

https://dspace.org/

The chemotaxonomy,phylogeny and biological activity of the genus Eriocephalus. L. (Asteraceae)

Njenga, Elizabeth Wanjiku

2006-11-01T13:12:01Z
http://hdl.handle.net/10539/1582

Downloaded from a DSpace repository. https://dspace.org/

THE CHEMOTAXONOMY, PHYLOGENY AND BIOLOGICAL ACTIVITY OF THE GENUS *ERIOCEPHALUS* L. (ASTERACEAE)

Elizabeth Wanjiku Njenga

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the Degree

Of

Doctor of Philosophy

Johannesburg, 2005.

DECLARATION

I declare that this thesis is my own work. It is submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted for any degree or examination at any other university. The abstracts and copies of paper(s) included are part of this work.

.....

Signature

.....

Date

DEDICATION

To Joy, Shalom and George, my lifetime friends, for their love, courage, strength and prayers that inspired me to face all the challenges…

ABSTRACT

The genus *Eriocephalus* commonly known as 'wild rosemary', 'Cape snow bush', or 'kapokbos' is a member of the family Asteraceae (tribe Anthemideae). The genus is endemic to southern Africa, with the highest concentration of species in the Western and Northern Cape. The genus comprises 32 species and a total of 42 taxa, which are distributed in South Africa, Namibia, Botswana, and Lesotho. The characters used in species delimitation are purely based on morphological variation in floral and foliar parts and are highly homoplastic due to phenotypic plasticity. In many cases these features are not sufficiently distinctive, as some taxa tend to exhibit dimorphism in some character states such as the presence of opposite and alternate leaves. In some species there is extensive intergrading of the major diagnostic characters leading to uncertainty in species delimitation. Both chemical and molecular characters were used in this study in an attempt to evaluate current species delimitations in the genus, along with species-level relationships and affinities. The genus is also economically important with some of its members used as medicinals, fodder, perfumes, and cosmetics. This warrants investigation into the phytochemistry and biological activity of these species in order to determine a scientific rationale for their traditional uses. For this reason, the antimicrobial, antiinflammatory, antioxidant activities, and inhibition of acetylcholinesterase by the volatile oils and leaf extracts of the genus, which are relatively unknown for most members of the genus, were also investigated.

Representatives of 22 species of the genus, eight of which were from Namibia and 14 from South Africa were collected from wild populations. In most cases multiple collections per population per species were considered. Aerial plant parts were hydrodistilled to obtain the essential oils, and phenolics were extracted from leaves using acetone. Essential oils were analysed by thin layer chromatography (TLC), gas chromatography (GC), gas chromatography coupled to mass spectroscopy (GC/MS), and phenolics were analysed using thin layer chromatography (TLC) and high performance liquid chromatography (HPLC/UV). Biological assays were carried out using the 5-lipoxygenase enzyme to evaluate antiinflammatory activity; disc diffusion and microtitre plate dilution assays were used to assess antimicrobial activities of selected fungi and bacteria; the TLC-DPPH and DPPH-microtitre methods were used to investigate antioxidant activities and a TLC-bioautographic assay was used for testing the inhibition of the acetylcholinesterase enzyme. Total genomic DNA was extracted from silica dried leaf material. The non-coding plastid DNA regions, the

*psb*A-*trn*H intergenic spacers and the internal transcribed spacer (ITS) region of nuclear ribosomal DNA were amplified, and sequenced and analysed using the parsimony algorithm.

The essential oils are largely comprised of acyclic, monocyclic, and bicyclic regular and irregular mono- and sesquiterpenes of various structural groups. Two hundred compounds were noted in the essential oils with some of the common constituents being; α- and β-pinene, yomogi alcohol, ρ-cymene, 1,8-cineole, camphor, 4-terpineol, spathulenol, caryophyllene oxide, α-copaene and β-caryophyllene. Most of the species have a relatively high content of 1,8-cineole and camphor. Twenty-two chemotypes were noted and the potential for commercial development in the flavour, fragrance and pharmaceutical industries has been recorded. Among the favourable chemotypes noted includes the camphor, 1,8-cineole, bisabolol oxide B and nerolidol rich oils. However, due to the extensive variability in the essential oil profiles, standardization of oils in commercial development is crucial.

The leaf extracts comprised of flavonoids with the flavones and flavanones as the major structural types present in most species. The terpene and flavonoid chemistry of the genus is highly divergent even among multiple individuals of the same species and hence not a good taxonomic marker for specific delimitation as no coherent groups was evident although some phytochemical congruence has been noted between some of the taxa.

The DNA sequence data revealed lack of variability in the non-coding regions *psb*A-*trn*H and *trn*L-F among species of the genus. The nuclear DNA region (ITS) was variable but the number of characters separating taxa was too few for resolution of relationships between taxa. Presence of highly divergent paralogous repeats of ITS were also noted in some taxa. The combination of molecular and chemical data did not resolve the species delimitation problems due to the highly variable distribution of characters within a single species. The patterns of variation observed in the genus may be attributed to chemical convergence, divergence, hybridisation, differential gene expression, polymorphism and allelochemical diversification among other factors. The lack of coherence in the phylogenetic and phenetic groupings of the various taxa implies that the current species boundaries may not be a true reflection of natural taxonomic entities. The use of multiple taxa in taxonomic studies is strongly recommended due to the extensive variability noted in the chemical profiles of the taxa that is also depicted in the phylogenetic histories. It also implies that caution should be taken in bioprospecting for new natural products for commercial development, as plant chemical profiles especially from

the same species can be very variable. This implies carrying out exhaustive population and genetic studies for evaluation of diversity in the study group.

In the antimicrobial assay, the oils were more active against the Gram-positive bacteria (2-16 mg/ml) and yeasts (1-16 mg/ml). *Bacillus cereus* and *Cryptococcus neofomans* were the most susceptible pathogens to the oils. The extracts exhibited low activity against the test pathogens except *E. aromaticus* and *E. pinnatus* with activity of 0.2 mg/ml against *Staphylococcus aureus* and *Bacillus cereus* respectively. The susceptibility of the fungal pathogens *Cryptococcus neoformans* and *Candida albicans* and the Gram-positive bacteria *Bacillus cereus* to the oils and extracts is an indication of the potential for use of the members of the genus as natural antibiotics. The essential oils exhibited antiinflammatory activities with IC_{50} values ranging between 19.0-98.6 μg/ml. The oils did not show antioxidant activity at the starting concentration of 100 μg/ml but the acetone leaf extracts exhibited antioxidant activities with IC_{50} values ranging between 21.5-79.6 μg/ml. The essential oils showed inhibitory activity against acetylcholinesterase enzyme. The biological activity of the oils indicates that most of the traditional uses are influenced by the presence of the oils. The *in vitro* biological activity of the essential oils and extracts against the test pathogens provides a scientific basis for the use of some of the members in traditional herbal remedies and validates the use of some of the members of the genus for treatment of respiratory tract infections, gastro-intestinal disorders, mental conditions, dermal infections, and inflammation. The study records the biological activities for some of the species for the first time and their potential for use in flavourings, perfumery, cosmetics, as sources of antimicrobial drugs, permeability enhancers in pharmaceutical formulations and for use as industrial oils.

PUBLICATIONS AND PRESENTATIONS

EW Njenga, G Reeves, SF van Vuuren and AM Viljoen (2004). The biological activity, essential oil composition, and molecular phylogenetic reconstruction of *Eriocephalus* L. (Asteraceae). *South African Journal of Botany* 70: 347 (Presentation. Abstract, see appendix III).

EW Njenga, SF van Vuuren and AM Viljoen (2005). Antimicrobial activity of *Eriocephalus* L. species. *South African Journal of Botany* 71: 81-87 (Full PDF article, see appendix II).

EW Njenga and AM Viljoen (2005). *In vitro* antioxidant and anti-inflammatory activity of species of *Eriocephalus* L (Asteraceae). (Submitted to *South African Journal of Botany*). Abstract, see Appendix III.

EW Njenga AM Viljoen and C Bicchi (2005). Essential oil composition of species of *Eriocephalus* L. (Asteraceae). (To be submitted to *South African Journal of Botany*). Abstract, see Appendix III.

EW Njenga, AM Viljoen, SF van Vuuren and C Bicchi (2005). Chemical composition and biological activities of some Namibian species of *Eriocephalus* L. (Submitted to *Journal of Ethnopharmacology*). Abstract, see Appendix III.

EW Njenga, G Reeves and AM Viljoen (2005). Phylogenetic reconstruction of the genus *Eriocephalus* L. (Asteraceae). (To be sumitted to *Molecular Phylogenetics and Evolution*). Abstract, see Appendix III.

ACKNOWLEDGEMENTS

I sincerely acknowledge the role my supervisor Professor Alvaro Viljoen played in ensuring the speedy conclusion of the project (May 2002-May 2005) as well as the guidance, valuable criticisms and patience in the course of the study and the write-up. His unwavering support and help in sourcing of material for the study is hereby acknowledged.

I also acknowledge the contribution and guidance of Dr. Gail Reeves (SANBI, Cape Town) in the molecular studies; I appreciate the great lessons and memorable experiences in the Leslie Hill Molecular Systematics Laboratory, Cape Town. All the staff, especially Ferozah Conrad in the same laboratory are thanked for their patience and encouragement especially those moments when things just didn't work! To Margaret a thank you for your help, friendship, and company during my long stay in Fynbos Lodge. The staff in the Compton Herbarium and library (Kirstenbosch Research Centre, Cape Town) are hereby acknowledged for allowing my continued use of the facilities.

I am indebted to my greatest friends Joy, Shalom and George for their love, encouragement, patience, understanding and travailing prayers during my long absence from them. It took God's grace for them to remain brave during those telephonic conversations and the brief moments we had together during the course of the study. To my husband George thanks for your sacrificial love and greatness and for everything you did for me, I am eternally grateful. To my sister Alice, God bless you for all your labour and sacrifice for my family and for your presence in my absence.

I am indebted to Professor Richard Pienaar and Professor David Mycock for the role they played in ensuring my smooth academic transition.

Since this project was multidisciplinary, many people were involved in one way or the other and it may not be possible to mention everyone; to those unwritten heroes and heroines I extend my eternal gratitude. To the written few, I hereby register my appreciation to following:

Mr Jan van Vlok and Dr. John Manning are hereby acknowledged for sourcing and identification of plant material.

Professor Kevin Balkwill, the head A.P.E.S. and curator, C.E. Moss Herbarium is acknowledged for allowing my use of the Herbarium research facilities for part of the phylogenetic analysis. Thanks to Dr. Donald Otieno for guidance in the PAUP analyses. To Renee, Dickson, MacCallum and Mando of the Biodiversity Museum much gratitude for the kindness and assistance.

Mrs Herta Kolberg and the staff of the National Herbarium, National Botanical Research Institute (NBRI), Namibia are acknowledged for the collection and identification of the Namibian taxa.

Mr Paul Herman of the South African National Biodiversity Institute (SANBI), Pretoria is hereby acknowledged for assisting in identification and determination of plant material.

The kind assistance of Professor Carlo Bicchi (Italy), for assisting in the GC/MS confirmation of the essential oil constituents is hereby acknowledged. I am indebted to Mr. Paul Steenkamp and Mr. Nial Harding of the Forensic Chemistry Laboratory, (Johannesburg) for their guidance with the high performance liquid chromatography analysis.

Sandy van Vuuren is sincerely acknowledged for her enthusiasm and guidance in microbiology assays. My special appreciation to all the staff and fellow students in the Department of Pharmacy and Pharmacology for their assistance, friendship, and the light moments we had together.

The Third World Organization for Women in Science (TWOWS) is hereby acknowledged for the generous study fellowship with special thanks to Professor Lydia Makhubu for her kindness and understanding. The National Research Foundation (NRF) and Medical Faculty Research Endowment Fund are hereby acknowledged for research funding.

My home institution, Moi University, Kenya, is acknowledged for granting study leave for my whole period of study.

To all who prayed for me God bless you. Above all, if God's grace had not sustained me, I would never have come this far, to Him be All the Glory, the Honour, the Power, Praise and adoration, for all the great things He has done! Amen.

TABLE OF CONTENTS

DECLARATION ii
DEDICATION iii
ABSTRACT iv
PUBLICATIONS AND PRESENTATIONS vii
ACKNOWLEDGMENTS viii
TABLE OF CONTENTS x
LIST OF FIGURES xv
LIST OF TABLES xvii
LIST OF ACRONYMS xviii

Chapter 1: General introduction 1

1.1. Introduction 2

1.2. Characteristics of the tribe Anthemideae: Asteraceae 4

1.3. Taxonomy of the genus *Eriocephalus* 4

1.4. Phytochemistry of the genus 9

1.5. Economic importance of the genus 9

1.5.1. Medicinal uses 9

1.5.2. Industrial uses 10

1.5.3. Other uses 10

1.5.4. Antiinflammatory properties 10

1.6. Phylogenetic relationships of *Eriocephalus* 11

1.6.1. Phytochemistry 11

1.6.2. DNA 11

1.7. Aims and objectives of the study 14

Chapter 2: Phytochemistry of the genus *Eriocephalus* 15

2.1. Introduction 16

2.1.1. Terpenes 18

2.1.2. Sesquiterpene lactones 20

2.1.3. Flavonoids 21

2.2. Phytochemistry and chemotaxonomy of the genus *Eriocephalus* 22

2.3. Importance of the study 24

2. 3.1. Objectives of the study 24

2.4. Materials and methods 25

2.4.1. Field sampling and vouchers 25

2.4.2. Chemical extraction and analysis 25
2.4.2.1. Volatile compounds 25
2.4.2.2. Non-volatile compounds 27
2.5. Results 28
2.5.1. Essential oil and leaf extract yields 28
2.5.2. TLC analysis 29
2.5.2.1. Volatile compounds 29
2.5.3. GC/MS analysis 31
2.5.3.1. *E. africanus* 34
2.5.3.2. *E. capitellatus* 35
2.5.3.3. *E. decussatus* 37
2.5.3.4. *E. ericoides* subsp. *eriocoides* 37
2.5.3.5. *E. klinghardtensis* 38
2.5.3.6. *E. pauperrimus* 40
2.5.3.7. *E. pinnatus* 41
2.5.3.8. *E. punctulatus* 41
2.5.4. Variation in composition between the radiate and disciform taxa 42
2.5.5. Cluster analysis 44
2.5.5.1. Quantitative analysis 44
2.5.5.2. Qualitative analysis 46
2.5.6. Analysis of non-volatile extracts. 50
2.5.6.1. TLC screening 50
2.5.6.2. HPLC analysis 50
2.5.6.3. Cluster analysis 51
2.6. Discussion 58
2.6.1. Chromatographic screening 58
2.6.2. Volatile and non-volatile yields 59
2.6.3. Phytochemistry of the genus 59
2.6.3.1. Essential oil chemistry 59
2.6.3.2. Non-volatile compounds 64
2.6.4. Relationships between species 68
2.6.4.1. Overall species variation 68
2.6.4.2. Affinity groupings 71
2.7. Conclusion 71

Chapter 3: Phylogenetic reconstruction of *Eriocephalus* 74
3.1 Introduction 75
3.2. Molecular systematics and use of DNA sequence data in the reconstruction of phylogeny 75
3.3. Secondary metabolites in phylogenetic studies 78
3.4. Importance of the study 79
3.4.1. Objectives of the study 80
3.5. Materials and methods 81
3.5.1. Plant material for DNA and essential oil extraction 81
3.5.2. PCR and DNA sequencing 81
3.5.3. Phylogenetic and chemical analysis 82
3.5.3.1. Molecular analysis 82
3.5.3.2. Chemical analysis 83
3.5.3.3. Combined analysis 83
3.6. Results 84
3.6.1. Analysis of ITS 84
3.6.2. Analysis of *psb*A-*trn*H intergenic spacer 86
3.6.3. Chemical analysis 86
3.6.3.1 Restricted taxon set 86
3.6.3.2. Complete taxon set 86
3.6.4. Combined analysis of ITS sequences and chemical data 88
3.6.4.1. Combined restricted taxon set 88
3.6.4.2. Complete combined chemical and molecular taxon set 90
3.7. Discussion 99
3.7.1. Molecular and chemical evolution 99
3.7.2. Phylogenetic relationships within *Eriocephalus* 101
3.7.2.1. ITS phylogeny 101
3.7.2.2. Combined ITS and chemical phylogeny 104
3.7.3. Molecular and chemical diversification 109
3.8. Conclusions 114
3.8.1. General conclusions 114
3.8.2. Conclusions on ITS phylogeny 115
3.8.3. Conclusions on combined ITS and chemical phylogeny 115

Chapter 4: Biological properties of *Eriocephalus* species 117
4.1. Introduction 118
4.1.1. Herbal remedies and traditional medicine 118
4.1.2. Antimicrobial activity 119
4.1.3. Antioxidants and free radicals 121
4.1.4. Antiinflammation 123
4.1.5. Alzheimer's disease and inhibitors of acetylcholinesterase 125
4.2. Biological properties of the genus *Eriocephalus* 127
4.3. Importance of the study 129
4.3.1. Aim and objectives of the study 130
4.4. Materials and methods 131
4.4.1. Antimicrobial activity 131
4.4.1.1. Test organisms 131
4.4.1.2. Disc diffusion assay 131
4.4.1.3. Determination of minimum inhibitory concentration (MIC) 132
4.4.2. Determination of antioxidant activity 133
4.4.2.1. Preliminary DPPH radical scavenging activity 133
4.4.2.2. Quantitative evaluation of antioxidant activity 133
4.4.3. Determination of antiinflammatory activity 134
4.4.4. Preliminary screening for detection of acetylcholinesterase 135
4.4.4.1. TLC bioautography 136
4.5. Results and discussion 136
4.5.1. Antimicrobial activity 136
4.5.2. Antioxidant activity 143
4.5.3. Antiinflammatory activity 150
4.5.4. Acetylcholinesterase inhibition 157
4.6. Chemical composition and biological properties 158
4.6.1. Volatile compounds 158
4.6.2. Non-volatile compounds 162
4.7. Conclusions 162

Chapter 5: Discussions, conclusions and recommendations 164
5.1. Preview 165
5.2. Phytochemistry and chemotaxonomy 167
5.3. Phylogenetic inference using ITS and terpenes 169
5.3.1. *E. purpureus* 171
5.3.2. *E. punctulatus-E. aromaticus* 171
5.3.3. *E. pinnatus-E. microphyllus* 171
5.3.4. *E. spinescens-E. decussatus* 172
5.3.5. *E. racemosus, E. africanus, E. eximius,* and *E. luederitzianus* 173
5.3.6. *E. racemosus-E. klinghardtensis* 173
5.3.7. *E. brevifolius-E. grandiflorus* 173
5.3.8. *E. aromaticus-E. grandiflorus* 173
5.3.9. *E. brevifolius, E. africanus, E. punctulatus,* and *E. scariosus* 174
5.3.10. *E. africanus-E. merxmuelleri* 174
5.3.11. *E. punctulatus-E. purpureus* 174
5.3.12. *E. ericoides* subsp. *ericoides-E. microphyllus-E. purpureus* 174
5.4. Biological properties and traditional remedies 175
5.4.1. Relationships based on biological and chemical properties 179
5.5. Favourable chemotypes 180
5.6. Conclusions 180
5.6.1. Phytochemistry and chemotaxonomy 180
5.6.2. Phylogenetic conclusions 181
5.6.3. Biological properties 182
5.7. Recommendations 183
References 184
Appendices 197
Appendix I-Monographs 197
Appendix II 296
Appendix III 304

LIST OF FIGURES

Chapter 1: General introduction

Figure 1.1. *E. namaquensis* showing an erect branched habit and woolly heads.....8
Figure 1.2. *E. africanus* showing a spreading habit in a rocky coastal habitat 8
Figure 1.3. *E. grandiflorus* showing radiate capitula 8
Figure 1.4. *E. microphyllus* showing opposite leaves.. 8
Figure 1.5. *E. spinescens* showing disciform capitula 8
Figure 1.6. *E. africanus* var *paniculatus* showing alternate leaves 8

Chapter 2: Chemistry of the genus *Eriocephalus*

Figure 2.1. Possible biosynthetic routes to some irregular monoterpenes 19
Figure 2.2. A commercial plantation and habit of *E. punctulatus* showing white rays.....26
Figure 2.3. Commercial blue oil of *E. punctulatus* 26
Figure 2.4. Advertisement for the oil of *E. punctulatus* by Grassroots Natural Products (GNP). 26
Figure 2.5. A clevenger apparatus used in hydrodistillation of essential oils. 26
Figure 2.6. Variation in essential oil yields of three representative species of *Eriocephalus* with their constituent taxa from different populations 30
Figure 2.7. Variation in oil colour of essential oils of *E. africanus* 31
Figure 2.8. A TLC plate of selected essential oils of various species of *Eriocephalus* 34
Figure 2.9. Variation in percentage composition of some of the common compounds found in the essential oils of populations of *E. africanus* 36
Figure 2.10. Variation in percentage composition of some common compounds found in the essential oils of populations of *E. ericoides* subsp. *ericoides* 39
Figure 2.11. The percentage composition of the various bisabolol derivatives in three individuals of *E. pauperrimus* 40
Figure 2.12: Variation in percentage composition of some of the compounds found in the essential oils of individuals of *E. punctulatus*.....43
Figure 2.13. Dendrogram of quantitative matrix based on 200 terpene chemical characters for 86 OTUs.....47
Figure 2.14. Dendrogram of qualitative matrix based on 200 terpene chemical

characters for OTUS 49
Figure 2.15: HPLC chromatogram of *E. aromaticus* from Ladismith 51
Figure 2.16. Representative HPLC/UV chromatograms showing common peaks for flavanones (RT 29.77 minutes) and flavones (RT 30.36 minutes) 53
Figure 2.17. A HPLC dendrogram of acetone leaf extracts based on Euclidean distances 54

Chapter 3: Phylogenetic reconstruction of *Eriocephalus*

Figure 3.1. Strict consensus of 2735 equally parsimonious trees from analysis of ITS sequences of 37 taxa 94
Figure 3.2. One of the equally most parsimonious trees found from analysi of ITS sequences 95
Figure 3. 3. Strict consensus of five equally most parsimonious tre of 91 taxa for the chemical dat set with equal weights 96
Figure 3.4. Strict consensus of three equally most parsimonious trees from the analysis of 37 taxa for the combined chemical and molecular data set with successive weighting (SW) 97
Figure 3. 5. Strict consensus of five equally most parsimonious trees from the analysis of 91 taxa for the combined data set with equal weights. 98

Chapter 4 Biological properties of *Eriocephalus* species

Figure. 4.1. Arachidonic acid cascade and the process of inflammation. 125
Figure 4.2. A representative microtitre plate showing the MIC values (mg/ml) 132
Figure 4.3. Variation in IC_{50} values (μg/ml) in the antioxidant test of seven representative species of *Eriocephalus* from different localities and populations 152
Figure 4.4. A representative graph showing the percentage inhibition of 5-lipoxygenase by the essential oil of *E. punctulatus* 153
Figure 4.5. Variation in IC_{50} values (μg/ml) in the 5-lox test of seventeen species of *Eriocephalus* from different localities and populations. 154
Figure 4.6. A TLC bioautographic profile for the presence of inhibitors of acetylcholinesterase 157

LIST OF TABLES

Chapter 1: General introduction

Table 1.1: Species of *Eriocephalus* and their uses 12

Chapter 2: Chemistry of the genus *Eriocephalus*

Table 2.1. Types of commonly known terpenes..... 19
Table 2.2: Voucher specimen information for species of *Eriocephalus,* percentage yields of essential oils, acetone leaf extract, localities, and oil colour 32
Table 2.3. Variation in chemical composition of major compounds of essential oils of taxa of *E. africanus* from different localities..... 36
Table 2.4. Major compounds found in the essential oils of six populations of *E. ericoides* subp. *ericoides* from five localities 39
Table 2.5. Variation in major compounds in essential oils of *E. punctulatus* 43
Table 2.6. Variation patterns of compounds present in radiate and disciform taxa 44
Table 2.7. A summary of the HPLC/UV data of 16 species of *Eriocephalus* showing retention time and concentration of non-volatile components..... 55
Table 2.8. A summary of the 22 chemotypes 65

Chapter 3: Phylogenetic reconstruction of *Eriocephalus*

Table 3.1. *Eriocephalus* taxa and outgroups used for the molecular systematics. 84
Table 3.2. A summary of the tree statistics for the separate and combined analyses 92
Table 3.3. Bootstrap percentages (Fitch weights) for some of the clades 93
Table 3.4: Species of *Eriocephalus*, their major diagnostic features 110

Chapter 4: Biological properties of *Eriocephalus* species

Table 4.1. Preliminary antimicrobial screening of essential oils and acetone leaf extracts..... 139
Table: 4.2. Antimicrobial activity of essential oils and acetone leaf extracts 144
Table 4.3: Minimum inhibitory concentration of essential oils and leaf extracts 147
Table 4.4. Antioxidant activity of acetone leaf extracts of species of *Eriocephalus* ... 149
Table 4.5. Antiinflammatory activity of some selected species of *Eriocephalus* on 5-lipoxygenase. 153

LIST OF ACRONYMS

AA-arachidonic acid
AD-anno domini
AIDS-acquired immunodeficiency syndrome
ATCC-American type culture collection
CFU-colony forming unit
COPH-cophenetic (ultrametric) values
CpDNA-chloroplast DNA
CTAB-cetyltrimethylammonium bromide
DNA-deoxyribonucleic acid
DPPH-2, 2-diphenyl-1-picryhydrazyl
EW-equally weighted
GC/MS-gas chromatography coupled to mass spectroscopy
GC-gas chromatography
HCL-hydrochloric acid
HIV-human immunodeficiency virus
HPLC-high performance liquid chromatography
ITS-internal transcribed spacer
KCL-potassium chloride
LC-liquid chromatography
LHMS-Leslie Hill Molecular Systematics Laboratory
LT-leukotriene
MIC-minimum inhibitory concentration
MXCOMP-matrix comparison
NBRI-National Botanical Research Institute
NCTC-National collection of type cultures
NTSYS-pc-Numerical taxonomy and multivariate analysis system
PAUP-phylogenetic analysis using parsimony
PPM-parts per million
RNA-ribonucleic acid
RT-retention time
SAHN- sequential agglomerative hierarchical nested cluster analysis
SANBI-South African National Biodiversity Institute
SIMINT-similarity
SUBSP- subspecies
SW-successively weighted
TBE-trizma base
TLC-thin layer chromatography
TMD-thermabeam mass selective detector
UPGMA-unweighted pair mean average
UV-ultra violet
OTUS-Operational taxonomic units
SM-secondary metabolites

CHAPTER 1

General Introduction

1.1. Introduction

Plants have always formed an integral component of human life by providing the basic survival necessities such as food, shelter, fuel, and medicine. Over and above fulfilling these basic needs, they also serve as environmental aesthetics. Ethnobotanical research revolves around these uses of plants and a substantial body of information has been garnered on the subject. Some of the earliest written accounts on plant uses were largely on the medicinal uses recorded in herbals. In 1770 B.C, the Sumerian and famous lawgiver of the Middle Ages, Hammurabi, wrote on stone tablets documenting the medicinal uses of plants such as henbane, licorice and mint. Hippocrates, the father of medicine, from whom the Hippocratic oath, the traditional code of physicians, was named, gave a written account of between 300-400 species of medicinal plants (Simpson and Ogorzaly, 1995).

Dioscorides, (1 AD) made an important contribution to the knowledge of plant use by writing the encyclopaedic *De Materia Medica*, in which he listed approximately 1000 simple drugs. Though poorly organized and often inaccurate, his work became the prototype for future pharmacopoeias. The Bible offers descriptions of approximately 30 healing plants with frankincense and myrrh probably the most recognized due to their value and medicinal properties (Stace, 1989; Simpson and Ogorzaly, 1995; Cowan, 1999).

The above mentioned are cases of some of the most rudimentary attempts by man to classify plant species according to their uses, and it is no wonder that scientists have continued to perfect this art for the purposes of easy and quick identification of useful plants. This forms in part the basis for taxonomy and systematics. The former is defined as the study and description of the variation of organisms involving classification, nomenclature, descriptions, and identification. The latter is broader than taxonomy and includes the basic tenets of taxonomy as well as biological interrelations such as breeding systems, genetics, phylogeny and evolutionary processes, biogeography and synecology (Stace, 1989; Hawksworth, 1988; Minelli, 1993; Woodland, 1997). Taxonomy and systematics are widely used in classification systems with the latter greatly influenced by the former in identification of organisms.

It is not possible to understand the diversity and extensive variation existing in plants without first naming them and then grouping them into recognizable categories. Classification is a process which mankind naturally and instinctively has been carrying out from the beginning

for accurate recognition (identification) of food, fuel, and building material etc. To simplify this aspect, several methods of classification have been proposed and used which categorize plants into hierarchies or, classifications, the most widely used are the phenetic and phylogenetic or cladistic methods (Minelli, 1993; Woodland, 1997). Both approaches have been adopted in this study.

Chemotaxonomy, which is the application of natural product chemistry as evidence in solving taxonomic problems employs various basic evidentiary characters. Some of these include flavonoids, terpenoids, carotenoids polysaccharides, alkaloids, amino acids, fatty acids and the basic C3 and C4 pathways in photosynthesis (Radford, 1986). The chemical evidence is useful in establishing relationships among taxa and providing clues for alternative interpretations concerning relationships among study groups (Radford, 1986).

Use of characters from many sources for descriptive purposes, identification and classification of organisms is useful in determination of phenetic, genetic, and phylogenetic relationships (Heywood and Moore, 1984; Radford, 1986; Stace, 1989). In this case, one type of evidence should be used in conjunction with others to be able to establish definitive taxonomic correlations and relationships. Many authors advocate that no single type of evidence is more reliable and meaningful than another but different evidences complement each other in strengthening taxonomic conclusions (Radford, 1986), but sometimes it may not be so.

Classification systems are useful in aiding with the identification of plant groups, but the understanding of the chemistry and biological properties of any economically important plant is also crucial. This is important in the light of increased interest in use of herbal remedies from plants, and the need to provide a scientific rationale for the use of traditionally used plants. In cases where plants are potentially useful as sources of natural products, phytochemical screening is crucial in understanding the chemistry of the study groups (Grayer *et al*., 1996; Skaltsa *et al*., 2001;Van Wyk, 2002).

The focus of this study is the genus *Eriocephalus* L. (Asteraceae), which is widely used in traditional herbal remedies and in the fragrance industry, and has a centre of endemism in southern Africa. This genus is riddled with species delimitation problems arising from the intergrading of the major diagnostic characters. This study therefore, seeks to use chemical and molecular characters to elucidate and clarify the relationships between the species in the

genus. A brief overview of the characteristics and taxonomy of the genus *Eriocephalus*, its chemistry, economic importance (including biological properties) and phylogeny are hereby given. The details are discussed under the appropriate chapters in the study.

1.2. Characteristics of the tribe Anthemideae: Asteraceae

Asteraceae is one of the most widely distributed families of Angiosperms, and is found in abundance on every continent except Antarctica. The family occupies a wide range of habitats, occurring in montane, sub-tropical to tropical latitudes and semi-arid areas (Heywood and Humphries, 1977). Globally, Asteraceae constitutes over ca. 23,000 species representing almost 10% of the total Angiosperm flora. The family is most concentrated in southern Africa (Botswana, Lesotho, Namibia and South Africa) with 253 genera and 2253 species (Koekemoer, 1996). Of the 17 tribes recognized by Bremer (1994), 15 are represented in Southern Africa, with the two remaining tribes Barnadesieae (Benth.) K. Bremer & Jansen and Liabeae (Cass.) Rydb. restricted to South and Central America (Koekemoer, 1996).

The tribe Anthemideae Cass., comprising 109 genera and about 1740 species, is primarily old world, and occurs mainly in temperate regions with centres of endemism in the Mediterranean regions of Europe, North Africa, Southern Africa, South America and Australia (Heywood and Humphries, 1977; Koekemoer, 1996; Bremer, 1994). It contains approximately 8% of the total number of genera in Asteraceae globally, and 12% of the species of Asteraceae in southern Africa (Bremer, 1994; Koekemoer, 1996). Most of the members of this tribe, particularly those from the Northern Hemisphere e.g. *Pentzia* and *Seriphidium*, share clear synapomorphies. In general, the tribal conspectus has not changed considerably with taxonomic revision, but some generic concepts have been significantly modified. Members of the tribe are recognized by their aromatic scent, dissected leaves, the non-bristly paleaceous pappus (which are sometimes absent), scarious margined involucral bracts, the ecaudate or the short tailed-anther appendages and the truncate penicillate style-branches with parallel stigmatic lines. The general aromatic odours are mainly due to the presence of monoterpenes and sesquiterpenes, which are diagnostic of the tribe (Hegnauer, 1977).

1.3. Taxonomy of the genus *Eriocephalus*

The genus *Eriocephalus* commonly known as ‘wild rosemary’, ‘Cape snowbush’, ‘kapokbos’, or ‘asmabossie’ (Dyson, 1998) is considered to be a distinct genus in the tribe Anthemideae (Adamson and Salter, 1950; Bremer and Humphries, 1993) and is characterized by its

aromatic scent and dissected leaves (Müller *et al.* 2001). The aromatic odours are caused by the presence of high concentrations of volatile constituents, which include mono- and sesquiterpenes (Swanepoel, 1997). The genus derives its name from the Greek word *'erion'* for wool and *'cephale'* for head (Figure 1.1) (Adamson and Salter, 1950) and is endemic to southern Africa. Its distribution covers the whole of the Flora of southern Africa (FSA) region except Gauteng and KwaZulu-Natal, with the highest concentration of taxa in the Western and Northern Cape (Müller *et al.,* 2001). It currently comprises 32 species, all of which are endemic to southern Africa. In total 42 taxa, including subspecies have been recorded from South Africa, Botswana, Lesotho and Namibia (Bremer, 1994; Müller *et al*., 2001).

The species of *Eriocephalus* occur in a variety of habitats ranging from coastal (Figure 1.2) to inland on plains, mountains, and the desert. Morphologically, the members of the genus are many-stemmed, sparsely to much-branched (Figure 1.1 and 1.2), erect to spreading (Figure 1.2), sometimes spinescent shrubs, rarely suffrutices, and 0.2-2.0 m high and in diameter and are often aromatic. All members of *Eriocephalus,* with the exception of *E. pinnatus* and *E. longifolius,* display anomalous secondary growth, resulting in the splitting of older plants into independent daughter plants (Müller, 1988). The branches comprise of long 'normal' shoots (dolichoblasts) with either opposite or alternate leaves and the dwarf shoots (brachyblasts) containing leaf tufts (Müller *et al.,* 2001). Diagnostic characters used to delimit taxa in the genus include whether paleae of marginal florets are free or connate, capitula disciform or radiate, indumentum on leaves felty or sericeous and whether leaves are opposite or alternate (Figures 1.3-1.6). Previous studies (De Candolle 1838; Harvey 1865; Bentham 1873) indicate that the involucre consists of two rows. However, an intensive ontogenetic and anatomical investigation of the capitula showed that the involucre consists of only one row of involucral bracts (Müller, 1988). The inner one represents the paleae of the marginal female florets, which are either free or connate (Müller *et al.,* 2001).

The latest revision of the genus broadly grouped taxa into heterogamous radiate forms (zygomorphic, with fertile pistillate rays and disc florets with functional stamens and sterile pistils) and the heterogamous disciform group (actinomorphic, with fertile, marginal pistillate florets and disc florets with functional stamens and sterile pistil) (Watson *et al*., 2002). The former group comprises 20 species and the latter comprises 12 species. Species delimitations within the genus are complex, and have led to varying treatments often characterised by substantial confusion in the ranking of some of the constituent taxa. Revision of the genus by

Harvey and Sonder (1865) in Flora Capensis recorded 17 species. Unfortunately, later studies have used Harvey and Sonder's (1865) old groupings, resulting in gross misidentification and misplacement of taxa. Bremer and Humphries (1993) reported 26 species based on these old groupings. Goldblatt and Manning (2000) recorded 34 species occurring in southern Africa while Leistner (2000) noted the existence of ca. 32 endemic species in the genus in Southern Africa. The most recent revision recorded the same number of species with many being reduced to subspecific and varietal status (Müller *et al*., 2001). However, the status and ranking of some of the taxa could still not be determined with certainty due to high level of similarities between them. For instance, among the new taxa recognized by Müller (1988), the delimitation of *E. ambiguus*, *E. microphyllus* var. *pubescens* and *E. merxmuelleri*, remained confusing due to the intergrading nature of their diagnostic features. Some of these features are phenotypically plastic and hence, separation of taxa was based on their allopatric distribution. *Eriocephalus namaquensis* and *E. karooicus* are difficult to distinguish from *E. spinescens,* as is *E. ericoides* from *E. purpureus* and *E. glandulosus*.

The paleae character is very confusing, being either free or partly to fully connate and in some taxa (e.g. *E. kingesii, E. eximius*, and *E. brevifolius, E. purpureus* and *E. scariosus*) tend to display both character states (Müller *et al*., 2001).

Other taxa have both opposite and alternate leaves and therefore this character cannot be effectively used to separate some of the closely related taxa such as *E. pedicellaris, E. purpureus, E. capitellatus,* and *E. macroglossus.* The *E. africanus* complex is intriguing. Müller (1988) recognized two subspecies within it; *E. africanus* subsp. *africanus* and subsp. *paniculatus* but Herman in Müller *et al*., (2001) reduced them to varietal status due to their occurrence in similar habitats and altitudinal range. *Eriocephalus ericoides* has two subspecies; *E. ericoides* subsp. *ericoides* and *E. ericoides* subsp. *griquensis.* The former shows a wide range of morphological variation and distribution with disjunct taxa in Namibia, while the latter is restricted to the Northern Cape Province, from the Orange River to near the border of Botswana. The two subspecies bear very close affinities such that they can only be separated on the basis of leaf length and indumentum type (Müller *et al.,* 2001). However, most of these characters are subject to phenotypic plasticity across the species geographic range.

Eriocephalus pinnatus differs from the rest of the members of the genus in having distinctly pinnatisect leaves, large golden yellow ray florets and being herbaceous. These characters are not found in the remaining species. This species is also said to be scarce due to its very low seed set (Müller *et al*., 2001). *Eriocephalus klinghardtensis* is reported to be restricted to the Klinghardt Mountains and growing in the isolated mountain range within the desert and succulent steppe in Namibia (Müller *et al.,* 2001). It grows in association with *E. giessii* (Müller *et al*., 2001) and is thought to be closely related to *E. scariosus* (Müller *et al*., 2001). Other species, for example, *E. pedicellaris* and *E. africanus* var *africanus* are reported to have succulent leaves whereas *E. scariosus, E. klinghardtensis,* and *E. brevifolius* have semi-succulent leaves. The remaining taxa do not exhibit these two characters.

Within the genus *Eriocephalus*, most taxa are reported to have a wide geographic distribution where the ranges overlap extensively. Species-level relationships within the genus are complex and this is further complicated by the possibility of hybridization among sympatric taxa. *Eriocephalus dinteri* is reported to hybridize with *E. luederitzianus*, *E. merxmuelleri* and with *E. ambiguus* (Müller *et al.,* 2001). *Eriocephalus africanus*, *E. punctulatus*, and *E. ericoides* have a wide distribution range and a high likelihood of extensive hybridization. *Eriocephalus africanus* closely resembles *E. eximius, E. scariosus, E. grandiflorus, E. brevifolius* and *E. klinghardtensis,* which have varying number of chromosomes (Table 1.1). Hence this implies that the *E. africanus* group has many closely related taxa with which it can potentially hybridize.

Eriocephalus africanus, apart from being widely distributed in a variety of vegetation types is also characterised by extensive phenotypic plasticity in life form, leaf shape, indumentum on the leaves, chromosome numbers 2n = 18, 36 (Table 1.1), and flower composition within the capitula. Two varieties have been demarcated within this species (Müller *et al*., 2001) (1): *E. africanus* var *africanus,* occurring in sand dunes of the coastal fynbos, from sea level to 100m inland, or on rocks arising from the sea and having succulent leaves and spreading habits. (2): *E. africanus* var *paniculatus,* occurring at a higher altitude and comprising of plants with erect habits and semi-succulent to non-succulent leaves. Within the second variety, five subgroups based on their distribution within the Eastern and Western Cape regions are recognised (Müller *et al*., 2001).



Figure 1.1. *E. namaquensis* (from between Clanwilliam and Perdefontein) showing an erect branched habit and woolly heads.



Figure 1.2. *E. africanus* (Mossel Bay) showing a spreading habit in a rocky coastal habitat.



Figure 1.3. *E. grandiflorus* from between Laingsburg and Matjiesfontein showing radiate capitula.



Figure 1.4. *E. microphyllus* from between Sutherland and Fraserburg showing opposite leaves.



Figure 1.5. *E. spinescens* from between Sutherland and Ceres showing disciform capitula.



Figure 1.6. *E. africanus* var *paniculatus* from Sutherland showing alternate leaves.

Eriocephalus punctulatus resembles *E. aromaticus, E. tenuifolius*, and *E. pedicellaris*. Its distribution range and that of *E. aromaticus*, overlap in Wittenberg and the Klein Roggeveld mountains. *Eriocephalus purpureus* and *E. ericoides* also intersect in the eastern boundary of Matjiesfontein and Loeriesfontein areas.

1.4. Phytochemistry of the genus

With the exception of the nine Namibian taxa, whose chemistry is briefly described by Zdero *et al.*, (1987) and *E. africanus* and *E. punctulatus,* which are used commercially, the chemistry of the remaining species is relatively unknown and undocumented. A further investigation of the terpene chemistry is therefore crucial as there may be more species with potentially useful compounds for commercial development. An overview of the phytochemistry is presented in Chapter 2.

1.5. Economic importance of the genus

Plants are a valuable source of natural products and will always remain one of the reservoirs for new and novel drugs. Recent advances in therapeutic research have contributed to the knowledge of natural plant products. The Asteraceae tribe Anthemideae comprises several genera with medicinal uses. Among them, *Artemisia, Achillea, Chamaemelun, Chamomilla* and *Tanacetum* are well known. The volatile components of the essential oils of these genera exhibit strong odours due to the presence of terpenes that are reported to possess antimicrobial activities (Alvarez-Castellanos *et al*., 2001). There has been a recent increase in the use of volatile oils in folk medicine, and this has prompted several studies into the therapeutic effects of these oils (Szentmihalyi *et al*., 2001). *Eriocephalus* has not been widely studied in relation to its medicinal uses. The limited information available on the medicinally important species has been recorded by Watt and Breyer-Brandwijk, (1962); Van Wyk *et al.,* (1997); Van Wyk and Gericke, (2000) for just a few of the species. Some of the medicinal properties of the rest of the species are as yet unknown and will be investigated in this study.

1.5.1. Medicinal uses

The recorded medicinal uses of some of the members of the genus confer potential economic importance to the genus *Eriocephalus.* The Griqua and the Nama are known to have used some of the species as diaphoretics and diuretics (Watt and Breyer-Brandwijk, 1962) while *E. africanus*, *E. racemosus* and *E. punctulatus* have also been used for treatment of respiratory ailments, gastro-intestinal disorders and various skin inflammation diseases (Watt and Breyer-

Brandwijk, 1962; Van Wyk *et al*., 1997; Dyson, 1998; Van Wyk and Gericke, 2000). The Griqua used *E. tenuifolius* as a substitute for 'buchu' (*Agathosma betulina*, a traditional herbal remedy used by the San and the Khoi) and its efficacy may be due to the presence of compounds with diuretic effects. *Eriocephalus karooicus* was also used locally as 'wild dagga' (Müller *et al.,* 2001) probably this species contains chemical compounds which induce psychotropic effects such as linalyl acetate and β-caryophyllene (Nakatsu *et al*., 2000). *Eriocephalus africanus* was used as a substitute for rosemary in flavouring of dishes such as fish, poultry, and lamb (Dyson, 1998). A detailed account of the uses of the important species is given in Chapter 2 and 4.

1.5.2. Industrial uses

The strong aromatic odours characteristics of the genus are as a result of high concentrations of terpenes. The presence of these aromatic terpenes makes *E. punctulatus* in particular, a very useful species for extraction of its characteristic bright blue oil, which is due to the presence of azulenic compounds (Van Wyk *et al.,* 1997). The oil, commercially known as 'Cape chamomile', marketed under the same name, is used in making of perfumes and cosmetics. This oil is internationally recognized as the 'fourth chamomile' after German, Roman and Moroccan chamomiles. (Watt and Breyer-Brandwijk, 1962; Van Wyk *et al*., 1997; Dyson, 1998; Van Wyk and Gericke, 2000). *Eriocephalus africanus* yields a yellow oil termed 'Cape snow bush oil' and is an important constituent of cosmetics and is also used as a blend in skin care products (Table 1.1). A detailed account of the important commercial species is given in Chapter 2.

1.5.3. Other uses

Among the species of *Eriocephalus,* those that are palatable and readily browsed by ungulates include; *E. pedicellaris* (browsed by domesticated and wild animals), *E. pinnatus, E. purpureus, E. tenuifolius, E. brevifolius, E. scariosus* (browsed despite being highly aromatic), *E. eximius, E karooicus*, *E. racemosus*, and *E. microphyllus* var *carnosus* (Table 1.1), (Watt and Breyer-Brandwijk, 1962; Van Wyk *et al*., 1997; Dyson, 1998; Van Wyk and Gericke, 2000).

1.5.4. Antiinflammatory properties

Only two species namely *E. punctulatus* and *E. africanus* are reported to have antiinflammatory activities (Watt and Breyer-Brandwijk, 1962; Swanepoel, 1997; Dyson,

1998). Therefore it is important to screen more species in an attempt to determine if there are other potentially active species in the genus with inhibitory activities against 5-lipoxygenase, the determinant enzyme of inflammation.

1.6. Phylogenetic relationships of *Eriocephalus*

1.6.1. Phytochemistry

The phenetic and cladistic methods of classification are useful in systematic studies of a large array of molecules at micro- and macromolecular levels. This is especially so, where these methods have been used in response to economic demands such as bioprospecting, pharmaceutical and cosmetics technologies, which require systematic information on several classes of compounds. The results can be used to understand the intra- and interspecific variation in groups containing these compounds at various hierarchical levels as well as shedding light on the various biosynthetic pathways in plants (Minelli, 1993). An understanding of the phylogenetic relationships between the species of the genus would be crucial especially in the light of prospecting for novel compounds for commercial development.

1.6.2. DNA

To date, no phylogenetic reconstruction of *Eriocephalus* has been attempted using DNA sequence data as a source of phylogenetic information. This study will therefore constitute the very first comprehensive study of the plastid *psb*A-*trn*H intergenic spacer and the nuclear internal transcribed spacer (ITS) DNA regions. An attempt will also be made to combine the chemical data with the molecular data to try and clarify species-level relationships within the genus.

Table 1.1. Species of *Eriocephalus* and their uses

SPECIES	VERNACULAR NAME	CHROMOSOME NUMBER	ECONOMIC USES
E. africanus L var *africanus* var *paniculatus* (Cass.) M.A.N. Müller	Kapokbossie, wilde roosmaryn, rosemary, renosterveldkapok	18 and 36	Diuretic, diaphoretic, tincture for heart trouble, oedema, colic, dropsy, flatulence, inflammation, used for delayed menstruation and gynaecological conditions, foot baths, dandruff hair-rinse, coughs, colds, asthma, used as fragrance in pillow cushions, substitute for wild rosemary in flavouring of dishes, browsed. Cape snowbush oil used in cosmetics and as a blend in skin care products, oil also used for stress related ailments, depression.
E. ambiguus (DC) M.A.N. Müller	Kapokbos	18	-
E. aromaticus C.A.Sm.	Kapokbos	18	-
E. brevifolius (DC) M.A.N. Müller	Kapokbos	54	Browsed
E. capitellatus DC	Kapokbos	18	Browsed
E. decussatus Burch.	Kapokbossie	18	-
E. dinteri S. Moore	Kapokbos	36	
E. ericoides (L.F.) Druce subsp *ericoides* subsp *griquensis* M.A.N. Müller	Kapokbos	18	Diuretic, diaphoretic
E. eximius DC	Grootbergkapok	18	Browsed
E. giesii M.A.N. Müller	Kapokbos	18	
E. glandulosus M.A.N. Müller	Kapokbos	18	-
E. grandiflorus M.A.N. Müller	Kapokbos	54	Browsed
E. karooicus M.A.N. Müller	Doringkapok (bossie), kleinkapokbossie, kleindoringkapokbos, silwerkapokbossie, veerkapok (bossie) and volstruiskapok	18	Used as a substitute for wild dagga, browsed
E. kingesii Merx & Eberle	Kapokbos	54	-
E. klinghardtensis M.A.N. Müller	Kapokbos	-	-
E. longifolius M.A.N. Müller	Kapokbos	18	
E. luederitzianus O.Hoffm.	Kapokbos	36	-
E. macroglossus B. Nord	Kapokbos	36	-
E. merxmuelleri M.A.N. Müller	Kapokbos	54	-
E. microcephalus DC	Kapokbossie	18	-
E. microphyllus DC. var *microphyllus*	Kapokbos	36	Browsed

SPECIES	VERNACULAR NAME	CHROMOSOME NUMBER	ECONOMIC USES
var *pubescens* (DC) M.A.N. Müller var *carnosus* M.A.N. Müller			
E. namaquensis M.A.N. Müller	Kapokbos	18	-
E. pauperrimus Merx & Eberle	Kapokbos	18	-
E. pedicellaris DC.	Kapokbos	72	Browsed
E. pinnatus O. Hoffm	Kapokbossie	18	Browsed
E. punctulatus DC.	Kapokbos	36	Diuretic, diaphoretic, also used for oedema, flatulence sometimes used with *Metalasia muricata* in after-death cleansing rituals, also used as fragrance in pillow cushion. Cape chamomile (blue oil) used in high class perfumes and as a blend oil, antiinflammatory, treatment of stress related ailments, dermal complications and gastro-intestinal disorders, browsed, aromatherapy.
E. purpureus Burch.	Kapokbos	36	Browsed
E. racemosus L. var *racemosus* var *affinis* (DC) Harv.	Sandveldkapok, strandveldkapok, rivierkapok and kapkappie, kapokbos	36	Diuretic, diaphoretic, gastro-intestinal and respiratory ailments, skin inflammation
E. scariosus DC.	Kapkbossie	72	Browsed
E. spinescens Burch	Kapokbos	36	-
E. tenuifolius DC.	Boegoekapok, klein-bergkapokbossie	-	Used in the past as a substitute for buchu by the Griquas (boegoekapok)
E. tenuipes C.A.Sm.	Kapokbos	36	-

1.7. Aims and objectives of the study

Based on the aforementioned factors, it is evident that this genus is important medicinally and industrially. This therefore necessitates further investigation into its chemistry, biological properties, and evolutionary trends using molecular and chemical data. The suggested morphological grouping of *Eriocephalus* taxa is still riddled with uncertainties as a result of the intergrading of the major diagnostic features. It is therefore imperative to search for alternative methods of understanding species delimitation and relationships in this genus. This study aims to investigate affinities and relationships within *Eriocephalus* at a chemical and molecular level. Given the extensive use in traditional medicine and the biological activity associated with the phytochemicals abundant in *Eriocephalus*, the pharmacological properties will also be investigated. To achieve the aims of the study, the following objectives have been considered:

- To clarify or try to resolve the specific and infra-specific delimitation problems within the genus using chemical data from essential oils and non-volatile compounds and to infer phylogenetic relationships and evolutionary trends from DNA sequence data.
- To record the biological activity (antimicrobial, antiinflammatory and anti-oxidant activities) of *Eriocephalus* species.
- To establish the rational usage of some of the members of the genus in traditional herbal medicine.
- To make recommendations to the flavour and fragrance industries on the selection of favourable chemotypes for commercial development.

CHAPTER 2

Phytochemistry of the genus *Eriocephalus*

2.1. Introduction

Chemical characteristics of plants have been noted and used by taxonomists for many centuries. The development of newer and faster screening techniques such as chromatography and electrophoresis has led to rapid identification of large number of compounds which in turn and has led to natural products receiving more attention from chemists and pharmacologists (Heywood, 1976; Hostettmann, 1999; Ohsaki *et al.*, 1999). Despite the availability of different approaches for the discovery of drugs, natural products remain the best reservoirs of new structural types. This has led to more advanced phytochemical investigations of their properties and the biosynthetic pathways which are an important prerequisite in the screening of plants for new drugs, cosmetics and nutraceuticals (Kubitzki, 1984; Van Wyk, 2002).

In plant systematics, the phytochemical components of any given plant carry some crucial information that may be diagnostic of the group to which they belong as well as for phylogenetic inference on basis of biosynthetic considerations (Ohsaki *et al*., 1999). This information is largely used in chemosystematics (Harbourne, 1984), hence forming an essential ingredient of the taxonomic process in infraspecific ranking (Merle *et al*., 2004). In higher plants, the use of chemosystematics in instances where morphological information has failed to resolve delimitation problems is a reliable alternative. Other uses include cultivar discrimination and hybrid recognition where documentation of parental origin is not provided by other data sources (Skaltsa *et al*., 2001). What were once believed to be 'waste products' of various metabolic processes in plants have now become integral components in phytochemical research.

Secondary plant metabolites are defined as naturally occurring substances that are involved in plant reproduction (signals for attracting pollinators e.g. fragrant monoterpenes, coloured anthocyanins or carotenoids and for seed dispersal), defense against herbivory, against pathogenic attacks (microbes and viruses) and effective in allelopathy. In other instances, some of the secondary metabolites concomitantly carry out physiological functions such as serving as mobile and toxic nitrogen transport and storage compounds or UV-protectants (Manthey and Busling, 1998; Wink, 2003; Wink and Mohamed, 2003). These metabolites are known to possess high structural diversity and as a rule, a single group of the metabolites dominates within a given taxon. A few major compounds are accompanied by several derivatives and minor components. On the overall, the pattern of secondary metabolites in a

given plant is complex and changes depending on the plant part where they are stored. Differences in concentration and composition of secondary metabolites can be seen between different developmental stages (e.g. organs important for survival and reproduction have the highest and most potent SM), between individuals and between populations. These metabolites can be present in a plant in an active state or a 'prodrug' that becomes activated upon wounding, infection or in the body of an herbivore (Wink, 2003).

Secondary metabolites are derivatives of glucose and acetyl-CoA known as isoprenoids of C_5 units and are therefore adaptive characters that have been subjected to natural selection over a period during the course of evolution. It has been reported in literature that the distribution of these compounds has some value for taxonomy and that their occurrence is a reflection of adaptations and particular life strategies embedded in a phylogenetic framework (Wink, 2003). Secondary metabolites vary considerably in composition, quantity, and hence sometimes resulting in conflicting interpretation of data arising from their analysis. Choice of analytical methods, inherent variability of substances under investigation, insufficient data or even the genetic variability of individual plants may contribute to variability of results in any phytochemical study (Swain, 1963; Grayer *et al*., 1996).

In plants, variation in composition and yields of secondary metabolites is due to several factors some of which are intrinsic namely; genetic, diurnal, ontogenetic, and seasonal. Other factors are extrinsic, like soil and climate types (Swain, 1963). Genetic factors largely influence the quantity and distribution of chemical compounds, and thus the chemical non-uniformity of taxa is quantitative. The diurnal factors affect processes like photosynthesis and the quantities of the metabolites produced especially essential oils due to evaporation and resinification. This is common in plants whose glands are located on the surface. The time of harvesting of plant material, whether morning, afternoon or evening is critical as it affects the yields obtained (Wink and Mohamed, 2003). Studies of *Matricaria chamomilla* demonstrated two maxima in oil content, one in early morning and the second late in the afternoon. The same species showed maximum concentration of azulenogenic substances at noon. Hence, it appears that the factors affecting the composition and the yields of secondary metabolites vary, thus it is not surprising that these variations may give very different patterns of the chemistry of individuals of species from a population and in other cases may offer insight into phytochemical relationships between taxa.

Secondary metabolites comprise various classes of compounds but the most commonly used for taxonomic purposes in Asteraceae are the aromatic terpenes, sesquiterpene lactones and flavonoids (Heywood and Humphries, 1977).

2.1.1. Terpenes

Terpenes are widely distributed in the plant kingdom (Ikan, 1991). The strong aromatic odours of many species of Anthemideae are mainly based on high concentrations of terpenes, which are water insoluble, acyclic and cyclic compounds (Swanepoel, 1997). Terpenes consist of (C_5) units of original carbon skeleton compounds. Most terpenes found in Anthemideae are products of isoprenoid synthesis derived by condensation of isopentlyl pyrophosphate and dimethylallyl pyrophosphate in a head-to-tail fashion. They range from five to several hundred carbons. The terpenes mainly constitute the essential oils among other compounds. Essential oils are volatile products deposited in dead cells (oil idioblasts), in oil cavities and ducts or in subcuticular spaces of glandular hairs. Once formed, essential oils are not metabolized hence their continued accumulation. Members of Asteraceae, with the exception of the Cichorieae, have secreting glandular hairs and schizogenous ducts (Swain, 1963; Swanepoel, 1997; Skaltsa *et al*., 2001). The essential oils found in Asteraceae include monoterpenes (C_{10}), sesquiterpenes (C_{15}) and diterpenes (C_{20}) among many others (Table 2.1).

Several genera in Anthemideae have irregularly distributed monoterpenes with a possible biosynthetic relationship to chrysanthemic acid and derivatives of this biosynthesis are of systematic importance. The suggested biosynthetic pathway is shown in Figure 2.1.

The monoterpenes are major compounds of oils obtained by distillation of plant material. They are characteristically volatile, insoluble in water and very fragrant or aromatic. They may be acyclic (no ring, e.g. geraniol), monocyclic (one ring, e.g. α-terpineol, limonene, menthol, etc) and bicyclic (two rings, e.g. α-pinene, camphor etc) (Smith, 1976; Ikan, 1991; Swanepoel, 1997).

Some of the species in the genera of the tribe Anthemideae are widely used medicinally and industrially. Their monoterpenes contain thujane and camphane derivatives such as thujone, camphor and borneol, as well as 1,8-cineole as the major and most widespread structural types of compounds. The phytotoxic effects of 1,8-cineole, camphor and isothujone probably

contribute to the allelopathic effects of some species in the genera of the tribe Anthemideae (Heywood and Humphries, 1977; Swanepoel, 1997).

Table 2.1. Types of commonly known terpenes (Swanepoel, 1997; Dewick, 2001; Skaltsa *et al*., 2001)

Number of isoprene units	Type/group of compounds	Representatives
1	Hemiterpenes	Combined compounds, e.g. coumarins, quinines etc
2	Monoterpenes	Essential oils, iridoids
3	Sesquiterpenes	Essential oils, sesquiterpene lactones (bitter principles). Gibberellins, abscisic acid, juvenile hormone
4	Diterpenes	Resins, phytol, gibberellins, vitamin A
5	Sesterterpenes	Unsaponifiable lipids, extracts, wastes
6	Triterpenes	Sterols, steroids, saponins,
8	Tetraterpenes	Carotenes, xanthophylls
N	Polyterpenes	Rubber, gutta

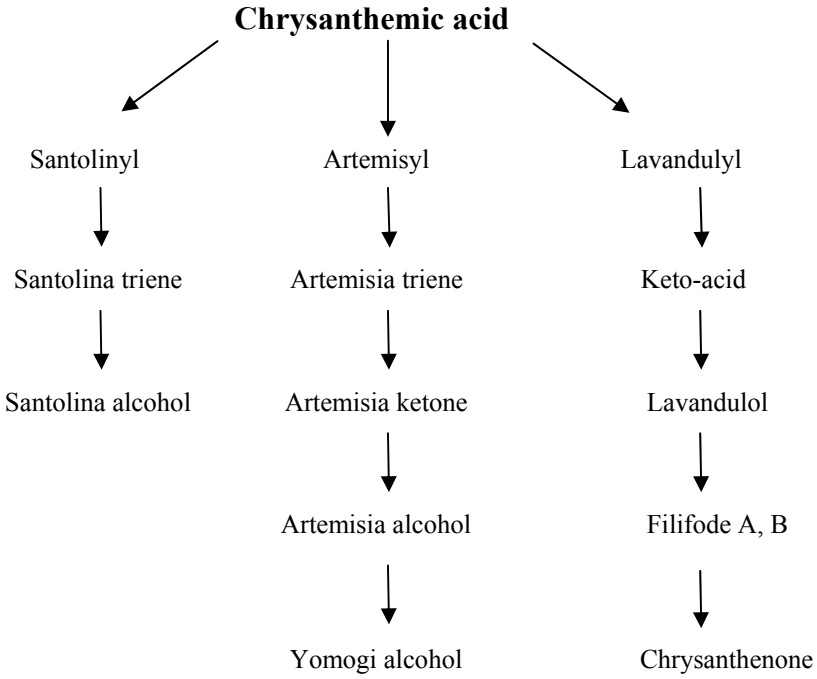


Figure 2.1. Possible biosynthetic routes to some irregular monoterpenes characteristic of the Anthemideae (Source: Heywood and Humphries 1977. Vol: II).

In Anthemideae, the insecticidal effects of some of the species are attributed to the presence of six constituents: pyrethrin I and II; cinerin I and II; and jasmolin I and II. Monoterpenes have been studied extensively in several genera and besides the environmental variabilities; it has been shown that their chemical composition is highly dependent upon the plant's

genotype. Hence, since their quantitative expression and chemical patterns are under genetic control, they are variable at infraspecific level and can be used as taxonomic markers (Greger, 1977; Hegnauer, 1977; Swanepoel, 1997; Roussis *et al.,* 2000). This important aspect can be used to delimit taxa in the genus *Eriocephalus*. Most of the isolated monoterpenes have been linked to a possible biosynthetic relationship to chrysanthemic acid (Figure 2.1). The other important group of terpenes is the sesquiterpenes.

Sesquiterpenes are volatile terpenes comprised of three isoprene units having 15 carbons. They are acyclic (e.g. nerolidol), monocyclic (e.g. humulene and α-bisabolene) or bicyclic (e.g. guaiol and lactone santonin). The most common sesquiterpenes in Anthemideae are caryophyllene and cadinene with their closely related derivatives (Heywood and Humphries, 1977, Swanepoel, 1997). The occurrence of bisabolol and the oxidation products, which are pharmaceutically important in treatment of inflammatory ailments, has been reported in *Marticaria chamomilla*. When oxidation occurs in the volatile sesquiterpenes, the resultant products are the sesquiterpenes lactones that are systematically important in Asteraceae.

2.1.2. Sesquiterpene lactones

This is a natural class of sesquiterpenes, which is chemically distinct from other members of the group by the presence of γ-lactone system (Swanepoel, 1997). They comprise volatile components of essential oils that have been rendered non-volatile by oxidation (Hegnauer, 1977). They are characteristic constituents of Asteraceae and the major structural features include α, β-unsaturated-γ-lactone. It is reported that the presence of these compounds confers pharmacological properties to the group of plants where they are present (Swanepoel, 1997).

Sesquiterpene lactones are colourless, bitter, and relatively stable lypophilic constituents genetically derived from *trans*, *cis* farnesyl pyrophosphate. Over 3000 lactones have been described of which the majority are from Asteraceae. The major class of lactones includes germacranolides, guaianolides, pseudoguaianolides, eudesmanolides, eremophilanolides and xanthonolides. It is reported that species of plants yield only one skeletal type of the structural lactones with varying oxidations in the skeleton. In genera with wide geographical distribution, it is likely that the species may exhibit considerable infra-specific variation in their sesquiterpene lactone structures. Many species and genera of Asteraceae are reported to be versatile with regard to lactone production. This variation within species and genera

renders the constituents suitable for taxonomic studies at specific and sectional levels and for study of speciation and plant migration (Heywood and Humphries, 1977; Swanepoel, 1997).

2.1.3. Flavonoids

Among the groups of secondary metabolites used by chemotaxonomists, probably none has provided more taxonomic data than the phenolics. The most taxonomically important include the flavonoids. It is reported in literature that there is considerable diversity of flavonoids in plants (Stace, 1989). Flavonoids are an important class of compounds that constitute the naturally occurring phenolics with a characteristic C_{15} nucleus in a three-ring system. They have an A-benzene ring formed by acetate units and a B-benzene ring formed by the shikimic acid pathway. The flavonoid nucleus is usually attached to a sugar resulting in a polar compound known as a glycoside (Heywood and Humphries, 1977; Swanepoel, 1997). They occur in all parts of the plant, including the fruit, pollen, roots and hearth wood (Ikan, 1991; Dewick, 2001).

Flavonoids are divided into structurally distinct classes. They include the 3-hydroxyl substituted flavonols (e.g. quercetin, kaempferol, myricetin and rutin); flavones (e.g. luteolin and apigenin) which are the most widespread flavone aglycones and occur in leaves and flowers of angiosperms. The other classes are flavanones (e.g. naringenin and hesperetin); isoflavones and isoflavonoids are isomeric with flavone compounds due to the presence of the B ring on the 3-position and they differ from isoflavones by reduction of the 2, 3-double bond (e.g. orobol). Anthocyanidins which lack the carbonyl group at the 4-position (e.g. cyanidin and pelargonidin) and catechins (flavanols), chalcones and aurones which are yellow pigmentation flavonoids (e.g. butein) also flavonoids and the biflavonoids which are dimers of flavone apigenin and have a restricted occurrence in gymnosperms (e.g. amentoflavone) (Ikan, 1991; Dewick, 2001).

The difference in the classes includes changes in the pyrone ring (absence or presence of double bond, presence of 3-hydroxy and / or 2-oxy groups) and the number of hydroxyl groups in the rings. Flavonoids may be monomeric, dimeric or oligomeric and vary greatly in molecular weight (Manthey and Busling, 1998). The distinct patterns of flavonoid constituents often represent taxonomically valuable characters (Hegnauer, 1977). Flavonoids have a broad spectrum of biochemical activities such as immune and inflammatory response, including direct inhibitory activity of cyclooxygenases, protein kinases lipoxygenases and

phospholipases (Manthey and Busling, 1998). They are also natural dietary biological response modifiers hence their use as anti-oxidants, anti-allergics, antivirals, anticancer among many other uses (Dewick, 2001).

Terpenoids and flavonoids have been reported to occur in the genus *Eriocephalus* in some of the species in South Africa and Namibia and the chemistry of the genus is hereby discussed.

2.2. Phytochemistry and chemotaxonomy of the genus *Eriocephalus*

The genus *Eriocephalus* is one of the genera in Anthemideae that has not received a lot of attention in scientific investigations. Apart from the commercially used essential oils of *E. punctulatus* 'Cape chamomile' (Figure 2.2 and 2.3) and *E. africanus* 'Cape snowbush', and the few species from Namibia and South Africa that have been investigated (Zdero *et al*., 1987), the chemistry of the rest of the species remains relatively unexplored. It is possible that some of the unexplored species could be potentially useful for commercial development if their chemistry is documented.

Cape chamomile oil obtained from *E. punctulatus* (Figure 2.3) has a striking blue colour, which is believed to be due to the presence of azulenic compounds. It also has a fine fruity fragrance, with a roman chamomile-like aroma. Commercially, it has been rated as one of the four chamomiles of the world, after the Roman, the German and the Morrocan chamomiles. The Cape and Moroccan chamomiles are not true chamomiles as they are from different plants. The oil has a high potential as a fruity flavour enhancer with a low threshold value. The essential oil is reported to have antiinflammatory, antispasmodic and antimicrobial activities. This is probably due to the presence of compounds such as bisabolol derivatives and chamazulene. The latter is formed from matricin during steam distillation of essential oils (Heywood and Humphries, 1977; Povh *et al*., 2001; Szoke *et al.,* 2004).

However, the oils of *E. punctulatus* investigated by Grassroots Natural Products (Figure 2.4) (http://www.gnp.co.za) were found to contain of 2-methylpropyl 2-methylpropionate, 2-methylylbutyl 2-methylpropionate and the linalyl acetate as the major constituents. The blue colour is due to the azulene compounds; 1,4-dimethylazulene and 1,4-dimethyl-7-ethyl azulene. A study on the commercial oils of the same species by Mierendorff *et al*., (2003), yielded 200 compounds some of which included α-thujone, α-pinene, camphene, sabinene, linalool, camphor, borneol, 1,8-cineole, cymene and limonene among many others. The oils

of *E. africanus* are reported to be composed of linalyl acetate, cymene, and 1,8-cineole as the major constituents among several other sesquiterpenes.

In the genus *Eriocephalus*, the sesquiterpene lactones have been studied in a few of the species. *Eriocephalus africanus*, whose yellow essential oil yield is reported to be 10-15% from steam distillation, contains a mixture of sesquiterpene lactones of which 4, 11 eudesmanediol is the major constituent (Watt and Breyer-Brandwijk, 1962; Zdero *et al*., 1987; Swanepoel, 1997; Van Wyk *et al*, 1997).

Terpenes such as camphor, linalyl acetate, nerolidol and spathulenol were also reported in some of the species studied by Zdero *et al*., (1987). In the same study, the aerial parts of *E. merxmuelleri* only accumulate camphor and those of *E. ambiguus* caryophyllene epoxide and taraxasteryl acetate.

Among the flavonoids reported from species of the genus *Eriocephalus* is the widespread C_{17}-compound, dehydrofalcarinone (DF) and ivangustine. The former is characteristic of the species of *Eriocephalus*. Among the members of the genus *Eriocephalus* examined for flavonoids; *E. giesii* was shown to have pectolinarigenin, salvigenin. *E. kingesii* afforded only 5,6,4'-trihydroxy-7, 3'-dimethoxyflavone (Zdero *et al*., 1987). *E. pauperrimus* was shown to have phloracetophenone and a mixture of eudesmane derivatives. An extract of aerial parts of *E. scariosus* gave squalene, dehydrofalcarinol, ivangustin and other derivatives. The aerial parts of *E. ericoides* yielded only germacranolides. Three other flavones were reported from the leaf resin of *E. punctulatus*; hispidin, jaceosidin and eupatilin. Additional information has added the following compounds to the list namely; apigenin, luteolin, luteolin-3', 4' dimethyl ether, (which is rare), 5,7-dihydroxy-6, 4'-dimethoxyflavone (pectolinarigenin) quercetin, isorhamnetin, naringenin and eriodictyol which constitute the minor flavonoids (Swanepoel, 1997; Wollenweber and Mann, 1989; Bohm and Stuessy, 2001).

Traditionally, *Eriocephalus* is placed in the tribe Anthemideae and phylogenetically assigned to the South African grade in the same clade with *Hymenolepsis* and a sister to *Cotula* and *Lasiospermum* (Watson *et al*., 2000). The only chemotaxonomic study of the genus carried out by Zdero *et al*., (1987), supported the placement of *Eriocephalus* in the tribe Anthemideae. The co-occurrence of dehydrofalcarinol and several types of sesquiterpene lactones was reported in *Artemisia* species but never in *Tarchonanthus* and related genera or

from the representatives of the *Lasiospermum* group of which *Eriocephalus* is a close ally. The presence of dehydrofalcarinol and the related compounds in the genus clearly indicates and supports its relationship with other genera in the tribe. However, the chemotaxonomic relationships between and within the species in the genus have not been studied. There is a lot of morphological similarities in the genus and intergrading of major morphological diagnostic characters used to delimit the taxa. Thus a chemotaxonomic attempt to clarify the relationships in the genus based on the terpene constituents was undertaken.

2.3. Importance of the study

It is clear that the genus *Eriocephalus* is economically important and a study aimed at unveiling the chemistry of the species is crucial in understanding the whole group and the identification of potential species for commercial development.

This study aims at clarifying the relationships between and within the taxa in the genus using terpenes and non-volatile compounds as taxonomic markers. The data will also be used to understand the biological activity of the members of the genus. On the overall the chemical data will be superimposed onto the molecular species level phylogeny tree for evaluation and understanding of evolutionary trends in the genus.

For the first time, a comprehensive survey and analysis of the essential oils of the species of *Eriocephalus* was attempted in this study. It is envisaged that this information will be very useful in clarification of relationships between the taxa and in the identification of favourable chemotypes for commercial development. Emphasis has been placed in the study of essential oil composition as GC/MS allows for rapid identification of compounds in the oil. Despite recent advances in LC/MS, the rapid identification of non-volatile (phenolic) extracts remains a laborious and challenging task.

2. 3.1. Objectives of the study

1. To record the chemical profiles of the non-volatile and volatile extracts for several taxa and to use the data in;
2. An attempt to resolve the specific and infra-specific delimitation problems within the genus using chemotaxonomic data.
3. To identify favourable chemotypes suitable for commercial development in the flavour and fragrance industries.

2.4. Materials and methods

2.4.1. Field sampling and vouchers

Fresh plant material was collected from the wild populations of *Eriocephalus* during their flowering and fruiting periods from different localities in South Africa and Namibia. As the study includes aspects of variation at specific and population levels, multiple collections were made in most cases and voucher specimens were also prepared (Table 2.2). Taxonomic verification was carried out at the South African National Biodiversity Institute (SANBI) Pretoria, Compton Herbarium (Kirstenbosch) and NBRI (Windhoek). The voucher specimens are deposited in the Department of Pharmacy and Pharmacology of the University of the Witwatersrand, Johannesburg, South Africa and the duplicates of Namibian taxa are deposited in the Herbarium of the National Botanical Research Institute, Windhoek, (NBRI) Namibia.

2.4.2. Chemical extraction and analysis

2.4.2.1. Volatile compounds

The plant material was hydrodistilled immediately upon arrival from the collection sites. Between 20-750g of the aerial plant parts (wet or dry material) were hydrodistilled for four hours using a Clevenger apparatus (Figure 2.5). The distillate was collected in a pre-weighed amber vial, which was later, weighed, capped (Teflon cover) and the percentage yields tabulated. The oils were labelled accordingly and refrigerated at 4 ^{o}C for further analyses.

Thin layer chromatography (TLC) is the simplest method for detecting plant constituents. It is reproducible and requires unsophiscated equipment. A selection of the essential oil samples was analyzed using TLC to observe the patterns of variation within and between different populations. One part essential oil was diluted with seven parts of hexane. About 3 µl of the mixture was applied to a silica gel plate (Alugram Sil G/UV_{254}) and compounds eluted in a solvent system comprising toluene/ethyl acetate (93:7). The plate was developed by spraying with either vanillin (1% alcoholic vanillin and 10% sulphuric acid) or anisaldehyde (sulphuric acid) spray and heated in an oven for a few minutes at 100 ^{o}C for improved visualization.

A Schimadzu GC-17A was used for gas chromatography (GC). About 0.8 µl of hexane was mixed with 0.2 µl of essential oil and injected into GC comprising a capillary column (J & W-DBI; 30 m x 0.25 mm x 0.25 mm film thickness). The temperature was set at 60 ^{o}C for one



Figure 2.2. A commercial plantation and habit of *E. punctulatus* showing white rays.

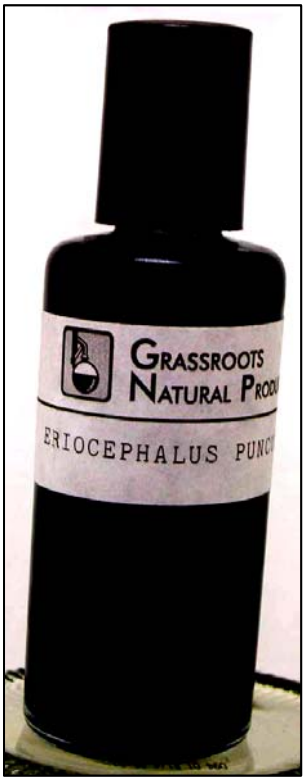


Figure 2.3. Commercial blue oil of *E. punctulatus.*

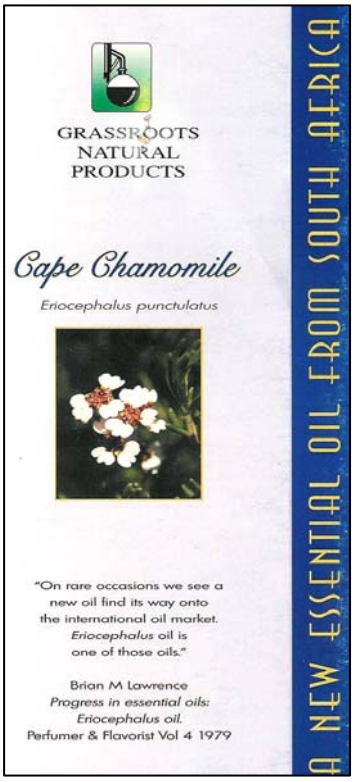


Figure 2.4. Advertisement for the oil of *E. punctulatus* by Grassroots Natural Products (GNP).

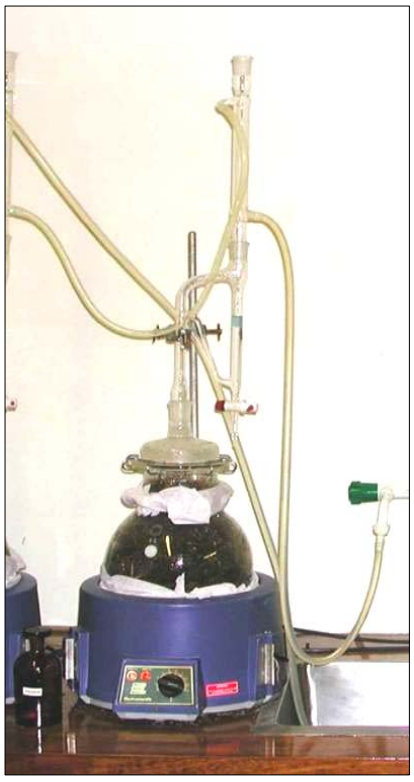


Figure 2.5. A clevenger apparatus used in hydrodistillation of essential oils.

minute and later raised to 180 ^{o}C for complete analysis of 90 minutes. The operating temperature for the injection port and the flame ionization detector (FID) was maintained at 250 ^{o}C. The flame gas comprised hydrogen and air and the carrier gas was helium.

For the GC/MS analysis, the column used was 25 m OV1; the initial temperature of the column was 50 ^{o}C for 1 min and was then heated to 220^{o}C for 5 min with a 3 ^{o}C/min rate. The carrier gas used was helium (He) with a flow rate of 1 ml/min. The samples were injected using a split sampling mode, ratio 1:10. The sample concentration was 1µl essential oil diluted to 1/200. The components were characterized and identified through their retention indices on OV-1 and mass spectra and by use of available standards from the commercial libraries.

The data obtained was analysed using NTSYS-pc Version 2.0 (Rohlf, 1998). The OTUs represented the individual populations of the species and number of compounds sampled. The variables were standardized, and then similarity (SIMINT) using Euclidean distance was computed. Clustering (SAHN) was carried out using the UPGMA algorithm, followed by computation of cophenetic values (COPH) and the correlation (MXCOMP) to obtain goodness-of-fit of the data sets. Finally, the dendrogram was constructed using TREE PLOT and results interpreted accordingly.

2.4.2.2. Non-volatile compounds

Between 0.5-9.4 g of air-dried plant parts (ground or whole) were weighed and 30ml of acetone added. The mixture was left to extract in a water bath (37 ^{o}C) for four hours. The extract was filtered using cotton wool and pipetted into pre-weighed polytubes. The solvent was evaporated and the residue resuspended in methanol and was later passed through a Sephadex LH 20 column rinsed with methanol to remove the overwhelming terpenoids (Wollenweber and Mann, 1989). The mixture was left to evaporate and the residue weighed and refrigerated at 4 ^{o}C.

TLC screening of the extract was carried out using different volumes of the extract until a suitable volume was recorded. 10 µl of extract of a concentration of 100 mg/ml was loaded onto the silica gel plates (Alugram Sil G/UV_{254}) and eluted in a solvent system comprising toluene/dioxane/acetic acid (90: 25: 5). The plates were sprayed using natural spray A (1% methanolic diphenylborinic acid/ 2-aminoethyl ester 98%) and natural spray B (5%

polyethylene glycol). The plates were allowed to develop and dry before observing them under UV_{254nm} and UV_{366nm}.

The acetone extracts were further analysed using high performance liquid chromatography (HPLC). A Waters 2690 HPLC (Phenomex Aqua C18 column, 250 mm x 2.1 mm) equipped with a 996 photo iodide array detector (PDA) and a thermabeam mass selective detector (TMD), operated at 70 eV with a gain of 10 and scanning a mass range of 50-550 amu was used. The thermabeam produces classical electron impact spectra, which can be compared against commercial MS libraries like NIST® and Wiley®.

The samples were diluted in methanol and the injection of 1 µl was done. The flow rate was 0.2 ml/min and gas flow in the nebuliser was 30 l/h with the temperature at 80 ^{o}C and source temperature at 225 ^{o}C, expansion region 90 ^{o}C. The mobile phase started with 10% acetonitrile, 90% water containing 100 mM formic acid. The solvent ratio was changed through a linear gradient to 90% acetonitrile, 10% water (with 100 mM formic acid) at 40 minutes. This ratio was maintained for 10 minutes after the solvent ratio was changed back to the initial starting conditions. The retention time (RT) and UV spectra were recorded using Empower® Software.

2.5. Results

2.5.1. Essential oil and leaf extract yields

There was quantitative variation noted in the oil yields even within individuals of the same species from the same population and from different populations (Figure 2.6). The results obtained from the essential oils yields showed a consistent trend of low yields within species and even between populations e.g. *E. punctulatus* and *E. purpureus* (Table 2.2). The highest oil yield was for *E. purpureus* from Kamiesberg (0.49%), then *E. scariosus* (0.42%) from Namibia and *E. capitellatus* (0.41%) from Swartberg. The lowest yields were between 0.01-0.05% for *E. decussatus* from the Sutherland, *E. spinescens*, *E. purpureus* from Papkuilsfontein, *E. namaquensis* and *E. eximius* among many others. The highest yield of the acetone leaf extracts was noted for *E. aromaticus* (14.2%) from Swartberg and the for *E. spinescens* (0.7%) (Table 2.2). The notable variation in the yields was observed in individuals of *E. punctulatus* (Table 2.2) from different localities as was in the individuals of *E. africanus, E. ericoides* subsp. *ericoides* among other species. However, the individuals of *E. africanus* var *paniculatus, E. aromaticus, E. capitellatus, E. namaquensis* and *E. purpureus*

from Laingsburg had almost the same yields for the three individuals per given species. The oil colour varied greatly between individuals of the same species as observed in *E. africanus* from Citrusdal and from Melkbosstrand (Figure 2.7) among many others (Table 2.2). However, in other cases, the oil colour was noted to be uniform e.g. *E. capitellatus* and *E. brevifolius.*

2.5.2. TLC analysis

2.5.2.1. Volatile compounds

Preliminary TLC screening of essential oils of representative species of *Eriocephalus* indicated variability between individuals of the same species and between populations (Figure 2.8). This is not surprising as the species in this genus depict interesting variation even in the oil colour of individuals of the same species from the same population. Taking into account that same quantities of oils were spotted on the TLC plate, it is worthy noting how diverse the chemistry of a given species can be. These results shed light into the extent of variation in the chemistry of the genus and this was an indication that a single collection per species would not be representative of this extensive variation. Therefore, collection of multiple taxa would be required to understand the chemical diversity of *Eriocephalus.* This strategy was adopted for the rest of the study.

TLC was only used as a prescreening method for recording qualitative variation and for verification of existence of infraspecific variation but the major technique used in this study for essential oil analysis was GC/MS. Despite the variability, some of the individuals of the species showed similarity as was noted in *E. racemosus* var *racemosus* from Velddrif but was different from the individual from Koeberg (Figure 2.8) on track 1A, B and C. Interpopulation variation was noted in individuals of populations of *E. africanus* from Melkbosstrand, Malmesbury and Mossel Bay as was their oil colour (Table 2.2). The TLC profiles for *E. capitellatus* (Figure 2.8) were almost similar for the four individuals from Swartberg but the individuals of *E. microphyllus* from Sutherland and Nieuwoudtville had different profiles.

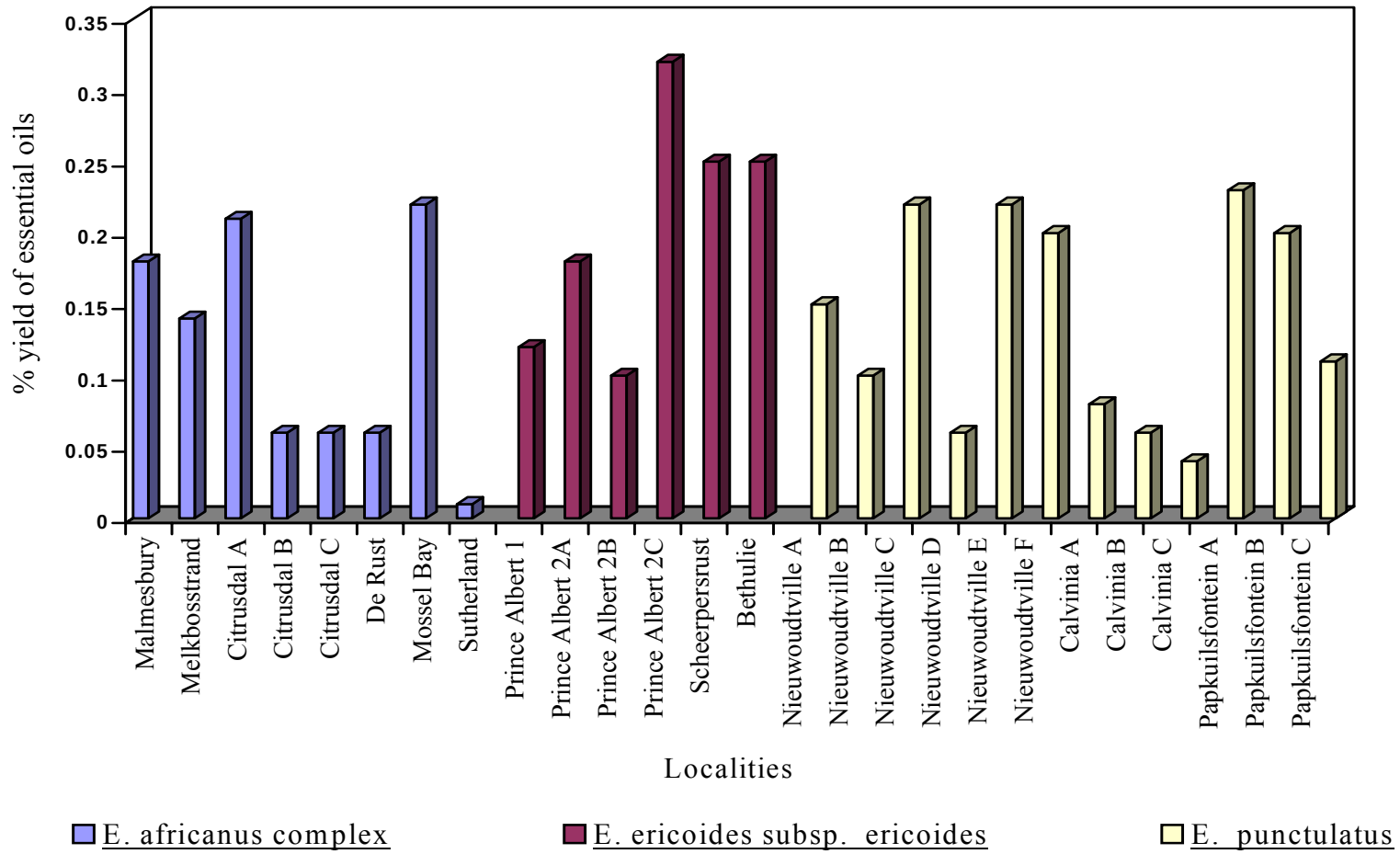
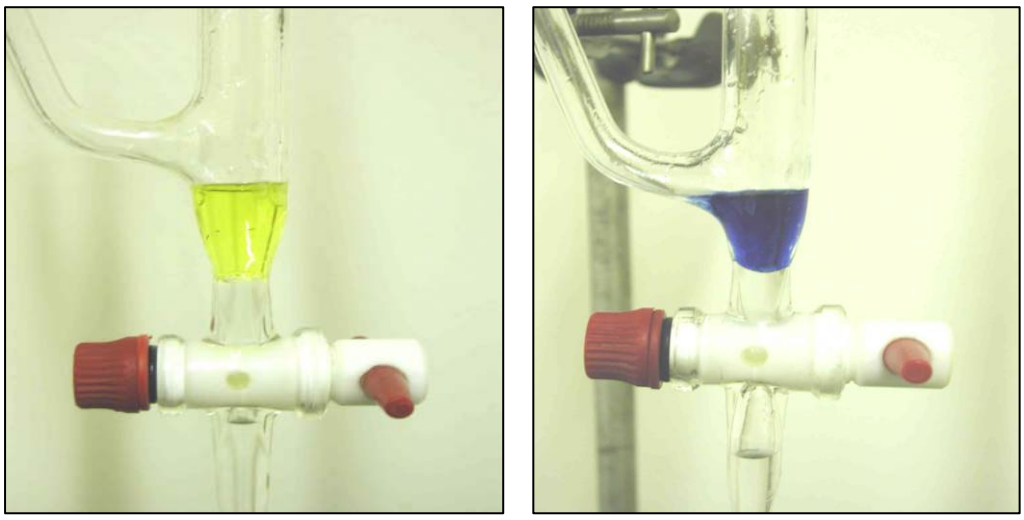


Figure 2.6. Variation in essential oil yields of three representative species of *Eriocephalus* with their constituent taxa from different populations and localities. Note the variation in yields between individuals of the same species from the same population.



A **B**

Figure 2.7. Variation in oil colour of essential oils of *E. africanus* from Malmesbury (A) and Melkbosstrand (B).

2.5.3. GC/MS analysis

The GC/MS analysis of essential oils of 86 taxa of *Eriocephalus* from different localities (Table 2.2) yielded 200 compounds, inclusive of 91 compounds, which could not be identified convincingly using commercial GC/MS libraries. It should be noted that the number of samples analysed is less than in Table 2.2. This was due to low yields for some of the taxa. The common constituents of essential oils present in almost all of the taxa studied included; α- and β-pinene, yomogi alcohol, ρ-cymene, 1,8-cineole, camphor, 4-terpineol, spathulenol, caryophyllene oxide, α-copaene and β-caryophyllene. Most of the species had a relatively high content of 1,8-cineole and spathulenol and a few with a high percentage of camphor.

The summaries of the essential oil composition for each species and constituent taxa are given in the alphabetical monographs in Appendix I (monographs 1-22).

The patterns of variation noted in the essential oil profiles points out that the genus is not only complex at morphological level but also at chemical level. This great chemical diversity was noted between individuals of the same species and between individuals from different populations. Apart from the common compounds mentioned above, other compounds of interest in the genus included, bicyclogermacrene, the azulenic compounds (aromadendrene, alloaromadendrene and chamazulene), bergamotene, eugenol, thymol, eudesmol, bisabolol

Table 2.2: Voucher specimen information for species of *Eriocephalus,* percentage yields of essential oils, acetone leaf extract, their localities, and oil colour of *Eriocephalus* species. Unless indicated essential oils values are based on wet weight and acetone leaf extracts on dry weight. The letters A, B, and C represent individuals of a species from the same population. Nd-not determined; EO-essential oil; AE- acetone extract. *-Values based on dry weight.

