroughly with 2 ml 10% ammonia solution before being extracted for 10 min with 10 ml methanol under reflux. The extract was filtered under vacuum through Whatman No. 1 filter paper and the filtrate collected and concentrated to 1 ml through evaporation. Twenty μ l were strip-loaded onto silica gel plates (Merck) and run in ethyl acetate: methanol: water (100:13.5:10 v:v:v) and toluene: ethyl acetate: diethylamine (70:20:10 v:v:v) solvent systems. The plates were sprayed with Dragendorff reagent (WAGNER, BLADT and ZGAINSKI 1984) and examined under visible light for brown or orange-brown zones.

3.3.2.3. Method C

Two g of dried *M. natalensis* bulb material were extracted under constant stirring with 20 ml 96% ethanol for 30 min. The extract was filtered under vacuum through Whatman No. 1 filter paper and 15 ml water added. The ethanol was removed carefully by evaporation using a hot plate. Five ml 2 N NaOH were added to basify the extract. The basic extract was partitioned with 10 ml dichloromethane. The organic phase was collected and filtered through Whatman No. 1 filter paper before being partitioned against 2 ml 0.1 N HCl. The aqueous phase was divided in two and a few drops of Dragendorff and Meyer's reagent (WAGNER, BLADT and ZGAINSKI 1987) were added respectively. The extracts were observed for a precipitate.

3.3.3. Results and Discussion

In all cases no distinct brown or orange-brown zones were noted. Neither were any white precipitates observed with the Meyer's reagent. According to FARNSWORTH (1966), the presence of alkaloids in plant material can vary depending on the age, climate, habitat, plant part tested, season, time of harvest, chemical races of plants and the sensitivity of the alkaloid type to the reagents used. The plant material used had not been stored for a long period of time and so any alkaloids that were present should still be in the material. Therefore it can be

concluded that there are no detectable levels of alkaloids present in the bulbs of *M. natalensis*.

3.4. Tannins

3.4.1. Introduction

Tannins can be placed into two groups on the basis of their phenolic constituents. The first group is the hydrolysable tannins or pyrogallol tannins, which are yellow-brown amorphous substances. These tannins form colloidal dispersions after dissolving in hot water. Tannins are very astringent and have the ability to tan hide. They are esters that can be hydrolyzed by boiling with dilute acids and produce phenolic compounds that are usually derivatives of gallic acid and a sugar. The second group is known as condensed tannins or phlobatannins. These are polymers of phenolic compounds that are related to the flavonoids. They are similar in general properties to the hydrolysable tannins, however, these tannins are not very soluble in water. Insoluble polymers known as phlobaphenes are formed once these tannins are treated with boiling dilute acid.

Tannins like the previously discussed phytochemicals, have been reported to have pharmacological properties. Tannin isolated from the fruits of *Punica granatum* showed antibacterial activity when tested against *Staphylococcus aureus* (MACHADO, LEAL, AMARAL, DOSSANTOS, DASILVA and KUSTER 2002). In an evaluation of tea-polyphenols, HASHIMOTO, ONO, MASUOKA, ITO, SAKATA, SHIMIZU, NONAKA, NISHIOKA and NOHARA (2003) reported tannins to have an antioxidative effect *in vitro*. WANG, CHEN and YANG (2002) found the tannin Cuphiin D-1 to have antitumor activity. The activity was reported in both *in vitro* and *in vivo* studies. Tannins have also been shown to have antiviral activity. Casuarinin, a tannin isolated from the bark of *Terminalia arjuna*, was active against Herpes simplex virus type-2 (CHENG, LIN and LIN 2002).

3.4.2. Method of Detection

Tannins were screened for, by using the gelatin-salt block test (WILSON and MERRIL 1931). Five hundred mg of dried powdered *M. natalensis* bulb material were extracted for 30 min with 5 ml distilled water, using an ultrasound bath. The extract was filtered under vacuum through Whatman No. 1 filter paper before 1-2 drops of 10% sodium chloride solution was added. This is done to eliminate any non-tannin compounds that may give a false-positive result. The extract was refiltered through Whatman No. 1 filter paper and the filtrate collected. Four drops of the filtrate were placed into four wells in a porcelain cavity plate. One drop of 1% gelatin solution was added to one of the wells and observed for a precipitate. One drop of 1% gelatin solution and one drop of 10% sodium chloride were added to another of the extract wells and observed for a precipitate. One drop of 10% ferric chloride solution was added to a third extract well and observed for colour change.

3.4.3. Results and Discussion

The development of a precipitate with the addition of gelatin or gelatin and sodium chloride is indicative of the presence of tannins. The basis for the reaction is that tannins precipitate proteins. In this case the protein was gelatin. The addition of sodium chloride makes the reaction more sensitive by enhancing the "salting out" of the protein-tannin complex. Sodium chloride was used as the control. If a precipitate is only observed in the well containing NaCl, then the results would be a false-positive. Ferric chloride is used to confirm a positive test by inducing a precipitate colour change to blue, green or green-blue. This colour change is the result of a reaction between ferric chloride and the phenolic functions of the tannins. If the extracts contained phenolic or polyphenolic compounds other than tannins, a negative gelatin-salt test would be observed, but there would be a colour change with the addition of ferric chloride. No precipitate was observed with the addition of gelatin or sodium chloride. The addition of ferric chloride did not cause a colour change. Although there are no documented indications that there are tannins present in the bulbs of *M. natalensis*, the lack of tannins had never been confirmed. A negative result from this screening indicates that there are no or very little tannins in the bulbs of *M. natalensis*.

3.5. Summary

- The bulbs of *M. natalensis* were screened for the following phytochemicals: saponins, cardiac glycosides, alkaloids and tannins.
- Fresh and dry material were screened for saponins using the froth and haemolysis tests.
- Positive results were obtained for both tests. After shaking the material in water, a layer of froth that remained for 12 h, was observed.
- The haemolysis test resulted in clear zones encircling the extracts. These zones were as a result of the saponins causing the erythrocytes to rupture.
- Dry material was screened for both cardenolides and bufadienolides.
- No unsaturated lactone ring was detected with Kedde's reagent, which indicates that there are no cardenolides present within the bulbs of *M. natalensis*.
- Bufadienolide type cardiac glycosides were detected with antimony trichloride stain reagent. One of the TLC bands matched chromatographically the reference bufadienolide, proscillaridin A.
- No alkaloids were detected within the bulbs of *M. natalensis* using both Dragendorff and Meyer's stain reagents.
- No tannins were precipitated out using the gellatin-salt block test.
- It can be concluded that saponins and bufadienolides are present in the bulbs of *M. natalensis*. The pharmacological activity shown in Section 2 may be a result of these types of phytochemicals. Both alkaloids and tannins were not detected in the bulbs with the techniques used.

CHAPTER 4 GROWTH ANALYSIS OF MATERIAL COLLECTED FROM FIELD TRIALS

4.1. Introduction

Since the beginning of recorded history, approximately 40 000 plant species that are used for ethnomedical purposes have been collected and gathered from the wild instead of being grown in cultivated fields (MÁTHÉ 1988). In recent times, people have become more aware of the need to protect plant genetic resources especially now when there is an increase in the destruction of natural ecosystems. The necessity to ensure a continued supply of plants and plant products has led to the introduction of wild species into cultivation. In a South African context, the increasing demand for medicinal plants has resulted in over-exploitation of many of the wild populations (MANDER, MANDER and BREEN 1996). There is a growing need for sustainable use of natural resources. However, with the increase in non-specialist gatherers and commercial traders, the demand for medicinal plants is already too large to be met by sustainable harvesting. VAN STADEN (1999) mentioned that the only real solution would be to develop medicinal plants as crops through small-scale farming. Cultivation of medicinal plants would not only lead to the sustainable development and protection of natural resources, but also to assist in generating employment through small entrepreneurs. The successful domestication of wild medicinal plant species suggests that there would be an improvement in the quality of the raw, crude plant extracts. This would enable the standardization of the content of the active ingredients and homogeneous plant populations necessary for modern production, processing and utilization (MÁTHÉ 1988).

The introduction of medicinal plants into cultivation is not something new. Many species have been cultivated by different ancient civilizations. As early as the 16th century, medicinal plants have been cultivated as cash crops. However, the movement of medicinal plants from natural habitats to cultivated fields is not as simple as transplanting the plants into a new location. According to BERNATH (1986) medicinal plants have coevolved with their natural ecosystems. Any changes in the growth environments of the plant, such as those associated with transplanting from a wild

ecosystem to a cultivated field, can result in modifications in plant growth, development, and natural product content. MÁTHÉ (1988) stated that the problems of introduction are similar whether the material being transplanted is from cultivation or from a wild population. Transplanted plants must acclimatize to the new environment, which is associated with a new set of abiotic and biotic ecological factors that may exert significant selection pressures that are different from those of the native habitat.

The domestication of wild species requires the manipulation of the field ecology to match the environmental requirements that are necessary for the plants to grow and reproduce. In terms of medicinal plants, the ecological goal would be to eliminate the differences between the wild and cultivated habitats to prevent any detrimental affect on the production of economically important natural products (MÁTHÉ 1988). Most assumptions suggest that the introduction of a plant to a new habitat would have a detrimental affect on the economic production of the species by decreasing growth and the chemical yield, however MÁTHÉ (1988) suggests that this can be prevented. It is often possible to adjust the planting time, soil conditions and several other aspects of cultivation to mimic the natural system. Various species are adapted to specific phytogeographical systems and any attempt in trying to introduce them into different climatic regions would fail. Factors such as the time of frost, water availability, day length, and growing season temperatures can determine the suitability of a new location for specific species.

Although the cultivation of medicinal plants has been suggested before, there remains a lack of understanding with respect to the cultivation and the economics of producing medicinal plants by small-scale farmers. MANDER, MANDER and BREEN (1996) suggested that it is this lack of understanding that is one of the most limiting factors in the commercialization of indigenous medicinal plants. JÄGER and VAN STADEN (2000) mentioned the need for basic information on how to grow the plants, planting to harvest periods, yield, expected financial return, storage and shelf-life are needed before small-scale farmers can be expected to venture into cultivating medicinal plants. It is essential that field trials be conducted in order to establish this information. One of the problems with cultivation of medicinal plants is obtaining material to cultivate. There are problems associated with the availability of seed stocks and the harvesting of seed from the wild (JÄGER and VAN STADEN 2000). Vegetative propagation is one

possible solution (MANDER, MANDER, CROUCH, McKEAN and NICHOLS 1995), while micropropagation is another. Micropropagation protocols have been established for many South African medicinal plant species including the *Scilla* species (APPLETON and VAN STADEN 1995; McCARTAN and VAN STADEN 1998; RABE and VAN STADEN 1999; ZSCHOCKE and VAN STADEN 2000). As mentioned by JÄGER and VAN STADEN (2000), sophisticated facilities are not required for hardening off microplants. It is possible then for small-scale farmers to take plants directly from tissue culture and acclimatize them themselves. This would reduce the price of the stock material making it more feasible for the farmers.

4.2. Methodology

4.2.1. Cultivation Sites

Two sites in South Africa were selected to cultivate *M. natalensis* bulbs as a crop on a small-scale basis. The two sites selected were the smallholding H₂A Botanicals and the premises at the University of Fort Hare (Figure 4.1).

4.2.1.1. H₂A Botanicals

H₂A Botanicals is a private farm specializing in growing cash crop vegetables. The farm is situated near Hillcrest in the province of KwaZulu-Natal, South Africa. The geography of the region is “hilly” terrain with steep valleys (Figure 4.2C). It is positioned approximately 29° 47' S and 30° 50' E at an altitude of 488 m above sea level. It is situated in the “rain belt” and has a high occurrence of mist. The mean annual rainfall for the area is over 1000 mm. Temperatures range from 18-26°C in summer to 9-21°C during winter.

The plants used were taken directly from tissue culture to be hardened off in seedling trays under 80% shade cloth (Figure 4.2A). The soil mixture used in the hardening off process was 33% Umgeni River sand and 66% Gromor® (National Plant Food) Seedling Mix. After three weeks the seedlings were placed under 30% shade cloth. Once the seedlings had grown large enough, they were removed from the trays and repotted into seedling bags with the same soil mix (Figure 4.2B).

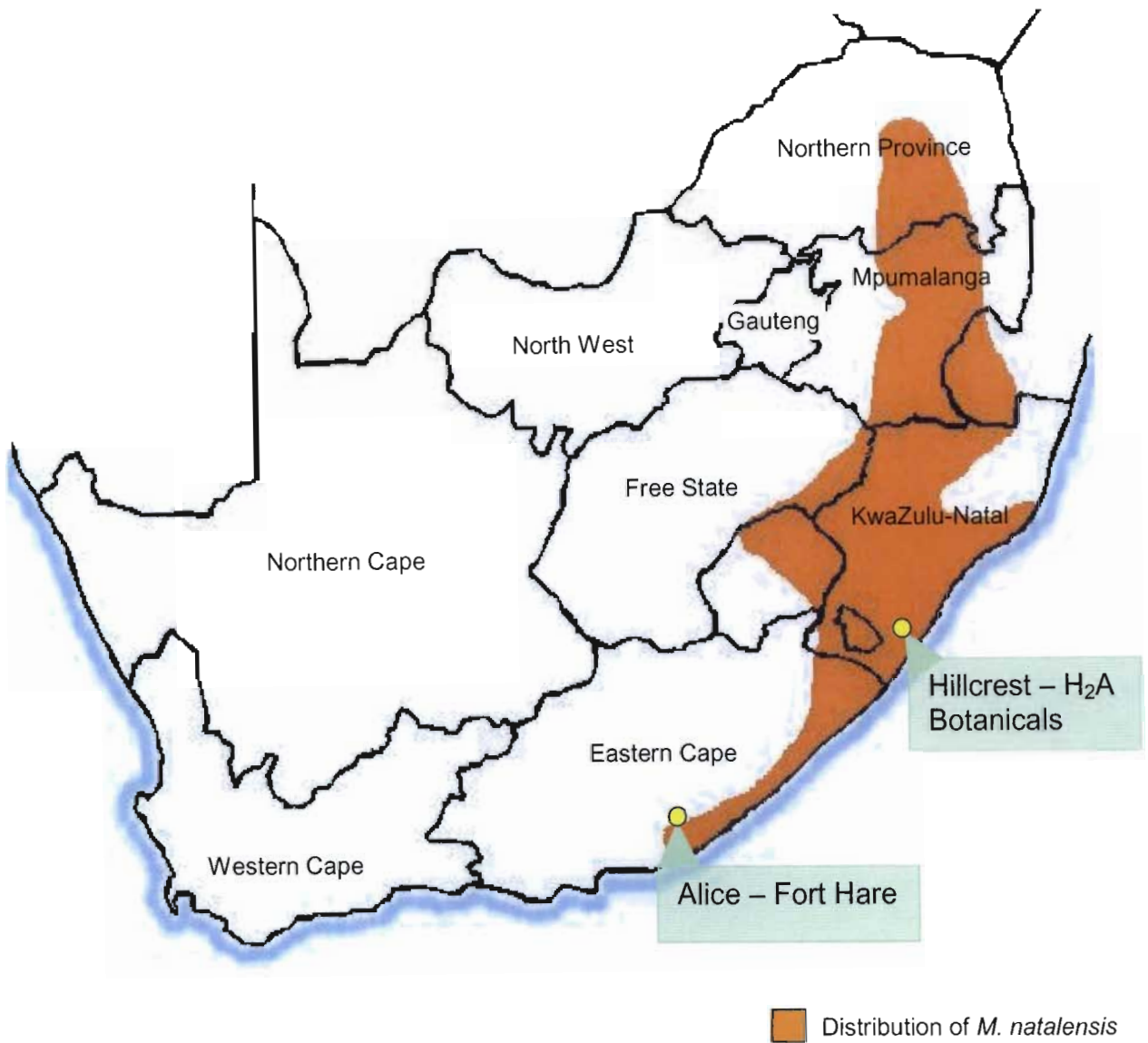


Figure 4.1. Provincial map of South Africa showing the distribution of wild populations of *M. natalensis* and the two cultivation sites, H₂A Botanicals and the University of Fort Hare where field trials were conducted.

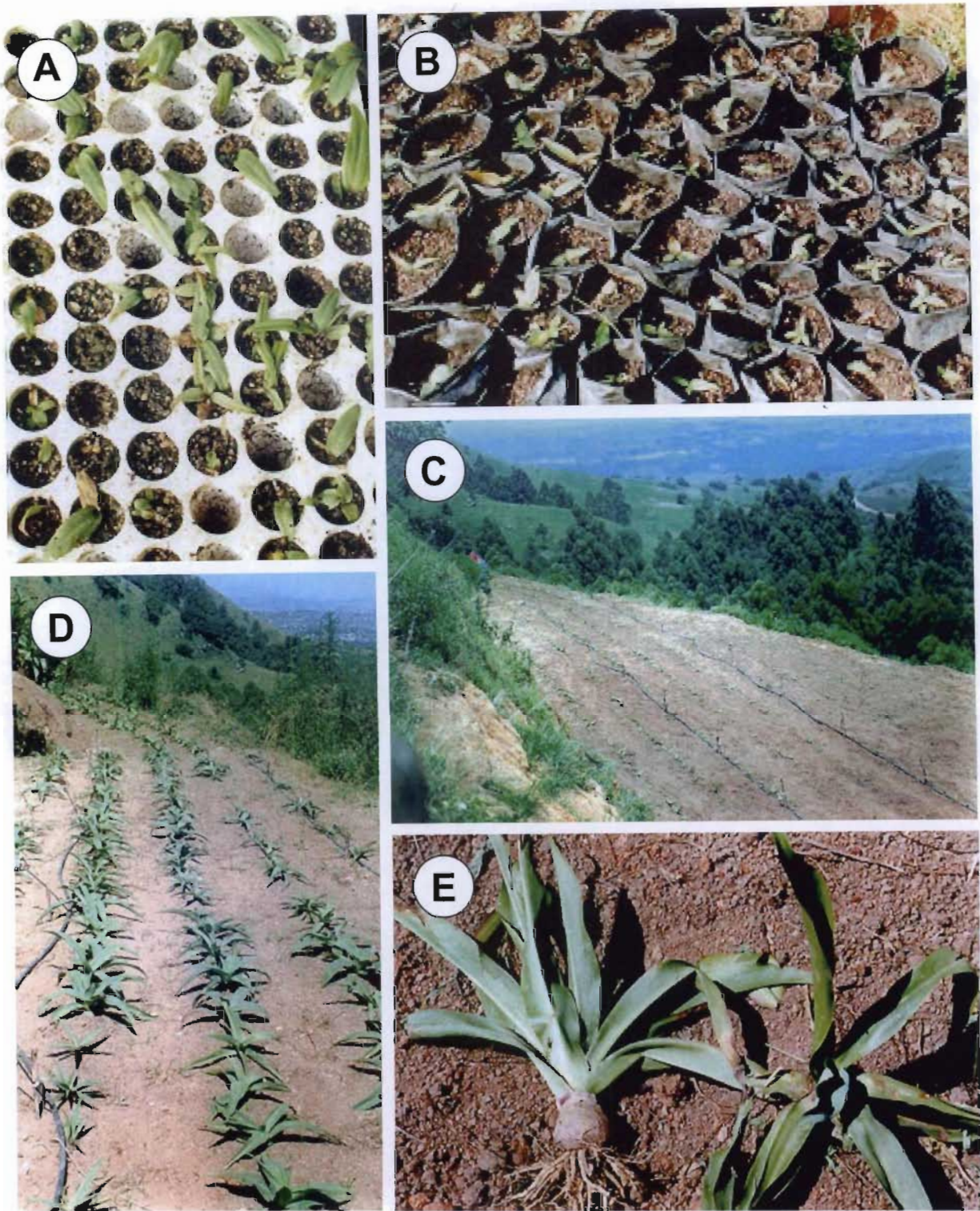


Figure 4.2. (A) *M. natalensis* bulblets being acclimatized in seedling trays under 80% shade cloth at H₂A Botanicals. (B) Young plants were put into bags after acclimatized to allow the plants to establish and grow before planting out into the field. (C) Overview of the terraced site at H₂A Botanicals where plants were planted out. (D) Rows of *M. natalensis* plants growing in the irrigation and fertilizer plot. (E) Bulbs harvested after two years of growing in the field.

