

**AN INVESTIGATION OF
ANTIBACTERIAL COMPOUNDS IN**

Combretum microphyllum

[KLOTZSCH]

MAGDELEN KOTZÉ

B. Pharm (P) M. Sc. (H)

Dissertation submitted to the faculty of medicine (Department of

University of Pretoria in partial fulfilment of the requirements of the degree of

M. KOTZÉ

(9831029)

MAGISTER SCIENTIARUM

Promoter: Prof JN Eloff

UNIVERSITY OF PRETORIA

Date of submission: November 2000

2000

AN INVESTIGATION OF ANTIBACTERIAL COMPOUNDS IN *C. microphyllum* [Klotzsch]

MAGDELEEN KOTZÉ

B. Pharm (PU vir CHO)

Dissertation submitted to the faculty of Medicine (Department of
Pharmacology),
University of Pretoria, Pretoria, in partial fulfilment of the
requirements of the degree of

MAGISTER SCIENTIAE

Promoter: Prof JN Eloff

Date of submission: November 2000

21.587 3945
131 321 7808174

ACKNOWLEDGEMENTS PREFACE

**I hereby confirm that this is my own work and have not
been submitted to any other institution**


.....

Magdeleen Kotze

ACKNOWLEDGEMENTS

I would like to extend a warm word of thanks to the following:

- Prof JN Eloff, my promoter, without whose support and wisdom I would not have been able to complete this investigation.
- The Department of Pharmacology, University of Pretoria, for making their facilities available at all hours throughout the three years of this research.
- My fellow students and Dr I von Teichman who assisted me in the laboratory and Julia Becker for all her help.
- My parents, brothers and sister for their love and encouragement.
- Francois for his patience and understanding.
- My Creator for His abundant blessings.

SUMMARY

Acetone, which efficiently removes compounds from finely ground *Combretum* spp leaves, was compared with 1 % aqueous sodium bicarbonate as extractants using intact dried leaves of *Combretum microphyllum*.

Acetone gave more promising results, than sodium bicarbonate. The acetone extract yielded the highest concentration, as well as the greatest diversity of compounds visible after TLC. The sodium bicarbonate extract inhibited Gram-positive bacteria to a larger extent than Gram-negative bacteria. *E. coli* was inhibited much more than *E. faecalis* by the acetone extract. This indicated that there may be some selectivity.

The total quantity extracted by acetone from intact leaves was substantially lower (3.8 %) than values found earlier (6.2 %) for finely ground material. The MIC values for the extracts of intact leaves were lower, indicating that fewer inactive compounds were co-extracted. I decided to continue working on finely ground leaf material. I experienced difficulties in the acid precipitation of the bicarbonate extracts and decided not to continue with sodium bicarbonate as extractant.

Eleven different extractants (hexane, carbon tetrachloride, isopropyl ether, diethyl ether, methylene dichloride, tetrahydrofurane, ethyl acetate, acetone, ethanol, methanol and water) were used on finely ground, dried leaf material to extract antibacterial compounds from *C. microphyllum*.

There were large differences in the chemical composition of compounds extracted. Methylene dichloride and ethanol extracted the most compounds. The extracts of the intermediate polarity extractants isopropyl ether, ethyl ether and methylene dichloride gave the best separation with BEA as eluent, whereas the carbon tetrachloride and tetrahydrofurane extracts gave pronounced streaking. Carbon tetrachloride surprisingly extracted a large quantity of polar compounds (low R_f-value) and a small quantity of non-polar compounds in comparison to the more polar isopropyl ether and diethyl ether.

On the other hand, a relatively polar extractant such as ethanol, extracted several non-polar compounds. With CEF as eluent the carbon tetrachloride and tetrahydrofurane extracts gave serious streaking. Acetone extracted the highest concentration and most diverse number of compounds that reacted with the vanillin spray reagent. Although it did not extract the most antibacterial activity, I decided to continue with acetone as extractant due to its relatively low toxicity to test organisms and the good TLC separation of compounds with this extractant.

Solvent/solvent fractionation was used to simplify extracts because of the initial complexity of the extracts. The most non-polar fractions (hexane, carbon tetrachloride and chloroform) gave the best separation of compounds as well as having the greatest diversity of components when separated by TLC. The highest quantity, c. 42 %, was present in the hexane fraction and nearly two-thirds of the total antibacterial activity was also present in the hexane fraction.