Species	Source/locality	Voucher number	Oil colour	% Yield EO	% Yield AE*
E. africanus	Malmesbury	AV 444	Yellow	0.18	8.26
E. africanus	Melkbosstrand	AV 445	Deep blue	0.14	3.84
E. africanus A	Citrusdal	AV 452	Deep yellow	0.21	4.5
E. *africanus* B	Citrusdal	AV 453	Blue	0.06	3.8
E. africanus C	Citrusdal	AV 454	Deep blue	0.06	3.9
E. africanus	De Rust	AV 500	Pale yellow	0.06	1.9
E. africanus	Mossel Bay	AV 504	Pale yellow	0.22	Nd
E. africanus var *paniculatus* A	Sutherland/Farm Koornlandshloof	AV 515 A	Clear	0.01	3.5
E. africanus var *paniculatus* B	Sutherland/Farm Koornlandshloof	AV 515 B	Clear	0.01	1.7
E. africanus var *paniculatus* C	Sutherland/Farm Koornlandshloof	AV 519	Clear	0.02	7
E. ambiguus	Schakalsberge (ex NBRI)	AV 868	Nd	Nd	1.0
E. aromaticus	Swartberg	AV 484	Nd	0.03	14.2
E. aromaticus A	Ladismith/Seweweekspoort	AV 524	Very pale yellow	0.03	10.7
E. aromaticus B	Ladismith/Seweweekspoort	AV 521	Clear	0.03	11.1
E. aromaticus C	Ladismith/Seweweekspoort	AV 520	Clear	0.03	6.8
E. brevifolius	Oudtshoorn	AV 483	Yellow	0.11	6
E. brevifolius A	De Rust/Vergelegen	AV 491	Pale yellow	0.14	Nd
E. brevifolius B	De Rust/Vergelegen	AV 492	Pale yellow	0.27	Nd
E. brevifolius C	De Rust/Vergelegen	AV 493	Pale yellow	0.15	3.3
E. brevifolius	Sutherland/Kamiesberg	AV 835	Pale yellow	0.13	4.7
E. capitellatus	Swartberg Pass	AV 482	Pale yellow	0.10	6.2
E. capitellatus A	Swartberg Pass	AV 497	Pale yellow	0.41	3.7
E. capitellatus B	Swartberg Pass	AV 498	Pale yellow	0.36	Nd
E. capitellatus C	Swartberg Pass	AV 499	Pale yellow	0.38	Nd
E. decussatus A	Sutherland/Fraserburg	AV 532	Pale yellow	0.04	3.6
E. decussatus B	Sutherland/Fraserburg	AV 529	Pale yellow	0.02	2.6
E. decussatus C	Sutherland/Fraserburg	AV 522	Pale yellow	0.03	3.2
E. decussatus	Sutherland/Kamiesberg	AV 836	Deep blue	0.21	4.3
E. dinteri	Near Aus	AV 871	Pale yellowish green	0.19*	2.8
E. ericoides subsp. *ericoides*	Windhoek dist. (ex NBRI)	AV 866	Pale yellow	0.23*	5.9
E. ericoides subsp. *ericoides*	Farm Hohenheim	AV 867	Blue	0.19*	7.3
E. ericoides subsp. *ericoides*	Prince Albert	AV 481	Deep blue	0.12	4.8
E. ericoides subsp. *ericoides* A	Scheepersrust	AV 488	Pale yellow	0.25	2.9
E. ericoides subsp. *ericoides* B	Scheepersrust	AV 489	Pale blue	0.17	Nd
E. ericoides subsp .*ericoides* C	Scheepersrust	AV 490	Deep blue	0.33	Nd
E. ericoides subsp. *ericoides* A	Prince Albert	AV 494	Deep blue	0.18	3.2
E. ericoides subsp. *ericoides* B	Prince Albert	AV 495	Pale yellow	0.10	Nd
E. ericoides subsp. *ericoides* C	Prince Albert	AV 496	Pale yellow	0.32	Nd
E. ericoides subsp. *ericoides* A	Bethulie	AV 747	Clear	0.22	4
E. ericoides subsp. *ericoides* B	Bethulie	AV 748	Clear	0.28	5.3
E. eximius A	Sutherland/Bo-visrivier	AV 528	Light or pale blue	0.04	1.6
E. eximius B	Sutherland/Bo-visrivier	AV 535	Pale blue	0.04	1.4
E. eximius C	Sutherland/Bo-visrivier	AV 534	Pale blue	0.01	1.9
E. eximius	Sutherland/Kamiesberg	AV 837	Deep blue	0.04	2.1
E. grandiflorus A	Laingsburg/Matjiesfontein	AV 525	Clear	0.07	1.5
E. grandiflorus B	Laingsburg/Matjiesfontein	AV 533	Pale yellow	0.04	3.6
E. grandiflorus C	Laingsburg/Matjiesfontein	AV 526	Clear	0.03	2.4
E. klinghardtensis	Neiaab Mountain	AV 870	Greenish yellow	0.17*	3.9
E. luederitzianus A	12 km E of Windhoek	AV 865 A	Pale yellow	0.06*	2.5
E. luederitzianus B	12 km E of Windhoek	AV 865 B	Nd	Nd	2.6

Species	Source/locality	Voucher number	Oil colour	% Yield EO	% Yield AE*
E. merxmuelleri	Buschmanberge	AV 869	Deep blue	0.16*	2.8
E. microphyllus A	Sutherland/Fraserburg	AV 531	Pale brown to yellow	0.08	1.8
E. microphyllus B	Sutherland/Fraserburg	AV 530	Pale brown to yellow	0.09	8.6
E. microphyllus C	Sutherland/Fraserburg	AV 536	Pale brown to yellow	0.12	15
E. microphyllus A	Nieuwoudtville/Loeriesfontein	AV 542	Pale green	0.16	2.9
E. microphyllus B	Nieuwoudtville/Loeriesfontein	AV 543	Dark green	0.11	6.01
E. microphyllus C	Nieuwoudtville/Loeriesfontein	AV 544	Pale green	0.04	5.01
E. microphyllus	Kamiesberg	AV 794	Blue	0.24	5.7
E. microphyllus	Spektakel Pass	AV 795	Blue	0.08	9.5
E. namaquensis A	Clanwilliam/Farm Perdefontein	AV 545	Pale yellow	0.05	1.4
E. namaquensis B	Clanwilliam/Farm Perdefontein	AV 546	Pale yellow	0.05	2.9
E. namaquensis C	Clanwilliam/Farm Perdefontein	AV 547	Pale-greenish yellow	0.05	2.7
E. pauperrimus A	Nieuwoudtville/Loeriesfontein	AV 539	Cloudy white	0.15	1.4
E. pauperrimus B	Nieuwoudtville/Loeriesfontein	AV 540	Pale green	0.39	4.2
E. pauperrimus C	Nieuwoudtville/Loeriesfontein	AV 541	Cloudy white	0.21	4.4
E. pinnatus	Brandberg (ex NBRI)	AV 864	Greenish yellow	0.09*	3.7
E. punctulatus A	Nieuwoudtville	AV 439	Greenish blue	0.15	6.4
E. punctulatus B	Nieuwoudtville	AV 441	Greenish blue	0.10	3.1
E. punctulatus C	Nieuwoudtville	AV 442	Cloudy blue	0.22	2.6
E. punctulatus D	Nieuwoudtville	AV 443	Cloudy blue	0.06	4.2
E. punctulatus E	Nieuwoudtville	AV 447	Blue	0.22	2
E. punctulatus F	Nieuwoudtville	AV 448	Deep green blue	0.20	8.8
E. punctulatus A	Nieuwoudtville/Calvinia	AV 449	Greenish blue	0.08	1.9
E. punctulatus B	Nieuwoudtville/Calvinia	AV 450	Nd	0.06	3.3
E. punctulatus C	Nieuwoudtville/Calvinia	AV 451	Nd	0.04	3.7
E. punctulatus A	Nieuwoudtville/Papkuilsfontein	AV 548	Pale yellow	0.23	4.1
E. punctulatus B	Nieuwoudtville/Papkuilsfontein	AV 549	Pale yellow	0.20	5.8
E. punctulatus C	Nieuwoudtville/Papkuilsfontein	AV 550	Pale yellow	0.11	5.9
E. purpureus A	Laingsburg/Matjiesfontein	AV 516 A	Clear	0.02	1.5
E. purpureus B	Laingsburg/Matjiesfontein	AV 516 B	Clear	0.02	7.2
E. purpureus C	Laingsburg/Matjiesfontein	AV 516 C	Clear	0.02	5.9
E. purpureus	Nieuwoudtville	AV 440	Nd	0.01	2.3
E. purpureus A	Nieuwoudtville/Papkuilsfontein	AV 551	Greenish blue	0.01	6.1
E. purpureus B	Nieuwoudtville/Papkuilsfontein	AV 552	Greenish blue	0.01	5.1
E. purpureus C	Nieuwoudtville/Papkuilsfontein	AV 553	Pale green	0.05	5.4
E. purpureus	Kamiesberg	AV 796	Pale yellow	0.49	11
E. racemosus	Koeberg	AV 446	Light- bluish green	1.16	3.2
E. racemosus var *racemosus* A	Velddrif	AV 455	Deep yellow	0.10	1.4
E. racemosus var *racemosus* B	Velddrif	AV 456	Deep yellow	0.13	6.5
E. racemosus var *racemosus* C	Velddrif	AV 457	Deep yellow	0.18	8.1
E. scariosus	Near Aus	AV 872	Pale yellow	0.42*	5.4
E. spinescens A	Sutherland/Ceres	AV 523	Pale yellow	0.01	0.7
E. spinescens B	Sutherland/Ceres	AV 517	Clear	0.03	2.9
E. spinescens C	Sutherland/Ceres	AV 518	Pale yellow	0.03	2.1

products, linalyl acetate, linalool, nerolidol, and α-phellandrene. Most of these compounds have medicinal properties and are commercially used in flavour, fragrance and cosmetic industries. However, most of these compounds, apart from linalool, linalyl acetate and nerolidol, were in very low amounts in the essential oils of the various species. Their presence could be responsible for the various biological activities noted in Chapter 4.

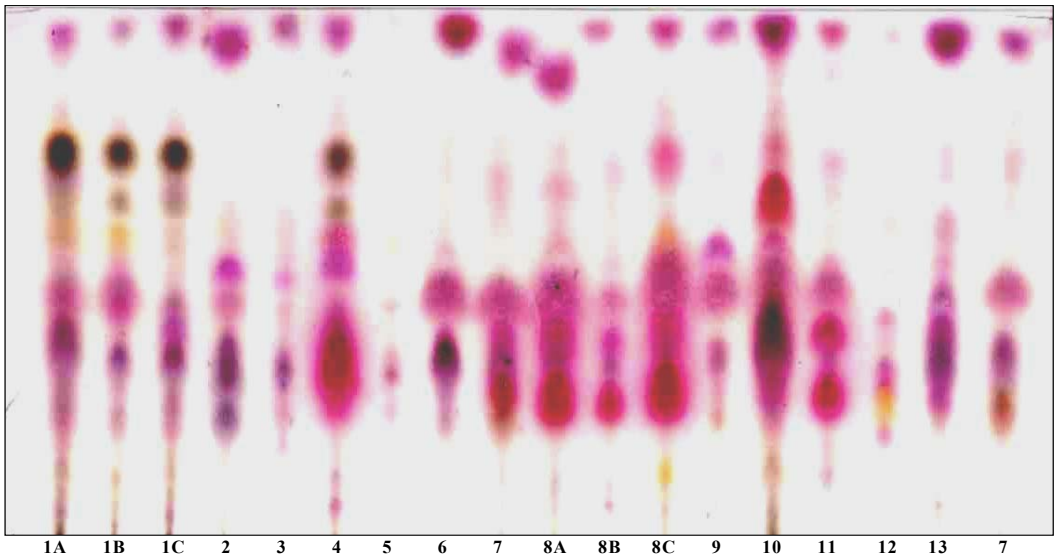


Figure 2.8. A TLC plate of selected essential oils of various species of *Eriocephalus* showing variation between individuals of same species and between populations 1A, B and C: *E. racemosus* var *racemosus* (Velddrif); 2: *E. racemosus* var *racemosus* (Koeberg); 3-5: *E. africanus* (Melkbosstrand, Malmesbury and Mossel Bay); 6: *E. ericoides* subsp. *ericoides* (Prince Albert); 7: *E. capitellatus* (Swartberg Pass); 8A, B and C: *E. punctulatus* (Nieuwoudtville); 9: *E. punctulatus* (Nieuwoudtville/Papkuilsfontein); 10: *E. microphyllus* (Sutherland/Fraserburg); 11: *E. microphyllus* (Nieuwoudtville/Loeriesfontein); 12: *E. pauperrimus* (Nieuwoudtville/Loeriesfontein); 13: *E. purpureus* (Nieuwoudtville/ Papkuilsfontein).

The results for representatives of some of the species in the study are hereby discussed, and include some of the commercially used species. The species discussed include *E. africanus, E. capitellatus, E. ericoides* subsp. *ericoides, E. microphyllus, E. pauperrimus, E. punctulatus, E. purpureus* and *E. spinescens*.

2.5.3.1. *E. africanus*

Six populations of this species were included in this study including the individuals of *E. africanus* var *paniculatus* from Sutherland (Farm Koornlandshloof) (Appendix I, monograph 1). Eighty-one compounds, including 20 unknown were recorded in the essential oil. Compounds present in most of the taxa included α- and β-pinene (0.5-7.5% and 0.3-2%) respectively (with the latter absent from the Citrusdal population); ρ-cymene (0.5-7.4%); 1,8-cineole (0.7-23.6%); limonene (0.2-0.8%) except in one individual from Citrusdal and Mossel Bay. Other compounds included camphor (0.4-13.3%), which was absent in the individuals

from Melkbosstrand, Malmesbury and Mossel Bay; 4-terpineol (0.30-7.3%); α-copaene (1.1-2.5%); spathulenol (9.5-40%), which was absent in the population from Malmesbury and caryophyllene oxide (3.8-16.8%). The latter two compounds constituted the major compounds in almost all the taxa, though they could be probable artifacts due to storage. The rest of the major compounds present in the taxa have been summarized in Table 2.3. It is clear that apart from the population from Malmesbury, one individual from Citrusdal and the population from Mossel Bay with artemisia ketone, santolina alcohol and 1,8-cineole as major compounds respectively, the rest of the populations had spathulenol and caryophyllene oxide as major compounds. Two individuals from Citrusdal had piperitone (0.4-17.2%). Bicylogermacrene was present in the Citrusdal and Mossel Bay populations. Present in the populations from Malmesbury and Sutherland was α-longipinene.

Santolina triene was present in the populations from Melkbosstrand and Malmesbury (0.7-2.8%). One individual from Citrusdal had α-bisabolol (2.4%) and chamazulene was present in the Melkbosstrand and Citrusdal populations (1.1-2.4%). A summary of major compounds in each taxa and their total percentage has been given in Table 2.3.

Variation in some of the major components noted in the essential oils has been summarised in Figure 2.9. Spathulenol was present in relatively higher percentages than were 1,8-cineole and camphor in most of the populations (Figure 2.9). Camphor, spathulenol and caryophyllene oxide were conspicuously absent in the population from Melkbosstrand (Figure 2.9).

2.5.3.2. *E. capitellatus*

Thirty-four (34) compounds inclusive of four unidentified were realized from the analysis of the essential oils of two populations from Swartberg (Appendix I, monograph 5). Among the species of *Eriocephalus* studied, this species had characteristically very high contents of camphor (47-50.3%) and derivatives and conspicuous presence of camphene groups that distinguished it from the rest of the members in the group. The compounds present in almost all of the individuals included α- and β-pinene (0.6-1.3%; 1.2-5.9%) respectively, camphene (1.8-3.4%), 1,8-cineole (8.5-11.4%), camphor (47-50.3%), 4-terpineol (1.1-2.1%), limonene (0.3-0.6%), spathulenol (0.9-2.1) and sabinene (0.3-0.5%) among other several compounds (Appendix I, monograph 5).

Table 2.3. Variation in chemical composition of major compounds of the essential oils of taxa of *E. africanus* from different localities. The compound with the highest (%) in each taxon is indicated in bold case.

Major compounds	**Pop 1** **Mmy**	**Pop. 2** **Mkb**	**Pop. 3** **Cdl**			**Pop. 4** **Dr**	**Pop. 5** **Msy**	**Pop 6** **Stl**	
			A	**B**	**C**			**A**	**C**
α-Pinene	0.8	3.1	<0.46			0.5	7.5	0.5	2.4
Yomogi alcohol	2.2	0.4	1.0				0.4		
Sabinene	0.2	8.4		<0.70	<0.41		2.0		
p-Cymene	1.4	7.4	0.6	1.2	0.9	3.8	3.4	0.5	0.8
1,8-Cineole	1.8	4.1	2.0	1.2	0.7	3.8	**23.6**	3.5	4.6
Santolina alcohol			**29.9**						
Artemisia ketone	**11.8**						4.5		
Chrysanthenone							18.5		
Camphor			12.1	1.1		7.1		0.9	0.4
Borneol	1.6		10.0			1.9	2.7	1.2	0.9
Piperitone				17.2	0.4				
4-Terpineol	0.3	3.7	0.6	3.5	5.0	1.7	7.3	0.8	0.6
Neryl acetone					5.7				
β-Caryophyllene		5.1		1.1	1.2	1.5	0.6	2.0	
α-Longipinene	0.6							3.7	
Bicyclogermacrene		3.3	0.7	13.6	10.6				
Spathulenol		**9.5**	0.6	**25.5**	**30.3**	**19.3**	1.2	**17.2**	**40.0**
Caryophyllene oxide		6.8		3.8		9.8	1.0	4.5	10.1
α-Cadinol	2.5								
δ-Cadinene			9						
α–Cadinol or τ-Muurolol			2.7			8.4	<0.35		1.5
β -Eudesmol						2.9			2.1
Chamazulen + MW =220		1.2		1.1	2.4				
En-in-dicycloether	2.3								
Total	**25.5**	**53**	**69.7**	**70**	**57.6**	**60.7**	**77.5**	**34.8**	**63.4**

MMY - Malmesbury; MKB - Melkbosstrand; CDL – Citrusdal; DR – De Rust; MSY – Mossel Bay ; STL – Sutherland/Farm Koornlandshloof.

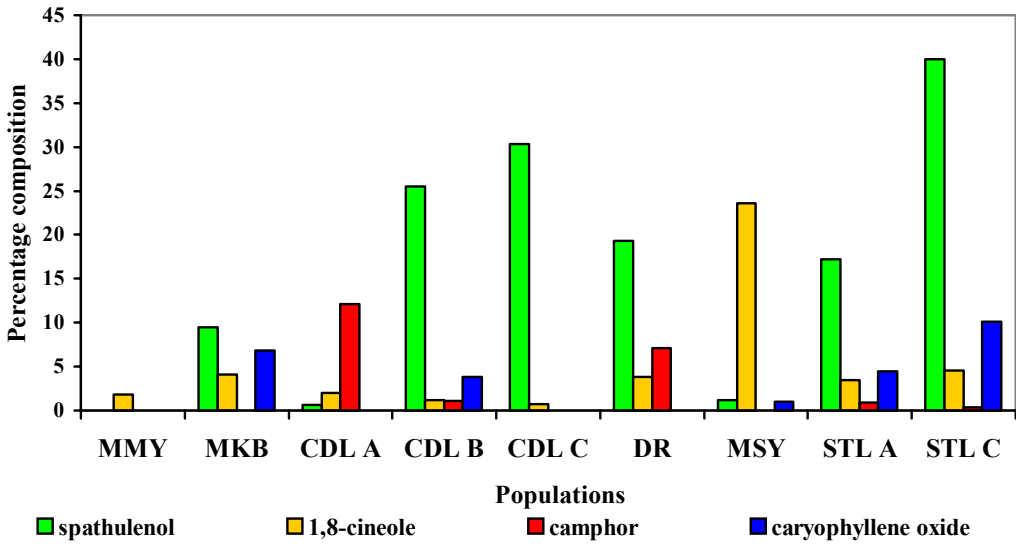


Figure 2.9. Variation in percentage composition of some of the common compounds found in the essential oils of populations of *E. africanus*. MMY - Malmesbury; MKB - Melkbosstrand; CDL - Citrusdal; DR - De Rust; MSY - Mossel Bay; STL - Sutherland.

This species had taxa that showed very little variation in the composition of the essential oils, a deviation from the normal trend noted in most taxa in other species with variable chemical profiles. The two populations from the same geographical range (Swartberg) were analysed and all the individuals had camphor as the major compound and in relatively high amounts (47-50%). The oils were largely comprised of camphene groups such as borneol, pinocamphone and camphene in varying amounts. Also present in the essential oils was caryophyllene alcohol, carvone, bicyclogermacrene and santolina triene but in low amounts.

2.5.3.3. *E. decussatus*

The GC/MS analysis of the essential oils recorded 32 compounds inclusive of seven unidentified ones (Appendix I, monograph 6). The essential oil composition in this species varied considerably in individuals from Sutherland (Fraserburg) where individual B was different from individuals A and C, and was characterized by the presence of high content of linalool and derivatives. This was an example of extreme variation of chemical profiles in individuals from the same locality. However, there were common compounds present in the essential oils despite this discrepancy namely; 1,8-cineole (1.4-12.1%) ρ-cymene (0.7-3.7%), 4-terpineol (0.63-2.7%), isocomene (1.0-2.5%), spathulenol (12.4-30.9%) and caryophyllene (2.6-9.8%). Present only in individual B were compounds such as fenchene and camphene, *trans*-pinocarveol, borneol, α-pinene, nerolidol and pinocarveol among others. The major compounds characterizing the species were spathulenol (12-30%) and linalyl acetate (19-30%). Linalool was also noted in the essential oils (9-12%) and small amounts of linalool enantiomers, *cis* and *trans*-linalool oxide. This species shared similar chemistry with *E. spinescens* (Appendix I, monograph 22). The major compounds in the latter included spathulenol (11-22%), linalyl acetate (7-21%) and 1,8-cineole (5-11%) and linalool (4-8%). As in *E. decussatus,* small amounts of linalool enantiomers *cis* and *trans*-linalool oxide were recorded in the essential oils of *E. spinescens.* The *cis*- and *trans*-linalool oxides, linalool and linalyl acetate were also recorded in the essential oils of *E. microphyllus, E. grandiflorus, E. brevifolius, E. eximius* and *E. dinteri*.

2.5.3.4. *E. ericoides* subsp. *eriocoides*

This was the most widely distributed species in the genus and with very variable chemistry. Six populations of this species were studied, two of which were from Namibia. Eighty-six compounds including 27 unidentified were recorded (Appendix I, monograph 8). The taxa of

this species had characteristically high percentages of 1,8-cineole (23.4-46.9%). Some of the compounds present in all the taxa studied included; α-pinene and spathulenol. Most of the taxa had camphene, pinane, and fenchene groups. Other notable compounds present in the group included β-eudesmol, chamazulene, limonene, linalyl acetate, linalool and alloaromadendrene with the latter, an azulenic compound, probably responsible for the blue colour of the oils.

One of the individuals from Namibia had similar chemistry to the collection from Bethulie (South Africa). The Namibian individuals, the two populations from Prince Albert and one from Scheepersrust were characterized by the presence of high percentages of 1,8-cineole as shown in Figure 2.10 compared to the rest of the taxa in the group. The population from Bethulie had the least amount of this compound. β-eudesmol was absent in the Namibian and Bethulie populations. A summary of all the major compounds found in all the populations studied has been given in Table 2.4 and the summary of chemical composition of each individual given in (Appendix I, monograph 8).

2.5.3.5. *E. klinghardtensis*

Among the taxa of *Eriocephalus* from Namibia, this species was the only one that had chrysanthenone as the major compound. Only one individual was analyzed and nineteen compounds were recorded including one unidentified compound (Appendix I, monograph 11). The major compounds included chrysanthenone (24.4%), ρ-cymene (8.9%), α-pinene (7.9%), *trans*-chrysantemyl acetate (5.6%) and 4-terpineol (5.4%). The chemical profiles of this species compared closely to those of *E. luederitzianus* and *E. merxmuelleri* with the latter species characterized by a high percentage of pinane groups (α-pinene = 30.8%; β-pinene = 10.3%) in the essential oil (Appendix I, monographs 12 and 13 respectively). α-phellandrene was found mostly in the Namibian species; *E. dinteri, E. merxmuelleri, E. pinnatus* and *E. ericoides* subsp. *ericoides* and in two of the South African species namely; *E. ericoides* subsp. *ericoides* from Scheepersrust and *E. microphyllus* from Kamiesberg. Other notable differences were the comparatively low percentages of 1,8-cineole in some of the Namibian species e.g *E. klinghardtensis* and *E. pinnatus* (1.36-5.1%).

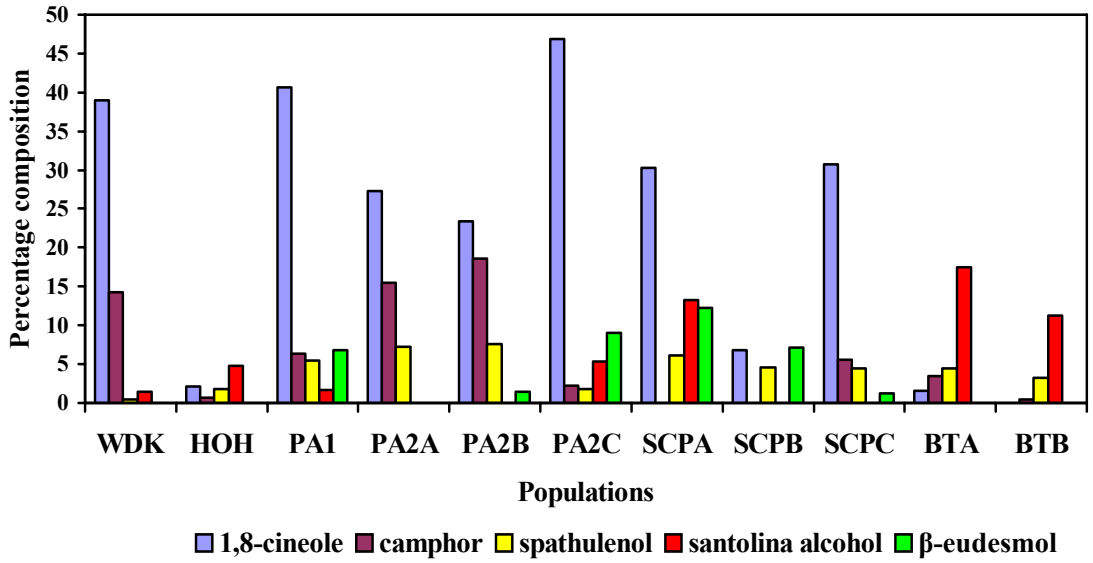


Figure 2.10. Variation in percentage composition of some common compounds found in the essential oils of populations of *E. ericoides* subsp. *ericoides*. WDK - Windhoek; HOH-Hohenheim; PA - Prince Albert (2); SCP - Scheepersrust; BT - Bethulie.

Table 2.4. Major compounds found in the essential oils of six populations of *E. eriocoides* subp. *ericoides* from five localities*. Values are given in percentages.

Major compounds	**Pop.1 Wdk**	**Pop. 2 Nmb**	**Pop. 3 Pa**	**Pop. 4 Pa**			**Pop. 5 Scp**			**Pop. 6 Bt**	
				A	**B**	**C**	**A**	**B**	**C**	**A**	**B**
3,5–heptadien-2-ol, 2,6-dimethyl	5.4	1.2									
Yomogi alcohol	5.6	4.4	1.8			0.8	8.9			7.6	**22.4**
1,8-Cineole	**39.0**	2.1	**40.6**	**27.3**	**23.4**	**46.9**	**30.3**	6.8	**30.7**	1.6	
p-Cymene	1.3		1.3	0.6	2.2	2.9	1.8	9.5	4.1	0.7	0.7
Santolina alcohol	1.5	4.8	1.7			5.4	13.2			**17.5**	11.3
Artemisia alcohol	3.4	1.6	0.4			<0.45	2.0			2.0	14.1
Linalool		**10.4**				0.5		1.3			
Camphor	14.3	0.7	6.4	15.5	18.5	2.2			5.6	3.5	0.5
Linalyl acetate		5.0						3.9		1.0	
Borneol		3.0							5.1	0.8	
4-Terpineol			4.6	2.7	3.4	3.0	3.1	**33.2**	2.3		1.0
Artemisyl acetate	3.2	3.2								5.0	
Trans-Chrysantemyl acetate									4.2		
Bornyl acetate	0.9	4.3	1.2	9.6	16.0	1.6					0.6
α-Cadinol or τ-Muurolol		1.4	2.1		1.3	8.0		2.7		1.8	4.1
γ-Cadinene						4.0					
Spathulenol	0.5	1.8	5.5	7.2	7.6	1.8	6.1	4.6	4.5	4.4	3.2
Borneol + Pinocamphone	3.2			3.3	5.1						
β-Eudesmol			6.8		1.4	9.0	12.3	7.1	1.2		
γ-Terpinene			1.7	0.9			0.7	8.1			
Bicyclogermacrene			3.5	3.5	0.7			0.5			
Total	**78.3**	**43.9**	**77.6**	**70.6**	**79.6**	**86.6**	**78.4**	**77.7**	**57.7**	**45.9**	**57.9**

*- For full names of localities, refer to legend in Figure 2.10

2.5.3.6. *E. pauperrimus*

This was one of the twenty-two species studied that showed a unique essential oil composition consisting of relatively high contents of bisabolol derivatives (Figure 2.11). Three individuals from between Nieuwoudtville and Loeriesfontein were analysed and 36 compounds were identified (Appendix I, monograph 16). The common compounds in all three individuals included α- and β-pinene (0.6-1.8%) and bisabolol oxide A and B (0.5-22.8% and 1.5-45.3%) respectively. Individual B had the lowest quantity of the bisabolol derivatives when compared to the other two individuals that had bisabolol oxide A as the major compound (Figure 2.11).

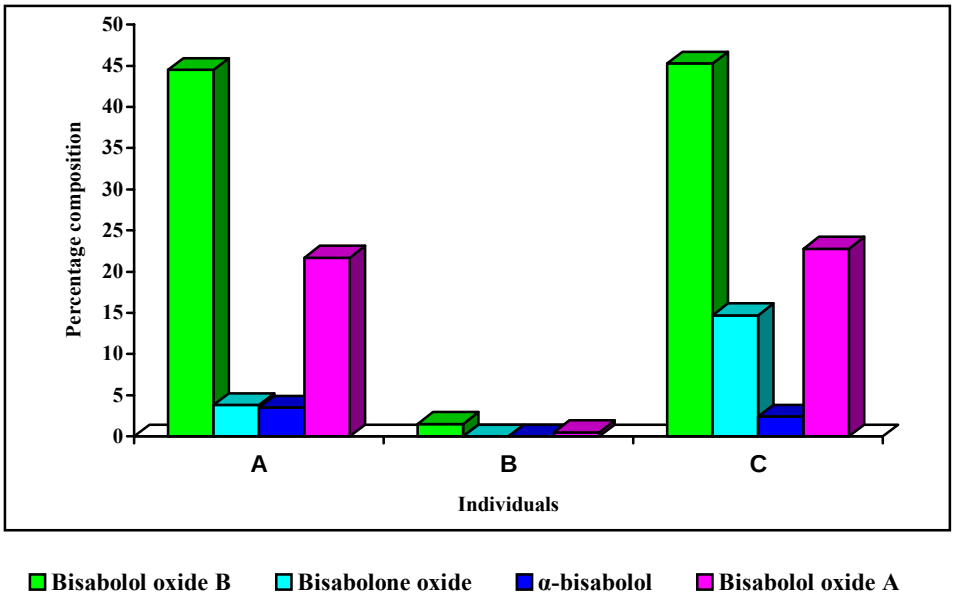
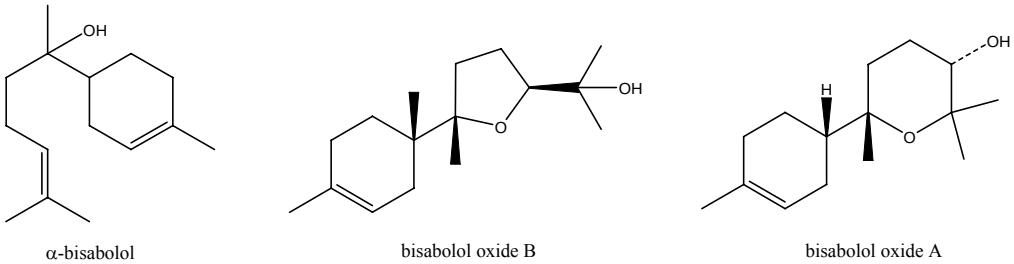


Figure 2.11. The percentage composition of the various bisabolol derivatives in three individuals of *E. pauperrimus* from Nieuwoudtville/Loeriesfontein. The structures of some of the compounds are shown below.



α-bisabolol bisabolol oxide B bisabolol oxide A

The major constituents in the essential oils in the three individuals were bisabolol derivatives; bisabolol oxide B (45%), nerolidol (5-36%), bisabolol oxide A (0.5-22%), bisabolone oxide

(4-15%) and α-bisabolol (2-4%). Individual A and C had bisabolol oxide B as the main components as B had nerolidol. There was conspicuous absence of spathulenol and camphor in the three individuals, which was present in almost all of the other species. Individuals A and C had α-bisabolol which had also been detected in *E. africanus* from Citrusdal and *E. racemosus* var *racemosus* from Velddrif. The chemical composition of individual A and C was very similar.

2.5.3.7. *E. pinnatus*

This was one of the species in the genus that had unique autapomorphies such as yellow rays, pinnatisect leaves and absence of secondary growth in the habit. The GC/MS analysis of the essential oils recorded 36 compounds (Appendix I, monograph 17). The percentage composition of most of the compounds was relatively low and ranged between (0.5-7.9%). The major constituents included; isoamyl-2-methyl butyrate (7.9%), β-pinene (7.3%), isoamyl valerate (6.5%), 1,8-cineole (5.1%) and ρ-cymene (4.5%).

Chemically, the species had a few compounds not common in the rest of the taxa studied such as propanoic acid-2-methyl-3-butyl, isopentyl isobutanoate, isoamyl-2-methyl butyrate and isoamyl valerate. Some of the compounds found in this species that were also present in the rest of the taxa in the genus included pinane groups, *cis*- and *trans*-piperitol, limonene, 4-terpineol and 1,8-cineole among many others. The essential oil composition of this species had some similarities with that of *E. microphyllus* (Appendix I, monograph 14).

2.5.3.8. *E. punctulatus*

This was one of the species with the highest number of individuals (six) from one particular population included in the study. It is also one of the commercially used species. The three populations included in the study were from a similar geographical range, Nieuwoudtville, Nieuwoudtville (near Calvinia) and Nieuwoudtville (near Papkuilsfontein) from the Northern Cape Province and there were some similarities in their chemical profiles. Seventy-four compounds were recorded inclusive of 11 unidentified ones (Appendix I, monograph 18). The essential oils were characterized by the presence of a high number of acetates and alcohols. Also present were few azulenic compounds such as aromadendrene and alloaromadendrene. The population from Nieuwoudtville had individuals A, B and E having piperitone as the main major compound while C, D and F had 1,8-cineole as the major constituent. The population from Nieuwoudtville/Calvinia was characterized by the presence of α-cadinol or τ-

muurolol as the major compound while the population from Nieuwoudtville (Papkuilsfontein) was characterized by the presence of 1,8-cineole as the major compound.

Only 4-terpineol (1.5-5.5%) and spathulenol (0.9-7.8%) were present in all the taxa studied. Other compounds were sometimes absent in one or two individuals or completely absent from a given population. For instance, artemisia and yomogi alcohols were absent in the population from Nieuwoudtville (Papkuilsfontein). α- and β-pinene were conspicuously absent from the Nieuwoudtville (Calvinia) population (Appendix I, monograph 18). Most of the artemisyl derivatives, and bicycogermacrene were absent in NVPP population. The variation in the percentage composition of the various compounds has been shown in (Figure 2.12). Most of the taxa had relatively high contents of 1,8-cineole. A summary of the major compounds found in all the taxa studied has been given in Table 2.5. *Cis*-piperitol γ-terpinene, artemisia ketone, limonene, aromadendrene and alloaromadendrene, β-eudesmol and many other camphene and pinane groups were also present in the oils.

The composition of the essential oils for the three populations of *E. punctulatus* did not entirely resemble that of the commercially exploited species as given by Mierendorff *et al*., (2003). It is clear that the material used commercially is developed from very specific clones.

2.5.4. Variation in composition between the radiate and disciform taxa

The overall pattern emerging in the essential oil profiles of the two groups was the obvious absence of artemisia ketone, carvacrol and α-cedrene in the disciform taxa. In the radiate taxa, the following compounds were conspicuously absent namely: *Cis* and *trans*-linalool oxide, nerol oxide, *cis* and *trans*-chrysantemyl acetate, *trans*-sabinene hydrate and α-thujenal (Table 2.6). The common compounds and their percentages in the two groups included; α- pinene (0.1-31%) β-pinene (0.2-15%), yomogi alcohol (0.2-22%), 1,8-cineole (0.2-53%), camphor (0.3-52%), 4-terpineol (0.3-33%), spathulenol (0.2-40%) and caryophyllene oxide (0.1-11%). The radiate taxa had the highest percentage of 1,8-cineole, camphor and spathulenol while the disciform taxa had relatively high percentages of α- and β-pinene.

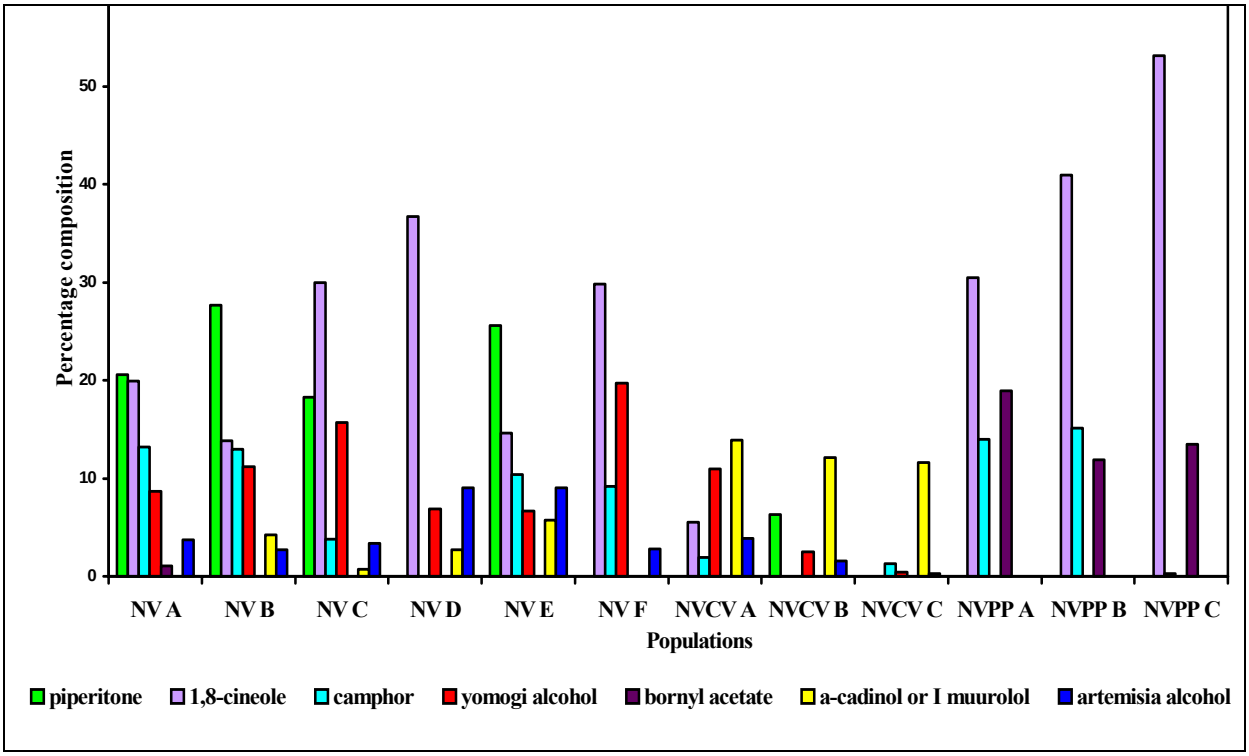


Figure 2.12: Variation in percentage composition of some of the compounds found in the essential oils of individuals of *E. punctulatus* from three different localities. NV - Nieuwoudtville; NVCV - Nieuwoudtville/Calvinia; NVPP - Nieuwoudtville/Papkuilsfontein.

Table 2.5. Variation in major compounds found in essential oils of taxa of *E. punctulatus* from different localities.

Major compounds	**Pop. 1 Nv**						**Pop. 2 Nvcv**			**Pop. 3 Nvpp**		
	A	**B**	**C**	**D**	**E**	**F**	**A**	**B**	**C**	**A**	**B**	**C**
α-Pinene	0.8	1.0	0.5		0.4	3.4				3.2	3.9	2.1
Camphene	1.3	0.7	0.5		<0.42	1.0				2.3	1.6	0.5
Yomogi alcohol	8.7	11.2	15.7	6.9	6.7	19.7	11.0	2.5	0.4			
1,8-Cineole	19.9	13.8	**30.0**	**36.7**	14.6	**29.8**	5.5			**30.5**	**40.9**	**53.1**
p-Cymene	1.5	1.1	1.4	1.8	1.2	0.8	0.3			2.3	3.3	3.8
Santolina alcohol				7.3								
Artemisia alcohol	3.7	2.7	3.4	9.0	0.9	2.8	3.9	1.6	0.3			
Camphor	13.2	13.0	3.8		10.4	9.2	1.9		1.3	14.0	15.1	<.29
4-Terpineol	2.6	1.5	2.0	2.8	2.5	1.9	5.6	3.1	1.5	2.3	5.5	5.5
α-Cadinol or τ-Muurolol		4.2	0.7	2.7	5.7		**13.9**	12.1				
Spathulenol	1.5	2.7	1.1	1.6	0.9	2.4	1.3	7.8	**7.7**	1.5	1.6	1.6
Caryophyllene oxide	1.2	2.4	1.0	2.6	1.5	2.2	2.4	**8.2**	6.6		1.7	1.0
Pinocamphone				3.8								
Borneol + Pinocamphone							5.8					
Borneol	5.2								3.1	4.6	3.9	3.4
Piperitone	**20.6**	**27.7**	18.3		**25.6**			6.3				
Bornyl acetate	1.1									18.9	11.9	13.5
β-Caryophyllene	0.6	1.4	1.1	2.0	1.9	1.4	2.6	4.7	4.9		0.5	0.6
β-Eudesmol		1.4				0.8			6.3			
Total	**81.9**	**84.8**	**68.5**	**77.2**	**72.7**	**75.4**	**54.2**	**46.3**	**43.7**	**79.6**	**89.9**	**85.39**

NV - Nieuwoudtville; **NVCV** - Nieuwoudtville/Calvinia; **NVPP** - Nieuwoudtville/Papkuilsfontein

Table 2.6. Variation patterns of compounds present in radiate and disciform taxa of *Eriocephalus.* (+) Present; (-) Absent.

Capitula type	Compounds							
	Artemisia ketone	Carvacrol	α-cedrene	*Cis* and *trans*-linalool oxide	Nerol oxide	*Cis* and *trans*-chrysanthemate acetate	*Trans*-sabinene hydrate	α-thujenal
Radiate	+	+	+	-	-	-	-	-
Disciform	-	-	-	+	+	+	+	+

These variations noted in the chemical profiles in the radiate and disciform taxa are further expounded under the discussion.

2.5.5. Cluster analysis

2.5.5.1. Quantitative analysis

The cluster analysis of all chemical compounds (200) in all the 86 OTUs resulted in two clusters at the highest level of dissimilarity, 11.36 (Figure 2.13). The major cluster I comprised nearly all the taxa and cluster II had one individual of *E. ericoides* subsp. *ericoides* from Scheepersrust with *E. luederitzianus* as the outlier. The clustering of the OTUs did not follow the species pattern; grouping was rather based on the chemistry of individual OTUs irrespective of the species. The chemical profiles of the species in the genus were highly complex and this phenomenon was reflected in the clustering patterns. In most cases there was no coherent patterns emerging that would be used to support the infraspecific delimitation. A few groups (A-F) based on Figure 2.13 were hereby considered.

Group A had OTUs of *E. africanus* var *paniculatus, E. aromaticus, E. spinescens, E. pauperrimus* and two taxa of *E. purpureus* characterized by presence spathulenol (11.9-22.2%) in their essential oils. Morphologically, *E. africanus* var *paniculatus, E. aromaticus* and *E. purpureus* have radiate capitula and connate paleae while *E. spinescens* and *E. pauperrimus* have disciform capitula and free paleae of marginal florets. All these species have opposite, decussate to alternate leaves with variants of the indumentum on the leaf surface.

Group B was dominated by five OTUs of *E. punctulatus* from Nieuwoudtville and one of *E. brevifolius* B from De Rust. This relationship was characterized by the presence of relatively high levels of piperitone (18.3-27.7%) in the OTUs of the former species and presence of

comparatively high levels of 1,8-cineole (5.5-30%), camphor (0.6-13.22%) and yomogi alcohol (5.2-15.7%) in all the OTUs in this subcluster. These species showed some chemical affinities and morphologically have radiate capitula. The affinities between *E. brevifolius* and *E. punctulatus* have been discussed in Chapter 5.

Group C was comprised of OTUs of *E. ericoides* subsp. *ericoides*, *E. microphyllus, E merxmuelleri, E. pauperrimus,* two OTUs of *E. namaquensis, E. spinescens* and *E. scariosus.* All these OTUs were characterized by presence of high contents of 1,8-cineole (27.3-30%) as major compound in their essential oils apart from *E. pauperrimus.* All the OTUs apart from *E. scariosus* have disciform capitula and the paleae of their marginal florets are free. In the recent account of the genus, *E. microphyllus, E. ambiguus, E. luederitzianus* and *E. merxmuelleri* have been considered very similar due to the intergrading of the characters used to delimit them. There were obvious chemical affinities between *E. microphyllus* and *E. merxmuelleri* as they grouped next to each other in this cluster. *Eriocephalus namaquensis* and *E. spinescens* have also been reported as similar and sometimes confused with each other. It is clear they share some chemical affinities as they clustered together, but they are also distinct species as depicted by their chemistry. Next to this group were OTUs of *E. aromaticus* and *E. grandiflorus* that featured prominently in all the analyses (phenetic) as an indication of close morphological and chemical affinities. They shared camphor (12.3-12.8%) as the major compound and are morphologically related in having radiate capitula, connate paleae and decussate leaves. This relationship is further discussed in Chapter 5.

Group D had OTUs of *E. ericoides* subsp. *ericoides* and three of *E. punctulatus* from Nieuwoudtville/Papkuilsfontein characterized by the presence of high contents of 1,8-cineole (23.4-53%), bornyl acetate (1.2-19%) and camphor (5.6-18.5%) as major compounds. The OTUs of the latter species were distinctively characterized by the presence of α-cadinol as the major compounds and they shared other similar compounds hence clustering together. Morphologically, the two species have opposite leaves but other morphological characters are different despite the similarities in their essential oil chemical profiles.

Group E had OTUs of *E. capitellatus, E. dinteri, E. microphyllus* and *E. purpureus,* which were characterized by the presence of high levels of camphor (25-52%) and its derivatives. In this dendrogram, the OTUs of *E. capitellatus* were clustered in different positions despite them having high contents of camphor. Among all the species of *Eriocephalus,* the

aforementioned species had the highest amount of camphor recorded in individuals. Morphologically the species in this group have opposite to decussate leaves that have sericeous to felty indumentum on their surfaces.

Group F had OTUs of *E. decussatus* and *E. spinescens* that were characterized by the presence of relatively high contents of linalyl acetate (19.3-31.1%), which was the major compound in their essential oils. These species had linalool and derivatives present in reasonable amounts in the oils. Morphologically, these species have disciform capitula and sericeous, decussate leaves. The affinities between these species have been discussed in Chapter 5.

The rest of the OTUs in the dendrogram had relationships based on varying percentages of the constituent compounds in their essential oils. Some of these compounds included spathulenol (25.5-30.3%) in *E. africanus* and *E. eximius* respectively with the latter having relatively high levels of caryophyllene oxide (10.5-10.6%) as major compound in the oil. The grouping together of the OTUs of *E. africanus* from Citrusdal with one of *E. africanus* var *paniculatus* was an indication of close chemical affinities and the former population could be *E. africanus* var *paniculatus.* This was supported by their geographical proximity to each other. The recurrent relationship between *E. africanus* and *E. eximius* is a clear indication that these species have close chemical affinities. This group has been discussed in Chapter 5. The grouping patterns in the various clusters indicated the presence of intricate relationships in the taxa of *Eriocephalus* and it comes out clearly that some of the OTUs have very different chemical profiles from their own relations.

The cophenetic correlation coefficient was 0.92 indicating goodness-of-fit between the cophenetic value matrix and the original similarity matrix being clustered.

2.5.5.2. Qualitative analysis

The cluster analysis of all chemical compounds (200) in all the 86 OTUs resulted in two clusters I and II at the highest level of similarity, 6.29 with *E. pinnatus* as the outlier (Figure 2.14). The pattern of the grouping of the OTUs was different from the quantitative

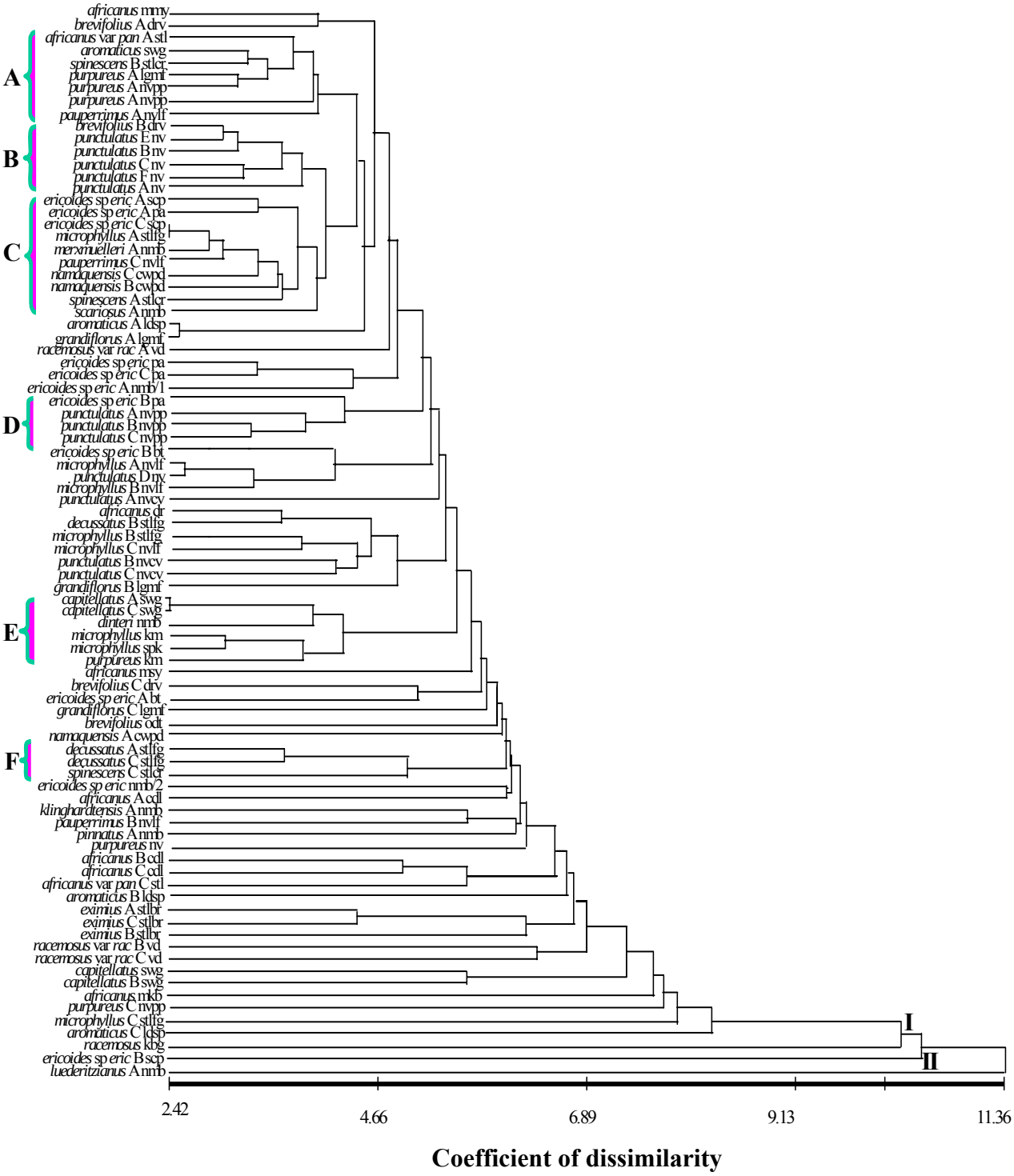


Figure 2.13. Dendrogram of quantitative matrix based on 200 terpene chemical characters for 86 OTUs. Cophenetic correlation r = 0.92. Abbreviated names of taxa and geographical localities are indicated. Euclidean distance was used as a measure of dissimilarity.

dendrogram. However, as previously mentioned the grouping of the OTUs rarely followed a coherent taxonomic pattern and hence cannot be used to define the species boundaries. The most that can be achieved is to tentatively recognize the consistent affinity groups that are not necessarily based on multiple taxa coherence. However, in the qualitative analysis, some of the groups showed clearer relationships than others in all the analyses and a few of these have been considered below.

Group A had OTUs of *E. pauperrimus* from one population and one of *E. africanus* (Malmesbury). The relationship was characterized by the presence of α- and β-pinene, yomogi alcohol and 1,8-cineole as common compounds among these taxa. Individual A and C of *E. pauperrimus* showed closer chemical affinities than did individual B to the former two OTUs.The OTUs of *E. pauperrimus* were uniquely characterized by the presence of bisabolol and derivatives in relatively high amounts especially bisabolol oxides A and B.

Group B had 24 OTUs with complex relationships based on chemical similarities. The most notable compounds included the camphane, pinane, santolinyl and artemisyl groups and 1,8-cineole in their essential oils. Caryophyllene oxide and β-caryophyllene and ρ-cymene featured prominently in the clusters as some of the components in the essential oils. Other notable relationships included the taxa of *E. capitellatus* that were clustered together due to their similar chemistry especially the very high contents of camphor and derivatives. In the same group, *E. dinteri* and *E. merxmuelleri* from Namibia shared similarities in their chemical profiles. Morphologically, the species in this group have opposite, decussate to alternate leaves and felty to sericeous indumentum on the leaf surfaces. On the overall, the relationships depicted especially between *E. brevifolius* and *E. grandiflorus, E. scariosus, E. africanus, E. microphyllus. E. ericoides* subsp. *ericoides* among others are an indication of presence of similar enzymes systems for synthesis of similar compounds present in their essential oils. Some of the affinity groups realized in this group are discussed in Chapter 5.

Group C and D had OTUs of *E. africanus, E. aromaticus, E. capitellatus, E. purpureus*, *E. brevifolius, E. decussatus, E. spinescens, E. eximius* and *E. punctulatus* in relationships largely based on similar chemical profiles. The essential oils in these OTUs had spathulenol, 1,8-cineole, linalool, and derivatives among other common compounds. The affinities between some of the species e.g. *E. decussatus-E. spinescens* and *E. africanus-E. eximius* have been discussed in Chapter 5.

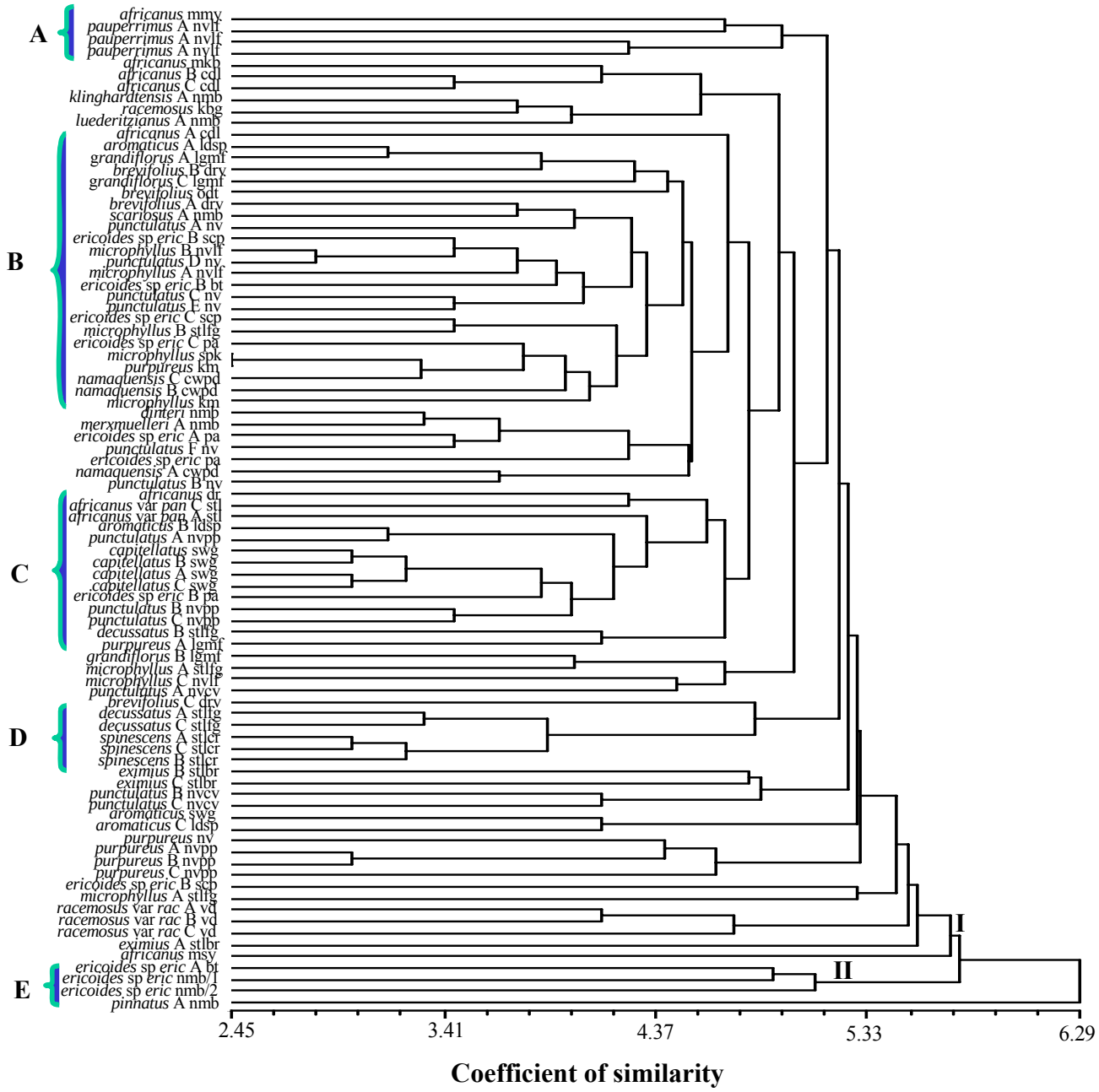


Figure 2.14. Dendrogram of qualitative data matrix based on 200 terpene chemical characters for 86 OTUs. Cophenetic correlation coefficient r = 0.74.

Group E had three OTUs of *E. ericoides* subsp. *ericoides* from Namibia and Bethulie in South Africa in a relationship indicating absence of chemical diversification in these taxa from geographically different localities.

The cophenetic correlation coefficient for this analysis was 0.74 indicating poor-fit between the cophenetic value matrix and the original similarity matrix being clustered.

2.5.6. Analysis of non-volatile extracts.

2.5.6.1. TLC screening

Results from the preliminary TLC screening of leaf extracts of some representatives of the species, showed the same phenomenon observed in the patterns of variation of essential oils at specific and population levels. Variation between populations was a common occurrence. However, it was noted that the leaf extracts comprised of various classes of flavonoids as evidenced by their fluorescence under UV_{254nm} and UV_{366nm}. The most notable flavonoids being the predominantly orange and yellow-green fluorescence for the flavone type, and dark green spot for the flavanone type. Phenol carboxylic acids, which frequently occur in flavonoids, appeared as intense, light blue zones after treating the plate with the natural spray (Wagner and Bladt, 1996). These spots were noted in *E. punctulatus, E. ericoides* subsp. *ericoides, E. purpureus, E. aromaticus, E. eximius, E. microphyllus, E. grandiflorus, E. racemosus* and *E. africanus* var *paniculatus.*

2.5.6.2. HPLC analysis

Preliminary results obtained from the TLC screening of acetone leaf extracts supported the presence of variation of phenolic compounds at species and population levels. A HPLC analysis of selected 30 taxa of the genus indicated the presence of flavonoids of various classes such as flavanones and flavones. A summary of the HPLC data is given in the monographs (Appendix I, monographs 1-22). Examples of the flavanone and flavone type have been given in Figure 2.15. Variation within and between species and their populations was observed in the taxa studied (Table 2.7).

The HPLC analysis of the acetone leaf extracts (Table 2.7) indicated that the genus is characterized by the presence of various classes of flavonoids. The major structural groups included flavones and flavanones. Flavones (at retention time 30.36 minutes) and the

flavanones (at retention time 29.77 minutes) were the most abundant in most of the species studied as shown in Table 2.7 and Figure 2.16.

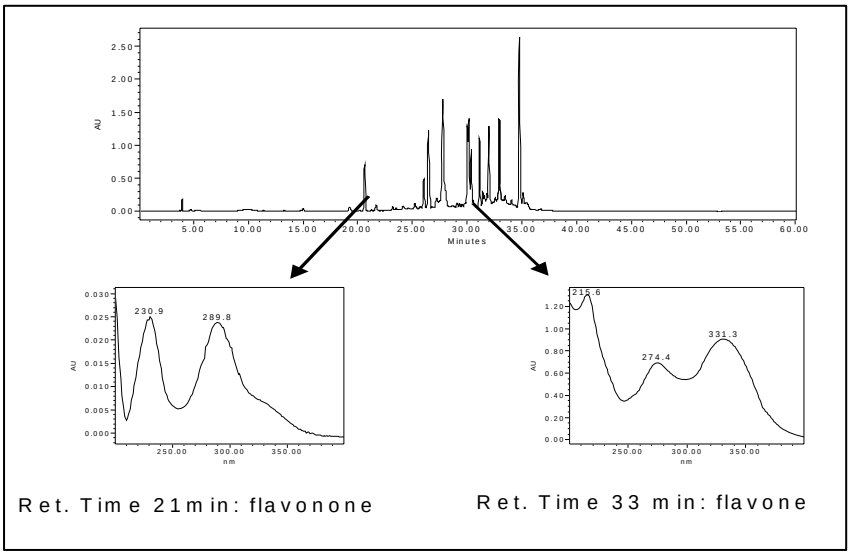


Figure 2.15: HPLC chromatogram of *E. aromaticus* from Ladismith showing some of the flavonoids in the acetone leaf extract at retention time 21 minutes and 33 minutes

As noted for the essential oil chemistry, variation existed in the flavonoid profiles between individuals of the same species from the same populations e.g. in *E. punctulatus* (Table 2.7) and between individuals from different populations in *E. africanus* (Table 2.7 and Figure 2.16). However, there were some similarities noted in the flavonoid patterns of *E. punctulatus, E. aromaticus, E. africanus, E. pauperrimus* and *E. namaquensis* as shown in Table 2.7 and Figure 2.16.

2.5.6.3. Cluster analysis

A cluster analysis of the HPLC/UV data (Table 2.7) for 30 OTUs and 86 compounds (Figure 2.17) produced two clusters one (containing majority of the OTUs) and the second one (with one individual of *E. racemosus* var *racemosus*) at the highest level of dissimilarity (7.62). An OTU of *E. microphyllus* from Sutherland was the outlier. As noted in the essential oils analysis, the flavonoid data did not result in coherent patterns for infraspecific delimitation. Four groups have been noted (A, B, C, D) for the discussion below.

Group A had two OTUs of *E. africanus* from Sutherland and De Rust. An individual of *E. punctulatus* from Nieuwoudtville was nested in this cluster. There was some affinity between

these two species, which were also grouped in same clusters. Morphologically these species have radiate capitula, connate paleae and leaves opposite with sericeous indumentum.

Group B had several OTUs among which an individual of *E. punctulatus* shared similar flavonoid chemistry with one of *E. spinescens* in a cluster where OTUs of *E. africanus, E. ericoides* subsp. *ericoides* and *E. aromaticus* were nested. There seemed to be an obvious affinity between the taxa of *E. punctulatus* and *E. aromaticus.* (Figure 2.16 and 2.17) and this relationship was also noted in the terpene chemistry. Morphologically, both species have radiate capitula, connate paleae and opposite to decussate leaves. The former species also showed similar flavonoid chemistry with the taxa of *E. spinescens* though morphologically the former has radiate capitula and the latter disciform capitula. They have opposite to decussate leaves with sericeous indumentum.

Group C comprised OTUs of *E. capitellatus* and *E. racemosus.* These species have morphological differences with the former having radiate capitula and the latter having disciform type but they both have connate paleae and alternate leaves.

Group D had an OTU of *E. eximius* and *E. punctulatus* in a relationship supported by the flavonoid chemistry and morphological affinities such as radiate capitula, connate paleae, opposite and sericeous leaves. These species also occur within the same geographical range, characterized by severe temperature fluctuations (Sutherland/Nieuwoudtville). The rest of the OTUs namely: *E. decussatus, E. namaquensis, E. purpureus, E. pauperrimus* and *E. racemosus* var *racemosus* were nested in the cluster.

The cophenetic correlation coefficient for this analysis was 0.96 indicating goodness-of-fit between the cophenetic value matrix and the original similarity matrix being clustered.

The flavonoid chemistry was as variable as the essential oil chemistry between individuals of the same species as are individuals from different populations, as observed in the dendrogram. This was clear from observing how the OTUs of *E. africanus* and *E. ericoides* subsp. *ericoides* were distributed erratically in the dendrogram. As previously noted in the terpenoid chemistry, the flavonoid profiles did not give coherent patterns for infra-specific delimitation in the genus.

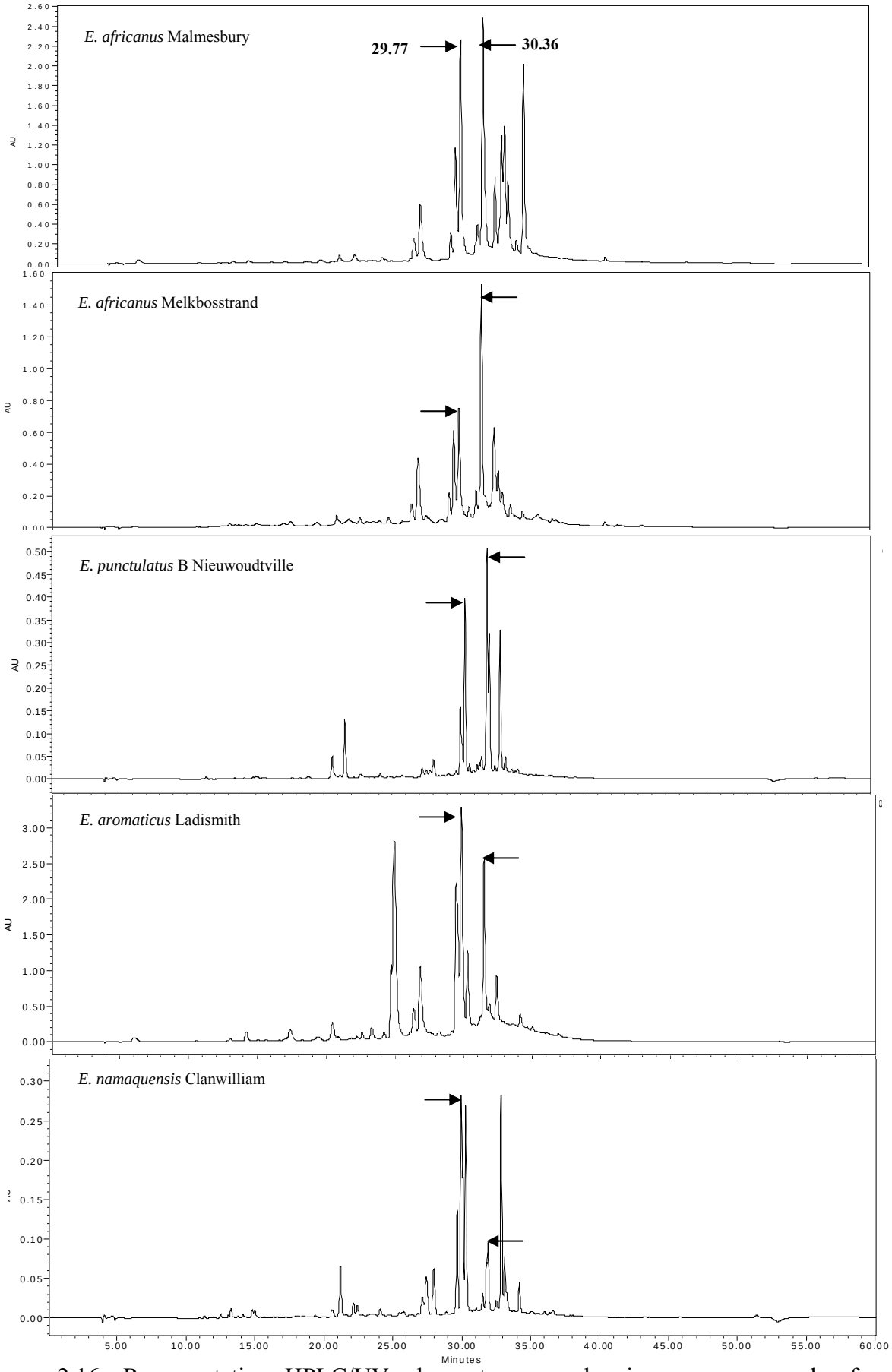


Figure 2.16. Representative HPLC/UV chromatograms showing common peaks for flavanones (RT 29.77 minutes) and flavones (RT 30.36 minutes) for some selected species.

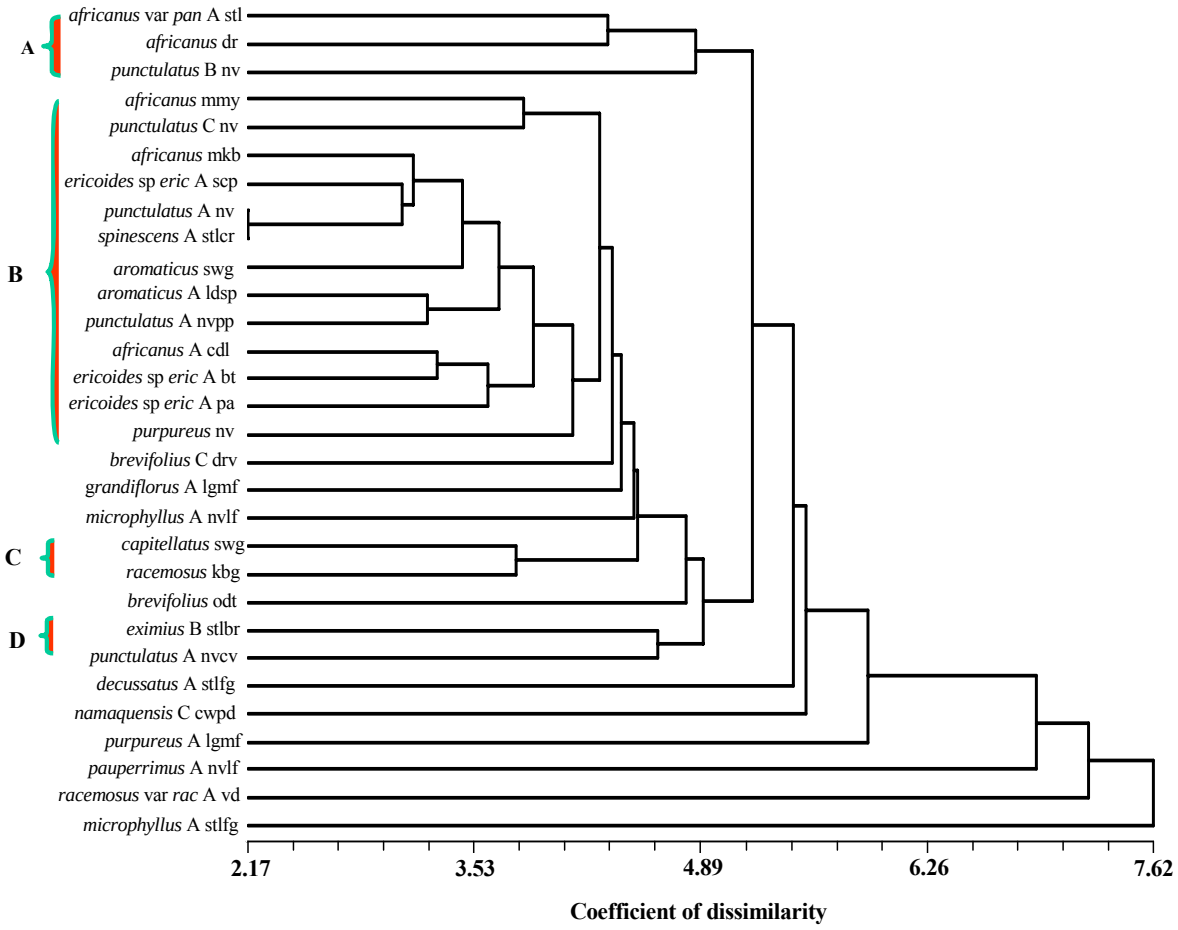


Figure 2.17. A HPLC dendrogram of acetone leaf extracts based on Euclidean distances showing variation in relationships of 30 taxa of the genus *Eriocephalus* from different localities. r = 0.96.

.

Table 2.7. A summary of the HPLC/UV data of 16 species of *Eriocephalus* showing retention time and concentration of non-volatile components from the acetone leaf extracts. The symbols represent: low concentration (+); medium concentration (++) and high concentration (+++).

Retention time	**UV_{max}**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**	**29**	**30**
19.49	206, 287											+		+							+					+		+			
20.59	219, 278										+	+																			
20.65	289	+	+	+	+	+	+	+	+				+			+	+	++	++	+	++	+	+	+	++		+	+		+	+
20.95	328		+									+		+												+					
21.10	226, 259, 297									++							+		+												
21.25	231, 297, 325	++		+	+	+					+		+		+	+	+	+++		++	+			+	+		+	+	+	+	+
21.45	238, 287, 390												+											+			+				
21.59	240, 369								++									+++	+			+	++		+++						
21.62	222, 329											++				+				++								++			
21.89	210, 285		+	+								+					+		+								+	++			
22.06	222, 267, 332	+				+				++	+															+			+		
22.15	220, 298, 327											+			+	+	+	+		+					+			++			+
22.43	219, 266, 291, 329	+	+											+															+		
22.57	225, 287							+				+											+	+	+						
23.15	217, 291		+										+		+												+				
23.36	202, 241, 293,		+					+				+				+	+		+	+++							+				
23.56	215, 259, 297							+				+																			
23.82	215, 258, 392														+																+
23.99	227, 286													+																	
24.12	220, 259, 297		+				+		+	+	+				+	+	+	+	+				+								+
24.60	220, 286			+								+																			
24.81	287																+		+							+					
24.93	207, 237, 298							+++				+															+				
25.23	211, 249																										+				
25.50	220, 273										+							+		+	+										
25.75	218, 287, 393		+		+										+		+	+		+									+	+	+
26.00	241, 295						++									++															
26.16	254,297, 369											+		+			+										++				
26.48	209, 287, 339						+++	++				+		+		+++	+				+					++	+++	+			
26.88	251, 312							++																			+++				
26.95	213, 253, 271, 346							++				+		+			+				+					+++		++			
27.19	254, 293, 368						+		+	++							+	+	+	++			+	++	+						
27.30	289		++	++	+							++			+												++	+++			
27.52	222, 253, 291, 348	++		+		+	+		+		+		+			++		++	+	++	+		+	++	++				+	+

Retention time	UV_{max}	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
27.62	218, 287, 345		++	+		+												++	+			+	+		++		+			+	
27.99	215, 271, 346		+	++					+									++	+	++										+	
28.02	213, 253, 271, 346	++		++		+	+			+	+++		+		++	++						+	+	++	++			++	+++		+
28.06	216, 272, 346				+																+										
28.60	217, 289										+++		+	+	++			+												+	
28.83	222, 254, 287					+																		++							+
28.98	290																				++						+				
29.21	223, 267, 292, 336	+++	+++			+++						++		+++			+									+++		++			
29.48	225, 289													+++							++			++				++		+	
29.54	218, 273, 325							+++				+++					+				+					+++					
29.63	207, 253, 288, 362																										++				
29.77	227, 267, 290, 333	+++	+++	+++	+++	++	+++		++		+++				+++	+++		+++	+++	++	++			+++	+++			+++	+++	+	+++
29.98	216, 273, 336	++	+++		+++	++		+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	++		++	+++	+++	+++			+++	++	+++
30.04	215, 273, 346						+++					+++									+++	+++				+++		+++			
30.14	254, 293, 366						+++			+++	+++	+						+++									++				
30.36	215, 273, 346	++	+++	+++	+++	++	+++	+++	+++	+++	+++	++	+++		+++	+++	+++	+	+++	+++	+++	+++	+++	+++	+++			++	+++	++	+++
30.55	244, 293,341						+++						+++			+++		+												
30.57	218, 260, 296														+++													++			
30.65	216, 287																						+++				+			++	
30.93	223, 273										+++																				
31.12	242, 289, 363						+++									++		+			+										
31.19	230, 268, 332											++					+++											+			
31.32	225, 290, 361																	+++			+		+++	+++			+				
31.38	229, 256, 364				++		+++								+++															++	
31.47	222, 271, 336			++				+++			+		++				++										++	++	++		
31.57	233, 270, 336	++	++		+++	+++			++	+		++		+++	+++	++		+++	++	++	+	++	+	+	+++	+++	++	+++			+
31.92	215, 273, 342	+++		+++	+++	+++					+++	++				+++		+++	++	++	+		+++		+++	+++			++	++	
31.95	206, 243, 333						+++		+++								++				+						+++				
31.98	215, 233, 274, 342	+++	+++						+	+++				+++	+++	+++						+++		+++				+++			
32.00	223, 279, 333									+++			++		+++		+++														
32.34	240, 317		+++		++																										
32.43	231, 384																				+										
32.45	215, 277, 330							+++								+	+++									+++		+++			
32.51	229, 267, 328	++				+++			+++			+++	++	+++	+++			+++		+++			+		+++						
32.69	230, 266, 363														+++												+				
32.88	216, 278, 330	+++	++		++	+++			+++		+++	+++	+++				+++	+++	+++	+++			+++	++	+++	+++		+++		+	
32.93	216, 234, 278, 330			++			+++			+++				+++	+++	++	+++				+							+++		++	
33.00	252, 356																										+				

Retention time	UV_{max}	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
33.20	234, 329																					+									
33.33	233, 278, 339	+++	++	++	+++	+++			+++	+++	++	+++	+++		+++	+		+	+++	+++	+		++	+	+			+++	+		
33.45	229, 252, 318		++											+												++					
33.61	216, 278, 329											+++					++													+	
33.78	236, 312		++		++																										
33.87	233, 272, 336						+											+											+		
33.98	241, 324		++																												
34.04	241, 311								++																					++	
34.17	251, 328		++					+	+	+++		+++							+												
34.22	218, 236, 279, 330	++		+	+	+						+++	+							+	+		+					++		+++	
34.76	223, 317		++									+				+					+									+++	
35.24	237, 318		+++		++																									++	
35.53	233											+																			
35.78	236, 336																												+		

Index to abbreviations in the table

1-*E. africanus* var *paniculatus* (Sutherland); **2, 3, 4, 5**-*E. africanus* (Malmesbury, Melkbosstrand, Citrusdal, De Rust); **6, 7**-*E. aromaticus* (Swartberg, Ladismith); **8, 9**-*E. brevifolius* (Oudtshoorn, De Rust; **10**-*E. capitellatus* (Swartberg); **11**-*E.decussatus* (Sutherland); **12, 13, 14**-*E. ericoides* subp. *ericoides* (Scheerpersrust, Prince Albert, Bethulie); **15**-*E. eximius* (Sutherland/Bo-visrivier); **16**-*E. grandiflorus* (Laingsburg/Matjiesfontein); **17, 18**–*E. microphyllus* (Sutherland/Loeriesfontein); **19**-*E. namaquensis* (Clanwilliam/Perdefontein); **20**-*E. pauperrimus* (Nieuwoudtville/Loeriesfontein); **21, 22, 23, 24, 25**-*E. punctulatus* (Nieuwoudtville, Nieuwoudtville/Calvinia, Nieuwoudtville/Papkuilsfontein); **26, 27**-*E. purpureus* (Nieuwoudtville, (Laingsburg/Matjiesfontein); **28, 29**-*E. racemosus* var *racemosus* (Koeberg, Velddrif); **30**-*E. spinescens* (Sutherland/Ceres).

2.6. Discussion

2.6.1. Chromatographic screening

Since plants are an important source of natural products, phytochemical and pharmacological investigations are important in establishing the presence of medicinally important compounds. In the recent past, chemical characters of plant constituents have also become a practical aid in plant systematics. This has been greatly enhanced by chromatographic and spectroscopic methods. These methods have proved useful in preliminary chemical screening that allows localized and targeted isolation of new or useful types of constituents with potential activities. The procedures allow for early recognition of known metabolites in extracts hence economically viable (Hostettmann, 1999).

Secondary metabolites are also crucial in providing not only phenetic information but also phylogenetic inference on the basis of biosynthetic considerations (Ohsaki *et al*., 1999). In this study, the thin layer chromatography (TLC), gas chromatography (GC) and high performance liquid chromatography (HPLC) techniques were used to provide insight into the diversity of the chemistry of the taxa used in the study and for adoption of plant collection and sampling strategy. It was apparent from the results obtained in the preliminary TLC screening of selected samples, that the chemistry of the genus is very complex, taking into consideration the variation patterns observed in individuals of a species from the same population. The TLC profiles of the samples tested depicted intra- and infraspecific variation as well as similarity for some taxa. For example, the similar chemical profiles of individuals of *E. racemosus* var *racemosus* from Velddrif and their obvious difference with the individual of the same species from Koeberg are reflected in the phenetic relationships (Figure 2.13; 2.14).

The above aspect helps in limiting the assumption of uniformity of chemical profiles unless proved otherwise through chemical screening. This phenomenon was observed in all the other results from the various analyses. This has greatly shaped the direction of the study as well as introducing a new parameter of phytochemical studies. The high level of chemical diversity observed in the various species is just a momentary glimpse into nature's diversification of resources and a big challenge to ethno-medicine and bioprospecting for new sources of natural products and medicines. Grayer *et al*., (1999), notes that assessment of chemical relationships of a taxon such as a family should be carried out using several species taken at random of that family or else a distorted picture of relationships may be obtained. This also

raises doubt on the validity of results based on just one individual representative of a particular plant group in any study, especially at specific level. One would argue that these results are specific to a particular group of plants under study. When viewed from a tribal perspective, and taking into account that, members of a tribe do share some common features *sensu lato*; then, it is obvious that one needs to carefully evaluate and consider the plant sampling strategies to be employed in any phytochemical study. It is therefore crucial that multiple samples of any groups of plant under study be considered for a clear understanding of the extent of variation within the group.

2.6.2. Volatile and non-volatile yields

It is well known that the volatile and non-volatile yields from any plant are influenced by a number of factors. The extrinsic factors comprising the physical, chemical and microbiological factors of the soil and the radiation, temperature and moisture (climate) of the growth environment of the plants contribute to the quantities of the yields of secondary metabolites (Swain, 1963; Grayer *et al*., 1996; Echeverrigaray *et al*., 2003)

The above factors coupled with intrinsic factors such as gene expression directly affect the quantitative and qualitative properties of the secondary metabolites. Other factors that could have affected the yields are the phenological stages (growth stages at time of harvesting) (Juteau *et al*., 2002). On the other hand, diurnal variation and greater volatilization during the day are some of the other factors affecting the quantities of yields as well as the time of harvesting of the plants (Smith, 1976).

The extensive interplay of the above factors could be responsible for the variation observed in the yields for volatile and non-volatile components of the members of *Eriocephalus*. The leaf extracts however, showed consistent weights in comparison to the volatile extracts.

2.6.3. The phytochemistry of the genus

2.6.3.1. Essential oil chemistry

The chemistry of volatile compounds has proved particularly useful is assessing taxonomic relationships at generic and family levels (Grayer *et al*., 1996; Skaltsa *et al*., 2001; Wink, 2003). Essential oils have also been used in clarification of species delimitation in the South African species of the genus *Vitex* (Nyiligira *et al.,* 2004) and are thus a useful tool in taxonomy. The chemistry of *Eriocephalus* is complex at specific and at population levels. The

essential oils were characterized by the presence of acyclic, monocyclic and bicyclic mono- and sesquiterpenes of various functional groups. Some of the functional groups included acetates, alcohols, ketones, aldehydes, ethers, phenols etc. However, it was clear that all the species studied had some common compounds that defined their intricate relationships. These were present in almost all of the taxa studied and they included; α- and β-pinene, yomogi alcohol, ρ-cymene, 1,8-cineole, camphor, 4-terpineol, spathulenol, caryophyllene oxide, α-copaene and β-caryophyllene. Some of the major and common terpenes reported in tribe Anthemideae (thujone, camphor, borneol and 1,8-cineole) were also present in relatively high amounts in some taxa, especially 1,8-cineole and camphor (Greger, 1977). Caryophyllene and cadinene derivatives were also present. The percentage of the azulenic compounds namely; aromadendrene, alloaromadendrene, and chamazulene was relatively low in most of the taxa or absent in most cases.

The taxa studied showed diversity in their chemistry especially with regard to the percentage composition of major compounds. In some cases, individuals of the same species had different compounds in their essential oil or some compounds are absent, for example, in *E. punctulatus, E. spinescens, E. racemosus* var *racemosus* and *E. purpureus* where individuals had different major compounds. There was almost lack of specific coherence in the grouping of the taxa in the dendrogram. This was due to the taxa grouping according to the similarities in their chemical composition irrespective of the species.

Unrelated taxa may have had similar enzyme systems hence production of similar compounds. This could be an indication of relationships existing between the relevant plants. On the other hand, related plants may have different enzyme systems that may have been caused by mutation. When such changes occur, they may give rise to large differences in production of secondary metabolites due to blocking of some of the biosynthetic routes and thus resulting in 'abnormal' chemistry (Swain, 1963). This phenomenon may have occurred in *Eriocephalus* species in the course of evolution. The chemical profiles observed in most of the taxa studied are an indication of presence of enzymes associated with the general terpenoid biosynthesis in the mevalonate and deoxyxylulose phosphate pathways (Dewick, 2001). Also present are enzyme systems associated with the biosynthesis of irregular monoterpenes in the chrysanthemic acid pathway. The artemisyl, santolinyl and lavandulyl derivates were noted in most of the taxa studied and are characteristic of most members of the family Asteraceae (Heywood and Humphries, 1977).

Chemical similarity in terpenes can also be due to free gene flow and lack of coherence can be attributed to restricted gene flow, hence reproductive isolation (Skaltsa *et al*., 2001). On the other hand, the chemical variation at intraspecific level of essential oils has been described in various aromatic and medicinal plants in various plant groups (Echeverrigaray, *et al*., 2003) and it is possible to characterize and classify plants on basis of chemical constituents. Plants, like any other living systems or organisms, are known to vary and different individuals of the same species can differ considerably. Many factors may be responsible for this variation and among them includes the effects of soil conditions or seasonal (climatic) factors. This therefore necessitates examination of several individuals of any plant group under study to examine the extent of variation within such a group (Swain, 1963). This phenomenon was observed in the *Eriocephalus* and the constituent taxa where taxa of the same species from the same population were distributed discordantly in the dendrograms.

Despite all these variations, it is evident that chemical characters are important markers in understanding the economic uses of plant especially in industries and in research. In any case, the medicinal properties and biological activity of a plant are due to its chemical profile (Grayer *et al.,* 1999). Hence, phytochemical studies are an integral component for understanding the diversity in plants.

It was noted that there was an obvious absence of artemisia ketone, carvacrol and α-cedrene in the disciform taxa. This was a clear indication of the absence of enzymes responsible for the synthesis of these compounds. On the hand, the absence of *cis*- and *trans*-linalool oxide, nerol oxide, *cis*- , *trans*-chrysantemyl acetate and α-thujenal in the radiate taxa was also indicative of the absence of enzyme systems responsible for the synthesis of the aforementioned compounds. However, these differences were not reflected in the cluster analysis and hence could not be used in clarification of species relationships in the genus. Nevertheless, this was an important finding that can be explored further in future studies of this genus.

Another phenomenon common in a plant's chemistry is chemical convergence in plants with different evolutionary histories. The co-occurrence of structurally similar compounds is also attributed to differential gene expression where genes encoding for the enzymes for the production of a given chemical compound have evolved during evolution. The genes are not lost during phylogeny but might be "switched on" later at some point in the phylogeny (Wink and Mohamed, 2003 and Wink, 2003).

This leads to diversity in the chemical composition of secondary metabolites. The variation in chemistry of individuals of one species in a given population provokes one to seek explanations for such discrepancies. It is well documented that the primary functions of secondary metabolites are defense against microbes, herbivores, and competition from other plants. They are also signal compounds for pollination and seed dispersal (Wink, 2003). In some cases, allelochemical diversification may occur due to pressure from other plants resulting in production of new defense or allelopathic compounds (Kubitzki, 1984). This forces individuals in a given population to be different from such other populations or even individuals within this population become different from each other. In such cases, allopatric taxa will tend to have similar chemistry than the sympatric ones (Kubitzki, 1984). Other factors contributing to the different chemistry of individual are the chemical polymorphisms, which are adaptive in nature and may follow environmental gradients (Kubitzki, 1984; Grayer *et al*., 1999; Wink and Mohamed, 2003; Wink, 2003). This may explain the differences observed in the essential oils profiles of the populations of *E. punctulatus* and *E. ericoides* subsp. *ericoides.*

It is quite clear from the above-mentioned factors that the variation observed within and between species even at population levels has greatly influenced the grouping of species of *Eriocephalus* in the various dendrograms. *Eriocephalus africanus,* is one of the most variable among the species of *Eriocephalus* and second most widely distributed after *E. ericoides* subsp. *ericoides* and is characterized by extensive phenotypic plasticity (Müller *et al*., 2001).

The extensive diversity observed in the various species and their constituent taxa makes it difficult to assign any infra-specific ranks to the species in the genus. These results are an indication that the current species boundaries in the members of the genus *Eriocephalus* are not supported by the terpene chemistry. It is therefore difficult to clarify the interspecific relationships, but despite the diverse chemistry, 22 chemotypes (based on quantitative analysis of chemical compounds ≥5% of the total essential oil composition) were recognised from the 86 samples analysed and have been summarized in Table 2.8.

Some of the chemotypes recognized from this study are potentially useful in traditional medicine as well as in the flavour, fragrance and pharmaceutical industries. One has to be cautious as the essential oils of the genus are highly variable and if they were to be explored further for commercial development, *ex-situ* cultivation would be a better option. This would

involve cloning for production of oils with uniform composition majoring in the commercially important components.

Among the 22 chemotypes recognized in this study, there a few notable ones including those rich in camphor, 1,8-cineole, nerolidol, 4-terpineol, chrysanthenone, linalyl acetate, bisabolol derivatives, α-pinene and spathulenol. Most of these compounds are widely used industrially, in flavouring and in aromatherapy. Camphor, for example, is useful for its soothing, antiinflammatory and antiseptic properties (Ikan, 1991; Dewick, 2001) and the composition falls within the range of those found in camphor oil of *Cinnamomum camphora* (27-45%). It is clear that the taxa of *E. capitellatus* have consistently high levels of camphor and low variability in the essential oil composition and are hence, valuable source of camphor. Other species with high levels of camphor include *E. dinteri, E. purpureus* and *E. aromaticus.*

It is well documented that essential oils are also very important in pharmaceutical formulations. The most widely used are limonene, 1,8-cineole, nerolidol, α-bisabolol and caryophyllene oxide, which are used to increase the permeability of the stratum corneum during drug administration (Cornwell and Barry, 1994; Cornwell *et al*., 1996). The taxa of *E. eximius* and *E. pauperrimus* are a potential source of nerolidol with the latter species also having high contents of bisabolol products. Most of the taxa of the genus *Eriocephalus* have relatively high contents of 1,8-cineole, especially the taxa of *E. ericoides* subsp. *ericoides, E. microphyllus* and *E. punctulatus* and are hence potentially useful sources of this compound.

The bisabolol products and derivatives are widely used in cosmetics for their antiinflammatory properties. In this study, the consistently high percentages of bisabolol derivatives of *E. pauperrimus,* distinguish this species as a favourable source of these industrially important classes of sesquiterpenes.

Linalyl acetate is a mild sedative that is widely used in aromatherapy and treatment of mind related disorders. It can also be used in flavourings and perfumery, as it is an aromatic ester. From the study, it is evident that the species of *Eriocephalus* have varying amounts of this compound with the most notable ones being *E. spinescens* and *E. decussatus.* These species had comparatively higher contents of linalyl acetate and other linalool derivatives in their essential oils and are potential sources of this compound.

α-pinene has a wide range of industrial applications (Ikan, 1991; Nakatsu *et al*., 2000; Dewick, 2001). *Eriocephalus klinghardtensis* and *E. luederitzianus* from Namibia had chrysanthenone and α-pinene as the major compounds respectively with the latter being the only species with a pinane group constituting the highest percentage. Each of them formed a unique chemotype. The oil of the latter species has a potential for use in flavouring, perfumery and as industrial oil due to the presence of relatively high content of α-pinene (31%).

The terpene chemistry of this genus is indeed very complex from the mere fact that there are 22 chemotypes and the number is likely to increase when more taxa are added. As observed above, the species have a potential for use as sources of cosmetics, perfumes, flavouring agents, antiseptics and industrial oils. Other potential uses include antimicrobial drugs and as primary sources for cosmetics oils (Mangena and Muyima, 1999; Nakatsu *et al*., 2000; Cimanga *et al*., 2002; Tepe *et al*., 2004).

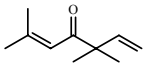
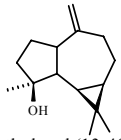
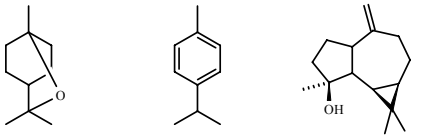
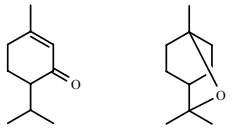
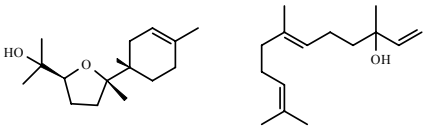
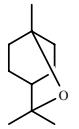
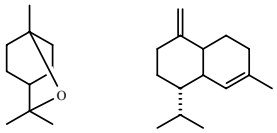
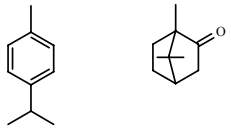
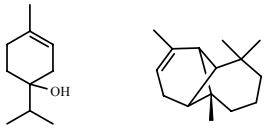
The overall essential oil chemistry of the genus is riddled with extensive variation. However, this study is the most recent account of the terpene chemistry of the 22 species of *Eriocephalus* and it is thus a useful reference document for future studies.

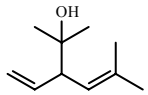
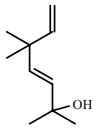
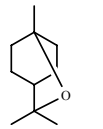
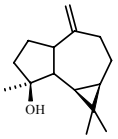
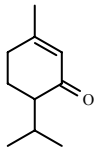
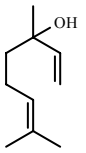
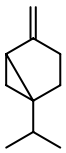
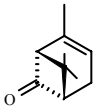
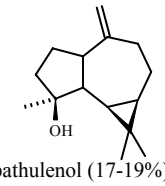
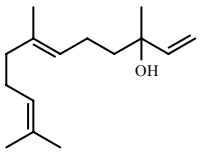
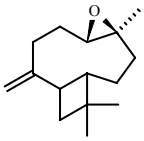
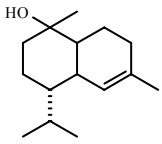
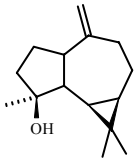
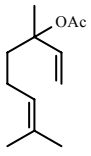
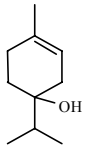
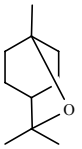
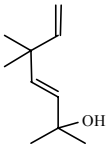
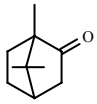
2.6.3.2. Non-volatile compounds

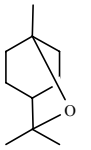
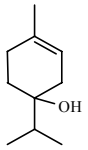
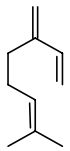
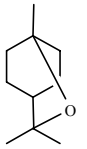
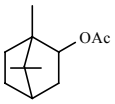
The composition of the non-volatile components revealed that majority of the species tested had relatively high levels of flavones and flavanones as the major structural types of flavonoids as is common in most members of Asteraceae (Harbourne, 1977) with a lesser concentration of flavonols. It is clear from the preliminary TLC analysis of the extracts that the aforementioned compounds were abundant in the taxa studied and notably in the populations of *E. africanus, E. ericoides, E. brevifolius, E. punctulatus* and *E. microphyllus* among several others. This probably explains the medicinal use and importance of *E. africanus* and *E. punctulatus* as diuretics and diaphoretics. These species showed relatively high concentrations of flavones that are reported to be cardiac stimulants as well as diuretics and antioxidants (Ikan, 1991).

