Isolation and characterization of antibacterial compounds in *Combretum apiculatum* Sond subsp. *apiculatum* Exell

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Isolation and characterization of antibacterial compounds in *Combretum apiculatum* Sond subsp. *apiculatum* Exell

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PREFACE

This represents an experimental record for the work carried out in the Department of Pharmacology, University of Pretoria, under the supervision of Prof. J.N. Eloff and Dr. D.R.P. Katerere.

I, the undersigned Serage Sekgoro Andrew present this document as my authentic material and acknowledge that it has not been submitted in any form to any other institution. I also acknowledge that I have consulted many publications in compiling this work and the references are all listed.

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ACKNOWLEDGEMENTS

- A special word of thanks to my family, friends and relatives for their endless support, guidance and passion for learning.

- Word of thanks to Prof: J.N. Eloff for his endless support and parental dedication.

- I also owe it to my co-promoter, Dr.D.R.P.Katerere for his contributions.

- And, of course, thanks and glory be to God.

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ABSTRACT

Because microorganisms develop resistance towards antibiotics, the fear of reaching a "post antibiotic era" has stressed the value of searching for new antibiotic moieties. Many plants show promising antimicrobial activity. Antimicrobial agents were found in several *Combretum* species but *Combretum apiculatum* subspecies *apiculatum* has not been investigated in detail yet.

In this study dried and ground leaves were extracted and fractionated to isolate and characterize antibacterial compounds from the plant. Ten solvents of varying polarities were used to extract compounds from the leaves of *C. apiculatum* i.e.: hexane, diisopropyl ether, diethyl ether, methylene dichloride, ethyl acetate, tetrahydrofuran, acetone, methanol, ethanol and water. The purpose of extracting the leaf material with the ten different solvents was to establish which one would extract most antibacterial components in the least chemically complex extract.

Tetrahydrofuran and acetone extracted the largest quantities of 14.4% and 13.6% respectively compared to the other extractants, indicating that leaves contain many compounds with an intermediate polarity. With the exception of hexane and water the chemical composition determined by thin layer chromatography using vanillin-sulphuric acid as detection agent was surprisingly similar.

Minimum inhibitory concentrations were determined by a serial dilution microplate technique using *Staphylococcus aureus* (ATCC 29213), *Pseudomonas aeruginosa* (ATCC 27853), *Escherichia coli* (ATCC 25922) and *Enterococcus faecalis* (ATCC 21212), recommended by the National Committee for Clinical Laboratory Standards as test organisms. These bacteria are also responsible for most nosocomial diseases in hospitals.

All the extracts except water and hexane inhibited the growth of test organisms. Although tetrahydrofuran extracted the highest quantity, acetone was selected for the large-scale extraction in the rest of the study.

The dried acetone leaf extract was further fractionated by solvent-solvent fractionation into the following six fractions of varying polarities: water, butanol, 35% water/methanol,

chloroform, carbon tetrachloride and hexane. The highest proportion of the acetone extract was in the chloroform (26.9%), and the lowest (6.0%) was in the water/methanol fraction. The chloroform fraction was the most active and was further fractionated by silica gel column chromatography to isolate antibacterial compounds. Some pure components mainly active against *E. faecalis* and *S. aureus* were isolated by crystallization after the first column chromatography, but to analyse complex fractions a second column chromatographic separation was required.

Fractions were tested for activity against *E. faecalis* and *S. aureus* The structures and characterization of four active pure fractions were elucidated by nuclear magnetic resonance and mass spectroscopy. Three were flavonoids i.e. pinocembrin [two samples] and flavokawain-A and one was a chalcone i.e. alpinetin. Not one of these compounds has yet been found in Combretaceae, but was isolated from other plants. The antibacterial activities of these compounds were unknown. All isolates compounds had a low to reasonable activity with MIC values for the two Gram-negative pathogens averaging at 268 µg/ml and 100 µg/ml for the two Gram-positive bacteria.

The activities of extracts and individual compounds in this plant supports the rationale for using it in treating human or animal infection related diseases.

OPSOMMING

Omdat mikroorganismes weerstand teen antibiotika opbou, het die gevaar van 'n nuwe "post antbiotikum era" navorsers genoop om na antibakteriese verbindings in plante te soek. Antibakteriese verbindings is in verskeie lede van die Combretaceae in ons navorsingsgroep gevind, maar *Combretum apiculatum* subspesie *apiculatum* is nog nie in diepte ondersoek nie.

In hierdie studie is gedroogde fyngemaalde blare geekstraheer en gefraksioneer om die antibakteriese verbindings in hierdie plant te ondersoek. Tien oplosmiddels met verskillende polariteite is gebruik naamlik: heksaan, di-isopropieleter, di-etiel eter, metileendichloried, etielasetaat, tetrahidrofuraan, asetoon metanol etanol en water. Die doel was om te bepaal watter ekstraheermiddel die meeste antibakteriese verbindings in die mins komplekse matriks sou ekstraheer.

Tetrahidrofuraan [14.4%]en asetoon [13.6%] het die grootste massa uit die blare geekstraheer wat daarop dui dat blare baie verbindings met 'n intermediêre polariteit bevat. Met die uitsondering van die heksaan en water ekstrakte was die chemiese samestelling soos deur dunlaagchromatografie, met vanillien swawelsuur as kleurreagens bepaal, verbasend ooreenstemmend.

Minimum inhiberende konsentrasies is bepaal deur 'n verdunningreeks mikroplaatmetode met *Staphylococcus aureus* (ATCC 29213) , *Pseudomonas aeruginosa* (ATCC 27853) , *Escherichia coli* (ATCC 25922) en *Enterococcus faecalis* (ATCC 21212), soos aanbeveel deur die National Committee for Clinical Laboratory Standards, as toetsorganismes. Hierdie bakteriespesies is ook verantwoordelik vir die belangrikste nosokomiale siektes in hospitale.

Al die ekstrakte behalwe die water en heksaanekstrakte het die bakteriese groei onderdruk. Alhoewel tetrahidrofuraan die grootste hoeveelheid verbindings geïsoleer het, is asetoon gekies vir die grootskaalekstraksie in die res van die studie.

Die asetoonekstrak is verder deur vloeistof-vloeistof fraksionering verdeel in ses fraksies gebaseer op polariteit naamlik water, butanol, 35% water in metanol, chloroform, koolstoftetrachloried enheksaan. Die hoogste massa was in die chloroform [26.9%] en die

laagste in die water/metanol [6.0%] fraksie. Die chloroformfraksie was ook die mees aktiewe en is verder deur silika gel chromatografie gefraksioneer om antibakteriese verbindings te isoleer. Sekere verbindings hoofsaaklik aktief teen *E. faecalis* en *S. aureus* is na die eerste kolomchromatografie geisoleer deur kristallisasie, maar in ander gevalle was verdere kolomskeidings en kristallisasie nodig.

Die struktuur van vier aktiewe suiwer verbindings is deur kernmagnetieseresonsspektroskopie en massaspektroskopie opgeklaar. Drie van die verbinding was flavonoïede nl. pinosembrien [twee monsters] en flavokawain-A en een verbinding was 'n sjalkoon nl alpinetien. Nie een van die verbindings is tot dusver in Combretaceae gevind nie alhoewel almal al uit ander plante geïsoleer is. Die minimum inhiberende konsentrasies was tot dusver onbekend. Daar was effense tot gemiddelde aktiwiteit in al die verbindings met waardes tussen 40 en 600 µg/ml. Die gemiddelde waardes vir die twee Gram-negatiewe organismes was 268 µg/ml en vir die twee Gram-positiewe bakterieë was 100 µg/ml. Daar is aanduidings gevind dat hier 'n sinergisties antibakteriese aktiwiteit betrokke mag wees.
Die resultate bevestig die rasioneel vir die gebruik van die plant vir die behandeling van infeksieverwante siektes

LIST OF ABBREVIATIONS

In this thesis where ***C. apiculatum*** is used it refers to ***Combretum apiculatum*** subspecies ***apiculatum* Exell**

β	Beta
WHO	World Health Organisation
TLC	Thin Layer Chromatography
CC	Column chromatography
HIV	Human immune deficiency virus
MIC	Minimum inhibitory concentration
CEF	Chloroform ethylacetate formic acid [20:16:4], (v:v:v)
EMW	Ethylacetate methanol water [40:5,4:4,0], (v:v:v)
BEA	Benzene ethanol ammonia [36:4,0:0,4], (v:v:v)
R_f	Resolution factor
µl	Microlitre
INT	*para*-Iodonitrotetrazolium violet
HE	Hexane
IE	Isopropyl ether
EE	Di-ethyl ether
MD	Methylene dichloride
EA	Ethyl acetate
TH	Tetrahydrofuran
AC	Acetone
ET	Ethanol
ME	Methanol
WA	Water
MW	35% water in methanol fraction
B	Butanol fraction
CF	Chloroform fraction
CT	Carbon tetrachloride fraction
UV-light	Ultraviolet light
Anisaldehyde SR	*para*-anisaldehyde spray reagent. (1 ml *para*-anisaldehyde, 18 ethanol and 1 ml sulphuric acid)

Vanillin SR	Vanillin spray reagent, (0.3 g vanillin, 84 ml methanol and 3 ml sulphuric acid)	
E. coli	*Escherichia coli*	(ATCC 25922)
S. aureus	*Staphylococcus aureus*	(ATCC 292163)
P. aeruginosa	*Pseudomonas aeruginosa*	(ATCC 25922)
E. faecalis	*Enterococcus faecalis*	(ATCC 29212)

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Combretum apiculatum subsp. *apiculatum*

Painting of *C. apiculatum* from Carr 1998

CHAPTER 1

LITERATURE SURVEY

1.1. The problem of antibiotic resistance

Antibiotics are substances that suppress the growth of other microorganisms eventually killing them (Goodman and Gilman, 1998). However, common usage often extends the term ''antibiotics'' to include products derived from plants as well as synthetic antibacterial agents, such as the sulfonamides and quinolones, which are not products of microbes. Antibiotics differ in chemical, physical, and pharmacological properties, antibacterial spectra, and mechanisms of action.

The development of antimicrobial drugs represents one of the most important advances in therapeutics, both in the cure and control of serious infections and in the prevention and treatment of infectious complications arising from other forms of medical therapy such as cancer chemotherapy and surgery, (Katzung, 1998). The discovery of the antimicrobial activity of penicillin turned the attention of investigators to antibiotics as potentially useful chemotherapeutic compounds. In the 1940's and 1950's streptomycin, chloramphenicol, tetracyclines, polymyxin, bacitracin and neomycin greatly increased the range of effectiveness of antibacterial chemotherapy (Clark *et al.*, 1992). However, there is increasing evidence that antimicrobial agents are vastly over-prescribed in many outpatient settings globally (Katzung, 1998). This as well as the availability of antimicrobial agents without a prescription in many developing countries has facilitated the development of resistance, severely limiting the therapeutic options in the treatment of life-threatening infections.
In a small study at Pretoria Academic Hospital, it was established that 60% of patients were prescribed antimicrobials as part of their treatment regimen (Serage *et al.* 1999, unpublished). In addition, as the population increases, more funds are spent on antimicrobial therapy alone. Worldwide spending on finding new anti-infective agents is expected to increase by 60% from the spending levels in 1993. The public is becoming increasingly aware of problems with the over-prescribing and traditional misuse of antibiotics (Cowan, 1999).

The cornerstone of treatment for an infected patient is the isolation and identification of the microorganism involved. The susceptibility of the microorganism(s) to the antimicrobial agents is then determined. However, because of time lag involved in this process, often about 48 hours, the initial treatment must frequently be based entirely on clinical impression. This is deduced from the history of the patient, physical examination, symptomology, microscopic examination, epidemiology and, if possible, rapid laboratory tests. The antimicrobial therapy is started with agents that cover the suspected microorganism (Wiener and Pepper, 1985). It is also therapeutically recommended to treat with the single agent that is most specific for the infecting organism. This strategy reduces the emergence of resistance and minimizes toxicity (Mycek, Harvey and Champe, 2000).

The rational use of antimicrobials must involve a consideration of several aspects. Antimicrobial agents should be indicated based on thorough clinical findings. Clinical specimens should be obtained in order to establish the sensitivity of the suspected pathogens to the available antimicrobial regimen. The etiologic factors for the patient's illness should be established. Lastly, the individuals exposed to the index case should be protected in order to prevent secondary or nosocomial infections. Unfortunately antimicrobial agents are usually used before the causative pathogen for a particular illness or the susceptibility to a particular antimicrobial agent is known. This use of antimicrobial therapy is called empirical (presumptive) therapy, and is based upon the experience with a particular clinical entity (Katzung, 1998). The principal justification for empirical therapy is that infections are best treated early. To withhold therapy until the results on culture and susceptibility tests are available may expose the patient to risks of serious morbidity or death. Initiation of empirical therapy to a certain extend should however conform to a well-defined protocol (Barrie and Jacobs, 1996).

Several schemes have been proposed to classify and group antimicrobial drugs by their mode of action, (Katzung, 1998). Some of the proposed mechanisms of action are as follows:

- Agents that inhibit the synthesis of bacterial cell walls, e.g. the penicillins and cephalosporins.

- Agents that act directly on the cell membrane of the microorganism, affecting permeability and thus leading to leakage of intracellular constituents; e.g. detergents, polymyxin, and the polyne antifungal agents, nystatin and amphoteracin B, that binds to cell wall sterols.
- Agents that affect the function of the 30s or the 50s ribosomal subunits to cause a reversible inhibition of protein synthesis, this includes chloramphenicol and the tetracyclines.
- Agents that affect the nucleic acid metabolism and thus inhibiting DNA-dependent RNA polymerase, such as rifampicin.
- Antimetabolites, which block specific steps that are essential to the growth of microorganisms, eg. trimethoprim and sulfonamides. They may also be nucleic acid analogues, which inhibit viral enzymes that are essential for DNA synthesis thus hindering viral replication. e.g. zidovudine, acyclovir and vidarabine.

Microorganisms can adapt effectively to environmental pressures in a variety of ways. Their response to antibiotics is no exception. It is reported that penicillins are by far the most widely used antibiotics and therefore account for some of the most drastic consequences of antibiotic misuse (Jawetz 1996). Although pneumococci have long been considered an example of the total and regular susceptibility to penicillins, this is no longer entirely true. In Papua New Guinea and South Africa, outbreaks of meningitis and pneumonia due to penicillin-resistant pneumococci have been observed (Jawetz, 1996). The increase in resistant strains to current regimens of antimicrobials has led to increased efforts in the search of new antimicrobial agents.

An inevitable consequence of antimicrobial usage is the selection of resistant microorganisms. Thus, an important part of the pharmacology of antibiotics is the study of mechanisms of resistance to them. Some of the ways through which resistance is brought about are as follows:

- The increased production of enzymes by the microorganisms. The β-lactam ring of the penicillins and cephalosporins stimulate production of the enzyme β-lactamase, which inactivate the antimicrobials,

- Changing of the target site, e.g. penicillin binding site by a single mutation,
- Antibiotic exclusion, resulting from the inability of the drug to penetrate the outer membrane of the organisms.
- The cell wall of Gram-negative organisms is more difficult to penetrate than those of the Gram-positive, so in general the former present greater problems with resistance.

1.2. Can plant constituents play a role in resolving the problem of antibacterial resistance?

Man has largely depended on plants as the main source of medicine for thousands of years (Husain, 1991). It has been estimated that approximately 25 % of modern drugs are originally derived from higher plants. The use of plant extracts (botanicals or phytomedicines) and other forms of alternative medicinal treatments has gained popularity in the late 1990's. In 1996 alone sales of phytomedicines for antimicrobial usage increased by 37% over sales in 1995 (Kinghorn and Balandrin, 1993).

It was only since the Second World War that scientists embarked on the synthesis of medicine at a pharmaceutical level. The use of herbal medicines has however remained the mainstay of health-care in most of the world (Husain, 1991). Noristan Pharmaceutical company was involved in the screening of South African medicinal plants. Many of the plants investigated did exhibit a potential antimicrobial activity, (Fourie *et al.* 1992). Plants can be used either as dried or as fresh material depending on the type of cure they are intended for. The leaves, roots and bark are the most useful parts of the plants because of their accessibility.

Early man developed the doctrine of signatures; this states that God created plants so that the natural appearance of plant parts signifies what they can cure. For example, plants with heart-shaped leaves would cure heart-related disorders while kidney-shaped leaves will be useful for kidney malfunctions. As expected, scientific evidence could not confirm this general theory although in some cases there was efficacy, e.g. digitalis for cardiac failure (Huxley, 1984). Some plants are used to cure multiple diseases, effective

against many ills e.g. *Ephedra* species. When the twigs are made into tea they allay fever and coughs, and may increase blood pressure. Reserpine derived from *Rauwolfia serpentina* lowers blood pressure in hypotensive patients, controls arrhythmia and calms the central nervous system.

Herbal medicines may have little or no pharmacological effect and could be mere placebos that might do little good but do not cause any harm, in contrast to the pharmaceuticals (Tyler and Robbers, 2000). However, one major threat to the use of plants in herbal industry is if, the natural vegetation fails to cope with the ever-increasing demand. A good portion of currently used medicines is derived either directly or indirectly, from active principles that have been isolated from plants [Table 1.1]. Most of these substances do not occur in plants individually but in groups of compounds, such as caffeine in the group of methylxanthines, digoxin in the group of cardiac glycosides, and morphine in the group of opium alkaloids.

Table 1.1 Active principles presently used for medicinal purposes and the plants from which they are derived (Husain, 1991 and Cowan, 1999).

Plant constituent	**Plant raw material**	**Pharmacological activity**
Cocaine	*Erythrophyllum coca*	Local anaesthetic, Analgesic
Digoxin lanatosides	*Digitalis lanata*	Cardiotonic
Emetine	*Cephaelis ipecacuanha*	Emetic
Ephedrine, Pseudoephedrine	*Ephedra species*	Antihypertensive, Sympathomimetics
Ergotamine	*Claviceps purpurea*	Migraine
Quinidine	*Cinchona species bark*	Antiarrythmic
Quinine	*Cinchona species bark*	Antimalarial
Reserpine	*Rauwolfia serpentina*	Antihypertensive
Tannin	*Eucalyptus globulus*	Antibacterial, antiviral
Totarol	*Podocarpus nagi*	*Pseudomonus acne*
Curcumin	*Curcuma longa*	Antiprotozoal, antibacterial
Thymol	*Thymus vulgaris*	Antibacterial, antiviral, antifungal
β-Resercyclic acid	*Cannabis sativa*	Antibacterial, antiviral
Catechin	*Camellia sinesis*	*Shigella, Vibrio, Streptococcus mutans*
Combretastatins	*Combretum caffrum*	Anti-angionesis

Plants have been used for centuries to treat infections and other illnesses in humans. In some cases traditional healers working together with scientists were keeping records on the safety and effectiveness of phytochemical treatments, but these were generally uncontrolled and unrandomised studies. However, a few reviewed trials of the use of phytochemicals are briefly reported e.g. mouth-rinses containing antimicrobials were evaluated in humans and compared with listerine or chlorhexidine. The investigation of plant extracts effective against methicillin-resistant *S. aureus* provides an example of prospects in the search for new compounds which may be effective against infections that are currently difficult to treat (Cowan, 1999). In the next chapter the possible use of members of Combretaceae as the source of antibacterial compounds will be discussed.

CHAPTER 2

BOTANICAL AND PHYTOCHEMICAL OVERVIEW

2.1. Taxonomy and habitat

Combretaceae consists of 18 genera distributed mainly in Africa and Asia. The largest genus wolrdwide is *Combretum* with about 370 species and *Terminalia* is second largest with about 200 species (Lawrence, 1951). The species of the genus *Combretum* and *Terminalia* are widely used for medicinal purposes and occur in most parts of Africa (Rogers and Verotta, 1996). The other genera are small and include: *Calopyxes, Carnocarpis, Quisqualis, Buchenavia* and *Pteleopsis* (Rogers and Verotta, 1996). This study will focus on *C. apiculatum.* It is divided into, *Combretum apiculatum* Sond subspecies *apiculatum* Exell and *Combretum apiculatum* Sond subsp. *leutweinii* (Schinz) Exell. (Fig.2.1). Some plant taxonomists doubt the validity of *Combretum apiculatum* subsp. *leutweinii*, which is extremely hairy and only occurs in Namibia. In this report *Combretum apiculatum* will refer to subsp. *apiculatum* Exell as distinguished by Carr, (1998).