Organic fertilizer, R.U.M. (Pvt) Ltd., was applied as a soil drench. The bagged young plants were placed under 20% shade cloth until planting out into the fields. Field trial planting was done in September 2000.

4.2.1.2. *Fort Hare*

The University of Fort Hare is situated outside the town of Alice in the Eastern Cape, South Africa. Positioned 32° 47' S and 26° 51' E, at an altitude of 520 m above sea level, it is outside of the "eastern coast rain belt". The mean annual precipitation for the area is 612 mm. Temperatures range from 16-28°C in the summer months to 6-20°C during the winter months.

The young *M. natalensis* plantlets were hardened off from tissue culture at the University of Natal, Pietermaritzburg. The seedlings were hardened off in seedling trays in a mist house under high humidity. Fully hardened off plants were sent to Fort Hare to be planted out in the fields. Field trial planting out was done in mid-October 1999.

4.2.2. *Cultivation Methods*

Cultivation was carried out on three plots. The first plot was the control treatment, which received no fertilizer additives or supplementary irrigation. The plants were grown in tilled topsoil and relied on rain for their water source. The second plot was fertilized only and was not irrigated (referred to as the fertilizer treatment). The fertilizer used at the H₂A Botanicals site was a mixture of composted horse and chicken manure at a ratio of 2:1. The fertilizer used at the Fort Hare was inorganic fertilizer NPK 232(22) (Nitrochem). The third plot was irrigated and fertilized (referred to as the irrigation treatment). The plants were planted approximately 30 cm apart in rows with 50 cm spacing between rows.

4.2.3. *Harvesting*

Twenty bulbs were removed from the initial planting out batches, weighed and dried to form the initial samples. After six months of growing in the field 20 plants

from each of the three treatments were harvested and fresh weights of the bulbs recorded. Due to harvesting being done during summer and then again after winter, the total fresh weights of the plants were not used for growth analysis as leaf senescence affects the results. Instead bulb weight was analysed as the growth variable. Harvesting was then done on a six-monthly basis for a period of two years. Average dry weights and standard errors of the bulbs were calculated and compared using Fisher's pairwise comparisons at 5% level of significance.

4.2.4. *Drying and Storage*

The harvested bulbs were dried in ovens at 50°C for 72 h, after which dry weights were recorded. Bulbs were grouped into fives to form four sample groups. The five bulbs were ground together and stored in honey jars in the dark at 24°C. This was done for each of the treatments for each harvest from both H₂A Botanicals and Fort Hare.

4.3. Results and Discussion

4.3.1. *H₂A Botanicals*

The young *M. natalensis* plantlets were found to harden-off very successfully using very basic and economical methods. With the use of different shade cloth densities, the plantlets can be hardened off directly from tissue culture with 83% survival. This is important in terms of providing stock plants for small-scale farmers. Plants can be obtained directly from tissue culture and hardened off by the farmers themselves at low cost. This would also reduce the cost of the stock plantlets as the tissue culture supplier would not have to harden the plants off before selling them.

One of the problems that were noticed with many of the plants growing in the field plots was chlorosis of the leaves. Many of the leaves showed signs of leaf yellowing. The cause of the chlorosis was linked to a combination of chicken manure and excessive watering. The addition of chicken manure resulted in a change in soil pH from 5 to almost 8. During the summer months, excessive rainfall and poor run-off or drainage resulted in water logging. *M. natalensis* are found mainly in slightly acidic

soils that are usually well drained. Once the chicken manure was broken down and leached from the system and the pH returned to around 5, the chlorosis disappeared and the plants had a more healthy appearance. The leaves of *M. natalensis* are known to be infected by the badnavirus (GEORGE, BANDU and VAN STADEN 1999), which is a retrovirus that infects many horticultural crops. However, this virus was not observed on the leaves of any of the harvested material.

The average dry weight of the bulbs increased exponentially once planted out for cultivation. Results from the control plot indicated that the relative growth rate (RGR) increased sharply over the period of acclimatization and establishment to six months (March 2001) of growing in the field (Figure 4.3). The RGR decreased sharply over the next six months (March 2001 -September 2001). This decrease occurred during the months April to August, which are the winter months in the southern Hemisphere during which the eastern half of South Africa experiences very low rainfall. Like most monocotyledons, especially bulbous species, *M. natalensis* follows a seasonal growth pattern. During the dry winter months the leaves senesce, and the bulbs resprouts in the following spring as seen in the picture taken of the bulbs in September 2001 (Figure 4.3). The bulbs remain in a state of dormancy, using what nutrients and water it has in reserve to maintain the basic physiological systems needed to survive the winter months. During these months very little growth occurs which is evidenced by the average dry weights (Figure 4.3). No significant differences ($p \leq 0.05$) were found between average dry weights of the harvests March 2001 and September 2001, for plants growing in the field for six and 12 months respectively. No significant differences ($p \leq 0.05$) were found between average dry weights of the 18 month (March 2002) and 24 month (September 2002) harvests. Growth did increase over the summer season between September 2001 and March 2002. However, the RGR although increasing, was not as pronounced as during the first growing season.

Bulbs that had been growing in the field and received no irrigation or fertilizer other than from natural sources reached almost marketable size (± 300 g fresh weight) after only two years in the field. After 24 months cultivation, the average dry weight of bulbs grown under these conditions was 38.4 g (Figure 4.3). The average dry weight of the bulbs grown in the fertilizer treatment (Figure 4.4) and the irrigation

treatment (Figure 4.5) were not significantly different ($p \leq 0.05$) from the control treatment (Figure 4.3). The average dry weights of the bulbs growing in fertilizer treatment and irrigation treatment after 24 months were 36.5 g and 37.2 g respectively, which were lower than the average dry weight of the bulbs from the control treatment. The plants grown with fertilizer had similar growth trends (Figure 4.4) as those from the control treatment. It is very clear then that there were no relationships between fertilizer and bulb weight. Whether fertilizer is added to the soil or not did not seem to have an effect on the size of the bulbs or the rate of growth. However, the bulbs harvested from the irrigation treatment after 12 months (September 2001) were significantly different ($p \leq 0.05$) from those harvested in March of the same year (Figure 4.5). The difference in average dry weight shows that the bulbs actually increased in weight over the winter months. Bulbs harvested from the irrigation treatment still had leaves as seen in the photograph of the September 2001 harvest (Figure 4.5). The irrigation treatment was irrigated during the winter months between March 2001 and September 2001, which seems to have caused the plants to readjust their annual rhythm. This adjustment resulted in the plant leaves not senescing and the plants continuing to grow as they would do during summer. However, this adjustment only occurred in the first year in the field. The second year the leaves senesced at the start of winter and the average dry weight of the bulbs showed no significant difference ($p \leq 0.05$) before and after winter.

Analysis of the effect that the time of harvest had on growth was highly significant ($p < 0.001$). This would be expected as growth is relative to time. The type of treatment had no significant effect on the dry weight of the bulbs ($p = 0.249$) although it was shown that irrigation can effect growth while bulbs are still young. The interaction between the treatment and the time of harvest was not significant ($p = 0.140$).

One of the noticeable differences between treatments was the number of plants that flowered. After 24 months of growing in the field, 10% of the plants harvested from the control treatment were flowering. Only 15% of the plants harvested from the fertilizer treatment were flowering while 45% of the plants harvested from the irrigation treatment were flowering. It seems that the continued growth during winter 'matured' the plants. This could be important if one also wants

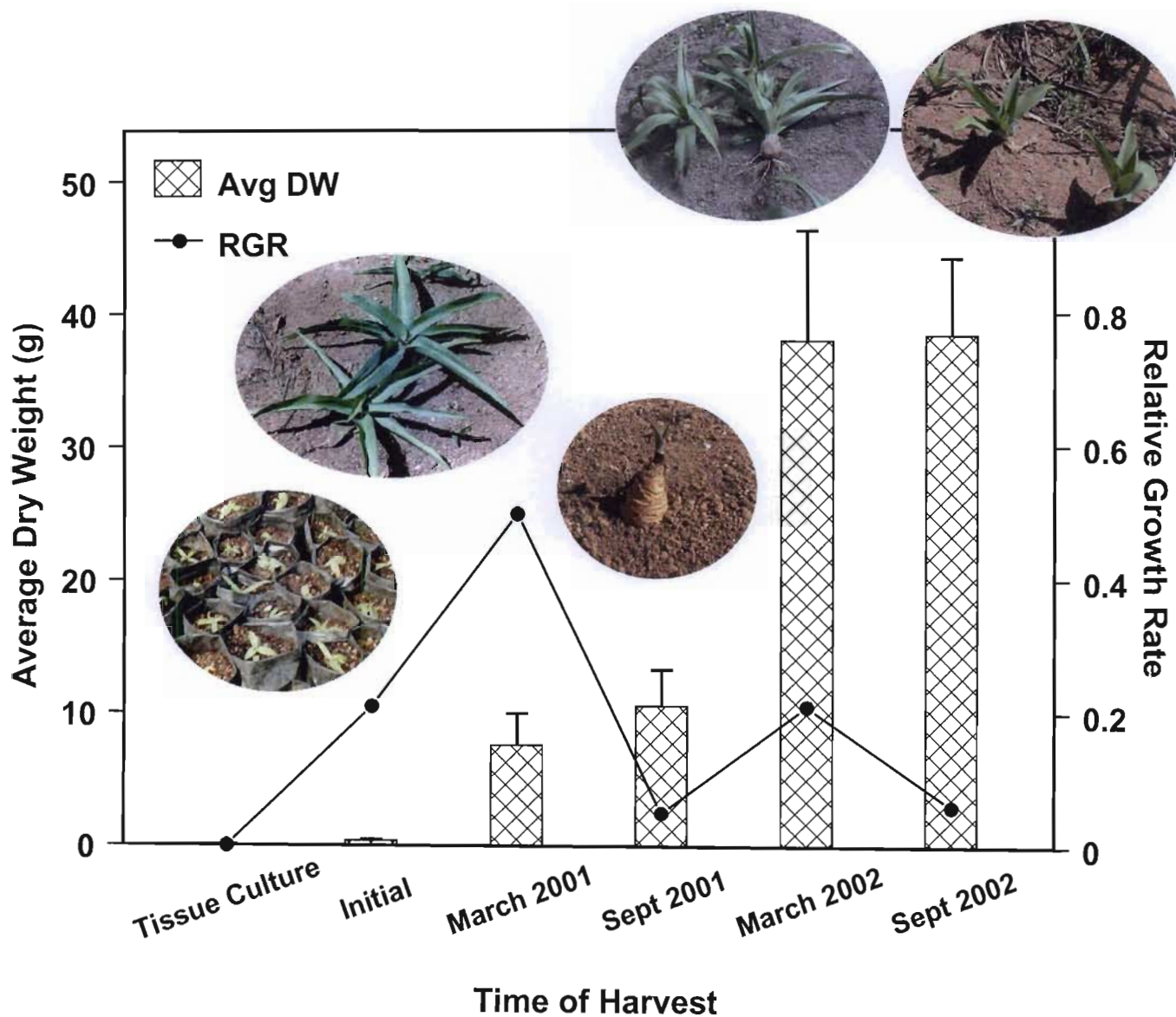


Figure 4.3. Average dry weight and relative growth rate of *M. natalensis* bulbs harvested from the control treatment at the H₂A Botanicals site.

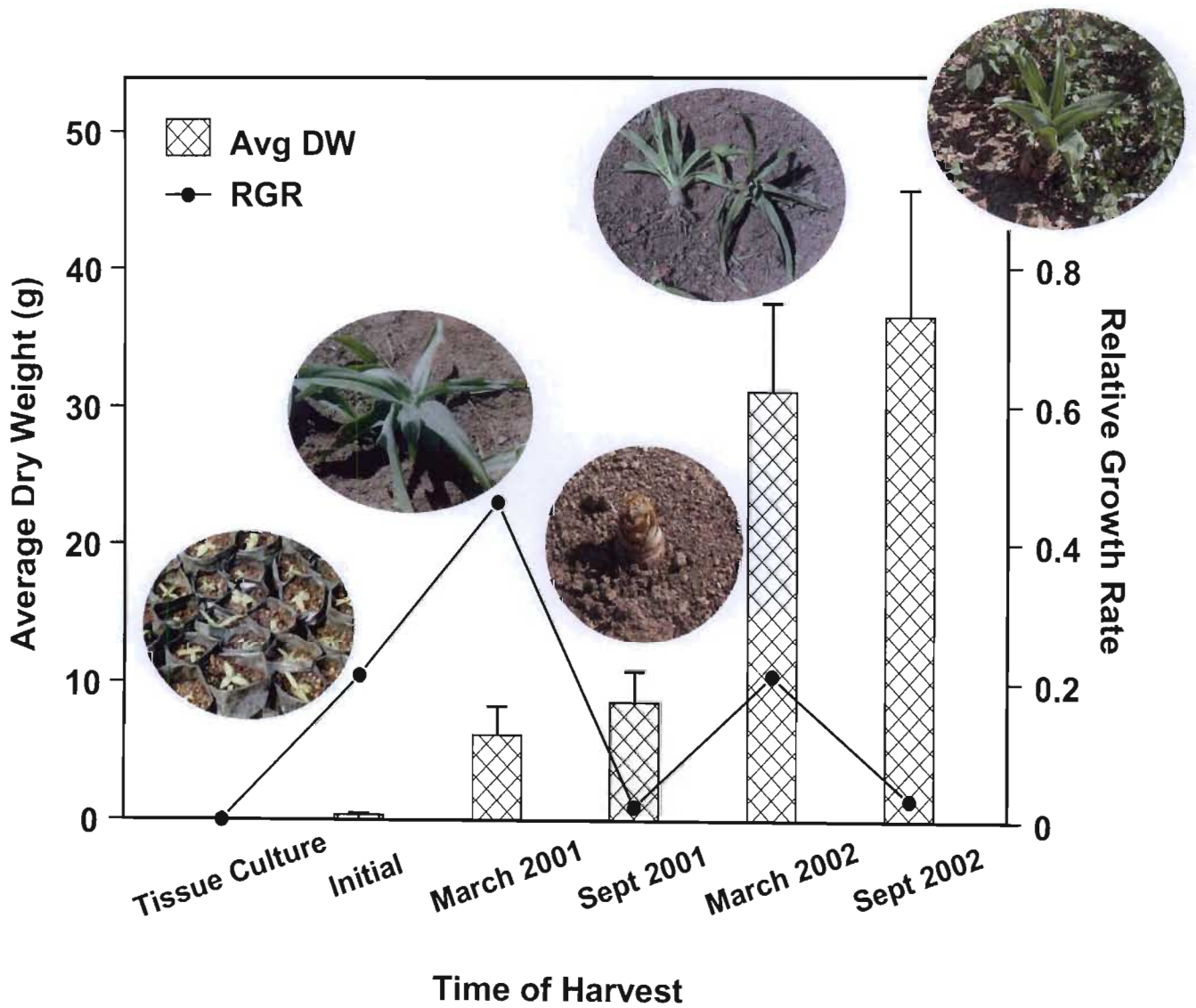


Figure 4.4. Average dry weight and relative growth rate of *M. natalensis* bulbs harvested from fertilizer treatment at the H₂A Botanicals site.

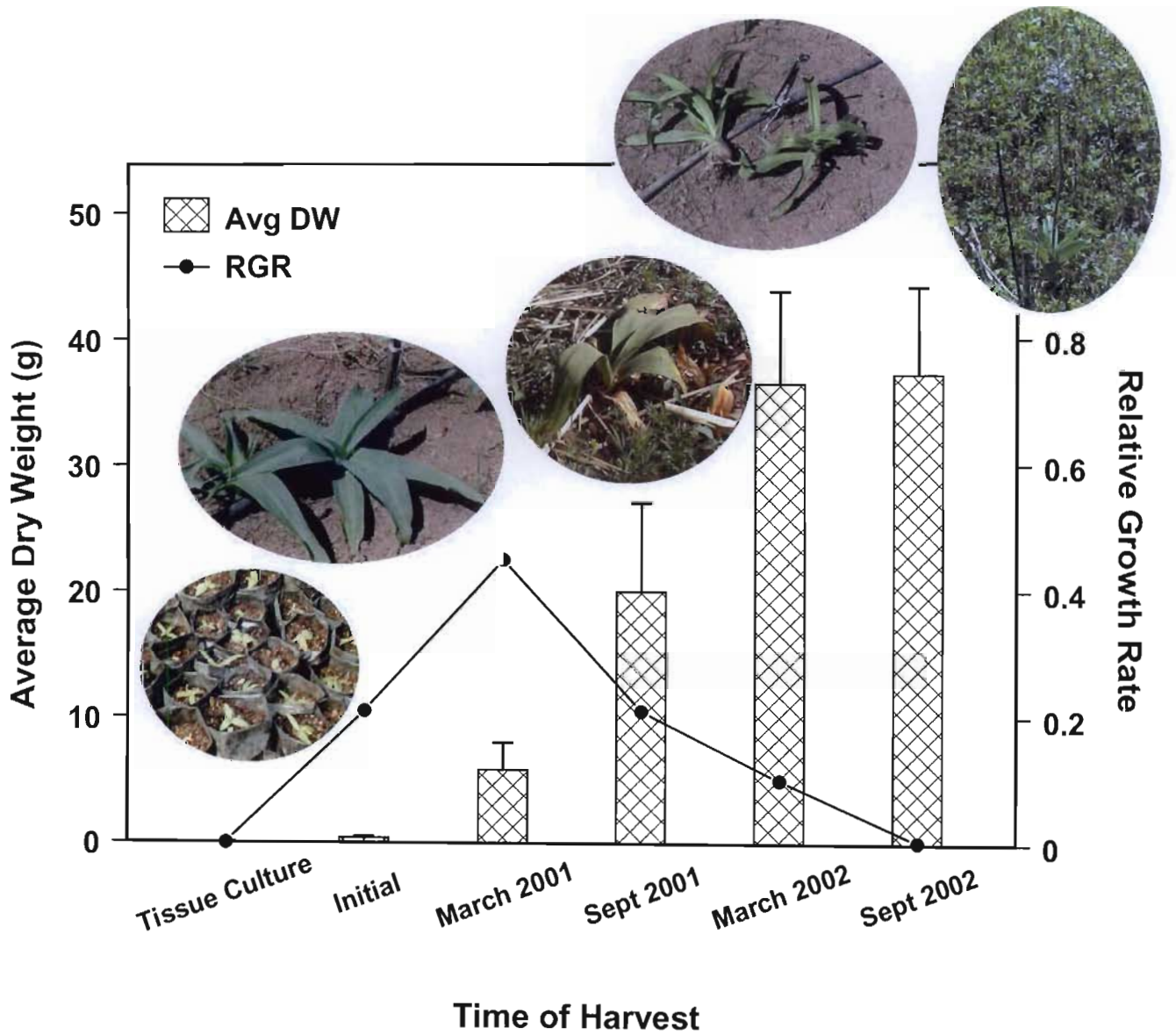


Figure 4.5. Average dry weight and relative growth rate of *M. natalensis* bulbs harvested from irrigation treatment at the H₂A Botanicals site.

the crop to flower before the bulbs are harvested. MATHE (1988) suggested that morphological traits are frequently modified in the course of introduction to field cultivation. However, several species that have been transplanted from the wild to cultivated environments have not had any morphological modifications. No noticeable morphological changes were found when cultivated *M. natalensis* plants were compared to plants growing under natural conditions. The only difference that was noticed between the cultivated plants and plants growing under natural conditions was in the length of the flowering stalks. In the natural ecosystem, these stalks grow to around 1 m in length. Plants flowering in the cultivated fields produced flowers on stalks of between 30-40 cm in length. It is not known whether this is due to a genetic trait that was passed on from the ex-plant during tissue culture, or whether it was the result of cultivation.

