

**Evaluation of anthelmintic,
antiamoebic and antibacterial activity
in traditional South African medicinal
plants**

by

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DECLARATION

The experimental work described in this thesis was conducted in the Research Centre for Plant Growth and Development, School of Botany and Zoology, University of Natal Pietermaritzburg, from May 1998 to May 2001, under the supervision of Professor J. van Staden and Doctor A. K. Jäger.

These studies are the result of my own investigations, except where the work of others is acknowledged, and have not been submitted in any other form to another University.

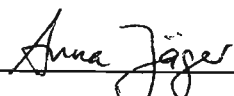


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ABSTRACT

Traditional medicine in southern Africa draws upon a vast selection of plants to treat gastrointestinal disorders such as diarrhoea and intestinal parasites. The evaluation of these plants for biological activity is necessary, both to substantiate the use of these plants by healers, and also as a possible lead for new drugs or herbal preparations.

After a survey of the existing ethnobotanical literature, plants used to treat stomach ailments such as diarrhoea, dysentery or intestinal worm infestations were selected and submitted to bioassays according to their traditional uses. Extracts of the chosen plants were made using the solvents hexane, ethanol and water, to ensure the extraction of compounds with a wide range of polarity. In total, 138 extracts were tested for antibacterial activity, 72 for anthelmintic activity, and 42 for antiamoebic activity. Antibacterial activity was evaluated using the disc-diffusion assay, and Minimal Inhibitory Concentration (MIC) values were determined using a microdilution assay. The extracts were tested against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, and the Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae*. Ethanolic extracts showed the greatest activity and Gram-positive bacteria were the most susceptible microorganisms.

The free-living nematode *Caenorhabditis elegans*, which is morphologically similar to parasitic nematodes, was used in two different assays to evaluate anthelmintic activity. A microdilution technique was employed to investigate antiamoebic activity against the enteropathogenic *Entamoeba histolytica*, the causal organism of amoebic dysentery. These assays were suitable for the screening of a large number of extracts at one time. Several plants exhibited significant activity against these test organisms.

Many species of plants belonging to the family Combretaceae are used in southern African traditional medicine against a variety of ailments, including abdominal complaints, bilharzia and diarrhoea. Extracts of powdered leaf material of 24 species

belonging to the Combretaceae were prepared using the solvents ethyl acetate, acetone, methanol and water. These extracts were screened for anthelmintic activity. Significant activity was exhibited by *C. apiculatum*, *C. hereroense* and *C. mossambicense*. The most anthelmintic activity was shown by acetone extracts, followed by ethyl acetate, water and then methanol extracts.

The aromatic rhizomes of *Acorus calamus* L. are used extensively in traditional medicine worldwide. They reportedly relieve stomach cramps and dysentery, and are used as anthelmintics. Rhizome extracts of *A. calamus* growing in KwaZulu-Natal, South Africa, exhibited anthelmintic and antibacterial activity in the initial general screening. Using bioassay-guided fractionation, the phenylpropanoid β -asarone was isolated from the rhizome. This compound possessed both anthelmintic and antibacterial activity. It has previously been isolated from *A. calamus*, and a related species, *A. gramineus*. Different varieties of *A. calamus* exhibit different levels of β -asarone, with the diploid variety containing none of the compound. Mammalian toxicity and carcinogenicity of asarones has been demonstrated by other researchers, supporting the discouragement of the medicinal use of *Acorus calamus* by traditional healers in South Africa.

Schotia brachypetala was another plant to show good antibacterial activity in the initial screening. The roots and bark of *S. brachypetala* are used in South African traditional medicine as a remedy for dysentery and diarrhoea. The lack of pharmacological and chemical data on this plant prompted a further investigation into its antibacterial activity. The differences in activity of ethanol and water extracts with respect to plant part, season and geographical position were analysed. No extreme fluctuations in activity were noted. Two other *Schotia* species, *S. afra* and *S. capitata*, were included in the study, and both displayed good antibacterial activity. The storage of the plant, either as dried, ground plant material at room temperature, or as an extract residue at -15°C , had little effect on the antibacterial activity. Preparing the extracts from fresh or dry material also did not notably affect the activity. In general, the ethanolic extracts were more active than the aqueous extracts. The chemical profiles on TLC chromatograms were compared and found to

be very similar in the case of ethanol extracts prepared in different months of the year, and from different trees. The extracts of the three species, and of the leaves stored under various conditions, as well as extracts prepared from fresh or dry material, also showed similar TLC fingerprints. However, various plant parts of *S. brachypetala* showed distinctly different chemical compositions.

The leaves of *S. brachypetala* showed slightly higher antibacterial activity than the roots. Fractionation of the ethanol extract of the dried leaves using liquid-liquid partitioning and chromatographic techniques yielded 9,12,15-octadecatrienoic (linolenic) acid and methyl-5,11,14,17-eicosatetraenoate. These fatty acids displayed antibacterial activity against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, and activity to a lesser extent against the Gram-negative *Escherichia coli* and *Klebsiella pneumoniae*. Linolenic acid is known to have antibacterial activity.

The screening of plants for biological activity yielded valuable preliminary information about the plants used by traditional healers to treat gastrointestinal illnesses. The isolation of biologically active compounds from two highly active plants was achieved.

PAPERS PREPARED FROM THIS THESIS

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TABLE OF CONTENTS

| | |
|---|------------|
| Declaration..... | i |
| Acknowledgements | ii |
| Abstract | iii |
| Papers prepared from this thesis | vi |
| Conference contributions from this thesis..... | vii |
| Table of contents | ix |
| List of Figures and Plates | xvi |
| List of Tables | xix |
| List of Abbreviations | xxi |

CHAPTER 1 LITERATURE REVIEW

| | |
|--|----|
| 1.1 Introduction..... | 1 |
| 1.2 Plants as sources of useful drugs..... | 1 |
| 1.3 Selection of plants for biological activity screening..... | 6 |
| 1.4 Plants as sources of antimicrobial drugs..... | 8 |
| 1.5 Traditional medicine in South Africa..... | 10 |
| 1.6 Gastrointestinal disorders..... | 13 |
| 1.7 The effect of intestinal parasites in humans..... | 15 |
| 1.8 Aims and objectives..... | 16 |

CHAPTER 2 PLANT COLLECTION AND EXTRACTION

| | |
|---------------------------------------|----|
| 2.1 Introduction..... | 17 |
| 2.2 Materials and Methods | 18 |
| 2.2.1 Plant collection..... | 18 |
| 2.2.2 Plant extract preparation | 19 |
| 2.3 Results | 20 |
| 2.4 Discussion | 30 |
| 2.5 Conclusion | 30 |

CHAPTER 3 ANTHELMINTIC SCREENING

| | |
|--|----|
| 3.1 Introduction | 31 |
| 3.1.1 Helminthiasis | 31 |
| 3.1.2 Control of helminth infection | 33 |
| 3.1.3 Anthelmintic assays | 34 |
| 3.2 Materials and Methods | 36 |
| 3.2.1 Culture of <i>Caenorhabditis elegans</i> | 36 |
| 3.2.2 Mortality assay | 37 |
| 3.2.3 Reproductive ability assay | 37 |
| 3.3 Results | 38 |
| 3.3.1 Standardization of results | 38 |
| 3.3.2 Anthelmintic activity of plant extracts | 38 |
| 3.4 Discussion | 44 |
| 3.4.1 Comparison of anthelmintic assays | 44 |
| 3.4.2 Anthelmintic activity of plant extracts | 44 |
| 3.5 Conclusion | 45 |

CHAPTER 4 ANTIAMOEBIIC SCREENING

| | |
|--|----|
| 4.1 Introduction | 47 |
| 4.1.1 Amoebiasis | 47 |
| 4.1.2 Control of amoebiasis | 48 |
| 4.1.3 Antiamoebic assays | 50 |
| 4.2 Materials and Methods | 51 |
| 4.2.1 Antiamoebic assay | 51 |
| 4.3 Results | 52 |
| 4.3.1 Standardization of assay | 52 |
| 4.3.2 Antiamoebic activity of plant extracts | 53 |
| 4.4 Discussion | 55 |
| 4.4.1 Antiamoebic activity of plant extracts..... | 55 |
| 4.5 Conclusion | 55 |

CHAPTER 5 ANTIBACTERIAL SCREENING

| | |
|--|----|
| 5.1 Introduction | 56 |
| 5.1.1 Infectious diarrhoea and dysentery | 56 |
| 5.1.2 Control of enteropathogens causing infectious diarrhoea and dysentery | 57 |
| 5.1.3 Antibacterial assays | 58 |
| 5.1.3.1 Diffusion methods | 59 |
| 5.1.3.2 Dilution methods | 60 |
| 5.1.3.3 Bioautographic methods | 60 |
| 5.2 Materials and Methods | 61 |
| 5.2.1 Quantification of bacteria | 61 |
| 5.2.2 Disc-diffusion assay | 62 |
| 5.2.3 Microdilution assay | 62 |
| 5.3 Results | 64 |
| 5.3.1 Quantification of bacteria | 64 |
| 5.3.2 Antibacterial activity of plant extracts | 64 |
| 5.4 Discussion | 68 |
| 5.4.1 Antibacterial activity of plant extracts | 68 |
| 5.5 Conclusion | 70 |

CHAPTER 6 ANTHELMINTIC ACTIVITY IN THE FAMILY COMBRETACEAE

| | |
|---|----|
| 6.1 Introduction | 71 |
| 6.1.1 The family Combretaceae | 71 |
| 6.1.2 Traditional medicinal usage | 71 |
| 6.1.3 Biological activity and chemical constituents | 72 |
| 6.2 Materials and Methods | 73 |
| 6.2.1 Plant extract preparation | 73 |
| 6.2.2 Screening of plant extracts for anthelmintic activity | 73 |
| 6.3 Results | 74 |
| 6.3.1 Anthelmintic activity of Combretaceae extracts..... | 74 |
| 6.4 Discussion | 78 |

| | |
|--|-----|
| 9.2.2 Bioassay-guided fractionation for isolation of active compounds..... | 118 |
| 9.2.2.1 Liquid-liquid partitioning | 119 |
| 9.2.2.2 Vacuum Liquid Chromatography..... | 119 |
| 9.2.2.3 Chlorophyll extraction | 120 |
| 9.2.2.4 Gravity-assisted Column Chromatography..... | 120 |
| 9.2.2.5 Preparative Thin Layer Chromatography..... | 120 |
| 9.2.3 Identification of purified active compound..... | 121 |
| 9.3 Results | 121 |
| 9.3.1 Bulk extraction | 121 |
| 9.3.2 Bioassay-guided fractionation for isolation of active compounds..... | 121 |
| 9.3.2.1 Liquid-liquid partitioning..... | 122 |
| 9.3.2.2 Vacuum Liquid Chromatography..... | 122 |
| 9.3.2.3 Chlorophyll extraction..... | 122 |
| 9.3.2.4 Gravity-assisted Column Chromatography..... | 122 |
| 9.3.2.5 Preparative Thin Layer Chromatography..... | 123 |
| 9.3.3 Identification of purified active compound..... | 123 |
| 9.4 Discussion | 125 |
| 9.4.1 Isolation and identification of active compounds..... | 125 |
| 9.4.2 Fatty acids as constituents of plants..... | 125 |
| 9.4.3 Compound A: 9,12,15-octadecatrienoic acid..... | 126 |
| 9.4.4 Compound B: methyl-5,11,14,17-eicosatetraenoate..... | 128 |
| 9.4.5 Fatty acids as antimicrobial agents..... | 128 |
| 9.4.6 Structure-function relationships of fatty acids..... | 130 |
| 9.4.7 Mechanism of antimicrobial action of fatty acids..... | 132 |
| 9.5 Conclusion | 139 |

CHAPTER 10 GENERAL CONCLUSIONS

| | |
|--|-----|
| 10.1 Introduction | 141 |
| 10.2 Screening of plants for biological activity..... | 142 |
| 10.3 Variation in activity and chemical composition of plant extracts..... | 143 |
| 10.4 Isolation and identification of active compounds in plants..... | 144 |
| 10.5 Conclusion | 144 |

REFERENCES.....146

APPENDIX 1174

LIST OF FIGURES AND PLATES

CHAPTER 3

Figure 3.1. Inhibition of nematodes by levamisole (2 h mortality assay).....39

Figure 3.2. Inhibition of nematodes by levamisole (7 day reproductive ability assay).....39

CHAPTER 4

Figure 4.1. Amoebicidal activity of metronidazole.....52

CHAPTER 7

Figure 7.1. Structure of β -asarone.....87

Plate 7.1. *Acorus calamus* (A) uprooted from a shallow pond in the Botanic Gardens, UNP and (B) rhizome. The length of the plant from rhizome to leaf tip was 72 cm, and the length of the rhizome portion displayed was 17 cm. TLC fingerprints of ethanol extracts of *A. calamus* leaf, rhizome and root viewed (C) under visible light, (D) under UV₂₅₄ and (E) under UV₃₆₆. The solvent system used was hexane:ethyl acetate (2:1). Marked with an arrow is the compound isolated (β -asarone).....89

Plate 7.2. TLC of Vacuum Liquid Chromatography (VLC) fractions in the bioassay-guided fractionation of *A. calamus* ethanolic rhizome extract. Viewed (A) under UV₂₅₄, (B) under UV₃₆₆ and (C) after staining with anisaldehyde. The bioautography plate (D) shows white areas of no bacterial growth, indicating the presence of antibacterial compounds. The active compound of interest (β -asarone) is marked with an arrow.....90

CHAPTER 8

Plate 8.1. A flowering *Schotia brachypetala* tree in Umfolozi Nature Reserve,

KwaZulu-Natal (A), a close-up view of the flowers, leaves and seed pods with seeds (B), and photographs of a tree from which bark had recently been harvested (C, D and E) in Pietermaritzburg..... 101

Plate 8.2. TLC separation of the ethanol extracts (A) of different plant parts of *S. brachypetala*, viewed (1) under visible light, (2) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}. The solvent system used was toluene:ethyl acetate (4:1).

[lf = leaf, st = stem, bk = bark, rt = root, rb = root bark, fl = flower, sp = seeds plus pods]

TLC separation of the ethanol extracts (B) of *S. brachypetala* (Sb), *S. afra* (Sa) and *S. capitata* (Sc) viewed (1) under visible light, (2) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}..... 102

Plate 8.3. TLC separation of the ethanol extracts of leaves of genetically different trees of *S. brachypetala*, viewed (1) under visible light, (2) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}. The solvent system used was toluene:ethyl acetate (4:1).

[Source of plant material: a - g = different trees growing in the Botanic Gardens, UNP; h = Silverglen Nature Reserve; i = Umfolozi Nature Reserve; j = Scottsville, Pietermaritzburg; k = Hilton, Pietermaritzburg]..... 103

Plate 8.4. TLC separation of the ethanol extracts of *S. brachypetala* leaf material collected during different months, viewed (1) under visible light, (2) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}. The solvent system used was toluene:ethyl acetate (4:1).

[Collection month: a = January, b = February, c = March, d = April, e = May, f = June, g = July, h = August, i = September, j = October, k = November, l = December]..... 104

Plate 8.5. TLC separation (A) of the ethanol extracts of *S. brachypetala* leaf extracts prepared from fresh (F) and dry (D) material, viewed (1) under visible light, (2) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}.

TLC separation of (B) the ethanol extracts of *S. brachypetala* leaf material after

being stored under various conditions, viewed (1) under visible light, (2) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}. The solvent system used was toluene:ethyl acetate (4:1).

[a = extract screened immediately after collection, drying and extraction of material; b = extract stored in freezer at -15°C for 18 months; c = dried, intact plant material stored in dark cupboard at room temperature before being ground, extracted and screened]..... 105

CHAPTER 9

Plate 9.1. TLC separation (A) of the VLC fractions of the hexane fraction from the liquid-liquid partitioning step, viewed (1) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}. The bioautography plate (2) shows white areas where bacterial growth was inhibited. The arrows indicate the two fatty acids, compounds a (9,12,15-octadecatrienoic acid) and b (methyl-5,11,14,17-eicosatetraenoate) isolated from *S. brachypetala*. The solvent system used was hexane:ethyl acetate (2:1).

[H = hexane extract from the liquid-liquid partitioning step before being submitted to VLC]

TLC separation (B) of the different plant parts of *S. brachypetala* viewed (1) after staining with anisaldehyde. The bioautography plate (2) shows the position of the two subsequently isolated fatty acids, indicated by the arrows. It is clear that these compounds are present in the leaves, bark and roots; the roots possess additional antibacterial compounds, as do the flowers. The solvent system used was hexane:ethyl acetate (2:1).

[lf = leaf, st = stem, bk = bark, rt = root, rb = root bark, fl = flower, sp = seeds plus pods]..... 124

LIST OF TABLES

CHAPTER 2

| | |
|--|----|
| Table 2.1. South African medicinal plants investigated for anthelmintic, antiamebic and antibacterial activity..... | 20 |
|--|----|

CHAPTER 3

| | |
|--|----|
| Table 3.1. Inhibition of nematodes by plant extracts (2 h and 7 day anthelmintic assays)..... | 40 |
|--|----|

CHAPTER 4

| | |
|--|----|
| Table 4.1. Antiamebic activity in South African medicinal plants..... | 54 |
|--|----|

CHAPTER 5

| | |
|--|----|
| Table 5.1. Correlation of bacterial numbers and optical density readings of overnight cultures..... | 64 |
|--|----|

| | |
|--|--|
| Table 5.2. Determination of the antibacterial activity of South African medicinal plants with the disc-diffusion and microdilution assays (MIC recorded in mg ml ⁻¹)...65 | |
|--|--|

CHAPTER 6

| | |
|--|----|
| Table 6.1. Results of the anthelmintic screening of Combretaceae leaf extracts..... | 75 |
|--|----|

CHAPTER 7

| | |
|---|----|
| Table 7.1. Comparison of MIC values obtained using the microplate method (ELOFF, 1998) for <i>Acorus calamus</i> ethanol extracts, isolated β -asarone and the antibiotic neomycin against various bacteria..... | 88 |
|---|----|

CHAPTER 8

| | |
|--|--|
| Table 8.1. Antibacterial activity of different plant parts of <i>Schotia brachypetala</i> (voucher number <i>McGaw58NU</i>). Treatments in each column denoted by the same | |
|--|--|

letters are not significantly different at the 5 % level, with a standard error (SE) for the treatment means = 1.618.....106

Table 8.2. Antibacterial activity of leaves from different trees (*Schotia brachypetala*). Treatments in each column denoted by the same letters are not significantly different ($p < 0.05$), with S.E. (treatment means) = 1.3043.....107

Table 8.3. Antibacterial activity of *Schotia brachypetala* leaves (voucher number McGaw85NU) collected monthly from the same tree. Treatments in each column denoted by the same letters are not significantly different at the 5 % level, with SE (treatment means) = 1.593.....108

Table 8.4. Antibacterial activity of leaves of different *Schotia* species. Treatments in the same column denoted by the same letters are not significantly different ($p < 0.05$), with SE (treatment means) = 1.457.....109

Table 8.5. Antibacterial activity of *Schotia brachypetala* leaf extracts prepared from fresh and dry plant material. Treatments in the same column denoted by the same letters are not significantly different ($p < 0.05$), with SE (treatment means) = 0.614.....109

Table 8.6. Antibacterial activity of *Schotia brachypetala* leaf extracts immediately after being prepared, after being stored in the freezer for 18 months, and extract made from leaf material stored in the dark for 18 months. Treatments in the same column denoted by the same letters are not significantly different ($p < 0.05$), with SE (treatment means) = 0.844.....110

CHAPTER 9

Table 9.1. MIC values (mg ml^{-1}) of fatty acids.....123

LIST OF ABBREVIATIONS

| | |
|----------------------|--|
| ABFA..... | albumin bound fatty acids |
| ANOVA..... | analysis of variance |
| AS..... | anisaldehyde/sulphuric acid spray reagent |
| ATP..... | adenosine triphosphate |
| EIMS..... | Electron Impact Mass Spectrometry |
| FFA..... | free fatty acids |
| HPLC..... | High Performance Liquid Chromatography |
| INT..... | <i>p</i> -iodonitrotetrazolium violet ✓ |
| LPS..... | lipopolysaccharide |
| M..... | molar |
| MH..... | Mueller-Hinton ✓ |
| MIC..... | Minimal Inhibitory Concentration ✓ |
| MRC..... | Medical Research Council |
| MRSA..... | methicillin-resistant <i>Staphylococcus aureus</i> |
| MS..... | Mass Spectrometry |
| NEFA..... | non-esterified fatty acids |
| NG..... | nematode growth |
| NMR..... | Nuclear Magnetic Resonance |
| OD..... | Optical Density |
| R _f | mobility relative to front ✓ |
| SE..... | standard error ✓ |
| TLC..... | Thin Layer Chromatography ✓ |
| TZC..... | 2,3,5-triphenyltetrazolium chloride |
| UNP..... | University of Natal Pietermaritzburg |
| UV..... | ultraviolet ✓ |
| VFA..... | volatile fatty acid |
| VLC..... | Vacuum Liquid Chromatography |
| WHO..... | World Health Organization ✓ |

CHAPTER 1

LITERATURE REVIEW

1.1 INTRODUCTION

The use of medicinal plants in seeking relief from illness can be traced back over five millennia to documents produced by the early civilizations in China, India and the Near East, but it indisputably stretches even earlier into the history of mankind (HAMBURGER and HOSTETTMAN, 1991). The major role played by plants as sources of pharmacologically active substances is beyond dispute (PRINCIPE, 1989). Some have questioned the future need for plants as sources of these substances, but new and important plant-based drugs are still being introduced, with considerable economic significance attached (PRINCIPE, 1989).

No accurate data are available to assess the value and extent of the use of plants or active constituents derived from them in worldwide health care (FARNSWORTH *et al.*, 1985). The World Health Organization (WHO) has estimated that 80 % of the inhabitants of the world rely chiefly on traditional medicines for their primary health care needs, and it may be presumed that a major part of traditional healing involves the use of plant extracts or their active principles (FARNSWORTH *et al.*, 1985).

1.2 PLANTS AS SOURCES OF USEFUL DRUGS

Ecological awareness and an increased demand for alternative therapies after the emphasis on synthetic pharmaceutical chemistry from 1945, among other reasons, has resulted in a resurgence of interest in drugs of plant origin (HAMBURGER and HOSTETTMAN, 1991). Major pharmaceutical companies are focussing renewed attention on higher plants as a source for lead structures. The economic value of

both current plant-based pharmaceuticals, and those as yet undiscovered is considerable (PRINCIPE, 1989). Since the number of plant species that are likely to become extinct by the year 2000 is approximately 50 000 (PRINCIPE, 1989), the analysis of plants for active compounds assumes great importance. The number of marketable prescription pharmaceuticals that will be lost can be estimated to be about 25, notwithstanding the other lost benefits, such as insight into biological mechanisms or new over-the-counter drugs (PRINCIPE, 1989).

In industrialized countries, about 25 % of all prescription drugs contain active principles that are still extracted from higher plants (FARNSWORTH, 1984; FARNSWORTH *et al.*, 1985). FARNSWORTH *et al.* (1985) reported that at least 119 compounds derived from 90 plant species can be considered as important drugs currently in use in one or more countries, with 77 % of these being derived from plants used in traditional medicine. Close to half of the best selling pharmaceuticals in 1991 were either natural products or their derivatives (O'NEILL and LEWIS, 1993), which provides further evidence for the importance of natural products. PHILLIPSON (2001) reported that six of the top twenty prescriptions dispensed in 1996 were natural products, and the clinical use of drugs such as artemisinin, etoposide and taxol has once again focussed attention on plants as sources of novel drug entities. Taxol is an anticancer taxane diterpenoid derived from the Pacific or western yew tree, *Taxus brevifolia* (BALANDRIN *et al.*, 1993). Etoposide is a relatively new semisynthetic antineoplastic agent based on podophyllotoxin, a constituent of the mayapple, *Podophyllum peltatum* (BALANDRIN *et al.*, 1993). Artemisinin, from *Artemisia annua*, is a sesquiterpene lactone with an unusual endoperoxide group that is essential for its antimalarial activity (KLAYMAN, 1993). FARNSWORTH (1984) noted that less than 10 well-established plant-derived drugs are produced commercially by synthesis, even though laboratory syntheses have been described for most of them. Although the percentage of plant-based drugs varies from country to country, for example 15-20 % in Japan and 35-40 % in Germany in the 1980's according to PRINCIPE (1989), 25 % is a good estimate of the average.

The potential of higher plants as sources for new drugs is still mostly unexplored (HOSTETTMAN *et al.*, 1996). Approximately 5 to 15 % of the estimated 250 000 existing species of higher plants have been systematically surveyed for the presence of biologically active compounds (BALANDRIN *et al.*, 1993). Even plants that are considered to have been investigated have often been screened for only a single type, or at best a few types, of biological activity (BALANDRIN *et al.*, 1993). Plants may contain hundreds or thousands of metabolites, so phytochemical analysis of a given plant will reveal only a narrow spectrum of its constituents (HAMBURGER and HOSTETTMAN, 1991; HOSTETTMAN *et al.*, 1996). A multidisciplinary collaboration of botanists, pharmacognosists, chemists, pharmacologists, toxicologists and others such as microbiologists is optimal for the investigation of medicinal agents from higher plants, leading to isolation of the pure constituents (VLIETINCK, 1987; HOSTETTMAN *et al.*, 1996). Botanical knowledge is essential for the conservation of medicinal plants, particularly with the present problems of over-harvesting and loss of natural habitats caused by over-population, resulting in loss of potentially useful plants (HEDBERG, 1993).

A significant development in natural product research is the introduction of high-throughput assays for biological activity, used in drug discovery by industry but also on a smaller scale in academic research (HOUGHTON, 1999). This lends the advantage of allowing the investigation of a large number of samples in a shorter time. The amount of sample required is usually small. The process enables the investigation of a large number of plants and also fractions from individual plant extracts so that the active chemicals can be determined (HOUGHTON, 1999). A major drawback of using bioactivity screening to find novel compounds is the possibility that known compounds may be responsible for the activity detected in the plant under study (HOUGHTON, 1999).

Bioassay-guided fractionation of crude plant extracts has led to the isolation of numerous compounds with interesting biological activities (HOSTETTMAN *et al.*, 1996). The availability of suitable bioassays to detect the biological activity of interest is essential. To facilitate bioassay-guided fractionation, these assays must

have the capacity for high sample throughput in order to cope with the numbers of extracts and fractions generated by the process (HOSTETTMAN *et al.*, 1996). The test system should be simple, rapid, inexpensive and reproducible (HOSTETTMAN *et al.*, 1996). The bioassays have to be sensitive enough to detect activity in active principles present at low concentrations in the crude extracts so as to avoid false negative results; at the same time the occurrence of false positives should be reduced to a minimum (RASOANAIVO and RATSIMAMANGA-URVERG, 1993; HOSTETTMAN *et al.*, 1996). O'NEILL and LEWIS (1993) stated that the presence in higher plant extracts of polyphenols, saponins, certain pigments or fatty acids may result in false positives, particularly in the case of enzyme-based assays.

In a bioassay-guided fractionation, failure to isolate active constituents from an active extract is sometimes a problem. This may occur if the active compound is labile under certain conditions, if there is a synergistic effect with other constituents, or if the fractionation procedure is inadequate to isolate the bioactive constituents (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). Synergism between different components would be lost upon separation, and chemical breakdown of the active compound may occur after removal of compounds present in the total extract, which may prevent its hydrolysis, oxidation or some other cause of decomposition (HOUGHTON, 1999).

Plants synthesize very original or highly complex natural products, useful directly as medicinal agents, and also as new models or lead substances for medicinal chemists and pharmacologists to synthesize large quantities of new compounds (ANTON *et al.*, 1987). Any biological molecule is a natural product, but the term is usually reserved for secondary metabolites, which are small molecules produced by an organism that are not absolutely necessary for the survival of the organism, unlike the more prevalent macromolecules such as proteins, nucleic acids and polysaccharides that are necessary for the more fundamental processes of life (CANNELL, 1998). Secondary metabolites are a very broad group of metabolites. For instance, they may be products of overflow metabolism as a result of nutrient limitation, or shunt metabolites produced as defence mechanisms (CANNELL,

1998). If a secondary metabolite has no adverse effect on the organism, it may be conserved for a relatively long period, during which time it may come to confer a selective advantage (CANNELL, 1998). This concept is supported by the fact that secondary metabolites are often unique to a particular species or group of organisms, and while many act as antifeedants, attractants or antibiotic agents, many have no apparent biological role (CANNELL, 1998).

Phytochemical research linked with a pharmacological study can be the key to the discovery of new biologically active compounds. Plants have a remarkable ability to produce complicated molecules with very specific stereochemistry and it would be difficult for synthetic chemists to conceive or synthesize many of the structures found in plants (HOUGHTON, 1999). These new structures may have novel modes of action, possess better activity, or have fewer detrimental side-effects than presently-used compounds (HOUGHTON, 1999). HOUGHTON (1999) concluded that it is reasonable to assume that a large number of phytochemicals remain to be isolated. Another reason for investigating plants for new chemicals is that they may provide insights into biosynthetic pathways to known molecules (HOUGHTON, 1999).

VLIETINCK (1987) contended that the potential of traditional drugs for the discovery of substances with valuable pharmacological activities is considerable. Several factors are involved in deciding the ability of such compounds to play a role in the development of new drugs, as templates for synthesis or semi-synthesis, and as biochemical tools (VLIETINCK, 1987). CRAGG *et al.* (1997) warned that the renewed interest of the past 15 years in the investigation of natural materials may be waning in favour of new approaches to drug discovery, such as combinatorial chemistry and computer-based molecular modelling designs. Biochemical manipulation in tissue culture to produce novel compounds is an interesting technique as it offers prospects for the discovery of new compounds, since cultures sometimes produce substances different from those found naturally (HOUGHTON, 1999). PRINCIPE (1989) pointed out the argument made by some that those plants with the highest probability of containing novel useful bioactive compounds have

already been investigated, and that this ratio cannot be expected to remain constant. PRINCIPE (1989) contended that it is more likely for the ratio to increase in favour of discovering more plants with interesting properties, as geographical areas that have yet to be explored for new plants are often the ones producing the highest percentage of pharmacologically active plants. There is still a solid base for the claims made for the importance of natural products in drug discovery and development. Also, there is the significance of scientifically validating the choice and use of plants by traditional healers.

1.3 SELECTION OF PLANTS FOR BIOLOGICAL ACTIVITY SCREENING

The cost of making a new drug is estimated to be from 50 to 100 million dollars, and approximately 10 years is needed to develop it (FARNSWORTH, 1984; LEWINGTON, 1990). It is impossible to screen each plant for biological activity in a world with limited financial resources; a reliable collection strategy of useful medicinal plants is required. It is important for each researcher, who can only deal with a relatively small number of species at a time, to select the most suitable plants for biological screening and possible isolation of novel compounds. There are three main approaches to select plants for biological investigation: the ethnopharmacological, chemotaxonomical and random selection approaches.

Ethnopharmacology has been defined as the study of the use of local materials for medicinal purposes by ethnic groups, usually designated as those indigenous to a geographical area (HOUGHTON, 1999). Using the 'ethnopharmacological approach', many bioactive constituents have been discovered through scientific investigations of traditional medicinal plants (FARNSWORTH, 1984). The 'chemotaxonomical approach' relies upon correlations between plant taxonomy and the occurrence of specific chemical constituents (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). In the 'random selection approach', a variety of plants (and plant parts) are submitted to routine extraction and bioassay without preconceived selection on the basis of ethnobotanical knowledge or

chemotaxonomical data (RASOANAIVO and RATSIMAMANGA-URVERG, 1993).

KHAFAGI and DEWEDAR (2000) described five different approaches of selecting plants for pharmacological screening. Along with the above-mentioned more classical techniques, these researchers included 'phytochemical targeting', which involves the collection of all members of a plant family known to be rich in bioactive compounds, and a method based on 'specific plant parts', such as seeds. Another interesting approach augmenting the variety is the 'ecological approach', which concentrates on species growing where competition may necessitate the mobilisation of chemical defence systems (HOUGHTON, 1999). HOUGHTON (1999) emphasized the need to explore the diversity within one species owing to the existence of chemical races, but cautioned against ignoring chronological factors, as the identity and quantity of secondary metabolites vary throughout the life of the plant. HOUGHTON (1999) described intraspecific variation as the variation in a species which occurs owing to its genetics, growth conditions, location, stage in its life cycle or the part of it which is being investigated.

Tropical ecologists have proposed that tropical plants should produce a wider range of defensive chemicals because herbivores and pathogens are active throughout the year, so the selective pressures are higher (NASH, 2001). However, in temperate regions, the extremes of seasonality and the ferocity of herbivore attack, combined with the necessity to survive frost and drying winds, could be argued to increase the requirement for good chemical defences and production of interesting natural products (NASH, 2001). GENTRY (1993) anticipated that most pharmacologically active compounds would come from tropical forests because an estimated half to two-thirds of the world's plant species grow there.

The ethnobotanical approach to drug discovery has proven successful on many occasions (COX, 1994). Results obtained by KHAFAGI and DEWEDAR (2000) indicated that plant sampling based on the ethnobotanical approach produced a greater number of plants showing antimicrobial activity, compared to plants collected using the random method. FOURIE *et al.* (1992) screened for pharmacological

activity in about 300 plants used in South African traditional medicine. It was found that at least 31 % of plants showed marked activity, whereas only 21 % could be considered to be inactive and 48 % were moderately active (FOURIE *et al.*, 1992). FOURIE *et al.* (1992) suggested that the majority of the plants used in folk medicine in southern Africa possess some pharmacological activity, making folk medicine an excellent starting point for effective research. Of some concern was the finding that 9 % of the plants reputed to have medicinal properties investigated by FOURIE *et al.* (1992) showed definite toxic effects. FARNSWORTH (1984) maintained that virtually all the currently useful drugs derived from plants were discovered through scientific investigation of traditional medicinal usage and claims of human efficacy. However, the random selection approach may be valuable for identifying the presence of bioactive compounds from plants with unknown medicinal uses (KHAFAGI and DEWEDAR, 2000).

COX (1994) noted that not all Western diseases are equally likely to be diagnosed by traditional medical practitioners. Diseases such as gastrointestinal maladies, inflammation, skin infections and some viral diseases are probably fairly easily detected by healers, whereas cancer and cardiovascular illnesses, among others, are unlikely to be as easily recognized (COX, 1994). Possibly then, the likelihood of obtaining effective plant remedies from indigenous knowledge systems against the recognizable ailments is relatively high, and these remedies may provide sound leads for the development of new pharmaceutical preparations. Therefore, while the strength of the ethnobotanical method of plant selection appears to be its great potential for discovering potent new compounds, it may be limited in the type of drugs it is likely to provide (COX, 1994).

1.4 PLANTS AS SOURCES OF ANTIMICROBIAL DRUGS

Natural products have played, and continue to play, an invaluable role in the drug discovery process, particularly in the area of infectious diseases (CRAGG *et al.*, 1997). Many phytochemical laboratories are actively involved in the search for

antimicrobial agents (HAMBURGER and HOSTETTMAN, 1991). Although no plant derived compound has been discovered which can compete with clinically used antibiotics, the search for compounds exhibiting a spectrum of activity complementary to existing drugs and novel lead structures remains an important task (HAMBURGER and HOSTETTMAN, 1991).

The crisis of new and reemerging infectious diseases for which no effective therapies are available, and the development of resistance of many pathogens to currently used drugs is widely recognized as being of serious and immediate concern (CRAGG *et al.*, 1997). There is an urgent need to identify novel, active chemotypes as leads for effective drug development, and nature is an incomparable source of such lead discoveries, as demonstrated by the discovery of the “wonder” antibiotics of the 1940s and 1950s (CRAGG *et al.*, 1997). Higher plants and marine sources, as well as microbial sources apart from the Actinomycetes, which have been well investigated and have already produced many novel microbial metabolites, have great potential for the origination of new chemicals (CRAGG *et al.*, 1997). HOSTETTMAN *et al.* (1995) emphasized the importance of field observations, stating that if a bush or tree shows no signs of being attacked by pests and has neither pieces eaten out of the leaves nor discolourations owing to the presence of some foreign organism, then there is a good chance that some metabolites are present which act as insecticides or antimicrobial agents.

Western market demands heavily influence pharmaceutical companies, which are based primarily in Western cultures and economies (SHELDON *et al.*, 1997). The largest market for pharmaceuticals comprise Western patients, the needs of whom therefore guide pharmaceutical research. As a result, far more research emphasis is placed on treatments for afflictions such as cancer, heart disease and chronic stress, than on health problems of developing countries, such as basic infections, dysentery and parasitic diseases (SHELDON *et al.*, 1997). The purchasing power of those affected is simply not sufficient to justify or encourage a company's investment. Research emphasis in developing countries should then perhaps be focussed on evaluating traditional medicinal preparations used to treat illnesses

important in these countries for subsequent incorporation into the national health care system.

1.5 TRADITIONAL MEDICINE IN SOUTH AFRICA

The African continent is fortunate to contain an extraordinary diversity of plant species, and aligned with this are a large number of traditional healers who exploit this resource (MARSTON *et al.*, 1993). In South Africa, up to 80 % of Zulu patients seen by medical practitioners also consult traditional healers (GUMEDE, 1990), and many people in rural areas rely completely on traditional healers owing to the lack of Western medical facilities. FARNSWORTH (1984) estimated that two-thirds of the people in developing countries rely on plants as sources of drugs.

Medicinal plants are an important part of South African culture. Well over 30 000 species of higher plants may be found in southern Africa (VAN WYK *et al.*, 1997). With the remarkable plant and cultural diversity in South Africa, it is unsurprising that approximately 3 000 species of plants are used as medicines, and of these, about 350 species are the most commonly used and traded medicinal plants (VAN WYK *et al.*, 1997).