The presence of the various structural types of flavonoids is an indication of the heterogeneous flavonoid pattern characteristic of the Asteraceae (Harbourne, 1977). The taxa

Table 2.8. A summary of the 22 chemotypes recognized from the quantitative cluster analysis (with values of 5% and above) of the 86 taxa of the genus *Eriocephalus.*

Chemotype	Representative taxa	Essential oil constituents
1	• *E. africanus* • *E. brevifolius*	 artemisia ketone (12-30%)
2	• *E. africanus* • *E. aromaticus* • *E. decussatus* • *E. eximius* • *E. namaquensis* • *E. purpureus*	 spathulenol (13-40%)
3	• *E. africanus* • *E. aromaticus,* • *E. namaquensis* • *E. purpureus*	 1,8-cineole (20-24%) *p*-cymene (11%) spathulenol (12%)
4	• *E. punctulatus* • *E. scariosus*	 piperitone (21%) 1,8-cineole (24%)
5	• *E. pauperrimus*	 α-bisabolol oxide B (45%) nerolidol (36%)
6	• *E. microphyllus,* • *E. punctulatus,* • *E. ericoides* subsp. *ericoides, E. namaquensis,* • *E. racemosus,* • *E. merxmeulleri.*	 1,8-cineole (12-53%)
7	• *E. ericoides* subsp. *ericoides,* • *E. grandiflorus,*	 1,8-cineole (27%) γ-cadinene (23%)
8	• *E. microphyllus* • *E. aromaticus,*	 *p*-cymene (14%) camphor (25%)
9	• *E. microphyllus* • *E. purpureus*	 4-terpineol (15%) α-longipinene 14-20%)

Chemotype	Representative taxa	Essential oil constituents
10	• *E. brevifolius* • *E. ericoides* subsp. *Ericoides*	 santolina alcohol (12-18%)
11	• *E. brevifolius* • *E. spinescens* • *E. grandiflorus* • *E. racemosus* var *racemosus*	 yomogi alcohol (9%)  1,8-cineole (11%)  camphor (13%)  spathulenol (9%)
12	• *E. punctulatus*	 piperitone (26-28%)
13	• *E. ericoides* subsp. *ericoides*	 linalool (10%)
14	• *E. racemosus* var *racemosus*	 sabinene (7.4%)
15	• *E. klinghardtensis*	 chrysanthenone (24%)
16	• *E. africanus* • *E. decussatus* • *E. eximius*	 spathulenol (17-19%)  nerolidol (12%)  caryophyllene oxide (13%)
17	• *E. namaquensis* • *E. punctulatus* • *E. spinescens* • *E. decussatus*	 α-cadinol (11-15%)  spathulenol (10-22%)  linalyl acetate (21-30%)
18	• *E. brevifolius* • *E. ericoides* subsp. *ericoides* • *E. microphyllus* • *E. punctulatus*	 4-terpineol (21%)  1,8-cineole (30-40%)  yomogi alcohol (22%)
19	• *E. capitellatus* • *E. dinteri* • *E. purpureus* • *E. aromaticus*	 camphor (12-52%)

Chemotype	Representative taxa	Essential oil constituents
20	• *E. leuderitzianus*	 α-pinene (31%)
21	• *E. ericoides* subsp. *ericoides* • *E. microphyllus* • *E. racemosus*	   1,8-cineole (31%) 4-terpineol (27%) myrcene
22	• *E. ericoides* subsp. *ericoides* • *E. grandiflorus*	  1,8-cineole (30%) borneol acetate (14%)

with radiate flowers e.g. *E. eximius, E. africanus, E. aromaticus, E. brevifolius; E. punctulatus* and *E. purpureus* have relatively high levels of flavonoids. This is not surprising as some of the functions of these compounds include screening of UV light, *in situ* radical scavenging and anti-feeding effects (astringency) among many uses (Ikan, 1991; Dewick, 2001).

A qualitative cluster analysis of the flavonoid data revealed variability within and between the taxa analysed. The chemical profiles did not seem to follow the specific boundaries but rather the chemistry of individuals and rarely the morphological affinities. In cluster A Figure 2.17, for instance, the two individuals of *E. africanus* and one of *E. punctulatus* grouped together according to their chemistry. Morphologically, they have radiate flowers with connate paleae of the marginal florets. The rest of the taxa for *E. africanus* and *E. punctulatus* were distributed erratically in the dendrogram. In cluster B, the flavonoid profiles of one individual of *E. africanus* had chemical similarities with taxa of *E. ericoides* subsp. *ericoides, E. punctulatus* and *E. spinescens.* The latter two taxa had identical profiles and similarities with *E. aromaticus* too. The patterns emerging were not those of the same taxa grouping together in coherent clusters. This implies that the expected patterns of grouping of the taxa were not obvious probably due to their chemical diversity. It was therefore difficult to assign infra-specific ranks or to clarify the specific relationships from the results obtained in this study.

In some cases, different plants are known to contain substances from different classes of chemical compounds but appear to be biosynthetically analogous. The compounds they

produce indicate presence of relationships between the concerned plants (Swain, 1963). On the other hand, it is probable that related plants may have different enzyme systems hence the differences in their individual chemical profiles.

All the above factors may have largely contributed to the diversity of flavonoid profiles noted in taxa studied. It is undoubtly clear that the flavonoid chemistry of the genus is also complex and highly variable and in this study, they did not offer guidance in infra specific ranking of the taxa studied.

2.6.4. Relationships between species

2.6.4.1. Overall species variation

The value of phenetic methods in systematics lies in the translation of results into defensible taxonomic decisions and in the absence of agreement among biologists on a universally acceptable species concept, the initiative remains with the individual taxonomist to define species level taxa. The phenetic species concept (Sneath and Sokal, 1973) is an empirical approach that considers distinct phenetic clusters as species without making assumptions about speciation. The formation of clusters is produced by overall similarity between objects as a function of their individual similarities in each of the many characters in which they are being compared. Therefore, phenetic clusters may not necessarily include a fixed character, but are recognized by the possession of groups of partially correlating (polythetic) characters.

If, however, infraspecific taxa are to be recognized, and named, they should be clearly delimited from other taxa by non-overlapping discontinuity in one or more characters, and have a geographic basis. Any intraspecific variation that does not meet this criterion should not be formally named (Brunell and Whitkus, 1999).

Species that exhibit high levels of inter- and intra-population variability pose a special challenge for systematists both in classification and in deciphering the most probable pathways of evolutionary radiation (Kephart *et al*., 1999). It is believed that differences among populations of a single species, whether arising independently or under similar selective regimes, can produce complex patterns of diversification at spatial scales. This is further complicated by genetic differentiation if there is restricted gene flow, natural geographical barriers and habitat fragmentation (Kephart *et al*., 1999; Skaltsa, *et al.*, 2001). Habitat heterogeneity and genetic drift may lead to morphological differentiation even where

there are reproductive isolating mechanisms and in other cases, the populations may be too variable to warrant taxonomic recognition.

This study used terpene and non-volatiles compounds chemistry as supporting evidence in an attempt to delimit the taxa in the genus *Eriocephalus.* In the tribe Anthemideae, attempts have been made to delimit the genera in the tribe using secondary metabolites such as sesquiterpene lactones and flavonoids and meaningful relationships have emerged from such attempts (Emerenciano *et al*., 1987).

The results obtained from the cluster analysis of quantitative and qualitative data indicate that the relationships between the species of *Eriocephalus* are quite complex. This is aggravated by the fact that individuals of the same species within the same population have different chemical profiles and their overall chemistry group them with other species with distant relations. This may not be surprising since during evolution, conditions for the production of some compounds or groups of biosynthetically related substances may have developed separately in many plants. Therefore, it is probable that totally unrelated groups of plants would have enzyme prerequisites for synthesizing several chemically unrelated compounds of intermediate distribution. This may be a case of probable biochemical convergence (Swain, 1963; Dural *et al*., 2003). Therefore, the interpretation of the relationships between and within species is a matter of the patterns observed in the various OTUs considered in this study rather than the actual taxonomic circumscription or specific boundaries.

Lack of resolution in most of the species relationships observed in the clusters does not in any way imply that there is nothing to grasp from the study. It may be that, the characters used are not the most suitable for defining the specific and infraspecific relationships in a given group. When individuals of species fail to show taxonomic coherence, this is an indication of inherent variation and chemical polymorphism (Dural *et al*., 2003). It is also probable that small genetic changes may have given rise to large differences in the production of secondary metabolites resulting in blockage of some synthetic routes. This may result in plants having very “abnormal chemistry” and hence making it difficult to derive taxonomic conclusions. As it stands out, the species of *Eriocephalus* depict large chemical variation as evidenced by the extreme variations between individuals from the same population.

Cluster analysis of quantitative and qualitative data revealed that the taxa rarely grouped according to their infraspecific designation or geographical region but mainly according to their individual chemistry except for a few of the species and their taxa. An example of taxa from the same population grouping together is that of *E. punctulatus* from Nieuwoudtville / Papkuilsfontein in Figure 2.13.

In chemotaxonomy, the presence or absence of chemical compounds is an indication of common descent or relatedness and presence of enzymes responsible for their biosynthesis (Kubitzki, 1984; Wink, 2003). In the qualitative analysis, some of the individuals of species that were randomly dispersed in the quantitative dendrogram, group together in the clusters due to divergent chemistry. Examples include taxa of *E. pauperrimus, E. capitellatus, E. spinescens, E. purpureus* and *E. racemosus* var *racemosus* most of these groupings if not all are realized in the chemical and combined phylogenies while some of the previous groupings shifted positions. An example includes the taxa of *E. punctulatus* from Nieuwoudtville and taxa of *E. eximius*. Most of the relationships emerging in this analysis are discussed in Chapter 5. In some instances, there is morphological relevance in the way the taxa group in the dendrogram as observed in *E. africanus* and *E. brevifolius* which have sericeous indumentum on their leaves apart from having radiate capitula and connate paleae of the marginal paleae.

Geographical isolation may not have played a major role in defining the populations of *E. ericoides* subsp. *ericoides,* which have extremely variable chemistry. In the latest revision of the genus (Müller *et al*., 2001), the taxa from Namibia are described as disjunct but this study has revealed that the chemistry of the Namibian taxa resembles their South African counterparts as noted in the cluster analysis. This implies presence of similar biosynthetic pathways in their chemistry despite being geographically isolated. Therefore, the taxa have not undergone infraspecific differentiation to be accorded subspecific status.

The taxa of *E. decussatus* and *E. spinescens* grouped together in a relationship supported by their geographical locality, Sutherland. The essential oil composition of these two species is similar probably due to their habitats, which are characterised by extreme temperature variations.

The relationship between an individual of *E. aromaticus* and *E. grandiflorus* is realized in the chemical and combined phylogenies. The two taxa have similar major compounds in almost similar proportions; these are camphor, methyl *trans*-chrysanthemate, spathulenol, yomogi alcohol and 1,8-cineole. This explains their grouping together in the clusters. This could be another interesting case of chemical convergence and it is not surprising that the rest of the individuals of these species are so different among themselves yet similar to other non-relatives. A possibility of allelochemical diversification cannot be ruled out either (Kubitzki, 1984).

It is clear that the relationships in the genus are a reflection of the quantitative and qualitative chemistry of the groups within which certain compounds are present irrespective of the species and the population considerations. As previously mentioned, the real interpretation of the relationships depend entirely on the taxonomists own opinion, especially in this case where there is almost lack of coherence at specific and interpopulational levels. It emerges then from this study that it was difficult to assign infraspecific ranking due to the discordant placement of the taxa in the dendrograms and by the fact that the current species boundaries are not supported by the chemistry of *Eriocephalus.*

2.6.4.2. Affinity groupings

Despite the inconsistencies observed in the terpene and flavonoid chemistry of the genus *Eriocephalus,* it is apparent that there are some chemical affinities between the following taxa based on qualitative and quantitative analysis. These groups are listed below and are discussed in detail in Chapter 5.

1. *E. brevifolius-E. africanus-E. punctulatus.*
2. *E. aromaticus-E. grandiflorus-E. brevifolius*.
3. *E. decussatus-E. spinescens*.
4. *E. ericoides* subsp. *ericoides-E. microphyllus-E. purpureus.*
5. *E. eximius-E. africanus.*

2.7. Conclusion

It is evident from this study that terpenoid and flavonoid patterns are highly complex to the extent that the original objective of infra specific ranking of the taxa was not achievable in the current study. The diverse and anomalous chemical patterns observed may be due to genetic mutations resulting in major changes in the enzyme systems in the major biosynthetic

pathways. Chemical convergence, divergence, allelochemical diversification, polymorphisms, and a host of several environmental variables may also have played a role in the chemical diversification. Differential gene expression may probably be responsible for the major variation in the terpene composition in individuals of the same species from the same locality.

Despite the discrepancies, the following major conclusive outcomes were deduced;

- A comprehensive chemistry of 22 species of *Eriocephalus* is presented for some of the South African and Namibian taxa for the first time.
- Two hundred (200) compounds were recorded in the GC/MS analysis comprising largely of acyclic, monocyclic and bicyclic mono- and sesquiterpenes. The common constituents present in the oils of almost all of the taxa studied were found to be; α-and β-pinene, yomogi alcohol, ρ-cymene, 1,8-cineole, camphor, 4-terpineol, spathulenol, caryophyllene oxide, α-copaene and β-caryophyllene. Most of the species have a relatively high content of 1,8-cineole and camphor.
- The study has defined 22 chemotypes based on the highest percentage value for the major compounds (singularly or in combination) present in the 86 taxa of the species studied as summarised in Table 2.8.
- The essential oils of the species have compounds that make them potentially useful in flavourings, perfumery, cosmetics, antimicrobial drugs, and pharmaceutical formulations and as sources of industrial oils. The favourable chemotypes include; oils rich in camphor, bisabolol oxide B and nerolidol, linalool, linalyl acetate and spathulenol and α-cadinol, 1, 8-cineole and α-pinene rich oils that are major components in industrial oils. However, standardization of these oils is required for commercial development.
- This study has proved that the chemistry of *Eriocephalus* is intricate and the data to some extent indicates a lack of coherent patterns of variation consistent with expectations of infraspecific differentiation. However, the character discontinuity within the taxa of *Eriocephalus* and the inconsistent secondary metabolite profiles mean that the systematic value of terpene characters in *Eriocephalus* becomes a matter of interpretation in the same way as traditional morphological markers.
- The taxa group according to the similarities in their chemistry even with taxa of unrelated species.

- Relationships based on phenetic analyses resulted in incoherent clustering for most of the individuals of the various species except for some taxa that were consistently recurring together in the various analyses though. The affinity relationships recognized include 1.*E. brevifolius-E. africanus-E.punctulatus* 2. *E. aromaticus-E. grandiflorus-E. brevifolius* 3. *E. decussatus-E. spinescens* 4. *E. ericoides* subp. *ericoides-E. microphyllus-E. purpureus* 5. *E. eximius-E. africanus*
- The study eludes to the intraspecific chemical diversity in the group attributed to various factors such as chemical divergence, polymorphism as well as the differential gene expression and allelochemical diversification. An attempt should be made to use sesquiterpenes lactones chemistry for specific and infraspecific delimitation as they have been reported useful in the family Asteraceae in solving delimitation problems.
- The terpenes in the genus are highly variable and not consistent taxonomic markers. Therefore, it was not possible to clarify the taxonomic relationships between the species in the genus in line with the objective of the study.
- The study also proves that the non-volatile components are largely comprised of flavonoids of various structural groups. The flavones and the flavanones are the most abundant.
- Cluster analysis of the flavonoid data did not reveal coherent species patterns and can therefore not be used to clarify relationships in the genus.
- The study has noted absence in the essential oils of artemisia ketone, carvacrol and α-cedrene in the disciform taxa and conspicuous absence of *cis* and *trans*-linalool oxide, nerol oxide, *cis* and *trans*-chrysantemyl acetate, *trans*-sabinene hydrate and α-thujenal in the radiate taxa.

`

CHAPTER 3

Phylogenetic reconstruction of *Eriocephalus*

3.1. Introduction

Despite its commercial importance, little is known with regard to species-level relationships within the Southern Africa endemic genus *Eriocephalus*. Having mentioned the economic uses of the genus elsewhere, it is clear that the question of species delimitation and relationships are vital to well directed economic use of this genus. Relationships to date have largely been inferred based upon morphological characteristics and in some cases on chemotaxonomic variation (Zdero *et al*., 1987). However, neither suite of characters have been analysed within a cladistic framework. DNA sequence data are now commonly exploited as a source of characters for phylogenetic reconstruction at various hierarchical levels, including the inference of species-level phylogenies. The following section details the use of molecular data in phylogenetic reconstruction.

3.2. Molecular systematics and use of DNA sequence data in the reconstruction of phylogeny

In recent years, molecular studies have been extensively used to clarify relationships between various taxa where alpha taxonomy alone has proved inadequate to do so. Molecular phylogenetic analysis can use restriction site data, comparative DNA sequencing, analysis of DNA arrangements (e.g. inversions) and gene intron loss. DNA sequencing has largely replaced cpDNA (chloroplast DNA) restriction site analysis for phylogenetic inference even at lower taxonomic levels (Sang *et al*., 1997; Soltis *et al.,* 1998). Plant molecular systematics has relied primarily on plastid coding and non-coding sequences for phylogenetic tree reconstruction. The mitochondrial genome is a much less versatile molecule, is difficult to purify, less abundant in leaves and has high rates of rearrangements and low rates of point mutations, compared to plastid DNA. Overall, the plastid genome remains the most widely used in molecular systematics for resolving taxonomic problems in plants. DNA coding and non-coding sequences have therefore provided a large number of molecular characters to address numerous phylogenetic issues (e.g. Soltis *et al*., 1992; Soltis *et al.,* 1998; Hind and Beentje, 1996; APG II, 2003).

With respect to phylogenetic analyses within the Asteraceae, restriction site data along with sequences of the *ndh*F gene have supported the monophyly of subfamily Asteroideae that includes the tribe Anthemideae (Jansen *et al*., 1992; Jansen and Kim, 1996). Within the subfamily, 13 monophyletic tribes including Anthemideae were recognized. Phylogenetic analysis of the plastid gene *ndh*F and internal transcribed spacer (ITS) of nrDNA resulted in

the placement of most of the South African genera of the tribe Anthemideae in a paraphyletic grade at the base of the tree (Watson *et al*., 2000; Watson *et al*., 2001). These studies also supported a South African origin of the tribe in contrast to previous hypothesis, which had suggested that the tribe originated from Eurasia, with an early vicariant event resulting in relictual members occurring in Southern Africa. The *ndh*F gene sequences also supported the monophyly of subtribes Chrysantheminae and Gonosperminae in Anthemidae. Members of the Southern Hemisphere clade of the subtribe Matricariinae (*Soliva, Hippia, Schistotephium, Cotula* and *Eriocephalus*) were embedded with other non-Matricariinae genera (*Lasiospermum, Hymenolepsis, Lidbeckia, Athanasia* and *Osmitopsis*) at the basal grade of the Southern Hemisphere clade with 96% bootstrap support for the clade. However, the relationship between *Eriocephalus* and the rest of the members of the tribe inferred from *ndh*F sequences remained unresolved (Watson *et al*., 2000).

Comparative studies between plastid and nuclear DNA phylogenetic trees have been useful in identifying incongruence arising from effects of hybridization and introgression in plastid DNA studies and for shedding light on the origin of polyploid species (Baldwin, 1992). However, it can be problematic to identify nuclear DNA regions that are useful for phylogenetic comparisons with plastid DNA. Such regions must be evolutionary conservative, that is, evolving by point mutation at a level comparable to that in the plastid DNA; they should be phylogenetically interpretable (evolving such as to allow resolution of speciation); must be easily examinable in the laboratory and they should be sufficiently large enough to offer potentially useful characters for phylogeny reconstruction (Baldwin, 1992). However, it is evidently difficult to meet all the criteria due to sexual recombination and segregation in nuclear DNA (Baldwin, 1992).

The plastid genome is used in phylogenetic reconstruction as it is relatively small (120-200 kb), exhibits a high single copy number per individual, a relatively conservative rate of evolution both structurally and in DNA sequence, and shows predominantly uniparental inheritance and thus the absence of genetic recombination (Hind *et al*., 1995; Soltis *et al*., 1998). It is therefore well suited for evolutionary and phylogenetic studies as it is relatively abundant in total DNA extracted from plants, and is easy to extract and analyze. There is also an extensive background of molecular information on the plastid genome which makes it easy to investigate changes in gene content, structural organization and the rates of evolution of closely related taxa (Soltis *et al.,* 1992; Soltis *et al.,* 1998).

Variation in plastid DNA has also been used to evaluate phylogenetic relationships at tribal and generic levels in Asteraceae using DNA sequences from the coding regions *rps*2, *rps*4, *ndh*F, *rbc*L, *atp*B and *mat*K, and non-coding *trn*L intron, *trn*L-F and *psb*A-*trn*H intergenic spacers for various taxa. The phylogenies reconstructed for the genus *Paeonia* from sequences of the *mat*K coding region, *psb*A-*trn*H, and *trn*L-*trn*F intergenic spacers were well resolved and supported at sectional level (Clegg and Zurawski, 1992; Sang *et al.,* 1997; Kornkven *et al.,* 1998, 1999; Watson *et al.,* 2000, 2002; Schmidt and Schilling, 2000; Samuel *et al*., 2003). This example supports the use of coding and non-coding DNA regions for inference of phylogenetic relationships in plant groups.

Plastid and nuclear DNA sequences have also been used at interspecific levels to clarify relationships within the genera *Artemisia*, *Microseris, Eupatorium* and *Hypochaeris* where ITS was sequenced for the three former genera and, ITS, *trn*L intron, *trn*L-F intergenic spacer and *mat*K for the latter genus. The resultant phylogenetic trees based on the four gene regions resulted in well-resolved topologies (Jansen *et al*., 1992; Kornkven *et al*., 1998; Schmidt and Schilling, 2000; Samuel *et al.,* 2003).

Intraspecific plastid DNA variations are rare. However, they have been documented in *Artemisia, Eupatorium, Helianthus,* and *Microseris.* They arise because of the different rates of plastid DNA mutations and when present, provide insight into population history where diploids and tetraploids from the same geographical regions do or do not group together (Jansen *et al*., 1992; Kornkven *et al*., 1998; Schmidt and Schilling, 2000).

Nuclear DNA is also a powerful tool in the interpretation of variation among closely related species. The internal transcribed spacer (ITS) region is important for phylogenetic studies as it is non-coding and therefore contains high levels of variability, evolves rapidly, is subject to concerted evolution and is easily amplified using universal primers in the flanking genes (Baldwin, 1992; Baldwin *et al.,* 1995). Sequences from the ITS region, may yield important characters for phylogenetic reconstruction among species. It has been sequenced for phylogenetic reconstruction of some of the tribes, genera, and species in Asteraceae including ITS 1 and 2 sequences of *Eriocephalus africanus* (Baldwin, 1992; Kornkven *et al*., 1998, 1999; Watson *et al*., 2000, 2002; Schmidt and Schilling, 2000; Samuel *et al*., 2003). Bremer and Humphries (1993) in their phylogenetic revision of Anthemideae based on cladistic analysis of 184 morphological characters, described *Eriocephalus* as one of the most distinct

and specialized genera in Anthemideae. Their analysis placed *Eriocephalus* with *Hippia* in the same clade, although the two genera were not previously thought to be closely related morphologically. According to Bremer and Humphries (1993), the flattened fruits in *Eriocephalus* were an indication of a relationship with the *Cotula* group and its South African relative *Hippia*. Within the *Cotula* group, *Eriocephalus* was most parsimoniously placed as sister to *Hippia* based on morphological characters. Müller (1988) reported *Eriocephalus* as being closely related to *Lasiospermum* Lag. and belonging to the subtribe Anthemidinae. Studies by Watson *et al.,* (2000), using *ndh*F gene sequences, placed the genus embedded with other non-Matricariinae genera within a basal grade of Southern Hemisphere (primarily South African) genera.

In addition to molecular data, chemical characters may also provide useful information in inference of phylogenetic trends in plant groups. The following section therefore details the use of phytochemical data in phylogenetic reconstruction.

3.3. Secondary metabolites in phylogenetic studies

Chemical characters have in the recent past become potent practical aids in plant systematics with the improvement of chromatographic and spectroscopic methods. This is no surprise then that chemical features have often provided not only phenetic information but also phylogenetic inferences based on biosynthetic considerations (Ohsaki *et al*., 1999). Apart from molecular studies of plant groups, the chemistry of a species is equally crucial in understanding the patterns of chemical evolution. The phenomenal introduction of chemotaxonomic studies in the early sixties was important in understanding the importance of chemical compounds in species delimitation. It had become quite apparent that there was frequently a correlation between the chemical constituents of plants and their geographical distribution (Kubitzki, 1984). Recent studies in flavonoids, alkaloids and terpenes, among other compounds, have focused on the role of these secondary metabolites as defence mechanisms in plants as well as their evolutionary significance in groups in which they are reported to occur. Their distribution often shows taxon specific patterns, and therefore the sharing of compounds or biogenetically related compounds may contain valuable information on phylogenetic relationships.

Chemical characters can be used to complement or improve molecular trees. At lower than generic level, secondary metabolites can be, useful in providing sufficient number

of characters for evaluation of species relationships (Grayer *et al.,* 1999).

Chemical variation in volatile constituents is also attributed to the different biosynthetic pathways as well as environmental variables. This renders them some taxonomic importance (Juteau *et al*., 2002) in study of relationships between plant groups. It is quite evident that presence of certain chemicals in a plant is an indication of the presence of the enzyme(s) responsible for their biosynthesis. Experimental evidence strongly supports the view that secondary metabolites are adaptive traits that have diversified during evolution by natural selection. Several of these metabolites have evolved as defence mechanisms against plant pathogenic attacks by viruses, bacteria and fungi, and excessive herbivory (e.g. by slugs, snails, arthropods and vertebrates), against interspecific competition and as signal compounds for pollination and seed dispersal. Presence, absence, or co-occurrence of a particular compound may have some evolutionary significance in inference of phylogeny but in most cases, this is shrouded by convergent evolution and differential gene expression. Other factors that may interfere include parallelism and divergence, a common phenomenon in secondary metabolites. (Kubitzki, 1984; Grayer *et al*., 1999; Wink and Mohamed, 2003; Wink, 2003). Nevertheless, despite these disparities, chemical characters may contain important information for phylogenetic inference.

3.4. Importance of the study

Therefore in this study an attempt is being made for the first time to elucidate the phylogenetic relationships between taxa of the genus *Eriocephalus* using both DNA sequence and chemical characters. This will be crucial in understanding the patterns of variation in chemical composition as well as biological activity. The results of this study will be useful in identifying the favourable chemotypes suitable for commercial development hence contributing directly to conservation and reduction of wasteful harvesting of plant resources from the wild.

In order to better understand relationships in *Eriocephalus,* this study attempted to utilise DNA sequence data from two non-coding regions to reconstruct species-level relationships in the group. The nuclear ribosomal RNA genes (rDNAs) of higher plants are organized in long tandem repeatable units comprising a single transcribed region for the 18S, 5.8S, 26S ribosomal RNA gene, two small internal transcribed spacers (ITS1 and ITS2) and a large external non-transcribed intergenic spacer (IGS). Therefore the ITS (internal transcribed

spacer) of the nuclear DNA region is particularly useful for studies involving a number of species of the same genus and between genera as these gene regions evolve more rapidly than the coding regions (Schmidt and Schilling, 2000; Van der Bank *et al*., 2002). In some cases, the *psb*A-*trn*H intergenic spacer has been demonstrated to evolve more rapidly than ITS, and has been used to assess interspecific relationships in the genus *Paeonia* along with the *trn*L-*trn*F intergenic spacer. Previous studies therefore imply that, this region is a source of useful phylogenetic information at lower taxonomic levels. Therefore, it was envisioned that phylogenetic analysis of ITS and *psb*A-*trn*H intergenic spacer DNA sequence data would be useful in phylogenetic reconstruction and inference of evolutionary relationships among species of the genus *Eriocephalus.* This phylogenetic framework was then used to evaluate the phylogenetic coherence of observed chemotypes that yield high-grade oils, in addition to the evolution of antimicrobial activities that can be potentially used in traditional herbal remedies.

The aim of this study is therefore to reconstruct a species-level phylogeny of *Eriocephalus* using DNA sequence data from the ITS and *psb*A-*trn*H intergenic spacer regions. These gene regions should be sufficiently variable to explore species boundaries using multiple representatives of each species. The resulting phylogenetic framework will also be used to address the evolutionary patterns of biological activity and essential oil composition in *Eriocephalus.* Eventually, this information will be helpful in identifying chemotypes from the essential oils data that are, or can be exploited commercially, in the genus. (Sang *et al.,* 1997; Kornkven *et al.,* 1998; Watson *et al.,* 2000; 2002).

3.4.1. Objectives of the study

1. To reconstruct a species-level phylogeny for the genus using DNA sequence data and chemical characters.
2. To infer phylogenetic relationships and evolutionary trends from the above analyses.

3.5. Materials and methods

3.5.1. Plant material for DNA and essential oil extraction

Fresh leaf material was collected from wild populations and stored in silica gel. Sources of plant material and the voucher specimens for the taxa used in the study are given in Table 3.1. The voucher specimens are housed in the Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg. Duplicate voucher specimens for the Namibian taxa are in the National Botanical Research Institute (NBRI), Windhoek, Namibia. Total genomic DNA was extracted from 1-2 g of fresh leaf material or 0.15-0.2 g of silica dried or herbarium material using the 2 X CTAB (cetyltrimethylammonium bromide) procedure of Doyle & Doyle (1987). Total DNA was purified using QIAquick silica columns (Qiagen Inc.) and resuspended in 1 X TE and stored at 4 ^{o}C. The total genomic DNA duplicate samples are deposited in the LHMS DNA bank, South Africa National Biodiversity Institute (SANBI), Cape Town, South Africa.

The data used in the chemical analysis was generated by GC/MS analysis of volatile components obtained from the fresh and dried aerial parts of the species of *Eriocephalus* through hydrodistillation. The procedure and the experimental conditions are described in Chapter 2.

3.5.2. PCR and DNA sequencing

The plastid *psb*A-*trn*H intergenic spacer and the internal transcribed spacer (ITS) of the nrDNA were amplified for 40 taxa of *Eriocephalus.* Between one to four individuals from 22 species were sampled (Table 3.1). Two outgroups; *Lasiospermum pedunculare* and *Cotula macroglossus* were also amplified. About 10-50 ng of total genomic DNA were used as template for amplification. The reaction mixture of 100 µl comprised promega magnesium-free thermophilic buffer (50 mM KCL, 10 mM Tris-HCL, 0.1% Triton X-100), 3 mM $MgCl_2$, 0.004% BSA (Savolainen *et al*., 1995), 0.2 mM each dNTP (dGTP, dATP, dCTP, dTTP), 0.75 µl of each primer, and 2.5 U Taq polymerase.

The ITS region was amplified using forward primer AB101F, and either ITS4R (Baldwin, 1992) or AB102R reverse primer. Where amplification of the entire region was not possible primers AB101F and ITS2R, and ITS3F (Baldwin, 1992) and AB102R were used to amplify the region in two non-overlapping halves (Baldwin, 1992).

For amplification of the ITS region, the PCR procedure comprised an initial denaturation for two minutes at 94 ^{o}C; followed by 25 or 28 cycles of 94 ^{o}C for one minute; annealing, 52 ^{o}C for one minute and extension, 72 ^{o}C, one minute with a final seven minute extension at 72 ^{o}C.

Amplification of the *psb*A-*trn*H intergenic spacer was carried out using primers *psb*AF and *trn*HR (Sang *et al*., 1997) for 30 cycles. The PCR procedure comprised an initial denaturation for two minutes at 94 ^{o}C; followed by 30 cycles of 94 ^{o}C for one minute; annealing, 52 ^{o}C for one minute and extension, 72 ^{o}C, one minute, with a final seven minute extension at 72 ^{o}C. For both regions, the resultant amplified fragments were visualized on 1% agarose gel and purified using QIAquick silica columns (Qiagen Inc.). For ITS the following primers were used as cycle sequencing primers (AB101F and ITS4R) and for the *psb*A-*trn*H intergenic spacer, PCR primers were used. The purified products were directly sequenced on an ABI 377 automated sequencer using standard dye terminator chemistry following the manufacturer's protocol (Applied Biosystems). Assembling and editing of the complimentary strands was carried out using Sequencher 4.1 (Gene Codes) and the assembled sequences transferred to the software package PAUP* (phylogenetic analysis using parsimony; *and other methods; Swofford, 2000) version 4.02b and aligned and edited visually.

3.5.3. Phylogenetic and chemical analyses

3.5.3.1. Molecular analysis

All cladistic analyses were carried out using the parsimony algorithm of the software package PAUP* 4.02b (Swofford, 2000). The data matrices for each of the two gene regions (ITS and *psb*A-*trn*H intergenic spacer) were analyzed using 1000 replicates of random taxon-addition, tree bisection-reconnection (TBR) branch swapping, with MULPARS on. All characters were treated as equally likely (Fitch parsimony; Fitch 1971). A limit of five trees was set for each replicate to reduce the time spent swapping on large numbers of equally parsimonious trees. Internal support was assessed by performing 1000 jacknife and bootstrap replicates (Felsenstein, 1985) using simple taxon addition and TBR branch swapping with a tree limit of five trees per replicate. Only those groups with frequency >50% were reported. A modified criteria for bootstrap and jackknife statistical support percentages was adopted from Van der Bank *et al*., (2002) and Langström and Oxelman, (2003), which is as follows: 50-74%, weak; 75-84% moderate; 85-94% well supported and 95-100%, strongly supported.

3.5.3.2. Chemical analysis

The volatile constituents of the species of *Eriocephalus* were all analysed for their chemical composition using GC/MS analysis. The experimental conditions are described in Chapter 2. Two hundred terpene compounds were obtained. Among these, were 91 unidentified compounds, which were included in the analysis. Each character carries information that is crucial and therefore all known and unknown characters were used in the analysis. Very little information on the chemistry of the outgroup taxa, *Lasiospermum pedunculare* and *Cotula macroglossus* is available, hence only two compounds of *Lasiospermum pedunculare* are included in this analysis giving the total number of characters as 202.

A data matrix for chemical characters was generated using MacClade 4.0b, (Maddison and Maddison, 1999). Data was scored as binary; presence (1) or absence (0) of a chemical compound and missing data was coded as (?). The presence or absence criterion, inappropriate as it may appear from theoretical view, is useful in studies of low hierarchical levels. This is because substances or chemical compounds differ in such a way as to reflect the steps of biosynthetic diversification (Kubitzki, 1984).

Analysis of 202 characters was carried out for complete taxa (91) for which the chemical data was available and another analysis for restricted taxa (37) for which the ITS data was available (Table 4. 1). A heuristic search was carried out using the parsimony algorithm of the software package PAUP* 4.0b10 (Swofford, 1998) following the same procedure as described in the ITS analysis.

3.5.3.3. Combined analysis

The combined matrix of the ITS and chemical data was analyzed using the same procedure as in the ITS and chemical analysis. For the restricted taxon set (comprising 37 taxa), successive approximations weighting (SW; Farris, 1969) was carried out according to rescaled consistency index based on best-fit criterion and a base weight of one. Using Fitch trees from the heuristic search as the basis for calculating the initial weights, the search reweighting process was repeated until the tree length remained the same in two successive rounds. The complete taxon combined (molecular and chemical) analysis included 89 taxa. The rest of the analysis is as described for the ITS analysis. Taxa not represented in the molecular but present in the chemical matrix and vice versa were scored as missing for these data.

Table 3.1. *Eriocephalus* taxa and outgroups used for the molecular systematics studies.

LHMS Accession NO.	Species	Locality/Population	Voucher specimen
535	*E. africanus*	Malmesbury (MMY)	AV 444 - DPP
546	*E. africanus*	Melkbosstrand (MKB)	AV 445 - DPP
544	*E. africanus* B	Citrusdal (CDL)	AV 453 - DPP.
1223	*E. africanus* var *paniculatus* A	Sutherland/Koornladshloof (STL)	AV 515- DPP
1300	*E. ambiguus* A	Schakalsberge (ex NBRI)- Namibia (NMB)	AV 868 - DPP, NBRI
539	*E. aromaticus*	Swartberg (SWG)	AV 484 - DPP
1266	*E. aromaticus* A	Ladismith/Seweweekspoort (LDSP)	AV 524 - DPP
540	*E. brevifolius*	Oudtshoorn (ODT)	AV 483 - DPP
1286	*E. brevifolius*	Kamiesberg (KMG)	AV 835 - DPP
541	*E. capitellatus*	Swartberg (SWG)	AV 482 - DPP
1225	*E. decussatus* A	Sutherland/Fraserburg (STLFG)	AV 532 - DPP
1285	*E. decussatus*	Sutherland/Kamiesberg (STLKM)	AV 836 - DPP
1301	*E. dinteri* A	Near Aus- Namibia (NMB)	AV 871 - DPP, NBRI
542	*E. eriocoides* subsp *ericoides* A	Prince Albert (PA)	AV 494 - DPP
1272	*E. eriocoides* subsp *ericoides* A	Bethulie (BT)	AV 747 - DPP
1296	*E. ericoides* subsp *ericoides* A	Windhoek dist. (ex NBRI)- Namibia (NMB/1)	AV 866, NBRI
1297	*E. ericoides* subsp. *ericoides* A	Farm Hohenheim- Namibia (NMB/2)	AV 867 - DPP, NBRI
1224	*E. eximius* A	Sutherland/Bosrivier (STLBR)	AV 528 - DPP
1284	*E. eximius*	Sutherland/ Kamiesberg (STLKM)	AV 837 - DPP
1248	*E. grandiflorus* B	Laingsburg/Matjiesfontein (LGMF)	AV 533 - DPP
1299	*E. klinghardtensis* A	Neiaab Mountain- Namibia (NMB)	AV 870 - DPP, NBRI
1295	*E. luederitzianus* A	12 km East of Windhoek- Namibia (NMB)	AV 865 - DPP, NBRI
1298	*E. merxmuelleri* A	Buschmanberge- Namibia (NMB)	AV 869 - DPP, NBRI
1263	*E. microphyllus* A	Sutherland/Fraserburg (STLFG)	AV 531 - DPP
1250	*E. microphyllus* B	Nieuwoudtville/ Loeriesfontein (NVLF)	AV 543 - DPP
1244	*E. namaquensis* B	Clanwilliam/Perdefontein (CWPD)	AV 546 - DPP
1246	*E. pauperrimus* B	Nieuwoudtville/ Loeriesfontein (NVLF)	AV 540 - DPP
1294	*E. pinnatus* A	Brandberg (ex NBRI)- Namibia (NMB)	AV 864 - DPP, NBRI
1255	*E. punctulatus* A	Nieuwoudtville (NV)	AV 449 - DPP
531	*E. punctulatus* C	Nieuwoudtville (NV)	AV 442 - DPP
1252	*E. punctulatus* B	Nieuwoudtville (NV)	AV 441 - DPP
538	*E. purpureus*	Nieuwoudtville (NV)	AV 440 - DPP
1257	*E. purpureus* A	Nieuwoudtville/ Papkuilsfontein (NVPP)	AV 551 - DPP
1260	*E. purpureus* A	Laingsburg/Matjiesfontein (LGMF)	AV 516 - DPP
534	*E. racemosus*	Koeberg (KBG)	AV 446 - DPP
543	*E. racemosus* var *racemosus* C	Velddrif (VD)	AV 457 - DPP
1302	*E. scariosus* A	Near Aus- Namibia (NMB)	AV 872 - DPP, NBRI
1242	*E. spinescens* B	Sutherland/ Ceres (STLCR)	AV 517 - DPP
1293	*Lasiospermum pedunculare* Lag	-	J Manning 2900- NBG
	Cotula macroglossus	-	J Manning 2891-NBG

LHMS #. SANBI DNA bank accession number
DPP – Department of Pharmacy and Pharmacology, University of the Witwatersrand, Johannesburg
NBRI – National Botanical Research Institute, Windhoek, Namibia
SANBI – South African National Biodiversity Institute, Cape Town
NBG-National Botanical Garden, Compton Herbarium, Kirstenbosch, Cape Town.

3.6. Results

3.6.1. Analysis of ITS

The aligned matrix included 37 taxa (35 ingroup and two outgroup taxa) and 640 characters of which 171 (26.7 %) were variable and 53 (8.28 %) were potentially parsimony informative. Three taxa, namely *E. ambiguus, E. decussatus* and *E. punctulatus,* produced sequences

containing multiple paralogous copies of the ITS region, and were therefore impossible to align and were not included in the analysis. 1000 replicates of random taxon addition yielded 2735 equally most parsimonious trees of 237 steps and CI = 0.82 and RI = 0.74. The strict consensus of the 2735 trees is shown in Figure 3.1. Only Jacknife (plain text) and bootstrap (bold text) percentage values >50% are indicated below the branches; groups with percentages <50% have nothing indicated. One of the equally most parsimonious trees was chosen at random and is shown in Figure 3.2. The ITS topology had two major clades A and B but relationships within these groups were not well resolved in the strict consensus. Five smaller clades have been defined for the purposes of describing the tree topology below. (Groups I-V; Figure 3.1).

Relationships within *Eriocephalus* were not well resolved but some nodes did receive moderate to weak support values (Figure 3.1). Several polytomies occurred within *Eriocephalus* in each major clade (due to a lack of informative characters), and many groups were defined by a single change (Figure 3.2).

In Figure 3.1 clade I, a weakly supported relationship between two representatives each of *E. punctulatus* and *E. purpureus* received weak support (BS = 64%), but relationships within this clade were unresolved.

In clade II, *E. racemosus* var *racemosus* and *E. decussatus* were moderately supported as sister taxa (JK = 61%, BS = 82%).

In clade III, a weakly supported relationship (JK = %, BS = 58%) between two of individuals of *E. eximius* and one individual of *E. africanus* was recovered with the two former taxa receiving moderate support (JK = 63%, BS = 78%) as sister taxa.

Clade IV included an unsupported relationship between *E. spinescens* and *E. luederitzianus.*

Clade V was comprised of individuals of *E. brevifolius, E. grandiflorus, E. africanus, E. ericoides* subsp. *ericoides, E. merxmuelleri* and *E. scariosus* with the clade receiving weak bootstrap support (BS) of 63%. Two individuals of *E. brevifolius* from Kamiesberg and Oudtshoorn received weak support (BS = 62%) as sister taxa, and in turn were sister to *E. grandiflorus*, together these taxa received support values of JK =75% and BS = 86%.

It is also noteworthy that in some instances individuals of the same species did group together as noted in *E. eximius, E. brevifolius, E. punctulatus* and *E. purpureus.* This trend was also observed in Chapter 2 whereby individuals of the same species displayed different chemical profiles in some cases.

3.6.2. Analysis of *psb*A-*trn*H intergenic spacer

The matrix included 39 taxa (38 ingroup and 1 outgroup) and 506 characters of which 421 were included in the analysis. 31 characters were variable but only 3 were potentially parsimony informative resulting in an insufficient amount of phylogenetic information with which to build a tree. This region was therefore not included in the final analysis due to lack of variability present in this data set.

3.6.3. Chemical analysis

3.6.3.1. Restricted taxon set

The matrix included 37 taxa (35 ingroup taxa and two outgroups) and 202 characters. The number of characters included in the analysis was 202, of which 159 (79%) were variable and 94 (47%) were potentially parsimony informative. 1000 replicates of random taxon addition yielded 635 equally most parsimonious trees of 407 steps and CI = 0.39 and RI = 0.42. The strict consensus from the equally weighted (EW) data formed polytomies, hence not shown. Successive character reweighting (SW) resulted in 720 equally most parsimonious trees of Fitch length = 108 steps, CI = 0.59 and RI = 0.44. However, all nodes collapsed in the strict consensus tree and hence the relationships among the taxa were completely unresolved. For this reason the trees are not shown.

3.6.3.2. Complete taxon set

The complete taxon chemical matrix included 91 taxa (89 ingroup taxa and two outgroups) and 202 characters, of which 197 (98%) characters were variable and 136 (67%) were potentially parsimony informative. The analysis yielded five equally most parsimonious trees of 878 steps and CI = 0.22 and RI = 0.47. The strict consensus of the equally weighted (EW) trees is shown in Figure 3.3. The low consistency index is indicative of a high level of homoplasy in the chemical characters used.

The chemical phylogeny resulted in two unsupported major clades (marked as A and B) with five smaller clades nested within B (Groups I-VI; Figure 3.3). Within these clades were

weakly to strongly supported smaller clades. It should be noted that the complete taxon set includes triplicates of individual species per population except *E. punctulatus* from Nieuwoudtville where six individuals were sampled. Some of the species have more than one population included in the analysis. This sampling strategy was adopted during collection of plant material for hydrodistillation of essential oils. Thin layer chromatography and preliminary GC (Gas Chromatography) analysis indicated presence of variation in some individuals of the same species from the same population and therefore all the subsequent studies and analyses have concentrated on the multiple taxa sets.

Variation between individuals of the same species is also reflected in the chemical tree except for the taxa of *E. racemosus* var *racemosus* and *E. capitellatus,* which group together in clade III. The remaining multiple individuals of the same species rarely group together (Figure 3.3).

Several of the clades observed in the ITS tree (Figure 3.1) were recovered in the chemical analysis e.g. in clade II, taxa of *E. purpureus* from Laingsburg/Matjiesfontein and Nieuwoudtville/Papkuilsfontein grouped together as sister taxa (JK = 76%; BS = 80%) and in clade V, *E. brevifolius* and *E. grandiflorus* grouped together but the placement of some taxa in the two separate topologies were not identical.

Clade A received <50% support, and all but one of the relationships between these taxa were unsupported. Two individuals of *E. punctulatus* from Nieuwoudtville/Calvinia were strongly supported (JK = BS = 98%) as sister taxa.

Clade II contained weakly supported smaller clades comprising two individuals of *E. aromaticus* as sister taxa (JK = 61%; BS = 66%) with a third individual of this species grouping with *E. punctulatus* (JK = 53%; BS = 57%). Two individuals of *E. purpureus* from Laingsburg and Nieuwoudtville were moderately supported (JK = 76%; BS = 80%) as sister taxa.

Several of the relationships between groups within clade III were unresolved with only relationships between two individuals of *E. africanus* and three individuals of *E. racemosus* var *racemosus* receiving weak support (70% and 53% BS respectively). All individuals of *E. capitelllatus* formed a monophyletic group but without any support.

Clade IV received support of <50% as did all the relationships among taxa in this clade.

Clade V included an individual of *E. aromaticus* and of *E. grandiflorus* as sister taxa in a weakly supported (BS = 58%) relationship. The relationships of the rest of the taxa in this clade were unsupported.

Clade VI of the strict consensus tree included a weakly supported clade (JK = 57%; BS = 52%) comprising two individuals of *E. pauperrimus* as sister taxa. A clade comprising individuals of *E. spinescens* and *E. decussatus* received weak support (JK = 59%; BS = 58%) as did a further two individuals of the former species as sister taxa (JK = 71%, BS = 74%). Two individuals of *E. ericoides* subsp. *ericoides* from Namibia were sister taxa in an unsupported clade with an individual from Bethulie as sister to this clade. The second individual from Bethulie was placed at the base of a paraphyletic grade with the remainder of clade B as the terminal group.

3.6.4. Combined analysis of ITS sequences and chemical data

3.6.4.1. Combined restricted taxon set

The apparent lack of resolution in the pruned chemical tree (comprising 37 taxa) does not imply that the data contains no signal; rather there may be some masked phylogenetic signals that may only be revealed by direct combination of the data sets (De Queiroz *et al*., 1995). Therefore, an attempt was made to combine the ITS and chemical data for the restricted taxon set to evaluate the resulting tree topology for increased support and/or resolution.

The issue of incongruence between the two data sets was not considered a hindrance to their direct combination. Reeves *et al*., (2001) noted that soft incongruence may arise from sampling error (caused by both lack of taxa and/or phylogenetically informative characters). Combination of the two data sources was chosen as the best measure of incongruence, whereby increased support in the combined tree would be interpreted as additive phylogenetic signal, not conflict.

The combined matrix of molecular and chemical characters for the restricted taxon set included 37 taxa (35 ingroup and two outgroups) and 1067 characters. The number of characters included in the analysis was 842 of which 330 (39.2%) were variable and 147 (17.5%) were potentially parsimony informative. 1000 replicates of random taxon addition

yielded 37 equally most parsimonious trees of 693 steps and CI = 0.51 and RI = 0.42. However, relationships between taxa of *Eriocephalus* in the strict consensus tree were not fully resolved except for a few groups at the tips (tree not shown). Successive character reweighting (SW) resulted in 3 trees of 257 steps and CI = 0.75 and RI = 0.67 and the strict consensus tree is shown in Figure 3.4 The underlined bootstrap values above the branches were obtained from successive reweighting of characters and the values below the branches are Jacknife (plain) and bootstrap (bold) from EW analysis.

The resultant tree provided improved resolution compared to the ITS tree and there is some notable increase in the bootstrap support for some of the clades (Figure 3.4 and Table 3.3). A summary of the tree lengths, phylogenetically informative characters, CI and RI for all analyses are given in Table 3.2. Clades receiving >50% support in the ITS, chemical and combined analyses are summarized in Table 3.3. The topology derived from SW analysis of the combined data (Figure. 3.4) resulted in two weakly supported (both with BS = 59%) major clades (marked as groups A and B). Five smaller clades were retrieved and have been identified for the purposes of the following description of the tree topology (Groups I-V, Figure 3.4). Arrows in Figure 3.4 indicate six newly resolved clades that were recovered in the combined analysis, and three clades that were present in the ITS tree are marked in bold. The placement of taxa in the combined tree remains almost the same as in ITS topology except for the taxa with unresolved relationships at the base of the ITS strict consensus tree and for the taxa in the six clades indicated by arrows.

The affinities between *E. africanus/E. eximius* and *E. brevifolius/E. grandiflorus* were maintained in the combined analysis with improved support (Figure 3.4). On the other hand, new relationships emerge as noted in all the clades in the combined topology with most of them weakly to moderately supported with exception of clade IV that is well supported.

The affinity between *E. spinescens* and *E. luederitzianus* in the ITS topology changed as the former species found a new position with *E. decussatus* in the combined analysis. Other changes in position were observed between the taxa of *E. ericoides* subsp. *ericoides* and *E. aromaticus* in the combined analysis.

The pattern of grouping reflected in the combined topology is similar to that noted in the topologies derived from the separate analyses; where individuals of the same species do not

always group together. This was observed in taxa of *E. africanus, E. ericoides* subsp. *ericoides* and *E. punctulatus* in clades I, II and IV.

The improved resolution arising from the combined data and the associated increase in support for some of the clades in Figure 3.4 is an indication that the chemical data may be contributing some phylogenetic signal not evident when analysed separately.

3.6.4.2. Complete combined chemical and molecular taxon set

This study involved multiple taxa for each species of *Eriocephalus* and combination of molecular and chemical data for the restricted taxon set resulted in a fully resolved topology with some support. This gave some indication of the presence of phylogenetic signal from the chemical data. Based on this, all the chemical data for the complete taxon set were combined with the molecular data (available for only a subset of taxa - 37). It is evident that there is a substantial amount of missing ITS data that may influence the grouping of taxa in the resulting topology. However, I have attempted to use all data available in a single analysis in order to achieve the best estimate of phylogenetic relationships in this group.

The aligned matrix for the combined molecular and complete chemical data included 91 taxa (89 ingroup and 2 outgroups) and 1067 characters. The number of characters used in the analysis was 842 of which 368 (43%) were variable and 189 (22%) were potentially parsimony informative. 1000 replicates of random taxon addition yielded five equally most parsimonious trees of 1161 steps and CI = 0.34 and RI = 0.47. The strict consensus of the five equally most parsimonious trees is shown in Figure 3.5 and a summary of the tree statistics, along with clade support for the separate, and combined analyses are shown in Table 3.2 and 3.3 respectively.

In Figure 3.5, two unsupported major clades A and B have been identified for the purposes of the tree description along with eleven smaller clades nested within these (Figure 3.5; Groups I-XI). Some of the clades from the separate analyses were retrieved with improved statistical support, (Table 3.3) while support for others decreased minimally, possibly due to the high level of homoplasy in the chemical characters.

The combined topology in Figure 3.5 is used as the major reference for the discussion, as all the multiple taxa of species of *Eriocephalus* are represented. However, reference is made of

the other analyses in the discussion. The correlation between biological properties and the chemical and molecular trees is discussed in chapter 5.

Table 3.2. A summary of the tree statistics for the separate and combined analyses.

	Fitch lengths (EW)					Successive weights (SW)				
Analysis	**Steps**	**Phylogenetically Informative characters**	**CI**	**RI**	**Number of trees**	**Steps**	**Phylogenetically Informative characters**	**CI**	**RI**	**Number of trees**
ITS sequences[a]	237	53	0.82	0.74	2735					
Chemical data[a]	407	94	0.39	0.42	635	108	94	0.44	0.6	720
Chemical data[b]	878	136	0.23	0.47	5					
ITS + chem. data[a]	693	147	0.51	0.42	37	257	147	0.7	0.74	3
ITS+ chem. data[b]	1161	189	0.34	0.47	5					

[a]Data for a restricted (37) taxon set

[b]Data for a complete (91) taxon set

Table 3.3. Bootstrap percentages (Fitch weights) for some of the clades in the separate and combined analyses. The symbol (-) denotes values <50%; *- denotes absence of the clade in a given analysis.

Clade	**ITS**	**Combined ITS + Restricted chemical**		**All chemical**	**Combined ITS + All chemical**
		EW	**SW**	**EW**	**EW**
E. purpureus A NV-*E. purpureus* A LGMF	*	-	74	*	*
E. punctulatus C NVCV-*E. punctulatus* A NVCV	*	*	*	98	95
E. punctulatus A NVPP-*E. aromaticus* B LDSP	*	*	*	57	60
E. aromaticus SWG-*E. aromaticus* C LDSP	*	*	*	66	65
E. aromaticus SWG-*E. grandiflorus* A LGMF	*	*	*	54	54
E. africanus MKB-*E. eximius* STLKM	58	84	94	*	-
E. eximius A STLBR-*E. eximius* STLKM	78	90	92	*	63
E. decussatus A STLFG-*E. spinescens* B STLFG	*	70	92	56	*
E. spinescens A STLCR-*E. spinescens* C STLCR	*	*	*	74	69
E. racemosus var *racemosus* C VD-*E. racemosus* var *racemosus* B VD	*	*	*	53	62
E. racemosus var *racemosus* A VD-*E. racemosus* var *racemosus* B VD	*	*	*	57	-
E. racemosus var *racemosus* C VD-*E. decussatus* A STLFG	82	*	*	*	*
E. africanus A CDL-*E. africanus* C CDL	*	*	*	70	70
E. pauperrimus A NVLF-*E. pauperrimus* C NVLF	*	*	*	54	54
E. brevifolius KM-*E. brevifolius* ODT	76	77	74	*	-
E. brevifolius KM-*E. grandiflorus* B LGMF	86	77	77	*	-

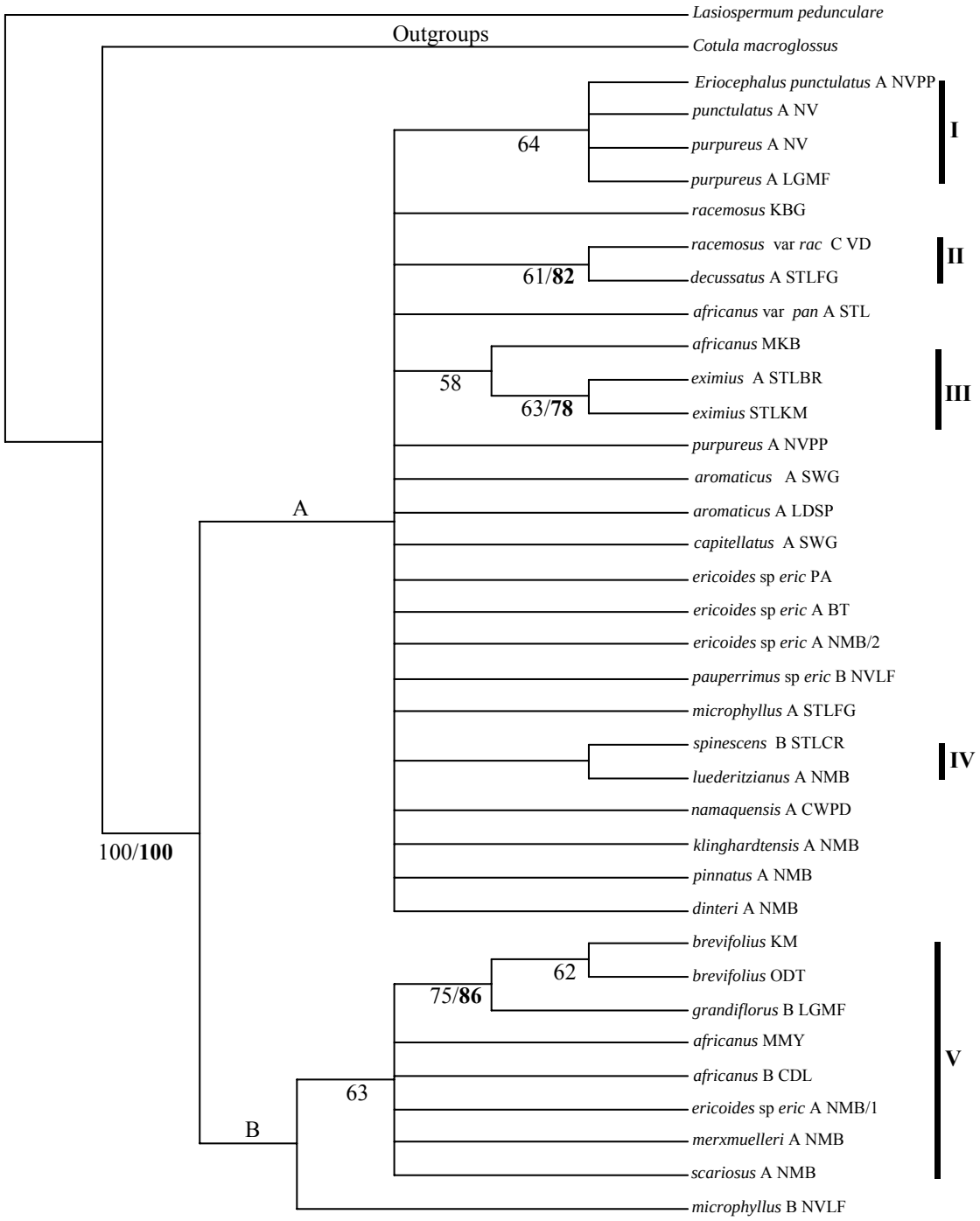


Figure 3. 1. Strict consensus of 2735 equally parsimonious trees from analysis of ITS sequences of 37 taxa. Tree length = 237, CI = 0.82 RI = 0.74. Jacknife (plain) and bootstrap (bold) percentages are indicated beneath branches. A and B represent two major clades. The roman numerals and bars represent clades identified in the results section. The abbreviations given after the species names are individual taxon labels (A, B, C) and locality. Locality abbreviations are as follows: NVPP-Nieuwoudtville/Papkuilsfontein; NV-Nieuwoudtville; NVLF- Nieuwoudtville/Loeriesfontein; LGMF-Laingsburg/Matjiesfontein; MKB-Melkbosstrand; MMY-Malmesbury; STL-Sutherland; STLBR-Sutherland/Bosrivier; STLKM-Sutherland/Kamiesberg; STLFG-Sutherland/Fraserburg; KBG-Koeberg; VD-Velddrif; PA-Prince Albert; NMB-Namibia; BT-Bethulie; CWPD-Clanwilliam/Perdefontein; STLCR-Sutherland/Ceres; KM-Kamiesberg; ODT-Oudtshoorn; CDL-Citrusdal; SWG-Swartberg.

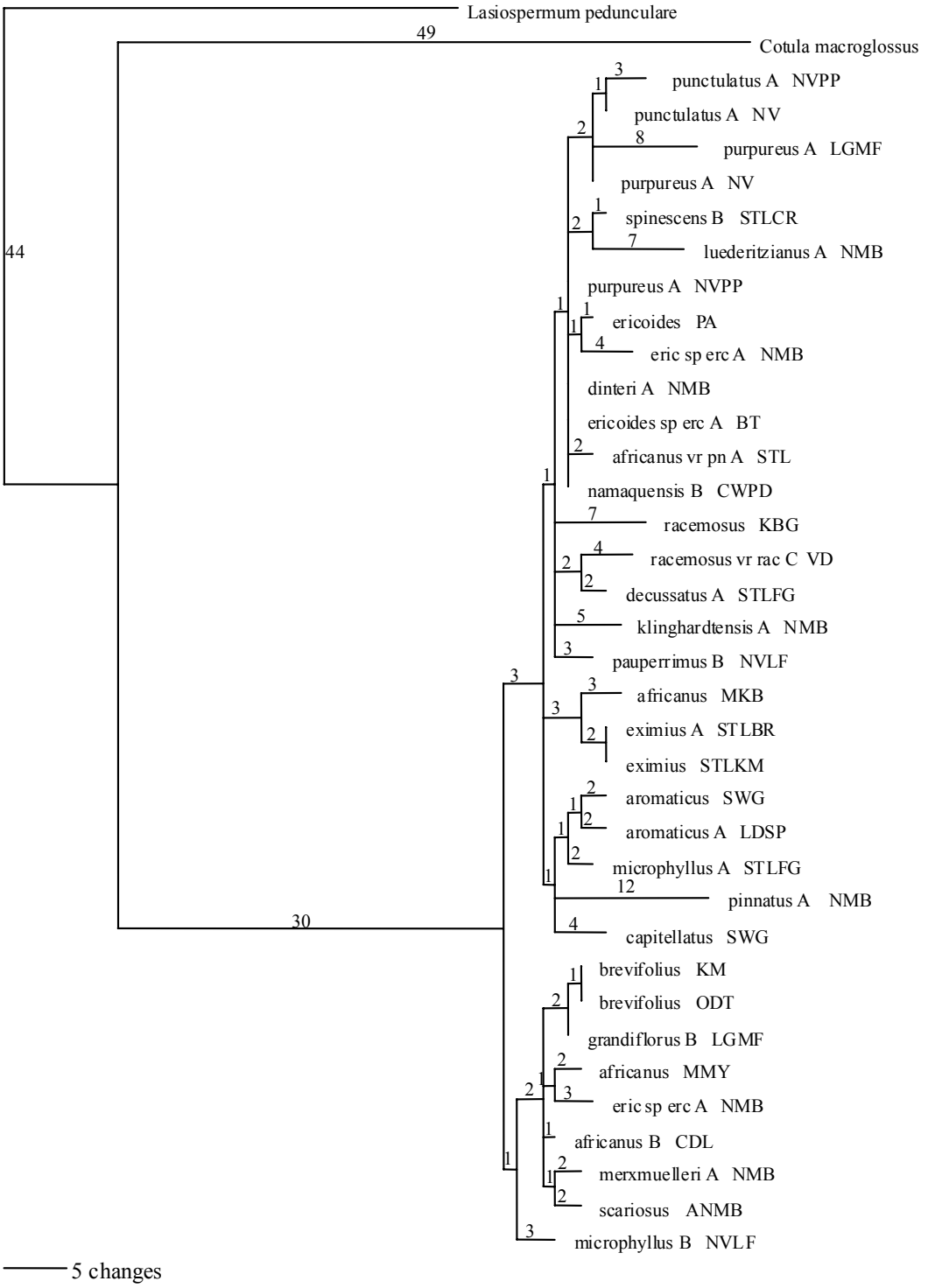


Figure 3.2. One of the equally most parsimonious trees found from analysis of ITS sequences. Branch lengths are indicated above branches. The abbreviations given after the species names are individual taxon labels and locality (expanded abbreviations are given in Figure 3.1).

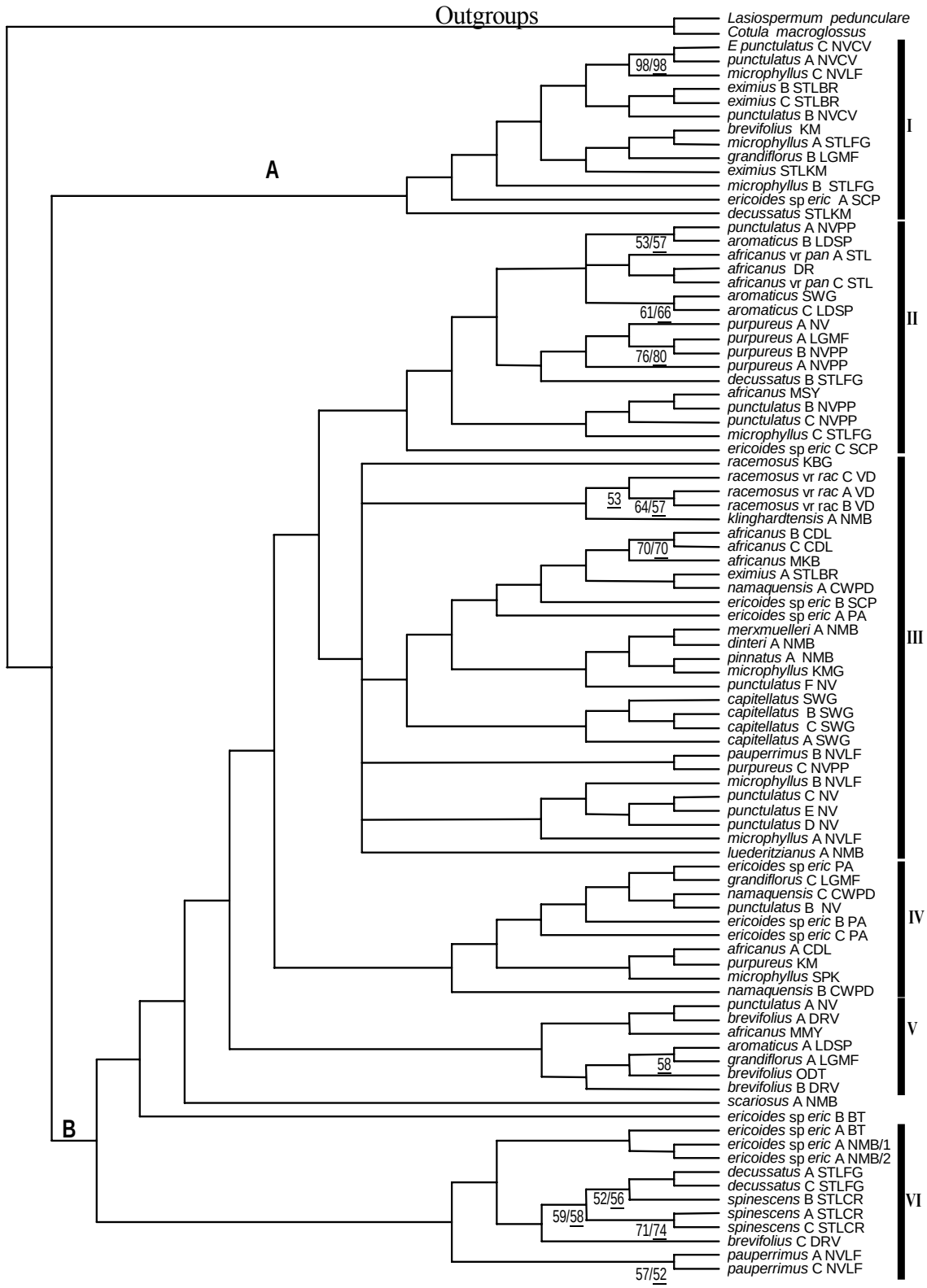


Figure 3. 3. Strict consensus of five equally most parsimonious trees from analysis of 91 taxa for the chemical data set with equal weights. Tree length = 878, CI = 0.22, RI = 0.47. Jackknife (plain) and bootstrap (underlined) percentages are indicated below the branches. The letters A and B represent major clades. The roman numerals and bars represent clades identified in the results section. The abbreviations given after the species names are individual taxon labels (A B, C) and locality. Locality abbreviations are as follows: NVPP-Nieuwoudtville/Papkuilsfontein; NV-Nieuwoudtville; NVLF-Nieuwoudtville/Loeriesfontein; LGMF-Laingsburg/Matjiesfontein; MKB-Melkbosstrand; MMY-Malmesbury; MSY-Mossel Bay ; STL-Sutherland; STLBR-Sutherland/Bosrivier; STLKM-Sutherland/Kamiesberg; STLFG-Sutherland/Fraserburg; KBG-koeberg; VD-Velddrif; PA-Prince Albert; NMB-Namibia; BT-Bethulie; CWPD-Clanwilliam/Perdefontein; STLCR-Sutherland/Ceres; KM-Kamiesberg; ODT-Oudtshoorn; CDL-Citrusdal; SWG-Swartberg; DRV-De Rust Vergelegen; DRV- De Rust; SCP-Scheerpersrust; LDSP-Ladismith/Seweweekspoort.

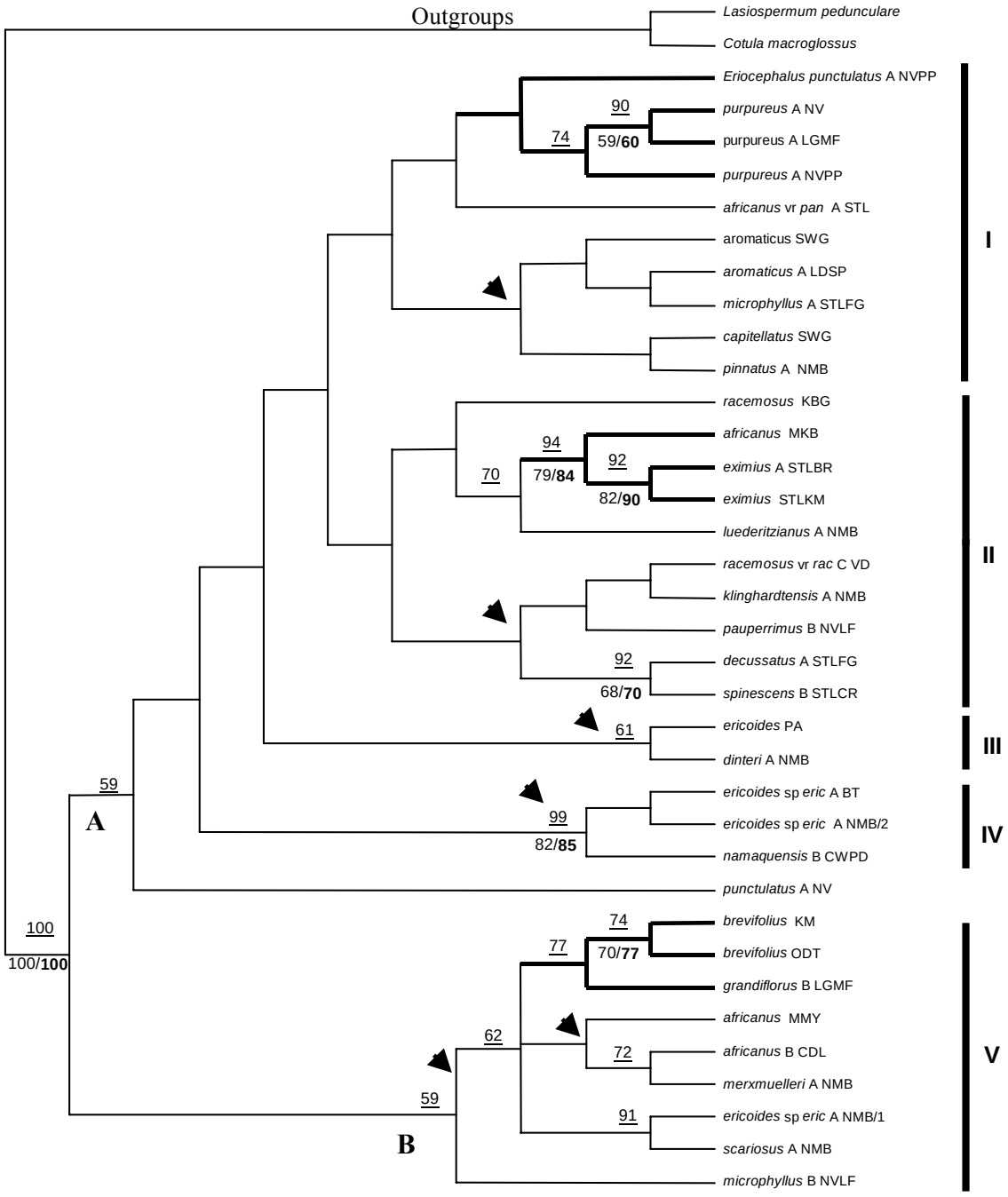


Figure 3.4. Strict consensus of three equally most parsimonious trees from analysis of 37 taxa from combined analysis of the chemical and molecular data with successive weighting (SW). Bootstrap percentages underlined above branches are for SW and those of equally weighted EW (bold) and jackknife (plain) are below the branches. Letters A and B represent the major clades. The arrows indicate newly resolved clades and the bold lines the recovered clades from ITS analysis. The roman numerals and bars represent clades identified in the results section. The abbreviations given after species names are individual taxon labels (A, B, and C) and locality. Locality abbreviations are as follows: NVPP-Nieuwoudtville/Papkuilsfontein; NV-Nieuwoudtville; NVLF-Nieuwoudtville/Loeriesfontein; LGMF-Laingsburg/Matjiesfontein; MKB-Melkbosstrand; MMY-Malmesbury; MSY-Mossel Bay ; STL-Sutherland; STLBR-Sutherland/Bosrivier; STLKM-Sutherland/Kamiesberg; STLFG-Sutherland/Fraserburg; KBG-koeberg; VD-Velddrif; PA-Prince Albert; NMB-Namibia; BT-Bethulie; CWPD-Clanwilliam/Perdefontein; STLCR-Sutherland/Ceres; KM-Kamiesberg; ODT-Oudtshoorn; CDL-Citrusdal; SWG-Swartberg; DRV-De Rust Vergelegen; DRV- De Rust; SCP- Scheerpersrust; LDSP-Ladismith/Seweweekspoort

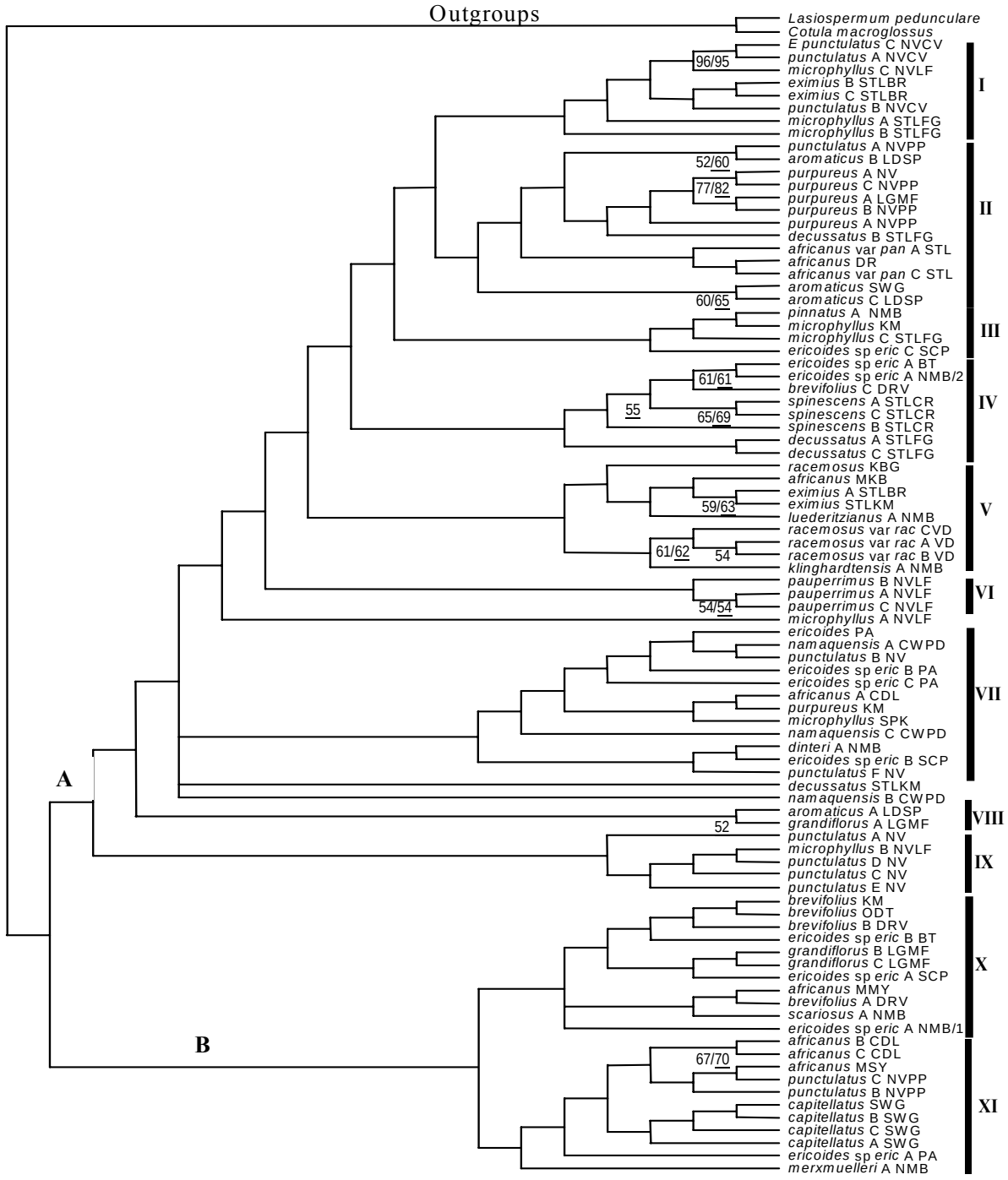


Figure 3. 5. Strict consensus of five equally most parsimonious trees from analysis of 91 taxa for the combined data set with equal weights. Tree length = 1161, CI = 0.34, RI = 0.47. Jackknife (plain) and bootstrap (underlined) percentages are indicated below the branches. The letters A and B represent major branches. The roman numerals and bars represent clades identified in the results section. The abbreviations given after species names are taxon labels (A, B, C) and locality. Locality abbreviations are as follows: NVPP-Nieuwoudtville/Papkuilsfontein; NV-Nieuwoudtville; NVLF-Nieuwoudtville/Loeriesfontein; LGMF-Laingsburg/Matjiesfontein; MKB-Melkbosstrand; MMY-Malmesbury; MSY-Mossel Bay; STL-Sutherland; STLBR-Sutherland/Bosrivier; STLKM-Sutherland/Kamiesberg; STLFG-Sutherland/Fraserburg; KBG-koeberg; VD-Velddrif; PA-Prince Albert; NMB-Namibia; BT-Bethulie; CWPD-Clanwilliam/Perdefontein; STLCR-Sutherland/Ceres; KM-Kamiesberg; ODT-Oudtshoorn; CDL-Citrusdal; SWG-Swartberg; DRV-De Rust Vergelegen; DRV- De Rust; SCP-Scheerpersrust; LDSP-Ladismith/Seweweekspoort.

3.7. Discussion

3.7.1. Molecular and chemical evolution

The aim of any systematic study is to reconstruct a true phylogenetic system that reflects natural relationships amongst taxa (Grayer *et al.,* 1999). In such studies, single data sets, whether molecular, chemical, morphological, cytological, ecological, or geographical are sometimes not sufficient on their own for inference of phylogeny. However, phylogenetic reconstruction can be enhanced by conducting analyses of independent data sets and later combining them to observe the overall effects. Other authors have noted that reliance on a single data set may result in lack of resolution, misleading conclusions or both (Reeves *et al*., 2001), and particularly in molecular studies, a single gene, despite being a definable functional unit, may not constitute a reliable source for phylogenetic reconstruction on its own (Savolainen *et al*., 2000; Reeves *et al*., 2001).

Robust phylogenetic trees are much needed in order to efficiently explore the medicinal and economic uses and potential of plant groups (Paton *et al*., 2004). In some cases combination of data sources may reveal groups not present in the separate analyses because with increased number of characters, the phylogenetic signal is likely to assert itself over the noise, resulting in a more accurate estimate of phylogeny (De Queiroz *et al*., 1995).

In this study it was clear that the ITS tree obtained in the molecular analysis (Figure 3.1) was not fully resolved, especially for those taxa at the base of clade A. The strict consensus tree of the ITS and chemical data (37 taxa) resulted in a fully resolved topology. This was despite the fact that the chemical topology for the restricted taxon set (37 taxa) was unresolved. It appears that phylogenetic signal was present in the separate data sets that was only manifested in the combined analyses. The increase in the bootstrap support values (Table 3.3) for equally and successively weighted analyses support this observation. However, the combination of all the chemical data for 91 taxa with the ITS data for 37 taxa resulted in minimal increase and decrease in the bootstrap support values for some groups (Table 3.3 and Figures 3.3 and 3.5). This phenomenon may be due to the high level of homoplasy in the chemical data as indicated by the very low CI = 0.24. Reeves *et al.*, (2001) noted in their molecular analyses of Iridaceae, that direct combination of data sets resolved more relationships with enhanced bootstrap support regardless of the negative combinability test (these authors used the partition homogeneity test). According to them, the latter tests are often unreliable in

addressing cases of disagreements between topologies from different analyses, and thus I did not attempt to apply them as a test of congruence here.

As mentioned above, combining data sets can enhance detection of real phylogenetic groups, and analysis of separate data can be used to detect incongruences, which might affect phylogenetic estimations giving misleading results (De Queiroz *et al.,* 1995). From whichever perspective one views it from, use of both approaches is crucial in enhancing phylogenetic considerations. There is also greater descriptive and explanatory power of phylogenetic hypotheses generated from combined data and there is potentially greater ability of combined analyses to uncover real phylogenetic groups.

One aspect that emerges from this study is the fact that multiple taxa of individual species studied rarely grouped together as would be expected. The erratic patterns of the grouping of these multiple representatives of taxa, especially in the chemical tree may imply that the current species delimitations are not supported by the chemistry. This makes it difficult assign any infra specific ranks to the taxa studied but affinity groups were noted in the various analyses and have been discussed in Chapter 5.

However, the ITS phylogeny is discussed here as no molecular account of the ITS phylogeny of the genus in Southern Africa has been recorded elsewhere. Incongruences between trees derived from the various analyses were noted but the data sets were still combined because the low support values made it impossible to determine whether these were due to sampling error (lack of phylogenetic signal) or hard incongruence (real differences in phylogenetic signal). Despite these discrepancies, there was some agreement among the various topologies when the data was combined with an obvious increase in bootstrap and/or jacknife support (Table 3.3).

In some exceptional cases, taxa of the same species that occur from populations with allopatric distributions group together rather than with their respective multiple individuals from the same population. This was observed in *E. brevifolius* from Kamiesberg and Oudtshoorn (Figures 3.1 and 3.5) *E. aromaticus* from Swartberg and Ladismith, (Figure 3.3 and 3.5), *E. purpureus* from Nieuwoudtville and Nieuwoudtville/Papkuilsfontein and *E. eximius* from Sutherland/Bosrivier and Kamiesberg among other species (Figure 3.4 and 3.5).

Kubitzki, (1984) noted that allelochemical diversification could be one of the reasons for this discrepancy.

Since secondary metabolites are often similar within a clade, their occurrence or absence might be taken as an indication of common descent and thus relatedness (Wink, 2003), consequently, the co-occurrence of structural class of compounds in two taxa, could, but not necessarily be an indication of monophyletic relationship. Alternatively, it could be due to convergent evolution or to differential gene expression. It is likely that in some cases the genes that encode enzymes for the production of a given structure or skeletons have remained during diversification. These genes are not lost during diversification but might be 'switched off'. On the other hand, such genes might be 'switched on' again at a later point (Wink, 2003).

As previously mentioned, no study has been carried out on the ITS phylogeny of the genus *Eriocephalus* and neither has anyone attempted a study using multiple taxa of the species in this economically important genus. Based on this, a detailed discussion of relationships between the species based on ITS and combined phylogenies is hereby given.

3.7.2. Phylogenetic relationships within *Eriocephalus*

3.7.2.1 ITS phylogeny

Parsimony analysis of ITS sequences resulted in a not well-resolved topology of the genus *Eriocephalus* with only three moderately supported clades with (70-86%) bootstrap and jacknife values. High support values are unlikely to be obtained with so few phylogenetically informative characters. The relationships depicted in the topology agreed to some extent with proposed ideas of relationships based on morphological account (Müller *et al*., 2001) and those observed in the chemistry of the individual taxa (Chapter 2). These patterns are discussed in more detail below.

In the genus *Eriocephalus* two capitula forms exist, the radiate and the disciform types, with variants of the two forms also present. It has been suggested that in Asteraceae the differentiation of heads is a recapitulation of the evolutionary sequence of solitary flowers, in a process driven by plant-pollinator interaction (Leppik, 1977). However, Watson *et al.,* (2002), in their molecular study of subtribe Artemisiinae, concluded that the capitular morphology was not a reliable taxonomic character for the study of the Artemisiinae group

due to its inconsistency and parallel divergence. Similarly, the groupings in the ITS topology here were inconsistent with capitula type with the exception of clade I where the taxa have a radiate capitula and have 2n = 36, opposite leaves and paleae that is connate. This relationship however is inconsistent with the most recent morphological account of the genus, where *E. purpureus* and *E. punctulatus* are not considered closely related (Müller *et al.* 2001). These species do however produce blue oils (due to conversion of matricin to chamazulene during hydrodistillation) and they phenetically cluster in adjacent clusters in an analysis of chemical characters (see Chapter 2; Heywood and Humphries, 1977; Szoke *et al*., 2004).

The relationship between *E. africanus* and *E. eximius* in clade III (Figure 3.1) is characterised by them having radiate capitula, and sericeous and opposite leaves. They also produce blue oils among other shared characteristics. However, other representatives of *E. africanus* in this analysis were not included in this clade. The grouping of *E. racemosus* var *racemosus* and *E. decussatus* in clade II, Figure 3.1 can be defined by presence of disciform capitula and paleae of the marginal florets that is connate (Table 3.1). Their relationship is not fully supported by the morphology and in the most recent revision of the genus (Müller *et al*., 2001) *E. decussatus* is reported as being morphologically close to *E. microphyllus* but this is not supported by the ITS tree.

A new relationship emerged in the ITS phylogeny between *E. spinescens* and *E. luederitzianus* (clade IV). These species are not related according to the latest morphological account; however, they have disciform capitula, free paleae, and decussate and sericeous leaves. They have eight nucleotide character differences separating them (Figure 3.2). The relationships between the taxa of *E. africanus* complex were unresolved as the other members are embedded in the clade A and B. This is one of the most variable species in the genus and with the second widest distribution in South Africa after *E. ericoides* subsp. *ericoides*.

Clade V is comprised of members of the genus that are polyploids as they have multiple sets of chromosomes: these are *E. africanus* (2n = 18 or 36); *E. brevifolius, E. merxmuelleri* and *E. grandiflorus* (2n = 54) and *E. scariosus* (2n = 72) (Table 3.1). A new species relationship emerged in the clade comprising *E. brevifolius* and *E. grandiflorus.* They are not mentioned as related but they do occur in the same geographical regions (Laingsburg and Oudtshoorn, in Western Cape), have radiate capitula, connate paleae and sericeous indumentum on their leaves. However, morphologically *E. brevifolius* resembles *E. africanus* var *paniculatus*,

which shares the same characteristics, and is only separated, based on the dense felty sericeous indumentum on the leaves giving it a grey-green appearance as opposed to the silvery white appearance of the latter species. They also have semi-succulent leaves.

Clade resolution in the phylogeny does not reflect well-known morphological affinities between taxa of *Eriocephalus.* This is expected, as molecular phylogenies do not always agree with morphological data and similarity patterns based on observed phenotypic traits (Judd *et al*., 1999). Since a gene tree is not necessarily a species tree (Brower *et al.* 1996), more support from other gene sequences will be necessary to strengthen the tree obtained from the ITS sequences. A clearer pattern of relationships based on the ITS phylogeny would probably emerge if all the species and constituent taxa in the genus were included.

Hybrids between radiate and discoid taxa have been reported in the genus *Senecio* and *Aster* especially where their taxa are sympatric and flower during the same periods. The resultant hybrids have intermediate characters. Speciation in plants is said to occur as allopatric populations first adapt to a novel growth environment, followed by secondary contact of populations, and flowers are modified to prevent erosion of genotype through hybridization (Johnson, 1996). Some plant families are believed to have radiated primarily in vegetative characters, reflecting adaptation to the environment, while others have radiated mainly in floral characters, reflecting an adaptation to the pollinators. Infraspecific variation in floral characters therefore provides some evidence of the active role played by pollinators in the diversification of taxa (Johnson, 1996). In the genus *Eriocephalus,* there is a whole range of complex relationships, which are based on just more than floral diversification and pollinators. It is probable that environmental variables such as soil types, climate among others factors may have contributed to the evolution of the genus.

Apart from a few species, which depict relationships that are also supported on morphological grounds, the relationships of most taxa in *Eriocephalus* are unresolved based on ITS sequence data. More molecular data probably from other gene regions as well other data from other sources are required for a robust phylogeny of the group to be achieved. However, characters from two non-coding plastid regions (the *trn*L-F region and *psb*A-*trn*H intergenic spacer) were also gathered during this study and revealed minimal sequence variation. This would suggest that collecting sufficient DNA sequence data to resolve species level relationships in this genus may be an extremely time consuming exercise. Indeed if diversification in this

genus has been sufficiently recent it may not be possible to reconstruct a bifurcating tree from DNA sequence data since insufficient time has elapsed for lineages to coalesce.

3.7.2.2. Combined ITS and chemical phylogeny

The major reference topology is Figure 3.5, other trees are referred to whenever necessary as there are some similarities in the grouping of some taxa. Using the combined trees as a phylogenetic framework, there is an opportunity to examine and discuss similarities and dissimilarities of secondary metabolite profiles and increase understanding of their evolutionary patterns.

Clade I

This clade (*E. eximius, E. microphyllus* and *E. punctulatus*) is characterized by the presence of yomogi and santolina alcohols as synapomorphies. This is an indication of presence of similar enzymes responsible for the synthesis of the two alcohols and the possible presence of chrysanthemic acid biosynthetic pathway responsible for the synthesis of santolinyl and artemisyl derivatives (Chapter 2, Figure 2.1). The species in this clade have radiate and disciform capitula and are from similar geographical localities known to have extreme temperature variations (Sutherland and Nieuwoudtville) (Appendix I, monographs 9, 14 and 18), respectively. However, the constituent taxa of these species are scattered all over the tree.

Clade II

This clade is characterised by the presence of limonene, *trans*-pinocarveol, borneol, myrtenol, myrtenal, and bornyl acetate as synapomorphies. The presence of these monocyclic and bicyclic monoterpenes in the taxa of five species (*E. punctulatus*, *E. decussatus*, *E. purpureus, E. africanus* and *E. aromaticus*) in this clade is an example of unrelated plants having enzyme prerequisites for synthesizing similar compounds, and also these compounds are shared by other taxa in the rest of the topology, hence not diagnostic of these species. The same groups were recovered in the phenetic analysis (Chapter 2, Figure 2.14).

In the same clade, taxa of *E. purpureus* from different populations form a monophyletic group supported by presence of *cis*-carvyl acetate and aromadendrene compounds. *Eriocephalus punctulatus* and *E. aromaticus* have camphene, caryophyllene oxide, and jatamansone as synapomorphies and are sympatric in their distribution range and are morphologically distinguished by presence of opposite leaves in the former and deccusate leaves in the latter.

In the same clade two individuals of *E. aromaticus,* (Swartberg and Ladismith) are characterized by the presence of α-pinene and camphor. This relationship was also retrieved in the chemical topology (Figure 3.3) An individual of *E. africanus* var *paniculatus* and that of *E. africanus* from De Rust are sister taxa whereas the second individual of *E. africanus* var *paniculatus* is sister to this clade in a grouping characterized by the presence of β-elemene and β-caryophyllene. It is probable that the individual from De Rust is a var *paniculatus* too. α-terpinene differentiates this clade from the rest of the taxa in the major clade. Morphologically, the rest of the taxa except *E. decussatus* with disciform capitula, have radiate capitula with the connate paleae, (Table 3.4) with the latter being a consistent character in this clade.

Clade III

This clade is distinguished by the presence of camphene, sabinene, and β-eudesmol as synapomorphies, although this clade has <50% support. The relationship between *E. pinnatus* and *E. microphyllus* is also retrieved in the chemical phylogeny and is characterized by the presence of sabinene, α-phellandrene, α-terpinene, γ-terpinene, *cis*-piperitol, and bicyclogermacrene. The two species are not thought to be morphologically related - the former has radiate and connate paleae and the latter has disciform and free paleae (Table 3.4). Despite the former having unique autapomorphies and being morphologically different from all the species in the genus, it is evident that it shares similar chemistry with the latter species.

Clade IV

This clade is characterized by the presence of acyclic monoterpenes: α-fenchene, *cis*- and *trans*-linalool oxides, linalool, linalyl acetate, geranyl acetate, and isocomene. The relationships in this clade were also recovered in the phenetic analysis. Apart from the taxa of *E. brevifolius* with radiate capitula and camphor, *cis*- and *trans*-linalool oxides, artemisia and santolina alcohols, borneol and isocomene as autapomorphies, the rest of the taxa in this clade have disciform capitula and sericeous indumentum with *E. spinescens* having free paleae and *E. decussatus* having connate paleae (Table 3.4). In the chemical analysis, the taxa of *E. decussatus* and *E. spinescens* have reportedly high contents of linalool and derivatives hence forming unique chemotypes and their affinities are enhanced by their occurrence in geographically similar areas characterized by extreme temperature variations (Sutherland) (Appendix I, monographs 6 and 22). The fact that these two species group together phylogenetically and are phenetically clustered together is an indication of sharing similar

chemical evolutionary histories. They also have a great potential for use as sources of linalool derivatives, which are important medicinally and in cosmetic industries. In this clade the relationship between an individual of *E. ericoides* subsp. *ericoides* from Bethulie and one from Namibia as sister taxa is characterized by presence of α-fenchene, α-terpineol, neryl acetate, geranyl acetate and α-cadinol or muurolol. The latter taxa have disjunct distributions and present a case of allopatric taxa being more closely related than sympatric taxa (Kubitzki, 1984).

Clade V

This clade has <50% support and is characterized by the presence of two monocyclic monoterpenes, limonene and γ-terpinene as synapomorphies. This is a clade depicting relationships between taxa of *E. africanus, E. racemosus, E. eximius, E. luederitzianus* and *E. klinghardtensis* from different localities. Two individuals of *E. eximius* from Sutherland are sister taxa and one individual of *E. africanus* from Melkbosstrand is sister to the clade sharing santolina triene, yomogi alcohol, myrtenol, isocomene, β-elemene and δ-cadinene as synapomorphies. This relationship was recovered in all the analyses including the phenetic analyses. *E. racemosus* from Koeberg and *E. luederitzianus* are in turn sister to the clade and share the presence of β-myrcene, α-humulene and bicyclogermacrene as synapomorphies. The individuals of *E. racemosus* from Velddrif are monophyletic and *E. klinghardtensis* is sister to this clade. The affinities between the latter and the former species are defined by the presence of α-thujene, α-fenchene, and *cis*-sabinene hydrate. The relationships in this clade were also recovered in the restricted taxon combined analysis. The affinities between species in this clade are discussed in Chapter 5.

Clade VI

The three taxa of *E. pauperrimus* form a monophyletic group characterised by presence of nerolidol, spathulenol, bisabolol oxide A and B as synapomorphies. The same grouping was noted in the phenetic analysis (Chapter 2, Figure 2.14). The latter compounds are unique synapomorphies and together with nerolidol, they distinguish this species as having a unique chemotype of bisabolol derivatives. These are important components in cosmetics and pharmaceutical formulations due to their antiinflammatory properties and there is potential for use in flavour and fragrance industries for future exploitation.

Clade VII

The relationships between the taxa in this clade are unsupported comprising taxa of *E. ericoides* subsp. *ericoides, E. namaquensis, E. punctulatus, E. africanus, E. microphyllus,* and *E. dinteri.* Most of the relationships among the terminal taxa were also recovered in the phenetic analysis. The major clade is defined by the presence of artemisyl acetate, bicyclogermacrene, and β-eudesmol as synapomorphies. The *E. ericoides*-*E. ericoides* clade is defined by β-caryophyllene, δ-cadinene, caryophyllene oxide, limonene, and γ-terpinene as apomorphies. The *E. africanus*-*E. namaquensis* clade has α-copaene as synapomorphy and the *E. dinteri*-*E. punctulatus* clade is defined by the presence of α-terpinene, γ-terpinene, linalyl acetate, bornyl acetate and δ-cadinene.