Frequently Gram-negative bacteria are more resistant to plant extracts than Gram-positive bacteria, but this was not true for the compounds present in *C. microphyllum*.

Bioautography worked well with *S. aureus*, but was not as reproducible with the other test organisms. Unfortunately *S. aureus* was the least sensitive of the four test organisms, especially for the hexane and carbon tetrachloride fractions.

Because the hexane fraction had the highest antimicrobial activity by far for all four test organisms used, I chose this fraction for the next steps in the isolation of bioactive compounds with column chromatography.

Attempts to use Extrelut, a diatomaceous earth product, instead of the solvent/solvent fractionation, were not successful.

Column chromatography on silica gel 60 [15-40 μm] gave good results. Four volatile eluents, which worked well on TLC, were used on the column in the following order: BEA, CEF, acetone and methanol.

Fractions were combined based on the analysis by TLC. Fraction ABC gave the most definite bands and best separation on TLC. By far the largest antibacterial activity (ABC: 66 % and DEF: 15 %) was in the first highly non-polar pooled fractions. *S. aureus* was not as sensitive as the other test organisms.

I developed a TLC system, using volatile eluents that would separate the components of fractions ABC and DEF well in order to employ this system in a next silica gel chromatographic separation. Good results were obtained with a 2:1 hexane-acetone mixture. I decided to continue with only the ABC fraction using this eluent. To increase the resolution, a finer grade silica gel [15-25 μm] with a narrower size range was used as packing material.

The fractions were analyzed by TLC and were combined based on the TLC

analysis and the quantity present in each fraction. Some of the pooled minor fractions seemed to be relatively pure based on the TLC analysis of these fractions and had significant antibacterial activity based on bioautography with *S. aureus* as test organism.

When these active pooled fractions were dried under vacuum in a vacuum dessicator prior to NMR spectroscopy, c. 80 % was lost. According to the NMR analysis, the remaining sample consisted mainly of phthalate, a common component of plasticizers used in making plastics and also a common contaminant in certain solvents such as ethyl acetate.

Phthalic acid did not react with the vanillin spray reagent, but it gave clear quenching in UV light after TLC. According to the R_f-value, phthalic acid was not responsible for the antibacterial activity. It was also not volatile under high vacuum.

High concentrations of phthalic acid had a slight antibacterial activity, possibly due to a pH effect.

The procedures developed therefore led to the isolation of an antibacterial compound from *C. microphyllum*. Unfortunately personal circumstances made it impossible to repeat the last part of the work or expand on any other part of the results.

A major avenue for further work exists in the non-polar fractions of the final separation containing most of the dry mass and biological activity. There is also the possibility to capitalize on the suspected volatility of the bioactive compound to purify it by vacuum distillation.

SAMEVATTING

Asetoon, wat verbindings baie goed verwyder vanuit fyngeaalde *Combretum* spp blare, is vergelyk met 1 % waterige natriumbikarbonaat, as ekstraheermiddels deur heel, gedroogde blare van *Combretum microphyllum* te gebruik.

Asetoon het meer belowende resultate gelever as natriumbikarbonaat. Die asetoonekstrak het die hoogste konsentrasie asook die meeste verskillende verbindings sigbaar na dunlaagchromatografie gelever. Die natriumbikarbonaat ekstrak het Gram-positiewe bakterieë meer geinhibeer as Gram-negatiewe bakterieë, maar *E. coli* was meer onderdruk as *E. faecalis* deur die asetoonekstrak. Dit het daarop gedui dat daar wel selektiwiteit mag bestaan.

Die totale hoeveelheid geëkstraheer deur asetoon vanaf heel blare, was aansienlik laer (3.8 %) as waardes wat vroeër gevind is (6.2 %) vir fyngeaalde blare. Die minimum inhiberende konsentrasie waardes vir heel blare was laer, wat daarop gedui het dat minder onaktiewe verbindings geëkstraheer is. Ek het besluit om eerder op fyngeaalde blare te werk. Ek het ook probleme ondervind met die suur presipitering van die bikarbonaat ekstrakte en het besluit om nie verdere studies op natriumbikarbonaat as ekstraheermiddel te doen nie.

