AN INVESTIGATION OF ANTIBACTERIAL COMPOUNDS IN *Combretum microphyllum* [KLOTZSCH]

M. KOTZÉ

(9831029)

UNIVERSITY OF PRETORIA

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

MAGDELEEN KOTZÉ

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PREFACE

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

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Magdeleen Kotze

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

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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. microhyllum.*

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 Rf-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 [15-25μm] 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 Rf-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 fyngemaalde *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 gelewer as natriumbikarbonaat. Die asetoonekstrak het die hoogste konsentrasie asook die meeste verskillende verbindings sigbaar na dunlaagchromatografie gelewer. 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 fyngemaalde 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 fyngemaalde 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, fyngemaalde 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 diëtieleter.

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 heksaan en koolstoftetrachloried fraksies.

Omdat die heksaan 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 heksaan 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 Rf-waarde kon ftalsuur nie verantwoordelik gewees het vir die antibakteriese aktiwiteit nie. Dit was ook nie vlugtig onder hoë vakuum 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 vakuumdistillasie.

CONFERENCE ATTENDED

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

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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.	CCl_4	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.	H_2O_2	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.	*P. aeruginosa*	*Pseudomonas aeruginosa*
28.	*S. aureus*	*Staphylococcus aureus*
29.	THF	Tetrahydrofurane extractant
30.	TLC	Thin layer chromatography
31.	UV	Ultraviolet
32.	W	Water fraction
33.	WA	Water extractant

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Combretum microphyllum

Combretum microphyllum

CHAPTER 1

LITERATURE BACKGROUND

1.1 MOTIVATION

The increasing use of antibiotics and misuse by over prescribing and/or insufficient patient compliance, have led to the development of bacteria resistant to antibiotics. This resistance may be unavoidable (Spratt, 1994).

Berkowitz (1995) indicates that this resistance is a result of the rate at which bacteria multiply and disperse, as well as the ease with which their genetic material can change, thus inactivating the curative or remedial treatment, or changing the target area. Berkowitz (1995) and Leggiadro (1995) warn that we may reach a post-antibiotic era where antibiotics are no longer effective and they emphasize the need for the development of new antimicrobial compounds to control resistant organisms. Berkelman *et al.* (1994) also stress the importance of finding new antibiotics because more and more resistant pathogenic organisms are identified.

Many plants contain antibacterial compounds. Some of these compounds may be useful in controlling resistant bacteria. Several scientists are therefore investigating plants as alternative sources of antibacterial compounds. In 1967, 58 % of all antibiotics were produced by Actinomycetes, 18 % by other fungi, 12% by higher plants, 9 % by bacteria and the remaining 3 % by algae, lichens and animals (Edwards, 1980).

The pharmaceutical industry has a renewed interest in the development of chemicals of medicinal value from natural products, as combinational chemistry did not deliver the expected yield of lead compounds. As the interest in natural products is increasing, many laboratories are focussing on this type of research. Screening and analysis of plants for pharmacological activity are carried out on a routine basis by teams of scientists in different disciplines at universities and pharmaceutical companies (Alexander *et al.*, 1992).

Plants in southern Africa can be of great value as a source of medicinal components. South Africa has c. 10 % of the plant diversity of flowering plants in the world. With the diminishing of species diversity, the international community is becoming increasingly aware of the potential medicinal value of chemical compounds in plants. Alexander *et al.* (1992) warn against the isolation of research units in South Africa. South African universities should establish natural product research units to prevent the bulk of the natural products of South African plants to be investigated and exploited overseas. The rights of the country of origin concerning the economic advantages involved with the development of secondary chemical compounds from plants have recently been acknowledged. The development of this valuable resource in southern Africa will become increasingly important in the future (Eloff, 1998a).

There is consequently value in investigating southern African plants for antimicrobial activity.

1.2 SELECTION OF PLANT TO INVESTIGATE

1.2.1 Introduction

According to ethnobotanical literature, the genus *Combretum* is used widely and for a variety of conditions. Members of the genus have, inter alia, the following biological activities: anti-inflammatory, diuretic, hypotensive, antibiotic, antifungal, molluscicidal, antimicrobial, as well as being poisonous, having an antihepatitic B virus effect, and the inhibition of gastric ulcers (Hutchings *et al.*, 1996).

Several members of the Combretaceae have been used for treating bacterial diseases in southern Africa (Watt and Breyer-Brandwijk, 1962). Traditional healers in southern and central Africa have used certain *Combretum* species for the treatment of a wide range of disorders (Rogers and Verotta, 1996). Leaves and bark are used extensively by traditional healers, but the winged fruits, which are produced in great abundance, are never used in medicine, nor eaten by wild animals because they are apparently poisonous. A characteristic symptom of this type of poisoning is violent and continuous hiccupping, hence the common name of 'hiccup nut' for *C. bracteosum.* It is suggested that the toxins are common to the fruit of all *Combretum* species. Isolation of these toxins has not yet been accomplished (Panzini *et al.*, 1993).

Carr and Rogers (1986) showed that there are differences in the chemical composition between *Combretum* species. They developed a TLC method to identify *Combretum* species. This method is useful because of the presence of abundant polar compounds in the genus. In our laboratory we have subsequently developed several TLC systems that indicate the extreme complexity of Combretaceae extracts.

1.2.2 Combretaceae

Combretaceae, with some 19 genera and 600 species, is a family of the tropics and subtropics. In South Africa its members are widespread, other than in the extreme southern and south western areas. They rival the *Acacias* in the frequency of their occurrence.

The Combretaceae is one of the prominent plant families in southern Africa. Members of the family include as attractive trees and creepers. Easily observed features of the Combretaceae such as leaves, stems, flowers and fruits are useful in field identification. The leaves are entire, alternate or opposite, and without stipules. The main stem produces whorls of horizontal lateral branches, which in turn are made up of a succession of branchlet units with the tips turned up with a cluster of leaves at its apex. Flowers are usually clustered in axillary heads or spikes, and fruits are characteristically 4-winged (Van Wyk and Van Wyk, 1997).

Carr (1988) has worked on Combretaceae in southern Africa. He has made a substantial contribution to the indigenous flora of the subcontinent with information on propagation, descriptions of each species including synonyms, common names, distribution, habitat, bark foliage, inflorescence, fruit and also keys to identification.

Essential characteristics of Combretaceae (Carr, 1988) are summarized in **Table 1.1**.

Table 1.1 Characteristics of Combretaceae.

Plant type	**Trees, shrubs, climbers, sometimes spinose mangrove**
Inflorescences	Axillary, terminal, spicate (sometimes subcapitate) or paniculte.
Flowers	Sessile, or pedicellate, bisexual, or bisexual and male on the same inflorescence, receptacle usually in 2 parts, the lower containing the ovary, the upper terminating in 4 or 5 sepals.
Petals	Number 4 or 5, or absent.
Stamens	Number 8 or 10, but 4 with *Meiostemon*. Style centrally situated on disc and free, except with *Quisqualis* where the lower portion is attached to the calyx tube.
Fruit	A stipiate or sessile samara with single (surrounding) wing, 4 or 5 or more wings, or a nut.
Seedling cotyledons	Mostly 2, but sometimes 3 or 4, petiolate (sometimes connate) or sessile, arising above or below ground.

Table 1.2 The subgeneric classification of species of the genus *Combretum* occurring in southern Africa.

***Combretum* Loefl**	
Subgenus *Combretum*	**Subgenus *Cacoucia***
Section *Hypocrateropsis*	Section *Lasiopetala*
• *C. celastroides*	• *C. obovatum*
• *C. imberbe*	
• *C. padoides*	
Section *Combretastrum*	
• *C. umbricola*	
Section *Angustimarginata*	Section *Conniventia*
• *C. caffrum*	• *C. microphyllum*
• *C. erythrophyllum*	• *C. paniculatum*
• *C. kraussii*	• *C. platypetalum*
• *C. vendae*	
• *C. woodii*	
Section *Macrostigmatea*	Section *Oxystachya*
• *C. engleri*	• *C. oxystachyum*
• *C. kirkii*	
• *C. sp. nov.*	
Section *Metallicum*	Section *Megalantherum*
• *C. collinum*	• *C. wattii*
Section *Glabripetala*	Section *Poivrea*
• *C. fragrans*	• *C. bracteosum*
Section *Spathulipetala*	• *C. mossambicense*
• *C. zeyheri*	
Section *Ciliatipetala*	
• *C. albopunctatum*	
• *C. apiculatum*	
• *C. edwardsii*	
• *C. moggii*	
• *C. molle*	
• *C. petrophilum*	
• *C. psidioides*	
Section *Fusca*	
• *C. coriifolium*	
Section *Breviramea*	
• *C. hereroense*	
Section *Elaeagnoida*	
• *C. elaeagnoides*	

The Combretaceae family in southern Africa is divided into six genera: *Combretum, Lumnitzera, Meiostemon, Quisqualis, Pteleopsis* and *Terminalia*. Of the six genera in southern Africa, *Combretum* Loefl and *Terminalia* L. are the most important.

The subgenera and sections of the genus *Combretum* are presented in **Table 1.2** (Carr, 1988).

1.2.3 The genus *Combretum*

The taxonomy of *Combretum* is complex. Flowering plants are grouped into families and families are divided up into genera, which may also have subgenera. Each genus is split into species and some species may have two or more subspecies or varieties (Carr, 1988).

The genus *Combretum* Loefl has two subgenera, these being subgenus *Combretum* and subgenus *Cacoucia*. Subgenus *Combretum* is divided into 11 sections and subgenus *Cacoucia* is divided into 5 sections. The genus *Terminalia* L. is divided into 3 sections only, *Abbreviatae, Psidioides* and *Platycarpae* (Carr, 1988).

The genus *Combretum* comprises some 180 species of trees, shrubs and climbers of which c. 33 species occur in southern Africa. It is the largest genus in the Combretaceae.

In the subgenus *Combretum*, trichomes present are scales with or without hairs, but in the subgenus *Cacoucia* the trichomes are stalked glands, accompanied or not by hairs. Carr (1988) argues that characteristics of the trichomes provide an additional aid to identification as even fragmentary specimens of *Combretum* can usually be assigned at least to sectional level under a microscope.

Of the six genera in southern Africa, *Combretum*, being the focus of this study can be identified according to the following essential characteristics (Carr, 1988).

Table 1.3 Characteristics of *Combretum*.

Leaves	Usually opposite or verticillate and having scales or stalked glands.
Inflorescences	Axillary or terminal, spicate or paniculate.
Flowers	Sessile, 4- to 5-merous, bisexual, the receptacle having 2 parts, lower and upper.
Petals	Number: 4 or 5.
Stamens	Number: 8 or 10.
Fruit	With one exception, a 4- or 5-winged samara.

1.2.4 Previous studies

Only about 25 of the *Combretum* species have hitherto been subjected to even a superficial scientific study. The following classes of compounds were found in the genus: alkaloids, tannins, flavonoids, amino acids, substituted phenanthrenes from various heartwoods, a series of unique stilbenes and their glucosides and macrocyclic lactones known as 'combretastatins', 'triterpenoid acids' and their saponins, mainly of the cycloartane and oleanane types (Rogers and Verotta, 1996).

Combretum species occurring in southern Africa are used for many medicinal purposes. These include treating abdominal disorders, abdominal pains, bilharziasis, chest coughs, colds, conjunctivitis, diarrhoea, dysmenorrhoea, earache, fattening babies, fever, headache, hookworm, infertility in women, leprosy, pneumonia, scorpion bite, snake bite, swelling caused by mumps, syphilis, toothache and general weakness (Gelfand, *et al*, 1985; Hutchings, *et al*, 1996; Kokwaro, *et al*, 1976 and Watt, *et al*, 1962).

Breytenbach and Malan (1989) isolated three antimicrobial compounds from *C. zeyheri* and Alexander, *et al.* (1992) found antimicrobial activity in six species of *Combretum.*

Eloff (1998a; 1999) investigated leaf material from 27 southern African members of the Combretaceae. Based on the minimal inhibitory concentration (MIC) values of extracts and the total content of each plant, the species with the highest antibacterial activity (for the four bacteria used) are *C. molle, C. petrophilum, C. moggii, C. erythrophyllum, C. padoides, C. paniculatum, C. mossambicense, C. nelsonii, C. apiculatum,* subspecies *apiculatum, P. myrtifolia* and *C. woodii* (Eloff, 1999).

All the members of Combretaceae examined contain antibacterial compounds. Martini and Eloff (1998) found at least 14 antibacterial compounds in *C. erythrophyllum* and some of these had activities higher than chloramphenicol and ampisillin.

1.2.5 Why *C. microphyllum* was selected

I could not find any publication or report on the antibacterial activity of *C. microphyllum. C. microphyllum* possesses different chemical and antibacterial compounds from other *Combretum* species as well as a relatively high antibacterial activity (unpublished results).

C. microphyllum falls in another section (*Conniventia*) of the subgenus *Cacoucia.* Other students in our laboratory are investigating species from other sections in an effort to provide chemotaxonomic evidence for the sectional classification and to confirm the occurrence of different antibacterial compounds in the different species.

1.2.6 *Combretum microphyllum*

C. microphyllum falls under the subgenus *Cacoucia* which is divided into five sections and *C. microphyllum* forms part of the section *Conniventia.* A short overview of *Combretum microphyllum* from Carr, 1988 and Van Wyk and Van Wyk, 1997 is given.

◆ Description

Features of *C. microphyllum* are typically those of the family Combretaceae. Stems have petiole bases, which often persist as spines. *C. microphyllum* possesses bark with rather rectangular flakes, lifting, light to medium grey; climbers can reach 10 m, stems twine, spines spread to 25 mm and is found in woodland, savannah, and riverine at low altitudes up to 1500 m. Leaves are ovate, oblong-elliptic to almost circular. The average sizes of leaves are 65 mm (lamina length) x 41 mm (lamina width) x 12 mm (petiole length). Principal lateral veins are yellowish varying from 4 to 6 pairs. Basal portions of petioles are thickened and often persist after leaves fall to form blunt spines. They are produced in terminal or axillary panicles, usually before the new leaves.

Flowers are showy and crimson-red. *C. microphyllum* flowers from September to October with an abundance of scarlet blossoms [**Fig. 1.1**] (Joffe, 1993).

C. microphyllum produces 4-winged fruit. The average sizes of fruit are up to 25 mm (length over wing/s) x 23 mm (width over wing/s) x 5 mm (stipe length), averaging respectively 19 mm x 19 mm x 3 mm. They are green tinged with red or pink when young, drying to pale yellowish brown [**Fig. 1.2**] (Van Wyk and Van Wyk, 1997).

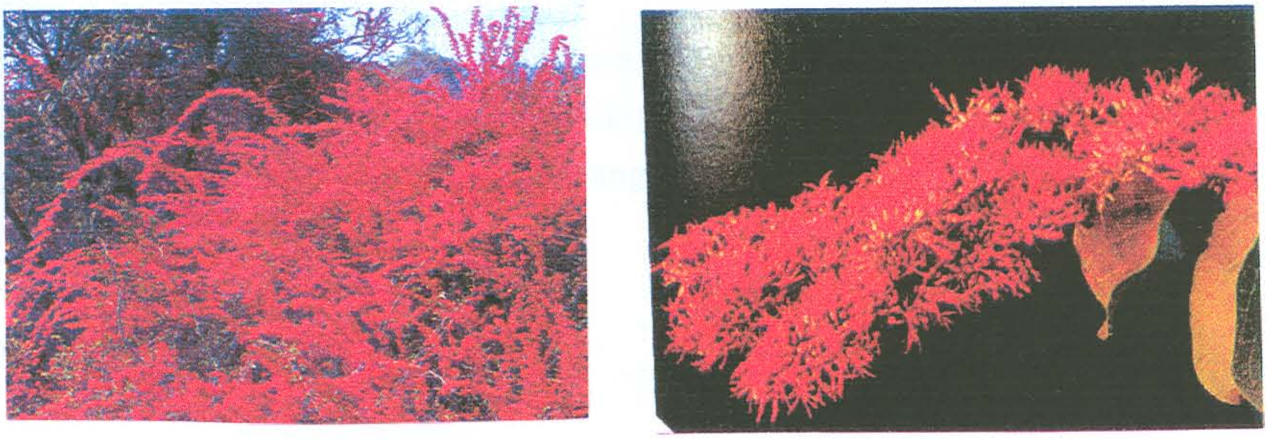

Figure 1.1 ***Combretum microphyllum:*** **Flowers.**

Figure 1.2 ***Combretum microphyllum:*** **Fruit at an early and late stage.**

♦ Synonyms

Synonyms are *Combretum lomuense* and *Combretum paniculatum*, subspecies *microphyllum.*

♦ Common names

Combretum microphyllum in South Africa and Zimbabwe is commonly known as 'flame creeper' ('vlamklimop'), 'mukopokopo', 'burning bush combretum', 'mupfurura', 'bambagwenya' (or 'bambangwenya') and 'muzutsu'.

♦ Distribution

In southern Africa *C. microphyllum* grows in the north eastern parts of South Africa, the Swaziland region and scattered in the northern parts of Kwazulu-Natal, in Mozambique, and in Zimbabwe, mostly in the eastern highlands **[Fig. 1.3]** (Van Wyk and Van Wyk, 1997).

The species is mainly confined to the lowveld in Mpumulanga, extending from north of the Soutpansberg over to the east and down to the extreme south. Isolated occurrences of *Combretum microphyllum* are noted elsewhere on the Tambotie river near Ellisras and at Doorndraai Dam Nature Reserve. In Kwazulu Natal it is mainly confined to the north-eastern part to the east of the Lebombo range and reaching as far south as the Mkuze valley. There is also a record from the Kranskop area.

Although there are no records of occurrences in Botswana it seems likely that it occurs in the north western area. Occurrences are further recorded in Swaziland and in all 5 geographical divisions of Zimbabwe. The species is also found in Zambia, Mozambique, Malawi and Tanzania.