Clade VIII

This clade is comprised of an individual of *E. aromaticus* and *E. grandiflorus*, in a weakly, supported (BS=52%) relationship. The clade is defined by the presence of monoterpenes, linalool, *trans*-pinocarveol, pinocarveol, borneol, and geranyl acetate as synapomorphies. The two species are distinguished based on the former having santolina triene, limonene, myrtenol and δ-cadinene and the latter having δ-cadinene. Morphologically, they have radiate capitula, connate paleae, and decussate leaves, which are glabrous on the surface in the former and sericeous in the latter. The latter has distinctively large pedunculate capitula compared to the former (Table 3.4). The affinity between these two species is noted in the phenetic analysis hence confirming the affinities between these two species.

Clade IX

This clade comprises four individuals of *E. punctulatus* and one of *E. microphyllus* from Nieuwoudtville in a relationship with <50% support. The major clade is defined by the presence of santolina triene, *cis*-sabinene hydrate, piperitone, γ-terpinene, δ-cadinene and α-copaene. *Eriocephalus microphyllus* is embedded within a clade comprised of three individuals of *E. punctulatus.* In the chemical phylogeny, (Figure 3.3) and phenetic analyses (Chapter 2) these individuals group together variously. The grouping is one of the rare cases whereby all the taxa concerned are from the same geographical location.

Clade X

This clade has individuals of *E. brevifolius, E. grandiflorus, E ericoides* subsp. *ericoides, E. africanus* and *E. scariosus* whose relationships are characterized by the presence of santolina

triene, camphene, sabinene, and santolina alcohol. Some of the relationships in this clade were also noted in the phenetic analysis. In the restricted taxa combined analysis, the relationship between *E. brevifolius* and *E. grandiflorus* is moderately supported (Figure 3.4) but is incongruent with the current topology. However, these species have close affinities and have been discussed further under the ITS tree and in Chapter 5. In the same clade, three individuals of *E. brevifolius* from three populations group together along with an individual of *E. ericoides* subsp. *ericoides* in a relationship defined by the presence of lavandulyl acetate, methyl chrysanthemate and β-eudesmol. A corresponding sister clade has taxa of *E. grandiflorus* and *E. ericoides* subsp. *ericoides* in a relationship characterized by the presence of β-eudesmol. The two taxa of the former are also recovered in the phenetic analysis. The relationships between *E. africanus, E. brevifolius,* and *E. scariosus* with the rest of the members of the clade are unresolved. Nevertheless, the relationships within the latter clade are defined by the presence of sabinene, borneol, α-copaene, and β-caryophyllene. An individual of *E. ericoides* subsp. *ericoides* from Namibia forms a polytomy along with the *E. africanus*-*E. scariosus* clade.

Clade XI

This clade has individuals of *E. africanus, E. punctulatus, E. capitellatus, E. ericoides* subsp. *ericoides* and *E. merxmuelleri* in relationships characterized by the presence of bicyclogermacrene, limonene, borneol, pinocamphone, γ-terpinene and α-terpinene as synapomorphies most of which were also recovered in the phenetic analysis (Chapter 2, Figure 2.11). The two individuals of *E. africanus* from Citrusdal group in a relationship also noted in the phenetic analysis as an individual of *E. africanus* from Mossel Bay grouped together with an individual of *E. punctulatus* in a relationship also noted in the phenetic analysis. These taxa share limonene, *cis*-sabinene hydrate, chrysanthenone and *trans*-chrysantemyl acetate. Morphologically, the two species have radiate capitula, connate paleae, sericeous indumentum on the leaves which are also opposite (Table 3.4). The individuals of *E. capitellatus* grouped together in a clade while an individual of *E. ericoides* subsp *ericoides* and *E. merxmeulleri* grouped as sisters to the clade sharing α-terpinene, isopinocamphone and myrtenal. These relationships were also noted in the chemical phylogeny and in the qualitative phenetic analysis. This species has characteristically high levels of camphor and variable amounts of camphor derivatives. (Appendix 1, monograph 5). Hence, potentially a viable source of camphor, which is an important counter-irritant, anaesthetic, and mild antiseptic (Ikan, 1991) and it is widely used in the cosmetics industry due to its soothing

properties. At the base of this clade are *E. ericoides* subsp. *ericoides* from Prince Albert and *E. merxmuelleri* in turn placed as sister to the main clade. These taxa share yomogi alcohol, artemisia alcohol, myrtenol and α-copaene with the remaining taxa in the clade. These two species have disciform capitula and free paleae of marginal florets. In the combined restricted analysis *E. merxmuelleri* and one individual of *E. africanus* group together (Figure 3.4).

3.7.3. Molecular and chemical diversification

It is evident that *Eriocephalus* is a chemically variable genus as evidenced by the distribution of the triplicate taxa of individual species from the same populations in the separate and combined analyses. Apart from triplicate taxa of *E. racemosus, E. pauperrimus* and *E. capitellatus* that form monophyletic groups, the remaining species show scattered positioning of their individuals in the topology. This phenomenon is observed in the chemistry of the individuals and it is perhaps not surprising that the same pattern is observed in the ITS tree.

One explanation for the lack of coherence in the phylogenetic groupings of the various taxa is that the species boundaries as they are currently defined are not a true reflection of natural taxonomic entities. The phylogeny based on chemical data indicates that the current species delimitations are not supported by their chemistry, and as such, the chemical characters as shown here may not be used generally as taxonomic markers (as the taxonomy is currently defined). The same phenomenon was noted in the phenetic analysis where specific and infraspecific circumscription of the taxa was difficult due to lack of coherent groupings.

The complex patterns of relationships between the species and constituent taxa are difficult to explain and the following discussion attempts to highlight some of the probable reasons for the existence of such complexities.

At the tribal level, breeding systems in Anthemideae show remarkable diversity with self-incompatibility being the most widespread genetic device for promoting cross-fertilization. However, despite this being a common phenomenon in diploid plants, polyploids show weak self-incompatibility. It is highly probable that the influence of the pollinators in the two floral forms (radiate and disciform capitula) influences the rate and amount of gene flow resulting in speciation. The possible breakdown of incompatibility barriers in the genus could also explain the complex relationships that exist amongst its members, especially in the *E. africanus* complex.

Table 3.4: Species of *Eriocephalus*, their major diagnostic features. R = radiate, D = disciform, F = free, PC = partly connate, C = connate, FT = felty sericeous, S = glabrous, O = opposite, A = alternate.

Species name	Capitula R/D	Paleae F/PC/C	Indumentum FT/S/G	Leaves O/A	Chromosome Number (2n)
E. africanus var *africanus* var *paniculatus*	R	C	S	O/A	18 and 36
E. ambiguus	D	F	FT	A	18
E. aromaticus	R	C	G	O	18
E. brevifolius	R	C/PC	FT/S	O	54
E. capitellatus	R	C	S	A/O	18
E. decussatus	D	C	S	O	18
E. dinteri	R	F	S	O	36
E. ericoides subsp *ericoides* subsp *griquensis*	D	F	FT	O	18
E. eximius	R	C/PC	S	O	18
E. giesii	R	F	S	O	18
E. glandulosus	D	F	FT	O	18
E. grandiflorus	R	C	S	A	54
E. karooicus	R	F	S	O	18
E. kingesii	D	F/PC	S	O	54
E. klinghardtensis	R	C	FT/S	O	-
E. longifolius	R	C	FT	A	18
E. luederitzianus	D	F	S	O	36
E. macroglossus	R	C	S	O/A	36
E. merxmuelleri	D	F	S	O	54
E. microcephalus	D		FT	O	18
E. microphyllus var *microphyllus* var *pubescens* var *carnosus*	D	F	FT	O	36
E. namaquensis	D	F	S	O	18
E. pauperrimus	D	F	FT	A	18
E. pedicellaris	R	C	FT	O/A	72
E. pinnatus	R	C	S	A	18
E. punctulatus	R	C	FT	O	36
E. purpureus	R	C/PC	FT	O/A	36
E. racemosus var *racemosus* var *affinis*.	D	C	FT	A	36
E. scariosus	R	C/PC	S	A	72
E. spinescens	D	F	S	O	36
E. tenuifolius	R	C	FT	O	-
E. tenuipes	R	C	S	A	36

Sources: (Müller *et al*., 2001).

It is also well known that in any molecular data the mutation rate, alignment, analytic technique and the relationships between the history of genes and the history of the organisms (gene trees versus species tree) are very crucial.

If a species has a single history, then it is expected that all parts of the plant should reflect that history in their genes, but this is rarely so. Some of the reasons for this anomaly include mutation. Being a random process, some of the phylogeny reconstructed for a particular gene

may differ from another by chance alone; the process of hybridization may transfer some DNA into a different lineage, especially for organelles not linked to particular nuclear genomes; and lastly polymorphisms in ancestral species can be lost in descendant species. When this happens, the history of the gene becomes different from that of the organisms (Judd *et al*., 1999).

Another complication observed between organismal and gene phylogeny is lineage sorting. The presence of ancestral polymorphism coupled with the differential survival of alleles can result in a phylogeny not matching organismal phylogeny (Judd *et al*., 1999). This is especially so, when the time taken for the alleles to coalesce is greater than the interval between successive speciation events.

Hybridization also plays a major role in the reconstruction of the phylogeny. In evolution, it may reinforce reproductive isolating mechanisms; or lead to formation of hybrid swarm through reproduction by hybrids at one site; or fusion of two species; or creation of genetic diversity or to evolution of new species. Hence, the process can maintain biodiversity, destroy it, or create it. All these factors may have contributed to the overall ITS phylogeny of *Eriocephalus* and hence making it difficult to define the species boundaries.

The genus *Eriocephalus* has a complex chemistry pattern as previously mentioned and as indicated by the TLC and GC/MS results. The extensive variation noted in the taxa was also reflected in the resulting phylogenetic tree. The complex chemical profiles comprising regular and irregular terpenes did not support the current specific delimitations and hence are not strong systematic characters. This has serious implications for bioprospecting, as one needs to understand the diversity within a potentially useful plant group to minimize trial and error in *in situ* harvesting of plant resources. It is probable that the obvious differences noted in the essential oil composition of the three populations of *E. punctulatus* included in this study with that of the commercially exploited species could be a case of the above-mentioned factor. Therefore, extensive population and genetic studies would be required to understand the complex relationships between and within this important genus.

This then raises the question of reliability of data from secondary metabolites to address infraspecific delimitation and phylogenetic reconstruction. Combination of molecular and chemical data sets has not adequately addressed the phylogenetic problems in this genus, as

most relationships were not well supported. It is noteworthy, that an examination of the chemical composition of each taxa studied reveals that different chemical histories exist even within individuals of the same species resulting in erratic grouping of these taxa. It is astounding how complex the chemistry of individuals of the same species collected in the same locality can be and this poses the question of the credibility of some chemotaxonomic conclusions based on just one representative of a particular group. On this note, it is apparent that broader sampling including duplicates of the same taxa is one way forward in addressing the chemical diversity in plants - but may not be a solution to delimitation problems. As observed in the molecular and combined phylogenies of *Eriocephalus*, multiple taxa from the same species have very different phylogenetic histories. Even though *a priori,* one would expect the duplicate taxa to be closely related, they are not and most of them have been shown to have very different chemical histories.

Other anomalies arise when chemical characters are used in addressing phylogenetic problems. These include chemical convergence, parallelism, and divergence (Grayer *et al.,* 1999; Wink, 2003; Wink and Mohamed, 2003). If the groupings are strongly supported by the concordance in phenetic and phylogenetic groupings this could be because of common ancestry. When the reverse is true, it is as a result of convergence or parallelism or it may be due to different evolutionary rates - among several other reasons (Heywood, 1976).

Allelochemical diversification may cause sympatric taxa to have more variation than allopatric taxa (Kubitzki, 1984). This phenomenon occurs where presence of certain metabolites is a reflection of steps of biosynthetic diversification, which are subject to continuous selection. When selective pressures becomes fully operational, a shift to new defense chemicals not present in the rest of the population is inevitable forcing these populations to be chemically different from all others. The affected plants partition the realm of chemical diversity for co-existence purposes (Kubitzki, 1984). This implies that the pathways for phytochemical change within each lineage do not depend only on the co-evolutionary relationship involved but also on the nature of defences already deployed by members within the group. Finally, within that population similar species deploy the same biogenetic group of secondary metabolites as key chemical barriers. The result is differential chemical diversification in sympatric species hence, making them more different from each other than from allopatric taxa (Kubitzki, 1984). This factor could be responsible for the diversity of chemistry noted in the individuals from the same population in most of the taxa

studied. This would also lead to these characters being unreliable phylogenetic markers at the species level.

One of the major uses of secondary metabolites is defense against pathogenic attacks by bacteria and fungi and against excessive herbivory. Coevolution sometimes results in the plant attackers also evolving by producing chemicals to detoxify the SM from plants, this in turn provokes plants to evolve new chemicals to counter attack their predators. When the threat declines the genes responsible for the production of these chemicals are switched off rather than lost, and may be switched on later in the plant life history if the original threat is sensed (Kubitzki, 1984; Grayer *et al*., 1999; Wink and Mohamed, 2003; Wink, 2003). The absence of such a trait in phylogenetically derived groups is probably due to differential gene expression, in that corresponding genes are not lost but switched off.

As a result chemical switchovers are known to blur chemical similarity. The ability of a plant to synthesize the same chemical may have originated independently in two or more unrelated taxa, and if monophyletic clades share a chemical characteristic this would favour its use as a taxonomic marker. In other instances, a particular SM may occur in several unrelated clades and /or plant families. The erratic SM distribution can be due to simple convergence in that genes that encode a particular biosynthetic pathway evolved independently in several parts of the phylogeny (Grayer *et al.,* 1999; Wink, 2003). Chemical divergence is likely in members of a population where intense allelochemical diversification has occurred to the point where such species cease being similar to their related groups. The triplicate taxa used in this study may have diverged so much as to be so different and especially where selective pressures may be skewed towards favouring of certain secondary metabolites.

It is evident that from the current results it is extremely difficult to define species groups in *Eriocephalus* due to absence of natural taxonomic groupings at the species-level. What remains is to tentatively recognize the affinity of groups arising in the study as listed below:

1. *E. purpureus*-different populations
2. *E. punctulatus* and *E. aromaticus*
3. *E. pinnatus* and *E. microphyllus*
4. *E. spinescens* and *E. decussatus*
5. *E. racemosus* and *E. africanus*
6. *E. eximius*, and *E. luederitzianus*

7. *E. racemosus* and *E. klinghardtensis*
8. *E. brevifolius* and *E. grandiflorus*
9. *E. aromaticus* and *E. grandiflorus*
10. *E. brevifolius, E. africanus* and *E. scariosus*
11. *E. purpureus* and *E. punctulatus*
12. *E. africanus* and *E. merxmuelleri*

All the above-mentioned taxa feature prominently in the separate and the combined analyses and similar affinities were noted in the phenetic analysis. The full discussion of the affinities of these groups is given in Chapter 5.

3.8. Conclusions

3.8.1. General conclusions

The study reveals that relationships between the species of *Eriocephalus* are very intricate and highly diversified and current species boundaries were not supported by the ITS, the chemical and the combined chemical and molecular data. In isolated cases, the taxa grouped according to their geographical localities, as observed in *E. spinescens* and *E. decussatus.* Sympatric taxa were noted to be more diversified than their corresponding allopatric taxa in most cases.

The importance of using multiple taxa in any study is clearly illustrated by the diversity noted within and between the taxa of the genus *Eriocephalus* to avoid making incorrect conclusions based on study of a single taxon. This calls for revision of sampling strategies to include multiple taxa of the study groups to accommodate the extensive variation in plants.

The results from this study emphasise the great need to carry out extensive and exhaustive population and genetic studies for evaluation of the extensive diversity noted in the study genus. It is also crucial to establish the existence of hybridization in the group, as this has not been proved experimentally. These results would be crucial when considering bioprospecting for important phytoconstituents in this genus.

The morphological characters should be evaluated within a cladistic framework and more gene regions sequenced in an attempt to resolve the delimitation problems in the group, which the current study was not able to achieve because of complexity in the relationships of the taxa studied.

3.8.2. Conclusions on ITS phylogeny

This study forms the first major attempt to reconstruct the phylogeny of the endemic South African genus *Eriocephalus.* Though the relationships within the genus are not fully resolved, the clades reconstructed shed light to how molecular groupings can differ from morphological groupings in any one group and this genus is not an exception. As previously mentioned, the relationships among the species of *Eriocephalus* are quite complex and need to be re-evaluated using other data sets. It is clear that lack of coherence in grouping of taxa means the current species boundaries are flawed and do not represent true natural taxonomic entities.

From the study it is clear that the plastid DNA genes selected for the study were not variable enough for this particular genus. At times, it was completely impossible to predict the outcome of the study, as what may have seemed to work well for one genus in a tribe may not work for another genus in the same tribe. As demonstrated the *psb*A-*trn*H intergenic spacer and *trn*L-F region were not sufficiently variable to allow phylogenetic reconstruction in this genus.

Even though ITS demonstarted some variability the number of characters separating the taxa were too few for adequate resolution and support of relationships. Another problem observed in using the ITS region was the presence of divergent paralogous repeats for some of the taxa studied like one taxa of *E. punctulatus, E. decussatus* and *E. ambiguus* (as a result these taxa could not be included in the analysis). If possible, more variable cpDNA and nrDNA gene regions will need to be sequenced. In addition, to comprehensively clarify phylogenetic relationships in *Eriocephalus* a much wider sampling of taxa will have to be carried out as the current tree only included 22 out of the 32 recognized species.

3.8.3. Conclusions on combined ITS and chemical phylogeny

The combined analysis for the complete taxon set resulted in a fairly resolved tree but support for most of the relationships was lacking. The inconsistent SM profiles mean that the systematic value of chemical characters becomes a matter of interpretation in the same way as traditional morphological markers despite the fact that they can be defined unambiguously in terms of both origin and structure. The distribution of SM apparently has some value for taxonomy but it has to be analyzed carefully and critically, as any adaptive trait. It is clear that the lack of coherent groupings makes it difficult to assign infraspecific ranks to taxa and recognise any favourable chemotypes based on the phylogeny. Rather it is preferable to

recognise them from looking into individual chemistry of the species. Despite the high level of diversity in the genus, certain affinity groups were noted in all the analyses and are discussed in Chapter 5.

In summary, it was not possible to define species boundaries using the combined chemical and molecular data due to the highly variable distribution of characters within a single species. These patterns could be due to chemical convergence and divergence, differential gene expression, allelochemical diversification, mutation, hybridization or lineage sorting. The similarities in some of the relationships noted in the combined phylogenies and the phenetic dendrograms is an indication of presence of similar enzymes for biosynthesis of the compounds present in these groups. Chemical characters from terpenes were noted to be highly homoplasious and hence not useful taxonomic markers.

CHAPTER 4

Biological properties of *Eriocephalus* species

4.1. Introduction

4.1.1. Herbal remedies and traditional medicine

The rural population, since the era of our forefathers, has always used herbal remedies for treatment of all kinds of diseases, ranging from respiratory tract ailments, gastro-intestinal disorders, dermal infections to ritual uses and for treatment of mystical spiritual ailments (Shale *et al.,* 1999). The World Health Organization (WHO) through the Traditional Medicine Programme has globally addressed this on-going practice of traditional medicine since 1976. The WHO defines traditional medicine as "the sum total of all knowledge and practices, whether explicable or not, used in diagnosis, prevention and elimination of physical, mental or social imbalance and relying exclusively on practice, experience and observation handed down from generation to generation, whether verbally or in writing". Therefore, traditional herbal medicine plays a vital role in provision of primary health care especially for rural communities (Shale *et al.,* 1999; Dorman and Deans, 2000; Rukangira, 2000).

The WHO officially recognized the importance of traditional medicine as a source of primary healthcare in 1978 in the Primary Health Care Declaration of Alma Ata. It is estimated that 80% of the world's population relies on various traditional medical practices. Herbal remedies are widely used in South Africa where more than 80% of people use plants for therapeutic reasons or visit traditional healers such as the 'sangomas' and 'inyangas' for herbal administration. It is reported that most households in KwaZulu-Natal spend between 4 and 8% of their annual income on traditional medicine services (Mulholland and Drewes, 2004). In the recent past, there has been an increase in interest in pharmacological studies of the most widely used plants in herbal remedies in South Africa and scientific validation of their uses (Marius, 1995; Rabe and Van Staden, 1997; Dyson, 1998; Mulholland and Drewes, 2004). This is an on-going process with a bright prospective future given the ongoing discoveries of new phytoconstituents with therapeutic potential.

The cost of manufactured drugs has continued to escalate rendering it almost impossible for many citizens to afford them. This has led to people seeking alternative remedies from medicinal plants, which are relatively cheap and readily available for use. There is therefore a great need to screen plants for their pharmacological activity to be able to accrue the knowledge of their active ingredients, levels of toxicity and efficacy. It is also important to investigate the plants used traditionally for potential novel antimicrobial compounds and as sources of valuable new natural products. This will in the long run confer credibility or

establish the 'rational usage' to what healers have known and used for centuries in traditional therapy. Besides this, only a fraction of the world's plant diversity has been investigated for pharmacological properties and extraction of active constituents (Swanepoel, 1997; Hammer *et al.,* 1999; Hostettmann, 1999; Http://www.botany.unp.ac.za). Such knowledge is also important in conservation of indigenous medicinal plant resources since only plants with known efficacy will be harvested from the wild.

Volatile and non-volatile plant products have become very important in combating diseases such as cancer, thrombogenesis, cell damage, inflammation, viral infection, allergic responses etc. Flavonoids, for example, are non-volatile and have the ability to scavenge reactive oxygen species (ROS) involved in a number of key body physiological processes, including in part, inflammation and immune responses. Damage caused by these reactive species is a major source of tissue damage during chronic inflammation. Flavonoids have a broad spectrum of biochemical activities including ability to inhibit a number of enzymes responsible for production of free radicals in the body e.g. protein kinases, lipoxygenases, phosphatases, phospholipases, cyclo-oxygenases, topoisomerase, NADH oxidase and others (Manthey and Buslig, 1998; Grabmann *et al.,* 2000) and are important in human diets and curbing of ailments. Therefore, it is important to screen more plants in search of alternative sources of remedies with antioxidants and antiinflammatory properties as well as the inhibitors of many enzyme mediated ailments such as Alzheimer's disease.

The genus *Eriocephalus* is one of the South African endemics with very little information on the biological properties apart from the commercially used species. It is clear from the scanty literature available that a lot of scientific information on the biological properties of *Eriocephalu*s is largely unknown. Therefore this study will be a major contribution towards understanding the biological properties of the genus as well as unravelling the potential of members of the genus particularly in the search for natural antimicrobial, antioxidants, antiinflammatory activities and inhibitory effects on acetylcholinesterase enzyme, the precursor of Alzheimer's disease. These activities are briefly discussed below.

4.1.2. Antimicrobial activity

Among the well-known and documented natural plant products are the volatile oils from aromatic medicinal plants. Most of these volatile oils have biological activities namely; antibacterial, antifungal and antioxidant properties. These activities are attributed to the

presence of the various classes of terpenes, with the most common being the mono- and sesquiterpenes of various structural groups (Williams, 1996; Svoboda and Hampson, 1999; Nakatsu *et al*., 2000; Pauli, 2001; Oladimeji *et al*., 2004). Industrially, they are important as antimicrobial agents in cosmetics, fragrances, food preservation and pharmaceutical formulations. (Baratta *et al*., 1998).

The volatile and the non-volatile plant components are useful, whether in crude or in purified forms both medicinally and industrially. In the past, the Greek, French, Roman, Indian and Egyptian people made use of the plant volatile components in almost every aspect of their lives such as bathing, relaxation, footbaths, curing diseases, culinary enhancers, skin care and in embalming of their dead among many uses. Some essential oils such as rosemary (*Rosmarinus officinalis*), chamomile (*Matricaria chamomilla*) and sage (*Salvia officinalis*) were widely used and still are even today. Chamomile extracts are used in the cosmetic industry to impart fragrance to skin care products as they contain chamazulene and bisabolol derivatives, which are also reported to have antimicrobial, antiinflammatory, antispasmodic, soothing and anti-allergic properties among others (Povh *et al.,* 2001).

Antimicrobial agents, whether from oils or extracts are important in combating various forms of bacterial and fungal infections. These two are the most prevalent diseases posing greater risks to human health. Recent advances in research and the resurgence of interest in use of natural therapies coupled to increasing consumer demand necessitates thorough investigation into the antimicrobial activities, safety, mode of action and potential uses of plant extracts (Hammer *et al.,* 1999). Infectious and inflammatory diseases are among those treated using herbal remedies (Shale *et al.,* 1999) and the number of people using the latter has continued to increase day by day (Dorman and Deans, 2000). With the increase in opportunistic infections associated with HIV, especially Candidiasis of the mouth and oesophagus (*Candida albicans* and related species) and Cryptococcosis (*Cryptococcus neoformans*), a search for antifungal drugs from plant sources is crucial. The existing antibiotics are limited (Viollon and Chaumont, 1994; Hostettmann, 1999) or their overuse has led to development of pathogenic resistance (Sokmen *et al.,* 2004). Some microorganisms that have become resistant to the commonly used antibiotics include methicilin resistant *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* (VRE) and are causing major concerns in hospitals (Williams, 1998). Hence screening of plants in search of volatile and non-volatile components

with antifungal and antibacterial properties would go a long way towards alleviating human suffering.

Since the demand for natural therapeutics continues to increase and the cost of manufactured drugs is too high for most people to afford, *in vitro* screening for new antimicrobial agents from oils or extracts with broad spectrum of activity, stable and non irritants to sensitive or damaged skins is therefore very crucial (Williams, 1998). The methods used in screening for antimicrobial agents such as disc diffusion and the minimum inhibitory concentration (MICs) go a long way in providing qualitative and quantitative measures of the agents required in combating microorganisms (Williams, 1998). Reports from these screenings should be made available to allow other researchers to conduct more confirmatory tests and estimation of safety indices.

4.1.3. Antioxidants and free radicals

The other major application of natural products is in their use as antioxidants in combating free radicals. Free radicals are atoms or group of atoms that contain one or more unpaired electrons that makes them very reactive and capable of independent existence. Examples include trichloromethyl ($CCL_3^{\cdot}$), superoxide ($O_2^{\cdot}$), hydroxyl ($HO^{\cdot}$), peroxyl ($ROO^{\cdot}$) and nitric oxide ($NO^{\cdot}$), which are produced metabolically in living organisms. Other non-radical derivatives of oxygen molecules include hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCL).

Biological free radicals are by-products of biochemical reactions and aerobic metabolism when oxygen is used to oxidize the food we eat to produce energy. The free radicals can also enter the body from a polluted environment, radiation, alcohol, and smoking or even from stress. These free radicals create chain reactions, as they are unstable and react quickly with other compounds to gain electrons for stability hence initiating the production of more free radicals. These free radicals cause oxidative damage to protein enzymes, cell membranes, DNA and RNA, unsaturated fatty acids and they can also cause cell death. They are associated with age dependant diseases such as cancer, diabetes, cataract, heart diseases, impaired brain function and premature symptoms of aging such as wrinkling of skin, hair graying, chronic fatigue, absent-mindedness, antherosclerosis, immune system impairment and neuro-degenerative diseases such as Parkinson's and Alzheimer's diseases (Sanchez-Moreno, 2002).

The respiratory tract is also highly vulnerable to potentially toxic and infective airborne molecules and particles such as ozone, sulphur dioxide, nitrous oxides, organic/inorganic aerosols, smoke, soot, mineral fibres, bacteria and viruses. All these factors can cause inflammation of the lungs as the reactive oxygen species (ROS) are generated by several types of inflammatory cells, e.g. neutrophils or alveolar macrophages. Their action results in formation of more free radicals.

Antioxidants have ability to neutralize these free radicals by donating one of their own electrons, ending the electron 'robbing chain'. They act as free-radical scavengers and chain breakers as well as complexers of pro-oxidant metals ions and quenchers of single-oxygen formation (Trouillas *et al.,* 2003; Amarowicz, *et al.,* 2004). The antioxidants do not lose stability hence they are able to break the free radical production chain. Antioxidants or free radical scavengers, especially those containing flavonoids play an important role in preventing cell membrane from oxidative damages induced by active oxygen radicals in living systems and have gained significant interest due to their pharmacological properties (Braca *et al.,* 2003). They have also been reported to inhibit oxidation-reduction enzymes such as cycloxygenase and lipoxygenase. Some flavonoids are reported to suppress non-enzymatic lipid oxidation caused by oxygen radicals. Natural food usually contains natural antioxidants that can scavenge free radicals. Small dietary antioxidants such as vitamin C, vitamin E and carotenoids act as defences against diseases (Aljadi and Kamaruddin, 2004).

The human body has the ability to fight free radicals through production of natural antioxidant enzymes, which absorb vitamin A, B, C, and E from the food to scavenge the free radicals. Other natural antioxidants include carnosine, selenium, and polyphenols. As the body ages the activity of the enzymes decreases and the absorbing function of the intestines decreases leading to the accumulation of the free radicals in the body. This results in a stressful state referred to as 'Oxidative Stress State' (OSS) leading to the previously mentioned diseases. This condition can be contained if foods such as raw vegetables, fresh fruits and herbs are consumed, as they are rich in natural antioxidants.

The oxidative deterioration of fats and oils in foods is responsible for rancid odours and flavours. This consequently decreases the nutritional quality and safety caused by the formation of secondary, potentially toxic compounds. Synthetic antioxidants used in foods

such as butylated hydroxytoluene (BHT), butylated hydroxyanisole (BHA), propyl gallate (PG) and tert-butyl hydroquinone (TBHQ) have been suspected to have side toxic effects when used by man. This has led to an increasing perceptible consumer preferences and awareness in regard to food additive safety, especially of the synthetic preservatives (Grabmann *et al.,* 2000; Kulisic *et al.,* 2004; Sokmen *et al.,* 2004). It is therefore important that safer, natural and more readily available antioxidants be used in food industry and this has prompted extensive screening of plants for discovery of natural additives and potential antioxidants.

In view of the importance of natural plant products in everything regarding life, *in vitro* screening of the members of *Eriocephalus,* which are reported to have various classes of flavonoids, will be a major contribution to discovery of the antioxidative potential of the genus as a novel source of antioxidants.

Apart from having compounds with antioxidant properties, plants are also important sources of compounds with antiinflammatory properties.

4.1.4. Antiinflammation

Apart from being useful as antimicrobials and antioxidants, volatile and non-volatile plant components are also very important due to their ability to regulate the process of inflammation. 'Inflammation is a physiological body response to attacks by external infectious organisms, or response to environmental aggressions such as sun burn, pollution, wind, pollen and mechanical shock, resulting in a complex cascade of biochemical events culminating in symptoms such as redness, swelling, irritation, pain edema, heat and disturbed tissue function' (Fig.4.1) (Ammon *et al.,* 1991; Baylac and Racine, 2003.).

The symptoms of inflammation are caused by a variety of mediators, some of which include prostaglandins (PG), leukotrienes (LT) and histamines (Ammon, 1996). 5-lipoxygenase is the main determinant of inflammation as it initiates the conversion of membrane phospholipids derived fatty acid, arachidonic acid into a number of compounds like leukotrienes.

Leukotrienes are naturally occurring 5-lipoxygenase products of arachidonic acid metabolism with potent biological actions and are considered mediators of many diseases including inflammation (e.g. psoriatic lesions), bronchitis, rheumatism, skin diseases, microvascular

leakiness, airway edema, asthma and pain. The 5-lipoxygenase catalyses the oxidation of arachidonic acid to 5-hydroperoxyeicosatetraenoic acid (5-HPETE) and then to LTA_4. This compound is converted by LTA_4 hydrolase into the potent phagocycte chemo attractant LTB_4. Alternatively, LTC_4 synthase catalyzes the conjugation of LTA_4 with glutathione to form LTC_4, which can be modified further to LTD_4 and LTE_4. Thus, search for potent inhibitors of the 5-lipoxygenase from plants is crucial because many diseases are mediated by this enzyme (Fig.4.1) (Kumar *et al.,* 2000; Benrezzork *et al.,* 2001).

Production of LTs during inflammatory responses depends upon availability of activating protein (FLAP), which facilitates the transfer of arachidonic acid to initiate LT synthesis. This is followed by cell stimulation, after which the mediators are synthesized and released quickly to act on the cell surface G protein coupled receptors. LTB_4 promotes the migration and activation of phagocytes at inflammation sites and in some species acts in cooperation with vasodilators to provoke plasma exudation dependant on circulating leukocytes (Benrezzork *et al.,* 2001). The implications of inflammatory diseases are enormous on the human well-being and a search for plants with potential to alleviate the suffering is a major way forward. A search for new plants with antiinflammatory properties or validation of the ones already in use is an urgent necessity.

Some of the traditional uses of the *Eriocephalus* species include treatment of inflammation and other dermal infections and most of these uses have not been validated scientifically. Therefore, this study seeks to validate the uses of the genus in treatment of inflammation by *in vitro* screening of the essential oils for potential inhibitory activity against 5-lipoxygenase enzyme, the major determinant of most of the antiinflammatory activities. Apart from the latter, the potential of other species not used in herbal remedies will be documented for the first time in this study.

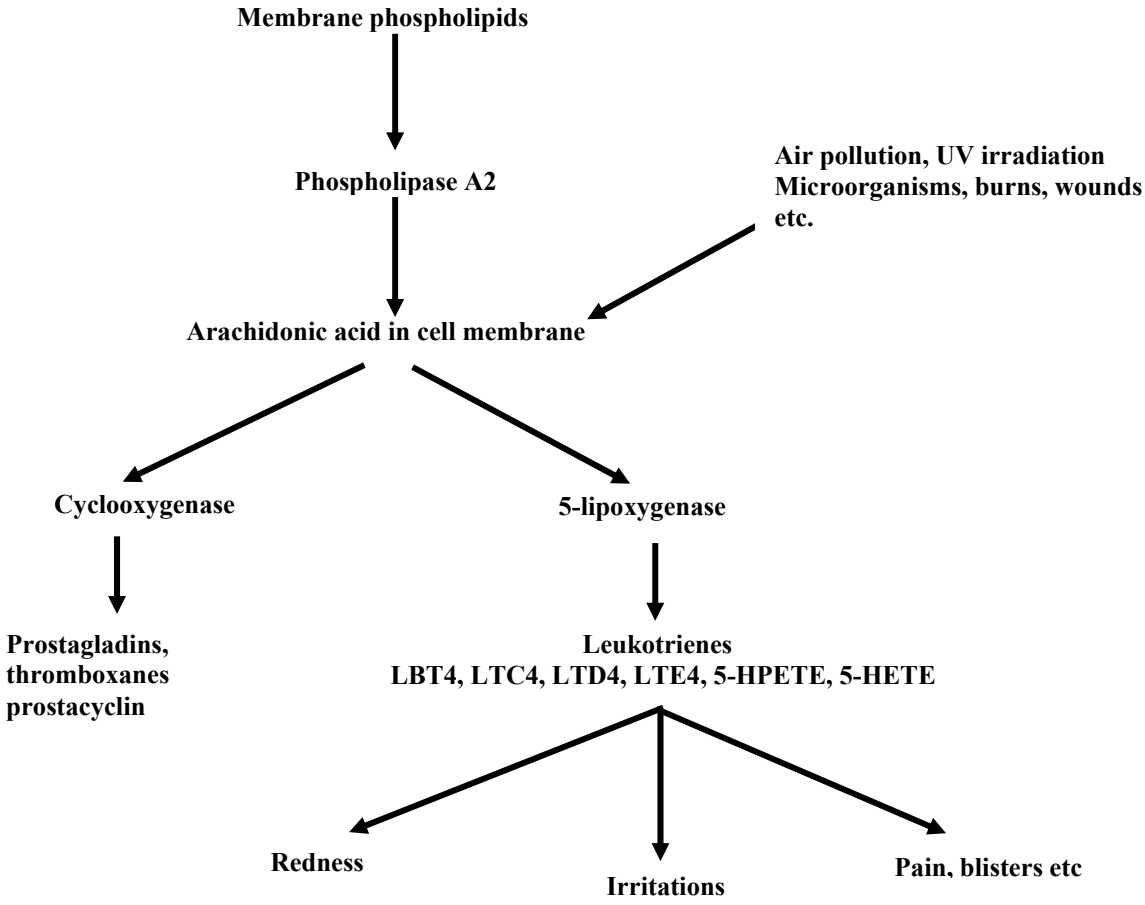


Figure. 4.1. Arachidonic acid cascade (source, Ammon, 1996) and the process of inflammation.

4.1.5. Alzheimer's disease and inhibitors of acetylcholinesterase

The usefulness of plants in treatment of diseases cannot be exhausted as every now and then, new discoveries are being made of new uses of herbal remedies. Apart from their usefulness in treatment of free radical mediated and inflammatory diseases, they are also useful in regulation and treatment of various mental conditions.

The use of complimentary medicines such as plant extracts in dementia therapy may vary from locality to locality and is dependant on the cultural traditions. In the African continent, use of plants for treatment of mind related complications is a long and well established tradition and has been in existence even before the modern drugs were discovered (Perry *et al.,* 1999). There are many mental conditions prevalent in any given human population; among such is Alzheimer's disease.

Alzheimer's disease is the most common cause of senile dementia in later life. Statistics show

that up to 4 million people are affected in the USA. However, in the western world, pharmacological properties of traditional cognitive- or memory-enhancing plants have not been widely investigated in relation to the Alzheimer's disease. An exception is *Gingko biloba* whose gingkolides have antioxidant, neuroprotective and cholinergic activities relevant to the disease management and treatment. The extracts have been shown to have therapeutic efficacy in the disease in placebo controlled clinical trials whose results are similar to those of the currently prescribed drugs such as tacrine or donepezil (Perry *et al.,* 1999).

Other species whose extract have been reported to have mind-improving properties and cholinergic activities include *Salvia officinalis* (sage) and *Melissa officinalis* (balm). With recent major advances in understanding of the neurobiology of the Alzheimer disease, it is important to find alternative sources of inhibitors of acetylcholinesterase from plants not yet explored for such possibilities (Marston *et al.,* 2002).

Inhibitors of acetylcholinesterase currently form the basis of the newest drugs available for the management of the disease. They function by correcting a deficiency of the neurotransmitter acetylcholine in the synapses of the cerebral cortex. Other properties include restricting oxidative stress and inflammatory reactions; prevention of β-amayloid formation or toxicity; elevation of oestrogen circulation and other levels of neutrophic agents such as nerve growth factors (Perry *et al*., 1999; Marston *et al.,* 2002).

Galanthamine, isolated from plants of the family Amaryllidaceae, such as snow drop (*Galanthus nivalis*) or from snow-flake (*Leucojum vernum*), is the latest addition to drugs used for the treatment of the disease (Marston *et al.,* 2002).

From the literature, some of the members of the genus *Eriocephalus* namely; *E. punctulatus* and *E. africanus* are reported to have mind-improving properties like relieving stress and depression and warming up of emotions but no written accounts exist on any tests for presence of acetylcholinesterase inhibitors. Hence, there is a great possibility that they may have acetylcholinesterase inhibitors and for this reason, a preliminary screening has been carried out in this study to verify this aspect. This test was not part of the original objectives but it emerged as a matter of interest into the mind-improving properties of the genus as recorded in literature.

As previously mentioned elsewhere, the biological properties for most of the species in the study genus are unknown and the few that that are known are discussed below.

4.2. Biological properties of the genus *Eriocephalus*

There is very little information with respect to the biological activities of most of the species of *Eriocephalus,* apart from the previously mentioned and commercially used species and the few that are recorded in literature as medicinally useful. Thus, the biological properties of the rest of the species in the genus remain relatively unknown and unexplored.

From the recorded accounts, some of the members of the genus were used by the people in the Cape region for treatment of various ailments (Chapter 1, Table 1.1) including respiratory ailments, gastro-intestinal disorders and a variety of dermal infections. The Griqua and the Nama used *E. ericoides, E. africanus, E. racemosus* and *E. punctulatus* as diaphoretics and diuretics. *Eriocephalus africanus* is used as a substitute for rosemary in flavouring of dishes such as fish, poultry and lamb (Dyson, 1998). Leaf infusions of *E. africanus*, decoctions and tinctures are used as diuretics and diaphoretics, as tincture for heart troubles, for oedema, dropsy, coughs, flatulence and delayed menstruation. The infusions were also used for swelling and pain arising from gynaecological conditions, for foot baths and also as a hair rinse to treat dandruff and itchy scalps and for reducing inflammation of the skin (Chapter 1, Table 1.1).

Honey and lemon are usually added to the tea made from *E. africanus* to make a refreshing drink for treatment of colds and coughs. An infusion of the leaves is used by the Rastafarians to cure chest complaints hence the name '*asmabossie'* (Dyson, 1998). A mixture of infusion of *E. africanus* and *Rosmarinus officinalis* is used for bathing to invigorate the skin and hair. The Khoi use the fluffy seed heads to stuff cushions and give them a long-lasting fragrance. *E. africanus* is also used as a substitute for ordinary rosemary in flavouring dishes such as fish, poultry and lamb. The Nama used this species as a colic remedy.

Eriocephalus tenuifolius was used by the Griqua as a substitute for 'buchu' and may be due to presence of compounds with diuretic effects. *E. karooicus* was also used locally as 'wild dagga' (Müller *et al.,* 2001) probably this species contains chemical compounds with psychotropic effects. Some members of the genus are used as fodder for livestock namely; *E. punctulatus, E. capitellatus, E. pedicellaris, E scariosus, E. karooicus* and *E. africanus* (Watt

and Breyer-Brandwijk, 1962; Van Wyk *et al.,* 1997; Dyson, 1998; Van Wyk and Gericke, 2000).

E. punctulatus is used by the southern Sotho with *Metalasia muricata* to fumigate the hut of a sick person or after death of a person (Http://www.africasgarden.com/library.htm). This could be indicative of presence of compounds in the plant with antiseptic properties.

E. punctulatus was produced and marketed in the early 1970's and was enthusiastically accepted by the perfumery trade. However, crafted material of the same failed to regenerate due to severe defoliation and the project was abandoned. The Grassroots Natural Products Company has been able to successively clone material having unique chemical and organoleptic properties. This resulted in establishment of commercial plantations and development of sustainable harvesting systems (Http://www.gnp.co.za/essential oils/ chamomile).

Industrially, the oils from *E. punctulatus* and *E. africanus* "Cape chamomile" and "Cape snowbush" respectively have had a wide application in skin care preparations and as blend oils in certain beauty care products (e.g. bergamot, ylang-ylang, geranium and ginger among many others). The blue azulene compound (chamazulene) that is produced from matricin during steam distillation, via the unstable chamazulene carboxylic acid is reported to be present in the oils of *E. punctulatus.* The commercial oil 'Cape chamomile' oil has a fine fruity fragrance similar to that of the Roman chamomile. The oil has a low taste threshold value and is said to have a great potential for use as a flavour enhancer. It is widely used for making high-class perfumes and in cosmetics it displays excellent properties due to the antiinflammatory and soothing properties. It is widely used in cleansers, toners and moisturizing facial products e.g. 'Sea-sational products'. Its aromatherapeutic properties include being an analgesic, anti-allergic, anti-depressant (due to presence of linalyl acetate), antiseptic, and antiinflammatory and as a diuretic among its numerous applications (Http:/www.gnp.co.za/essential oils/ chamomile).

The 'Cape snowbush' yellow oil is also widely used. It is believed to have similar properties as rosemary (*Rosmarinus officinalis*), hence, the reference 'wild rosemary'. The earliest account of an essential oil extract from a South African plant was made by Schveyver in (circ.1677) when he distilled from a company garden, oil of what is believed to have been *E.*

africanus of which he referred to as 'roosmaryn'. The species is commercially cultivated and is also reported to be suited in flavourings as it has bitter-spiced taste that also has a potential for the making of fragrances. It has similar properties as the oil of *E. punctulatus* except it is used more for treatment of gastro-intestinal disorders, gynecological complications, and respiratory related ailments. It is also used for treating stress related ailments, depression and as blend oil in skin care products.

Some members of the genus are used as fodder for livestock namely; *E. punctulatus, E. capitellatus, E. pedicellaris, E scariosus, E. karooicus* and *E. africanus.* It is interesting to note that some of the species that are browsed moderately to heavily may have compounds that are not pungent while the ungrazed species may have toxic compounds in high proportions. All these factors are crucial in elucidating the biological properties of the members of the genus.

4.3. Importance of the study

It is clear that medicinal plants form an integral part in the provision of primary health care. In the advent of increasing resistance of some of the pathogens to conventional drugs, due to overuse or misuse, there is an urgent need to search for alternative remedies to alleviate human suffering. The issue of foods, their preservation and safety in uses of preservatives, necessitates a search for natural antioxidants that will not only be used to preserve food but also to be used in treatment of some of diseases and their management. Such would be remedies for cure of life threatening diseases such as cancer and HIV/AIDS. The antiinflammatory properties of plants have a lot to offer in treatment of various dermal infections as well as their use in cosmetic and fragrance industries.

Eriocephalus is an economically important genus as noted in the various medicinal and industrial uses. As previously mentioned, the paucity of literature on the biological properties of the species in the genus necessitates further investigations for documentation of its potency. Therefore, this study provides an opportunity to screen and document the potential uses of these species with undocumented uses and provide a scientific basis for the uses of the members of the genus in traditional herbal remedies for future research. In view of all these needs and the importance of phytochemical research, the present research on the members of the genus *Eriocephalus* is a contribution to the search of novel and potential antimicrobial, antioxidant, antiinflammatory and acetylcholinesterase inhibiting properties of the genus,

through bioassay *in vitro* screening. It will also be the first comprehensive recording of the biological properties of the genus and provision of a scientific basis for use of some of the species in traditional herbal therapies.

4.3.1. Aim and objectives of the study

It is important to provide a scientific basis for the use of some members of the this genus in herbal remedies and based on the traditional uses, commercial importance and the need to screen more species for new antimicrobials, the following study was undertaken aimed at:

1. At investigating and recording, the biological activity (antiinflammatory, antimicrobial, antioxidant and acetylcholinesterase inhibition) properties of the species of *Eriocephalus* through bioassay *in vitro* screening.

2. And to establish the rationale for their use in traditional herbal remedies by preliminary *in vitro* screening.

4.4. Materials and methods

The details for the materials and voucher numbers are given in Chapter 2, under materials and methods pages 25-28.

4.4.1. Antimicrobial activity

4.4.1.1. Test organisms

Preliminary screening was carried out using 19 test pathogens, whose reference numbers are given in (Table 4.1). Based on susceptibility patterns, seven of these were selected for further study (Table 4.1). Only a few of the taxa of the genus were used in the preliminary screening due to the limitation of samples, especially the essential oils, which were low in quantity. Activity was measured as growth inhibition in millimetres from the edge of the disc (Table 4.1 and 4.2).

4.4.1.2. Disc diffusion assay

The disc diffusion assay was used to determine the growth inhibition of the bacteria and selected fungi. Tryptone Soya agar (Oxoid) was prepared by dissolving 30 g of the agar in 750 ml of water and autoclaved for 15 min at 121 ^{o}C and cooled to 55 ^{o}C in a water bath. A base layer of 100 ml of agar was poured into the plate and inoculated with a top layer of 100 ml of agar containing an inoculum of approximately 1 x 10^{6} CFU/ml. Sterilized paper discs (6 mm) were saturated with either 8 µl of essential oils or 25 µl the acetone leaf extracts (50 mg/ml) and loaded onto the agar plates. The plates were refrigerated for one hour to pre-diffuse the oil and extracts into the seeded agar layer, and then incubated at 37 ^{o}C for 24 hours for bacterial isolates. The yeasts were incubated for 48 hours. Neomycin (30 µg/disc, Oxoid) and Nystatin (100 IU/ disc, Oxoid) were used as positive controls for the anti-bacterial and anti-fungal activities, respectively. Activity was measured as growth inhibition zones in millimeters from the edge of the disc (Table 4.1 and 4.2).

Based on the disc diffusion results, and the available quantities (oils) of test samples, two Gram-positive bacteria *Bacillus cereus*, *Staphylococcus aureus*, two Gram-negative bacteria *Klebsiella pneumoniae, Escherichia coli* and the yeasts, *Cryptococcus neoformans* and *Candida albicans* were selected for minimum inhibitory concentration (MIC) assay.

4.4.1.3. Determination of minimum inhibitory concentration (MIC)

The micro-titre plate dilution method (Eloff, 1998, Figure 4.2) was used for testing inhibitory activities of the oils and extracts that showed some activity in the disc diffusion assay. Test cultures were inoculated in Tryptone Soya (Oxoid) broth and incubated for 24 hours. Stock solutions for the leaf extracts were obtained by resuspending in acetone (50 mg/ml) and the inoculum by transferring one milliliter of stock into 100 ml of sterile broth. Then 51.2 mg of oil was weighed and made up in 400 µl of acetone (128 mg/ml). The starting concentration in the first wells was 32 mg/ml for the oils and 12.5 mg/ml for the extracts. Afterwards, 100 µl of sterile water were pipetted into the wells followed by 100 µl of the leaf and essential oil extracts, the mixture was then eight-fold serially diluted. Finally, 100 µl of actively growing culture of test pathogen was added into all the wells. The plates were incubated at 37 ^{o}C for 24 hours for the bacterial strains and 48 hours for the fungal strains. The negative control involved the presence of the culture material and the positive controls were ciprofloxacin (0.01 mg/ml stock solution, Merck) for bacterial strains and amphotericin B (0.01 mg/ml stock solution, Merck) for yeasts.

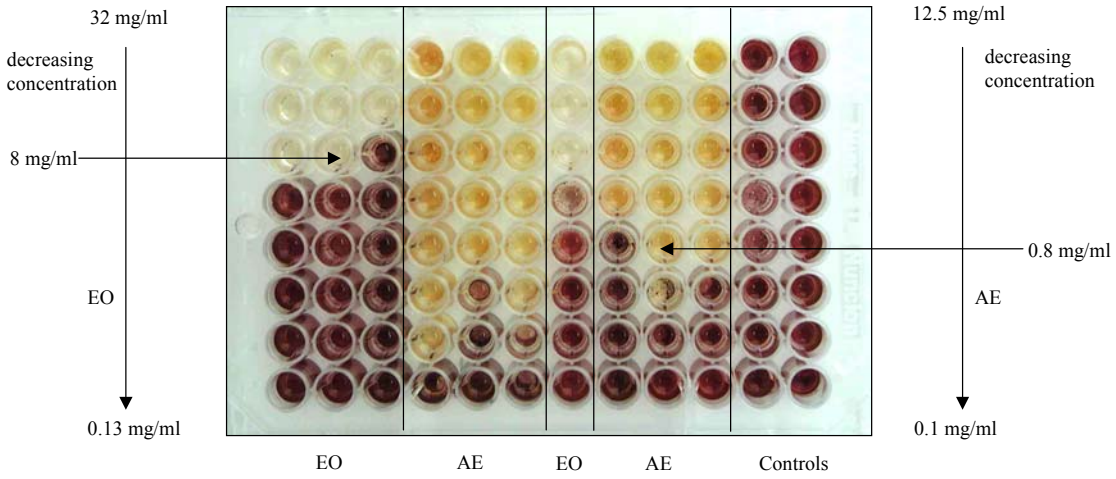


Figure 4.2. A representative microtitre plate showing the MIC values (mg/ml) of essential oils (EO) and acetone leaf extracts (AE) against *Staphylococcus aureus.*

Culture growth was visualized by adding 40 µl of 0.2 mg/ml *p*-iodo-nitrotetrazolium violet (INT) solution (Sigma) into all the inoculated wells. The plates were examined after six hours for bacteria and 24 hours for yeasts. Minimum inhibitory concentration was determined as the lowest concentration (the first clear well) inhibiting the growth of the test pathogen. The tests were repeated twice.

4.4.2. Determination of antioxidant activity

4.4.2.1. Preliminary DPPH radical scavenging activity

Several methods to determine free scavenging have been reported but the reaction with 2, 2, diphenyl-1-picrylhydrazyl (DPPH) is the most studied. This is a radical generating substance, which is widely used to monitor the free radical scavenging abilities (the ability of a compound to donate an electron) of various antioxidants. The DPPH radical has a deep violet colour due to unpaired electron and radical scavenging can be followed spectrophotometrically by the loss of absorbance at 517-525 nm, as the pale yellow non-radical form is produced. Screening for antioxidant properties in plant phenolic or essential oil extracts involves testing for radical capturing properties with DPPH test. TLC plates are spotted with the phenolic extracts and developed in a suitable solvent system comprising of chloroform, ethyl acetate, benzene and formic acid or any accepted solvent system. A known standard is used. The plates are then sprayed with the DPPH radical. Antioxidants reduce the purple or violet DPPH radical producing white spots (DPPH-H) on a purple background and the spots develop better 2 minutes after spraying and continue to improve their intensity with time. The reaction is a homolytic substitution of a hydrogen atom in a *para*-position of one of the phenyl rings thus nitrogen oxide affords 2-(4-nitrophenyl)-2-phenyl-1-picrylhydrazine and/or the corresponding free radical. Alternatively, bleaching of crocin (which normally gives a yellow colour on the plate) can be used to distinguish components of plant extracts with potential, antioxidant radical scavenging properties.

The preliminary screening for antioxidant properties of acetone plant extracts involved testing for radical capturing properties with 2,2 diphenyl-1-picryhydrazyl (DPPH) using TLC bioautographic assay. A concentration of (50 mg/ml) per sample was tested. TLC plates were spotted with 10 µl of the extract and developed in a solvent system comprising of chloroform (2.4): ethyl acetate (2): benzene (1): formic acid (0.6) v/v. Rosmarinic acid was used as the standard. The plates were dried and sprayed with 0.4% DPPH (Fluka) solution in methanol and allowed to develop for 10 minutes. Antioxidant compounds reduced the purple or violet DPPH radical producing white spots (DPPH-H) on a purple background.

4.4.2.2. Quantitative evaluation of antioxidant activity

The radical scavenging activity of the acetone leaf extracts was determined spectrophotometrically. The DPPH stock solution was made by dissolving 0.01896 g of

DPPH in 0.5 L HPLC grade methanol and made to a concentration of 96.2 µM, and covered with foil to avoid photo-oxidation.

Extracts (5 mg) were dissolved in 500 µl of dimethyl sulfoxide (DMSO, Saarchem) to give an initial stock of 10,000 ppm. The mixture was vortexed to dissolve the extract. 50 µl of the stock was diluted (1:1 dilution) with 950 µl of DMSO. Then 50 µl of this stock solution was pipetted into a 96 well micro-titre plate in triplicate. Then 50 µl of DMSO was added to rows A and H as controls followed by addition of 200 µl of HPLC grade methanol to even columns (2, 4, 6, 8, 10 and 12) and 200 µl of DPPH was added to odd columns (1, 3, 5, 7, 9 and 11). The plates were shaken on an automated micro-titre plate reader (Labsystems Multiskan RC) for 2 minutes and then kept in the dark at room temperature for 30 minutes. The changes in colour from deep violet to light yellow were measured at 550 nm on an UV/visible light spectrophotometer (Labsystems Multiskan RC) linked to the computer equipped with GENESIS® software. The radical scavenging activity was measured as the decolourization percentage of the test sample. All determinations were made in triplicate. Ascorbic acid was used as the positive control. The IC_{50} which is the concentration at which there is 50% decolourization of the DPPH by the test sample was determined using the Enzfitter® 1.05 version software where the decolourization % was determined using the following formula:

$$\% \text{ Decolourisation} = \frac{(\text{Av controls} - (\text{Av sample}_{DPPH} - \text{Av sample}_{MEOH})) \times 100}{\text{Controls}}$$

Where Av controls = average absorbance of all DPPH control wells-average absorbance of all methanol control wells; Av sample_{DPPH} average absorbance of sample wells with DPPH and Av sample_{MEOH} = average absorbance of sample wells with methanol.

4.4.3 Determination of antiinflammatory activity

In this study, the antiinflammatory activity of essential oils of *Eriocephalus* was determined using a modified method of Baylac and Racine (2003) with linoleic acid as the substrate for the 5-lipoxygenase enzyme (Cayman). In normal biological systems, 5-lipoxygenase enzyme catalyses the oxidation of unsaturated fatty acids containing 1-4 pentadiene structures with arachidonic acid as the biological substrate converting them into conjugated dienes which result in continuous increase in absorbance at 243 nm.

Standardization was first carried out using the reference sample made up of 10 µl of DMSO, 2.95 ml of phosphate buffer (pH 6.3), prewarmed in a water bath at 25 ^{o}C. 50 µl of linoleate solution (100 µM final concentration) was added and 12µl enzyme and 12 µl of phosphate buffer. The production of the conjugated dienes was measured over a period of 10 minutes at 234 nm. Two sets of controls were run with the reference samples.

For the essential oils of *Eriocephalus*, 0.022 g of oil was weighed and made up to 1.1 g with DMSO with Tween 20. The final concentration was calculated (100 µg/ml). In a 3 ml cuvette maintained at 25 ^{o}C in a thermostat bath was added 10 µl of extract, 2.95 ml of phosphate buffer (pH) 6.3, followed by 48 µl of linoleate acid (> 99%, Fluka). The mixture was shaken and 12 µl of the aliquoted enzyme (stored at –80 ^{o}C) and 12 µl of the phosphate buffer stored at 4 ^{o}C were pipetted to initiate enzymatic reaction. Absorbance was measured at 234 nm over a period of 10 minutes using a single beam spectrophotometer (Specord 40-analytikjena) connected to a computer with Winspect® software. Linoleic acid was enzymatically converted to conjugated dienes resulting in an increase in absorbance at 234 nm. Absorbance was plotted graphically against the different concentrations used. The slopes of the straight-line portions of the sample and the control curves were used to determine the percentage activity of the enzyme (Lourens *et al.,* 2004). The IC_{50} (the concentration that gives 50% enzyme inhibition) was determined using the Enzfitter® 1.05 software. Nordihydroguaiaretic acid (NDGA) was used as the positive control.

4.4.4. Preliminary screening for detection of acetylcholinesterase

Several methods are used for screening of plants with inhibitors of acetylcholinesterase enzyme. Among such methods is thin layer chromatography (TLC). The TLC assay is one of the quick methods of screening plant extracts as it gives quick access to information concerning the activity and localization of the activity in complex plant matrices. Presence of compounds in question can be easily detected on the TLC plate. The test relies on the cleavage by acetylcholinesterase of 1-naphthly acetate to form 1-naphthol, which in turn reacts with fast blue to give a purple coloured diazonium dye. Regions of the TLC plate, which contain acetylcholinesterase inhibitors, show up as white spots against a purple background.

Preliminary screening for presence of inhibitors of acetylcholinesterase was carried out on 17

species of *Eriocephalus* using thin layer chromatography (TLC) bioautography. Essential oils were diluted 1:7 with hexane. The acetone leaf extracts were resuspended in acetone to a concentration of 50 mg/ml.

4.4.4.1. TLC bioautography

Acetylcholinesterase (1000 U) was dissolved in 150 ml 0.05 M Tris-hydrochloric acid buffer at pH 7.8; bovine serum albumen (150 mg) was added to the solution in order to stabilize the enzyme during the bioassay. The stock solution was kept at 4 ^{o}C.

TLC plates were eluted with appropriate solvent (acetone or isopropanol) in order to wash them, and were thoroughly dried just before use. 3 µl of essential oils and 10 µl acetone leaf extracts were loaded on separate plates and eluted with a solvent system comprising toluene (93): ethyl acetate (7) and toluene (90): dioxane (25): acetic acid (5) respectively. After migration of the sample in the above solvent systems, (or direct deposition of sample), the TLC plate was dried with a hair drier for complete removal of the solvent.

For incubation of the enzyme, the plate was laid flat on plastic plugs in a plastic tank containing a little water to avoid the plate coming into contact with the water but the atmosphere was kept humid. The tank was covered and incubation performed at 37 ^{o}C for 20 minutes (Perry *et al*., 1999; Marston *et al.,* 2002).

The enzyme had satisfactory stability under these conditions. For detection of the enzyme, solutions of 1-naphthyl acetate (250 mg) in ethanol (100 ml) and of fast blue B salt (400 mg) in distilled water (160 ml) were prepared immediately before use (to prevent decomposition). After incubation of the TLC plate, 10 ml of naphthyl acetate solution and 40 ml of fast blue B salt solution were mixed and sprayed onto the plate to give a purple coloration after 1-2 minutes.

4.5. Results and discussion

The format for this section is different from the rest of the thesis as part of it was prepared for publication and hence the results and discussion sections were combined.

4.5.1. Antimicrobial activity

The results from the broad *in vitro* screening of the essential oils and the acetone leaf extracts

of *Eriocephalus* species indicate that the species were strongly active only against a few of the 19 test pathogens (Table 4.1). The results for *Staphylococcus aureus* (ATCC 612600) were not included in the Table 4.1 as only a few extracts were tested. The test pathogens, *Staphylococcus aureus* (ATCC 6538, ATCC 612600, methicillin resistant *Staphylococcus aureus* (clinical strain), *Staphylococcus epidermidis* (ATCC 2223), *Pseudomonas aeruginosa* (ATCC 9027), *Enterobacter*, *Yersinia enterocolitica* (ATCC 23715), *Salmonella typhimurium* and *Salmonella enteriditis* (clinical strains), *Proteus vulgaris* (clinical strain), *Serratia odorifera* (ATCC 33132), *Enterococcus faecalis* (ATCC 29212) and *Alternaria alternata* (clinical strain) did not show promising results and were not studied further. Of the four strains of *Staphylococcus aureus,* only the most sensitive was selected for further study.

The most susceptible pathogens (of which were seven) observed from the broad preliminary screening (Table 4.1) for antimicrobial activity of *Eriocephalus* species were selected for further study as shown in Table 4.2. The *in vitro* antimicrobial activities of the essential oils and the extracts against these selected test pathogens were assessed qualitatively and quantitatively by sizes of inhibition zones and MIC values respectively.

According to the results in Table 4.2, the essential oils of *E. purpureus* (Nieuwoudtville), *E. ericoides* subsp *ericoides*, *E. pauperrimus, E. microphyllus* (Sutherland), *E. africanus* (Malmesbury), *E. punctulatus, E. racemosus* var *racemosus* and all the taxa from Namibia exhibited at least 50% activity against the total number of the pathogens tested even though the activity was relatively low.

The leaf extracts however, showed lower activity ranging from 20-40% against the total number of the test pathogens with the exception of *E. aromaticus, E. microphyllus* (Nieuwoudtville), *E. punctulatus* (Nieuwoudtville population 1 and 2), *E. dinteri, E. pinnatus, E. purpureus* (Kamiesberg), *E. scariosus* and *E. africanus* (Melkbosstrand), which showed at least 50% activity. However, the taxa from Namibia demonstrated increased activity compared to their South African counterparts.

Table 4.2 presents a summary of the results of essential oil and leaf extracts that exhibited antimicrobial activities against the seven selected test pathogens. This confirms that some species of the genus have antimicrobial properties and supports their use in traditional herbal remedies. The species show variation in activity within individuals of the same species and

between different populations of the same species in addition to between the species. Variation in activity between individuals of the same population was observed in several of the taxa studied. This was observed in the essential oils of *E. punctulatus* from Nieuwoudtville (population 1) and *E. brevifolius* from Vergelegen against *Cryptococcus neoformans* where three individuals (A, B and C) had inhibition of 2, 9 and 3 mm and <1, 3 and 5.5 mm respectively.

This pattern was observed among taxa where three individuals were tested for activity against the test pathogens (Table 4.2) and the same phenomenon was observed in the leaf extracts as observed in taxa of *E. africanus* var *paniculatus* against *Cryptococcus neoformans*. This variation in antimicrobial activity of essential oils and leaf extracts in regard to chemical composition is addressed in Chapter 5.

Variation in activity against the test pathogens was also evident amongst the essential oils and the extracts of populations of the same species as observed in the populations of *E. africanus, E. punctulatus, E. aromaticus, E. microphyllus, E. brevifolius, E. ericoides* subsp. *ericoides* and *E. racemosus* var *racemosus.* This intra-specific variation was also observed within the remaining species of *Eriocephalus* (Table 4.2).

Essential oils were active against most of the test pathogens and the highest activity noted against the Gram-positive bacteria *Bacillus cereus* was 8 mm noted in *E. microphyllus* from Sutherland and moderate activity against *Bacillus subtilis* and *Staphylococcus aureus* was noted in the remainder of the oils.

Low inhibition (1-2.5 mm) against the Gram-negative bacteria *Klebsiella pneumoniae* and *Escherichia coli* was noted for all the essential oils. This may be due to the fact that the Gram-negative bacteria are more resistant due to their lipophilic and polysaccharide membrane structure (Martin, 1995; Rabe and Van Staden 1998; Mangena and Muyima, 1999).

The essential oils showed relatively good activity against the yeast *Cryptococcus neoformans* with *E. namaquensis* having the highest activity (10 mm). The activity of the oils against

Table 4.1. Preliminary antimicrobial screening of essential oils and acetone leaf extracts of the species of *Eriocephalus*. Activity is measured in millimetre (mm) as growth inhibition from the edge of the disc.

Taxon	Locality	Extract	Activity in millimeter (mm) from the edge of the disc																
			Cn	Bc	Ef	Bs	Ca	Sa-1	Sa-2	Se	Kp	Ec	YePa	St	Snt	Pv	So	MR SA	An
E. africanus	Melkbosstrand	EO	1	3	R	1	1	1	2	R	R	R	R	<1	R	<1	R	R	1	R
E. africanus	Citrusdal (A)	EO	2	2	<1	<1	1	<1	1	R	1	R	<1	<1	<1	<1	<1	<1	1	<1
E. africanus	Citrusdal (B)	EO	1	5.5	R	2	R	1	1	R	<1	R	R	<1	R	R	R	R	1	R
E. africanus	Mossel Bay	EO	1.5	R	1	1	<1	<1	<1	R	<1	<1	<1	<1	1	1	R	2	1.5	R
E. aromaticus	Ladismith (B)	EO	R	3	R	1	R	<1	2	<1	R	R	R	R	R	<1	R	R	1	1
E. brevifolius	De Rust (A)	EO	<1	1.5	<1	1	<1	<1	4	<1	<1	R	1.5	<1	R	<1	R	R	1	1
E. brevifolius	De Rust (B)	EO	3	3	<1	<1	<1	<1	1	R	R	R	<1	<1	R	R	R	R	<1	1
E. brevifolius	De Rust (C)	EO	5.5	4	R	1	1	<1	2	R	<1	R	1	<1	R	R	R	R	<1	R
E. capitellatus	Swartberg Pass (A)	EO	4	3.5	R	<1	R	1	R	R	<1	R	R	<1	R	<1	ND	1	<1	<1
E. capitellatus	Swartberg Pass (B)	EO	2	3.5	1	1	1	<1	1	<1	1	<1	<1	<1	ND	1	1	1	1	R
E. capitellatus	Swartberg Pass (C)	EO	3	2.5	2	2	2	R	2	R	1	<1	<1	<1	2	1	2	1	<1	R
E. spinescens	Sutherland/Ceres (B)	EO	2	2	R	<1	R	<1	1	R	R	R	R	<1	R	R	R	R	1	5
E. ericoides subsp. *ericoides*	Scheerpersrust (A)	EO	3	2.5	<1	2	<1	<1	1.5	<1	<1	R	<1	<1	<1	<1	R	R	1	4
E. ericoides subsp. *ericoides*	Scheerpersrust (B)	EO	2	3	3	1	1	1	3	R	R	1	2	<1	2	1	1.5	2	1	R
E. ericoides subsp. *ericoides*	Scheerpersrust (C)	EO	3	2	R	1	1	1	1.5	R	<1	R	R	<1	<1	<1	<1	1.5	<1	R
E. ericoides subsp. *ericoides*	Prince Albert (A)	EO	4.5	4	R	<1	R	<1	1	R	R	R	R	<1	R	R	R	R	1	<1
E. eximius	Sutherland/Ceres (A)	EO	R	3	R	<1	R	<1	<1	R	<1	R	R	<1	R	R	R	R	<1	R
E. punctulatus	Nieuwoudtville (A)	EO	2	3	1	1	<1	1	2.5	R	1	1.5	<1	<1	<1	<1	R	1	1	R
E. racemosus var *racemosus*	Velddrif (A)	EO	2	4	<1	1	1	1	1	<1	<1	R	<1	<1	<1	<1	R	R	<1	R
E. racemosus var *racemosus*	Velddrif (B)	EO	2	4	R	1	R	<1	1	R	<1	R	R	<1	R	R	R	R	<1	R
E. racemosus var *racemosus*	Velddrif (C)	EO	5	3.5	<1	1	1	1	2	R	2	R	R	<1	R	R	R	R	<1	R
E. africanus	Citrusdal (A)	AE	2	1	R	<1	R	R	R	R	R	R	R	<1	R	R	R	R	2	R
E. brevifolius	Oudtshoorn	AE	3	R	R	4	R	<1	R	R	R	R	R	<1	R	R	R	R	R	R
E. capitellatus	Swartberg Pass	AE	<1	R	R	<1	R	<1	<1	1	R	R	R	<1	R	R	<1	<1	1	R
E. decussatus	Sutherland/Fraserburg (C)	AE	<1	1	R	<1	R	R	R	R	R	<1	R	<1	1	<1	R	R	<1	R
E. ericoides subsp. *ericoides*	Prince Albert	AE	3	1	R	<1	R	<1	R	R	R	R	R	<1	R	R	R	R	<1	R
E. eximius	Sutherland/Ceres (A)	AE	R	1	R	<1	R	R	<1	R	R	<1	R	R	1	<1	R	<1	2	R
E. grandiflorus	Laingsburg/Matjiesfontein (A)	AE	R	1	R	2	R	R	R	R	R	R	R	<1	1	R	R	R	R	R
E. punctulatus	Nieuwoudtville/Calvinia (A)	AE	3	1	R	2	R	<1	<1	R	R	R	R	<1	R	R	R	R	<1	R
E. purpureus	Laingsburg (A)	AE	1	1	<1	R	R	R	R	<1	<1	R	R	<1	1	R	R	<1	R	R
E. microphyllus	Sutherland/Fraserburg (A)	AE	R	1	<1	1	R	R	R	R	<1	<1	R	<1	1	R	R	<1	R	R
E. racemosus	Koeberg	AE	2	1	R	1	R	1	<1	<1	R	R	R	<1	R	R	R	R	<1	R
E. spinescens	Sutherland/Ceres (A)	AE	R	<1	R	1	R	R	R	R	<1	R	R	R	1	1	<1	R	R	R
		Control	12	6	3	5	7	6	5	6	2	2	3	1.5	2	2	2.5	2	5	5

Cn-*Cryptococcus neoformans* ATCC 90112
Ye-*Yersinia enterocolitica* ATCC 23715
Ef-*Enterococcus faecalis* ATCC 29212
Sa-1-*Staphylococcus aureus* ATCC 25923
Sa-2- *Staphylococcus aureus* ATCC 6538
Kp-*Klebsiella pneumoniae* NCTC 9633
Ye-*Yersinia enterocolitica* ATCC 23715
Se-*Staphylococcus epidermidis* ATCC 2223
So- *Serratia odorifera* ATCC 33132

Ca-*Candida albicans* ATCC 10231
Bs-*Bacillus subtilis* ATCC 6051
Snt-*Salmonella enteriditis*
Pv-*Proteus vulgaris*
AE-Acetone extract
Pa-*Pseudomonas aeroginosa* ATCC 9027
MRSA-methicilin resistant *Staphylococcus aureus*
ND-not determined due to insufficient sample
R-resistant

Bc-*Bacillus cereus* ATCC 11778
St-*Salmonella typhimurium*
Ec-*Escherichia coli* ATCC 8739
EO-Essential oil

Candida albicans was moderate (5 mm) as noted in *E. punctulatus* (Nieuwoudtville population 1) and *E. ericoides* subsp *ericoides* (Bethulie). This is in agreement with the activity of essential oils against yeasts where they are reported to be more active than against the bacteria as noted in Bağci and Diğrath (1996).

Among the species of *Eriocephalus* studied, some individuals of *E. punctulatus, E. ericoides* subsp *ericoides, E. brevifolius, E. purpureu*s and *E. microphyllus* showed varying degrees of activity against all the test pathogens.

The leaf extracts were not as active as the essential oils against the Gram-positive *Bacillus subtilis* except *E. aromaticus* with an activity of 2-6 mm zone of inhibition. The same species was active against *Bacillus cereus* (4-8 mm) and *Staphylococcus aureus* (4-6 mm). The extracts showed very low (1 mm or less inhibition) or no activity against the Gram-negative bacteria *Klebsiella pneumoniae* and *Escherichia coli.* However, most of the extracts were active against the yeast *Cryptococcus neoformans* with the highest activity of 7 mm noted for *E. purpureus* (Nieuwoudtville), 6 mm for *E. racemosus* var *racemosus* (from Velddrif) and *E. punctulatus* from (Nieuwoudtville population 1). The extracts were mostly inactive against *Candida albicans* except for some species namely, *E. microphyllus* and *E. purpureus* that showed some inhibition (1-2 mm). The extracts of *E. aromaticus* were however, active against at least four out of the seven test pathogens and the same group recorded the highest activity among the extracts of *Eriocephalus* species. Promising results were also observed in leaf extracts of the taxa of *E. punctulatus*, *E. africanus*, *E. racemosus* var *racemosus*, *E. spinescens*, *E. purpureus*, *E. microphyllus* and *E. pauperrimus* but on the overall, the essential oils were comparatively more active than the leaf extracts (Table 4.2). This implies that biological activity of members of the genus that are used in herbal remedies is mainly influenced by the presence of essential oils.

In other studies, Salie *et al.,* (1996) reported the petroleum ether stem and methanol root extracts of *E. africanus* to be slightly active against *Staphylococcus aureus.* In this study a similar pattern was observed as the acetone leaf extracts of *E. africanus* had little or no activity against *Staphylococcus aureus*. The essential oils of the same species showed very low antimicrobial activity against *Staphylococcus aureus*. The essential oils of *E. africanus* were observed to be active against *Candida albicans* but the acetone leaf extracts were not

active against the aforementioned. However, Salie *et al.,* (1996) reported the lipophilic extracts of the same species to be active against *Candida albicans.*

Following the results from the disc diffusion assay, the minimum inhibitory concentration (MIC) was determined for six selected test pathogens (Table 4.3). The 18 species of *Eriocephalus* (Table 4.3) that showed promising activity in the disc diffusion screening assay (Table 4.2, species in bold text) and those with sufficient oil quantities were selected for the MIC assay. The antimicrobial effect for essential oils ranged between 2–32 mg/ml for the Gram-positive bacteria; 4->32 mg/ml for the Gram-negative bacteria and 1->32 mg/ml for the fungal strains for the essential oil (Table 4.3).

The MIC for the leaf extracts for the Gram-positive bacteria was 0.2-3.1 mg/ ml, 3.1 mg/ml for Gram-negative bacteria and 0.5-6.3 mg/ml for the fungal strains.

It is well documented that testing and evaluation of antimicrobial activity of essential oils is difficult because of their volatility, their water insolubility, and their complexity. The results are greatly influenced by the choice of assay technique; growth medium, the test pathogen and the oil extract (Janssen *et al.,* 1987). Studies to establish if there is any correlation between the inhibition diameters and MIC values for essential oils have been carried out and it is evident that qualitative screening methods and quantitative minimum inhibitory concentration methods are not necessarily comparable as indicated in Janssen *et al.,* (1987). The nature of diffusion of the leaf extracts and the essential oil in water or culture medium differs considerably. Hence, the results obtained may vary qualitatively and quantitatively. In this study, the same phenomenon was observed with the results obtained for the MIC test not confirming or tarrying with those obtained for inhibition diameters in the disc diffusion assay (Brantner and Grein, 1994; Lourens *et al.,* 2004).

In herbal remedies, species of *Eriocephalus* are mainly used for treatment of respiratory related ailments, skin inflammation, stomach disorders and as diuretics and diaphoretics. From the broad screening of taxa in the genus it was observed that most of essential oils were active against the respiratory pathogen *Cryptococcus neoformans. Eriocephalus racemosus* var *racemosus* and *E. ericoides* subsp *ericoides* had an MIC of 2 mg/ml and 1 mg/ml respectively compared to the rest of the species tested (Table 4.3). The leaf extracts of *E. scariosus*, *E. punctulatus*, *E. aromaticus* and *E. ericoides* subsp. *ericoides* had activity of 0.5

mg/ml, 0.8 mg/ml, 1.6 mg/ml, and 1.6 mg/ml respectively against *Cryptococcus neoformans.* The MIC of *E. ericoides* subsp *ericoides* and *E. dinteri*/*E. microphyllus* was 8-16 mg/ml for the former and 8 mg/ml respectively for the latter for the essential oils against *Klebsiella pneumoniae*. Therefore, the activity of the essential oils and the extracts against the aforementioned pathogens supports the use of *Eriocephalus* species for treatment of respiratory related ailments.

Most of the species studied here demonstrated essential oil and the leaf extracts activity against *Bacillus cereus* and *Staphylococcus aureus,* both of which may be associated with dermal infections. The essential oils of *E. dinteri, E. klinghardtensis, E. pinnatus, E, scariosus, E. punctulatus*, *E. ericoides* subsp *ericoides*, *E. africanus* and *E. brevifolius*, had an MIC of between 4 mg/ml to 16 mg/ml for effective inhibition of the test pathogen. The leaf extracts of *E. pinnatus, E. dinteri, E. merxmuelleri*, *E. aromaticus, E. punctulatus, E. microphyllus* and *E. purpureus* had an MIC range of 0.2 mg/ml to 0.8 mg/ml (Table 4.3). These results support the use of some of the species in treating dermal related infections.

For gastro-intestinal disorders or infections, the essential oils and extracts of *E. africanus* and *E. punctulatus* showed activity of 4-16 mg/ml and 1.6 mg/ml respectively. This supports the use of these species in treatment of the aforementioned ailments in traditional remedies. The essential oils of *E. dinteri, E. klinghardtensis, E. punctulatus, E. microphyllus*, *E. racemosus*, *E. brevifolius* and *E. ericoides* subsp. *ericoides* indicated potential, as these species showed some activity against *Escherichia coli* and *Candida albicans* (Table 4.2 and 4.3). The leaf extract of *E. decussatus, E. microphyllus* and *E. purpureus* showed some activity against the previously mentioned pathogens.

From the results obtained from this study, the essential oils of *E. dinteri, E. klinghardtensis, E. luederitzianus, E. merxmuelleri, E. pinnatus, E. scariosus, E. punctulatus, E. ericoides* subsp. *ericoides, E. purpureus, E. microphyllus, E. decussatus* and *E. brevifolius* were active against nearly all the test pathogens and can be used to treat respiratory related ailments, dermal infections and gastro-intestinal disorders in traditional herbal remedies. The other notably biologically active species include; *E. pauperrimus, E. microphyllus, E. racemosus* and *E. capitellatus* and these are therefore potentially useful as a source of herbal remedies. *E punctulatus, E. africanus* and *E. racemosus* are traditionally used for treatment of respiratory,

skin and stomach problems and the results from the disc diffusion assay and the MIC values obtained in this study confirm their efficacy in traditional uses.

This study, albeit *in vitro,* confirms that *Eriocephalus* species have broad and varied antimicrobial activity within their essential oils and leaf extracts. The results obtained from the broad screening with various test pathogens confirm their use in traditional herbal remedies. The essential oils have proved to be more antimicrobially active in comparison to the leaf extracts. This study showed antimicrobial activity for selected test pathogens, which clearly indicate that there are more potentially active species of the genus not initially documented. It should also be noted that nearly all of the essential oils and most of the leaf extracts were active against the yeast *Cryptococcus neoformans* and *Bacillus cereus.* This forms a basis for an alternative source of remedies for treatment of fungal and bacterial infections. More research needs be carried out to isolate the active compound(s) by bioassay-guided fractionation for some of the species like *E. aromaticus*, which showed good inhibitory activity in the preliminary screening. However, if these species are to be used for medicinal purposes, their chemical composition and issues of safety and toxicity will need to be investigated further.

4.5.2. Antioxidant activity

Essential oils did not show any antioxidant activity in the DPPH assay. In the TLC screening for detection radical scavenging properties, leaf extracts showed white spots against the purple background after spraying with 0.4% DPPH. This implies that they have ability to scavenge free radicals but when the quantification analysis was carried out spectrophotometrically, a different pattern emerged. Among the taxa tested, the strongest effect was observed in *E. punctulatus* from Nieuwoudtville 2 and *E. klinghardtensis* from Namibia with an IC_{50} of 21.5 $\pm$ 1.3 and 28.1 $\pm$ 1.8 µg/ml respectively (Table 4.4 and Figure 4.3). The weakest effect was noted in some of the taxa of *E. punctulatus* (IC_{50} 65 and 79 µg/ml) (Table 4.4). The rest of the taxa showed moderate concentrations that reduced the DPPH radical ranging from IC_{50} 30-50 µg/ml.

A similar pattern of variation in activity was noted between individuals of same species as was observed in antimicrobial activity involving intraspecific differences in activity e.g. in *E. africanus* from Citrusdal and *E. punctulatus* from Nieuwoudtville 2 (Figure 4.3). Variation was also noted between populations of the same species (Table 4.4).

Table. 4.2. Antimicrobial activity of essential oils and acetone leaf extracts of *Eriocephalus.* Activity is measured in millimetres (mm) from the edge of the disc.

Taxon	Locality	Extract	Activity in mm from edge of the disc						
			Cn	***Ca***	***Bc***	***Bs***	***Sa***	***Kp***	***Ec***
E. africanus	Mossel Bay	EO	1.5	<1	R	1	<1	<1	<1
E. africanus*	**Malmesbury**	**EO**	**5**	**2**	**6**	**2**	**2**	**1**	**R**
E. africanus	Melkbosstrand	EO	1	1	3	1	1	R	R
E. africanus	Citrusdal (A)	EO	2	1	2	<1	<1	1	R
E. africanus	Citrusdal (B)	EO	1	R	5.5	2	1	<1	R
E. africanus	Citrusdal (C)	EO	8	1	4	<1	1.5	R	1
E. aromaticus	Swartberg	EO	R	2	3.5	3	2	<1	<1
E. aromaticus	Ladismith (B)	EO	R	R	3	1	<1	R	R
E. aromaticus	Ladismith (C)	EO	8	R	4	<1	R	R	R
E. brevifolius	Vergelegen (A)	EO	<1	<1	1.5	1	<1	<1	R
E. brevifolius	Vergelegen (B)	EO	3	<1	3	<1	<1	R	R
E. brevifolius	Vergelegen (C)	EO	5.5	1	4	1	<1	<1	R
E. brevifolius	**Oudtshoorn**	**EO**	**R**	**2**	**5.3**	**4**	**5**	**2**	**2**
E. brevifolius	**Kamiesberg**	**EO**	*	**2**	**2.5**	**1**	**2.8**	**1**	**R**
E. capitellatus	**Swartberg Pass (A)**	**EO**	**4**	**R**	**3.5**	**<1**	**1**	**<1**	**R**
E. capitellatus	Swartberg Pass (B)	EO	2	1	3.5	1	<1	1	<1
E. capitellatus	**Swartberg Pass (C)**	**EO**	**3**	**2**	**2.5**	**2**	**R**	**1**	**<1**
E. decussates	Sutherland	EO	4	2	2	2	3	<1	<1
E. decussatus	**Kamiesberg**	EO	**5.2**	**2**	**3.5**	**1.5**	**2.5**	**<1**	**<1**
E. dinteri	**Aus-Namibia**	**EO**	**6.6**	**1.5**	**3.6**	**1.2**	**3.2**	**1.5**	**1**
E. ericoides subsp. *ericoides*	Scheerpersrust (A)	EO	3	<1	2.5	2	<1	<1	R
E. ericoides subsp. *ericoides*	Scheerpersrust (B)	EO	2	1	3	1	1	R	1
E. ericoides subsp. *ericoides*	Scheerpersrust (C)	EO	3	1	2	1	1	<1	R
E. ericoides subsp. *ericoides*	Prince Albert-1	EO	R	1	7.25	3	1	<1	<1
E. ericoides subsp. *ericoides*	Prince Albert (A)-2	EO	4.5	1	4	<1	<1	R	R
E. ericoides subsp. *Ericoides*	Prince Albert (B)	EO	5	1	3	1.2	1	R	R
E. ericoides subsp. *ericoides*	Prince Albert (C)	EO	3	2	4	3	3	1	1
E. ericoides* subsp. *ericoides	**Bethulie (A)**	**EO**	**9**	**5**	**3**	**2**	**1**	**1**	**1.2**
E. ericoides* subsp. *ericoides	**Bethulie (B)**	**EO**	**4**	**2**	**2**	**3**	**2**	**1**	**1.5**
E. ericoides* subsp. *ericoides	**Windhoek-Namibia**	**EO**	**3.2**	**2**	**4**	**1.5**	**2**	**1**	**1**
E. ericoides* subsp. *ericoides	**Hohenheim-Namibia**	**EO**	**7**	**2.5**	**3**	**1**	**2.1**	**1.5**	**R**
E. eximius	Sutherland (A)	EO	R	R	3	<1	<1	<1	R
E. eximius	Sutherland (B)	EO	5	R	2	<1	1.5	R	R
E. eximius	**Kamiesberg**	EO	**4.5**	**1.5**	**4.7**	**1.5**	**3**	**R**	R
E. klinghardtensis	**Neiaab Mt.-Namibia**	**EO**	**6.2**	**2**	**2.8**	**1.2**	**2.6**	**2.4**	1
E. luederitzianus	**Windhoek- Namibia**	**EO**	**2.8**	**1.5**	**2.1**	**1.2**	**3.8**	**<1.0**	**R**
E. merxmuelleri	**Buschmanberge-Namibia**	**EO**	**6**	**1.5**	**3.5**	**2**	**1.5**	**1.5**	**R**
E. microphyllus	Sutherland (A)	EO	R	3	8	2	3	<1	<1
E. microphyllus	Sutherland (B)	EO	6	3	2	1	2	1	R
E. microphyllus	Sutherland (C)	EO	7	3	3	1.5	1.5	1.5	2
E. microphyllus	Nieuwoudtville (B)	EO	4	2	3.5	3	1.5	<1	<1
E. microphyllus	Nieuwoudtville (C)	EO	6	<1	3	R	1	1	1
E. microphyllus	**Kamiesberg**	**EO**	**5**	**1**	**2**	**1**	**<1**	**2**	**1**
E. microphyllus	Spektakel Pass	EO	6	1	4	<1	<1	1.5	R
E. namaquensis	Clanwilliam (A)	EO	10	1	3.5	3	1.5	<1	<1
E. namaquensis	Clanwilliam (C)	EO	6	2	R	R	1	<1	R

Taxon	Locality	Extract	Activity in mm from edge of the disc						
			Cn	***Ca***	***Bc***	***Bs***	***Sa***	***Kp***	***Ec***
E. pauperrimus	Nieuwoudtville (A)	EO	6	3	4	2	2	R	R
E. pauperrimus	Nieuwoudtville (C)	EO	3	2	2	1.5	1.5	R	R
E. pinnatus	**Brandberg-Namibia**	**EO**	**3.8**	**1.5**	**5**	**<1**	**2.5**	**<1.0**	R
E. punctulatus	**Nieuwoudtville (A)-1**	**EO**	**2**	**<1**	**3**	**1**	**1**	**1**	**1.5**
E. punctulatus	Nieuwoudtville (B)	EO	9	2	3	2	1	1	1
E. punctulatus	**Nieuwoudtville (C)**	**EO**	**3**	**5**	**2.5**	**3**	**1**	**1**	**1**
E. punctulatus	Calvinia	EO	R	2	3	4	1.5	<1	<1
E. punctulatus	Nieuwoudtville -2	EO	5	2	5.5	2	1.5	1	2.5
E. purpureus	Nieuwoudtville	EO	5	2	3.5	4	2	<1	<1
E. purpureus	Kamiesberg	EO	5	R	2	1.5	<1	1	1
E. racemosus	**Koeberg**	**EO**	**R**	**1**	**1**	**4**	**3**	**1**	**1**
E. racemosus var *racemosus*	Velddrif (A)	EO	2	1	4	1	1	<1	R
E. racemosus var *racemosus*	Velddrif (B)	EO	2	R	4	1	<1	<1	R
E. racemosus* var *racemosus	**Velddrif (C)**	**EO**	**5**	**1**	**3.5**	**1**	**1**	**2**	**R**
E. scariosus	**Aus-Namibia**	**EO**	**3.5**	**1.5**	**1.3**	1	**1**	**1.5**	1
E. spinescens	Sutherland (B)	EO	2	R	2	<1	<1	R	R
E. spinescens	Sutherland (C)	EO	4	1	4	<1	2	R	R
E. africanus	De Rust	AE	4	R	R	R	R	<1	<1
E. africanus	Malmesbury	AE	3	R	1	2	R	<1	R
E. africanus	Melkbosstrand	AE	3	1	2	2	<1	<1	R
E. africanus	Citrusdal (A)	AE	2	R	1	<1	R	R	R
E. africanus	Citrusdal (B)	AE	1.5	R	1	2	R	<1	<1
E. africanus	Citrusdal (C)	AE	3	R	2	R	<1	R	R
E. africanus var p*aniculatus*	Sutherland (A)	AE	4	R	R	1	R	<1	<1
E. africanus var *paniculatus*	Sutherland (B)	AE	5	R	R	R	R	R	R
E. africanus var *paniculatus*	Sutherland (C)	AE	2	R	<1	R	R	R	R
E. ambiguous	**Schakalsberge-Namibia**	**AE**	**R**	**R**	**R**	**R**	**2.5**	**R**	<1.0
E. aromaticus	**Swartberg**	**AE**	**R**	**R**	**8**	**4**	**5**	**<1**	**<1**
E. aromaticus	**Ladismith (A)**	**AE**	**1**	**<1**	**4**	**2**	**4**	**R**	**<1**
E. aromaticus	**Ladismith (B)**	**AE**	**5**	**R**	**7.3**	**6**	**6**	**R**	**R**
E. aromaticus	Ladismith (C)	AE	1	<1	R	1	4	R	<1
E. brevifolius	Vergelegen	AE	2	R	3	R	R	R	R
E. brevifolius	Oudtshoorn	AE	3	R	R	4	<1	R	R
E. brevifolius	Kamiesberg	AE	1.5	1.5	1.5	<1	2.5	R	<1
E. capitellatus	Swartberg Pass-1	AE	R	R	1	1	R	<1	<1
E. capitellatus	Swartberg Pass -2	AE	<1	R	R	<1	<1	R	R
E. decussatus	Sutherland (A)	AE	5	R	R	1	R	<1	<1
E. decussatus	Sutherland (B)	AE	R	R	1	R	R	R	R
E. decussatus	Sutherland (C)	AE	<1	R	1	<1	R	R	<1
E. decussatus	**Kamiesberg**	**AE**	**1.5**	**R**	**1**	**<1**	**2.5**	**R**	**<1**
E. dinteri	**Aus-Namibia**	**AE**	**2.8**	**R**	**1.7**	**R**	**3**	**R**	**<1.0**
E. ericoides subsp. *ericoides*	Scheerpersrust (A)	AE	2	R	3	1.5	1	R	R
E. ericoides subsp. *ericoides*	Prince Abert-1	AE	3	R	1	<1	<1	R	R
E. ericoides subsp. *ericoides*	Prince Albert-2	AE	3	R	2	1.5	1	R	R
E. ericoides subsp. *ericoides*	Bethulie (A)	AE	R	R	<1	1	1	1	R
E. ericoides subsp. *ericoides*	Bethulie (B)	AE	R	R	1	1	2	<1	R
E. ericoides* subsp. *ericoides	**Windhoek-Namibia**	**AE**	**1.5**	**R**	**1**	**R**	**2.5**	**R**	R
E. ericoides* subsp. *ericoides	**Hohenheim-Namibia**	**AE**	**2**	**R**	**1.3**	**<1**	**2**	**R**	**R**
E. eximius	Sutherland (A)	AE	R	R	1	<1	R	R	<1

Taxon	Locality	Extract	Activity in mm from edge of the disc						
			Cn	***Ca***	***Bc***	***Bs***	***Sa***	***Kp***	***Ec***
E. eximius	Sutherland (B)	AE	2	R	R	1	R	R	R
E. eximius	Sutherland (C)	AE	2	R	R	1	R	R	R
E. eximius	Kamiesberg	AE	1	R	1	<1	1.5	R	1
E. grandiflorus	Laingsburg (A)	AE	R	R	1	2	R	R	R
E. grandiflorus	Laingsburg (B)	AE	3	R	<1	1	<1	R	R
E. grandiflorus	Laingsburg (C)	AE	3	<1	1	1	1	R	R
E. klinghardtensis	**Neiaab Mt.-Namibia**	**AE**	**<1**	**R**	**1.8**	**<1**	**1.5**	**R**	<1.0
E. luederitzianus	**Windhoek- Namibia**	**AE**	**R**	**R**	**R**	**R**	**1.5**	**R**	**R**
E. luederitzianus	**Windhoek- Namibia**	**AE**	**R**	**R**	**1**	**R**	**1.5**	**R**	**R**
E. merxmuelleri	**Buschmanberge-Namibia**	**AE**	**2.4**	**R**	**1**	**R**	**1.5**	**R**	**<1.0**
E. microphyllus	Sutherland (A)	AE	R	R	1	1	R	<1	<1
E. microphyllus	Sutherland (B)	AE	2	R	1	R	<1	R	R
E. microphyllus	Sutherland (C)	AE	2	R	1	R	<1	R	R
E. microphyllus	Nieuwoudtville (A)	AE	4	R	2	3	1	<1	<1
E. microphyllus	Nieuwoudtville (B)	AE	4	R	1	1	R	<1	<1
E. microphyllus	Nieuwoudtville (C)	AE	1	R	2	2	R	R	R
E. microphyllus	Kamiesberg	AE	2	1	<1	1	1	R	R
E. microphyllus	**Spektakel Pass**	**AE**	**3**	**2**	**3**	**1**	**1.5**	**R**	**<1**
E. namaquensis	Clanwilliam (A)	AE	4	R	2	1	<1	<1	<1
E. namaquensis	Clanwilliam (C)	AE	R	R	R	1	R	R	R
E. pauperrimus	Nieuwoudtville (A)	AE	1	R	R	2	R	<1	R
E. pauperrimus	Nieuwoudtville (B)	AE	4	R	R	R	R	<1	R
E. pauperrimus	**Nieuwoudtville (C)**	**AE**	**4**	**R**	**3**	**3**	**3**	**1**	**R**
E. pinnatus	**Brandberg-Namibia**	**AE**	**3.3**	**R**	**1.3**	**1**	**1.5**	**R**	<1.0
E. punctulatus	**Nieuwoudtville (A)-1**	**AE**	**6**	**1**	**3**	**2**	**R**	**<1**	**R**
E. punctulatus	Nieuwoudtville (B)	AE	3	R	3	R	<1	R	R
E. punctulatus	Nieuwoudtville (C)	AE	R	R	1	2	2	<1	R
E. punctulatus	Calvinia	AE	3	R	1	2	<1	R	R
E. punctulatus	Nieuwoudtville (A)-2	AE	3	R	R	R	R	<1	R
E. punctulatus	Nieuwoudtville (B)	AE	R	R	R	R	R	<1	R
E. punctulatus	Nieuwoudtville (C)	AE	R	R	1	R	R	R	<1
E. purpureus	Laingsburg (A)	AE	3	R	1	R	R	<1	R
E. purpureus	Laingsburg (B)	AE	5	R	1	1	<1	<1	<1
E. purpureus	Nieuwoudtville-1	AE	3	R	R	1	R	<1	<1
E. purpureus	Nieuwoudtville -2	AE	7	R	1	2	R	<1	1
E. purpureus	**Kamiesberg**	**AE**	**4**	**2**	**4**	**1**	**1.5**	**R**	**<1**
E. racemosus	Koeberg	AE	2	R	1	1	1	R	R
E. racemosus var *racemosus*	Velddrif (A)	AE	R	R	1	1	R	<1	<1
E. racemosus var *racemosus*	Velddrif (B)	AE	6	<1	2	R	1	R	R
E. racemosus var *racemosus*	Velddrif (C)	AE	R	R	2.5	1.5	1	R	R
E. scariosus	**Aus-Namibia**	**AE**	**2**	**R**	**1.5**	**R**	**2**	**R**	**<1.0**
E. spinescens	Sutherland (A)	AE	R	R	<1	1	R	<1	R
E. spinescens	Sutherland (B)	AE	2	R	2	1.5	1	R	R
E. spinescens	Sutherland (C)	AE	1	R	R	R	R	R	R
		Control	11	7	8	6.5	7	3.5	2

*The samples in bold were selected for further MIC assays (Table 4.3).
Cn-*Cryptococcus neoformans* ATCC 90115; Bc-*Bacillus cereus* ATCC 11778.
Bs-*Bacillus subtilis* ATCC 6051; Sa-*Staphylococcus aureus* ATCC 5923.
Ec-*Escherichia coli* ATCC 8739; Ca-*Candida albicans* ATCC 10231.