There are an estimated 200 000 indigenous traditional healers in South Africa and about two-thirds of South Africans consult these healers, usually in addition to using modern biomedical services (VAN WYK *et al.*, 1997). The South African Medical Association has estimated that in the cities South Africa has one doctor for every 700 people, but in rural areas, it has one doctor for every 10 000 people (SUNDAY TIMES BUSINESS TIMES, 2001). With the acute shortages of Western medical doctors and health clinics in rural areas, people in these districts often have to rely solely on traditional healers for treatment. Indigenous healers therefore have an indispensable role to play in the Primary Health Care System of South Africa. It is essential for these traditional healers to be registered and regulated so they may be given official recognition and support. They must also be held accountable for

malpractice and fraud, as are Western medical doctors.

Most of the health problems in Africa are attributable to unsafe drinking water, lack of personal hygiene and malnutrition (ABEBE, 1987). Only by educating the community about ways to prevent the outbreak and spread of diseases can we overcome these and many other health problems (ABEBE, 1987). The herbalists and traditional medical practitioners are in a good position to fulfil this function, as they have the acceptance of the public to change the existing health outlook of the rural communities (ABEBE, 1987). More scientific work on traditional remedies needs to be performed to prove the efficacy of plant remedies, so they may be used at least at the primary health care level. Also of concern is the possible toxicity, manifested as possible short term or long term effects, of medicinal plants. Scientific investigation into the standardization of natural remedies, as well as toxicity testing and evaluation of potential side effects is therefore necessary.

Another problem is the conservation of medicinal plant resources. In the past, plants were protected from over-harvesting as traditional healers collected and stored their medicinal plants in accordance with traditions and taboos (VAN WYK *et al.*, 1997). Modern times have seen the emergence of urbanized healers who may be less rigorously trained than their forebears, and who mainly purchase their plants from gatherers who would be otherwise unemployed, or street markets and stores, providing an economic incentive for the destructive harvesting of vulnerable medicinal plants (VAN WYK *et al.*, 1997). The exponential population growth in South Africa has resulted in more people demanding medicinal plants, with the consequence of increased collection from wild plant populations. Some sangomas and farmers are cultivating rare and valuable plants used for medicinal purposes, thus reducing the pressure on wild populations (VAN WYK *et al.*, 1997).

Traditional healers in South Africa are known commonly as "inyanga" and "sangoma" (Zulu), "ixwele" and "amaqira" (Xhosa), "nqaka" (Sotho), "bossiedokter" and "kruiedokter" in the Western and Northern Cape (VAN WYK *et al.*, 1997). The designations "inyanga" and "sangoma" previously referred exclusively to herbalist

and diviner respectively, but in modern times the distinction has become blurred (VAN WYK *et al.*, 1997). Supporting the herbalists and diviners, who are believed to be spiritually empowered, are the traditional birth attendants, prophets, spiritual healers, spirit mediums, intuitives and dreamers (VAN WYK *et al.*, 1997). In rural areas, most of the elderly members of the community have a knowledge of herbal remedies (VAN WYK *et al.*, 1997).

Although held by traditions of the past, indigenous medicine systems are adaptive and changing (VAN WYK *et al.*, 1997). This is illustrated by the incorporation into the *materia medica* of introduced medicinal herbs such as liquorice root (*Glycyrrhiza glabra*) and calamus root (*Acorus calamus*) (VAN WYK *et al.*, 1997). SIMON and LAMLA (1991) described the incorporation of Western pharmaceutical products such as penicillin into the traditional dispensaries of Xhosa healers. Also significant is the interest in modern Primary Health Care training programmes shown by traditional healers' associations (VAN WYK *et al.*, 1997). This ability to adapt suggests that with the necessary official support and recognition, traditional medicine will survive in the future, and not be overwhelmed by modern science (VAN WYK *et al.*, 1997). For several years, the World Health Organization (WHO) has been promoting the resolution adopted in 1977 by the Thirtieth World Health Assembly urging interested governments to give adequate importance to the utilization of their traditional systems of medicine, with appropriate regulations as suited to their national health systems (AKERELE, 1984). According to AKERELE (1984), the WHO will continue to promote the development, teaching, and application of analytical methods that can be used to evaluate the safety and efficacy of various elements of traditional medicine.

Indigenous plants make an extremely important contribution to the Primary Health Care systems in South Africa (VAN WYK *et al.*, 1997). However, there is a lack of detailed documentation on the use of medicinal plants in this country. Some recent publications have set out to alleviate this problem by recording plant use by South African healers, including HUTCHINGS *et al.* (1996), VAN WYK *et al.* (1997) and VAN WYK *et al.* (2000). The esteemed work by WATT and BREYER-BRANDWIJK

(1962) is also essential to any literature search on the topic of southern African medicinal plant usage. The wealth of indigenous knowledge of the medicinal value of plants is the result of centuries of experimentation - essentially what amounts to many lifetimes of empirical testing (MALONE, 1983). The urgency of committing to written record the knowledge of plants used medicinally and otherwise, passed down the generations by fragile oral tradition cannot be underestimated. VAN WYK *et al.* (1997) reported that the informal oral-tradition medical systems of the Khoi-San people, the Nguni and the Sotho-speaking people have not yet been systematized. VAN WYK *et al.* (1997) believe that with formal documentation, scientific research and official support, the beneficial components of Africa's indigenous systems of medicine will someday claim their rightful position among the accepted and recognized healing traditions of the world.

1.6 GASTROINTESTINAL DISORDERS

Diarrhoea is an increase in the fluidity and frequency of stools and is one of the most common disorders of man. Fortunately in many cases the disorder is short-lived or responds satisfactorily to a number of well-tried remedies (LEWIS and ELVIN-LEWIS, 1977). There are numerous causes of diarrhoea: acute diarrhoea results from bacterial and viral enteritis, food and toxin poisoning, chemical poisoning, and gastrointestinal allergy; chronic diarrhoea may be caused by chronic intestinal infections among other things (LEWIS and ELVIN-LEWIS, 1977). Diarrhoea and dysentery epidemics are mostly common where living conditions are crowded and unhygienic (OTSHUDI *et al.*, 2000). Dysentery is a microbial infection of the gastrointestinal tract, and symptoms include fever, vomiting, abdominal pain, and diarrhoea (OTSHUDI *et al.*, 2000). The onset of the disease usually occurs within 2-3 days after infection and lasts for up to several weeks. Dehydration occurs rapidly, especially in children, and can cause death if treatment is not given (OTSHUDI *et al.*, 2000).

Many intestinal parasites may cause diarrhoea. It is common in trichuriasis (caused

by the nematode *Trichuris trichiura*) and giardiasis (caused by the amoeba *Giardia lamblia*), especially when infestation is heavy (TAYLOR *et al.*, 1995). The cause of diarrhoea may be treated by the administration of an antibiotic or antiamoebic drug, or by replacing abnormal losses of water, electrolytes, protein and blood.

Symptomatic relief may be obtained by reducing the number of bowel actions with drugs, or harmful substances in the bowel may be neutralized (LEWIS and ELVIN-LEWIS, 1977).

Diseases such as diarrhoea and dysentery are the main causes of the high mortality rate in developing countries (OTSHUDI *et al.*, 2000). In these countries, the World Health Organization has estimated that over five million children under the age of five die each year from severe diarrhoeal diseases (WHO, 1996). Socioeconomic status plays an important role in the incidence of diarrhoeal disease, with poor rural children experiencing a greater number of diarrhoeal episodes than more affluent urban children (BERN *et al.*, 1992). A recent outbreak of cholera (causal organism *Vibrio cholerae*) greatly impacted on rural areas of KwaZulu-Natal, South Africa, while urban areas were largely unaffected. In this epidemic, 192 people died and 90 888 cases were reported from mid-August 2000 to 1 May 2001. The mortality rate was decreased significantly by the setting up of a number of rehydration centres where patients not within easy reach of a health clinic could obtain treatment.

Most rural people have limited access to formal and adequate health care, and the assistance of traditional medical practitioners is sought (OTSHUDI *et al.*, 2000). Encouragingly, BERN *et al.* (1992) reported that over the last 20 years, there have been many studies documenting a decline in diarrhoeal mortality. This is probably owing to diarrhoeal disease control programmes in many countries, as well as general improvements in standards of living, improved nutrition and better immunization. However, despite the indication of a decline in diarrhoeal mortality in many developing countries, there are also some small-scale studies that give clear evidence of continuing high mortality in certain areas (BERN *et al.*, 1992). Overall, the estimated global mortality has declined substantially, although the estimated global morbidity from diarrhoea has remained constant over the last ten years

(BERN *et al.*, 1992). To maintain the reduction in mortality, and to have an impact on morbidity statistics, BERN *et al.* (1992) advised the intensification of diarrhoeal disease control efforts, including promotion of the correct management of acute watery diarrhoea and dysentery, and adequate access to treatment.

1.7 THE EFFECT OF INTESTINAL PARASITES IN HUMANS

Parasite infestations, aside from dysentery and acute or chronic diarrhoea, are rarely associated with mortality, but cause significant impaired physical and mental development. Intensity of infestation affects morbidity (TAYLOR *et al.*, 1995). In medical terms, morbidity is defined as being of the nature of or indicative of disease. Academically less able students in Jamaica were found by NOKES and BUNDY (1992) to have greater prevalence and intensity of infection with the nematode *Trichuris trichiura*. The effect of worm load on children's nutritional status is also important. Research on children suffering from mild to moderate undernutrition has shown that treatment of helminth infestation improved height for age and weight for height indices (SIMEON and GRANTHAM-MCGREGOR, 1990). Improvement in physical fitness has been noted as a result of the successful treatment of ascariasis and trichuriasis, caused by the nematodes *Ascaris lumbricoides* and *Trichuris trichiura* respectively (STEPHENSON *et al.*, 1990).

A significant improvement in spatial memory demonstrated by children in the Congo was noted by BOIVIN *et al.* (1993) after successful treatment for infestation with serious types of chronic intestinal parasites. In KwaZulu-Natal, KVALSVIG (1986) reported that spontaneous energetic activity increased in children treated for schistosomiasis relative to controls. Parasite infection has also been shown to be associated with poor performance on an attention task (KVALSVIG *et al.*, 1991a). In addition, many individuals host more than one parasite infestation simultaneously (KVALSVIG *et al.*, 1991a). In the 1920s, white children in the former eastern Transvaal were treated for parasitic diseases by the Medical Officer of Health attached to the Transvaal Education Department on the grounds that these diseases

affected their schoolwork, but many decades later prevalence rates among black children remain high and for the most part the diseases remain untreated (KVALSVIG *et al.*, 1991b).

Lack of clean water and adequate sanitation are major contributors to infestation and reinfestation (TAYLOR *et al.*, 1995). TAYLOR *et al.* (1995) investigated whether targeted chemotherapy can reduce parasite prevalence in rural black preschool children in southern KwaZulu-Natal. They reported unacceptably high rates of reinfestation with *A. lumbricoides*, *T. trichiura* and *Giardia lamblia* 12 weeks after initial treatment. Reinfestation is a problem because in the southern KwaZulu-Natal area where the study was conducted, most people do not have clean water or adequate sewerage facilities. TAYLOR *et al.* (1995) suggested that, while ongoing repeated courses of chemotherapy may help, provision of pure water and acceptable sanitation facilities is vital in ensuring the long-term health of communities. In addition, TAYLOR *et al.* (1995) maintained that regular treatment with a suitable anti-parasitic agent may have a major impact on morbidity reduction, as the effects of repeated intervention may change both parasite transmission characteristics and the pattern of infestation.

1.8 AIMS AND OBJECTIVES

Intestinal parasites are an important problem in South Africa, particularly in rural communities. An astounding number and variety of plants are used by traditional healers to treat stomach ailments. The discovery of new anthelmintic, antiamebic or antibacterial compounds from traditional plant remedies would assist in the development of new preparations to combat intestinal parasites. It would also help in validating the work of traditional healers.

The aim of this study was to evaluate South African medicinal plants for anthelmintic, antiamebic and antibacterial activity using bioassay screening, and to identify active compounds in plants showing high activity in the initial testing.

CHAPTER 2

PLANT COLLECTION AND EXTRACTION

2.1 INTRODUCTION

In this project, the ethnopharmacological approach was chosen because gastrointestinal ailments are readily recognized and treated by traditional healers, who use a large number of plants for this purpose. This approach is a proven method of plant selection for active biological compound screening and isolation (CHAPTER 1), and, importantly, it is also a means of validating the work of traditional South African healers.

Various solvents have been used to extract plant metabolites (ELOFF, 1998b). Many scientists employ Soxhlet extraction of dried plant material using solvents with increasing polarity, but this technique cannot be used if the plants contain thermolabile compounds (ELOFF, 1998b). It is desirable to extract a large variety of compounds from the plant to improve the chances of extracting the biologically active components if a specific class of chemical is not targeted (ELOFF, 1998b). The use of a range of extracting solvents differing in polarity would be suitable for a screening procedure, ensuring the extraction of both hydrophilic and lipophilic compounds.

SHIMIZU (1998) warned that simple maceration of fresh biological material with water or aqueous buffers fails to extract water-soluble compounds because they are mostly stored in protected states. The mechanism of such protection is varied: binding to the membranes, compartmentalization, or protection by lipophilic material (SHIMIZU, 1998). This problem is usually not encountered in the extraction of lipophilic compounds because organic solvents break up the compartmental structures (SHIMIZU, 1998). Methods which interrupt the compartmentalization

include sonication, freezing-thawing, freeze-drying, heating, and enzyme digestion (SHIMIZU, 1998).

Fresh or dried plant material may be used as a source for secondary plant components (ELOFF, 1998b). Most scientists have tended to use dried material because there are fewer problems associated with the large scale extraction of dried rather than fresh plant material (ELOFF, 1998b). The time delay between collecting and processing the plant material makes it difficult to work with fresh material because differences in water content may affect solubility (ELOFF, 1998b). Also, the secondary metabolites should be relatively stable if they are to be used as antimicrobial agents, and most plants are used in the dried form (or as an aqueous extract) by traditional healers (ELOFF, 1998b).

SILVA *et al.* (1998) recommended drying plant material at room temperature or in an oven away from direct sunlight, because ultraviolet radiation may produce chemical reactions giving rise to compound artifacts. The plant material should be well-ventilated to avoid fungal infestation and elevated temperatures by fermentation (SILVA *et al.*, 1998). Grinding the plant material assists the penetration of the solvent to the cellular structure of the plant tissues, thereby helping to dissolve the secondary metabolites and increase the yields of extraction (SILVA *et al.*, 1998). Generally, it has been found that the smaller the particle size of the plant material the more efficient the extraction (SILVA *et al.*, 1998).

2.2 MATERIALS AND METHODS

2.2.1 Plant collection

A list of plants used by Zulu, Xhosa and Sotho traditional healers for treatment of intestinal worms, diarrhoea and dysentery was compiled from work done by various researchers (WATT and BREYER-BRANDWIJK, 1962; HUTCHINGS *et al.*, 1996; VAN WYK *et al.*, 1997). From this list, plants were collected, predominantly from the

KwaZulu-Natal region of South Africa. Herbarium voucher specimens for each plant were deposited at the Herbarium of the University of Natal, Pietermaritzburg. The traditional usage and voucher specimen numbers of the plants collected are recorded in Table 2.1. The plant material for use in the screening procedures was dried in a 50°C oven for 3 days, or until dry, and stored at room temperature until extraction.

2.2.2 Plant extract preparation

Dried plant material was ground to a powder using a Wiley mill. Two separate samples of 1 g were extracted with 10 ml water and ethanol respectively. Four g of plant material was extracted with 40 ml hexane. Extraction was performed by sonication for 30 min in a Julabo ultrasound bath. The plant extracts were filtered through Whatman No. 1 filter paper into pill vials. The filtrates were evaporated in front of a fan and the residues stored at -15°C.

2.3 RESULTS

Table 2.1. South African medicinal plants investigated for anthelmintic, antiamoebic and antibacterial activity

| Family and botanical name (voucher specimen ¹) | Plant part ² assayed | Traditional uses and administration |
|--|---------------------------------|--|
| ALLIACEAE (LILIACEAE) | | |
| <i>Tulbaghia violacea</i> Harv. (McGaw50NU) | TB | Pounded tuber decoctions used as enemas for stomach ailments (HULME, 1954). Tubers used as anthelmintics (WATT and BREYER-BRANDWIJK, 1962) |
| ANACARDIACEAE | | |
| <i>Harpephyllum caffrum</i> Bernh. ex Krauss (McGaw38NU) | BK | Bark decoctions used as emetics (PUJOL, 1990) |
| <i>Sclerocarya birrea</i> (A. Rich.) Hochst. (McGaw44NU) | BK | Bark decoctions taken as enemas for diarrhoea (GERSTNER, 1938; PUJOL, 1990) and dysentery (WATT and BREYER-BRANDWIJK, 1962) |
| APIACEAE (UMBELLIFERAE) | | |
| <i>Heteromorpha trifoliata</i> (Wendl.) Eckl. & Zeyh. (Zschocke2NU) | LF | Infusions given as enemas for abdominal disorders (WATT and BREYER-BRANDWIJK, 1962; BRYANT, 1966). Leaf decoctions given for intestinal worms in children and roots used for dysentery (WATT and BREYER-BRANDWIJK, 1962) |
| <i>Pimpinella caffra</i> (Eckl. & Zeyh.) D. Dietr. (McGaw67NU) | WH | Unspecified parts used against intestinal worms (WATT and BREYER-BRANDWIJK, 1962) |

Table 2.1 continued...

| Family and botanical name (voucher specimen ¹) | Plant part ² assayed | Traditional uses and administration |
|---|---------------------------------|--|
| APOCYNACEAE | | |
| <i>Acokanthera oblongifolia</i> (Hochst.) Codd (McGaw49NU) | LF | Unspecified parts used as an anthelmintic (HUTCHINGS <i>et al.</i> , 1996) |
| <i>Rauvolfia caffra</i> Sond. (McGaw42NU) | LF | Latex used as an emetic for abdominal complaints (WATT and BREYER-BRANDWIJK, 1962) |
| ARACEAE | | |
| <i>Acorus calamus</i> L. (McGaw47NU) | RH | Rhizomes used as carminatives, stomachics, for dysentery (WATT and BREYER-BRANDWIJK, 1962) and diarrhoea (VAN WYK <i>et al.</i> , 1997) |
| ARALIACEAE | | |
| <i>Cussonia spicata</i> Thunb. (McGaw56NU) | LF | Emetics made from the decorticated base of the fruit, stem or root taken for nausea (WATT and BREYER-BRANDWIJK, 1962) |
| ASCLEPIADACEAE | | |
| <i>Asclepias fruticosa</i> L. (McGaw36NU) | LF | Leaf infusions administered for diarrhoea and stomach pain in children (HULME, 1954; WATT and BREYER-BRANDWIJK, 1962). Root decoctions used for stomach ailments (MABOGO, 1990) |
| ASPHODELACEAE (LILIACEAE) | | |
| <i>Aloe arborescens</i> Mill. (McGaw48NU) | LF | Leaf infusions used for stomach ache (HUTCHINGS and JOHNSON, 1986) |

Table 2.1 continued...

| Family and botanical name (voucher specimen ¹) | Plant part ² assayed | Traditional uses and administration |
|---|---------------------------------|--|
| <i>Aloe marlothii</i> Berger (McGaw62NU) | LF | Leaf and root decoctions administered orally or as enemas against roundworms; shoot decoctions widely used for stomach ailments (WATT and BREYER-BRANDWIJK, 1962) |
| <i>Bulbine latifolia</i> (L. f.) Roem. & Schult. (McGaw73NU) | TB | Tuber decoctions used for dysentery and diarrhoea (PUJOL, 1990) |
| ASTERACEAE | | |
| <i>Artemisia afra</i> Jacq. ex Willd. (McGaw30NU) | LF | Enemas made from ground plants administered for constipation and intestinal worms (ROBERTS, 1990). Plants widely used in southern Africa as anthelmintics and emetics (HUTCHINGS <i>et al.</i> , 1996) |
| <i>Bidens pilosa</i> L. (McGaw74NU) | LF | Leaf or root infusions taken or administered as enemas for stomach complaints (BRYANT, 1966). Flowers used for diarrhoea (HUTCHINGS <i>et al.</i> , 1996) |
| <i>Brachylaena discolor</i> DC. (McGaw84NU) | LF | Infusions of pounded leaves taken as purgatives against intestinal parasites (BRYANT, 1966; MABOGO, 1990) |
| <i>Tarchonanthus camphoratus</i> L. (McGaw55NU) | LF/TW | Leaf infusions used for abdominal pain (HUTCHINGS <i>et al.</i> , 1996) |
| BIGNONIACEAE | | |
| <i>Kigelia africana</i> (Lam.) Benth. | LF | Leaves, stems and twigs used for |

Table 2.1 continued...

| Family and botanical name (voucher specimen ¹) | Plant part ² assayed | Traditional uses and administration |
|--|---------------------------------|---|
| (McGaw57NU) | | dysentery; roots used for constipation and tapeworm; fruit and ground bark decoctions taken or administered as enemas to children with stomach ailments (HUTCHINGS <i>et al.</i> , 1996) |
| <i>Tecomaria capensis</i> (Thunb.) Spach (McGaw52NU) | BK | Dried powdered bark infusions used for diarrhoea, dysentery and stomach pains (ROBERTS, 1990) |
| CAESALPINACEAE | | |
| <i>Senna didymobotrya</i> (Fresn.) Irwin + Barneby (McGaw64NU) | LF | <i>Senna</i> spp. are used pharmaceutically in laxative preparations (HUTCHINGS <i>et al.</i> , 1996) |
| CELASTRACEAE | | |
| <i>Cassine transvaalensis</i> (Burt Davy) Codd (McGaw70NU) | BK | Bark infusions taken or administered as emetics or enemas for stomach ache (GERSTNER, 1939). Decoctions of powdered bark taken for diarrhoea and intestinal cramps (PUJOL, 1990) and as an anthelmintic (MABOGO, 1990). Roots used for diarrhoea (HUTCHINGS <i>et al.</i> , 1996) |
| <i>Catha edulis</i> (Vahl) Forssk. Ex Endl. (McGaw63NU) | RT | Roots used for stomach ailments (HUTCHINGS <i>et al.</i> , 1996) |
| COMBRETACEAE | | |
| <i>Combretum apiculatum</i> Sond. subsp. <i>apiculatum</i> LF (McGaw78NU) | | Leaf decoctions used as steam baths or administered as enemas for abdominal disorders (WATT and |

Table 2.1 continued...

| Family and botanical name (voucher specimen ¹) | Plant part ² assayed | Traditional uses and administration |
|---|---------------------------------|--|
| | | BREYER-BRANDWIJK, 1962) |
| CORNACEAE | | |
| <i>Curtisia dentata</i> (Burm. f.) C.A. Sm. (McGaw53NU) | BK | Bark used for stomach ailments including diarrhoea (PUJOL, 1990) |
| DRACAENACEAE (LILIACEAE) | | |
| <i>Sansevieria hyacinthoides</i> (L.) Druce (McGaw51NU) | LF | Used for intestinal worms (WATT and BREYER-BRANDWIJK, 1962), stomach disorders and diarrhoea (PUJOL, 1990) |
| EBENACEAE | | |
| <i>Euclea divinorum</i> Hiern (McGaw60NU) | BK | Bark and roots used as anthelmintics, tonics and purgatives (KOKWARO, 1976) |
| EUPHORBIACEAE | | |
| <i>Clutia pulchella</i> L. (McGaw61NU) | LF | Leaf infusions taken for stomach ache (BRYANT, 1966), diarrhoea and dysentery (HUTCHINGS <i>et al.</i> , 1996) |
| <i>Croton sylvaticus</i> Hochst. (Zschocke1NU) | BK | Bark used for abdominal disorders (BRYANT, 1966) |
| <i>Ricinus communis</i> L. (McGaw28NU) | LF | Leaf infusions administered orally or as enemas for stomach ache (GERSTNER, 1939). Roots chewed as anthelmintics, root decoctions taken for abdominal complaints, stems and leaves used for stomach ache and diarrhoea (KOKWARO, 1976) |

Table 2.1 continued...

| Family and botanical name (voucher specimen ¹) | Plant part ² assayed | Traditional uses and administration |
|--|---------------------------------|--|
| <i>Spirostachys africana</i> Sond. (McGaw45NU) | LF | Roots or stems used for diarrhoea, dysentery and stomach pains (MABOGO, 1990) |
| FABACEAE | | |
| <i>Albizia adianthifolia</i> (Schumach.) W.F. Wight (McGaw34NU) | LF | Unspecified parts used for stomach ailments (GERSTNER, 1939). Leaves and roots used for stomach ache and dysentery (MABOGO, 1990) |
| <i>Erythrophleum lasianthum</i> Corbishley (Zschocke5NU) | BK | Bark taken internally for abdominal pains (WATT and BREYER-BRANDWIJK, 1962) and as an anthelmintic (PALMER and PITMAN, 1972) |
| <i>Schotia brachypetala</i> Sond. (McGaw58NU) | LF | Roots used for dysentery and diarrhoea (BRYANT, 1966) |
| GUNNERACEAE | | |
| <i>Gunnera perpensa</i> L. (McGaw39NU) | RH/RT | Root decoctions used for stomach ailments (WATT and BREYER-BRANDWIJK, 1962) |
| ICACINACEAE | | |
| <i>Apodytes dimidiata</i> E. Mey. ex Arn. (McGaw32NU) | LF | Decoctions of root bark administered as enemas for intestinal parasites (BRYANT, 1966) |
| IRIDACEAE | | |
| <i>Crocoshmia paniculata</i> (Klatt) Goldbl. (McGaw83NU) | CM | Corm decoctions taken for dysentery and diarrhoea, followed by enemas of the same decoctions (GERSTNER, 1941; WATT and |

Table 2.1 continued...

| Family and botanical name (voucher specimen ¹) | Plant part ² assayed | Traditional uses and administration |
|---|---------------------------------|--|
| BREYER-BRANDWIJK, 1962) | | |
| LAMIACEAE | | |
| <i>Leonotis leonurus</i> (L.) R. Br. (McGaw35NU) | LF | Infusions of leaves and stems taken for dysentery (GERSTNER, 1941; WATT and BREYER-BRANDWIJK, 1962). Leaves and flowers used for tapeworm (HUTCHINGS <i>et al.</i> , 1996) |
| <i>Tetradenia riparia</i> (Hochst.) Codd (McGaw31NU) | LF | Leaves used for diarrhoea (WATT and BREYER-BRANDWIJK, 1962) |
| LAURACEAE | | |
| <i>Cinnamomum camphora</i> (L.) J. Presl. (McGaw65NU) | LF | Used as an antibacterial and to treat diarrhoea (WATT and BREYER-BRANDWIJK, 1962) |
| <i>Ocotea bullata</i> (Burch.) Baill. (Jäger16NU) | BK | Infusion of the powdered bark used for stomach trouble (PUJOL, 1990) and infantile diarrhoea (VAN WYK <i>et al.</i> , 1997) |
| LOGANIACEAE | | |
| <i>Buddleja salviifolia</i> (L.) Lam. (McGaw43NU) | LF | Root decoctions used to treat stomach upsets and diarrhoea (ROBERTS, 1990) |
| <i>Strychnos spinosa</i> Lam. (McGaw79NU) | LF | Unspecified parts used for dysentery (WATT and BREYER-BRANDWIJK, 1962) |
| MELIACEAE | | |
| <i>Ekebergia capensis</i> Sparrm. (McGaw69NU) | LF | Roots used for dysentery (POOLEY, 1993); leaf infusion used as a |

Table 2.1 continued...

| Family and botanical name (voucher specimen ¹) | Plant part ² assayed | Traditional uses and administration |
|---|---------------------------------|--|
| | | purgative parasiticide (HUTCHINGS <i>et al.</i> , 1996) |
| <i>Melia azedarach</i> L. (McGaw77NU) | LF | Cold water leaf infusions taken for abdominal pains and as anthelmintics (HUTCHINGS <i>et al.</i> , 1996) |
| MYRSINACEAE | | |
| <i>Maesa lanceolata</i> Forssk. (McGaw75NU) | LF | Powdered whole fruit or seeds used as anthelmintics (WATT and BREYER-BRANDWIJK, 1962) |
| OLEACEAE | | |
| <i>Olea europaea</i> L. (McGaw59NU) | LF | Immature fruits used as astringents against diarrhoea (IWU, 1993) |
| PEDALIACEAE | | |
| <i>Ceratotheca triloba</i> (Bernh.) Hook. f. (McGaw81NU) | LF | Leaf infusions administered for diarrhoea and gastrointestinal cramps (WATT and BREYER-BRANDWIJK, 1962; ROBERTS, 1990) |
| PERIPLOCACEAE | | |
| <i>Mondia whitei</i> (Hook. f.) Skeels (McGaw82NU) | LF | Roots chewed for stomach ache (GÉRSTNER, 1941; BRYANT, 1966) |
| PITTOSPORACEAE | | |
| <i>Pittosporum viridiflorum</i> Sims (McGaw37NU) | BK | Bark decoctions administered as emetics or enemas for stomach troubles (WATT and BREYER-BRANDWIJK, 1962). Roasted bark used for dysentery (HUTCHINGS |

Table 2.1 continued...

| Family and botanical name (voucher specimen ¹) | Plant part ² assayed | Traditional uses and administration <i>et al.</i> , 1996) |
|---|---------------------------------|--|
| POLYGONACEAE | | |
| <i>Rumex lanceolatus</i> (McGaw29NU) | RT | Cold root infusions used for tapeworm (WATT and BREYER-BRANDWIJK, 1962) |
| RHAMNACEAE | | |
| <i>Ziziphus mucronata</i> Willd. (McGaw80NU) | LF | Root infusions used for dysentery (HUTCHINGS <i>et al.</i> , 1996) |
| RUBIACEAE | | |
| <i>Canthium inerme</i> (L. f.) Kuntze (McGaw68NU) | LF | Leaves used to cure stomach complaints (BRYANT, 1966); leaf infusions in milk taken for dysentery and diarrhoea (HUTCHINGS <i>et al.</i> , 1996) |
| <i>Psychotria capensis</i> (Eckl.) Vatke (McGaw72NU) | RT | Root infusions taken as emetics (WATT and BREYER-BRANDWIJK, 1962). Used for gastric complaints (POOLEY, 1993) |
| RUTACEAE | | |
| <i>Clausena anisata</i> (Willd.) Hook. f. ex. Benth. (McGaw66NU) | RT | Roots used as tapeworm remedy (BRYANT, 1966); leaf infusions taken as parasiticides and purgatives (HUTCHINGS <i>et al.</i> , 1996) |
| <i>Zanthoxylum capense</i> (Thunb.) Harv. (McGaw41NU) | LF | Leaves are an ingredient in infusions used as purgative parasiticides and for stomach complaints (HUTCHINGS <i>et al.</i> , 1996) |

Table 2.1 continued...

| Family and botanical name (voucher specimen ¹) | Plant part ² assayed | Traditional uses and administration |
|--|---------------------------------|---|
| SAPINDACEAE | | |
| <i>Deinbollia oblongifolia</i> (E. Mey. ex Arn.) Radlk. (McGaw33NU) | LF | Roots used for dysentery and diarrhoea (BRYANT, 1966) |
| STANGERIACEAE | | |
| <i>Stangeria eriopus</i> (Kunze) Baill. (Zschocke7NU) | LF | Tubers used as emetics and purgatives (WATT and BREYER-BRANDWIJK, 1962; OSBORNE and GROVE, 1992) |
| STERCULIACEAE | | |
| <i>Dombeya rotundifolia</i> (Hochst.) Planch. (McGaw54NU) | LF | Decoctions from unspecified parts taken for diarrhoea and intestinal upsets (HUTCHINGS <i>et al.</i> , 1996) |
| TYPHACEAE | | |
| <i>Typha capensis</i> (Rohrb.) N.E. Br. (McGaw46NU) | RH | Decoctions used for dysentery and diarrhoea (HUTCHINGS <i>et al.</i> , 1996) |
| ULMACEAE | | |
| <i>Trema orientalis</i> (L.) Blume (McGaw40NU) | BK/WD | Bark used as an anthelmintic against hookworm and roundworm; wood used for dysentery (HUTCHINGS <i>et al.</i> , 1996) |
| VERBENACEAE | | |
| <i>Clerodendrum glabrum</i> E. Mey. (McGaw71NU) | LF | Leaves used as ingredients in infusions against intestinal parasites (HUTCHINGS <i>et al.</i> , 1996) |
| <i>Lippia javanica</i> (Burm. f.) Spreng. (McGaw76NU) | LF/TW | Leaf infusions used as anthelmintics and as prophylactics against dysentery and diarrhoea (MABOGO, 1990) |

¹Voucher specimen: NU = Natal University Herbarium, Pietermaritzburg

²Plant part: BK = bark, BL = bulb, CM = corm, FR = fruit, LF = leaf, LX = latex, RH = rhizome, RT = root, RT BK = root bark, ST = stem, TB = tuber, TW = twig, WD = wood, WH = whole plant

2.4 DISCUSSION

Hexane, ethanol and water were chosen as extracting solvents because of their wide range in polarity. This selection of solvents would be expected to extract both water-soluble and lipophilic compounds, allowing a greater possibility of discovering biologically active compounds in the subsequent screening procedure. It was decided to use dried plant material to eliminate the possibility of the water content of different plants influencing the amount of residue resulting from the extraction. The dried plant material was ground to a powder to allow the extracting solvent improved access to plant cell contents. The extracts were stored in a freezer at -15°C to prevent decomposition of natural products before the screening procedure was undertaken.

2.5 CONCLUSION

* The selection of plants for screening in bioassays is important to maximise the potential for discovering plants with high biological activity. Selecting plants based on ethnopharmacological leads serves a dual purpose in both substantiating the claims of traditional healers, and revealing pharmacologically interesting plants for further investigation. This may involve isolating the active components using bioassay-guided fractionation, or producing galenical preparations for the herbal medicine market.

CHAPTER 3

ANTHELMINTIC SCREENING

3.1 INTRODUCTION

3.1.1 Helminthiasis

Helminthiasis is a disease characterized by the presence of parasitic worms in the body. Gastrointestinal helminthiasis are estimated to be the most common of all parasitic infections (BUNDY and COOPER, 1989). There are several types of worms found inhabiting the intestines of humans and their domesticated animals, of which the most important are nematodes or roundworms. Nematodes are widely distributed and are found in almost every type of environment (LEE, 1965). There is much morphological uniformity throughout nematode species. Differences in behaviour and physiology have been instrumental in the success of the group (CROLL, 1970). Most nematodes are free-living, but many species are parasitic on insects, fish, birds, fungi, plants or animals including man. It has been estimated that about 16 000 nematode species have been described and that at least 40 000 species exist (ANDERSON, 1992). About 33 % of all the nematode genera which have been described occur as parasites of vertebrates (ANDERSON, 1992). The economic importance of parasitic nematodes has focussed attention on these, rather than on free-living species.

Most of the worms that affect man live unobtrusively in the intestine, doing little to impair the health of their host, with symptoms and signs usually appearing only in cases of heavy infection (LEWIS and ELVIN-LEWIS, 1977). The World Health Organization (WHO) estimates that one billion people are infected with *Ascaris lumbricoides* and 500 million with *Trichuris trichiura* (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). EVANS *et al.* (1987) reported prevalences in

KwaZulu-Natal children of 64.5 % for *A. lumbricoides* and 61.1 % for *T. trichiura*. Tissue-living nematodes are also an important health problem, with 100 million people being infected with filarial worms such as *Wucheraria bancrofti* and *Onchocerca volvulus* (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). The prevalence of helminthiasis is particularly intense in children in countries with poor sanitation, a low standard of education, and little health education (ENWEREM *et al.*, 2001). The extent of debility and ill health caused by worms is related to the worm load, type and duration of the infection, with treatment being directed primarily at reduction of worm load (ENWEREM *et al.*, 2001). Although the mortality rate associated with parasite infestations is negligible, morbidity such as impaired physical and mental development is significant (TAYLOR *et al.*, 1995). The major impact of parasitic infection is the impairment of nutritional status in the patient (CROMPTON, 1991). A commonly reported consequence of parasitic infection is lethargy, connected not only with a lack of energy for physical activity, but also for mental effort (CONNOLLY and KVALSVIG, 1993). FINCHAM *et al.* (1998) stated that the "vast mass" of helminth infection is contributing to impairment of nutrition, growth, learning and physical performance of South African children. Since the population has multiplied exponentially, and the consequent deterioration in environmental conditions has enhanced infection by intestinal parasites, it is likely that clinical cases are being seen increasingly frequently at most hospitals and clinics (FINCHAM *et al.*, 1998). In addition to the direct impact of nematodes on human health, helminthiasis is a major factor adversely influencing animal breeding (ASUZU and NJOKU, 1996).

The nematode life cycle includes an egg, four larval stages, each separated by a moult, and the adult stage (CROLL, 1970). In the parasitic nematodes most studied, the adults are usually parasitic, and the larval stages free-living or parasitic in an intermediate host (CROLL, 1970). One stage has usually been developed which completes the transition from free-living to parasitic - the infective stage (CROLL, 1970). The free-living infective stage in animal parasitic nematodes is often a long-lived ensheathed, resistant, non-feeding stage, bridging the gap between the free-living and the parasitic habits (CROLL, 1970). Roundworms are soil-transmitted,

each species spending an obligatory period of development on the soil, free from its host (CROMPTON, 1991).

3.1.2 Control of helminth infection

A range of control measures for soil-transmitted helminthiases is available based on chemotherapy, sanitation and health education (CROMPTON, 1991). NJOKU *et al.* (1996) proposed that the most practical way to control helminth infection is by chemotherapy using broad spectrum anthelmintics. There are several anthelmintics available for treating helminthiasis, but a major setback to their continued usage is the development of resistance (ASUZU and NJOKU, 1996). In addition, some currently used synthetic anthelmintics are expensive or not readily available (ASUZU and NJOKU, 1996). There is thus an urgent need for new, inexpensive drugs which will be able to act for longer periods before resistance sets in. Much effort is being made at the discovery of new anthelmintics of plant origin (NJOKU *et al.*, 1996).