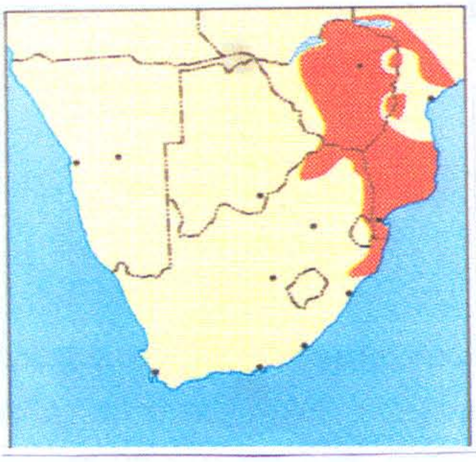

Figure 1.3 Distribution map of *C. microphyllum.*

♦ Habitat

C. microphyllum is a robust climber, sometimes a scrambling shrub or small tree, growing in bushveld and forest and often along rivers. The habitat of this species is in the tropical and warm regions of Africa, in the savanna and forests of warmer areas and woodland in the hotter areas. It flourishes especially in thickly forested regions along water sources at low to medium altitudes, and occasionally up to 1 500 meter in tropical parts (Carr, 1988).

♦ General

The species is usually a several-stemmed deciduous liane, which may grow into a dense dome-shaped mass 2 - 3 m high without supporting vegetation. It spreads to a width of 12 m or more with sturdy, almost straight, unsupported stems of up to 3,5 m extending upwards seeking support. Irvine (Carr, 1988) reports that single-stemmed specimens in the presence of supporting large trees have stems up to 240 mm diameter at breast height. Two adjacent stems will coil around each other to form a right-hand helix. Stems, which are supported by nearby vegetation, will climb up over the crowns of these, thus providing floral displays up to 15 m or more above the ground. The species, when in full flower with vast expanses of brilliant scarlet provides a powerful show which last for 3 weeks or more.

♦ Bark

Bark on the main stem near the base may have a crocodile skin pattern with light to medium grey flakes curling up at the edges. Stems are about 50 mm in diameter and are fairly smooth. Current growth stems are green and puberulous, even pubescent at the terminals and thus grey.

♦ Foliage

A typical season's extension comprises a substantial straight stem reaching lengths of several metres with a few other long stems emerging from the basal portion. Leaf size varies as already indicated. Emerging leaves and exposed portions of their petioles are initially purple-tinged while venation on the upper side is a lighter green and slightly recessed.

♦ Spines

Most stems are armed with blunted, slightly recurved spines, occurring singly or in pairs.

♦ Inflorescence

The species is in full flower during September or October, rarely in November and sometimes as early as the end of August. The brilliant showy display of non-scented flowers, make it a useful horticultural subject in warm climates.

- **Fruit**

Fruit is a 4- (or sometimes 5-) winged samara with a broadly elliptic to subcircular outline. The base is rounded and usually broadly notched, as is the apex.

- **Propagation and cultivation**

The fruit matures rapidly and can be ripe by mid December. To hasten the process seeds should be removed from the fruit for sowing. Soaking beforehand will lead to earlier emergence of seedlings, usually from 10 to 21 days after sowing. Having established its head well above the ground *C. microphyllum* will withstand a fair amount of cold as well as resisting drought as little or no artificial watering of the established plant is necessary.

1.3. AIM OF THE STUDY

The aim of this study is to investigate the antibacterial compounds by following a bio-assay guided approach.

Different extractants will be used on dried, powdered leaves of *C. microphyllum* to determine:

- If there is any specificity in extracting the antibacterial compounds,
- Which extractant extracts the highest antibacterial activity,
- Which extractant extracts the most antibacterial compounds.

I will attempt to isolate one or more antibacterial compounds by:

- Simplifying the extracts through several group separation techniques and following the fractionation quantitatively and qualitatively,

- Applying relevant column chromatographic techniques.

CHAPTER 2

MATERIALS AND METHODS

2.1 COLLECTION OF PLANT MATERIAL

Leaves of *C. microphyllum*, a creeper, were collected in June 1998 from a plant growing in the Lowveld National Botanical Garden, Nelspruit (Eloff, 1999). The reference number of the plant is 151/72 indicating that it was collected in 1972 and was the 151st collection of that year. The origin of the plant is recorded in the database of the botanical garden and a voucher specimen from the plant is deposited in the garden's herbarium.

2.2 PREPARATION OF LEAF MATERIAL

Leaves were dried at room temperature (c. 20 °C) by spreading out on a table in the laboratory. All the old, brown and diseased leaves were removed. The leaves were ground to a fine powder in a Jankel and Künkel Model A10 mill, which ensured homogenized tissue. The ground leaf material was stored at room temperature in the dark in tightly closed glass containers.

Intact dried leaves were used for the initial extraction with acetone and sodium bicarbonate.

2.3 EXTRACTION

Initially bicarbonate extraction was attempted. In a preliminary experiment, 3 g intact, dried leaves were extracted with 100 ml 1 % aqueous sodium bicarbonate ($NaHCO_3$) (Rogers, 1998) by shaking for 1 hour on an ultrasonic sound bath (Bransonic 52 ultrasonic bath). After filtration through Whatman # 1 filter paper, the extract was acidified by adding up to 4 ml concentrated HCl, to precipitate the extracted acidic compounds. The solution was left in a cold room.

The same quantity of leaves was also extracted with acetone under the same conditions without acidifying. The acetone extract was dried under reduced pressure and the precipitate was dissolved in 1 ml acetone. The process was repeated three times and the extracts were decanted and combined.

The procedure subsequently used was extracting 500 mg of finely ground, dried leaf material with 5 ml of the extractant. The following extractants were used (polarity and selectivity group, according to Snyder and Kirkland, 1979, given in brackets): hexane [0.1, 0], carbon tetrachloride [1.6, 0], isopropyl ether [2.4, I], diethyl ether [2.8, I], methylene dichloride [3.1, V], tetrahydrofurane [4.0, III], acetone [5.1, VIa], ethanol [4.3, II], methanol [5.1, II] and water [10.2, VIII]. Later ethyl acetate [4.4, VIa] was also used. Extractants used were of technical grade.

The material was extracted in polyethylene centrifuge tubes while shaking vigorously in a Vortex model K-500-4 test tube mixer for 5 minutes. After balancing and centrifuging at 5300 x g for 5 minutes, the extract was decanted in a pre-weighed container. The process was repeated three times on the same plant material. (Filtration was used with carbon tetrachloride because it could not be separated from the solid materials by centrifugation, due to its high density.) The combined extracts were dried in a stream of air at room temperature.

Due to problems with drying the water extract in a stream of air, water was removed by vacuum distillation in a Büchi rotary evaporator and placed in a dessicator overnight to determine yield. The dried extracts were dissolved in acetone to yield c. 25 mg/ml of the extract and stored at c. 7 °C in sealed, labeled containers.

2.4 TLC ANALYSIS OF EXTRACT

Thin layer chromatography (usually 5 - 10 µl of a 50 - 100 mg extract/ml solution) was on Merck TLC F_{254} plates with one or more of the following eluents:

- benzene/ethanol/ammonium hydroxide (90/9/1, v/v/v) [BEA],
- chloroform/ethyl acetate/formic acid (5/4/1, v/v/v) [CEF],
- methylene dichloride/acetone (3/2, v/v) [MA].

Extracts were applied to the plates 1 cm from the bottom and allowed to develop by ascending chromatography for c. 9 cm. Different volumes of the same concentration of each set of samples were applied with a micropipette. Development of chromatograms took place in a saturated, closed TLC tank. The atmosphere was saturated by placing filter paper, wetted with the eluent, against the walls of the tank. Separated components were investigated under visible and ultraviolet light (245 and 360 nm, Camac Universal UV lamp TL-600). TLC plates were sprayed with one of the following spray reagents (Stahl, 1969):

- 0,59 g vanillin dissolved in 100 ml sulphuric acid : ethanol (4 : 1) [vanillin spray reagent],
- 20 % aqueous perchloric acid [perchloric spray reagent],
- 15 ml 85 % phosphoric acid diluted to 100 ml with methanol [phosphoric acid spray reagent],
- 5 ml *p*-anisaldehyde dissolved in 90 ml ethanol and 5 ml concentrated sulphuric acid (Carr and Rogers, 1986) [*p*-anisaldehyde spray reagent].

The plates were heated at 105 °C until the development of colour bands was complete.

2.5 MINIMUM INHIBITORY CONCENTRATION BY INT MICROPLATE BIOASSAY

Minimum inhibitory concentration (MIC) was determined with a microplate serial dilution method (Eloff, 1998c). The following test organisms were used: *Staphylococcus aureus* (American Type Culture Collection number 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922) and *Enterococcus faecalis* (ATCC 21212). The National Committee also recommends these bacterial strains for Clinical Laboratory Standards. These bacteria are also responsible for most nosocomial diseases in hospitals. These cultures were grown at 37 °C and regularly subcultured (1 % inoculum). Approximately every 6 months, fresh cultures were obtained from Dr. F. Huygens, Department Medical Microbiology, University of Pretoria to ensure that no contamination of cultures influenced our results.

2.5.1 Dilution of extract

The 96-well microplate was labeled with sample codes after deciding on two-fold serial dilutions of 8 times (landscape mode) or 12 times (portrait mode). Distilled water (100 µl) was placed in each of the wells with a Socorex multichannel micropipette and 100 µl of extract was added with a single channel micropipette to the first well in the relevant series producing 50 % of the original concentration. The pipette was rinsed between applications with a small quantity of acetone.

After all extracts have been diluted in the first well, the multichannel pipette was used to remove 100 µl from the first well and was placed into the next well. The plunger was pushed up and down three to four times to ensure that the content of the well was properly mixed. (This produced 50 % of the original extract concentration; the next well 25 %, etc at this stage.) The process was repeated all the way to the bottom of the plate. The first 100 µl from the last row of wells was discarded to ensure that all the wells contained 100 µl of the extract. The first column had a series of two-fold dilutions of extract number 1, the second of extract number 2, etc.

2.5.2 Addition of bacteria

The growth of bacteria was arranged in advance to ensure having an active culture of the bacteria required. The active culture used, was never older than 10 days (Eloff, 1998c). To ensure that the active culture was not contaminated, activities were completed within a laminar flow cabinet. After 100 µl of the relevant bacterial culture was placed into each of the wells, it was mixed thoroughly. This yielded 25 % of the original extract concentration in the first row, 12.5 % in the second row, etc. Either ampicillin (4 mg/ml) or gentamicin (1 mg/ml) solutions (100 µl) was added to well A12 to serve as a positive control of inhibitory activity of the extract. The microplate was sealed in a plastic bag and incubated overnight at 37 °C at 100 % relative humidity in an incubator.

The following morning 40 µl of a 0,2 mg/ml solution of INT (*p*-iodonitrotetrazolium violet) was added to each row with a multichannel micropipette. The plate was returned to the incubator for at least 30 minutes to ensure adequate colour development and was then examined. The microplates were again examined for colour development after 60 minutes and after 120 minutes. (*E. faecalis* took much longer to react - up to 16 hours).

A clear solution or a definite decrease in colour reaction compared to the next two-fold dilution, indicated inhibition of growth. The minimum inhibitory concentration (MIC) of the extract was calculated from the original concentration of the extracts.

2.6 BIOAUTOGRAPHY

The bioautography procedure described by Begue and Kline (1972) was used. The developed thin layer chromatogram plates were dried overnight in an air stream to remove any remaining eluent. The TLC plates were sprayed with a concentrated suspension of actively growing *S. aureus* cells, *P. aeruginosa* cells, *E. coli* cells and *E. faecalis* cells. The plates were incubated overnight at 38 °C in a chamber at 100 % relative humidity. Plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet [Sigma Chemicals] in water.

Inhibition of growth was indicated by clear zones on the chromatogram after incubating for 30 - 90 minutes. Toxicity of solvents of the different test organisms used was determined by serial dilution as medium to levels where growth was not inhibited.

2.7 COMPARISON OF DIFFERENT SOLVENTS

The different solvents used were methanol, ethanol, acetone, water, methylene dichloride and methanol : chloroform : water (12 : 5 : 3). The total quality extracted, rate of extraction, total number of components extracted, number of inhibitory components extracted and the toxicity to the micro-organisms were determined.

Plant material (0,5 g) was weighed into each of six centrifuge tubes and 5 ml of each solvent was added to the respective tubes, to test the rate of extraction and the quantity extracted [**Section 2.3** for procedure]. The process was repeated two more times. After each extraction the extracts were placed in pre-weighed glass vials for separate analysis. Solvents were removed at room temperature for quantitative analysis. The vials with the dry material were weighed and the quantity extracted was calculated. The quantity obtained after each extraction was divided by the total amount extracted, and the rate of extraction was calculated.

2.7.1 Solvent/solvent fractionation

The solvent/solvent group separation procedure used by the USA National Cancer Institute as described by Suffness and Dourus (1979) was applied with a slight variation.

Fine, dry plant material (90 g) was extracted with 900 ml acetone by vigorously shaking for 30 minutes and repeated three times. The acetone extract was taken to dryness in a pre-weighed round-bottomed flask in a Büchi PE120 rotary evaporator under reduced pressure, rotating at c. 100 r.p.m. and the water bath temperature at c. 50 °C. When the extracts were nearly dry, the round-bottomed flask was lifted from the water and taken to dryness at ambient room temperature to prevent possible heat inactivation or change.

The acetone extract was dissolved in 100 ml water and 100 ml chloroform and the two layers were separated in a separatory funnel. The lighter fraction (water) was extracted with an equal volume of *n*-butanol in a separatory funnel to yield the water (W) and the butanol (B) fractions. The heavier fraction (chloroform) was taken to dryness in a rotary evaporator (in a pre-weighed round-bottomed flask) under reduced pressure and extracted with a 1 : 1 mixture of hexane and 10 % water in methanol.

The hexane (H) fraction was recovered with a separatory funnel. The 10 % water in methanol extract was diluted to 20 % water in methanol by adding water and extracted with carbon tetrachloride (CT) fraction. The 20 % water in the methanol extract was diluted to 35 % methanol in water and extracted with chloroform to yield the chloroform (CF) fraction and the 35 % water in methanol (MW) fraction. Equal volumes of the different solvents were used each time.

In all cases, the upper phase was re-extracted with the lower phase two or three times with c. 10 % of the original lower phase volume to ensure adequate separation. A khaki colour appeared when water was added to the 10 % and 20 % water in methanol solutions. The mass of each round-bottomed flask used on the rotary evaporator was determined before and after with the dry fractions to determine the difference. The extracts were redissolved in acetone or acetone : water (1 : 1) in pre-weighed amber flasks with tight sealing lids and the concentration of each fraction was determined. The process is schematically represented in **Figure 2.1**.

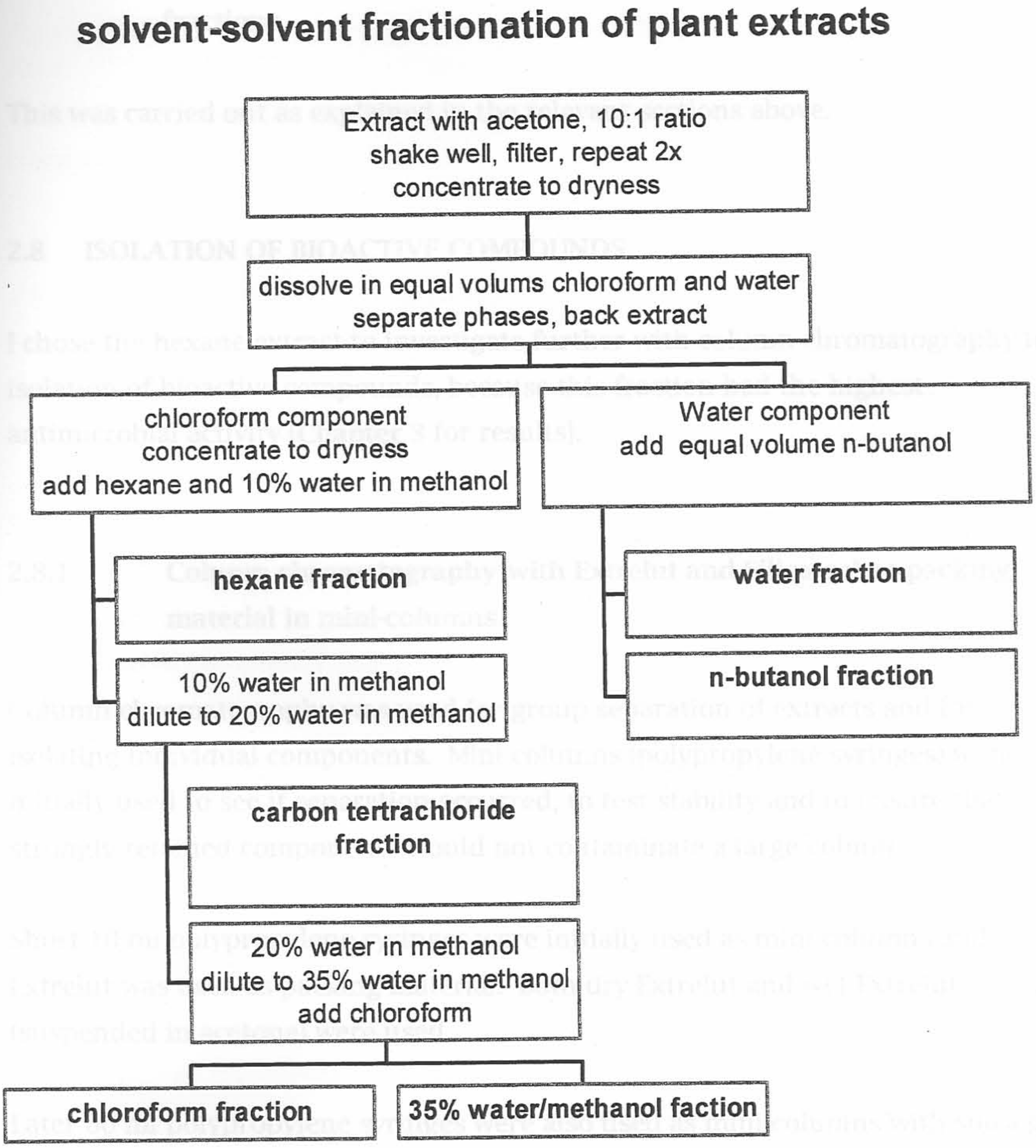

Figure 2.1 The procedure used for solvent/solvent extraction and fractionation of the components in *C. microphyllum* into six fractions.