Elf verskillende ekstraheermiddels [heksaan, koolstoftetrachloried, isopropieleter, diëtieleter, metileendichloried, tetrahidrofuraan, etielasetaat, asetoon, etanol, metanol en water) is gebruik op gedroogde, fyngeaalde blare om antibakteriële verbindings vanuit *C. microphyllum* te ekstraheer.

Daar was groot verskille in die chemiese samestelling van verbindings wat geëkstraheer is. Metileendichloried en etanol het die meeste verskillende verbindings geëkstraheer. Die ekstrakte van die intermediêre polariteit ekstraheermiddels, isopropieleter, etieleter en metileendichloried het die beste skeiding met BEA as elueermiddel gegee, terwyl die koolstoftetrachloried en tetrahidrofuraan ekstrakte onbevredigend gestreep het. Koolstoftetrachloried het tot my verbasing 'n groot hoeveelheid polêre verbindings (lae Rf-waarde) geëkstaheer en 'n klein hoeveelheid nie-polêre verbindings in vergelyking met die meer polêre isopropieleter en diëtleter.

Aan die ander kant het 'n relatiewe polêre ekstraheermiddel soos etanol verskeie nie-polêre verbindings geëkstraheer. Met CEF as elueermiddel het die koolstoftetrachloried en tetrahidrofuraan ekstrakte duidelik gestreep. Asetoon het die hoogste konsentrasie en die grootste hoeveelheid verbindings geëkstraheer wat met die vanillien spuitstof gereageer het. Alhoewel asetoon nie die meeste antibakteriële aktiwiteit geëkstraheer het nie, het ek besluit om steeds voort te gaan met asetoon as ekstraheermiddel as gevolg van asetoon se relatiewe lae toksisiteit vir die toetsorganismes asook die duidelike skeiding van verbindings.

Die oplosmiddel-fraksionerings proses is gebruik om ekstrakte te vereenvoudig na aanleiding van die aanvanklike kompleksiteit van die ekstrakte. Die mees nie-polêre drie fraksies (heksaan, koolstoftetrachloried en chloroform) het die beste skeiding van verbindings asook die meeste verskillende verbindings gegee deur dunlaagchromatografie. Die hoogste hoeveelheid, ongeveer 42 %, was teenwoordig in die heksaan fraksie en amper twee derdes van die totale antibakteriële aktiwiteit was ook teenwoordig in die heksaan fraksie.

Gewoonlik is Gram-negatiewe bakterieë meer weerstandbiedend vir plant ekstrakte as Gram-positiewe bakterieë, maar dit was nie die geval met verbindings wat teenwoordig is in *C. microphyllum* nie.

Bioautografie het goed gewerk met *S. aureus*, maar nie so goed met die ander toetsorganismes nie. Ongelukkig was *S. aureus* die minste sensitief van die vier die toetsorganismes, veral vir die hekasaan en koolstoftetrachloried fraksies.

Omdat die hekasaan fraksie verreweg die hoogste antimikrobiese aktiwiteit vir al vier die toetsorganismes gehad het, het ek besluit om verder te gaan met eksperimente vir die isolering van bio-aktiewe verbindings deur kolom chromatografie deur die hekasaan fraksie te gebruik.

Pogings om Extrelut, 'n diatoomaarde produk te gebruik in plaas van die oplosmiddel-fraksionerings proses, was nie suksesvol nie.

Kolom chromatografie op silika gel 60 [15-40 μm] het goeie resultate gelewer. Vier vlugtige elueermiddels, wat goed gewerk het met dunlaagchromatografie, is gebruik in die volgorde BEA, CEF, asetoon en metanol.

Fraksies is gekombineer, gebaseer op die analise deur dunlaagchromatografie. Fraksies ABC het die duidelikste bande en beste skeiding gegee op dunlaagchromatografie. Die meeste antibakteriese aktiwiteit (ABC: 66 % en DEF: 15 %) was in die eerste hoogs nie-polêre gekombineerde fraksies. *S. aureus* was nie so sensitief soos die ander organismes nie.

Ek het 'n dunlaagchromatografie stelsel ontwikkel met vlugtige elueermiddels, wat die verbindings in fraksies ABC en DEF goed sou skei met die doel om hierdie stelsel dan te gebruik op 'n volgende silikagel kolomskeiding. Goeie resultate is verkry met 'n 2:1 heksaan-asetoon mengsel. Ek het besluit om slegs met die ABC fraksie voort te gaan deur hierdie elueermiddel kombinasie te gebruik. Om die resolusie te verhoog is 'n fyner graad silika gel [15-25 μm] met 'n smaller grootte verspreiding te gebruik as pakkingsmateriaal.