Table 2.1 The subgeneric classification of the genus *Combretum* in South Africa according to Carr 1988

Combretum Loefl	
Subgenus *Combretum*	**Subgenus *Cacoucia***
Section *Hypocrateropsis*	**Section *Lasiopetala***
C. celastroides	*C. obovatum*
C. imberbe	Section Conniventia
C. padoides	*C. microphyllum*
Section *Combretastrum*	*C. paniculatum*
C. umbricola	*C. platypetalum*
Section *Angustimarginata*	Section *Oxystachya*
C. caffrum	*C. oxystachytum*
C. erythrophyllum	**Section *Megalantherum***
C. kraussili	*C. wattii*
C. vendae	**Section *Poivrea***
C. woodii	*C. bracteosum*
Section *Macrostigmatea*	*C. mossambicense*
C. zeyheri	
Section *Ciliatipetala*	
C. albopunctatum	
C. apiculatum	
C. edwardsii	
C. moggii	
C. molle	
C. petrophilum	
C. psidioxides	
Section *Fusca*	
C. coriifolium	
Section *Breviramea*	
C. hereroense	
Section *Elaeagnoida*	
C. elaeagnoides	

Common names of *C. apiculatum* include, Bush willow (English), Rooiblaar, Rooiboswilg (Afrikaans) *Mohwelere* (Northern Sotho) and *Umbondwe* or *Umbondwe omnyama* (Zulu), (Hutchings, 1996). It occurs as a tree, shrub or climber. Generally, the tree is small about 4 – 6 m tall or even 10 m. Main branches commence 1 – 3 m above the ground, mainly bushy and shrub-like. The tree is deciduous and provides good shade when in leaf (Carr, 1998). The leaves are simple, sessile or petiolate, opposite, alternate verticulate, whorled, without stipules, with margins, endumentum comprising of hairs,

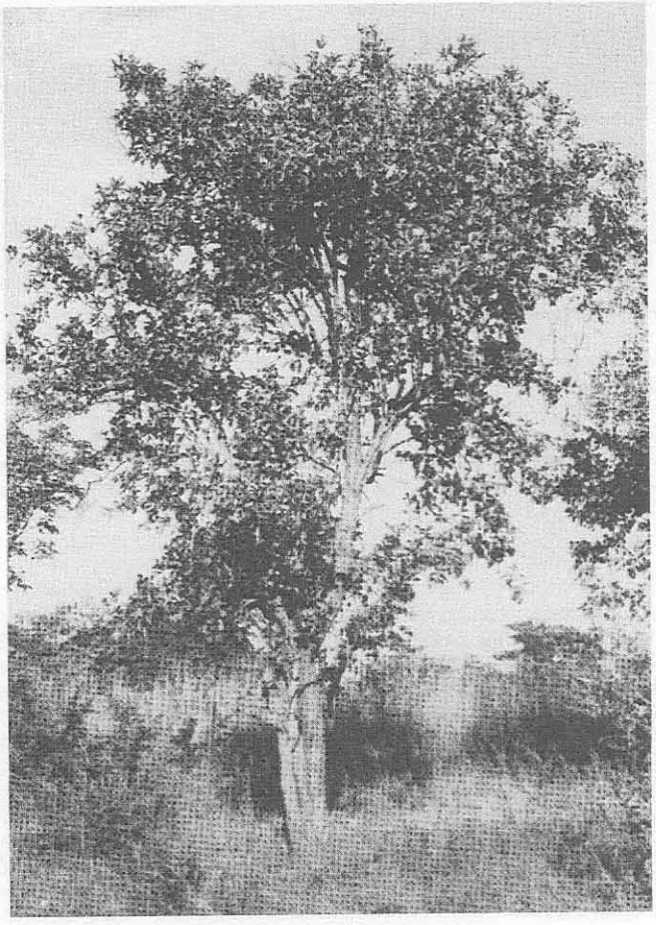
stalked glands and scales. The leaves are oblong and broadly obviate spaced in an opposite arrangement on the branch. They may have hairs on both sides. The flowers are sessile or pedicillate. Seeding cotyledons mostly two but sometimes three or four petriplate or sessile, arising above or below the ground (Carr, 1998). Flowers are present for only a short period along the annual cycle; they are yellow to creamy green with reddish brown winged fruits when matured. Fruit serves as best aid for plant identification because they occur for a long period in contrast to the leaves and flowers. Bark found on the main stem, black and comparatively smooth. On young trees, it is light brown in colour particularly in the highest rainfall areas.

C. apiculatum occurs in various savannah situations under medium rainfall to semi-arid conditions. Found on soils varying from dark and heavy to Kalahari sands, mainly at low altitudes but up to 1400 m in the Northern parts of South Africa (formerly known as Transvaal) and 1500 m in Zimbabwe. It is well distributed in Southern Africa, [fig. 2.1].

2.2. Ethnomedicinal use of *Combretum* species

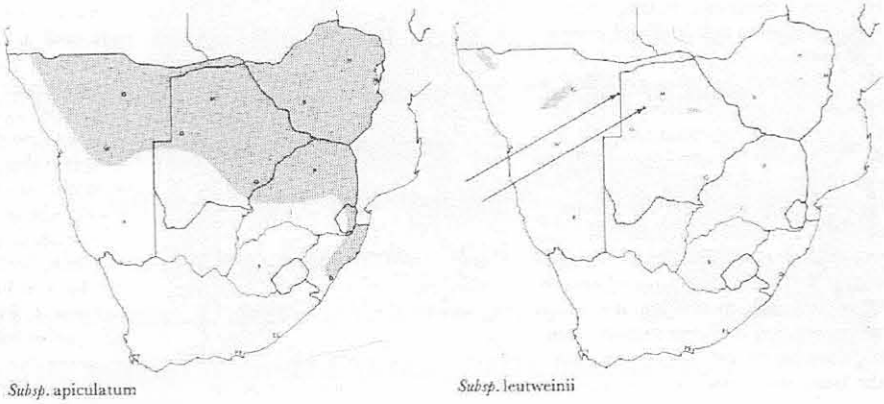
Three antimicrobial agents were found in *Combretum zeyheri*, and six more species of *Combretum* have been found to contain antimicrobial agents. (Breytenbach and Malan, 1989, Alexander *et al.*, 1992). The 27 members of Combretaceae assayed had antimicrobial activity against *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa and Enterococcus faecalis* (Eloff, 1999b). *C. erythrophyllum* was found to contain at least fourteen antimicrobial agents with some having an activity superior to that of chloramphenicol and ampicillin (Martini and Eloff, 1998). Several members of the Combretaceae also had anti-inflammatory activity (Eloff at el 2000) as well as anthelmintic activity (McGaw *et al.*, 2001).

Combretum apiculatum Sond.



Habit: subsp. apiculatum, *Selous, Zimbabwe.*

Synonyms
These are as listed in *Flora Zambesiaca:*
Combretum apiculatum Sond. forma *sulphureum* Heurck & Müll. Arg.
Combretum apiculatum Sond. forma *viscosum* Heurck & Müll. Arg.
Combretum apiculatum Sond. var. *parvifolium* Bak. f.
Combretum glutinosum Wood
Combretum apiculatum Sond. var. *sulphureum* (Heurck & Müll. Arg.) Duemmer
Combretum apiculatum Sond. var. *viscosum* (Heurck & Müll. Arg.) Duemmer
Combretum apiculatum Sond. subsp. *boreale* Exell



Subsp. apiculatum *Subsp.* leutweinii

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Figure 2.1 Distribution of the two subspecies of *Combretum apiculatum* (Carr, 1998)

The West African drug, "kinkeliba" isolated from the leaves of *C. micranthum* was found to have diuretic activity, antimicrobial activity against both Gram-positive and Gram-negative pathogens. It is used in Nigeria, Sudan and Senegal for the treatment of biliary fever, colic and vomiting, (Paris, 1942). Species of Combretaceae have been used by traditional healers throughout Africa for the treatment of various diseases e.g. pneumonia, syphilis, leprosy, wound healing, colds, chest cough, diarrhea, conjunctivitis and mumps (Hutchings *et al.* 1996) [Table 1.2]. *C. erythrophyllum* has been used by the communities of Venda of Northern South Africa for the treatment of worms, infertility and wound healing (Mabogo, 1990). And also was used as a purgative by the Zulu communities (Watt and Breyer-Brandwijk, 1962).

Table 2.2. Some of the medicinal uses of some species of Combretaceae (Rogers and Verotta, 1996)

Combretum species	**Uses**
C. apiculatum	snake bite, diarrhea, conjunctivitis, abdominal disorders
C. fragrans	chest cough, syphilis
C. erythrophyllum	fattening, tonic of dogs
C. molle	helmintics, leprosy, headaches, fever
C. hereroense	bilharziasis, headaches, infertility
C. imberbe	coughs, colds, diarrhea
C. zeyheri	toothache, cough, eye lotion, abdominal disorders.

Approximately 100 Combretaceae species occur in Africa; only about 25 species have been subjected to any scientific studies (Rogers and Verotta 1994). It is evident that this family represents an important class of potential sources of medicine. Amongst others, *C. apiculatum* has been found to have antibacterial activity although the active compounds have not been isolated yet (Eloff *et al.* 2000). This study is intends to follow up on this previous work. Other students in our laboratory have isolated antibacterial compounds from the section Angustimarginata: *C. erythrophyllum* (Martini, 1998), *C. woodii* (Famakin, 2002); from the section Conniventia: (Kotze, 2002); from the section Hypocrateropsis (Angeh, 2003). This study is the first on the section Ciliatipetala in our laboratory.

2.3. Phytochemical overview

The following classes of compounds were among those found in *Combretum* species: tannins, amino acids, phenanthrenes, stilbenoids, triterpenoids, and flavonoids (Rogers and Verotta, 1996). Some of the metabolites show cytotoxic, molluscicidal, anti-HIV and general antimicrobial and anti-inflammatory activity. Among the known angiogenesis inhibitors are flavonoids and triterpenoids, (Paper, 1998).
A brief overview of compounds isolated from Combretaceae to date, will be discussed.

2.3.1. Tannins

Tannins are water-soluble polyphenols with an ability to precipitate proteins such as gelatin from solution (Scalbert, 1991). This astringent property is the reason for their past and present use in the tanning of animal skins. Tannins are toxic towards microorganisms. Tannins play a role in food science, wood science, soil science, plant pathology, therapeutics and human and animal nutrition (Scalbert, 1991).

The action of tannins on bacteria appears to be similar to that of related synthetic phenolics such as diphenyl and diphenylalkane compounds, which have found wide application as disinfectants (Scalbert, 1991). The obvious interactions of tannins with enzymes or substrates may have led to a failure to examine other possible mechanisms involved in their biological activity. Their mode of action probably depends on the individual microorganism. Despite the antimicrobial properties of tannins, many microorganisms can grow and develop on tannin-rich materials.

Tannins are found in a variety of woody and herbaceous plants. They can be found in high quantities in any part of plants. They are classified in two groups according to their structures, hydrolysable and condensed. Hydrolysables are esters of phenolic acids and a polyol which is usually glucose (Scalbert, 1991). When dissolved in hot water they form colloidal dispersions, appearing as yellow-brown amorphous substances. They are astringent and have the ability to tan hide (Farnsworth 1966).

Condensed tannins are polymers of phenolic compounds related to the flavonoids and are similar to the hydrolysable tannins. Their toxicity towards filamentous fungi and bacteria were not found to differ significantly (Scalbert, 1991). In general *Combretum* and *Terminalia* species are the source of tannins, [fig 2.3].

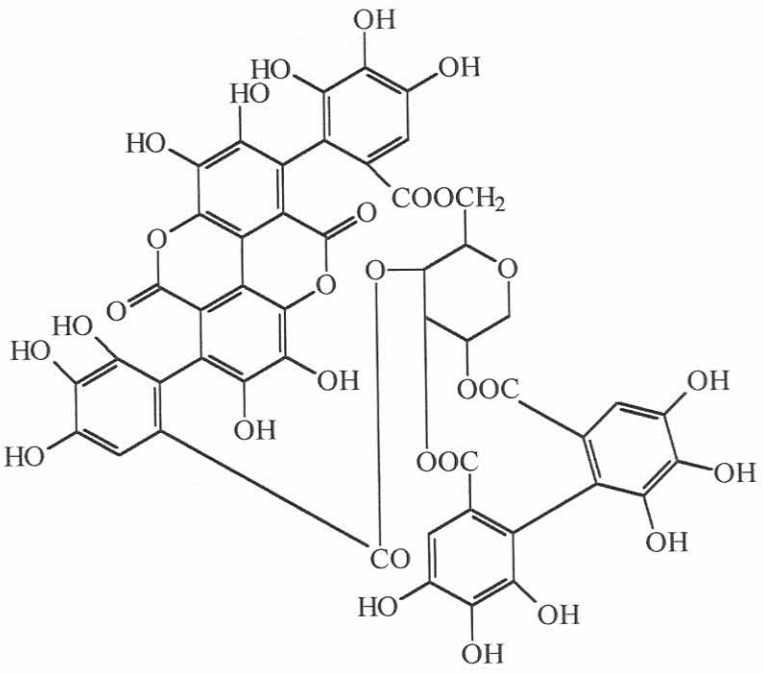


Fig.2.2. 2,3-(S)-Hexahydroxydiphenol-4,6-(S,S)-gallagylglucose (puncalagin) from *Terminalia oblongata* (Scalbert, 1991)

2.3.2. Amino acids and other nitrogen compounds

Choline, betaine and combretins A and B are nitrogenous compounds responsible for the activity of the drug "Kinkaleba" extracted from the leaves of *C. micranthum*. The amino acids N-methyl-L-tyrosine, 3-amino-methyl-2-phenylalanine was isolated from the fruits of *C. zeyheri*. The amino acids have been attributed to fungal intrusion. (Mwauluka et al., 1975; Panzini et al., 1993; Perosa, 1992).

2.3.3. Phenanthrenes

Seventeen substituted phenanthrenes and about 10 dihydrophenanthrenes have been isolated from the heartwood of *C. molle, C. psidioides,* and *C. hereroense*, (Letcher et al 1971 and 1973). Dihydrophenanthrenes, isolated from *C. caffrum*, showed reasonable antileukemic activity and inhibition of penicillin in antifungal tests, (Malan and Swinny, 1993).

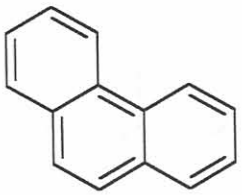


Fig.2.3. Phenanthrene ring

2.3.4. Stilbenoids

The stilbenoids isolated from *Combretum* species are known as combretastatins, (Pettit et al, 1998; Malan and Swinny, 1993). The seed of *Combretum kraussi* and *Combretum hereroense* showed the presence of combretastatins A and B as well as their glycosides. Interest in the family of Combretaceae has increased as a result of the therapeutic potential of the combretastatins, which is being clinically evaluated, (Pettit et al 1995). Combretastatins have cytotoxic activity, inhibit tubulin polymerisation and are antimitotic agents. Stilbenes have been also found in *C. caffrum, C. bracteosum* and *C. zeyheri* (Famakin, 2002).

2.3.5. Terpenoids

A variety of acidic triterpenoids and their glycosides have been isolated from the scale-like trichomes of the leaf surface of *C. molle* and *C. imberbe* [Fig. 2.5]. Many *Combretum* species also contain these substances. Isolated triterpenoids from *Combretum*

species fall under two groups, namely cycloartanes and oleananes (Pegel and Rogers 1976, 1985). They have demonstrated molluscicidal activity (Rogers 1995), exhibit good activity *in vitro* against *Listeria* species and also *Mycobacterium fortuitum* (Katerere 2001).

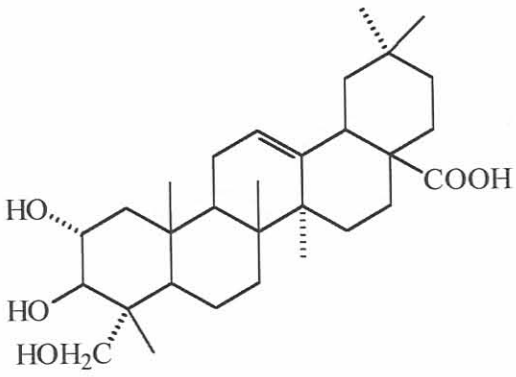


Fig 2.4. Pentacyclic terpenoid from *C. molle* (Saleh, 1994)

2.3.6. Flavonoids

In the broad sense of the word, flavonoids are virtually universal plant pigments (Farnsworth 1966). Almost always water soluble, they are responsible for the colour of flowers, fruits and sometimes leaves. A number of specific colour reactions for various types of flavonoids have been reported, colours ranging from orange to red (flavones), red to crimson (flavanols) crimson to magenta (flavanones) and occasionally to green or blue (Farnsworth, 1966). Flavonoids are universally present in the leaf cuticle and epidermal cells where they ensure tissue protection against the damaging effects of UV radiation. All flavonoids have a common biosynthetic origin and therefore possess the same structural frame, namely the 2-phenylchromane skeleton [fig 2.6.].
Flavones and flavonols represent the two largest classes of flavonoids. They occur in the plant either in the glycosidic form (in leaf, stem, flower and fruit) or in the free aglycone form (in root, bark, heartwood or external leaf surface). Common flavones such as apigenin, luteolin and chrysoeriol are encountered in the African flora. (Saleh, 1994).

Flavonoids isolated from *C. miranthum* leaves have shown antimicrobial activity against both Gram-positive and Gram-negative microorganisms, (Rogers and Verotta, 1996).

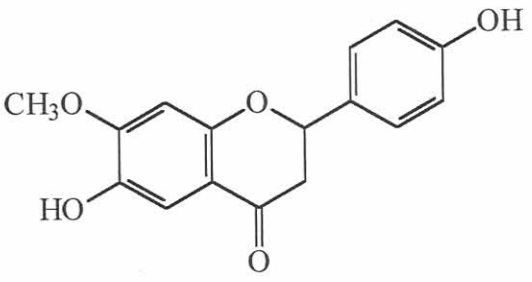


Fig.2.5. Structure of a flavone from *Terminalia arjuna* (Saleh, 1994)

2.4. Aims and objectives of this study

Previous studies (Alexander et al, 1992; Eloff, 1999) have indicated antibacterial activity in *Combretum apiculatum* leaf extracts. This is a follow up bioassay-guided study. *C. apiculatum* may contain antimicrobial compounds, that may be useful in anti-infective therapy. Furthermore studies in our laboratory have shown that other members of the same section of the genus contain several flavonoids and stilbenes with substantial antibacterial activity. A preliminary bioautographic experiment indicates that the antibacterial compounds of *C. apiculatum* differ from the other members of the Angustimarginata (Eloff, unpublished data).

This study aims to isolate and characterize antibacterial compounds from *C. apiculatum*. This will be attempted by:

- Determining the best extractant for antibacterial compounds present in *C. apiculatum*.
- Using different techniques to isolate antibacterial compounds by bioassay guided fractionation.

- Determining the chemical structure of isolated compounds.
- Determining antibacterial activity of isolated compounds.

CHAPTER 3

MATERIALS AND METHODS

3.1. Sample Preparation and selection of best extractant

C. apiculatum leaves were collected from a tree in the Lowveld National Botanical Gardens, Nelspruit. The plant label and a site map identified the tree. A voucher specimen is kept in the Garden Herbarium. The leaves were collected in April 2001, allowed to dry for 2 months in the shade at room temperature. Stems and thick veins were removed. The leaves were ground to a fine powder in a Junkel and Kunkel Model A10 mill.

The powder was initially extracted on a shaking machine with a 10:1 solvent to dry weight ratio for 4 hours, 6 hours and 24 hours. Later 0,5 g of powder was extracted with 5 ml of the extractant in a centrifuge tube by vigorous shaking and the extract was decanted after centrifuging at 3000-x g for 5 minutes. The solvents used were of technical grade (Merck). For quantitative determination, solvent from extracts placed in pre-weighed glass vials was removed by a stream of air at room temperature. The ten different solvents used were Hexane (HE), Isopropyl ether (IE), Diethyl ether (EE), Methylene dichloride (MD), Ethyl acetate (EA), Tetrahydrofuran (TH), Acetone (AC), Methanol (ME), Ethanol (ET) and Water (WA). To afford quantitative extraction; the procedure was repeated twice. The purpose of extracting the leaf material with the ten different solvents was to establish which one would extract most antibacterial components in the least chemically complex fraction.

3.2. Microorganisms used in Bioassay

The following served as test organisms in this study.

Staphylococcus aureus *(ATCC 292163),* Gram-positive

Enterococcus faecalis (ATCC 29212), Gram-positive
Escherichia coli (ATCC 27853), Gram-negative
Pseudomonas aeruginosa *(ATCC 25922),* Gram-negative

These microorganisms were chosen on the basis that they are the common cause of nosocomial infections in hospitals, (Sacho et al 1993). They are the reference strains recommended by the National Committee for Clinical Laboratory Standards, Villanova, Pennsylvania. (NCCLS, 1990). Bacteria were grown at 37°C in Mueller-Hinton broth for 4 to 6 hours, then stored at 8°C. Every 6 months a new liquid culture was established from agar cultures.

3.3. Thin layer chromatography (TLC)

Thin layer chromatography was carried out using the TLC F254 MERCK plates with the following solvent systems developed in our laboratory:

- Chloroform/ethyl acetate/formic acid, (CEF) [5:4:1],
- Benzene/ethanol/ammonia (BEA), [36:4:0:4],
- Ethyl acetate/methanol/water (EMW), [40: 5.4: 4.0].

The solvent systems separate components with a wide range of polarities and different R_f values.

In this study; 20 x 20 cm aluminium plates were cut in half and prepared in duplicate for each of the solvent systems described above. To each plate, 5 µl of a 20 mg extract/ml solution (ie: 100 µg) different extracts was applied in a line of about 1 cm length. The plates were then put into the TLC tanks to develop for 10-20 minutes. The tanks were saturated before development by lining with filter paper moistened with the eluent. The separated components were visualized under UV light at 240 nm and 360 nm. They were also sprayed with spray reagents and heated at 100°C. Spray reagents (SR) used were:

- Vanillin SR, was prepared as follows: 0.3 g of vanillin powder dissolved in 84 ml of methanol to which 3 ml of sulphuric acid was added carefully.

- Anisaldehyde SR, was prepared as follows: 1 ml of *para*-anisaldehyde was added to 18 ml of ethanol and 1 ml of sulphuric acid was added carefully.

3.4. Microplate Bioassay

In the assay of extracts for antimicrobial activity, some scientists have been using an agar plate diffusion technique. An extract is placed in a hole punched in a nutrient agar plate seeded with the test organism. A zone of growth inhibition indicates bioactivity. The pitfalls with this agar diffusion assay are that, agar is hydrophilic and the components of a complex extract may diffuse at different rates. The correlation between zone of inhibition and MIC values is very low (Eloff, unpublished data).

In this study, a serial dilution microplate technique was used to determine the minimum inhibitory concentration (MIC) values of the extracts to the microorganisms (Eloff, 2000). In brief, 100 µl of water was added to all the wells of the microplate in relevant order using multichannel micropipette. To the 100 µl of water in each of the first wells, 100 µl of different extracts at 10 mg/ml concentrations was added. This reduced the concentration of each well by 50% of the original concentration. When all extracts have been diluted in this manner, multichannel micropipette was used to remove 100 µl from the first wells into the wells of the next row. This was done all the way down to the last well. The concentration was therefore reduced from 100% down to 0.39% in the wells of the eight row. Caution was taken not to contaminate extracts between the wells by rinsing the pipette thoroughly with small quantity of water between each application. To each of the diluted wells, 100 µl of actively growing culture of bacteria was added, see section 3.2 above. The microplate was covered and incubated overnight at 37°C, after which 40 µl of a 0.2 mg/ml *para*-iodonitrotetrazolium (INT) (Sigma) solution was added to each well. The microplates were examined for colour changes after 30, 60, and 120 minutes of incubation at 37 °C. The lowest concentration at which a decrease in red colour is apparent compared to the next dilution is taken as the MIC value. Bacterial growth is indicated by the red color of the INT reduced to formazan, (Eloff, 2000).