4.3.2. Fort Hare

The plants planted at Fort Hare were acclimatized under controlled conditions within mist houses. This method of acclimatization resulted in minimal losses, however, it is more costly than the method used at H₂A Botanicals and therefore if the plants were to be sold after acclimatization, the price per unit would be much higher than if the plants were sold directly from tissue culture. The only difference was that the plants that were obtained at Fort Hare were older and better established and so the plants that were planted out into the field were slightly larger than the plants that were planted out at H₂A Botanicals.

The bulb weights from the 24 month (November 2001) harvest were not available to be included in the analysis. Therefore, growth was only analysed up to 18 months (April 2001). The plant material from the 24 month harvest was fingerprinted and screened for pharmacological activity.

The average dry weights of bulbs grown under the control treatment (Figure 4.6A), showed similar growth patterns to the bulbs grown under the control treatment from the H₂A Botanicals site. Growth increased exponentially over the summer months, with no significant difference ($p \leq 0.05$) in average dry weight of the bulbs over the winter months. The average dry weights of the bulbs harvested from

the fertilizer treatment (Figure 4.6B) also showed similar growth patterns to those from the H₂A Botanicals site. The average dry weight of bulbs harvested in November 2000 from the irrigation treatment (Figure 4.6C), were significantly higher ($p \leq 0.05$) than the average dry weight of bulbs harvested in November 2000 from both control and fertilizer treatments. These findings suggest that it may be possible to cause a plant to override its natural annual rhythms while still young, but as the plant gets older these rhythms are not easily manipulated. This difference was also noticeable at the H₂A Botanicals plot. Relative growth rates all showed similar patterns to those plants cultivated at H₂A Botanicals, except for the irrigation treatment where RGR remained constant at Fort Hare between November 2000 and April 2001. H₂A Botanicals showed a decrease in RGR during the second growing season.

After 18 months of growing in the field, the average dry weights of bulbs grown under the control treatment were 52.2 g. The average dry weight of bulbs grown under the fertilizer treatment were 56.7 g, while those growing under the irrigation treatment were on average larger than both the other plots (60.2 g). Since the bulbs that were sent to Fort Hare to be planted out were much larger and already established plants, the average dry weights were higher than those from H₂A Botanicals. Plants from Fort Hare were of marketable size (± 300 g fresh weight) after 18 months in the field.

Analysis of the effect that the time of harvest had on growth was highly significant ($p < 0.001$). This would be expected as growth is relative to time. The type of treatment had no significant effect on dry weight of the bulbs ($p = 0.110$). The interaction between the treatments and the time of harvest was not significant ($p = 0.230$). These findings suggest that if this species is to be grown as a crop for commercial sale by farmers, the cost of cultivation would be very low as the plants seem to be very hardy and requires little irrigation or fertilizer supplements. This would be the ideal type of crop for rural farmers or small-scale farmers that do not have the economic capital to grow other crops that need these supplements.

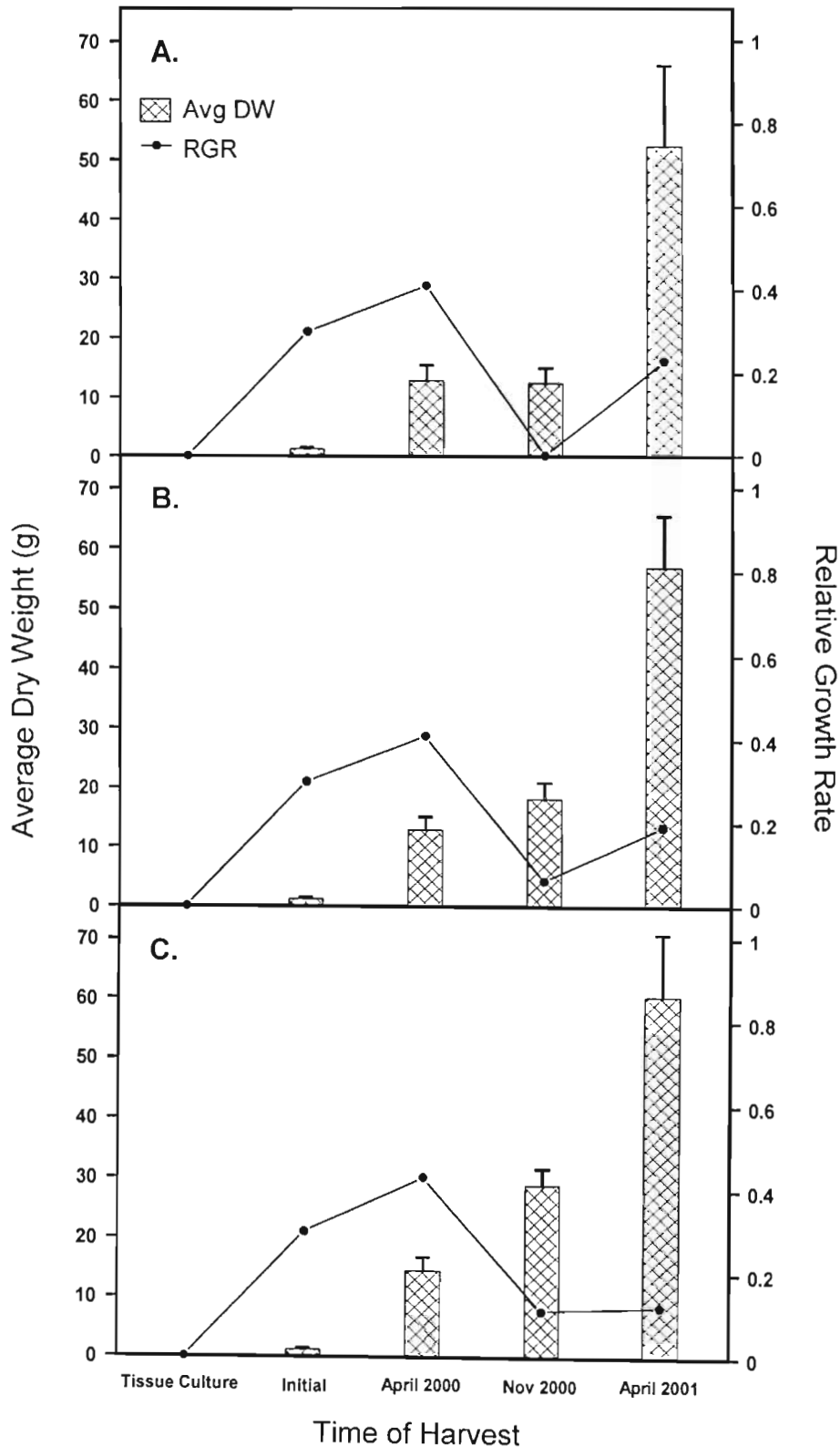


Figure 4.6. Average dry weights and relative growth rates of harvested *M. natalensis* bulbs from Fort Hare field trials. Measurements were taken from bulbs harvested from (A) Control treatment, (B) Fertilizer treatment, and (C) Irrigation treatment.

4.4. Summary

- Tissue cultured *M. natalensis* plantlets were hardened off by means of simple and cost effective methods and successfully grown in cultivation.
- Plants were grown successfully at two different geographical regions with different climatic conditions.
- Bulbs grew exponentially during the summer growing seasons, reaching almost marketable size after 24 months if taken directly from tissue culture and hardened off and then planted out into the field.
- Bulb growth is not dependent on additional fertilizer (organic or inorganic) or irrigation as there were no significant differences ($p \leq 0.05$) in the average dry weight of the bulbs grown in the three treatments.
- Irrigation had an effect on growth of young plants, preventing senescence and causing the plants to grow during the dormancy period.
- Introduction into cultivated ecosystems did not result in any morphological modifications.
- Bulbs started flowering after two years of growing in cultivated fields. Bulbs seem to flower more when irrigated. However, there were noticeable differences in flowering stalk length when compared to plants growing in natural ecosystems.

CHAPTER 5 CHEMICAL FINGERPRINTING BY TLC

5.1. Introduction

Thin-layer chromatography (TLC) is a rapid method of drug analysis that can be used to efficiently demonstrate the characteristic constituents of a drug or raw plant extract. TLC can be used to assess the quality of a drug by providing semi-quantitative information of the active constituents (WAGNER, BLADT and ZGAINSKI 1984). TLC also enables the quantitative and qualitative detection of known natural products or compounds, and the associated metabolites or breakdown products (GIBBONS and GRAY 1998). This has resulted in the development of secondary metabolite fingerprinting which allows for the classification of metabolites within the same range, and highlights those that are different in profile (VAN MIDDLESWORTH and CANNELL 1998). The use of photographic plates can be used to develop catalogue spectra of different phytochemicals. The use of stain reagents can facilitate secondary metabolite identification. Generally stains are used that give striking colours (WAGNER and BLADT 1996).

Reproducibility is very important when fingerprinting a plant extract. This can only be guaranteed if standardized absorption layers are used. Silica gel is generally regarded as an efficient absorbent for most TLC separations of most drug extracts. Silica gel 60 F₂₅₄ precoated TLC plates (MERCK) are used in most cases. Compounds that have been separated on TLC can be detected by viewing the TLC plate under UV light. The advantage of UV detection is that it is non-destructive and compounds can be detected at any stage in the separation process (GIBBONS and GRAY 1998). However, a major disadvantage of UV detection is that not all compounds absorb UV light at wavelengths of 254 or 366 nm. These compounds are therefore not visible under UV. Such compounds need to be stained with spray reagents to facilitate visualization (GIBBONS and GRAY 1998). Although easy to apply, spray reagents are not always specific in that they seldom detect only the compounds of a specific class, and often do not react with every compound of the class (VAN MIDDLESWORTH and CANNELL 1998).

Another problem encountered with TLC fingerprinting is over-loading of extracts. By restricting the quantity of material applied to the plates, sharp zones (bands) can be obtained. However, it is sometimes necessary to apply larger sample volumes if the compounds are present in low concentrations. This can lead to the broadening and overlapping of compounds. Compounds are located on the TLC plates through the calculation of R_f values. R_f values represent the ratio of the distance traveled by the compounds (bands) to the distance traveled by the solvent front. These R_f values should be consistent for different TLC separations run under standard conditions.

According to BERNATH (1986) ecological factors have a decisive impact on the chemical composition of the plant. Therefore, the variability in natural product content and composition are functions of both growth and development, and plays a role in determining the optimal harvest time of certain species. PRANCE (1994) suggested that the chemistry within individual plant species vary according to the ecology, the soil and the climate. YANIV and PALEVITCH (1982) stated that depending on the species, the quantity of secondary metabolites increase, decrease or remain constant when under water stress. Variations in natural product content and composition has been investigated for numerous species. Seasonal variation of alkaloids in *Crinum moorei* was observed by ELGORASHI, DREWES and VAN STADEN (2002). No clear trend in seasonal variation was noted with alkaloid yields varying from organ-to-organ as well as from season-to-season. DONG, CUI, SONG, ZHUO, JI, LO and TSIM (2003) showed that the active constituents of *Panax notoginseng*, including saponins, varied when the plants were grown in different geographical regions. The levels of compounds also varied seasonally.

Essential oil yield in *Tagetes minuta* varies during the plants growth, with young plants having higher contents of certain oils than older plants (CHALCHAT, GARRY and MUHAYIMANA 1995). CHALCHAT, GARRY and MUHAYIMANA (1995) also found that the nature of the soil, the climate and how the plants were grown had little influence on the chemical composition of the oils. Comparative analysis of the essential oils of *Acanthospermum hispidum* showed there to be only slight variations over the different seasons (MENUT, MOLANGUI and LAMATY 1995). LOPES, KATO, de AGUIAR ANDRADE, MAIA and YOSHIDA (1997) found that the essential

oils from leaves of *Virola surinamensis* showed circadian variation in elemicin and in monoterpenes during the rainy season. SOUTHWELL and BOURKE (2001) showed that the hypericin content of *Hypericum perforatum* varied seasonally. The concentration of hypericin decreases from 3000 mg/l in summer to 100 mg/l in winter. A quantitative investigation by BINNS, LIVESEY, ARNASON and BAUM (2002) on *Echinacea* a widely cultivated taxa that is traded for both medicinal and horticultural purposes, showed variations in phytochemistry between wild and cultivated populations. Plant age and the type of growth conditions had an effect on phytochemical composition and content.

5.2. Methodology

5.2.1. Extract Preparations

Aqueous, methanol and dichloromethane extracts were made of each of the four samples for each treatment of each harvest. Two grams of dried material was extracted with 20 ml extraction solvent. The material was left to extract for 24 h under constant agitation with a magnetic stirrer. The extracts were filtered under vacuum through Whatman No. 1 filter paper discs using a Büchner funnel. The filtered extracts were taken to dryness and stored in sealed pill vials in the dark at 5°C.

5.2.2. Sample Preparation

The extracts residues were dissolved to a concentration of 10 mg/ml with the relevant extraction solvents.

5.2.3. TLC Separation

Extracts were applied in 5 µl aliquots as 10 mm bands (5 mm spacing between extracts). Ten mm margins were left on either side and the origin was situated 15 mm from the base. One sample from each treatment was strip-loaded onto the TLC plates. Samples were grouped according to the time of harvest. All TLC separations were performed at room temperature ($\pm 24^\circ\text{C}$).

5.2.3.1. *Methanol Extracts*

Methanol extracts were applied to (20x20) Silica gel 60 F₂₅₄ TLC plates (MERCK). The chloroform: glacial acetic acid: methanol: water (64:32:12:8 v:v:v:v) system described in Section 3.2 gave the best separation with methanol extracts and was subsequently used for preparation of methanol extract TLC fingerprints. Plates were developed over 170 mm in the solvent system. These plates were allowed to dry before being stained with anisaldehyde-sulphuric acid reagent (0.5 ml anisaldehyde, 10 ml sulphuric acid, 85 ml methanol and 5 ml concentrated sulphuric acid mixed in this order) and antimony trichloride reagent (20% antimony trichloride in chloroform). The stain reagents were sprayed over the plates, which were then heated at 110°C for 8 min. The anisaldehyde-sulphuric acid stained plate was observed and photographed under visible light. The antimony trichloride stained plate was observed and photographed under both visible light and ultra violet (UV) light (366 nm).

5.2.3.2. *Dichloromethane Extracts*

Dichloromethane extracts were applied to (20x20) Silica gel 60 F₂₅₄ TLC plates (MERCK). Plates were developed with a dichloromethane: chloroform: methanol (10:1:1 v:v:v) solvent system. The plates were allowed to dry before being stained with antimony trichloride reagent (20% antimony trichloride in chloroform). The stained plates were heated at 110°C for 8 min and then observed and photographed under UV light (366 nm).

5.2.3.3. *Water Extracts*

Water extracts were applied to (20x20) precoated RP-18 F₂₅₄ s TLC plates (MERCK). Plates were developed with a dichloromethane: methanol: water (1:3:1 v:v:v) solvent system. The plates were allowed to dry before being observed under UV light (366 nm). Plates were stained with anisaldehyde-sulphuric acid reagent (0.5 ml anisaldehyde, 10 ml sulphuric acid, 85 ml methanol and 5 ml concentrated sulphuric acid mixed in this order). The stained plates were heated at 110°C for 8 min before being examined and photographed under visible light.

5.3. Results and Discussion

5.3.1. Methanol Extracts

TLC fingerprints of the methanol extracts are seen in Figures 5.1 for H₂A Botanicals and Figure 5.2 for Fort Hare. Results of the methanol bulb extracts from the H₂A Botanicals site show few differences in the chemical compositions between mature (M) *M. natalensis* bulbs and the cultivated plants (Figure 5.1). The only noticeable differences in chemical composition occur between R_f 0.4 and R_f 0.5. Methanol extracts of bulbs harvested after 12 months (C₁₂, F₁₂ and IF₁₂) and 24 months (C₂₄, F₂₄ and IF₂₄) contained compounds that were also visible in the mature plants (Figure 5.1A).

Results of the methanol bulb extracts from the Fort Hare site showed many more differences in chemical composition between the mature bulbs and the cultivated plants (Figure 5.2). Noticeable differences were also seen between harvests. The 12 month (C₁₂, F₁₂ and IF₁₂) and 24 month (C₂₄, F₂₄ and IF₂₄) harvests were found to be very similar in chemical composition. The 6 month (C₆, F₆ and IF₆) and 18 month (C₁₈, F₁₈ and IF₁₈) harvests were also very similar chemically. A pink compound was observed at R_f 0.4 when stained with anisaldehyde-sulphuric acid (Figure 5.2A). The 12 and 24 month harvests had a greenish compound at R_f 0.4 that matched the mature plant extract. Staining the plates with antimony trichloride also produced differences in the chemical composition between R_f 0.25 and R_f 0.5. Again the 6 and 18 month harvests differed in chemical profile from the 12 and 24 month harvests (Figure 5.2B). Another noticeable difference was observed between R_f 0.8 and R_f 0.9. A compound, also observed in the bulbs harvested from the H₂A Botanicals site when stained with antimony trichloride were observed in the 12 and 24 month harvests only (Figure 5.2B and C).

Results from both sites suggest that possibly the chemical composition of the plants differ according to the time of harvest. Plants harvested during the rainy season differed chemically from the plants harvested going into the dry season. These differences seem to be more pronounced at Fort Hare, which has half the mean annual rainfall that H₂A Botanicals site.

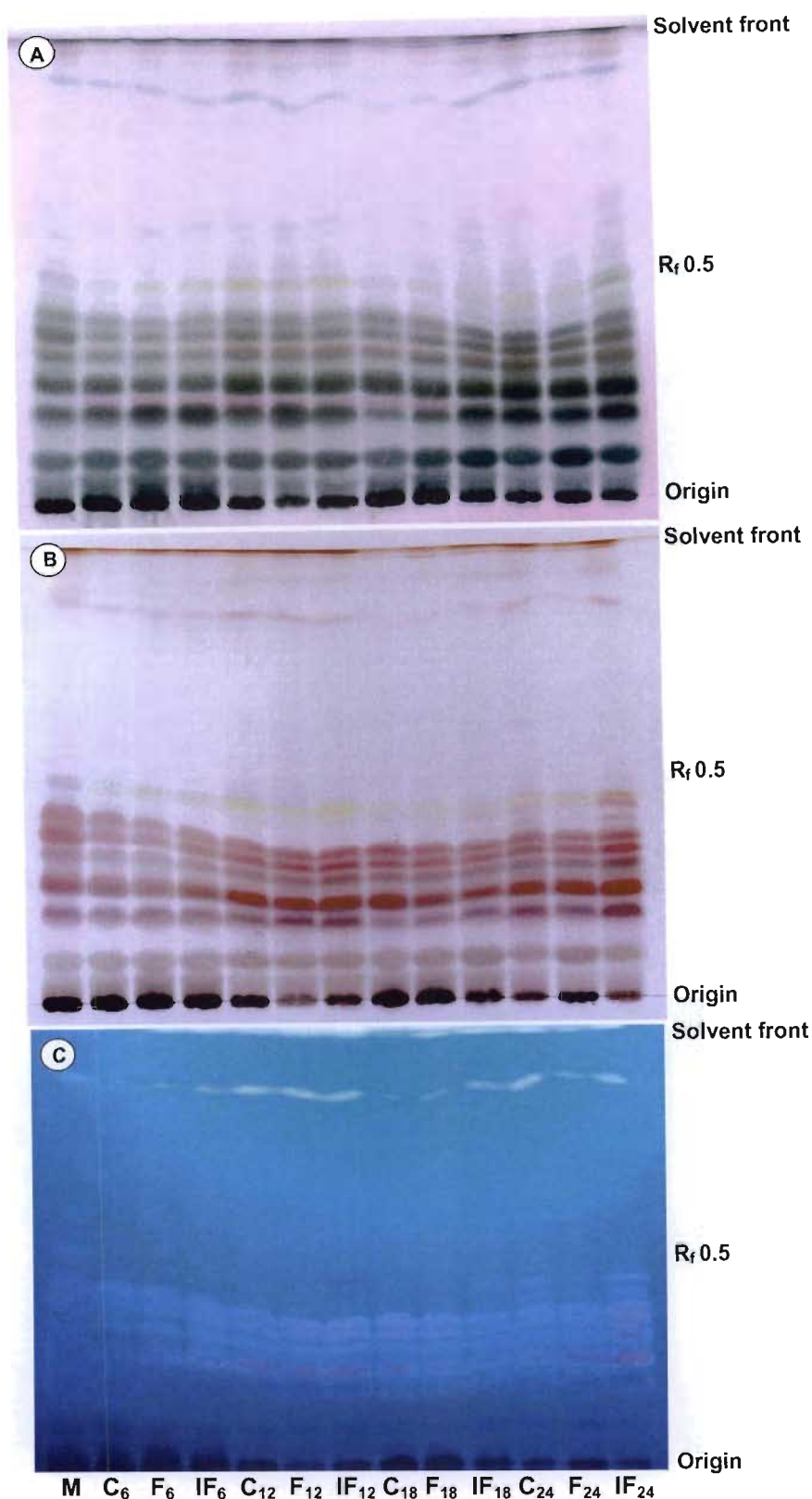


Figure 5.1. TLC Fingerprinting profiles of methanol extracts of *M. natalensis* bulbs harvested from the H₂A Botanicals site. TLC plates were developed in chloroform: glacial acetic acid: methanol: water (64:32:12:8 v:v:v:v) solvent system. (A) Anisaldehyde-sulphuric acid stained TLC plate viewed under visible light. (B) Antimony trichloride stained TLC plate viewed under visible light. (C) Antimony trichloride stained TLC plate viewed under UV₃₆₆ nm. M = mature *M. natalensis* plant, C = control treatment, F = fertilizer treatment, IF = irrigation treatment and 6-24 = month of harvest.