Table 4.3: Minimum inhibitory concentration (mg/ml) of essential oils and leaf extracts of *Eriocephalus* species.

Species	Locality	Extract	Minimum inhibitory concentration mg/ml					
			Cn	***Ca***	***Bc***	***Sa***	***Kp***	***Ec***
E. africanus	Malmesbury	EO	4	4	8	32	*	16
E. brevifolius	Oudtshoorn	EO	*	8	8	16	>32	*
E. brevifolius	Kamiesberg	EO	16	>32	8	4	*	*
E. capitellatus	Swartberg Pass (A)	EO	4	*	16	*	*	*
E. capitellatus	Swartberg Pass (C)	EO	4	*	*	*	*	*
E. decussates	Kamiesberg	EO	16	32	8	4	*	*
E. dinteri	Aus-Namibia	EO	32	32	16	4	8	8
E. ericoides subsp. *ericoides*	Bethulie (A)	EO	1	4	4	8	16	*
E. ericoides subsp. *ericoides*	Bethulie (B)	EO	*	4	*	*	8	16
E. ericoides subsp. *ericoides*	Windhoek-Namibia	EO	16	16	8	4	16	*
E. ericoides subsp. *ericoides*	Hohenheim-Namibia	EO	8	16	8	4	8	*
E. klinghardtensis	Neiaab Mt.-Namibia	EO	32	32	8	4	8	16
E. merxmuelleri	Buschmanberge-Namibia	EO	16	16	8	2	8	*
E. microphyllus	Kamiesberg	EO	*	*	*	*	8	16
E. pinnatus	Brandberg-Namibia	EO	16	16	8	8	*	*
E. punctulatus	Nieuwoudtville (C)	EO	4	8	*	8	*	*
E. punctulatus	Nieuwoudtville (A)-1	EO	*	*	*	*	*	16
E. racemosus	Koeberg	EO	*	*	*	*	*	16
E. racemosus var *racemosus*	Velddrif	EO	2	*	16	*	16	*
E. scariosus	Aus-Namibia	EO	8	>32	12	4	8	8
E. aromaticus	Swartberg	AE	*	*	0.4	0.2	*	*
E. aromaticus	Ladismith (A)	AE	*	*	3.1	0.8	*	*
E. aromaticus	Ladismith (B)	AE	1.6	*	0.8	0.4	*	*
E. ambiguous	Schakalsberge	AE	*	*	*	3.1	*	*
E. brevifolius	Kamiesberg	AE	1.8	*	0.9	0.9	*	*
E. decussates	Kamiesberg	AE	2.4	*	0.9	1.6	*	3.1
E. dinteri	Aus-Namibia	AE	6.3	*	0.4	3.1	*	*
E. ericoides subsp. *ericoides*	Windhoek-Namibia	AE	1.6	*	1.6	1.6	*	*
E. ericoides subsp. *ericoides*	Hohenheim-Namibia	AE	1.6	*	1.3	3.1	*	*
E. klinghardtensis	Neiaab Mt.-Namibia	AE	*	*	0.9	0.8	*	*
E. luederitzianus	Windhoek- Namibia	AE	*	*	*	0.8	*	*
E. luederitzianus	Windhoek- Namibia	AE	*	*	*	0.8	*	*
E. merxmuelleri	Buschmanberge-Namibia	AE	3.1	*	0.4	0.4	*	*
E. microphyllus	Spektakel Pass	AE	6.3	1.6	3.1	0.8	*	*
E. pinnatus	Brandberg	AE	6.3	*	0.2	0.4	*	*
E. pauperrimus	Nieuwoudtville	AE	*	*	*	1.6	*	*
E. punctulatus	Nieuwoudtville -1	AE	0.8	*	0.8	*	*	*
E. purpureus	Kamiesberg	AE	*	1.6	*	0.8	*	*
E. scariosus	Aus-Namibia	AE	0.5	*	0.5	1.6	*	*
	Controls		$1x10^{-3}$**	$1x10^{-3}$	$6x10^{-4}$*	$1x10^{-3}$	$3x10^{-3}$	$3x10^{-3}$

*Not determined due to insufficient sample or lack of activity
**- Ciprofloxacin positive control for bacteria; Amphotericin B–positive control for fungi.

The antioxidative ability of the genus *Eriocephalus* is more pronounced at higher concentrations 30-80 μg/ml. The patterns of the various activities in this genus are intriguing and this explains the deviation from the normal patterns of activity against the DPPH.

Previous studies (Zdero *et al.,* 1987; Wollenweber and, 1989; Bohm and Stuessy, 2001) indicate that the genus contains nearly all classes of flavonoids. Flavonoids are among the naturally occurring plant secondary metabolites that have been reported to have broad pharmacological activity. Species of *Eriocephalus* have various classes of flavonoids noted in the leaf extracts and these compounds are known to have strong antioxidant properties. The activity noted in most of the species could be attributed to the presence of flavones, isoflavones and flavanones that were abundant in the leaf extracts. For instance, the IC_{50} of *E. africanus* is between 37.4 μg/ml and 49.9 μg/ml (Figure 4.3) and the flavonoid data in Chapter 2, Table 2.7, shows that most of these taxa have close to similar concentrations of flavones and flavanones in their extracts that may be responsible for the activity e.g. *E. africanus* (Figure 4.3). Further study should be conducted to evaluate their toxicity profiles and safety indices.

The IC_{50} values for most of the taxa studied are not very different and this may de due to the presence of similar classes of flavonoids. An example includes the individuals of *E. aromaticus* from Ladismith (Figure 4.3) that show similar IC_{50} values. As previously mentioned, the majority of species have flavones and flavanones almost in the same range of concentration, however, quantitative analysis of taxa shows that there is variation between individuals of the same species from the same population. These erratic patterns were noted in the terpene chemistry and it appears that the general trend in the chemical profiles of the species of *Eriocephalus* is that the allopatric taxa share more similar chemistries to each other than with sympatric taxa of the same species. A number of factors influencing such patterns have been discussed in Chapters 2, 3 and 5. It then follows that the flavonoids in this genus are as complex as the terpenes such that they may not be helpful in delimitation or clarification of relationships between species. Therefore, the current species boundaries in the genus are not supported by the flavonoid chemistry. It may also be worth testing the polar extracts as they were not considered in the study and they may give different patterns of variation.

Table 4.4. Antioxidant activity of acetone leaf extracts of species of *Eriocephalus.* IC_{50} values are given (μg/ml).

Species	Source/locality	DPPH IC_{50} (μg/ml)**
E. africanus	Malmesbury	47.2 ± 7.2
E. africanus	Melkbosstrand	46.4 ± 6.7
E. africanus	Citrusdal A	49.9 ± 10.0
E. africanus	Citrusdal B	37.4 ± 8.8
E. africanus	Citrusdal C	38.1 ± 4.3
E. africanus	De Rust	41.9 ± 7.1
E. africanus var *paniculatus*	Sutherland A	42.5 ± 5.4
E. africanus var *paniculatus*	Sutherland B	49.4 ± 4.4
E. africanus var *paniculatus*	Sutherland C	45.8 ± 2.5
E. ambiguus	Schakalsberge	32. 9 ± 2.8
E. aromaticus	Swartberg	31.8± 2.0
E. aromaticus	Ladismith A	43.6 ± 4.0
E. aromaticus	Ladismith B	45.3 ± 4.8
E. aromaticus	Ladismith C	42.5 ± 4.5
E. brevifolius	Oudtshoorn	49.7 ± 7.2
E. brevifolius	Vergelegan C	47.9 ± 6.2
E. brevifolius	Kamiesberg	30.9 ± 2.0
E. capitellatus	Swartberg Pass	*
E. capitellatus	Swartberg Pass A	40.5 ± 3.2
E. decussatus	Sutherland A	47.2 ± 7. 8
E. decussatus	Sutherland B	42.3± 4.5
E.decussatus	Sutherland C	45.9 ± 9.0
E. decussatus	Kamiesberg	44.1 ± 4.3
E. dinteri	Near Aus	34.9 ± 2.7
E. ericoides subsp. *ericoides*	Windhoek Namibia	45.1 ± 5.0
E. ericoides subsp. *ericoides*	Hohenheim-Namibia	43.7 ± 4.4
E. ericoides subsp. *ericoides*	Prince Albert	47.9 ± 2.5
E. ericoides subsp. *ericoides*	Scheepersrust A	48.8 ± .0
E. ericoides subsp. *ericoides*	Prince Albert A	56.7± 1.2(01)
E. ericoides subsp. *ericoides*	Bethulie A	52.7 ± 8.5
E. ericoides subsp. *ericoides*	Bethulie B	44.8 ± 3.7
E. eximius	Sutherland A	56.9 ± 2.1(01)
E. eximius	Sutherland B	50.3 ± 3. 6
E. eximius	Sutherland C	43.8 ± 8.5
E. eximius	Kamiesberg	39.3 ± 3.6
E. grandiflorus	Laingsburg A	45.6 ± 5.1
E. grandiflorus	Laingsburg B	46.0 ± 5.9
E. grandiflorus	Laingsburg C	42.5 ± 4.1
E. klinghardtensis	Neiaab Mountain	28.1 ± 1.8
E. luederitzianus	Windhoek A	48.1 ± 5.9
E. luederitzianus	Windhoek B	45.0 ± 4.9
E. merxmuelleri	Buschmanberge	39.9 ± 4.5
E. microphyllus	Sutherland A	43.2 ± 4.2
E. microphyllus	Sutherland B	46.2 ± 5.0
E. microphyllus	Sutherland C	45.35 ± 5.89
E. microphyllus	Nieuwoudtville A	44.03 ± 3.53

Species	Source/locality	DPPH IC_{50} (μg/ml)**
E. microphyllus	Nieuwoudtville B	41.58 ± 3.87
E. microphyllus	Nieuwoudtville C	45.56 ± 5.17
E. microphyllus	Kamiesberg	46.96 ± 6.67
E. microphyllus	Spektakel Pass	47.67 ± 4.34
E. namaquensis	Clanwilliam A	45.3 ± 6.47
E. namaquensis	Clanwilliam B	44.37 ± 4.88
E. namaquensis	Clanwilliam C	44.62 ± 6.78
E. pauperrimus	Nieuwoudtville A	46.57 ± 5.82
E. pauperrimus	Nieuwoudtville B	50.0 ± 10.84
E. pauperrimus	Nieuwoudtville C	46.46 ± 6.15
E. pinnatus	Brandberg	53.04 ± 4.36
E. punctulatus	Nieuwoudtville A	43.19 ± 3.47
E. punctulatus	Nieuwoudtville B	65.65 ± 2.76(01)
E. punctulatus	Nieuwoudtville C	*
E. punctulatus	Nieuwoudtville 2 A	44.97 ± 4.95
E. punctulatus	Nieuwoudtville B	32.42 ± 2.6
E. punctulatus	Nieuwoudtville C	21.46 ± 1.29
E.punctulatus	Nieuwoudtville 3 A	79.63 ± 2.02(01)*
E. punctulatus	Nieuwoudtville B	38.8 ± 2.57
E. punctulatus	Nieuwoudtville C	37.9 ± 4.06
E. purpureus	Laingsburg A	42.33 ± 4.33
E. purpureus	Laingsburg B	37.56 ± 4.4
E. purpureus	Laingsburg C	37.26 ± 3.76
E. purpureus	Nieuwoudtville 1	36.15 ± 2.99
E. purpureus	Nieuwoudtville 2 A	40.05 ± 5.27
E. purpureus	Nieuwoudtville B	39.54 ± 4.41
E. purpureus	Nieuwoudtville C	38.52 ± 3.99
E. purpureus	Kamiesberg	41.46 ± 6.03
E. racemosus	Koeberg	42.88 ± 2.39
E. racemosus var *racemosus*	Velddrif A	59.2 ± 9.92
E. racemosus var *racemosus*	Velddrif B	40.61 ± 3.72
E. racemosus var *racemosus*	Velddrif C	58.81 ± 1.7(01)
E. scariosus	Aus-Namibia	35.39 ± 3.77
E. spinescens	Sutherland A	41.14 ± 2.13
E. spinescens	Sutherland B	45.29 ± 3.88
E. spinescens	Sutherland C	46.47 ± 3.20
Control	Vitamin C	2.9 ± 0.01

**-Values are means + SE of three replicates

4.5.3. Antiinflammatory activity

In this assay, only essential oils were screened for antiinflammatory activity. Since most of the uses reported for some of the commercially and traditionally used species are attributed to the essential components with antiinflammatory properties, an attempt was made to carry out the *in vitro* screening of the essential oils from the wild species to determine if they too have the same properties. The oils were selected based on availability of the oil samples as most of

the taxa yielded very little oils. The antiinflammatory activity of one of the taxa of *E. punctulatus* at different concentrations is shown in Figure 4.4.

The lowest effective concentration that inhibited the enzyme was 19 μg/ml of *E. africanus* followed by taxa of *E. brevifolius* 25 and 30 μg/ml (Table 4.5 and Figure 4.5). The most inactive oil was noted as that of *E. scariosus* with IC_{50} of over 100 μg/ml (Table 4.5 and Figure 4.5). The two individuals of *E. punctulatus* from Nieuwoudtville have almost similar activities, as are *E. pauperrimus* and *E. microphyllus.* It is surprising that the former species did not show good activity despite having relatively high contents of bisabolol derivatives (Figure 4.5).

Of the 17 species tested for antiinflammatory activity, it is clear that activity varies greatly between populations (Figure 4.5) as observed in the three populations of *E. africanus.* In traditional remedies *E. africanus* and *E. punctulatus* are used to treat inflammatory diseases and this is supported by the values obtained in this assay.

The results also show that there are other potentially active species of *Eriocephalus* which have not been used traditionally, but have inhibitory ability against the enzyme e.g. *E. dinteri* (35 μg/ml), *E. brevifolius* (25 and 30 μg/ml), *E. eximius* (37 μg/ml) and *E. decussatus* (39 μg/ml) (Table 4.5).

The results from this study support the use of members of *Eriocephalus* in treatment of inflammatory diseases mediated by 5-lipoxygenase products, i.e. leukotrienes in traditional remedies. Further research should be focused on the development of these ethnomedicines, as they are easily accessible in areas where modern medicine is not readily available. There is support for the use of the members of the genus in cosmetic industries as one of the properties considered is the antiinflammatory effect. In traditional herbal remedies, some of the species are used for their soothing effects, which make them suitable for cosmetics. A further discussion based on the essential oil composition and the antiinflammatory activities is included under chemical composition and biological properties.

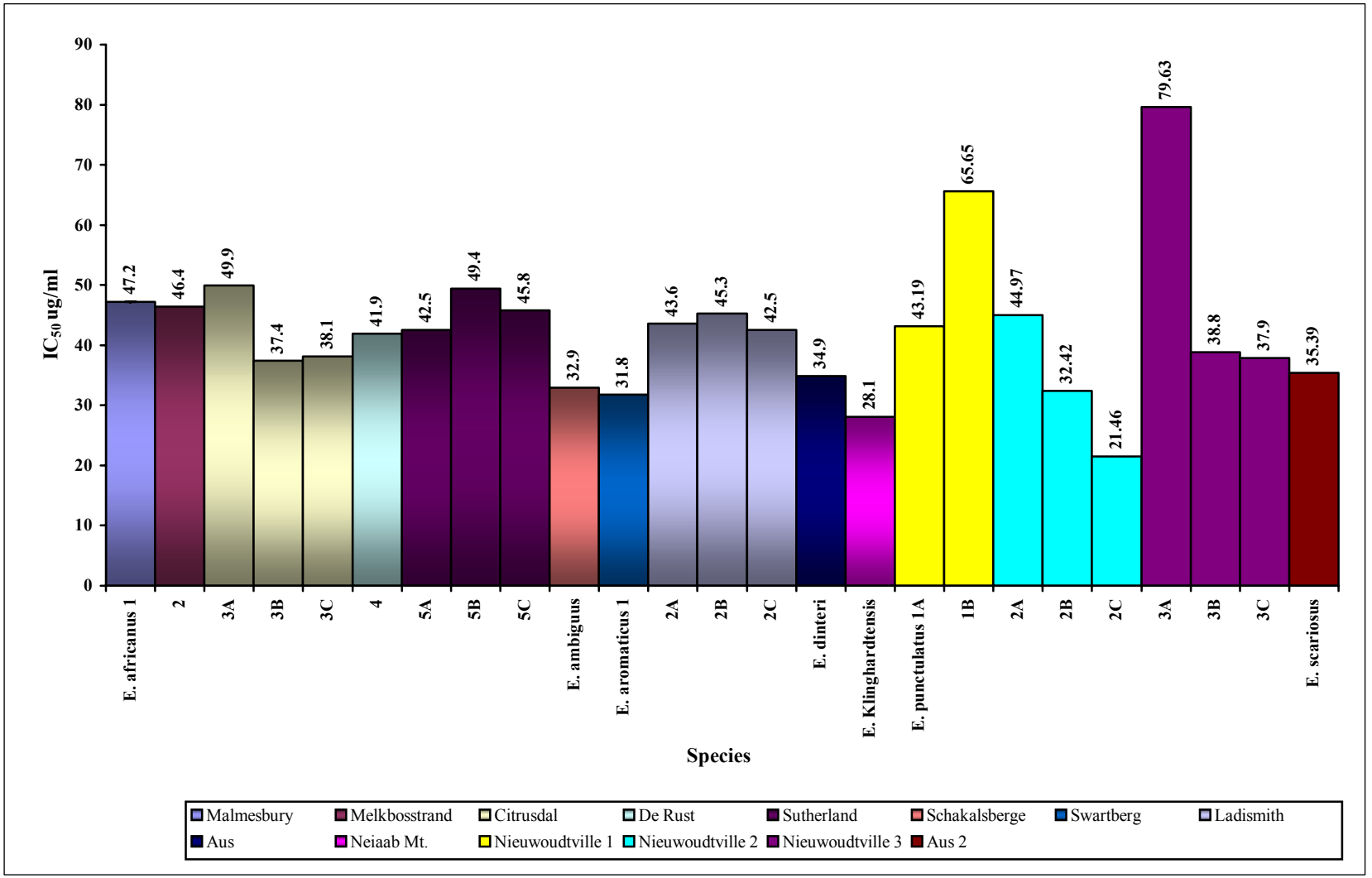


Figure 4.3. Variation in IC_{50} values (µg/ml) in the antioxidant test of seven representative species of *Eriocephalus* from different localities and populations.

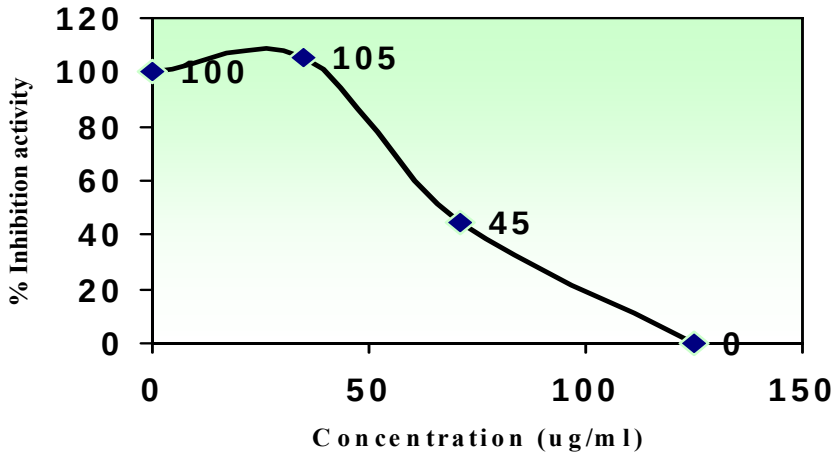


Figure 4.4. A representative graph showing the percentage inhibition of 5-lipoxygenase activity by the essential oil of *E. punctulatus* at different concentrations.

Table 4.5. Antiinflammatory activity of some selected species of *Eriocephalus* against 5-lipoxygenase. The IC_{50} values are given with (n=1).

Species	Locality	5-LOX IC_{50} (µg/ml)**	Oil colour
E. africanus	Malmesbury	32.8	Yellow
E. africanus	Melkbosstrand	19.0	Blue
E. africanus	Citrusdal A	31.8	Yellow
E. brevifolius	Oudtshoorn	30.2	Yellow
E. brevifolius	Kamiesberg	25.4	Yellow
E. capitellatus	Swartberg Pass A	43.1	Yellow
E. decussatus	Kamiesberg	39.6	Blue
E. dinteri	Aus-Namibia	35.4	Yellow
E. ericoides subsp. *ericoides*	Hohenheim Namibia	43.1	Blue
E. ericoides subsp. *ericoides*	Scheepersrust A	55.4	Yellow
E. eximius	Kamiesberg	37.9	Blue
E. klinghardtensis	Neiaab Mountain	59.7	Yellow
E. luederitzianus	Windhoek-Namibia A	40.5	Yellow
E. merxmuelleri	Buschmanberge	44.5	Blue
E. microphyllus	Kamiesberg	69.4	Blue
E. pauperrimus	Nieuwoudtville C	69.9	Cloudy
E. pinnatus	Brandberg -Namibia	58.7	Yellow
E. punctulatus	Nieuwoudtville A	63.0	Blue
E. punctulatus	Nieuwoudtville F	63.8	Blue
E. purpureus	Kamiesberg	98.9	Yellow
E. racemosus var *racemosus*	Velddrif B	32.8	Yellow
E. scariosus	Aus-Namibia	>100	Yellow
Control	NDGA	5 ± 0.5	*

NDGA-nordihydroguaiaretic acid.

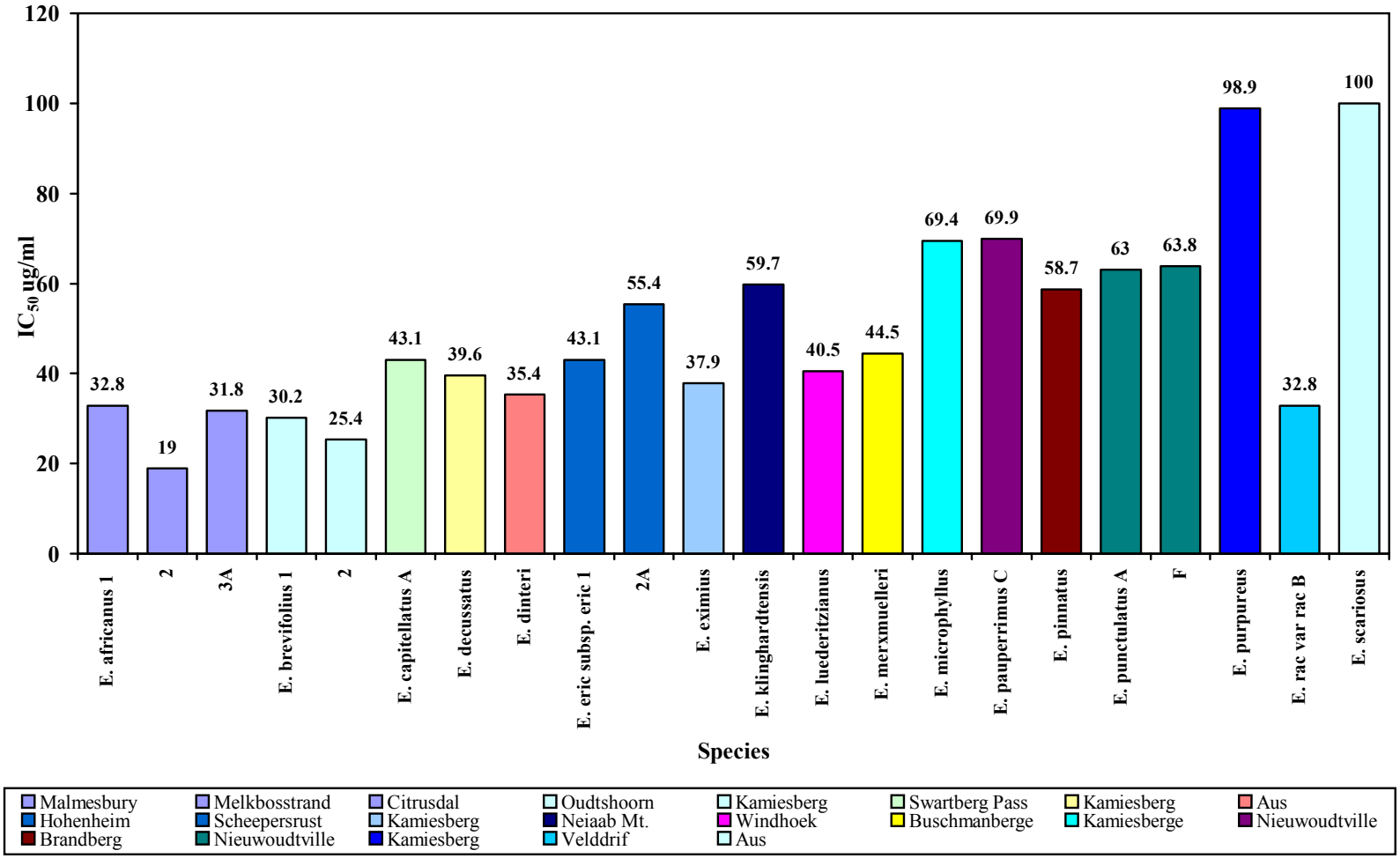


Figure 4.5. Variation in IC_{50} values (µg/ml) of the 5-lox test for seventeen species of *Eriocephalus* from different localities and populations.

4. 5.4. Acetylcholinesterase inhibition

The essential oils of seventeen species of *Eriocephalus* screened for detection of the presence of inhibitors of the enzyme showed several white spots on the TLC plate (Figure 4.6) as an indication of presence of the enzyme inhibitors. The acetone leaf extracts did not show any activity probably implying absence of the inhibitors or their presence in trace amounts.

The essential oils appear to have more of the inhibiting compounds, as evidenced by the numerous spots on the TLC plate. Perhaps when lower dilutions are used for the screening it will be possible to tell at what actual concentrations these compounds present in the samples would still inhibit the enzyme.

Importantly, the results of the qualitative screening give a new dimension to the biological activities of the members of the genus. Firstly, they support the use of the species in traditional remedies as mind-improving plants and their use in treatment of stress related ailments.

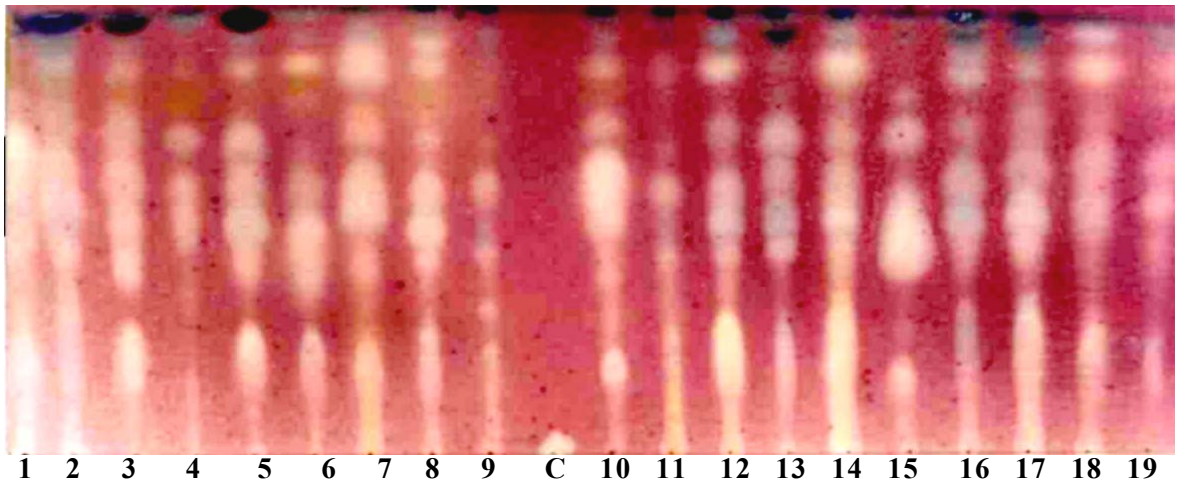


Figure 4.6. A TLC bioautographic profile of essential oils of 17 species of *Eriocephalus* tested for the presence of inhibitors of acetylcholinesterase enzyme. The white spots against the purple background indicate presence of inhibitory compounds. C is a control (Galathamine). **1** and **10**-*E. punctulatus*; **2** and **19**-*E. purpureus*; **3** and **16**-***E****. africanus*; **4**-*E. racemosus*; **5** and **18**-***E.*** *ericoides*; **6**-***E.*** *capitellatus*; **7**-***E.*** *brevifolius*; **8**-***E.*** *aromaticus*; **9**-***E****. spinescens*; **11**-***E.*** *decussatus*; **12**-***E****. grandiflorus*; **13**-***E.*** *eximius*; **14**-*E. microphyllus*; **15**-*E. pauperrimus*; **17**-*E. namaquensis*.

Secondly, their ability to inhibit acetylcholinesterase adds to the list of plants that can be

explored for the treatment of Alzheimer's disease. The results of the study also support the use of the plants in fumigation of houses after death, although it is more of a psychotropic ritual than actual therapy. A further discussion based on the essential oils composition and their inhibitory activities is included in the next section.

4.6. Chemical composition and biological properties

4.6.1. Volatile compounds

Essential oils form an important component of our daily lives since they are used in almost everything including foodstuffs, pharmaceuticals, cosmetics, perfumes, and other household items. They are also widely used in aromatherapy due to their pharmacological activities (Pauli, 2001), and a search for natural therapeutics with sufficient antimicrobial properties is an added advantage. All these uses and many other unlisted ones make the oils useful in combating microorganisms that cause deterioration of several products and therefore any form of investigative research into the inhibitory properties and antimicrobial potentials of oils or extracts is crucial.

The susceptibility and sensitivity of the test pathogens is discussed at length in previous sections. These findings need to be supported by the chemical composition data of the various species studied. The GC/MS analysis of the volatile constituents for the 22 species studied shows clearly that these oils are largely comprised of acyclic, monocyclic, and bicyclic mono- and sesquiterpenes. There are reports in literature supporting the activity of some of these essential oil components.

As previously mentioned, all the essential oils of the species of *Eriocephalus*, were active against *Cryptococcus neoformans* and *Bacillus cereus* and moderate activity against the rest of the pathogens. The antimicrobial activities of the essential oils of genus *Eriocephalus* noted in the study as a result of the numerous chemical compounds, validates their use in traditional herbal remedies. It is clear that the presence of camphor, 1,8-cineole, 4-terpineol, limonene, α- and β-pinene, camphene, linalool, ρ-cymene, α-terpinene, α-terpineol, γ-terpinene β-caryophyllene, pinocarvone, α-bisabolol, bisabolol oxide A and B, caryophyllene oxide, artemisia ketone, santolina, yomogi and artemisia alcohols, β-eudesmol, piperitone, α-cadinol, borneol, artemisia acetate and bicyclogermacrene among several other compounds, contributes to the antimicrobial activity of the taxa studied.

There was favourable activity noted in taxa where camphor, 1,8-cineole, α-cadinol, piperitone and 4-terpineol, santolina, yomogi and artemisia alcohols constituted the highest percentage of the total essential oil composition. This was observed in taxa of *E. ericoides* subsp *ericoides, E. punctulatus, E. brevifolius,* and *E. microphyllus*. This is in agreement with some authors who have observed that camphor and 1,8-cineole have antimicrobial activities, which are enhanced by their synergistic association than when each of the compounds was tested independently (Nakatsu *et al.,* 2000; Candan *et al*., 2003; Viljoen *et al*., 2003). The presence of 1,8-cineole and camphor in varying amounts could be the rationale behind the use of *E. africanus* in treatment of respiratory ailments in traditional herbal remedies.

All the taxa studied showed varying activity against the two yeasts, *Cryptococcus neoformans* and *Candida albicans* probably due to the presence of 1,8-cineole, camphor, α- and β-pinene, limonene, linalool, ρ-cymene, α-terpinene, γ-terpinene and 4-terpineol, that are reported to have antimicrobial properties (Williams *et al*., 1998; Nakatsu *et al*., 2000; Cimanga *et al*., 2002; Oladimeji *et al*., 2004; Tepe *et al.,* 2004). In instances where the activity was notably high against *Cryptococcocus neoformans* as in *E. africanus, E. aromaticus, E. ericoides* subsp *ericoides, E. microphyllus, E. punctulatus* and *E. namaquensis*, the oils were observed to be mostly comprised of ketones, aldehydes and alcohols which are reported to have higher antimicrobial activities (Nakatsu *et al*., 2000; Griffin *et al*., 1999) than the mono-and sesquiterpene hydrocarbons and acetates (Griffin *et al*., 1999; Houghton and Raman, 1998). Comparatively, *Cryptococcocus neoformans* was more susceptible than *Candida albicans* with 1-10 mm zone of inhibition and MIC of 1-32 mg/ml with the latter ranging from 1-5 mm zone of inhibition and MIC of 4-32 mg/ml respectively.

The activity of the essential oils against the three Gram-positive bacteria was interesting as all the taxa studied with exception of one individual of *E. africanus* and *E. namaquensis* were active against *Bacillus cereus.* On the other hand the activity of oils against the other two bacteria namely *Bacillus subtilis* and *Staphylococcus aureus* was moderate to low. The activities of the oils against these test pathogens could be attributed to the presence of α- and β-pinene, camphene, limonene, linalool, ρ-cymene, α-terpinene, α-terpineol, γ-terpinene β-caryophyllene, pinocarvone, α-bisabolol, caryophyllene oxide and 4-terpineol which are reported to have antimicrobial properties (Carson and Riley, 1995; Magena and Muyima, 1999; Nakatsu *et al.,* 2000; Aliagiannis *et al*., 2001; Cimanga *et al*., 2002; Viljoen *et al*., 2002; Tepe *et al.,* 2004).

The essential oils containing α-terpineol, camphene and α- and β-pinene are active against *Staphylococcus aureus* as reported by several authors. The presence of high contents α- and β-pinene in *E. luederitzianus* could be responsible for the activity of the essential oils against *Staphylococcus aureus*. In the traditional herbal remedies, *E. africanus* was used in treatment of dermal infections. The essential oils of the taxa of this species have among other compounds 4-terpineol, α- and β-pinene, α-bisabolol, limonene, linalool and caryophyllene oxide which have inhibitory activity against *Bacillus cereus* and *Staphylococcus aureus*. The activity of the essential oils against these two dermal infection-causing microorganisms validates the use of the species in treatment of the skin conditions in herbal medicine.

The activity of the oils against the Gram-negative bacteria *Klebsiella pneumoniae* and *Escherichia coli* was relatively low or absent in some cases. The poor activity that was observed could be attributed to the presence of compounds such as caryophyllene oxide β-pinene, pinocarvone, *trans*-pinocarvone, and α-terpineol among such other antimicrobially active compounds (Nakatsu *et al.,* 2000; Dorman and Deans, 2000; Viljoen *et al*., 2002). Several reports in literature indicate that low to no activity of the oils against the Gram-negative bacteria is normally associated with the cell wall membrane structure made up of lipopolysaccaride chains that acts as a barrier to hydrophobic essential oils (Mangena and Muyima, 1999; Oladimeji *et al*., 2004).

Since no tests on single compounds were carried out to confirm which compounds were responsible for the activities, the synergistic effects of the above listed compounds with each other and with minor constituents of the essential oils could be responsible for the overall activities of the various taxa. It has been reported in (Cimanga *et al*., 2002) that the compounds present in relatively high amounts may not be necessarily responsible for the greatest share of activity. This phenomenon is clearly displayed in some of the taxa where presence of high percentage of active compounds did not seem to contribute to the noted activities; on the contrary, the activities were noted to be lower. An example is in the individual of *E. microphyllus* from Sutherland with a high percentage of 1,8-cineole, which is supposed to be active against the fungal species yet the oil was inactive against *Cryptococcus neoformans.*

In other cases it was noted that the presence of particular compounds such as α-bisabolol and derivatives as in *E. pauperrimus* and *E. racemosus* var *racemosus* could have contributed to the activity against the Gram-positive and fungal pathogens as was the presence of linalool

and derivatives in the species of *E. decussatus* and *E. spinescens.* The presence of oils with alcohol functional groups, in the taxa of *E. ericoides* subsp. *ericoides* from Bethulie could be responsible for the fairly good activities noted against most of the test pathogens (Table 4.3).

This study proves that *Eriocephalus* has a great potential for use in combating microbe related ailments and other biological problems. It is evident that the essential oils had stronger activity and a broader spectrum than the extracts against the test pathogens with the Gram-positive bacteria more sensitive to oils and extracts than the Gram-negative bacteria (Martin, 1995; Mangena and Muyima, 1999; Sokmen *et al.,* 2004). The latter are reported to be more resistant due to their impermeable membrane structure. The essential oils also showed good activity against the yeast *Cryptococcus neoformans* in agreement with other authors (Bağci and Diğrath, 1996) who noted the high susceptibility of this yeast to the essential oils. *Candida albicans* however, showed lower susceptibility towards the oils and extracts. The high susceptibility of the former pathogen is a good sign that the species of the genus have a potential for use as antibiotics in combating various fungal infections.

Many types of industries are keen on finding natural products with environmentally friendly antimicrobials, antibiotics, antioxidants, and antiinflammatory properties for their products (Svoboda and Hampson, 1999). The preliminary screening of the essential oils of 17 species of this genus showed some of the oils as having some antiinflammatory properties. The compounds responsible for the activity were mono- and sesquiterpenes noted in the essential oils namely; α-bisabolol, bisabolol oxide A, and B, 1,8-cineole, thymol, linalool, chamazulene and other azulenic compounds, β-caryophyllene, γ-terpinene α-pinene and limonene which (Peana *et al*., 2002; Baylac and Racine, 2003; Standen and Myers, 2004; Szoke *et al*., 2004). These compounds were noted in some of the species namely; *E. pauperrimus, E. africanus, E. racemosus, E. eximius, E. aromaticus, E. punctulatus, E. purpureus, E. ericoides,* and *E. namaquensis* though some are in very low amounts.

The species that showed inhibitory activity against 5-lipoxygenase enzyme included an individual of *E. africanus* from Melkbosstrand that had the most promising activity (19 μg/ml) among all the taxa tested. Two other species, *E. brevifolius,* and *E. racemosus* showed moderate activity ranging from 25.4-32.8 μg/ml. Among the compounds present in their oils are α-pinene, β-caryophyllene, γ-terpinene, 1,8-cineole, limonene, linalyl acetate and linalool. Nerolidol and α-bisabolol were present in the essential oil of *E. racemosus* with the latter compound in relatively high amounts and probably responsible for the activity. Some of the

factors responsible for the variation noted in the antimicrobial activity of the volatile components are discussed in Chapter 5.

The inhibitory activity of the essential oils against acetylcholinesterase may have been due to the presence of compounds that are used in treatment of mental related conditions such as linalool, linalyl acetate, β-eudesmol and β-caryophyllene. More details are discussed in Chapter 5.

4.6.2. Non-volatile compounds

The activity of the acetone leaf extracts against the test pathogens namely; *Cryptococcus neoformans, Bacillus cereus,* and *Staphylococcus aureus* may be attributed to the various classes of flavonoids. The most common ones noted were the flavones and the flavanones. Flavonols were also present as noted in the HPLC/UV analysis. Among the species tested, the extracts of *E. aromaticus* and *E. pinnatus* showed good activity in the disc diffusion and the MIC assays and the good activity against the Gram-positive bacteria may be attributed to the presence of the flavones and flavanones. Probably the presence of the flavones could be responsible for the diuretic and diaphoretic effects in some of the species used in the traditional herbal remedies.

The presence of flavonoids in the extracts may also be responsible for the antioxidant activities noted in the various extracts of the 22 species tested. As in most species, the moderate antioxidant activity in the extracts of *E. dinteri, E. brevifolius, E. aromaticus, E. aromaticus, E. ambiguus, and E. scariosus* may be attributed to the presence of the numerous flavones and flavanones. The trends observed in the antioxidant activities were consistent with the other analyses where variation between individuals of the same species was observed. An example includes the taxa of *E. punctulatus* from different localities (Table 4.4), which have varying activities.

4.7. Conclusions

This study represents the very first comprehensive survey of the biological properties of the genus *Eriocephalus* and it is a pacesetter for future investigations. References on this genus are scarce as no major work on biological properties has been undertaken for the genus for a long time and the outcome of this study forms a future reference document for any further studies. In most traditional herbal remedies, the various plant parts used are either used in their raw forms or as tinctures, infusions, and mixtures of various decoctions. It is possible

that the effects of a certain plant are due to the synergistic effects of various compounds. The following conclusions can be drawn from the study:

- The genus is biologically active as supported by *in vitro* qualitative and quantitative results in the various assays and there is great variation observed in activity within and between populations.
- The essential oils are more active relative to the acetone leaf extracts against the microbial pathogens used in this study implying that the activity of the medicinally used species may be largely influenced by the presence of essential oils.
- The Gram-positive and fungal pathogens were more susceptible to the oils and extracts than the Gram-negative bacteria.
- The study provides an *in vitro* scientific validation for the use of some of the members of the genus in treatment of respiratory ailments, dermal infections and the various gastro-intestinal disorders in traditional herbal practices.
- The antiinflammatory and the antioxidant properties in the genus are recorded here for the first time. There is still great potential for exploitation of the genus in herbal remedies. The antiinflammatory properties also confer credibility to the uses of the essential oils of some species in fragrances and cosmetics.
- The inhibitory activity of the essential oils against acetylcholinesterase enzyme has been recorded for the first time and in a way validates the use of some of the species in CNS related conditions.

In general, the study has highlighted the biological importance of the genus and accorded support to the use of the genus in traditional herbal remedies. It is interesting to note the potential of the genus in food preservation, treatment of Alzheimer's disease, and the antiinflammatory properties have been realized in this study. It therefore implies more research and isolation of the active compounds responsible for the various activities is necessary in the future.

CHAPTER 5

Discussion, Conclusions and Recommendations

5.1 Preview

The study of medicinal plants for discovery of new bioactive components is a progressive trend that entails use of information on plants from various disciplines. Among these includes ethnobotany. Ethnbotany encompasses a very broad field with few publications available that deal with South African ethnobotany in general (Van Wyk, 2002; Heitzman, *et al*., 2005). Among the genera of Anthemideae used in herbal preparations, *Eriocephalus* happens to be one of the South African endemics that has not received much attention regarding knowledge of its chemistry, phylogeny, and biological properties hence necessitating further studies on the group.

Basic research in pharmacology, chemotaxonomy, and biosystematics of medicinal plants represents an important priority because it provides a deeper understanding without which no innovation will be possible (Van Wyk, 2002). The advancement of such studies would be largely useful in understanding the diversity of the most commonly used economic plants in cultivation and *in situ*. An additional component of intra- and infraspecific variability can be introduced by examination of more than one population per taxon. Results obtained can be compared with the biology, phylogeny and taxonomy of the taxa, to extract the possible phylogenetic and chemotaxonomic significance (Skaltsa *et al*., 2001; Grayer *et al*., 1996).

The chemistry of a plant may also provide insights into observed morphological variations. In other cases, the essential oil chemistry may be useful in defining the limits of the given taxa, where morphological relationships are reticulate. However, this phenomenon may be complicated if infraspecific chemical variation is heterogeneous as in the case of the present genus under study. This is not unusual, as the same pattern has been observed in the families Lamiaceae and Scrophulariaceae (Grayer *et al.,* 1996). Terpenes are amongst the chemicals responsible for the medicinal, culinary and fragrant uses of aromatic and medicinal plants. Several studies have been carried out to determine the antimicrobial properties of plant volatile oils (Janssen *et al* 1987; Viljoen *et al*., 2003). The search for novel drugs and cosmetics from natural plant products is broad and requires contribution from various fields. It is therefore crucial to adopt a multidisciplinary approach in handling such a study (Hostettmann, 1999; WHO, 2002). There is also an urgency to characterize and validate through scientific rationale, the use of numerous traditional remedies that have been in use for a long time (Dorman and Deans, 2000; Van Wyk, 2002). All the above-mentioned aspects have been adopted in this study to highlight the inherent diversity in *Eriocephalus.*

The genus *Eriocephalus,* which is one of South Africa's endemics, has received very little attention regarding ethnobotanical uses and antimicrobial properties, with the exception of *E. africanus* and *E. punctulatus* that are commercially exploited in perfumes, cosmetics and also in herbal remedies. There is much scope for systematic investigations into this endemic genus that can provide new insights and contribute towards the knowledge of the group. Information generated from such studies is crucial as starting points for further investigations into other areas of the group in question. Such information is lacking for *Eriocephalus* and indeed it has been extremely difficult to get scientific references to compare results from the various analyses. The findings from this study are my original contribution to furthering our knowledge of this interesting group of the family Asteraceae.

This study has brought together the chemistry, phylogeny and biological activities of *Eriocephalus* to help in the understanding of intra- and infraspecific variation in the genus. Other studies have shown that intraspecific variability can be examined by processing more than one population per taxon. The results are compared with the biology and taxonomy of the taxa, to extract possible chemotaxonomic characters of evolutionary significance (Skaltsa *et al*., 2001). The present study has employed the same criteria and intriguing results have been realised.

The secondary metabolites which have been investigated in the study (terpenes and flavonoids) as previously mentioned elsewhere, are adaptive traits that have undergone diversification and selection over evolutionary time, and hence, may have a lot to offer in terms of phylogenetic inference (Wink, 2003). This includes their presence or absence being an indication of common descent and relatedness, alternatively, co-occurrence of similar structural class of compounds in two unrelated taxa may represent a case of chemical convergence or differential gene expression. The occurrence of similar essential oils profiles in unrelated taxa is not uncommon as has been evidenced in the current study. Hence when chemical data was combined with molecular data, the resultant phylogenetic trees were used to examine similarities and dissimilarities of secondary metabolites (Wink, 2003, Grayer *et al*., 1996).

As previously mentioned, sometimes members of a monophyletic clade share a defence character, or sometimes a given trait within a taxon is substituted by another chemical (Wink and Mohamed, 2003). In such cases, the trait would be recognised as a valuable taxonomic marker. Hence, the study of the distribution of secondary metabolites in plants, along with

their phylogenetic relationships, offers information on the underlying evolutionary and ecological processes driving diversification. It also gives insight into the life strategies and taxonomic relationships as well as providing guidance into the understanding of the patterns of variation observed in the biological properties of a given taxon (Grayer *et al*., 1996). The basic tenets responsible for information of the evolutionary past of any plant of a given taxonomic grouping are embedded in the genomic constitution of such a plant. Hence, it is not surprising that, the terpenoid diversity is an explicit display of the complex relationships emerging from the various gene expressions (Grayer *et al*., 1996, Wink and Mohamed, 2003).

The following discussion outlines the outcome of the study based on the aims and objectives outlined in Chapter 1. The recent account of the genus (Müller *et al*., 2001), gives an indication of morphological polymorphism in the genus and from the current study it is clear that the relationships within and between members of the genus are highly complex as noted in their chemical profiles and DNA phylogeny.

5.1. Phytochemistry and chemotaxonomy

The first objective of this study was to attempt to clarify infra-specific delimitation problems using chemical (terpene and flavonoid) and molecular data. It is interesting to note that the chemical profiles in the genus are largely comprised of regular and irregular terpenes. This implies that there are several biosynthetic pathways present in the group and the most common being the mevalonic and chrysanthemic acid pathways. The latter pathway is common in the members of Anthemideae and the essential oils of most species studied showed a considerable amount of artemisyl, santolinlyl and lavandulyl derivatives that characterize the chrysanthemic acid pathway. The presence of various enzymes responsible for the synthesis of most of the compounds characterised in the essential oils are not species-specific as evidenced by the lack of grouping of individuals of a the species in the phenetic and phylogenetic analyses.

It is clear then, that intra-specific variation in essential oils and extracts has resulted in a complex pattern of relationships even between individuals of the same species from the same population (as depicted in the dendrograms and phylograms). The detailed implications and possible reasons for these patterns in the various dendrograms and phylogenetic trees have been addressed in details in Chapter 2 and 3.

Despite all these anomalies some affinity groups were noted in the analyses despite some of the taxa of the same species not forming monophyletic groups within themselves. Cluster

analysis based on Euclidean distances with unweighted pair group average linking for qualitative and quantitative data resulted in dendrograms depicting species groups that were largely incongruent with existing taxonomy in most of the taxa studied. This made it difficult to assign any infra-specific ranks or define the relationships between the species of *Eriocephalus*. As previously mentioned, terpene and flavonoid chemistry in the species was so divergent that the current species delimitation in the genus is not fully supported by the chemistry of the genus.

No obvious variation was noted in the taxa from Namibia, which seem to have similar chemistry to their South African counterparts. Then one would take note that geographical isolation has not resulted in distinct differentiation in terpene chemistry of the species from the two countries. However, the Namibian taxa have fewer number of compounds released from the GC/MS analysis (83) out of a total of (200). It is then clear that 117 compounds present in the South African species are absent in the Namibian species. Nevertheless, some chemical affinities were noted between some of the species from these two different geographical regions.

It is apparent that the expression of secondary metabolites of a given structural type has almost invariably arisen on a number of occasions. In normal circumstances, related species share genetically controlled features such as many chemical constituents (Judd *et al*., 1999) but in the case of *Eriocephalus* species, such a generalization is not applicable as proved by the complex chemistry of each individual in the study. The connection between genes and characters is so complex as are the developmental pathways, which are susceptible to both internal and external factors, of which little is known. When chemical characters are considered for delimitation purposes, it is in the hope that one will achieve definable groups but it is clear from this study that they are fallible to parallelism, convergence and environmental factors just like any other taxonomic markers and they also evolve at different rates. At the end of it all they remain a facet in a complex evolutionary scenario, and the interpretation of their behaviour remains a matter of interpretation based on the objectives of the given study.

The magnitude and pattern of infra-specific variation within any species depends upon the definition of a species. Despite the extensive and objective sampling of the taxa of the genus, the infra-specific variation is not clearly definable (Kubitzki, 1984). It proves difficult in such a situation to represent adequately, the variation existing between species in theoretical basis,

resulting in ambiguity in what essentially constitutes taxonomic units (Judd *et al*., 1999). As such this was a major problem encountered in the study of the genus *Eriocephalus.*

5.3. Phylogenetic inference using ITS and terpenes

This section is part of objective 1. An attempt to reconstruct the phylogeny of the genus using two non-coding plastid regions, *trn*L-F and *psb*A-*trn*H intergenic spacers was unsuccessful as these two regions revealed minimal sequence variation. This is despite the fact that the same regions have been reported as variable in the phylogenetic reconstruction in some of the genera in the tribe Anthemideae, e.g. in the genus *Paeonia* (Clegg and Zurawski, 1992; Sang *et al.,* 1997; Kornkven *et al.,* 1998, 1999; Watson *et al*., 2000, 2002; Schmidt and Schilling, 2000; Samuel *et al*., 2003). The ITS region was variable but the number of characters separating the taxa was too few for phylogenetic resolution and support of the relationships between taxa and species. Hence the expectation that the molecular analysis would lead to clarification of the specific and intraspecific relationships was not met. Another anomaly observed in the ITS region was the presence of highly divergent paralogous repeats for some of the taxa which were not included in the analysis.

It was not possible to use the molecular data for assessment of species delimitation and a further attempt to combine the molecular data with the chemical data did not make it any easier to achieve the objective. This was due to the highly variable histories depicted by multiple representatives of each species hence no obvious solution was available to the phylogenetic problems encountered in the genus based on these data. It must be emphasized that most of the bootstrap/jacknife support in the phylogenetic analyses was low or below 50%. Apart from the analysis of the restricted taxon data set of combined chemical and ITS analyses, where the support values retrieved were fairly high (Chapter 3, Figure 3.4), the analyses of the complete taxon data sets (both separate and combined) received relatively low support values (Chapter 3; Figures 3.1, 3.3, and 3.5). This may be due to the loss of phylogenetic signals as a result of high level of homoplasy. It became clear that allopatric taxa of the same species were in most cases showing closer affinities than the sympatric taxa especially in cases where multiple taxa were analysed for phylogenetic inference. Overall, the molecular and chemical characters used did not provide adequate support values to address the problems of species delimitation and therefore it was not possible to assess their reliability as phylogenetic markers at the species level.

However, despite the lack of strong support, all data sources points towards the conclusion that some species concepts in the group are flawed, as the species do not conform to the biological, phenetic, genetic, phyletic or ecological species definition. Despite use of superior chemical and molecular data to attempt infra-specific delimitation of taxa, the resultant relationships are not coherent, and this is aggravated by the fact that most of the chemical characters are homoplasious, hence giving unreliable phylogenetic signals (Donoghue and Sanderson, 1992).

It was not possible to assign any infra-specific groups to species of *Eriocephalus* using the molecular and chemical data due to lack of coherence in the grouping of taxa from the same species in the trees. However, the uncertainty surrounding the delimitation *E. merxmuelleri, E. luederitzianus* and *E. microphyllus* are hereby tentatively resolved as these species occupy unrelated positions in the trees. Since only one individual was studied for *E. merxmuelleri* and *E. luederitzianus,* the recognition of their affinity is only tentative. Other species with delimitation problems such as *E. spinescens, E. capitellatus, E. purpureus* and *E. namaquensis* also occupied unrelated positions in the phylogeny though the taxa of the latter species were erratically placed. In the latest revision of the genus (Müller *et al.,* 2001), it was noted that *E. spinescens* and *E. namaquensis* are sometimes difficult to separate. However, in this study, the two species are unrelated in the various trees presented here. They were phylogenetically placed as distinct species in the various analyses. The same applied to *E. klinghardtensis* and *E. scariosus* which are reported to be similar but in the current study, the former (which is only represented by one individual here) is placed as sister to *E. racemosus* var *racemosus* and the latter (also represented by one individual) is placed in a group with individuals of *E. brevifolius, E. africanus* and *E. ericoides* subsp. *ericoides. Eriocephalus ericoides* subsp. *ericoides,* which is reported as being morphologically, close to *E. purpureus* and *E. purpureus* to *E. capitellatus,* occupy unrelated positions in the trees.

Despite the incongruence with the taxonomy and species delimitation as it currently stands, some affinity groups between species and some of the multiple individuals per taxon were noted in each analysis have been discussed below. These were groups recovered in almost all of the phenetic and phylogenetic analyses and appeared to reflect some molecular, chemical, and morphological affinities. It must be noted that these affinity groups did not necessarily involve all the multiple taxa of the species in consideration and are thus tentative groupings that will need further support from other analyses in any future studies.

5.3.1. *E. purpureus*

This species showed affinities between individuals of the same from different populations and was considered for discussion as most of the specie studied did not show this type of association. The taxa of this species from Nieuwoudtville, Nieuwoudtville/Papkuilsfontein and Laingsburg/Matjiesfontein grouped together in nearly all the analyses, an indication of closeness characterised by the presence of mainly bicyclic monoterpenes; myrtenal, myrtenol, bornyl acetate, α-cocaine and isocomene as synapomorphies. In the phylogenetic analysis, the individual from Kamiesberg is nested elsewhere which is not surprising as its chemistry is slightly different but closer to that of the individuals of *E. africanus, E. microphyllus* and *E. namaquensis*. The grouping of the taxa of *E. purpureus* is quite unique in the analyses of this genus considering that most of the species with representatives from more than one population did not group these individuals together in the phylogenetic trees. Probably one would argue that the geographical proximity of their respective localities is the reason for close relationships, this was also noted for individuals of *E. capitellatus* that were collected from the same locality. However, this is not necessarily our expectation since Kubitzki, (1984) has argued that in some cases, allopatric taxa may be more similar chemically than sympatric taxa due to localized diversification of chemical compounds in plants in the latter.

5.3.2. *E. punctulatus-E. aromaticus*

The close relationship between these species for some of the individuals included is characterised by the presence of camphene, caryophyllene oxide and jatamansone. Morphologically, both taxa have radiate capitula and the paleae of the marginal florets are connate. The two species also overlap in their ranges of distribution in the Western Cape (Klein Roggeveld and Witteberg Mountains) and the latter species used to be confused with the former. They have been distinguished on the basis of the latter species having consistently opposite leaves even on the flowering shoots. Chemically the two species are separated by the former having sabinene, *cis*-carvyl acetate and several unknown compounds and the latter having α-terpineol, carvacrol, α-longipinene and viridiflorol or globulol. This close relationship between the two species was recovered for some of the populations in the phenetic and phylogenetic analyses. The same relationship was also noted in the flavonoid dendrogram.

5.3.3. *E. pinnatus-E. microphyllus*

Among the species of *Eriocephalus, E. pinnatus* is one of the species characterised by unique autapomorphies such as large golden yellow ray florets, distinctively pinnatisect leaves and a

herbaceous habit all of which are absent in the rest of the species. Morphological variation is not always correlated to chemical variation as they follow different evolutionary pathways. This could be the reason the former species shares close chemical affinities with the latter species. Though they are morphologically different with the former having a radiate capitula and the latter having a disciform capitula, chemically they have *cis*-sabinene hydrate, 1-terpineol, α-terpineol, *trans*-piperitol, δ-cadinene, α-cadinol or muurolol, α-phellandrene and bicyclogermacrene as apomorphies among many other compounds. However, they are distinguished from each other by the former having isomethylbutyrate, isoamylvalerate, neryl acetate among many other compounds and the latter species has artemisyl derivatives, α-thujene, *cis*- and *trans*-sabinene hydrate, *trans*-pinocarveol, pinocamphone among many other compounds. The two species are placed as sister taxa in the combined chemical and molecular phylogeny. The affinity between these species was specifically noted to point out that the former species is chemically similar to the latter despite it being morphologically different from the rest of the species in the genus.

5.3.4. *E. spinescens*-*E. decussatus*

Morphologically, the two species have disciform capitula but the former has free paleae and the latter connate and both have sericeous and opposite leaves. Taxonomically, the latter species has been misidentified on occasions as the former on the basis of spines, which are actually remains of the terminal racemose peduncles that are sometimes present (Müller *et al*., 2001). The two species share similarities in their chemistry and are closely related. De Candolle (1838) and Harvey (1865) recognised these species as closely related. In the combined phylogenetic tree the species group together based on the presence of α-fenchene, *cis*- and *trans*-linalool oxides, linalool, linalyl acetate, geranyl acetate and isocomene as synapomorphies. The individual species are distinguished by the presence of 4-terpineol, α-copaene, ar curcumene, α-cadinol or muurolol in *E. spinescens* and α-pinene, limonene, camphor, geranyl acetate and chrysanthenone in *E. decussatus.* The two species have relatively high contents of linalool and derivatives among all the species studied.

5.3.5. *E. racemosus, E. africanus, E. eximius,* and *E. luederitzianus*

These species are related through presence of β-myrcene as a synapomorphy despite their multiple taxa grouping elsewhere in the tree and dendrograms. *Eriocephalus racemosus* has α-terpinene, α-copaene, methyleugenol and bergamotene while *E. africanus, E. eximius* and *E. luederitzianus* are characterised by the presence of α-humulene and bicyclogermacrene. *Eriocephalus africanus* and *E. eximius* resemble each other morphologically by having radiate

capitula, sericeous and opposite leaves and paleae of the marginal florets that is connate. Chemically they have santolina triene, yomogi alcohol, myrtenol, isocomene, β-elemene, and δ-cadinene as apomorphies. The two species clearly grouped together in the ITS, chemical and combined chemical and molecular phylogenies. Their grouping together is an indication of their close affinities. *Eriocephalus luederitzianus* has α-fenchene, sabinene, 1,8-cineole, 4-terpineol and α-longipinene linking it to the other three species. Morphologically this species only resembles *E. racemosus* in having disciform capitula and no morphological affinities with the other two species. The relationships between these four species are largely based on the chemical convergence evidenced by the chemical compounds they share in common.

5.3.6. *E. racemosus-E. klinghardtensis*

The two species have connate paleae but the former has disciform capitula and the latter species has radiate capitula. These species were noted to group together in most of the analyses and chemically share α-thujene, α-fenchene and *cis*-sabinene hydrate as synapomorphies. *Eriocephalus racemosus* is distinguished by having α-bisabolol, *trans*-sabinene hydrate, *trans*-pinocarveol, nerolidol and caryophyllene oxide among many other compounds. *E. klinghardtensis* is distinguished by the presence of α-terpinene, filifolone, chrysanthenone, *trans*-chrysantemyl acetate, spathulenol and β-eudesmol.

5.3.7. *E. brevifolius-E. grandiflorus*

These species are morphologically similar in having radiate capitula, connate paleae, sericeous indumentum on their leaves and are found in similar geographical localities. They also have the same chromosome number 2n = 54. However, they are chemically distinguished from each other by the presence of methyl *trans*-chrysanthemate, lavandulol acetate and β-eudesmol in *E. brevifolius* and nerolidol, santolina triene, *trans*-pinocarveol, pinocarvone and myrtenol among many other compounds in *E. grandiflorus.* Some of the individuals of these species grouped together in all the analyses and it is clear that they share some affinities.

5.3.8. *E. aromaticus-E. grandiflorus*

The species have radiate capitula, paleae that are connate and decussate leaves. They also have linalool, *trans*-pinocarveol, pinocarvone, borneol and geranyl acetate as synapomorphies among several other compounds. *E. grandiflorus* is distinguished morphologically from the former by possession of large and showy pedunculate capitula and rigid-branched habit, while the former species has erect branched habit with smaller capitula. Chemically, the former has δ-cadinene and the latter has santolina triene, limonene, and myrtenol among other

compounds as distinguishing features. It was evident from the grouping together of these species in the phenetic and phylogenetic analyses that there were some close affinities between them.

5.3.9. *E. brevifolius, E. africanus, E. punctulatus,* and *E. scariosus*

Though the relationships between these species with the rest of the members of the genus are unresolved in the phylogeny, it is worthwhile to mention that they are reported in Müller *et al*., (2001), as having close morphological affinities. These are peduncles, connate paleae, and felty sericeous indumentum on the leaves. Chemically, they have α-fenchene and camphene, sabinene, borneol, α-copaene and β-caryophyllene as synapomorphies. *E. brevifolius* and *E. africanus* share artemisia ketone, caryophyllene oxide, and α-cadinol. All the relationships discussed are based on the recovery of the groupings in the various analyses, despite the inconsistencies and discrepancies noted in the phylogenetic trees, these species groupings were reflected in the phenetic and phylogenetic analyses hence deserving tentative recognition.

5.3.10. *E. africanus-E. merxmuelleri*

These species grouped together in the same clade in the combined phylogeny for complete and restricted taxon sets in a relationship defined by the presence of α-terpinene, limonene, piperitone, and bicyclogermacrene as apomorphies. Morphologically, the former has radiate capitula and connate paleae while the latter has disciform capitula and free paleae of the marginal florets. However, they both have sericeous indumentum on leaf surfaces. The latter species occurs in Namibia and the association could be a case of convergent evolution.

5.3.11. *E. punctulatus-E. purpureus*

These species are characterized by the presence of pinocarvone, *cis*-carvyl acetate and β-caryophyllene as synapomorphies. They also occupy similar geographical ranges, they have radiate capitula, connate paleae and 2n = 36. Both produce variants of blue oils although as mentioned elsewhere, oil colour is not really a distinguishing character as it is also highly diversified within a species. The two species group together in the ITS phylogeny and in the combined restricted taxa analysis though the relationship receives <50% support.

5.3.12. *E. ericoides* subsp. *ericoides-E. microphyllus-E. purpureus*

Morphologically, these species have disciform capitula and free paleae of the marginal florets. Their chemistry is characterised by the presence of 1,8-cineole, santolina alcohol, camphor,

yomogi alcohol, and spathulenol and artemisia alcohol. *Eriocephalus ericoides* subsp. *ericoides* and *E. microphyllus* are very variable as evidenced by the erratic placement of the individuals of the two species in the various phylogenetic topologies. The latter species is the most widely distributed in the genus and probably this accounts for its erratic grouping in the phylogenetic analyses.

The above-discussed affinity groups as previously mentioned represent patterns of grouping of taxa irrespective of the placement of the remainder of the multiple taxa in the various analyses. It appears that the species lineages in the genus are quite recent to have differentiated from each other. This phenomenon has also been noted in the study of other species rich groups in the Cape Floral Kingdom. It is evident that most of the species in the Cape flora radiated within the Miocene and Pliocene era. Some of the genera with such recent radiations include *Moraea*, *Phylica* and *Ferraria* (Goldblatt et al., 2002).

Most of the patterns observed in the various phylogenetic trees do not give a clear pattern based on chemistry with the exception of *E. purpureus, E. racemosus* var *racemosus, E. capitellatus* and *E. pauperrimus.* These species appear to have some affinities between their multiple taxa but they rarely formed chemotypes on their own except for *E. pauperrimus* (Chapter 2, Table 2.8). Bioprospecting of the members of this genus for new natural products is quite a challenge taking into account the presence of extreme heterogeneity noted in the chemistry of the species and their multiple taxa. Besides, as previously mentioned the percentage of most of the compounds such as camphor is consistently high and may have a potential for further investigations. The same applies to *E. pauperrimus* whose individuals showed characteristically high contents of bisabolol derivatives. The individuals of *E. punctulatus* (Nieuwoudtville/Papkuilsfontein) and *E. ericoides* subsp. *ericoides* from Scheerpersrust and Prince Albert were noted to have consistently high contents of 1,8-cineole (31-53%) while the individuals from (Nieuwoudtville/Calvinia) have relatively high contents of α-cadinol. The aforementioned species are worth investigating further for commercial development.

5.4. Biological properties and traditional remedies

In line with the second and third objective of the study, an attempt was made to record the biological properties of 22 species of the genus *Eriocephalus* and to provide a scientific basis for the traditional uses of some of the species. Most of these ethnobotanical uses have not been validated through scientific experiments. This aspect has been covered extensively in

Chapter 4 and it is evident that medicinal species are used to treat respiratory ailments, gastro-intestinal disorders, and dermal infections. The study revealed to a great extent the antimicrobial potential of the species as evidenced by their activity against the selected test pathogens as listed in Chapter 4. Comparatively, the volatile components were more active against the bacterial and the fungal pathogens than were the leaf extracts. This is in part indicates that the reported antimicrobial activity of the traditionally used species is mainly influenced by the presence of essential oils. The traditional use of *E. africanus* and *E. punctulatus* in fumigation of huts after death of a person or of one suffering from colds could be linked to the presence of compounds with antiseptic properties, e.g. camphor. The compound is reported to be a counterirritant, anaesthetic and is a mild antiseptic (Ikan, 1991; Viljoen *et al*., 2003). Other compounds with similar properties such as p-cymene, linalool, and 1,8-cineole were also present in the essential oil of this species (Dewick, 2001). This validates the use of the aforementioned species in the traditional herbal practices. Other species from this study that were noted to have high contents of camphor are *E. capitellatus, E. dinteri, E. purpureus,* and *E. microphyllus* from Kamiesberg. They are thus potentially useful sources of this compound, which is also widely used in cosmetics due to its skin soothing properties.

Some of the uses of the species in the traditional herbal remedies (e.g. *E. punctulatus*) include psychotropic inducing effect and as anti-depressants leading to emotional relaxation. The presence of linalool, linalyl acetate, β-eudesmol, and β-caryophyllene, which are associated with treatment of mental conditions, was noted in the essential oils of some of the species. The compounds are known to affect the central nervous system (Nakatsu *et al.,* 2000). The volatile oils are either inhaled or applied to the skin and they act by means of their lipophilic fraction reacting with the lipid parts of the cell membranes. As a result, they modify the activity of the calcium ion channels. At certain levels of dosage, the volatile oils saturate the membrane and show effects similar to those of local anaesthetics. They can interact with the cell membranes by means of their physiochemical properties and molecular shapes, and can influence their enzymes, carriers, ion channels, and receptors. Hence, inducing effects such as brain stimulation, anxiety relieving sedation and anti-depressant activities, as well as increasing the cerebral blood flow (Svoboda and Hampson, 1999).

Probably these same compounds were responsible for the inhibition of acetylcholinesterase noted in the preliminary qualitative TLC screening of the essential oils of 17 species of this genus. The essential oils, and especially those of *E. spinescens* and *E. decussatus*, have a

potential for use in combating mental illnesses such as Alzheimer's disease due to the high percentages of linalyl acetate and other linalool derivatives among other compounds. However, further quantitative studies will be required to verify this. On the other hand, the *in vitro* screening results from the screening of essential oils for antiinflammatory properties confers credibility to the use of *E. punctulatus* and *E. africanus* in treatment of inflammation and other related diseases in traditional herbal remedies.

It is possible that the presence of the non-volatile components in the leaf extracts, especially the flavonoids were responsible for the antimicrobial activity noted against the test pathogens. The flavones and the flavanones may be responsible for the diuretic and the diaphoretic effects reported in some of the species used in traditional herbal remedies.

There was obvious variation in the antimicrobial activities of the oils and extracts observed within and between individuals of the same population and different populations, which may be due to a complex interplay of various factors. The presence of trace components in essential oils, even the unidentified ones can influence the odour, flavour, and the biological activities of the oil to a significant extent. The activity of most oils is the result of the combined effect of both the active and inactive compounds. These inactive compounds might influence re-adsorption, rate of reactions and bioavailablity of the active compounds. Several of these active minor compounds may have synergistic effects, hence introducing a complex interplay in the reactions. In other instances, these compounds may also exhibit antagonistic effects thus lowering the overall activities of the oils (Carson and Riley 1995; Houghton and Raman, 1998; Cimanga *et al*., 2002; Tepe *et al*., 2004). In other cases, it has been reported that other factors like time of harvest and stage of growth prior to harvesting, genetic constitution, environmental conditions and the ecological pressures may influence the composition of the oils, yield and consequently the potency of their biological activity (Knobloch *et al*., 1989; Baratta *et al*., 1998; Echeverrigaray *et al*., 2001; Szoke *et al.,* 2004).

The limited water solubility of the test compounds and diffusion into the reaction media, vapourisation, and the nature and presence of the various structural groups may be another source of variability in antimicrobial activity of oils and extracts (Janssen *et al*., 1987; Griffin *et al*., 1999). The low hydrogen bonding capacity of some of these compounds renders them less soluble in water. The mono- and sesquiterpene hydrocarbons and the acetates have been shown to have lower H-bonding capacity and hence are less soluble (Griffin *et al*., 1999) whereas the aldehydes, ketones, and alcohols were found to be more active due to their higher

H-bonding capacity and higher solubility. However, the activity in this study was specific and the levels of activity were rarely defined by the functional groups present but rather by the aforementioned reasons. This phenomenon was observed in the patterns of activity of the essential oils against the test pathogens. For the Gram-negative bacteria the sensitivity or the susceptibility of the pathogen, apart from being influenced by solubility and H-bonding capacity, the molecular size parameters do apply (Houghton and Raman, 1998; Griffin *et al*., 1999; Roussis *et al*., 2000).

The chemistry of the compounds also plays a crucial in understanding the patterns of biological activities. According to Dormans and Deans (2000), the activity of essential oils of plants would be expected to relate to the respective composition of the plant volatiles, the structural configuration of the constituent components and their functional groups and possible synergistic interactions between components. This implies that the activity of the esters, aldehydes, ketones, alcohols, oxides, and hydrocarbons among such other compounds may differ considerably. It has been documented that in most cases the terpene acetates and hydrocarbons tend to be relatively inactive regardless of the structural groups and this phenomenon could be associated with their limited hydrogen-bond capacity and water solubility. On the other hand the ketones, aldehydes and alcohols tend to be more active with varying levels of specificity (Knobloch *et al*., 1989; Griffin *et al*., 1999).

The stereochemistry also has an influence on bioactivity. It has been observed that α-isomers are inactive relative to β- isomers e.g. α-pinene as *cis*-isomers are inactive contrary to *trans*-isomers e.g. geraniol and nerol as noted in *E. spinescens* (Griffin *et al*., 1999; Dorman and Deans, 2000).

Another source of variation in activity is the test methods which are to known vary considerably, especially the disc diffusion method, which is a qualitative method devoid of easy reproducibility, hence there is always some variation in the values obtained (Carson and Riley, 1995; Hammer *et al*., 1999; Griffin *et al*., 1999). The lack of reproducibility of the disc diffusion method is, however, a distinct disadvantage and it should only be used as a screening test. The size of the zone of inhibition produced by a compound in a disc diffusion test is determined by the inherent antimicrobial activity of the compound, its solubility in and diffusion through the medium and the various characteristic of the organism (Carson and Riley, 1995). On the other hand, the agar, pH, content, volume and the type of microbial strains contribute largely to the result or the activity. This may introduce variations in the end

results (Hammer *et al*., 1999). These factors may have had some contribution to the variation noted in the activities of the oils and leaf extracts but their role is minor.

The non-volatile components that showed activity in the DPPH assay are indicative of potential of *Eriocephalus* species in combating free radicals and related ailments and can also be used in food preservation. Similarly, the ability of the essential oils and the extracts to inhibit the activities of the food-borne pathogens such as *Bacillus* sp., *Escherichia coli* and *Staphylococcus aureus* (Sokmen, *et al.,* 2004), makes them a potential source for natural food preservatives. The results are also supportive of the culinary uses of *E. africanus.* However, further quantitative and toxicity analyses need to be carried out.

The qualitative and quantitative *in vitro* results from this study provide a scientific basis and validation of use of some of the members of the genus in traditional herbal remedies including the treatment of dermal infections, respiratory related ailments, and gastro-intestinal disorders. On the other hand, the ability of the oils to inhibit the 5-lipoxygenase enzyme confers credibility to the use of the 'Cape chamomile' and 'Cape snowbush' oils in cosmetics. The presence of inhibitors of acetylcholinesterase supports the use of some of the members in treatment of mental related conditions.

5.4.1. Relationships based on biological and chemical properties

The biological activities of essential oils of any one plant are an expression of its chemical composition, structural configuration of its constituent compounds, their functional groups, and not excluding the synergistic interactions between the components. Solubility and molecular properties are also crucial (Griffin *et al*., 1999; Dorman and Deans, 2000).

An attempt to establish if there is any correlation between the antimicrobial activity and the essential oil composition using cluster analysis (not shown) of the quantitative chemical and antimicrobial data did not produce coherent groupings. This is not surprising considering the complex chemistry between individuals of the same species in the genus. Many factors are involved in expression of antimicrobial activities of different plant extracts as discussed previously (Houghton and Raman, 1998; Griffin *et al*., 1999; Dorman and Deans, 2000; Cimanga *et al*., 2002). Hence, the relationships of the taxa based on antimicrobial activity did not conform to the evolutionary groupings observed in the combined ITS and chemical phylogeny.

In general, one can cautiously conclude that the antimicrobial activities of the species of *Eriocephalus* are affected by both extrinsic and intrinsic factors, which exert their influences differently. In this study, no direct and obvious correlation between chemical composition and the antimicrobial activities was noted and neither did the clustering of the taxa based on antimicrobial activity relate directly to the phylogenetic groupings.

5.5. Favourable chemotypes

From the study of essential oils profiles 22 chemotypes were recognized and details are included in Chapter 2. Among these are the camphor, 1,8-cineole, nerolidol, linalyl acetate, bisabolol derivatives, and the pinane groups that are widely used industrially in cosmetics, fragrances, pharmaceutical formulations, flavouring, and aromatherapy. The chemotypes can be further explored for commercial development**.**

5.6. Conclusions

Several conclusions drawn from the various components of this study and recommendations are hereby listed.

5.6.1. Phytochemistry and chemotaxonomy

This section covered objective 1 and 4 of the study and the following conclusions were made:

- The essential oil yields for the genus are relatively low compared to the non-volatile yields and may be affected by genetic, environmental, edaphic and agronomic variables.
- The TLC screening of oils and leaf extracts showed variability within individuals of a species from a population. Hence, it is advisable that multiple samples of a given species be collected to enable understanding of the extent of variation in the group and to avoid reporting of misleading results.
- The essential oils (86 samples) of the 22 species of *Eriocephalus* studied are comprised of acyclic, monocyclic and bicyclic mono- and sesquiterpenes. Two hundred (200) compounds were realized from the GC/MS analysis with the most common compounds being α- and β-pinene, yomogi alcohol, ρ-cymene, 1,8-cineole, camphor, 4-terpineol, spathulenol, caryophyllene oxide, α-copaene and β-caryophyllene with most species having high content of 1,8-cineole and camphor.
- The study has defined 22 chemotypes based on the highest percentage value for the major compounds present in the taxa of the species studied (Chapter 2, Table 2.8).

- The essential oils have a potential for use in flavouring, fragrances, cosmetics, industrial chemicals, pharmaceutical formulations and aromatherapy.
- The quantitative and qualitative essential oil chemistry of this group is very complex and lack of coherence in grouping of taxa makes it difficult to assign infra-specific ranks. The taxa grouped according to similarities in their chemistry irrespective of specific boundaries. The current species boundaries are not supported by the terpene chemistry.
- Cluster analysis of qualitative and quantitative data revealed that the relationships between species of *Eriocephalus* are highly complex and lack complete coherence at intra- and infra-specific groupings hence rendering it difficult to assign any ranks to the groups. However, affinity groupings were noted; *E. brevifolius-E. africanus-E. punctulatus, E. aromaticus-E. grandiflorus-E. brevifolius, E. decussatus-E. spinescens, E. ericoides* subp. *ericoides-E. microphyllus-E. purpureus, E. eximius-E. africanus.*
- Several factors affecting the complex chemistry include differential gene expression, chemical polymorphism, allelochemical diversification, convergence and divergence.
- Terpenes are highly complex and may not be suitable taxonomic markers in the genus.
- The non-volatile components of the acetone leaf extracts are characterized by the presence of various classes of flavonoids as shown by the TLC screening. The most abundant being the flavones and the flavanones.
- The quantitative flavonoid analysis did not reveal coherent specific limits of the group.

Phylogenetic conclusions

- The study has provided a preliminary ITS phylogeny of 21 species of *Eriocephalus*
- The plastid *psb*A-*trn*H intergenic spacer and *trn*L-F region were not variable among species of the genus.
- The nuclear ITS gene did not provide variable characters for phylogenetic reconstruction but the number of characters were too few for resolution of most relationships between species.
- The combined chemical (terpenes) and ITS phylogeny gave some resolved relationships but the pattern of the grouping of taxa was incongruent with existing species delimitation that it was difficult to assign infra-specific ranks to the taxa. However, several affinity groups were realized namely: *E. purpureus, E. punctulatus-E. aromaticus, E. pinnatus-E. microphyllus, E. spinescens-E. decussatus, E.*

racemosus-E. africanus-E. eximius-E. luederitzianus, E. racemosus-E. klinghardtensis, E. brevifolius-E. grandiflorus and *E. aromaticus-E. grandiflorus* and *E. brevifolius-E. africanus-E. scariosus.*

- The chemical phylogenetic tree did not give coherent groups, thus an indication that terpene characters are not strong taxonomic markers for delimitation of the taxa of *Eriocephalus*.
- It was difficult to define the specific relationships within the genus due inconsistent groupings that may be due to chemical convergence and divergence, hybridization, differential gene expression, chemical polymorphism and allelochemical diversification. The current species boundaries are not supported by the ITS and the terpene chemistry.

5.6.2. Biological properties

This part covered objective 2 and 3 in the study and the following conclusions were made:

- This study provides the first comprehensive record of biological properties of the genus through *in vitro* screening.
- The essential oils are more active than the acetone leaf extracts. The essential oils showed profound activity against *Cryptococcus neoformans* and *Bacillus cereus* and moderate to low activity against the rest of the test pathogens. Therefore, they have potential for use in combating fungal and bacterial infections that pose great health risks.
- No obvious direct correlation was noted between chemical composition of the oils and antimicrobial activity. Hence, the activity of the oils is most likely due to the interplay of major and minor compounds exerting synergistic or antagonistic effects, and other intrinsic and extrinsic factors.
- Variability in activity exists in the essential oils and acetone leaf extracts in individuals of the same species as was observed between individuals from different populations.
- The antimicrobial activities noted in the essential oils and extracts against the test pathogens provide a scientific rationale or validation of the use of some of the taxa in traditional herbal remedies for treatment of respiratory ailments, dermal infections, and gastro-intestinal disorders.
- The essential oils of the genus have potential as a source of natural products with inhibitory effects against the enzymes responsible for mental related diseases and as disinfectants as was noted by their inhibition of acetylcholinesterase.

- The essential oils of *Eriocephalus* have antiinflammatory properties as evidenced by their inhibitory activity against 5-lipoxygenase enzyme. This makes them valuable for use in cosmaceuticals as well as providing the scientific support for the already commercially exploited species.
- The antioxidant activities of the acetone leaf extracts have been recorded for the first time in the genus but the essential oils were inactive in the DPPH assay.

5.7. Recommendations

- Further research needs be carried out using other chemical characters (e.g. sesquiterpene lactones) to see whether relationships between the species can be better determined.
- The collection of the remaining species should be made for completion of the ITS phylogeny and chemistry profile of the genus.
- Water and methanol leaf extracts should be tested for antimicrobial properties as in the current study only apolar extracts were tested and results obtained may be different.
- Toxicity of the oils and the leaf extracts should be tested.
- Further analysis and identification of flavonoids should be carried out to help in understanding the relationships among species of this genus.
- There is need for bioassay-guided fractionation of the active compounds in the acetone leaf extracts of *E. aromaticus.*
- Further studies on the genus to determine if there is hybridization and the extent of the same is important.
- From the results of the study, there is great need to adopt multiple samples sampling strategies as this gives a clearer view of the range of variation in a particular plant group.

References

Adamson, R.S. and Salter, T.M. (1950). Flora of the Cape Peninsula. Juta, Cape Town.

Aligiannis, N., Kalpoutzakis, E., Mitaki, S. and Chinou, I.B. (2001). Composition and antimicrobial activity of the essential oils of two *Origanum* species. *Journal of Agriculture Food Chemistry* 49: 4168–4170.

Aljadi, A.M. and Kamaruddin, M.Y. (2004). Evaluation of the phenolic contents and antioxidant capacities of two Malaysian floral honeys. *Food Chemistry* 85: 513–518.

Alvarez-Castellanos, P.P., Bishop, C.D. and Pascual-Villalobos, M.J. (2001). Antifungal activity of the essential oil of flowerheads of garland Chrysanthemum (*Chrysanthemum coronarium*) against pathogens. *Phytochemistry* 57: 99–102.

Amarowicz, R., Pegg, R.B., Rahimi-Moghaddam, Barl, B. and Weil, J.A. (2004). Free radical scavenging capacity and antioxidant activity of selected plant species from the Canadian praires. *Food Chemistry* 84: 551–562.

Ammon, H.P.T. (1996). Salai Guggal- *Boswellia serrata*: from a herbal medicine to a specific inhibitor of leukotriene biosynthesis. *Phytomedicine* 3: 67–70.

Ammon, H.P.T., Mack, T., Singh, G.B. and Safayhi, H. (1991). Inhibition of leukotriene B_4 formation in rat peritoneal neitrophils by an ethanolic extract of the gum resin exudates of *Boswellia serrata. Planta Medica* 57: 203–207.

APG II, (2003). An update of the Angiosperm phylogeny group classification for the orders and families of flowering plants. *Botanical Journal of the Linnean Society* 141:399–436.

Bağci, E. and Diğrak, M. (1996). Antimicrobial activity of essential oils of some *Abies* (Fir) species from Turkey. *Flavour and Fragrance Journal* 11: 251–256.

Baldwin, B.G. (1992). Phylogenetic utility of the internal transcribed spacers of nuclear ribosomal DNA in plants: An example of the Compositae. *Molecular Phylogenetics and Evolution* 1: 3–16.

Baldwin, B.G., Sanderson, M.J., Porter, J.M., Wojciechowski, M.F., Campbell, C.S. and Donoghue, M.J. (1995). The ITS region of nuclear ribosomal DNA. A valuable source of evidence on Angiosperm phylogeny. *Annuals of Missouri Botanical Garden* 82: 247–277.

Baratta, M.T., Dorman, H.J.D., Deans, S.G., Figueiredo, A.C., Barroso, J.G. and Ruberto, G. (1998). Antimicrobial and antioxidant properties of some commercial essential oils. *Flavour and Fragrance Journal* 13: 235–244.

Baylac, S. and Racine, P. (2003). Inhibition of 5-lipoxygenase by essential oils and other natural fragrant extracts. *The International Journal of Aromatherapy* 13: 138–142.

Benrezzork, R., Terencio, M.C., Ferrandiz M.L., Hernandez-Perez, M., Rabanal, R. and Alcaraz, M.J. (2001). Inhibition of 5-lipoxygenase activity by the natural antiinflammatory compound aethiopinone. *Inflammation Research* 50: 96–101.

Bentham, G. (1873). Ordo 88. In Bentham, G. and Hooker, J.D., *Genera Plantarum* 2:163-533. Reeve, London.

Bohm, B.A. and Stuessy, T.F. (2001). Flavonoids of Sunflower Family (Asteraceae). Springer Wien, NewYork.

Braca, A., Fico, G., Morelli, I., De Simone, F. and De Tommasi, N. (2003). Antioxidant and free radical scavenging activity of flavonol glycosides from different *Aconitum* species. *Journal of Ethnopharmacology* 86: 63–67.

Brantner, A. and Grein, E. (1994). Antibacterial activity of plant extracts used externally in traditional medicine. *Journal of Ethnopharmacology* 44: 35–40.

Bremer, K. (1994). Asteraceae, Cladistics and Classification. Timber Press, Oregon.

Bremer, K. and Humphries, C. (1993). Generic monograph of the Asteraceae- Anthemideae. *Bulletin of Natural History Museum* 23:1–77.

Brower, A.V.Z., Desalle, R. and Vogler, A. (1996). Gene trees, species trees and systematics. *Annual Review of Ecology and Systematics* 27: 423–450.

Brunell, M.S. and Whitkus, R. (1999). Assessment of morphological variation in *Eriastrum densifolium* (Polemoniaceae): Implications for subspecific delimitation and conservation. *Systematic Botany*: 23(3): 351–368.

Candan, F., Unlu, M., Tepe, B., Daferera, D., Polissiou, M., Sokmen, A. and Akpulat, H. (2003). Anti-oxidant and antimicrobial activity of the essential oil and methanol extracts of *Achillea millefolium* subsp. *millefolium* Afan. (Asteraceae). *Journal of Ethnopharmacology* 87: 215–220.

Carson, C.F. and Riley, T.V. (1995). Antimicrobial activity of the major componenets of the essential oil of *Melaleuca alternifolia*. *Journal of Applied Bacteriology*, 78: 264–269.

Cimanga, K., Kambu, K., Tona, L., Apers, S., De Bruyne, T., Hermans, N., Joffe, J., Pieters, L. and Vlietinck, A.J. (2002). Correlation between chemical composition and anti-bacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. *Journal of Ethnopharmacology* 79: 213–220.

Clegg, M.T. and Zurawski, G. (1992). Chloroplast DNA and the Study of Plant Phylogeny: Present Status and Future Prospects. *In* Soltis, P.S., Soltis, D.E. and Doyle, J.J. (Eds) Molecular Systematics of Plants. Chapman and Hall, London.

Cornwell, P.A. and Barry, B.W. (1994). Sesequiterpene components of volatile oils as skin penetration enhancers for the lipophilic permeant 5-fluorouracil. *Journal of Pharmacy and Pharmacology*, 46: 261-269.

Cornwell, P.A., Barry, B.W., Bouwstra, J.A. and Gooris, G.S. (1996). Modes of action of terpene penetration enhancers in human skin: differential scanning calorimetry, small-angle x-ray diffraction and enhancer uptake studies. *International Journal of Pharmaceutics*, 127: 9–26.

Cowan, M.M. (1999). Plant products as antimicrobials. *Clinical Microbiology Reviews* 12: 564–582.

De Candolle, A. P. (1838). Compositae. Prodromus systematis naturalis regni vegetalis 6. Treuttel and Wurtz, Paris.

De Queiroz, A., Donoghue, M.J. and Kim, J. (1995). Separate versus combined analysis of phylogenetic evidence. *Annual Review of Ecology and Systematics* 26: 657–681.

Dewick, P.M. (2001). Medicinal Natural Products. A Biosynthetic Approach. 2nd Edition. John Wiley and Sons, England.

Donoghue, M. J. and Sanderson, M.J. (1992). The suitability of molecular and morphological evidence in reconstructing plant phlogeny. In, Soltis, P.S., Soltis, D.E. and Doyle, J.J. eds. Molecular systematics II. NewYork and London. Chapman and Hall.

Dorman, H.J.D. and Deans, S.G. (2000). Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *Journal of Applied Microbiology* 88: 308–316.

Doyle, J.J. and Doyle, J.L. (1987). A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry Bulletin* 19: 11–15.

Dural, H., Bagci, Y., Ertugrul, K., Demirelma, H., Flamini, G., Cioni, P.L. and Morelli, I. (2003). Essential oil composition of two endemic *Centaurea* species from Turkey, *Centaurea chrysantha,* collected in the same habitat. *Biochemical Systematics and Ecology* 31: 1417–1425.

Dyson, A. (1998). Discovering Indigenous Healing Plants of Herb and Fragrance Gardens at Kirstenbosch, National Botanical Garden. National Botanical Institute, Cape Town.

Echeverrigaray, S., Fracaro, F., Atti dos Santos, A.C., Paroul, N., Wasum, R. and Atti Serafini, L. (2003). Essential oil composition of South Brazilian populations of *Cunila galioides* and its relation with geographic distribution. *Biochemical Systematics and Ecology* 31: 467–475.

Eloff, J.N. (1998). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica* 64: 711–713.

Emerenciano, V.P., Ferreira, Z.S., Kaplan, M.A.C. and Gottlieb, O. R. (1987). A chemosystematic analysis of tribes of Asteraceae involving sesquiterpene lactones and flavonoids. *Phytochemistry* 26: 3103–3115.

Farris, J.S. (1969). A successive approximations approach to character weighting. *Systematic Zoology* 18: 274–385.

Felsenstein, J. (1985). Confidence limits in phylogenies: an approach using the bootstrap *Evolution* 39: 783–791.

Fitch, W.M. (1971). Toward defining the course of evolution: minimum change for a specific tree topology. *Systematic Zoology* 20:406–416.

Goldblatt, P. and Manning, J. (2000). Cape Plants. A Conspectus of the Flora of South Africa. NBI, South Africa & Missouri Botanical Garden, U.S.A.

Goldblatt, P., Savolainen, V., Porteous, O., Sostaric, I., Powell, M., Reeves, G., Manning, .C., Barraclough, T.G. and Chase, M.W. (2002). Radiation in the Cape Flora and the phylogeny of the peacock irises *Moraea* (Iridaceae) based onfour plastid DNA regions. *Molecular Phylogenetics and Evolution.* 25: 341–360.

Grabmann, J., Hippeli, S., Dornisch, K., Beuscher, N. and Elstner, E. F. (2000). Antioxidant properties of essentials oils. Possible explanations for their anti-inflammation effects. *Arzneim-Forsch/drug Research*. 50: 135–139.

Grayer, R.J., Chase, M.W. and Simmonds, M.S.J. (1999). A comparison between chemical and molecular characters for the determination of phylogenetic relationships among plant families: An appreciation of Hegnauer's "Chemotaxonomic Der Pflanzen". *Biochemical Systematics and Ecology* 27: 369–393.

Grayer, R.J., Kite, G.C., Goldstone, F.J., Bryan, S.E., Paton, A. and Putievsky, E. (1996). Infraspecific taxonomy and essential oil chemotypes in sweet basil, *Ocimum basilicum. Phytochemistry,* 43: 1033–1039.

Greger, H. (1977). Anthemideae-Chemical Review. *In* Heywood, V.H., Humphries, C. and Turner, B.L. Eds The Biology and Chemistry of the Compositae. Vol II. Academic Press, (London) Ltd.

Griffin, S. G., Wyllie, S.G., Markham, J.L. and Leach, D.N. (1999). The role of structure and the molecular properties of terpenoids in determining their antimicrobial activity. *Flavour and Fragrance Journal* 14: 322–332

Hammer, K. A., Carson, C.F. and Riley, T. V. (1999). Antmicrobial activity of essential oils and other plant extracts. *Journal of Applied Microbiology* 86: 985–990.

Harbourne, J.B. (1977). Flavonoids in the compositae. *In* Heywood, V. H., Humphries, C. and Turner, B.L. Eds The Biology and Chemistry of the Compositae. Vol I. Academic Press Inc. (London) Ltd.

Harbourne, J.B. (1984). Chemical data in plant taxonomy. *In* Heywood, V.H. and Moore, D.M. Eds. Current Concepts in Plant Taxonomy. The Systematics Association Special Volume No. 25. Academic Press, London.

Harvey, W.H. (1865). *Lasiospermum* and *Eriocephalus*. In W.H.Harvey and O.W. Sonder, *Flora Capensis* 3: 153–154.

Harvey, W.H. and Sonder, O.W. (1865). *Flora Capensis* 3: 199-204.

Hawsksworth, D.L. ed. (1988). Prospects in Systematics. Claredon Press Oxford.

Hegnauer, R. (1977). The Chemistry of the Compositae. *In* Heywood, V. H., Humphries, C. and Turner, B.L. Eds The Biology and Chemistry of the Compositae. Vol I. Academic Press Inc. (London) Ltd.