3.5. Bioautographic assay

Bioautography is used to determine the number of active compounds present. The bioautographic procedure described by Begue and Kline (1972) was used. The TLC plates were spotted with the extracts, and developed using the eluants described in section 3.3. The plates were dried overnight in a stream of air to remove all traces of the eluents. The following day, the plates were sprayed with a concentrated suspension of an actively growing culture of the four test organisms discussed in section 3.2. The plates were then incubated overnight at 37°C and 100% relative humidity. This was followed by the spraying of the plates with a solution of INT to detect biological activity on the chromatograms. Clear zones on the chromatograms indicated inhibition of growth.

3.6. Solvent/solvent fractionation

Solvent/solvent fractionation was used as a preliminary separation to simplify the complex extracts. The USA National Cancer Institute has used this method (Suffness and Douros, 1979). The *C. apiculatum* leaf powder (mg) was extracted on a shaking machine with a 10:1(mg:ml) ratio of acetone to powder. This was repeated three times. The extracts were decanted and filtered, combined and dried under reduced pressure using a rotary evaporator. The dried acetone extract was dissolved in a 1:1 mixture of chloroform and water. The separated water fraction was extracted with an equal volume of n-butanol in a separatory funnel to yield the water (W) and the butanol (B) fractions. The chloroform fraction was taken to dryness in a rotary evaporator under reduced pressure and extracted with a 1:1 mixture of hexane (HE) and 10% water in methanol (10%W/M). The hexane fraction was recovered in a separatory funnel while the 10% W/M was diluted to 20% water in methanol and extracted with carbon tetrachloride to yield the carbon tetrachloride (CT) fraction. The 20% water in methanol was further diluted to a 35% water in methanol and extracted with chloroform to yield the 35% water in methanol (W/M) and the chloroform (CF) extracts. In all cases, equal volumes of the solvents were used and the extraction process repeated with a small volume approximately two or more times. TLC and bioautographic assay of the six fractions obtained was carried out as

described in sections 3.3 and 3.5 respectively. R_f values were also determined from the bioautographic plates.

3.7. Column chromatography

Column chromatography is used in the isolation of different components from crude extract. The column is prepared by packing a solid stationery phase onto which the sample mixture is applied. A mobile phase is then allowed to move down the column by gravity. The constituents of the mixture elute at different rates through the column as bands.

In this study, a one meter by 25 mm diameter glass column was packed with silica gel slurry in hexane. Caution was taken to prevent the formation of bubbles within the column. The glass column was then clamped in an upright position with its tap carefully controlled to regulate the flow. The CF fraction of about 4.44 g was applied onto the top of the column and the mobile phase allowed to move down the column through gravity. The mobile phase was added in portions to fill the top of the column.

Elution was started with 100% hexane and followed with a mixture of increasing proportions of dichloromethane in hexane until 100% of dichloromethane, then a mixture of dichloromethane and methanol until 100% methanol is reached. Fractions of 10 ml were collected each time. The collected fractions were concentrated in a stream of air at room temperature and analyzed by TLC.
Fractions were then pooled based on similar chemical composition. Fractions 19 to 24 appeared to have complex chemical composition. These fractions were combined and separated in the second run of the column. The column was prepared in exactly the same manner as the first one, except that this one 50 cm long with 1 cm diameter.
Bioautography using *S. aureus* and *E. coli* was carried out on the fractions. Active compounds were isolated by crystallization and subsequent washing with different solvents to remove contaminants.

3.8. Spectroscopic analysis

Nuclear magnetic resonance (NMR) is used to determine the structure of organic compounds by measuring the magnetic moments of their hydrogen and carbon atoms. In most compounds H-atoms are attached to different functional groups such as –CHO, -CH_2, -CH_3, -NH_2, -CHOH etc and the NMR spectrum provides a record of the number of H-atoms in the different positions. At least 5 – 10 mg of a sample is needed for NMR analysis. However, the advantage of using NMR over the other methods like mass spectroscopy is that the sample can be recovered and used for other tests. In practice, a sample is placed in a solution of an inert deuterated solvent, between the poles of a powerful electromagnet. The protons or carbon atoms undergo chemical shifts according to their molecular environment within the bulk molecule, upon being subjected to the radio waves. The complexity of the spectrum is related to the number of different protons and functional groups. Interpretation of the spectrum leads to the determination of the chemical structure. The spectra in this study were obtained on a 300 MHz Varian (Oxford instruments) machine at Medunsa.

Mass spectroscopy depends on the production of ions in a gaseous phase from the sample. These ions then separate according to mass to charge ratio (*m/e*). The principle of mass spectroscopy is that when an organic molecule is bombarded with electrons of sufficient energy, it loses an electron and so becomes a positive ion. The imparted excess energy accumulates in a particular bond which then cleaves. Different bonds require different energies to break. Each molecule will give rise to unique fragments. The mass spectrometer separates and records positive ions according to their mass to charge ratio (*m/e*).

CHAPTER 4

EXTRACTION AND ISOLATION OF ANTIBACTERIAL COMPOUNDS

4.1. Extraction efficiency of ten extractants

To determine the best extractant for isolating antibacterial compounds, ten different solvents were used to extract 500 mg of a finely ground leaf material of *C. apiculatum*. The extraction was done three times with 5 ml of each solvent on the same plant material. The extracting efficiency for antibacterial compounds of each solvent was determined. This was measured in terms of the percentage yield of the extracts from the original 500 mg leaf material, the chemical complexity of the extract and lastly the antibacterial activity of the components extracted by a particular extractant. The same plant material was extracted twice more to ensure maximal extraction. The individual extracts were placed in pre-weighed containers and dried in an air stream at room temperature.

Tetrahydrofuran and acetone were the best extractants by extracting 14.4% and 13.2% respectively [Table 4.1]. Water extracted only 0.8% of the original powder mass of the leaf. This indicates that *C. apiculatum* leaves contain a low concentration of very polar components because water is highly polar and hydrophilic therefore its extracting spectrum was limited to very polar and water-soluble compounds only.

The rate of extraction is calculated as the mass of the first extract divided by the total mass extracted by three successive extractions and multiplied by 100. The rate of extraction varied from 50% using water, hexane 60%, methanol 63%, methylene dichloride 72%, ethyl ether 78%, isopropyl ether 80%, acetone 85%, ethanol 86%, ethyl acetate 88% and tetrahydrofuran 89% [Fig. 4.1]. TH and AC extracted the largest quantities of 72 mg and 66 mg respectively compared to the other extractants. This indicates that *C. apiculatum* leaves contain a high concentration of intermediate polarity compounds. According to fig 4.2 the extractants with intermediate polarity were by far the most efficient in extraction. They extracted metabolites over a wide range of polarities.

Table 4.1. Extraction of 500 mg leaf material of *C. apiculatum* by three successive extractions with 5 ml of different extracts. Rate of extraction (percentage) = first extract / total extracted. Hexane (HE), Isopropyl ether (IE), Ethyl ether (EE), Methylene dichloride (MD), Ethyl acetate (EA), Tetrahydrofuran (TH), Acetone (AC), Methanol (ME), Ethanol (ET) and Water (WA).

Solvents	**HE**	**IE**	**EE**	**MD**	**EA**	**TH**	**AC**	**ET**	**ME**	**WA**
1st	6	12	14	23	21	64	56	19	10	2
2nd	3	2	2	5	2	7	9	2	3	1
3rd	1	1	2	4	1	1	1	1	3	1
Total (mg)	*10*	*15*	*18*	*32*	*24*	*72*	*66*	*22*	*16*	*4*
% Extracted	2	3	3.6	6.4	4.8	14.4	13.2	4.4	3.2	0.8
Rate Extraction	60%	80%	78%	72%	88%	89%	85%	86%	63%	50%

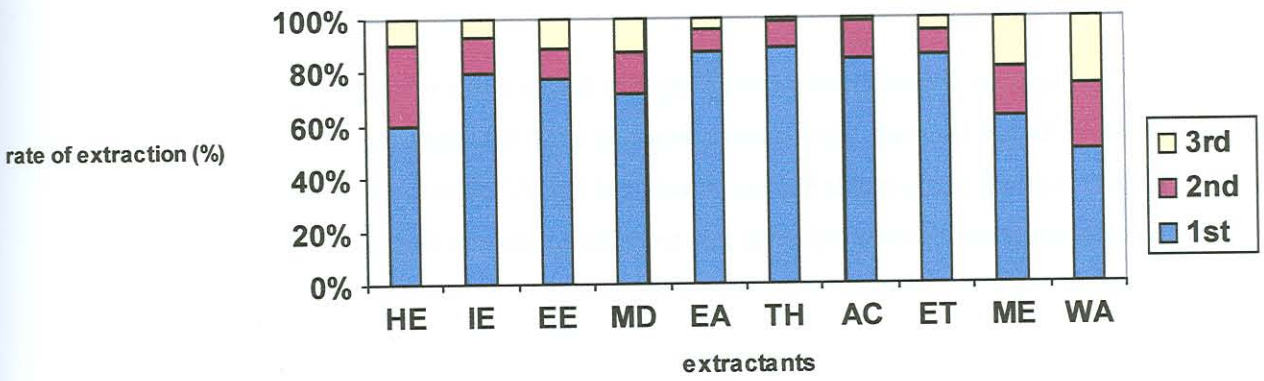


Fig.4.1 Quantities extracted as percentage of the total quantity extracted in three rounds. (Extractants arranged from non-polar to polar)

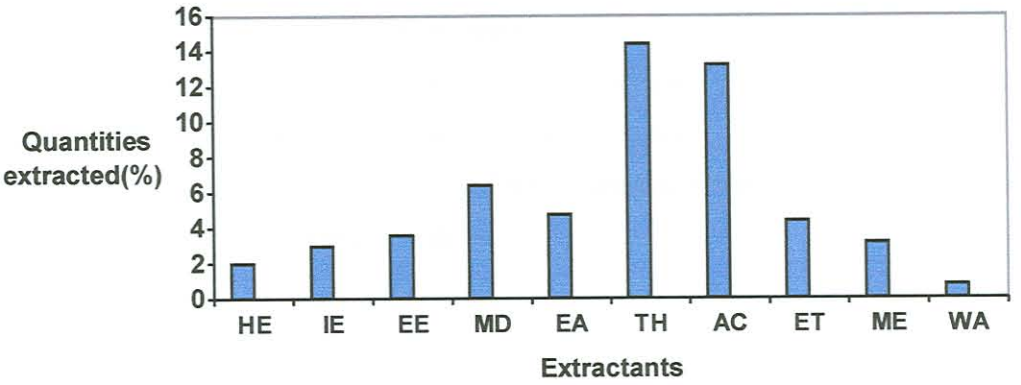


Fig.4.2. Quantities extracted by the ten different extractants in percentage of dry mass.

One would have thought that there would be a good correlation between the rate of extraction and the total quantity extracted because if the extraction rate is high, the total extraction should also be high. In some cases notably with EA, ET but also IE and EE this was not the case. This may mean that whatever EA extracts, it extracts quickly but that there are several components that are not extracted and that the chemical composition must differ. The complexity of the different extracts were therefore examined next.

4.1.1. Complexity of extracts

The different extracts were separated by developing TLC plates in EMW, CEF and BEA (section 3.3). The plates were dried and sprayed with vanillin SR and anisaldehyde SR to detect separated compounds. The components were separated well by CEF and EMW solvent systems. The following colours of components were common to all of the extractants: green, red, yellow and purple. The yellow compound appeared in all the extracts except with water. The red compound was present in all extractants except hexane, when the components were separated by BEA and CEF. Purple and green compounds appeared sparingly under the CEF and BEA on IE, EE, ET, TH and MD extractants. The BEA solvent system yielded the same colours on the constituents but the separation was not good. The water extractant showed only the red colour on all the TLC plates under the three solvent systems. Therefore, the red colour could indicate a polar constituent.

The purpose was to determine the complexity of compounds present in the extract. The larger the variety of compounds extracted by the different extractants, the better the chance that biologically active components are present in screening studies. On the other hand, the extractants that extracted the bioactive compounds preferably to non-active compounds would be the best in isolation studies.

The BEA did not give good separation of compounds in these extracts; hence it was not used as extensively as the other solventsfor further work in this study. Of the spray reagents used, anisaldehyde SR led to better detection of colours of the constituents on the chromatogram than vanillin SR and was used for further work.

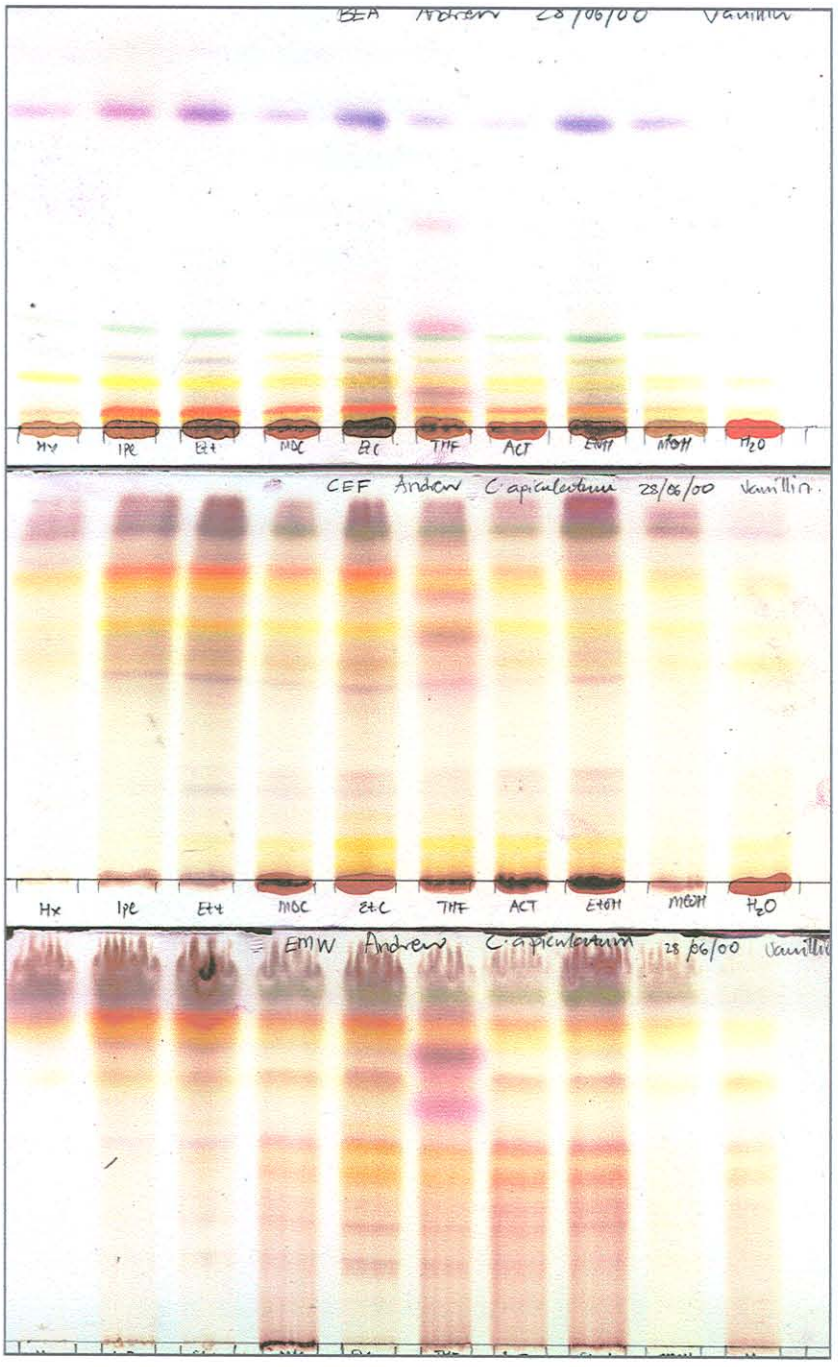


Fig.4.3. Separation of components present in 100 µg of 10 different extracts with BEA (top), EMW (bottom) and CEF (middle) as eluents using vanillin SR as spray reagent. Extractants from left: (HE), (IE), (EE), (MD), (EA), (TH), AC), (ME), (ET) and (WA).

Since different solvents are able to extract certain classes of phytochemicals based on similar polarity, one can argue that the *C. apiculatum* powdered leaves contain less fixed oils, waxes and

fats, since only solvents of low polarity, e.g. hexane can extract this. Hexane (HE) and methylene dichloride (MD) extracted fewer compounds from the extraction mixture. In addition the non-polar eluent, BEA did not separate any visible compounds after spraying with anisaldehyde SR. According to table 4.2. the spectrum of compounds present in large quantities in *C. apiculatum* may possibly be narrowed to aglycones, alkaloids, chalcones, flavonoids, glycosides and other related phytochemicals, (Houghton and Rama, 1998).

Table 4.2. Types of phytochemicals extracted by different extractants (Houghton and Rama, 1998)

Polarity	Solvent	Types of chemicals extracted
Low	Hexane	waxes, fats, fixed oils
Moderate	Dichloromethane	alkaloids, aglycones, volatile oils
	Diethyl ether	alkaloids, aglycones
	Ethyl acetate	alkaloids, aglycones, glycosides
	Acetone	alkaloids, aglycones, glycosides
	Ethanol	glycosides
	Methanol	sugars, amino acids, glycosides
High	Water	sugars, amino acids, glycosides

4.2. Bioassay of extracts using different extractants

The ten extracts were tested for antimicrobial activity. Cultures of the four bacteria described in section 3.2 were used. Minimum inhibitory concentration and total activity were determined for all extractants on the test microorganism, [Table 4.3 and 4.4] and [Figure 4.9 and 4.10]. The water and hexane extracts only had slight inhibition to the four microorganisms hence were left out on Figure 4.9 and 4.10 below.

4.2.1. Bioautography

The procedure followed is explained in section 3.5 of chapter 3. The bioautographic technique worked better with the *S. aureus* and *E. coli* than with the other two test organisms. These two were more sensitive. Most of the activity on the test organisms was exhibited by components in IE and EA extracts. There was little or no activity on extracts of very high or very low polarity. Only those with intermediate polarity showed good activity. Bioautography plates developed in EMW showed one compound, which inhibited the growth of *S. aureus*. This was found with the following extracts: IE, EE, MD, EA and ME. The same activity was exhibited by IE, EE, MD and EA using CEF. The BEA bioautogram had no clear zones of inhibition. The inhibition of the growth of *E. coli* was more distinct than that of *S. aureus* in all the solvent systems. All the extractants under EMW and CEF inhibited the growth of *E. coli* by one compound. The zones of inhibition in CEF were clearer than those in EMW. Only IE, EE, MD and EA inhibited the growth of *E. coli* with one compound.

P. aeruginosa was more resistant to all extracts. It is a Gram-negative microorganism with known ability to resist antimicrobial regimens due to the nature of its cell wall (Nakae et al, 1986 in Goodman and Gilman, 1996). Polar and non-polar compounds contain yellow or brown colours and some of these compounds may mask the inhibition of the growth of the test organism (Martini and Eloff, 1998).

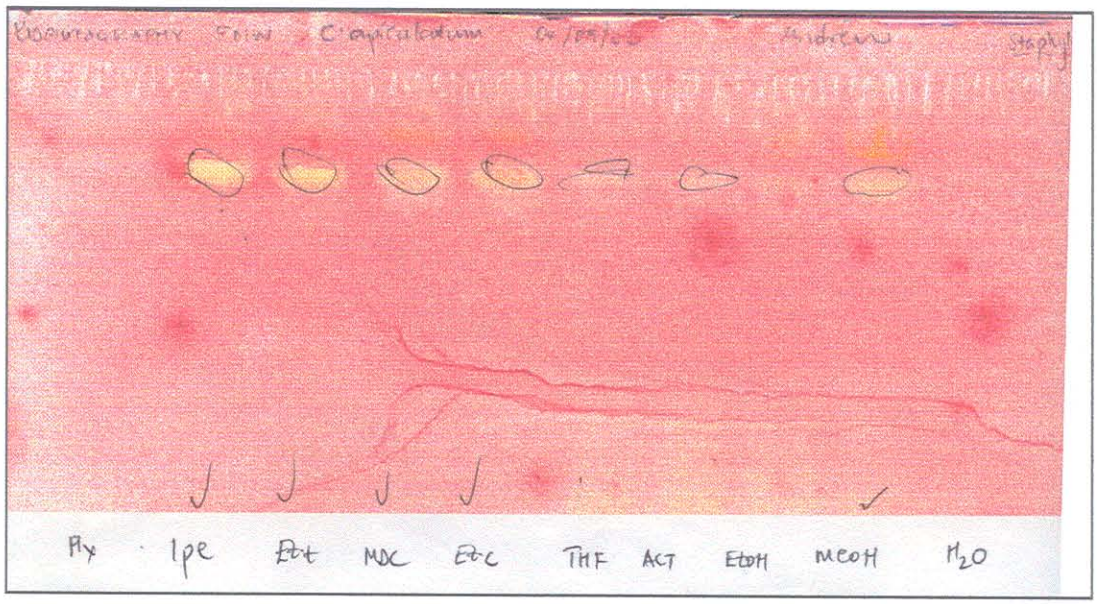


Fig. 4.4 Bioautogram of *C. apiculatum* leaves extracted by 10 different extractants. TLC developed in EMW and sprayed with *S. aureus* culture, incubated overnight then sprayed with INT solution.

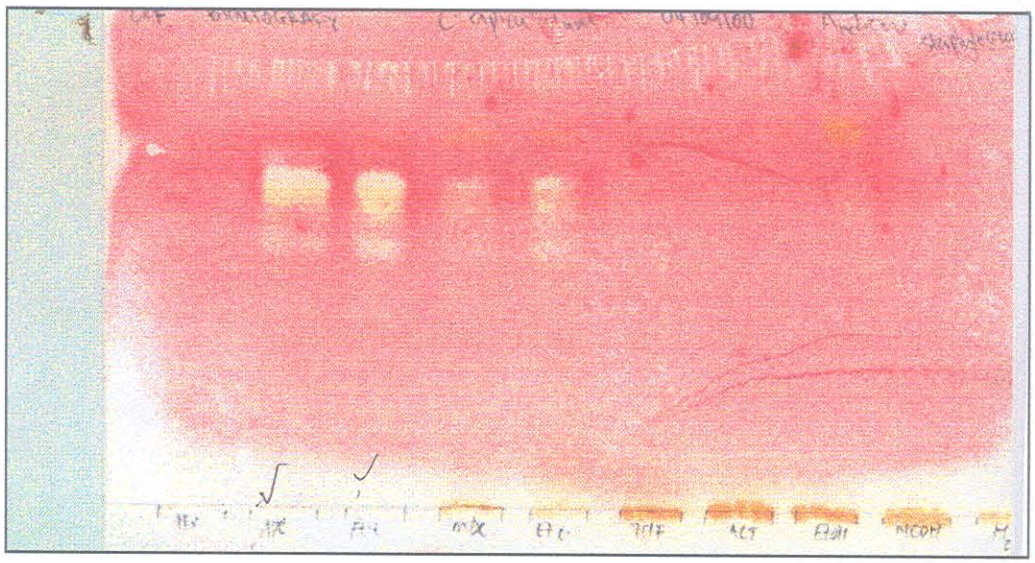


Fig. 4.5 Bioautogram of *C. apiculatum* leaves extracted by 10 different extractants. TLC developed in CEF and sprayed with *S. aureus* culture, incubated overnight then sprayed with INT solution.