An anthelmintic drug must have a wide margin of safety between its toxicity to the worm and its toxic side effect on the host. LEWIS and ELVIN-LEWIS (1977) stated that anthelmintic properties of many naturally occurring products have been known since the beginning of civilization and are still widely used today. To be effective they should be orally active, produce results in a single dose, and be cheap.

Chemotherapeutic agents are administered widely today to rid the host of helminth parasites, acting either by reducing intestinal flora, which may serve as food for certain worms, or by direct toxic action on the worms (LEWIS and ELVIN-LEWIS, 1977). Often the use of these drugs is followed by a purge (COLE, 1971, cited by LEWIS and ELVIN-LEWIS, 1977). Therapeutic agents may only kill certain stages of the life cycle, so if the adult stage is killed but the eggs persist, a purge is necessary to cleanse the intestine of eggs to prevent the recurrence of infection.

Generally-used anthelmintics include the benzimidazoles such as albendazole and thiabendazole, and the non-benzimidazoles such as levamisole, pyrantel and ivermectin (SIMPKIN and COLES, 1981). Levamisole continues to be a major

broad spectrum anthelmintic for the treatment of gastrointestinal nematodes in man and animals despite the occurrence of some side effects (AMERY and BRUYNSEELS, 1992). Levamisole and pyrantel act by disrupting neuromuscular transmission in nematodes (ATCHISON *et al.*, 1992). Levamisole can penetrate the cuticle of nematodes and then act on the cholinergic receptors at the neuromuscular junctions to paralyse the worms in a contractile state, and once rendered immobile, the worms are excreted (FRAYHA *et al.*, 1997).

Anthelmintic properties of microbial secondary metabolites, such as avermectins and milbemycins, have been discovered in members of the genus *Streptomyces* (MISHRA *et al.*, 1987). The avermectins (which include ivermectin) and milbemycins are structurally related and belong to the family of macrolide antibiotics with activity against a wide range of nematodes (MISHRA *et al.*, 1987). The avermectins and milbemycins have a similar mode of action in that they are able to stimulate the conductance of chloride ions across muscle membranes (HABER *et al.*, 1991). Benzimidazoles, which include thiabendazole, mebendazole and albendazole, appear to act by binding to tubulin and disrupting the assembly of microtubules, and by altering transmembrane proton discharge (GROVE, 1996). Paraherquamide is a nematocidal indole alkaloid first isolated from *Penicillium paraherquei* (SCHAEFFER *et al.*, 1992). Ivermectin-resistant and thiabendazole-resistant nematodes remain sensitive to paraherquamide treatment, suggesting a novel mode of action for the alkaloid (SCHAEFFER *et al.*, 1992). The study of SCHAEFFER *et al.* (1992) supported the hypothesis that the biological activity is mediated via a ligand-receptor interaction. The action of many, if not all anthelmintics is due to their ability to reduce larval motility (ENWEREM *et al.*, 2001).

3.1.3 Anthelmintic assays

NEAL in 1980 (cited by RASOANAIVO and RATSIMAMANGA-URVERG, 1993) stated that about 50 new compounds need to be evaluated every week for 4 years to stand a good statistical chance of success in finding a new parasitocidal compound (1/10 000). To achieve this goal, a rapid and relevant first stage screen

using 15 mg or less of compound or plant extracts would be easier than any animal model. Animal tests require relatively large quantities of test chemicals and plenty of space for the animals, which can themselves be expensive (SIMPKIN and COLES, 1981). ASUZU and NJOKU (1996) indicated that medicinal plants may be the most important sources of new anthelmintics. Many vascular plants are in common use in domestic medicine in tropical areas where they are readily available and inexpensive (LEWIS and ELVIN-LEWIS, 1977).

A number of *in vitro* test systems have been reported, using various nematodes as test organisms. These systems have been briefly reviewed by SIMPKIN and COLES (1981). In 1958, TINNER used eggs and larvae of trichostrongyles from rabbits but the rate of screening was relatively low. TINNER (1958) also reported that cultured *Caenorhabditis briggsae* did not respond reliably to the anthelmintic phenothiazine. *Cooperia punctata* responded to anthelmintics *in vitro*, but long pre-test incubation procedures with several media changes were required, and it is not certain whether the method is sufficiently selective for use as a random screen (LELAND *et al.*, 1975). COLES and MCNEILLIE (1977) used eggs of *Nematodirus spathiger* to detect benzimidazole-like drugs and cultured adult *Nippostrongylus brasiliensis* to detect other anthelmintics, but a single step test would be more convenient. PLATZER *et al.* (1977) evaluated nine benzimidazoles against the free-living nematode *Caenorhabditis elegans* and suggested that it could be used in a test for anthelmintics.

Caenorhabditis elegans is a self-reproducing hermaphrodite, each animal producing both sperm and eggs (BRENNER, 1974). Occasionally, cultures are found with a few males, which may be mated with the hermaphrodites. The adults are about 1 mm in length and the life cycle for worms grown on *E. coli* is 3.5 days at 20°C (BRENNER, 1974). *C. elegans* has been adopted as a convenient model to study important problems in the developmental genetics and biology of metazoans (BRENNER, 1974). WHARTON (1986) predicted that knowledge of nematode biology as a whole will increasingly be based on this species. The essentially complete DNA sequence of *C. elegans* has been determined, and this knowledge

will be a significant aid to understanding and controlling nematode parasites (BLAXTER, 1998). Manipulations of this sequence will be important in studies of gene function, as genes involved in development are often homologous or shared by the worm and other multicellular organisms (PENNISI, 1998).

SIMPKIN and COLES (1981) recommended screening anthelmintic compounds using free-living nematodes, as the culture and screen are quite simple and do not require sophisticated equipment. After successfully using *Caenorhabditis elegans* for *in vitro* screening for potential anthelmintics and for screening some standard anthelmintics, SIMPKIN and COLES (1981) suggested the use of *C. elegans* in high through-put *in vitro* assays for anthelmintics, as this test system has a remarkable degree of selectivity, and is very cheap and easy to operate.

3.2 MATERIALS AND METHODS

3.2.1 Culture of *Caenorhabditis elegans*

Caenorhabditis elegans var. Bristol (N2) nematodes were cultured on nematode growth (NG) agar (APPENDIX 1) seeded with *E. coli* as a food source for the nematodes, according to the method of BRENNER (1974). Subculturing was performed on a laminar flow bench to prevent contamination of the nematode cultures. An aliquot (50 μ l) of *E. coli* culture grown overnight at 35°C was pipetted onto each NG agar plate and spread with a glass spreader. Five ml of sterile M9 buffer (APPENDIX 1) was added to a previous nematode culture plate to wash the nematodes off the plate. Portions of this nematode suspension (500 μ l) were pipetted onto each fresh NG agar plate seeded with *E. coli* and incubated in the dark at 20°C.

The extracts of plants used by traditional healers for treating intestinal worm infestations were tested at concentrations of 1 and 2 mg ml⁻¹. Ethanol and water extracts were resuspended in their extracting solvents, while hexane extracts were

resuspended in acetone or dimethylsulfoxide (DMSO).

3.2.2 Mortality assay

Two types of anthelmintic assay were carried out. The first was a simple bioassay described by RASOANAIVO and RATSIMAMANGA-URVERG (1993) where nematode mortality after addition of nematocidal compound or plant extract was assessed. In this assay, 500-1000 nematodes (7 to 10-day old cultures) in M9 buffer (APPENDIX 1) were incubated with plant extract for 2 h at 25°C in the dark. The assay was first standardized using the anthelmintic drug levamisole in place of plant extract. A standard concentration of 5 $\mu\text{g ml}^{-1}$ levamisole was used as a control in all subsequent experiments. A second control, or blank, consisted of nematodes incubated with no plant extract or levamisole. Using a dissecting microscope, the percentage of live worms was estimated, and their movement noted and compared to the controls.

3.2.3 Reproductive ability assay

The second anthelmintic assay to determine the effect of plant extracts on reproductive ability of nematodes was performed following the protocol of SIMPKIN and COLES (1981). Nematodes for the test were washed from 4 to 8-day old cultures with M9 buffer and held in M9 supplemented with 1 $\mu\text{g ml}^{-1}$ chlorhexidine digluconate for 1 hour. This treatment does not harm the worms and helps to avoid contaminating bacteria overgrowing the test medium. The test medium was made by adding 10 ml of 3 to 5-day old *E. coli* (grown at 20°C) to 100 ml M9 buffer along with 5 mg ampicillin and 10 000 U nystatin. The ampicillin inhibits the *E. coli* so that multiplication is reduced and the bacteria are less likely to metabolize the added compounds. After 2 h at room temperature, 2 ml of the test medium was placed in each well of a sterile repli-dish (Sterilin) and 10 μl of extract or levamisole solution was added to each well. A drop of the worm suspension containing about 20 nematodes was added to the wells after a few minutes so the worms did not drop into a region of high concentration of extract or levamisole. In each repli-dish were

negative control wells containing test medium, nematodes and water, ethanol, DMSO or acetone as well as blank wells containing test medium only. In the positive control, 5 $\mu\text{g ml}^{-1}$ levamisole was added in place of plant extract. Two wells were allocated to each concentration of plant extract or levamisole. The plates were incubated in the dark at 20°C for 7 days. After incubation the number and movement of nematodes in each well was assessed compared to the controls using a dissecting microscope.

In both assays, the number and movement of nematodes as compared to the control (nematodes incubated with no plant extract or levamisole) was noted and scored.

3.3 RESULTS

3.3.1 Standardization of results

Standard curves (Figures 3.1 and 3.2) were prepared using levamisole as the standard anthelmintic drug for each of the assays. Using regression analysis, the 50% inhibitory concentration (IC_{50}) values for levamisole were calculated to be 4.674 and 6.901 $\mu\text{g ml}^{-1}$ for the 2 h and 7 day assays respectively. SIMPKIN and COLES (1981) reported the minimum detectable dose of levamisole on *C. elegans* as 0.5 $\mu\text{g ml}^{-1}$; no literature available reports IC_{50} values.

3.3.2 Anthelmintic activity of plant extracts

Inhibition of *C. elegans* by plant extracts in both anthelmintic assays is recorded in Table 3.1.

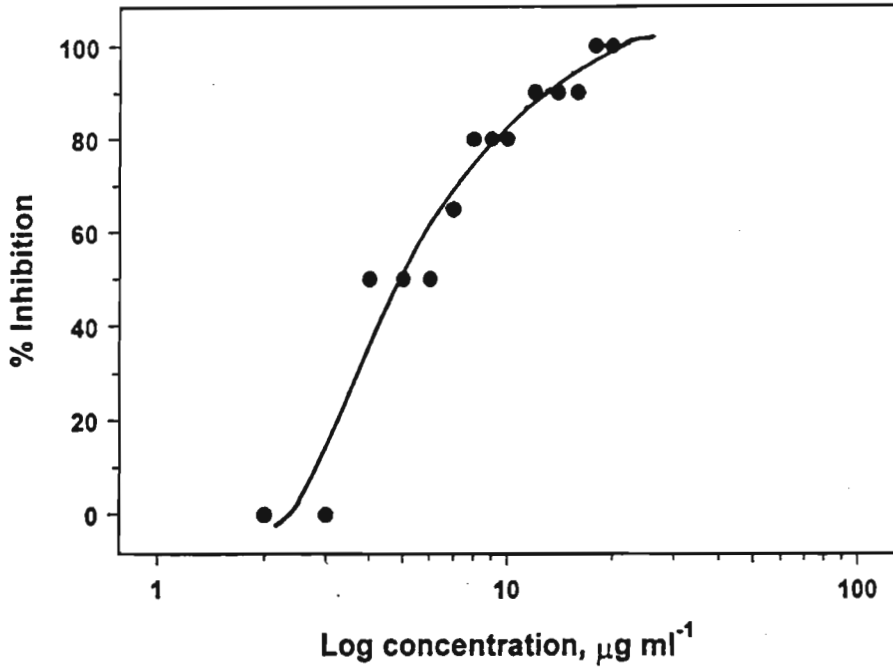


Figure 3.1. Inhibition of nematodes by levamisole (2 h mortality assay)

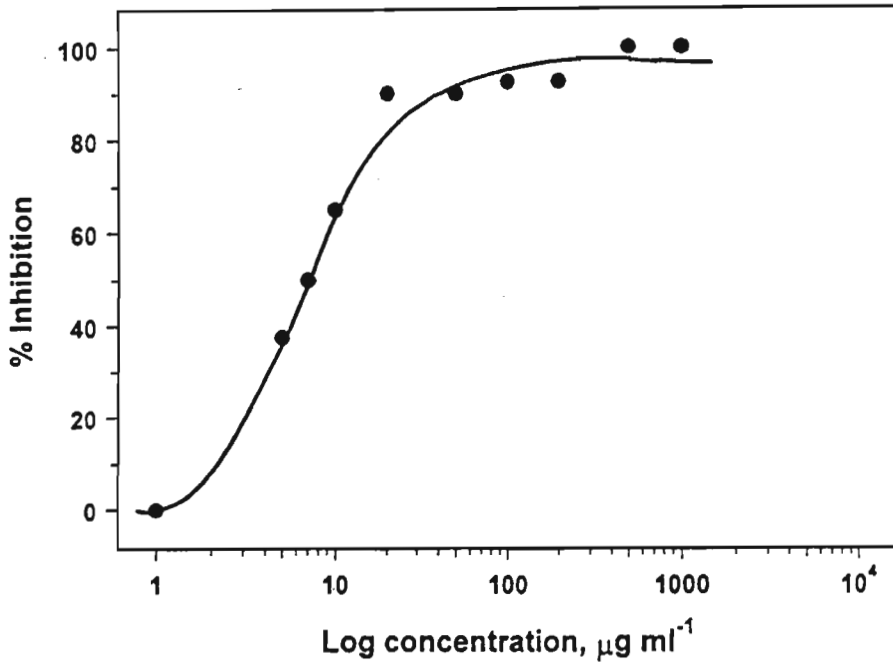


Figure 3.2. Inhibition of nematodes by levamisole (7 day reproductive ability assay)

Table 3.1. Inhibition of nematodes by plant extracts (2 h and 7 day anthelmintic assays)

| Plant name | Plant part ¹ | Extract ² | Concentration (mg ml ⁻¹) | Activity ³ | |
|---------------------------------|-------------------------|----------------------|---|-----------------------|-------|
| | | | | 2 h | 7 day |
| <i>Acokanthera oblongifolia</i> | LF | H | 2 | - | - |
| | | | 1 | - | - |
| | | E | 2 | - | - |
| | | | 1 | - | - |
| | | | W | 2 | - |
| <i>Acorus calamus</i> | RH/RT | H | 2 | + | ++ |
| | | | 1 | + | + |
| | | E | 2 | ++ | +++ |
| | | | 1 | + | ++ |
| | | | W | 2 | -+ |
| <i>Albizia adianthifolia</i> | LF | H | 2 | - | + |
| | | | 1 | - | - |
| | | E | 2 | + | + |
| | | | 1 | -+ | - |
| | | | W | 2 | + |
| <i>Aloe marlothii</i> | LF | H | 2 | -+ | - |
| | | | 1 | -+ | - |
| | | E | 2 | ++ | ++ |
| | | | 1 | + | ++ |
| | | | W | 2 | + |
| <i>Apodytes dimidiata</i> | LF | H | 2 | - | - |
| | | | 1 | - | - |
| | | E | 2 | + | ++ |
| | | | 1 | + | + |
| | | | W | 2 | -+ |
| <i>Artemisia afra</i> | LF | H | 2 | - | - |
| | | | 1 | - | - |
| | | E | 2 | + | ++ |
| | | | 1 | + | ++ |
| | | | W | 2 | ++ |
| <i>Brachylaena discolor</i> | LF | H | 2 | + | + |
| | | | 1 | -+ | - |
| | | E | 2 | + | ++ |
| | | | 1 | - | ++ |
| | | | W | 2 | -+ |

Table 3.1 continued...

| Plant name | Plant part ¹ | Extract ² | Concentration (mg ml ⁻¹) | Activity ³ | |
|---------------------------------|-------------------------|----------------------|---|-----------------------|-------|
| | | | | 2 h | 7 day |
| <i>Clausena anisata</i> | LF | H | 1 | - | - |
| | | | 2 | - | - |
| | | | 1 | - | - |
| | | E | 2 | + | ++ |
| | | | 1 | + | ++ |
| | | | 2 | + | +++ |
| <i>Clerodendrum glabrum</i> | LF | H | 1 | + | + |
| | | | 2 | - | - |
| | | | 1 | - | - |
| | | E | 2 | -+ | ++ |
| | | | 1 | - | - |
| | | | 2 | - | + |
| <i>Ekebergia capensis</i> | LF | H | 1 | - | - |
| | | | 2 | - | + |
| | | | 1 | - | - |
| | | E | 2 | -+ | + |
| | | | 1 | - | - |
| | | | 2 | - | -+ |
| <i>Erythrophleum lasianthum</i> | LF | H | 1 | - | -+ |
| | | | 2 | - | - |
| | | | 1 | - | - |
| | | E | 2 | -+ | ++ |
| | | | 1 | - | + |
| | | | 2 | -+ | ++ |
| <i>Euclea divinorum</i> | BK | H | 1 | - | + |
| | | | 2 | - | - |
| | | | 1 | - | - |
| | | E | 2 | + | - |
| | | | 1 | -+ | - |
| | | | 2 | -+ | ++ |
| <i>Heteromorpha trifoliata</i> | LF | H | 1 | -+ | ++ |
| | | | 2 | - | - |
| | | | 1 | - | - |
| | | E | 2 | -+ | - |
| | | | 1 | - | - |
| | | | 2 | - | + |
| <i>Kigelia africana</i> | LF | H | 1 | - | - |
| | | | 2 | -+ | -+ |
| | | | 1 | - | -+ |
| | | E | 2 | - | - |
| | | | 1 | - | - |
| | | | 2 | -+ | ++ |
| <i>Leonotis leonurus</i> | LF | H | 1 | - | + |
| | | | 2 | -+ | - |

Table 3.1 continued...

| Plant name | Plant part ¹ | Extract ² | Concentration (mg ml ⁻¹) | Activity ³ | |
|----------------------------------|-------------------------|----------------------|---|-----------------------|-------|
| | | | | 2 h | 7 day |
| | | | 1 | - | - |
| | | E | 2 | -+ | ++ |
| | | | 1 | - | ++ |
| | | W | 2 | - | ++ |
| | | | 1 | - | ++ |
| <i>Lippia javanica</i> | LF/TW | H | 2 | - | -+ |
| | | | 1 | - | - |
| | | E | 2 | -+ | + |
| | | | 1 | - | - |
| | | W | 2 | - | +++ |
| | | | 1 | - | +++ |
| <i>Maesa lanceolata</i> | LF | H | 2 | -+ | - |
| | | | 1 | -+ | - |
| | | E | 2 | - | + |
| | | | 1 | - | -+ |
| | | W | 2 | - | - |
| | | | 1 | - | - |
| <i>Melia azedarach</i> | LF | H | 2 | -+ | - |
| | | | 1 | - | - |
| | | E | 2 | - | - |
| | | | 1 | - | - |
| | | W | 2 | -+ | +++ |
| | | | 1 | - | ++ |
| <i>Pimpinella caffra</i> | WH | H | 2 | - | - |
| | | | 1 | - | - |
| | | E | 2 | - | - |
| | | | 1 | - | - |
| | | W | 2 | - | ++ |
| | | | 1 | - | + |
| <i>Ricinus communis</i> | LF | H | 2 | -+ | ++ |
| | | | 1 | -+ | + |
| | | E | 2 | - | ++ |
| | | | 1 | - | + |
| | | W | 2 | + | ++++ |
| | | | 1 | - | +++ |
| <i>Sansevieria hyacinthoides</i> | LF | H | 2 | - | - |
| | | | 1 | - | - |
| | | E | 2 | - | - |
| | | | 1 | - | - |
| | | W | 2 | - | +++ |
| | | | 1 | - | ++ |
| <i>Trema orientalis</i> | BK/WD | H | 2 | -+ | ++ |
| | | | 1 | -+ | + |

Table 3.1 continued...

| Plant name | Plant part ¹ | Extract ² | Concentration (mg ml ⁻¹) | Activity ³ | |
|----------------------------|-------------------------|----------------------|---|-----------------------|-------|
| | | | | 2 h | 7 day |
| <i>Tulbaghia violacea</i> | TB | E | 2 | -+ | ++ |
| | | | 1 | -+ | ++ |
| | | W | 2 | - | +++ |
| | | | 1 | - | +++ |
| | | H | 2 | - | - |
| | | | 1 | - | - |
| | | E | 2 | + | +++ |
| | | | 1 | -+ | ++ |
| | | W | 2 | -+ | ++++ |
| | | | 1 | - | +++ |
| <i>Zanthoxylum capense</i> | LF | H | 2 | - | - |
| | | | 1 | - | - |
| | | E | 2 | - | + |
| | | | 1 | - | + |
| | | W | 2 | - | - |
| | | | 1 | - | - |

¹Plant part: BK = bark, LF = leaf, RH = rhizome, RT = root, TB = tuber, TW = twig, WD = wood, WH = whole plant

²Extract: H = hexane, E = ethanol, W = water

³Activity:

- same number of nematodes alive as control (nematodes incubated without levamisole or plant extract), worms move vigorously
- + about 80% of nematodes compared to control, worms move slightly slower
- + about half number of nematodes compared to control, worms move slowly
- ++ less than 20% of nematodes compared to control, worms move slowly
- +++ less than 10% of nematodes compared to control, worms move very slowly
- ++++ no movement of worms

The number of extracts tested for anthelmintic activity was 72, derived from 24 genera (18 families). Several crude extracts used by traditional healers for treating intestinal worms displayed activity against *C. elegans*.

3.4 DISCUSSION

3.4.1 Comparison of anthelmintic assays

The results of the two anthelmintic assays correlated well, with the 7 day incubation assay appearing to be more sensitive than the shorter assay. A larger number of extracts revealed anthelmintic activity in the 7 day assay. This may indicate that some extracts exerted an effect on the ability of the nematodes to reproduce rather than having a direct killing effect, which was monitored in the 2 h assay.

3.4.2 Anthelmintic activity of plant extracts

The anthelmintic test systems using *C. elegans* are cheap and easy to use. A large number of extracts or compounds may be tested at one time, leaving few requiring screening in infected animals. Many extracts exhibited activity at a concentration of 2 mg ml⁻¹ but a more selective effect was noted at 1 mg ml⁻¹. In general, the water extracts were more active than ethanol extracts, and very little anthelmintic activity was shown by the hexane extracts.

Acokanthera oblongifolia was the only plant displaying no anthelmintic activity.

Anthelmintic activity has been reported in ethanol extracts of root bark of *Albizia anthelmintica*, and the active principle was identified as musennin (TSCHESCHE and FORSTMAN, 1957). However, *Albizia adianthifolia* did not display much anthelmintic activity.

Extracts of *Acorus calamus* exhibited a high level of anthelmintic activity. There are possibly carcinogenic and toxic compounds in *Acorus calamus*, with toxic effects owing to the presence of β -asarone, a phenylpropanoid (LANDER and SCHREIER, 1990; BRUNETON, 1995). This compound may cause liver and duodenal cancer (BRUNETON, 1995). The anthelmintic activity of *Acorus calamus* may possibly be attributed to the presence of the toxic β -asarone. *Aloe marlothii* displayed some activity against the nematodes. Other species of *Aloe* are known to have a laxative

action, for example *Aloe ferox* (VAN WYK *et al.*, 1997). The main purgative principle of *A. ferox* is the anthrone C-glucoside aloin, or barbaloin (VAN WYK *et al.*, 1997). This compound has been shown to occur in *A. marlothii* (LINDSEY *et al.*, 2001). *A. marlothii* also contains aloesin, aloeresin A and aloeresin D (LINDSEY *et al.*, 2001).

Other plants with marked activity against *C. elegans* included *Artemisia afra*, *Brachylaena discolor*, *Clausena anisata*, *Ricinus communis*, *Trema orientalis* and *Tulbaghia violacea*. Many chemical constituents have been isolated from different parts of *Clausena anisata* (HUTCHINGS *et al.*, 1996). The volatile oil of the leaves yields the toxic estragole (OKUNADE and OLAIFA, 1987). The alkaloids imperatorin and xanthoxyletin, isolated from the root or root bark, have molluscicidal activity (HUTCHINGS *et al.*, 1996). *Ricinus communis*, the castor oil plant, contains the fatty acid ricinoleic acid, which stimulates intestinal peristalsis (VAN WYK *et al.*, 1997). This purgative action assists in clearing the body of intestinal parasites, but the direct effect of the plant extract on the nematodes remains unexplained. The bark and wood of *Trema orientalis* contain tannins (WATT and BREYER-BRANDWIJK, 1962). Many chemical constituents of *Tulbaghia violacea* have been isolated, including several sulphur compounds and steroidal saponins (BURTON, 1990) but it is not known whether these compounds are responsible for the biological activity of the plant.

3.5 CONCLUSION

The anthelmintic assays in this study analysed the direct killing effect as well as the effect on the reproductive ability of plant extracts on free-living nematodes. Owing to the similarity in morphology of free-living and parasitic nematodes, it is probable that extracts displaying anthelmintic activity against free-living nematodes will inhibit parasitic nematodes. Many of the extracts tested revealed anthelmintic activity, supporting their use by traditional healers for the treatment of helminth infestations. Plants are often used by indigenous healers for their purgative action, physically ridding the patient of intestinal parasites. As a result of this, a lack of a direct killing

effect on the nematodes does not necessarily mean that the plants are of no use in treating nematode infestations. Combinations of plants are usually employed by healers, some plants possibly having direct anthelmintic activity, and others with a purgative action to clear the body of parasites.

The widespread nature of worm infestations in rural parts of southern Africa, where sanitation is poor or nonexistent, and where health clinics are sparse, indicates an important role for the treatments administered by traditional healers. It would be worthwhile to investigate further the plants used by local healers for efficacy and toxicity in an effort to maximise the beneficial influence on primary healthcare in these areas.

Prevention of parasite infestation is a priority, especially with the unacceptably high reinfestation rates in children after treatment (TAYLOR *et al.*, 1995). Improvement in the socio-economic status of poor communities, and the provision of clean water and adequate sewerage facilities are high priorities in South Africa.

CHAPTER 4

ANTIAMOEBIIC SCREENING

4.1 INTRODUCTION

4.1.1 Amoebiasis

Amoebiasis is a major disease caused by infections with the protozoan *Entamoeba histolytica*, which exists in the large intestine as either mobile trophozoites or as cysts. The protozoa usually live and multiply in the lumen of the large intestine, living in symbiosis with the bacterial flora as commensal organisms. The host, who may exhibit no symptoms of the disease, will pass infective cysts in the faeces (KEENE *et al.*, 1986). If the cysts are transferred to another host as a result of poor hygiene, they withstand the acidic conditions of the stomach and release trophozoites in the small intestine (KEENE *et al.*, 1986). Mild cases of amoebiasis cause little disturbance to the patient, but a heavy infection results in amoebic dysentery (KEENE *et al.*, 1986). Invasive trophozoites cause local ulceration in the large intestine resulting in dysentery, possibly leading to perforation and fatal peritonitis; if trophozoites move to other organs such as the liver, brain or lung, they form abscesses, and such widespread infections may be fatal (PHILLIPSON *et al.*, 1987). Amoebiasis occurs throughout the world, and is more closely related to sanitation and socio-economic status than to climate, although the occurrence of the disease is most severe in tropical and subtropical regions (DI STASI, 1995).

In 1986, WARREN (cited by PHILLIPSON *et al.*, 1987) reported that for 1977-1978 in Asia, Latin America and Africa there were an estimated 400 million infections annually with 30 000 deaths and 1.5 million cases of the disease. WRIGHT *et al.* (1988) reported that an estimated 12 % of the world's population harbour *Entamoeba histolytica*.

4.1.2 Control of amoebiasis

Since the turn of the century, the isoquinoline alkaloid emetine from *Cephaelis ipecacuanha* (ipecac) has been used successfully to treat amoebic dysentery, and in 1959 its analogue dihydroemetine (which is less toxic on the heart muscle) was introduced (LEWIS and ELVIN-LEWIS, 1977; PHILLIPSON *et al.*, 1995). The severe side effects of emetine include abdominal cramps, dizziness, fainting, vomiting, neuromuscular and cardiovascular effects, and pain or even necrosis at the site of injection (KEENE *et al.*, 1987). Although emetine has been superseded by newer drugs with fewer side effects, it is still considered to be one of the most potent amoebicidal agents and is used in the initial treatment of severe cases of acute amoebic dysentery (KEENE *et al.*, 1987). The synthetic 2,3-dihydroemetine has a similar activity but is less toxic than emetine owing to faster rates of metabolism and excretion (KEENE *et al.*, 1987). Chloroquine and antibiotics such as paromomycin also have direct amoebicidal action; tetracycline and erythromycin indirectly affect survival of amoebae by modifying the intestinal flora necessary for the existence of the organisms (SEATON, 1971, cited by LEWIS and ELVIN-LEWIS, 1977).

PHILLIPSON *et al.* (1987) described a series of pharmaceuticals available for the treatment of amoebiasis, including emetine, chloroquine, diloxamide furoate, paramomycin, 8-hydroxyquinolines and metronidazole. Metronidazole is currently the preferred drug, but it has disadvantages in that it is not well tolerated by some patients, causing side effects such as severe nausea (PHILLIPSON *et al.*, 1987; WRIGHT *et al.*, 1988). Metronidazole is metabolized only in the cytosol of the *Entamoeba* trophozoite stage (FRAYHA *et al.*, 1997). The toxic reduction products exert their killing effects on the parasite cell by reacting and damaging intracellular macromolecules, including DNA (FRAYHA *et al.*, 1997). The enzyme oxidoreductase, which is responsible for the reduction of metronidazole in the anaerobic parasites, does not have a counterpart in the aerobic mammalian cell, thus accounting for the low toxicity of the drug to the host (FRAYHA *et al.*, 1997). Metronidazole has been reported to have mutagenic effects in bacteria, and to cause tumours in experimental animals (PHILLIPSON *et al.*, 1987; WRIGHT *et al.*,

1988). Hence, it appears that no ideal drug exists for the treatment of amoebiasis (PHILLIPSON and O'NEILL, 1987). Additionally, the possibility of the future development of resistant strains, as has been demonstrated by other protozoa, cannot be excluded (WRIGHT *et al.*, 1987).

WRIGHT *et al.* (1988) discovered highly anti-amoebic quassinoids from *Brucea javanica* fruits and *Simarouba amara* stems, which are both used as Chinese traditional remedies against dysentery. A number of naturally occurring alkaloids have amoebicidal activity, including alstonine from *Alstonia scholaris* and *A. macrophylla*, conessine from *Holarrhena antidysenterica*, as well as tubulosine, cryptopleurine and berberine (KEENE *et al.*, 1986). The major modes of action of antiprotozoal drugs include intercalation with DNA (e.g. alstonine and berberine), inhibition of protein synthesis (e.g. emetine and quassinoids), alkylation and oxidant stress (quinones) (PHILLIPSON and WRIGHT, 1991).

A number of natural products which have anticancer activities are also active against protozoa (PHILLIPSON *et al.*, 1995). In addition to *in vitro* amoebicidal assays, PHILLIPSON *et al.* (1995) advised the use of a mammalian cell line *in vitro* for comparison with antiprotozoal activity to establish that compounds are not acting solely as cytotoxic agents. There is a considerable lack of knowledge concerning the effects of total extracts which may act by more than one mechanism and in which different compounds may potentiate or antagonize the activity of antiprotozoal components (PHILLIPSON *et al.*, 1995).

In traditional medicine, a range of plant species is used for the treatment of amoebiasis. Worldwide, species from about 139 plant genera have been reported to be used for the treatment of amoebiasis (FARNSWORTH, 1985, pers. comm. to PHILLIPSON *et al.*, 1987). Useful leads for new anti-amoebic drugs may be obtained by the systematic investigation of plants used in the traditional treatment of amoebiasis (PHILLIPSON *et al.*, 1987; PHILLIPSON and WRIGHT, 1991). It is vital that the efficacy and safety of traditional medicines be validated and their active constituents identified so that reliable quality controls may be established

(PHILLIPSON and O'NEILL, 1987). PHILLIPSON *et al.* (1995) anticipated that research into antiprotozoal natural products from higher plants will lead to novel chemotherapeutic agents and to the selection and standardization of plants used in traditional medicine.

4.1.3 Antiamoebic assays

In vitro test methods have relatively recently been developed with *Entamoeba histolytica* (DIAMOND *et al.*, 1978; NEAL, 1983). KEENE *et al.* (1986) demonstrated that such an *in vitro* test is effective in assessing the activity of plant extracts. A rapid and sensitive procedure for assessing *in vitro* activity of plant extracts against *E. histolytica* using microdilution techniques in microtiter wells has been developed by WRIGHT *et al.* (1988). In this test, metronidazole and emetine were shown to be highly active against *E. histolytica*, while some antibacterial and antifungal drugs were not active. PHILLIPSON *et al.* (1987) stated that sensitive *in vitro* tests for assessing activity against *E. histolytica* are applicable to the bioassay-guided fractionation of plant extracts.

Antiamoebic activity *in vitro* has previously been assessed utilizing techniques such as observing clonal growth of amoebae in semisolid medium to determine viability (GILLIN *et al.*, 1982). Another method incorporated using a haemocytometer to count amoebae grown in flat-sided tissue culture tubes (KEENE *et al.*, 1986). WRIGHT *et al.* (1988) judged these techniques to be time-consuming, producing variable results, and needful of relatively large quantities of test compounds and materials. CEDENO and KROGSTAD in 1983 described a micromethod based on measuring the incorporation of [³H] thymidine into *E. histolytica* grown in microtiter plates. The results with this method correlated well with quantitative parasite counts. A similar test has been developed by WRIGHT *et al.* (1988), but assessment of amoebae present in the microtiter plate wells is made by staining with eosin. The growth of amoebae may alternatively be visualized as turbidity in the wells, and numbers of amoebae may be estimated using a haemocytometer (WRIGHT, pers. comm., 1999). This simple micromethod does not require the use of radiolabeled

compounds or special gas mixtures as in the method of CEDENO and KROGSTAD (1983). The microdilution assay has proved to be highly sensitive and more reproducible than the flat-sided test tube method (PHILLIPSON, 1991). In addition, it is not as expensive or difficult as *in vivo* amoebicidal testing of natural products (PHILLIPSON, 1991).

4.2 MATERIALS AND METHODS

4.2.1 Antiamoebic assay

Ethanol and aqueous extracts of plants used in traditional medicine for treating dysentery were tested for antiamoebic activity against *E. histolytica* using the micromethod of WRIGHT *et al.* (1988). This test was validated with the standard amoebicidal drug metronidazole. Amoebae were cultured at the Medical Research Council (MRC) by Mr S. Suparsad at 35.5°C in liquid Diamond's TYI-S-33 medium (DIAMOND *et al.*, 1978). In the screening procedure, 10 mg extract was placed in a pill vial and allowed to evaporate. Ethanol or water (50 µl) was added to the ethanol and water extracts respectively, followed by culture medium to result in concentrations of 10 mg ml⁻¹. Plant extracts were dissolved or suspended for 5-10 min by sonication. Two-fold serial dilutions were made in wells of 96-well microtitre plates in 170 µl culture medium. Each plate included metronidazole as a standard amoebicidal drug, control wells (culture medium with amoebae), and a blank (culture medium only).

The tubes of amoebal culture were placed in ice-cold water for 5 min to allow detachment of amoebae from the glass culture tube wall. The tubes were centrifuged for 5 min at 1800 rpm to form a pellet. The supernatant was poured from the tube and fresh culture medium was added until approximately 10⁵ amoebae ml⁻¹ were present in each tube. Amoebal suspension (170 µl) was added to each of the control and test wells. Plates were covered with expanded polystyrene (approximately 5 mm thick), partially sealed with tape and gassed for 10 min with

nitrogen before being sealed and incubated at 35.5°C for 72 hours.

After incubation, the growth of amoebae was checked with a microscope. Numbers of amoebae in each well were estimated using a haemocytometer and compared to the metronidazole standard and control wells.

4.3 RESULTS

4.3.1 Standardization of assay

A standard curve (Figure 4.1) was prepared for the amoebicidal compound metronidazole. The IC_{50} value of $0.202 \mu\text{g ml}^{-1}$ (calculated using regression analysis) was close to the values of 0.22 and $0.32 \mu\text{g ml}^{-1}$ obtained in previously published experiments (KEENE *et al.*, 1986; WRIGHT *et al.*, 1988).