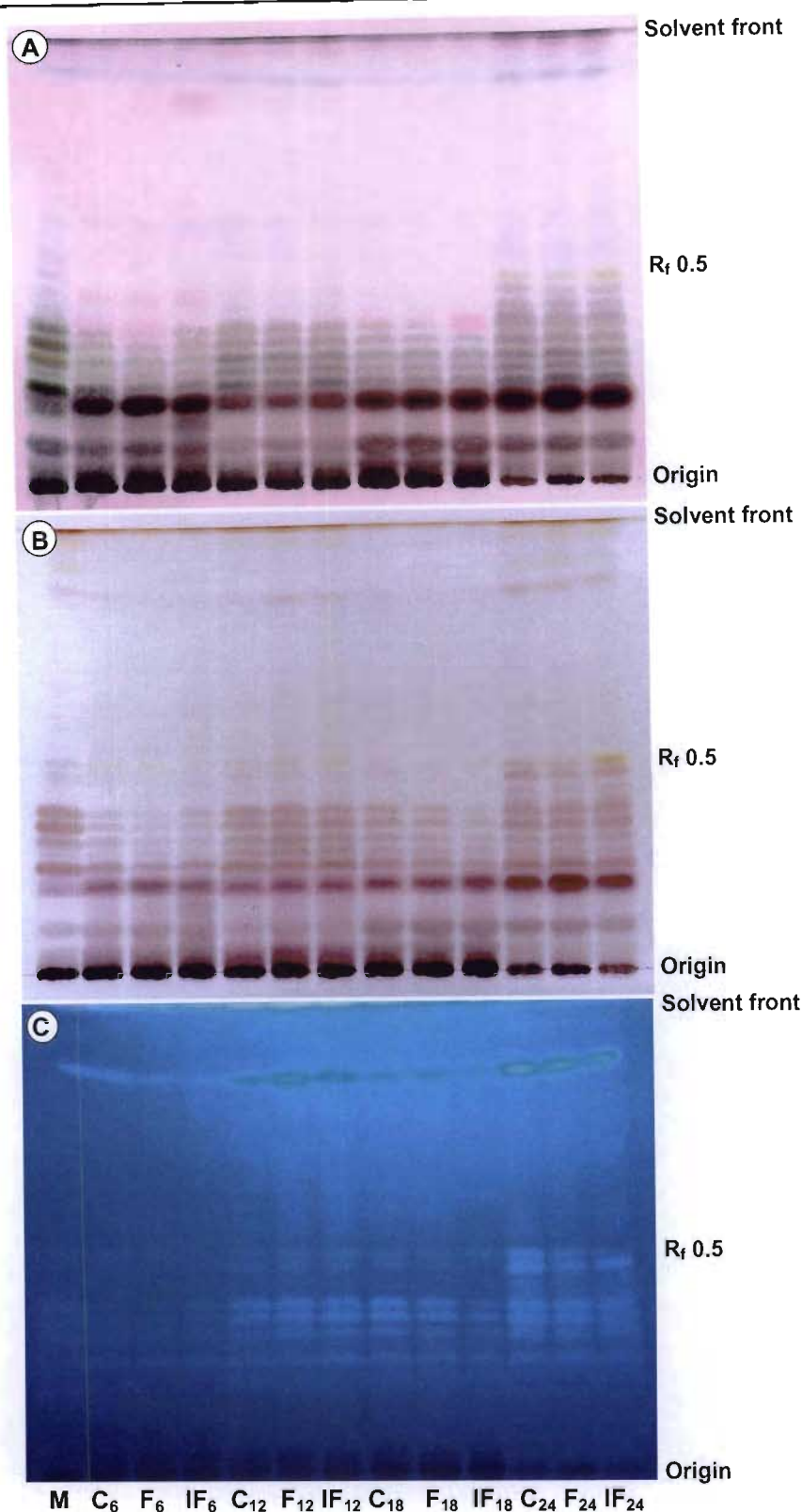


Figure 5.2. TLC Fingerprinting profiles of methanol extracts of *M. natalensis* bulbs harvested from the Fort Hare site. TLC plates were developed in chloroform: glacial acetic acid: methanol: water (64:32:12:8 v:v:v:v) solvent system. (A) Anisaldehyde-sulphuric acid stained TLC plate viewed under visible light. (B) Antimony trichloride stained TLC plate viewed under visible light. (C) Antimony trichloride stained TLC plate viewed under UV₃₆₆ nm. M = mature *M. natalensis* plant, C = control treatment, F = fertilizer treatment, IF = irrigation treatment and 6-24 = month of harvest.

There were noticeable differences in the concentrations of the chemicals between the extracts of bulbs from the different cultivation treatments and the different harvests. Concentration differences are indicated by the intensity of the bands of the same compounds. However, no differences in chemical composition were observed between bulbs cultivated in the different plots. This indicates that it may not necessarily be irrigation that had an effect on the chemical composition but rather other factors e.g. temperature, or a combination of factors.

5.3.2. Dichloromethane Extracts

The TLC fingerprinting profiles of the dichloromethane extracts of bulbs harvested from both the H₂A Botanicals and the Fort Hare sites are seen in Figure 5.3. No visual differences in chemical compositions were observed between the cultivated bulbs and the mature *M. natalensis* bulbs. No differences were noticeable between bulbs harvested from the different treatment plots or at different harvests. No differences in the chemical constituents were observed between bulbs cultivated at the two sites. The only differences that were found were in the concentration of some of the compounds. Bulbs harvested after 24 months from the fertilizer only plot at the Fort Hare site showed some chemical differences from the other bulbs (Figure 5.3B).

5.3.3. Water Extracts

The TLC fingerprinting profiles of the water extracts of bulbs harvested from both the H₂A Botanicals and the Fort Hare sites are presented in Figure 5.4. No differences in chemical composition were observed between the water extracts of the bulbs harvested from the H₂A Botanicals site and the mature bulbs. There were slight differences in the concentration of certain compounds (Figure 5.4A).

The water extracts of bulbs harvested at the Fort Hare site showed differences in chemical composition with each harvest (Figure 5.4B). These differences were observed between R_f 0.6 and 0.8. The TLC fingerprinting profiles of the 24 month harvest was similar to that of mature reference bulbs.

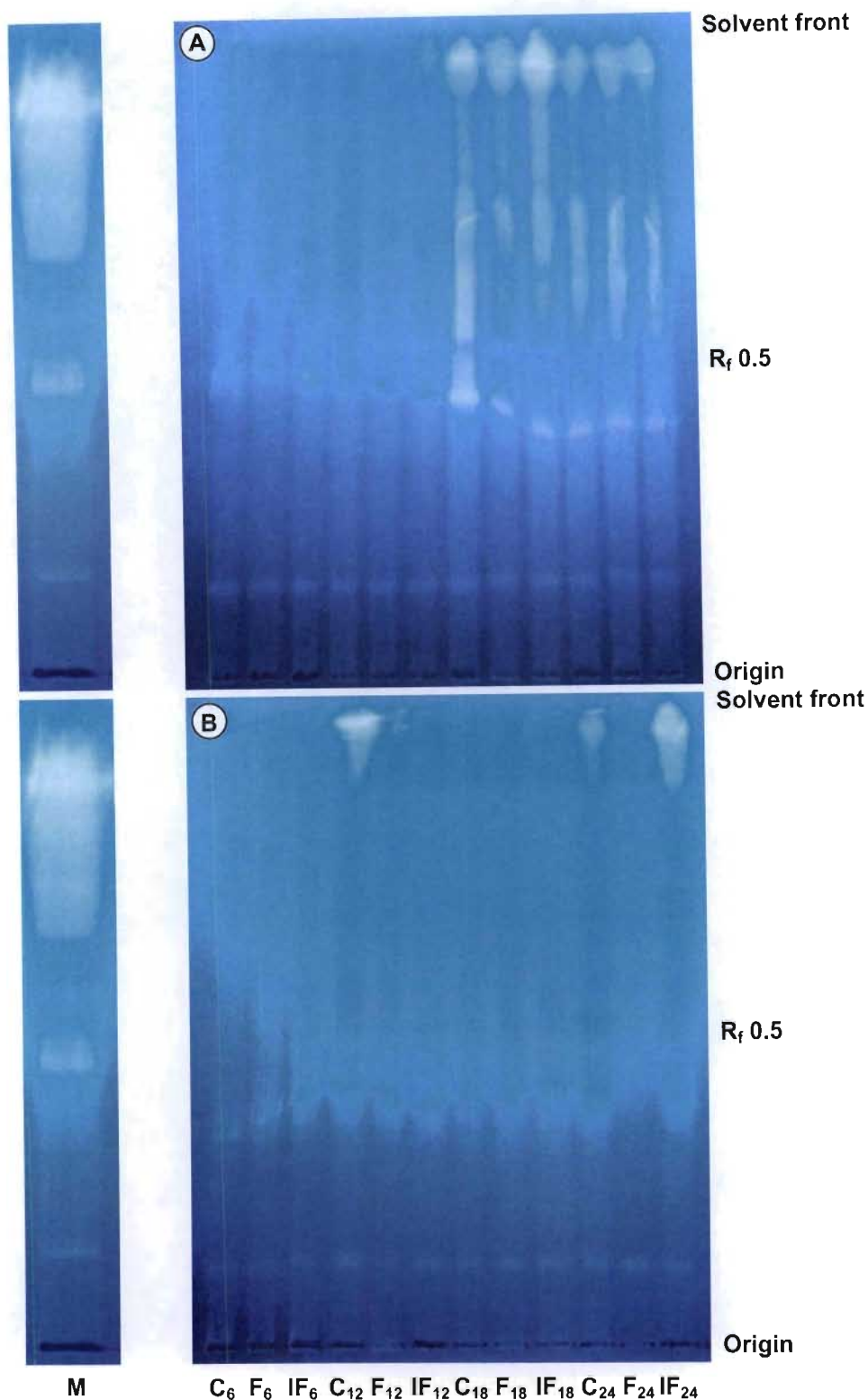


Figure 5.3. TLC Fingerprinting profiles of dichloromethane extracts of *M. natalensis* bulbs harvested from the (A) H₂A Botanicals site and (B) Fort Hare site. TLC plates were developed in dichloromethane: chloroform: methanol (10:1:1 v:v:v) solvent system. TLC plates stained with antimony trichloride reagent and viewed under UV₃₆₆ nm. M = mature *M. natalensis* plant, C = control treatment, F = fertilizer treatment, IF = irrigation treatment and 6-24 = month of harvest.

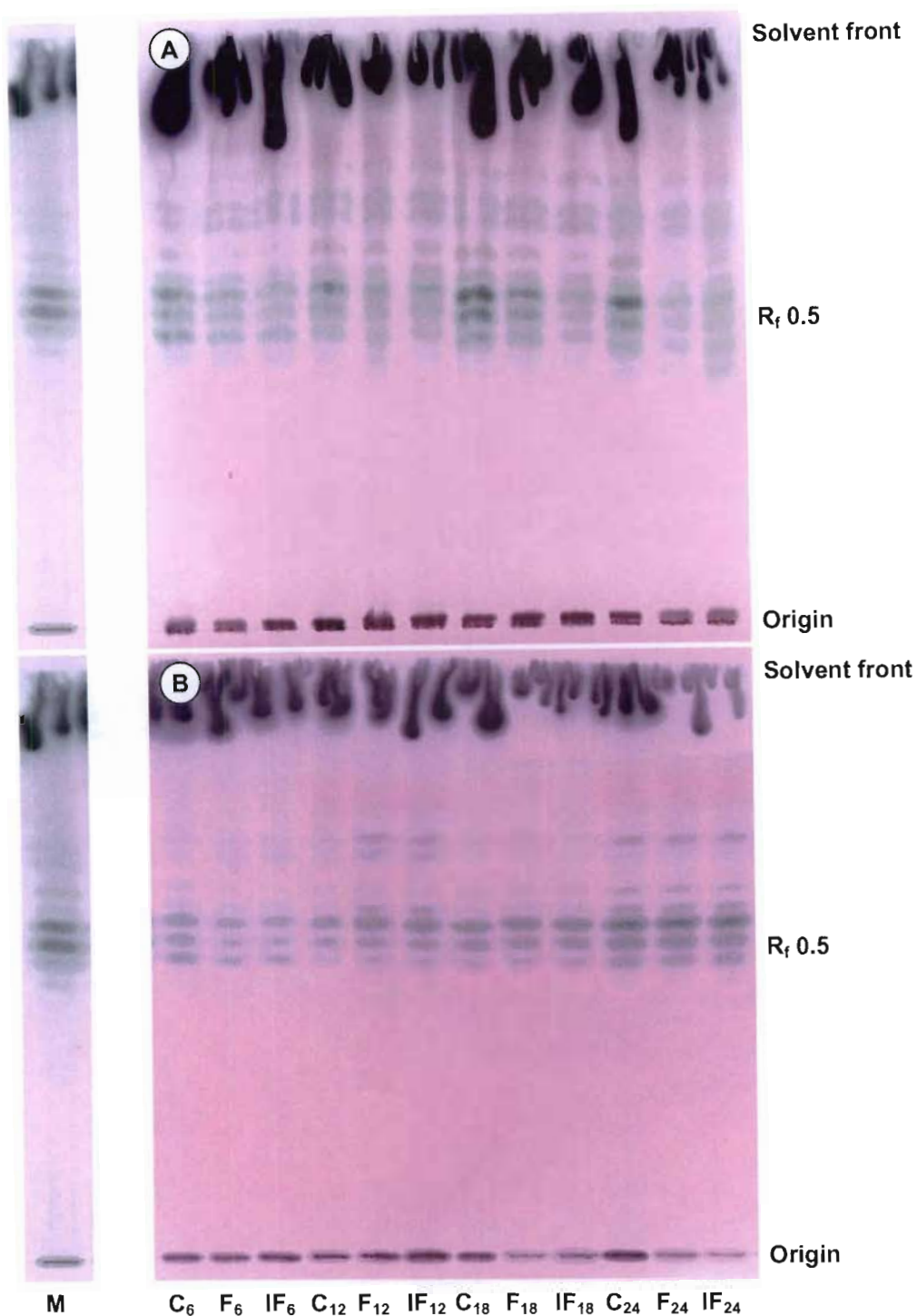


Figure 5.4. TLC Fingerprinting profiles of water extracts of *M. natalensis* bulbs harvested from the (A) H₂A Botanicals site and (B) Fort Hare site. TLC plates were developed in dichloromethane: methanol: water (1:3:1 v:v:v) solvent system. TLC plates stained with anisaldehyde-sulphuric acid reagent and viewed under visible light. M = mature *M. natalensis* plant, C = control treatment, F = fertilizer treatment, IF = irrigation treatment and 6-24 = month of harvest.

5.4. Summary

- TLC fingerprinting profiles were made of methanol, dichloromethane and water extracts of *M. natalensis*.
- Solvent systems were developed for each extract to allow for comparisons to be made between wild mature plants and cultivated plants.
- Methanol extracts were developed with chloroform: glacial acetic acid: methanol: water (64:32:12:8 v:v:v:v) solvent system and stained with anisaldehyde-sulphuric acid and antimony trichloride reagents. TLC plates were viewed under UV light (366 nm) and visible light.
- Differences were found between methanol extract profiles of bulbs for each harvest from both cultivation sites. Differences were more noticeable in bulbs harvested from the Fort Hare site.
- Dichloromethane extracts were developed with dichloromethane: chloroform: methanol (10:1:1 v:v:v) solvent system and stained with antimony trichloride reagent. TLC plates were viewed under UV light (366 nm).
- No differences were found between dichloromethane extract profiles of bulbs harvested from both cultivation sites. TLC profiles were the same as the mature *M. natalensis* reference plant.
- Water extracts were developed with dichloromethane: methanol: water (1:3:1 v:v:v) solvent system and stained with anisaldehyde-sulphuric acid reagent. TLC plates were viewed under visible light.
- No differences in chemical composition between the water extract profiles of bulbs harvested from the H₂A Botanicals site and the mature reference plants were observed
- Differences were found between water extract profiles of bulbs harvested from the Fort Hare site and the mature reference plant. Bulbs from the 24 month harvest matched the TLC profile of the mature reference bulb.

CHAPTER 6 PHARMACOLOGICAL ANALYSIS OF MATERIAL COLLECTED FROM FIELD TRIALS

6.1. Introduction

Very little research has been done on seasonal variation in pharmacological activity or the effect that plant growth (age) has on the pharmacological activity of *M. natalensis*. In a study by LIGHT, McGAW, RABE, SPARG, TAYLOR, ERASMUS and VAN STADEN (2002) the effect of senescence in *Siphonochilus aethiopicus* on its pharmacological activity was investigated. TAYLOR and VAN STADEN (2001) investigated the effect of age, season and growth conditions on the anti-inflammatory activity of *Eucomis autumnalis* plant extracts. Significant differences in activity were found between plants harvested before and after the growing season. The effect of cold storage on the anti-inflammatory activity of *E. autumnalis autumnalis* also showed differences in activity (TAYLOR and VAN STADEN 2002).

6.2. Extract Preparations

The dried harvested bulbs (20 in each case) were divided into groups of fives, to form four sample groups for each harvest. The five bulbs were ground together and stored in honey jars in the dark at $\pm 24^{\circ}\text{C}$ (room temperature). This was done for each of the treatments from both Fort Hare and H₂A Botanicals growing sites, at each harvest time.

Aqueous, methanol and dichloromethane extracts were prepared for of each of the four samples for each treatment of each harvest. Two g of dried material were extracted with 20 ml extraction solvent. The material was allowed to extract for 24 h with constant agitation. The extracts were filtered under vacuum through Whatman No. 1 filter paper discs using a Büchner funnel. The filtered extracts were taken to dryness and stored in sealed pill vials in the dark at 5°C.