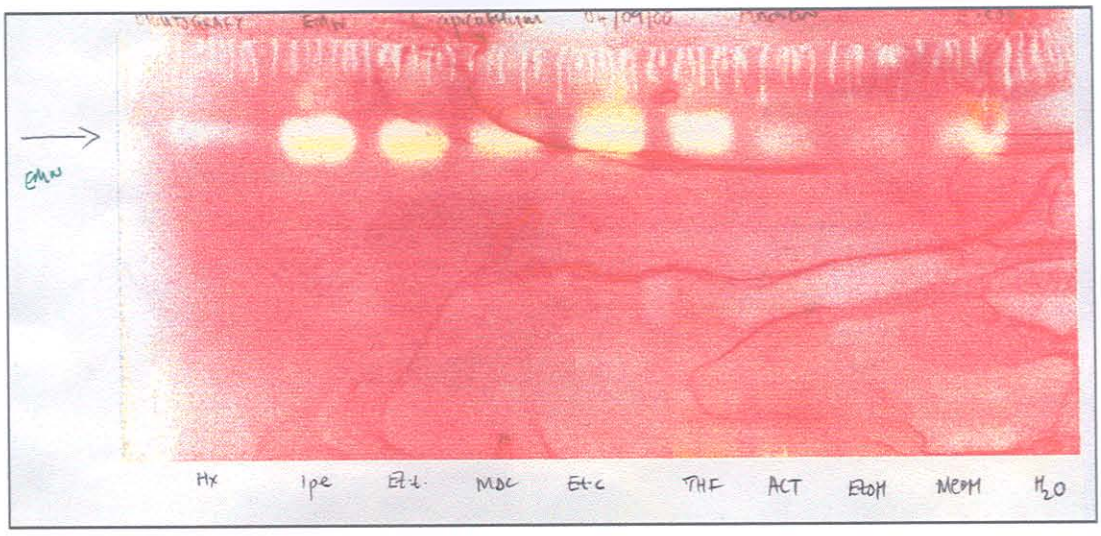


Fig. 4.6 Bioautogram of *C. apiculatum* leaves extracted by 10 different extractants. TLC developed in EMW and sprayed with *E. coli* culture, incubated overnight then sprayed with INT solution.

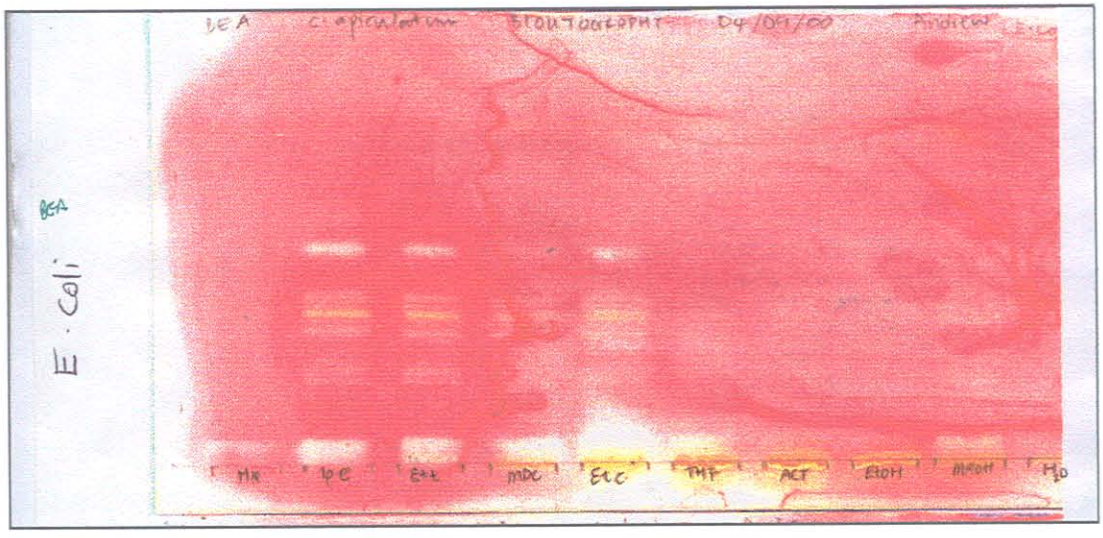


Fig. 4.7 Bioautogram of *C. apiculatum* leaves extracted by 10 different extractants. TLC developed in BEA and sprayed with *E. coli* culture, incubated overnight then sprayed with INT solution.

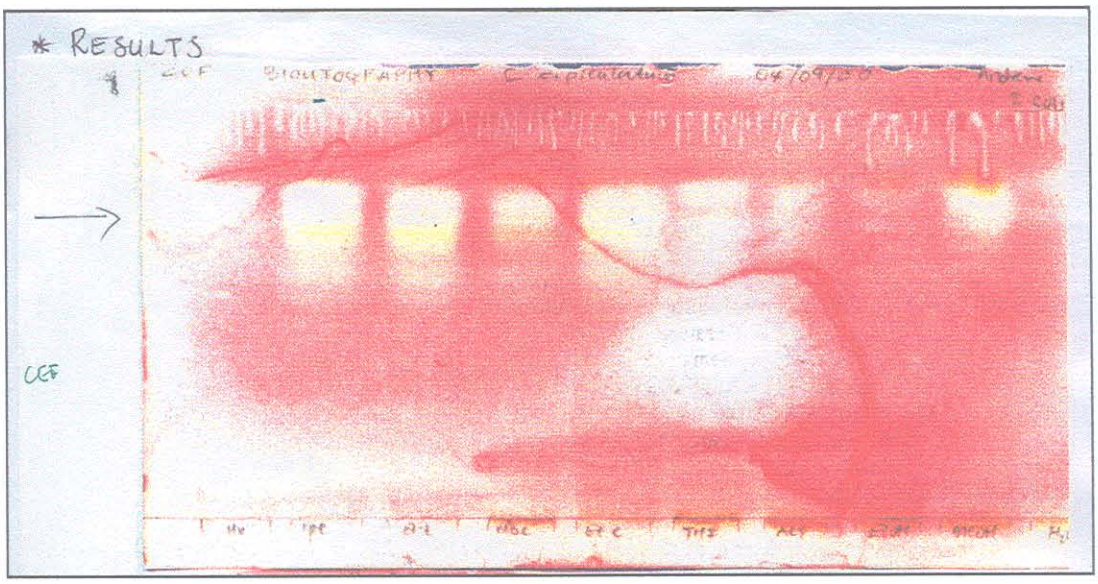


Fig. 4.8 Bioautogram of *C. apiculatum* leaves extracted by 10 different extractants. TLC developed in CEF and sprayed with *E. coli* culture, incubated overnight then sprayed with INT solution.

4.2.2. Minimum inhibitory concentration (MIC)

The MIC was determined as explained in section 3.4 above. Extracts were dried and reconsituted in acetone in all cases. Acetone control did not inhibit the bacterial growth rate. All the extractants except water and hexane inhibited the growth of *E. coli* at relatively low concentrations of 0.1 mg /ml. *E. faecalis* and *S. aureus* followed with an average MIC value of 0.2 mg/ml. The MIC values of on all the extractants ranged from 0.04 mg /ml to 2.5 mg/ml. EE and EA had the lowest MIC values of 0.04 mg /ml on the activity of *E. faecalis*. This however differs with the results obtained in the bioautography on the same microorganism. Water on the contrary had the highest value of 2.5 mg/ml. The average MIC value of 0.11 mg /ml was exhibited by EA. *P. aeruginosa* was resistant to most of the extracts.

Table 4.3. MIC values in mg/ml of *C. apiculatum* leaves, extracted with ten different extractants (HE), (IE), (EE), (MD), (EA), (TH), (AC), (ME), (ET) and (WA).

Solvents	IE	EE	MD	EA	TH	AC	ET	ME	HE	WA
MIC values (mg/ml)										
E. coli	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.6	0.6
P.aeruginosa	0.2	0.2	0.2	0.1	0.6	0.6	0.2	0.1	0.3	2.5
E. faecalis	0.1	0.04	0.2	0.04	0.2	0.2	0.3	0.3	0.3	0.5
S. aureus	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.1	0.6	1.3
Average	0.15	0.4	0.18	0.11	0.3	0.3	0.2	0.15	0.45	1.23

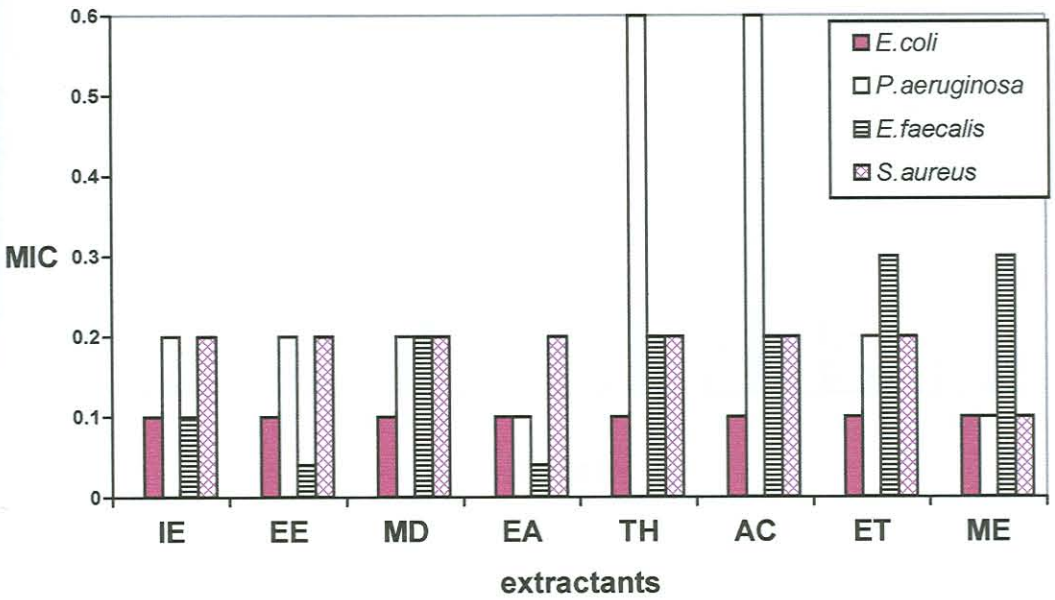


Figure 4.9. Minimum inhibitory concentrations (mg/ml) of the different extracts on the test organisms: *S. aureus, E. coli, P. aeruginosa and E. faecalis.*

Total activity is defined as the total volume (ml) to which the bioactive compounds present in 1 g of dried leaf material or extract can be diluted and still inhibit the growth of the test microorganism. Total activity = Quantity extracted ($mg.g^{-1}$) / MIC ($mg.ml^{-1}$) (Eloff, 2000).

Table 4.4. Total activity in ml/g of *C. apiculatum* leaves, extracted with ten different extractants. (HE), (IE), (EE), (MD), (EA), (TH), (AC), (ME), (ET) and (WA).

Solvents	IE	EE	MD	EA	TH	AC	ET	ME	HE	WA
Total activity (ml)										
E. coli	307	358	588	537	163	1433	486	256	19	6
P.aeruginosa	153	179	294	537	204	179	243	255	38	2
E. faecalis	307	716	294	1075	819	717	121	64	38	1
S. aureus	153	179	294	268	820	717	243	256	19	3
Average	230	358	368	604	502	762	273	208	29	3

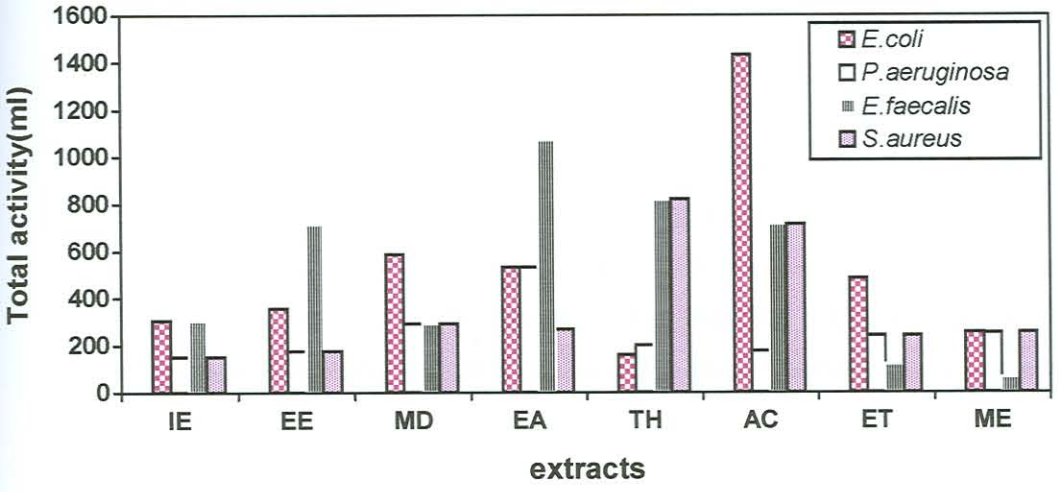


Figure 4.10. Total activity in ml/g of the different extractants on the test organisms: *S. aureus, E. coli, P. aeruginosa and E. faecalis.*

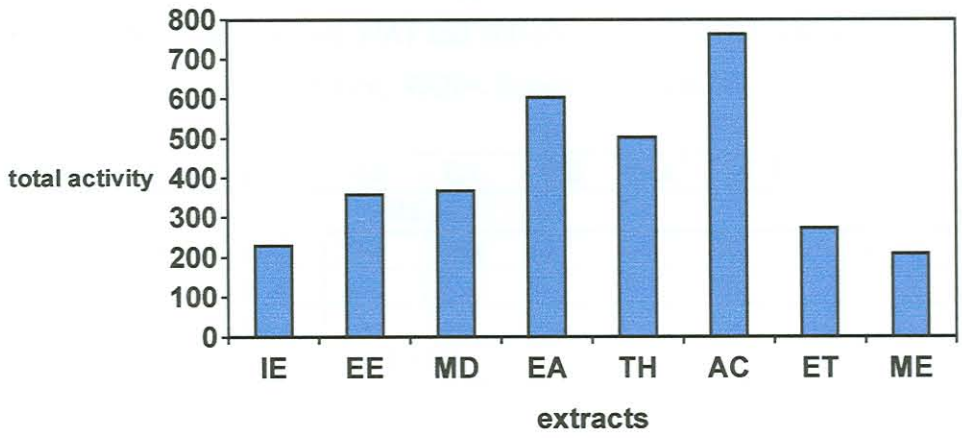


Figure 4.11. Average total activity of the different extracts on the test organisms: *S. aureus, E. coli, P. aeruginosa* and *E. faecalis.*

Even though AC did not show high activity against *P. aeruginosa*, it had low MIC values on the other entire test organism, hence high activity. AC and EA had high average total activity of 762 and 604 respectively. Consequently AC was used in the large-scale extraction to isolate bioactive compounds. EA would also have been a good candidate as extractant especially if compounds active against *E. faecalis* were to be isolated. The lower volatility and non-water miscibility of EA, however decreases its usefulness.

It appears that the active principles shown by the chromatograms could be acidic compounds because the R_f value in the relatively non-polar acidic CEF was nearly as high as in the much polar neutral EMW. In acidic medium compounds with an ionizable hydroxyl group will be in the unionized (non-polar) form whereas in a neutral solvent system the compounds would be ionized (polar).

The compounds that were active against *E. coli* had R_f values ranging from 0.4 to 0.8; against *S. aureus.* R_f values from 0.5 to 0.7 and against *E. faecalis* R_f values from 0.1 to 0.8. This was using the three solvent systems, BEA, EMW and CEF. The compound that exhibited activity against both *E. coli* and *S. aureus* had the same R_f value of 0.8. This compound showed this activity using EMW and CEF. Compounds active against *E. coli* only had R_f values of 0.1 and 0.5 with BEA as solvent system.

Table 4.5. R_f values of compounds inhibiting *E. coli* growth using different extractants (HE, IE, EE, MD, EA, TH, AC, ME, ET and WA) and solvent systems. (CEF, BEA and EMW). X=. Slight inhibition, XX=. Moderate inhibition, XXX=. Strong inhibition.

	HE	IE	EE	MD	EA	TH	AC	ET	ME	WA
BEA										
0.1	x	xx	xx	xxx	xxx	x			x	
0.3			x	x						
0.5		x	xx							
EMW										
0.8	xx	xx	xx	xx	xx	xx	x	x	x	x
CEF										
0.4						x				
0.7						x				
0.8	xx	xx	xx	x	xx	x	x	x		

Acetone, water and ethanol extracts did not show activity against *E. coli* in the non-polar BEA, solvent system. Slight activity was exhibited on the plates developed in EMW and CEF. This shows that the active components appear to be non-polar because they could only be separated by non-polar solvent systems. This is in contrast to other extracts, which showed a slight inhibition.

Table 4.6. R_f values of compounds inhibiting *S. aureus* growth using different extractants (HE, IE, EE, MD, EA, TH, AC, ME, ET and WA) and solvent systems. (CEF, BEA and EMW). X=. Slight inhibition, XX=. Moderate inhibition,
XXX=. Strong inhibition.

	HE	IE	EE	MD	EA	TH	AC	ET	ME	WA
CEF										
0.7		xx	xx	x	x					
0.4		x	x							
EMW										
0.8		x	x	x	x				x	

Inhibition of growth could only be seen under EMW and CEF. The BEA chromatogram did not show any clear zone that could be associated with inhibition. The active extracts in this regard were isopropyl ether (IE), ethyl ether (EE), methylene dichloride (MD) and ethyl acetate (EA).

Table 4.7. R_f values of compounds inhibiting *E. faecalis* growth using different extractants (HE, IE, EE, MD, EA, TH, AC, ME, ET and WA) and solvent systems. (CEF, BEA and EMW). X=. Slight inhibition, XX=. Moderate inhibition, XXX=. Strong inhibition.

	HE	IE	EE	MD	EA	TH	AC	ET	ME	WA
BEA										
0.1		x	x		x					
EMW										
0.8		x	x	x	x				x	

E. faecalis was only slightly inhibited by a compound with a R_f value of 0.1 in BEA and 0.8 in EMW. No inhibition was found when CEF was used as solvent system.

4.2.3. Conclusion

The most effective extractants are TH and AC according to total quantity extracted and AC and EA according to the total antibacterial activity. Although the bioautography results with acetone were not as good as with other extractants, acetone was used for the large-scale extraction for the following reasons (Eloff, 1998):

- Acetone dissolves both hydrophilic and lipophilic compounds.
- It is miscible with water
- It has low toxicity to the test microorganisms in the bioassay
- It is easy to remove by evaporation
- It is non-toxic to humans
- Other students in our group have used it and it makes comparisons with their results easier

4.3. Large-scale extraction and group separation of extracts

4.3.1. Fractionation of extracts

The purpose was to extract and separate the extracts of the *C. apiculatum* leaf into groups of varying polarities by solvent/solvent fractionation. By simplifying the extracts, it may be easier to isolate the antibacterial compounds, if it were present in only one or two fractions, containing fewer non-active compounds.

4.3.2. Extracted quantities

The finely ground *C. apiculatum* leaf powder (100 g) was extracted with 1000 ml of acetone three times. The extract was dried under vacuum in a Buchi; PE 120 rotary evaporator to yield 16.484 g. The dried extract was dissolved with equal volumes of chloroform and water in a separating funnel. The procedure was followed as discussed in section 3.6. The exact quantities of fractions are shown in Fig.4.12 and Table 4.8 below.

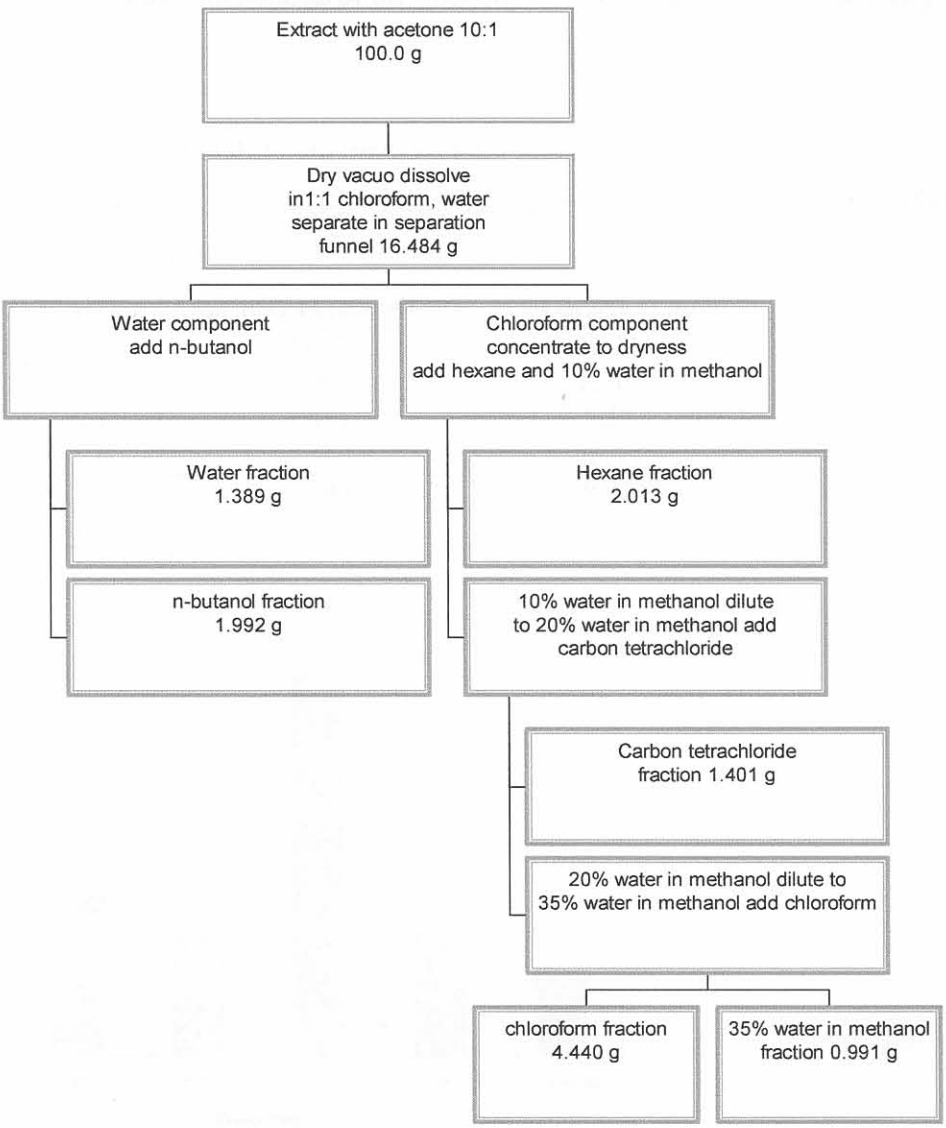


Figure 4.12. The procedure and quantities obtained during the solvent/solvent fractionation of the components in the *C. apiculatum* leaf extract.