Die fraksies is met dunlaagchromatografie geanaliseer en gekombineer, gebaseer op die dunlaagchromatografie analise en die hoeveelheid teenwoordig in elke fraksie. Sommige van die gekombineerde kleiner fraksies het redelik suiwer voorgekom volgens die dunlaagchromatografie resultate van hierdie fraksies en het 'n beduidende antibakteriese aktiwiteit gehad volgens bioautografie met *S. aureus* as toetsorganisme.

Ongeveer 80 % van hierdie gekombineerde fraksies het verlore gegaan toe hierdie fraksies gedroog is onder vakuum in 'n vakuumdessikator net voor KMR spektroskopie uitgevoer is.

Volgens die KMR resultate het die oorblywende monster hoofsaaklik bestaan uit ftalsuur, 'n algemene komponent van plastiseerders wat gebruik word in die maak van plastiek. Dit is ook 'n algemene kontaminant in sekere oplosmiddels byvoorbeeld etielasetaat.

Ftalsuur het nie met die vanillien spuitstof gereageer nie, maar die het baie duidelike blussing van UV lig gegee na dunlaagchromatografie met F_{254} plate. Volgens die R_f -waarde kon ftalsuur nie verantwoordelik gewees

het vir die antibakteriese aktiwiteit nie. Dit was ook nie vlugtig onder hoë vakuüm nie.

Die prosedures wat dus ontwikkel is, het gelei tot die isolasie van 'n antibakteriese verbinding uit *C. microphyllum*. Ongelukkig het persoonlike omstandighede dit vir my onmoontlik gemaak om die werk te herhaal of om uit te brei op enige ander deel van die resultate.

Groot ruimte vir verdere studies bestaan in die nie-polêre fraksies van die finale skeiding wat die meeste van die droë gewig en biologiese aktiwiteit besit het. Daar is ook 'n moontlikheid om te kapitaliseer op die vermoedelike vlugtigheid van die bio-aktiewe verbinding deur dit te suiwer deur middel van vakuümdistillasie.

CONFERENCE ATTENDED

M. Kotze and J.N. Eloff: Extraction and investigation of antibacterial compounds from *Combretum microphyllum*. Indigenous Plant Use Forum. Nelspruit, July 2000.

ACKNOWLEDGEMENTS

SUMMARY

SANDA ATTING

CONFERENCE ATTENDED

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

LIST OF FIGURES

LIST OF TABLES

1. LITERATURE BACKGROUND

1.1 Introduction

1.2 Selection of plant to investigate

TABLE OF CONTENTS

PREFACE	ii
ACKNOWLEDGEMENTS	iii
SUMMARY	iv
SAMEVATTING	viii
CONFERENCE ATTENDED	xiii
TABLE OF CONTENTS	xiv
LIST OF ABBREVIATIONS	xx
LIST OF FIGURES	xxii
LIST OF TABLES	xxviii
1. LITERATURE BACKGROUND	
1.1 Motivation	2
1.2 Selection of plant to investigate	3

1.2.1	Introduction	3
1.2.2	Combretaceae	4
1.2.3	The genus <i>Combretum</i>	6
1.2.4	Previous studies	8
1.2.5	Why <i>C. microphyllum</i> was selected	9
1.2.6	<i>Combretum microphyllum</i>	10
1.3	Aim of the study	15

2. MATERIALS AND METHODS

2.1	Collection of plant material	17
2.2	Preparation of leaf material	17
2.3	Extraction	18
2.4	TLC analysis of extract	19
2.5	Minimum inhibitory concentration by INT microplate bioassay	20
2.5.1	Dilution of extract	20
2.5.2	Addition of bacteria	21
2.6	Bioautography	22
2.7	Comparison of different solvents	22
2.7.1	Solvent/solvent fractionation	23
2.7.2	Thin layer chromatography, bioautography and bioassay of fractions	26
2.8	Isolation of bioactive compounds	26