2.7.2 Thin layer chromatography, bioautography and bioassay of fractions

This was carried out as explained in the relevant sections above.

2.8 ISOLATION OF BIOACTIVE COMPOUNDS

I chose the hexane extract to investigate further with column chromatography for isolation of bioactive compounds, because this fraction had the highest antimicrobial activity [**Chapter 3** for results].

2.8.1 Column chromatography with Extrelut and Silica gel as packing material in mini-columns

Column chromatography was used for group separation of extracts and for isolating individual components. Mini columns (polypropylene syringes) were initially used to see if separation occurred, to test stability and to ensure that strongly retained components would not contaminate a large column.

Short 10 ml polypropylene syringes were initially used as mini-columns and Extrelut was used as packing material. Both dry Extrelut and wet Extrelut (suspended in acetone) were used.

Later 60 ml polypropylene syringes were also used as mini-columns with silica gel as packing material.

Gravitational force was used to elute the components through the column, in some cases and air pressure from a membrane pump in other cases.

2.8.2 Combination of fractions after TLC and bioassay

An aliquot of the different fractions were separated by TLC using one of the three eluent systems and the results were used to pool fractions with similar composition.

2.9 TLC OF COMBINED FRACTIONS

2.9.1 TLC in Seprachrom containers

Seprachrom consists of plastic 5 cm x 10 cm containers, which can be closed. Several combinations of volatile eluents were tested to develop an eluent system for column chromatography. The best separation on column chromatography is if the wanted compound had an Rf-value of c. 0.5 on TLC. Later only glass TLC-tanks were used due to the unsatisfactory results in the Seprachrom containers.

2.9.2 TLC in small glass tanks

Attempts were made to develop a TLC system that would separate the components of fractions well so that this system could be used in subsequent column chromatography. Initially different combinations of carbon tetrachloride and methylene dichloride were tested as eluents.

Good results were eventually obtained with a hexane-acetone combination. The combination of hexane : acetone in a 2 : 1 ratio, gave the best separation on TLC.

2.9.3 Column chromatography on preparative Silica gel columns

A Michel-Miller glass preparative column, specially designed for high performance low pressure liquid chromatography, was used (length: 60 cm and diameter: 25 mm) to scale up TLC separation. These columns have Teflon couplings, fittings and adapters which resolves problems of plastic incompatibility with halogenated aliphatic compounds and ketones.

A finer silica gel, LiChroprep 15-25 μm [Merck] was used as packing material. The silica gel was suspended in the eluent mixture and was left to swell. Air was removed by placing the suspension in a Bransonic 52 ultrasonic bath for five minutes. The silica gel slurry was carefully poured into the column containing the eluent mixture.

In order to protect the top of the column from disturbance, a filter paper disk was cut to fit the column-diameter and was placed on top of the column. To minimise dead volume, a Michel-Miller adjustable bed height fitting was attached to the top of the column and adjusted to remove all the free space on the top of the column.

The selected fraction was applied under pressure from an injector to the top of the column through a Rheodyne 5020 low pressure injection valve injector in a closed column system.

Pressure from a membrane pump was used to elute components through the column and the column was developed at a rate of c. 3 ml/min. Fractions were collected by an Isco Foxy Junior fraction collector. A gradient was formed by joining two Schott bottles at the bottom and taking eluents off to the column from the near side bottle while adding a more polar eluent to the far side.

2.9.4 Combination of collected fractions

Some of the collected fractions were combined, based on the analysis by TLC and the quantity present in each fraction. Bioautography was carried out on the combined fractions, using *S. aureus*, *E. coli* and *P. aeruginosa* as test organisms.

NMR spectroscopic analysis was carried out on combined fractions based on the TLC results.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 EXTRACTION

3.1.1 Comparing sodium bicarbonate and acetone as extractants

3.1.1.1 Introduction

Rogers (1998) proposed that a hot 1 % $NaHCO_3$ solution should be used for the extraction of compounds from *Combretum* spp. The basis of this extraction procedure is that many of the compounds are acidic and occur on the surface of the leaf. At a low pH, these compounds are insoluble in water. By decreasing the pH, the acidic compounds are changed to water soluble sodium salts and are then extracted into water. Subsequently the water extract is acidified with HCl and the sodium salts are changed to the water insoluble acidic compounds and precipitated out. The process is cheap and it delivers good results.

In a first experiment, I compared acetone, which efficiently removes compounds from finely ground leaves (Eloff 1998a), with sodium bicarbonate, which dissolves outside compounds from intact leaves, as extractants [**Section 2.3** for procedure].

3.1.1.2 Quantity extracted with initial extractants

The extraction on 500 mg intact, dried leaves of *C. microphyllum* was repeated three times. The acetone extract (A) was bright green and yielded 19 mg and the sodium bicarbonate extract (B) was black and yielded 27 mg from the intact dried leaves.

3.1.1.3 Thin layer chromatography of acetone and bicarbonate extracts

Initially quantities between 100 and 600 µg (5 - 30 µl) were placed on TLC plates. The CEF-eluent was used and the plates were sprayed with the *p*-anisaldehyde spray reagent [**Section 2.4** for eluent and spray reagent formula]. Little fluorescence could be detected under the UV-light at 350 nm.

Later, quantities between 600 and 1 200 µg (20 - 60 µl) were chromatographed using the same eluent. Some plates were sprayed with the vanillin spray reagent. To investigate whether other spray reagents would show better separated components, plates were sprayed after one week with the vanillin, perchloric and phosphoric acid spray reagents. All plates were placed in an oven at 110 °C after spraying for colour development.

Better isolated and more definite bands could be observed (also fluorescence at different wavelengths under UV-light) with higher concentrations of the acetone extract. There was a slight decrease in colour intensity after one week. The vanillin and perchloric spray reagents showed the best separation of compounds for the acetone extract. Neither the higher concentrations used, the different spray reagents nor the time sprayed, gave better results on TLC for the sodium bicarbonate extract [**Fig. 3.1 and 3.2**].

From these results I decided to use the vanillin spray reagent in future work. Because I am primarily interested in isolating bioactive compounds, the antibacterial activity of the extracts was determined next.

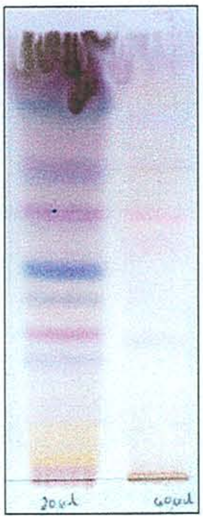

Figure 3.1 Chromatogram showing the separation of compounds present in 380 µg of the acetone extract (left) and 408 µg of the bicarbonate extract (right). The CEF system was used as eluent and the plate was sprayed directly after TLC with the vanillin spray reagent.

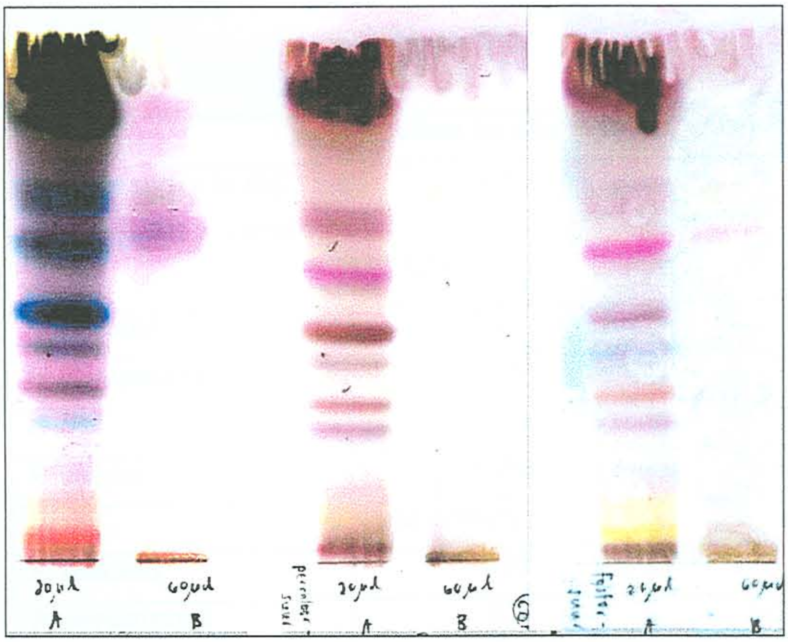

Figure 3.2 Three chromatograms of 380 µg acetone extract (left) and 408 µg bicarbonate extract (right) sprayed one week after chromatography: vanillin spray reagent (left), perchloric spray reagent (centre) and phosphoric spray reagent (right).

3.1.1.4 Minimum inhibitory concentration by INT microplate bioassay of acetone and $NaHCO_3$ extracts

MIC was determined in triplicate by two-fold dilution of extracts beyond the level where no inhibition of growth of the four test organisms occurred [**Section 2.5.1** for procedure].

Growth of bacteria was not easily visible before INT was added due to the inherent turbidity in different wells. After INT addition, growth at low extract concentrations was visible. Acetone extracts inhibited growth most in all four cultures. $NaHCO_3$ fractions inhibited *E. faecalis* and *S. aureus* growth the strongest [**Table 3.1**].

Table 3.1 MIC values in mg/ml and total activity in ml for different test organisms of intact *C. microphyllum* leaves extracted with sodium bicarbonate and acetone.

	Sodium bicarbonate	Acetone
Total quantity in mg extracted from 1 gram	9.1	38
	MIC	
E. faecalis	0.07	0.11
P. aeruginosa	0.16	0.23
E. coli	0.16	0.03
S. aureus	0.06	0.01
	Total activity	
E. faecalis	130	77
P. aeruginosa	57	37
E. coli	57	283
S. aureus	152	850
Average total activity	99	312

Total activity was calculated by dividing the quantity extracted from 1 gram of leaves with the MIC value (Eloff, 2000). This makes it possible to compare the total quantity of antibacterial activity extracted. Because the quantity extracted per gram is divided by the MIC in mg/ml, the unit of total activity is ml. This represents the volume to which the antibacterial compounds in the extract can be diluted and still inhibit growth of the bacteria.

In a preliminary study (Eloff, 1999), 27 southern African members of Combretaceae were compared with acetone as extractant. The values found for *C. microphyllum* were calculated and are presented in **Table 3.2**.

Table 3.2 MIC values in mg/ml and total activity in ml for different test organisms for *C. microphyllum* leaves extracted with acetone calculated from Eloff (1999). (Total quantity extracted from 1 gram was 62 mg).

Test organisms	MIC	Total activity
E. faecalis	0.8	78
P. aeruginosa	1.6	39
E. coli	0.8	78
S. aureus	0.4	155
Average		88

3.1.1.5 Discussion

The average total activity for fresh *C. microphyllum* acetone extracts calculated from Eloff (1999) was 88 ml, which is similar to the value I found for $NaHCO_3$ (99 ml), but is substantially lower than my average total activity of 312 ml for the acetone extraction of intact leaves. Although the total quantity extracted by acetone from intact leaves was substantially lower (38 mg vs. 62 mg), the MIC values for the extracts of intact leaves were much lower. This indicates that fewer inactive compounds were extracted when intact leaves were used.

Acetone as extractant gave more promising results than sodium bicarbonate. It also compared well with preliminary results found earlier (Eloff, 1999). My acetone extract yielded 38 mg from 1000 mg intact dried leaves by triple acetone extraction and the average quantity obtained from 1000 mg of finely ground leaf material by triple acetone extraction was c. 62 mg (Eloff, 1999). The sodium bicarbonate extract inhibited Gram-positive bacteria to a larger extent than Gram-negative bacteria, but *E. coli* was inhibited much more than *E. faecalis* by the acetone extract. This indicates that there may be some selectivity.

I experienced difficulties in the acid precipitation of the bicarbonate extracts and decided not to continue with sodium bicarbonate as extractant.

Because extracts of *Combretum* spp are complex, I decided to test extractants with varying polarity to determine if I could simplify extracts and/or extract more antibacterial activity with different extractants.

Consequently I decided to continue working on finely ground leaf material. Our experience with other Combretaceae indicated that as little as 50 μg gave good separations on TLC compared to larger quantities required with whole leaves. A major part of the compounds extracted from whole leaves may not have reacted with the spray reagents while contributing to the mass extracted.

3.1.2 Eleven different extractants tested for best screening and isolation of antimicrobial components from *C. microphyllum*

3.1.2.1 Extraction with 11 different extractants

When acetone, methanol, methylene dichloride and a methanol-chloroform-water mixture were used as extractants of *C. erythrophyllum* powdered leaves, acetone gave the best results (Eloff, 1998a). I decided to compare more extractants with differing polarity on *C. microphyllum* powdered leaves. Finely ground plant material (0.5 g) was extracted with 5 ml of each of the following extractants: hexane (HE), carbon tetrachloride (CCl_4), isopropyl ether (IPE), diethyl ether (EE), methylene dichloride (MDC), tetrahydrofurane (THF), acetone (A), ethanol (E), methanol (M) and water (WA). [Procedure as described in **Section 2.3**].

Later ethyl acetate (EA), with a polarity intermediate between tetrahydrofurane and acetone, was also tested as extractant. The carbon tetrachloride extraction was repeated because it is not possible to collect the extract by centrifugation because the density of carbon tetrachloride is higher than that of the plant material. The extract had to be collected by filtration.

All the extracts were made up to 10 mg/ml in acetone and 5 µl (c. 50 µg) of each extract was analyzed in duplicate by TLC. The BEA and CEF eluents were used. The different extracts chromatographed were arranged more or less from more non-polar to polar extractants [**Fig. 3.3- 3.7**]. Even before spraying the plates it was clear that there were differences in the composition of the different extracts based on the presence of green and yellow substances [**Fig. 3.3**].

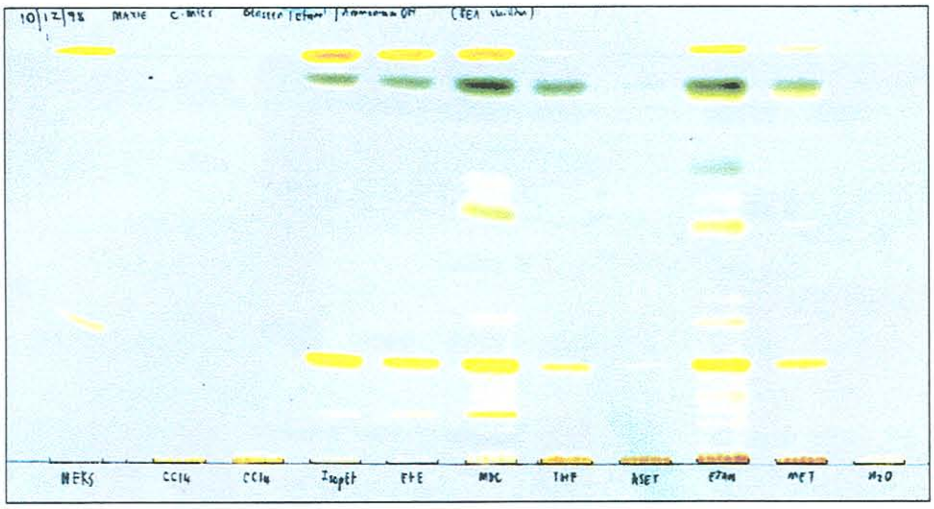

Figure 3.3 Separation of components present in 50 µg of 11 different extracts with BEA as eluent and viewed in daylight before spraying with spray reagent. Lanes from left to right extracted by hexane, carbon tetrachloride [centrifuged], carbon tetrachloride [filtered], isopropyl ether, diethyl ether, methylene dichloride, tetrahydrofurane, ethanol, methanol and water.

Methylene dichloride and ethanol extracted the most compounds. With hexane, carbon tetrachloride, acetone and water, no coloured compounds were visible with the 50 μg applied to the plates.

Plates were susequently sprayed with the vanillin spray reagent. A dark band occurred on the BEA-plate [**Fig. 3.4**]. This might have been due to a problem with the eluent, a dirty TLC tank, a secondary reaction with light or more likely due to absorption of vapours on the TLC plate before chromatography. In subsequent separations we ensured that plates were not exposed to laboratory vapour and the problem was not experienced again.

As could be expected, there were large differences in the compounds extracted. The CEF chromatogram showed distinct bands. The intermediate polarity extractants isopropyl ether, ethyl ether and methylene dichloride extracts gave the best separation, whereas the carbon tetrachloride and tetrahydrofurane extracts gave pronounced streaking. Carbon tetrachloride surprisingly extracted a large quantity of polar compounds [low Rf-value] and small quantity of non-polar compounds in comparison to the more polar isopropyl ether and diethyl ether [**Fig. 3.4, 3.5**].