The different fractions were dried in a pre-weighed round bottom flask. The following quantities of extracts were collected, CF (4.440 g), HE (2.013 g), CT (1.4001 g), B (1.992 g), W (1.389 g) and W/M (0.991 g) [table 4.8]. The fractionation started with 16.484 g crude extract of acetone. Total quantity extracted by different extracts was 12.226 g. The highest percentage of acetone extract was in chloroform (26.9%), the lowest was 6.0% extracted by 35% W/M. The quantity of the extract lost during fractionation was approximately 4.258 g. It is not easy to explain how nearly 25% of the extract was lost. The pellicle formed in some cases could not represent such a large loss. Possibly some of the components of the fractions were volatile and were lost during drying.

Table 4.8. Quantities (mg) present in different fractions after solvent/solvent fractionation of *C. apiculatum* leaf extract, water (W), butanol (B), hexane (H), carbon tetrachloride (CT), chloroform (CF) and 35% water in methanol (W/M)

Fraction	Quantity extracted (g)	% of original mass
HE	2.013	12.21
B	1.992	12.10
CT	1.401	8.50
CF	4.440	26.93
W	1.389	8.42
W/M	0.991	6.01

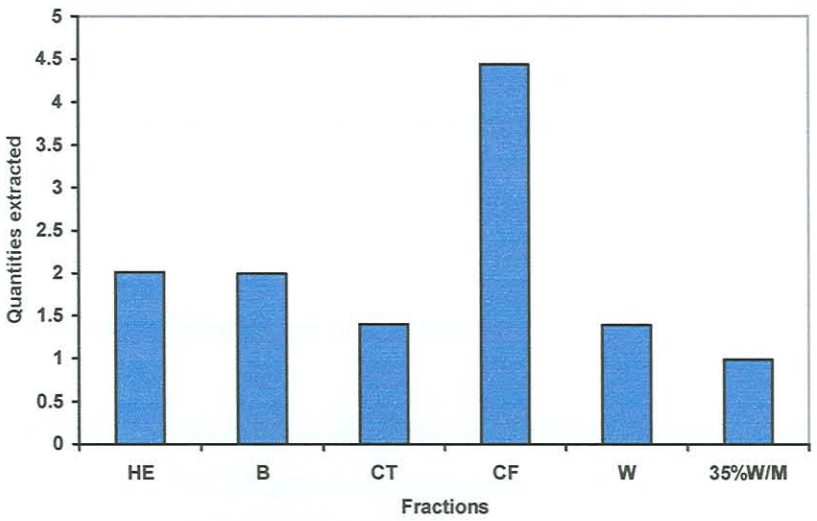


Figure 4.13. Quantities (mg) separated into different fractions by the solvent/solvent fractionation of *Combretum apiculatum* leaf extract.

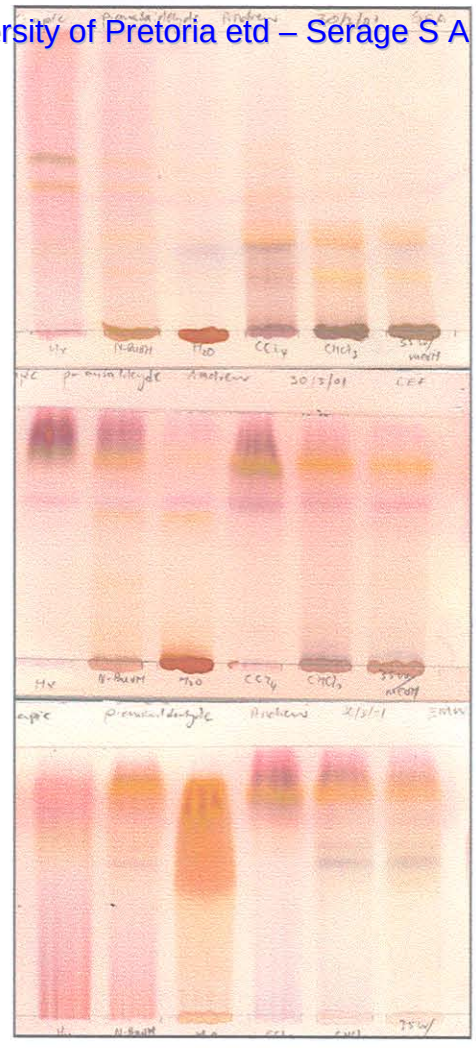


Fig.4.14. Chemical composition of solvent/solvent fractions separated by BEA (top), CEF (middle) and EMW (bottom) as eluants. Compounds were visualized using anisaldehyde SR. Fractions from left Hexane (H), butanol (B), water (W), carbon tetrachloride (CT), chloroform (CF) and 35% water in methanol, (W/M)

4.3.3. Chemical composition of fractions

To determine the chemical complexity of the different fractions, 100 µg of each fraction was separated using TLC [Figure 4.14]. Anisaldehyde SR was used to spray the TLC plates. The EMW and CEF separated the components better than BEA did. The CF, CT and M/W fractions contained more components than the other fractions. As observed with the plates from the preliminary extraction, the most dominating constituents were those with a red, purple and yellow colours. The three colours were distinct on the CF and CT fractions. The water fraction was slightly separated in

the BEA and CEF solvent systems. However, all the fractions were separated well with EMW and CEF as solvent systems. Consequently the major constituents present in the extracts were moderately polar to polar.

4.3.4. Bioassay of fractions

The different fractions were tested for antimicrobial activity using the test organisms discussed above, section 3.2. The compounds, which inhibited *S. aureus*, had the same R_f values as obtained under extraction. *E. coli* was inhibited by compounds with R_f values of 0.1, 0.3, 0.4 and 0.8, table 4.9. According to chromatograms the acetone extract contains more than one compound that inhibit *S. aureus* and *E. coli*. The compounds with R_f values of 0.8, 0.4 and 0.5 correspond to the yellow and purple compounds obtained under extraction. Constituents in the CF extract showed good inhibition. Inhibition of *E. coli* was clearer than that on *S. aureus*.

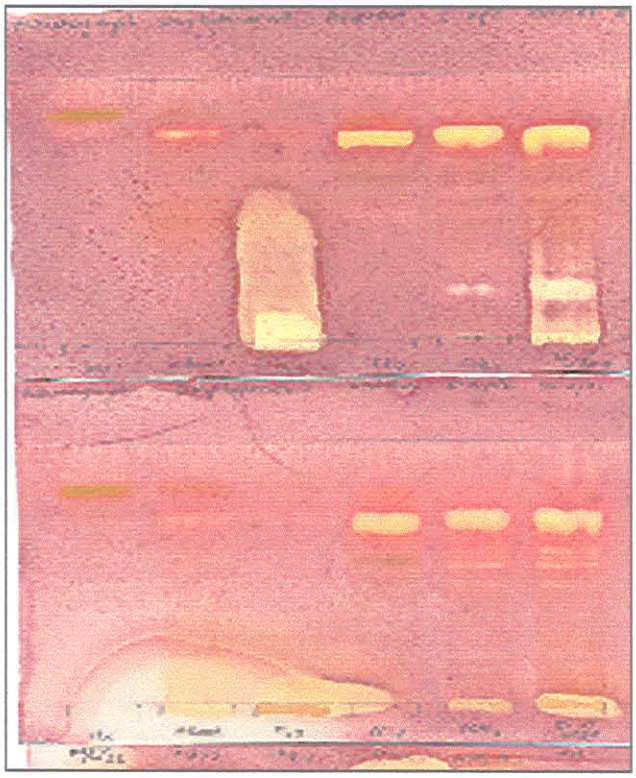


Fig.4.15. Bioautogram of six different fractions of *C. apiculatum* extract separated by solvent/solvent extraction. TLC plates were sprayed with *S. aureus.* Solvent systems used EMW (top) and CEF (bottom). Fractions from left hexane (H), butanol (B), water (W), carbon tetrachloride (CT), chloroform (CF) and 35% water in methanol, (W/M).

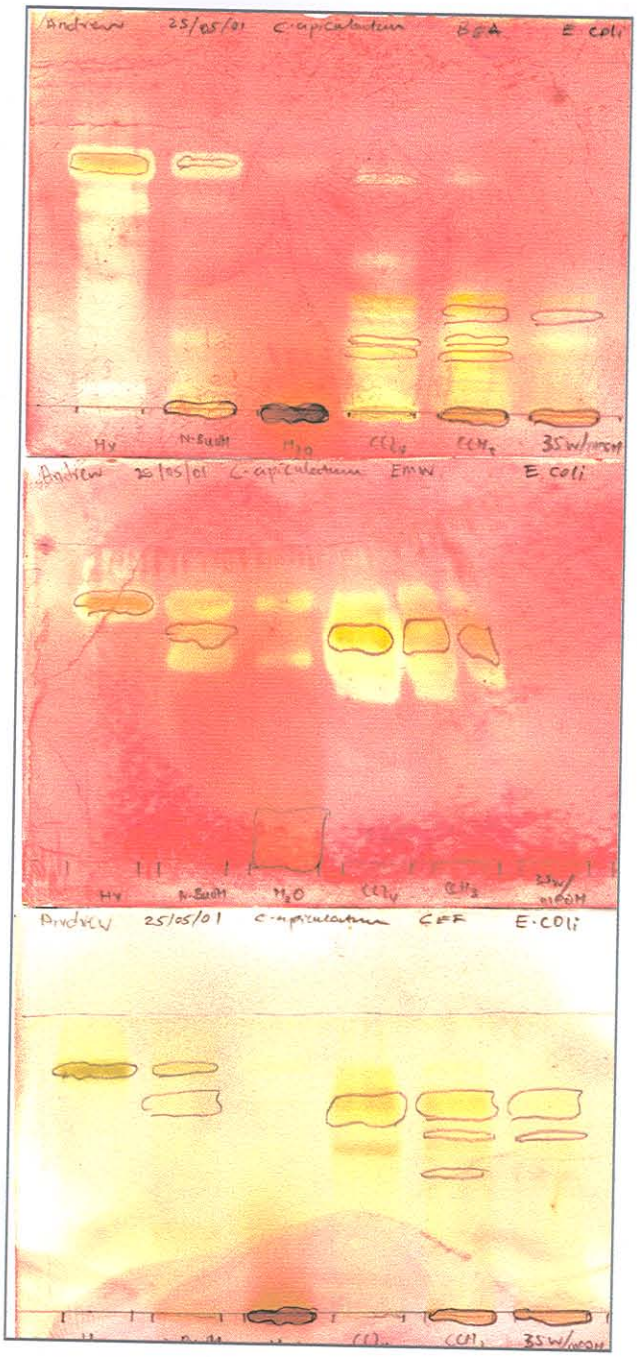


Fig.4.16. Bioautogram of six different fractions of *C. apiculatum* extract separated by solvent/solvent extraction. TLC plates were sprayed with *E. coli.* Solvent systems used BEA (top), EMW (middle) and CEF (bottom). Fractions from left hexane (H), butanol (B), water (W), carbon tetrachloride (CT), chloroform (CF) and 35% water in methanol, (W/M).

Table 4.9. R_f values of antibacterial compounds from CF, CT and 35%W/M fractions using *E. coli* as test organism, and BEA as solvent system. (X = slight inhibition, XX= moderate inhibition, XXX= strong inhibition)

R_f values	CF	CT	W/M
0.8		x	
0.4	x		x
0.3	x	x	
0.1	x	x	

Table 4.10. R_f values of antibacterial compounds from CF, CT and 35%W/M fractions using *S. aureus* as test organism and EMW as solvent system. (X= slight inhibition, XX= moderate inhibition, XXX= strong inhibition)

R_f values	CF	CT	35%W/M
0.8	xx	xx	xx
0.2	x		xx

4.3.5 Selecting best fraction

Further work was done on the chloroform extract because it displayed good activity against the test organisms. In comparing the bioautograms it must be kept in mind that the same quantity was separated from each fraction. The selection of the fraction to be investigated further, total quantity was considered. The CF fraction contained nearly the same quantity as the B, CT and W fractions combined. Based on the antibacterial activity and mass of each fraction, the CF fraction was the most promising and this was used for further work.

4.4. Isolation by bioassay guided fractionation

4.4.1. Introduction

Further work using column chromatography to isolate bioactive constituents was carried out on the chloroform (CF) fraction obtained from the group separation of extracts (section 3.6). The procedure for isolating the compounds is summarized in section 3.7 and shown schematically by figure 4.18 below.

The 100 x 2.5 cm glass column was packed with freshly prepared silica gel slurry. About 4.44 g of CF extract was loaded onto the column. The column was developed with hexane, chloroform and methanol. Fractions were collected into test tubes in volumes of approximately 50 ml. A total of 248 test tubes were collected at the end of the run. These were combined into 29 fractions based on similar chemical complexity after analyzing the fractions by TLC.
Fractions 1-18 and 25-29 were relatively simple and were dried in a stream of air in a fume cupboard and crystals formed, which were recrystalized from methanol, chloroform and hexane to yield relatively pure compounds. The complexity of fractions 19 to 24 necessitated the setting up of another column for further separation.

The second column of 1 x 50 cm long was set up using silica gel slurry made in hexane. Chloroform and methanol mixtures were used as mobile phases. Many fractions were collected and after TLC were pooled into fractions which were combined with fractions containing the same compounds. Bioautography was carried out on the same 29 fractions. Ten fractions, which had biological activity from both the 1st and 2nd columns, were selected. The components were separated by TLC and antibacterial activity determined by bioautography on these selected fractions fig 4.21

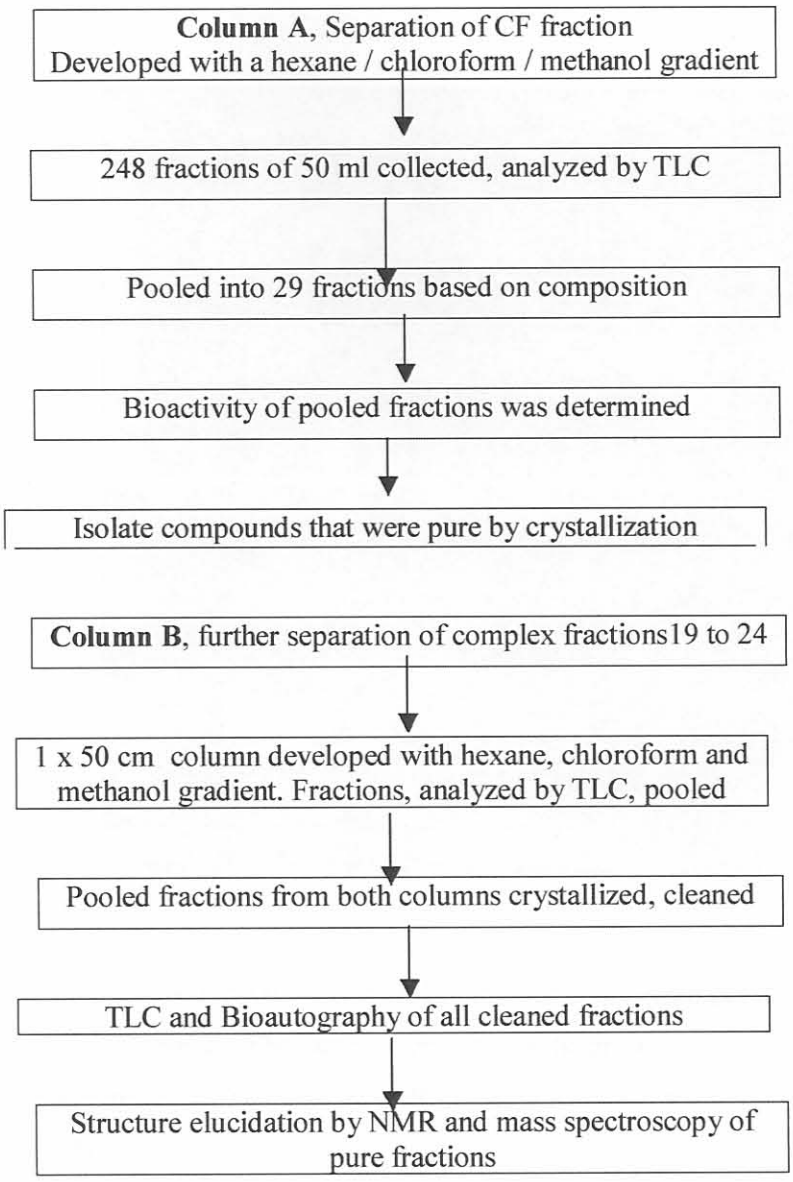


Figure 4.17. Steps followed in the extraction and characterization of active compounds

4.4.2. Antibacterial activity of fractions

Bioautography was carried out on the 29 pooled fractions to establish which of the fractions had bioactive compounds. Test microorganisms, *E. coli* and *S. aureus* were used for the bioassay, because these microorganisms were more sensitive to the chloroform extract (Fig 4.15 and 4.16). An aliquot from the fractions was spotted onto the TLC plates and the procedure followed as described in section 3.3 and 3.5. This revealed the presence of compounds of interest. Figure 4.21.

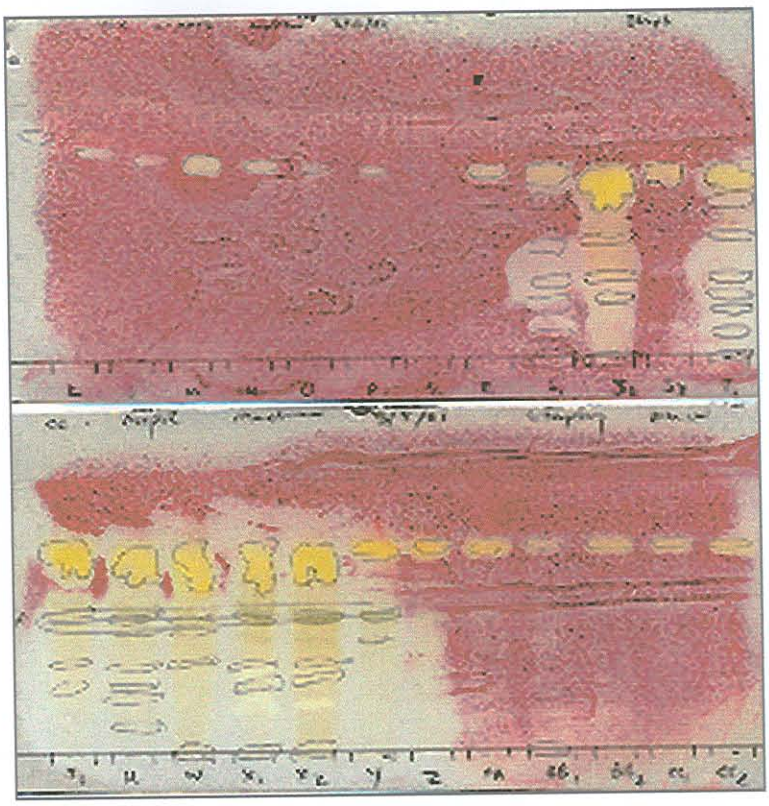


Fig.4.18. Bioautogram of pooled fractions of *C.apiculatum* obtained from column chromatography, EMW as an eluent using *S. aureus*. The clear zones indicate the growth inhibition of *S. aureus* by the fractions. Top fractions: K, L, M, N, O, P, Q, R, S_1, S_2 and bottom fractions: T, U, W, $X_{.1}$, X_2, Y, Z, AA, BB, CC, DD.

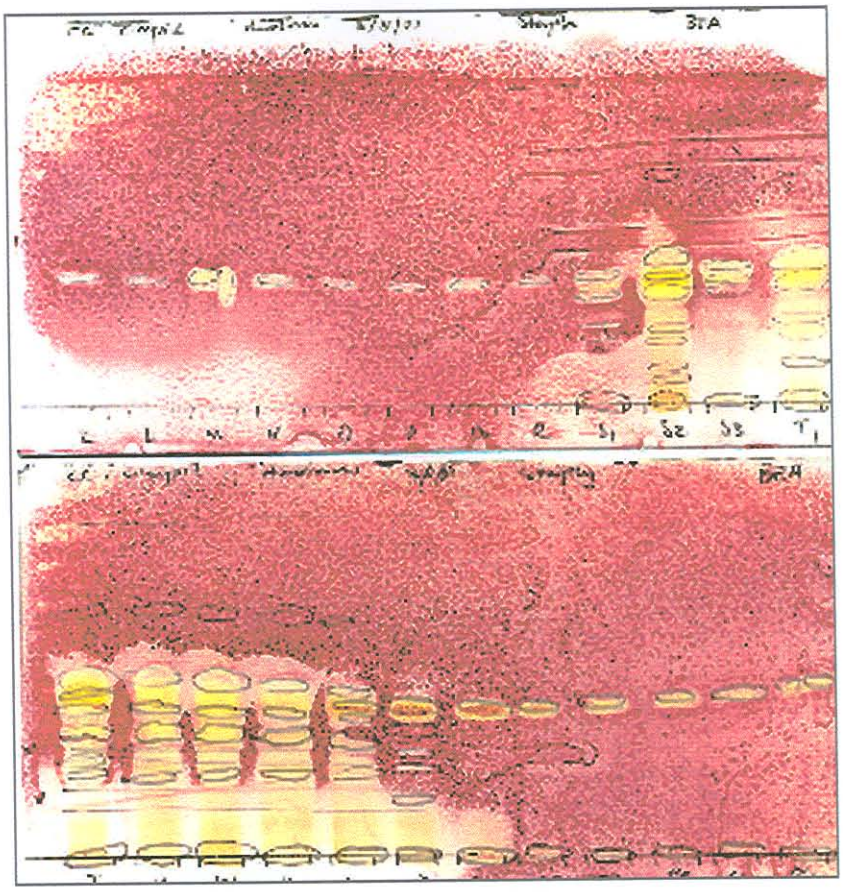


Fig 4.19. Bioautogram of pooled fractions of *C. apiculatum* obtained from column chromatography, BEA as an eluent using *S. aureus.* The clear zones indicate the growth inhibition of *S. aureus* by the fractions. Top fractions: K, L, M, N, O, P, Q, R, S_1, S_2 and bottom fractions: T, U, W, X_1, X_2, Y, Z, AA, BB, CC, DD.