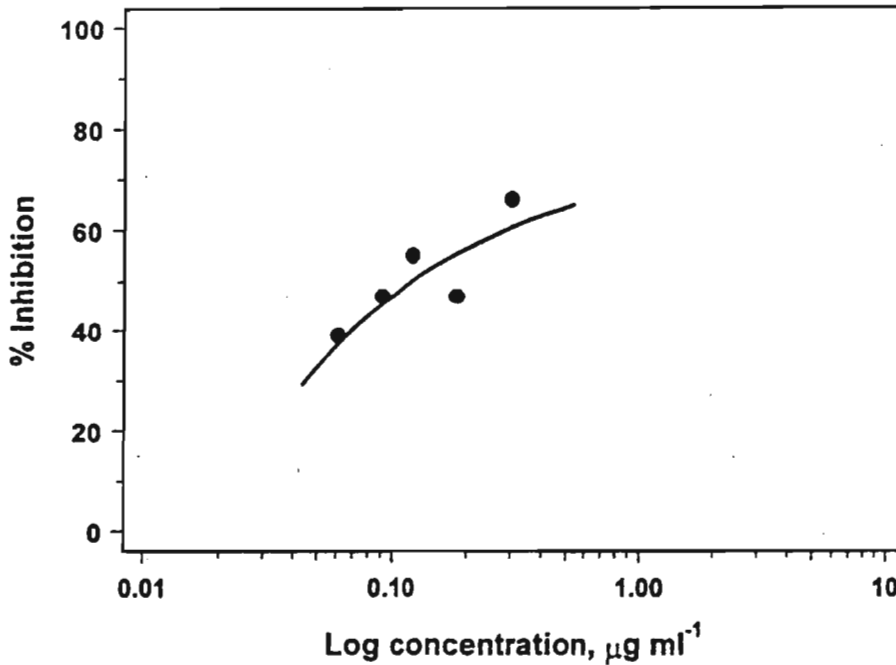


Figure 4.1. Amoebicidal activity of metronidazole

4.3.2 Antiamoebic activity of plant extracts

The activity of plant extracts against *Entamoeba histolytica* is shown in Table 4.1. Forty-two extracts from 21 genera (18 families) were assayed. Plants exhibiting no activity in both the ethanol and water extracts are not displayed in Table 4.1, and were as follows: *Bulbine latifolia*, *Canthium inerme*, *Clusia pulchella*, *Ekebergia capensis*, *Heteromorpha trifoliata*, *Kigelia africana*, *Leonotis leonurus*, *Lippia javanica*, *Senna didymobotrya*, *Spirostachys africana*, *Tecomaria capensis* and *Trema orientalis*. WRIGHT *et al.* (1988) found that the extracts of many plant species were inactive, attributing this to the selectivity of the antiamoebic test. These researchers also reported that most activity was found in methanol extracts, with water extracts of the same plants displaying less activity. This is reflected to an extent in the present study with the plants *Acorus calamus* and *Sclerocarya birrea*, where the ethanol extracts were more active than the water extracts. In total, 8 ethanol extracts showed antiamoebic activity while 5 water extracts were active.

Table 4.1. Antiamoebic activity in South African medicinal plants

| Botanical name | Plant part ¹ | Extract | IC ₅₀ ² (mg ml ⁻¹) |
|---------------------------------|-------------------------|---------|--|
| <i>Acorus calamus</i> | RH | E | 0.3125 |
| | | W | 2.5 |
| <i>Albizia adianthifolia</i> | LF | E | >5.0 |
| | | W | >5.0 |
| <i>Bidens pilosa</i> | LF | E | - |
| | | W | >5.0 |
| <i>Deinbollia oblongifolia</i> | RT | E | >5.0 |
| | | W | >5.0 |
| <i>Pittosporum viridiflorum</i> | BK | E | >5.0 |
| | | W | - |
| <i>Schotia brachypetala</i> | RT | E | >5.0 |
| | | W | - |
| <i>Sclerocarya birrea</i> | BK | E | 1.25 |
| | | W | >5.0 |
| <i>Typha capensis</i> | RH | E | 5.0 |
| | | W | - |
| <i>Ziziphus mucronata</i> | LF | E | >5.0 |
| | | W | - |
| Metronidazole | | | 0.202 x 10 ⁻³ |

¹Plant part: BK = bark, LF = leaf, RH = rhizome, RT = root, TB = tuber, TW = twig, WD = wood

²- = no activity (no effect on amoebae)

4.4 DISCUSSION

4.4.1 Antiamoebic activity of plant extracts

The high activity of *Acorus calamus* may be attributed to the presence of the toxic phenylpropanoid β -asarone (LANDER and SCHREIER, 1990). Other active plants included *Albizia adianthifolia*, *Deinbollia oblongifolia* and *Sclerocarya birrea*. Several compounds have been isolated from the bark and roots of *Albizia adianthifolia* (HUTCHINGS *et al.*, 1996) but little chemical work has been initiated on the leaves. Little chemical investigation has been carried out on *Deinbollia oblongifolia*. The bark of *Sclerocarya birrea* contains tannins and traces of alkaloids (WATT and BREYER-BRANDWIJK, 1962). GALVEZ *et al.* (1991) and GALVEZ *et al.* (1993) reported that procyanidin from the bark inhibits peristaltic reflexes in guinea-pig colon and shows antidiarrhoeal activity on isolated guinea-pig ileum and against various induced diarrhoeas in mice.

4.5 CONCLUSION

Traditional healers in South Africa use a number of plants to treat dysenteric diseases, but it is clearly not possible for the healers to ascertain whether the illness occurs as a result of amoebic or bacterial pathogens. Consequently, lack of antiprotozoal activity by the plant extracts tested in this study does not rule out the possibility that the plant preparation may be efficacious against dysentery. Although amoebic dysentery is not a commonly encountered affliction in South Africa, its occurrence in poverty-stricken rural areas warrants the investigation of plants used by indigenous healers. The discovery of novel antiprotozoal compounds with possibly new modes of action from plants would provide impetus for the development of new antiprotozoal drugs effective against resistant pathogens.

CHAPTER 5

ANTIBACTERIAL SCREENING

5.1 INTRODUCTION

5.1.1 Infectious diarrhoea and dysentery

Diarrhoeal disease ranges from a mild and inconvenient illness to a major cause of malnutrition, particularly among children in developing countries. In some cases diarrhoea protects the body from harmful substances in the intestine by hastening their removal. The most common causes of diarrhoea are viral infection, food poisoning and parasite infestation, but diarrhoea also occurs in other illnesses, as a side effect of some drugs for example.

Diarrhoeal diseases are a leading cause of morbidity and mortality in developing countries (MECKES *et al.*, 1997). Gastroenteritis is an inflammation of the stomach and intestines, characterized by abdominal distress, nausea, vomiting and diarrhoea (LEWIS and ELVIN-LEWIS, 1977). The cause of the disease may be viral, but outbreaks produced by bacteria are either intoxications resulting from ingesting food contaminated with toxic bacterial products or infections resulting from the ingestion of viable bacteria (LEWIS and ELVIN-LEWIS, 1977). Among the latter are the enteropathogenic strains of *Escherichia coli*, *Vibrio parahaemolyticus*, *V. cholerae*, *Salmonella* species (particularly *S. typhimurium*) and *Shigella* species, which cause bacillary dysentery (LEWIS and ELVIN-LEWIS, 1977). TORRES *et al.* (1995) cited by MECKES *et al.* (1997) stated that most cases of bloody diarrhoea are owing to bacterial enteropathogens such as *Shigella* spp. (the most virulent), *Salmonella* spp. and *Vibrio parahaemolyticus*, whereas *E. coli* and *V. cholera* generally cause watery diarrhoea. *Shigella* spp. cause severe dysentery in children (MECKES *et al.*, 1997). All of these bacteria are Gram-negative species.

Diarrhoea and dysentery epidemics are common where living conditions are crowded and hygiene is poor (OTSHUDI *et al.*, 2000). Dysentery, a microbial infection of the gastrointestinal tract, has symptoms of fever, vomiting, abdominal pain and diarrhoea which often contains blood and pus (OTSHUDI *et al.*, 2000). The onset of the disease usually occurs within 2-3 days after infection and lasts for up to several weeks. Dehydration occurs rapidly, especially in children and may cause death if treatment is not given (OTSHUDI *et al.*, 2000). As mentioned in CHAPTER 1, parasitic diseases such as dysentery and diarrhoea are the main causes of the high mortality rate in developing countries, where over five million children under the age of five die annually from severe diarrhoeal diseases (WHO, 1996).

Food poisoning may be caused by *Clostridium perfringens* and its toxins, *Bacillus cereus*, species of *Proteus*, *Klebsiella*, *Providencia* (*Paracolon*), *Citrobacter*, *Pseudomonas*, *Enterobacter*, and *Actinomyces* (LEWIS and ELVIN-LEWIS, 1977). Overgrowth of organisms such as *Staphylococcus aureus*, *Candida albicans*, *Streptococcus faecalis*, *Pseudomonas aeruginosa* and *Proteus mirabilis* may occur when there is suppression of gut flora owing to antibiotic therapy, resulting in diarrhoea among other symptoms (LEWIS and ELVIN-LEWIS, 1977).

5.1.2 Control of enteropathogens causing infectious diarrhoea and dysentery

A mechanism to counteract infectious diarrhoea is by inhibiting the growth of the enteropathogen causing the disease (MECKES *et al.*, 1997). This is usually done by prescribing antibiotic therapy. However, MECKES *et al.* (1997) warned that enteropathogens have a high level of resistance to commonly used antibiotics and resistance to new drugs emerges very fast. Bacteria have become resistant to antimicrobial agents as a result of chromosomal changes or the exchange of genetic material via plasmids and transposons (NEU, 1992). Fuelling this crisis is the extensive and often inappropriate use of antibiotics in the community and hospitals, leading to increased selective pressure on microorganisms (NEU, 1992). The widespread use of antibiotics in animal feed may explain the high antibiotic

resistance of *Salmonella* species (NEU, 1992). Strategies employed to reduce the emergence of resistant bacteria in animals should include the prohibition of the use for growth promotion in animals of antimicrobials approved for human therapy (WHO, 1996). *Streptococcus* species and staphylococci, organisms that cause respiratory and cutaneous infections, as well as members of the Enterobacteriaceae and *Pseudomonas* families, organisms that cause diarrhoea, urinary infection and sepsis, are now resistant to virtually all of the older antibiotics (NEU, 1992). NEU (1992) recommended the adoption of mechanisms such as antibiotic control programs, better hygiene, and synthesis of agents with improved antimicrobial activity in order to limit bacterial resistance. The need for new antibiotics will continue because bacteria have a remarkable ability to overcome each new agent synthesized (NEU, 1992). The search for new drugs with activity against enteropathogens is a priority. The antimicrobial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in treatment of resistant microbial strains (ELOFF, 1999a).

5.1.3 Antibacterial assays

Antimicrobial activity of medicinal plants has been evaluated previously using various methods, which are classified into three groups, i.e. the disc-diffusion, dilution and bioautographic assays. According to RIOS *et al.* (1988) many factors can influence the results, including the extraction method, inocula volume, culture medium composition, pH and incubation temperature. In addition, there is no standardized method for expressing the results of antimicrobial screening. The diffusion and dilution methods are also used to determine the degree of susceptibility of a test microorganism to a given antimicrobial agent (susceptibility tests), while bioautographic methods are mainly applied to bioassay screening and bioassay-guided fractionation (RASOANAIVO and RATSIMAMANGA-URVERG, 1993).

The diffusion method enables several plant extracts to be screened against various microorganisms at the same time, and is therefore suitable for preliminary bioassay

scanning (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). The dilution methods are used to determine more precisely the antibacterial activity of extracts, giving the MIC values of test extracts for a given microorganism (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). Bioautographic methods allow a rapid identification of bioactive constituents of plant extracts (RASOANAIVO and RATSIMAMANGA-URVERG, 1993) and are valuable in the bioassay-guided fractionation of active extracts.

5.1.3.1 Diffusion methods

Diffusion methods make use of discs, holes or cylinders containing samples to be tested, which are brought into contact with an inoculated medium. After incubation, the diameter of the clear zone around the reservoir (inhibition diameter) is measured. In the disc method, or disc-diffusion assay, a concentration of different plant extracts is applied to filter paper discs, which are placed onto the surface of inoculated agar medium in Petri dishes. Discs impregnated with plant extracts exhibiting antibacterial activity will be surrounded with a clear zone of inhibition after incubation. Inhibition diameters higher than 8 mm are generally considered as positive results (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). In the hole-plate assay method, holes are punched in inoculated agar in a Petri dish, plant extract or antibiotic is placed in the holes, and the zone of inhibited growth of the test organism is measured (RIOS *et al.*, 1988). The cylinder method is similar to the hole plate assay. Antibiotic or plant extract is applied to stainless steel or porcelain cylinders, placed on inoculated agar, and the resulting zones of inhibition measured. The inhibition diameters produced by the plant extracts in the diffusion assays may be compared to those obtained by antibiotic controls. Diffusion assays are useful in the preliminary screening for antimicrobial activity of plant extracts. It is not possible to relate MIC values with inhibition diameters as there is no relationship between the two (RIOS *et al.*, 1988).

5.1.3.2 Dilution methods

Dilution assays are those which require a homogeneous dispersion of the sample in water (RIOS *et al.*, 1988). They are mainly used to determine the Minimum Inhibitory Concentration (MIC) values of an extract or pure compound. These values are taken as the lowest concentration of the extract that completely inhibits bacterial growth after incubation for 24 h.

In the liquid dilution method, turbidity is taken as an indication of bacterial growth, so where the sample is inactive against the microorganism tested, the liquid will appear turbid (RIOS *et al.*, 1988). The grade of inhibition is related to the turbidity of the medium, and may be measured by spectrophotometry (RIOS *et al.*, 1988). With the agar dilution method, a fixed amount of an antibiotic- or extract-containing mixture is mixed with nutrient agar and allowed to set (RIOS *et al.*, 1988). The advantages of this method are its simplicity and speed, and the possibility of using it in the antimicrobial study of water-soluble or insoluble samples such as essential oils (RIOS *et al.*, 1988). Up to six microorganisms can be seeded in each Petri dish, and antimicrobial activity is indicated when the bacteria do not grow (RIOS *et al.*, 1988).

ELOFF (1998a) developed a microdilution technique using 96-well microtitre plates. A two-fold serial dilution of the extract, pure compound or antibiotic is prepared in the wells of the microplate, and bacterial culture added. After incubation, *p*-iodonitrotetrazolium violet is added, and in the wells where bacterial growth occurs, a deep red colour develops. Wells containing antibacterial compounds remain clear. This scaled-down technique requires only a small amount of test compound, and is quick, sensitive and reproducible. It is useful for screening plants for antimicrobial activity, for the bioassay-guided fractionation of extracts, and for the MIC determination of extracts or pure compounds (ELOFF, 1998a).

5.1.3.3 Bioautographic methods

Bioautography is an important detection method for new or unidentified antimicrobial

compounds (RIOS *et al.*, 1988). In the agar-overlay bioautographic assay, a plant extract is separated into individual compounds on a TLC plate. A molten agar overlay containing a suspension of bacteria and a tetrazolium salt such as 2,3,5-triphenyltetrazolium chloride (TZC) is applied to the TLC plate. The separated compounds on the TLC plate diffuse into the agar and, if they possess antibacterial activity, will inhibit the growth of bacteria. Bacteria reduce the tetrazolium salt through dehydrogenase activity to the corresponding intensely coloured formazan (HAMBURGER and CORDELL, 1987; SLUSARENKO *et al.*, 1989), so where bacterial growth is inhibited, a colourless zone appears against a dark pink background when TZC is used as an indicator. In the direct bioautography assay, a suspension of microorganisms in liquid medium is sprayed on a developed TLC plate and incubated overnight. A solution of tetrazolium salt is then sprayed on the plate and incubated to detect the areas of bacterial growth inhibition. With this method, there are no problems associated with differential diffusion of compounds through the agar (HAMBURGER and CORDELL, 1987). An advantage of bioautography is that it allows the localization of activity, even in complex mixtures (HAMBURGER and CORDELL, 1987).

5.2 MATERIALS AND METHODS

5.2.1 Quantification of bacteria

The test organisms in the investigations of antibacterial activity, *Bacillus subtilis*, *Escherichia coli*, *Klebsiella pneumoniae* and *Staphylococcus aureus*, were obtained from the bacterial collection of the Department of Microbiology, University of Natal, Pietermaritzburg. The number of cells in Mueller-Hinton (MH) broth cultures of each bacterial species was estimated using a serial dilution technique (LECH and BRENT, 1987). Ten-fold serial dilutions of overnight MH broth cultures were prepared, and 100 μ l of each dilution were spread onto MH agar plates using a glass spreader. The plates were incubated overnight at 37°C and colonies were counted using an Anderman Colony Counter. Following the assumption that each

living bacterial cell will grow into a separate colony on the plate, the number of cells present per ml of the original overnight cultures was calculated. The Optical Density (OD) at 600 nm (OD_{600}) for each dilution was determined using a Varian Cary 50 Spectrophotometer, and used to indicate numbers of bacterial cells in cultures for the antibacterial screening and MIC determination.

5.2.2 Disc-diffusion assay

The disc-diffusion assay (RASOANAIVO and RATSIMAMANGA-URVERG, 1993) was used in the antibacterial screening procedure. Plants used for treating diarrhoea, dysentery and unspecified stomach ailments were tested for antibacterial activity. Residues of plant extracts were resuspended in their extracting solvents to a concentration of 100 mg ml^{-1} . MH agar base plates were prepared using sterile 90 mm Petri dishes. MH agar at 48°C was inoculated with a MH broth culture ($10^6 - 10^8$ bacteria ml^{-1}) of each bacterial species and poured over the base plates to form a homogenous layer. Filter paper discs (Whatman No. 3, 6 mm diameter) were sterilized by autoclaving. One mg of plant residue ($10 \mu\text{l}$ of 100 mg ml^{-1} suspension) was applied to each filter paper disc and allowed to air-dry. The dry discs were placed on the seeded MH agar plates; each extract was tested in quadruplicate, with 4 discs of one extract per plate. Each plate also contained a reference antibiotic, neomycin ($500 \mu\text{g ml}^{-1}$), as a positive control. Air-dried solvent (hexane, ethanol and water) saturated discs were used as negative controls. The plates were incubated at 37°C overnight, after which the zones of inhibition, if present, around each disc were measured. The ratio of the inhibition zone (mm) produced by the plant extract and the inhibition zone around the neomycin reference (mm) was used to express antibacterial activity (VLIETINCK *et al.*, 1995).

5.2.3 Microdilution assay

The microplate method of ELOFF (1998a) was used with slight modifications to determine the Minimal Inhibitory Concentration (MIC) values for plant extracts with antibacterial activity. Residues of plant extracts active in the disc-diffusion assay

were made up to 50 mg ml⁻¹ with the extracting solvent in the case of ethanol and water. Hexane extracts were resuspended in acetone to a concentration of 50 mg ml⁻¹. The plant extracts (100 µl) were serially diluted 50 % with water in 96-well microtitre plates. Overnight MH broth cultures (grown at 37°C) of the test bacteria were diluted 1:100 (overnight bacterial culture:MH broth), and 100 µl of the resulting culture were added to each well. Neomycin was used as the reference antibiotic and controls with no extract and solvent only, or MH broth only were used. The covered microplates were incubated overnight at 37°C. As an indicator of bacterial growth, 40 µl of *p*-iodonitrotetrazolium violet (INT) dissolved in water were added to the microplate wells and incubated at 37°C for 30 min. MIC values were recorded as the lowest concentration of extract that completely inhibited bacterial growth, i.e. a clear well. The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active organisms (ELOFF, 1998a). Where bacterial growth is inhibited, the solution in the well will remain clear after incubation with INT.

In an effort to determine the effect of culture age on MIC determination, ELOFF (1998a) showed that *S. aureus* culture age from 1 to 6 h, and storing cultures up to 10 days in a cold room, had little or no effect on the MIC values. This contrasts with larger volume serial dilution assays where the number of cells inoculated has an effect on the MIC (HEWITT and VINCENT, 1989; cited by ELOFF, 1998a). ELOFF (1998a) suggested that the 50 % inoculum size used in the microdilution assay compared to the approximate 50 times lower inoculum used in the standard tube MIC method (ELOFF, 1998b) may explain the difference.

5.3 RESULTS

5.3.1 Quantification of bacteria

The OD₆₀₀ of overnight cultures was compared with the estimated number of bacteria in the overnight cultures of each bacterial species (Table 5.1). This supplied an indication of the inoculum of bacteria used in the antibacterial assays, as the Optical Density of each culture was confirmed before each assay to ensure that differences in inoculum size did not become a factor in the MIC determination.

Table 5.1. Correlation of bacterial numbers and optical density readings of overnight cultures

| Bacteria | OD ₆₀₀ | Number of bacteria |
|----------------------|-------------------|-----------------------|
| <i>B. subtilis</i> | 0.71 | 1.1 x 10 ⁸ |
| <i>E. coli</i> | 1.13 | 3.5 x 10 ⁹ |
| <i>K. pneumoniae</i> | 1.28 | 6.4 x 10 ⁹ |
| <i>S. aureus</i> | 1.63 | 5.7 x 10 ⁸ |

5.3.2 Antibacterial activity of plant extracts

The antibacterial activity of crude extracts showing positive results (hexane, ethanol and water) is presented in Table 5.2. In total, 138 extracts from 45 genera (31 families) were tested. Plants exhibiting no antibacterial activity were *Albizia adianthifolia*, *Aloe arborescens*, *Aloe marlothii*, *Buddleja salviifolia*, *Bulbine latifolia*, *Canthium inerme*, *Ceratotheca triloba*, *Cinnamomum camphora*, *Clutia pulchella*, *Croton sylvaticus*, *Deinbollia oblongifolia*, *Kigelia africana*, *Lippia javanica*, *Melia azedarach*, *Mondia whitei*, *Ocotea bullata*, *Olea europaea*, *Ricinus communis*, *Stangeria eriopus*, *Strychnos spinosa*, *Tarchonanthus camphoratus*, *Tetradenia riparia*, *Tulbaghia violacea* and *Zanthoxylum capense*.

Table 5.2. Determination of the antibacterial activity of South African medicinal plants with the disc-diffusion and microdilution assays (MIC recorded in mg ml⁻¹)

| Botanical name | Plant part ¹ | Extract ² | Bacteria tested ³ | | | | | | | |
|---------------------------------|-------------------------|----------------------|------------------------------|------------------|------|------|-------|------|------|-------|
| | | | B.s. | | E.c. | | K.p. | | S.a. | |
| | | | Dif ⁴ | MIC ⁵ | Dif | MIC | Dif | MIC | Dif | MIC |
| <i>Acorus calamus</i> | RH | H | 0.29 | 0.39 | 0 | - | 0 | - | 0 | - |
| | | E | 0.33 | 0.78 | 0 | - | 0 | - | 0 | - |
| | | W | 0 | - | 0 | - | 0 | - | 0 | - |
| <i>Artemisia afra</i> | LF | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0.2 | 0.39 | 0 | - | 0 | - | 0.33 | 0.39 |
| | | W | 0 | - | 0 | - | 0 | - | 0 | - |
| <i>Asclepias fruticosa</i> | LF | H | 0 | - | 0.22 | 6.25 | 0 | - | 0 | - |
| | | E | 0 | - | 0 | - | 0 | - | 0 | - |
| | | W | 0 | - | 0 | - | 0 | - | 0 | - |
| <i>Cassine transvaalensis</i> | BK | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0.23 | 0.195 | 0 | - | 0 | - | 0.58 | 0.098 |
| | | W | 0.18 | 0.78 | 0 | - | 0 | - | 0.33 | 0.195 |
| <i>Catha edulis</i> | RT | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0.21 | 0.012 | 0 | - | 0 | - | 0.50 | 0.012 |
| | | W | 0 | - | 0 | - | 0 | - | 0 | - |
| <i>Combretum apiculatum</i> | LF | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0.17 | 0.049 | 0 | - | 0 | - | 0.29 | 0.049 |
| | | W | 0.15 | 0.39 | 0 | - | 0 | - | 0.43 | 0.39 |
| <i>Curtisia dentata</i> | BK | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0.28 | 0.78 | 0 | - | 0 | - | 0 | - |
| | | W | 0.13 | 3.13 | 0 | - | 0 | - | 0 | - |
| <i>Cussonia spicata</i> | LF | H | 0.14 | 12.5 | 0.29 | 6.25 | 0 | - | 0.14 | 12.5 |
| | | E | 0.29 | 3.13 | 0.29 | 3.13 | 0.125 | 12.5 | 0 | - |
| | | W | 0 | - | 0 | - | 0 | - | 0 | - |
| <i>Dombeya rotundifolia</i> | LF | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0.40 | 1.56 | 0 | - | 0 | - | 0.33 | 1.56 |
| | | W | 0.40 | 1.56 | 0 | - | 0 | - | 0.36 | 1.56 |
| <i>Erythrophleum lasianthum</i> | LF | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0.17 | 0.78 | 0 | - | 0 | - | 0 | - |
| | | W | 0 | - | 0 | - | 0 | - | 0 | - |
| <i>Gunnera perpensa</i> | RT/RH | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0 | - | 0 | - | 0 | - | 0.23 | 3.13 |

Table 5.2 continued...

| Botanical name | Plant part ¹ | Extract ² | Bacteria tested ³ | | | | | | | |
|----------------------------------|-------------------------|----------------------|------------------------------|------------------|------|------|------|------|------|-------|
| | | | B.s. | | E.c. | | K.p. | | S.a. | |
| | | | Dif ⁴ | MIC ⁵ | Dif | MIC | Dif | MIC | Dif | MIC |
| | | W | 0 | - | 0 | - | 0 | - | 0.23 | 0.78 |
| <i>Harpephyllum caffrum</i> | BK | H | 0.33 | 0.78 | 0 | - | 0 | - | 0 | - |
| | | E | 0.50 | 0.098 | 0.17 | 1.56 | 0.17 | 3.13 | 0.17 | 3.13 |
| | | W | 0.25 | 1.56 | 0 | - | 0 | - | 0 | - |
| <i>Heteromorpha trifoliata</i> | LF | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0.25 | 0.78 | 0 | - | 0 | - | 0 | - |
| | | W | 0 | - | 0 | - | 0 | - | 0 | - |
| <i>Pittosporum viridiflorum</i> | BK | H | 0.17 | 3.13 | 0 | - | 0 | - | 0 | - |
| | | E | 0.50 | 1.56 | 0 | - | 0 | - | 0.29 | 1.56 |
| | | W | 0 | - | 0 | - | 0 | - | 0 | - |
| <i>Psychotria capensis</i> | RT | H | 0 | - | 0 | - | 0 | - | 0.43 | 12.5 |
| | | E | 0 | - | 0 | - | 0 | - | 0.71 | 0.39 |
| | | W | 0 | - | 0 | - | 0 | - | 0.43 | 3.13 |
| <i>Rauvolfia caffra</i> | LF | H | 0 | - | 0 | - | 0 | - | 0.13 | 0.78 |
| | | E | 0 | - | 0 | - | 0 | - | 0.13 | 1.56 |
| | | W | 0 | - | 0 | - | 0 | - | 0 | - |
| <i>Sansevieria hyacinthoides</i> | LF | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0 | - | 0 | - | 0 | - | 0 | - |
| | | W | 0.42 | 6.25 | 0 | - | 0 | - | 0 | - |
| <i>Schotia brachypetala</i> | LF | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0.29 | 0.024 | 0 | - | 0 | - | 0.29 | 0.195 |
| | | W | 0.25 | 1.56 | 0 | - | 0 | - | 0.14 | 6.25 |
| <i>Sclerocarya birrea</i> | BK | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0.33 | 0.012 | 0 | - | 0 | - | 0.29 | 0.049 |
| | | W | 0.33 | 0.78 | 0 | - | 0 | - | 0.43 | 0.78 |
| <i>Spirostachys africana</i> | RT/ST | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0 | - | 0 | - | 0 | - | 0.36 | 3.13 |
| | | W | 0 | - | 0 | - | 0 | - | 0 | - |
| <i>Tecomaria capensis</i> | BK | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0 | - | 0 | - | 0 | - | 0.17 | 3.13 |
| | | W | 0 | - | 0 | - | 0 | - | 0 | - |
| <i>Typha capensis</i> | RH | H | 0 | - | 0 | - | 0 | - | 0 | - |
| | | E | 0.17 | 3.13 | 0 | - | 0 | - | 0 | - |

Table 5.2 continued...

| Botanical name | Plant part ¹ | Extract ² | Bacteria tested ³ | | | | | | | |
|----------------|-------------------------|----------------------|------------------------------|------------------|------------------------|-----|------|------------------------|------|------------------------|
| | | | B.s. | | E.c. | | K.p. | | S.a. | |
| | | | Dif ⁴ | MIC ⁵ | Dif | MIC | Dif | MIC | Dif | MIC |
| | | W | 0 | - | 0 | - | 0 | - | 0 | - |
| Neomycin | | | 2.0 x 10 ⁻⁴ | | 1.3 x 10 ⁻² | | | 7.8 x 10 ⁻⁴ | | 3.9 x 10 ⁻⁴ |

¹Plant part: BK = bark, LF = leaf, RH = rhizome, RT = root, ST = stem, TB = tuber, WH = whole plant

²Extract: H = hexane, E = ethanol, W = water

³Bacteria: B.s. = *Bacillus subtilis*, E.c. = *Escherichia coli*, K.p. = *Klebsiella pneumoniae*, S.a. = *Staphylococcus aureus*

⁴Dif: Results obtained in the disc-diffusion assays. Antibacterial activity is expressed as the ratio of the inhibition diameter around the extract to the inhibition zone around the reference neomycin antibiotic. The symbol 0 indicates no activity, i.e. no inhibition zone around the extract discs

⁵MIC: Results obtained in the dilution assays. Antibacterial activity is expressed as the Minimum Inhibitory Concentration (MIC) in mg ml⁻¹. The symbol - is used where the MIC was not determined

Gram-positive bacteria (*B. subtilis* and *S. aureus*) were significantly more susceptible to the extracts than the Gram-negative bacteria (*E. coli* and *K. pneumoniae*). This is to be expected as Gram-negative bacteria are generally more resistant to antibiotic agents compared with Gram-positive bacteria (VLIETINCK *et al.*, 1995). Only four extracts showed activity against *E. coli*, while two extracts were active against *K. pneumoniae*. Concerning the Gram-positive bacteria, 27 extracts were active against *B. subtilis* and 24 against *S. aureus*. The ethanolic extracts displayed the most antibacterial activity, with 20 active extracts. Ten aqueous extracts showed activity while only seven hexane extracts were active.

5.4 DISCUSSION

5.4.1 Antibacterial activity of plant extracts

The activity of *Acorus calamus* may be attributed to the presence of the toxic compound β -asarone in this plant (LANDER and SCHREIER, 1990). *Cassine transvaalensis* bark contains tannins, which are sometimes used for their antidiarrhoeal properties (BRUNETON, 1995). No antibacterial compounds have been reported from *Catha edulis*. MARTINI and ELOFF (1998) isolated several unidentified antibacterial compounds from *Combretum erythrophyllum*. BREYTENBACH and MALAN (1989) isolated three antibacterial compounds from *Combretum zeyheri*. These researchers ascribed the ethnopharmacological use of this plant against diarrhoea to its antibacterial properties, particularly towards Gram-positive species. Antimicrobial components have been found in several other *Combretum* species (ALEXANDER *et al.*, 1992; ELOFF, 1999b). *Harpephyllum caffrum* is reported to contain phenolic compounds which may be responsible for its biological activity (EL SHERBEINY and EL ANSARI, 1976).

Antimicrobial activity has been detected in stembark aqueous extracts of *Kigelia africana* (synonym *K. pinnata*) (AKUNYILI *et al.*, 1991). This activity has been partially attributed to the presence of iridoids present in the bark (AKUNYILI *et al.*, 1991). Ethanol and ethyl acetate extracts of the fruit and bark of *K. africana* displayed antibacterial activity against Gram-positive and Gram-negative extracts, but aqueous extracts had no activity (GRACE *et al.*, 2001). In the present study no activity was found in leaf extracts of the same species. *Tetradenia riparia* leaves have been reported to inhibit several mycobacteria (VAN PUYVELDE *et al.*, 1986). A diterpene diol from the same species exhibited significant antimicrobial activity against several bacteria and fungi (VAN PUYVELDE *et al.*, 1986). No activity was displayed by leaf extracts of *T. riparia* in this investigation.

The chemical compounds of *Schotia* species have not been well investigated. Polyhydroxystilbenes have been isolated from the heartwood of *S. brachypetala*

(DREWES, 1971; DREWES and FLETCHER, 1974). Many stilbenes have antibiotic properties but the biological activity of the *Schotia* stilbenes is not known (VAN WYK *et al.*, 1997). Astringent tannins are present in the bark (BRUNETON, 1995), and activity may be partly attributed to these compounds. In preliminary studies, the antidiarrhoeal effects of *Sclerocarya birrea* bark have been linked to procyanidins (GALVEZ *et al.*, 1993). Extracts from stem bark have shown antibacterial activity against several species of bacteria (HUSSEIN and DEENI, 1991).

Polyphenols including tannins, which are known to have antimicrobial activity, are soluble in water, and these may explain the activity of some of the water extracts (IEVEN *et al.*, 1979). Tannins, which have the ability to precipitate proteins from solution (a property known as astringency), are commonly found in a large array of higher plant species, and may accumulate in large amounts in almost any plant part (SCALBERT, 1991). Tannins may deter herbivores from predation, and they may also increase resistance against microbial pathogens (SCALBERT, 1991). Increasing attention is being paid to the use of tannins as antimicrobial agents, as the toxicity of tannins towards microorganisms is well documented (SCALBERT, 1991). The astringent character of tannins may induce complexation with enzymes or substrates, and tannins may also directly affect the metabolism of microorganisms, in addition, possibly acting on membranes (SCALBERT, 1991). NISHIZAWA (1990) isolated from *Nuphar variegatum* gallotannins and elagitannins with antibacterial activity against *Staphylococcus aureus* and *Proteus vulgare*.

Amongst the plants tested, *Cassine transvaalensis*, *Catha edulis*, *Combretum apiculatum*, *Harpephyllum caffrum*, *Heteromorpha trifoliata*, *Schotia brachypetala* and *Sclerocarya birrea* showed the best activities with MICs $< 200 \mu\text{g ml}^{-1}$ and could provide useful leads for the discovery of antibacterial compounds. The only species displaying activity against Gram-negative bacteria were *Cussonia spicata*, *Harpephyllum caffrum* and *Asclepias fruticosa*. Emphasis should be placed on the discovery of new antimicrobials with activity against Gram-negative bacteria, as many species from this group are pathogenic, and there is a serious lack of antibiotics effective against them.

5.5 CONCLUSION

The evaluation of plants used by traditional healers to treat diarrhoeal disease is a necessity in South Africa. Diarrhoea is a common affliction, especially in settlements in rural areas that depend upon rivers as a source of water. Members of poor rural communities may not have ready access to expensive antibiotics to treat stomach ailments, particularly if health clinics are far away. Traditional healers are relied upon to provide treatment, and the efficacy of the plant remedies given to dispel pathogens causing diarrhoea and bacterial dysentery necessitates investigation. Scientific exploration of these plants may lead to the isolation of novel compounds with different mechanisms of action to those presently in use. With the compelling issue of emerging drug-resistant bacterial pathogens, Western medicine is searching for new and effective antimicrobial substances to combat this world-wide threat.

In this study, a number of plants exhibited promising activity against a variety of bacteria. These *in vitro* experiments are a step towards validating the use of these plants by indigenous healers in South Africa. Most extracts showed activity against Gram-positive bacteria, which are implicated in causing various stomach ailments. However, Gram-negative enteropathogenic bacteria pose a greater threat by causing more serious, life-threatening diarrhoeal and dysenteric diseases, warranting further research into the discovery of plant extracts active against these bacteria.

CHAPTER 6

ANTHELMINTIC ACTIVITY IN THE FAMILY COMBRETACEAE

6.1 INTRODUCTION

6.1.1 The family Combretaceae

The Combretaceae consists of 18 genera. The largest genus is *Combretum*, with about 370 species, and *Terminalia* is the second largest, with about 200 species (LAWRENCE, 1951). The other genera are much smaller, including *Calopyxes* and *Buchenavia* with 22 species each, *Quisqualis* with 16, *Anogeissis* with 14, *Conocarpis* with 12 and *Pteleopsis* with 10 species (ROGERS and VEROTTA, 1996). Species from the genus *Combretum* and to a lesser extent *Terminalia* are most widely used for medicinal purposes. These genera are widespread in parts of Africa where they are often the most abundant species (ROGERS and VEROTTA, 1996). They are easily characterised by the wing-shaped appendages of the fruits, and may be trees, shrubs or climbers (ROGERS and VEROTTA, 1996).

6.1.2 Traditional medicinal usage

Indigenous healers throughout southern Africa employ species of the Combretaceae for many medicinal purposes. These include the treatment of abdominal pains, backache, bilharzia, chest coughs, colds, conjunctivitis, diarrhoea, dysmenorrhoea, earache, fever, headache, hookworm, infertility in women, leprosy, pneumonia, scorpion and snake bite, swelling caused by mumps, syphilis, toothache and general weakness (WATT and BREYER-BRANDWIJK, 1962; KOKWARO, 1976; GELFAND *et al.*, 1985; HUTCHINGS *et al.*, 1996; VAN WYK *et al.*, 1997). The leaves and bark

of *Combretum* species are predominantly used. Fruits do not feature in medicine owing to their reported toxicity to humans (ROGERS and VEROTTA, 1996).

6.1.3 Biological activity and chemical constituents

Several investigations into the antimicrobial activity of members of the Combretaceae have been undertaken in recent years. BREYTENBACH and MALAN (1989) found that most antibacterial activity resided in the leaves, twigs and fruits with the latter being the most active. For reasons of availability, further investigation was done on leaf material. These researchers isolated three unidentified antibacterial compounds from leaves of *Combretum zeyheri*. ALEXANDER *et al.* (1992) reported six species of *Combretum* with antimicrobial activity. In 1998, MARTINI and ELOFF demonstrated the presence of at least 14 different unidentified bacterial inhibitors, of widely differing polarity, in leaves of *C. erythrophyllum*. ELOFF (1999b) examined other members of the Combretaceae, namely 27 species of *Combretum*, *Terminalia*, *Pteleopsis* and *Quisqualis*. Acetone leaf extracts of all the species inhibited the growth of the test bacteria to varying degrees. BABA-MOUSSA *et al.* (1999) investigated seven species of the West African Combretaceae for antifungal activity, reporting that all species displayed a degree of activity. These researchers suggested that the tannins and saponins, detected in the phytochemical screening of the plant extracts, may be responsible for the antifungal activity.