2.8.1	Column chromatography with Extrelut and Silica gel as packing material in mini-columns	26
2.8.2	Combination of fractions after TLC and bioassay	27
2.9	TLC of combined fractions	27
2.9.1	TLC in Seprachrom containers	27
2.9.2	TLC in small glass tanks	27
2.9.3	Column chromatography on preparative Silica columns	28
2.9.4	Combination of collected fractions	29

3. RESULTS AND DISCUSSION

3.1	Extraction	30
3.1.1	Comparing sodium bicarbonate and acetone as extractants	30
3.1.1.1	Introduction	30
3.1.1.2	Quantity extracted with initial extractants	30
3.1.1.3	Thin layer chromatography of acetone and bicarbonate extracts	31
3.1.1.4	Minimum inhibitory concentration by INT microplate bioassay of acetone and NaHCO ₃ extracts	33
3.1.1.5	Discussion	34

3.1.2	Eleven different extractants tested for best screening and isolation of antimicrobial components from <i>C. microphyllum</i>	35
3.1.2.1	Extraction with 11 different extractants	35
3.1.2.2	Investigation of alternative microplate serial dilution technique	40
3.1.2.3	Bioassay of extracts found with different extractants	40
3.1.2.4	Discussion	42
3.2	Group separation of extracts by solvent/solvent fractionation	43
3.2.1	Introduction	43
3.2.2	Quantities obtained and antibacterial activity of different fractions	43
3.2.3	Chemical composition of the six fractions obtained	46
3.2.4	Bioautography and bioassay of fractions	50
3.3	Group separation by using Extrelut as packing material	52
3.3.1	Introduction	52
3.3.2	Wet adsorbent	53
3.3.3	Dry adsorbent	54
3.3.4	TLC of Extrelut fractions	54
3.4	Column chromatography	56
3.4.1	Silica gel as packing material	56
3.4.2	Combination of fractions	59
3.5	Combination of fractions after TLC and bioassay	62

3.5.1	Developing a system to separate components by column chromatography	62
3.5.2	TLC in glass eluent tanks	63
3.6	Column chromatography on a preparative column	66
3.6.1	Chemical composition of different fractions	67
3.6.2	Further work on isolated compounds	70
3.6.3	Bioautography with pooled fractions 21-30 and phthalic acid	72
3.6.4	Quantitative evaluation of results	73
4.	CONCLUSIONS	
4.1	Introduction	75
4.2	Extraction	76
4.3	Preliminary separation of extracts	78
4.3.1	Group separation of extracts by solvent/solvent fractionation	78
4.3.2	Group separation by using Extrelut as packing material	80
4.4	Column chromatography	80
4.4.1	Initial column chromatography with silica gel [15-40 μm] as packing material on a small column	80
4.4.2	Developing a system to separate components by column chromatography on a large column	82
4.4.3	Final column chromatography on a preparative column	82
4.5	Isolation of a bioactive fraction	83

4.6	Recommendations on future work	85
5.	REFERENCES	86

LIST OF ABBREVIATIONS

1. A Acetone extractant
2. ATCC American Type Culture Collection
3. B Butanol fraction
4. BEA Benzene/ethanol/ammonium hydroxide
[90/9/1, v/v/v]
5. CCl₄ Carbon tetrachloride extractant
6. CEF Chloroform/ethyl acetate/formic acid
[5/4/1, v/v/v]
7. CF Chloroform fraction
8. CT Carbon tetrachloride fraction
9. E Ethanol extractant
10. EA Ethyl acetate extractant
11. *E. coli* *Escherichia coli*
12. EE Diethyl ether extractant
13. *E. faecalis* *Enterococcus faecalis*
14. H Hexane fraction
15. HE Hexane extractant
16. HCl Hydrochloric acid
17. H₂O₂ Hydrogen peroxide
18. INT *p*-iodonitrotetrazolium violet
19. IPE Isopropyl ether extractant

20.	M	Methanol extractant
21.	MA	Methylene dichloride/acetone [3/2, v/v]
22.	MDC	Methylene dichloride extractant
23.	MIC	Minimum inhibitory concentration
24.	MW	35 % water in methanol fraction
25.	NaHCO ₃	Sodium bicarbonate
26.	O ₂	Oxygen
27.	<i>P. aeruginosa</i>	<i>Pseudomonas aeruginosa</i>
28.	<i>S. aureus</i>	<i>Staphylococcus aureus</i>
29.	THF	Tetrahydrofurane extractant
30.	TLC	Thin layer chromatography
31.	UV	Ultraviolet
32.	W	Water fraction
33.	WA	Water extractant