6.3. Antibacterial Analysis of Cultivated Bulbs

6.3.1. Methodology

6.3.1.1. Extracts

Antibacterial screening of extracts from mature *M. natalensis* bulbs (Section 2.2) showed that the methanol extracts had the highest antibacterial activity. Methanol extracts from the harvested bulbs were therefore routinely screened for antibacterial activity. Extracts were dissolved to a concentration of 50 mg/ml.

6.3.1.2. Antibacterial Bioassay

The microdilution antibacterial assay (MIC bioassay) of ELOFF (1998) as described in Section 2.2.2.3 was used to screen the extracts for antibacterial activity. Four extracts per treatment per harvest were tested. The assay was repeated a second time giving a total of eight replicates for each treatment for each harvest. The extracts were tested against two Gram-positive bacteria, *Bacillus subtilis* (ATCC No. 6051) and *Staphylococcus aureus* (ATCC No. 12600), and two Gram-negative bacteria *Escherichia coli* (ATCC No. 11775) and *Klebsiella pneumoniae* (ATCC No. 13883).

6.3.1.3. Bioautographic Assay

Fingerprinting results of the methanol extracts showed no visual differences between the different treatments, therefore, only the extracts from bulbs harvested from the control plots were spotted and developed on TLC. Two μl of the control methanol extracts from each harvest were spotted onto 2 TLC plates (silica gel 60 F₂₅₄ TLC plates, Merck). The TLC plates were developed with a chloroform: methanol: water (8:5:1 v:v:v) solvent system. One plate was stained with anisaldehyde-sulphuric acid reagent and heated at 110°C for 8 min. The second plate was used in the bioautographic assay.

The bioautographic assay as described by MARTINI and ELOFF (1998) was used. The test bacterium *S. aureus* (ATCC No. 12600) was cultured overnight in MH broth medium. The cultures were centrifuged at 3000 *g* for 10 min and the supernatant discarded. The bacterial pellet was resuspended in 10 ml fresh MH broth and sprayed onto the developed TLC plate. The plate was then incubated overnight at 37°C in 100% humidity. The plate was removed and allowed to dry slightly before being sprayed with a 2 mg/ml solution of iodinitrotetrazolium chloride (INT) (Fluka). The plate was re-incubated for a further 2 h to allow for colour development. Zones of inhibition appear as white spots against a pink coloured background.

6.3.2. Results and Discussion

Minimum inhibitory concentrations (MIC) were calculated for an average of eight replicates for each treatment of each harvest. The MIC values of individual extracts were compared using Fisher's pairwise comparisons at 5% level of significance. The lower the MIC value, the higher the antibacterial activity. ANOVA was used to determine interaction and the effect of plot treatments and time of harvests on the MIC values. The neomycin control had MIC of 1.6×10^{-2} mg/ml against *B. subtilis* and *K. pneumoniae*, 3.1×10^{-2} mg/ml against *S. aureus*, and 6.3×10^{-2} mg/ml against *E. coli*.

The results from the antibacterial screening of bulbs cultivated at the H₂A Botanicals site showed no significant differences ($p \leq 0.05$) in activity between treatments against both the Gram-positive bacteria. Bulbs harvested from the fertilizer treatment after 12 months (September 2001) of growing in the field gave the highest activity (3.9 mg/ml) against *B. subtilis* (Figure 6.1A). Analysis of the results showed there to be no interaction between the treatments and the time of harvest ($p = 0.137$). Both the plot treatments (Figure 6.1B) and the time of harvest (Figure 6.1C) had no significant effect on the antibacterial activity ($p = 0.221$ and $p = 0.184$ respectively). Bulbs harvested after just 6 months (March 2001) from the fertilizer treatment had the highest activity (2.34 mg/ml) against *S. aureus* (Figure 6.2A). Analysis of the antibacterial results against *S. aureus* showed there to be no interaction between the treatments and the time of harvest ($p = 0.278$). The treatments had no significant effect on the antibacterial activity (Figure 6.2B). However, the time

of harvest (Figure 6.2C) did have a significant effect on the antibacterial activity ($p=0.002$). The antibacterial activity decreased as the plants matured. The 6 month (March 2001) harvest had significantly higher antibacterial activity against *S. aureus* than the 24 month (September 2002) harvest.

The screening against the Gram-negative bacteria did not show a similar pattern to the results for the Gram-positive bacteria. There were significant differences ($p\leq 0.05$) in activity between treatments (Figure 6.3B). Analysis of the results showed that the type of treatment had a significant effect on the antibacterial activity against *E. coli* ($p=0.008$). Bulbs harvested from the fertilizer treatment were more active against *E. coli* than the bulbs from the other treatment. The bulbs harvested in March 2001 (6 months) from the fertilizer treatment had the highest activity (MIC = 2.73 mg/ml) (Figure 6.3A). Analysis of the results also showed that the time of harvest (Figure 6.3C) had a significant effect on the antibacterial activity ($p=0.001$). The antibacterial activity decreased as the plants matured. However, there was no interaction between the type of treatment and the time of harvest ($p=0.887$). Bulbs harvested in March 2001 (6 months) from the irrigation and fertilizer plot had the highest activity against *K. pneumoniae* (MIC = 2.73 mg/ml) (Figure 6.4A). There was also no significant difference in activity between bulbs harvested from the fertilizer treatment for all harvest times (MIC values ranged from 2.92 mg/ml to 3.13 mg/ml). Analysis of the results showed that both the type of treatment (Figure 6.4B) and the time of harvest (Figure 6.4C) had no significant effect on the activity ($p=0.193$ and $p=0.221$ respectively). However, there was a significant interaction between the treatment and the time of harvest ($p<0.001$) (Figure 6.4A).

The results from bulbs cultivated at Fort Hare showed no significant differences ($p\leq 0.05$) in activity between treatments against both Gram-positive test bacteria (Figure 6.5B and Figure 6.6B). Analysis of the results showed that the type of treatment had no significant effect on the activity against *B. subtilis* ($p=0.579$). The bulbs harvested in April 2000 (6 months) from the fertilizer treatment, had the highest activity against *B. subtilis* (Figure 6.5A) with average MIC value of 4.69 mg/ml. All the other harvests and treatments ranged between 5.08 mg/ml to 6.25 mg/ml, except for the bulbs harvested in April 2001 (18 months) from the control treatment (MIC = 7.03 mg/ml).

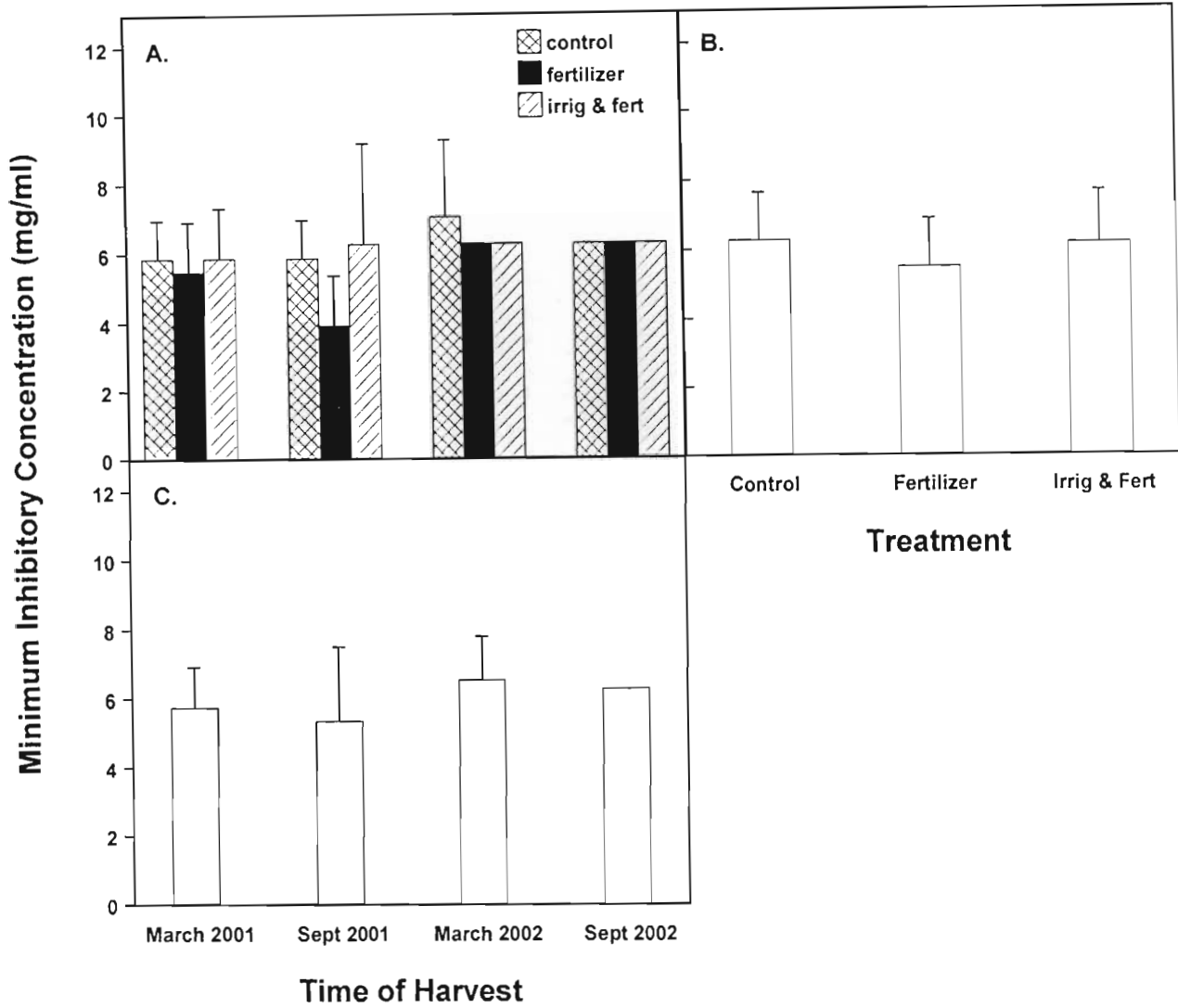


Figure 6.1. Mean MIC values of *M. natalensis* bulb extracts harvested from the H₂A Botanicals site over a period of two years against *B. subtilis*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.137$). The treatments and time of harvest had no significant effect on the antibacterial activity of the bulbs ($p=0.221$ and $p=0.184$ respectively).

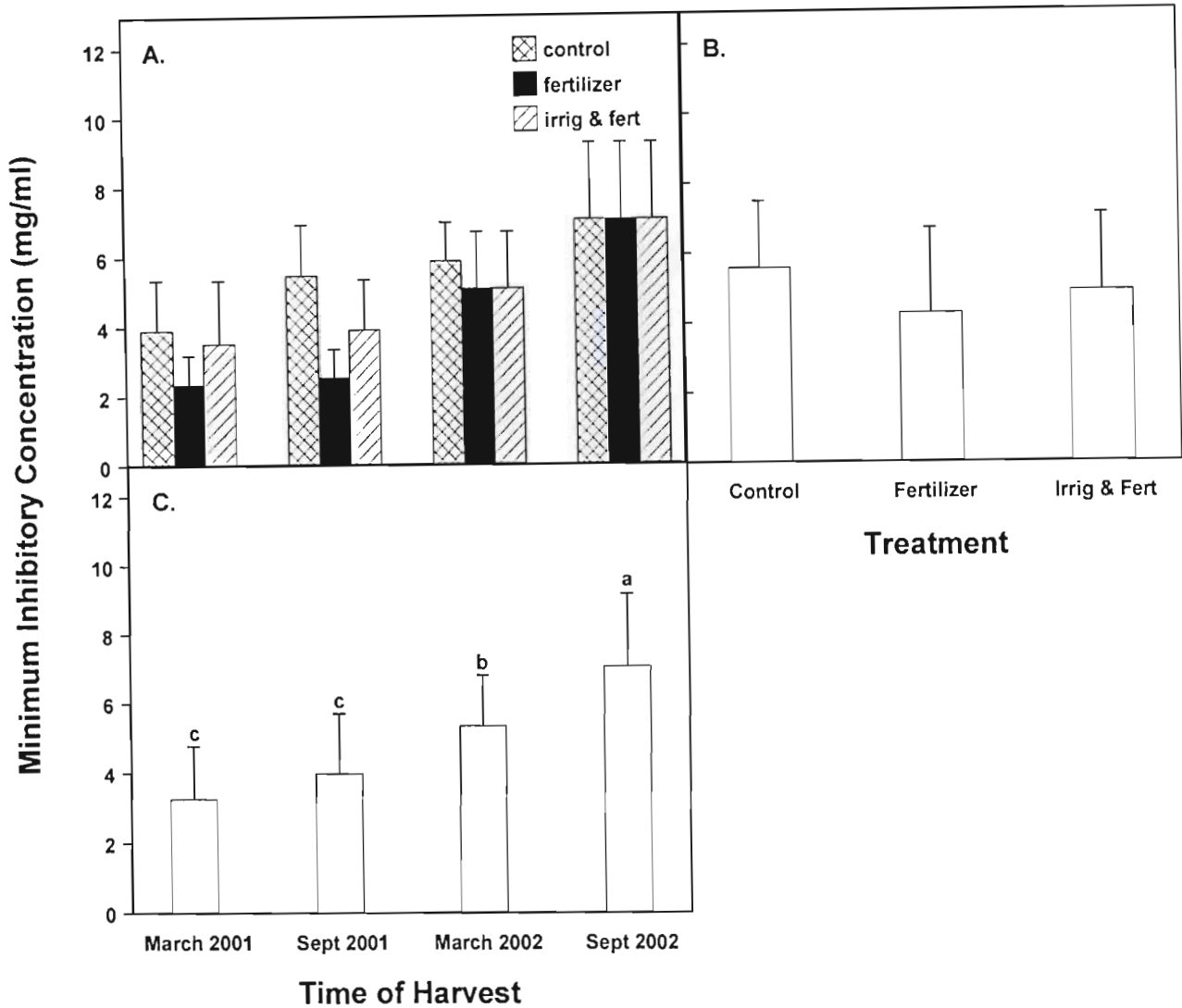


Figure 6.2. Mean MIC values of *M. natalensis* bulb extracts harvested from the H₂A Botanicals site over a period of two years against *S. aureus*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.278$). The treatments had no significant effect on the antibacterial activity ($p=0.076$). Time of harvest had a significant effect on the antibacterial activity of the bulbs ($p=0.002$). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other.

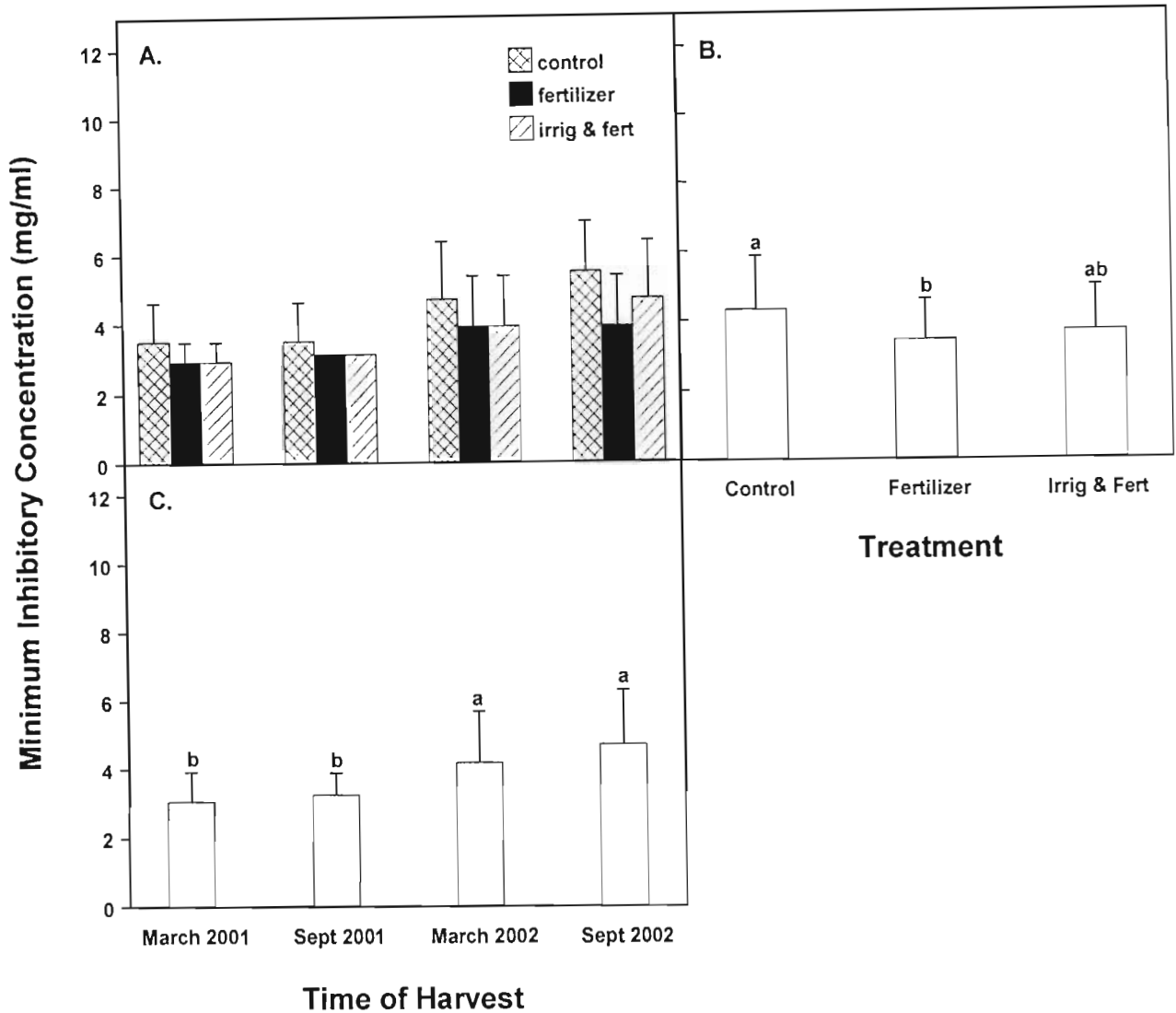


Figure 6.3. Mean MIC values of *M. natalensis* bulb extracts harvested from the H₂A Botanicals site over a period of two years against *E. coli*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.887$). Both the treatments and time of harvest had a significant effect on the antibacterial activity of the bulbs ($p=0.008$ and $p=0.001$ respectively). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other.