The chemistry of *Combretum* species has been studied by a number of researchers. Substituted phenanthrenes, dihydrophenanthrenes and bibenzyls have been isolated from *C. apiculatum* (LETCHER and NHAMO, 1971), *C. molle* (LETCHER *et al.*, 1972) and *C. hereroense* (LETCHER and NHAMO, 1973). CARR and ROGERS (1987) demonstrated the presence of triterpenoids and flavonoids in leaf extracts of several *Combretum* species. Several stilbenes and dihydrostilbenes (the combretastatins) with cytotoxic activity, as well as acidic triterpenoids and their glycosides with molluscicidal, antifungal, antimicrobial and anti-inflammatory activity have also been isolated from species of *Combretum* (PETTIT *et al.*, 1988; ROGERS, 1989a; ROGERS, 1989b).

The widespread use of *Combretum* species in indigenous medicine for many different ailments, as well as the significant antimicrobial and other activity exhibited by several species, justifies the further investigation on other possible biological activities, such as anthelmintic activity, of this group of plants.

6.2 MATERIALS AND METHODS

6.2.1 Plant extract preparation

Leaf material of various Combretaceae species, predominantly that of *Combretum*, was supplied by Professor J. N. Eloff of the Department of Pharmacology, University of Pretoria, P.O. Box 2034, Pretoria 0001, South Africa. This material was collected in late summer (February) from plants growing in the Lowveld National Botanical Garden in Nelspruit. A voucher specimen from each tree is present in the herbarium of this Botanical Garden. The collection numbers and year in which each specimen was collected are shown in Table 6.1.

Extracts of powdered leaf material were prepared using the solvents ethyl acetate, acetone, methanol and water. For each species, separate samples of 0.5 g were extracted in 5 ml of each solvent, sonicated for 30 min and filtered through Whatman No. 1 filter paper. The plant material was re-extracted twice more, and the filtrate was air-dried at room temperature.

6.2.2 Screening of plant extracts for anthelmintic activity

Anthelmintic activity of the ethyl acetate, acetone, methanol and water extracts was determined against the free-living nematode *Caenorhabditis elegans* var. Bristol (N2) as described in CHAPTER 3 (Section 3.2.2), with slight modifications. Plant extracts at concentrations of 1 and 0.5 mg ml⁻¹ plant extract were tested in the 2 h mortality assay. Nematodes from 6 to 7-day old cultures were incubated with 0.5 and 1 mg ml⁻¹ plant extract for 2 h at 25°C in the dark. The standard nematocidal

drug levamisole was used as a positive control in all experiments. A second control consisted of nematodes incubated with no plant extract or levamisole. Solvent blanks were included. Using a dissecting microscope, the percentage of living worms was estimated, and their movement recorded and compared to the controls.

6.3 RESULTS

6.3.1 Anthelmintic activity of Combretaceae extracts

The results of the anthelmintic screening of Combretaceae leaf extracts are presented in Table 6.1. Several extracts out of 88 water, acetone, methanol and ethyl acetate extracts had an effect on the mortality and movement of nematodes. At 0.5 mg ml⁻¹, one water, one ethyl acetate and nine acetone extracts were active, while at 1 mg ml⁻¹, one water, five ethyl acetate and 14 acetone extracts exhibited a nematocidal effect. The only methanol extract to display activity was that of *Combretum apiculatum*, which was active at both concentrations tested. *C. apiculatum* extracts had by far the most detrimental effect on the nematodes, with water, acetone, methanol and ethyl acetate extracts possessing varying degrees of anthelmintic activity. *C. hereroense* and *C. mossambicense* showed activity in both the acetone and ethyl acetate extracts. The only species apart from *Combretum* to show activity was *Quisqualis littorea*, the ethyl acetate extract of which was mildly nematocidal.

Table 6.1. Results of the anthelmintic screening of Combretaceae leaf extracts

| Species and reference number ¹ | Concentration of plant extract (mg ml ⁻¹) | Anthelmintic activity ² using extraction solvents: | | | |
|---|--|---|---------|---------------|----------|
| | | water | acetone | ethyl acetate | methanol |
| <i>C. apiculatum</i> Sond. subsp. | 0.5 | 1 | 4 | 4 | 4 |
| <i>apiculatum</i> 36/86 | 1 | 1 | 5 | 7 | 5 |
| <i>C. bracteosum</i> (Hochst.) | 0.5 | 0 | 0 | 0 | 0 |
| Brandis ex Engl. 127/88 | 1 | 0 | 0 | 0 | 0 |
| <i>C. celastroides</i> Welw ex Laws | 0.5 | 0 | 0 | 0 | 0 |
| subsp. <i>celastroides</i> 49/91 | 1 | 0 | 0 | 0 | 0 |
| <i>C. collinum</i> Fresen. subsp. <i>suluense</i> | 0.5 | 0 | 1 | 0 | 0 |
| (Engl. & Diels) Okafor 134/85 | 1 | 0 | 1 | 0 | 0 |
| <i>C. edwardsii</i> Exell | 0.5 | 0 | 0 | 0 | 0 |
| 55/83 | 1 | 0 | 1 | 0 | 0 |
| <i>C. erythrophyllum</i> (Burch.) Sond. | 0.5 | 0 | 0 | 0 | 0 |
| 274/85 | 1 | 0 | 1 | 1 | 0 |
| <i>C. hereroense</i> Schinz | 0.5 | 0 | 0 | 0 | 0 |
| 82/70 | 1 | 0 | 2 | 1 | 0 |
| <i>C. imberbe</i> Wawra | 0.5 | 0 | 0 | 0 | 0 |
| 260/76 | 1 | 0 | 1 | 0 | 0 |
| <i>C. kraussii</i> Hochst. | 0.5 | 0 | 1 | 0 | 0 |
| 89/70 | 1 | 0 | 2 | 0 | 0 |
| <i>C. microphyllum</i> Klotzsch | 0.5 | 0 | 1 | 0 | 0 |
| 151/72 | 1 | 0 | 1 | 0 | 0 |
| <i>C. mkuzense</i> Carr & Retief | 0.5 | 0 | 1 | 0 | 0 |

Table 6.1 continued...

| Species and reference number ¹ | Concentration of plant extract (mg ml ⁻¹) | Anthelmintic activity ² using extraction solvents: | | | |
|--|--|---|---------|---------------|----------|
| | | water | acetone | ethyl acetate | methanol |
| 142/90 | 1 | 0 | 2 | 0 | 0 |
| <i>C. moggii</i> Exell | 0.5 | 0 | 0 | 0 | 0 |
| 312/81 | 1 | 0 | 0 | 0 | 0 |
| <i>C. molle</i> R. Br. ex G. Don | 0.5 | 0 | 1 | 0 | 0 |
| 263/85 | 1 | 0 | 2 | 0 | 0 |
| <i>C. mossambicense</i> (Klotzsch) Engl. 76/77 | 0.5 1 | 0 0 | 0 2 | 0 1 | 0 0 |
| <i>C. nelsonii</i> Dümmer | 0.5 | 0 | 1 | 0 | 0 |
| 313/81 | 1 | 0 | 1 | 0 | 0 |
| <i>C. padoides</i> Engl. & Diels | 0.5 | 0 | 0 | 0 | 0 |
| 70/83 | 1 | 0 | 0 | 0 | 0 |
| <i>C. paniculatum</i> Vent. | 0.5 | 0 | 1 | 0 | 0 |
| 42a/95 | 1 | 0 | 1 | 0 | 0 |
| <i>C. petrophilum</i> Retief | 0.5 | 0 | 1 | 0 | 0 |
| 175/93 | 1 | 0 | 1 | 0 | 0 |
| <i>C. woodii</i> Dümmer | 0.5 | 0 | 0 | 0 | 0 |
| 5/93 | 1 | 0 | 0 | 0 | 0 |
| <i>C. zeyheri</i> Sond. | 0.5 | 0 | 0 | 0 | 0 |
| 287/71 | 1 | 0 | 0 | 0 | 0 |
| <i>Pteleopsis myrtifolia</i> (Laws.) Engl. & Diels 255/85 | 0.5 1 | - ³ - | - - | 0 0 | 0 0 |

Table 6.1 continued...

| Species and reference number ¹ | Concentration of plant extract (mg ml ⁻¹) | Anthelmintic activity ² using extraction solvents: | | | |
|--|---|---|---------|---------------|----------|
| | | water | acetone | ethyl acetate | methanol |
| <i>Quisqualis littorea</i> (Engl.) Exell. 41a/95 | 0.5 1 | - - | - - | 1 2 | 0 0 |
| <i>Terminalia brachystemma</i> Welw. ex Hiern. 167/96 | 0.5 1 | - - | - - | 0 0 | 0 0 |
| <i>Terminalia sericea</i> Burch. ex DC. 159/85 | 0.5 1 | - - | - - | 0 0 | 0 0 |

¹Reference number: first part indicates collection number and second part the year in which collected.

²Anthelmintic activity scoring system: 0 = same as blank, 1 = 80-90% nematodes alive, 2 = 70-80% alive, 3 = 60-70% alive, 4 = 50-60% alive, 5 = 40-50% alive, 6 = 30-40% alive, 7 = 20-30% alive, 8 = 10-20% alive, 9 = 0-10% alive.

³ -: not tested

6.4 DISCUSSION

6.4.1 Anthelmintic activity of Combretaceae extracts

Several species exhibited significant activity in the anthelmintic mortality assay. Of particular interest was *Combretum apiculatum*. The ethyl acetate, acetone and methanol extracts of this plant displayed excellent activity and some activity was present in the water extract, implying a wide range in polarity of active compounds. *C. mossambicense* and *C. hereroense* also showed good activity. *C. mossambicense* has also been shown to possess good antibacterial activity (ELOFF, 1999b). Acetone extracts were the most active with respect to anthelmintic activity followed by ethyl acetate and water, and lastly methanol.

6.5 CONCLUSION

The results obtained provide impetus for the further investigation of highly active *Combretum* species to discover the compounds responsible for their biological activity. Similar compounds are likely to be present in several species, providing support for chemotaxonomic studies (ALEXANDER *et al.*, 1992; ROGERS and VEROTTA, 1996). More investigations into the biological activity of compounds already isolated from active species are justified. In addition, the results support the use of many *Combretum* species by traditional healers for a wide range of common ailments in southern Africa.

CHAPTER 7

ISOLATION AND IDENTIFICATION OF β -ASARONE FROM *ACORUS CALAMUS*

7.1 INTRODUCTION

7.1.1 *Acorus calamus*

7.1.1.1 Description and traditional medicinal uses

Acorus calamus, of the family Araceae, is a reed-like, semi-aquatic, perennial plant with a stout aromatic rhizome. The inconspicuous flowers are compactly arranged on a long, fleshy axis, surrounded by a large leaf-like spathe. All parts of the plant have a pungent, not unattractive smell. It is indigenous to South East Asia, but is now widely distributed in Europe, eastern North America and parts of Africa (VAN WYK *et al.*, 1997). It has been cultivated in South Africa for many years and has become a popular component of Zulu medicine. The plant is used extensively in traditional medicine worldwide. Photographs of the plant growing in the Botanic Gardens (UNP), and a portion of the rhizome, are shown in Plate 7.1.

Rhizomes have been used as carminatives, stomachics and to treat dysentery in southern Africa, Europe and Asia (WATT and BREYER-BRANDWIJK, 1962). They are used as tonics, stimulants and aphrodisiacs, and to treat rheumatism, toothache and respiratory ailments (HUTCHINGS *et al.*, 1996). In the Cape, the use of the rhizome as a carminative and diarrhoea remedy has been recorded (WATT and BREYER-BRANDWIJK, 1962). In India, the powdered rootstock and rhizome are used as an antispasmodic, anthelmintic and insecticide (WATT and BREYER-BRANDWIJK, 1962). The fragrant oils obtained from the rhizome are also used as flavouring in alcoholic beverages and as fragrant essences in perfumes and sacred

oils (MOTLEY, 1994).

7.1.1.2 Biological activity

Much work has been done on investigating the biological activity of *Acorus calamus*, particularly in India and Europe. Extracts from the plant show larvicidal activity (CHAVAN *et al.*, 1976, cited by HUTCHINGS *et al.*, 1996). The antigonadal activity of β -asarone is potentially useful in insect control (SAXENA *et al.*, 1977). The ethanolic extract has shown anti-secretagogue, anti-ulcer and cytoprotective properties in rats (RAFATULLAH *et al.*, 1994), supporting the use of calamus for the treatment of gastropathy in traditional medicine. Rhizomes and roots have sedative, hypotensive, analgesic and hallucinogenic effects (AGARWAL *et al.*, 1956; SHARMA and DANDIYA, 1969). The active hallucinogenic principles are presumed to be α -asarone (chemically similar to mescaline, a psychoactive alkaloid) and the cis-isomer β -asarone (chemically similar to myristicin and kava alkaloids) (LEWIS and ELVIN-LEWIS, 1977).

Fungicidal effects of *A. calamus* have been reported (ALANKARARAO and RAJENDRA PRASAD, 1981; VASHI and PATEL, 1987; SAXENA *et al.*, 1990; JATISATIENR and JATISATIENR, 1999). An anti-mycotic principle was identified as β -asarone, with α -asarone displaying a similar level of activity (OHMOTO and SUNG, 1982). The essential oil and alcoholic extracts of the rhizomes have antibacterial activity against many Gram-positive and Gram-negative bacteria (KAR and JAIN, 1971; ALANKARARAO and RAJENDRA PRASAD, 1981; VASHI and PATEL, 1987). The compounds responsible for the antibacterial activity have not been well investigated.

The dried rhizome gave positive results as an antiamoebic agent when tested on *Paramecium caudatum* (CHOPRA *et al.*, 1957). Alcoholic extracts of the rhizomes were active against the human nematode *Ascaris lumbricoides* (KALEYSA RAJ, 1974) and the rhizome oil was active against the larva of the root knot nematode *Meloidogyne incognita* (SINGH *et al.*, 1991). Both forms of asarone showed a dual

effect on the larvae of the nematode *Toxocara canis* (SUGIMOTO *et al.*, 1995). The first was a fast acting temporary paralytic effect, and the other was a slowly emerging killing effect. Anthelmintic and pesticidal activity of a related species, *Acorus gramineus*, has been reported to be associated with the phenylpropanoids α - and β -asarone (PERRETT and WHITFIELD, 1995). Both isomers of asarone, particularly the β form, are therefore associated with a wide range of biological activity.

Extracts of *Acorus calamus* rhizomes are active in the antibacterial, anthelmintic and antiamebic tests (CHAPTERS 3, 4 and 5). Bioassay-guided fractionation for the isolation of the active compound(s) was attempted using the antibacterial agar overlay technique (SLUSARENKO *et al.*, 1989) and an anthelmintic assay (RASOANAIVO and RATSIMAMANGA-URVERG, 1993).

7.1.1.3 Chemical constituents

Essential oils are found in the leaves, rhizomes and roots, tannins occur in the rhizome and roots, and ascorbic acid is found in the leaves and rhizomes (PAMAKSTYTE-JUKNEVICIENE, 1971, cited by HUTCHINGS *et al.*, 1996). Owing to its wide usage as a crude drug, much research has been undertaken on the constituents of the oil. The major chemical constituents of the essential oils are phenylpropanes, monoterpenes and thermolabile sesquiterpenoids (RÖST and BOS, 1979, cited by MOTLEY, 1994). Methyleugenol, cis-methylisoeugenol, β -asarone, geranylacetate, β -farnesene, shyobunone, epishyobunone and isoshyobunone are the most abundant chemical compounds (RÖST and BOS, 1979, cited by MOTLEY, 1994). Other chemical components include α -asarone, γ -asarone, calamenene, asaronaldehyde, acorenone, calamenone, *n*-heptanic acid, calamendiol, many sesquiterpenes, and trace amounts of other compounds (MAZZA, 1985). The proportion of each chemical constituent of the oil, particularly in the case of β -asarone, varies between the varieties of *A. calamus*, corresponding to the ploidy (RÖST and BOS, 1979, cited by SCHMIDT and STRELOKE, 1994). The diploid caryotype grows in North America and in parts of Asia (Siberia), and the

rhizomes contain little or no β -asarone. In the triploid caryotype, present in central Europe and Kashmir, the β -asarone content of the rhizomes varies from 9 - 13 %. In the tetraploid caryotype, found in India, east Asia and Japan, the essential oil of the rhizomes is 70 - 96 % β -asarone. The ploidy of African plants has not been investigated.

β -Asarone is toxic (ABEL, 1987; LANDER and SCHREIER, 1990), and there is evidence that it may induce duodenal and liver cancer in rats (HABERMAN, 1971, cited by GÖGGELMAN and SCHIMMER, 1983). As a result, many countries have discontinued the use of *Acorus* in digestive medicines. Calamus has been banned by the FDA as a food additive and within the last few years many herbal shops have stopped recommending or dispensing it. Although calamus and its products are banned in the United States, they are authorized in Europe, where the highest acceptable concentration in foods and beverages is 0.1 mg kg⁻¹ and in alcoholic beverages, 1 mg kg⁻¹ (LANDER and SCHREIER, 1990).

7.2 MATERIALS AND METHODS

7.2.1 Plant extraction

Acorus calamus L. plants were collected from a shallow (approximately 0.3 m deep) pond at Silverglen Nursery in Durban, South Africa in October 1999. A voucher specimen (McGaw47NU) was deposited at the Herbarium of the University of Natal, Pietermaritzburg. The leaves, roots and rhizomes were separated and dried at 50°C. The dried, powdered plant parts (4.33 g, 13.27 g and 17.56 g for the leaves, roots and rhizomes respectively) were extracted separately with 100 ml ethanol. Extraction was performed by sonication for 30 min in a Julabo ultrasound bath, followed by overnight maceration. The extracts were filtered through Whatman No. 1 filter paper and the procedure was repeated twice with the same plant material. The filtrates were air-dried and stored at -15°C.

7.2.2 Antibacterial assays

The antibacterial activity of fractions resulting from each purification stage was tested using the agar-overlay bioautographic assay (SLUSARENKO *et al.*, 1989) as described in CHAPTER 5 (Section 5.1.3.3). The inhibition of bacterial growth by compounds separated on the TLC plate was visible as white spots against a deep red background. The test organism was the Gram-positive *Staphylococcus aureus* (ATCC 12600).

The Minimal Inhibitory Concentration (MIC) values of the pure compound were determined against the Gram-positive *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 12600) and the Gram-negative *Escherichia coli* (ATCC 11775) and *Klebsiella pneumoniae* (ATCC 13883). The microplate method of ELOFF (1998a) as described in CHAPTER 5 (Section 5.2.3) was used. The pure compound was dissolved in ethanol and serially diluted twofold with water in microtiter plate wells. The antibiotic neomycin and extract-free solutions were included as standard and blank controls. MIC values were recorded as the lowest concentration resulting in complete inhibition of bacterial growth.

7.2.3 Anthelmintic assay

The anthelmintic activity of extracts, fractions and the pure compound was determined against *Caenorhabditis elegans* var. Bristol (N2) nematodes using the mortality assay described in CHAPTER 3 (Section 3.2.2). Solvent blanks, and levamisole as a standard anthelmintic drug, were included. The percentage and movement of live nematodes after incubation with the plant extracts was recorded and compared to that of the controls.

7.2.4 Thin Layer Chromatography (TLC)

Thin Layer Chromatography (TLC) analysis was used to compare the chemical composition of the crude ethanol extracts of *Acorus* leaves, roots and rhizomes. A

spot (0.5 mg) of each extract was loaded on a silica gel TLC plate (Merck Silica gel 60 F₂₅₄, 0.25 mm thick) and developed using the solvent system hexane:ethyl acetate (2:1). The TLC plate was visualized under ultraviolet light at 254 nm and 366 nm, under visible light, and after staining with anisaldehyde/sulphuric acid (AS) spray reagent (465 ml ethanol, 5 ml glacial acetic acid, 13 ml concentrated sulphuric acid and 13 ml *para*-anisaldehyde mixed in order). The TLC plate strip was heated for 10 min at 100°C to allow development of the stain.

7.2.5 Bioassay-guided fractionation for isolation of active compound

All solvents were re-distilled using a rotary evaporator.

7.2.5.1 Vacuum Liquid Chromatography

Bioassay-guided fractionation was performed using the rhizome extract. The ethanolic rhizome extract (1.14 g) was separated by Vacuum Liquid Chromatography (VLC) over silica gel (Merck 230-400 mesh) using a hexane:ethyl acetate gradient solvent system of increasing polarity. A column 13 cm in length and with an internal diameter of 2 cm was packed with 20 g silica. The gradient solvent system was increased by 5 % increments from 100 % hexane to 60 % hexane in ethyl acetate, and subsequently by 10 % increments to 100 % ethyl acetate. At each concentration, 100 ml solvent was flushed through the column. Bioautography established that the most active fraction (270 mg) was eluted with 5 % and 10 % ethyl acetate in hexane.

7.2.5.2 Preparative Thin Layer Chromatography

The active fraction was further separated by preparative TLC on three silica gel plates (Merck Silica gel 60 F₂₅₄, 0.25 mm thick) using toluene:ethyl acetate (93:7). The active fraction (identified as a dark purple band under UV₂₅₄) was scraped off the TLC plates and eluted from the silica with ethanol. The active compound in ethanol was filtered through Millipore filters (0.45 μm and 0.22 μm) to remove the

silica. To ensure complete removal of the silica particles, the filtrate was passed through a Sephadex LH-20 column prepared in a Pasteur pipette. The Sephadex was loaded to a height of 30 mm, and the pipette had an internal diameter of 5 mm. The purity of the isolated compound, a yellow oil, was confirmed by TLC using various solvent systems.

7.2.3 Identification of purified active compound

High Resolution Mass Spectrometry (MS) using a VG70-SEQ spectrometer was performed. Nuclear Magnetic Resonance Spectroscopy (^1H NMR and ^{13}C NMR) was carried out to confirm the structure of the compound. A Varian Unity Inova 500 MHz spectrometer was used.

7.3 RESULTS

7.3.1 Plant extraction

The extraction from the dried, powdered plant material yielded residues of 0.5546 g (12.81 %) for the leaves, 0.2976 g (2.24 %) for the roots and 1.1357 g (6.47 %) for the rhizomes.

7.3.2 Antibacterial and anthelmintic activity in different plant parts

The leaf, root and rhizome ethanol extracts of *Acorus calamus* exhibited antibacterial and anthelmintic activity. The rhizome extract possessed a lower Minimal Inhibitory Concentration (MIC) than the leaf and root extracts (Table 7.1), against *Bacillus subtilis* and *Staphylococcus aureus*. Therefore, the rhizome extract was selected to perform bioassay-guided fractionation for isolation of active compound(s). Additionally, TLC and bioautography displayed fewer compounds, other than the target active compound, in the rhizome extract than in the leaf and root extracts. Photographs of the TLC fingerprints of the leaf, rhizome and root

extracts are presented in Plate 7.1. The compound of interest, which was shown using bioautography to have antibacterial activity, is marked with an arrow.

7.3.3 Bioassay-guided fractionation for isolation of active compound

VLC on the rhizome ethanol extract resulted in a good separation of compounds. Photographs of the TLC plates prepared from the VLC fractions, viewed under ultraviolet light and after staining with AS spray reagent, are shown in Plate 7.2. Also represented in Plate 7.2 is the bioautography plate of the VLC fractions. As previously mentioned, the white areas indicate the presence of antibacterial compounds, as the lack of bacterial growth cannot convert the indicator tetrazolium salt to a red product. Marked with an arrow is the compound of interest, which eluted in the second and third fractions. It is apparent that several other antibacterial compounds, indicated by further white areas on the plate, are present in the remaining VLC fractions. However, when the solvent was evaporated from these fractions, there was insufficient residue to continue separations leading to identification of these compounds. There was limited source plant material, so the focus of the investigation remained on the compound of interest. The third fraction was selected to continue the isolation as TLC indicated the presence of fewer contaminating compounds than in the second fraction.

The activity-directed fractionation, ending with preparative TLC, of 1.14 g of rhizome extract yielded 15.6 mg of oily yellow β -asarone. This represented 1.4 % of the extract and 0.09 % of the rhizome. This pure compound possessed antibacterial and anthelmintic activity. The structure of β -asarone is presented in Figure 7.1. The MIC values for the isolated β -asarone against the test bacteria are presented in Table 7.1.

The anthelmintic activity of the crude rhizome extract and isolated β -asarone was very similar, both resulting in the death of 60-70 % of nematodes after incubation. No other fractions resulting from the fractionation of the rhizome extract exhibited anthelmintic activity.

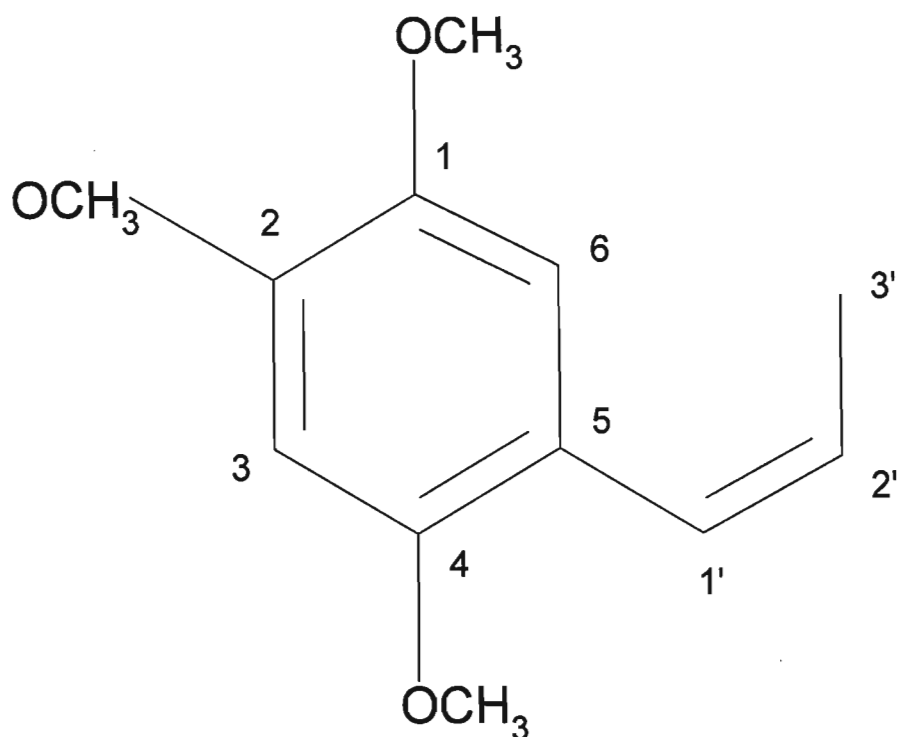


Figure 7.1. Structure of β -asarone

7.3.4 Identification of purified active compound

The compound identified as β -asarone was a yellow oil, $C_{12}H_{16}O_3$ $[M]^+$ 208.11016. EIMS m/z (rel. int.): 208 $[M]^+$ (100), 193 (47), 177 (3), 165 (27), 162 (12), 150 (6), 134 (3), 103 (4), 91 (11), 77 (7), 69 (3), 51 (2). The 1H NMR spectra in $CDCl_3$ were δ 1.84 (3H, dd, $J = 7.3$ Hz & 1.8 Hz, $-CH=CH-CH_3$), 3.81, 3.83 and 3.89 (each 3H, s, three OCH_3), 5.76 (1H, dq, $J = 12$ Hz & 6.8 Hz, H-2'), 6.49 (1H, dq, $J = 11.4$ Hz & 1.83 Hz, H-1'), 6.53 (1H, s, H-3) and 6.84 (1H, s, H-6). The ^{13}C NMR spectra were recorded in $CDCl_3$, δ 151.5 s (C-4), 148.5 s (C-2), 142.3 s (C-1), 125.8 d (C-1'), 124.7 d (C-2'), 118.0 s (C-5), 114.1 d (C-6), 56.6 q, 56.4 q, 56.0 q (OCH_3), 14.6 q (C-3').

Table 7.1. Comparison of MIC values obtained using the microplate method (ELOFF, 1998a) for *Acorus calamus* ethanol extracts, isolated β -asarone and the antibiotic neomycin against various bacteria

| Extract or compound tested | Microorganism ¹ tested (MIC in mg ml ⁻¹) | | | |
|----------------------------|---|------------------------|------------------------|-----------------------|
| | B.s. | E.c. | K.p. | S.a. |
| rhizome | 0.78 | - | - | 1.56 |
| root | 1.56 | - | - | 3.13 |
| leaf | 1.56 | - | - | 3.13 |
| isolated β -asarone | 3.13 | >12.5 | >12.5 | 3.13 |
| neomycin | 0.39x10 ⁻³ | 0.098x10 ⁻³ | 0.098x10 ⁻³ | 0.39x10 ⁻³ |

¹Microorganism tested: B.s., *Bacillus subtilis* (ATCC 6051); E.c., *Escherichia coli* (ATCC 11775); K.p., *Klebsiella pneumoniae* (ATCC 13883); S.a., *Staphylococcus aureus* (ATCC 12600); -, extract not tested

Plate 7.1. *Acorus calamus* (A) uprooted from a shallow pond in the Botanic Gardens, UNP and (B) rhizome. The length of the plant from rhizome to leaf tip was 72 cm, and the length of the rhizome portion displayed was 17 cm. TLC fingerprints of ethanol extracts of *A. calamus* leaf, rhizome and root viewed (C) under visible light, (D) under UV₂₅₄ and (E) under UV₃₆₆. The solvent system used was hexane:ethyl acetate (2:1). Marked with an arrow is the compound isolated (β -asarone).

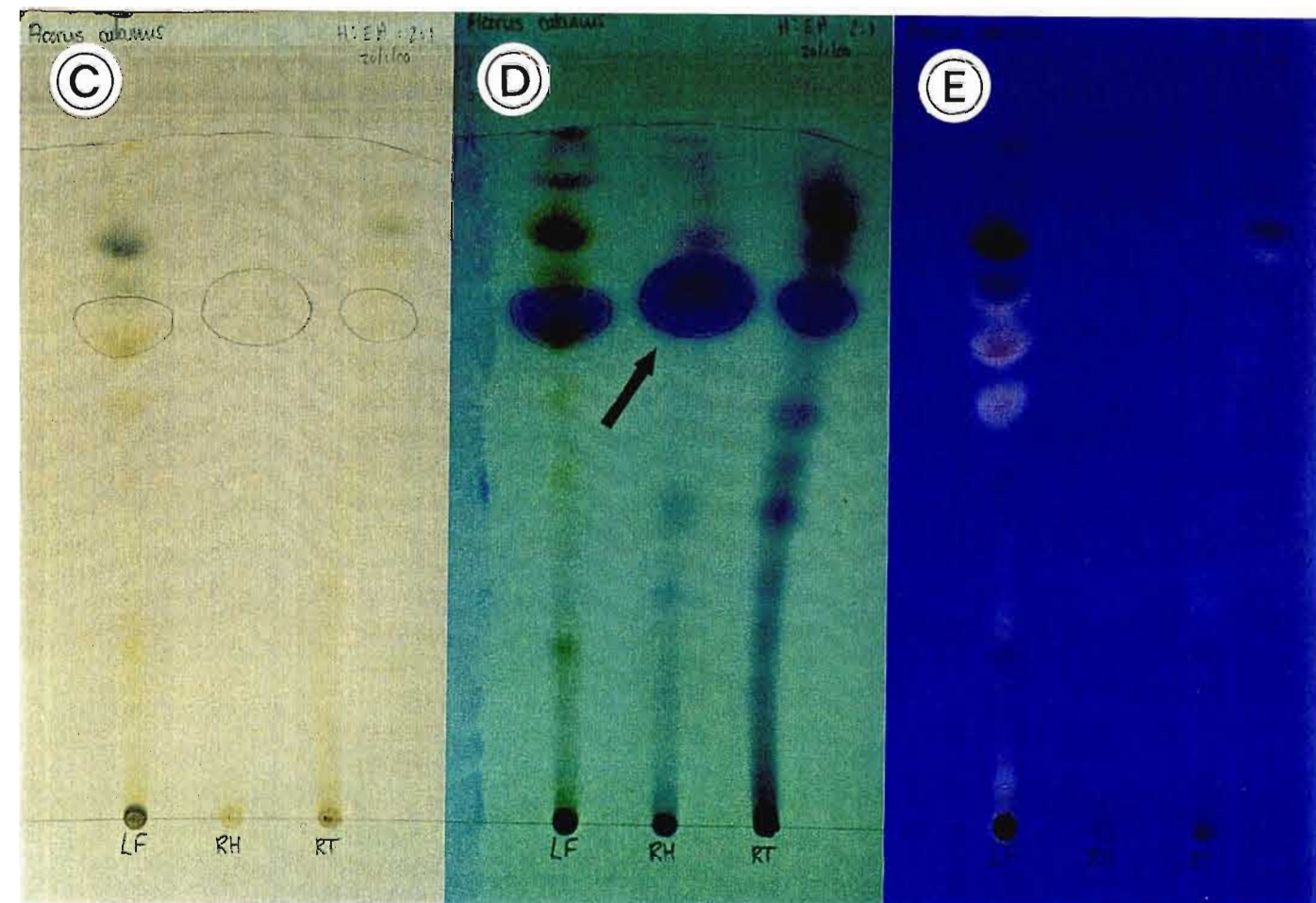
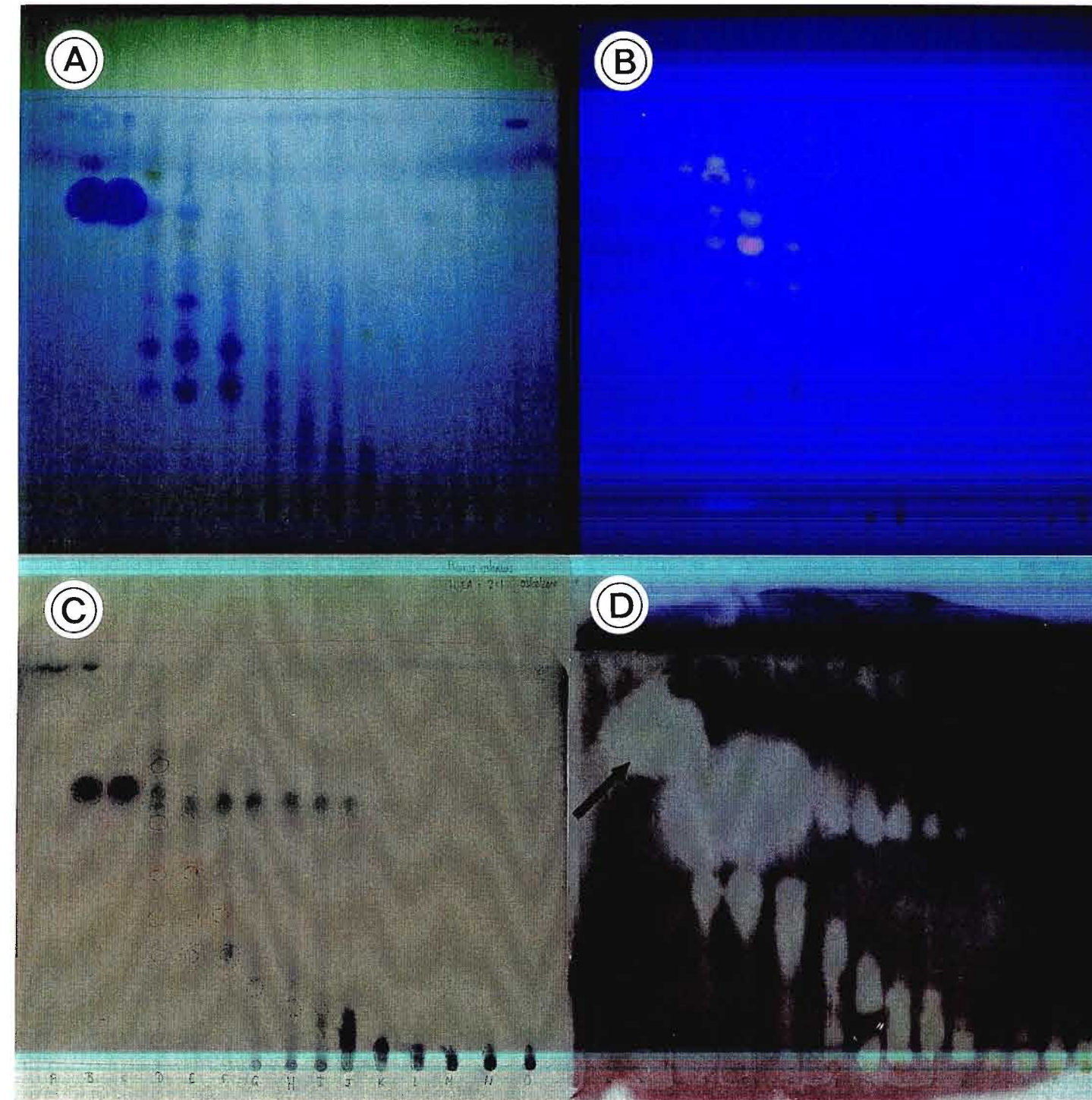


Plate 7.2. TLC of Vacuum Liquid Chromatography (VLC) fractions in the bioassay-guided fractionation of *A. calamus* ethanolic rhizome extract. Viewed (A) under UV₂₅₄, (B) under UV₃₆₆ and (C) after staining with anisaldehyde. The bioautography plate (D) shows white areas of no bacterial growth, indicating the presence of antibacterial compounds. The active compound of interest (β -asarone) is marked with an arrow.