LIST OF FIGURES

- Figure 1.1** *Combretum microphyllum*: Flowers. 11
- Figure 1.2** *Combretum microphyllum*: Fruit at an early and late stage. 11
- Figure 1.3** Distribution map of *C. microphyllum*. 13
- Figure 2.1** The procedure used for solvent/solvent extraction and fractionation of the components in *C. microphyllum* into six fractions. 25
- Figure 3.1** Chromatogram showing the separation of compounds present in 380 μg of the acetone extract (left) and 408 μg of the bicarbonate extract (right). The CEF system was used as eluent and the plate was sprayed directly after TLC with the vanillin spray reagent. 32
- Figure 3.2** Three chromatograms of 380 μg acetone extract (left) and 408 μg bicarbonate extract (right) sprayed one week after chromatography: vanillin spray reagent (left), perchloric spray reagent (center) and phosphoric spray reagent (right). 32
- Figure 3.3** Separation of components present in 50 μg of 11 different extracts with BEA as eluent and viewed in daylight before spraying with spray reagent. Lanes from left to right extracted by hexane, carbon tetrachloride

[centrifuged], carbon tetrachloride [filtered], isopropyl ether, diethyl ether, methylene dichloride, tetrahydrofurane, ethanol, methanol and water. 36

Figure 3.4 Separation of components present in 50 µg of 11 different extracts with BEA as eluent and vanillin as spray reagent. Lanes from left to right as in Figure 3.3. 37

Figure 3.5 Separation of components present in 50 µg of 11 different extracts using the CEF eluent and vanillin spray reagent. Lanes as in Figure 3.3. 38

Figure 3.6 Separation of components present in 100 µg of 11 different extracts using the BEA (top) and CEF (bottom) eluents and the vanillin spray reagent. Lanes as in Figure 3.3. No components separated in the water extract [results not shown]. 39

Figure 3.7 Total antibacterial activity to four test organisms of finely ground *C. microphyllum* leaves extracted with 11 different extractants. From left to right: hexane, carbon tetrachloride, isopropyl ether, diethyl ether, methylene dichloride, tetrahydrofurane, ethyl acetate, acetone, ethanol, methanol and water. 41

Figure 3.8 The relative quantities obtained by solvent/solvent fractionation and the relative antibacterial activities of the different fractions. H = hexane, CT = carbon tetrachloride, CF = chloroform, B = butanol, MW = 35 % methanol/water and W = water. 45

Figure 3.9 Total antibacterial activity to four test organisms of *C. microphyllum* leaves extracted with solvent/solvent fractions. (Fractions from left to right: H = hexane, CT = carbon tetrachloride, CF = chloroform, B = butanol, MW = 35 % methanol/water and W = water.) 46

Figure 3.10 Chemical composition of solvent/solvent fractions [200 µg] separated by MDC/Acetone as eluent (top left and top right), BEA as eluent (middle left and right) and CEF as eluent (bottom left and right). Compounds visualized by *p*-anisaldehyde as spray reagent (top left, middle left and bottom left) and vanillin as spray reagent (top right, middle right and bottom right). 48

Figure 3.11 Chemical composition of solvent/solvent fractions [100 µg] separated by MA as eluent (top and middle) and CEF as eluent (bottom). Compounds visualized by *p*-anisaldehyde (top) and vanillin (middle and bottom) spray reagents. 49

Figure 3.12 Bioautogram of six different fractions of *C. microphyllum* extract by solvent/solvent extraction. TLC using BEA [left] and CEF [right] as eluent, sprayed with *S. aureus* cell suspension, incubated and sprayed with INT. White areas indicate bacterial growth inhibition. (Fractions from left to right: hexane, carbon tetrachloride, chloroform, butanol, 35 % methanol/water and water.) 50

Figure 3.13 Separation of components in 50 µg of the different Extrelut fractions using the BEA eluent and the vanillin spray reagent. From left to right: H = hexane fraction, D -D4 = dry adsorbent

fractions eluted with hexane, D5 = dry adsorbent fraction eluted with acetone, N1-N5 = wet adsorbent fractions eluted with hexane and N5 = wet adsorbent fraction eluted with acetone. 55