The region of activity had the similar R_f values as those found on one of the CF compounds in solvent/solvent fractionation. It was however observed that fractions 19 to 24 had a high complexity of different components, which appeared red, yellow and brownish upon spraying the TLC plates with vanillin SR. Therefore, this necessitated the setting up of another column to further separate fractions, 19 – 24. The separated fractions from 19 to 24 had the same biological activity as those of the first column. Ten fractions, which had biological activity from both the 1st and 2nd columns, were selected. TLC was run on all the selected fractions [figure 4.20].

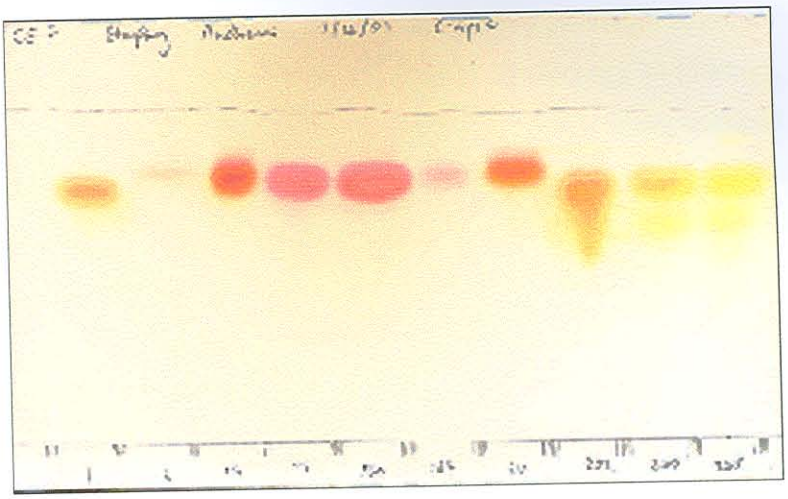


Fig.4.20. TLC of the 10 active samples from column chromatography using CEF as an eluent. Fractions from left, 1, 13, 19, 75, 130, 164, 34, 202, 207, and 238.

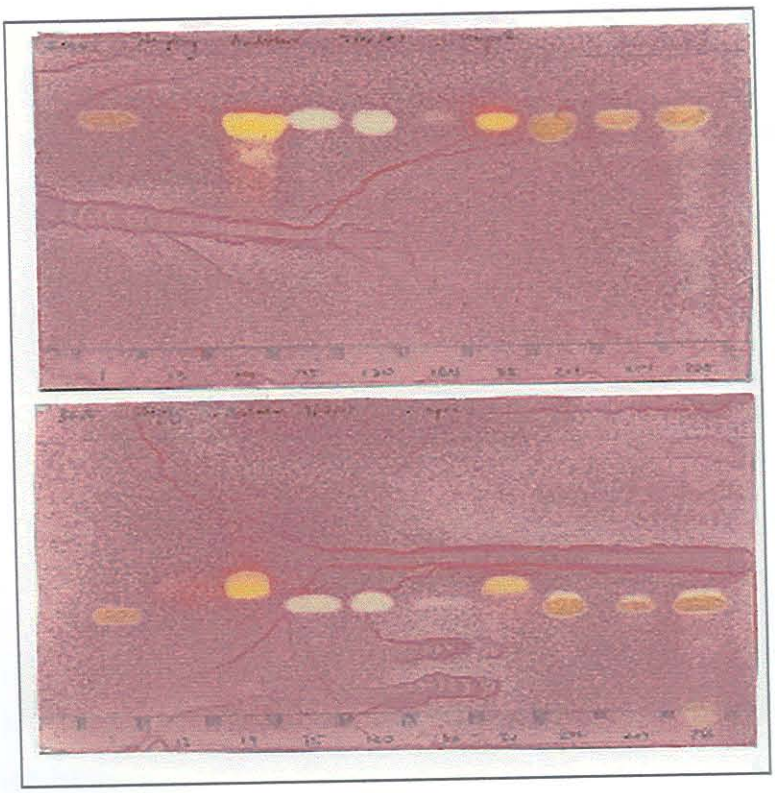


Fig.4.21. Bioautogram of the 10 active samples obtained from column chromatography, using EMW (top) and BEA (bottom) as eluants. The plates were sprayed with *S. aureus*. Fraction numbers from left, 1, 13, 19, 75, 130, 164, 34, 202, 207, and 238

Bioautography was carried out with the ten active fractions obtained from column chromatography. Fractions and their quantities were [1 (37 mg), 13 (40 mg), 19 (35 mg), 34 (40 mg), 75 (17 mg), 130 (24 mg), 164 (20 mg), 202 (68 mg), 207 (47 mg), and 238 (20 mg)]. Some fractions showed good activity on *S. aureus* and *E. faecalis,* but *P. aeruginosa* and *E. coli* appeared to be resistant. The active fractions were analyzed using NMR on a Varian 300 MHz machine. Four were sufficiently pure for structure elucidation. The spectra showed them to be flavonoids.

4.4.3. R_f values of isolated compounds

It is important to record the R_f values of isolated compounds to make it easier to identify bioactive compounds present in other Combretaceae extracts. It also makes it possible to dereplicate data, i.e. to ensure that known compounds are not isolated again. Based on the behaviour during column chromatography the compounds isolated were probably different, but the similar R_f values after TLC using EMW indicates a possible degree of similarity or that the solvent system is not useful in separating these compounds.

Table 4.11. R_f values of the bioactive compounds obtained from column chromatography

Samples	**AS-1**	**AS-13b**	**AS-164**	**AS-130**
BEA	0.36	0.4	0.38	0.4
EMW	0.8	0.8	0.8	0.79

4.4.4. MIC values of isolated compounds

MIC values were determined on the isolated compounds as described in section 3.4.
Good inhibition was shown by samples AS-1, AS-13b and AS-130 on *E. faecalis* and *S. aureus* with MIC value of 40 µg/ml. AS-13b inhibited both *E. faecalis* at the same MIC value of 40 µg/ml. AS-164 inhibited *E. faecalis* and *S. aureus* at 80 µg/ml. If the MIC values of the isolated compounds are compared to that of the crude extract (Table 4.3) in most cases the MIC values are two to five times lower with the exception of *E. coli* results. With *E. coli* the isolated compounds are up to 5.4 times less active that the crude extract. This may be due to not isolating the important

bioactive compounds present in the crude extract, but the R_f values of the isolated compounds were similar to that of the crude extract according to bioautography. A more likely explanation is that in the crude extract there are synergistic effects, i.e. two or more compounds may inhibit *E. coli* substantially more than the single isolated compounds. This would also explain the difference obtained between bioautography where compounds are isolated and MIC of crude extracts where several compounds are present.

Table 4.12. MIC values (µg/ml) of the four active samples on four test organisms: *E. faecalis, S. aureus. E. coli* and *P. aeruginosa*.

Samples	**AS-1**	**AS-13b**	**AS-130**	**AS-164**
P. aeruginosa	300	130	300	300
E. faecalis	400	40	40	80
E. coli	600	250	130	130
S. aureus	40	40	80	80
Average MIC's	**335**	**106**	**138**	**148**

AS-13b was the least active against *P. aeruginosa* and *E. coli.* Based on the bioassay results below, using the four test organisms, AS-1, AS-13b, AS-130 and AS-164 can be regarded as having substantial antibacterial activity against some of the pathogens. The values of AS-130 and AS-164 were very similar.

One difficulty with the presentation in Table 4.12 is that the higher activity is, the lower the value is. To compensate for this the total activity per mg of compound can be calculated. This has been done to compare the plant extracts (Eloff, 2000). In this case total activity per mg can be defined as 1/MIC in mg/ml. This value represents the volume in ml into which the one mg of the active compound can be diluted and still inhibit the growth of bacteria. One mg of AS-13b can therefore be diluted to 25 ml and still inhibit the growth of *S. aureus*.

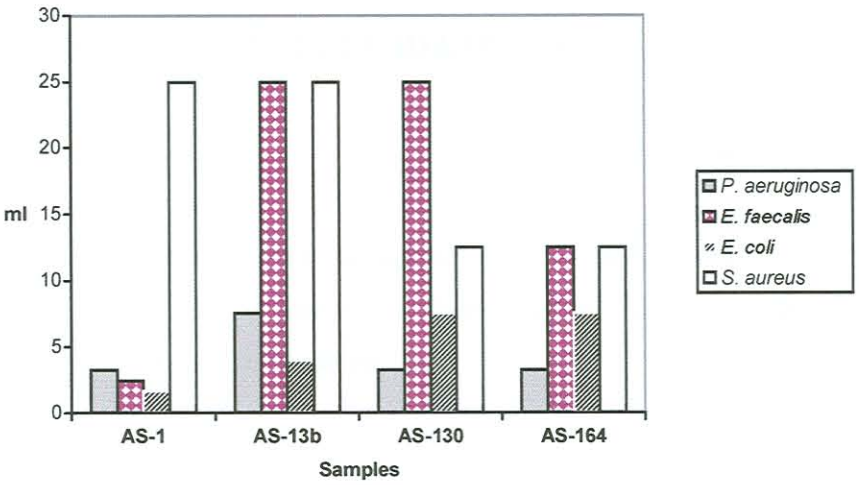


Figure 4.22. Total activity in ml per mg of the four bioactive samples

It is strange that the MIC value of the EE and EA extracts had a MIC value of 40 µg/ml, the same value as the isolated compounds. This may indicate that the compounds isolated may have a minor antibacterial activity. In the crude extracts, R_f values of the bioactive compounds, according to the bioautography results, were 0.4 and 0.8 the same as the isolated compounds. This indicates that the compounds isolated were the major bioactive compounds present in the original extract and not minor bioactive compounds. The only logical explanation at t his stage is that there may have been synergistic effects. Unfortunately there was not enough material available to test this hypothesis and elucidate the chemical structure.

CHAPTER 5

STRUCTURE ELUCIDATION OF ISOLATED COMPOUNDS

5.1 Introduction

NMR data was obtained by Mr NF Makhubela at the Department of Chemistry, MEDUNSA and the Mass Spectra were obtained by Dr. Boshoff at Cape Technikon. Dr DR. Katerere assisted with the interpretation of the information.

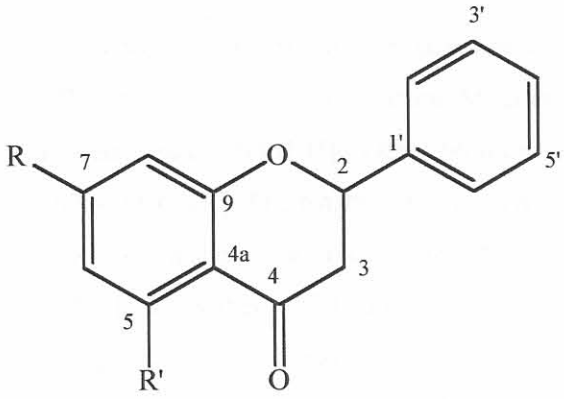
5.2 Overview of spectra of all compounds

The NMR spectra of AS-1, AS-13b, AS-130 and AS-164 are similar and are typical of flavonoids. They possess 2-phenyl chromanone as the parent skeleton. The heterocyclic ring has three carbon resonances, namely; oxymethine (C-2); resonating between 71.3 - 80.3 ppm, aliphatic methylene (C-3); resonating between 39.5 - 46.4 ppm, and carbonyl (C-4); resonating between 186.4 - 198.5 ppm (Agrawal, 1989). C-2 of the molecules is a centre of asymmetry and two forms of each structure are possible. However most of the naturally occurring flavanones acquire phenyl substituent at C-2 position in the pseudo-equatorial position. They bear a hydroxyl substituent at C-5 and C-7 positions hence the assignments of carbon resonances of ring A in case of 5,7-dihydroxyflavanone are of significance importance (Wagner *et al,* 1976). The carbonyl resonance (C-4) depends on the presence or the absence of the *para*-substituted C-5. In case of 5-unsubstituted flavanones C-4 resonance absorbs between 189.7 - 191.7 ppm except for the 7,8,3`, 4`-tetrahydroxyl flavanone, where it resonates at appreciably low field position δ194.5. In 5-hydroxylated flavanones, C-4 absorbs at deshielded position 195.6 - 197.3 ppm because of hydrogen bonding (Agrawal, 1989). The chemical shift of C-3 is independent of the substituents in the aromatic rings and should there be a shift it will be upfield.

1-2 ppm in 5-hydroxylated flavanones compared to the 5-unsubstituted.

All the six carbons of ring-A in flavanones do not superimpose with each other hence give rise to six signals unless there is symmetry.

Based on the spectra, two flavanones and one chalcone were isolated from the leaves of *C. apiculatum* Fig.4.24. The structures were elucidated by NMR and confirmed by spectrometry section. 3.



Compounds	R	R'
AS-130	OH	OH
AS-13b	OMe	OH

Fig. 5.1 The basic structure of flavanones isolated in this study

Table 5.1. Spectral data of flavanones isolated in this study.

	AS-130[x]		AS-13b[y]
Carbon position	**^{1}H resonance**	**^{13}C resonance**	**^{1}H resonance**
1	-	-	-
2	5.33 (dd, J= 3.0; 13.1)	79.5	7.80 (d, J=15)
3	2.71 (dd, J=3; 17.1) 2.98 (q, J=3.9; 15.2)	43.7	3.41 (q, J=1.8; 7.5) 8.26 (d, 15)
4	-	196.2	-
4a	-	103.6	-
5	-	164.8	-
6	5.96 (s)	97.2	6.04
7	-	164.9	-
8	5.96 (s)	95.9	5.99
9	-	163.6	-
1'	-	138.7	-
2'/6'	7.34 (m)	126.5	7.69 (m)
3'/5'	7.34 (m)	129.3	7.45 (m)
4'	7.34 (m)	128.8	7.27 (m)
7-OCH_3	-	-	3.82 (s)

[x] Spectra obtained in d-chloroform, [y]Obtained in d-acetone; ^{13}C not done
J is the coupling constant in Hz.

5.3 Structure of compound AS-130

About 4.7 mg of AS-130 was isolated as a cream-coloured compound. High Resolution Electron Impact Mass Spectroscopy (HREIMS) gave the molecular ion M^+ at *m/z* 256 corresponding to $C_{15}H_{12}O_4$. The base peak was seen at *m/z* 256 $[M-H]^+$. Other prominent peaks appeared at *m/z* 179 (67%) $[M-C_6H_5]^+$ and 167 (28%) $[M-C_7H_5]$. The fragmentation is typical of flavanones as illustrated by fig.4.23. ^1H-NMR spectra show a double doublet at 2.71 ppm (J = 3, 17.1 Hz), a quartet at 2.98 ppm (J = 3.9, 15.2 Hz), another double doublet at 5.33 ppm (J = 3.0, 13.1 Hz). Each integrates to one proton and is typical of the H-2 and H-3 *cis-trans* protons in a flavanone moiety. The signal at 5.96, a singlet integrating to two protons as well as the complex multiplet at 7.28 – 7.40ppm confirm the suspicion that this is a flavonoid with a mono-substituted C-ring. The signal at 5.96 ppm is typical of the H-6/8 protons in ring-A, implying that C-5 and C-7 have hydroxyl substituents. ^{13}C-NMR shows signals typical of an aromatic ring (126 – 129 ppm). The signal at 196.2 ppm is due to the ketonic carbon, C-4, which characteristically resonates between 186 and 199 ppm (Agrawal, 1989). In this case it shows up near the extreme downfield end because of the deshielding effect of hydrogen bonding with the hydroxyl group attached at C-5. C-6 and C-8 resonate at 97.2 and 95.9 ppm, while the signal at 103 ppm is due to C-4a. These signals are typical of flavonoids. In most cases, the C-4a signal is small (or absent all together) (Katerere, 2001) as was seen later on with AS-1. This appears to depend on the relaxation time set during the acquisition mode on the machine.

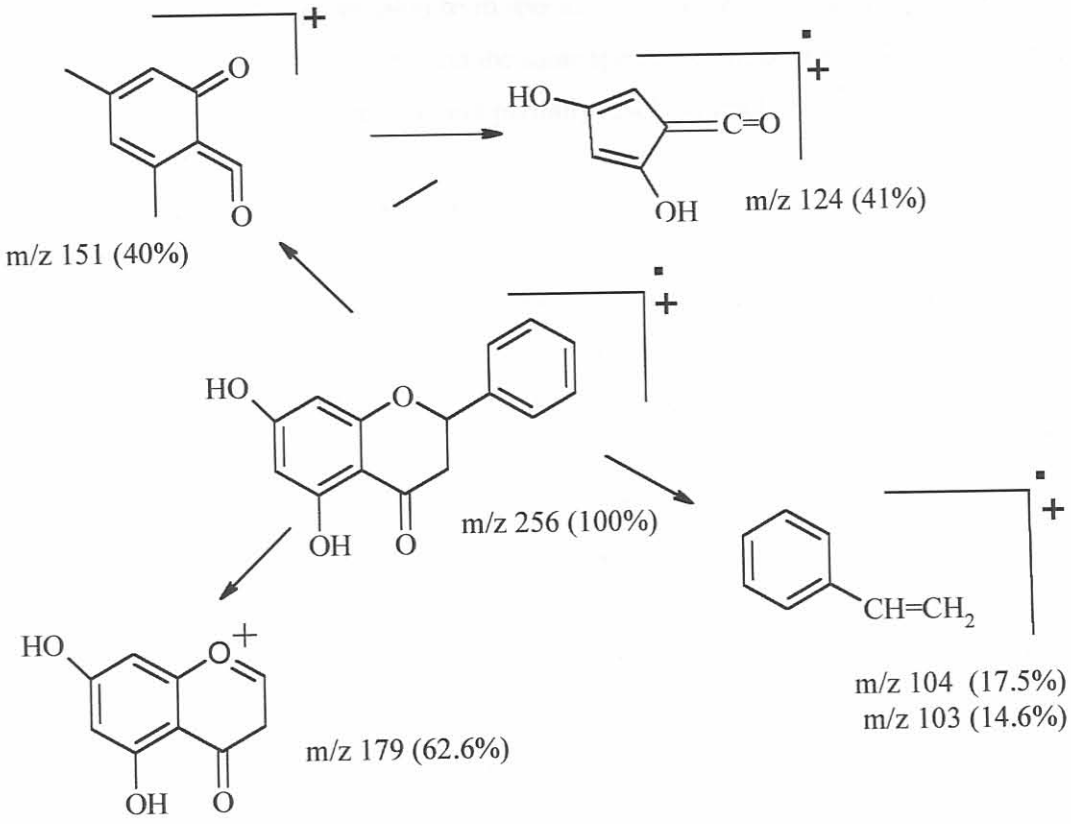


Fig 5.2 The fragmentation pattern of AS-130, which is typical of flavanones.

Based on the MS and NMR data as well as comparison with the literature (Agrawal, 1989), AS-130 was characterized as 5,7-dihydroxyphenyl flavanone, with commonly called pinocembrin.

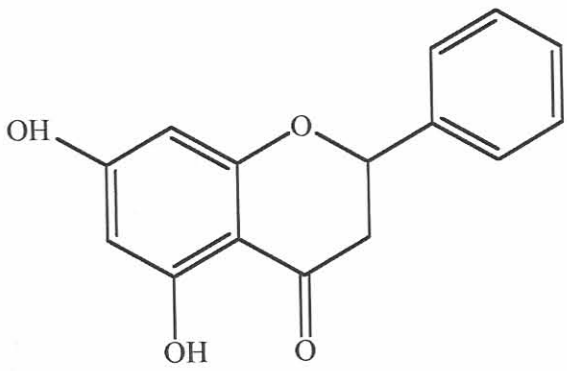


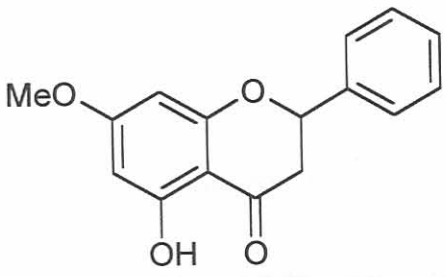
Fig.5.3.Chemical structure of Pinocembrin

Pinocembrin has also been isolated from species of *Alnus; Pinus; Eucalyptus* and *Populus*, (Itokawa, 1981). Fraction AS-164 had the same spectroscopic data as AS-130 and appears to be the same compound isolated from different partially resolved fractions.

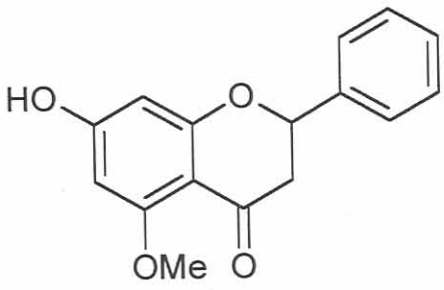
5.4. Structure of compound AS-13b

AS-13b (5.9 mg) was isolated as a white crystalline solid. HREIMS showed molecular ion M^+ at *m/z* 270,1 implying the molecular formula to be $C_{16}H_{14}O_4$. Other prominent peaks appeared at 167 $[M\text{-}C_8H_9]^+$ and at *m/z* 193 $[M\text{-}C_6H_7]^+$. 1H-NMR showed two doublets at 8.26 and 7.80 ppm, both with a coupling constant, J = 15 Hz signifying that these protons are oriented *trans* to each other. Their *cis* partner is at 3.41 (q, J = 1.8; 7.5). Together with the multiplets below 7.00pp, the singlets at 5.99 ppm and 6.04 pp (H-6 and H-8), the ^{1}H-NMR spectrum points to a flavonone. The region around 4.00 ppm shows two singlets implying that there are two methoxyl substituents. However the MS data supports the presence of only one showing the molecular ion to be at *m/z* 270 rather then 284. It could be suggested that the other peak may be due to an impurity whose other signals appear upfield i.e. a relatively non-polar contaminant.