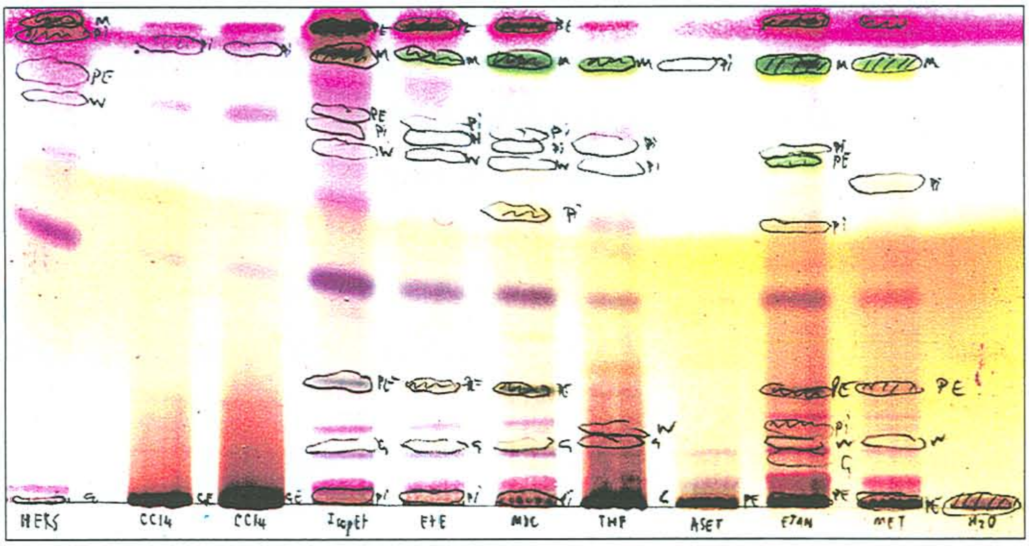

Figure 3.4 Separation of components present in 50 μg of 11 different extracts with BEA as eluent and vanillin as spray reagent. Lanes from left to right as in Figure 3.3.

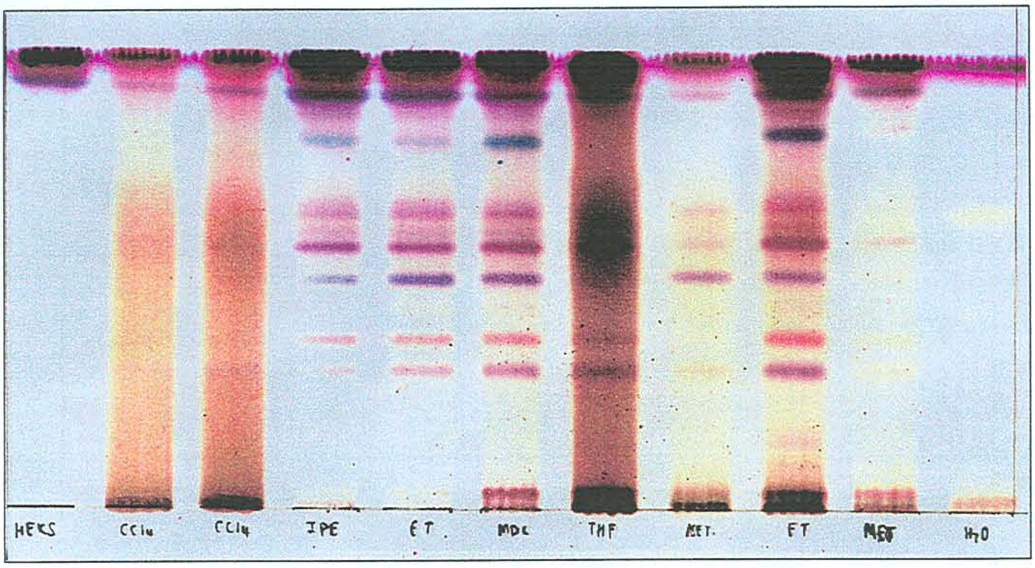

Figure 3.5 Separation of components present in 50 μg of 11 different extracts using the CEF eluent and vanillin spray reagent. Lanes as in Figure 3.3.

The results obtained with acetone as extractant was not in line with results obtained previously in our laboratory and I decided to repeat the extraction and to separate a larger quantity [100 μg] of all extracts [**Fig, 3.6**].

Better chromatograms with more definite bands and better separation were obtained especially with the acetone extract. The separation of the more non-polar compounds, using BEA as eluent yielded many compounds. One would have expected that the more polar extractants would have extracted few of the very non-polar compounds. It has to be kept in mind that the same quantity of all the extracts was chromatographed. If all extracts were made up to the same volume irrespective of the quantity extracted, different results might have been obtained.

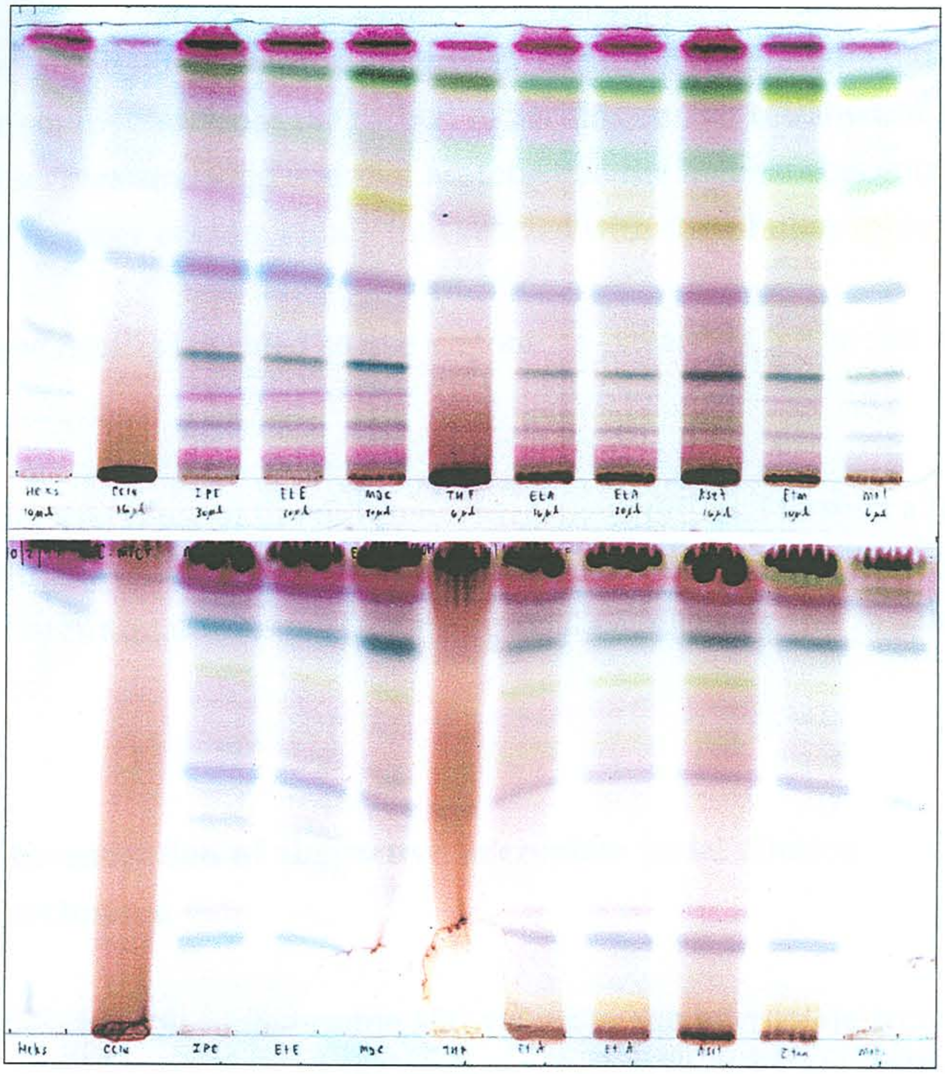

Figure 3.6 Separation of components present in 100 µg of 11 different extracts using the BEA (top) and CEF (bottom) eluents and the vanillin spray reagent. Lanes as in Figure 3.3. No components separated in the water extract [results not shown].

Surprisingly, a relatively polar extractant as ethanol, extracted similar concentrations of non-polar compounds [high Rf-values in normal phase chromatography]. This can probably be explained by the presence of saponin-like components present in the plant material. Similar to a soap with a polar and non-polar end, these compounds could make non-polar compounds soluble in polar extractants. This also explains how relatively non-polar medicinal compounds can be present in aqueous herbal preparations by traditional healers.

TLC with the intermediate polarity eluent CEF indicated that hexane did not extract polar compounds, that the carbon tetrachloride and tetrahydrofurane extracts had serious streaking and that acetone had the highest concentration and most diverse number of compounds that reacted with the vanillin spray reagent.

Based on these results I decided to use acetone as extractant in the rest of this study.

It is surprising that most of the different extractants differed to such a low degree. The important question was however, if the different extractants extracted different quantities of the antibacterial compounds and this was examined next.

3.1.2.2 Investigation of alternative microplate serial dilution technique

It is sometimes difficult to determine MIC values by the microplate technique, because the precipitated form of the extracts of some plants has a dark colour, which masks the red colour of the INT formazan. Because all actively growing bacteria contain catalase, I investigated whether addition of hydrogen peroxide (H_2O_2) would lead to visible oxygen (O_2) production and facilitate the determination of MIC values.

There was vigorous bubbling in the microwells in which the bacteria grew well, but the technique was not as sensitive as the INT technique.

3.1.2.3 Bioassay of extracts found with different extractants

MIC values were determined using the technique as described in **Section 2.5.1.** Gentamycin was used as positive control. (The MIC values for Gentamycin were 4 μg/ml for *E. coli*, 8 μg/ml for *P. aeruginosa* and *S. aureus* and 16 μg/ml for *E. faecalis*.) [**Table 3.3** for MIC values for different test organisms.]

Table 3.3 MIC values in mg/ml and total activity in ml of *C. microphyllum* leaves extracted with 11 different extractants.

	HE	CCl_4	IPE	EE	MD	TH	EA	A	E	M	W
Total quantity in mg extracted from 1 gram	36	48	20	24	106	64	24	32	36	174	48
MIC											
E. faecalis	0.08	0.15	0.21	0.15	0.30	0.28	0.40	0.13	0.07	0.18	1.20
P. aeruginosa	0.63	0.15	0.11	0.15	0.15	0.28	0.20	0.13	0.14	0.18	1.20
E. coli	0.32	0.60	0.05	0.15	0.14	0.20	0.20	0.27	0.07	0.18	1.20
S. aureus	1.25	0.60	0.21	0.30	0.15	0.57	0.20	0.27	0.14	0.18	1.20
Total activity											
E. faecalis	450	320	95	160	353	229	600	246	514	967	15
P. aeruginosa	57	320	182	160	707	229	120	246	257	967	15
E. coli	113	80	400	160	707	457	120	119	514	967	15
S. aureus	29	80	95	80	707	112	120	119	257	967	15

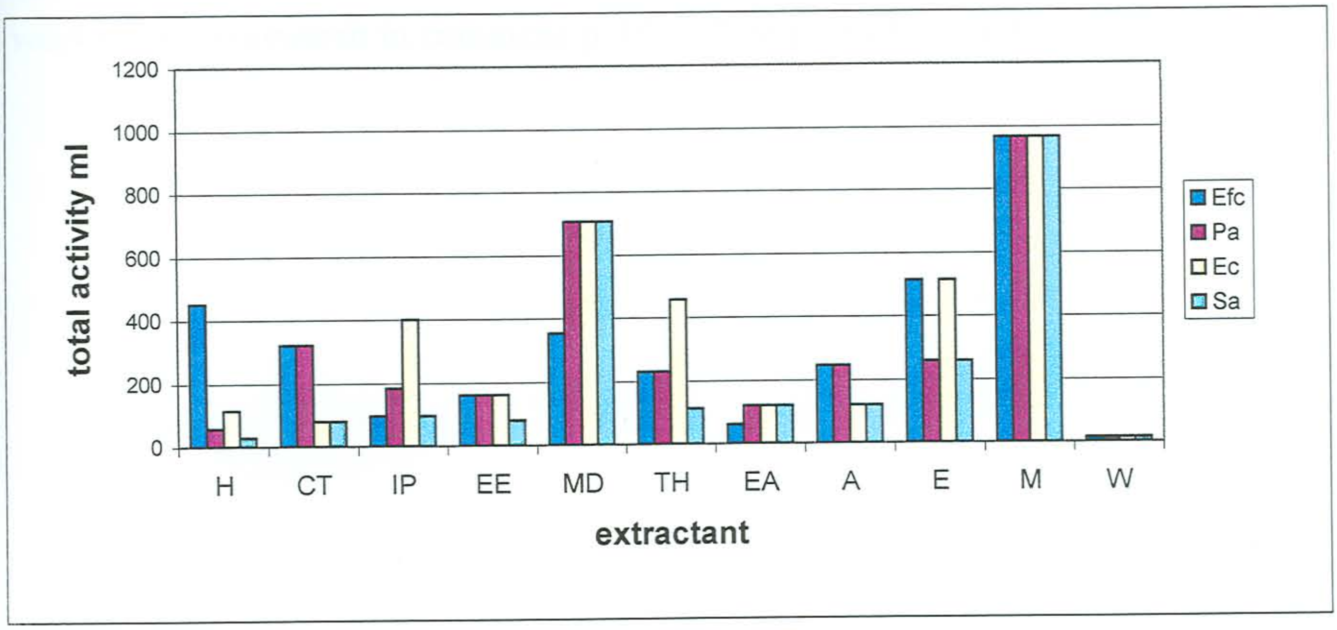

Figure 3.7 Total antibacterial activity to four test organisms of finely ground *C. microphyllum* leaves extracted with 11 different extractants. From left to right: hexane, carbon tetrachloride, isopropyl ether, diethyl ether, methylene dichloride, tetrahydrofurane, ethyl acetate, acetone, ethanol,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

3.1.2.4 Discussion

Although methanol and methylene dichloride extracted more activity [**Fig. 3.7**], I decided to continue with acetone as extractant due to its relatively low toxicity to test organisms and good TLC separation of compounds obtained with acetone as extractant and ease of removal from extracts.

Because methylene dichloride is not miscible with water, the use of this extractant complicates the handling of extracts.

Furthermore, methanol may extract polar compounds such as polysaccharides, polyphenols and tannins, which may have antibacterial activity. Major pharmaceutical companies are not interested in these compounds, because most polysaccharides break down to glucose on oral administration. Polyphenols/tannins bind to protein so that if it is absorbed, a large proportion binds to serum and is not bioavailable. These molecules are also very difficult to work with (Farnsworth in comment p. 19 - 20 on paper by Balick, 1994).

3.2 GROUP SEPARATION OF EXTRACTS BY SOLVENT/SOLVENT FRACTIONATION

3.2.1 Introduction

From the results obtained with sodium bicarbonate and acetone as extractants, it appeared that there is substantial selectivity using different extractants. By using 11 different extractants of varying polarity, little evidence of selectivity was however found. Because the extracts are very complex, I investigated whether solvent/solvent fractionation could simplify extracts without reducing antibacterial activity. This is a mild technique, which should not lead to inactivation of compounds by chemical changes. If the activity is caused by a single compound, i.e. is not caused by two interacting compounds that are separated by the solvent/solvent fractionation, there should not be a reduction of antibacterial activity.

The solvent/solvent fractionation of the components in *C. microphyllum* into six fractions was a variation on the group separation procedure developed in the National Cancer Institute as described by Suffness and Douros [1979] [procedure as described in **Section 2.7**].

3.2.2 Quantities obtained and antibacterial activity of different fractions

The quantities present and the antibacterial activity of the different fractions were determined [**Table 3.4**]. The highest quantity, nearly 42 % [**Table 3.5**], was present in the hexane fraction. Nearly two-thirds of the total antibacterial activity was also present in the hexane fraction. This is different from other *Combretum* species investigated thus far, where most of the activity was in the carbon tetrachloride and/or chloroform fractions. If the more polar components, which may contain uninteresting polysaccharides and polyphenols/tannins are ignored, the hexane fraction contained more than 88 % of the interesting non-polar antibacterial compounds. A comparison of the degree of purification can be obtained by dividing the % activity in each fraction with the % of the total mass [**Table 3.5**]. The procedure enriched the antibacterial activity of the polar compounds in the butanol and water fractions and the antibacterial activity of the non-polar fractions in the hexane fractions to a high degree. It had little effect on the methanol/water fraction and removed active compounds from the carbon tetrachloride and chloroform fractions.

Table 3.4 MIC values in mg/ml and total activity in ml of *C. microphyllum* leaves extract fractions obtained by solvent/solvent fractionation.

	H	CT	CF	B	MW	W
Total quantity obtained	1166	386	664	100	300	169
MIC						
E. faecalis	0.17	0.77	2.66	0.80	2.40	2.70
P. aeruginosa	0.33	0.77	2.66	0.05	0.15	0.08
E. coli	0.17	0.77	2.65	1.60	1.20	2.70
S. aureus	0.67	6.18	2.66	0.40	0.60	5.41
Total activity in ml						
E. faecalis	7000	500	250	125	125	62.5
P. aeruginosa	3500	500	250	2000	2000	2000
E. coli	7000	500	250	62.5	250	62.5
S. aureus	1750	62.5	250	250	500	31.3
Total for all organisms	19250	1563	1000	2438	2875	2156

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

	H	CT	CF	B	MW	W
% of total mass	41.9	13.9	23.8	3.6	10.8	6.1
% of total activity	65.5	5.4	3.4	8.4	9.9	7.4
ratio activity/mass	1.6	0.4	0.1	2.3	0.9	1.2

The degree of enrichment is also apparent from the pie charts shown in Figure 3.8. Not all the bacteria had the same sensitivity to the compounds present in the different fractions. Especially the *P. aeruginosa* cultures were more sensitive to the polar fractions [Fig.3.9].

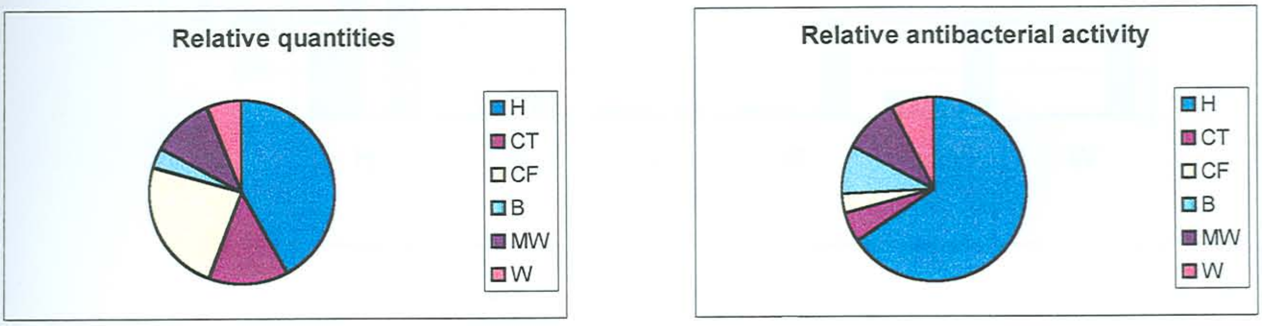

Figure 3.8 The relative quantities obtained by solvent/solvent fractionation and the relative antibacterial activities of the different fractions. H = hexane, CT = carbon tetrachloride, CF = chloroform, B = butanol, MW = 35 % methanol/water and W = water.