Heitzman, M.E., Neto, C.C., Winiarz, E., Vaisberg, A.J. and Hammond, G.B. (2005). Ethnobotany, phytochemistry and pharmacology of *Uncaria* (Rubiaceae). *Phytochemistry* 66: 5-29.

Heywood, V.H. (1976). Plant Taxonomy. 2nd edition. Edward Arnold, Britain.

Heywood, V.H. and Humphries, C.J. (1977). Anthemideae, Systematic Review. *In* Heywood, V.H., Humphries, C. and Turner, B.L. Eds. The Biology and Chemistry of the Compositae. Vol II. Academic Press Inc. (London) Ltd.

Heywood, V.H. and Moore, D.M. (1984). Current Concepts in Plant Taxonomy. The Systematics Association Special volume No. 25. Academic Press, London.

Heywood, V.H., Harbourne, C.J. and TurnerB.L. (1977). An Overture to the Compositae. The Biology and Chemistry of the Compositae. Vol I. Academic Press Inc. (London) Ltd.

Hind, D.J.N. and Beentje, H.J. Eds. (1996). Compositae: Systematics. Proceedings of the International Compositae Conference, 1994, Kew. Vol. 1. Royal Botanic Gardens, Kew.

Hind, D.J.N. Jeffrey, C. and Pope, G.V. Eds. (1995). Advances in Compositae Systematics. The Royal, Botanic Gardens, Kew.

Hostettmann, K. (1999). Strategy for the biological and chemical evaluation of plant extracts. Http://www.iupac.org/symposia/proceedings/phuket97/hostettmann.html.

Houghton, P.J. and Raman, A. (1998). Laboratory Handbook for the Fractionation of Natural Extracts. Chapman and Hall, London.

Http://www.africasgarden.com/library.htm 2002/05/13.

Http://www.biomedcentral.com/1471–2148/2/17.

Http://www.botany.unp.ac.za/rcpge/eb_current_research.htm 2002/05/13.

Http://www.gnp.co.za/essentialoils/chamomile.2002.

Http://www.gnp.co.za/May 2002.

Ikan, R. (1991). Natural Products. A Laboratory Guide. 2nd Edition. Academic Press, Inc., USA.

Jansen, R.K. and Kim, K.J. (1996). Implications of chloroplast DNA data for the classification and phylogeny of Asteraceae. In Hind, D.J.N., and Beentje, H.J. (Eds). Compositae: Systematics. Proceedings of the International Compositae Conference, 1994, Kew. Vol. 1. Royal Botanic Gardens, Kew.

Jansen, R.K., Michaels, H.J., Wallace, R.S. and Palmer, J.D. (1992). Chloroplast variation in Astereceae: Phylogenetic and Evolutionary implications. In Soltis, P.S., Soltis, D.E. and Doyle, J.J. Eds. Molecular Systematics of Plants. Chapman and Hall, London.

Janssen, A. M., Scheffer, J. J. C. and Svendsen, B.A. (1987). Antimicrobial Activity of Essential oils: A 1976–1986 Literature Review. Aspects of the Test Methods. *Planta Medica* 53: 395–398.

Johnson, S.D. (1996). Pollination, adaptation, and speciation models in the Cape flora of South Africa. *Taxon* 45: 59–66.

Judd, W.S., Campbell, C.S., Kellogg, E.A. and Stevens, P.F. (1999). Plant Systematics. A Phylogenetic Approach. Sinaeur Associates, Inc. Sunderland, Massachusetts, U.S.A.

Juteau, F., Masotti, V., Bessiere, J-M. and Viano, J. (2002). Compositional characteristics of essential oil of *Artemisia campestris* var *glutinosa. Biochemical Systematics and Ecology* 30:1065–1070.

Kephart, S., Sturgeon, K., Lum, J. and Bledsoe, K. (1999). Varietal relationships in *Silene douglasii* (Caryophyllaceae): Morphology variability at population level. *Systematic Botany*, 24:529–544.

Knobloch, K., Paul, A., Iberl, B., Weigand, H. and Weis, N. (1989). Antibacterial and antifungal properties of essential oil components. *Journal of Essential Oils Research* 1:119–128.

Koekemoer, M. (1996). An Overview of Asteraceae of Southern Africa. In Hind, D.J.N., and Beentje, H.J. eds. Compositae: Systematics. Proceedings of the International Compositae Conference 1994, Kew. Vol. 1. Royal Botanic Gardens, Kew.

Kornkven, A.B., Watson, L.E. and Estes, J.R. (1998). Phylogenetic analysis of *Artemisia* section *Tridentate* (Asteraceae) based on the sequences from internal transcribed spacer (ITS) of nuclear ribosomal DNA. *American Journal of Botany*. 85: 1787–1795.

Kornkven, A.B., Watson, L.E. and Estes, J.R. (1999). Molecular phylogeny of *Artemisia* sect. Tridentate (Asteraceae) based on chloroplast DNA restriction site variation. *Systematic Botany* 24: 69–84.

Kubitzki, K. (1984). Phytochemistry in Plant Systematics and Evolution. *In* Heywood, V.H. and Moore, D.M.Eds. Current Concepts in Plant Taxonomy. The Systematics Association Special Volume No. 25. Academic Press, London.

Kulisic, T., Radonic, A., Katalinic, V and Milos, M (2004). Use of different methods for testing antioxidative activity of oregano essential oil. *Food Chemistry*, 58:633–640.

Kumar, S., Ziereis, K., Wiegrebe, W and Muller, K. (2000). Medicinal plants from Nepal: evaluation as inhibitors of leukotriene biosynthesis. *Journal of Ethnopharmacology*, 70: 191-195.

Langström, E. and Oxelman, B. (2003). Phylogeny of *Echiochilon* (Echiochileae, Boranginaceae) based on ITS sequences and morphology. *Taxon* 52: 725–735.

Leistner, O. A. (ed). (2000). Seed plants of Southern Africa: Families and genera. Sterlitzia 10. National Botanical Institute, Pretoria.

Leppik, E.A. (1977). The Evolution of Capitulum Types of the Compositae in the light of Insect-flower Interaction. In Heywood, V.H., Harbourne, J.B. and Turner, B.L. Eds. (1977). The Biology and Chemistry of the Compositae. Vol. I. Academic Press Inc. (London) Ltd.

Lourens, A.C.U., Reddy, D., Başer, K.H.C., Viljoen, A.M. and Van Vuuren, S.F. (2004). *In vitro* biological and essential oil composition of four indigenous South African *Helichrysum* species. *Journal of Ethnopharmacology* 95: 253–258.

Maddison, W.P. and Maddison, D.R. (1999). MacClade, Version 4.0b, Analysis of Phylogeny and Character Evolution. Sinaeur Associates, Inc. Sunderland, Massachusetts, U.S.A.

Mangena, T. and Muyima, N. Y. O. (1999). Comparative evaluation of the antimicrobial activities of essential oils of *Artemisia afra, Pteronia incana* and *Rosmarinus officinalis* on the selected bacteria and yeast strains. *Letters in Applied Microbiology* 28, 291–296.

Manthey, J.A. and Busling, B.S. (1998). Flavonoids in the Living Systems. Advances in Experimental Medicine and Biology. Vol 439. Plenum Publishing Corporation.

Marius, B. (1995) a leaf from the Herbarium notes from Selmar Schonland Herbarium. Issue 2, September 1995. Http://www.ru.ac.za/affiliates/herbarium/leaf2c.html (02/22/2001).

Marston, A., Kissling, J. and Hostettmann, K. (2002). A rapid TLC bioautographic method for the detection of acetylcholinenesterase and butyrylcholinesterase inhibitors in plants. *Phytochemical Analysis* 13:51–54.

Martin, G.J. (1995). Ethnobotany. A People and Plants Conservation Manual. Chapman and Hall, London, U.K.

Merle, H., Movon, M., Blazquez, M.A. and Boira, H. (2004). Taxonomical contribution of essential oil in Mandarin cultivars. *Biochemical Systematics and Ecology* 32: 491–497.

Mierendorff, H-G., Stahl-Biskup, E., Posthumus, M.A. and van Beek, T.A. (2003). Composition of commercial Cape chamomile oil (*Eriocephalus punctulatus*). *Flavour and Fragrance Journal* 18:510–514.

Minelli, A. (1993). Biological Systematics. The State of the Art. Chapman and Hall, London.

Mulholland, D.A. and Drewes, S.E. (2004). Global phytochemistry: indigenous medicinal chemistry on track in southern Africa. *Phytochemistry* 65: 769–782.

Müller, M.A.N. (1988). `n Morfologiese en taksonomiese studie van die genusse *Lasiospermum* Lag. en *Eriocephalus* L. (Asteraceae) in suidelike Afrika. Unpublished PhD. thesis, University of Stellenbosch, Stellenbosch.

Müller, M.A.N., Herman, P.P.J. and Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum*. *Flora of Southern Africa*, Vol.33: 1-63.

Nakatsu, T., Lupo, A.T., Chinn, J.W. and Kang, R.K.L. (2000). Biological activity of essential oils and their constituents. *Studies in Natural Products Chemistry*, 21: 571–631.

Nyiligira, E., Viljoen, A.M., Başer, K.H.C. and Van Vuuren, S.F. (2004). Essential oil composition and *in* vitro antimicrobial and antiinflammatory activity of South African *Vitex* species. *South African Journal of Botany* 70:610-616.

Ohsaki, A., Shibata, K., Kubota, T. and Tokoroyama, T. (1999). Phylogenetic and chemotaxonomic significance of diterpenes in some *Portulaca* species (Portulaceae). *Biochemical Systematics and Ecology* 27: 289–296.

Oladimeji, F.A., Orafidiya, L.O. and Okeke, I.N. (2004). Physical properties and antimicrobial activities of leaf essential oil of *Lippia multiflora* Moldenke. *The International Journal of Aromatherapy* 14: 162–168.

Paton, A.J., Springate, D., Suddee, S., Otieno, D., Grayer, J.R., Harley, M.M., Willis, F., Simmonds, M.S.J., Powell, M.P. and Savolainen, V. (2004). Phylogeny and evolution of basils and allies (Ocimeae, Labiatae) based on three plastid DNA regions. *Molecular Phylogenetics and Evolution* 31: 277–299.

Pauli, A. (2001). Antimicrobial properties of essential oil constituents. *The International Journal of Aromatherapy* 11: 26–33.

Peana, A.T., D'Aquila, P.S., Panin, F., Serra, G., Pippia, P. and Moretti, M.D.L. (2002). Antiinflammatory activity of linalool and linalyl acetate constituents of essential oils. *Phytomedicine* 9: 721–726.

Perry, E.K., Pickering, A.T., Wang, W.W., Houghton, P.J. and Perry, N.S.L. (1999). Medicinal plants and Alzheimer's disease: from ethnobotany to phytotherapy. *Journal of Pharmacy and Pharmacology* 51: 527–534.

Povh, N.P., Marques, M.O.M. and Meireles, M. A.A. (2001). Supercritical CO_2 extraction of essential oil and Oleoresin from Chamomile (*Chamomilla recutita* (L.) Rauschert). *Journal of Supercritical Fluids* 21:245–256.

Rabe, T. and Van Staden, J. (1997). Antibacterial activity of South African Plants used for medicinal purposes. *Journal of Ethnopharmacology* 56: 81–87.

Radford, A.E. (1986). Fundamentals of Plant Systematics. Harper and Row Publishers, New York.

Reeves, G., Chase, M.W., Goldblatt, P., Rudall, P., Fay, M.F., Cox, A.V., Lejeune, B. and Souza-Chies, T. (2001). Molecular systematics of Iridaceae: Evidence from four plastid DNA regions. *American Journal of Botany* 88: 2074–2087.

Rohlf, F.J. (1998). NTSYSpc, Numerical Taxonomy, and Multivariate Analysis System. Version 2, User Guide. Exeter soft ware. Applied Biostatistics Inc., New York.

Roussis, V., Tsoukatou, M., Petrekis, I.C., Skoula, M. and Harbourne J.B. (2000). Volatile constituents of four *Helichrysum* species growing in Greece. *Biochemical Systematics and Ecology* 28: 163–175.

Rukangira, E. (2000). Medicinal plants and traditional medicine in Africa: constraints and challenges. Sustainable development international (2000). 179–184. Http://www.mtnforum.org/calender/events/0005mpta.htm.

Salie, F., Eagles, P.F.K. and Leng, H.M.J. (1996). Preliminary antimicrobial screening of four South African Asteraceae species. *Journal of Ethnopharmacology* 52:27–33.

Samuel, R., Stuessy, T.F., Tremetsberger, K., Baeza, C.M. and Siljak-Yakovlev, S. (2003) Phylogenetic relationships among species of *Hypochaeris* (Asteraceae, Chichorieae) based on ITS, plastid *trnL* intron, *trnL-F* spacer, and *matK* sequences. *American Journal of Botany* 90: 496–507.

Sanchez-Moreno, C. (2002). Review: Methods to evaluate the free radical scavenging activity in foods and biological systems. *Food Science and Technology International* 8: 121–137.

Sang, T., Crawford, D.J., Kimand, S-C. and Stuessy, T.F. (1997). Chloroplast DNA phylogeny, reticulate evolution, and biogeography of *Paeonia* (Paeoniaceae). *American Journal of Botany* 84:1120–1136.

Savolainen, V., Cuenoud, P., Spichinger, R., Martinez, M.D.P., Crevecouer. M. and Manen, J.F. (1995). The use of herbarium specimens in DNA phylogenetics: evaluation and improvement. *Plant systematics and Evolution* 197: 87–98.

Schmidt, G.J. and Schilling, E.E. (2000). Phylogeny and Biogeography of *Eupatorium* (Asteraceae: Eupatorieae) based on nuclear ITS sequence data. *American Journal of Botany* 87: 716–726.

Shale, T.L., Stirk, W.A. and Van Staden, J. (1999). Screening of medicinal plants used in Lesotho for anti-bacterial and antiinflammatory activity. *Journal of Ethnopharmacology* 67: 347–354.

Simpson, B.B. and Ogorzaly, M.C. (1995). Economic Botany, Plants of the World. 2nd edn. McGraw Hill Inc. U.S.A.

Skaltsa, H.D., Mavrommati, A. and Constantinidis, T. (2001). A chemotaxonomic investigation of volatile constituents in *Stachys* subsec. Swainsonianeae (Labiatae). *Phytochemistry* 57: 235–244.

Smith, P.M. (1976). The Chemotaxonomy of Plants. Edward Arnold, London.

Sneath, P.A. and Sokal R.R. (1973). Numerical Taxonomy. W.H.Freeman and Company. San Franscisco.

Sokmen, A. (2004). Antimicrobial and antioxidative activities of the essential oils and methanol extracts of *Salvia cryptantha* (Montbret et Aucher ex Benth.) and *Salvia multicualis* (Vahl). *Food Chemistry* 84: 519–525.

Sokmen, A., Gulluce, M., Akpulat, H.A., Daferera, D., Tepe, B., Polissiou, M., Sokmen, M. and Sahin, F. (2004). The *in vitro* antimicrobial activities of the essential oils and methanol extracts of endemic *Thymus spathulifolius. Food Control* 15: 627–634.

Soltis, D.E., Soltis P.S. and Doyle J.J. (1998). Molecular Systematics of Plants. DNA Sequencing. Kluwer Academic Publishers, U.S.A.

Soltis, P.S., Soltis, D.E. and Doyle, J.J. Eds. (1992). Molecular Systematics of Plants. Chapman and Hall, London.

Stace, C.A (1989). Plant Taxonomy and Biosystematics. 2nd edition. Cambridge, University Press.

Staden, M.D. and Myers, S.P. (2004). The roles of essential oils in the modulation of immune function and inflammation: survey of aromatherapy educators. *The International Journal of Aromatherapy* 14: 150–161.

Svoboda, K.P. and Hampson, J.B. (1999). Bioactivity of essential oils of selected temperate aromatic plants: antibacterial, antioxidant, antiinflammatory and other related pharmacological activities. Http://www.cls.gov.uk/Svoboda.pdf.

Swain, T. (1963). Chemical Plant Taxonomy. Academic press. London and New York.

Swanepoel, D.P. (1997). The medicinal value of the South African Asteraceae. Masters Thesis - Faculty of Biological and Agricultural Sciences of the University of Pretoria, Pretoria.

Swofford, D. L. (1998). PAUP* 4.0b 10. Phylogenetic analysis using parsimony (*and other methods). Sinaeur, Sunderland, Massachussets, U.S.A.

Swofford, D.L. (2000). PAUP* 4.02b. Phylogenetic analysis using parsimony (*and other methods). Sinaeur, Sunderland, Massachussets, U.S.A.

Szentmihalyi, K., Forgacs, E., Hajdu, M. and Then, M. (2001). *In vitro* study on the transfer of volatile oil components. *Journal of Pharmaceutical and Biochemical Analysis* 24: 1073-1080.

Szoke, E., Maday, E.,M Tyihak, E., Kuzovkina, I. N. and Lemberkovics, E. (2004). New terpenoids in cultivated and wild Chamomile (*in vivo* and *in vitro*). *Journal of Chromatography* 800: 231–238.

Tepe, B., Donmez, E., Unlu, M., Candan, F., Daferera, D., Vardar-Unlu, G., Polissiou, M. and Sokmen, A. (2004). Antimicrobial and antioxidative activities of the essential oils and methanol extracts of *Salvia cryptantha* (Montbret et Aucher ex Benth.) and *Salvia multicualis* (Vahl). *Food Chemistry* 84:519–525.

Trouillas, P., Calliste, C-A., Allais, D-P., Simon, A., Marfak, A., Delage, C. and Duroux, J-L. (2003). Antioxidant, antiinflammatory and antiproliferative properties of sixteen water plant extracts used in Limousin countryside as herbal teas. *Food Chemistry* 80: 399–407.

Van der Bank, M., Chase M.M., Van Wyk, B-E., Fay, M.F., Van Der Bank, F.H., Reeves,G. and Hulme, A. (2002). Systematics of the tribe Podalyrieae (Fabaceae) based on DNA, morphological and chemical data. *Botanical Journal of the Linnaean Society*, 139: 159–170.

Van Wyk, B-E. (2002). A review of ethnobotanical research in southern Africa. *South African Journal of Botany* 68: 1–13.

Van Wyk, B-E. and Gericke, N. (2000). People's Plants. A Useful Guide to Useful Plants of Southern Africa. Briza Publications, South Africa.

Van Wyk, B-E., Van Oudtshooorn, B. and Gericke, N. (1997). Medicinal Plants of South Africa. Briza Publications. South Africa.

Viljoen, A., Van Vuuren, S., Ernst, E., Klepser, M., Dermici, B., Baser, H. and Van Wyk, B-E. (2003). *Osmitopsis asteriscoides* (Asteraceae) - the antimicrobial activity and essential oil composition of a Cape-Dutch remedy. *Journal of Ethnopharmacology* 88: 137–143.

Viljoen, A.M., Klepser, M.E., Ernst, E.J., Keele, D., Roling, E., van Vuuren, S., Dermici, B., Baser, K.H.C. and van Wyk, B-E. (2002). The composition and antimicrobial activity of the essential oil of resurrection plant *Myrothamnus flabellifolius. South African Journal of Botany* 68: 100–105.

Viollon, C. and Chaumont, J-P. (1994). Antifungal properties of essential oils and the main components upon *Cryptococcus neoformans. Mycopathologia* 129: 151–153.

Wagner, H. and Bladt, S. (1996). Plant Drug Analysis. A Thin Layer Chromatography atlas. 2nd edition. Springer, Germany.

Watson, L.E, Bates, P, Evans, T.M., Unwin, M.M. and Estes, J.R (2002). Molecular phylogeny of subtribe Artemisiinae (Asteraceae), including *Artemisia* and its allied and segregate genera. *BMC Evolutionary Biology*, Vol 2:17. Http://www.pubmedcentral.nih.gov/articlerender.fcgi?tool=pmcentrez&artd=130036. 5/5/05

Watson, L.E., Evans, T.M. and Boluarte, T. (2000). Molecular phylogeny and Biogeography of tribe Anthemideae (Asteraceae) based on chloroplast gene *ndh*F. *Molecular Phylogenetics and Evolution* 1: 59–69.

Watson, L.E., Evans, T.M., Unwin, M.M. Korkven, A.B. (2001). Origin and diversification of the tribe Anthemideae (Asteraceae) based on molecular phylogenies of *ndh*F and ITS. Botany 2001 "Plants and People" Conference paper. Albuquerque Convention Centre, New Mexico.

Watt, J.M. and Breyer-Brandwijk, M.G. (1962). The Medicinal and Poisonous Plants of Southern and Eastern Africa. 2 edition Livingstone, London

WHO (World Health Organization). (2002) Monographs on Selected Medicinal plants, Vol.2. World Health Organization, Geneva.

Williams, L. (1996). Ranking antimicrobial activity. *The International Journal of Aromatherapy* 7: 32–35.

Williams, L.R., Stockley, J.K., Yan, W. and Home, V.N. (1998). Essential oils with high antimicrobial activity for therapeutic use. *The International Journal of Aromatherapy* 8: 30–40.

Wink, M. (2003). Evolution of secondary metabolites from an ecological and molecular phylogenetic perspective. *Phytochemistry* 64: 3–19.

Wink, M. and Mohamed, G.I.A. (2003). Evolution of chemical traits in Leguminosae: mapping of distribution patterns of secondary metabolites on a molecular phylogeny inferred from nucleotide sequences of *rbc*L gene. *Biochemical Systematics and Ecology*, 31: 897–917.

Wollenweber, E. and Mann, K. (1989). Exudate flavonoids in three essential oil plants from the Ciskei (South Africa). *Fitoterapia* Volume LX No. 3.

Woodland, D.W. (1997). Contemporary Plant Systematics. 2nd edition. Andrews University Press, London.

Zdero, C., Bohlmann F. and Müller, M. (1987). Sesquiterpene lactones and other constituents from *Eriocephalus* species. *Phytochemistry* 26: 2763–2775.

APPENDICES

APPENDIX I: Monographs of the species of *Eriocephalus*



2005

Monographs in alphabetical order

1. ***E. africanus***
2. ***E. ambiguus***
3. ***E. aromaticus***
4. ***E. brevifolius***
5. ***E. capitellatus***
6. ***E. decussatus***
7. ***E. dinteri***
8. ***E. ericoides* subsp. *ericoides***
9. ***E. eximius***
10. ***E. grandiflorus***
11. ***E. klinghardtensis***
12. ***E. luederitzianus***
13. ***E. merxmuelleri***
14. ***E. microphyllus***
15. ***E. namaquensis***
16. ***E. pauperrimus***
17. ***E. pinnatus***
18. ***E. punctulatus***
19. ***E. purpureus***
20. ***E. racemosus* var *racemosus***
21. ***E. scariosus***
22. ***E. spinescens***

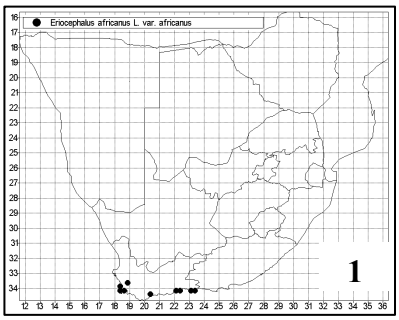
1. *E. africanus* L.

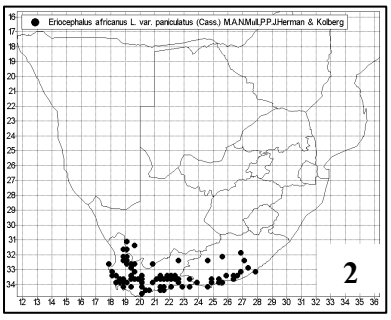
Synonyms
E. corymbosus Moench; *E. variifolius* Salisb.; *E. frutescens* R.Br.; *E. septifer* Cass.; *E. septulifer* DC; *E. paniculatus* Cass.; *E. racemosus* Gaertn.; *E. umbellulatus* Cass.; *E. umbellulatus* Cass. var. *glabriusculus* DC; *E. umbellulatus* Cass. var. *argenteus* DC; *E. sericeus* Gaudich; *Monochlaena racemosus* Cass.

Common name
Clustery leaved scentwort and wild rosemary (English), 'kapokbossie', 'wilde roosmaryn', 'renosterveld kapok' (Afrikaans).

Geographical distribution

Two varieties are recognized: *E. africanus* var *africanus* and *E. africanus* var *paniculatus.* The former is restricted to the coast of the Cape peninsula and occurs from Mossel Bay to Kynsna. The latter has the second widest distribution of all the species of *Eriocephalus* extending over the Northern, Western and Eastern Cape in Malmesbury, Melkbosstrand, Citrusdal, De Rust, and Sutherland and in various veld types. Distribution maps of *E. africanus* var *africanus* and *E. africanus* var *paniculatus* (Maps 1 and 2 respectively) (Müller *et al*., 2001).⇨





Botanical description
Much branched, spreading to erect, conical shrubs, 0.3-0.9 m high and up to 4 m in diameter. Old stems with anomalous secondary growth. Leaves mostly opposite, sometimes in whorls and alternate on flowering shoot; palmatisect or pinnatisect, succulent or not with grey-green to silver grey sericeous to felty sericeous indumentum. Capitula heterogamous radiate in terminal or lateral umbellate racemes or paniculate, peduncles almost absent. Ray florets white to red-purple. Paleae of marginal florets connate. Chromosome number 2n = 18, 36. Flowering periods correlated with rainfall and varying between July to September and January to March in summer rainfall areas (Müller *et al*., 2001).

Ethnobotanical uses

Medicinal
Traditionally used as a diaphoretic and diuretic. It is also used as a tincture for heart trouble, and in treatment of respiratory ailments, gastro-intestinal disorders, dermal infections including use as a dandruff rinse, footbaths and for gynecological conditions (delayed menstruation). It is also used in treatment of stress related ailments and depression.

Culinary uses
It is used as substitute for wild rosemary in flavouring of dishes such as meat, fish, and chicken e.t.c. The leaf infusion is used as tea.

Industrial
It is the source of commercial 'Cape snowbush oil', which is used as blend oil in skin care products and in aromatherapy.



The spreading habit of *E. africanus* var *africanus* in a coastal habitat (Mossel Bay).



The erect habit of *E. africanus* var *paniculatus* in an inland habitat (Sutherland).



Radiate capitula of *E. africanus* var *africanus* and succulent leaves.



Radiate capitula of *E. africanus* var *paniculatus.*

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.1% (wet weight) clear, pale yellow, yellow and deep blue oils.

GC/MS

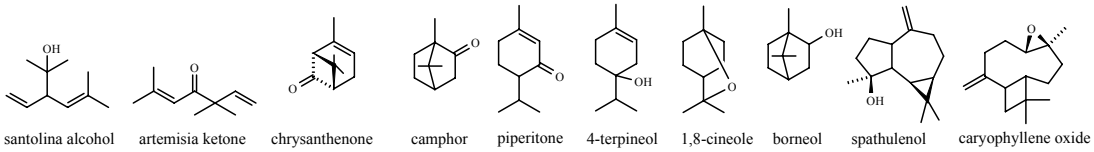
Major constituents
The essential contain approximately 81 compounds, which are summarised in Table 1.

Table 1. Essential oil composition and retention index (RI) of six populations of *E. africanus.* Pop 1-Malmesbury; Pop 2-Melkbosstrand; Pop 3-Citrusdal; Pop 4-De Rust; Pop 5-Mossel Bay; Pop 6-Sutherland/Farm Koornlandshloof (var *paniculatus*). Values are given in percentages. Compounds in bold represent some of the major compounds.

		Pop 1	Pop 2	Pop 3			Pop 4	Pop 5	Pop 6	
RI	**Compound**			**A**	**B**	**C**			**A**	**C**
901	Santolina triene	0.7	2.8							
922	α-Thujene		0.3					<0.35		
928	**α-Pinene**	0.8	3.1	<0.46			0.5	7.5	0.5	2.4
940	Camphene	0.6		<0.46				0.4		
963	**Sabinene**	0.2	8.4		<0.70	<0.41		2.0		
966	β-Pinene	0.3	1.3				1.3	1.3	1.2	2.0
984	β-Myrcene		0.8		<0.70	<0.41	0.9	0.4		
988	Yomogi alcohol	2.2	0.4	1.0				0.4		
1005	α-Terpinene		0.9		<0.70			1.0		
1008	**p-Cymene**	1.4	7.4	0.6	1.2	0.9	3.8	3.4	0.5	0.8
1016	**1,8-Cineole**	1.8	4.1	2.0	1.2	0.7	3.8	23.6	3.5	4.6
1018	Limonene	0.2	0.8		<0.70	<0.41	<0.48		0.2	0.5
1024	**Santolina alcohol**			29.9						
1045	**Artemisia ketone**	11.8		1.6				4.5		
1047	γ-Terpinene		4.3	<0.46	1.1	0.9		1.8		
1050	*Cis*-Sabinene hydrate							0.9		
1070	**Artemisia alcohol**	2.0		0.6						
1075	α-Terpinolene		0.4			<0.41		0.4		
1077	Filifolone						0.6	1.6		
1083	Linalool					0.6				0.3
1092	**Chrysanthenone**						3.6	18.5		
1099	MW=154					0.4		0.6		
1107	MW=154							1.2		
1110	**Camphor**			12.1	1.1		7.1		0.9	0.4
1114	*Trans*-pinocarveol						1.4	1.5	0.5	0.5
1129	Isopinocamphone								1.0	
1130	Pinocarvone							1.0		
1141	**Borneol**	1.6		10.0			1.9	2.7	1.2	0.9
1155	**4-Terpineol**	0.3	3.7	0.6	3.5	5.0	1.7	7.3	0.8	0.6
1160	Myrtenal							<0.35	0.3	0.4
1166	α-Terpineol				0.7	0.6	<0.48	<0.35	0.6	
1172	Myrtenol		0.7		2.7	0.6	0.9		0.3	
1210	O-Methylthymol	0.4								
1215	**Piperitone**				17.2	0.4				
1239	*Trans*-Chrysantemyl acetate							1.4		
1256	p-Cymen-7-ol									
1262	Bornyl acetate			0.5				<0.35	0.5	0.4
1336	**α-Longipinene**	0.6							3.7	
1357	***Cis*-Jasmone**		0.6							
1360	Geranyl acetate							0.8	0.8	
1362	α-Copaene	1.4		1.8		1.1	2.5		1.6	1.9
1363	**MW=204**		2.0							
1369	Isocomene	0.6	1.4			0.9	0.5			
1376	β-Elemene		1.3		0.7	0.9	0.6		0.4	0.8
1398	β-Caryophyllene		5.1		1.1	1.2	1.5	0.6	2.0	
1424	Neryl acetone					5.7				
1432	α-Humulene		0.7							
1461	Ar-Curcumene		1.0		1.6	2.4				
1471	MW=222									1.2
1474	Bicyclogermacrene		3.3	0.7	13.6	10.6				
1480	MW=222									1.3
1489	MW=222									1.0
1491	γ-Cadinene						1.0			
1500	δ-Cadinene		1.7					<0.35		
1520	MW=220									1.6
1530	MW=236									1.2
1535	Unknown 1							0.6		
1536	MW=220						2.1			
1542	Nerolidol			1.3						
1544	Spathulenol		9.5	0.6	25.5	30.3	19.3	1.2	17.2	40.0
1548	Caryophyllene oxide		6.8		3.8		9.8	1.0	4.5	10.1
1556	Viridiflorol or Globulol						1.0			1.8
1579	**MW=220**								10.4	
1581	MW=222			3.4						

		Pop 1	Pop 2	Pop 3			Pop 4	Pop 5	Pop 6	
RI	**Compound**			**A**	**B**	**C**			**A**	**C**
1582	MW=222			2.1						
1597	MW=222							1.1		
1599	MW=222								1.2	
1599	Main peak=136						5.0	1.4		
1607	α-Cadinol	2.5								
1608	α-Cadinol or τ-Muurolol			2.7			8.4	<0.35		1.5
1611	ß-Eudesmol						2.9			2.1
1616	MW=204				3.4			3.0		
1618	MW=222			2.7		6.0			2.9	
1632	Jatamansone	0.7								
1654	α-Bisabolol				2.4					
1680	Chamazulen+MW=220		1.2		1.1	2.4				
1697	Unknown 2								2.0	
1715	Unknown 3								1.7	
1812	En-in-dicycloether	2.3								
1892	MW=214			7.6						
	Total %	**31.61**	**59.53**	**81.1**	**64.73**	**49.95**	**78.3**	**92.26**	**58**	**74**

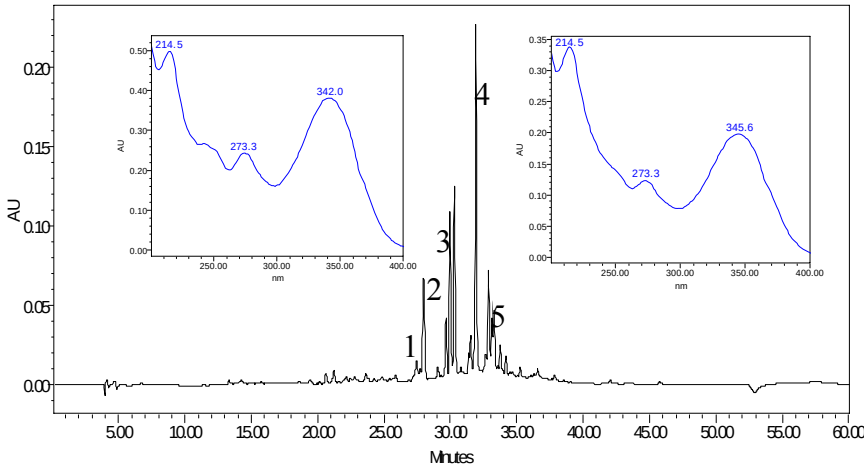
The essential oils are largely comprised of monocyclic and bicyclic mono- and sesquiterpenes in varying concentrations. The six populations have different chemistry as noted in most of the species in the genus. Examples include the presence of santolina triene in the Melkbosstrand and Malmesbury populations and even the major compounds are different in each population. Notably present in the essential oils are compounds such as piperitone, bicyclogermacrene, α-bisabolol and chamazulene. The structure for the major compounds is shown below.



Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various classes especially flavones as shown in the HPLC chromatogram. The UV spectra of the main peaks are also shown. The rest of the data is summarized in Table 2. The recognition of flavonoid classes was adapted from Markham, 1982 and the same criterion is used for rest of the species in the study.



A HPLC/UV chromatogram of leaf extracts of *E. africanus* (Melkbosstrand). The UV spectra of the major components (3 and 4) at retention time 30.30 and 31.91 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. africanus* from individuals from five populations. STL-Sutherland (var *paniculatus)*; MMY-Malmesbury; MKB-Melkbosstrand; CDL-Citrusdal; DR-De Rust. Only the (%) of the major peaks is noted.

Peak number	Retention time	$UV_{(\lambda max)}$	STL	MMY	MKB	CDL	DR
1	28.00	213, 271, 346	5.80	5.18	10.65	-	-
2	29.94	216, 273, 336	7.99	9.48	10.90	8.80	16.81
3	30.27	215, 273, 345	8.48	17.81	15.00	16.65	13.31
4	31.95	218, 273, 341	29.03	18.45	28.06	37.21	43.90
5	32.89	220, 277, 330	14.60	5.13	7.85	9.41	25.99

Biological properties

Antimicrobial activity

Essential oils were comparatively more active than extracts. Highest activity noted was against *Cryptococcus neoformans* (*Cn*) and *Bacillus cereus* (*Bc*), moderate activity against *Candida albicans* (*Ca*), *B. subtilis* (*Bs*) and *Staphylococcus aureus* (*Sa*) and lowest activity against *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). Despite the oils being more active, the extracts showed relatively good activity against *Cryptococcus neoformans* (*Cn*) but low to moderate activity against the rest of the pathogens. A summary of the activities is given in Table 3.

Minimum inhibitory activity (MIC)

The essential oil from an individual of the Malmesbury population was tested as it showed relatively good activity against most of the test pathogens. It had an MIC of 4-32 mg/ml.

Table 3. A summary of biological properties of individuals from six populations *E. africanus.* Pop 1-Malmesbury; Pop 2-Melkbosstrand; Pop 3-Citrusdal; Pop 4-De Rust; Pop 5-Mossel Bay; Pop 6-Sutherland (var *paniculatus*). EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	Extract	Activity in mm from the edge of the disc							5-Lox IC_{50} µg/ml	DPPH IC_{50} µg /ml
		Cn	Ca	Bc	Bs	Sa	Kp	Ec		
Pop 1	EO	5	2	6	2	2	1	R	32.8	*
Pop 2	EO	1	1	3	1	1	R	R	19	*
Pop 3 indiv A	EO	2	1	2	<1	<1	1	R	31.8	*
Pop 1	AE	3	R	1	2	R	<1	R	*	47.2
Pop 2	AE	3	1	2	2	<1	<1	<1	*	46.4
Pop 3 indiv A	AE	2	R	1	1	<1		R	*	49.9
Pop 4	AE	4	R	R	R	R	<1	<1	*	41.9
Pop 6 indiv A	AE	4	R	R	1	R	<1	<1	*	42.5
MIC mg/ml	EO	4	4	8	*	32	*	16	*	*

*-Not tested.

Antioxidant activity
The essential oils showed no activity at the starting concentration of 100 μg/ml but extracts were fairly active in the DPPH assay with activity ranging from 41.9-49.9 μg /ml. A summary of activities is included in Table 3.

Antiinflammatory activity
Three samples comprising representatives from three populations were tested and activity ranged from 19.0-32.8 μg /ml. This species showed the best antiinflammatory activity of 19.0 mg/ml recorded in the genus. This was noted in an individual collected from Melkbosstrand.

Acetylcholinesterase enzyme inhibition
The preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme in individuals from Melkbosstrand and Sutherland.

Phylogenetic studies
The ITS (internal transcribed spacer) of nuclear DNA and *psb*A-*trn*H regions of plastid DNA have been sequenced for this species and used in phylogenetic reconstruction with chemical data. This is one of the most variable species in the genus and the individuals from the six populations are variously placed in the topology. An individual from Sutherland is sister taxa to one from De Rust as the second individual of the former is sister to the clade. The individual from Melkbosstrand is most parsimoniously placed in a sister clade to two individuals of *E. eximius.* One of the individuals from Citrusdal is parsimoniously placed as sister taxa to an individual of *E. purpureus.* The other two individuals of the former group as sister taxa elsewhere in the topology. The individual from Malmesbury groups with one of *E.brevifolius* as sister taxa. This species shows affinities with *E. eximius, E. purpureus, E. scariosus, E. punctulatus, E. brevifolius,* and *E. merxmuelleri.*

References
Markham, K.R. (1982). Techniques of Flavonoid Identification. Academic press, London.
Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

2. *E. ambiguus* (DC.) M.A.N. Müller.

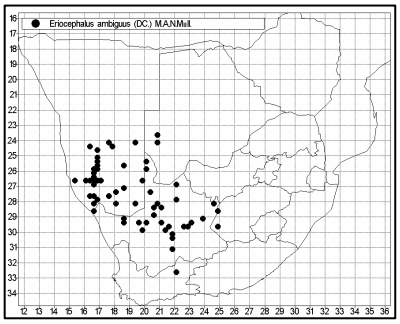
Synonym

E. aspalathoides DC var *ambiguus* DC., *E. aspalathoides* DC.

Common name

'Doringkapok'.

Geographical distribution



Distribution extends from central parts of Namibia (Schakalsberge) to Botswana and Northern, Western and Eastern Cape (see map) in areas receiving less than 200 mm of rainfall annually. Distribution map of *E. ambiguus* (Müller *et al.*, 2001).⇨

Botanical description

Many-stemmed, erect and much-branched, spinescent shrub, 0.3-0.6 m high and 450 mm in diameter. Old stems with anomalous secondary growth. Leaves on dolichoblasts alternate, entire with densely silver-grey shortly pilose to pilose indumentum. Capitula heterogamous disciform, peduncles 1-11 mm long. Paleae of marginal florets free. Chromosome number 2n = 18. Flowering periods January to April. (Müller *et al.*, 2001).

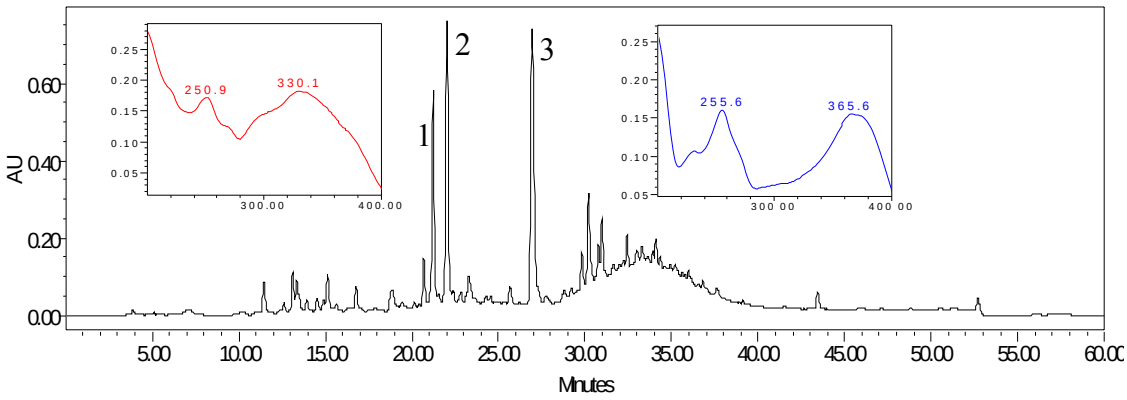
Chemical composition

Non volatile phytoconstituents

Extraction using acetone yielded 1.0% (dry wt) crude extract.

HPLC

The leaf extracts contain flavonoids of flavones and flavonols type as shown in the chromatogram below. The UV spectra of some of the main peaks are shown. The rest of the data is summarized in Table 1.



A HPLC/UV chromatogram of leaf extracts of *E. ambiguus*. The UV spectra of the major components mainly flavone (peak 2) and flavonol (peak 3) at retention time 22.05 and 26.95 minutes respectively are shown.

Table 1. A summary of the HPLC/UV data for acetone leaf extracts of *E. ambiguus.* Only the (%) of the major peaks are noted.

Peak number	Retention time	UV $_{(\lambda max)}$	% Area
1	21.23	218, 243, 328	20.71
2	22.05	202, 251, 330	35.03
3	26.95	203, 256, 366	44.26

Biological properties

Antimicrobial activity

The acetone leaf extract was tested for antimicrobial activities and was fairly active against *Staphylococcus aureus* (*Sa*), with an inhibition zone of 2.5 mm and weakly active against *Escherichia coli* (*Ec*) (<1.0 mm). No activity was noted against the rest of the test pathogens namely; *Cryptococcus neoformans* (*Cn*), *Candida albicans* (*Ca*), *Klebsiella pneumoniae* (*Kp*), *Bacillus cereus* (*Bc*) and *B. subtilis* (*Bs*). A summary of the activities is given in Table 2.

Minimum inhibitory activity (MIC)

The acetone extracts showed an MIC of 3.1 mg/ml against *Staphylococcus aureus.*

Table 2. A summary of biological properties of *E. ambiguus.* AE-acetone extract. The full names of pathogens are given in the text above. R-resistant.

Extract	Activity (mm) from the edge of the disc							DPPH
	Cn	Ca	Bc	Bs	Sa	Kp	Ec	IC_{50} µg/ml
AE	R	R	R	R	2.5	R	<1	32.9
MIC mg/ml	*	*	*	*	3.1	*	*	*

*-Not tested.

Antioxidant activity

The acetone leaf extract was active in the DPPH assay with activity of 32.9 µg /ml as shown in Table 2.

Phylogenetic studies

The ITS (internal transcribed spacer) of nuclear DNA and *psb*A-*trn*H regions of plastid DNA have been sequenced for this species but the sequences obtained for the ITS region were multiple paralogous copies of the gene and were impossible to align and hence were not used in the phylogenetic reconstruction. However, the non-variable *psb*A-*trn*H sequences are available for the species in Leslie Hill Molecular Systematics Laboratory, Cape Town.

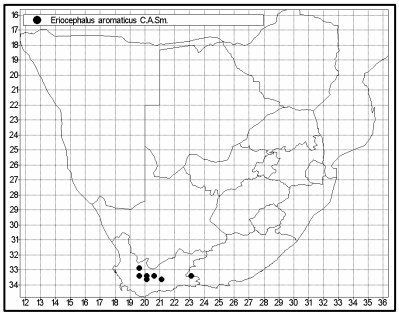
References

Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

3. *E. aromaticus* C.A.Sm.

Common name

'Kapokbos'.



Geographical distribution

Distribution restricted to mountains of winter rainfall areas of Western (Ladismith and Swartberg) and Eastern Cape, 900m above sea level as shown in the map below. Most of these areas have rocky and dry terrain. Distribution map of *E. aromaticus* (Müller *et al*., 2001).⇨

Botanical description

Erect, much-branched shrub up to 0.6 m high. Old stems with anomalous secondary growth. Leaves decussate and glabrous and whole surface with cavities, sometimes with glands, and glabrous. Capitula heterogamous radiate, racemose or umbellate-racemose, peduncles 3-5 (-12) mm long. Rays white or occasionally red-purple. Paleae of marginal florets partially connate. Chromosome number 2n = 18. Flowering periods (May to) June to October (to November) (Müller *et al*., 2001).



Erect habit of *E. aromaticus* in a rocky habitat (Seweweekspoort).



A sparsley branched *E. aromaticus* (Swartberg) in a dry habitat.



Radiate capitula of *E. aromaticus* with white rays.



E. aromaticus showing decussate leaves on flowering stem.

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.03% (wet wt) clear to pale yellow essential oils.

GC/MS

Major constituents

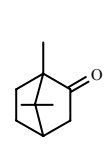
The essential oils contain approximately 54 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of two populations of *E. aromaticus.* Pop 1-Swartberg; Pop 2-Ladismith/Seweweekspoort (3 individuals). Values are given in percentages. Compounds in bold represent some of the major compounds.

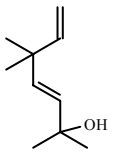
		Pop 1	Pop 2		
RI	**Compound**		**A**	**B**	**C**
901	Santolina triene		0.8		
928	α-Pinene		2.2	2.0	
940	Camphene		0.5	0.3	
963	Sabinene		<0.5		
966	β-Pinene	**0.3**	1.3	0.5	1.7
988	Yomogi alcohol		6.9		
1008	**p-Cymene**	3.5	1.0	14.2	10.8
1016	1,8-Cineole	1.4	6.2		0.7
1018	Limonene	<0.34	<0.5	0.2	0.2
1034	2-Nonanone				4.3
1047	γ-Terpinene				0.2
1070	Artemisia alcohol		2.0		
1083	Linalool		0.5		
1110	**Camphor**		12.3	0.4	
1114	*Trans*-pinocarveol	0.5	0.9	1.8	
1130	Pinocarvone		1.1	0.9	
1133	Mmean peak=100, PM=170	2.6			
1138	Mmean peak=100, PM=170	8.7			
1141	Borneol	1.8	1.2	1.5	1.5
1155	4-Terpineol	1.1	0.7	0.7	0.6
1158	Artemisyl acetate		4.9		
1160	Myrtenal	0.4		<0.16	0.6
1172	Myrtenol	0.7	0.4	0.3	1.1
1192	Methyl *trans*-chrysanthemate		8.2		
1262	Bornyl acetate	6.3	3.3	3.3	0.8
1267	Acetate MW=196		1.1		
1278	Carvacrol			0.7	0.8
1282	Unknown 1		1.8		
1325	MW=204				1.1
1336	α-Longipinene			12.7	
1360	Geranyl acetate		3.9		
1362	α-Copaene	1.6		2.1	7.2
1369	Isocomene				5.7
1385	MW=204				1.6
1392	α-Cedrene	1.0			
1398	β-Caryophyllene	1.9	0.7		
1419	Aromadendrene	0.8			
1449	MW=220	0.5			
1500	δ-Cadinene + MW=222		5.4		
1516	MW=220	1.5			1.1

		Pop 1	Pop 2		
RI	**Compound**		**A**	**B**	**C**
1522	MW=220	4.7			3.1
1534	MW=236				1.6
1544	**Spathulenol**	16.0	7.6	6.2	8.8
1548	Caryophyllene oxide	4.4	5.1		2.5
1556	Viridiflorol or Globulol			1.2	
1560	Viridiflorol				1.2
1581	MW=222			16.6	
1590	MW=222			3.1	
1591	MW=220				13.1
1592	MW=222	**13.1**			
1599	MW=222		2.5		
1599	Main peak=136	0.8			1.1
1618	MW=222		1.2		
1632	Jatamansone			4.5	
	Total %	**73.78**	**83.63**	**76.82**	**71.3**

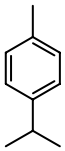
The essential oils are largely comprised of monocyclic, bicyclic mono- and sesquiterpenes in varying concentrations. The two populations studied depict different chemistry e.g. the presence of α-cedrene and aromadendrene in the Swartberg individuals and the conspicuous absence of these compounds in the individuals from Ladismith. The structure for the major compounds is shown below.

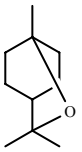


camphor

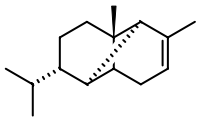


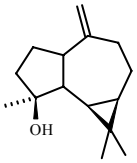
yomogi alcohol


p-cymene



1,8-cineole


α-copaene

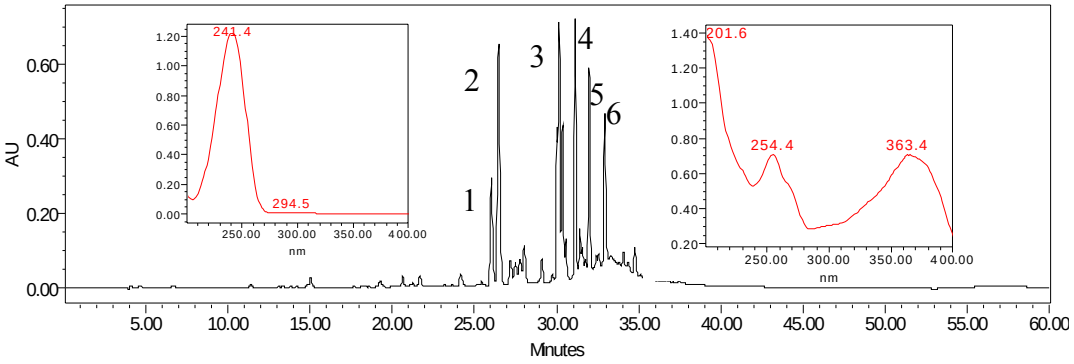


spathulenol

Non volatile phytoconstituents

HPLC

The leaf extracts contain classes of flavonoids as shown in the chromatograph below. The UV spectra of the main peaks are also shown. The chemistry of the two individuals studied is different as shown in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. aromaticus* from Swartberg. The UV spectra of the major components (2 and 3) at retention time 26.48 and 30.15 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone lea extracts of *E. aromaticus.* SWG-Swartberg; LD-Ladismith. Only the (%) of the major peaks is noted.

Peak number	Retention time	$UV_{(\lambda max)}$	SWG	LD
1	26.00	241, 295	8.07	-
2	26.48	209, 287, 339	19.44	-
3	30.15	202, 254, 363	14.27	7.19
4	31.95	206, 243, 333	10.57	-
5	32.93	216, 234, 278, 330	8.35	-
6	33.45	233, 272, 336	1.55	-

Biological properties

Antimicrobial activity

The acetone leaf extracts were more active than the essential oils. The leaf extracts of this species were the most active in the genus with activity noted against *Bacillus cereus* (*Bc*), *B. subtilis* (*Bs*) and *Staphylococcus aureus* (*Sa*)*;* moderate activity against *Cryptococcus neoformans* (*Cn*), and lowest activity against *Candida albicans* (*Ca*), *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). The essential oils were active against the same pathogens though with lower values but moderately active against *Candida albicans* (*Ca*), *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). A summary of the activities is given in Table 3.

Minimum inhibitory activity (MIC)

The acetone leaf extracts showed good activity against *Cryptococcus neoformans* (*Cn*), *Bacillus cereus* (*Bc*) and best overall activity against *Staphylococcus aureus* (*Sa*) as shown in Table 3.

Antioxidant activity

The essential oils showed no activity at the starting concentration of 100 µg /ml but extracts were active in the DPPH assay with activity ranging from 31.80-45.30 µg /ml. A summary of activities is included in Table 3.

Table 3. A summary of biological properties of individuals of *E. aromaticus* from two populations. Pop 1-Swartberg; pop 2-Ladismith/Seweweekspoort. EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	**Extract**	**Activity in mm from the edge of the disc**							**DPPH IC_{50} μg/ml**
		Cn	Ca	Bc	Bs	Sa	Kp	Ec	
Pop 1	EO	R	2	3.5	3	2	<1	<1	*
Pop 2 indiv B	EO	R	R	3	1	<1	R	R	*
Pop 2 indiv C	EO	8	R	4	<1	R	R	R	*
Pop 1	AE	R	R	8	4	5	<1	<1	31.8
Pop 2 indiv A	AE	1	<1	4	2	4	R	<1	43.6
Pop 2 indiv B	AE	5	R	7.3	6	6	R	R	45.3
Pop 2 indiv C	AE	1	<1	R	1	4	R	<1	42.5
MIC mg/ml	AE	1.6	*	0.4-3.1	*	0.2-0.8	*	*	*

*-Not tested.

Acetylcholinesterase enzyme inhibition

Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Phylogenetic studies

The Swartberg individual is most parsimoniously placed with individual C from Ladismith population as sister taxa. They share similar terpene chemistry while individual B groups with taxa of *E. punctulatus* and individual A groups with *E. grandiflorus* as sister taxa in the combined phylogeny. There is a possible relation between the species and *E. punctulatus* and *E. grandiflorus.* These species have radiate capitula, connate paleae and decussate/opposite leaves. This species has close affinities with *E. grandiflorus* and *E. punctulatus*.

References

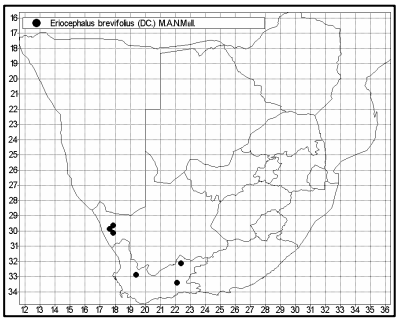
Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

4. *E. brevifolius* (DC.) M.A.N. Müller.

Synonym
E. punctulatus DC var *brevifolius.*

Common name
'Kapokbos'.

Geographical distribution



Distributed in Namaqualand (Kamiesberg), Swartruggens-Roggeveld and Swartberg (Oudtshooorn and De Rust) in an altitude of 900 m in the winter rainfall areas as shown in the map below. Distribution map of *E. brevifolius* (Müller *et al*., 2001).⇨

Botanical description
Erect conical shrubs up to 1.2 m high. Old stems with anomalous secondary growth. Leaves opposite except flowering shoots where they are sometimes alternate and with felty to shortly sericeous grey-green indumentum. Capitula heterogamous radiate, and solitary or in umbellate racemes, peduncles 5-10(-20) mm long. Ray florets white with variants of pink noted in population from De Rust. Paleae of marginal florets partly connate. Chromosome number 2n = 54. Flowering correlated with the rainy season, with the peak from July to September (Müller *et al*., 2001).

Economic uses
Browsed



Habit of *E. brevifolius* (De Rust).





Radiate capitula of *E. brevifolius* showing pink and white rays with red-purple disc florets.

Chemical composition

Essential oil

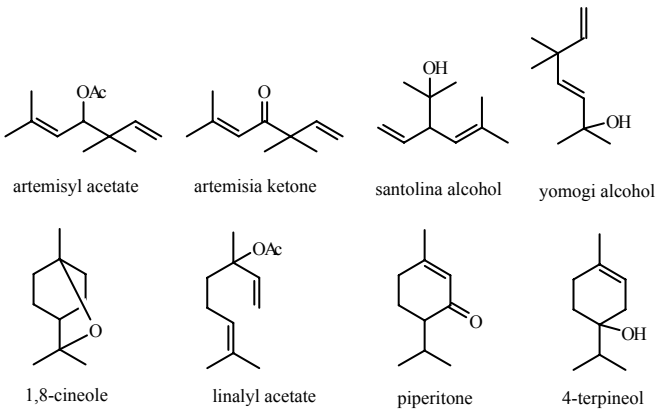
Extraction by hydrodistillation yielded 0.17% (wet wt) pale yellow essential oil.

GC/MS
Major constituents
The essential oils contain approximately 42 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of individuals of *E. brevifolius* from two populations. Pop 1 ODT-Oudtshoorn; Pop 2-DRV-De Rust/Vergelegen. Values are given in percentages. Compounds in bold represent some of the major compounds.

		Pop 1	**Pop 2**		
RI	**Compound**		**A**	**B**	**C**
901	Santolina triene	0.6	0.8	0.2	<0.25
921	Artemisia triene	0.3			
928	α-Pinene	0.5	1.1	0.8	0.3
939	α-Fenchene				0.5
939	α-Fenchene + camphene		0.5		
940	Camphene	1.4		0.3	
963	Sabinene		<0.28	<0.23	
966	β-Pinene	0.3	0.4	0.5	0.6
988	**Yomogi alcohol**	18.2	3.3	9.4	5.7
1008	p-Cymene	1.1	0.9	0.4	0.5
1016	**1,8-Cineole**	1.6	7.8	5.5	1.5
1024	**Santolina alcohol**		4.9	8.6	11.7
1045	**Artemisia ketone**	3.8	8.2	1.2	1.7
1070	Artemisia alcohol	4.5	1.1	3.3	1.5
1083	Linalool	2.0			3.1
1110	Camphor		2.0	0.6	0.5
1141	Borneol		1.5		0.5
1155	Lavandulol	1.0			
1155	**4-Terpineol**	20.8	1.5		
1158	**Artemisyl acetate**		5.3		10.6
1166	α-Terpineol				0.9
1192	Methyl *trans*-chrysanthemate	0.3		0.9	
1215	**Piperitone**	0.5	5.9		
1240	Linalyl acetate	4.4			8.4
1262	Bornyl acetate		0.3	1.6	
1342	Neryl acetate				0.4
1360	Geranyl acetate	2.2	0.8	1.8	2.3
1362	α-Copaene		0.5		
1398	β-Caryophyllene	1.3	0.7	0.8	
1432	α-Humulene	0.2			
1432	**Spathulenol**	1.8	5.5	1.9	3.1
1544	Mean peak=149		5.7		6.1
1545	Caryophyllene oxide	3.0			
1548	Viridiflorol				0.6
1560	MW=222				4.2
1581	MW=222		12.8		7.5
1597	Main peak=136			0.4	
1607	α-humulene	**0.2**			
1611	β-Eudesmol		1.1		
1618	MW=181			6.1	9.6
1618	MW=222		5.0		6.5
1632	Jatamansone				1.1
	Total %	**69.96**	**80.02**	**44.17**	**88.87**

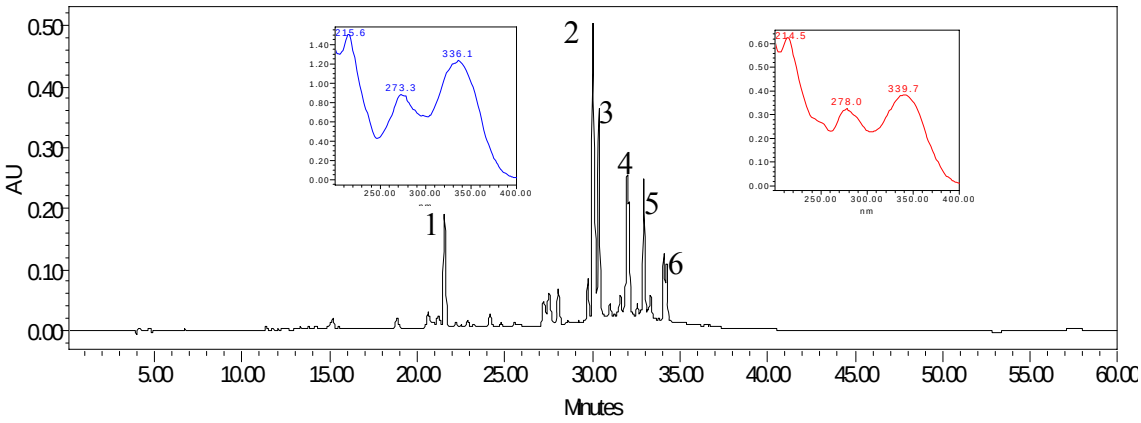
The essential oils are largely comprised of acyclic, monocyclic and bicyclic mono- and sesquiterpenes in varying concentrations. The chemistry of the individuals studied differs. For instance, the individual from Oudtshoorn has artemisia triene, lavandulol, α-humulene and caryophyllene oxide, which are absent in the individuals from De Rust. The oils of the four individuals are characterized by the presence of artemisyl acetate, linalool and camphane derivatives. They also have a number of acetates. The structure for the major compounds in the oils is shown in below.



Non volatile phytoconstituents

HPLC

The leaf extracts of this species contains flavonoids of various classes especially flavones as shown in the chromatogram below. The UV spectra of the main peaks are also shown. The data is summarized in Table 2.



The stereochemistry also has an influence on bioactivity. It has been observed that α-isomers A HPLC/UV chromatogram of leaf extracts of *E. brevifolius* (Oudtshoorn). The UV spectra of the major components (2 and 4) at retention time 30.03 and 31.98 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. brevifolius.* ODT-Oudtshoorn; DRV-De Rust/Vergelegen; C-one of the three individuals from De Rust. Only the (%) of the major peaks is noted.

Peak number	Retention time	UV$_{(\lambda max)}$	ODT	DRV C
1	21.55	240, 369	10.98	11.50
2	30.03	216, 273, 336	30.24	30.50
3	30.36	215, 273, 346	17.06	13.42
4	31.98	213, 278, 340	17.84	-
5	32.93	216, 277, 330	10.97	12.70
6	34.04	241, 328	9.71	-

Biological properties

Antimicrobial activity
Essential oils were more active than the extracts. Highest activity was noted against *Cryptococcus neoformans* (*Cn*) and *Bacillus cereus* (*Bc*) and moderate to low activity against *B. subtilis* (*Bs*), *Candida albicans* (*Ca*), *Staphylococcus aureus* (*Sa*), *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). The individual from Oudtshoorn showed better activity than those from De Rust. A summary of the activities is given in Table 3.

Minimum inhibitory activity (MIC)
The essential oil of the individual from the Oudtshoorn population was moderately active with an MIC ranging from 8-16 mg/ml against *Candida albicans, Bacillus cereus* and *Staphylococcus aureus.*

Table 3. A summary of biological properties of individuals of *E. brevifolius* from three populations. Pop 1-Oudtshoorn; Pop 2-De Rust; Pop 3-Kamiesberg. EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	**Extract**	**Activity in mm from the edge of the disc**							**5-Lox IC_{50} µg/ml**	**DPPH IC_{50} µg/ml**
		Cn	Ca	Bc	Bs	Sa	Kp	Ec		
Pop 1	EO	R	2	5.3	4	5	2	2	30.2	49.7
Pop 2 Indiv A	EO	<1	<1	1.5	1	<1	<1	R	*	*
Pop 2 Indiv B	EO	3	<1	3	<1	<1	R	R	*	*
Pop 2 Indiv C	EO	5.5	1	4	1	<1	<1	R	*	47.9
Pop 1	AE	3	R	R	4	<1	R	R	*	*
Pop 2 Indiv C	AE	2	R	3	R	R	R	R	*	*
Pop 3	AE	*	*	*	*	*	*	*	25.4	30.9
MIC mg/ml	EO	*	8	8	*	16	>32	*	*	*
MIC mg/ml	AE	1.8	0.9	*	*	0.9	*	*	*	*

*-Not tested.

Antioxidant activity

The essential oils showed no activity at the starting concentration of 100 µg/ml but extracts were active in the DPPH assay 30.9-49.7 µg/ml. A summary of activities is included in Table 3.

Antiinflammatory activity
The oil of the individual from Kamiesberg was moderately active against 5-lipoxygenase.

Acetylcholinesterase enzyme inhibition
Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Phylogenetic studies

Individuals from Kamiesberg and Oudtshoorn are most parsimoniously placed as sister taxa while individual B from De Rust is sister to this clade in the combined phylogeny. Individuals

A and C are placed elsewhere in the phylogeny. This species has similarities with *E. grandiflorus, E. africanus* and *E. scariosus* as they group in adjacent clades.

References

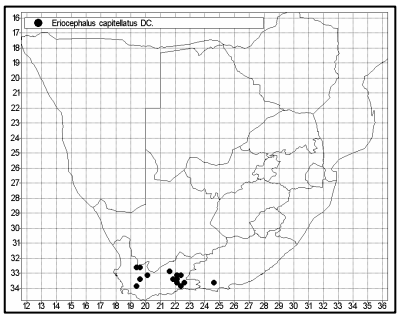
Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

5. *E. capitellatus* DC

Common name

'Kapokbos'

Geographical distribution



The species occurs on the high mountains of the Western (Swartberg) and Eastern Cape in an altitude of over 900 m in both summer and winter rainfall areas as shown in the map. Distribution map of *E. capitellatus* (Müller *et al*., 2001).⇨

Botanical description

Slender, erect, small conical shrubs, 0.25-1.2 m high. Old stems grey to grey-brown. Leaves alternate rarely opposite, mostly palmatisect to pinnatisect, with felty sericeous indumentum. Capitula heterogamous radiate, in terminal spikes or spicate-racemose and shortly pedunculate 0.3-0.5 mm long. Ray florets white in colour. Paleae of marginal florets connate. Chromosome number 2n = 18. Flowering and fruiting time April to September (winter rainfall areas) and February to May (summer rainfall areas) (Müller *et al*., 2001).

Uses

Browsed



The slender, erect habit of *E. capitellatus* (Swartberg).



Fruiting and spicate-racemose radiate capitula of *E. capitellatus.*

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.25% (wet wt) of cloudy white to pale green essential oil.

GC/MS

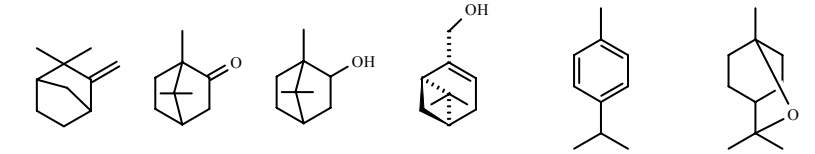
Major constituents

The essential oils contain approximately 36 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of three individuals of *E. capitellatus*. Pop 1 and 2-Swartberg. Values are given in percentages Compounds in bold represent some of the major compounds.

		Pop 1	Pop 2		
RI	**Compound**		**A**	**B**	**C**
901	Santolina triene			0.6	0.6
928	α-Pinene	1.3	0.7	0.6	0.6
940	Camphene	3.1	3.1	1.8	3.4
963	Sabinene	<0.57	0.3	0.4	0.3
966	β-Pinene	5.9	1.2	2.5	2.0
984	β-Myrcene				<0.34
1005	α-Terpinene	<0.57			
1008	**p-Cymene**	3.5	1.7	2.5	4.4
1016	**1,8-Cineole**	8.5	10.6	11.4	11.3
1018	Limonene	<0.57	0.5	0.3	0.6
1047	γ-Terpinene	0.6	0.9		0.8
1050	*Cis*-Sabinene hydrate				<0.34
1110	**Camphor**	49.0	48.0	46.9	50.3
1114	*Trans*-pinocarveol	0.7			
1129	**Isopinocamphone**	3.2		6.7	5.0
1141	**Pinocamphone**	12.2			
1141	**Borneol + Pinocamphone**		13.1		10.2
1141	**Borneol**			13.5	
1155	4-Terpineol	2.1	1.4	1.1	1.7
1160	Myrtenal	1.3	0.8	1.1	0.6
1166	α-Terpineol			<0.33	
1172	Myrtenol	2.5	2.3	2.2	1.7
1204	Carvone	1.0		0.9	0.8
1262	Bornyl acetate		0.3		
1362	α-Copaene	1.2	1.1	0.9	0.9
1398	β-Caryophyllene	<0.57	0.2	<0.33	0.4
1474	Bicyclogermacrene		0.3		
1536	MW=220	<0.57			
1544	Spathulenol	2.1	0.9	1.0	1.8
1548	Caryophyllene oxide	0.6	0.5	0.7	0.8
1559	Main peak=136				0.5
1598	Caryophyllene alcohol		0.7		
1599	Main peak=136	0.6		0.9	
1618	MW=222			1.1	
	Total %	**99.22**	**88.72**	**96.87**	**98.66**

The essential oils are largely comprised of bicyclic monoterpenes of camphane and pinane structural groups with few acyclic mono- and bicyclic sesquiterpenes. The four individuals have similarities and a few minor differences in their chemistry like the presence of *trans*-pinocarveol in the individual from population 1 and the presence of *cis*-sabinene hydrate in individual C from population 2. The structure for the major compounds is shown below.

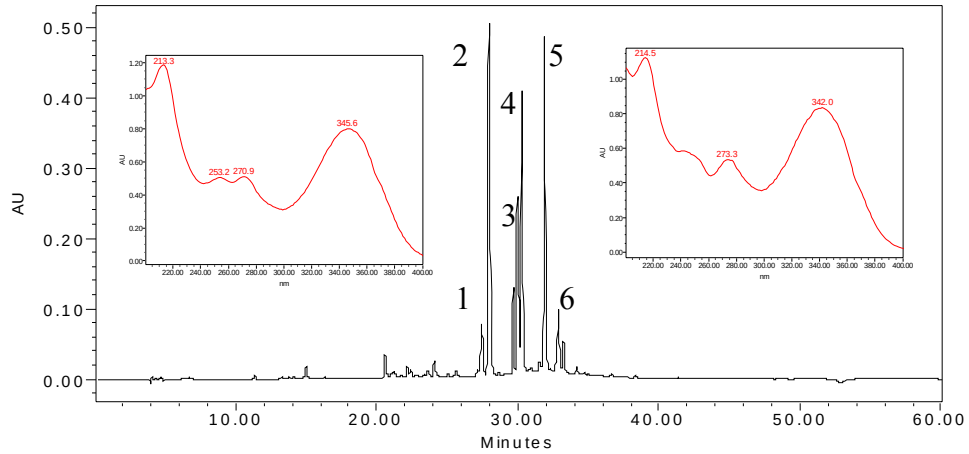


camphene camphor borneol myrtenol *p*-cymene 1,8-cineole

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various classes especially flavones, as shown in the chromatogram below. The UV spectra of the main peaks are also shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. capitellatus* The UV spectra of the major components mainly flavones (peak 2 and 5) at retention time 28.02 and 31.90 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. capitellatus.* Only major peaks are noted.

Peak number	Retention time	$UV_{(\lambda max)}$	% Area
1	27.52	222, 253, 291, 348	1.67
2	28.02	213, 253, 271, 346	34.73
3	29.67	211, 267, 336	4.81
4	29.98	216, 273, 336	13.63
5	31.92	215, 273, 342	23.88
6	32.88	216, 252, 278, 330	3.51

Biological properties

Antimicrobial activity

The essential oils were active to moderately active against *Cryptococcus neoformans* (*Cn*), *Bacillus cereus* (*Bc*), *Candida albicans* (*Ca*) and *Bacillus subtilis* (*Bs*) and low activity against *Staphylococcus aureus* (*Sa*), *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). The extracts showed low activity against all the test pathogens as shown in Table 3. A summary of the biological activities is given in Table 3.

Minimum inhibitory concentration

The essential oils showed low activity against *Cryptococcus neoformans* and *Bacillus cereus.*

Table 3. A summary of biological properties of individuals of *E. capitellatus* from two populations. Pop 1-Swartberg Pass; Pop 2-Swartberg. EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	**Extract**	**Activity in mm from the edge of the disc**							**5-Lox IC_{50} µg/ml**	**DPPH IC_{50} µg/ml**
		Cn	Ca	Bc	Bs	Sa	Kp	Ec		
Pop 1	EO	*	*	*	*	*	*	*	*	*
Pop 2 indiv A	EO	4	R	3.5	<1	1	<1	R	43.1	40.5*
Pop 2 indiv B	EO	2	1	3.5	1	<1	1	<1	*	*
Pop 2 indiv C	EO	3	2	2.5	2	R	1	<1	*	*
Pop 1	AE	R	R	1	1	R	<1	<1	*	*
Pop 2 indiv A	AE	<1	R	R	<1	<1	R	R		*
MIC mg/ml	EO	4	*	16	*	*	*	*	*	*

*-Not tested

Antioxidant activity

The extract was active in the DPPH assay with activity of 40.5 µg/ml. A summary of activities is included in Table 3.

Antiinflammatory activity

The essential oil of the individual A showed a moderate inhibitory activity against the 5-lipoxygenase enzyme.

Acetylcholinesterase enzyme inhibition

Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Phylogenetic studies

Since the chemistry of the four individuals is very so similar, they all grouped together in one clade with the individual of population 1 grouping with individual B from population 2 as sister taxa while the other individuals were sister to the this clade. The species has affinities with *E. merxmuelleri, E. punctulatus* and *E. ericoides* subsp. *ericoides.*

References

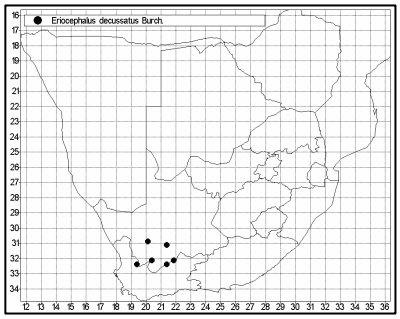
Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

6. *E. decussatus* Burch.

Synonym
E. aspalathoides DC.

Common name
'Kapokbossie'.

Geographical distribution
The distribution of this species extends over the summer and winter rainfall areas, over the central Karoo and parts of Namaqualand (Sutherland/Fraserburg) as shown in the map).



Distribution map of *E. decussatus* (Müller *et al*., 2001).⇨

Botanical description
Shrubs much-branched from the base, sometimes spinescent, 0.6-1.5 m high and in diameter and branches conspicuously opposite. Old stems with anomalous secondary growth. Leaves decussate, often alternate on flowering shoots and have permanently densely appressed sericeous indumentum. Capitula heterogamous disciform, solitary, rarely in terminal racemes and shortly pedunculate ((1.0-) 2.0-3.5(-60)) mm. Paleae of marginal florets connate. Marginal female florets creamy white. Chromosome number 2n = 18. Flowering time correlated with rainfall, extending from January to April and from July to September in different rainfall regions (Müller *et al*., 2001).



The much branched spinescent habit of *E. decussatus* (Sutherland).



The disciform capitula and a terminal branch showing the spine-like hardened remains of the terminal racemose peduncles.

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.08% (wet wt) of pale yellow and deep blue essential oils.

GC/MS

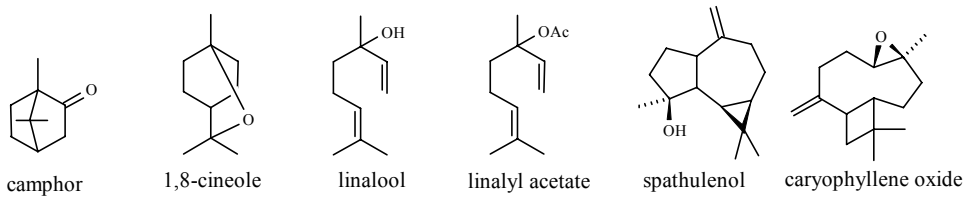
Major constituents

The essential oils contain approximately 32 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of three individuals of *E. decussatus* from Sutherland. Values are given in percentages. Compounds in bold represent some of the major compounds.

RI	Compound	A	B	C
928	α-Pinene		2.2	
939	**α-Fenchene**			3.2
939	α-Fenchene + camphene		1.7	
966	β-Pinene		1.8	<0.63
1008	p-Cymene	<2.45	3.7	0.7
1016	**1,8-Cineole**	6.9	12.1	1.4
1018	Limonene		0.9	<0.63
1057	*Cis*-linalool oxide	2.5		2.0
1070	*Trans*-linalool oxide	2.5		2.0
1083	**Linalool**	11.8		8.8
1092	Chrysanthenone	2.9		0.6
1110	**Camphor**		15.0	13.9
1114	*Trans*-pinocarveol		1.1	
1130	Pinocarvone		0.7	
1141	Borneol		2.5	
1155	4-Terpineol	<2.45	2.7	<0.63
1166	α -Terpineol		<0.72	
1240	**Linalyl acetate**	19.3		30.1
1323	Unknown 1			3.1
1326	Unknown 2			3.1
1360	Geranyl acetate	2.6		
1369	Isocomene	<2.45	1.0	1.2
1442	Unknown 3		2.3	
1452	Unknown 4		1.3	
1488	MW=220	2.6		
1497	MW=220	3.7		
1542	Nerolidol		3.3	
1544	**Spathulenol**	30.9	16.7	12.4
1548	**Caryophyllene oxide**	3.0	9.8	2.6
1598	Caryophyllene alcohol		1.4	
1608	**α–Cadinol or τ-Muurolol**		5.3	<0.63
1618	MW=181		2.3	5.3
	Total %	**88.82**	**87.59**	**90.3**

The essential oils are largely comprised of acyclic and bicyclic mono- and sesquiterpenes of camphane, pinane and linalool structural groups. The oils also have a relatively high amount of linalool and derivatives. The three individuals have similarities and differences in their chemistry like the presence of geranyl acetate in the individual A only, *trans*-pinocarveol in individual B only and α-fenchene in individual C only. On the overall, individual A and C have similar chemical profiles and B differs slightly. The structure for the major compounds is shown below.

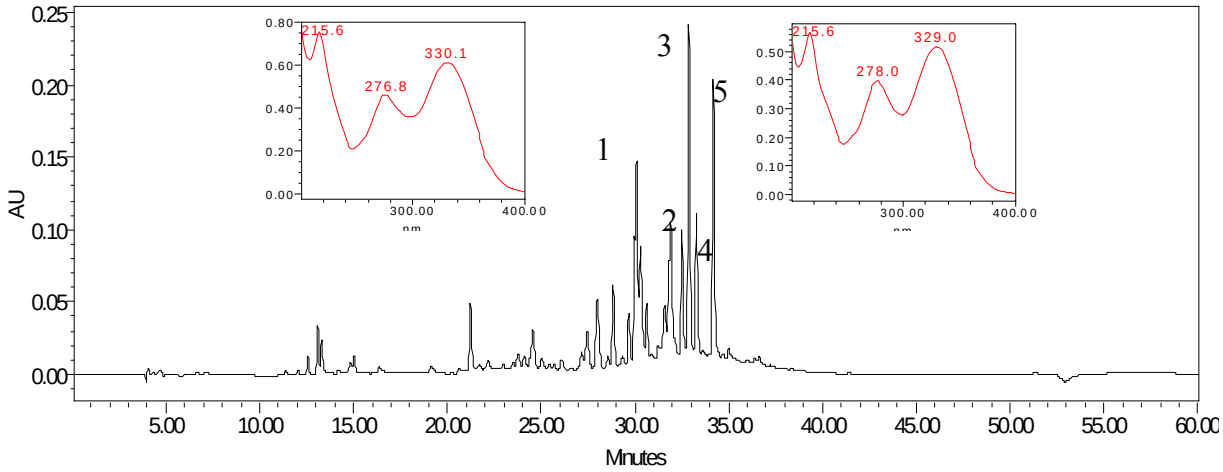


camphor 1,8-cineole linalool linalyl acetate spathulenol caryophyllene oxide

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various classes especially flavones as shown in the chromatogram below. The UV spectra of the main peaks are also shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. decussatus.* The UV spectra of the major components mainly flavones (peak 1 and 4) at retention time 30.09 and 32.87 minutes respectively are shown. Most of the major compounds are flavones as shown in the chromatogram.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. decussatus* from Sutherland. Only the major peaks are noted.

Peak number	Retention time	$UV_{(\lambda max)}$	% Area
1	30.09	202, 254, 366	15.09
2	32.50	215, 267, 329	6.42
3	32.87	229, 277, 330	16.42
4	33.27	215, 242, 278, 339	7.33
5	34.22	218, 236, 279, 330	13.53

Biological properties

Antimicrobial activity

The essential oils were active against all test pathogens with the highest activity noted against *Cryptococcus neoformans* (*Cn*), *Bacillus cereus* (*Bc*) and *Staphylococcus aureus* (*Sa*) and moderate to low activity against *Candida albicans* (*Ca*), *Bacillus subtilis* (*Bs*), *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). The extracts showed high to low activity against

some of the test pathogens as shown in Table 3. A summary of the biological activities is given in Table 3.

Minimum inhibitory concentration
The essential oils and the extracts showed moderate to low activity against the test pathogens as shown in Table 3.

Antioxidant activity
The extract was active in the DPPH assay with activity of 42.3-47.2 μg/ml. A summary of activities is included in Table 3.

Antiinflammatory activity
The essential oil of the individual from Kamiesberg showed a moderate inhibitory activity against 5-lipoxygenase enzyme.

Acetylcholinesterase enzyme inhibition
Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Phylogenetic studies
The chemistry of the four individuals included in the analysis differs except for individuals A and C, which share similar profiles and are most parsimoniously, placed as sister taxa in one clade in the combined phylogeny. This is a sister clade to that of *E. spinescens* with which the two species share similar chemistry and occur in close proximity to each other in their natural habitats. This species has close affinities to *E. purpureus* and *E. spinescens.*

Table 3. A summary of biological properties of individuals of *E. decussatus* from different populations. Pop 1-Kamiesberg; Pop 2-Sutherland. EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	**Extract**	**Activity in mm from the edge of the disc**							**5-lox IC_{50} μg/ml**	**DPPH IC_{50} μg/ml**
		Cn	Ca	Bc	Bs	Sa	Kp	Ec		
Pop 1	EO	5.2	2	3.5	1.5	2.5	<1	<1	39.6	*
Pop 2 indiv C	EO	4	2	2	2	3	<1	<1	*	*
Pop 1	AE	1.5	R	1	<1	2.5	R	1	*	44.1
Pop 2 indiv A	AE	5	R	R	1	R	<1	<1		47.2
Pop 2 indiv B	AE	R	R	1	R	R	R	R	*	42.3
Pop 2 indiv C	AE	<1	R	1	<1	R	R	<1	*	45.9
MIC mg/ml	EO	16	32	8	*	4	*	*	*	*
MIC mg/ml	AE	1.6	*	0.9	*	1.6	*	3.1		*

*Not tested.

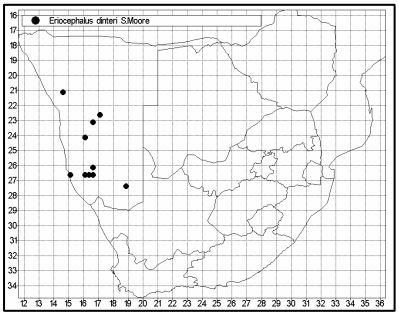
References

Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

7. *E. dinteri* S. Moore.

Synonym
E. parviflorus DC.

Common name
'Kapokbos'.



Geographical distribution
Distributed in summer rainfall areas. It is an endemic to Namibia and restricted to mountainous areas e.g. Brandberg, Auas and Aus Mountains 1000 m above sea level. Distribution map of *E. dinteri* (Müller *et al*., 2001).⇨

Botanical description
Slender erect many-stemmed, much-branched shrub, 0.3-1.0 m high, 300-500 mm in diameter. Old stems with anomalous secondary growth. Leaves decussate permanently appressed sericeous indumentum. Capitula heterogamous radiate, terminal, racemose or umbellate-racemose, peduncles (2.3-8.5 mm long). Rays white to red-purple. Paleae of marginal florets free. Chromosome number 2n = 36. Flowering time January to March and sometimes in May in northern summer rainfall areas, July to September and/or January to April in southern winter and summer rainfall areas (Müller *et al*., 2001).



Habitat of *E. dinteri* near Aus (Namibia).



Habit of *E. dinteri* showing erect branches.

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.19% (dry wt) pale yellowish essential oil.

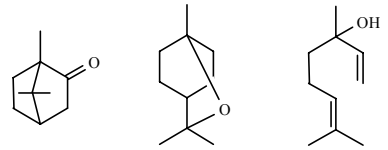
GC/MS

Major constituents
The essential oils contain approximately 26 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of *E. dinteri.* Values are given in percentages. Compounds in bold represent some of the major compounds.

RI	Compound	% Composition
922	α-Thujene	0.2
928	α-Pinene	0.9
940	Camphene	2.9
963	Sabinene	0.3
966	β-Pinene	2.5
988	Yomogi alcohol	2.3
992	α-Phellandrene	0.4
1005	α-Terpinene	0.7
1008	p-Cymene	4.2
1016	1,8-Cineole	4.4
1018	Limonene	1.3
1047	γ-Terpinene	2.2
1050	*Cis*-Sabinene hydrate	0.5
1070	Artemisia alcohol	1.9
1083	**Linalool**	4.6
1110	**Camphor**	38.4
1141	**Borneol + Pinocamphone**	4.6
1155	4-Terpineol	2.9
1158	**Artemisyl acetate**	4.6
1240	Linalyl acetate	4.1
1360	Geranyl acetate	0.7
1398	β-Caryophyllene	0.3
1474	Bicyclogermacrene	0.8
1500	δ-Cadinene	1.6
1544	Spathulenol	1.8
1548	Caryophyllene oxide	0.7
	Total %	**89.91**

The essential oils are largely comprised of acyclic and bicyclic monoterpenes and a few sesquiterpenes in varying concentrations. The species has relatively high content of camphor like *E. capitellatus.* Only one individual was studied. The structure for the major compounds is shown below.

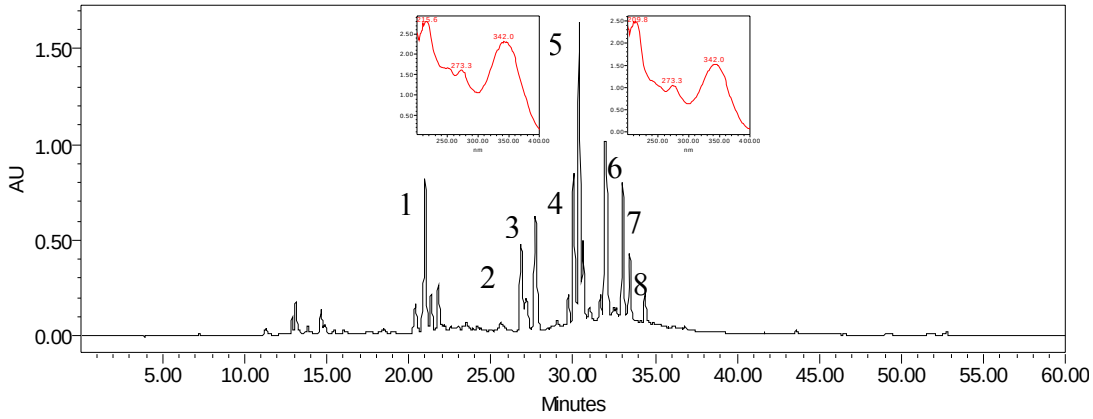


camphor 1,8-cineole linalool

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of flavones type as shown in the chromatogram below. The UV spectra of some of the main peaks are shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. dinteri.* The UV spectra of the major components mainly flavones (peak 5 and 6) at retention time 30.38 and 31.96 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. dinteri.* Only the (%) of the major peaks is noted.

Peak number	Retention time	UV $_{(\lambda max)}$	% Area
1	20.99	219, 243, 325	10.53
2	26.83	20.3, 256, 367	6.81
3	27.69	213, 254, 272, 346	9.17
4	30.03	216, 273, 336	8.99
5	30.38	217, 273, 342	16.66
6	31.96	215, 273, 343	15.36
7	33.03	217, 278, 329	6.98
8	34.38	216, 278, 329	1.74

Biological properties

Antimicrobial activity

The essential oil of this species was active against all the test pathogens with the highest activity noted against *Cryptococcus neoformans* (*Cn*), moderate activity against *Bacillus cereus* (*Bc*) and *Staphylococcus aureus* (*Sa*) and low activity against *Bacillus subtilis* (*Bs*), *Candida albicans* (*Ca*), *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). A summary of the activities is given in Table 2. The extract showed moderate to low activity against four of the test pathogens.

Minimum inhibitory activity (MIC)

The extract showed stronger inhibitory activity than the oil.

Antioxidant activity

The essential oil showed no activity at the starting concentration of 100 µg/ml but the extracts were active in the DPPH assay with activity of 34.9 µg/ml. A summary of activities is included in Table 3.

Antiinflammatory activity

The oil has a moderate inhibitory activity against 5-lipoxygenase enzyme.

Table 3. A summary of biological properties of *E. dinteri* from Aus (Namibia). EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. R-resistant.

Extract	**Activity in mm from the edge of the disc**							**5-Lox IC_{50} µg/ml**	**DPPH IC_{50} µg/ml**
	Cn	Ca	Bc	Bs	Sa	Kp	Ec		
EO	6.6	1.5	3.6	1.2	3.2	1.5	1.0	35	*
AE	2.8	R	1.7	R	3	R	<1.0	*	34.9
MIC EO	32	32	*	*	4	8	*	*	*
MIC AE	6.3	*	0.4	*	3.1	*	*	*	*

*-Not tested.

Phylogenetic studies

The species is most parsimoniously placed as sister taxa to an individual of *E. ericoides* from Scheerpersrust in the combined phylogeny. This species has close affinities with *E. merxmuelleri, E. ericoides* subsp. *ericoides*, and *E. punctulatus.*

References

Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

8. *E. ericoides* (L.F) Druce

Synonym

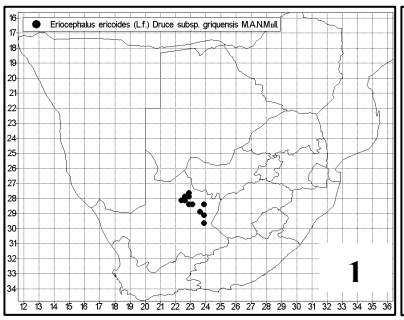
Tarchonanthus ericoides L.F, *E. glaber* Thunb., *E. glaber* Thunb. var *sessiliflorus* Sond. Ex Harv.

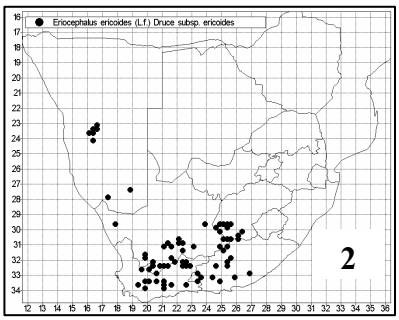
Common name

'Kapokbos', 'gewone kapokbossie', 'renosterveldkapok', 'roosmaryn' (rosemary), 'gladdekapokbos', 'regtekapok', 'gewonekapok', grootkapokbos' and 'sandveldkapokbos'.

Geographical distribution

This species has two subspecies. *E. ericoides* subp. *ericoides* with glabrescent, shiny, bright green leaves has the widest distribution of all *Eriocephalus* species. It extends from Namibia to Free State, Northern, Western and Eastern Cape, 300 m in altitude and occurs in summer and winter rainfall areas (200-500 mm). In Namibia, the species occurs in high areas (1000-1700 m altitude) in summer rainfall areas (250-300 mm). The Namibian species are fairly isolated from those in South Africa but there are no major morphological differences. *Eriocephalus ericoides* subsp. *griquensis* with permanently felty and dull green leaves is restricted to the Northern Cape, from Orange River to near the Botswana border (map 1). Distribution maps of *E. ericoides* subsp. *griquensis* (map 1) and *E. ericoides* subsp. *ericoides (*map 2) (Müller *et al*., 2001).⇨





Botanical description

Erect, many-stemmed, relatively sparsely branched, conical or broom-like shrubs, 0.3-1.0 m high, 300-400 in diameter, not or rarely spinescent. Old stems displaying anomalous secondary growth. Leaves mostly opposite, rarely alternate on flowering shoots with felty, glabrescent or permanent hairy indumentum. Capitula heterogamous disciform spicate racemose or racemose or solitary, and pedunculate (1.0-5.5 mm). Marginal florets yellow. Paleae of marginal florets free. Chromosome number 2n = 18. Flowering time correlated with rainfall, January to April, in summer rainfall areas and July to September in winter rainfall areas (Müller *et al*., 2001).

Ethnobotanical Uses

Medicinal

Traditionally used as a diaphoretic and diuretic.

Others

Used as fodder and browsed by wild animals.

The stereochemistry also has an influence on bioactivity. It has been observed that α-isomers



The erect, many-stemmed, and conical habit of *E. ericoides* subsp. *ericoides* in a relatively moist habitat (Prince Albert).



A spicate-racemose flowering and fruiting shoot showing disciform capitula.



A sparsley branched habit of *E. ericoides* subsp. *ericoides* (Windhoek, Namibia) in a very dry habitat.



Wooly heads of the disciform capitula.

Chemical composition

Essential oil
Extraction by hydrodistillation yielded 0.22% (wet wt) of clear to pale yellow and deep blue essential oils.

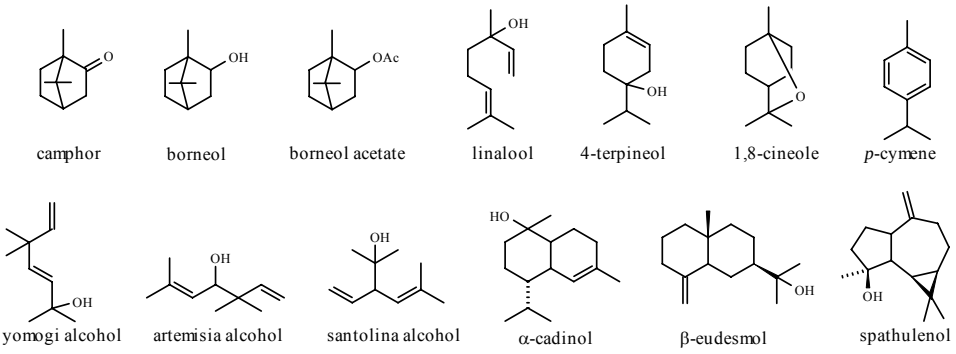
GC/MS
Major constituents
The essential oils contain approximately 86 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of individuals of *E. ericoides* subsp. *ericoides* for six populations. Pop 1 and 2 -Namibia (Windhoek, Hohenheim); Pop 3 and 4-Prince Albert; Pop 5-Scheepersrust; Pop 6-BT-Bethulie. Values are given in percentages. Compounds in bold represent some of the major compounds.