7.4 DISCUSSION

7.4.1 Isolation of an active compound

The isolated active compound β -asarone has been isolated previously by others from *Acorus calamus* growing in Europe and Asia (BAXTER *et al.*, 1960). In this study, the antibacterial and anthelmintic activity of β -asarone was demonstrated. The anthelmintic activity of the isolated β -asarone was very similar to that of the crude rhizome extract. The crude extract and isolated compound were both tested at the same concentration (1 mg ml^{-1}). It was expected that the isolated β -asarone would display higher anthelmintic activity than the original extract as it would be present in a higher concentration. However, this was not the case, and a possible explanation may be the instability of β -asarone after isolation, as some breakdown of the compound was visible after TLC analysis. No other anthelmintic compounds were apparent in the VLC fractions.

The antibacterial activity of the isolated β -asarone was lower than that of the crude extract. This may again be partly attributed to the lability of the isolated compound. Also, several other compounds with antibacterial activity in the VLC fractions of the crude extract were visualized in the bioautography assay. They made up such a small part of the overall composition that their identification was not attempted. β -Asarone appeared to be the major component of the crude extract (from TLC analysis, and with its yield of 1.4 % of the extract). However, if it were the major antibacterial compound, it would be expected to have a much lower MIC value. This was not the case, so other minor compounds may well have significantly higher antibacterial activity, contributing to a greater proportion of the overall activity even though they are not present in such high quantities as β -asarone. Unfortunately, it is not possible to quantify antibacterial activity from a bioautography assay, where the inhibition spot is by nature a positive/negative response, and does not reveal detail about the degree of activity of a compound.

7.4.2 Identification of isolated compound

The mass spectrometry, ^1H and ^{13}C NMR data were consistent with those previously reported for β -asarone (PATRA and MITRA, 1981; MAZZA, 1985; NIGAM *et al.*, 1990; OPREAN *et al.*, 1998). Asarone has been synthesized by two different methods (SHARMA and DANDIYA, 1969).

7.5 CONCLUSION

The evidence presented indicates that if the *Acorus calamus* tested in this study is representative of that found in southern Africa, then the South African *Acorus* is likely to be triploid or tetraploid, as these caryotypes of *A. calamus* are known to contain β -asarone (RÖST and BOS, 1979, cited by SCHMIDT and STRELOKE, 1994). Also, its origins possibly lie in Asia or Europe. Of some concern is the evidence implicating β -asarone in causing duodenal and liver cancer in test animals. Owing to the possible carcinogenic and toxic effects, the use of *Acorus* in digestive medicines has been discontinued in most countries. In South Africa, *Acorus calamus* is still widely used by Zulu traditional healers to treat a variety of ailments, including gastrointestinal disorders. The content of β -asarone substantiates the use of this plant as an anthelmintic and antibacterial remedy, but owing to the toxicity of the active compound, it is recommended that the use of the plant is restricted to external application for antibacterial use only, or discontinued altogether. *A. calamus* contains several additional compounds with antibacterial activity. The use of varieties of *A. calamus* which do not contain β -asarone, but which still have antibacterial activity, would be preferable.

CHAPTER 8

VARIATION IN ANTIBACTERIAL ACTIVITY OF *SCHOTIA BRACHYPETALA*

8.1 INTRODUCTION

8.1.1 *Schotia brachypetala*

Schotia brachypetala Sond. (Caesalpinaceae) is an attractive medium-sized to large deciduous tree, endemic to the north-eastern parts of southern Africa (COATES PALGRAVE, 1977). In South Africa, the roots are used for dysentery and diarrhoea (WATT and BREYER-BRANDWIJK, 1962; BRYANT, 1966). In Zimbabwe, roots are used to treat diarrhoea (GELFAND *et al.*, 1985). Decoctions of the bark or roots are used medicinally, alone or as an ingredient of a mixture (COATES PALGRAVE, 1977). An apparently emetic decoction of the bark is taken for heartburn and after an excess of beer (WATT and BREYER-BRANDWIJK, 1962). A photograph taken in July 2000 of a flowering *S. brachypetala* tree in Umfolozi Nature Reserve, KwaZulu-Natal is shown in Plate 8.1. Also shown in Plate 8.1 are photographs of a tree in Pietermaritzburg after a local labourer had harvested bark for his personal medicinal use.

There are four species of *Schotia* in southern Africa, namely *S. afra* (L.) Thunb var. *afra*, *S. brachypetala* Sond., *S. capitata* Bolle and *S. latifolia* Jacq. (POOLEY, 1993). The medicinal use of the bark of *S. afra* (WATT and BREYER-BRANDWIJK, 1962) and *S. capitata* (HUTCHINGS *et al.*, 1996) in traditional medicine has been recorded, but no details are supplied. *Schotia latifolia* is used by the Xhosa to treat dysentery, diarrhoea and asthma (SIMON and LAMLA, 1991). Little is known about the chemical constituents and biological activity of *Schotia* species.

A study of *Schotia brachypetala* was initiated after this plant displayed good antibacterial activity in a broad screening of South African medicinal plants (CHAPTER 5; MCGAW *et al.*, 2000a). The lack of chemical data and pharmacological knowledge concerning this plant was a further factor in the decision to focus investigation on *S. brachypetala*. Possible variations in antibacterial activity with respect to season, plant part and geographical location were investigated. The effect on antibacterial activity of storing the dry leaf material for an extended period at room temperature before screening, as well as storing a frozen, prepared plant extract for the same period, was studied. Extraction of fresh and dry material was carried out to see if this had an influence on the degree of activity in the leaves. Other available *Schotia* species were included in this investigation. TLC fingerprints of all the plant extracts, which gave information on the chemical constituents of each plant, were compared.

8.1.2 Variation in biological activity

The variation of activity in a species which occurs owing to its genetics, growth conditions, location, stage in its life cycle or the part of it which is being investigated is known as intraspecific variation (HOUGHTON, 1999). Biological activities are related to chemical compounds, particularly secondary metabolites (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). Some bioactive compounds are species-specific, and others may be found in several or many species of a genus, in several related genera, or even families. These active constituents may be present in small quantities in plant species from which they were first discovered. Other species in related genera may be superior sources of these compounds, or may lead to new bioactive derivatives. Chemotaxonomic relationships, therefore, are important in the possible discovery of similar compounds of interest in related species (RASOANAIVO and RATSIMAMANGA-URVERG, 1993; HOUGHTON, 1999).

Many plants concentrate certain secondary metabolites in specific organs (O'NEILL and LEWIS, 1993), so variation in bioactivity is often encountered between different parts of the same plant. Different parts of the plant may produce chemical

constituents quantitatively or qualitatively different from those in another part (HOUGHTON, 1999). The season and geographical location may also affect the presence and quantity of active compounds in higher plants (ELOFF, 1999a). Consequently, bioassays of extracts made from other parts of the plant, or of plant parts collected at a different time or from a different specimen, may yield novel active compounds.

8.1.3 Thin Layer Chromatography

Thin layer chromatography (TLC) is a rapid and effective means of obtaining a characteristic analytical fingerprint of a plant extract (WAGNER and BLADT, 1996). This technique clearly illustrates differences in chemical composition of plant extracts. Chemical data obtained from TLC are important additions to morphological features in taxonomic studies, as they reinforce relationships of plants at a different level. The chemical evidence may also assist in identifying the plants under study, if reference TLC chromatograms for known plants and plant parts are available.

8.2 MATERIALS AND METHODS

8.2.1 Collection of plant material

The plant parts, namely leaves, bark, root bark, roots, seed pods with seeds, flowers and young stems, were harvested individually from a small *S. brachypetala* tree (about 3 years old) growing in Hilton, Pietermaritzburg. Leaves were collected at about the same time of year from trees in different geographical locations in KwaZulu-Natal, i.e. Umfolozi Nature Reserve, Silverglen Nature Reserve near Durban, Scottsville (Pietermaritzburg), and the Botanic Garden of the University of Natal Pietermaritzburg (UNP). From a tree approximately 12 years old found in the Botanic Gardens (UNP), leaves were harvested in the middle of the month every month for a year. Leaf collections of *S. afra* (from Val-Lea Vista Nursery, Pietermaritzburg) and *S. capitata* (growing in the Botanic Gardens, UNP) were

made. Voucher specimens for each plant were deposited in the Herbarium of the University of Natal, Pietermaritzburg. The voucher numbers are recorded in Tables 8.1 to 8.4.

8.2.2 Plant extract preparation

The plant material to be assayed was dried at 50°C, and ground to a powder in a Wiley mill. Separate samples of 2 g were extracted with 20 ml ethanol and water respectively, while a further 8 g were extracted with 80 ml hexane. Extraction was performed by sonication for 30 min in a Julabo ultrasound bath, followed by filtration through Whatman No. 1 filter paper.

To determine whether drying the leaves affected the antibacterial activity or chemical profile on TLC, 10 g of freshly picked leaves were extracted with 100 ml hexane, and separate samples of 5 g each were extracted with 50 ml ethanol and 50 ml water respectively. Extraction was performed by blending the leaf material together with the extracting solvent in a Waring blender at high speed for 5 min. As before, the extracts were filtered through Whatman No. 1 filter paper. The filtrates were air-dried and stored at -15°C after being assayed for antibacterial activity. Also investigated was the effect on activity of storing intact plant material in the dark at room temperature for 18 months prior to grinding and extract preparation, and of storing the plant extracts at -15°C for 18 months.

8.2.3 Antibacterial activity screening

The Minimal Inhibitory Concentration (MIC) values of the plant extracts were determined against the Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus*, and the Gram-negative bacteria *Escherichia coli* and *Klebsiella pneumoniae*. The test organisms were obtained from the bacterial collection of the Department of Microbiology, UNP. The microplate method of ELOFF (1998a) in 96-well microtiter plates as previously described (CHAPTER 5, SECTION 5.2.3) was used to determine the Minimal Inhibitory Concentration (MIC) values. The MIC

values were used to compare the antibacterial activity of the ethanol and water extracts. As before, the MIC values were recorded as the lowest concentration resulting in complete inhibition of bacterial growth. The MIC determination experiments were repeated a further two times for each extract. The results were analysed by two-way analysis of variance (ANOVA), using least significant differences (LSD) to compare differences between the means of MIC values, at a 5 % level of significance ($p < 0.05$).

8.2.4 TLC fingerprinting

To determine the composition of the extracts, 0.5 mg of each of the ethanol extracts were loaded in a 1 cm band, with bands approximately 3 mm apart, on TLC plates (Merck Silica gel 60 F₂₅₄). The plates were developed using the solvent system toluene:ethyl acetate (4:1). The solvents were redistilled using a rotary evaporator. After evaporation of the solvent from the TLC plates, the separated components were visualized under visible and ultraviolet light at 254 and 366 nm. The TLC plates were then sprayed with AS spray reagent and heated at 110°C for 5-10 min to allow the development of colours resulting from the presence of various compounds.

8.3 RESULTS

The Minimal Inhibitory Concentration (MIC) values of ethanol and water extracts against the test bacteria are given in Tables 8.1 to 8.6. The MIC values reported are the means of three experiments. The TLC chromatograms of the ethanolic extracts are represented in Plates 8.2 to 8.4.

8.3.1 Plant part variation

With regard to the plant part variation (Table 8.1), there were no significant differences ($p < 0.05$) in antibacterial activity of the ethanol extracts against all four test bacteria. In contrast, there were significant differences ($p < 0.05$) for the water

extracts against the Gram-positive *B. subtilis* and *S. aureus*. The antibacterial activity of the water extracts ranged from 0.71 mg ml⁻¹ to more than 12.5 mg ml⁻¹. Only the leaves exhibited a small degree of activity against the Gram-negative bacteria.

The TLC chromatogram (Plate 8.2) comparing the ethanolic extracts of the different plant parts of the same tree indicated some differences in chemical composition. The leaf, seed plus pod, and stem ethanol extracts showed similar profiles, with more of the red fluorescence (UV_{366 nm}) typical of chlorophyll visible in the stem fingerprint than in that of the seed plus pod, and even more in that of the leaf extract. There were very few compounds present in the fingerprints of the root bark and flower extracts. The bark and root extracts produced similar chemical profiles on TLC. The profile of the leaf extract showed many compounds in common with the bark and root, but with the addition of chlorophyll compounds.

8.3.2 Locational variation

The results of the investigation on the antibacterial activity of genetically different leaf material are recorded in Table 8.2. There were significant differences ($p < 0.05$) in activity of ethanol extracts made from leaves of different trees against the Gram-negative bacteria *E. coli* and *K. pneumoniae*, but none against the Gram-positive bacteria. The water extracts showed significant differences ($p < 0.05$) in activity against all of the bacteria. The TLC chromatograms of the ethanol extracts (Plate 8.3) were again remarkably similar. The only noteworthy difference was that the extract prepared from a tree growing in the Umfolozi Game Reserve (KwaZulu-Natal) exhibited a bright apple-green compound seen under visible light at $R_f = 0.25$. This compound was not apparent in any of the other ethanol extracts.

8.3.3 Seasonal variation

Considering the monthly analysis of antibacterial activity (Table 8.3), there were significant differences ($p < 0.05$) in the activity of the water extracts against all four

test bacteria, and in the activity of ethanol extracts against the Gram-negative bacteria *E. coli* and *K. pneumoniae*. However, the range of activity was small, so good activity was constantly shown against the Gram-positive bacteria, and fair activity was present against the Gram-negative species most of the time. The antibacterial activity of ethanol extracts against the Gram-positive *B. subtilis* and *S. aureus* was not significantly different ($p < 0.05$) for all the months of the year. The TLC chromatogram (Plate 8.4) showed a similar range of compounds in ethanol extracts prepared from plant material collected in different months of the year. There were no striking differences in composition of the extract.

8.3.4 Species variation

The only significant differences ($p < 0.05$) in activity between the three *Schotia* species (Table 8.4) were apparent when the water extracts were tested against *B. subtilis* and *E. coli*. The TLC fingerprints of the ethanol extracts of the three species were similar (Plate 8.2). The fingerprint of *S. afra* showed two extra compounds not seen in the fingerprints of *S. brachypetala* and *S. capitata*. These compounds were apparent in visible light and under ultraviolet light at 366 nm, at $R_f = 0.27$ and $R_f = 0.22$.

8.3.5 Variation after extraction of fresh or dry material

There were no significant differences ($p < 0.05$) in activity between the fresh and dry material extracts when the ethanol extracts were tested against *B. subtilis* and when the water extracts were tested against *S. aureus* (Table 8.5). There were significant differences ($p < 0.05$) in all other cases. The antibacterial activity against all four test bacteria was stronger in the ethanol extracts of fresh material than in the ethanol extracts of dry material. The reverse was true in the case of the water extracts, where the dry leaf extracts were more antibacterial than those aqueous extracts prepared from fresh material.

The TLC profiles of the fresh and dry extracts shown in Plate 8.5 were similar except

for 3 additional compounds, seen under visible and UV_{366 nm}, which were present in the dried material extract at $R_f = 0.37, 0.32$ and 0.27 .

8.3.6 Effect of storage of plant material and extracts

Regarding the investigation into the effects of storage of dried leaf material and extracts of *Schotia brachypetala* (Table 8.6), there were significant differences ($p < 0.05$) in activity only when the water extracts were tested against *E. coli* and *S. aureus*. The TLC profiles (Plate 8.5) were similar with the only difference occurring where the dried material stored for 18 months before being ground and extracted lacked two compounds at $R_f = 0.32$ and 0.27 . The chromatograms of the extract screened immediately after preparation, and the extract stored in the freezer for 18 months after preparation were remarkably similar.

Plate 8.1. A flowering *Schotia brachypetala* tree in Umfolozi Nature Reserve, KwaZulu-Natal (A), a close-up view of the flowers, leaves and seed pods with seeds (B) and photographs of a tree from which bark had recently been harvested (C, D and E) in Pietermaritzburg.



Plate 8.2. TLC separation of the ethanol extracts (A) of different plant parts of *S. brachypetala*, viewed (1) under visible light, (2) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}. The solvent system used was toluene:ethyl acetate (4:1).

[lf = leaf, st = stem, bk = bark, rt = root, rb = root bark, fl = flower, sp = seeds plus pods]

TLC separation of the ethanol extracts (B) of *S. brachypetala* (Sb), *S. afra* (Sa) and *S. capitata* (Sc) viewed (1) under visible light, (2) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}.

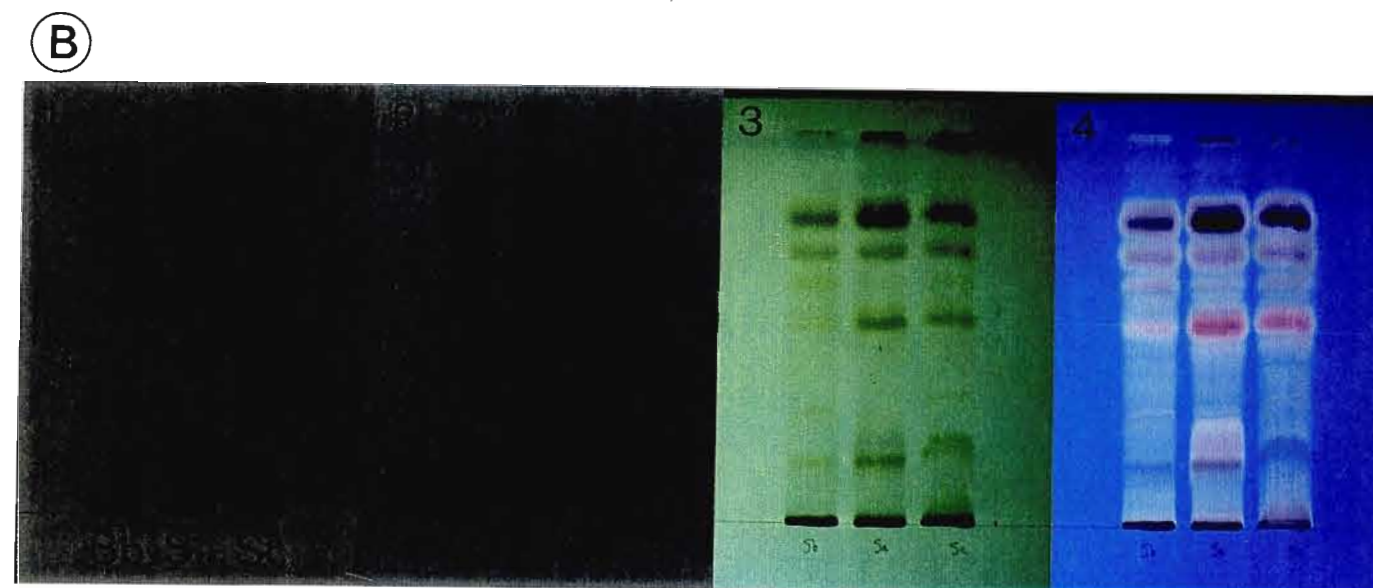
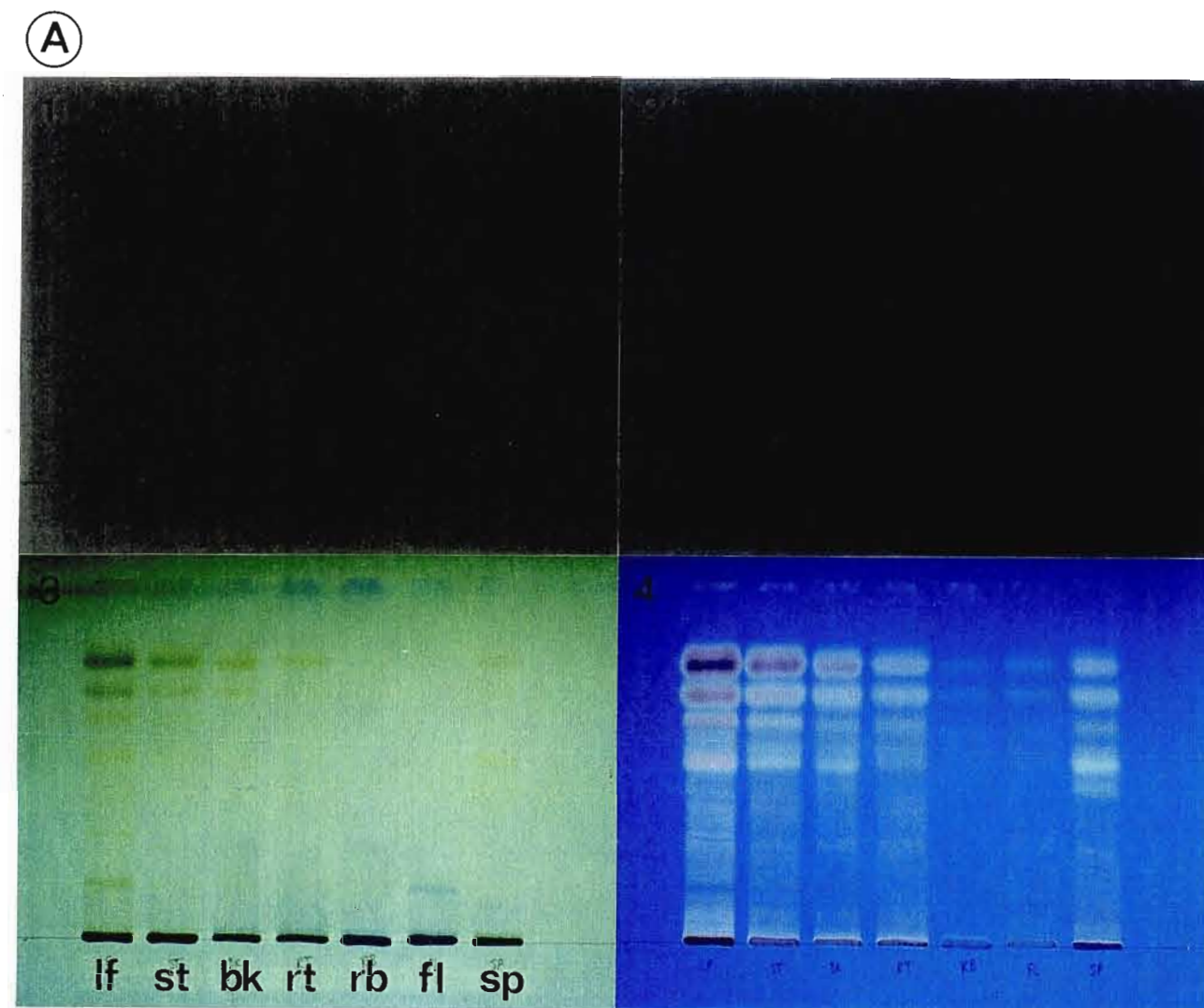


Plate 8.3. TLC separation of the ethanol extracts of leaves of genetically different trees of *S. brachypetala*, viewed (1) under visible light, (2) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}. The solvent system used was toluene:ethyl acetate (4:1).

[Source of plant material: a - g = different trees growing in the Botanic Gardens, UNP; h = Silverglen Nature Reserve; i = Umfolozi Nature Reserve; j = Scottsville, Pietermaritzburg; k = Hilton, Pietermaritzburg]

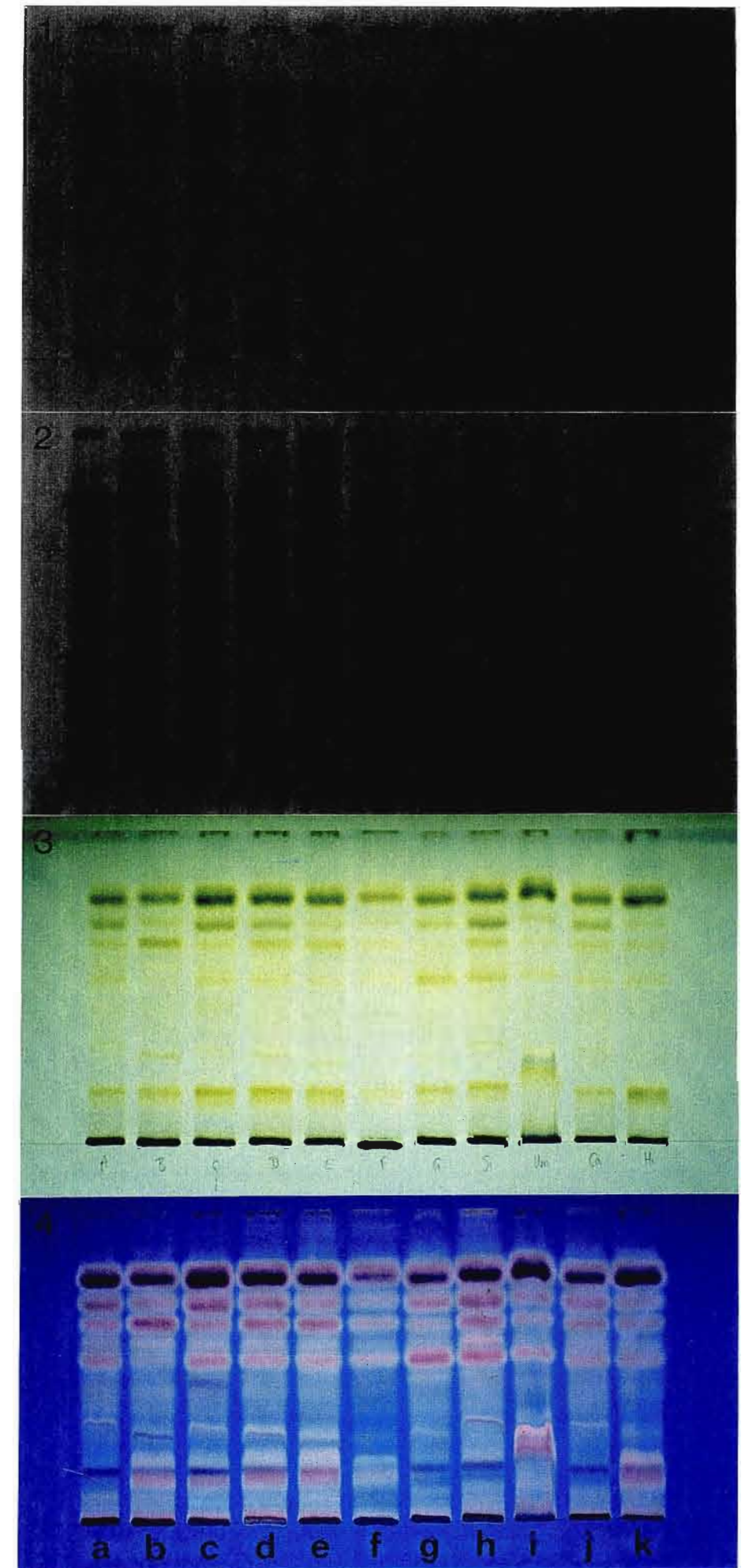


Plate 8.4. TLC separation of the ethanol extracts of *S. brachypetala* leaf material collected during different months, viewed (1) under visible light, (2) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}. The solvent system used was toluene:ethyl acetate (4:1).

[Collection month: a = January, b = February, c = March, d = April, e = May, f = June, g = July, h = August, i = September, j = October, k = November, l = December]

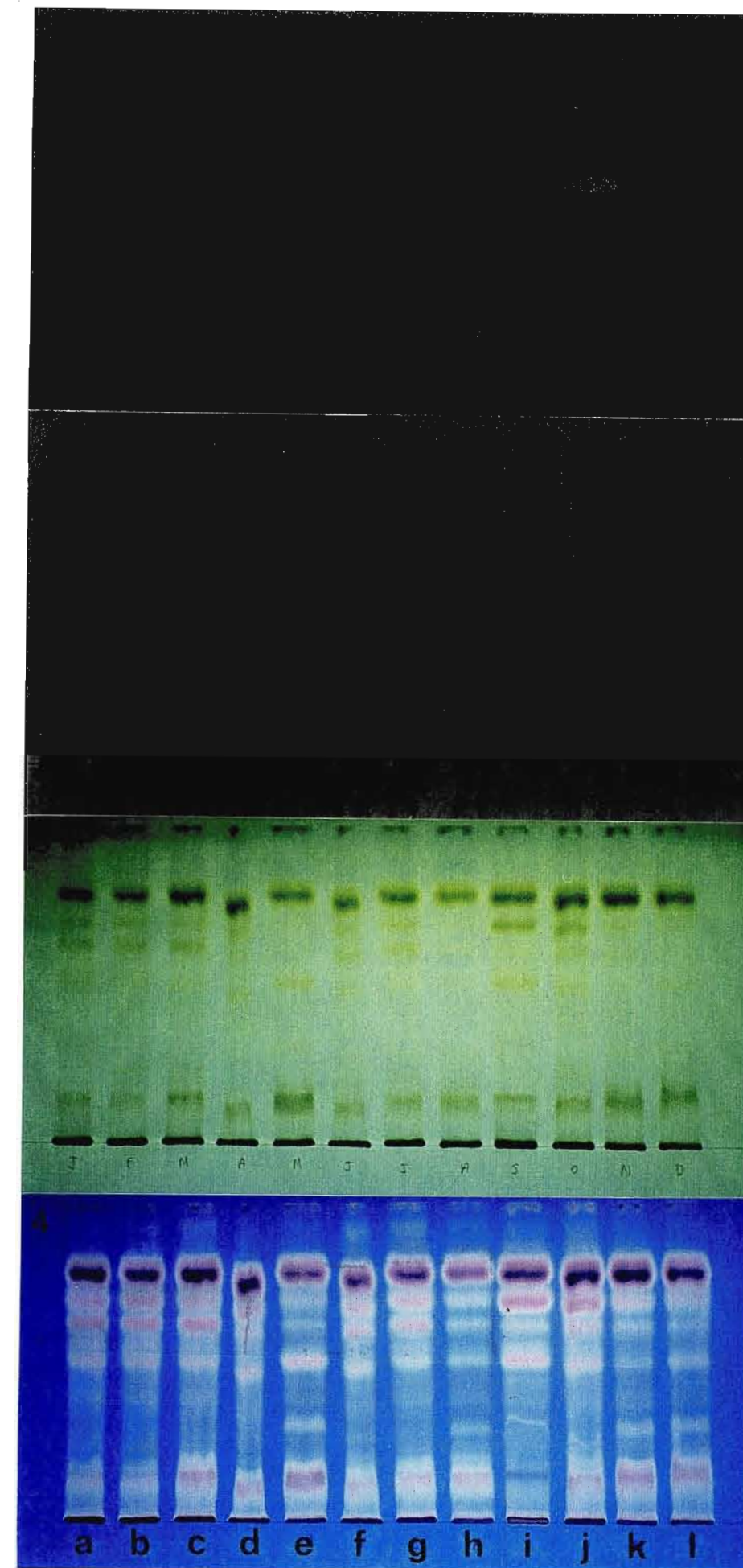


Plate 8.5. TLC separation (A) of the ethanol extracts of *S. brachypetala* leaf extracts prepared from fresh (F) and dry (D) material, viewed (1) under visible light, (2) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}.

TLC separation of (B) the ethanol extracts of *S. brachypetala* leaf material after being stored under various conditions, viewed (1) under visible light, (2) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}. The solvent system used was toluene:ethyl acetate (4:1).

[a = extract screened immediately after collection, drying and extraction of material; b = extract stored in freezer at -15°C for 18 months; c = dried, intact plant material stored in dark cupboard at room temperature before being ground, extracted and screened]

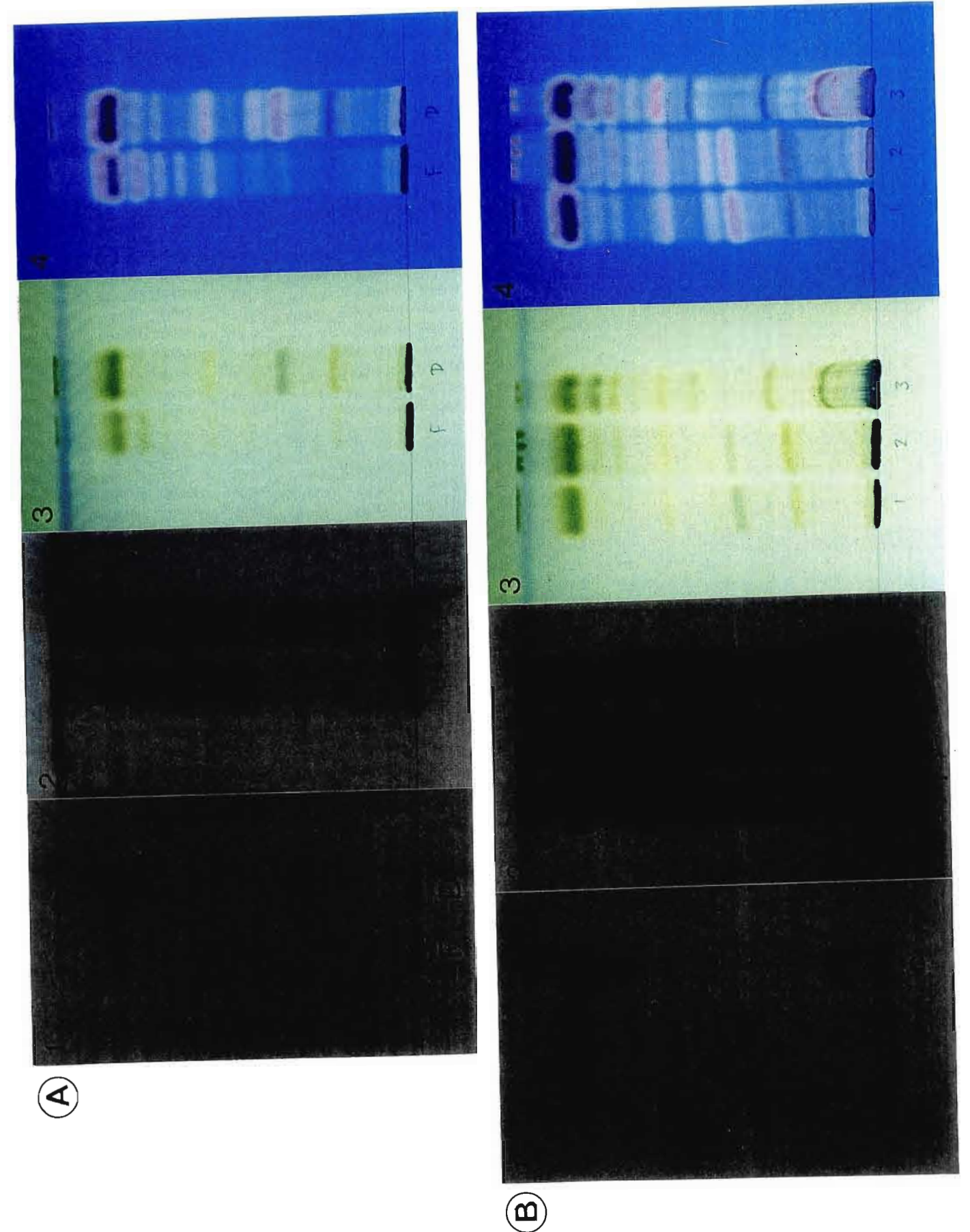


Table 8.1. Antibacterial activity of different plant parts of *Schotia brachypetala* (voucher number *McGaw58NU*). Treatments in each column denoted by the same letters are not significantly different at the 5 % level, with a standard error (SE) for the treatment means = 1.618

| Plant part ¹ | Extract and bacteria ² tested (MIC values in mg ml ⁻¹) | | | | | | | |
|-------------------------|---|-------------------|-------------------|-------------------|---------------------|-------------------|-------------------|-------------------|
| | Ethanol | | | | Water | | | |
| | B.s. | E.c. | K.p. | S.a. | B.s. | E.c. | K.p. | S.a. |
| ST | 0.45 ^a | 3.13 ^a | 3.13 ^a | 1.30 ^a | 12.5 ^a | - | - | 5.21 ^b |
| BK | 0.27 ^a | 3.13 ^a | 4.69 ^a | 1.43 ^a | 6.25 ^c | - | - | 6.25 ^b |
| RB | 0.39 ^a | 3.13 ^a | 3.13 ^a | 2.60 ^a | 8.33 ^{bc} | - | - | 4.69 ^b |
| RT | 0.32 ^a | 3.13 ^a | 4.69 ^a | 1.56 ^a | 10.42 ^{ab} | - | - | 12.5 ^a |
| FL | 0.65 ^a | 3.13 ^a | 4.17 ^a | 3.13 ^a | 1.30 ^d | - | - | 0.78 ^c |
| SP | 0.16 ^a | 1.30 ^a | 2.08 ^a | 0.08 ^a | 0.78 ^d | - | - | 0.71 ^c |
| LF | 0.16 ^a | 1.56 ^a | 3.13 ^a | 1.30 ^a | 1.04 ^d | 4.17 ^a | 12.5 ^a | 5.21 ^b |

¹Plant part: BK, bark; FL, flowers; LF, leaves; RB, root bark; RT, roots; SP, seeds and pod; ST, young stems

²Bacteria: B.s., *Bacillus subtilis*; E.c., *Escherichia coli*; K.p., *Klebsiella pneumoniae*; S.a., *Staphylococcus aureus*; -, MIC >12.5 mg ml⁻¹