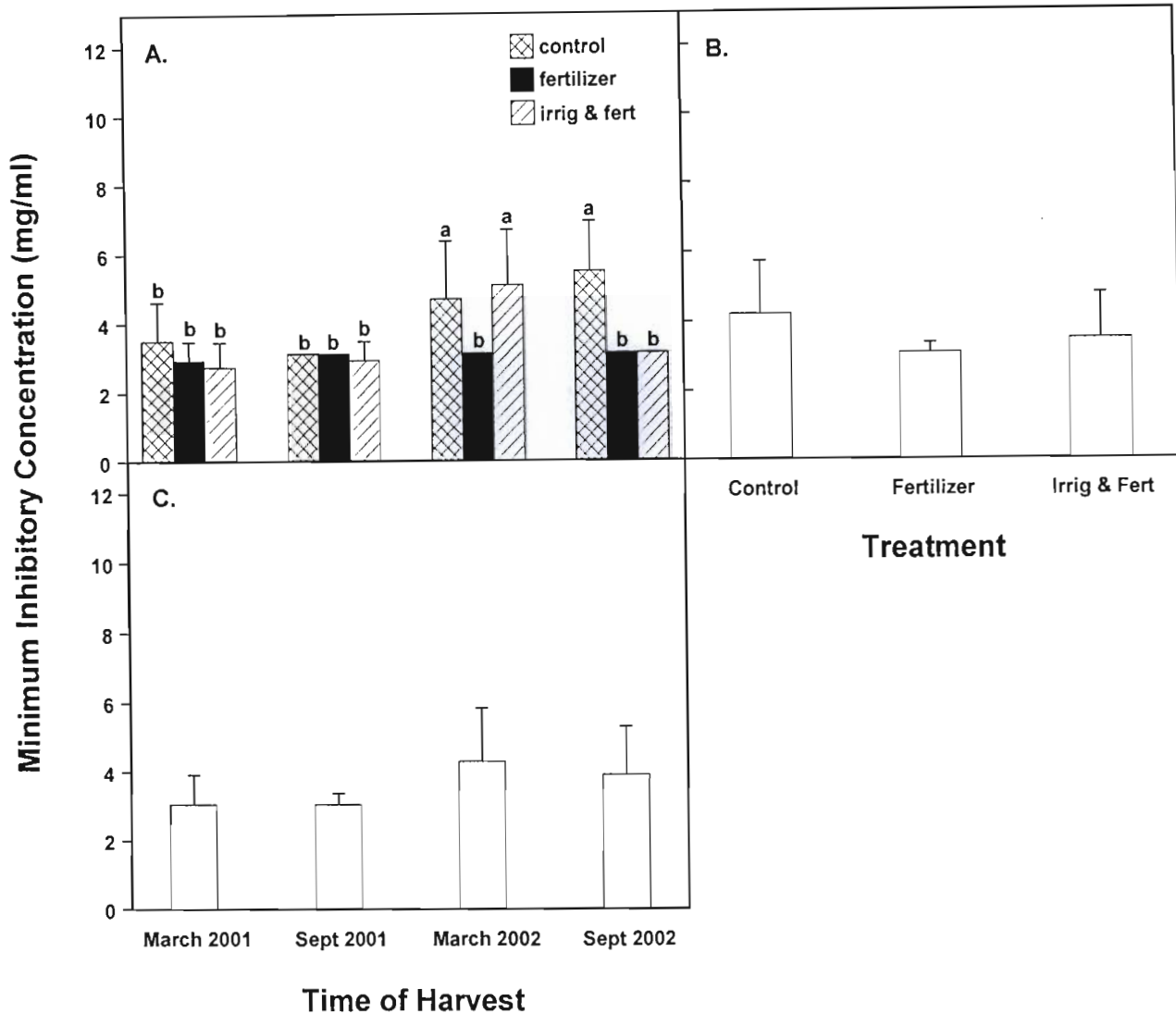


Figure 6.4. Mean MIC values of *M. natalensis* bulb extracts harvested from the H₂A Botanicals site over a period of two years against *K. pneumoniae*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was highly significant ($p < 0.001$). Both the treatments and time of harvest had no significant effect on the antibacterial activity of the bulbs ($p = 0.193$ and $p = 0.221$ respectively). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other.

Analysis of the results also showed that the time of harvest (Figure 6.5C) had a significant effect on the activity against *B. subtilis* ($p=0.002$). However, there was no interaction between the treatments and the time of harvest ($p=0.847$). Analysis of the results showed that the time of harvest (Figure 6.6C) had a highly significant effect ($p\leq 0.05$) on the activity against *S. aureus* ($p<0.001$). There was also no interaction between the type of treatment and the time of harvest ($p=0.866$). Analysis of the results showed that the type of treatment had no significant effect on the activity against *S. aureus* ($p=0.484$). The bulbs harvested from the fertilizer treatment and irrigation treatment had the highest activity (MIC = 2.15 mg/ml) against *S. aureus* (Figure 6.6A).

The results of bulbs harvested from the Fort Hare site against Gram-negative bacteria followed similar trends in activity to the results from the Gram-positive bacteria. Analysis of the results showed no significant ($p\leq 0.05$) interaction between treatments and time of harvest ($p=0.983$). The treatments had no significant effect on the antibacterial activity ($p=0.583$) (Figure 6.7B). There were no significant differences between the MIC values of the six month (April 2000) and 12 month (Nov 2000) harvests against *E. coli* (Figure 6.7C), but the MIC values of both harvests were significantly different from both the 18 month (April 2001) and 24 month (Nov 2001) harvests. Analysis of these results revealed the time of harvest to have a significant effect ($p\leq 0.05$) on the antibacterial activity against *E. coli* ($p<0.001$). Bulbs harvested from the irrigation treatment after 6 months growth, had the highest activity against *E. coli* (MIC = 2.34 mg/ml) (Figure 6.7A).

No real trends were noticeable from the results of *K. pneumoniae* as seen in Figure 6.8. There was no interaction between the treatments and the time of harvests ($p=0.847$). Analysis showed that the treatments (Figure 6.8B) and the time of harvest (Figure 6.8C) had no significant effect ($p\leq 0.05$) on the antibacterial activity ($p=0.071$ and $p=0.242$ respectively). On average, extracts were more active against *K. pneumoniae* than against *E. coli*. The highest activity was recorded with the bulbs harvested from the fertilizer treatment after six and 12 months, 2.73 mg/ml and 2.54 mg/ml respectively.

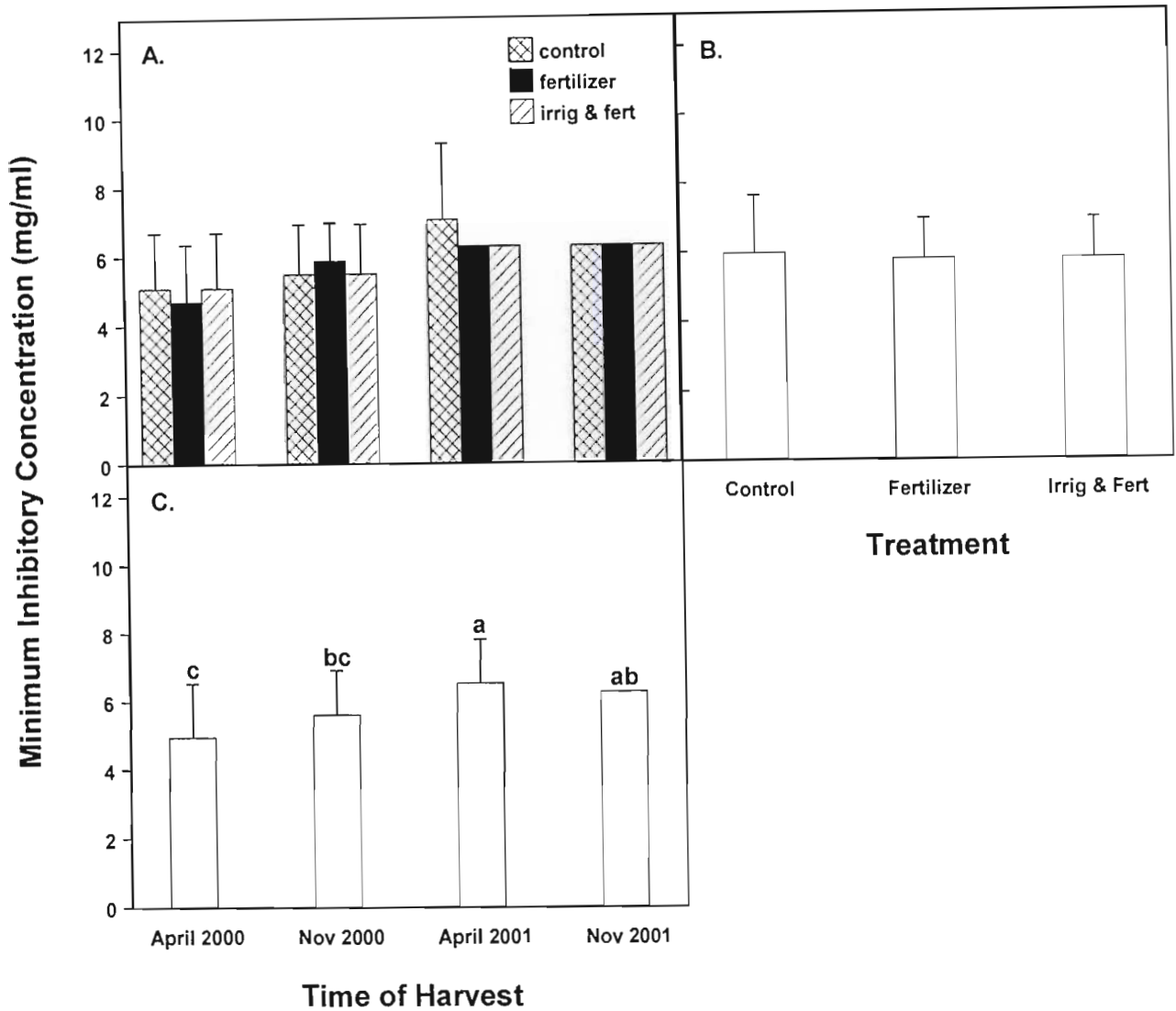


Figure 6.5. Mean MIC values of *M. natalensis* bulb extracts harvested from the Fort Hare site over a period of two years against *B. subtilis*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.847$). The treatments had no significant effect on the antibacterial activity of the bulbs ($p=0.579$). Time of harvest had a significant effect on the antibacterial activity of the bulbs ($p=0.002$). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other.

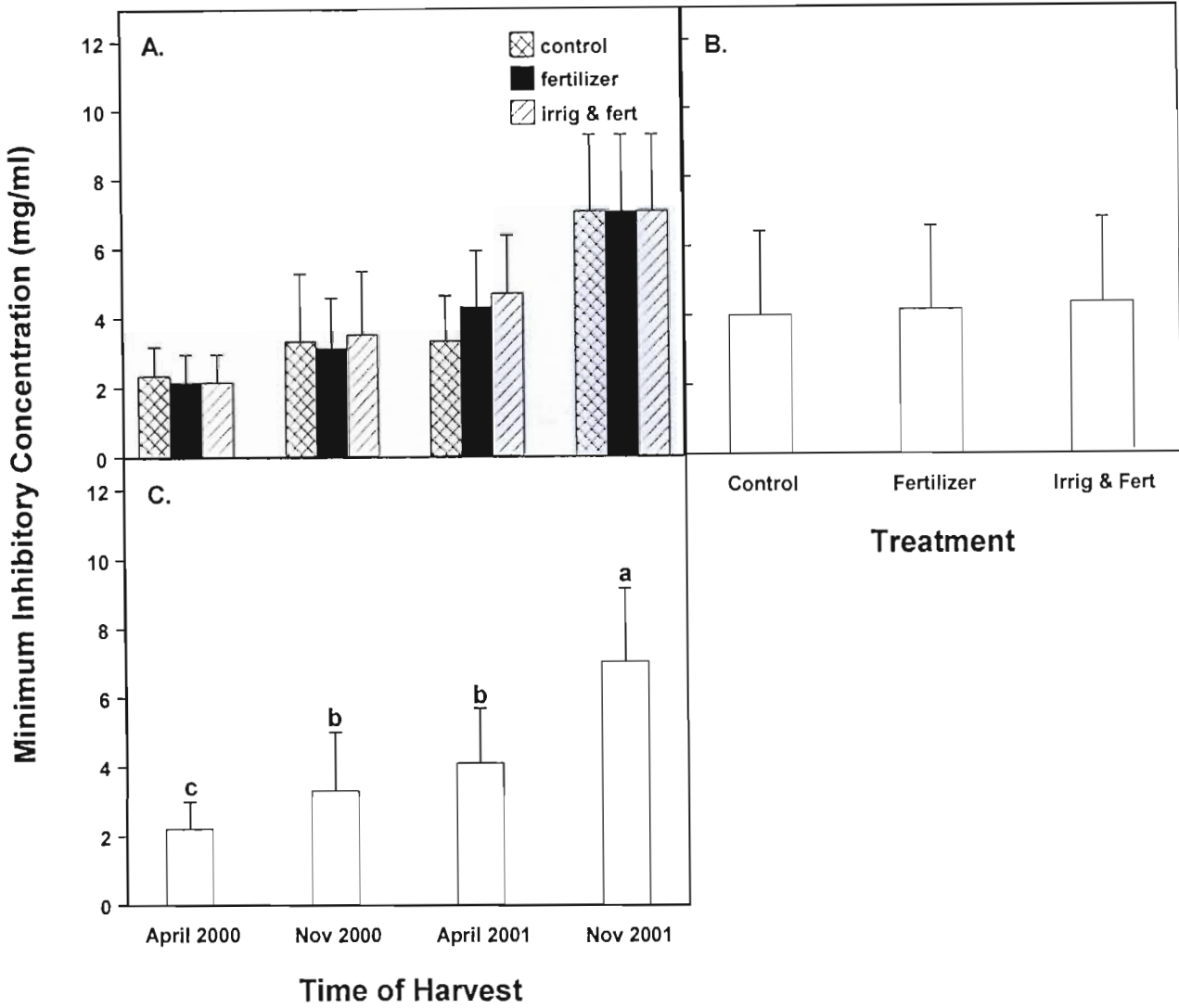


Figure 6.6. Mean MIC values of *M. natalensis* bulb extracts harvested from the Fort Hare site over a period of two years against *S. aureus*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.866$). The treatments had no significant effect on the antibacterial activity of the bulbs ($p=0.484$). Time of harvest had a significant effect on the antibacterial activity of the bulbs ($p<0.001$). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other.

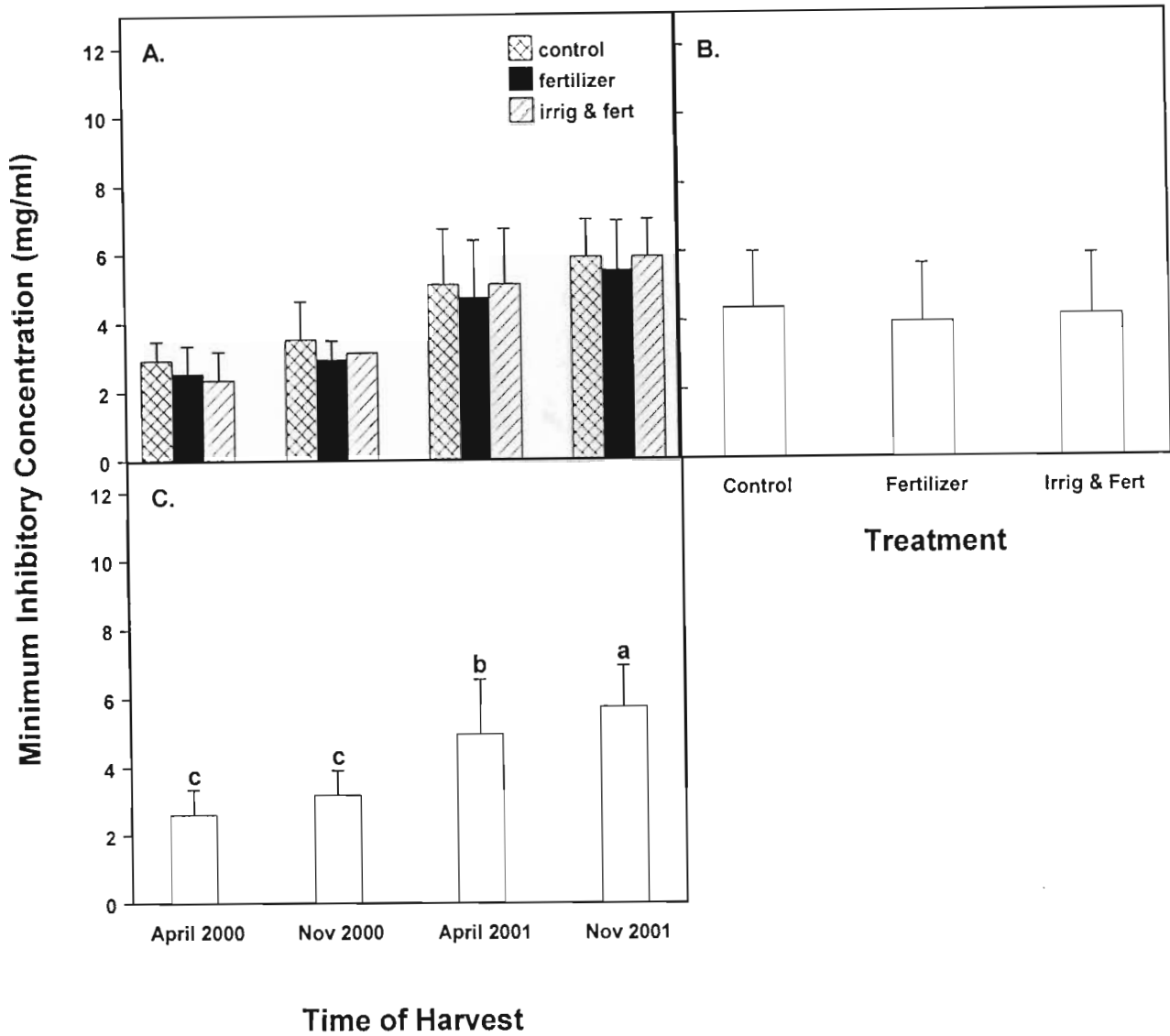


Figure 6.7. Mean MIC values of *M. natalensis* bulb extracts harvested from the Fort Hare site over a period of two years against *E. coli*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.983$). The treatments had no significant effect on the antibacterial activity of the bulbs ($p=0.583$). Time of harvest had a significant effect on the antibacterial activity of the bulbs ($p<0.001$). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other.

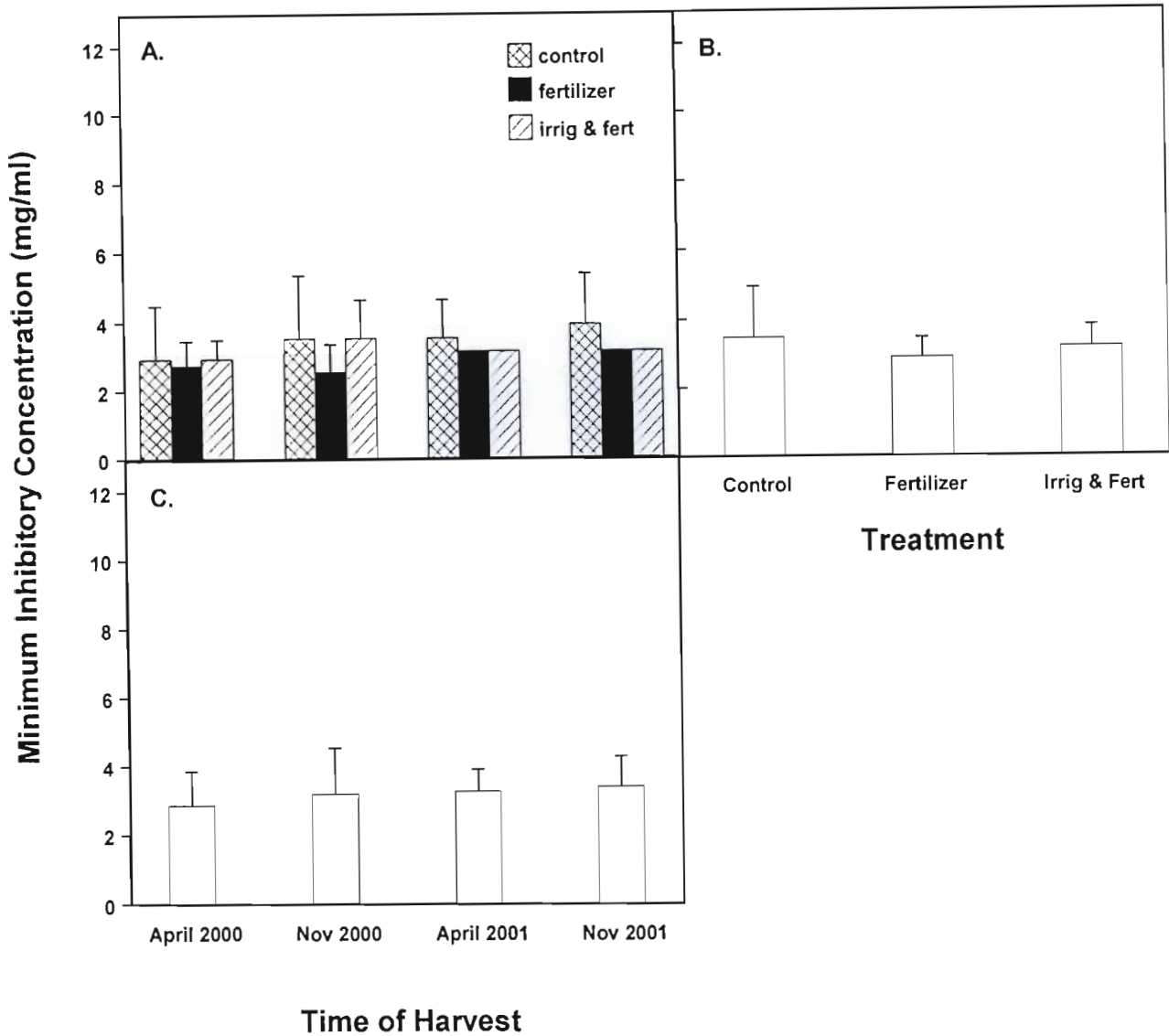


Figure 6.8. Mean MIC values of *M. natalensis* bulb extracts harvested from the Fort Hare site over a period of two years against *K. pneumoniae*. (A) Mean MIC for each extract. (B) Mean MIC of each treatment. (C) Mean MIC of each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.847$). The treatments and time of harvest had no significant effect on the antibacterial activity of the bulbs ($p=0.071$ and $p=0.242$ respectively).