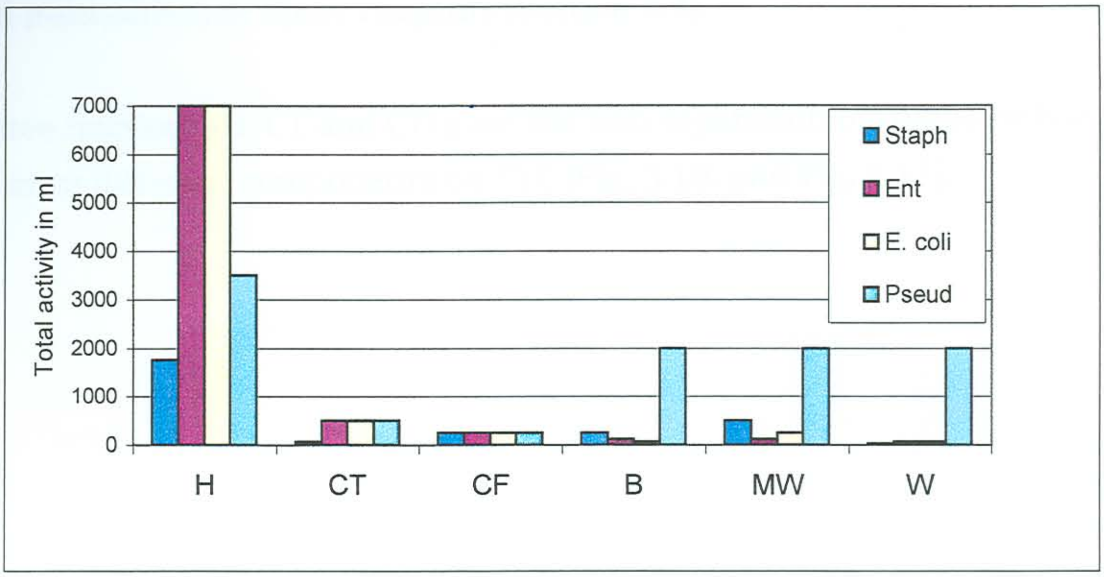

Figure 3.9 Total antibacterial activity to four test organisms of *C. microphyllum* leaves extracted with solvent/solvent fractions. (Fractions from left to right: H = hexane, CT = carbon tetrachloride, CF = chloroform, B = butanol, MW = 35 % methanol/water and W = water.)

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

3.2.3 Chemical composition of the six fractions obtained

Thin layer chromatography was on Merck TLC F_{254} plates. Initially 200 µg of the hexane fraction (H) was used. Due to the high concentration of the hexane fraction, the quantity of this fraction chromatographed was later reduced to 100 µg and gave better separation. Three different eluents were used: BEA, CEF and MA. Separated components were observed under visible and ultraviolet light (254 and 360 nm, Camac Universal UV lamp TL-600). Plates were sprayed with the vanillin and *p*-anisaldehyde spray reagents [**Section 2.4**].

The first three fractions (H, CT and CF) gave the best separation of compounds as well as the most different components on TLC [**Fig. 3.10. and Fig. 3.11**].

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).**

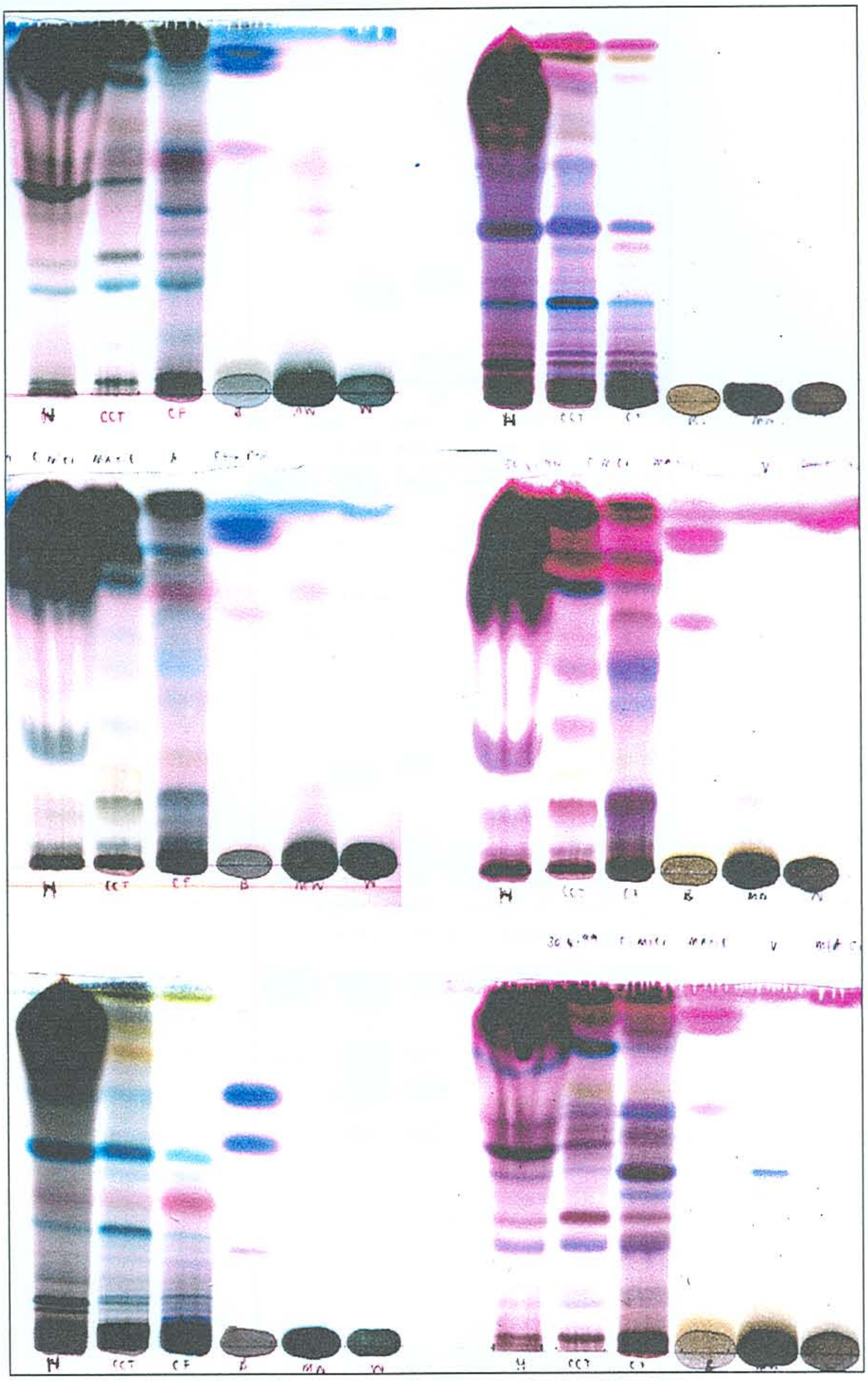

Figure 3.10 Chemical composition of solvent/solvent fractions [200 μg] separated by MDC/Acetone as eluent (top left and top right), BEA as eluent (middle left and right) and CEF as eluent (bottom left and right). Compounds visualized by *p*-anisaldehyde as spray reagent (top left, middle left and bottom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

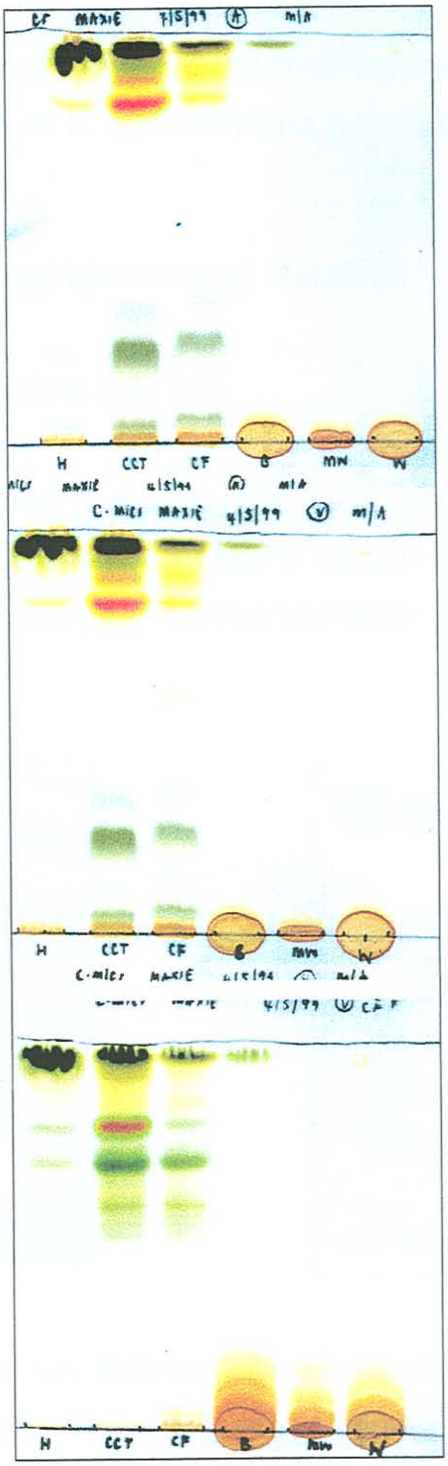

Figure 3.11 **Chemical composition of solvent/solvent fractions [100 μg] separated by MA as eluent (top and middle) and CEF as eluent (bottom). Compounds visualized by *p*-anisaldehyde (top) and vanillin (middle and bottom) spray reagents.**

MIC values were calculated in mg/ml and the total activity in ml was determined for the six different fractions of *C. microphyllum* [see **Table 3.4** above].

3.2.4 Bioautography and bioassay of fractions

Procedure as described in **Section 2.2**.

Bioautography worked well with *S. aureus* [**Fig. 3.12**], 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.

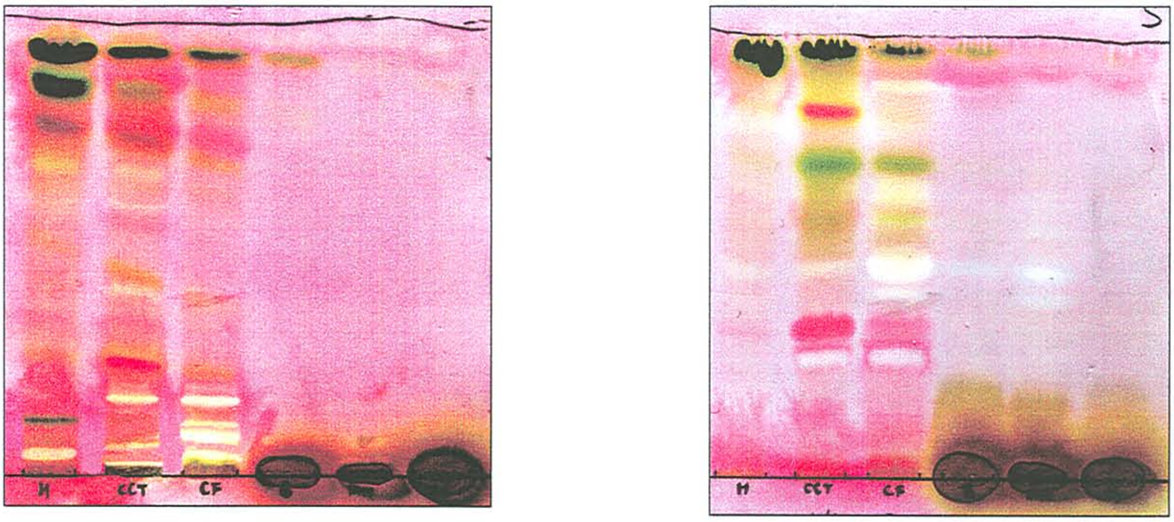

Figure 3.12 Bioautogram of six different fractions of *C. microphyllum* extract by solvent/solvent extraction. TLC using BEA [left] and CEF [right] as eluent, sprayed with *S. aureus* cell suspension, incubated and sprayed with INT. White areas indicate bacterial growth inhibition. (Fractions from left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

The hexane (H) fraction had the highest total antimicrobial activity by far for all four test organisms used [**Fig. 3.19**] and was chosen for the next steps in the isolation of bioactive compounds with column chromatography.

3.3 GROUP SEPARATION BY USING EXTRELUT AS PACKING MATERIAL

3.3.1 Introduction

Because the solvent/solvent fractionation is a tedious process, I decided to determine if Extrelut, sold by Merck, can be used instead of the solvent/solvent fractionation.

Extrelut is used as packing material for liquid-liquid extraction. It is used in analysing lipophillic substances in a complex matrix, e.g. body fluids where clean up is frequently necessary prior to the actual analysis. Conventional sample preparation, extraction using a separating funnel, is often associated with disadvantages: the formation of emulsion, poor phase separation, high solvent consumption, low degree of automation and high personnel costs. With Extrelut

- The eluate need not be dried prior to evaporation,
- Numerous published applications can be used for one's own laboratory problems,
- High rates of recovery, especially in the extraction of body fluids are obtained.

Liquid-liquid extraction becomes more efficient, easier and effective in routine laboratory work because:

- No emulsions are formed,
- Solvents, material and time are saved.

Extrelut consists of a specially processed wide-pore diatomaceous earth of high pore volume. The kieselguhr matrix of Extrelut is chemically inert and can be used within the pH-range 1 –13. Stringent initial control of raw material and equally stringent quality control of the final product guarantee uniform batch-to-batch quality, although Extrelut is a naturally occurring product and therefore subject to certain fluctuations.

The aqueous sample is applied to the Extrelut sorbent. It distributes itself in the form of a thin film over the chemically inert matrix and thus acts as a stationary phase. Subsequently, elution takes place using organic solvents that are non-miscible with water, solvents like e.g. diethyl ether, ethyl acetate or halogenated hydrocarbons. All the lipophillic substances are extracted from the aqueous into the organic phase. During this process, the aqueous phase remains on the stationary phase. The eluate is free from emulsions and can be evaporated for further analysis.

Extrelut has been used for quite some time in clinical chemistry and toxicology for the sample preparation of urine, whole blood, plasma, serum, gastric juice, liquor, amniotic fluid, faeces, animal and plant tissue. Other applications are in the areas of environmental and residue analysis, e.g. the analysis of industrial, domestic and wastewater. The fractionated elution of acidic and basic substances like e.g. drugs and their metabolites from body fluids is also possible (Merck, Chrom Book 2 provided by Merck).

Initially Extrelut [Merck] was packed in short 10 ml polypropylene syringes as small columns. Because the instructions were not clear, two procedures were followed. One syringe was packed with Extrelut that was suspended in acetone (wet treatment) and another syringe was packed with dry Extrelut only (dry treatment).

3.3.2 Wet adsorbent

A 10 ml polypropylene syringe was packed with Extrelut (Merck) by suspending in acetone and pouring into the small column. Approximately 200 μl of the hexane extract was placed on the column. The first four fractions were eluted with hexane (N1-N4) and collected by hand. This was followed by eluting with acetone (N5). The five collected fractions were dried in pre-weighed containers, dissolved in acetone and the components were separated by TLC [**Fig. 3.13**].

3.3.3 Dry adsorbent

The same procedure was followed, but in this case the adsorbent was not suspended in acetone before packing.

3.3.4 TLC of Extrelut fractions

Fractions N1-N5 (wet treatment), fractions D1-D5 (dry treatment) and a hexane fraction (c. 50 μg of each) were analyzed on TLC.

Very little fractionation took place with the dry column, but there was some fractionation of the more polar compounds by using Extrelut as packing material in the wet column [**Fig 3.13**]. The fractionation was not promising with this technique because it works best with aqueous extracts that are eluted with non-miscible organic solvents, and the column may have been overloaded.

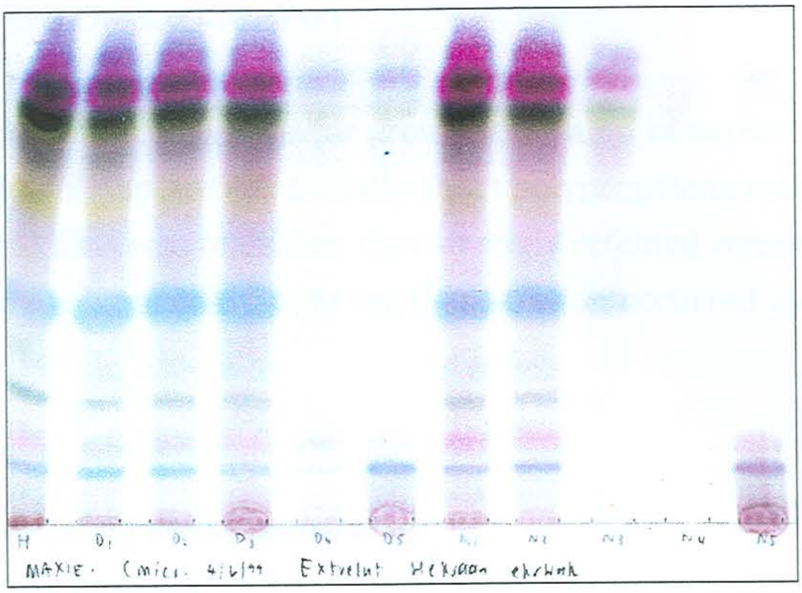

Figure 3.13 Separation of components in 50 μg of the different Extrelut fractions using the BEA eluent and the vanillin spray reagent. From left to right: H = hexane fraction, D1–D4 = dry adsorbent fractions eluted with hexane, D5 = dry adsorbent fraction eluted with acetone, N1–N5 = wet adsorbent fractions eluted with hexane and N5 = wet adsorbent fraction eluted with acetone.