Based on the MS and ^{1}H-NMR, two possible structures were were proposed,5-methoxy-7-hydroxy flavanone (I) and 5-hydroxy-7-methoxy flavanone (II). ^{1}H-^{1}H COSY (relayh) was performed. This showed cross-peaks between the methyl group at 3.82 ppm and both H-6 (6.04 ppm) and H-8 (5.99 ppm) implying that the methyl function is attached to C-7 rather than to C-5. With this data it was concluded that structure represented as (I) was more plausible than structure (II) because of the symmetry.



I



II

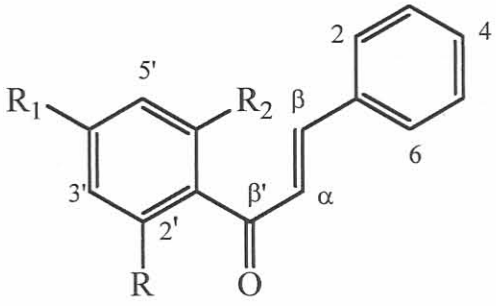
5.4. Possible structures of AS-13b.

AS-13b was elucidated as alpinetin. Alpinetin is one of the four flavonoids which were previously isolated from the crude extract of *Boesenbergia pandurata*. Its chemical structure was characterized by means of physical properties and spectroscopic data, and its antibacterial activity was apparently determined but no data is available (Tip-pyang, 2000). It was also isolated from *Mikania micrantha* (Jiang, 2001).

A number of other related flavanones have also been observed from Guinea *Piper* species, and it is entirely possible that at least two of them might have arisen through a chemical modification of chalcones as a consequence of isolation. Alpinetin is related similarly to alpinetinchalcone. Both of these compounds, regardless of their origins within the isolation procedures employed, represent several tenths of a percent of the total plant extract of Guinea *Piper* species (Alexander, 2001).

5.5 Structure of compound AS-1

About 7.2 mg of AS-1 was isolated as a yellow crystalline solid. HREIMS, showed the molecular ion M^+ at *m/z* 270 implying the molecular formula to be $C_{16}H_{14}O_4$. Prominent peaks appeared at *m/z* 167 $[M\text{-}C_8H_7]^+$ (52.3%) and the base peak at *m/z* 193 $[M\text{-}C_7H_7]^+$. Fig. 4.27. H-NMR spectrum shows a peak at 3.97 due to one O- methyl group. The peaks at 6.04 ppm (J = 2.1Hz) and 5.99 (2.1 Hz) are typical of H-6 and H-8 in flavonoids. In this case they are showing *meta*-coupling to each other. The complex multiplets at 7.27- 7.69 ppm are due to the mono-substituted ring B of the flavonoid.



Fig, 5.5. The basic structure and numbering of chalcones

The two doublets at 7.80 and 8.26 (J = 15.6 Hz) are alkenic protons which are deshielded by the conjugation arising from the phenylic ring on one side and the phenoyl group on the other side. Normally these protons appear between 5 - 6 ppm.

Table 5.2 Spectral data of chalcone isolated in this study

	AS-1	
Carbon position	**^{1}H resonance**	**^{13}C resonance**
α	8.26 (d, J = 15.6)	128.4
β	7.80 (d, J=15.6)	142.6
β'	-	193.1
1'	-	*
2'	-	164.4
3'	5.99 (d, J = 2.1	96.0
4'	-	166.0
5'	6.04 (d, J = 2.1)	92.2
6'	-	168.9
1	-	136.4
2/6	7.69 (m)	129.2
3/5	7.43 (m)	130.3
4	7.27 (m)	129.8
6'-OCH_3	3.97 (s)	56.4

The ^{13}C NMR spectra are typical of flavonoids and generally similar to that of AS-130. It shows a methoxyl group (56.4 ppm), the C-3'/ 5' signals at 92.2 and 96.0 ppm respectively, aromatic carbons at around 130 ppm, and the carbonyl at 193.1 pp (C-β'). In this case it is upfield relative to the similar group (C-4) in AS-130. This may be indicative of loss of hydrogen bonding. The signal expected at around 103 ppm is very small and almost indiscernible, again probably due to poor relaxation.

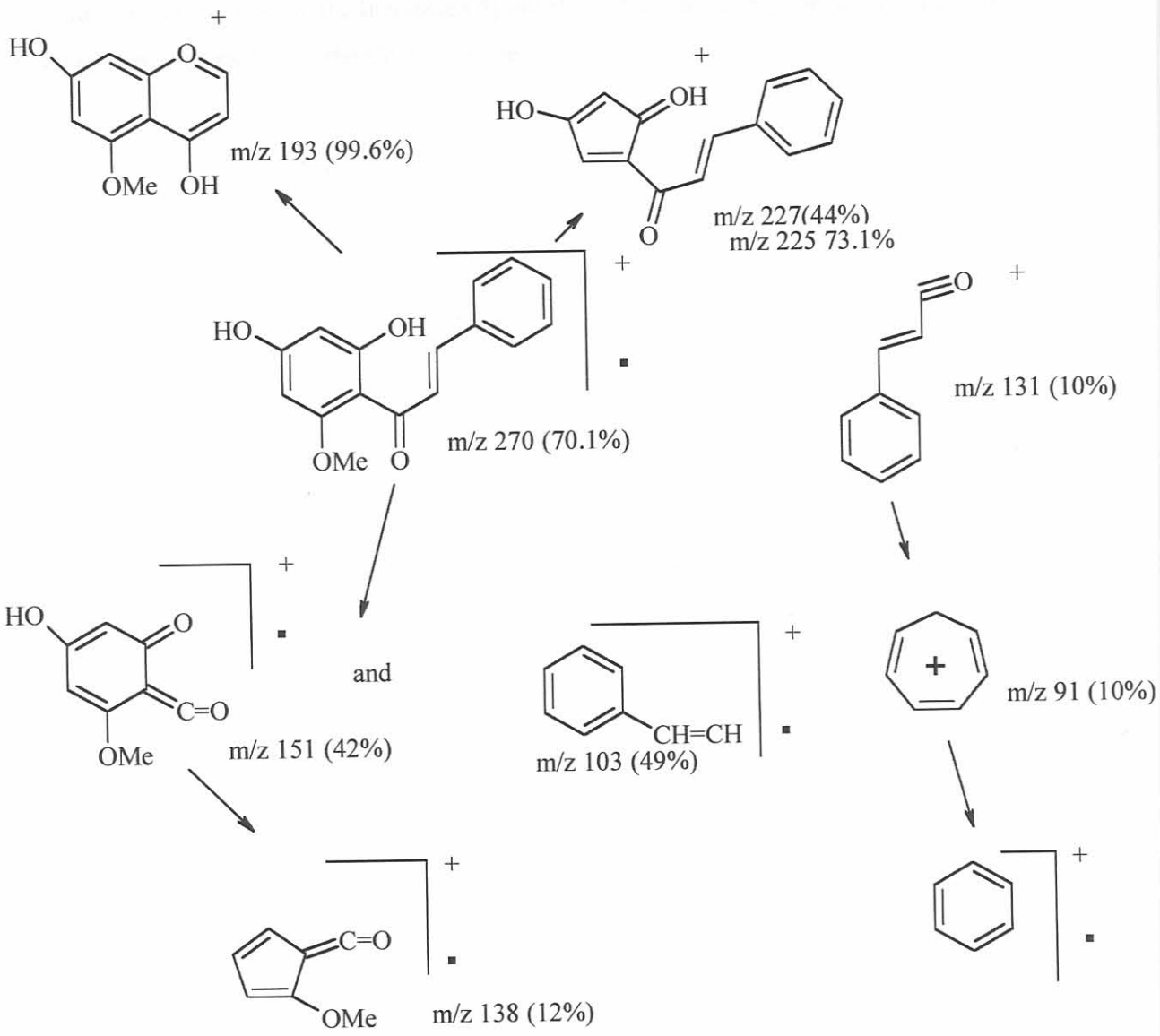


Fig.5.6. Typical fragmentation pattern of chalcones

From the NMR and MS obtained in the study, and by comparison with the literature (Wollenweber and Siegler 1982, Nascimento and Mors 1972; Agrawal, 1989; Katerere, 2001), AS – 1 was characterized as 2', 4' – dihydroxy – 6' – methoxychalcone. This is the chalcone of alpinetin (AS-13b) (which was also isolated in this study) and is known as cardamomin. Cardamomin has been used as a spice and as a flavouring agent in other medicines. The ancient Egyptians chewed it to whiten their teeth and also to sweeten their breath. In India it has found use in the cure of urinary and skin complaints. It has been previously isolated from *Boesebergia pandura, Pityrogramma*

chrysophylla, and species of *Alpinia, Piper and Populus* (Itokawa *et al*, 1981). The NMR data were considerably similar to those in the literature (Agrawal, 1989). This is only the second that this chalcone is being reported from the Combretaceae.

CHAPTER 6

DISCUSSION AND CONCLUSION

The aim of this study was to isolate and characterize antibacterial compounds from *C. apiculatum*. After determining the best extractant, an acetone extract was fractionated by solvent-solvent fractionation. The chloroform fraction had the highest antibacterial activity. By bio-assay guided fractionation three antibacterial compounds were isolated using silica gel column chromatography. The chemical structure and antibacterial activity of the isolated compounds were determined.

The need to develop new antimicrobial agents has become indispensable. Many scientists in different disciplines have embarked on the screening and analysis of plants for pharmacological activity (Alexander, 1992).
Member of the genus *Combretum* is used widely in traditional medicines. Members of this genus have shown the following biological activity including, anti-inflammatory, hypotensive, antifungal, molluscicidal and antimicrobial (Hutching et al 1996). This prompted the need to investigate the antibacterial activity of *C. apiculatum*. The leaves were extracted with different solvents of varying polarities. The solvents used were hexane (HE), isopropyl ether (IE), ethyl ether (EE), methylene dichloride (MD), ethyl acetate (EA), tetrahydrofuran (TH), acetone (AC), methanol (ME), ethanol (ET) and water (WA). Acetone, tetrahydrofuran, ethyl acetate and ethanol had a better extraction efficiency compared to the other extractants. TH and AC gave a good yield because they are moderately polar. Water was not a good extractant.

The solvent systems that resulted in the best chromatographic resolution were the EMW and CEF. With regard to the bioassay, IE, EE, EA, ME and MD had good inhibition on all microorganisms, (their MIC's ranging between 0.04 mg /ml and 0.6 mg /ml). TH and AC extracts were reasonably active against all microorganisms except *P. aeruginosa*. Total activity was good against *E. coli, E. faecalis* and *S. aureus,* while *P. aeruginosa* showed resistance towards almost all extracts. The average total activity for the extracts, EE, IE, EA, ME and MD was 354 ml. TH and AC extracts both inhibited *E. coli* and *S. aureus.*

These extracts were less complex and had a higher extraction yield than the other extracts. Compounds partitioning into this fraction also showed good activity against *S. aureus* and *E. coli.* Isolation work was carried out on the chloroform fraction. Seven antibacterial compounds which inhibited the *S. aureus* and *E. coli* were seen in bioautography. These compounds had R_f values of 0.4 and 0.8 and were later isolated in column chromatography. Even though the crude extracts inhibited *S. aureus*, *E. faecalis* and *E. coli*, most of the activity was seen using *S aureus. S. aureus* was therefore used for the bioassay guided fractionation of three antibacterial compounds. It is possible that there could be other active compounds against the rest of the bacteria. This phenomenon could be due to the possibility of synergistic effects among the active compounds in the crude extracts.

Three compounds were isolated by column chromatography and elucidated in this study. They were identified as alpinetin (5-hydroxy-7-methoxyflavanone), pinocembrin (5,7-dihydroxyflavanone), and flavokawain-A (4'-hydroxy-2',4'-dimethoxychalcone), which is a chalcone. These compounds were active against, *E. coli, E. faecalis*, *S. aureus,* and *P. aeruginosa.*

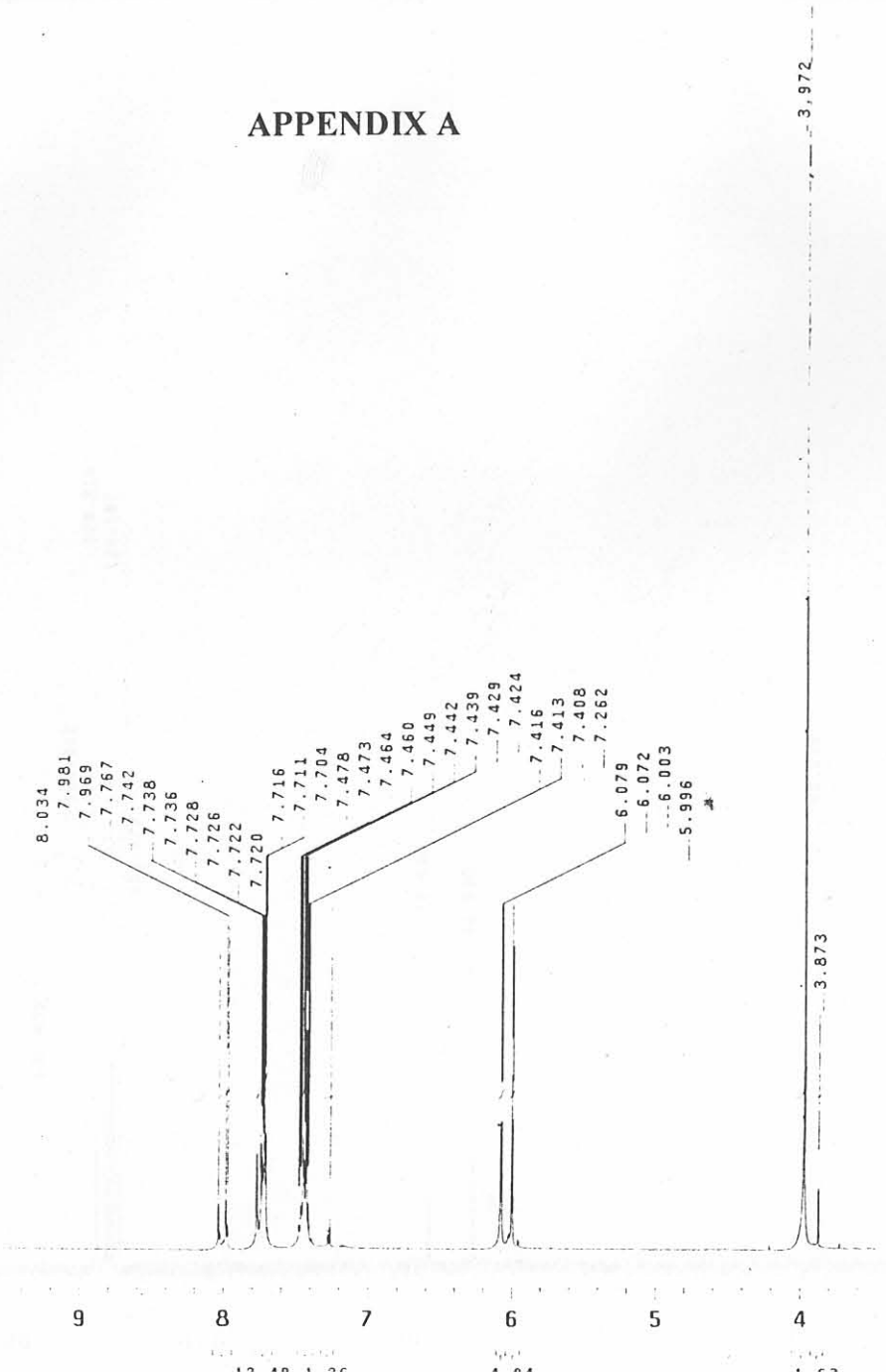
The biological activity found in propolis has been associated with pinocembrin that is one of the 38 flavonoids found in propolis and responsible for its wide therapeutic values. A large number of studies have confirmed propolis to possess antimicrobial activity on at least 21 species of bacteria, 9 species of fungi, 3 species of protozoa and a large range of viruses including the herpes and influenza (Anonymous, 2001). Apart from the antimicrobial value it has also shown to carry a range of other therapeutic properties like, anti-inflammatory, antioxidant, anti-allergic action (Van Wyk, 2000). Pinocembrin has also been found to act as a nutritional supplement, as a sweetener and a flavoring agent.

Flavokawain-A has been found as a constituent of *Piper methysticum.* It is known of anti-inflammatory and antiseptic activity has been used for the treatment of upper respiratory tract infections and cystitis (anonymous, 2001). It is also known for its antioxidant action, and the ability to inhibit cyclooxygenase enzyme. Anti-bacterial activity against *S. aureus* by this compound has been reported without providing MIC values (Bremner and Meyer, 1998).
The three active flavonoids isolated in this study have activities not much higher than crude extracts. The Rf values of the isolated compounds were similar to the Rf value of the active compound in the crude extract. The unexpected low antibacterial activity is therefore not because minor active compounds were isolated but rather indicates a synergistic effect in the crude extract.

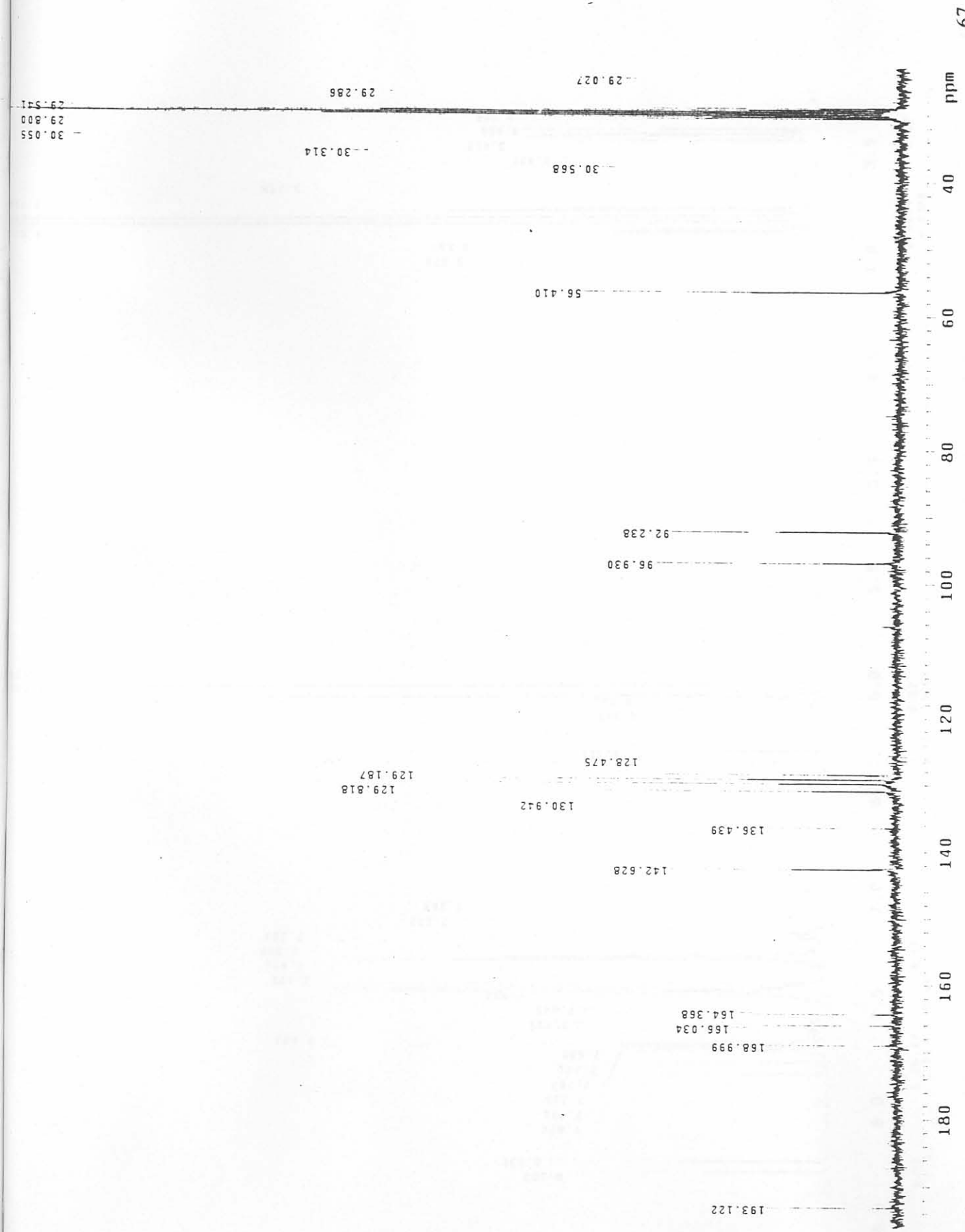
Although all of these compounds are known, not one of them has been isolated from the Combretaceae and the MIC values were not known.