In the study by LIGHT, McGAW, RABE, SPARG, TAYLOR, ERASMUS and VAN STADEN (2002) it was concluded that the time of harvest of *Siphonochilus aethiopicus* only had a minimal influence (two-fold difference) on the degree of antibacterial activity. Although the results of this study were four-fold decrease in activity, the extract concentration levels were not that low to suggest that there is good anti-bacterial activity. The initial testing done on mature bulbs (Section 2.2) resulted in higher antibacterial activity. The extracts from mature bulbs were more active at lower concentrations. From this study it is clear that there is a definite decrease in antibacterial activity with an increase in age with *M. natalensis*. Results of the bioautographic assay confirmed this finding (Figure 6.9). Zones of bacterial inhibition (clear zones) were seen in both the 6 and 12 month harvests (indicated by white arrows, Figure 6.9). These clear zones were observed in extracts of bulbs from both cultivation sites. Younger plants would be more susceptible to bacterial infections and would therefore need to have higher concentrations of antibacterial compounds. As was the case with the antibacterial screening (Section 2.2.3), extracts from *M. natalensis* are generally more active against Gram-negative bacteria. In many of the antibacterial studies that had been conducted with plant extracts, it is the Gram-positive bacteria that are more sensitive than Gram-negative. This is due to differences in the cell wall structure of the bacteria (PAGE, CURTIS, SUTTER, WALKER and HOFFMAN 1997).

6.4. Anti-inflammatory Analysis of Cultivated Bulbs

6.4.1. Methodology

6.4.1.1. Extracts

Dichloromethane extracts were tested for COX-1 inhibition only as these extracts showed the highest anti-inflammatory activity in initial screening (Section 2.3.3). The dichloromethane extracts were dissolved in ethanol to a concentration of 10 mg/ml (250 µg/ml per test solution).

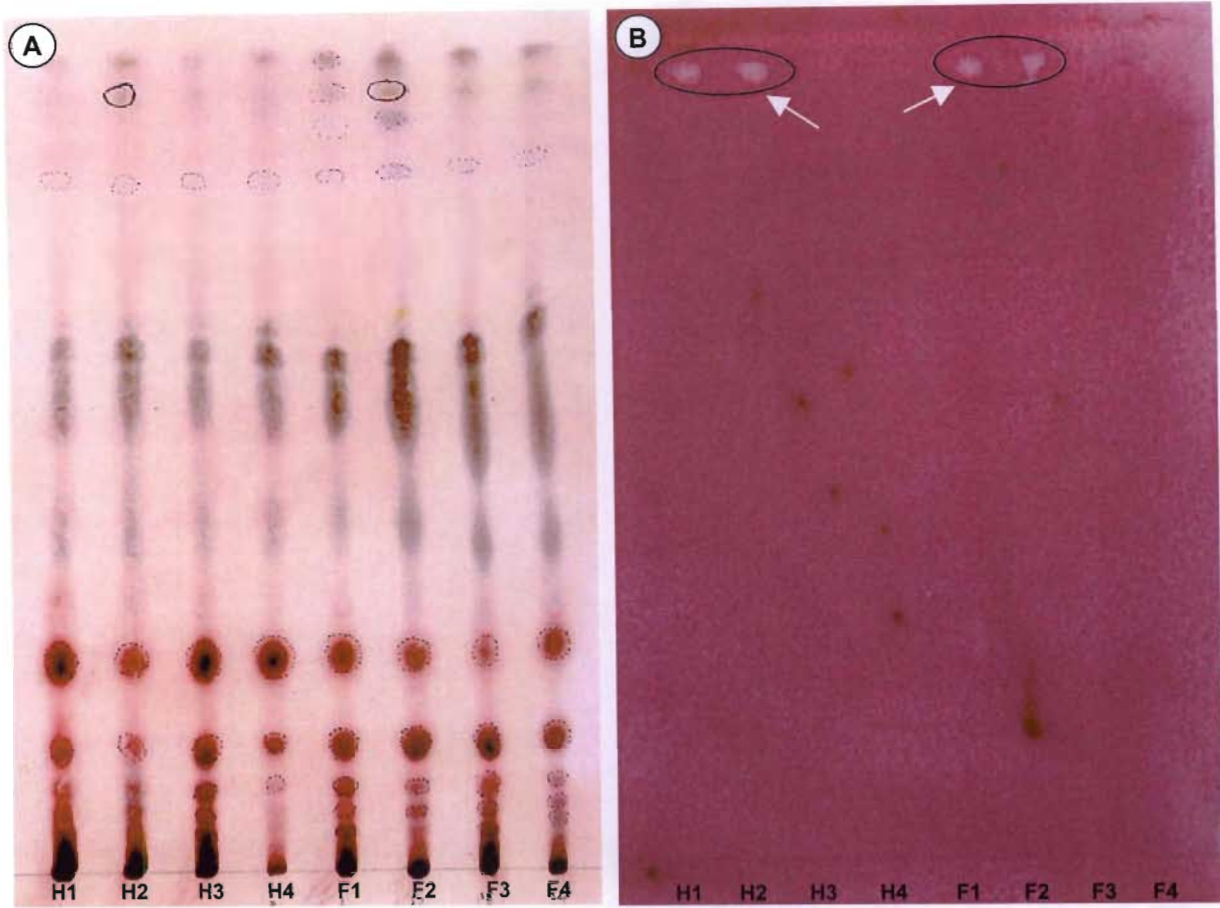


Figure 6.9. Bioautographic assay - TLC plates of *M. natalensis* methanol extracts from bulbs harvested from the control treatment from both the H₂A Botanicals and the Fort Hare sites. TLC plates were developed with a chloroform: methanol: water (8:5:1 v:v:v) solvent system. (A) Reference TLC plate stained with anisaldehyde-sulphuric acid reagent. (B) Corresponding TLC plate with *S. aureus* bacterial overlay. Clear zones indicate zones of bacterial inhibition. H = H₂A Botanicals. F = Fort Hare. 1 – 4 = Harvests (6, 12, 18 and 24 months).

6.4.1.2. *Anti-inflammatory Bioassay*

Extracts were screened using the COX-1 assay as described in Section 2.3.2. Four extracts per treatment per harvest were tested. One replicate of each of the extracts was performed. The assay was repeated a second time giving a total of 8 replicates for each treatment for each harvest.

6.4.2. *Results and Discussion*

The mean percentage inhibitions were calculated as a mean of eight replicates for each treatment of each harvest. The means were compared using Fisher's pairwise comparisons at 5% level of significance. ANOVA was used to determine the significance of interactions and effects of plot treatments and time of harvests on anti-inflammatory activity. The indomethacin control had 59% COX-1 inhibition.

Results from the screening indicate that COX-1 inhibition by both young and mature bulbs cultivated at H₂A Botanicals are high. No significant differences ($p \leq 0.05$) in activity were noticed between different treatments. The percentage inhibition ranged from 88% to 91% (Figure 6.10A). Analysis showed that treatments (Figure 6.10B) had no effect on COX-1 inhibition ($p = 0.613$). There was also no interaction between the treatments and the time of harvest ($p = 0.985$). The time of harvest (Figure 6.10C) also did not have a significant effect ($p \leq 0.05$) on inhibiting the action of the COX-1 enzyme in prostaglandin synthesis ($p = 0.613$).

The bulbs harvested from the Fort Hare site showed similar patterns as the bulbs harvested from H₂A Botanicals. The percentage COX-1 inhibition ranged from 88% to 96% (Figure 6.11A). The dichloromethane extracts from bulbs harvested from the control treatment had the highest activity, inhibiting 96% of the enzyme action in prostaglandin synthesis. The COX-1 inhibition was significantly different ($p \leq 0.05$) from all the other extracts. There was no interaction between the treatments and the time of harvest ($p = 0.550$). The type of treatment (Figure 6.11B) also showed no significant ($p \leq 0.05$) effect on the inhibition ($p = 0.881$). However, the time of harvest (Figure 6.11C) did have a significant effect ($p \leq 0.05$) on inhibiting the enzyme action in prostaglandin synthesis ($p = 0.011$).

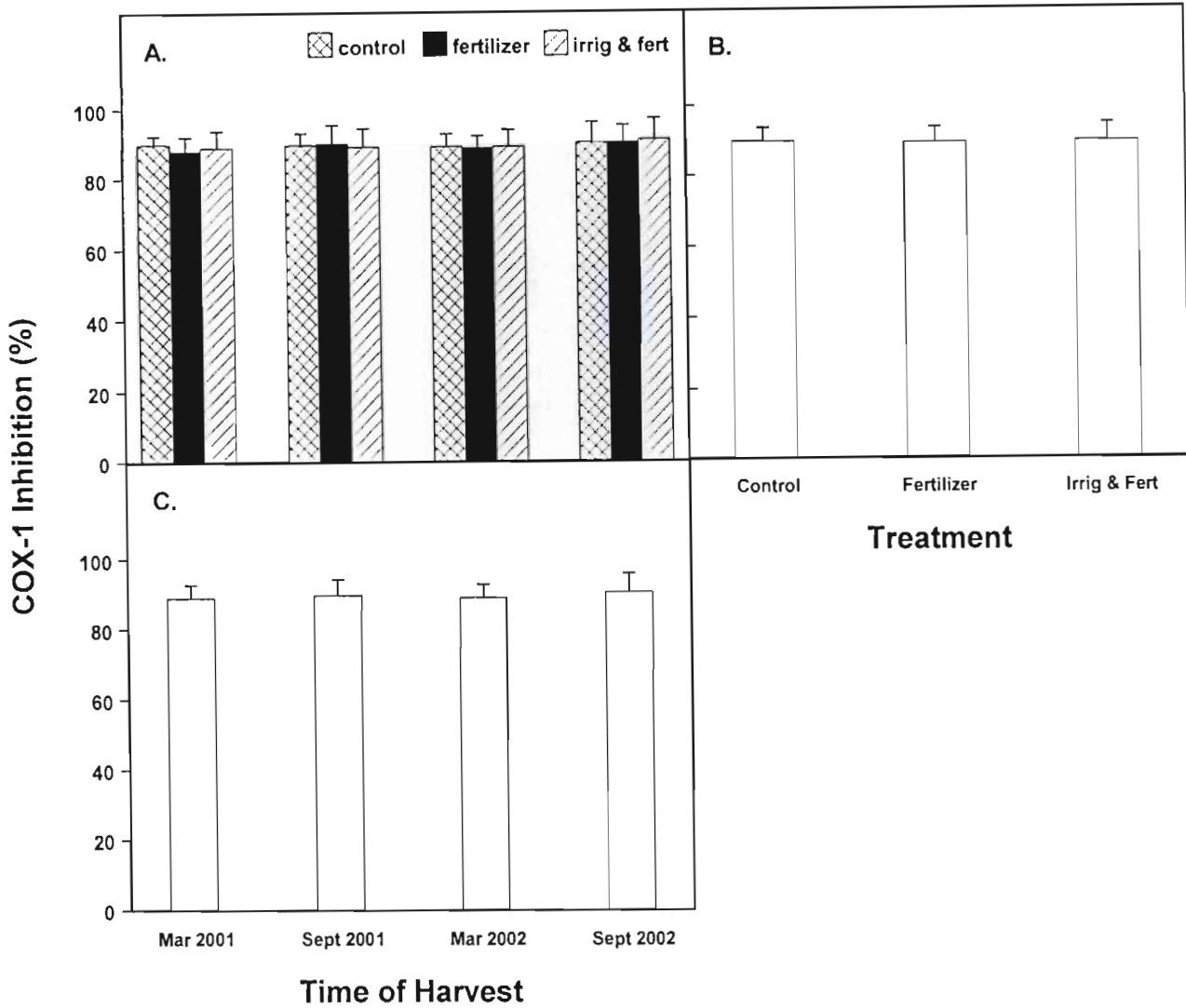


Figure 6.10. Mean percentage inhibition of COX-1 enzyme by *M. natalensis* bulb extracts. Bulbs harvested from the H₂A Botanicals site. (A) Mean inhibition for each extract. (B) Mean inhibition of each treatment. (C) Mean inhibition of each harvest. There was no significant ($p \leq 0.05$) interaction between the treatments and the time of harvest ($p = 0.985$). The treatments had no significant effect on COX-1 inhibition ($p = 0.613$). Time of harvest had no significant effect on COX-1 inhibition ($p = 0.128$).

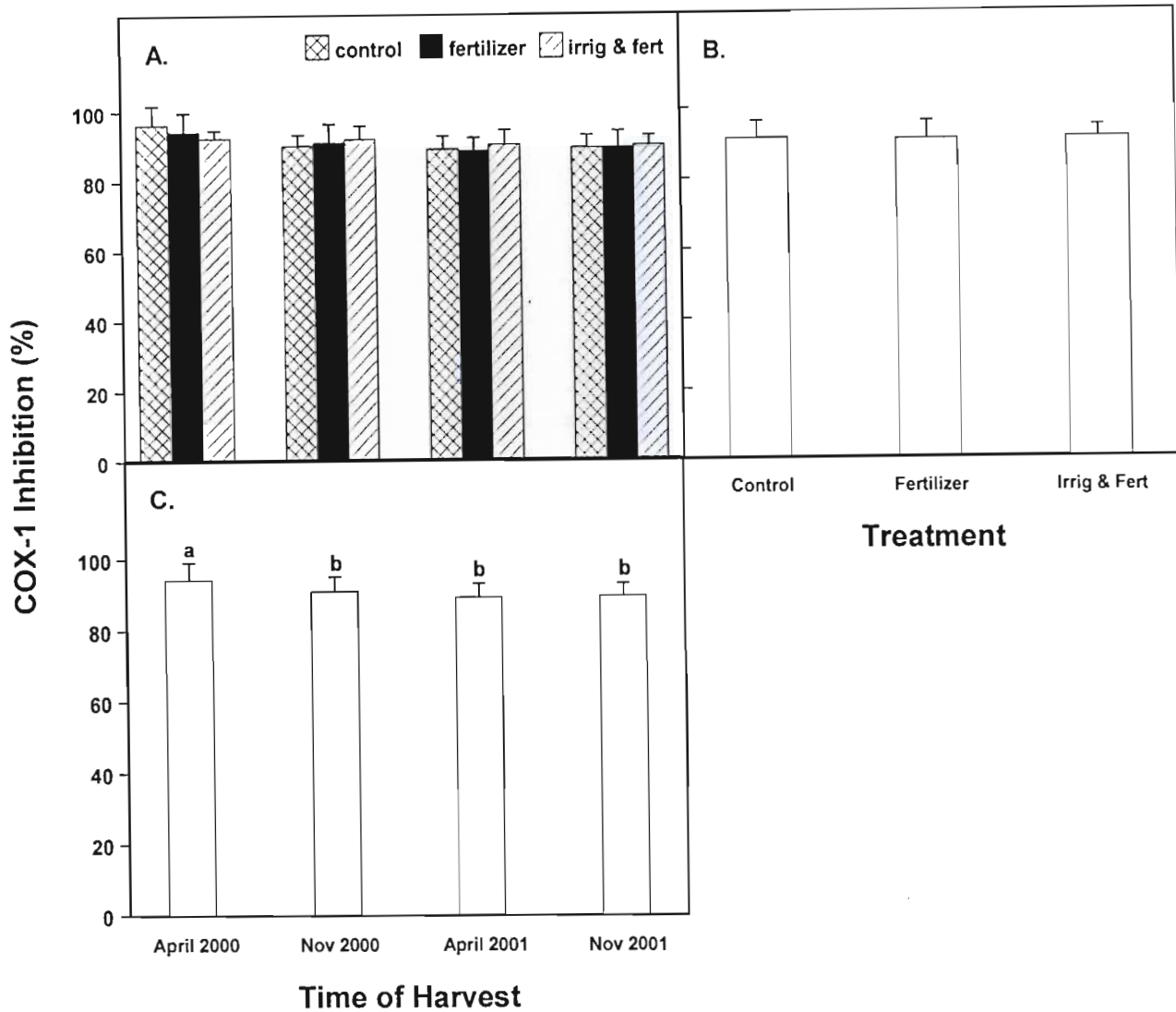


Figure 6.11. Mean percentage inhibition of COX-1 enzyme by *M. natalensis* bulb extracts. Bulbs harvested from the Fort Hare site. (A) Mean inhibition for each extract. (B) Mean inhibition of each treatment. (C) Mean inhibition of each harvest. There was no significant ($p \leq 0.05$) interaction between the treatments and the time of harvest ($p = 0.550$). The treatments had no significant effect on COX-1 inhibition ($p = 0.881$). Time of harvest had a significant effect on COX-1 inhibition ($p = 0.011$). Values within the same graph with the same letters are not significantly different (at 5% level of significance) from each other.

LIGHT, McGAW, RABE, SPARG, TAYLOR, ERASMUS and VAN STADEN (2002) showed that there was little seasonal variation on anti-inflammatory activity in *Siphonochilus aethiopicus*. However, TAYLOR and VAN STADEN (2001) showed that the age of *Eucomis autumnalis* bulbs had an effect on COX-1 inhibition. The bulbs of mature plants were more active than the bulbs of younger plants. There were also differences in activity over the seasons. The highest activity was detected towards the end of the growing season, shortly before the onset of dormancy.

Results from the Fort Hare site, indicates that the COX-1 inhibitory activity was effected by the time of harvest, and that the younger plants had higher activity. This would mean that young bulbs can potentially be used in the treating inflammation. TAYLOR and VAN STADEN (2002) showed that the environmental conditions during the period of winter dormancy effect the levels of anti-inflammatory activity that was exhibited by *Eucomis autumnalis autumnalis* bulbs. However, the results of *M. natalensis*, indicates that winter dormancy had no effect on the levels of COX-1 inhibitory activity.

6.5. Anthelmintic Analysis of Cultivated Bulbs

6.5.1. Methodology

6.5.1.1. Extracts

Dichloromethane extracts were tested against *Caenorhabditis elegans* only as these extracts showed the highest anthelmintic activity (Section 2.5.3). The dichloromethane extracts were dissolved in ethanol to a concentration of 100 mg/ml (1 mg/ml per test solution).

6.5.1.2. Anthelmintic Bioassay

The nematode *Caenorhabditis elegans* var. Bristol (N2) was cultured on nematode growth agar seeded with *E. coli* according to the method of BRENNER (1974). The anthelmintic assay used was the method of RASOANAIVO and

RATSIMAMANGA-URVERG (1993) as described by McGAW, JÄGER and VAN STADEN (2000) (Section 2.5.2). Four extracts per treatment per harvest were tested. One replicate of each of the extracts was performed. The assay was repeated a second time giving a total of 8 replicates for each treatment for each harvest.