3.4 COLUMN CHROMATOGRAPHY

Column chromatography was used for group separation of extracts and for isolating individual components. Initially short polypropylene syringes were used as small columns. This was to ensure that strongly retained components would not contaminate the large column, to see if separation occurred and to test stability.

3.4.1 Silica gel as packing material

Silica gel 60 [Merck] is a standardized silica gel widely used for preparative column chromatography that provides the user with unique method reliability. This is ensured by the fact that identical raw materials are used for all stationary phases, thus providing identical chromatographic selectivity. Practice has shown that for chromatographic separations in production, silica gel 60 types i.e. silica gels of a mean pore size of 60 A and in particle size ranges 40 - 63 μm and 63 - 200 μm are excellently suitable for the separation of complex mixtures (Merck Chrom Book 2 provided by Merck).

Subsequently polypropylene syringes (60 ml) were packed with silica gel 60 [Whatman 15- 40 μm]. Polypropylene was chosen because it has the best chemical resistance to the chemicals used and glass columns were not available at that stage. Glass wool was placed at the bottom of a 60 ml polypropylene syringe to prevent the silica gel from spilling.

Silica gel was suspended in a mixture of benzene : ethanol (9 : 1). A filter paper disk was placed on top of the silica gel after the silica gel suspension was poured into the small column to protect the top from disturbance.

Approximately half of the hexane extract (632.97 mg; 3.8 ml) was applied carefully in a narrow band to the top of the column with a pasteur pipette. It was mixed with the benzene-mixture. As soon as the hexane extract was absorbed into the silica gel, the BEA eluent was added form a raised solvent flask to facilitate solvent flow and the hexane extract was carefully washed into the column without disturbing the top of the column. A small quantity was added to wash the extract into the column.

Gravitational force was used to elute the components through the column. The first 22 test tubes (A-V) were collected by hand. The rest of the test tubes (1-25) were collected by the fraction collector [Isco Foxy Jr] (600 drops per fraction, c. 2 ml/min)

Four solvents were used in the following order: BEA, CEF, acetone and methanol, based on the separation by TLC. I chose volatile solvents to make drying of extracts at room temperature under air stream easier:

Solvent 1: c. 150 - 200 ml BEA (fractions A-V)
Solvent 2: c. 140 ml CEF (fractions 1-7)
Solvent 3: c. 150 ml acetone (fractions 8-15)
Solvent 4: c. 150 ml methanol (fractions 16-25)

Individual fractions were separated by different TLC systems. Fractions A-V (non-polar) were analyzed by TLC using the BEA system (c. 3 μl) and fractions 1-25 (polar) were analyzed by TLC using the CEF system (c. 9 μl of each fraction) [**Fig. 3.14**].

Fractions A-F were very concentrated and gave the most different components on TLC. The same component occurred in fractions A-P.

Fractions 1-10 gave the same component and fractions 9-20 and 25 gave the same component on TLC.

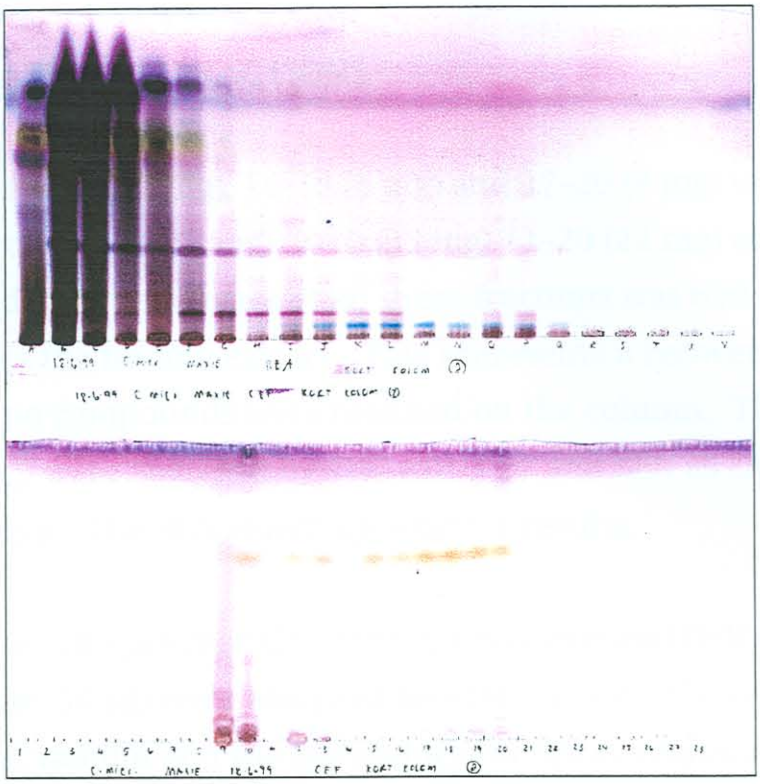

Figure 3.14 Top: Chromatogram of first highly non-polar fractions (A–V) using the BEA system as eluent and the vanillin spray reagent (c. 3 μl of each fraction placed on plates) and bottom: chromatogram of last polar fractions (1–25) using the CEF system as eluent and the vanillin spray reagent (c. 9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

3.4.2 COMBINATION OF FRACTIONS

Fractions 4-6 (4 mg), 11-13 (32 mg), 14-16 (4 mg) and 17-20 (8 mg) were combined. Later fractions 1-8 (15 mg), 9-10 (35 mg) 11-20 (22 mg) and 21-25 (17 mg) were re-combined. The total mass of all these fractions was 630 mg. I started with 633 mg of the hexane extract. This represents a recovery of 99.5 %, which indicates that no compounds were retained on the column. The combined fractions (c. 20 - 50 μg) were analyzed by TLC with CEF and BEA as eluents and vanillin as spray reagent. The BEA eluent gave better results.

Fractions ABC (431 mg), DEF (88 mg), GHI (9 mg), J-N (7 mg) and O-V (6 mg) were combined. Fractions (c. 50 μg) were analyzed several times by TLC using the BEA eluent system and the vanillin and the *p*-anisaldehyde spray reagents. Plates sprayed with the vanillin spray reagent, gave better separation of components [**Fig. 3.15**]. Fractions ABC and DEF gave the most definite bands and best separation on TLC.

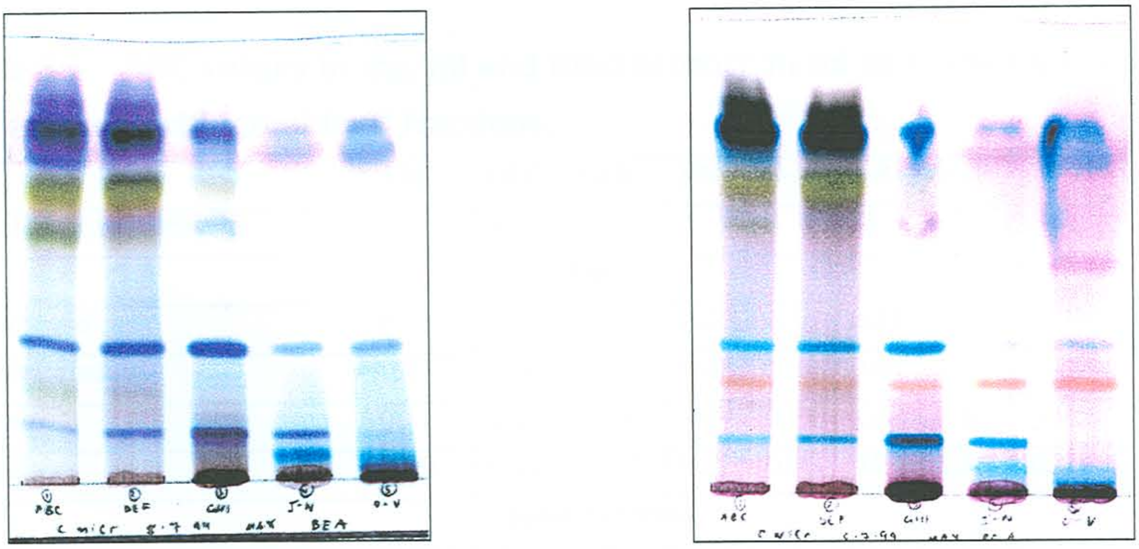

Figure 3.15 Separation of components present in 50 μg of fractions using the BEA eluent and the vanillin (left) and *p*-anisaldehyde (right) spray reagents. (From left to right: ABC, DEF, GHI, J-N and O-V combined fractions.)

The combined fractions were tested for antibacterial activity. By far the largest activity was in the first highly non-polar fractions [**Table 3.5**]. Lower activity was encountered with *S. aureus* [**Fig. 3.16**].

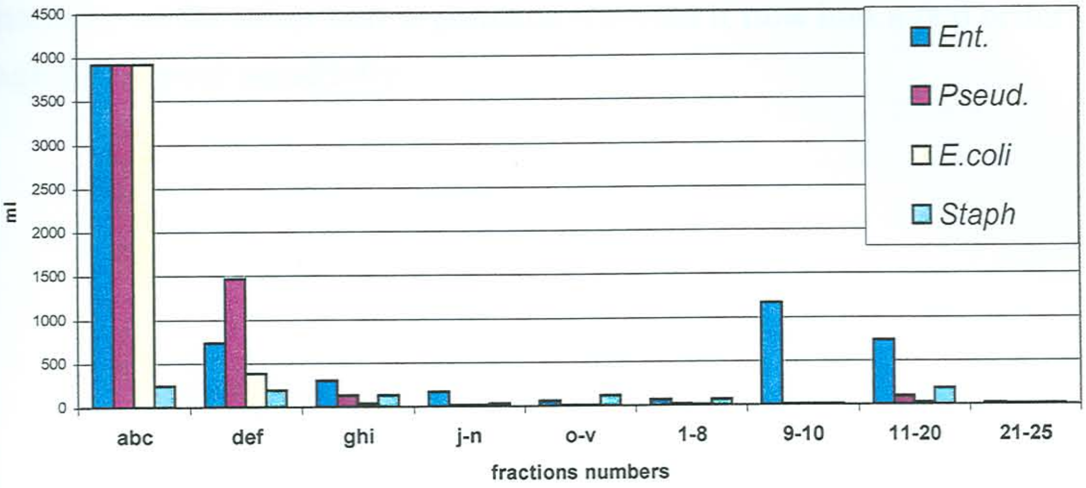

Figure 3.16 Total antibacterial activity in ml of *C. microphyllum* leaves extracted with combined fractions to four test organisms.

Table 3.5 MIC values in mg/ml and total activity in ml of *C. microphyllum* leaf extracts with combined fractions.

	ABC	DEF	GHI	J-N	O-V	1-8	9-10	11-20	21-25
Total quantity present	431	88	9	7	6	15	35	22	17
					MIC				
E. faecalis	0.11	0.12	0.03	0.06	0.10	0.24	0.03	0.03	1.42
P. aeruginosa	0.11	0.06	0.07	0.44	1.50	0.94	3.50	0.23	2.83
E. coli	0.11	0.23	0.26	.044	0.75	1.88	3.50	0.92	2.83
S. aureus	1.79	0.47	0.07	0.22	0.05	0.24	3.50	0.12	2.83
					Total Activity				
E. faecalis	3918	733	300	167	60	63	1167	733	12
P. aeruginosa	3918	1467	129	16	4	16	10	96	6
E. coli	3918	383	35	16	8	8	10	24	6
S. aureus	241	187	129	32	120	63	10	183	6
Total activity	11995	2770	593	231	192	150	1197	1036	30
Percentage of total	65.9	15.2	3.3	1.3	1.1	0.8	6.6	5.7	0.2

Results show that different fractions inhibited different organisms to varying degree, indicating that there is some specificity towards different test organisms. It is surprising that *S. aureus* was not nearly as sensitive as the other test organisms. In the original extract [**Table 3.3**] *S. aureus* had the same order of sensitivity as the other test organisms whereas it now had a two orders of magnitude lower sensitivity.

3.5 COMBINATION OF FRACTIONS AFTER TLC AND BIOASSAY

3.5.1 Developing a system to separate components by column chromatography

Attempts were made to develop a TLC system that would separate the components of fractions ABC and DEF well. This system would then be applied to column chromatography. Several combinations of volatile eluents were tested to develop an eluent system for column chromatography.

The best separation on column chromatography occurs if compound Rf is c. 0.5 on TLC. BEA as eluent has a relatively high solvent strength (c. 0.38). The higher the solvent strength, the more the components would occur at the top of the TLC plates. I was looking for a lower solvent strength. Toluene has a solvent strength of c. 0.29, but it is not volatile. Benzene has a solvent strength of 0.32, but is carcinogenic. Carbon tetrachloride may separate the components occurring at the top of the TLC plates.

Initially different combinations of carbon tetrachloride (CT) and methylene dichloride (MD) were tested as eluents in the following combinations:

CT : MD 1 : 2 (solvent strength c. 0.31)
CT : MD 3 : 2 (solvent strength c. 0.28)
CT : MD 2 : 1 (solvent strength c. 0.26)

Initially I used Seprachrom containers as eluent tanks. Fractions ABC and DEF were dissolved in acetone. (Initially fractions GHI were also tested). The volumes and concentrations of the combined fractions were determined. Approximately 50 μg of each fraction was applied to the plates. The combinations CT : MD 1 : 1, CT alone and MD alone were also tested as eluents. (Plates were sprayed with the vanillin spray reagent).

Separated components were visualized under visible and ultraviolet light. The different bands on TLC were not as prominent and well separated as I had hoped for. The bands flowed together at the side ends of the plates. I did not use the Seprachrom containers any more. The best separation occurred with the combination CT : MD 1 : 1.

3.5.2 TLC in glass eluent tanks

The experiment was repeated in glass TLC tanks with the same combinations and better results were obtained.

New combinations for eluents were tested. Initially methanol (ME) and methylene dichloride (MD) were combined as eluents in a ratio of 1 : 1. Methanol (ME) as eluent pushed the components to the top of the TLC plates. The ME proportion was initially reduced to MD : ME 2 : 1 and MD : ME 3 : 1 to ensure better separation. Later MD : ME 8 : 1, 9 : 1 and 10 : 1 as eluent combinations were used with good results. MD : ME 15 : 1 and 20 : 1 were also tested.

The combination MD : ME 15 : 1 gave the best separation of components and the most definite bands [**Fig. 3.17**]. All the plates were sprayed with the vanillin spray reagent.

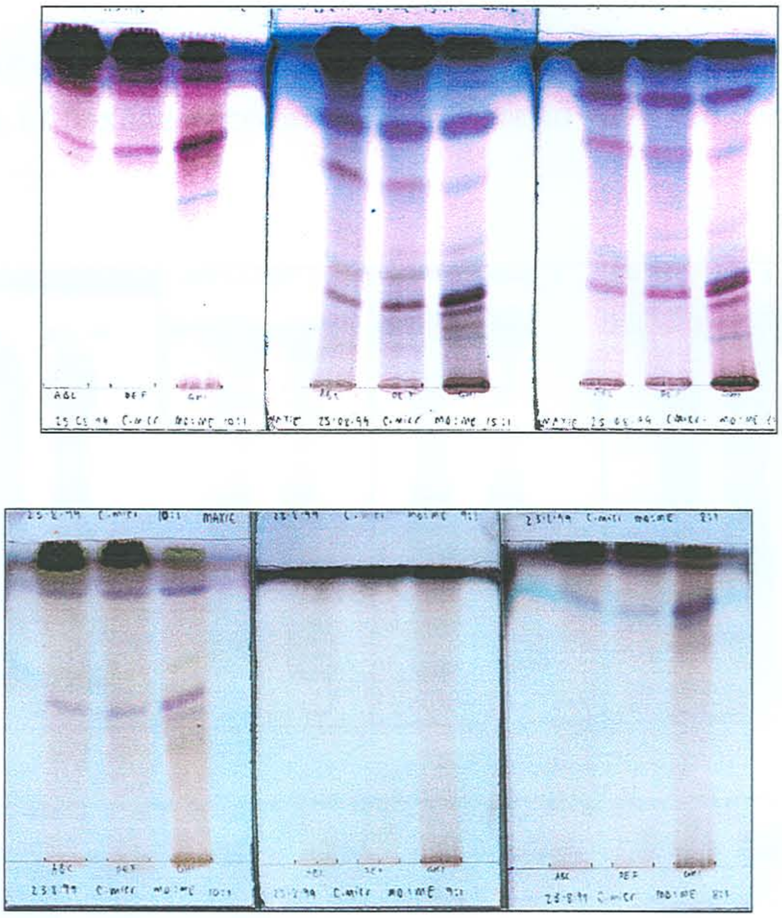

Figure 3.17 Different combinations of methylene dichloride (MD) and methanol (ME) as eluents for 50 μg of the ABC, DEF and GHI extracts. Plates were sprayed with the vanillin spray reagent. [MD : ME = 10 : 1 (top left), MD : ME = 15 : 1 (top middle), MD : ME = 20 : 1 (top right), MD : ME = 10 : 1 (bottom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

Later different combinations of acetone (A), hexane (H), methanol (ME), methylene dichloride (MD) and carbon tetrachloride (CT) were tested as eluents. Plates were sprayed with the vanillin and *p*-anisaldehyde spray reagents.

To determine the effect of heat on the components and the colour of the bands, some plates were put in the oven and the rest were dried with a hot air drier. The plates heated in the oven gave better results.

Good results were eventually obtained with a hexane-acetone mixture [**Fig. 3.18**]. The combination of hexane : acetone in a 2 : 1 ratio gave the best separation on TLC.