		Pop		Pop				Pop 5			Pop 6	
RI	**Compound**	**1**	**2**	**3**	**4A**	**4B**	**4C**	**A**	**B**	**C**	**A**	**B**
901	Santolina triene	0.7	1.7	0.2			0.5	0.7			<0.67	0.6
921	Artemisia triene	0.3										
922	α-Thujene			<0.23					0.2			
928	α-Pinene	1.0	0.3	2.2	0.3	1.0	1.0	0.9	0.3	1.9	<0.67	1.1
939	α-Fenchene								<0.2		<0.67	
939	α-Fenchene + camphene	2.6										
940	**Camphene**			0.9	1.4	1.5	0.4			1.2	0.9	
963	Sabinene			0.2	0.1	<0.47	<0.45		0.4	0.2		
966	β-Pinene	0.6		1.9	0.3	0.8	1.3	0.4	0.5	0.3		0.5
970	**3,5-heptadien-2-ol, 2,6-dimethyl**	5.4	1.2									
979	MW=152		3.3								1.0	
988	**Yomogi alcohol**	5.6	4.4	1.8			0.8	8.9			7.6	22.4
992	α-Phellandrene		2.8						0.4			
1005	α-Terpinene			0.8	0.4				3.7			
1008	**p-Cymene**	1.3		1.3	0.6	2.2	2.9	1.8	9.5	4.1	0.7	0.7
1016	**1,8-Cineole**	39.0	2.1	40.6	27.3	23.4	46.9	30.3	6.8	30.7	1.6	
1018	Limonene			0.7	0.4				<0.2			
1024	**Santolina alcohol**	1.5	4.8	1.7			5.4	13.2			17.5	11.3
1044	Unknown 1	0.8	2.2								1.0	
1047	**γ-Terpinene**			1.7	0.9			0.7	8.1			
1050	*Cis*-Sabinene hydrate								2.7			
1054	Unknown 2	1.8	5.5								2.4	
1070	**Artemisia alcohol**	3.4	1.6	0.4			<0.45	2.0			2.0	14.1
1075	α-Terpinolene			0.4	0.2				1.9			
1078	*Trans*-Sabinene hydrate								0.9			
1083	**Linalool**		10.4				0.5		1.3			
1091	MW=150									7.7		
1099	MW=154								2.0			
1110	**Camphor**	14.3	0.7	6.4	15.5	18.5	2.2			5.6	3.5	0.5
1114	*Trans*-pinocarveol			0.5		1.2	0.5				2.0	
1117	MW=154								1.3			
1130	Pinocarvone			0.4		1.1	0.7				1.3	
1133	Mean peak=100, PM=170		0.7								10.8	
1134	Nerol oxide		3.7									
1138	Mean peak=100, PM=170										2.3	
1141	Pinocamphone			1.9								
1141	**Borneol + Pinocamphone**	3.2			3.3	5.1						
1141	**Borneol**		3.0							5.1	0.8	
1155	**4-Terpineol**			4.6	2.7	3.4	3.0	3.1	33.2	2.3		1.0
1158	**Artemisyl acetate**	3.2	3.2								5.0	
1160	Myrtenal					0.5						
1166	α-Terpineol								1.5			
1172	Myrtenol			0.9	0.7	0.6	<0.45		1.5			
1183	*Cis*-Piperitol								0.8			
1192	Methyl *trans*-chrysanthemate											0.5
1204	Carvone					0.6						
1218	MW=152		2.6									
1240	**Linalyl acetate**		5.0						3.9		1.0	
1239	***Trans*-Chrysantemyl acetate**									4.2		
1262	**Bornyl acetate**	0.9	4.3	1.2	9.6	16.0	1.6					0.6
1267	Acetate MW=196											0.8
1272	Lavandulyl acetate											1.2
1282	Unknown 3	2.6										
1309	Unknown 4		1.4									
1328	MW=204		0.9									
1335	*Cis*-Carvyl acetate					0.5						

		Pop		Pop				Pop 5			Pop 6	
RI	**Compound**	**1**	**2**	**3**	**4A**	**4B**	**4C**	**A**	**B**	**C**	**A**	**B**
1357	*Cis*-Jasmone				0.4							
1360	Geranyl acetate			0.5								
1362	α-Copaene		1.1			2.8	1.5					1.1
1364	Unknown 5		1.9									
1369	Isocomene										1.0	
1383	Unknown 6		0.7									
1391	Unknown 7	0.9	0.9									
1398	β-Caryophyllene	0.3		0.7	0.6	0.7		0.9	<0.2			0.8
1439	Alloaromadendrene				0.7		<0.45	0.6	0.4			
1469	Unknown 8	1.2										
1470	Unknown 9		1.9									
1472	γ-Elemene						2.4					4.2
1474	**Bicyclogermacrene**			3.5	3.5	0.7			0.5			
1491	**γ-Cadinene**						4.0					
1495	Mean peak=159						0.8					
1500	δ-Cadinene		1.7	<0.23	0.8	<0.47			<0.2			1.2
1530	MW=236	2.3	0.3									
1534	MW=236										13.8	
1536	MW=220									1.3		
1544	Spathulenol	0.5	1.8	5.5	7.2	7.6	1.8	6.1	4.6	4.5	4.4	3.2
1548	Caryophyllene oxide	0.3	1.0		2.6	1.8		2.4	0.6	2.6	2.0	1.2
1581	MW=222			0.7						3.3		
1599	Main peak=136							4.6	0.5		1.3	
1608	**α-Cadinol or τ-Muurolol**		1.4	2.1		1.3	8.0		2.7		1.8	4.1
1611	**β-Eudesmol**			6.8		1.4	9.0	12.3	7.1	1.2		
1616	MW=204		2.7									
1618	MW=181	0.7									9.9	
1618	MW= 22							3.8	2.8			
1632	Jatamansone					0.2						
1680	Chamazulene			1.0	2.1							
	Total %	**94**	**81.2**	**91.35**	**82**	**93**	**94.9**	**92.5**	**99.68**	**76**	**95.7**	**70.9**

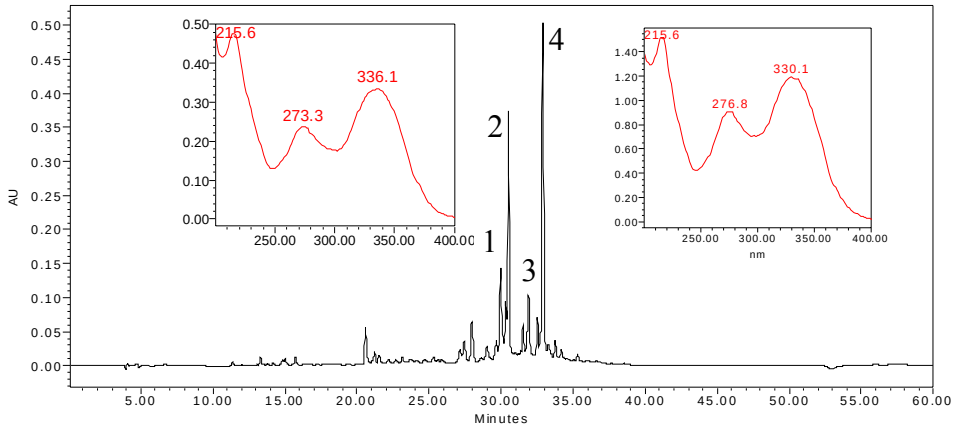
The essential oils are largely comprised of acyclic, monocyclic and bicyclic mono- and sesquiterpenes of pinane, camphane, linalool derivatives, artemisyl groups and azulenes. There is also characteristically high number of esters. Differences between individuals of the same population and between populations are clearly depicted in the essential oil profiles as observed in the table above. The overall chemistry of the species is highly variable but what emerges is the fact that the individuals studied have relatively high contents of 1,8-cineole. The structure for the major compounds is shown below.



Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various classes as shown in the chromatogram below. The UV spectra of the main peaks are also shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. ericoides* subsp. *ericoides* (Scheepersrust). The UV spectra of the flavonoids (peak 1 and 4) at retention time 29.98 and 32.88 minutes respectively are shown. Most of the major compounds are flavones as shown in the chromatogram.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. ericoides* subsp. *ericoides* from Prince Albert (PA); Scheepersrust (SP); Bethulie (BT); WDK (Windhoek); HOH (Hohenheim). A- represents individuals from a single population. Only the (%) of the major peaks is noted.

Peak number	Retention time	$UV_{(\lambda max)}$	SP A	PA A	BT A	WDK	HOH
1	29.98	216, 273, 336	10.78	24.87	14.52	7.81	19.85
2	30.51	244, 293, 341	27.91	–	10.75	14.56	15.47
3	31.91	216, 243, 274, 340	4.8	17.78	–	–	11.90
4	32.88	216, 278, 330	45.84	41.92	42.10	19.47	27.11

Biological properties

Antimicrobial activity

The essential oils were active against all the test pathogens. Good activity was noted for *Cryptococcus neoformans* (*Cn*), *Candida albicans* (*Ca*) and *Bacillus cereus* (*Bc*), moderate activity against *Bacillus subtilis* (*Bs*) and *Staphylococcus aureus* (*Sa*) and low activity against *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). The leaf extracts showed moderate to low activity against most of the test pathogens as shown in Table 3. A summary of the biological activities is given in Table 3.

Minimum inhibitory concentration

The essential oils and extracts showed strong to moderate inhibitory activity against the test pathogens as shown in Table 3.

Antioxidant activity
The extracts were moderate to weakly active in the DPPH assay with activity of 43.7-52.7 μg/ml. A summary of activities is included in Table 3.

Antiinflammatory activity
The essential oil showed moderate to low inhibitory activity against 5-lipoxygenase enzyme.

Acetylcholinesterase enzyme inhibition
Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Phylogenetic studies
The individuals from six populations are diverse in their chemistry as depicted by the erratic distribution of their taxa in the combined phylogeny. The individuals from Bethulie had closer relationships with those from Namibia and an individual of each are most parsimoniously placed as sister taxa in a clade. The erratic distribution of the taxa of this species is not surprising as it is the most widely distributed in the genus and therefore with extensive morphological variation in different habitat types. This species has close affinities with *E. africanus, E. brevifolius, E. capitellatus, E. dinteri, E. microphyllus, E. namaquensis* and *E. punctulatus* based on the chemistry.

Table 3. A summary of biological properties of *E. ericoides* subsp. *ericoides* from different populations. Pop 1 and 2- Windhoek and Hohenheim; Pop 3 and 4-Prince Albert; Pop 5-Scheepersrust and Pop 6-Bethulie (BT). EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	Extract	Activity in mm from the edge of the disc							5-lox IC_{50} μg/ml	DPPH IC_{50} μg/ml
		Cn	Ca	Bc	Bs	Sa	Kp	Ec		
Pop 1	EO	3.2	2	4	1.5	2	1	1	*	*
Pop 2	EO	7	2.5	3	1	2.1	1.5	R	43.1	*
Pop 3	EO	R	1	7.3	3	1	<1	<1	*	*
Pop 4 indiv A	EO	4.5	1	4	<1	<1	R	R	*	*
Pop 4 indiv B	EO	5	1	3	1.2	1	R	R	*	*
Pop 4 indiv C	EO	3	2	4	3	3	1	1	*	*
Pop 5 indiv A	EO	3	<1	2.5	2	<1	<1	R	55.4	*
Pop 5 indiv B	EO	2	1	3	1	1	R	1	*	*
Pop 5 indiv C	EO	3	1	2	1	1	<1	R	*	*
Pop 6 indiv A	EO	9	5	3	2	1	1	1.2	*	*
Pop 6 indiv B	EO	4	2	2	3	2	1	1.5	*	*
Pop 1	AE	1.5	R	1	R	2.5	R	R	*	45.1
Pop 2	AE	2	R	1.3	<1	2	R	R	*	43.7
Pop 3	AE	3	R	1	<1	<1	R	R	*	47.9
Pop 4 indiv A	AE	3	R	2	1.5	1	R	R	*	56.7
Pop 5 indiv A	AE	2	R	3	1.5	1	R	R	*	48.8
Pop 6 indiv A	AE	R	R	<1	1	1	1	R	*	52.7
Pop 6 indiv B	AE	R	R	1	1	2	<1	R	*	44.8
MIC mg/ml	EO	1-16	4-16	4-8	*	4-8	8-16	16	*	*
MIC mg/ml	AE	1.6	*	0.4-1.6	*	1.6-3.1	*	*	*	*

*Not tested.

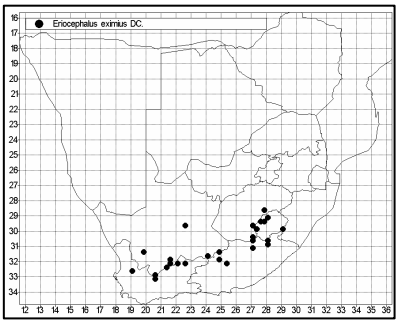
References
Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

9. *E. eximius* DC

Common name

'Grootbergkapok'.

Geographical distribution



The species is restricted to the high mountainous parts of the Free State, Lesotho, Northern (Sutherland, Bo-Visrivier and Kamiesberg) Western and Eastern Cape. It grows singly or in small groups. Distribution map of *E. eximius* (Müller *et al*., 2001).⇨

Botanical description

Much branched, rigid shrubs, 0.3-0.6 m high. Old stems and branches bare. Leaves opposite, entire and permanently silvery sericeous indumentum. Capitula heterogamous radiate, solitary, terminal and sessile or subsessile. Peduncles shorter than 0.5 mm long. Ray florets pale to dark red-purple or white. Paleae of marginal florets connate. Chromosome number 2n = 18. Flowering time correlated with rainfall, January to April in summer rainfall areas, July to August in winter–rainfall areas (Müller *et al*., 2001).

Uses

Browsed.



The multi–branched, rigid habit of *E. eximius* (Sutherland).



Radiate capitula with pale purple rays.

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.03% (wet wt) of pale blue essential oil.

GC/MS

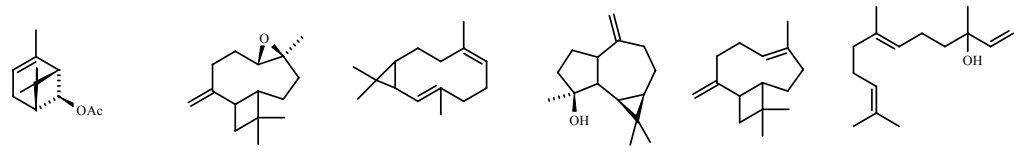
Major constituents

The essential oils contain approximately 49 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of *E. eximius* from Sutherland. Values are given in percentages. Compounds in bold represent some of the major compounds.

RI	Compound	A	B	C
901	Santolina triene	0.6		
928	α-Pinene	0.3	<0.17	
963	Sabinene	0.4		
966	**β-Pinene**	4.1	0.2	
984	β -Myrcene	<0.27		
988	Yomogi alcohol	0.3	0.4	
1008	p-Cymene	1.8	<0.17	0.1
1016	**1,8-Cineole**	7.5	1.5	0.2
1018	Limonene	<0.27		
1024	Santolina alcohol		0.4	
1047	γ-Terpinene	0.7		
1083	**Linalool**	<0.27		1.1
1114	*Trans*-pinocarveol			1.4
1141	Pinocamphone	0.5		
1141	Borneol			1.1
1143	MW=152		0.9	
1155	4-Terpineol	1.0	0.4	2.0
1158	Artemisyl acetate		0.3	
1160	Myrtenal	0.4		
1166	α-Terpineol	0.4		
1172	Myrtenol	<0.27		0.6
1234	Geraniol			0.6
1239	***Trans*-Chrysantemyl acetate**		12.8	
1262	Bornyl acetate			0.2
1267	Acetate MW=196	0.5		
1272	Lavandulyl acetate		0.5	
1272	Unknown 1	0.6		
1280	Unknown 2		8.1	
1360	**Geranyl acetate**	2.3	3.8	3.4
1362	α-Copaene		1.0	
1363	MW=204	1.9		
1366	Methyl eugenol			0.5
1369	Isocomene	0.9		
1376	β-Elemene	1.2		0.3
1398	**β-Caryophyllene**	9.6	6.8	8.8
1432	α-Humulene	2.3	2.4	2.9
1439	Alloaromadendrene	<0.27		0.3
1474	**Bicyclogermacrene**	2.5	8.1	2.0
1491	γ-Cadinene	0.6	2.6	
1500	**δ-Cadinene**	2.2	3.1	3.2
1536	MW=220	1.2		
1542	**Nerolidol**	12.1		
1544	Spathulenol	4.9	13.3	
1548	**Caryophyllene oxide**	10.2	4.2	10.6
1597	MW=222		4.7	
1599	Main peak=136	3.6	**2.8**	3.4
1608	α–Cadinol or τ-Muurolol		0.8	3.7
1618	MW=222	8.7	2.4	8.0
1636	MW=220			2.6
	Total %	**83.1**	**81.33**	**57.01**

The essential oils are largely comprised of bicyclic mono- and sesquiterpenes and a few acyclic and monocyclic mono- and sesquiterpenes in varying concentrations. The three individuals have similarities and some differences in their chemistry. Individual B and C have closer chemistry as shown in the table above. The structure for the major compounds is shown below.

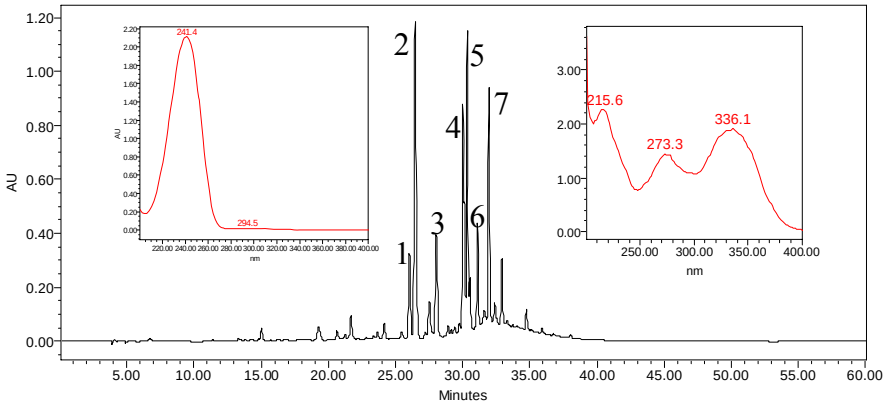


trans-chrysanthenyl acetate caryophyllene oxide bicyclogermacrene spathulenol β-caryophyllene nerolidol

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of flavone type as shown in the chromatogram below. The UV spectra of some of the main peaks are shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. eximius*. The UV spectra of the major components peak 2 and flavone (peak 4) at retention time 26.47 and 30.02 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. eximius*. Only the (%) of the major peaks are noted.

Peak number	Retention time	UV $_{(\lambda max)}$	% Area
1	26.00	218, 287, 393	6.01
2	26.47	209, 287, 339	24.64
3	27.52	222, 253, 291, 348	2.76
4	30.02	216, 273, 336	16.70
5	30.35	215, 273, 346	15.67
6	31.12	242, 289, 363	5.24
7	32.93	216, 24, 278, 330	9.21

Antimicrobial activity
The essential oils were active to moderatively active against *Cryptococcus neoformans* (*Cn*), *Bacillus cereus* (*Bc*) and *Staphylococcus aureus* (*Sa*) and low activity against *Bacillus subtilis* (*Bs*) and *Klebsiella pneumoniae* (*Kp*) and not active against *Candida albicans* (*Ca*) and *Escherichia coli* (*Ec*). The extracts showed moderate to low activity against five of the test pathogens as shown in Table 3. A summary of the biological activities is given in Table 3.

Table 3. A summary of biological properties of *E. eximius* of individuals from two populations. Pop 1-Sutherland; Pop 2-Kamiesberg. EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	**Extract**	**Activity in mm from the edge of the disc**							**5-Lox IC_{50} µg/ml**	**DPPH IC_{50} µg/ml**
		Cn	Ca	Bc	Bs	Sa	Kp	Ec		
Pop 1 indiv A	EO	R	R	3	<1	<1	<1	R	*	*
Pop 1 indiv B	EO	5	R	2	<1	1.5	R	R	*	*
Pop 1 indiv A	AE	R	R	1	<1	R	R	<1	*	56.9
Pop 1 indiv B	AE	2	R	R	1	R	R	R	*	50.3
Pop 1 indiv C	AE	2	R	R	1	R	R	R	*	43.8
Pop 2	EO	4.5	1.5	4.7	1.5	3	R	R	37.9	*
Pop 2	AE	1	R	1	<1	1.5	R	1	*	39.3

*-Not tested.

Antioxidant activity
The extracts showed moderate to low activity in the DPPH assay with activity ranging from 39.3-56.9 µg/ml. A summary of activities is included in Table 3.

Antiinflammatory activity
The essential oil of the individual from Kamiesberg showed a moderate inhibitory activity against 5-lipoxygenase enzyme.

Acetylcholinesterase enzyme inhibition
Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Phylogenetic studies
Individuals B and C from Sutherland grouped together in one clade as sister taxa in the combined phylogeny as they share similar chemistry while individual A and that from Kamiesberg grouped together in another clade as sister taxa with an individual of *E. africanus* as sister to the clade. This species has close relations with *E. africanus, E. punctulatus* and *E. luederitzianus.*

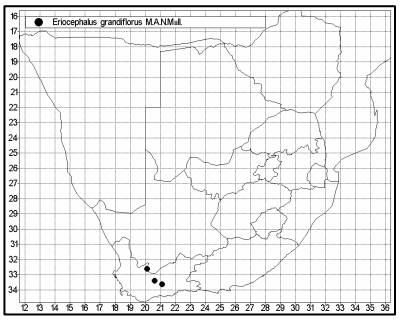
References
Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

10. *E. grandiflorus* M.A.N. Müller

Synonym
E. africanus L., *E. eximius* DC.

Common name
'Kapokbos'.

Geographical distribution
The species is confined to the mountainous area between the Roggeveld, Wittenberg and Swartberg Mountains. The species is also found in the Laingsburg and Matjiesfontein areas. Distribution map of *E. grandiflorus* (Müller *et al*., 2001).⇨



Botanical description
Robust, rigid, spinescent, woody and much-branched shrubs, 200-450 mm tall and the most aesthetic of the species of *Eriocephalus.* Old stems with grey to grey-black. Leaves decussate rarely alternate on flowering shoots with densely appressed sericeous indumentum. Capitula heterogamous radiate, terminally umbellate, pedunculate, 4-10 mm long. Ray florets 2-6 mm long, showy, white or pale to dark purple and red-purple disc florets. Paleae of marginal florets connate. Chromosome number 2n = 54. Flowering time is June to September (Müller *et al*., 2001).

Uses

Browsed



Habit of *E. grandiflorus* showing woody robust habit (Laingsburg).



The showy radiate capitula of *E. grandiflorus* with conspicuous large white ray florets.

Chemical composition

Essential oil
Extraction by hydrodistillation yielded 0.05% (wet wt) clear to pale yellow oil.

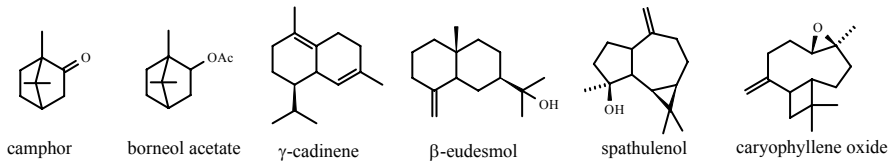
GC/MS

Major constituents
The essential oils contain approximately 36 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of three individuals of *E. grandiflorus* from Laingsburg. Values are given in percentages. Compounds in bold represent some of the major compounds.

RI	Compound	A	B	C
928	α-Pinene	2.4		1.5
940	Camphene	0.6		
963	Sabinene	<0.58		
966	β-Pinene	1.4		0.4
988	**Yomogi alcohol**	7.1	1.0	1.5
1008	p-Cymene	1.1	0.4	0.4
1016	1,8-Cineole	6.8	1.2	2.8
1018	Limonene			<0.4
1024	Santolina alcohol			0.5
1070	Artemisia alcohol	1.9	0.4	0.4
1083	Linalool	<0.58	0.4	
1110	**Camphor**	12.8	6.4	5.4
1114	*Trans*-pinocarveol	0.9	0.8	0.7
1130	Pinocarvone	1.1	0.7	0.9
1141	Borneol + Pinocamphone			1.2
1141	Borneol	0.7	0.7	
1155	4-Terpineol	0.6		0.7
1158	Artemisyl acetate	5.1		
1172	Myrtenol		0.9	0.7
1192	**Methyl *trans*-chrysanthemate**	9.1		
1239	*Trans*-Chrysantemyl acetate		2.4	
1262	**Bornyl acetate**	3.6	13.8	3.2
1295	Myrtenlyl acetate		1.9	
1342	Neryl acetate			0.5
1360	Geranyl acetate	3.9		3.8
1398	β-Caryophyllene	0.7		2.9
1432	α-Humulene			0.5
1474	Bicyclogermacrene			2.4
1491	**γ-Cadinene**			22.6
1500	δ-Cadinene	3.8		
1542	Nerolidol		5.5	
1544	**Spathulenol**	8.1	6.9	5.9
1548	**Caryophyllene oxide**	4.8	8.7	3.9
1599	Main peak=136	2.7	3.4	2.8
1611	**β-Eudesmol**		13.5	8.3
1618	MW = 222		4.8	3.2
	Total %	**79.24**	**73.51**	**77.12**

The three individuals have differences and similarities in chemistry as noted in most of the species in the genus. For instance, individual A has artemisyl acetate which is absent in the other two and likewise individual B has nerolidol and individual C has limonene; the former is absent in A and C and the latter is absent in A and B. They all have camphor, 1,8-cineole among several other compounds. The structure for the major compounds is shown below.

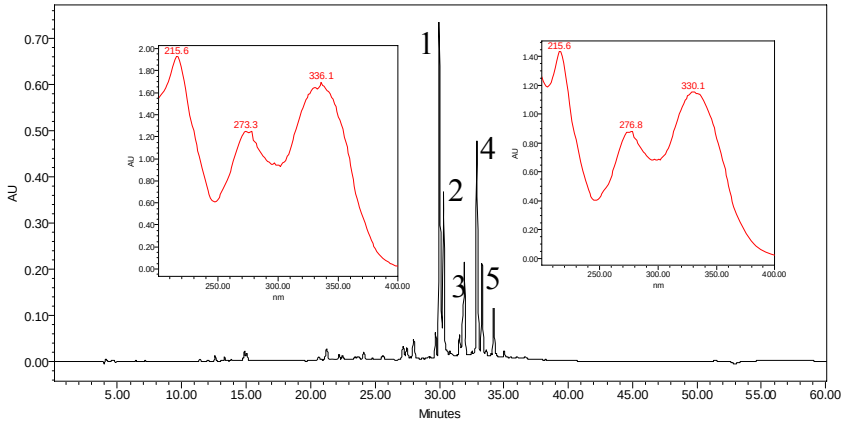


camphor borneol acetate γ-cadinene β-eudesmol spathulenol caryophyllene oxide

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various classes especially flavones as shown in the chromatogram below. The UV spectra of the main peak are also shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. grandiflorus.* The UV spectra of the major components mainly flavones (peak 1 and 4) at retention time 29.98 and 32.90 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. grandiflorus.* Only the (%) of the major peaks is noted.

Peak number	Retention time	$UV_{(\lambda max)}$	% Area
1	29.98	216, 273, 336	37.52
2	30.36	215 273, 346	14.57
3	32.00	223, 279, 333	10.70
4	32.90	216, 278, 330	22.06
5	34.21	215, 277, 330	6.09

Biological properties

Antimicrobial activity

The acetone leaf extracts were tested for antimicrobial activity. The activity was moderate against *Cryptococcus neoformans* (*Cn*) and *Bacillus subtilis* (*Bs*) but low to no activity against the rest of the test pathogens namely: *Bacillus cereus* (*Bc*), *Candida albicans* (*Ca*), *Staphylococcus aureus* (*Sa*), *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). A summary of the activities is given in Table 3.

Table 3. A summary of biological properties of *E. grandiflorus* from Laingsburg. EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	**Extract**	**Activity in mm from the edge of the disc**							**DPPH IC_{50} µg/ml**
		Cn	Ca	Bc	Bs	Sa	Kp	Ec	
Individual A	AE	R	R	1	2	R	R	R	45.6
Individual B	AE	3	R	<1	1	<1	R	R	46.0
Individual C	AE	3	<1	1	1	1	R	R	42.5

*****-Not tested**.**

Antioxidant activity

The extracts were active against DPPH with activity ranging from 42.5-46.0 µg/ml. A summary of activities is included in Table 3.

Acetylcholinesterase enzyme inhibition

Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Phylogenetic studies

Individual A is most parsimoniously placed as sister taxa to an individual of *E. aromaticus* from Ladismith in the combined phylogeny, while individual B and C group as sister taxa in agreement with a previous observation that they share similar chemistries. This species shows close affinities to *E. microphyllus, E. ericoides* subsp. *ericoides, E. brevifolius* and *E. aromaticus.*

References

Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

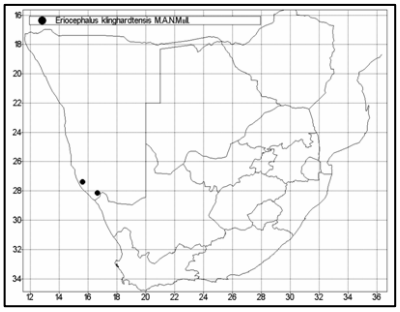
11. *E. klinghardtensis* M.A.N.Müller

Common name

'Kapokbos.'

Geographical distribution

Distribution restricted to the Klinghardt and Neiaab Mountains within the Namib Desert and the succulent steppe of Namibia (see map below) with 100 mm of rainfall annually in winter. The species is endemic to Namibia. Distribution map of *E. klinghardtensis* (Müller *et al*., 2001).⇨



Botanical description

Many-stemmed, much-branched aromatic shrub, 0.35-0.6 m high, 0.5 m in diameter. Old stems with anomalous secondary growth. Leaves opposite to subopposite even on flowering stems, semisucculent with densely felty-to-felty sericeous indumentum. Capitula heterogamous radiate in terminal umbellate racemes and pedunculate (7-10 mm long). Ray florets white. Paleae of marginal florets connate. Flowering time correlated with winter rainfall with peak from June to August (Müller *et al*., 2001).



Habitat of *E. klinghardtensis* (Namib Desert, Namibia).



Bushy habit of *E. klinghardtensis.*



Habit of *E. klinghardtensis* showing semi-succulent leaves.



Immature radiate capitula of *E. klinghardtensis.*

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.66% greenish yellowish essential oil.

GC/MS

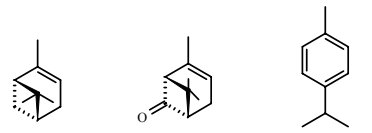
Major constituents

The essential oils contain approximately 19 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of *E. klinghardtensis* from Neiaab, Namibia. Values are given in percentages. Compounds in bold represent some of the major compounds.

RI	Compound	% Composition
922	α-Thujene	<1.02
928	**α-Pinene**	7.9
939	α-Fenchene	1.2
963	Sabinene	4.4
966	β-Pinene	3.8
1005	α -Terpinene	1.0
1008	**p-Cymene**	8.9
1016	1,8-Cineole	1.4
1018	Limonene	<1.02
1047	γ-Terpinene	4.3
1050	*Cis*-Sabinene hydrate	<1.02
1077	Filifolone	2.2
1092	**Chrysanthenone**	24.4
1143	MW=152	6.6
1155	**4-Terpineol**	5.4
1239	***Trans*-Chrysantemyl acetate**	5.6
1398	β-Caryophyllene	1.9
1548	Caryophyllene oxide	4.2
1611	**β-Eudesmol**	5.3
	Total %	**88.53**

The essential oil is largely comprised of bicyclic monoterpenes in varying concentrations. This is the only species in the current study with chrysanthenone as the major compound. Only one individual was studied and the structure for the major compounds is shown below.

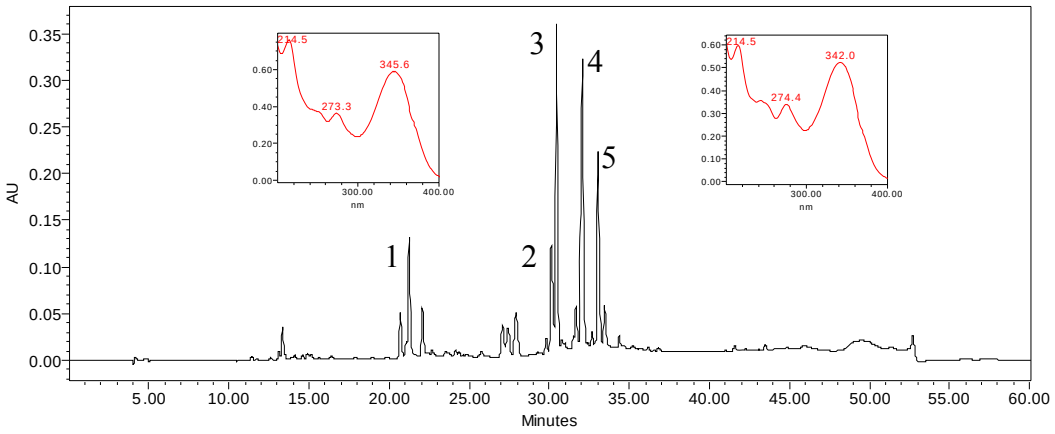


α-pinene chrysanthenone *p*-cymene

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of flavones as shown in the chromatogram below. The UV spectra of some of the main peaks are shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. klinghardtensis.* The UV spectra of the major components mainly flavones (peak 3 and 4) at retention time 30.43 and 32.06 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. klinghardtensis.* Only the (%) of the major peaks is noted.

Peak number	Retention time	UV $_{(\lambda max)}$	% Area
1	21.23	218, 243, 328	9.01
2	30.11	254, 293, 366	12.14
3	30.43	203, 256, 366	28.52
4	32.06	215, 274, 341	32.77
5	33.09	216, 274, 331	17.56

Biological properties

Antimicrobial activity

The essential oil of this species was active against all the test pathogens with the highest activity noted against *Cryptococcus neoformans* (*Cn*), moderate activity against *Bacillus cereus* (*Bc*), *Staphylococcus aureus* (*Sa*), *Klebsiella pneumoniae* (*Kp*), *Candida albicans* (*Ca*), and low activity against *Bacillus subtilis* (*Bs*), and *Escherichia coli* (*Ec*). The extract showed low activity against the test pathogens. A summary of the activities is given in Table 3.

Minimum inhibitory activity (MIC)

The oil showed moderate to low inhibitory activity all the test pathogens and the extract showed moderate inhibitory activity.

Antioxidant activity

The essential oil showed no activity at the starting concentration of 100 µg/ml but the extract was moderately active in the DPPH assay with activity of 28.1 µg/ml. A summary of activities is included in Table 2.

Table 3. A summary of biological properties of *E. klinghardtensis* from Neiaab Mountain (Namibia). EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. R-resistant.

Extract	Activity in mm from the edge of the disc							5-Lox IC_{50} µg/ml	DPPH IC_{50} µg /ml
	Cn	Ca	Bc	Bs	Sa	Kp	Ec		
EO	6.2	2	2.8	1.2	2.6	2.4	1	59	*
AE	<1	R	1.8	<1	2	R	R	*	28.1
MIC EO mg/ml	32	32	8	8	4	8	32	*	*
MIC AE mg/ml	*	*	2.0	*	*	*	*	*	*

*-Not tested.

Antiinflammatory activity

The oil has low inhibitory activity against 5-lipoxygenase enzyme.

Phylogenetic studies

The species is sister to the clade with individuals of *E. racemosus* var *racemosus* from Velddrif a relationship noted in almost all the analyses and in the phylogeny. Morphologically, they have connate paleae and have close chemical affinities.

References

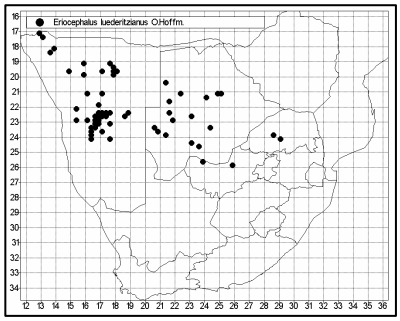
Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

12. *E. luederitzianus* O.Hoffm.

Synonym
E. eeni S.Moore, *E. squarrosus* Muschl, *E. hirsutus* Burtt Davy, *E. pubescens* sensu Merxm.

Common name
'Kapokbos.'

Geographical distribution
Distribution restricted to summer rainfall areas, extending over the Northern half of Namibia (12 km east of Windhoek) to most of Botswana into the Northern Province of South Africa. Distribution map of *E. luederitzianus* (Müller *et al*., 2001).⇨



Botanical description
Erect many-stemmed, sparsely branched aromatic shrubs, 300-500 m tall. Old stems with anomalous secondary growth. Leaves alternative, entire with permanently densly appressed silver-grey sericeous indumentum. Capitula heterogamous disciform, in terminal umbellate racemes and pedunculate (2-16 mm long). Marginal female and disc florets yellow. Paleae of marginal florets free. Chromosome number: 2n = 36. Flowering time October to May with a peak from January to March (Müller *et al*., 2001).



Habitat of *E. luederitzianus* (Neiaab Mountain, Namib Desert).



Many-stemmed and sparsely branched habit of *E. luederitzianus.*



Habit of *E. luederitzianus* showing disciform capitula.



Wooly heads of capitula of *E. luederitzianus.*

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.06% (dry wt) greenish yellowish essential oil.

GC/MS

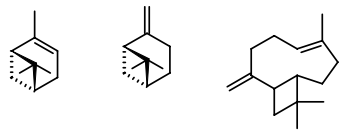
Major constituents

The essential oils contain approximately 15 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of *E. luederitzianus* from (Windhoek, Namibia). Values are given in percentages. Compounds in bold represent some of the major compounds.

RI	Compound	% Composition
928	**α-Pinene**	30.8
939	α-Fenchene	2.5
966	**β-Pinene**	10.3
984	β -Myrcene	1.5
1008	p-Cymene	6.7
1018	Limonene	2.1
1047	γ-Terpinene	3.6
1336	**α-Longipinene**	10.3
1368	Main peak=162	1.7
1398	**β-Caryophyllene**	13.3
1432	α-Humulene	1.3
1474	Bicyclogermacrene	1.3
1544	Spathulenol	1.7
1548	Caryophyllene oxide1	2.9
1946	MW=290	1.0
	Total %	**89.95**

This is the only species analysed with α-pinene as the major compound. Only one individual was studied and the structure for the major compounds is shown below.

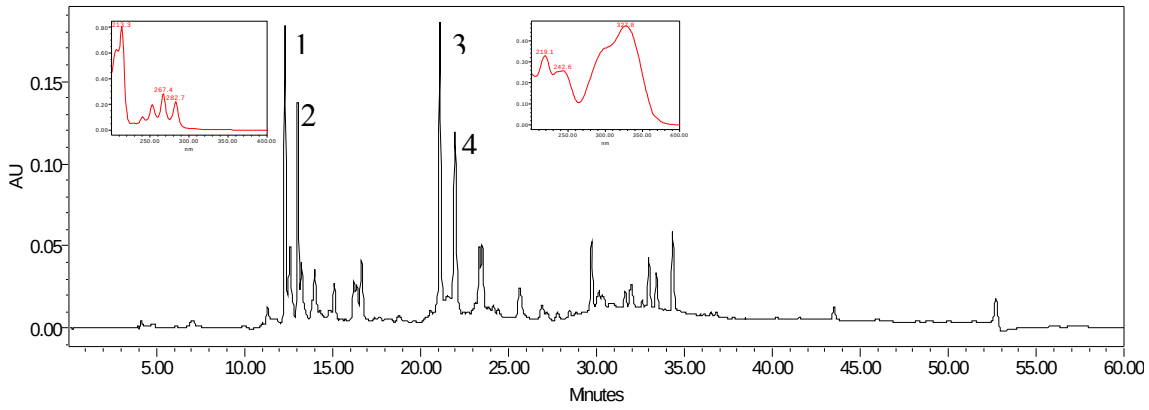


α-pinene β-pinene β-caryophyllene

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids as shown in the chromatogram below. The UV spectra of some of the main peaks are shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. luederitzianus.* The UV spectra of the major components peak 1 and 3 at retention time 12.32 and 21.12 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. luederitzianus.* Only the (%) of the major peaks is noted.

Peak number	Retention time	UV $_{(\lambda max)}$	% Area
1	12.32	213, 267,283	27.08
2	13.04	219, 265, 325	19.45
3	21.12	219, 243, 328	31.21
4	21.96	213, 267 283, 327	21.96

Biological properties

Antimicrobial activity

The essential oil of this species was active against nearly all the test pathogens except *Escherichia coli* (*Ec*). However, the activity was moderate *Cryptococcus neoformans* (*Cn*), *Bacillus cereus* (*Bc*) and *Staphylococcus aureus* (*Sa*) and low activity against *Klebsiella pneumoniae* (*Kp*), *Candida albicans* (*Ca*) and *Bacillus subtilis* (*Bs*). The extract showed low activity against the test pathogens. A summary of the activities is given in Table 3.

Minimum inhibitory activity (MIC)

The extract showed moderate to low inhibitory activity against two the test pathogens.

Antioxidant activity

The essential oil showed no activity at the starting concentration of 100 µg/ml but the extracts were fairly active in the DPPH assay with activity of 45.0-48.1 µg/ml. A summary of activities is included in Table 3.

Table 3. A summary of biological properties of *E. luederitzianus* from Windhoek (Namibia). EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. A and B are individuals of the species from same population. R-resistant.

Taxon	Extract	Activity in mm from the edge of the disc							5-Lox IC_{50} µg/ml	DPPH IC_{50} µg/ml
		Cn	Ca	Bc	Bs	Sa	Kp	Ec		
Individual	EO	2.8	1.5	2.1	1.2	3.8	<1.0	R	40.5	*
Individual A	AE	R	R	R	R	1.5	R	R	*	48.1
Individual B	AE	R	R	1	R	1.5	R	R	*	45.0
MIC mg/ml	AE		*	*	4		2-4	*	*	*

*-Not tested.

Antiinflammatory activity
The oil has low inhibitory activity against 5-lipoxygenase enzyme.

Phylogenetic studies
The species is a sister taxa to the individuals of *E. eximius* from two populations and one of *E. africanus* and is in the same clade with *E. klinghardtensis* in the combined phylogeny. Morphologically, these species have sericeous and opposite leaves**.** This species shows close affinities to *E. eximius, E. africanus* and *E. spinescens.*

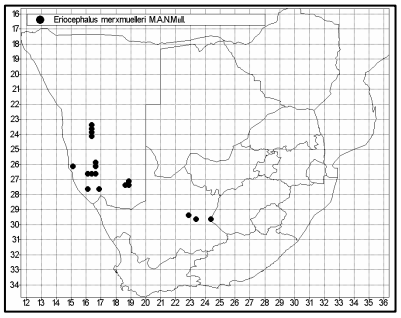
References
Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

13. *E. merxmuelleri* M.A.N. Müller

Synonym
E. microphyllus DC.

Common name

'Kapokbos'



Geographical distribution
Distributed in the summer and winter rainfall areas over the border between South Africa and Namibia (Buschmanberge) but restricted to the Namaqualand Broken Veld. Distribution map of *E. merxmuelleri* (Müller *et al*., 2001).⇨

Botanical description
Erect many-stemmed, much-branched shrubs, 0.4-1.2 m tall high, 0.3-0.6 m in diameter. Old stems with anomalous secondary growth. Leaves decussate, sometimes alternate on flowering shoots with felty sericeous indumentum to sometimes glabrous leaves. Capitula heterogamous disciform, in racemose panicles and pedunculate (2-7 mm long). Marginal female and discs florets cream coloured to yellow. Paleae of marginal florets free. Chromosome number: 2n = 54. Flowering time from December to April and from June to September (Müller *et al*., 2001).

Chemical composition

Essential oil
Extraction by hydrodistillation yielded 0.16% deep blue essential oil.

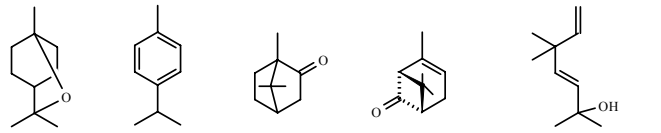
GC/MS
Major constituents
The essential oils contain approximately 25 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) for *E. merxmuelleri* from Buschmanberge (Namibia). Values are given in percentages. Compounds in bold represent some of the major compounds.

RI	Compound	% Composition
928	α-Pinene	2.0
940	Camphene	1.7
963	Sabinene	0.5
966	β-Pinene	1.8
988	**Yomogi alcohol**	6.1
992	α-Phellandrene	0.8
1005	α -Terpinene	0.6
1008	**p-Cymene**	3.5
1016	**1,8-Cineole**	17.4
1018	Limonene	0.7
1047	γ-Terpinene	1.3
1070	Artemisia alcohol	2.2
1092	**Chrysanthenone**	5.2
1110	**Camphor**	14.0
1141	Borneol + Pinocamphone	1.9
1155	4-Terpineol	2.9
1158	Artemisyl acetate	1.4
1210	o-Methylthymol	0.8

RI	Compound	% Composition
1215	Piperitone	0.5
1262	Bornyl acetate	2.8
1398	β-Caryophyllene	1.2
1474	Bicyclogermacrene	1.4
1530	MW=236	1.3
1544	Spathulenol	2.9
1548	Caryophyllene oxide	1.6
	Total %	**76.46**

The essential oil is largely comprised of camphane, pinane and artemisyl groups structural types in varying concentrations. Only one individual was studied and structure for the major compounds is shown below.

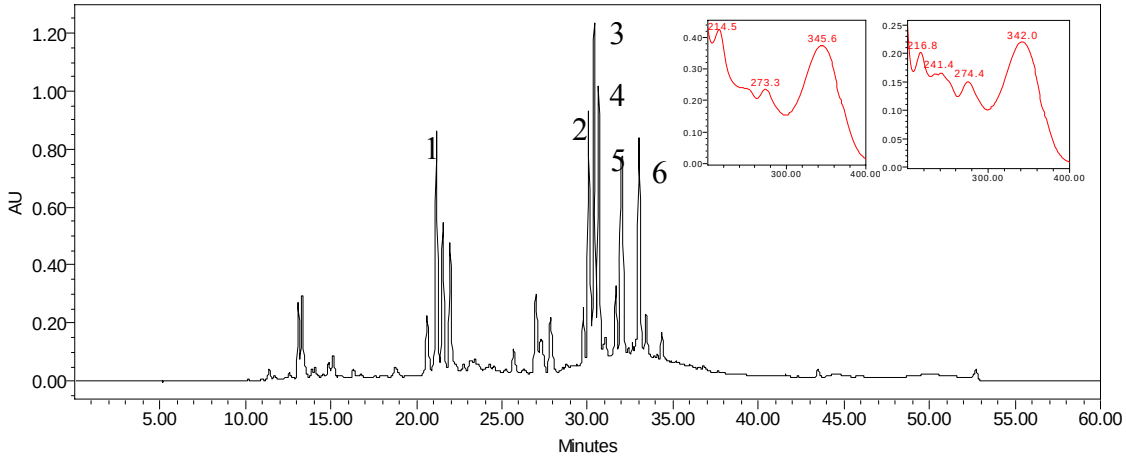


1,8-cineole *p*-cymene camphor chrysanthenone yomogi alcohol

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of flavones type as shown in the chromatogram below. The UV spectra of some of the main peaks are shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. merxmuelleri.* The UV spectra of the major components mainly flavones (peak 3 and 5) at retention time 30.41 and 32.01 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. merxmuelleri.* Only the (%) of the major peaks is noted.

Peak number	Retention time	UV $_{(\lambda max)}$	% Area
1	21.16	218, 243, 325	12.12
2	30.03	216, 273, 336	15.07
3	30.41	215, 273, 346	17.69
4	30.66	215, 273, 346	13.62
5	32.01	213, 274, 341	15.90
6	33.01	217, 274, 330	10.89

Biological properties

Antimicrobial activity

The essential oil of this species was active against most of the test pathogens with the highest activity noted against *Cryptococcus neoformans* (*Cn*), moderate activity against *Bacillus cereus* (*Bc*) low activity against *Staphylococcus aureus* (*Sa*), *Klebsiella pneumoniae* (*Kp*), *Candida albicans* (*Ca*) and *Bacillus subtilis* (*Bs*). The extract showed low activity against four of the test pathogens. A summary of the activities is given in Table 3.

Minimum inhibitory activity (MIC)

The oil showed good inhibitory activity against most of the test pathogens and the extract showed moderate inhibitory activity (2mg/ml) against *Staphylococcus aureus*, the best value observed among the species studied. The most notable activity for the extracts was against *Bacillus cereus.*

Table 3. A summary of biological properties of *E. merxmuelleri* from Buschmanberge (Namibia). EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. R-resistant.

Extract	Activity in mm from the edge of the disc							5-Lox IC_{50} µg/ml	DPPH IC_{50} µg/ml
	Cn	Ca	Bc	Bs	Sa	Kp	Ec		
EO	6	1.5	3.5	2	1.5	1.5	R	44.5	*
AE	2.4	R	1	R	1.5	R	<1.0	*	39.9
MIC EO	16	16	8	12	2	8	*	*	*
MIC AE	3.1	*	0.4	3.1	*	*	*	*	*

*-Not tested.

Antioxidant activity

The essential oil showed no activity at the starting concentration of 100 µg/ml but the extract was active in the DPPH assay with an IC_{50} value of 39.9 µg/ml. A summary of activities is included in Table 3.

Antiinflammatory activity

The oil has low inhibitory activity against 5-lipoxygenase enzyme.

Phylogenetic studies

The species is a sister to the clade with individuals of *E. africanus, E. punctulatus, E. capitellatus* and *E. ericoides* subsp. *ericoides* in the combined phylogeny, hence sharing some

affinities like opposite and decussate leaves.

References

Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

14. *E. microphyllus* DC.

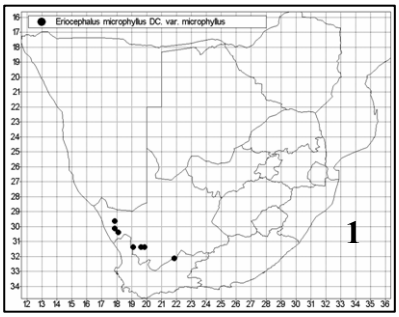
Synonym

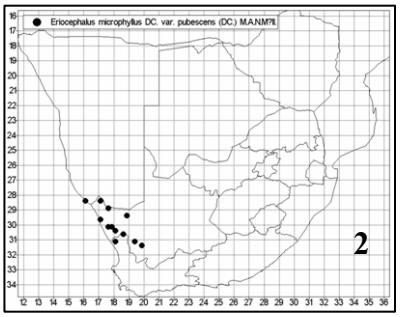
E. glaber Thunb. var *pubescens* Harv., *E. pubescens* DC.

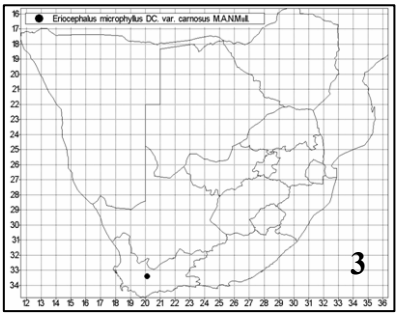
Common name

‘Kapokbos.’

Geographical distribution







This species has three varieties. *E. microphyllus* var *microphyllus* is typical of the Namaqualand (Northern Cape in Sutherland, Nieuwoudtville and Kamiesberg) and occurs mainly on low-lying plateau areas as shown in map 1. *E. microphyllus* var *pubescens* is concentrated mainly along the west coast in a more mountainous habitat than the former variety (map 2). The distribution of the third variety *E. microphyllus* var *carnosus* (map 3) is restricted mainly to the Worcester and Montagu Districts. It grows on shale and gravel plateaus (map 2). Distribution maps of *E. microphyllus* (Müller *et al*., 2001).⇨

Botanical description

Many-stemmed, markedly dichotomously but sparsely branched to densely intertwined shrub, 0.2-0.8 m high and 0.4-1.2 m in diameter. Old stems displaying anomalous secondary growth. Leaves opposite, decussate sometimes alternate on flowering shoots with felty-sericeous indumentum to glabrescent. Capitula heterogamous disciform, terminal, racemose or spicate racemose and pedunculate (1.5-11 mm long). Marginal female florets white. Paleae of marginal florets free. Chromosome number 2n = 36. Three varieties are recognised based on the peduncles length, leaves colour, indumentum and branching type. *E. microphyllus* var *microphyllus* flowering time is correlated with rainfall (summer and winter) with the peak in February to March and July to August in different rainfall areas. *E. microphyllus* var *pubescens* flowers mainly from July to September and var *carnosus* flowering period is correlated with the winter rainfall, June to September (Müller *et al*., 2001).

Uses

The species is reported as browsed by animals



The habit of *E. microphyllus* (Sutherland) showing the dichotomous branching.



A flowering and fruiting habit of *E. microphyllus* (Nieuwoudtville) in a dry habitat and with sparse branching.



A flowering stalk showing spicate and racemose arrangement of the pedunculate disciform capitula.



Fruiting heads of the disciform capitula. Note the opposite and decussate leaves on the flower stalks.

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.23% (wet wt) of pale brown to yellow, pale to dark green and blue essential oils.

GC/MS

Major constituents

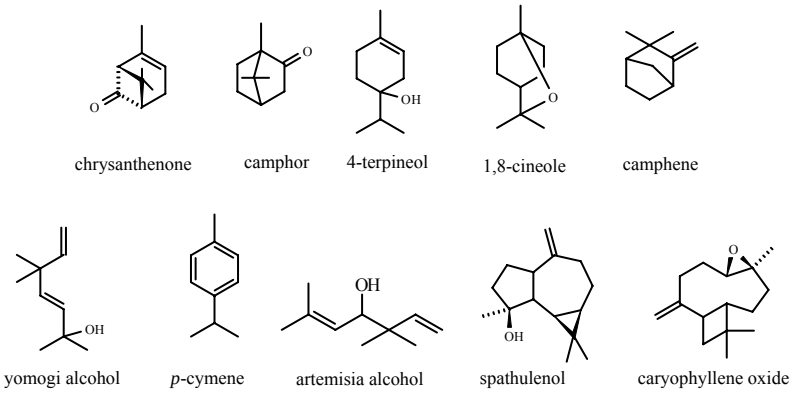
The essential oils contain approximately 56 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of individuals of *E. microphyllus* from four populations. Pop 1-Sutherland; Pop 2-Nieuwoudtville; Pop 3-Kamiesberg; Pop 4-Spektakel Pass. Values are given in percentages. Compounds in bold represent some of the major compounds.

		Pop 1			**Pop 2**			**Pop 3**	**Pop 4**
RI	**Compound**	A	B	C	A*	B	C		
901	Santolina triene					0.3			
922	α-Thujene			<0.29					
928	α-Pinene		1.2	0.9	0.4	0.4		1.2	1.0
940	**Camphene**			<0.29				3.5	3.8
963	Sabinene			0.3	0.3	0.3			
966	β-Pinene		0.6	0.6	0.2	1.8		1.4	1.3
988	**Yomogi alcohol**	4.7	1.0		15.1	19.1	0.7	4.1	3.7
1008	**p-Cymene**	2.5	6.3	18.0	1.5	2.0	0.2	1.8	0.3
1016	**1,8-Cineole**	32.4	11.8	12.3	25.5	39.5	<0.21	23.1	
1024	**Santolina alcohol**	5.7	0.9						
1047	γ-Terpinene					0.5		0.5	0.6
1050	*Cis*-Sabinene hydrate			0.8					
1057	*Cis*-linalool oxide	<1.25							
1070	**Artemisia alcohol**	2.4			10.2	8.0	0.2	2.3	1.2
1075	α-Terpinolene						0.4		
1077	Filifolone				1.3				
1078	*Trans*-Sabinene hydrate			0.4					
1083	Linalool	2.6							
1092	**Chrysanthenone**				14.0				
1091	MW=150		6.3						
1099	MW=154			1.9			1.2	4.9	
1110	**Camphor**	5.4	3.3	0.6			<0.21	25.0	27.4
1114	*Trans*-pinocarveol			0.5			0.4		
1116	1-Terpineol			1.2			1.6	3.5	
1141	Pinocamphone	3.0		2.3		1.3			
1155	**4-Terpineol**	3.5	10.2	26.8	2.7	4.7	15.4	1.4	1.2
1158	Artemisyl acetate				0.9	1.0			
1166	α-Terpineol			1.0			1.4		
1172	Myrtenol								0.9
1173	*Trans*-Piperitol			1.4			0.9	1.8	
1183	*Cis*-Piperitol						1.6	2.4	
1221	Unknown 1			2.4					
1240	Linalyl acetate	1.6							
1239	*Trans*-Chrysantemyl acetate		2.1						1.7
1262	Bornyl acetate	<1.25						0.7	2.6
1357	*Cis*-Jasmone						0.3		
1362	α-Copaene	1.3	2.2	0.4		0.7			0.8
1369	Isocomene								0.5
1398	β-Caryophyllene				0.6	0.7	0.4		
1442	Unknown 2	2.4							
1452	Unknown 2	1.4							
1472	γ-Elemene					0.6			
1474	Bicyclogermacrene							0.8	0.8
1500	δ-Cadinene + MW = 222		8.5	3.9				1.6	
1542	**Nerolidol**						3.7		
1544	**Spathulenol**	5.1	9.1	2.8	2.0	3.0	13.6	5.0	3.6
1548	**Caryophyllene oxide**	3.8	7.2	3.1	2.0	1.8	5.9	1.1	
1556	Viridiflorol or Globulol		1.1						
1560	Viridiflorol			1.1	0.9				
1581	MW=222					2.0	5.7		
1599	Main peak=136	1.5	6.1	1.2		2.9	4.6		
1607	α-Cadinol				3.6				
1608	**α-Cadinol or τ-Muurolol**			3.0		1.9	4.3	1.7	1.4
1611	β-Eudesmol	1.7		2.9				0.8	0.6
1632	Jatamansone					0.7	2.5		

		Pop 1			Pop 2			Pop 3	Pop 4
RI	**Compound**	A	B	C	A*	B	C		
1748	MW=248			1.2					
	Total %	**80.84**	**77.77**	**90.72**	**81.34**	**93.2**	**64.8**	**88.56**	**53.25**

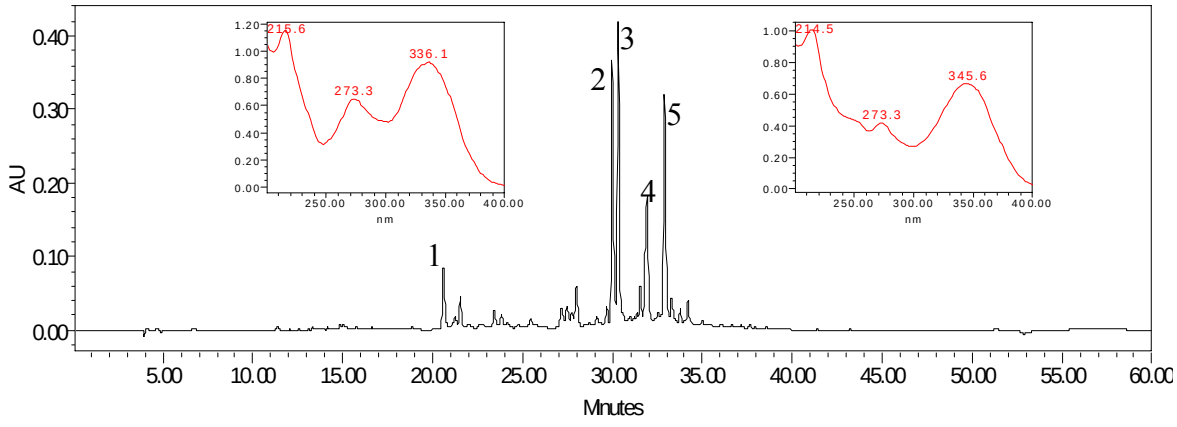
The essential oils are largely comprised of acyclic, monocyclic and bicyclic mono- and sesquiterpenes. The four populations have similarities and differences in their chemistry like the presence of α-thujene in the Sutherland population, the presence of santolina triene in the population from Nieuwoudtville only, presence of myrtenol in the Spektakel Pass population and presence of bicyclogermacrene in the population from Kamiesberg and Spektakel Pass only. The individuals within a population also differ in their chemical profiles as shown in Table 1. The structure for the major compounds is shown below.



Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various classes especially flavones as shown in the chromatogram below. The UV spectra of the main peaks are also shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. microphyllus* (Nieuwoudtville). The UV spectra of the major components mainly flavones (peak 2 and 3) at retention time 29.98 and 30.32 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. microphyllus* from STL/FG-Sutherland/Fraserburg; NV/LF-Nieuwoudtville/Loeriesfontein. Only the (%) of the major peaks is noted.

Peak number	Retention time	$UV_{(\lambda max)}$	STL/FG A	NV/LF A
1	20.59	202, 289	6.63	12.67
2	29.98	216, 273, 336	8.66	24.90
3	30.32	213, 273, 346	-	25.89
4	31.95	206, 243, 333	24.23	15.21
5	32.88	216, 278, 330	34.88	21.34

Biological properties

Antimicrobial activity

The essential oils showed activity against most of the test pathogens. The activity ranged from high to moderate against *Cryptococcus neoformans* (*Cn*), *Candida albicans* (*Ca*), *Bacillus cereus* (*Bc*) and *Staphylococcus aureus* (*Sa*) and low activity against *Bacillus subtilis* (*Bs*), *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). The extracts showed moderate to low activity against some of the test pathogens as shown in Table 3. A summary of the biological activities is given in Table 3.

Minimum inhibitory concentration

The essential oils and the extracts showed low inhibitory activity against the Gram-negative bacteria as shown in Table 3.

Antioxidant activity

The extract was active in the DPPH assay with activity of 41.58-47.67 µg/ml. A summary of activities is included in Table 3.

Antiinflammatory activity

The essential oil of the individual from Kamiesberg showed low inhibitory activity against 5-lipoxygenase enzyme.

Acetylcholinesterase enzyme inhibition

Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Table 3. A summary of biological properties of individuals of *E. microphyllus* from four populations. Pop 1-Sutherland; Pop 2-Nieuwoudtville; Pop 3-Kamiesberg; Pop 4-Spektakel Pass. EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	**Extract**	**Activity in mm from the edge of the disc**							**5-Lox IC_{50} µg/ml**	**DPPH IC_{50} µg/ml**
		Cn	Ca	Bc	Bs	Sa	Kp	Ec		
Pop 1 indiv A	EO	R	3	8	2	3	<1	<1	*	*
Pop 1 indiv B	EO	6	3	2	1	2	1	R	*	*
Pop 1 indiv C	EO	7	3	3	1.5	1.5	1.5	2	*	*
Pop 2 indiv B	EO	6	3	2	1	2	1	R	*	*
Pop 2 indiv C	EO	6	<1	3	R	1	1	1	*	*
Pop 3	EO	5	1	2	1	<1	2	1	69.4	*
Pop 4	EO	6	1	4	<1	<1	1.5	R	*	*
Pop 1 indiv A	AE	R	R	1	1	R	<1	<1	*	*
Pop 1 indiv B	AE	2	R	1	R	<1	R	R	*	46.20
Pop 1 indiv C	AE	2	R	1	R	<1	R	R	*	*
Pop 2 indiv A	AE	4	R	2	3	1	<1	<1	*	*
Pop 2 indiv B	AE	4	R	1	1	R	<1	<1	*	*
Pop 2 indiv C	AE	1	R	2	2	R	R	R	*	45.56
Pop 3	AE	2	1	<1	1	1	R	R	*	46.96
Pop 4	AE	3	2	3	1	1.5	R	<1	*	47.67
MIC mg/ml	EO	*	*	*	*	*	8	16	*	*
MIC mg/ml	AE	6.3	1.6	3.1	*	0.8	*	*	*	*

*-Not tested.

Phylogenetic studies

The chemistry of the individuals from the four populations included in the analysis differs largely and this phenomenon is reflected in the pattern of grouping of the taxa in the phylogeny. They are mostly erratically placed and are rarely most parsimoniously placed except in situations where two individuals A and B from Sutherland are sister to the one of the clades with individuals of *E. punctulatus* and *E. eximius.* This species has some affinities with *E. africanus, E. pinnatus, E. punctulatus, E. brevifolius, E. eximius,* and *E. purpureus* based on the chemistry.

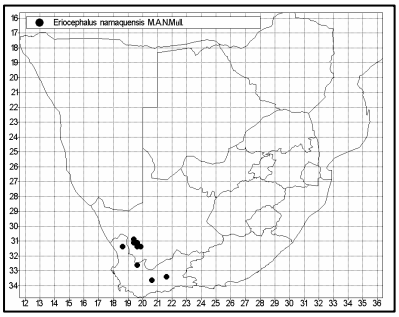
References

Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

15. *E. namaquensis* M.A.N. Müller

Synonym
E. microphyllus DC.

Common name
‘Kapokbos’.



Geographical distribution
Distributed in Namaqualand broken veld in summer and winter rainfall areas mainly in Clanwilliam and Perdefontein Farm as shown in the map. Distribution map of *E. namaquensis* (Müller *et al*., 2001).⇨

Botanical description
The habit is many stemmed and branched, 250-450 mm tall in diameter. Old stems with anomalous secondary growth. Leaves opposite-decussate with permanently densely silver sericeous indumentum. Capitula heterogamous disciform, solitary or in terminal racemes, peduncles 2.5-12 mm long. Marginal female flowers cream-colored. Paleae of marginal florets free. Chromosome number 2n = 18. Flowering periods varying between July to October and January to March (Müller *et al*., 2001).



Habit of *E. namaquensis* (Clanwilliam)



Disciform capitula of *E. namaquensis*

Chemical composition

Essential oil
Extraction by hydrodistillation yielded 0.05% (wet wt) pale yellow essential oil.

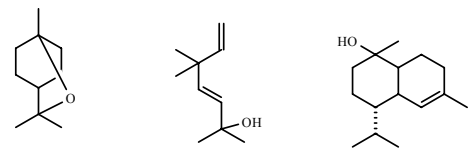
GC/MS

Major constituents
The essential oils contain approximately 39 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of three individuals of *E. namaquensis* from Clanwilliam. Values are given in percentages. Compounds in bold represent some of the major compounds.

RI	Compound	A	B	C*
928	α-Pinene	1.0		1.5
940	Camphene		<0.49	0.4
963	**Sabinene**	0.3	<0.49	0.5
966	β-Pinene	2.8	0.5	3.3
984	β -Myrcene	1.4		2.0
988	**Yomogi alcohol**	0.2	7.3	8.3
1008	ρ-Cymene	5.0	3.0	2.6
1016	1,8-Cineole	12.4	22.5	19.8
1018	Limonene	0.4		
1047	γ-Terpinene	0.2		
1070	Artemisia alcohol		2.2	4.0
1110	Camphor	0.7	6.1	3.6
1114	*Trans*-pinocarveol	0.7		
1129	Isopinocamphone	0.2		
1130	Pinocarvone	2.1		
1141	Pinocamphone		6.6	
1155	4-Terpineol	2.8	3.0	2.7
1158	Artemisyl acetate		4.0	
1172	Myrtenol	0.7	0.7	0.7
1192	Methyl *trans*-chrysanthemate			1.2
1262	Bornyl acetate	1.0	2.9	2.8
1362	α -Copaene	0.8		2.2
1369	Isocomene	1.2		0.8
1376	β-Elemene	0.5		
1398	β-Caryophyllene	2.1		4.2
1424	Neryl acetone		1.1	
1439	Alloaromadendrene	0.7		
1474	Bicyclogermacrene	0.7		1.0
1489	MW=222	1.4		
1491	γ-Cadinene		<0.49	
1500	δ-Cadinene	1.5		
1544	**Spathulenol**	13.6	5.5	7.0
1548	**Caryophyllene oxide**	3.5	6.1	7.0
1556	Viridiflorol or Globulol		0.9	
1599	MW=222		5.6	
1599	Main peak=136	2.4		3.8
1608	**α-Cadinol or τ-Muurolol**	15.0	12.6	
1611	β-Eudesmol			1.9
1618	MW=222	2.6		
	Total %	**78.03**	**90.57**	**81.22**

The essential oils are largely comprised of bicyclic mono- and sesquiterpenes in varying concentrations. The three individuals have different chemistry as noted in most of the species in the genus. An example is the presence of *trans*-pinocarveol and pinocarvone in individual A only. The structure for the major compounds is shown below.

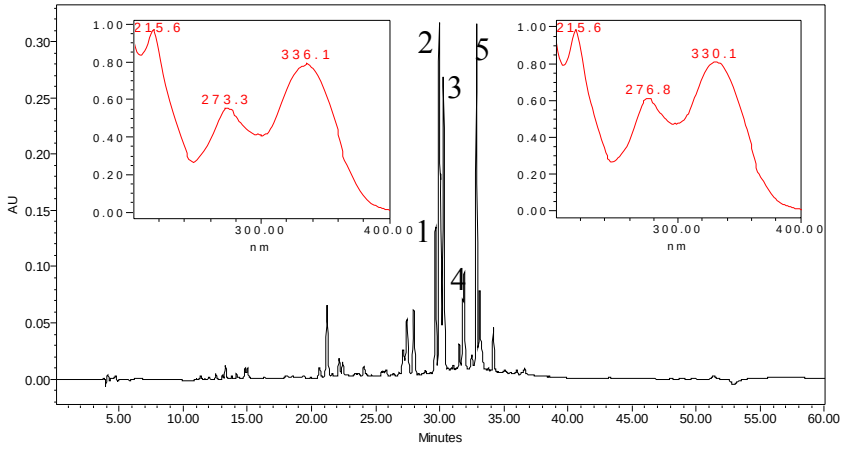


1,8-cineole yomogi alcohol α-cadinol

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various classes especially flavones as shown in the chromatogram below. The UV spectra of the main peak are also shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. namaquensis.* The UV spectra of the major components (2 and 5) at retention time 29.97 and 32.87 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. namaquensis.* Only the (%) of the major peaks is noted.

Peak number	Retention time	$UV_{(\lambda max)}$	% Area
1	29.67	227, 267, 336	8.74
2	29.97	216, 273, 336	32.62
3	30.30	215, 273, 346	19.57
4	31.90	215, 273, 342	9.14
5	32.87	216, 278, 330	22.41

Biological properties

Antimicrobial activity

Essential oils are more active than extracts. Highest activity noted against *Cryptococcus neoformans* (*Cn*), moderately active against *Bacillus cereus* (*Bc*) and *B. subtilis* (*Bs*) and lowest activity against *Candida albicans* (*Ca*), *Staphylococcus aureus* (*Sa*), *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). A summary of the activities is given in Table 3.

Antioxidant activity

The essential oils showed no activity at the starting concentration of 100 µg/ml but the extracts were active as free radical scavengers in the DPPH assay with activity ranging from 44.37-45.30 µg/ml. A summary of activities is included in Table 3.

Acetylcholinesterase enzyme inhibition

Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Table 3. A summary of biological properties of *E. namaquensis* from Clanwilliam. EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	**Extract**	**Activity in mm from the edge of the disc**							**DPPH IC_{50} µg /ml**
		Cn	Ca	Bc	Bs	Sa	Kp	Ec	
Individual A	EO	10	1	3.5	3	1.5	<1	<1	*
Individual C	EO	6	2	R	R	1	<1	R	*
Individual A	AE	4	R	2	1	<1	<1	<1	45.30
Individual B	AE	*	*	*	*	*	*	*	44.37
Individual C	AE	R	R	R	1	R	R	R	44.62

*-Not tested

Phylogenetic studies

Individual A is most parsimoniously placed as sister taxa to an individual of *E. punctulatus* from Nieuwoudtville as individual B is in a sister clade comprising *E. africanus, E. purpureus* and *E. microphyllus, E. ericoides* subsp. *ericoides* in the combined phylogeny. The species shows close affinities to the aforementioned species.

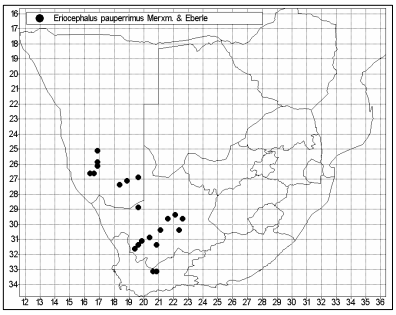
References

Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

16. *E. pauperrimus* Merxm. & Eberle.

Common name

'Kapokbos.'



Geographical distribution

The distribution of this species extends from southern Namibia through the Northern Cape to Matjiesfontein and Nieuwoudtville in the Western Cape in areas receiving less than 200 mm of rainfall per annum in summer and winter at an altitude of 300-600 m. Distribution map of *E. pauperrimus* (Müller *et al* ., 2001).⇨

Botanical description

An erect to spreading, many-stemmed, much-branched shrub, 350-450 mm tall and in diameter. Old stems with anomalous secondary growth. Leaves alternate and felty/glabrescent indumentum. Capitula heterogamous disciform, terminal and sessile. Paleae of marginal florets free. Chromosome number 2n = 18. Flowering time correlated with rainfall, January to March and June to September in summer and winter rainfall areas (Müller *et al*., 2001).



The spreading habit of *E. pauperrimus* (Nieuwoudtville).



The sessile disciform capitula of *E. pauperrimus*.

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.25% (wet wt) of cloudy white to pale green essential oil.

GC/MS

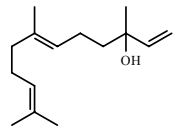
Major constituents

The essential oils contain approximately 36 compounds, which are summarized in Table 1

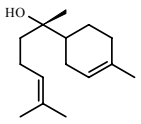
Table 1. Essential oil composition and retention index (RI) of *E. pauperrimus* Nieuwoudtville / Loeriesfontein. Values are given in percentages. Compounds in bold represent some of the major compounds. A, B and C are individuals from a single population.

RI	Compound	A	B	C*
901	Santolina triene	0.2		
922	α-Thujene		0.8	
928	α-Pinene	0.6	0.6	1.8
963	Sabinene		0.5	
966	**β-Pinene**	1.3	5.4	0.8
988	Yomogi alcohol	1.7	<0.5	
1008	**p-Cymene**	0.3	4.4	
1016	1,8-Cineole	0.2	0.6	
1018	Limonene		0.8	
1024	Santolina alcohol	1.2		
1070	Artemisia alcohol	0.3		
1076	Unknown 1	0.5		
1081	Unknown 2	0.5		
1118	*Cis*-Chrysanthemal	1.2		
1141	Pinocamphone		0.5	
1142	*Trans*-Chrysanthemal	1.0		
1155	Lavandulol	0.5		
1155	**4-Terpineol**		6.8	
1171	Verbenone			0.4
1362	α -Copaene		1.6	
1398	β-Caryophyllene	0.4	1.7	
1461	Ar-Curcumene			0.4
1472	γ-Elemene		3.5	
1474	Bicyclogermacrene	0.5		
1478	α-Muurolene		0.8	
1489	MW=222		5.3	
1495	Mean peak=159		1.2	
1500	δ-Cadinene		2.4	
1542	**Nerolidol**	5.1	36.1	
1548	**Caryophyllene oxide**	1.2	6.7	
1554	MW = 222			1.9
1607	α-Cadinol		3.6	
1628	**Bisabolol oxide B**	44.5	1.5	45.3
1644	**Bisabolone oxide**	3.8		14.7
1654	**α -Bisabolol**	3.5		2.4
1707	**Bisabolol oxide A**	21.7	<0.5	22.8
	Total %	**90.13**	**84.66**	**90.37**

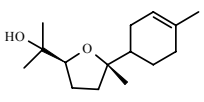
The three individuals have differences and similarities in chemistry as noted in most of the species in the genus. However, this species is the only one among the species studied with relatively high contents of bisabolol derivatives especially bisabolol oxide B, giving the species a distinct chemical profile. The structure for the major compounds is shown below.



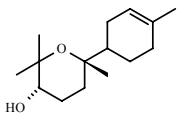
nerolidol



α-bisabolol



α-bisabolol oxide B

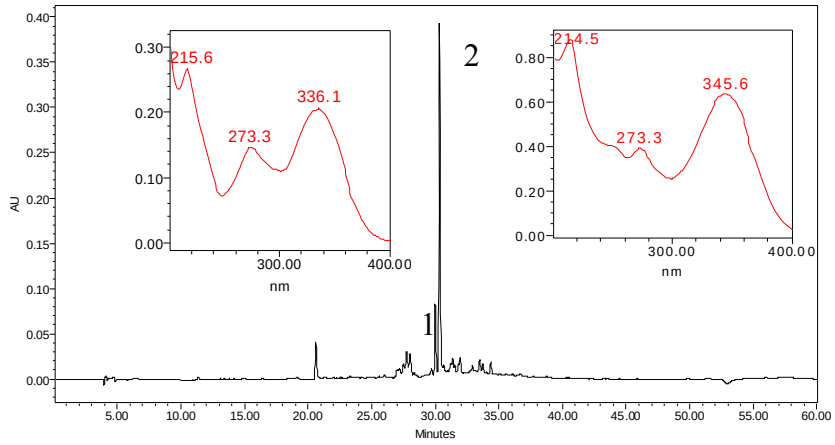


α-bisabolol oxide A

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various classes especially flavones as shown in the chromatogram below. The UV spectra of the main peaks are also shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. pauperrimus.* The UV spectra of the major components mainly flavones (peak 1 and 2) at retention time 29.99 and 30.32 and minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. pauperrimus.* Only the (%) of the major peaks is noted.

Peak number	Retention time	$UV_{(\lambda max)}$	% Area
1	29.99	215, 273, 336	10.99
2	30.32	215, 273, 346	54.77

Biological properties

Antimicrobial activity

In a disc diffusion assay, the essential oils were active against the yeasts *Cryptococcus neoformans* (*Cn*) and *Candida albicans* (*Ca*) and the Gram-positive *Bacillus subtilis* (*Bs*), *Bacillus cereus* (*Bc*) and *Staphylococcus aureus* (*Sa*) but showed no activity against the Gram-negative *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*) bacteria. The extracts showed moderate activity against three of the test pathogens as shown in Table 3. A summary of the activities is given in Table 3.

Minimum inhibitory concentration

One of the leaf extract tested had an inhibitory activity of 1.6 mg/ml against *Staphylococcus aureus.*

Table 3. A summary of biological properties of *E. pauperrimus* from Nieuwoudtville. EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	**Extract**	**Activity (mm) from the edge of the disc**							**5-lox IC_{50} µg/ml**	**DPPH IC_{50} µg/ml**
		Cn	Ca	Bc	Bs	Sa	Kp	Ec	*	
Individual A	EO	6	3	4	2	2	R	R	*	*
Individual C	EO	3	2	2	1.5	1.5	R	R	69.9	*
Individual A	AE	1	R	R	2	R	<1	R	*	46.6
Individual B	AE	4	R	R	R	R	<1	R	*	50
Individual C	AE	4	R	3	3	3	1	R	*	46.5
MIC mg/ml Individual C	AE	*	*	*	*	1.6	*	*	*	*

*-Not tested.

Antioxidant activity

The extracts were fairly active in the DPPH assay with activity ranging from 46.5-50 µg/ml. A summary of activities is included in Table 3.

Antiinflammatory activity

The essential oil showed low inhibitory activity against the 5-lipoxygenase enzyme.

Acetylcholinesterase enzyme inhibition

Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Phylogenetic studies

The three individuals grouped together in one clade with individuals A and C most parsimoniously placed as sister taxa due to their similar chemistry in combined phylogeny. The species shows some affinities with *E. klinghardtensis* and *E. racemosus* var *racemosus.*

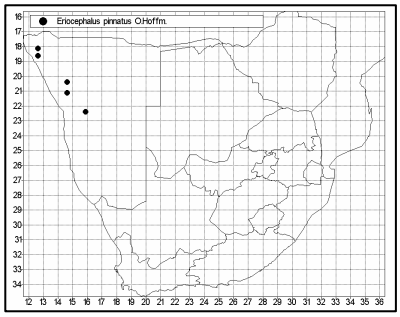
References

Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

17. *E. pinnatus* O.Hoffm.

Common name
'Kapokbossie.'

Geographical distribution
A Namibian endemic occurs in the northern and central Namib on the escarpment, in Brandberg and in Mopane savanna with annual rainfall of less than 200 mm. Distribution map of *E. pinnatus* (Müller *et al*., 2001).⇨



Botanical description
Erect many-stemmed, freely branched weakly woody shrubs or suffrutices, with annual regrowth, 350-450 mm tall, and 350 mm in diameter. Old stems without anomalous secondary growth and stems herbaceous only woody at base. Leaves alternate, distinctly pinnatisect with distinct petioles, with permanently felty grey-golden indumentum. Capitula heterogamous radiate, with racemose or umbellate terminal racemes and pedunculate (15-46 mm long). Distinct large golden yellow ray florets. Discs golden yellow. Paleae of marginal florets connate. Chromosome number 2n = 18. Flowering time March to May going into August and linked to rainfall (Müller *et al*., 2001).

Uses
Browsed

Chemical composition

Essential oil
Extraction by hydrodistillation yielded 0.09% (dry wt) greenish yellowish essential oil.

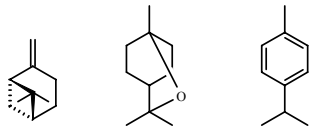
GC/MS
Major constituents
The essential oils contain approximately 33 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of *E. pinnatus* from Brandberg (Namibia). Values are given in percentages. Compounds in bold represent some of the major compounds.

RI	Compound	% Composition
928	α-Pinene	1.1
940	Camphene	0.5
960	Unknown 1	3.8
966	**β-Pinene**	7.3
992	α-Phellandrene	0.6
996	Propanoic acid, 2-methyl-3-butyl	2.3
1000	Isopentyl isobutanoate	1.1
1005	α-Terpinene	0.6
1008	**p-Cymene**	4.5
1016	**1,8-Cineole**	5.1
1018	Limonene	0.4
1047	γ-Terpinene	0.8
1050	*Cis*-Sabinene hydrate	1.1
1058	Unknown 2	0.8
1085	**Isoamyl-2-methylbutyrate**	7.9
1089	**Isoamyl valerate**	6.5
1094	Unknown 3	8.1

RI	Compound	% Composition
1097	Unknown 4	5.4
1099	MW=154	7.5
1110	Camphor	1.8
1116	1-Terpineol	3.8
1141	Borneol	2.0
1155	4-Terpineol	1.6
1160	Myrtenal	0.4
1166	α-Terpineol	0.7
1173	*Trans*-Piperitol	1.6
1183	*Cis*-Piperitol	4.1
1187	Unknown 5	1.7
1191	Unknown 6	2.0
1342	Neryl acetate	1.6
1363	MW=204	0.8
1368	Main peak=162	0.8
1474	Bicyclogermacrene	0.6
	Total %	**88.6**

The essential oil is largely comprised of acyclic, monocyclic and bicyclic mono- and sesquiterpenes of camphane and pinane groups. Only one individual was studied and the structure for the major compounds is shown below.

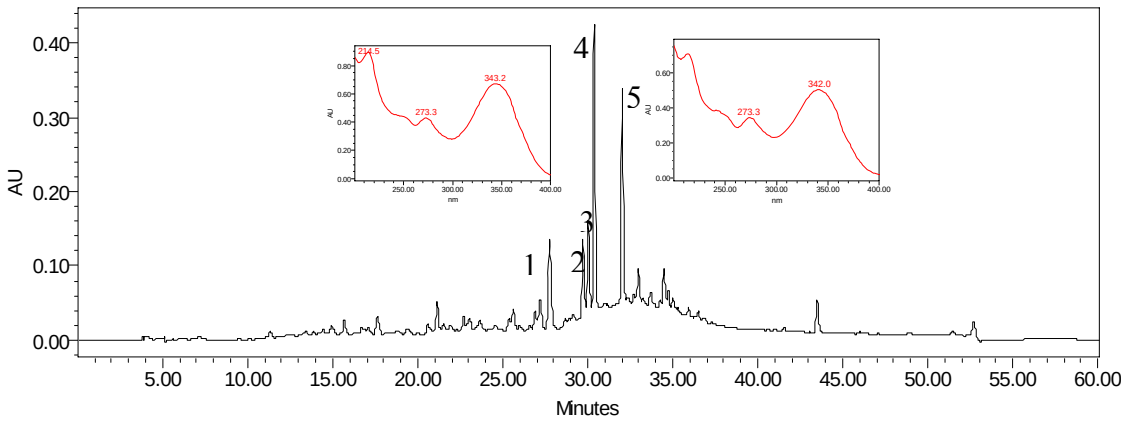


β-pinene 1,8-cineole *p*-cymene

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various structural types as shown in the chromatogram below. The UV spectra of some of the main peaks are shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. pinnatus*. The UV spectra of the major components mainly flavones (peak 4 and 5) at retention time 30.37 and 32.02 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. pinnatus.* Only the (%) of the major peaks is noted.

Peak number	Retention time	UV $_{(\lambda max)}$	% Area
1	27.76	271, 346	15.27
2	29.72	267, 336	10.57
3	30.03	216, 273, 336	11.95
4	30.37	215, 273, 343	35.72
5	32.02	273, 342	26.49

Biological properties

Antimicrobial activity

The essential oil of this species was active against nearly all the test pathogens with moderate activity noted for *Cryptococcus neoformans* (*Cn*) and *Staphylococcus aureus* (*Sa*) and low against *Bacillus cereus* (*Bc*), *Klebsiella pneumoniae* (*Kp*), *Candida albicans* (*Ca*) and *Bacillus subtilis* (*Bs*). The extract showed moderate activity against *Cryptococcus neoformans* (*Cn*) and low activity against the rest of the pathogens. A summary of the activities is given in Table 3.

Minimum inhibitory activity (MIC)

The oil showed moderate to low inhibitory activity against the test pathogens. The most notable activity (0.2 mg/ml) for the extracts was against *Bacillus cereus.*

Antioxidant activity

The essential oil showed no activity at the starting concentration of 100 μg/ml but the extract had low activity in the DPPH assay of 53.0 μg/ml. A summary of activities is included in Table 3.

Table 3. A summary of biological properties of *E. pinnatus* from Brandberg (Namibia). EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. R-resistant.

Extract	Activity in mm from the edge of the disc							5-Lox IC_{50} μg/ml	DPPH IC_{50} μg/ml
	Cn	Ca	Bc	Bs	Sa	Kp	Ec		
EO	3.8	1.5	5	<1.0	2.5	<1.0	R	58.7	*
AE	3.3	R	1.3	1	1.5	R	<1.0	*	53.0
MIC EO	16	16	8	*	8	8	*	*	*
MIC AE	6.3	*	0.2	*	*	*	*	*	*

*-Not tested.

Antiinflammatory activity

The oil has low inhibitory activity against 5-lipoxygenase enzyme.

Phylogenetic studies

This species is most parsimoniously placed as sister taxa with an individual of *E. microphyllus* from Kamiesberg and both species have felty indumentum. The species has close affinities to *E. microphyllus* and *E. capitellatus.*

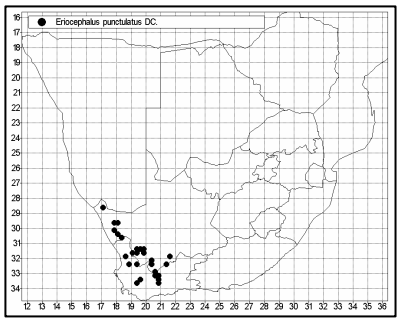
References

Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

18. *E. punctulatus* DC.

Common name
'Kapokbos.'

Geographical distribution
This distribution of this species extends from Springbok and Nieuwoudtville in the Northern Cape along the western parts of the Western Cape (The Roggeveld and Wittenberg Mountains). The species is found mostly in high-lying mountainous localities, above 300 m above sea level, mostly in winter rainfall areas. Slender, erect habits are known to occur in other veld types e.g. the Namaqualand Broken Veld, Succulent Karoo and Mountain Renossterbosveld with annual rainfall of 200 mm but in areas where rainfall exceeds 200 mm like the Fynbos and Coastal Renosterbosveld, habits that are more rigid are found. Distribution map of *E. punctulatus* (Müller *et al*., 2001).⇨



Botanical description
Slender, erect, sometimes spreading shrubs, 0.5-1.5 m high. Old stems displaying anomalous secondary growth. Leaves mostly opposite, but alternate on flowering shoots with felty indumentum to glabrescent. Capitula heterogamous radiate, umbellate-racemose, pedunculate (3-16 mm long). Ray florets white or occasionally pale red-purple. Paleae of marginal florets connate. Chromosome number 2n = 36. Flowering time correlated with rainfall, May to October with peak from July to September (Müller *et al*., 2001).

Ethnobotanical uses

Medicinal
Traditionally used as a diaphoretic and diuretic and in treatment of gastro-intestinal disorders, treatment of dermal complications and mental stress related ailments. Also used to treat inflammation and is used with *Metalasia muricata* in cleansing rituals after sickness or death and as fragrance in pillow cushions. Widely used in aromatherapy.

Industrial
Source of the commercial 'Cape chamomile' blue oil used in high class perfumes and as a blend oil in skin care products. There is likelihood that the commercially exploited species is not *E. punctulatus* based on the locality and the extreme differences noted between the essential oil composition of the taxa included in this study and that of the commercial essential oil profiles. It is probable that the commercial species is *E. tenuifolius,* which is morphologically so similar to *E. punctulatus* such that it is extremely difficult to tell them apart.

Others
Used as fodder and browsed by wild animals.



A habit of *E. punctulatus* showing alternate clavate-shaped leaves on flowering shoots.



Radiate capitula of *E. punctulatus* with white rays and red-purple disc florets.

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.13% (wet wt) of pale yellow and deep blue essential oils.

GC/MS

Major constituents

The essential oils contain approximately 74 compounds, which are summarized in Table 1.

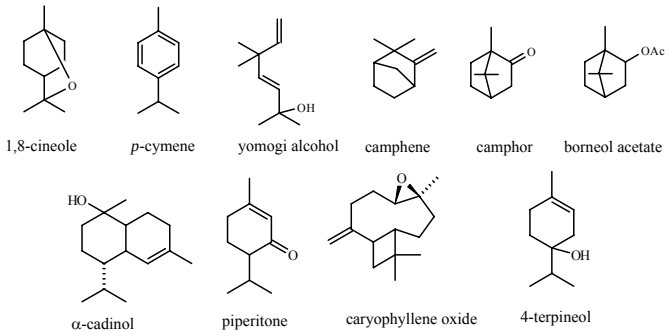
Table 1. Essential oil composition and retention index (RI) of individuals of *E. punctulatus* of three populations. Pop 1-Nieuwoudtville; Pop 2-Nieuwoudtville/Calvinia; Pop 3-Nieuwoudtville/Papkuilsfontein. Values are given in percentages. Compounds in bold represent some of the major compounds.

		Pop 1						Pop 2			Pop 3		
RI	**Compound**	**A**	**B**	**C**	**D**	**E**	**F**	**A**	**B**	**C**	**A**	**B**	**C**
901	Santolina triene	0.2		0.5	1.5	0.4							
922	α-Thujene											<0.47	
928	**α-Pinene**	0.8	1.0	0.5		0.4	3.4				3.2	3.9	2.1
940	**Camphene**	1.3	0.7	0.5		<0.42	1.0				2.3	1.6	0.5
963	Sabinene	0.4	<0.42	0.5	0.5	<0.42	0.8				0.6	1.7	1.1
966	β-Pinene	0.6	0.4	0.4	0.5	<0.42	1.2				2.4	1.9	1.0
984	β -Myrcene		<0.42									<0.47	<. 29
988	**Yomogi alcohol**	8.7	11.2	15.7	6.9	6.7	19.7	11.0	2.5	0.4			
1005	α-Terpinene						0.3					<0.47	
1008	**p-Cymene**	1.5	1.1	1.4	1.8	1.2	0.8	0.3			2.3	3.3	3.8
1016	**1,8-Cineole**	19.9	13.8	30.0	36.7	14.6	29.8	5.5			30.5	40.9	53.1
1018	Limonene										<0.44	0.9	
1024	**Santolina alcohol**				7.3								
1045	**Artemisia ketone**	0.5	0.5					1.0					
1047	γ-Terpinene	0.3	<0.42	0.8		<0.42	0.5	0.2				0.5	
1050	*Cis*-Sabinene hydrate	<0.24		0.3	0.6	<0.42							0.3
1070	**Artemisia alcohol**	3.7	2.7	3.4	9.0	0.9	2.8	3.9	1.6	0.3			
1075	α-Terpinolene							<0.27					
1083	Linalool			0.8				2.1	2.2				
1092	Chrysanthenone												0.5
1110	**Camphor**	13.2	13.0	3.8		10.4	9.2	1.9		1.3	14.0	15.1	<0.29

		Pop 1						Pop 2			Pop 3		
RI	**Compound**	**A**	**B**	**C**	**D**	**E**	**F**	**A**	**B**	**C**	**A**	**B**	**C**
1114	*Trans*-pinocarveol		1.1			0.8					0.4	0.5	0.4
1130	Pinocarvone	0.3	0.8								0.6	0.7	0.7
1141	**Pinocamphone**			1.4	3.8	1.0							
1141	**Borneol + Pinocamphone**							5.8					
1141	**Borneol**	5.2								3.1	4.6	3.9	3.4
1155	Lavandulol								0.8				
1155	**4-Terpineol**	2.6	1.5	2.0	2.8	2.5	1.9	5.6	3.1	1.5	2.3	5.5	5.5
1158	Artemisyl acetate			1.7	2.0			2.0					
1160	Myrtenal										<0.44		
1166	α-Terpineol							1.4	1.5		<0.44	0.8	0.3
1172	Myrtenol						0.7	1.9			<0.44		0.4
1183	*Cis*-Piperitol								0.4				
1215	**Piperitone**	20.6	27.7	18.3		25.6			6.3				
1240	Linalyl acetate			1.4			1.1	3.1	1.9				
1239	*Trans*-Chrysantemyl acetate												1.2
1262	**Bornyl acetate**	1.1									18.9	11.9	13.5
1272	Lavandulyl acetate				1.1								
1295	Myrtenlyl acetate							0.5					
1335	*Cis*-Carvyl acetate										0.7		
1342	Neryl acetate								0.6				
1357	*Cis*-Jasmone	<0.24		<0.31									
1360	Geranyl acetate								1.1			1.7	
1362	α-Copaene	<0.24	1.1	0.5	0.9	2.2		0.9	2.6	1.6	1.1	1.5	2.0
1368	Main peak=162							1.1					
1369	Isocomene		0.6						1.5	1.1			
1376	β-Elemene								0.9	0.4			
1398	**β-Caryophyllene**	0.6	1.4	1.1	2.0	1.9	1.4	2.6	4.7	4.9		0.5	0.6
1419	Aromadendrene											<0.47	
1432	α-Humulene		<0.42						0.4	0.7			
1434	MW=202								0.9				
1439	Alloaromadendrene	<0.24											
1469	Unknown 1										0.8		
1472	γ-Elemene					2.4							
1474	Bicyclogermacrene		0.6				0.9		4.2	2.3			
1484	Main peak=125			1.9									
1491	γ-Cadinene					3.8			0.6				
1494	Mean peak=125			1.2									
1495	Mean peak=159					1.0							
1500	δ-Cadinene	1.0	0.5			2.4	0.5	1.4	2.3	1.7			
1544	**Spathulenol**	1.5	2.7	1.1	1.6	0.9	2.4	1.3	7.8	7.7	1.5	1.6	1.6
1548	**Caryophyllene oxide**	1.2	2.4	1.0	2.6	1.5	2.2	2.4	8.2	6.6		1.7	1.0
1556	Viridiflorol or Globulol							1.3					
1560	Viridiflorol	1.2											
1581	MW=222							3.5			1.1		
1594	MW=222									4.7			
1597	MW=222					3.6					6.8		
1599	Main peak=136		1.7	0.9	**2.3**	1.8		5.5	5.0	5.8			1.1
1607	**α-Cadinol**	3.1					1.6			11.6			
1608	**α-Cadinol or τ-Muurolol**		4.2	0.7	2.7	5.7		13.9	12.1				
1611	**β-Eudesmol**		1.4				0.8			6.3			
1618	MW=222	0.9										<0.47	
1632	Jatamansone										<0.44		
1904	MW=246								2.8				
	Total %	**90.43**	**92.1**	**92**	**86.5**	**91.5**	**82.9**	**79.9**	**73**	**62**	**94.2**	**100**	**93.9**

The essential oils are largely comprised of acyclic, monocyclic and bicyclic mono- and sesquiterpenes of pinane, camphane, linalool derivatives, artemisyl groups and azulenes. There are also a characteristically high number of esters. Differences between individuals of the same population and between populations are clearly depicted in the essential oil profiles and the populations are distinguished on basis of the localities (Table 1). The population from Nieuwoudtville has piperitone and 1,8-cineole; the Nieuwoudtville/Calvinia is characterized

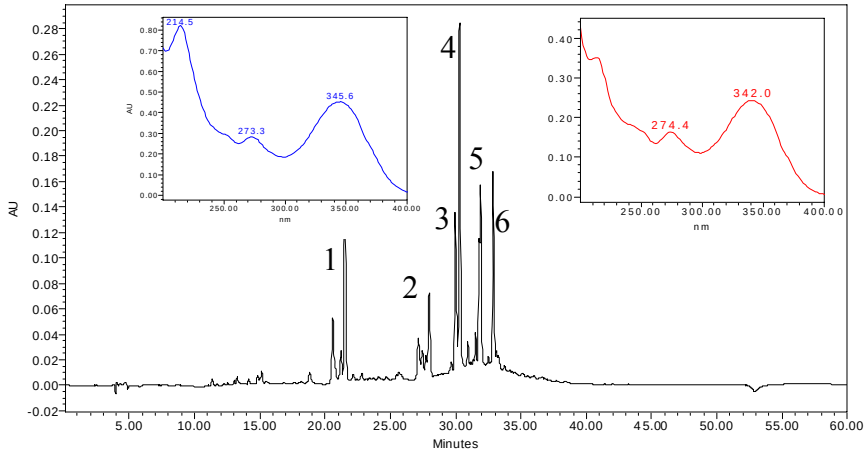
by the presence of α-cadinol or τ-muurolol and the Nieuwoudtville/Papkuilsfontein by 1,8-cineole as the major compound. The structure for the major compounds is shown below.



Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various classes especially flavones as shown in the chromatogram below. The UV spectra of the main peaks are also shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. punctulatus* (Nieuwoudtville/Calvinia). The UV spectra of the major components mainly flavones (peak 4 and 5) at retention time 30.29 and 31.89 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. punctulatus* from Nieuwoudtville (NV); Nieuwoudtville/Calvinia (NVCV) and Nieuwoudtville/Papkuilsfontein (NVPP). Only the (%) of the major peaks is noted.

Peak number	Retention time	UV (λmax)	NV			NV/CV	NV/PP
			A	B	C	A	A
1	21.59	240, 369	8.85	7.98	-	17.68	-
2	28.02	213, 253, 271, 346	7.07	-	8.87	1.56	17.17
3	29.96	215, 273, 336	21.63	10.66	33.23	15.63	28.22
4	30.29	215, 273, 346	29.97	21.68	27.56	29.32	18.65
5	31.89	212, 274, 342	-	16.26	-	21.78	-
6	32.87	216, 277, 330	1.97	17.20	-	15.60	-

Biological properties

Antimicrobial activity

The essential oils were active against all the test pathogens. Good activity was noted against *Cryptococcus neoformans* (*Cn*), *Candida albicans* (*Ca*), *Bacillus cereus* (*Bc*) and *Bacillus subtilis* (*Bs*) and moderate to low activity against *Staphylococcus aureus* (*Sa*), *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). The extracts showed good activity against *Cryptococcus neoformans* and *Bacillus cereus* and moderate to low activity against the rest of the test pathogens as shown in Table 3. A summary of the biological activities is given in Table 3.

Minimum inhibitory concentration

The essential oils and extracts showed moderate inhibitory activity against three test pathogens as shown in Table 3.

Antioxidant activity

The extracts showed some good activity in the population from Nieuwoudtville/Calvinia, and moderate to low activity in rest of the populations in the DPPH assay with activity of 21.46-79.63 μg/ml. A summary of activities is included in Table 3.

Antiinflammatory activity

The essential oil of the individual from population 1 showed low inhibitory activity against 5-lipoxygenase enzyme.

Acetylcholinesterase enzyme inhibition

Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Table 3. A summary of biological properties of *E. punctulatus.* Pop 1-Nieuwoudtville (NV); Pop 2-Nieuwoudtville/Calvinia (NVCV) and Pop 3-Nieuwoudtville/Papkuilsfontein (NVPP). EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	Extract	Activity in mm from the edge of the disc							5-Lox IC_{50} μg/ml	DPPH IC_{50} μg/ml
		Cn	Ca	Bc	Bs	Sa	Kp	Ec		
Pop 1 indiv A	EO	2	<1	3	1	1	1	1.5	*	*
Pop 1 indiv B	EO	9	2	3	2	1	1	1	*	*
Pop 1 indiv C	EO	3	5	2.5	3	1	1	1	*	*
Pop 1 indiv F	EO	*	*	*	*	*	*	*	63.8	*
Pop 2 indiv A	EO	R	2	3	4	1.5	<1	<1	*	*
Pop 3 indiv A	EO	5	2	5.5	2	1.5	1	2.5	*	*
Pop 1 indiv A	AE	6	1	3	2	R	<1	R	*	43.19
Pop 1 indiv A	AE	3	R	3	R	<1	R	R	*	65.65
Pop 1 indiv C	AE	R	R	1	2	2	<1	R	*	*
Pop 2 indiv A	AE	3	R	1	2	<1	R	R	*	44.97
Pop 2 indiv B	AE	*	*	*	*	*	*	*	*	32.42
Pop 2 indiv C	AE	*	*	*	*	*	*	*	*	21.46
Pop 3 indiv A	AE	3	R	R	R	R	<1	R	*	79.63
Pop 3 indiv B	AE	R	R	R	R	R	<1	R	*	38.8
Pop 3 indiv C	AE	R	R	1	R	R	R	<1	*	37.9
MIC mg/ml	EO	4	8	*	*	8	*	*	*	*
MIC mg/ml	AE	0.8	*	0.8	*	*	*	*	*	*

*- Not tested

Phylogenetic studies

The chemistry of the individuals from the three populations is similar and different to some extent. The individuals from Nieuwoudtville/Calvinia group in one clade with individuals A and C most parsimoniously placed as sister taxa as are individuals B and C from Nieuwoudtville/Papkuilsfontein but in a different clade in the combined phylogeny. Four individuals of population 1 group in one clade. It appears that this species is related to *E. aromaticus, E. microphyllus, E. namaquensis* and *E. ericoides* subsp. *ericoides* as most of its taxa group together with individuals of the aforementioned species.

References

Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

19. *E. purpureus* Burch.

Synonym

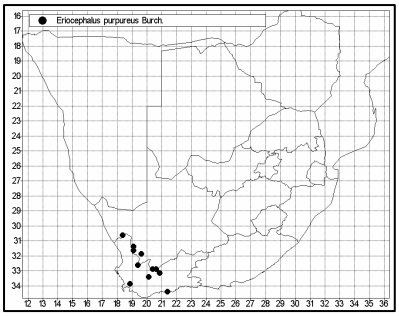
E. xerophilus Schltr.

Common name

'Kapokbos.'

Geographical distribution

The distribution of this species is restricted to the winter rainfall areas and extends from Kamiesberg to Nieuwoudtville, Loeriesfontein, Sutherland, Fraserburg, southwards to Matjiesfontein in the mountainous regions above 300 m. Distribution map of *E. purpureus* (Müller *et al*., 2001).⇨



Botanical description

Slender, erect, much-branched shrubs, 0.3-0.6 m high. Old stems displaying anomalous secondary growth. Leaves opposite, but alternate on flowering shoots with sparsely felty indumentum to glabrescent. Capitula heterogamous radiate in terminal umbellate-racemes, pedunculate (6-12 mm long). Ray florets pale to dark purple. Paleae of marginal florets connate. Chromosome number 2n = 36. Flowering time correlated with rainfall in June to September with a peak from July to August (Müller *et al*., 2001).

Uses

Used as fodder and browsed by wild animals.



The erect and many-branched habit of *E. purpureus* (Laingsburg).



Radiate capitula borne in terminal umbellate racemes. Note the purple ray florets.

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.08% (wet wt) of clear to pale greenish blue and yellow essential oils.

GC/MS

Major constituents

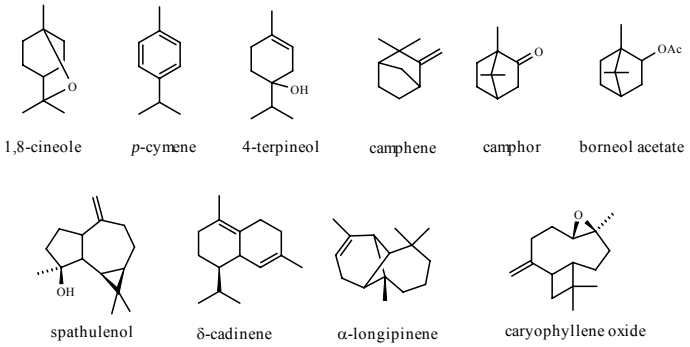
The essential oils contain approximately 57 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of individuals of *E. purpureus* from four populations. NV-Nieuwoudtville; LGMF-Laingsburg/Matjiesfontein; NVPP-Nieuwoudtville/Papkuilsfontein; KMG-Kamiesberg. Values are given in percentages. Compounds in bold represent some of the major compounds.

		NV	LGMF	NVPP			KMG*
RI	**Compound**			**A**	**B**	**C**	
901	Santolina triene						0.5
928	**α-Pinene**	<0.52	3.1	0.2	0.1	1.4	1.7
940	**Camphene**						5.3
963	Sabinene			**0.1**		0.1	
966	**β-Pinene**	<0.52	1.8	0.7		0.3	3.7
984	β-Myrcene			0.1			
988	Yomogi alcohol	<0.52		0.1	0.1	0.1	2.1
1005	α-Terpinene		0.2				
1008	**p-Cymene**	0.6	4.0	4.5	0.8	0.1	2.1
1016	1,8-Cineole	0.5	1.6	1.6	0.4	2.0	9.7
1018	Limonene		0.3	0.3	<.09	1.6	
1045	**Artemisia ketone**						2.7
1047	γ-Terpinene	<0.52	0.6				0.7
1070	Artemisia alcohol						1.1
1075	α-Terpinolene		0.2				
1076	Unknown 1					0.2	
1081	Unknown 2					1.3	
1110	**Camphor**		1.3				52.0
1114	*Trans*-pinocarveol	1.1	0.9	0.8	0.7		
1130	Pinocarvone	<0.52	0.6	0.5	0.3		
1141	Borneol + Pinocamphone			0.5			
1141	Borneol	0.8	0.7		1.0		
1155	**4-Terpineol**	2.0	3.7	6.8	3.5	1.2	1.1
1166	**α-Terpineol**		0.4	1.3	0.5	3.1	
1172	Myrtenol	<0.52					1.1
1204	Carvone					0.6	0.4
1215	Piperitone	<0.52				0.3	
1262	**Bornyl acetate**		3.9		0.4		1.2
1335	***Cis*-Carvyl acetate**		1.8	1.7	1.0		
1336	**α-Longipinene**	13.9				19.6	
1342	Neryl acetate	1.0					
1362	**α-Copaene**	2.9		1.0	0.8	3.8	0.8
1369	Isocomene	2.2	1.2	1.0	1.0		
1385	MW=204	1.1					
1398	**β-Caryophyllene**	2.0					
1419	**Aromadendrene**	2.2	1.0	0.7	0.6	2.5	
1461	**Ar-Curcumene**			1.5	1.2		
1468	MW=204	4.3	1.7	1.0	0.6		
1471	MW=222					0.7	
1474	Bicyclogermacrene		1.9				1.1
1476	Unknown 3		1.0				
1478	α–Muurolene				0.5	1.7	
1491	γ-Cadinene	1.4		0.9	0.9	1.4	
1495	Mean peak=159	2.2		1.0	1.2	4.1	
1500	**δ-Cadinene**	5.0				1.7	
1500	δ-Cadinene + MW = 222		6.8				
1544	**Spathulenol**	12.1	16.8	11.9	15.7	0.9	2.0
1548	**Caryophyllene oxide**	4.9	2.3	2.4	3.7	3.2	
1551	MW = 220	1.9					
1556	**Viridiflorol or Globulol**	3.4		1.4	3.6		
1560	Viridiflorol					1.9	
1581	MW=222			1.1	1.5		
1592	MW = 222	4.5					
1597	MW = 222				3.8		
1608	α–Cadinol or τ-Muurolol						1.6

		NV	LGMF	NVPP			KMG*
RI	**Compound**			**A**	**B**	**C**	
1611	β-Eudesmol						0.6
1714	MW=220				2.6		
	Total %	**69.8**	**57.79**	**43.22**	**46.6**	**53.77**	**91.44**

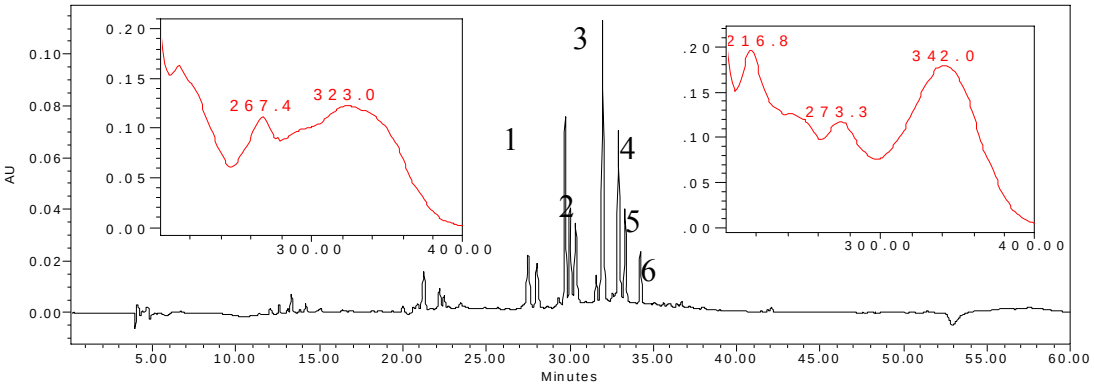
The four populations have similarities and differences in their chemistry like the presence of camphor in Laingsburg and Kamiesberg population only, absence of yomogi alcohol in the former population and the presence of bicyclogermacrene and ar-curcumene in Kamiesberg population only. Apart from the Kamiesberg population that seem to have slightly more variant chemistry, the rest of the population are more closely linked by their similarities in the chemical profiles. The structure for the major compounds is shown below.



Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various classes especially flavones as shown in the chromatogram below. The UV spectra of the main peaks are also shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. purpureus* (Laingsburg). The UV spectra of the major components mainly flavones (peak 1 and 3) at retention time 29.71 and 31.98 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. purpureus.* NV-Nieuwoudtville; LG/MF-Laingsburg/Matjiesfontein. Only the (%) of the major peaks is noted.

Peak number	Retention time	$UV_{(\lambda max)}$	Laingsburg	Nieuwoudtville
1	29.71	211, 267, 333	10.29	-
2	30.04	215, 273, 346	5.45	3.57
3	31.98	215, 233, 274, 342	17.00	41.57
4	32.91	215, 277, 330	9.10	-
5	33.33	233, 278, 339	5.01	3.46
6	34.22	218, 236, 279, 330	2.77	-

Biological properties

Antimicrobial activity

The essential oils were active against all the test pathogens. The activity ranged from high to moderate against *Cryptococcus neoformans* (*Cn*), *Bacillus cereus* (*Bc*) and *Bacillus subtilis* (*Bs*) and moderate to low activity against *Candida albicans* (*Ca*), *Staphylococcus aureus* (*Sa*), *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). The extracts showed good activity against *Cryptococcus neoformans* and *Bacillus cereus* and moderate to low activity against the rest of the test pathogens as shown in Table 3. A summary of the biological activities is given in Table 3.

Minimum inhibitory concentration

The extracts showed moderate inhibitory activity against the fungi and Gram-positive bacteria as shown in Table 3.

Antioxidant activity

The extract was active in the DPPH assay with activity of 36.15-42.33 µg/ml. A summary of activities is included in Table 3.

Table 3. A summary of biological properties of individuals of *E. purpureus* from four populations. Pop 1-Nieuwoudtville (NV); Pop 2-Laingsburg/Matjiesfontein (LGMF); Pop 3-Nieuwoudtville/Papkuilsfontein (NVPP); Pop 4-Kamiesberg (KMG). EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	Extract	Activity in mm from the edge of the disc							5-lox IC_{50} µg/ml	DPPH IC_{50} µg/ml
		Cn	Ca	Bc	Bs	Sa	Kp	Ec		
Pop 3 indiv C	EO	5	2	3.5	4	2	<1	<1	*	*
Pop 4	EO	5	R	2	1.5	<1	1	1	98.9	*
Pop 1	AE	3	R	R	1	R	<1	<1	*	36.15
Pop 2 indiv A	AE	3	R	1	R	R	<1	R	*	42.33
Pop 2 indiv B	AE	5	R	1	1	<1	<1	<1	*	37.56
Pop 2 indiv C	AE	*	*	*	*	*	*	*	*	37.26
Pop 3 indiv A	AE	*	*	*	*	*	*	*	*	40.05
Pop 3 indiv B	AE	*	*	*	*	*	*	*	*	39.54
Pop 3 indiv C	AE	7	R	1	2	R	<1	1	*	38.52
Pop 4	AE	4	2	4	1	1.5	R	<1	*	41.46
MIC mg/ml	AE	*	1.6	*	*	0.8	*	*	*	*

*-Not tested.

Antiinflammatory activity
The essential oil of the individual from Kamiesberg showed low inhibitory activity against 5-lipoxygenase enzyme.

Acetylcholinesterase enzyme inhibition
Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Phylogenetic studies
The chemistry of the individuals from the four populations included in the analysis is somehow similar to the extent that they group together phylogenetically except for the individual from Kamiesberg that is placed elsewhere in the topology. *E. purpureus* is placed in the same clade with *E. aromaticus, E. africanus* var *paniculatus* and an individual of *E. punctulatus.* All these species have radiate capitula.

References
Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

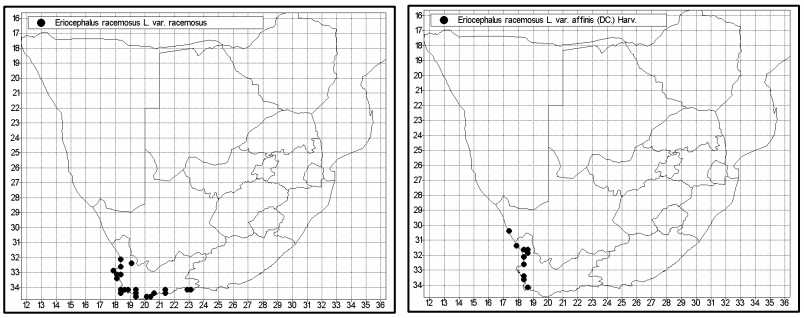
20. *E. racemosus* L.

Synonym
E. simplicifolius Salisb., *E. spicatus* Burm ex DC., *E. affinis* DC.

Common name
'Kapokbos', 'sandveldkapok', 'strandveldkapok', 'rivierkapok' (Bredasdorp area) and 'kapkappie.'

Geographical distribution

After *E. africanus, E. racemosus* is the oldest known species of *Eriocephalus.* The species has two varieties; var *racemosus,* which is distributed, along the coast forming part of the Coastal Fynbos. It extends as far east as Port Elizabeth and west to Lambert's Bay (see map). It also occurs in Velddrif and Koeberg. Var *affinis* distribution extends from near the coast to 50 km inland from Hondeklip Bay to Melkbosstrand. Distribution maps of *E. racemosus* (Müller *et al*., 2001).⇨



Botanical description
Many-stemmed, slender, erect shrubs, 1.2-2.0 m high. Old stems displaying anomalous secondary growth. Leaves alternate, rarely opposite, sessile, succulent and with permanently grey-felty indumentum. Capitula heterogamous disciform, racemose or paniculate, sessile to distinctly pedunculate (0-15 mm long). Marginal female florets with white to pink corolla. Paleae of marginal florets connate. Chromosome number 2n = 36. Flowering time June to September depending on the rain continuing until November sometime extending to April (Müller *et al*., 2001). Based on the obvious differences in capitulum structure together with differences in leaf shape, two varieties are recognized namely; *E. racemosus* var *racemosus* distinguished by the presence of sessile capitula that is shortly pedunculate (peduncle 5mm long) and disc florets (4-) 7-9. *E. racemosus* var *affinis* is characterized by the presence of distinctly pedunculate capitula (5-) 10-21 mm long and discs florets 13-21.

Ethnobotanical uses

Medicinal
The species is reportedly used to treat gastro-intestinal, respiratory ailments and for treating skin inflammation. It is also used as a diuretic and diaphoretic.

Chemical composition

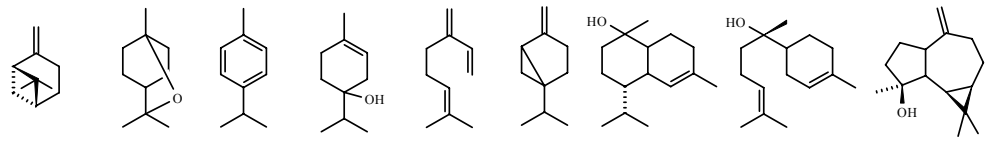
Essential oil
Extraction by hydrodistillation yielded 0.39% (wet wt) of light bluish green and deep yellow essential oil.

GC/MS
Major constituents
The essential oils contain approximately 51 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of individuals of *E. racemosus* var *racemosus* from two populations. Pop 1-Koeberg; Pop 2-Velddrif. Values are given in percentages. Compounds in bold represent some of the major compounds. Letter A, B and C represents individuals from a single population.

		Pop 1	**Pop 2**		
RI	**Compound**		**A**	**B***	**C**
922	α-Thujene				0.1
928	α-Pinene	2.3	0.4	0.6	0.9
939	α-Fenchene		0.9	1.9	1.1
963	**Sabinene**	1.1	4.2	10.3	7.4
966	**β-Pinene**	15.4	<0.31	0.5	0.8
984	**β-Myrcene**	19.7		0.3	
1005	α-Terpinene	0.3			
1008	**p-Cymene**	19.1	4.4	2.5	2.4
1016	**1,8-Cineole**	5.1	4.8	21.1	1.5
1018	Limonene	2.0	0.3	0.5	0.2
1047	**γ-Terpinene**	4.8	0.3	0.4	
1050	*Cis*-Sabinene hydrate		<0.31	1.1	1.9
1070	Artemisia alcohol				0.4
1078	*Trans*-Sabinene hydrate			0.9	1.7
1099	MW=154		0.4		
1110	Camphor		<0.31		
1114	*Trans*-pinocarveol		0.5		0.5
1116	1-Terpineol		0.6		
1129	Isopinocamphone				1.8
1130	Pinocarvone				2.1
1149	α-Thujenal		0.6		
1155	**4-Terpineol**	1.3	6.4	2.9	2.9
1160	Myrtenal				0.2
1172	Myrtenol		1.3	0.8	1.2
1256	p-Cymen-7-ol		0.5		
1336	α-Longipinene		1.1	0.7	
1362	α-Copaene	0.5			
1366	Methyl eugenol	<0.18			
1369	Isocomene		0.5		
1398	β-Caryophyllene	1.9			
1419	Aromadendrene				0.4
1422	α-Bergamotene	0.9			
1448	MW=220		9.3		5.9
1491	γ-Cadinene				0.3
1502	MW=220		0.9		
1506	MW=220		1.1		
1524	MW=220		1.4		
1542	Nerolidol			1.3	
1544	**Spathulenol**	2.1	8.7	7.0	4.1
1548	Caryophyllene oxide	3.8	1.2	1.5	
1577	MW=220		11.6		
1579	**MW=220**			5.3	
1581	MW=222				14.6
1607	α-Cadinol		1.3		
1608	α-Cadinol or τ-Muurolol				5.3
1632	Jatamansone				6.2
1654	**α-Bisabolol**		3.3	10.8	0.8
1812	En-in-dicycloether		1.2	2.3	
1840	MW=214		0.3	1.1	2.7
1892	MW=214		7.5	14.3	11.7
1964	MW=230		9.2		
	Total %	**80.25**	**75.19**	**88.02**	**78.99**

The four individuals have similarities and differences in their chemistry like the presence of α-bergamotene in the individual from Koeberg, the presence of α-thujenal in individual A only, nerolidol in individual B only and jatamansone in individual C only. On the overall, individual A and B have similar chemical profiles and C differs slightly. The chemistry of the Koeberg sample differs considerably from the Velddrif individuals. The structure for the major compounds is shown below.

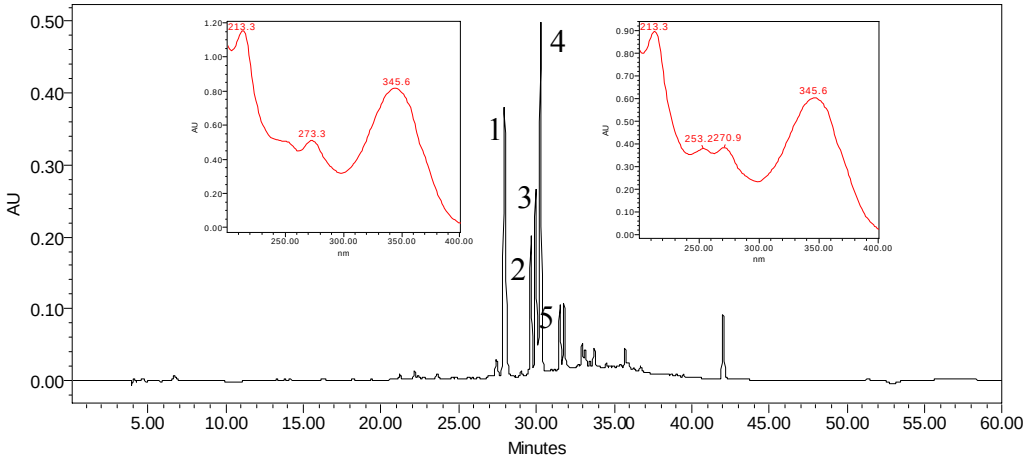


β-pinene 1,8-cineole *p*-cymene 4-terpineol myrcene sabinene α-cadinol α-bisabolol spathulenol

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various classes especially flavones as shown in the chromatogram below. The UV spectra of the main peaks are also shown. The rest of the data is summarized in Table 2.



A HPLC /UV chromatogram of leaf extracts of *E. racemosus* var *racemosus* (Koeberg). The UV spectra of the major components mainly flavones (peak 1 and 4) at retention time 27.94 and 30.28 minutes respectively are shown. Most of the major compounds are flavones as shown in the chromatogram.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. racemosus* var *racemosus* from Velddrif and Koeberg. Only the (%) of the major peaks is noted.

Peak number	Retention time	$UV_{(\lambda max)}$	Koeberg	Velddrif
1	27.94	213, 253, 270, 346	30.07	-
2	29.65	207 253, 288, 362	9.88	2.93
3	29.98	216, 273, 336	14.62	4.85
4	30.28	213, 273, 346	30.85	6.94
5	31.52	233, 270, 336	4.58	6.29
6	31.75	233, 270, 336	4.38	13.27

Biological properties

Antimicrobial activity
The essential oils were most active against *Cryptococcus neoformans* (*Cn*), *Bacillus cereus* (*Bc*), *Bacillus subtilis* (*Bs*) and *Staphylococcus aureus* (*Sa*) and low activity against *Candida albicans* (*Ca*), *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*). The extracts showed high to low activity against some of the test pathogens as shown in Table 3. A summary of the biological activities is given in Table 3.

Minimum inhibitory concentration
The essential oils and the extracts showed high to low activity against the test pathogens as shown in Table 3.

Table 3. A summary of biological properties of individuals of *E. racemosus* var *racemosus* from two populations. Pop 1-Koeberg; Pop 2-Velddrif. EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	Extract	Activity in mm from the edge of the disc							5-Lox IC_{50} μg/ml	DPPH IC_{50} μg/ml
		Cn	Ca	Bc	Bs	Sa	Kp	Ec		
Pop 1	EO	R	1	1	4	3	1	1	*	*
Pop 2 indiv A	EO	2	1	4	1	1	<1	R	*	*
Pop 2 indiv B	EO	2	R	4	1	<1	<1	R	32.8	*
Pop 2 indiv C	EO	5	1	3.5	1	1	2	R	*	*
Pop 1	AE	2	R	1	1	1	R	R	*	42.88
Pop 2 indiv A	AE	R	R	1	1	R	<1	<1	*	59.20
Pop 2 indiv B	AE	6	<1	2	R	1	R	R	*	40.61
Pop 2 indiv C	AE	R	R	2.5	1.5	1	R	R	*	58.81
Pop 1 MIC mg/ml	EO	2	*	16	*	*	16	*	*	*

*Not tested

Antioxidant activity
The extract was moderately active in the DPPH assay with activity of 40.61-58.81 μg/ml. A summary of activities is included in Table 3.

Antiinflammatory activity
The essential oil of the individual from Velddrif showed moderate inhibitory activity against 5-lipoxygenase enzyme.

Acetylcholinesterase enzyme inhibition
Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Phylogenetic studies
The chemistry of the four individuals included in the analysis is somewhat similar except for the individual from Koeberg whose profile is slightly different. Hence, individuals A and B with the closest chemical profiles are most parsimoniously placed as sister taxa while individual C and *E. klinghardtensis* are sister to this clade in the combined phylogeny. The individual from Koeberg is nested at a different position in the same clade. The species has affinities with *E. africanus, E. eximius, E. luederitzianus* and *E. klinghardtensis.*

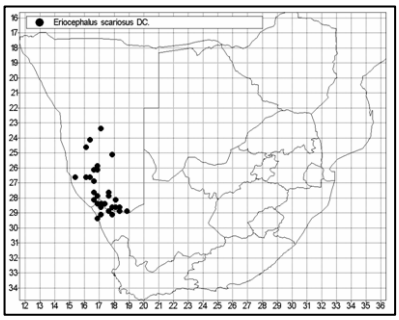
References

Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

21. *E. scariosus* DC.

Synonym
E. scariossisimus S.Moore, *E. rangei* Muschl., *E. virgatus* Dinter.

Common name
'Kapokbossie.'



Geographical distribution
Species distributed in summer and winter rainfall areas on mountains and forms part of the flora of sandstone hills and mountains extending from Namibia Naukluft Park and Aus southwards to Orange River. Distribution map of *E. scariosus* (Müller *et al.*, 2001).⇨

Botanical description
A slender erect, much-branched almost evergreen, strongly aromatic shrub, 0.5-1.5 m high, 1-2 m in diameter. Old stems deeply grooved. Leaves alternate, densely sericeous indumentum to glabrous, semisucculent with glands in leaf cavities. Capitula heterogamous radiate, with racemose or umbellate terminal racemes and pedunculate (6-12 mm long). Ray florets white. Paleae of marginal florets connate. Chromosome number: 2n = 72. Flowering time correlated with rainfall, varying from December to April and June to September (Müller *et al*., 2001).

Uses
Browsed by domesticated and wild animals.



Habitat of *E. scariosus* (Aus, Namib Desert).



Habit of *E. scariosus* with the inset showing radiate capitula with white rays.

Chemical composition

Essential oil

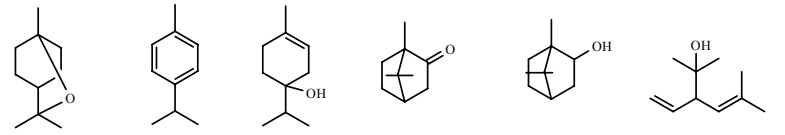
Extraction by hydrodistillation yielded 0.42% (dry wt) pale yellow-green essential oil.

GC/MS
Major constituents
The essential oils contain approximately 19 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of *E. scariosus* from Aus. Values are given in percentages. Compounds in bold represent some of the major compounds.

RI	Compound	%Composition
901	Santolina triene	1.7
928	α-Pinene	1.1
939	α-Fenchene + camphene	2.8
963	Sabinene	0.3
966	β-Pinene	1.1
988	Yomogi alcohol	0.9
1005	α-Terpinene	1.0
1008	**p-Cymene**	4.3
1016	**1,8-Cineole**	24.1
1024	**Santolina alcohol**	14.8
1047	γ-Terpinene	1.4
1070	Artemisia alcohol	1.7
1110	**Camphor**	17.2
1141	**Borneol**	5.8
1155	4-Terpineol	4.3
1262	Bornyl acetate	4.1
1362	α-Copaene	0.4
1544	Spathulenol	0.2
1548	Caryophyllene oxide	<0.19
	Total %	**87.07**

The essential oil is largely comprised of acyclic, monocyclic and bicyclic mono- and sesquiterpenes of camphane and pinane groups. Only one individual was studied and the structure for the major compounds is shown below.

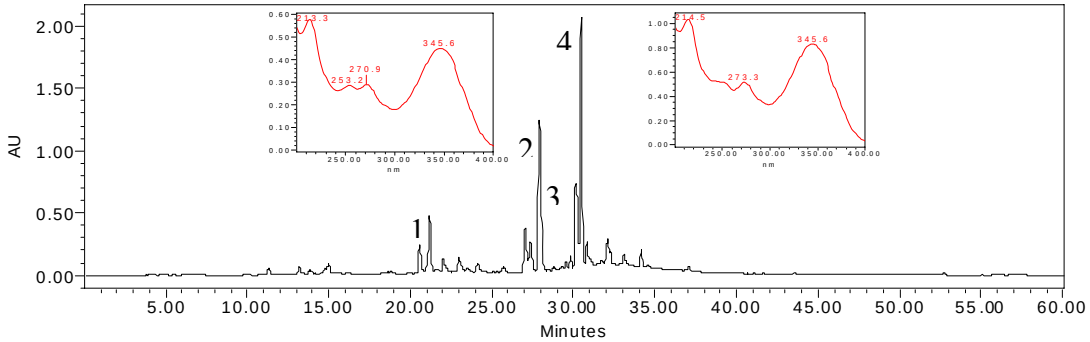


1,8-cineole *p*-cymene 4-terpineol camphor borneol santolina alcohol

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of flavones type as shown in the chromatogram below. The UV spectra of some of the main peaks are shown. The rest of the data is summarized in Table 2. respectively are shown.



A HPLC/UV chromatogram of leaf extracts of *E. scariosus.* The UV spectra of the major components mainly flavones (peak 2 and 4) at retention time 27.92 and 30.47 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. scariosus.* Only the (%) of the major peaks is noted.

Peak number	Retention time	UV $_{(\lambda max)}$	% Area
1	21.16	218, 328	7.23
2	27.92	213, 254, 271, 346	33.38
3	30.14	216, 273, 336	19.48
4	30.47	218, 273, 341	39.91

Biological properties

Antimicrobial activity

The essential oil of this species was active against all the test pathogens with moderate activity against *Cryptococcus neoformans* (*Cn*) and low activity against *Staphylococcus aureus* (*Sa*), *Bacillus cereus* (*Bc*), *Klebsiella pneumoniae* (*Kp*), *Candida albicans,* (*Ca*), *Bacillus subtilis* (*Bs*) and *Escherichia coli* (*Ec*). The extract showed moderate activity against *Cryptococcus neoformans* and *Staphylococcus aureus* and low activity against the rest of the pathogens. A summary of the activities is given in Table 3.

Minimum inhibitory activity (MIC)

The oil showed moderate to low inhibitory activity against the test pathogens. The extract was active against *Staphylococcus aureus* as shown in the Table 3.

Table 3. A summary of biological properties of *E. scariosus* from Aus. EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. R-resistant.

Extract	Activity in mm from the edge of the disc							5-Lox IC_{50} μg/ml	DPPH IC_{50} μg/ml
	Cn	Ca	Bc	Bs	Sa	Kp	Ec		
EO	3.5	1.5	1.3	1.0	1.0	1.5	1	>100	*
AE	2	R	1.5	R	2	R	<1.0	*	35.4
MIC EO mg/ml	10	>32	12	8	4	8	*	*	*
MIC AE mg/ml	*	*	*	*	1.6	*	*	*	*

*-Not tested.

Antioxidant activity

The essential oil showed no activity at the starting concentration of 100 μg/ml but the extract was active in the DPPH assay with activity of 35.4 μg/ml. A summary of activities is included in Table 3.

Antiinflammatory activity

The oil showed no inhibitory activity against 5-lipoxygenase enzyme.

Phylogenetic studies

The species is a sister to the clade with an individual of *E. africanus* and *E. brevifolius* in the combined phylogeny. Morphologically all these species have radiate capitula, connate paleae, opposite leaves and sericeous indumentum on the leaves.

References

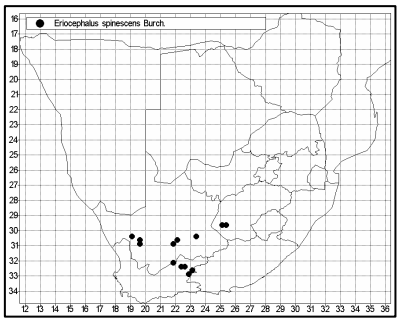
Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

22. *E. spinescens* Burch.

Common name

'Kapokbos.'

Geographical distribution



The distribution of this species falls in the transition zone between winter and summer rainfall areas and is allopatric to the close relatives *E. namaquensis* and *E. karooicus.* The distribution of *E. spinescens* extends from Calvinia eastwards in the Northern (Sutherland) and Western (Ceres) Cape in this very dry region receiving an average of less than 250 mm of rainfall annually. Distribution map of *E. spinescens* (Müller *et al*., 2001).⇨

Botanical description

Robust, many-stemmed, sympodially branched, spinesent shrubs, 0.5-1.2 m high and in diameter. Old stems with anomalous secondary growth. Leaves opposite decussate with permanent felty sericeous indumentum. Capitula heterogamous disciform, solitary, terminal and sessile or with very shortly pedunculate (1.0-3.5 (-5.0) mm long). Marginal female florets cream colored. Paleae of marginal florets free. Chromosome number 2n = 36. Flowering time varying from June to October or January to March depending on the time of rainfall (Müller *et al*., 2001).



The robust and many-stemmed habit of *E. spinescens* (Sutherland).



A habit showing sympodial branching with the inset showing the disciform pedunculate capitula.

Chemical composition

Essential oil

Extraction by hydrodistillation yielded 0.02% (wet wt) clear to pale yellow essential oil.

GC/MS

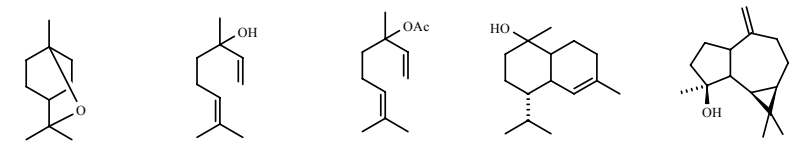
Major constituents

The essential oils contain approximately 27 compounds, which are summarized in Table 1.

Table 1. Essential oil composition and retention index (RI) of *E. spinescens* from between Sutherland/Ceres. Values are given in percentages. Compounds in bold represent some of the major compounds.

RI	Compound	A	B	C
901	Santolina triene	0.5		1.5
928	α-Pinene	0.1	1.5	<0.88
939	α-Fenchene	0.7	1.1	6.5
966	β-Pinene	0.9	0.6	0.9
979	MW=152			<0.88
988	Yomogi alcohol	**3.8**		2.1
1008	p-Cymene	2.0	1.3	3.1
1016	**1,8-Cineole**	10.7	6.0	5.2
1057	*Cis*-linalool oxide	1.1	2.1	1.9
1070	*Trans*-linalool oxide	2.1	2.0	3.2
1083	**Linalool**	3.9	5.7	7.8
1155	4-Terpineol	0.8		
1158	Artemisyl acetate	3.8		2.2
1166	α -Terpineol	1.0	1.7	1.9
1210	o-Methylthymol	1.9		1.4
1240	**Linalyl acetate**	7.4	11.4	20.9
1309	Unknown 1	0.8		
1342	Neryl acetate	0.5	0.6	
1360	Geranyl acetate	1.2	1.3	1.7
1362	α-Copaene	1.2		
1363	**MW=204**			3.4
1369	**Isocomene**	1.6	1.5	4.2
1461	Ar-Curcumene	1.8		
1499	MW=222		1.0	
1544	**Spathulenol**	8.4	22.2	18.5
1548	**Caryophyllene oxide**	4.6	1.5	1.9
1608	**α–Cadinol or τ-Muurolol**	7.9		
	Total %	**68.63**	**61.32**	**88.18**

The three individuals have similarities in their chemistry with very minor variations in the composition as shown in the table above. The structure for the major compounds is shown below.

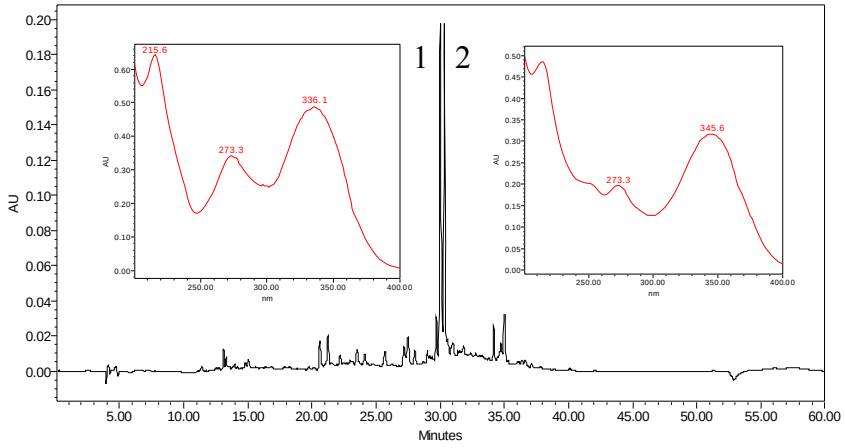


1,8-cineole linalool linalyl acetate α-cadinol spathulenol

Non volatile phytoconstituents

HPLC

The leaf extracts contain flavonoids of various classes especially flavones as shown in the chromatogram below. The UV spectra of the main peaks are also shown. The rest of the data is summarized in Table 2.



A HPLC/UV chromatogram of leaf extracts of *E. spinescens* The UV spectra of the major components mainly flavones (peak 1 and 2) at retention time 29.99 and 30.32 minutes respectively are shown.

Table 2. A summary of the HPLC/UV data for acetone leaf extracts of *E. spinescens.* Only the (%) of the major peaks is noted.

Peak number	Retention time	$UV_{(\lambda max)}$	% Area
1	29.99	215, 273, 336	36.41
2	30.32	215, 273, 345	29.23

Biological properties

Antimicrobial activity

The essential oils were moderately active against *Cryptococcus neoformans* (*Cn*) and *Bacillus cereus* (*Bc*), low activity against *Candida albicans* (*Ca*), *Staphylococcus aureus* (*Sa*) and *Bacillus subtilis* (*Bs*) showed no activity against *Klebsiella pneumoniae* (*Kp*) and *Escherichia coli* (*Ec*) bacteria. The extracts showed low activity against four of the test pathogens as shown in Table 3. A summary of the activities is given in Table 3.

Table 3. A summary of biological properties of individuals from *E. spinescens* from Sutherland/Ceres. EO-essential oil, AE-acetone extract. The full names of pathogens are given in the text above. Letters A, B and C represent three individuals from a single population. R-resistant.

Taxon	Extract	Activity in mm from the edge of the disc							DPPH IC_{50} µg/ml
		Cn	Ca	Bc	Bs	Sa	Kp	Ec	
Individual B	EO	2	R	2	<1	<1	R	R	*
Individual C	EO	4	1	4	<1	2	R	R	*
Individual A	AE	R	R	<1	1	R	<1	R	41.1
Individual B	AE	2	R	2	1.5	1	R	R	45.3
Individual C	AE	1	R	R	R	R	R	R	46.5

*-Not tested

Antioxidant activity

The extracts were active against DPPH with activity ranging from 41.1-46.5 µg/ml. A summary of activities is included in Table 3.

Acetylcholinesterase enzyme inhibition

Preliminary TLC screening of the essential oils indicated presence of inhibitors of acetylcholinesterase enzyme.

Phylogenetic studies

The ITS (internal transcribed spacer) of nuclear DNA and *psb*A-*trn*H regions of plastid DNA have been sequenced for this species and used in phylogenetic reconstruction with chemical data from terpenes. The three individuals grouped together in one clade with individuals A and C most parsimoniously placed as sister taxa due to their similar chemistry while individual B was sister to the clade. This species has close affinities with *E. decussatus.*

References

Müller, M.A.N., Herman, P.P.J., Kolberg, H.H. (2001). Fascicle 1: *Eriocephalus* and *Lasiospermum. Flora of Southern Africa,* Vol. 33: 1-63.

APPENDIX II



Copy of 10_Njenga.pdf

South African Journal of Botany 2005, 71(1): 81–87
Printed in South Africa — All rights reserved

Copyright © NISC Pty Ltd
SOUTH AFRICAN JOURNAL OF BOTANY
ISSN 0254–6299

Antimicrobial activity of *Eriocephalus* L. species

EW Njenga, SF van Vuuren* and AM Viljoen

Department of Pharmacy and Pharmacology, University of the Witwatersrand, 7 York Road, Parktown 2193, South Africa
** Corresponding author, e-mail: vanvuurensf@therapy.wits.ac.za*

Received 12 February 2004, accepted in revised form 30 July 2004

The genus *Eriocephalus,* commonly known as wild 'rosemary', 'Cape snow bush', 'kapokbush' or 'asmabossie', belongs to the family Asteraceae, of the tribe Anthemideae. It is endemic to southern Africa and is comprised of 32 species, of which several are economically important as traditional herbal remedies and as perfumes in fragrance industries. The species may be an important potential source for new and novel drugs for the treatment of various diseases, hence warrants further research. An investigation into the antimicrobial activity of the genus *Eriocephalus* using the disc diffusion assay against a range of Gram-positive and Gram-negative bacteria as well as a few selected fungi was carried out. The study included 15 *Eriocephalus* species with 113 essential oil and acetone leaf extract samples. Preliminary screening was carried out using 16 test pathogens: *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus* (four strains), *S. epidermidis*, *Klebsiella pneumoniae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Yersinia enterocolitica*, *Salmonella typhimurium*, *S. enteriditis*, *Proteus vulgaris*, *Serratia odorifera*, *Enterococcus faecalis*, *Cryptococcus neoformans*, *Candida albicans* and *Alternaria alternata.* From the preliminary screening, the most susceptible test pathogens selected for further study were: *Bacillus cereus*, *B. subtilis*, *Staphylococcus aureus* (one strain), *Klebsiella pneumoniae*, *Escherichia coli*, *Cryptococcus neoformans* and *Candida albicans*. The Gram-positive bacteria and two fungal pathogens showed inhibition for most of the essential oils and the leaf extracts while there was very little activity noted on the Gram-negative bacteria. Intra- and inter-population variation as well as inter-specific variation was observed in the antimicrobial activity for some species of *Eriocephalus*. The major variation was mainly observed in the activity of the essential oils and the leaf extracts against the yeast, *Cryptococcus neoformans* and the Gram-positive bacteria, *Bacillus cereus, B. subtilis* and *Staphylococcus aureus*. From the results obtained from the disc diffusion assay, the most active species were selected to determine the minimum inhibitory concentration against two Gram-positive and two Gram-negative bacteria and two fungal strains. The acetone extracts of *E. aromaticus* from Swartberg produced the most promising activity for all species studied with MIC values of 400µg ml^{-1} and 200µg ml^{-1} for *B. cereus* and *S. aureus* respectively.

Abbreviations: AE = acetone extract, ATCC = American Type Culture Collection, *Bc* = *Bacillus cereus*, *Bs* = *Bacillus subtilis*, *Ca* = *Candida albicans*, *Cn* = *Cryptococcus neoformans*, *Ec* = *Escherichia coli*, EO = essential oil, *Kp* = *Klebsiella pneumoniae*, MIC = minimum inhibitory concentration, NCTC = National Collection of Type Cultures, R = resistant, *Sa* = *Staphylococcus aureus*

Introduction

Traditional herbal medicine plays a vital role in the provision of primary health care, especially for the rural folk. Herbal remedies are widely used in South Africa and it is estimated that 70–80% of the people use plants for therapeutic purposes (Dyson 1998). The cost of manufactured drugs has continued to escalate, thus becoming unaffordable for many citizens. It is therefore important to investigate the plants used traditionally for potential novel antimicrobial compounds and confer credibility or establish the 'rational usage' upon what healers have known and used for centuries in traditional therapy, as noted by Hammer *et al*. (1999) and Swanepoel (1997). Infectious and inflammatory diseases are among those treated using herbal remedies Shale *et al*. (1999) and many people are reverting back to the traditional use of plants for treatment of such and other ailments (Dorman and Deans 2000).

The genus *Eriocephalus,* commonly known as wild 'rosemary', 'Cape snow bush', 'kapokbos' or 'asmabossie' belongs to the family Asteraceae, of the tribe Anthemideae, (Adamson and Salter 1950) and is characterised by aromatic and highly dissected leaves. It is comprised of 32 species endemic to southern Africa (Müller *et al.* 2001). The Griqua and Nama used some of the members of the genus as a diuretic and diaphoretic, a tincture for heart troubles, a colic remedy and for treatment of oedema and stomach ache. The species used include *E. africanus*, *E. ericoides*, *E.*

racemosus and *E. punctulatus.* Leaf infusions of *E. africanus*, decoctions and tinctures are used to treat coughs, flatulence and delayed menstruation, as well as for swelling and pain arising from gynaecological conditions. The plants are also popular ingredients for footbaths and as a hair rinse to treat dandruff and itchy scalps. They are also used to treat inflammation of the skin and for chest complaints, hence the name 'asmabossie' or 'asthma bush'. An infusion of *E. africanus* and *Rosmarinus officinalis* is used for bathing to invigorate the skin and hair, as recorded in Watt and Breyer-Brandwijk (1962), Salie *et al*. (1996), Van Wyk *et al.* (1997), Dyson (1998) and Van Wyk and Gericke (2000). *E. punctulatus* is used by the southern Sotho with *Metalasia muricata* to fumigate the hut of a person suffering from a cold or after the death of a person.

The chemistry of most *Eriocephalus* species is poorly studied, with the exception of *E. punctulatus* (Mierendorff *et al*. 2003), *E. africanus* and a few other species endemic to Namibia (Zdero *et al.* 1987). Some of the major compounds reported to occur in the species include various terpenoid aliphatic esters, camphor, linalyl acetate, nerolidol, spathulenol and several sesquiterpene lactones. Since the focus of this study was on the antimicrobial activity, the chemistry of the species will be addressed elsewhere (Njenga *et al.* in prep.).

As some of the conditions mentioned above may be microbe-related, this study is aimed at investigating the potential antimicrobial properties of the species of *Eriocephalus* and to verify the rationale for their use in traditional herbal remedies by *in vitro* screening.

Materials and Methods

Preparation of plant material

The 15 species tested in this study and their localities are given in Table 1. The voucher specimens are deposited in the Department of Pharmacy and Pharmacology at the University of the Witwatersrand. The aerial plant parts were collected from natural populations during their growing periods and the fresh material hydrodistilled in a Clevenger apparatus for three to four hours to obtain the essential oils. It should be noted at this juncture that the essential oils yields for several of the species of *Eriocephalus* were relatively low, hence it is only those species which yielded sufficient oil that were considered for the MIC assay. Due to variability aspects, essential oils were distilled from a single plant, thus explaining low yields of the oils. Dried plant material was crushed, weighed (0.5–3.0g) and 30ml of acetone added. The mixture was extracted for four hours in a water bath at 30°C, then filtered and evaporated. The residue was re-suspended in acetone to a concentration of 50mg ml^{-1}.

Disc diffusion assay

Preliminary antimicrobial screening was carried out using 16 test pathogens, and seven of these were selected for further study, based on susceptibility patterns. The selected test pathogens are given in Table 1.

The disc diffusion assay was used to determine the growth inhibition of the bacteria and selected fungi. Tryptone Soya agar was prepared by dissolving 30g of the agar in 750ml of water and autoclaved for 15min at 121°C and cooled to 55°C in a water bath. A base layer of 100ml of agar was poured into the plate and inoculated with a top layer of 100ml of agar containing an inoculum of approximately 1 x 10^6cfu ml^{-1}. Sterilised paper discs (6mm) were saturated with approximately 8µl of either essential oils or the acetone leaf extracts (50mg ml^{-1}) and loaded onto the agar plates. The plates were kept at 4°C for one hour to pre-diffuse the oil and extracts into the agar and then incubated for 24h at 37°C for bacterial isolates. The yeast and mould were incubated for 48h and seven days respectively. Neomycin (30µg) was used as a positive control for the bacterial strains and Nystatin (100IU) as a control for the fungal strains. Activity was measured as growth inhibition zones in millimetres from the edge of the disc (Table 1). Repetitions were made to confirm results.

Minimum inhibitory concentration

Based on the results obtained from the disc diffusion assays, two Gram-positive bacteria (*Bacillus cereus* ATCC 11778, *Staphylococcus aureus* ATCC 25923), two Gram-negative bacteria (*Klebsiella pneumoniae* NCTC 9633, *Escherichia coli* ATCC 8739) and the yeasts (*Cryptococcus neoformans* ATCC 90112, *Candida albicans* ATCC 10231) were selected for further study. The plant specimens were selected on the basis of activity resulting from the disc diffusion assays and availability of samples, especially the essential oils, most of whose quantities were not sufficient for minimum inhibitory concentration (MIC) determination.

The test cultures were inoculated in Tryptone Soya (Oxoid) broth and incubated for 24h. One millilitre of the inoculum was transferred into 100ml of sterile broth. The starting concentration of essential oils was 32mg ml^{-1} and 12.5mg ml^{-1} for the leaf extracts. The 96-well micro titre plates were aseptically prepared and serial dilutions carried out as outlined by Eloff (1998, 1999).

Results and Discussion

The most susceptible pathogens observed from the broad preliminary screening were selected for further study as shown in Table 1. Among the species studied, the essential oils of *E. purpureus* (Nieuwoudtville), *E. ericoides* subsp. *ericoides*, *E. pauperrimus*, *E. microphyllus* (Sutherland), *E. africanus* (Malmesbury), *E. punctulatus* and *E. racemosus* var. *racemosus* exhibited at least 50% activity against the total number of the pathogens tested, though the activity was relatively low. The leaf extracts, however, showed lower activity, ranging from 20–40% against the total number of the test pathogens with an exception of a few, e.g. *E. aromaticus*, *E. microphyllus* (Nieuwoudtville), *E. punctulatus* (Nieuwoudtville population 1 and 2) and *E. africanus* (Melkbosstrand), that showed at least 50% activity overall.

The test pathogens *Staphylococcus aureus* (ATCC 6538, ATCC 612600, methicillin-resistant *Staphylococcus aureus* (clinical strain), *Staphylococcus epidermidis* (ATCC 2223),

Table 1: Antimicrobial activity of essential oils and acetone leaf extracts of *Eriocephalus.* Activity is measured in millimetres (mm) from the edge of the disc

Taxon	Locality	Extract	Activity in mm from edge of the disc						
			Cn	*Ca*	*Bc*	*Bs*	*Sa*	*Kp*	*Ec*
E. africanus L.	Mossel Bay	EO	1.5	<1	R	1	<1	<1	<1
E. africanus*	**Malmesbury**	**EO**	**5**	**2**	**6**	**2**	**2**	**1**	**R**
E. africanus	Melkbosstrand	EO	1	1	3	1	1	R	R
E. africanus	Citrusdal (A)	EO	2	1	2	<1	<1	1	R
E. africanus	Citrusdal (B)	EO	1	R	5.5	2	1	<1	R
E. africanus	Citrusdal (C)	EO	8	1	4	<1	1.5	R	1
E. aromaticus C.A.Sm	Swartberg	EO	R	2	3.5	3	2	<1	<1
E. aromaticus	Ladismith (B)	EO	R	R	3	1	<1	R	R
E. aromaticus	Ladismith (C)	EO	8	R	4	<1	R	R	R
E. brevifolius (DC) M.A.N. Müller	Vergelegen (A)	EO	<1	<1	1.5	1	<1	<1	R
E. brevifolius	Vergelegen (B)	EO	3	<1	3	<1	<1	R	R
E. brevifolius	Vergelegen (C)	EO	5.5	1	4	1	<1	<1	R
E. brevifolius	**Oudtshoorn**	**EO**	**R**	**2**	**5.3**	**4**	**5**	**2**	**2**
***E. capitellatus* DC**	**Swartberg Pass (A)**	**EO**	**4**	**R**	**3.5**	**<1**	**1**	**<1**	**R**
E. capitellatus DC	Swartberg Pass (B)	EO	2	1	3.5	1	<1	1	<1
E. capitellatus	**Swartberg Pass (C)**	**EO**	**3**	**2**	**2.5**	**2**	**R**	**1**	**<1**
E. decussatus Burch	Sutherland	EO	4	2	2	2	3	<1	<1
E. ericoides subsp. *ericoides* (L.F) Druce	Scheepersrust (A)	EO	3	<1	2.5	2	<1	<1	R
E. ericoides subsp. *ericoides*	Scheepersrust (B)	EO	2	1	3	1	1	R	1
E. ericoides subsp. *ericoides*	Scheepersrust (C)	EO	3	1	2	1	1	<1	R
E. ericoides subsp. *ericoides*	Prince Albert-1	EO	R	1	7.25	3	1	<1	<1
E. ericoides subsp. *ericoides*	Prince Albert (A)-2	EO	4.5	1	4	<1	<1	R	R
E. ericoides subsp. *ericoides*	Prince Albert (B)	EO	5	1	3	1.2	1	R	R
E. ericoides subsp. *ericoides*	Prince Albert (C)	EO	3	2	4	3	3	1	1
E. ericoides* subsp. *ericoides	**Bethulie (A)**	**EO**	**9**	**5**	**3**	**2**	**1**	**1**	**1.2**
E. ericoides* subsp. *ericoides	**Bethulie (B)**	**EO**	**4**	**2**	**2**	**3**	**2**	**1**	**1.5**
E. eximius DC	Sutherland (A)	EO	R	R	3	<1	<1	<1	R
E. eximius	Sutherland (B)	EO	5	R	2	<1	1.5	R	R
E. microphyllus DC	Sutherland (A)	EO	R	3	8	2	3	<1	<1
E. microphyllus	Sutherland (B)	EO	6	3	2	1	2	1	R
E. microphyllus	Sutherland (C)	EO	7	3	3	1.5	1.5	1.5	2
E. microphyllus	Nieuwoudtville (B)	EO	4	2	3.5	3	1.5	<1	<1
E. microphyllus	Nieuwoudtville (C)	EO	6	<1	3	R	1	1	1
E. microphyllus	**Khamiesburg**	**EO**	**5**	**1**	**2**	**1**	**<1**	**2**	**1**
E. microphyllus	Spektakel Pass	EO	6	1	4	<1	<1	1.5	R
E. namaquensis M.A.N. Müller	Clanwilliam (A)	EO	10	1	3.5	3	1.5	<1	<1
E. namaquensis	Clanwilliam (C)	EO	6	2	R	R	1	<1	R
E. pauperrimus	Nieuwoudtville (A)	EO	6	3	4	2	2	R	R
E. pauperrimus	Nieuwoudtville (C)	EO	3	2	2	1.5	1.5	R	R
***E. punctulatus* DC**	**Nieuwoudtville (A)-1**	**EO**	**2**	**<1**	**3**	**1**	**1**	**1**	**1.5**
E. punctulatus	Nieuwoudtville (B)	EO	9	2	3	2	1	1	1
E. punctulatus	**Nieuwoudtville (C)**	**EO**	**3**	**5**	**2.5**	**3**	**1**	**1**	**1**
E. punctulatus	Calvinia	EO	R	2	3	4	1.5	<1	<1
E. punctulatus	Nieuwoudtville-2	EO	5	2	5.5	2	1.5	1	2.5
E. purpureus Burch	Nieuwoudtville	EO	5	2	3.5	4	2	<1	<1
E. purpureus	Khamiesburg	EO	5	R	2	1.5	<1	1	1
***E. racemosus* L.**	**Koeberg**	**EO**	**R**	**1**	**1**	**4**	**3**	**1**	**1**
E. racemosus var. *racemosus* L.	Velddrif (A)	EO	2	1	4	1	1	<1	R
E. racemosus var. *racemosus*	Velddrif (B)	EO	2	R	4	1	<1	<1	R
E. racemosus* var. *racemosus	**Velddrif (C)**	**EO**	**5**	**1**	**3.5**	**1**	**1**	**2**	**R**
E. spinescens Burch	Sutherland (B)	EO	2	R	2	<1	<1	R	R
E. spinescens	Sutherland (C)	EO	4	1	4	<1	2	R	R
E. africanus	De Rust	AE	4	R	R	R	R	<1	<1
E. africanus	Malmesbury	AE	3	R	1	2	R	<1	R
E. africanus	Melkbosstrand	AE	3	1	2	2	<1	<1	R
E. africanus	Citrusdal (A)	AE	2	R	1	<1	R	R	R
E. africanus	Citrusdal (B)	AE	1.5	R	1	2	R	<1	<1
E. africanus	Citrusdal (C)	AE	3	R	2	R	<1	R	R
E. africanus var. *paniculatus* (Cass.) M.M, H & K	Sutherland (A)	AE	4	R	R	1	R	<1	<1
E. africanus var. *paniculatus*	Sutherland (B)	AE	5	R	R	R	R	R	R
E. africanus var. *paniculatus*	Sutherland (C)	AE	2	R	<1	R	R	R	R

Table 1: (cont.)

Taxon	Locality	Extract	Activity in mm from edge of the disc						
			Cn	*Ca*	*Bc*	*Bs*	*Sa*	*Kp*	*Ec*
E. aromaticus	**Swartberg**	**AE**	**R**	**R**	**8**	**4**	**5**	**<1**	**<1**
E. aromaticus	**Ladismith (A)**	**AE**	**1**	**<1**	**4**	**2**	**4**	**R**	**<1**
E. aromaticus	**Ladismith (B)**	**AE**	**5**	**R**	**7.3**	**6**	**6**	**R**	**R**
E. aromaticus	Ladismith (C)	AE	1	<1	R	1	4	R	<1
E. brevifolius	Vergelegen	AE	2	R	3	R	R	R	R
E. brevifolius	Oudtshoorn	AE	3	R	R	4	<1	R	R
E. capitellatus	Swartberg Pass-1	AE	R	R	1	1	R	<1	<1
E. capitellatus	Swartberg Pass-2	AE	<1	R	R	<1	<1	R	R
E. decussatus	Sutherland (A)	AE	5	R	R	1	R	<1	<1
E. decussatus	Sutherland (B)	AE	R	R	1	R	R	R	R
E. decussatus	Sutherland (C)	AE	<1	R	1	<1	R	R	<1
E. ericoides subsp. *ericoides*	Scheerpersrust	AE	2	R	3	1.5	1	R	R
E. ericoides subsp. *ericoides*	Prince Albert-1	AE	3	R	1	<1	<1	R	R
E. ericoides subsp. *ericoides*	Prince Albert-2	AE	3	R	2	1.5	1	R	R
E. ericoides subsp. *ericoides*	Bethulie (A)	AE	R	R	<1	1	1	1	R
E. ericoides subsp. *ericoides*	Bethulie (B)	AE	R	R	1	1	2	<1	R
E. eximius	Sutherland (A)	AE	R	R	1	<1	R	R	<1
E. eximius	Sutherland (B)	AE	2	R	R	1	R	R	R
E. eximius	Sutherland (C)	AE	2	R	R	1	R	R	R
E. grandiflorus	Laingsburg (A)	AE	R	R	1	2	R	R	R
E. grandiflorus	Laingsburg (B)	AE	3	R	<1	1	<1	R	R
E. grandiflorus	Laingsburg (C)	AE	3	<1	1	1	1	R	R
E. microphyllus	Sutherland (A)	AE	R	R	1	1	R	<1	<1
E. microphyllus	Sutherland (B)	AE	2	R	1	R	<1	R	R
E. microphyllus	Sutherland (C)	AE	2	R	1	R	<1	R	R
E. microphyllus	Nieuwoudtville (A)	AE	4	R	2	3	1	<1	<1
E. microphyllus	Nieuwoudtville (B)	AE	4	R	1	1	R	<1	<1
E. microphyllus	Nieuwoudtville (C)	AE	1	R	2	2	R	R	R
E. microphyllus	Khamiesburg	AE	2	1	<1	1	1	R	R
E. microphyllus	**Spektakel Pass**	**AE**	**3**	**2**	**3**	**1**	**1.5**	**R**	**<1**
E. namaquensis	Clanwilliam (A)	AE	4	R	2	1	<1	<1	<1
E. namaquensis	Clanwilliam (C)	AE	R	R	R	1	R	R	R
E. pauperrimus	Nieuwoudtville (A)	AE	1	R	R	2	R	<1	R
E. pauperrimus	Nieuwoudtville (B)	AE	4	R	R	R	R	<1	R
E. pauperrimus	**Nieuwoudtville (C)**	**AE**	**4**	**R**	**3**	**3**	**3**	**1**	**R**
E. punctulatus	**Nieuwoudtville (A)-1**	**AE**	**6**	**1**	**3**	**2**	**R**	**<1**	**R**
E. punctulatus	Nieuwoudtville (B)	AE	3	R	3	R	<1	R	R
E. punctulatus	Nieuwoudtville (C)	AE	R	R	1	2	2	<1	R
E. punctulatus	Calvinia	AE	3	R	1	2	<1	R	R
E. punctulatus	Nieuwoudtville (A)-2	AE	3	R	R	R	R	<1	R
E. punctulatus	Nieuwoudtville (B)	AE	R	R	R	R	R	<1	R
E. punctulatus	Nieuwoudtville (C)	AE	R	R	1	R	R	R	<1
E. purpureus	Laingsburg (A)	AE	3	R	1	R	R	<1	R
E. purpureus	Laingsburg (B)	AE	5	R	1	1	<1	<1	<1
E. purpureus	Nieuwoudtville-1	AE	3	R	R	1	R	<1	<1
E. purpureus	Nieuwoudtville-2	AE	7	R	1	2	R	<1	1
E. purpureus	**Khamiesburg**	**AE**	**4**	**2**	**4**	**1**	**1.5**	**R**	**<1**
E. racemosus	Koeberg	AE	2	R	1	1	1	R	R
E. racemosus var. *racemosus*	Velddrif (A)	AE	R	R	1	1	R	<1	<1
E. racemosus var. *racemosus*	Velddrif (B)	AE	6	<1	2	R	1	R	R
E. racemosus var. *racemosus*	Velddrif (C)	AE	R	R	2.5	1.5	1	R	R
E. spinescens	Sutherland (A)	AE	R	R	<1	1	R	<1	R
E. spinescens	Sutherland (B)	AE	2	R	2	1.5	1	R	R
E. spinescens	Sutherland (C)	AE	1	R	R	R	R	R	R
Control			11	7	8	6.5	7	3.5	2

*The samples in bold were selected for further MIC assays (Table 2)

*Pseudomonas aeruginosa (*ATCC 9027), *Yersinia enterocolitica* (ATCC 23715), *Salmonella typhimurium* and *Salmonella enteriditis* (clinical strains), *Proteus vulgaris* (clinical strain), *Serratia odorifera* (ATCC 33132), *Enterococcus faecalis (*ATCC 29212) and *Alternaria alternata* (clinical strain) did not show promising results and were not studied further. Of the four strains of *Staphylococcus aureus*, only the most sensitive was selected for further study.

Table 1 presents a summary of the results of essential oil and leaf extracts which exhibited antimicrobial activity against the seven selected test pathogens. This confirms that some species of the genus have antimicrobial properties and supports their use in traditional herbal remedies. The species show variation in activity within individuals of the same species and between different populations of the same species and between the species. Variation in activity between individuals of the same population was observed in several of the taxa studied. This was observed in the essential oils of *E. punctulatus* from Nieuwoudtville (population 1) and *E. brevifolius* from Vergelegen against *Cryptococcus neoformans* where three individuals (A, B and C) had inhibition of 2mm, 9mm and 3mm and <1mm, 3mm and 5.5mm respectively. This pattern was observed among the taxa where three individuals were tested for activity against the test pathogens (Table 1) and the same phenomenon was observed in the leaf extracts. This variation in the antimicrobial activity of essential oils and leaf extracts should be investigated further in reference to the chemical composition of the oils and the extracts.

Variation in sensitivity patterns against the test pathogens was also evident amongst the essential oils and the extracts of populations of the same species as observed in the populations of *E. africanus*, *E. punctulatus*, *E. aromaticus*, *E. microphyllus*, *E. brevifolius*, *E. ericoides* subsp. *ericoides* and *E. racemosus* var. *racemosus.* This intra-specific variation was also observed within the rest of the species of *Eriocephalus* (Table 1).

Essential oils showed antimicrobial activity against most of the test pathogens with the highest activity noted against the Gram-positive bacteria *Bacillus cereus* (8mm in *E. microphyllus* from Sutherland and *E. aromaticus* from Swartberg), and moderate activity against *Bacillus subtilis* and *Staphylococcus aureus*. Little inhibition (1–2.5mm) against the Gram-negative bacteria *Klebsiella pneumoniae* and *Escherichia coli* was noted for all the essential oils. This may be due to the fact that the Gram-negative bacteria are more resistant because of their membrane structure, as mentioned in Martin (1995), Rabe and Van Staden (1998) and Mangena and Muyima (1999).

The essential oils showed relatively good activity against the yeast *Cryptococcus neoformans* with *E. namaquensis* producing a zone of 10mm. The activity of the oils against *Candida albicans* was moderate with the largest zone (5mm) in *E. racemosus* var. *racemosus* (Velddrif), *E. punctulatus* (Nieuwoudtville population 1) and *E. ericoides* subsp. *ericoides* (Bethulie). This is in agreement with the activity of essential oils against the yeasts where they are reported to be more active than against the bacteria as noted in Bagci and Digrak (1996).

Among the species of *Eriocephalus* studied, some individuals of *E. punctulatus*, *E. ericoides* subsp. *ericoides*, *E. brevifolius*, *E. purpureu*s and *E. microphyllus* showed varying degrees of sensitivity against all the test pathogens.

The leaf extracts were not as active as the essential oils against the Gram-positive *Bacillus subtilis* except *E. aromaticus,* with activity of 2–6mm zone of inhibition. The same species was active against *Bacillus cereus* (4–8mm) and *Staphylococcus aureus* (4–6mm). The extracts showed very low (1mm or less inhibition) or no activity against the Gram-negative bacteria *Klebsiella pneumoniae* and *Escherichia coli.* However, most of the extracts were active against the yeast *Cryptococcus neoformans*, with the highest activity of 7mm noted for *E. purpureus* (Nieuwoudtville), and 6 mm for *E. racemosus* var. *racemosus* (from Velddrif) and *E. punctulatus* from (Nieuwoudtville population 1). The extracts were mostly inactive against *Candida albicans* except for some species, namely *E. microphyllus* and *E. purpureus*, which showed some inhibition. *E. aromaticus*, however, was active against at least four out of the seven test pathogens and the same group recorded the highest activity among the extracts of *Eriocephalus* species. Promising results were also observed in the leaf extracts of the following taxa: *E. punctulatus*, *E. africanus*, *E. racemosus* var. *racemosus*, *E. spinescens*, *E. purpureus*, *E. microphyllus* and *E. pauperrimus*, but overall the essential oils were comparatively more active than the extracts (Table 1). This implies that the activity in the members of the genus used in herbal remedies is mainly influenced by the presence of essential oils.

In other studies, Salie *et al.* (1996) reported the petroleum ether stem and methanol root extracts of *E. africanus* to be slightly active against *Staphylococcus aureus.* In this study a similar pattern was observed, as the acetone leaf extracts of *E. africanus* had little or no activity against *Staphylococcus aureus*. The essential oils of the same species showed very low antimicrobial activity against *Staphylococcus aureus*. The essential oils of *E. africanus* were observed to be active against *Candida albicans* but the acetone leaf extracts were not active; however, Salie *et al.* (1996) reported the lipophilic extracts of the same species to be active against *Candida albicans.*

Following the results from the disc diffusion assay, the minimum inhibitory concentration (MIC) was determined for six selected test pathogens (Table 2). The 10 species of *Eriocephalus* comprising 18 samples (Table 2) that showed promising activity in the disc diffusion screening assay (Table 1, species in bold text) and those with sufficient oil quantities were selected for the MIC assay. The antimicrobial effect ranged between 4–32mg ml^{-1} for the Gram-positive bacteria, 8 32mg/ml^{-1} for the Gram-negative bacteria and 1–8mg ml^{-1} for the fungal strains for the essential oil (Table 2).

The MIC for the leaf extracts for the Gram-positive bacteria was 0.2–3.1mg ml^{-1} and 0.8–6.3mg ml^{-1} for the fungal strains. The Gram-negative bacteria were not tested for the extracts, as there was no notable activity observed from the disc diffusion assays. It is well documented that testing and evaluation of antimicrobial activity of essential oils is difficult because of their volatility, their water

Table 2: Minimum inhibitory concentration (mg ml^{-1}) of essential oils and leaf extracts of 10 *Eriocephalus* species

Species	Locality	Extract	Minimum inhibitory concentration					
			Cn	*Ca*	*Bc*	*Sa*	*Kp*	*Ec*
E. africanus	Malmesbury	EO	4	4	8	32	*	16
E. brevifolius	Oudtshoorn	EO	*	8	8	16	>32	*
E. capitellatus	Swartberg Pass (A)	EO	4	*	16	*	*	*
E. capitellatus	Swartberg Pass (C)	EO	4	*	*	*	*	*
E. ericoides subsp. *ericoides*	Bethulie (A)	EO	1	4	4	8	16	*
E. ericoides subsp. *ericoides*	Bethulie (B)	EO	*	4	*	*	8	16
E. microphyllus	Khamiesburg	EO	*	*	*	*	8	16
E. punctulatus	Nieuwoudtville (C)	EO	4	8	*	8	*	*
E. punctulatus	Nieuwoudtville (A)-1	EO	*	*	*	*	*	16
E. racemosus	Koeberg	EO	*	*	*	*	*	16
E. racemosus var. *racemosus*	Velddrif	EO	2	*	16	*	16	*
E. aromaticus	Swartberg	AE	*	*	0.4	0.2	*	*
E. aromaticus	Ladismith (A)	AE	*	*	3.1	0.8	*	*
E. aromaticus	Ladismith (B)	AE	1.6	*	0.8	0.4	*	*
E. microphyllus	Spektakel Pass	AE	6.3	1.6	3.1	0.8	*	*
E. pauperrimus	Nieuwoudtville	AE	*	*	*	1.6	*	*
E. punctulatus	Nieuwoudtville-1	AE	0.8	*	0.8	*	*	*
E. purpureus	Khamiesburg	AE	*	1.6	*	0.8	*	*

* Not determined due to insufficient sample or lack of activity

insolubility and their complexity. The results are greatly influenced by the choice of assay technique, growth medium, the test pathogen and the oil extract (Janssen *et al*. 1986). Studies to establish if there is any correlation between the inhibition diameters and MIC values for essential oils have been carried out and it is evident that qualitative screening methods and quantitative minimum inhibitory concentration methods are not necessarily comparable, as indicated in Janssen *et al*. (1986). The nature of diffusion of the leaf extracts and the essential oil in water or culture medium differs considerably. Hence, the results obtained may vary qualitatively and quantitatively. In this study, the same phenomenon was observed with the results obtained for the MIC test not confirming or tallying with those obtained for inhibition diameters in the disc diffusion assay as mentioned in Brantner and Grein (1994).

In herbal remedies, the species of *Eriocephalus* are mainly used for treatment of respiratory-related ailments, skin inflammation, stomach disorders and as diuretics and diaphoretics. From the broad screening of the taxa in the genus it was observed that most of the essential oils were active against the respiratory pathogen *Cryptococcus neoformans. Eriocephalus racemosus* var. *racemosus* and *E. ericoides* subsp. *ericoides* had an MIC of 2mg ml^{-1} and 1mg ml^{-1} respectively, compared to the rest of the species tested (Table 2). The leaf extracts of *E. punctulatus* and *E. aromaticus* had an MIC of 0.8mg ml^{-1} and 0.2–1.6mg ml^{-1} respectively. The MIC of *E. ericoides* subsp. *ericoides* and *E. microphyllus* was 8–16mg ml^{-1} for the former and 8mg ml^{-1} for the latter for the essential oils against *Klebsiella pneumoniae*. This supports the use of the species of *Eriocephalus* for treatment of respiratory-related ailments.

Most of the species studied showed activity in the essential oils and the leaf extracts against *Bacillus cereus* and *Staphylococcus aureus*, which may be associated with dermal infections. The essential oils of *E. punctulatus*, *E. ericoides* subsp. *ericoides*, *E. africanus* and *E. brevifolius* had an MIC of between 4mg ml^{-1} to 8mg ml^{-1} for effective inhibition of the test pathogen. For the leaf extracts, *E. aromaticus*, *E. punctulatus*, *E. microphyllus* and *E. purpureus* had an MIC range of 0.2mg ml^{-1} to 0.8mg ml^{-1} (Table 2).

For gastro-intestinal disorders or infections, *E. punctulatus*, *E. microphyllus*, *E. racemosus*, *E. brevifolius* and *E. ericoides* subsp. *ericoides* indicated potential, as these species showed activity against *Escherichia coli*, while the rest showed activity against *Candida albicans* (Tables 1 and 2).

From the results obtained from the disc diffusion screening, the essential oils of *E. punctulatus*, *E. ericoides* subsp. *ericoides*, *E. purpureus*, *E. microphyllus*, *E. decussatus* and *E. brevifolius* are active against nearly all the test pathogens and can be used to treat respiratory-related ailments, dermal infections and gastro-intestinal disorders recorded for the traditional herbal remedies. The other notably biologically- active species include *E. pauperrimus*, *E. microphyllus*, *E. racemosus* and *E. capitellatus* and are therefore potentially useful as a source of herbal remedies. *E. punctulatus*, *E. africanus* and *E. racemosus* are traditionally used for treatment of respiratory, skin and stomach problems and the results from the disc diffusion assay and the MIC values obtained in this study confirm their efficacy in traditional uses.

This study confirms that *Eriocephalus* species have broad and varied antimicrobial activity within their essential oils and leaf extracts. The results obtained from the broad screening with various test pathogens confirm their use in traditional herbal remedies. The essential oils have proved to be more antimicrobially active in comparison to the leaf extracts. This study showed antimicrobial activity for selected test pathogens, which clearly indicates that there are more potentially active species of the genus not initially documented. It should also be noted that nearly all the essential oils and most of the leaf extracts were active against the yeast *Cryptococcus neoformans.* This forms a basis for an alternative source of remedies for treatment of

fungal infections. More research will be carried out to isolate the active compound(s) by bioassay-guided fractionation for some of the species like *E. aromaticus*, which showed high inhibitory activity in the preliminary screening. However, if these species are to be used for medicinal purposes, their chemical composition and issues of safety and toxicity will need to be investigated further.

Acknowledgements — The National Research Foundation, the University of the Witwatersrand Research Committee and Faculty of Health Sciences Research Endowment Fund are hereby acknowledged for their financial support. We are indebted to Mr Jan Vlok, Mr Paul Herman and Dr John Manning for assisting in the identification of plant material.

References

Adamson RS, Salter TM (1950) Flora of the Cape Peninsula. Juta, Cape Town, pp 800–801

Bagci E, Digrak M (1996) Antimicrobial activity of essential oils of some *Abies* (fir) species from Turkey. Flavour and Fragrance Journal **11**: 251–256

Brantner A, Grein E (1994) Antibacterial activity of plant extracts used externally in traditional medicine. Journal of Ethnopharmacology **44**: 35–40

Dorman HJD, Deans SG (2000) Antimicrobial agents from plants: antibacterial activity of plant volatile oils. Journal of Applied Microbiology **88**: 308–316

Dyson A (1998) Discovering indigenous healing plants of the herb and fragrance gardens at Kirstenbosch National Botanical Garden. National Botanical Institute, Cape Town, pp 33–34. ISBN 1–919684–09–3

Eloff JN (1998) A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica **64**: 711–713

Eloff JN (1999) The antibacterial activity of 27 southern African members of the Combretaceae. South African Journal of Science **95**: 148–151

Hammer KA, Carson CF, Riley TV (1999) Antimicrobial activity of essential oils and other plant extracts. Journal of Applied Microbiology **86**: 985–990

Janssen AM, Scheffer JJC, Baerheim Svendsen A (1986) Antimicrobial activity of essential oils: a 1976–1986 literature review. Aspects of the test methods. Planta Medica **53**: 395–398

Mangena T, Muyima NYO (1999) Comparative evaluation of the antimicrobial activities of essential oils of *Artemisia afra*, *Pteronia incana* and *Rosmarinus officinalis* on the selected bacteria and yeast strains. Letters in Applied Microbiology **28**: 291–296

Martin GJ (1995) Ethnobotany. A People and Plants Conservation Manual. Chapman and Hall, London, UK, pp 67–93. ISBN 0–412–48370–X

Mierendorff H-G, Stahl-Biskup E, Posthumus MA, Van Beek TA (2003) Composition of commercial Cape chamomile oil (*Eriocephalus punctulatus*). Flavour and Fragrance Journal **18**: 510–514

Müller MAN, Herman PPJ, Kolberg HH (2001) Fascicle 1: *Eriocephalus* and *Lasiospermum*. Flora of Southern Africa **33**: 1–63

Rabe T, Van Staden J (1998) Screening of *Plectranthus* species for antibacterial activity. South African Journal of Botany **64**: 62–65

Salie F, Eagles PFK, Leng HMJ (1996) Preliminary antimicrobial screening of four South African Asteraceae species. Journal of Ethnopharmacology **52**: 27–33

Shale TL, Stirk WA, Van Staden J (1999) Screening of medicinal plants used in Lesotho for anti-bacterial and anti-inflammatory activity. Journal of Ethnopharmacology **67**: 347–354

Swanepoel DP (1997) The medicinal value of the South African Asteraceae. MSc Thesis, Faculty of Biological and Agricultural Sciences of the University of Pretoria, Pretoria

Van Wyk BE, Gericke N (2000) People's Plants. A Guide to Useful Plants of Southern Africa. Briza, Pretoria, South Africa, pp 218–219. ISBN 1–875093–19–2

Van Wyk BE, Van Oudtshoorn B, Gericke N (1997) Medicinal Plants of South Africa. Briza, Pretoria, South Africa, pp 122–123. ISBN 1–875093–09–5

Watt JM, Breyer-Brandwijk MG (1962) The Medicinal and Poisonous Plants of Southern and Eastern Africa (2nd edn). Livingstone, Edinburgh, UK, pp 228–389

Zdero C, Bohlmann F, Muller M (1987) Sesquiterpene lactones and other constituents from *Eriocephalus* species. Phytochemistry **26**: 2763–2775

Edited by JN Eloff

APPENDIX III

South African Journal of Botany, (SAJB) Vol 70 (2): 347

Abstract of paper presented at the 30th Annual Congress of the South African Association of Botanists and the 4th Botanical Artists' Association of Southern Africa Conference, Exhibition and Workshop. University of Kwa-Zulu Natal, Durban, South Africa, 18th-22nd, January 2004

The biological activity, essential oil composition, and molecular phylogenetic reconstruction of *Eriocephalus* L. (Asteraceae).

E. W. Njenga[1], G. Reeves[2,] A.M. Viljoen[1] and S.F. van Vuuren[1]
[1]-Department of Pharmacy and Pharmacology, Faculty of Health Sciences,
University of the Witwatersrand, 7 York Road, Park Town, 2193,
Johannesburg, South Africa.

[2]- Leslie Hill Molecular Systematics Laboratory, Kirstenbosch Research Centre, National Botanical Institute, Private Bag X7, Claremont 7735,
Cape Town, South Africa.

Abstract

The genus *Eriocephalus* L. commonly known as 'wild rosemary', 'Cape snow bush' or 'kapok bush' belongs to the family Asteraceae, tribe Anthemideae. It is endemic to southern Africa and comprises of 32 species of which several are economically important in the medicinal and fragrance industries. Species delimitations within the genus are complex and have led to varying treatments often characterised by substantial confusion in the ranking of some of its constituent taxa. This study has utilised essential oil composition, biological activity and DNA sequence data to help resolve species delimitations within the genus. Plant parts collected from wild populations were hydrodistilled using Clevenger apparatus to obtain essential oils, which were analysed using GC/MS to elucidate their composition. Disc diffusion assay and minimum inhibitory concentration (MIC) analyses were carried out to evaluate antimicrobial activity of plant extracts and essential oils against *Staphylococcus aureus, Bacillus cereus, Bacillus subtilis, Klebsiella pneumoniae, Escherichia coli*, *Cryptococcus neoformans* and *Candida albicans.* For the disc diffusion assay the essential oils generally showed higher activity than the extracts. However, the acetone extracts of *E. aromaticus* had the lowest MIC (0.4 mg/ml) for *B. cereus*. In a preliminary analysis, two plastid and nuclear DNA regions, comprising the *psbA-trnH* intergenic spacer and internal transcribed spacer (ITS) respectively, were sequenced to reconstruct species-level relationships within the genus. This phylogenetic framework was then used to address the evolutionary patterns of biological activity and essential oil composition in *Eriocephalus*.

Paper 2. To be submitted to the Journal of Ethnopharmacology.

Chemical composition and biological activities of some Namibian species of *Eriocephalus* L. (Asteraceae)

Elizabeth W Njenga[1], Alvaro M Viljoen[1*], Sandy F van Vuuren[1] and Carlo Bicchi[2],

[1]Department of Pharmacy and Pharmacology, Faculty of Health Sciences,
University of the Witwatersrand, 7 York Road, Parktown, 2193, South Africa.
[2]Department of Pharmacy and Pharmacology, Torino, Italy.
** Corresponding author, e-mail: viljoenam@therapy.wits.ac.za*

Abstract

The essential oils composition of seven species of *Eriocephalus* collected from various localities in Namibia were determined using gas chromatography coupled to mass spectra (GC/MS). Eighty-three (83) compounds were obtained. A quantitative analysis of the essential oil data revealed four chemotypes. A qualitative cluster analysis of essential oils resulted in three clusters based on affinity groupings. *In vitro* screening for antimicrobial activities of the species was carried out using the disc diffusion and minimum inhibitory concentration (MIC) assays on oils and extracts. Antioxidant and anti-inflammatory properties were determined for acetone leaf extracts and essential oils respectively. The essential oils showed some antimicrobial activity against the test pathogens in the disc diffusion assay. The lowest MIC was noted against *Staphylococcus aureus* (2 mg/ml) in *E. merxmuelleri* while the lowest in the extracts was (0.2 mg/ml) against *Bacillus cereus* in *E. pinnatus.* The rest of the species showed moderate activities in their essential oils and extracts. Antioxidant and anti-inflammatory activities ranged from IC_{50} (33-54 μg/ml) for acetone extracts and IC_{50} (35-100 μg/ml) for essential oils respectively. The essential oils and the extracts have a potential for use in treatment of fungal and bacterial diseases as evidenced by their activity against the Gram-positive and negative bacteria and the selected yeasts. The oils have potential for use in treatment of inflammation and for use in cosmetics as they inhibited 5-lipoxygenase enzyme. The extracts showed activity in the antioxidant assay and hence have a potential for use as sources of free radical scavengers. This study documents the essential oil composition of the seven Namibian species of *Eriocephalus.*

Paper 3 (To be submitted to *South African Journal of Botany*).

In vitro antioxidant and antiinflammatory activity of species of *Eriocephalus* L (Asteraceae).

Elizabeth W Njenga and Alvaro M Viljoen[*],
Department of Pharmacy and Pharmacology, Faculty of Health Sciences,
University of the Witwatersrand, 7 York Road, Parktown, 2193, South Africa
** Corresponding author, e-mail: viljoenam@therapy.wits.ac.za*

Abstract

The genus *Eriocephalus* is a South African endemic of the family Asteraceae and the tribe Anthemideae. Some of the species in the genus are traditionally used in treatment of dermal infections, gastro-intestinal disorders, and upper respiratory tract infections. Others are reportedly used as diuretics and diaphoretics and in treatment of inflammation. There are no written accounts to date validating the latter use and this study seeks to provide a scientific rationale for use of the members of the genus in treatment of inflammation. The use of some of the species for culinary purposes and the great need to explore plants for novel antioxidants for use in industries and management of free radical associated diseases provides a basis for the screening for presence of antioxidants in the group as well as validating their traditional uses.

In vitro screening for presence of antioxidants was carried out on acetone leaf extracts of 22 species collected from wild populations using 1,1, diphenyl-2-2-picryhydrazyl (DPPH) radical scavenging method. The essential oils were also tested but did not show activity at the starting concentration of 100 µg/ml. The extracts showed moderate activity with the IC_{50} values ranging from 21.5 µg/ml to 79 µg/ml in *E. punctulatus* with the values for rest of the species tested within the aforementioned range. Essential oils of seventeen species were screened for presence of inhibitors of 5-lipoxygenase enzyme with linoleic acid as the substrate. The results are indicative of the presence of enzyme inhibitors with the IC50 values ranging from 19 µg/ml in *E. africanus* and 98.9 µg/ml in *E. purpureus.*

The results albeit *in vitro* indicate the presence of antioxidants hence potential sources of natural antioxidants. The essential oils have compounds with inhibitory activity against the enzyme under study and the activity validates the traditional uses of some of the members in treatment of inflammation. The study provides a most recent account of the antioxidant and antiinflammatory properties of the species in the genus.

.

Paper 4 (To be submitted to *South African Journal of Botany*).

Essential oil composition of species of *Eriocephalus* L. (Asteraceae).

Elizabeth W Njenga[1], Alvaro M Viljoen[*1], and C Bicchi .
[1]Department of Pharmacy and Pharmacology, Faculty of Health Sciences,
University of the Witwatersrand, 7 York Road, Parktown, 2193, South Africa
[2]Department of Pharmacy and Pharmacology, Torino, Italy
** Corresponding author, e-mail: viljoenam@therapy.wits.ac.za*

Abstract

The terpene chemistry of the genus *Eriocephalus* L has not been recorded for most the species in the genus except for the commercially used species *E. africanus* (Cape snowbush oil) and *E. punctulatus* (Cape chamomile oil). This group of compounds are important in medicinally and in industries for flavour and fragrance and many other uses. This study seeks to record the terpene profiles for 21 species collected from the wild populations and an attempt to give some chemotaxonomic considerations is made using phenetic analysis. An attempt was also made to identify favourable chemotypes suitable for commercial development in flavour and fragrance industries.

Multiple collections of plant material were made and essential oils obtained through hydrodistillation of aerial plant parts using a Clevenger apparatus. The oils were analysed using thin layer chromatography (TLC) for detection of variability in the oils and were later subjected to gas chromatography coupled to mass spectroscopy (GC/MS).

The essential oil profiles are largely comprised of acyclic, monocyclic, and bicyclic regular and irregular mono- and sesquiterpenes of various structural groups. Two hundred compounds were noted in the essential oils with some of the common constituents being; α- and β-pinene, yomogi alcohol, ρ-cymene, 1,8-cineole, camphor, 4-terpineol, spathulenol, caryophyllene oxide, α-copaene and β-caryophyllene. Most of the species have a relatively high content of 1,8-cineole and camphor. Twenty-two chemotypes were noted and the potential for commercial development in the flavour, fragrance, and pharmaceutical industries has been recorded. Among the favourable chemotypes noted includes the camphor, 1,8-cineole, bisabolol oxide B and nerolidol rich oils. However, due to the extensive variability in the essential oil profiles, standardization of oils in commercial development is crucial.

A quantitive and qualitative cluster analysis of the multiple collections of the species in the genus did not reveal coherent groups in line with infraspecification delimitation in the group. The current species delimitation of the group is not supported by the terpene chemistry in the current study.

Paper 5 (To be sumitted to *Molecular phylogenetics and Evolution*).

Phylogenetic reconstruction of the genus *Eriocephalus* L. (Asteraceae).

E. W. Njenga[1], G. Reeves[2,] and A.M. Viljoen[1].
[1]-Department of Pharmacy and Pharmacology, Faculty of Health Sciences,
University of the Witwatersrand, 7 York Road, Park Town, 2193,
Johannesburg, South Africa.

[2]- Leslie Hill Molecular Systematics Laboratory, Kirstenbosch Research Centre, Southern African National Biodiversity Institute (SANBI), Private Bag X7, Claremont 7735,
Cape Town, South Africa.

Abstract

Despite its commercial importance, little is known with regard to species-level relationships within the Southern Africa endemic genus *Eriocephalus* L. This genus is economically important as a source of the 'Cape snowbush' and the 'Cape chamomile' oils and is also extensively used in traditional herbal remedies. It is clear that the question of species delimitation and relationships are vital to well directed economic use of this genus. The genus has extensive delimitation problems due to intergrading of the major diagnostic morphological characters. Relationships to date have largely been inferred based upon morphological characteristics and in some cases on chemotaxonomic variation. However, neither suite of characters have been analysed within a cladistic framework. Therefore in this study an attempt is being made for the first time to elucidate the phylogenetic relationships between taxa of the genus *Eriocephalus* using both DNA sequence and chemical characters. This will be crucial in clarification of relationships between species and the understanding of patterns of variation observed in the group and for commercial considerations.

Total genomic DNA was extracted from silica dried leaf material and the non-coding plastid DNA regions, the *psb*A-*trn*H and *trn*L-F intergenic spacers and the internal transcribed spacer (ITS) region of nuclear ribosomal DNA were amplified, and sequenced and analysed using the parsimony algorithm.

The DNA sequence data revealed lack of variability in the non-coding regions *psb*A-*trn*H and *trn*L-F among species of the genus. The nuclear DNA region (ITS) was variable but the number of characters separating taxa was too few for resolution of relationships between taxa. Presence of highly divergent paralogous repeats of ITS were also noted in some taxa. The combination of molecular and chemical data did not resolve the species delimitation problems due to the highly variable distribution of characters within a single species. The patterns of variation observed in the genus may be attributed to chemical convergence, divergence, hybridisation, differential gene expression and polymorphism and allelochemical diversification among other factors. The lack of coherence in the phylogenetic groupings of the various taxa implies that the current species boundaries may not be a true reflection of natural taxonomic entities. The use of multiple taxa in taxonomic studies is strongly recommended due to the extensive variability noted in the chemical profiles of the taxa that is also depicted in the phylogenetic histories. It also implies that caution should be taken in bioprospecting for new natural products for commercial development, as plant chemical profiles especially from the same species can be very variable. This implies carrying out exhaustive population and genetic studies for evaluation of diversity in the study group.