6.5.2. Results and Discussion

The percentage survival of the nematodes was calculated as a mean of eight replicates for each treatment of each harvest. The means were compared using Fisher's pairwise comparisons at 5% level of significance. ANOVA was used to determine the significance of interactions and effects of treatments and time of harvest on anthelmintic activity. The levamisole control killed approximately 40% of the nematodes (i.e. a survival rate of 60%).

Methanol extracts from the bulbs of cultivated *M. natalensis* were did not show high in activity as was observed during initial anthelmintic screening (Section 2.5.4). Results of bulbs from the H₂A Botanicals site are given in Figure 6.12. The methanol extracts killed between 30 and 40% (i.e. a survival rate of between 60 and 70%) of the *C. elegans* nematodes after 2 h (Figure 6.12A). There was no significant interaction between the treatments and the time of harvest ($p = 0.510$). The treatments as presented in Figure 6.12B had no significant ($p \leq 0.05$) effect on the anthelmintic activity ($p = 0.092$). There was a clear increase in activity as the plants matured. Figure 6.12C shows that the extracts of bulbs harvested after 24 months were more lethal to *C. elegans*. Analysis indicates that the time of harvest did have a significant effect ($p \leq 0.05$) on the anthelmintic activity ($p = 0.020$). Bulbs harvested from both the fertilizer treatment and the irrigation treatment after 24 months had the highest activity, killing approximately 40% of the nematodes. The 6 month harvest from the control treatment had the weakest activity leading to approximately 30% nematode mortality.

Similar results were shown for the methanol extracts of bulbs cultivated at Fort Hare (Figure 6.13). The extracts killed between 30 and 40% (i.e. a survival rate of between 60 and 70%) of the *C. elegans* nematodes after 2 h (Figure 6.13A). Similarly to the H₂A Botanicals results, no significant ($p \leq 0.05$) interaction between the treatments and the time of harvest ($p = 0.998$) was noted. However, the treatments were found to

have a significant effect ($p \leq 0.05$) on the anthelmintic activity ($p = 0.024$). The bulbs harvested from the irrigation treatment had the highest activity, killing on average 37% of the nematodes (Figure 6.13B). There were no significant differences between the activity from bulbs harvested from the control treatment and the fertilizer treatment. This suggests that irrigation could have an effect on the anthelmintic activity of the bulbs. The average annual rainfall at Fort Hare is lower than at H₂A Botanicals. Therefore, the irrigation treatment at the H₂A Botanical site did not have a significant effect on the anthelmintic activity. From the results of bulbs harvested from the Fort Hare site it can be seen that irrigation could increase the anthelmintic activity of *M. natalensis* bulbs if grown in an area where the mean annual rainfall is around 600 mm. However, because both cultivation sites used different fertilizers in their treatments, the sites cannot be compared properly. The inorganic fertilizer used at the Fort Hare site, combined with irrigation could have effected the chemical composition of the bulbs. The time of harvest had a significant effect ($p \leq 0.05$) on the anthelmintic activity ($p < 0.001$). The more mature bulbs were more active against *C. elegans*, killing on average 41% of the nematodes (Figure 6.13C). Young bulbs harvested after 6 months of cultivation killed only 30% of the nematodes.

Although the differences in anthelmintic activity were statistically significant, it must be remembered that the results are estimated at a 5% range. The results from this *in vitro* study indicate that mature plants could be used in the treatment of intestinal worms (nematodes). However, further *in vivo* testing would be necessary to investigate levels of toxicity.

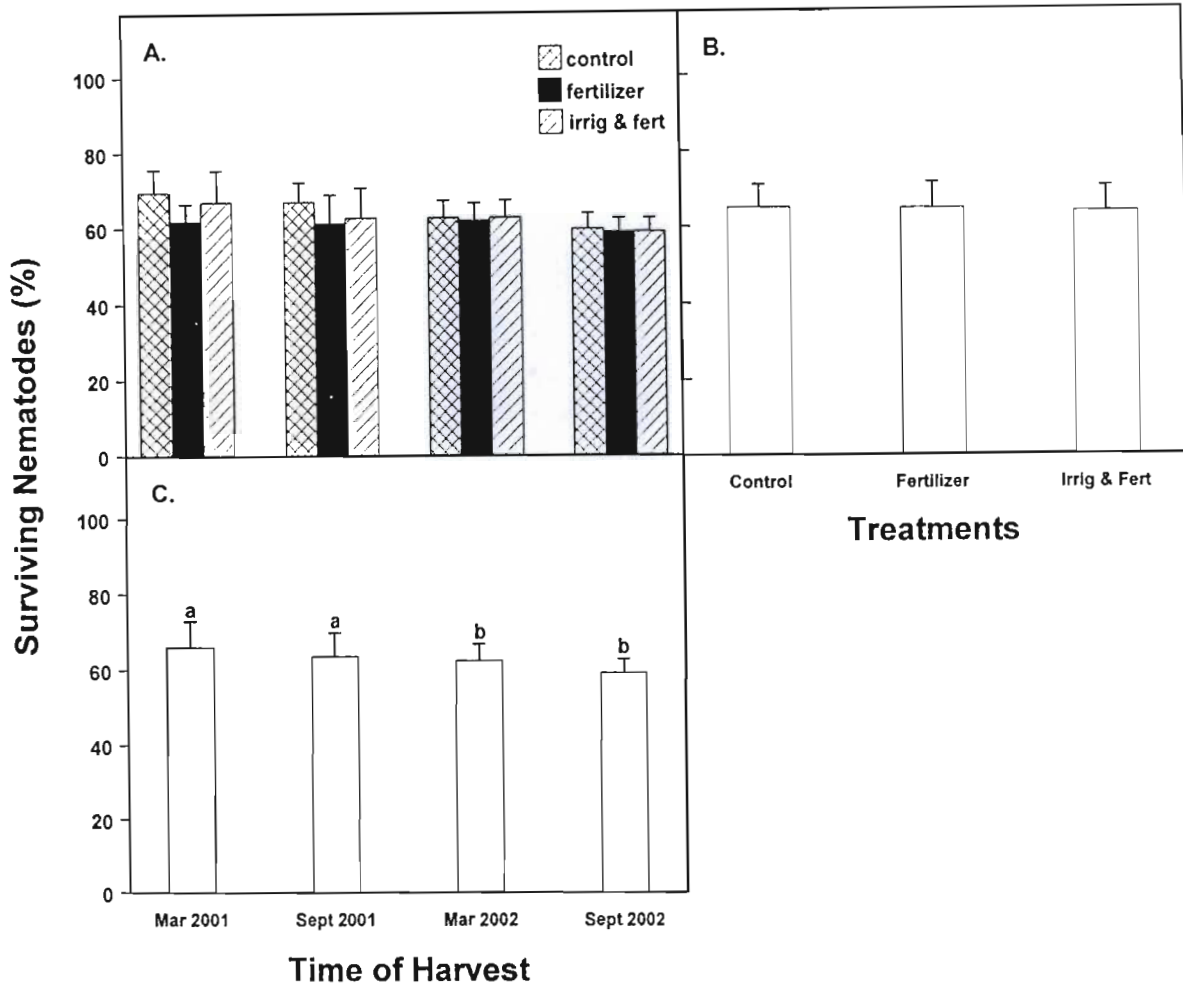


Figure 6.12. Percentage of *Caenorhabditis elegans* nematodes alive after being exposed to methanol extracts of *M. natalensis* bulbs harvested from the H₂A Botanicals site. (A) Mean percentage of nematodes surviving for each extract. (B) Mean percentage of nematodes surviving for each treatment. (C) Mean percentage of nematodes surviving for each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.510$). The treatments had no significant effect ($p\leq 0.05$) on the anthelmintic activity ($p=0.092$). The time of harvest had a significant effect on the anthelmintic activity ($p=0.020$).

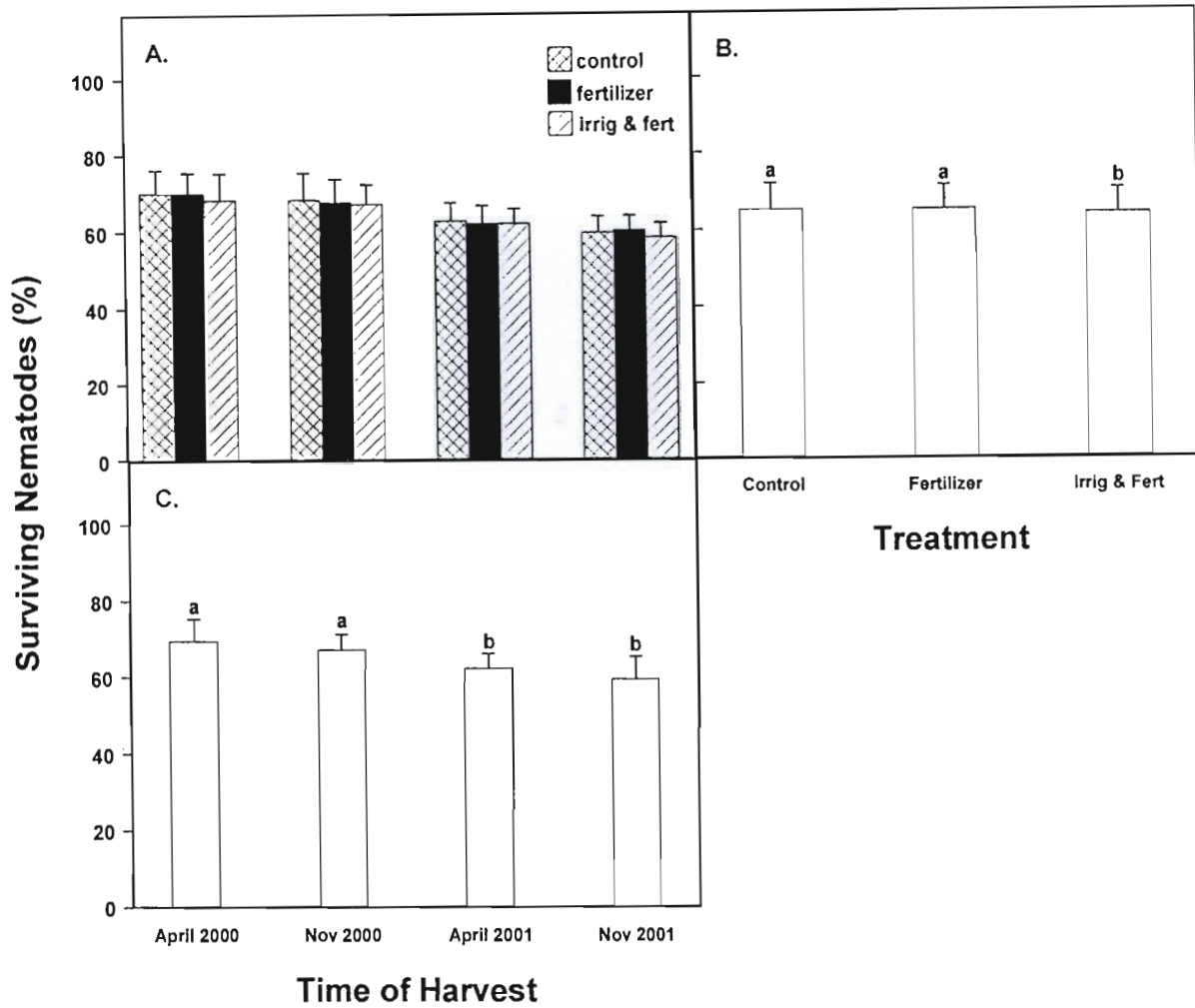


Figure 6.13. Percentage of *Caenorhabditis elegans* nematodes alive after being exposed to methanol extracts of *M. natalensis* bulbs harvested from the Fort Hare site. (A) Mean percentage of nematodes surviving for each extract. (B) Mean percentage of nematodes surviving for each treatment. (C) Mean percentage of nematodes surviving for each harvest. The interaction between the treatments and the time of harvest was not significant ($p=0.998$). The treatments and time of harvest had a significant effect on the anthelmintic activity ($p=0.024$ and $p<0.001$ respectively).

6.6. Summary

- Variations in antibacterial activity were recorded from bulbs cultivated at both growing site.
- In most cases, the treatments had no significant effect ($p \leq 0.05$) on the antibacterial activity against all test bacteria. However, the treatments did have a significant effect ($p \leq 0.05$) against *E. coli* with bulbs grown at the H₂A Botanicals site. The bulbs from the fertilizer treatment were more active against *E. coli* than the bulbs from the other treatments.
- The time of harvest had a significant effect ($p \leq 0.05$) against both Gram-positive bacteria (*B. subtilis* and *S. aureus*) as well as against *E. coli*. In cases where the time of harvest had a significant effect on antibacterial activity, the younger plants had higher activity. Activity seemed to decrease with age.
- Dichloromethane extracts from bulbs grown at both sites had high COX-1 inhibitory activity. Bulbs harvested from the H₂A Botanicals site showed between 88 and 91% inhibition. Bulbs harvested from the Fort Hare site had between 88 and 96% inhibition.
- There were no significant ($p \leq 0.05$) interactions between treatments and the time of harvest on the anti-inflammatory activity.
- The treatments had no significant effect ($p \leq 0.05$) on the anti-inflammatory activity.
- The time of harvest at the Fort Hare site had a significant effect ($p \leq 0.05$) on the inhibition of prostaglandin synthesis by COX-1. The younger plants had higher COX-1 inhibition activity than the mature plants.
- Methanol extracts from bulbs cultivated at both sites had slightly lower anthelmintic activity against *C. elegans* than the methanol extracts of mature bulbs (Section 2.5.4).
- There were no significant ($p \leq 0.05$) interactions between the treatments and the time of harvest with the anthelmintic activity.

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- The treatments had no significant effect ($p \leq 0.05$) on the anthelmintic activity of bulbs cultivated at the H₂A Botanicals site. However, the treatments did have a significant effect ($p \leq 0.05$) on the anthelmintic activity of bulbs cultivated at the Fort Hare site. Results suggest that irrigation might increase the anthelmintic activity of the bulbs if cultivated in areas of low rainfall.
 - The time of harvest at both sites was found to have a significant effect ($p \leq 0.05$) on the anthelmintic activity. Activity was found to increase with plant maturity.

CHAPTER 7 CONCLUSIONS

Until recently, medicinal plants have been primarily collected from the wild. With the increase in cultivation and the use of medicinal plants, a fundamental understanding of the processes involved in cultivating a medicinal plant such as *Merwillia natalensis* as a crop for small scale farming is needed. There are many problems that can be anticipated as more medicinal plants are domesticated and introduced into cultivation. The successful development of *M. natalensis* as a domestic crop indicates that some species can be introduced into cultivation with very little changes in growth, development, and chemical content or activity. However, before any plant can be introduced into cultivation, levels of pharmacological activity and basic chemical composition of wild populations need to be analysed.

The work presented in this thesis encompassed different areas of study that are important in obtaining a better understanding of plants used in southern Africa for traditional medicine and the processes involved in introducing bulbous medicinal plants into cultivation. The first investigation centred on the screening of *M. natalensis* for pharmacological activity. This involved a broad screening of different solvent extracts ranging in polarity. The second aspect was centred on the phytochemical screening of *M. natalensis* bulbs for saponins, cardiac glycosides, alkaloids and tannins. The third aspect of this project was centred around the cultivation of *M. natalensis* as a crop for small-scale farming. This study, involved the analysis of the growth of cultivated plants to determine rates of growth and suitable growing conditions. A fourth aspect of this project concentrated on developing a TLC fingerprinting system and TLC fingerprinting profiles of the cultivated plants. The final aspect of the project centred on screening the cultivated plants for pharmacological activity. This study involved analysis of the effect of growth conditions and times of harvest on the pharmacological activity of the product.

7.1. Pharmacological Investigation of *Merwillia natalensis*

M. natalensis bulb extracts exhibited a range of pharmacological activities. Methanol extracts had good antibacterial activity against both Gram-positive and Gram-negative test bacteria as well as good anthelmintic activity. Aqueous extracts (0.4 mg/ml) of both fresh and dry material yielded very high antischistosomal activity.

Non-polar extracts were found to have high anti-inflammatory activity against both COX-1 and -2. Although non-polar solvents are not accessible to traditional healers, the levels of activity shown in these screenings, confirmed the plants usage in traditional medicine. Cytotoxicity testing showed the bulbs to be highly cytotoxic against VK cells killing 44% of the cells at a concentration of 3.9 µg/ml.

7.2. Phytochemical Screening

Phytochemical screening revealed two major groups of compounds within the bulbs of *M. natalensis*. The bulbs contain saponins, which appear to make up a large portion of the chemical composition of the bulbs. The other group was bufadienolide-type cardiac glycosides of which it seems that proscillaridin A is one. The pharmacological activity shown by the bulb extracts could be attributed to these two groups of compounds. Screenings for alkaloids and tannins came up negative.

7.3. Growth Analysis of Material Collected From Field Trials

M. natalensis plants were cultivated at two different sites that experience different climatic conditions. Bulbs grew successfully at both sites, reaching almost marketable size (± 300 g fresh weight) two years after being planted out into the field. Bulb size was found not to be dependent on additional fertilizer or irrigation.

The plants started flowering after two years, with the highest percentage flowering in the irrigation and fertilizer plot. Leaf senescence and dormancy of young plants were prevented with the addition of irrigation. However, the annual growth cycles were found to resume as the plants matured.

7.4. Chemical Fingerprinting By TLC

TLC fingerprinting profiles were developed for methanol, dichloromethane and water extracts of mature wild *M. natalensis* bulbs. Comparisons between the mature wild bulbs and the harvested bulbs from both the cultivation sites were done. The methanol and water extracts showed some differences in chemical composition. Differences were most pronounced in the bulbs harvested from the Fort Hare site. No differences in the chemical constituents were found between the dichloromethane extracts. However, there were noticeable differences in the concentrations of certain compounds.

7.5. Pharmacological Analysis of Material Collected From Field Trials

Pharmacological analysis of the harvested material did demonstrate some statistically significant variations in the activity of the bulbs. Antibacterial screenings showed that the activity of younger plants is higher, with the activity decreasing as the plants mature. The treatments did not have a significant effect ($p \leq 0.05$) on the activity. Anti-inflammatory screenings against COX-1 showed no significant differences ($p \leq 0.05$) in activity with plant age. As with the antibacterial activity, the treatments had no significant effect ($p \leq 0.05$) on the anti-inflammatory activity. The anthelmintic screenings against *Caenorhabditis elegans* showed that the activity increased as the bulbs mature. Plants cultivated in areas of lower rainfall, under irrigation, had higher activity than plants that were not irrigated. However, in areas where the mean annual rainfall is higher than 1000 mm the application of irrigation did not increase the biological activity of *M. natalensis* bulbs.

The results indicate that *M. natalensis* is a hardy species. Generally the application of supplementary fertilizer or irrigation did not appear to affect the pharmacological activity of the bulbs. In most cases the time of harvest had a significant effect ($p \leq 0.05$) on the activity. However, the plants were only cultivated for a period of two years and the activity levels achieved were generally lower than the activity levels of the mature plants in the initial screenings (Chapter 2). This means that growing the plants to a fully mature stage would probably be more beneficial to farmers who wish to cultivate and sell the bulbs. The larger bulbs would fetch higher

prices at markets even though certain biological activity levels of the bulbs may be slightly lowered.

7.6. Conclusion

This thesis represents an extensive investigation into the medicinal properties of *M. natalensis* and rationalise its importance as a medicinal plant. This study provides scientific verification for the use of *M. natalensis* in medicinal preparations. The investigation into the introduction to cultivation has not only provided much of the information needed for cultivating this species as a crop by small-scale farmer, but it has also allowed collection of information useful to provide for ecological sustainability. The successful domestication along with the pharmacological evidence shows that through cultivation the activity of indigenous medicinal plants is not necessarily lost but is in most cases dependent on the time of harvesting. This work therefore provides important data for the potential conservation and exploitation of many over-harvested medicinal plants.

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