Table 8.2. Antibacterial activity of leaves from different trees (*Schotia brachypetala*). Treatments in each column denoted by the same letters are not significantly different ($p < 0.05$), with SE (treatment means) = 1.3043

| Collection site ¹ | Voucher number | Extract and bacteria ² tested (MIC values in mg ml ⁻¹) | | | | | | | |
|------------------------------|----------------|---|--------------------|--------------------|--------------------|--------------------|--------------------|-------------------|--------------------------------|
| | | Ethanol | | | | Water | | | |
| | | B.s. | E.c. | K.p. | S.a. | B.s. | E.c. | K.p. | S.a. |
| Hilton | McGaw58NU | 0.16 ^a | 1.56 ^c | 3.13 ^{bc} | 1.30 ^a | 1.04 ^c | 4.17 ^b | 12.5 ^a | 5.21 ^{ab} |
| UNP | McGaw85NU | 0.26 ^a | 2.61 ^c | 3.13 ^{bc} | 0.195 ^a | 0.78 ^c | 10.42 ^a | 12.5 ^a | 0.39 ^e |
| Silverglen | McGaw86NU | 1.17 ^a | 3.65 ^{bc} | 4.17 ^{bc} | 1.17 ^a | 3.13 ^{bc} | 6.25 ^b | 6.25 ^c | 2.61 ^{cd^e} |
| UNP | McGaw87NU | 0.39 ^a | 4.69 ^{bc} | 9.38 ^a | 0.33 ^a | 0.78 ^c | - | - | 0.39 ^e |
| UNP | McGaw88NU | 0.39 ^a | 8.33 ^a | 8.33 ^a | 0.26 ^a | 2.08 ^{bc} | - | - | 3.13 ^{bcd} |
| UNP | McGaw89NU | 1.30 ^a | 2.61 ^c | 2.61 ^c | 0.59 ^a | 6.25 ^a | - | - | - |
| UNP | McGaw90NU | 0.45 ^a | 1.56 ^c | 2.61 ^c | 0.33 ^a | - | - | - | 6.25 ^a |
| UNP | McGaw91NU | 0.33 ^a | 2.35 ^c | 3.13 ^{bc} | 0.195 ^a | 3.13 ^{bc} | - | - | 4.17 ^{abc} |
| UNP | McGaw92NU | 0.33 ^a | 2.61 ^c | 3.13 ^{bc} | 0.26 ^a | 4.17 ^{ab} | 6.25 ^b | 12.5 ^a | 4.17 ^{abc} |
| Scottsville | McGaw94NU | 0.78 ^a | 3.13 ^{bc} | 4.17 ^{bc} | 0.78 ^a | 1.56 ^c | 6.25 ^b | 6.25 ^c | 1.56 ^{de} |
| Umfolozi | McGaw95NU | 0.52 ^a | 5.21 ^b | 5.21 ^b | 0.33 ^a | 2.35 ^{bc} | 9.38 ^a | 9.38 ^b | 2.35 ^{cde} |

¹Collection site: UNP, Botanic Gardens, University of Natal Pietermaritzburg

²Bacteria: B.s., *Bacillus subtilis*; E.c., *Escherichia coli*; K.p., *Klebsiella pneumoniae*; S.a., *Staphylococcus aureus*; -, MIC >12.5 mg ml⁻¹

Table 8.3. Antibacterial activity of *Schotia brachypetala* leaves (voucher number *McGaw85NU*) collected monthly from the same tree. Treatments in each column denoted by the same letters are not significantly different at the 5 % level, with SE (treatment means) = 1.593

| Collection time | Extract and bacteria ¹ tested (MIC in mg ml ⁻¹) | | | | | | | |
|-----------------|--|--------------------|--------------------|--------------------|--------------------|---------------------|---------------------|--------------------|
| | Ethanol | | | | Water | | | |
| | B.s. | E.c. | K.p. | S.a. | B.s. | E.c. | K.p. | S.a. |
| January | 0.32 ^a | 0.41 ^c | 0.12 ^c | 0.195 ^a | 0.52 ^b | 6.25 ^{bc} | 6.25 ^c | 0.19 ^b |
| February | 0.195 ^a | 2.61 ^{bc} | 3.65 ^b | 0.13 ^a | 1.30 ^b | 3.13 ^c | 8.33 ^{bc} | 0.45 ^b |
| March | 0.15 ^a | 4.17 ^{ab} | 2.61 ^{bc} | 0.11 ^a | 0.78 ^b | 5.21 ^{bc} | 7.29 ^{bc} | 0.65 ^b |
| April | 0.16 ^a | 4.69 ^{ab} | 5.21 ^{ab} | 0.29 ^a | 0.39 ^b | 8.33 ^b | 10.42 ^{ab} | 0.58 ^b |
| May | 0.26 ^a | 7.29 ^a | 7.29 ^a | 0.39 ^a | 0.65 ^b | 7.29 ^b | 12.5 ^a | 0.91 ^b |
| June | 0.52 ^a | 3.65 ^b | 4.17 ^{ab} | 0.65 ^a | 3.12 ^{ab} | 8.33 ^b | 10.42 ^{ab} | 4.30 ^a |
| July | 0.13 ^a | 3.13 ^{bc} | 4.17 ^{ab} | 0.42 ^a | 4.69 ^a | 12.5 ^a | 12.5 ^a | 4.69 ^a |
| August | 0.52 ^a | 4.17 ^{ab} | 5.21 ^{ab} | 0.59 ^a | 1.82 ^{ab} | 12.5 ^a | 12.5 ^a | 2.35 ^{ab} |
| September | 0.33 ^a | 5.21 ^{ab} | 7.29 ^a | 1.17 ^a | 3.12 ^{ab} | 10.42 ^{ab} | 12.5 ^a | 4.43 ^a |
| October | 0.26 ^a | 3.65 ^b | 5.21 ^{ab} | 0.55 ^a | 0.78 ^b | 5.21 ^{bc} | 12.5 ^a | 1.10 ^b |
| November | 0.16 ^a | 2.61 ^{bc} | 2.61 ^{bc} | 0.55 ^a | 0.65 ^b | 12.5 ^a | 12.5 ^a | 0.84 ^b |
| December | 0.26 ^a | 4.69 ^{ab} | 4.69 ^{ab} | 0.29 ^a | 0.78 ^b | 6.25 ^{bc} | 7.29 ^{bc} | 0.91 ^b |

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

Table 8.4. Antibacterial activity of leaves of different *Schotia* species. Treatments in the same column denoted by the same letters are not significantly different ($p < 0.05$), with SE (treatment means) = 1.457

| Collection site | Voucher number | Extract and bacteria ¹ tested (MIC in mg ml ⁻¹) | | | | | | | |
|------------------------|----------------|--|-------------------|-------------------|--------------------|-------------------|---------------------|-------------------|-------------------|
| | | Ethanol | | | | Water | | | |
| | | B.s. | E.c. | K.p. | S.a. | B.s. | E.c. | K.p. | S.a. |
| <i>S. afra</i> | McGaw96NU | 0.39 ^a | 2.61 ^a | 2.08 ^a | 0.195 ^a | 6.25 ^a | 12.5 ^a | 12.5 ^a | 0.91 ^a |
| <i>S. brachypetala</i> | McGaw58NU | 0.26 ^a | 2.61 ^a | 3.13 ^a | 0.195 ^a | 0.78 ^b | 10.42 ^{ab} | 12.5 ^a | 0.39 ^a |
| <i>S. capitata</i> | McGaw93NU | 0.39 ^a | 2.61 ^a | 3.65 ^a | 0.195 ^a | 1.04 ^b | 8.33 ^b | 12.5 ^a | 0.78 ^a |

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

Table 8.5. Antibacterial activity of *Schotia brachypetala* leaf extracts prepared from fresh and dry plant material. Treatments in the same column denoted by the same letters are not significantly different ($p < 0.05$), with SE (treatment means) = 0.614

| Plant material | Extract and bacteria ¹ tested (MIC in mg ml ⁻¹) | | | | | | | |
|----------------|--|-------------------|-------------------|-------------------|-------------------|-------------------|-------------------|--------------------|
| | Ethanol | | | | Water | | | |
| | B.s. | E.c. | K.p. | S.a. | B.s. | E.c. | K.p. | S.a. |
| Fresh | 0.32 ^a | 1.56 ^b | 3.13 ^b | 0.26 ^b | 1.56 ^a | 12.5 ^a | 12.5 ^a | 0.46 ^a |
| Dry | 1.04 ^a | 4.17 ^a | 12.5 ^a | 5.21 ^a | 0.26 ^b | 2.61 ^b | 3.13 ^b | 0.195 ^a |

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

Table 8.6. Antibacterial activity of *Schotia brachypetala* leaf extracts immediately after being prepared, after being stored in the freezer for 18 months, and extract made from leaf material stored in the dark for 18 months. Treatments in the same column denoted by the same letters are not significantly different ($p < 0.05$), with SE (treatment means) = 0.844

| Plant material ¹ | Extract and bacteria ² tested (MIC recorded in mg ml ⁻¹) | | | | | | | |
|-----------------------------|---|-------------------|-------------------|-------------------|-------------------|--------------------|-------------------|-------------------|
| | Ethanol | | | | Water | | | |
| | B.s. | E.c. | K.p. | S.a. | B.s. | E.c. | K.p. | S.a. |
| 1 | 0.26 ^a | 2.61 ^a | 3.13 ^a | 0.20 ^a | 0.78 ^a | 10.42 ^a | 12.5 ^a | 0.39 ^b |
| 2 | 0.16 ^a | 1.56 ^a | 3.13 ^a | 1.30 ^a | 1.04 ^a | 4.17 ^c | 12.5 ^a | 5.21 ^a |
| 3 | 0.52 ^a | 2.61 ^a | 3.13 ^a | 0.39 ^a | 1.04 ^a | - | - | 0.52 ^b |

¹Plant material: 1 = extract prepared and screened immediately after collection and drying of material, 2 = extract stored in the freezer for 18 months after preparation, 3 = dry material stored in the dark at room temperature for 18 months before being ground and extracted

²Bacteria: B.s., *Bacillus subtilis*; E.c., *Escherichia coli*; K.p., *Klebsiella pneumoniae*; S.a., *Staphylococcus aureus*; -, MIC >12.5 mg ml⁻¹

8.4 DISCUSSION

It is known that the presence of biologically active compounds in a species may vary with the season, locality of the plant, and between plant parts. The active constituents may also be present to a greater or lesser extent in related species, or even genera. An investigation was instigated to analyse the effect of these factors on the antibacterial activity of *Schotia brachypetala*, a little-studied South African medicinal plant.

8.4.1 Plant part variation

Antibacterial activity of extracts prepared from different plant parts was similar, with most activity shown by the ethanol extracts against the Gram-positive bacteria. The antibacterial activity was highest in the leaves, seeds and pods, and bark. The TLC chromatogram showed that the ethanol extracts of these plant parts had several compounds in common. The absence of statistical differences in activity between the ethanol extracts of the plant parts was interesting, implicating the occurrence of antibacterial compounds throughout the plant. This may indicate that one or more of the active compounds are ubiquitous, and not localized to a specific plant part.

Traditional healers use decoctions of *S. brachypetala* bark or roots when treating gastrointestinal ailments. In this study, aqueous root and bark extracts displayed moderate antibacterial activity against Gram-positive bacteria, but none against the Gram-negative bacteria. The water extracts of the aerial parts (leaves, flowers, seeds and pods, but not stems) displayed much increased activity against the Gram-positive species, and in addition, the leaves showed some activity against the Gram-negative bacteria.

Although *Schotia brachypetala* is not a threatened medicinal plant, the collection of leaves instead of bark or roots for therapeutic purposes would be a less destructive and more aesthetic method of collection. The higher antibacterial activity of the leaf extracts than the bark and root extracts indicates that leaves may be preferable for medicinal use provided they have no toxicity. In southern Africa, the bark and

underground parts of medicinal plants are the most frequently utilized, leading to cases where popular medicinal plants become threatened by destructive harvesting. Plant part substitution has the potential for becoming an important strategy for the conservation of medicinal plants in South Africa (ZSCHOCKE *et al.*, 2000). Upon investigating the possible plant part substitution of four of the most threatened South African medicinal plants, *Eucomis autumnalis* (bulb), *Siphonochilus aethiopicus* (rhizome), *Ocotea bullata* (bark) and *Warburgia salutaris* (bark), ZSCHOCKE *et al.* (2000) concluded that the potential for plant part substitution is highly plant specific, but should be investigated, particularly in the case of threatened medicinal plants. The finding in this study that leaves possess superior antibacterial properties to the bark or roots provides impetus for this approach.

8.4.2 Locational variation

The MIC values of extracts prepared from different trees were comparable, with again more activity shown by the ethanol extracts against the Gram-positive bacteria. The statistically significant ($p < 0.05$) differences in activity of the aqueous extracts of leaves collected from different areas indicates that geographical location does affect the activity of *S. brachypetala*. However, the majority of extracts had low MIC values against Gram-positive bacteria.

The chromatograms of extracts from the genetically different trees were similar. The slightly different chromatogram shown by the ethanol extract of the tree growing in the Umfolozi Game Reserve may indicate additional compounds produced by the tree in response to herbivory. This tree was the only one in the study that grew in an area inhabited by browsers. *Schotia brachypetala* is a dietary plant utilised by giraffe, kudu and impala (VAN HOVEN and FURSTENBURG, 1992). In this area, *S. brachypetala* may retain secondary metabolites that confer an advantage by being unpalatable to predators.

8.4.3 Seasonal variation

Antibacterial activity was present to some degree at all times of the year, particularly in the case of ethanol and water extracts which were generally highly active against the Gram-positive bacteria *B. subtilis* and *S. aureus*. The TLC analysis revealed little variation in chemical composition of each of the twelve ethanol extracts prepared during each month of the year. This reasonable degree of constancy in antibacterial activity regardless of the season is desirable for traditional healers as they may be assured of little variation in medicinal value of the plant.

8.4.4 Species variation

Schotia afra and *S. capitata* showed similar antibacterial activity to that of *S. brachypetala*. ELOFF (1999a) advocated the practice of screening closely related taxa or different populations to discover plants with a high concentration of the target component. In this case, it appears that the *Schotia* species screened have a comparable degree of antibacterial activity, and hence similar levels of active compounds. The TLC fingerprints revealed that these *Schotia* species contain a closely related chemical composition, thus supporting the chemotaxonomic theory that similar compounds are likely to be present in plants that are taxonomically closely related.

8.4.5 Variation after extraction of fresh or dry material

There were significant differences ($p < 0.05$) in the antibacterial activity of ethanol and water extracts prepared from fresh or dried plant material when tested against most of the bacteria. It was interesting to note that the ethanol extract of the fresh material was more active than that of the dried material, while the reverse was true for the water extracts. The three additional compounds present in the dried material extract are possibly breakdown products, perhaps of chlorophyll as they fluoresced a deep red colour under ultraviolet light at 366 nm.

8.4.6 Effect of storage of plant material and extracts

The similarity of the TLC chromatograms of the ethanol extract screened immediately after preparation, and the extract stored in the freezer for 18 months after preparation is noteworthy. It indicates that the chemical composition of the prepared extracts remains fairly stable upon freezing. This is reflected also in the MIC values, as there were no significant differences ($p < 0.05$) in the antibacterial activity of the two different extracts. Upon storage at room temperature, the leaf material appeared to lose compounds, possibly owing to the breakdown of chemicals in the leaves. However, the antibacterial activity was not significantly different from that of the extracts stored in the freezer or assayed directly after preparation, so storage of the leaf material does not seem to affect the antibacterial activity of *S. brachypetala* leaves.

The effect of storage on the activity of medicinal plants is important as these plants are often stored in markets, or by traditional healers. ELOFF (1999a) showed that the antibacterial components of *Combretum erythrophyllum* are very stable in the dry state, as MIC values remained constant for different plants over a number of years. The compounds responsible for the antibacterial nature of *C. erythrophyllum* have not yet been identified. ELOFF (1999a) prepared extracts from herbarium specimens of *Helichrysum pedunculatum* (Asteraceae), which contains the fatty acids linoleic and oleic acids as the active antibacterial components (DILIKA *et al.*, 2000). ELOFF (1999a) analysed the antibacterial activity of the specimen extracts and concluded that fatty acids appear to be stable in dry specimens stored under herbarium conditions. The antibacterial compounds in *Schotia brachypetala* leaves are fatty acids (CHAPTER 9). It would seem that these fatty acids are stable when the dried, powdered plant material is stored in the dark at a constant temperature, and also as an extract stored at -15°C . Many types of secondary plant products are stable if stored under herbarium conditions in the dark and at relatively low temperatures (ELOFF, 1999a). Samples of herbarium material have been shown to still contain stable compounds such as amino acids, flavonoids, sweetening agents, volatile oils and alkaloids (PHILLIPSON, 1982).

8.4.7 TLC fingerprinting

TLC fingerprinting is a fast, relatively cheap and effective method to obtain a characteristic analytical fingerprint of a plant extract (WAGNER and BLADT, 1996), although a more sophisticated study could be performed using HPLC and GC-MS analysis in addition to TLC. TLC fingerprinting of the acidic triterpenoid fraction of leaf extracts in the genus *Combretum* (Combretaceae) has been used to assist in elucidating the taxonomy of the genus (CARR and ROGERS, 1987; ROGERS and COOMBES, 1999). ROGERS *et al.* (2000) showed that leaf extracts of fourteen species of *Maytenus* produced unique chemical fingerprints, supporting their specific distinctness, although close chemical relationships between certain species were established. In the present study of the three *Schotia* species, the TLC fingerprints were very similar, particularly in the case of *S. brachypetala* and *S. capitata*, so it would be difficult to distinguish between these species using only TLC.

TLC chromatograms of different samples of the same species from varying geographical locations showed a high level of consistency, both in the study of *Combretum* species (CARR and ROGERS, 1987) and *Maytenus* species (ROGERS *et al.*, 2000). The *Schotia* extracts in this location effect study also showed little genetic variation in chemical composition. TLC fingerprinting to assist in identification can therefore be applied to these genera and probably others, although not all plant taxa may be suitable for the application of this technique. ZSCHOCKE *et al.* (2000) successfully used TLC analysis to compare the chemical composition of various plant parts of four threatened South African medicinal plants. A similar chemical composition, as well as biological activity, is important if alternative plant parts such as leaves are to be used instead of bark or underground parts of the plant. In this investigation, TLC fingerprinting allowed the correlation of similar chemical composition with similar levels of antibacterial activity.

8.5 CONCLUSION

The TLC fingerprinting of the ethanol extracts proved to be useful, with the chromatograms supplying comparative information on the chemical composition of the *Schotia* extracts. Extracts with a similar chemical composition tended to display similar levels of antibacterial activity.

Most antibacterial activity was shown by *Schotia brachypetala*, *S. afra* and *S. capitata* extracts against Gram-positive bacteria. Gram-negative bacteria are more commonly associated with stomach ailments than Gram-positive species. The ethanol extracts were generally more active than the aqueous extracts, but despite this the water extracts still possessed good antibacterial activity. Traditional healers use aqueous decoctions in preparing treatments for stomach ailments. The antibacterial activity shown by *Schotia* extracts substantiates the use of the plants by indigenous healers for treating dysentery and diarrhoea.

The two other *Schotia* species, *S. afra* and *S. capitata* included in the study both displayed good antibacterial activity and similar chromatograms, lending support to the chemotaxonomic argument.

Little variation in the biological activity of the tree with respect to season and locality is important so that healers and scientific investigators may be sure of constant activity whenever and wherever material is collected. In this study, there was not much variation in activity with season, but some variation with differing locality. Despite this, there was still antibacterial activity present in all the specimens tested. The dried, ground plant material, and the frozen plant extracts of *S. brachypetala* retained their antibacterial activity over time. This implies that the active constituents are stable, both in the dried plant and as an extract. Plant material stored in a cool, dark, dry environment will therefore maintain these active properties, which has beneficial implications for traditional healers and collectors.

CHAPTER 9

ISOLATION AND IDENTIFICATION OF ANTIBACTERIAL FATTY ACIDS FROM *SCHOTIA BRACHYPETALA*

9.1 INTRODUCTION

9.1.1 *Schotia brachypetala*: biological activity and chemical constituents

There have been few reports on the chemistry and biological activity of *Schotia* species. Astringent tannins in the bark of *S. brachypetala* may account for some of the activity of the plant (BRUNETON, 1995). The wood dust and root are also believed to contain tannins (WATT and BREYER-BRANDWIJK, 1962). Stilbenes and phenolics [(E)-1-(3,5-dihydroxyphenyl)-2-(3,4,5-trihydroxyphenyl)-ethylene and (Z)-1-(3,5-dihydroxyphenyl)-2-(3,4,5-trihydroxyphenyl)-ethylene] are reported to be present in *S. brachypetala* (GLASBY, 1991). Polyhydroxystilbenes have been isolated from the heartwood of the tree (DREWES, 1971; DREWES and FLETCHER, 1974). The main constituent is *trans*-3,3',4,5,5'-pentahydroxystilbene, with small amounts of *trans*-3,3',4,5'-tetrahydroxystilbene, catechin, epicatechin, and trace amounts of *cis*-3,3',4,5,5'-pentahydroxystilbene. Many stilbenes have antibiotic effects (VAN WYK *et al.*, 1997), but the biological activity of the *Schotia* stilbenes has not been reported. BONNIER (1954) described the inhibition by *Schotia* species of a *Rhizobium* strain specific for alfalfa, while strains specific for soybeans, garden peas and clover were unaffected.

In the initial screening of plants used in South Africa for treatment of gastrointestinal disorders, *S. brachypetala* demonstrated good antibacterial activity against a range of bacteria (CHAPTER 5; MCGAW *et al.*, 2000a). This species was selected for further investigation using bioassay-guided fractionation to discover some of the

compounds responsible for antibacterial activity because of its wide usage in traditional medicine and because of the lack of previous investigation on this species. Although the roots are used in traditional medicine, the leaves possess antibacterial activity superior to that expressed in the roots (CHAPTER 8; MCGAW *et al.*, 2001). For reasons of availability, further investigation was continued with the leaves.

9.2 MATERIALS AND METHODS

9.2.1 Bulk extraction

Leaves of *Schotia brachypetala* Sond. were harvested from a young tree (about 12 years old) growing in the Botanic Gardens, UNP, in August 2000. A voucher specimen (McGaw85NU) was deposited at the Herbarium of the University of Natal, Pietermaritzburg. The leaves were dried at 50°C and ground to a powder using a Little Hippo mill.

The powdered leaves (1.2 kg) were extracted with 3 l ethanol by sonication for 30 min, and overnight maceration. The extract was filtered through a Büchner funnel and Whatman No. 1 filter paper, and the leaves were re-extracted twice, with 0.5 l ethanol each time. The filtration was repeated, and the solvent evaporated under reduced pressure at 40°C.

9.2.2 Bioassay-guided fractionation for isolation of active compounds

Throughout the isolation procedure, all solvents used were redistilled using a rotary evaporator.

The antibacterial activity of fractions after each purification stage was tested using the direct bioautography assay (HAMBURGER and CORDELL, 1987). In this assay, an overnight culture of test bacteria in 20 ml MH broth was pelleted by centrifugation

at 3 000 g for 10 min and resuspended in 10 ml fresh MH broth. This suspension was sprayed on a developed TLC plate and incubated at 37°C overnight. A 2 mg ml⁻¹ solution of INT (iodonitrotetrazolium violet) was then sprayed on the plate and incubated to detect the areas of bacterial growth inhibition. Antibacterial compounds on the TLC plate were visible as white spots against a deep red background, as bacterial growth reduces the tetrazolium salt to a red formazan product. The test organism was *Staphylococcus aureus* (ATCC 12600).

9.2.2.1 Liquid-liquid partitioning

The extract was dissolved in ethanol (400 ml) and water (100 ml), and partitioned with hexane (500 ml) in a 2 l separating funnel. This was repeated a further three times. The aqueous fraction was subsequently partitioned with dichloromethane (500 ml) three times.

9.2.2.2 Vacuum Liquid Chromatography

The hexane portion was fractionated by Vacuum Liquid Chromatography (VLC) on silica gel 0.04-0.063 mm (Merck). A column 27 cm in length and with an internal diameter of 6 cm was packed with 140 g silica. A hexane:ethyl acetate gradient solvent system with increasing amounts of ethyl acetate was used (400 ml per fraction); fr. I: 100 % *n*-hexane (490 mg), fr. II: 97.5% *n*-hexane (530 mg), fr. III: 95% *n*-hexane (7800 mg), fr. IV: 92.5% *n*-hexane (2320 mg), fr. V: 90% *n*-hexane (1570 mg), fr. VI: 87.5% *n*-hexane (2180 mg), fr. VII: 85% *n*-hexane (1570 mg), fr VIII: 82.5% *n*-hexane (790 mg), fr. IX: 80% *n*-hexane (890 mg), fr. X: 77.5% *n*-hexane (1100 mg), fr XI: 75% *n*-hexane (860 mg), fr. XII: 72.5% *n*-hexane (1000 mg), fr. XIII: 70% *n*-hexane (860 mg), fr. XIV: 67.5% *n*-hexane (940 mg), fr. XV: 65% *n*-hexane (1010 mg), fr. XVI: 62.5% *n*-hexane (970 mg), fr. XVII: 60% *n*-hexane (360 mg), fr. XVIII: 57.5% *n*-hexane (660 mg), fr. XIX: 55% *n*-hexane (520 mg), fr. XX: 52.5% *n*-hexane (420 mg), fr. XXI: 50% *n*-hexane (970 mg), fr. XXII: 0% *n*-hexane (1460 mg).

9.2.2.3 Chlorophyll extraction

Chlorophyll was separated from each of the two sets of combined fractions by column chromatography over Sephadex LH-20 (Pharmacia). A column of length 75 cm, and internal diameter of 2.5 cm was used. Sephadex was packed to a height of 45 cm in the column. The solvent system employed was cyclohexane:dichloromethane:methanol (7:4:1). Fractions of approximately 10 ml were collected using a fraction collector (Gilson FC 203B). The active compounds were detected using TLC and the bioautography assay.

9.2.2.4 Gravity-assisted Column Chromatography

Column chromatography was performed on the first combined set of active fractions, referred to as fraction A. A column of 71 cm length and 2.5 cm internal diameter packed with 125 g silica gel 0.04-0.063 mm (Merck) was used. One hundred ml of *n*-hexane was run through the column, followed by 500 ml each of 80% *n*-hexane, 75% *n*-hexane, 66.6% *n*-hexane and 50% *n*-hexane in ethyl acetate. The column was washed with 600 ml ethyl acetate. A fraction collector (Gilson FC 203B) was set to collect fractions of approximately 10 ml each. TLC and bioautography were again used to detect the presence of the active compound.

9.2.2.5 Preparative Thin Layer Chromatography

Approximately 20 mg of fraction A were applied to each of ten TLC plates (Merck glass plates, 20 x 20 cm, Silica gel 60 F₂₅₄). The solvent system was hexane:ethyl acetate (2:1), and plates were developed to a height of 15 cm. A strip of one TLC plate was cut off with a glass-cutting knife, and sprayed with AS spray reagent. The TLC plate strip was then heated for 10 min at 100°C. The active compound is not detectable under visible or ultraviolet light at 365 nm, but is faintly visible under ultraviolet light at 254 nm and is present as a vivid green stain after spraying with AS spray. The zone containing the active fraction on the unsprayed TLC plates was scraped off the plates, and the compound (A) eluted from the silica with ethanol.

Fraction B was also subjected to preparative TLC. The same procedure as for fraction A was followed. Compound B also appeared green upon spraying with AS spray.

The purity of the two compounds was confirmed by TLC using various solvent systems. Using a solvent system of hexane:ethyl acetate (2:1), compound A was visible at $R_f = 0.49$. With a solvent system of hexane:ethyl acetate (1:2), compound A appeared at $R_f = 0.81$, and compound B was visible at $R_f = 0.56$.

9.2.3 Identification of purified active compound

High Resolution Mass Spectrometry (MS) using a VG70-SEQ spectrometer was performed. Nuclear Magnetic Resonance Spectroscopy (^1H NMR and ^{13}C NMR) was carried out to confirm the structure of the compound. A Varian Unity Inova 500 MHz spectrometer was used.

The Minimal Inhibitory Concentration (MIC) values of the pure compounds were determined against the Gram-positive *Bacillus subtilis* (ATCC 6051) and *Staphylococcus aureus* (ATCC 12600) and the Gram-negative *Escherichia coli* (ATCC 11775) and *Klebsiella pneumoniae* (ATCC 13883). The microplate method of ELOFF (1998a) in 96-well microtiter plates (CHAPTER 5, Section 5.2.3) was used.

9.3 RESULTS

9.3.1 Bulk extraction

The bulk extraction of the leaves of *S. brachypetala* yielded a concentrated oily, dark green residue of 100.6 g (8.4 %).

9.3.2 Bioassay-guided fractionation for isolation of active compounds

9.3.2.1 Liquid-liquid partitioning

Bioautography revealed the presence of antibacterial compounds in the hexane extract after the partitioning step. The mass of the hexane residue after removal of the solvent was 34.95 g.

9.3.2.2 Vacuum Liquid Chromatography

After the VLC step in the isolation of compounds, bioautography revealed the presence of antibacterial compounds eluted in fractions 8 - 15 (which were combined to produce fraction A), and fractions 16 - 22 (combined separately to produce fraction B). These groups of fractions each contained an active compound at a different R_f value on a TLC plate. The TLC and bioautographic analysis of the VLC fractions is represented in Plate 9.1. The antibacterial compounds A and B are indicated with arrows on the TLC chromatograms.

9.3.2.3 Chlorophyll extraction

Filtration of each of the two residues (A and B) through Sephadex allowed the chlorophyll to be removed in the first few fractions, after which the active compounds eluted from the column. The active fractions from fraction A after elution through Sephadex were again combined and dried to produce a residue with a mass of 393.5 mg. The combined active fractions resulting from the separation of fraction B over Sephadex had a mass of 198 mg.

9.3.2.4 Gravity-assisted Column Chromatography

Bioautography on the fractions resulting from the gravity-assisted column chromatography of fraction A revealed the presence of the active compound in fractions 105-191. On combining and drying these fractions, a residue of 198.3 mg (again referred to as fraction A) was produced.

9.3.2.5 Preparative Thin Layer Chromatography

Following preparative TLC on fraction A, the mass of the isolated compound A was 18.7 mg. Preparative TLC on the residue of fraction B resulted in an antibacterial compound B of mass 9 mg.

9.3.3 Identification of purified active compound

NMR and MS resulted in the identification of two separate antibacterial mixtures of fatty acids. The major compounds were 9,12,15-octadecatrienoic acid (linolenic acid) (42%) in the case of compound A, and methyl-5,11,14,17-eicosatetraenoic acid (37%) in the case of compound B.

The MIC values of the isolated fatty acids, as well as those of linolenic acid purchased from Sigma, are presented in Table 9.1. The MICs of the isolated linolenic acid were not as low as those of the linolenic acid standard from Sigma. This was to be expected as the isolated fatty acids were not completely pure, but contaminated with smaller amounts of other fatty acids. Both compounds displayed good bactericidal activity against Gram-positive *B. subtilis* and *S. aureus*, and a lesser degree of activity against Gram-negative *E. coli* and *K. pneumoniae*.

Table 9.1. MIC values (mg ml^{-1}) of fatty acids

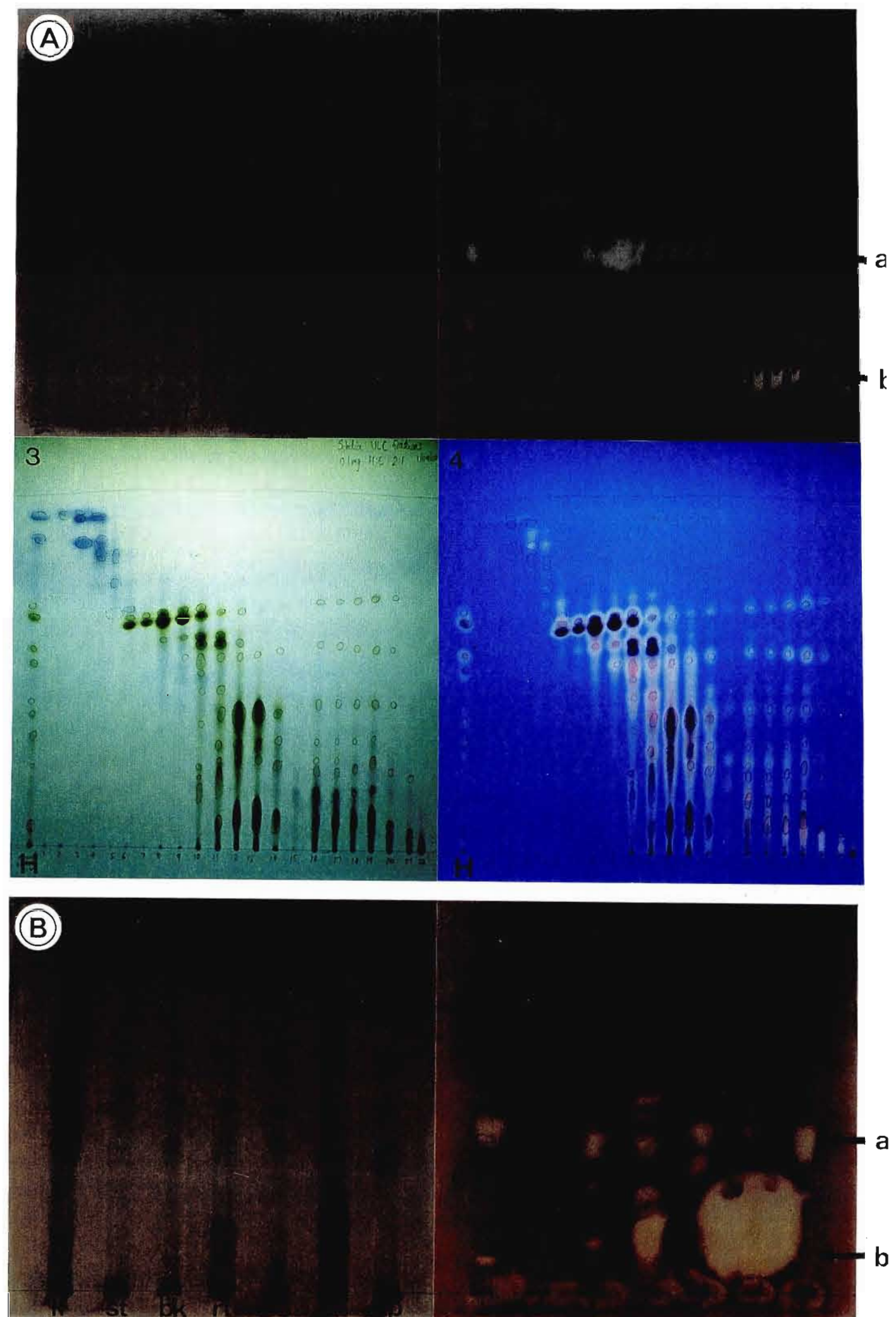
| Microorganism tested | MIC of compound | | |
|----------------------|---------------------------|------------------------|-------------------------------------|
| | linolenic acid (isolated) | linolenic acid (Sigma) | methyl-5,11,14,17-eicosatetraenoate |
| <i>B subtilis</i> | 3.13 | 0.049 | 0.78 |
| <i>E. coli</i> | 3.13 | 3.13 | 3.13 |
| <i>K. pneumoniae</i> | 3.13 | 1.56 | 3.13 |
| <i>S. aureus</i> | 1.56 | 0.39 | 0.78 |

Plate 9.1. TLC separation (A) of the VLC fractions of the hexane fraction from the liquid-liquid partitioning step, viewed (1) after staining with anisaldehyde, (3) under UV_{254 nm} and (4) under UV_{366 nm}. The bioautography plate (2) shows white areas where bacterial growth was inhibited. The arrows indicate the two fatty acid compounds a (9,12,15-octadecatrienoic acid) and b (methyl-5,11,14,17-eicosatetraenoate) isolated from *S. brachypetala*. The solvent system used was hexane:ethyl acetate (2:1).

[H = hexane extract from the liquid-liquid partitioning step before being submitted to VLC]

TLC separation (B) of the different plant parts of *S. brachypetala* viewed (1) after staining with anisaldehyde. The bioautography plate (2) shows the position of the two subsequently isolated fatty acids, indicated by the arrows. It is clear that these compounds are present in the leaves, bark and roots; the roots possess additional antibacterial compounds, as do the flowers. The solvent system used was hexane:ethyl acetate (2:1).

[lf = leaf, st = stem, bk = bark, rt = root, rb = root bark, fl = flower, sp = seeds plus pods]



9.4 DISCUSSION

9.4.1 Isolation and identification of active compounds

The bulk extraction of *S. brachypetala* leaves and the subsequent bioassay-guided fractionation to isolate antibacterial compounds resulted in the identification of two fatty acids, namely 9,12,15-octadecatrienoic acid and methyl-5,11,14,17-eicosatetraenoate. Unsaturated fatty acids are widespread in both leaf and seed oils of many plant species (HARBORNE and BAXTER, 1993). TLC analysis and bioautography have indicated that similar antibacterial compounds are present in the leaves, roots and bark of *S. brachypetala*. Photographic evidence is shown in Plate 9.1. The position of compounds A and B (9,12,15-octadecatrienoic acid and methyl-5,11,14,17-eicosatetraenoate, respectively) are indicated by arrows. The roots possessed additional antibacterial compounds than the bark or leaves. This warrants further investigation.

9.4.2 Fatty acids as constituents of plants

Fatty acids occur mainly in bound form in plants, esterified to glycerol, as fats or lipids (HARBORNE and BAXTER, 1993). These lipids comprise up to 7 % of the dry weight in leaves in higher plants, and are important membrane constituents in chloroplasts and mitochondria (HARBORNE and BAXTER, 1993). The seeds and fruits of many plants also contain lipids in considerable amounts, providing a storage form of energy to use during germination (HARBORNE and BAXTER, 1993).

The common fatty acids in plants are either saturated or simple unsaturated compounds of C₁₆ or C₁₈ chain length (HARBORNE and BAXTER, 1993). Palmitic acid (C₁₆) is the major saturated acid in leaf lipids, also occurring in some seed oils, while stearic acid (C₁₈) is less prominent in leaf lipids but is a major saturated acid in seed fats in several plant families (HARBORNE and BAXTER, 1993). Unsaturated acids based on C₁₆ and C₁₈ are widespread in leaf and seed oils (HARBORNE and BAXTER, 1993). The tri-unsaturated linolenic acid is common, as are linoleic and

oleic acids (HARBORNE and BAXTER, 1993). Whereas plant seeds contain a wide variety of fatty acids, those from leaf tissue are remarkably constant from plant to plant (HARWOOD, 1980). Just as the complex lipid composition of higher plant leaves is relatively constant, so the fatty acid content is also quite characteristic. Quantitatively, the major fatty acids are palmitic, linoleic and, in particular, α -linolenic acids (HITCHCOCK and NICHOLS, 1971; HARWOOD, 1980). The membranes of chloroplasts contain exceptionally high (about 90 % in some lamellae) percentages of α -linolenic acid (HITCHCOCK and NICHOLS, 1971; HARWOOD, 1980).