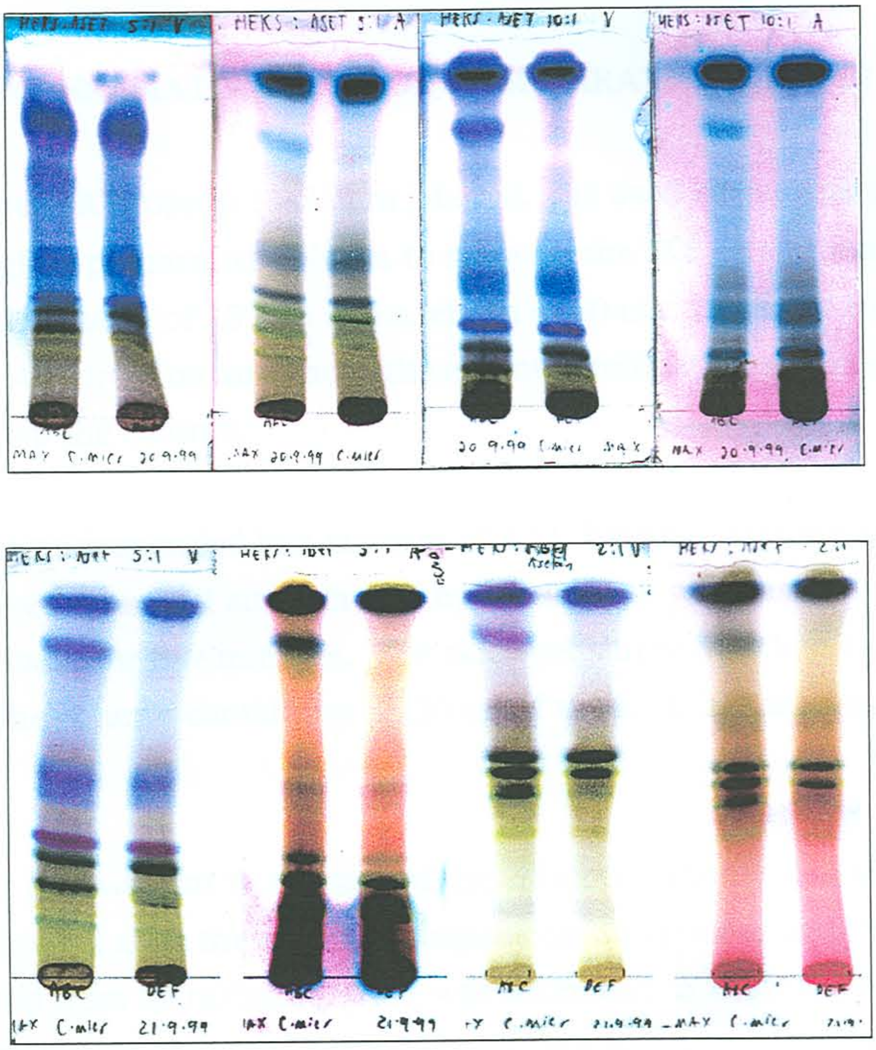

Figure 3.18 Different combinations of hexane (H) and acetone (A) as eluents for 50 μg of the ABC and DEF fractions. Plates sprayed with the vanillin spray reagent (top first, top third, bottom first and bottom third) and *p*-anisaldehyde spray reagent (top second, top fourth, bottom second and bottom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

This eluent combination (hexane : acetone 2 : 1) will be applied to the mixture in an attempt to isolate the bioactive compounds using a finer silica gel as packing material with column chromatography on a preparative column.

3.6 COLUMN CHROMATOGRAPHY ON A PREPARATIVE COLUMN

A finer silica gel, LiChroprep 15-25 µm [Merck], was used as packing material in a Michel-Miller glass preparative column to scale up the TLC separation. The column had a diameter of 25 mm and a length of 60 cm. I decided to continue with only the ABC fraction and chose the eluent combination of hexane : acetone (2 : 1) to develop the column.

The silica gel was suspended in a mixture of 2 : 1 hexane : acetone and was left to swell. I removed dissolved air in the gel by placing the suspension in a Bransonic 52 ultrasonic bath for five minutes. The silica gel slurry was then carefully poured into the column containing c. 150 ml of the 2 : 1 hexane : acetone mixture.

A filter paper disk was cut to the size of the column-diameter and was placed on top of the silica gel after the silica gel suspension was poured into the column to protect the top from disturbance. Care was taken not to allow the top of the column to dry out. A Michel-Miller adjustable bed height fitting was attached to the top of the column to minimize dead volume.

The ABC fraction was applied under pressure from a syringe to the top of the column through a Rheodyne 5020 low pressure injection valve injector in a closed column system.

Unfortunately I lost c. a quarter to a third of the ABC fraction, which was pushed back through the injector. This was probably caused by the volatile hexane which expanded in the injector and on the column due the higher temperature inside the column. It was a very hot night and the hexane volatility caused cracks in the column when pressure was released.

Approximately another two litres of the hexane : acetone mixture was placed in a Bransonic 52 ultrasonic bath to remove gas, which could cause problems to the column. Pressure from a membrane pump was used to elute the components through the column. The column was left to run overnight, filling the test tubes with c. 15 ml every 3 minutes. Seventy test tubes were collected by the fraction collector [Isco Foxy Junior]. As soon as the separation was completed, the test tubes were placed under a stream of cold air, in order to concentrate the fractions for further analysis by TLC, bioautography and bioassay.

3.6.1 Chemical composition of different fractions

The fractions were analysed by TLC, using BEA and CEF as eluents and the vanillin spray reagent. Based on the TLC analysis and the quantity present in each fraction [**Fig. 3.19 and Fig. 3.21**], some fractions were pooled as follows: 7-12, 13-20, 21-30, 31-40, 41-50, 51-60 and 61-70. The quantity present in the first 6 and the rest of the pooled fractions is shown in **Figure 3.20**.

The separation was very disappointing. This was probably caused by a malfunction of the injection system. This caused the top of the column to dry out. To remove the air in the top, c. 4 cm of the top of the column had to be stirred with a glass rod. Consequently the extract was placed in a very broad band on the column and thus destroyed much of the potential separation.

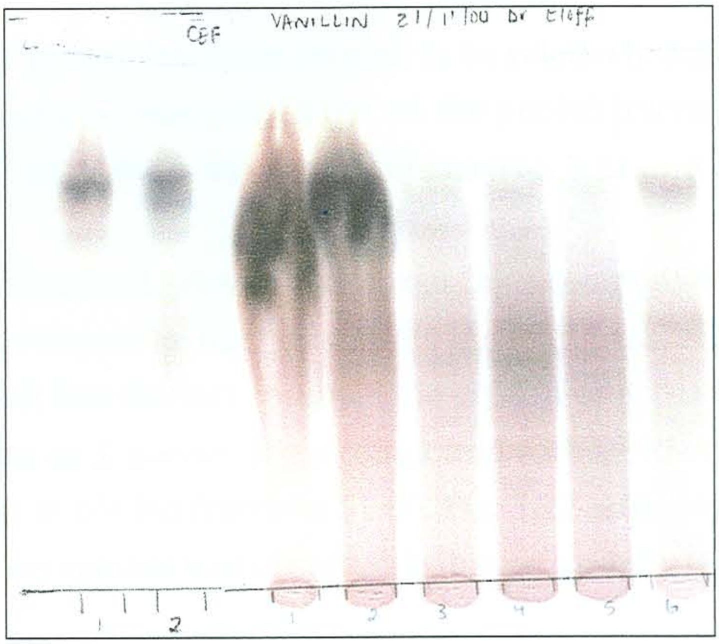

Figure 3.19 Separation of components present in 50 μg of fractions 1-6, using the CEF eluent and the vanillin spray reagent.

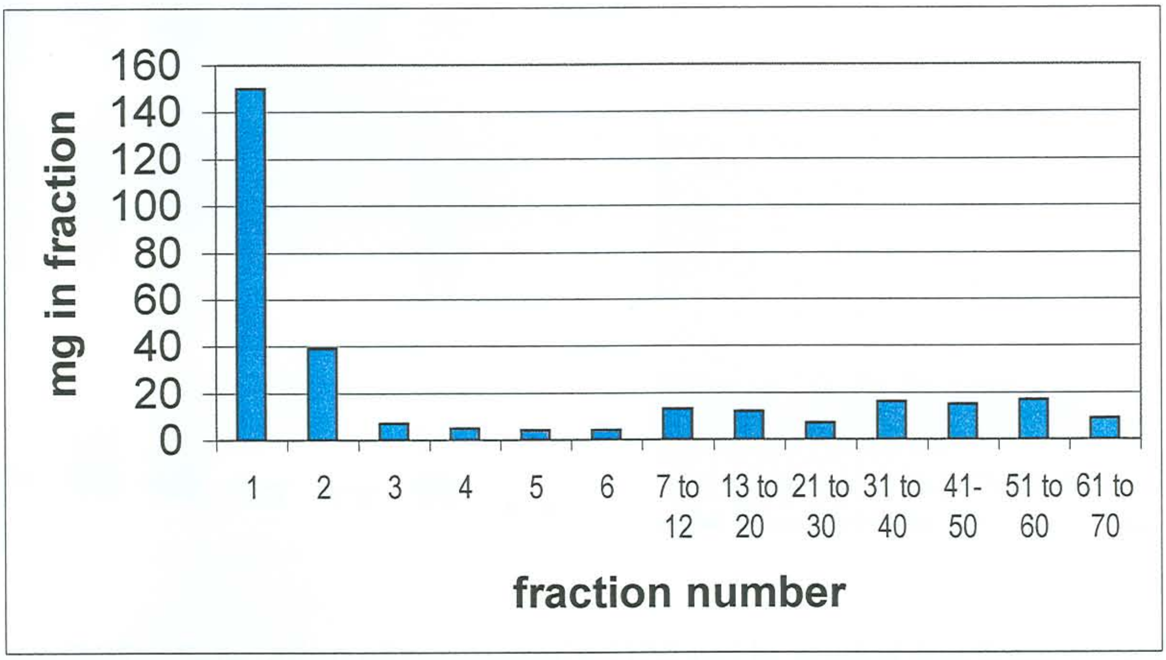

Figure 3.20 Separation of fractions from ABC by large scale column chromatography. (Fractions combined from fraction 7, based on quantity present in individual fractions.)

Because some of the pooled fractions seemed to be relatively pure based on the TLC results, bioautography was carried out on the pooled fractions using *S. aureus, E. coli* and *P. aeruginosa* as test organisms [**Fig. 3.21 and Fig. 3.22**].

There was clear inhibition of growth of *S. aureus* by a compound with an Rf-value of c. 0.74 with CEF as eluent by fractions 21-50. As found earlier, the other test organisms gave much less distinct results. The growth of *E. coli* was not inhibited by the same fractions as *S. aureus*, but a compound with an Rf-value of c. 0.84, gave clear inhibition in pooled fractions 51-60 [**Fig. 3.22 left**]. No clear inhibition of the growth of *P. aeruginosa* was observed by bioautography [**Fig. 3.22 right**].

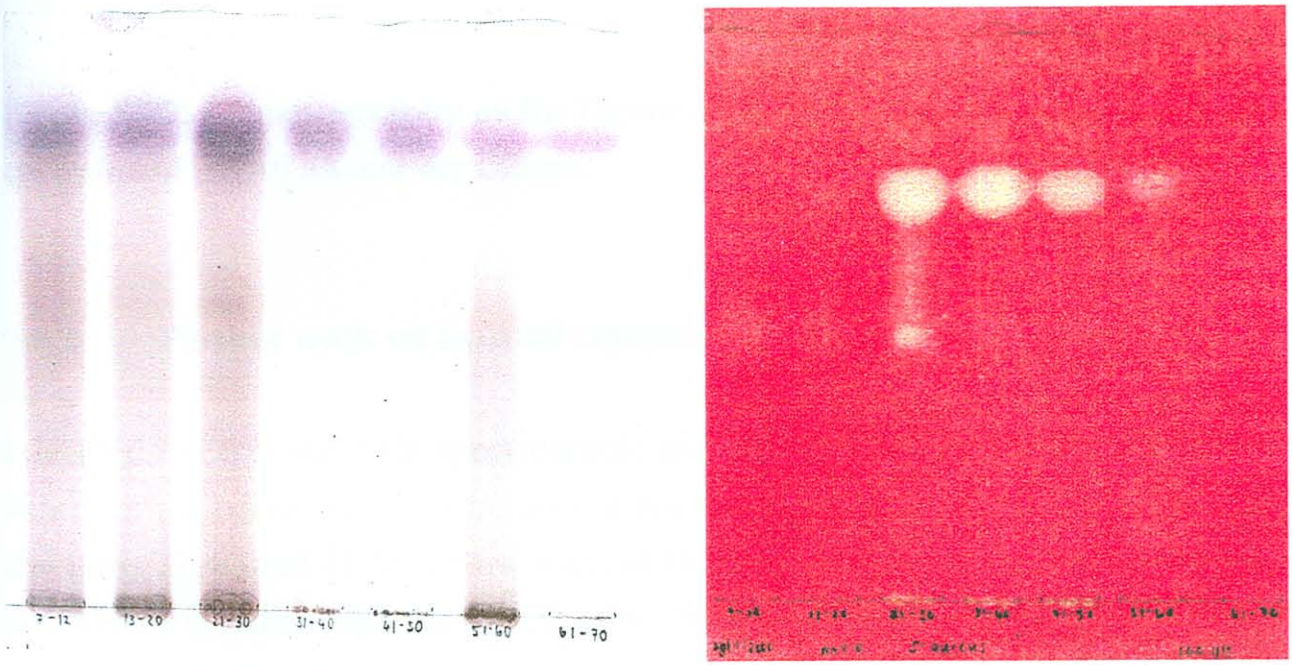

Figure 3.21 Separation of compounds [100 μg] in pooled fractions 7-20, 21-30, 31-40, 41-50 and 51-60 by TLC with CEF as eluent. Left sprayed with vanillin spray reagent and right bioautography with *S. aureus* as test organism.

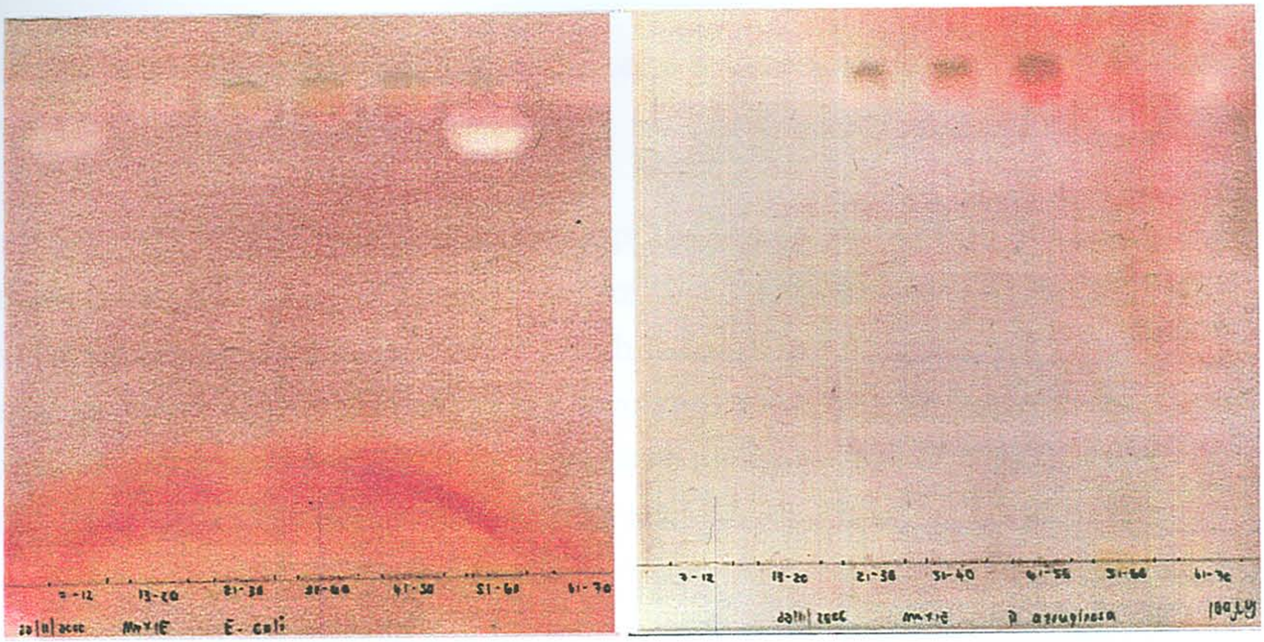

Figure 3.22 Bioautography as for Figure 3.21 with *E. coli* [left] and *P. aeruginosa* [right] as test organisms.

3.6.2 Further work on isolated compounds

I decided to carry out NMR spectroscopic analysis, before determining the MIC values of the active fractions because it is a non-destructive method. Pooled fractions 31-40 and 41-50, with a mass of 16 mg and 15 mg respectively, were apparently clean enough for NMR analysis based on the TLC results. These pooled fractions were dried over silica gel in a vacuum dessicator overnight to remove all traces of water that would give problems in the NMR analysis.

It was a big shock to find that only c. 3 mg of the material was left the next morning. Apparently the active compound was volatile enough to be removed by vacuumpump that was used for c. one hour before leaving the samples to dry overnight. Prof Fanie van Heerden, Chemistry Department, RAU was kind enough to carry out the NMR analysis on pooled fractions 31-40 and 41-50. She found that 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.

To test whether I could have been chasing phthalic acid and to find out if this compound had antibacterial activity, I performed a number of tests. In the first place I evacuated phthalic acid under a high vacuum in a vacuum dessicator for an hour to determine if this compound is volatile enough to be removed by high vacuum. It did not seem likely because it has a melting point of 230 °C according to the Merck Index. No loss of the phthalic acid [Merck Chemicals] was found.