Figure 3.14 Top: Chromatogram of first highly non-polar fractions (A-V) using the BEA system as eluent and the vanillin spray reagent (c. 3 µl of each fraction placed on plates) and bottom: chromatogram of last polar fractions (1-25) using the CEF system as eluent and the vanillin spray reagent (c. 9 µl of each fraction placed on plates.) 58

Figure 3.15 Separation of components present in 50 µg of fractions using the BEA eluent and the vanillin (left) and *p*-anisaldehyde (right) spray reagents. (From left to right: ABC, DEF, GHI, J-N and O-V combined fractions.) 59

Figure 3.16 Total antibacterial activity in ml of *C. microphyllum* leaves extracted with combined fractions to four test organisms. 60

Figure 3.17 Different combinations of methylene dichloride (MD) and methanol (ME) as eluents for 50 µg of the ABC, DEF and GHI extracts. Plates were sprayed with the vanillin spray reagent. [MD : ME = 10 : 1 (top left), MD : ME = 15 : 1 (top middle), MD : ME = 20 : 1 (top right), MD : ME = 10 : 1 (bottom left), MD : ME = 9 : 1 (middle bottom) and MD : ME = 8 : 1 (bottom right)]. 64

Figure 3.18 Different combinations of hexane (H) and acetone (A) as eluents for 50 µg of the ABC and DEF fractions. Plates sprayed with the vanillin spray reagent (top first, top third, bottom first

and bottom third) and *p*-anisaldehyde spray reagent (top second, top fourth, bottom second and bottom fourth). [H : A = 2 : 1 (bottom third and bottom fourth), H : A = 5 : 1 (top first and second, bottom first and second) and H : A = 10 : 1 (top third and top fourth)]. 65

Figure 3.19 Separation of components present in 50 µg of fractions 1-6, using the CEF eluent and the vanillin spray reagent. 68

Figure 3.20 Separation of fractions from ABC by large scale column chromatography. (Fractions combined from fraction 7, based on quantity present in individual fractions.) 68

Figure 3.21 Separation of compounds [100 µg] in pooled fractions 7-20, 21-30, 31-40, 41-50 and 51-60 by TLC with CEF as eluent. Left sprayed with vanillin spray reagent and right bioautography with *S. aureus* as test organism. 69

Figure 3.22 Bioautography as for Figure 3.21 with *E. coli* [left] and *P. aeruginosa* [right] as test organisms. 70

Figure 3.23 Separation of chemical components present in pooled fractions 21-30 [first, 50 µg and third, 100 µg] and phthalic acid [second, 50 µg and last, 100 µg] by TLC using CEF as eluent and visualizing components with vanillin spray reagent. Quenching of absorbance at 254 nm indicated by pencil circles. 71

Figure 3.24 Bioautogram of fractions 21-30 [50 µg first and 100 µg third] and phthalic acid [50 µg second and 100 µg last]. TLC using CEF as eluent, sprayed with *S. aureus* cell suspension, incubated

and sprayed with INT. White areas indicate bacterial growth inhibition.

73

LIST OF TABLES

Table 1.1 Characteristics of Combretaceae.	5
Table 1.2 The subgeneric classification of species of the genus <i>Combretum</i> occurring in southern Africa.	7
Table 1.3 Characteristics of <i>Combretum</i> .	8
Table 3.1 MIC values in mg/ml and total activity in ml for different test organisms of intact <i>C. microphyllum</i> leaves extracted with sodium bicarbonate and acetone.	33
Table 3.2 MIC values in mg/ml and total activity in ml for different test organisms for <i>C. microphyllum</i> leaves extracted with acetone calculated from Eloff (1999). (Total quantity extracted from 1 gram was 62 mg).	34
Table 3.3 MIC values in mg/ml and total activity in ml of <i>C. microphyllum</i> leaves extracted with 11 different extractants.	41
Table 3.4 MIC values in mg/ml and total activity in ml of <i>C. microphyllum</i> leaves extract fractions obtained by solvent/solvent fractionation.	44

Table 3.5 Distribution of mass and total antibacterial activity in different solvent/solvent fractions. 44

Table 3.6 MIC values in mg/ml and total activity in ml of *C. microphyllum* leaves extracted with combined fractions. 60



Elise
Zitendag

Combretum microphyllum