In general, fatty acids do not exist as free carboxylic acids because of their affinity for many proteins (GURR and JAMES, 1980). One result of this is an inhibitory action on most enzymes. Where free acids have been reported as major constituents they are usually artefacts due to cell damage which allows lipases to act on acyl lipids of the tissue (GURR and JAMES, 1980). One major exception to this rule lies in the albumin bound fatty acids (ABFA) of mammalian blood. These are also referred to as FFA (free fatty acids) or NEFA (non-esterified fatty acids) (GURR and JAMES, 1980).

Variation in the fatty acid and sterol composition of plants has long been of interest to chemotaxonomists. Fatty acids from seeds and leaves, more than sterols, have been previously used for plant family classification into classes and subclasses (PERDETZOGLU *et al.*, 1996). Since variation in fatty acid composition is rather small among plant families, quantitative differences of the fatty acid composition have been used as chemotaxonomic tools (PERDETZOGLU *et al.*, 1996).

9.4.3 Compound A: 9,12,15-octadecatrienoic acid

All-*cis*-9,12,15-octadecatrienoic acid (α -linolenic acid, 18:3) occurs in higher plants and algae, especially as a component of galactosyl diacylglycerol (GURR and JAMES, 1980). Linolenic acid, $\text{CH}_3\text{-CH}_2\text{-CH=CH-CH}_2\text{-CH=CH-CH}_2\text{-CH=CH-}[\text{CH}_2]_7\text{-COOH}$, is the most usual form of triethenoid C_{18} acid found in seed fats (HILDITCH, 1956). It forms 50% or more of the mixed fatty acids in linseed oil, and also occurs in

other seed oils (HILDITCH, 1956).

COOPER *et al.* (1985) described the antibacterial activity of linolenic acid against *Bacillus subtilis* and *Vibrio parahaemolyticus*. The marine bacterium, *V. parahaemolyticus*, is a major cause of gastroenteritis in the countries where sea fish and its products are consumed (COOPER *et al.*, 1985). Linolenic acid had inhibitory activity against spores of *Clostridium botulinum*, *Clostridium sporogenes* and *Bacillus cereus* (ABABOUCH *et al.*, 1992). LACEY and LORD (1981) reported that 25 strains of *Escherichia coli*, 25 *Pseudomonas aeruginosa*, 16 *Proteus* spp. and 25 *Klebsiella* spp. were all resistant to 0.25 % (v/v) linolenic acid.

GIAMARELLOS-BOURBOULIS *et al.* (1995) demonstrated the inhibition of the Gram-negative *E. coli* by gamma-linolenic acid (GLA). The possible mechanism of GLA action on *E. coli* could be attributed either to an alteration of cell membrane properties induced by GLA or to the generation of free radicals from GLA, which may lead to membrane damage and ultimately bacterial death (GIAMARELLOS-BOURBOULIS *et al.*, 1995). Further studies are necessary to clarify the mechanism of GLA action on *E. coli*, and the clinical importance of these findings.

Alpha-linolenic acid was found to have strong anti-conidial germination activity against blast fungus (SEKIZAWA *et al.*, 1981). Linoleic and linolenic acid have been identified as antialgal substances in the culture medium of the green alga *Chlamydomonas reinhardtii* (MCCRACKEN *et al.*, 1980, cited by OHTA *et al.*, 1995).

The extensive use of antibiotics by medical institutions in Japan (OHTA *et al.*, 1995) and the state of Victoria in Australia (MCDONALD *et al.*, 1981), has led to outbreaks of infections by methicillin-resistant *Staphylococcus aureus* (MRSA), against which antibiotics are often ineffective. This is most likely a world-wide phenomenon. The symptoms of infection with MRSA in immunosuppressed patients are fever and diarrhoea, followed by respiratory difficulty, damage to internal organs, and sometimes death (OHTA *et al.*, 1995). Using activity-directed fractionation of methanol extracts of the green algae *Chlorococcum* strain HS-101 and *Dunaliella*

primolecta, OHTA *et al.* (1995) isolated the polyunsaturated fatty acid, α -linolenic acid (C_{18:3}), which showed antibacterial activity against MRSA. LACEY and LORD (1981) showed that *S. aureus* was inhibited by linolenic acid, while *S. epidermidis* was relatively resistant. LACEY and LORD (1981) recommended that the possibility of therapeutic use of linolenic acid as an antibacterial agent should be explored. Linolenic acid, although present in small quantities in human skin (WILKINSON, 1972, cited by LACEY and LORD, 1981) may well be an important naturally occurring antibacterial agent, and its presence could explain why pathogenic staphylococci are rarely found on intact skin (LACEY and LORD (1981).

9.4.4 Compound B: methyl-5,11,14,17-eicosatetraenoate

KNAPP and MELLY (1986) found that several Gram-positive species, including *Bacillus megaterium*, *S. aureus* and *S. megaterium*, were susceptible to arachidonic acid, an isomer of 5c,11c,14c,17c-eicosatetraenoic acid (20:4). All-*cis*-5,8,11,14-eicosatetraenoic acid (arachidonic acid) occurs as a major component of animal lipids and some algae, especially as a component of phospholipids (GURR and JAMES, 1980).

RAKOFF (1993) stated that methyl 5,11,14,17-eicosatetraenoate (5,11,14,17 - 20:4) is potentially an intermediate in the conversion of linolenate (9,12,15 - 18:3) to eicosapentaenoate (5,8,11,14,17 - 20:5). DE ALANIZ *et al.* (1976) and GASPAR *et al.* (1977) reported that hepatoma cells converted α -linolenic acid to octadeca-6,9,12,15-tetraenoic acid (18:4), eicosa-11,14,17-trienoic acid (20:3), eicosa-8,11,14,17 and 5,11,14,17- tetraenoic acids (20:4) and eicosa-5,8,11,14,17-pentaenoic acid (20:5), and also to myristic, palmitic, palmitoleic, stearic and oleic acids.

9.4.5 Fatty acids as antimicrobial agents

Although they generally function as anionic surface agents, the bactericidal and antifungal properties of fatty acids are well known (KABARA *et al.*, 1972). There

exist volatile fatty acid- (VFA-) producing anaerobic bacteria (mainly *Bacteroides*) in the intestines of man and other animals, and these VFAs appear to be self-limiting substances of excessive multiplication of bacteria (PROHÁSZKA and BARON, 1982). There are insufficient data on the role of the antibacterial activity of VFA in enteric diseases of domestic animals and man. In an effort to address this, PROHÁSZKA and BARON (1982) studied the VFA-associated antibacterial activity of the large-intestinal contents of healthy rabbits and swine *in vitro*. The total detectable quantity of volatile fatty acids (VFA) represented the sum of 3 VFA components: acetic acid (C₂), propionic acid (C₃) and butyric acid (C₄) (PROHÁSZKA and BARON, 1982). The results indicated that the VFA in the large intestine of rabbits and swine have inhibitory activity not only towards *E. coli*, but also towards other members of the Enterobacteriaceae, including *Salmonella*, *Shigella*, *Proteus* and *Klebsiella* (PROHÁSZKA and BARON, 1982). From studies on human faeces, PROHÁSZKA and BARON (1982) drew the conclusion that the VFA concentration present in the large intestine of humans is similar to that found in the intestine of swine, so presumably the antibacterial effect of the human VFA is similar to that of the VFA of the swine.

OHTA *et al.* (1995) demonstrated the antibacterial activity of α -linolenic acid (18:3) against MRSA. MCDONALD *et al.* (1981) reported on the susceptibility of several strains of MRSA to linolenic acid and hydrolysed linseed oil (containing 52% linolenic acid in the ester form). MCDONALD *et al.* (1981) described the potential role of preparations containing hydrolysed linseed oil in the eradication of staphylococcal infection, and in the prophylaxis of infection in debilitated patients.

The possibility of the therapeutic use of linolenic acid as an antibacterial agent should be explored (LACEY and LORD, 1981). α -Linolenic acid is generally considered to have low toxicity, so it may potentially be administered to patients infected with MRSA as a dietary treatment (OHTA *et al.*, 1995). It is naturally occurring, and hence unfavourable reactions would not be anticipated, it should not destroy the commensal flora, resistance to it would not develop, and there would apparently be no risk of resistance to antibiotics developing during its use (LACEY

and LORD, 1981).

NIEMAN (1954) stated that some natural defence systems of higher organisms, such as the self-disinfection of the skin, are due at least partly to the presence of antibacterial fatty acids *in situ*. However, the potential antibacterial activity of fatty acids *in vivo* may be neutralized by adsorption on proteins in the bloodstream (NIEMAN, 1954).

Other fatty acids with antibacterial activity have been isolated from plants using bioassay-guided fractionation. CERDEIRAS *et al.* (2000) identified 11-O-(6'-O-acetyl- β -D-glucopyranosyl)-stearic acid as the main antibacterial component of aerial parts of *Ibicella lutea*. This fatty acid derivative showed an interesting antibacterial activity, being active against *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus* (CERDEIRAS *et al.*, 2000). DILIKA *et al.* (2000) described the antibacterial activity of linoleic and oleic acids isolated from the leaves of *Helichrysum pedunculatum*. Linoleic and oleic acids inhibited the growth of Gram-positive *Bacillus subtilis*, *Micrococcus kristinae* and *Staphylococcus aureus* and linoleic acid also showed activity against *B. cereus* and *B. pumilis* (DILIKA *et al.*, 2000). Both acids displayed no activity against Gram-negative *Enterobacter cloacae*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Serratia marcescens* (DILIKA *et al.*, 2000).

9.4.6 Structure- function relationships of fatty acids

There is an intimate relationship between the structure of fatty acids and their ability to function as antimicrobial agents. The most effective saturated, monounsaturated, and polyunsaturated fatty acids are those with chain lengths of C₁₂, C_{16:1} and C_{18:2}, respectively (KABARA, 1980). The number and position of double bonds is more important to fatty acids longer than 12 carbons than for fatty acids with fewer carbons (KABARA, 1980). Gram-positive bacteria are more susceptible to fatty acids than are Gram-negative bacteria. Yeasts are affected to a greater extent by fatty acids containing 10-12 carbons, while Gram-positive bacteria are more affected by

slightly longer chain lengths (KABARA, 1980). Gram-negative organisms are affected by very short chain fatty acids, that is C_6 or less (KABARA, 1980). Fatty acids with greater than eight carbons are not inhibitory to Gram-negative bacteria (KABARA, 1980).

Fatty acid esters of sucrose have been reported to have antimicrobial properties against Gram-negative bacteria, although to a much lower extent than that shown for Gram-positive bacteria (MARSHALL and BULLERMAN, 1994). Fungi are inhibited to a greater extent with acetylenic derivatives than with ethylenic derivatives of fatty acids (MARSHALL and BULLERMAN, 1994). Fatty acids esterified to monohydric alcohols have no antimicrobial activities, while esterification of fatty acids to polyhydric alcohols, such as glycerol or sucrose, increases the antimicrobial effectiveness of the fatty acids (KABARA, 1980). The fatty acid used to esterify the polyol dictates the potency of the ester. Lauric acid (C_{12}) and palmitoleic acid ($C_{16:1}$) form the most active saturated and unsaturated esters, respectively, and monoester forms are more potent than polyester forms (KABARA, 1980). MARSHALL and BULLERMAN (1994) concluded that the antimicrobial properties of sucrose fatty acid esters, in addition to other functional properties such as emulsification or stabilization, suggests potential use of these and related compounds in the food processing, cosmetic and pharmaceutical industries.

OHTA *et al.* (1995) tested ten fatty acids and their methyl esters against *Staphylococcus aureus* and MRSA. They found that palmitic acid ($C_{16:0}$), stearic acid ($C_{18:0}$) and oleic acid ($C_{18:1}$) had no activity, but fatty acids with two or more double bonds (starting with $C_{18:2}$) were active. Of the polyunsaturated fatty acids tested, the activity of γ -linolenic acid ($C_{18:3}$) was the highest, and α -linolenic acid ($C_{18:3}$), eicosapentaenoic acid ($C_{20:5}$), and docosahexaenoic acid ($C_{22:6}$) also had strong activity. The methyl esters were either inactive or much less active than the other form (OHTA *et al.*, 1995). In a similar experiment with *E. coli*, OHTA *et al.* (1995) found that all fatty acids were inactive.

In general, inhibitory properties of fatty acids appear more pronounced with longer

and more unsaturated compounds (NIEMAN, 1954). It appears that unsaturation is not essential for inhibition of fungi and mycobacteria by fatty acids (NIEMAN, 1954). The position of unsaturation affects biological activity (KABARA, 1986). The stereochemistry of the fatty acids also has an effect, with the (natural) *cis*-forms of unsaturated fatty acids exhibiting a greater antibacterial activity than the corresponding *trans*-isomers (NIEMAN, 1954; KABARA, 1986). Unsaturation in contrast to esterification is less effective with low chain fatty acids as compared to higher chain fatty acids (KABARA, 1986). Monoesters of fatty acids are more active than the corresponding fatty acid (KABARA, 1986).

9.4.7 Mechanism of antimicrobial action of fatty acids

The antimicrobial effects of fatty acids and their derivatives have been known for many years, and there have been various suggestions as to the mechanism of antibacterial action (KNAPP and MELLY, 1986). Fatty acids can inhibit the growth of numerous types of bacteria, as well as protozoans, viruses and fungi (NIEMAN, 1954; KNAPP and MELLY, 1986). In general, fatty acid sensitivity is considered to be a characteristic of Gram-positive bacteria, with few Gram-negative species being susceptible (KNAPP and MELLY, 1986). Members of the Gram-negative families Neisseriaceae, Enterobacteriaceae and Parvobacteriaceae are inhibited by small amounts of fatty acids (NIEMAN, 1954).

Short-chain saturated fatty acids and long-chain polyunsaturated fatty acids appear to exert their antimicrobial effects by different mechanisms. Short-chain fatty acids are generally toxic in high concentrations in a pH-dependent manner and are known to have an adverse effect on the energy metabolism of a wide variety of microorganisms (KNAPP and MELLY, 1986). On the other hand, polyunsaturated fatty acids affect only certain types of bacteria and are toxic at much lower concentrations in a more pH-independent fashion (KNAPP and MELLY, 1986).

MARSHALL and BULLERMAN (1994) reported that the mechanism of action of sucrose esters of fatty acids appears to be biostatic rather than biocidal.

BERGSSON *et al.* (1999) tested several fatty acids and their 1-monoglycerides against *Neisseria gonorrhoeae*, a Gram-negative coccus. Lauric acid (C_{12:0}), palmitoleic acid (C_{16:1}) and monocaprin (C_{10:0}) effectively killed the pathogen. BERGSSON *et al.* (1999) hypothesized that the lipids kill the bacteria by disruption of their cell membranes (visible with electron microscopy), and because of this lipid action on biological membranes, the emergence of resistance is unlikely.

TSUCHIDO *et al.* (1985) discovered that the addition of saturated C₆, C₈, C₁₀, and C₁₂ fatty acids appeared to lyse actively growing cells of *Bacillus subtilis* 168, as judged by a decrease in the optical density of the culture. TSUCHIDO *et al.* (1985) suggested that fatty acid-induced lysis of *B. subtilis* 168 is due to the induction of autolysis by an autolytic enzyme rather than massive solubilization of the cell membrane by the detergent-like action of the fatty acids. According to TSUCHIDO *et al.* (1985), it is unclear how fatty acids induce autolysis. TSUCHIDO *et al.* (1985) stated that it is unlikely that fatty acid directly activates autolytic enzymes in the cell wall, but rather, the primary target of action of fatty acids may be the cell membrane. One explanation for the induction of autolysis by fatty acids is that the fatty acids solubilize autolysins from the membrane at relatively low concentrations; this is supported by the results of experiments on protoplasts (TSUCHIDO *et al.*, 1985). In addition, the dependency of the lytic action of fatty acids on the carbon chain length of the molecule suggests that the surface-active action of the acid might participate in the dissociation of autolysins from the membrane (TSUCHIDO *et al.*, 1985). To summarize, the short and medium chain fatty acids tested by TSUCHIDO *et al.* (1985) seem to have a dual effect on cellular lysis, depending on their concentration, with the action on the cell membrane leading to the induction of autolysis being detectable at relatively low concentrations, and the association with the autolytic enzyme itself being strong at relatively high concentrations. The mechanism of lytic action of fatty acids seems to be different from that of their bactericidal action (TSUCHIDO *et al.*, 1985).

In a subsequent study, TSUCHIDO *et al.* (1987) examined the lytic action of glycerol and sucrose esters of fatty acids with different carbon chain lengths. It was found

that glycerol dodecanoate and sucrose hexadecanoate induced autolysis in cells of *B. subtilis* 168, and during treatment a great loss of viability occurred preceding lysis (TSUCHIDO *et al.*, 1987). The esters caused morphological changes in the cells, but an apparent adaptation of the cells to the esters was noted (TSUCHIDO *et al.*, 1987). As with short- and medium-chain fatty acids (TSUCHIDO *et al.*, 1985), the lysis of *B. subtilis* cells induced with glycerol dodecanoate and sucrose hexadecanoate may be due to the action of autolytic enzymes and not to the direct action of solubilization of the bacterial membrane (TSUCHIDO *et al.*, 1987). After lysis the surviving cells grew, suggesting that these cells adapted to esters, as indicated by their renewed growth and tolerance to esters if they were added again (TSUCHIDO *et al.*, 1987). The cell death observed by TSUCHIDO *et al.* (1987) during the treatment with esters was very rapid. This suggests that the mechanism that causes death is different from that which causes lysis and that direct interaction of esters with the cell membrane may cause the cell death, although the possibility that an irreversible triggering of cell lysis induces apparent rapid death cannot be ruled out (TSUCHIDO *et al.*, 1987). The fact that cells treated with esters altered cell morphology suggests the inhibition of some process of synthesis or regulation of the cell envelope which is possibly related to the induction of autolysis (TSUCHIDO *et al.*, 1987). Glycerol and sucrose esters of fatty acids are supposed to be effective nontoxic antimicrobial agents, but the adaptation seen in this study may be a problem if this phenomenon exists in microorganisms that cause food spoilage or that produce toxins (TSUCHIDO *et al.*, 1987).

Fatty acids inhibit the growth and oxygen consumption of *Bacillus subtilis* in nutrient medium by inhibiting the transport of substances such as amino acids and keto acids through the cellular membrane (FREESE *et al.*, 1973). The effectiveness of inhibition increased with increasing chain length (FREESE *et al.*, 1973). In contrast, the inhibitory effect on *E. coli* increased only up to a fatty acid chain length of six, while long-chain fatty acids had no effect (FREESE *et al.*, 1973).

With the exception of mycoplasmas, bacteria produce cell walls (NIKAIDO and VAARA, 1985). Gram-negative bacteria are typically surrounded by an outer

lipopolysaccharide (LPS) layer (OSBORN, 1969). This outer layer is very important in the physiology of Gram-negative bacteria, making them resistant to various host defence factors which are toxic to Gram-positive bacteria (NIKAIDO and VAARA, 1985). This is particularly relevant in the case of enteric Gram-negative bacteria living in the intestinal tract of animals. The outer membrane of enteric and some other Gram-negative bacteria acts as a strong permeability barrier to many antibiotics that are effective against other bacteria (NIKAIDO and VAARA, 1985). Even when the diffusion of the antibiotic is only slowed down by the presence of the outer membrane, the bacteria can then inactivate the small amount of penetrating antibiotic rather than attempt to inactivate the almost infinite amount of antibiotic present in the surrounding medium (NIKAIDO and VAARA, 1985).

The LPS layer prevents the entry and subsequent inhibition by intermediate and long-chain fatty acids (SHEU and FREESE, 1973). SHEU and FREESE (1973) suggested that this protection would be essential for the survival of bacteria in the intestinal tract where such fatty acids are produced by the digestion of fats. However, these bacteria are not resistant to short-chain fatty acids up to hexanoate, so these fatty acids may therefore be useful for the treatment of infection by Gram-negative bacteria.

KNAPP and MELLY (1986) found that the presence or absence of a polysaccharide capsule did not alter the susceptibility of *Staphylococcus aureus* to arachidonic acid. Upon treating Gram-negative *Neisseria gonorrhoeae* and *Haemophilus influenzae* with arachidonic acid, KNAPP and MELLY (1986) observed, using electron microscopy, a loss of ribosomes, increased irregularity of outer membranes, separation of outer and inner membranes, loss of cell shape and complete cell disorganization and disruption. There were no morphological changes in *E. coli* resistant to the toxic effects of arachidonate (KNAPP and MELLY, 1986). After treating the Gram-positive *Staphylococcus aureus* with arachidonate, KNAPP and MELLY (1986) saw no visible alteration in cell wall structure, but the arachidonate-induced cell killing was associated with the appearance of peripheral cytoplasmic condensations similar to those seen in the susceptible Gram-negative organisms.

The Gram-negative species sensitive to arachidonic acid were the same species known to have a more permeable outer membrane than other Gram-negative species (KNAPP and MELLY, 1986). The presence of LPS or other components of the Gram-negative cell envelope is probably not the sole factor in determining arachidonic acid resistance (KNAPP and MELLY, 1986). KNAPP and MELLY (1986) suggested the possibility that in the resistant species, arachidonic acid does not have access to critical cell structures or that potentially toxic metabolites of arachidonic acid are formed in a location or manner that causes less cell damage. According to KNAPP and MELLY (1986), the killing of *S. aureus* by arachidonic acid appears to involve a peroxidation of the fatty acid catalyzed by bacterial Fe^{2+} and H_2O_2 . A high concentration of arachidonic acid-derived free radicals could be expected to rapidly overcome local antioxidant defences and become involved in deleterious reactions with many bacterial macromolecules (KNAPP and MELLY, 1986).

GALBRAITH and MILLER (1973a) discovered that the bactericidal activity of long chain saturated fatty acids was antagonized by alkaline earth metals. Both Gram-positive and Gram-negative bacteria reversibly adsorbed fatty acids, and uptake increased with decreasing pH value and increasing chain length (GALBRAITH and MILLER, 1973a). Upon investigating the interaction between antibacterial fatty acids and the bacterial membrane, GALBRAITH and MILLER (1973b) recorded that fatty acids of chain length greater than 10 carbons induced lysis of protoplasts at pH 7.4. The experiments of GALBRAITH and MILLER (1973b) on protoplasts implicated the cytoplasmic membrane as the site of action of the fatty acids and reflected the relative physicochemical properties of the acids on the whole cells since the order of bactericidal activity coincided with that of lytic activity.

SHEU and FREESE (1972) found that fatty acids of different chain length inhibited growth of *Bacillus subtilis*, but the effect was reduced in the presence of glycolytic compounds and reversed by transfer to medium without fatty acids. The concentration required for inhibition of growth, oxygen consumption and adenosine triphosphate (ATP) synthesis increased with decreasing molecular weight of the fatty

acids (SHEU and FREESE, 1972). SHEU and FREESE (1972) postulated that the fatty acids reversibly react with the cell membrane or cell proteins in it; they could either alter the membrane structure or uncouple the electron transport chain from two types of proteins, those used for ATP regeneration and others needed for the transport of certain compounds into the cells. In another study, SHEU *et al.* (1972) reported that acetate and other short-chain fatty acids ($C_1 - C_6$) inhibited strongly the uptake of L-serine or other L-amino acids of *Bacillus subtilis*. It was concluded that the fatty acids “uncouple” the amino acid carrier proteins from the cytochrome-linked electron transport system, to which they may be coupled via protein interaction or via a cation gradient (SHEU *et al.*, 1972). FREESE *et al.* (1973) reported that the concentration of fatty acids required to produce a certain amount of growth inhibition of *Bacillus subtilis* (at pH 6.5) decreases with increasing chain length, with saturated and unsaturated fatty acids being about equal. FREESE *et al.* (1973) presumed that the more lipophilic long chain carbons are more effectively partitioned into the cell membrane. This membrane attachment was found to be reversible (FREESE *et al.*, 1973).

SAITO and TOMIOKA (1988) compared the susceptibilities of colonial variants of various strains of *Mycobacterium avium* complex to long-chain fatty acids with a strong hydrophobicity. Smooth T variants, which have an outer, regularly structured polysaccharide layer, showed a much higher resistance to various antimicrobial agents than do the smooth D variants, which lack the outer layer (SAITO and TOMIOKA, 1988). This polysaccharide layer may be related to an impaired permeability in smooth T variants, resulting in decreased susceptibility (SAITO and TOMIOKA, 1988). The smooth D variant was more susceptible to all the test fatty acids, which included capric, lauric, oleic and linolenic acids (SAITO and TOMIOKA, 1988). SAITO and TOMIOKA (1988) noted that for *M. avium*, the fatty acid susceptibility of a particular variant correlates well with its virulence, i.e. the smooth T variant is much more virulent than is the smooth D variant. An interesting observation was that activated macrophages with an enhanced mycobactericidal activity secreted a large amount of antimycobacterial fatty acids such as oleic and linolenic acids (HUI *et al.*, 1977). SAITO and TOMIOKA (1988) stated that, since

fatty acids penetrate through cell surface structures, including the outer membrane and cell wall, before they reach their target sites and act on the target molecules on the cell membrane, the permeability of the surface structures for fatty acids may dominate the susceptibility of a given organism. The antibacterial action of fatty acids has been explained by the insertion of the nonpolar moieties of the fatty acids into the phospholipid layer of the bacterial cell membrane, causing a change in membrane permeability, alteration of the activity of some membrane proteins essential for maintenance of cellular functions, and uncoupling of the oxidative phosphorylation system (SAITO and TOMIOKA, 1988). The hydrophilic moiety of the outer membrane of Gram-negative bacteria probably prevents fatty acids from penetrating the interior target sites of *E. coli* (SAITO and TOMIOKA, 1988). It is possible that the hydrophilic nature of the polysaccharide outer layer of the *M. avium* smooth T variants, which is lacking in the smooth D variants, might reject fatty acid penetration into the cell wall and thereby inhibit their transport to the target sites on the membrane (SAITO and TOMIOKA, 1988). It is also feasible that hindrance of the permeability of antimicrobial agents and bacterial inhibitors by the polysaccharide outer layer is based on unknown functions rather than on physical and hydrophilic barriers (SAITO and TOMIOKA, 1988).

KANETSUNA (1985) reported that unsaturated fatty acids showed strong bactericidal activity against *Mycobacterium smegmatis* in low concentrations, whereas saturated fatty acids, except for lauric and myristic acids, were not very effective. Palmitic, arachidonic and linoleic acids showed strong antibacterial activity, but the relationship between the degree of unsaturation of a fatty acid and its bactericidal activity was not clear (KANETSUNA, 1985). Among the saturated fatty acids, lauric and myristic acids showed mycobactericidal activity (KANETSUNA, 1985). Macrophages secrete mainly palmitic and stearic acids (saturated) along with the unsaturated oleic and linoleic acids (KANETSUNA, 1985). KANETSUNA (1985) did not observe an inhibition of bactericidal activity of the unsaturated fatty acids when examining the effect of coexistence with saturated fatty acids. KANETSUNA (1985) concluded that the coexistence of saturated fatty acids with unsaturated fatty acids in phagosomes should not interfere with the bactericidal effect of the latter

acids. Besides free fatty acids, reactive oxygen metabolites (O_2^- and H_2O_2) have been reported to be mycobactericidal substances secreted by macrophages (KANETSUNA, 1985). Free fatty acids and reactive oxygen metabolites possibly cooperate as potent mycobactericidal substances in phagosomes (KANETSUNA, 1985). Depending on the kinds of lymphokines which activate the macrophages and also on the stage of activation of the macrophages, either fatty acids or H_2O_2 may play the main role in the intracellular killing of mycobacteria (KANETSUNA, 1985). The susceptibilities of *M. tuberculosis*, *M. bovis* and *M. kansasii* were similar to that of *M. smegmatis* (KANETSUNA, 1985).

There are increasing indications that host fatty acids influence the types of bacteria that normally colonize in various sites within the host (KNAPP and MELLY, 1986). Further studies on the exact mechanism of bactericidal effects of polyunsaturated fatty acids may allow the determination of the role this phenomenon plays in bacterial ecology and host-bacterial interactions (KNAPP and MELLY, 1986).

9.5 CONCLUSION

The antibacterial and antifungal properties of fatty acids are well known (KABARA *et al.*, 1972). The linolenic acid isolated from *Schotia brachypetala* showed activity against the test bacteria similar to that displayed by methyl-5,11,14,17-eicosatetraenoate. This is to be expected as they are both all-*cis* polyunsaturated long-chain fatty acids. Inhibitory properties of fatty acids appear more pronounced with longer and more unsaturated compounds (NIEMAN, 1954; KABARA, 1986). Also, methyl-5,11,14,17-eicosatetraenoate (5,11,14,17 - 20:4) is potentially an intermediate in the conversion of linolenate (9,12,15 - 18:3) to eicosapentaenoate (5,8,11,14,17 - 20:5), lending further support to the close structural and hence possibly functional relationship of these fatty acids (RAKOFF, 1993). There is an intimate relationship between the structure of fatty acids and their ability to function as antimicrobial agents.

In general, fatty acid sensitivity is considered to be a characteristic of Gram-positive bacteria, with few Gram-negative species being susceptible (KNAPP and MELLY, 1986). This characteristic was reflected in the present study as both isolated fatty acids displayed greater activity against Gram-positive bacteria.

The presence of antibacterial fatty acids in *S. brachypetala* supports the use of the plant by traditional healers. The same active compounds in the leaves appear to be present in the roots and bark, with some additional antibacterial compounds being present in the roots. The isolation of known compounds from active plants is a problem in the bioassay-guided fractionation of plant extracts if the aim is to discover new constituents with interesting biological activity. In these situations, early recognition of plant metabolites, at the earliest stage of separation possible, is essential in order to avoid a time-consuming isolation of common constituents. In this case, the reason for the study of *Schotia brachypetala* was to substantiate the claims by traditional healers of antibacterial activity, and to reveal the nature of the antibacterial compounds in this plant. The discovery of a new antibacterial compound would have been an added benefit.

CHAPTER 10

GENERAL CONCLUSIONS

10.1 INTRODUCTION

Traditional healers in South Africa employ an extensive range of plants for the treatment of gastrointestinal diseases. The symptoms of these ailments are easily recognized by indigenous healers and consequently, the healers have generations of experience in effectively treating diarrhoea, dysentery and intestinal parasites. A large number of traditional healers operate in rural areas of the country where access to Western doctors is limited. Many people thus depend on the healers for treatment of common afflictions such as stomach upsets.

The research presented in this thesis commenced with screening available species of medicinal plants used by southern African traditional healers for treating gastrointestinal ailments. The plants were allocated to various bioassays in accordance with their traditional use. This initial study was extended to include an investigation on the anthelmintic activity of the family Combretaceae as many members of this family are used against abdominal complaints, and good antibacterial activity is known to exist in various species. Another aspect of this research involved analyzing the variation in levels of activity of *Schotia brachypetala*, taking various factors such as plant part, season and location into account. TLC fingerprinting was a technique used in this facet of the project to study possible variation in chemical composition, which might correlate with variations in antibacterial activity. The final investigation included in the thesis was the isolation and identification of active principles in plants showing promising activity in the initial screening.

10.2 SCREENING OF PLANTS FOR BIOLOGICAL ACTIVITY

The rationalization of the use of medicinal plants by indigenous healers is an important task, particularly in developing countries where the services of traditional healers play a role in the primary healthcare system. The results presented in CHAPTERS 3, 4 and 5 indicate that a significant proportion of plants used by traditional healers for treating stomach problems are effective in *in vitro* tests. Further studies, including *in vivo* experiments and toxicity tests are necessary to gain a full understanding of the effectiveness and possible toxic nature of these remedies. The ethnopharmacological approach is a proven method of obtaining new chemical lead structures from plants. With the emerging problem of antibiotic resistance to presently used drugs, new structures with different modes of action are being investigated. The activity of plants tested in this study provides possible leads for research on the active compounds in these plants, as there is a possibility of novel structures being responsible for the activity.

More plants displayed activity against Gram-positive bacteria than against Gram-negative bacteria. This was to be expected as the surrounding protective lipopolysaccharide layer unique to Gram-negative organisms provides them with additional protection against antibacterial substances. Future research into antimicrobials against Gram-negative bacteria is a priority, as many pathogenic bacteria that are resistant to current antibiotics belong to this group. Among these bacteria are the Enterobacteriaceae, which cause diarrhoeal diseases.

Lack of biological activity in the screening tests does not necessarily indicate lack of effectiveness of the plant remedies. They may act in other ways to effect a cure, such as by stimulating the immune system of the patient, or by manufacturing internal conditions unfavourable for the multiplication of the pathogen. Also, if plants are used as part of a mixture, the synergistic effects of principles in more than one plant may cause relief from the ailment.

10.3 VARIATION IN ACTIVITY AND CHEMICAL COMPOSITION OF PLANT EXTRACTS

Intraspecific variation is a factor to take into consideration in the investigation of medicinal plants, as this may influence the results of any research into biological activity. In CHAPTER 8, the effects of season, genetics and plant part on the antibacterial activity and on the chemical composition as visualized using TLC separations, or fingerprints, of *Schotia brachypetala* were compared. It was evident that the seasonal and genetic, or locational, effect was not important with respect to activity, so the time and place of collection are not important. The active constituents were stable, both in the dried plant and as an extract, which is beneficial as plants are often stored by healers.

Upon studying three *Schotia* species, it was found that there was a chemotaxonomic relationship reflected in constant levels of antibacterial activity, and chemical composition. In other cases it may be true that biological activity is higher in related species than in the plant in which the activity was first discovered. Similarities in biological activity and chemical composition provide evidence on another level for taxonomic relationships in plants.

The anthelmintic activity of 24 species of the Combretaceae was investigated, and a substantial range in activity among the different species emerged. Activity ranged from excellent in *Combretum apiculatum*, to none in most of the species tested. However, some leads deserving of further research were yielded, with good activity present in a few other species. The family Combretaceae is known to possess other biological activities, particularly antibacterial. This study demonstrated the use of screening members of a family known to possess good activity in one area for additional biological activities.

10.4 ISOLATION AND IDENTIFICATION OF ACTIVE COMPOUNDS IN PLANTS

In CHAPTER 7, the isolation and identification of the antibacterial and anthelmintic β -asarone from *Acorus calamus* was described. This compound is known to be toxic and carcinogenic, which has negative implications for its use by Zulu traditional healers. Bioautography revealed the presence of several other, possibly more active compounds, in the plant, and these deserve further study. The toxicity of medicinal plants is a serious consideration that demands more intensive research. In particular, the long-term effects of ingested plants are generally not known.

Two fatty acids were identified to be antibacterial components of the leaves of *Schotia brachypetala* (CHAPTER 9). These compounds were also present in the roots and bark, which are the plant parts utilized by traditional healers to treat dysentery and diarrhoea. Other antibacterial compounds deserving of future research were apparent in the roots and flowers of *S. brachypetala*. Also, there may be other active principles in the aqueous fractions, and as healers often use aqueous decoctions this aspect should be worth investigating.

In research where the principal objective is to identify novel bioactive structures, a preliminary screening for common constituents with known biological activity such as fatty acids or tannins would obviate the unnecessary isolation of known compounds.

10.5 CONCLUSION

The scientific investigation of the potential biological activity of plants used by traditional healers against stomach upsets is important, but other considerations must be taken into account. The use of certain plants may be justified and subsequently promoted, but it is essential to effectively impede the spread of infectious diarrhoea, and the infestation and reinfestation by intestinal parasites. To accomplish this, it is necessary to educate people on how to avoid becoming

infected by the faecal-oral route. There will be no permanent respite from intestinal diseases, whether they are caused by viruses, bacteria, protozoa or helminths, until culturally acceptable, functional and affordable sanitation systems are provided for the collection, treatment and disposal of human excrement. Many informal settlement areas in South Africa still lack access to clean water and sanitation facilities. The work of traditional healers in these areas is exceedingly important, in the role of both educator and healer.

The results presented in this thesis represent an extensive investigation into plants used by South African traditional healers to treat stomach ailments. The value of this research lies in the scientific verification of the use of many of these plants. In two of these species, some of the compounds responsible for activity have been identified. There is much potential for future research activities in this field, as investigation of the active principles of other plants with good biological activity may yield exciting discoveries.

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SUNDAY TIMES BUSINESS TIMES, 22 April 2001

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APPENDIX 1

Nematode Growth (NG) agar:

(BRENNER, 1974)

| | | |
|---------|-------|---------------------------------------|
| NaCl | 3 g | |
| peptone | 2.5 g | |
| agar | 17 g | dissolved in 975 ml dH ₂ O |

After autoclaving,

- 1 ml cholesterol in ethanol (5 mg ml⁻¹)
- 1 ml 1M CaCl₂
- 1 ml 1M MgSO₄
- 25 ml 1M potassium phosphate buffer (pH 6.0)

are added in order

Potassium phosphate buffer (pH 6):

(BRENNER, 1974)

| | | |
|---------------------------------|----------|-----------------------------|
| KH ₂ PO ₄ | 12.014 g | |
| K ₂ HPO ₄ | 2.807 g | in 100 ml dH ₂ O |

M9 buffer:

(BRENNER, 1974)

| | |
|--------------------------------------|-------|
| Na ₂ HPO ₄ | 6g |
| KH ₂ PO ₄ | 3g |
| NaCl | 5g |
| MgSO ₄ .7H ₂ O | 0.25g |

per litre