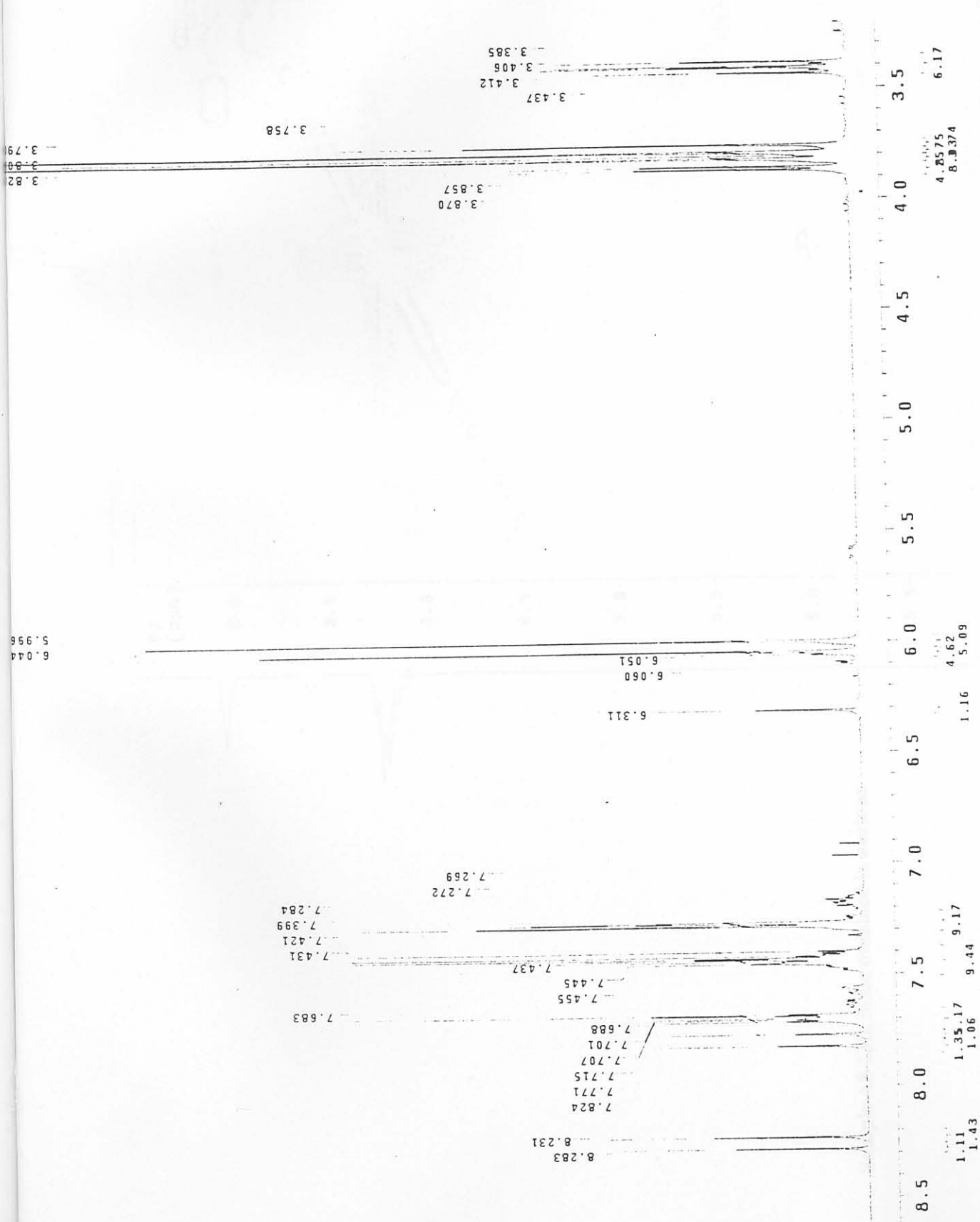
There is little prospect of developing pharmaceutical products based on these compounds. The bioassay was limited to only four microorganisms and there were no control experiments in this study. This study however, justifies the use of *C. apiculatum* for diarrhoea and conjunctivitis in traditional medicines. Further studies on the use of crude extracts in rural communities where the tree occurs, may be useful

APPENDIX A

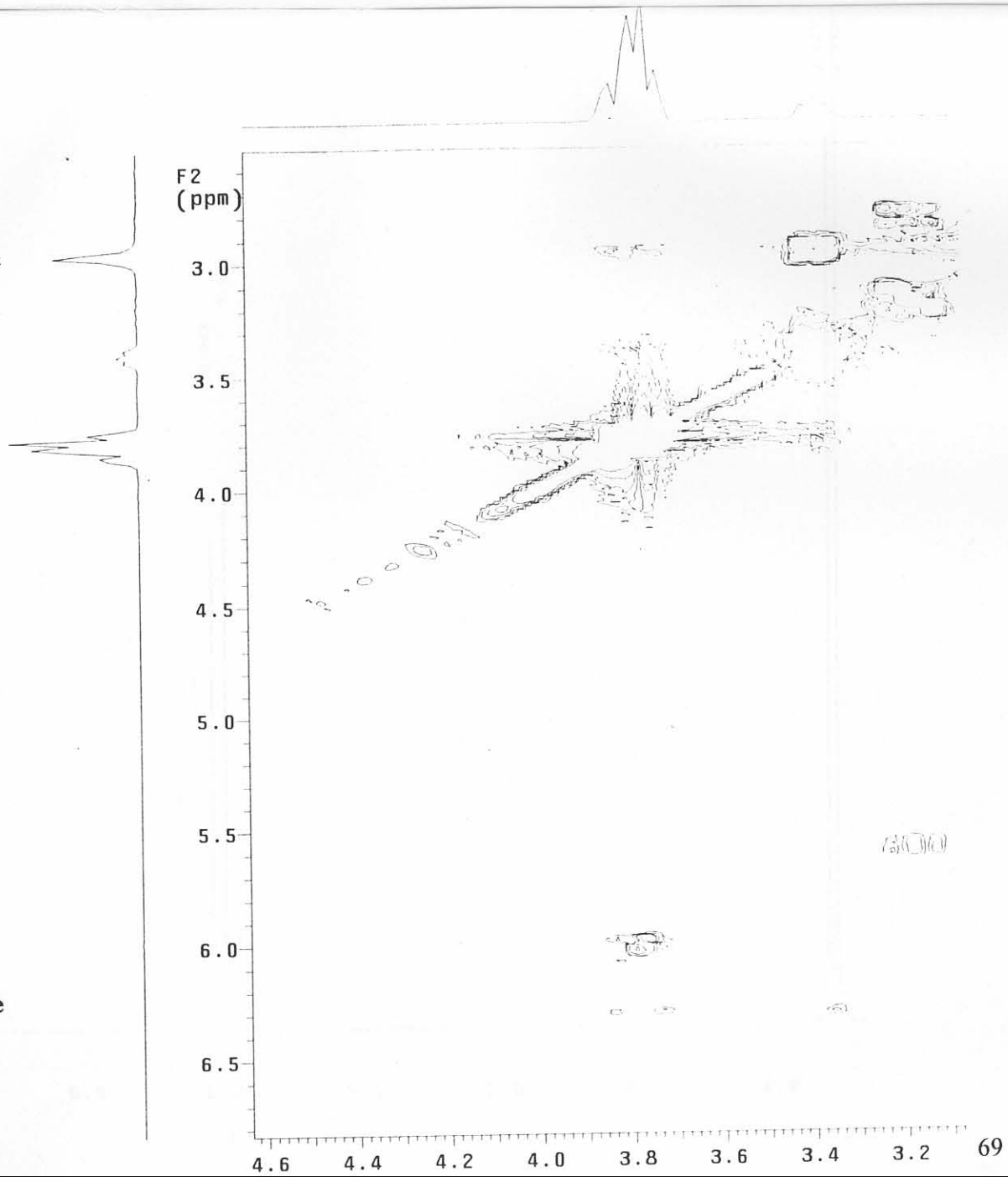


Sample AS-1 Mass spectrum

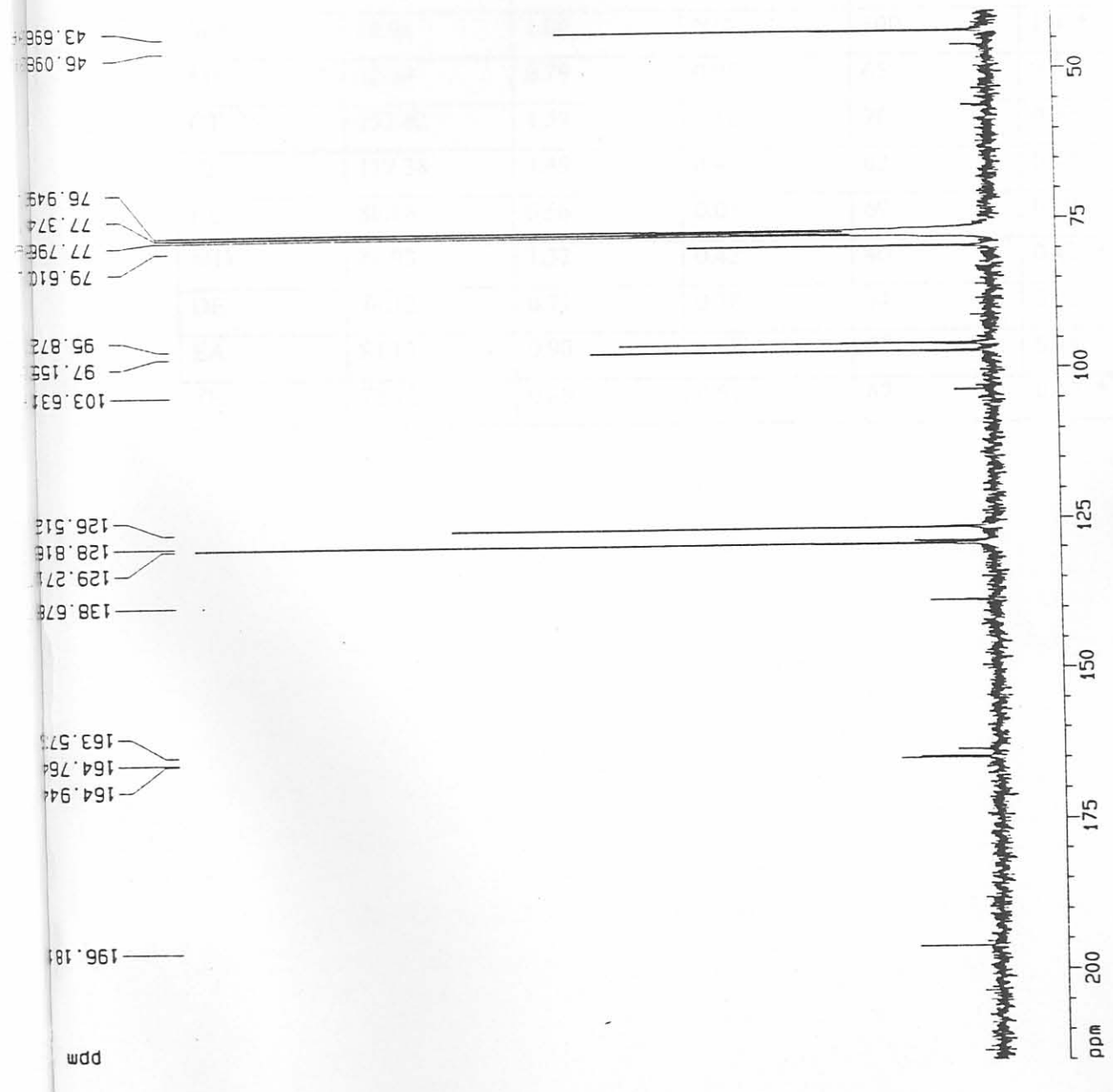




AS-13b, H-NMR spectrum obtained in acetone



Sample AS-13b, Mass obtained in acetone



Sample AS-130, mass spectrum

APPENDIX B

Molecular properties of solvents (All at 25 °C except those marked with *asterisks* which were at 20°C). Hexane (HE), Isopropyl ether (IE), Ethyl ether (EE), Ethylene dichloride (MD), Ethyl acetate (EA), Tetrahydrofuran (TH), Acetone (AC), Methanol (ME), Ethanol (ET) and Water (WA).

	MW (g/mol)	Density(kg/	Polarity	BP	Viscosity
AC	58.08	0.79	0.56	56	0.31
ET	46.07	0.79	0.88	78	1.2 *
WA	18.02	1.00	N/A	100	1.0 *
ME	32.04	0.79	0.95	65	0.55
CT	153.82	1.59	0.18	76	0.97 *
CF	119.38	1.49	0.4	62	0.54
HE	86.18	0.66	0.01	69	0.29
MD	84.93	1.32	0.42	40	0.43 *
DE	74.12	0.71	0.38	34	0.22
EA	88.12	0.90	0.58	77	0.44
TH	72.12	0.89	0.57	67	0.47 *

REFERENCES

Agrawal, P. K. 1989. NMR spectroscopy in the structural elucidation of oligsaccharides and glycosides. Carbon - 13 NMR of flavonoids. Studies in organic chemistry 39.

Alexander, D. M. Bhana, N. Bhika, K. H. and Rogers, C. B. 1992. Antimicrobial testing of selected plant extracts from *Combretum* species South African Journal of Science. 88, 342 - 344.

Alexander, T. 2001. The narcotic pepper - The chemistry and pharmacology of Piper methysticum and related species. *Scientific Consultant, 1483 Shulghin Road, Lafayette, California (94549) USA*

Anonymous 2001. http: // www, vespapower.com/ propolis. htm.

Begue, W. J. and Kline, R. N. 1972. The use of tetrazolium salts in bioautografic procedures Journal. Chromatograph, 64. 182-184.

Berkowitz, F. E. 1995. Antibiotic resistance in bacteria, South Med. Journal 88, 797 - 804.

Breytenbach, J. C. and Malan, S F. 1989. Pharmacochemical properties of *Combretum zeyheri*.85, 372 - 374.

Carr, J. D and Rogers, C. B. 1986. Chemosystematic studies of the genus *Combretaceae*. A convenient method of identifying species of this genus by comparison of the polar constituents extracted from leaf material. South African Journal of Botany

Carr, J. D. 1988. Combretaceae in Southern Africa. The Tree Society of Southern Africa, Johannesburg.

Clark, W. G, Bratter, D.C, Johnson, A.R. 1992. Medical pharmacology 13th edition. Introduction to chemotherapy, mechanisms of antimicrobial action. 628 - 631.

Cowan, M. M. 1999. Clinical microbiology reviews, American Society of Microbiology. Plant products as antimicrobial agents.

Eloff J.N. 1998. The presents of antimicrobial compounds in *Anthocleista Grandiflora* (Loganiacae) South. African Journal of Botany 64, 209 - 212.

Eloff, J.N. 1998a. A sensitive and quick method to determine the minimum inhibitory concentration of plant extracts for bacteria. Planta Medica, 64, 711 - 713.

Eloff, J.N. 1998b. Which extractant should be used for the screening and isolation of antimicrobial components from plants J. Ethnopharmacol, l60, 1-8.

Eloff, J.N. 1999a. It is possible to use herbarium specimens to screen antibacterial components in some plants. J. Ethnopharmacol 67. 355 – 36

Eloff, J.N. 1999b. The antibacterial activity of 27 Southern African members of the *Combretaceae*, South African journal of Science

Eloff, J. N. 2000. On expressing the antibacterial activity of plant extracts- a small first step in applying scientific knowledge to rural primary health care. South African journal of Science 96, 116 - 118.

Famakin, J.O. 2002. An investigation of antibacterial compounds in *Combretum woodii* Duemmer. Msc. thesis, University of Pretoria

Farnsworth, N. R., Morris, R W. 1976. Higher plants: the sleeping giants of drug development. Am. J. Pharm. 148, 46 - 52.

Farnsworth, N. R., 1984. The role of medicinal plants in drug development. In Natural products and Drug Development, eds P. Krogsgaard-Larsen, S. B. Christensen, H. Kofod, pp. 8 - 98. Balliere, Tindall and Cox, London.

Farnsworth, N. R., Akerele, O., Bingel, A. S., et al, 1985. Medicinal plants in therapy. Bull. World Health Organiz. 63, 965 - 981.

Farnsworth, N. R., Soejarto, D.D., 1991. Global importance of medicinal plants. In Conservation of Medicinal Plants, editors O. Akerele, V Heywood, H. Synge, pp 25 - 51. Cambridge University Press, Cambridge.

Farnsworth, N. R. 1966. Biological and phytochemical screening of plants. Journal of Pharmaceutical Sciences.

Foster, R.W, 1996, Basic pharmacology. Antiparasitic chemotherapy, 4th edition, 302 - 304.

Harborne, J. B. 1973, Phytochemical methods. A guide to modern techniques of plant analysis, Chapman and Hall, London.

Houghton, P. J. and Amala, R. 1998. Laboratory handbook for the fractionation of natural extracts.

Husain, A. 1991. Conservation of medicinal plants, (Economic aspects of exploitation of medicinal plants. Director, Central Institute of Medicinal and Aromatic Plants, Lucknow, India.

Hutchings, A., Scott, A. H., Lewis, G., Cunnigham, A. B., 1996. Zulu medicinal plants-an inventory. University of Natal Press Pietermaritsburg, South Africa.

Itokawa, H., Morita, M. and Mihashi, S. 1981. Phenolic compounds from rhizomes of Alpinia speciosa, Phytochemistry volume 20: 2503 – 2506.

Jiang, R. W, He, Z. D, But, P. P, Chan, Y. M, Ma, S. C, Mak, T. C. Chem Pharm Bull Tokyo 2001 Sep;49(9):1166-9. A novel 1:1 complex of potassium mikanin-3-O-sulfate with methanol. Department of Chemistry, The Chinese University of Hong Kong, Hong Kong SAR, PR China

Katerere, D.R.P. 2001, Phytochemical and pharmacological studies of species of African Combretaceae. PhD Thesis. University of Stratchelyde, UK.

Katzung, B.G., 1995. Basic and clinical pharmacology, 6th edition. Principles of antimicrobial drug action.

Katzung, B. G, 1998. Basic and clinical pharmacology 7th edtion. Principles of antimicrobial drug action.

Letcher, R. M. and Nhamo, L. R. M. 1971. Chemical constituents from the Combretaceae. Part I. Substituted phenanthrenes and 9, 10-dihydrophenanthrens from the heartwood of *Combretum apiculatum*. Journal of Chemical Society (C) 3070-3076

Malan, E., Swinny, E, 1993. Substituted bibenzyls, phenathrenes and 9,10-dihydrophenanthrenes from heartwood of *C. apiculatum*. Phytochemistry 34, 1139 – 1142.

Malone, M. H., 1983. The pharmacological evaluation of natural products. General and specific approaches to screening ethnopharmaceuticals. Journal of Ethnopharmacology 8: 127 - 147.

Martini, N. and Eloff, J. N. 1998, The preliminary isolation of several antimicrobial components from *Combretum erythrophyllum (*Combretaceae*)* Journal of Ethnopharmacology, 62, 255 - 263

McGaw, L. J., Rabe, T., Sparg, S. G., Jager, A. K., Eloff, J. N., Van Staden, J. 2001 An investigation on the biological activity of *Combretum* species. Journal of Ethnopharmacology. 25, 45 - 50.

Mycek, J. M, Harvey, R A, Champe, P. C 2000. Lippincott's illustrated reviews, Pharmacology, 2nd edition. Principles of antimicrobial therapy.

Mwaulauka, K., Charlwood, B., Briggs, J., Bell, E. 1975. Aminoethylphenylanine, a new amino acid from seeds o *Combretum zeyheri*. Biochem. physiol. Pflaven. 168, 15-18

National Committee for Clinical Laboratory Standards 1992. Performance standards for antimicrobial disk susceptibility tests- fourth edition; Approved Standard. NCCLS Document M2-A4, Villanora, Pa NCCLS.

Panzini, I, Pelizzoni, F, Verrotta, L, Rogers, C. B, 1993. Constituents of the fruit of South African *Combretum* species. South African Journal of Science , 89, 324 - 328.

Pettit, G. R, Singh, S. B. 1987. Isolation, structure, and synthesis of combrestatin A-2, A-3, and B-2. Can. J. Chem. 65, 2390 - 2395.

Pettit, G. R., Singh, S. B., Boyd, M. R., Hamel, E., Petit, R. K., Schmidt, J. M., Hogan, F. 1995 Antineoplastic agents. 291. Isolation and synthesis of combretastatins A-4, A-5 and A-6 (1a). J. Med Chem 1995. 38, 1666 - 1672.

Robbers, J. Tyler, V. 1997. Tyler's herbs of choice: The therapeutic use of Phytomedicinals, The Haworth Herbal Press, Bringhamton, New York.

Rogers, C.B. 1996. Chemistry and biological properties of African Combretaceae. Proceedings of the first international IOCD, Symposium. 25 – 28 February, Victoria Falls, Zimbabwe. In Hostettman K, Chinyanganya F, Millard M, Wolfer JL.

Saleh, N. A. M. 1994. Recent advances in the flavonoid chemistry of African plants. Phytochemistry vol. 36, No. 5

Scalbert, A. 1991. Antimicrobial properties of tannins, Phytochemistry vol. 30 No. 12, 3875 – 38833.

Schulz, V. Hansel, R. Tyler, V. 1997. Rational phytotherapy,3rd Edition, Springer-Verlag, Berlin, Heidelburg. New York.

Scott, A. H., Lewis, G. and Cunninghan, A. 1996. Zulu medicinal plants- an inventory. University of Natal Press, Pietermaritzburg, South A frica.

Stuffness, M. Duoros, J. 1979. Drugs of plant origin. Methods in cancer research.

Stuttgart, G. T. V. 1998, Plant medica-Natural products and medicilnal plant research. Journal 8, volume 64.

Thieme, G. 1998. Plant medica, natural products and medicinal plant research vol. 64, journal no. 8.

Taylor, R. S., Manadhar, N. P., Towers, G. H. N., 1995. Screening of selected medicinal plants of Nepal for antimicrobial activities. Journal of Ethnopharmacology. 46, 153 - 159.

Tyler, V. E. 1997. Rational phytotherapy, 3rd edition, Springer-Verlag, Berlin, Heidelberg, New York.

Van Wyk, B. E., Van Oudtshoorn, B. and Gericke, N. 1997. Medicinal plants of South Africa, Briza Publication, South Africa.

Van Wyk, B. E., Gericke, N. 2000. Peoples plants, A guide to useful plants of Southern Africa. Dedicated to the African renaissance.

Wiener, M. B., Pepper, G. A. 1985. Clinical pharmacology and therapeutics in nursing, 2nd edition. General principles of antimicrobial therapy and acute bacterial infections.

Wagner, H., Bladt, S. 1996. Plant drug analysis. A Thin layer chromatograph, Atlas. Springer-Verlag Berlin. Heidelburg New York.

Watt, J. M., Breyer-Brandwijk, M. G. 1962, The medicinal and poisonous plants of Southern and Eastern Africa. E. S. Livingstone, Edinburgh.

Watt, J. M., Breyer-Brandwijk, M. G. 1962. Medicinal and poisonous plants of Southern and Eastern Africa. Being an account of their medicinal and other uses, chemical composition, pharmacological effects and toxicology in man and animals. E. S. Livingstone, Edinburgh