I then determined the Rf-values of phthalic acid and the active compound. Phthalic acid did not react with the vanillin spray reagent, but it gave very clear quenching in 254 nm UV light when TLC was performed on F_{254} TLC plates. The Rf-value was c. 0.33. **Figure 3.23** shows that phthalic acid could not be responsible for the antibacterial activity noticed in **Figure 3.21 [right].**

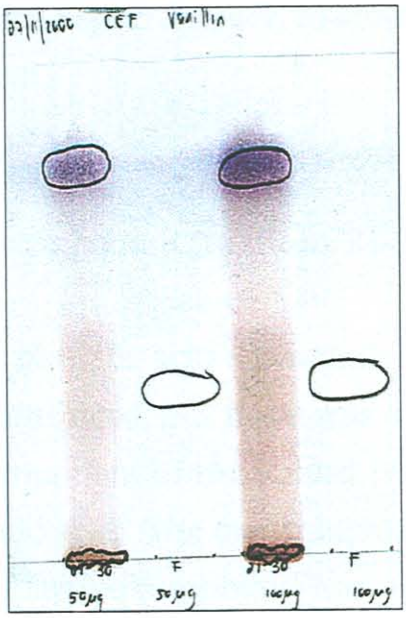

Figure 3.23 Separation of chemical components present in pooled fractions 21-30 [first, 50 µg and third, 100 µg] and phthalic acid [second, 50 µg and last, 100 µg] by TLC with CEF as eluent and visualizing components with vanillin spray reagent. Quenching of absorbance at 254 nm indicated by pencil circles.

To confirm the results, I also determined MIC values of phthalic acid and pooled fractions 21-30. In addition to this, bioautography with *S. aureus* was also carried out.

The MIC values for phthalic acid for the different test organisms were: *S. aureus* 2.5 mg/ml; *P. aeruginosa* 1.3 mg/ml; *E. faecalis* 0.6 mg/ml and *E. coli* 1.3 mg/ml. The MIC values for pooled fractions 21-30 were: *S. aureus* 0.9 mg/ml; *P. aeruginosa* 0.9 mg/ml: *E. faecalis* 1.8 mg/ml and *E. coli* 0.2 mg/ml.

Phthalic acid therefore does have a slight antimicrobial activity. Considering the structure [o-benzenedicarboxylic acid] this may even have been a localized pH inhibition effect at the high concentrations where inhibition took place.

The MIC values of all the fractions containing many non-active compounds, was usually below 0.5 mg/ml [**Table 3.5**]. It is therefore extremely unlikely that phthalic acid would represent a major portion of the antimicrobial activity measured. To investigate this aspect further, bioautography using *S. aureus* as test organism was carried out.

3.6.3 Bioautography with pooled fractions 21-30 and phthalic acid

The low quantity [50 μg] of phthalic acid separated, did not inhibit the growth of *S. aureus* under the conditions used, but there was a slight inhibition at 100 μg level. A minor bioactive component of the pooled fractions 21-30 had the same Rf-value of c. 0.33 as phthalic acid. The major bioactive compound was certainly not phthalic acid and the bioactive compound was also not the substance that quenched light at 254 nm indicated with the pencil lines. If the compound with an Rf-value of c. 0.33 is indeed phthalic acid, it means that the main bioactive compound must be present in a very low concentration and consequently must have a very high bioactivity because a relatively large quantity of phthalic acid is required for the growth inhibition noted [**Fig. 3.24**].

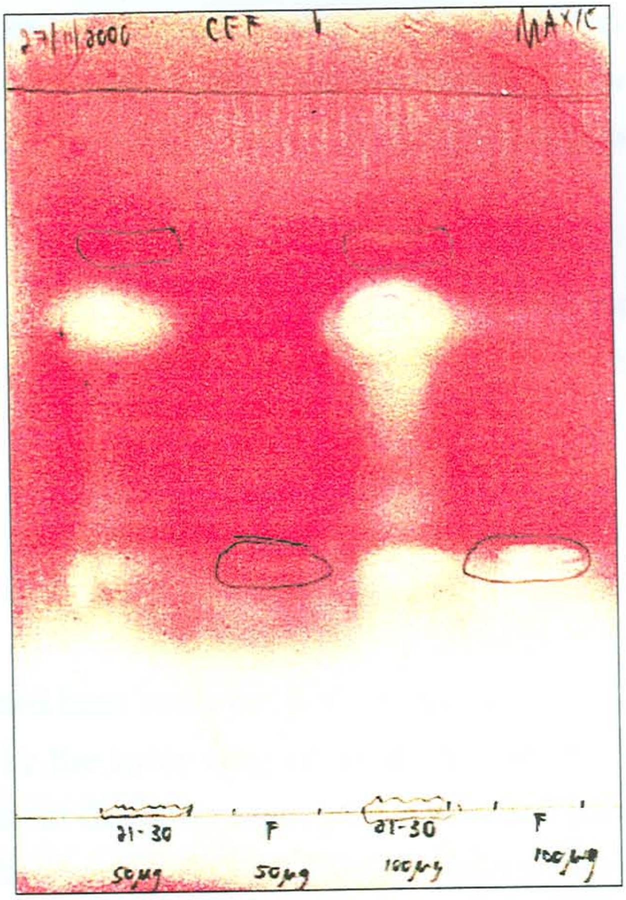

Figure 3.24 Bioautogram of fractions 21-30 [50 µg first and 100 µg third] and phthalic acid [50 µg second and 100 µg last]. TLC using CEF as eluent, sprayed with *S. aureus* cell suspension, incubated and sprayed with INT. White areas indicate bacterial growth inhibition.

3.6.4 Quantitative evaluation of results

Original 90 g of plant material was extracted with acetone. The acetone extract weighed 8.4 g. Solvent fractionation led to nearly 80 % of the non-aqueous activity being concentrated in the hexane fraction. The first column chromatography lead to 65.9 % of the total activity going into fractions ABC. The final chromatography did not yield good separation of the highly non-polar components, but it did yield an antibacterial compound in a relatively pure state.

If I estimate that this compound was 90 % pure, c. 30 mg would have been obtained from 90 g of the original plant material. With an estimated loss of 30 % in the final column chromatography, this compound was probably present at a quantity in the order of 40 mg. This means that it was present at a level of c. 0.04 % in the original material.

It is a pity that MIC values of these pooled fractions were not determined before it was lost, otherwise it would have been possible to calculate what percentage of the total activity is presented by this compound. It is in any case a minor bioactive component because, the majority of the activity resided in the first two fractions. The only reason for investigating the minor compound first was because it seemed to be relatively pure according to the TLC data.

The experience gained here stresses the fact that not all compounds separated by TLC are visualized by the spray reagent used, that one has to be careful to use high quality solvents in the latter stage of the isolation procedure and that there is a danger that volatile compounds can be lost by vacuum drying.

Unfortunately due to personal circumstances it was not possible to follow up on the results obtained thus far. A major avenue for further work exists in the concentrated fractions 1-3 of the final separation. There is also the possibility to capitalize on the suspected volatility of the bioactive fractions by separating these compounds from other compounds through a cold finger vacuum distillation process.

CHAPTER 4

CONCLUSIONS

4.1 INTRODUCTION

The aim of the study was to investigate whether *Combretum microphyllum* contains antibacterial compounds. I also attempted to isolate one or more of these antibacterial compounds.

I worked on *C. microphyllum*, which forms part of another section (*Conniventia*) of the subgenus *Cacoucia*. There has not been any publication on the antibacterial activity of *C. microphyllum* that I could find. *C. microphyllum* possesses different chemical and antibacterial compounds from other *Combretum* species as well as a reasonably high antibacterial activity. The chemical composition and Rf-values of antibacterial compounds present in extracts also differ from that of other sections of the genus according to preliminary studies.

Other students in our laboratory are investigating species from other sections in an effort to provide chemotaxonomic evidence for the sectional classification and to confirm the occurrence of different antibacterial compounds in the different species. Car and Rogers (1986) identified certain differences in the chemical composition of the *Combretum* species in a preliminary study. They did not however investigate *C. microphyllum*.

4.2 EXTRACTION

The extracts were very complex and I investigated whether extraction with different solvents could yield less complex extracts with high antibacterial activity. In order to find an extractant that would be optimally useful both in screening and isolation of antimicrobial components from plants, I decided to compare a number of extractants of varying polarity.

In a preliminary experiment, I compared acetone with aqueous sodium bicarbonate, as extractants using intact dried leaves of *C. microphyllum* as proposed by Carr and Rogers (1986).

Different spray reagents were examined. The vanillin spray reagent gave the best results and I decided to use it in future work.

Acetone as extractant gave more promising results than sodium bicarbonate. The acetone extract yielded the highest concentration, as well as the most different compounds on TLC. The acetone extract inhibited growth most in all four cultures. The sodium bicarbonate extract inhibited Gram-positive bacteria to a larger extent than Gram-negative bacteria, but *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 than values from Eloff (1998a) using finely ground leaf material and the MIC values for the extracts of intact leaves were much lower (thus more active). Consequently I decided to continue working on finely ground leaf material.

I also experienced difficulties in the acid precipitation of the bicarbonate extracts and decided not to continue with sodium bicarbonate as extractant.

Because extracts of *Combretum* spp are complex, I decided to test more extractants with varying polarity to determine if I could simplify extracts and/or extract more antibacterial activity with different extractants.

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

There were large differences in the compounds extracted. Methylene dichloride and ethanol extracted the most different compounds that could be separated by TLC at the quantity analyzed. With hexane, carbon tetrachloride, acetone and water, no coloured compounds were visible before spraying with the 50 μg applied to the plates.

The separation of the more non-polar compounds, using BEA as eluent yielded many compounds. Surprisingly, a relatively polar extractant as ethanol, extracted similar concentrations of non-polar compounds (high Rf-values in normal phase chromatography). TLC with the intermediate polarity eluent CEF indicated that hexane did not extract polar compounds, that the carbon tetrachloride and tetrahydrofurane extracts had serious streaking and that acetone had the highest concentration and most diverse number of compounds that reacted with the vanillin spray reagent.

MIC values were determined. Although methanol and methylene dichloride extracted more activity, I still decided to continue with acetone as extractant due to its relatively low toxicity to test organisms and good TLC separation of compounds obtained with acetone as extractant and ease of removal from extracts.

4.3 PRELIMINARY SEPARATION OF EXTRACTS

4.3.1 Group separation of extracts by solvent/solvent fractionation

I investigated to what extent solvent/solvent extraction could simplify extracts without reducing antibacterial activity. This fractionation procedure separated the components of *C. microphyllum* into six fractions: hexane, carbon tetrachloride, chloroform, butanol, 35 % methanol/water and water soluble.

The first three fractions (hexane, carbon tetrachloride and chloroform) gave the best separation of compounds as well as the most different components on TLC.

The quantities present and the antibacterial activity of the different fractions were determined. The highest quantity, nearly 42 %, was present in the hexane fraction. Nearly two-thirds of the total antibacterial activity was also present in the hexane fraction. This was different from other *Combretum* species investigated thus far where most of the activity was in the carbon tetrachloride and/or chloroform fractions. If the more polar components (which may contain uninteresting polysaccharides and polyphenols/tannins) are ignored, the hexane fraction contained more than 88 % of the interesting non-polar antibacterial compounds.

This procedure enriched the antibacterial activity of the polar compounds in the butanol and water fractions and the antibacterial activity of the non-polar fractions in the hexane fraction to a high degree. It had little enrichment effect on the methanol/water fraction and removed bioactive compounds from the carbon tetrachloride and water fractions. Not all the bacteria had the same sensitivity to the compounds present in the different fractions. Especially *P. aeruginosa* cultures were more sensitive to the non-polar fractions.

Frequently Gram-negative bacteria are more resistant to plant extracts than Gram-positive bacteria, but this is 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 future steps in the isolation of bioactive compounds with column chromatography.

4.3.2 Group separation by using Extrelut as packing material

Because the solvent/solvent fractionation is a tedious process, I decided to determine if Extrelut, a diatomaceous sold by Merck, can be used instead of the solvent/solvent fractionation.

Small columns were initially used to see if separation occurred, to test stability and to ensure that strongly retained components would not contaminate a large column. Both wet and dry adsorbents of Extrelut were used.

The Extrelut fractions were analyzed on TLC. Very little fractionation took place with the dry column, but there was some fractionation of the more polar compounds in the wet column. The fractionation was not promising with this technique because it works best with aqueous extracts that are eluted with non-miscible organic solvents, and the column may have been overloaded.

4.4 COLUMN CHROMATOGRAPHY

4.4.1 Initial column chromatography with silica gel [15-40 μm] as packing material on a small column

A 60 ml Polypropylene syringe was packed with a slurry of silica gel 60 [Whatman 15 - 40 μm] in a mixture of benzene : ethanol (9 : 1). Four solvents were used in the following order: BEA, CEF, acetone and methanol, based on the separation by TLC. I chose volatile solvents to make drying of extracts at room temperature under air stream easier.

The first 22 test tubes were collected by hand and the rest were collected by the fraction collector.

Individual fractions were separated by different TLC systems based on the expected polarity. The first non-polar fractions were analyzed using the BEA system and the more polar fractions with the CEF system. Fractions A-F were very concentrated and gave the most different components on TLC.

Fractions were analyzed by TLC and combined. Fraction ABC gave the most definite bands and best separation on TLC. The BEA system and the vanillin spray reagent gave the best results.

The combined fractions were tested for antibacterial activity. By far the largest activity was in the first highly non-polar fractions (ABC and DEF). *S. aureus* was not nearly as sensitive as the other test organisms. In the original extract, *S. aureus* had the same order of sensitivity as the other test organisms whereas it now had a two orders of magnitude lower sensitivity. It is not clear whether this change was due to experimental error or a change in activity due to the procedure followed.

The results showed that different fractions inhibited different organisms to varying degree, which indicated that there was some specificity towards different test organisms.

4.4.2 Developing a system to separate components by column chromatography on a large column

Attempts were made to develop a TLC system using volatile eluents that would separate the components of fractions ABC and DEF well, with the purpose of applying this system later in column chromatography.

Initially different combinations of carbon tetrachloride and methylene dichloride were tested as eluents. Later different combinations of acetone, hexane, methanol, methylene dichloride and carbon tetrachloride were also tested as eluents.

Good results were eventually obtained with a hexane-acetone mixture with a 2:1 ratio.

4.4.3 Final column chromatography on a preparative column

A finer silica gel, LiChroprep 15–25 μm, was used as packing material on a glass preparative column to scale up the TLC separation.

I decided to continue with only the ABC fraction and chose the eluent combination of hexane : acetone 2:1 in an attempt to isolate the bioactive compounds using this finer silica gel as packing material with column chromatography on a preparative column.

A syringe was used to apply the ABC fraction under pressure to the top of the column. Unfortunately I lost c. a quarter to a third of this fraction, which was pushed back through the injector. The reason for this could be due to the volatility of hexane which could have expanded in the injector and on the column due to the higher temperature inside the column.

4.5 ISOLATION OF BIOACTIVE FRACTION

The fractions were combined based on the TLC analysis and the quantity present in each fraction. Some of the combined fractions seemed to be relatively pure based on the TLC results and I decided to carry out bioautography on the combined fractions using *S. aureus*, *E. coli* and *P. aeruginosa* as test organisms.

The growth of *S. aureus* was inhibited by a compound with a Rf-value of c. 0.74 with CEF as eluent by fractions 21-50. As found earlier, bioautography with the other test organisms gave much less distinct results. The growth of *E. coli* was not inhibited by the same fractions as *S. aureus*, but a compound with an Rf-value of c. 0.84, gave clear inhibition in pooled fractions 51-60. This pool did not inhibit the growth of *S. aureus* indicating that there is some selectivity of the different bioactive compounds.

NMR spectroscopic analysis was carried out before determining the MIC values of the active fractions because it is a non-destructive method. Combined fractions 31-40 and 41-50, with a mass of 16 mg and 15 mg respectively, were apparently clean enough for NMR analysis based on the TLC results. These combined fractions were dried in a vacuum dessicator overnight after evacuating for one hour under high vacuum. Unfortunately only c. 3 mg of the material was left the next morning. The active compound was apparently volatile enough to be removed by vacuumpump.

NMR analysis showed that 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 was evacuated under vacuum to determine if this compound is volatile enough to be removed by high vacuum, but no loss of the phthalic acid was found. I determined the Rf-values of phthalic acid and the active compound. Phthalic acid did not react with the vanillin spray reagent, but it gave very clear quenching in UV light when TLC was performed. The Rf-value with CEF was c. 0.33 and phthalic acid could not be responsible for the antibacterial activity.

MIC of the pooled fractions 21-30 and phthalic acid was carried out as well as bioautography with *S. aureus*. Phthalic had a slight antimicrobial activity with an MIC in the order of 1 - 2 mg/ml. This could be an indirect pH related effect. The MIC values of all the fractions containing many non-active compounds were usually below 0.5 mg/ml. It is therefore unlikely that phthalic acid would represent a significant portion of the antimicrobial activity measured. A quantity of 50 µg pure phthalic acid did not exhibit any bacterial growth inhibition whereas 50 µg of an acetone leaf extract showed several antibacterial zones at Rf-values different from phthalic acid.

The main bioactive compound must have been present in a very low concentration and consequently must have a very high bioactivity, because a relative large quantity of phthalic acid would have been required for the growth inhibition noted.

4.6 RECOMMENDATIONS ON FUTURE WORK

There are still many aspects left to explore. The next logical step is that the non-polar fractions from the final column chromatographic separation should be investigated further.

There is also the possibility to capitalize on the suspected volatility of the bioactive fractions by separating these compounds from other compounds through a cold finger vacuum distillation process.

It should not be difficult to remove any contaminating phthalic acid from bioactive fractions by anion exchange chromatography.

At an international medicinal plant conference held in Zürich in September 2000, it was reported that *Combretum paniculatum* was the most active of all plants tested against the HIV virus replication (Anti-HIV-1 and –HIV-2 Activity of Ethnobotanically Selected Ethiopian Medicinal Plants. K. Asres, F. Bucar, T. Kartnig, M. Witvrouw, C. Pannecouque and E. De Clercq). Most taxonomists consider *C. paniculatum* to be synonymous with *C. microphyllum*. We found that the chemical fingerprints are similar. This result increases the importance of continuing the work initiated in this first modest attempt to isolate bioactive compounds from *C. microphyllum*.

5. REFERENCES

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