

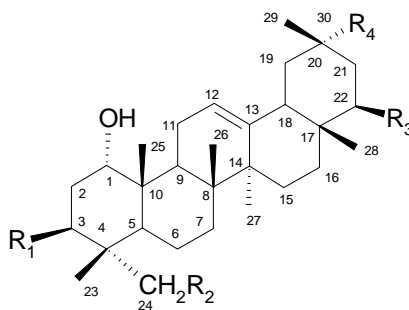
LIST OF ABBREVIATIONS USED

1D	1-dimentional
2D	2-dimentional
CC	Cytotoxic concentration
COSY	Correlation spectroscopy
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethyl sulfoxide
EI	Electron impact
ESI	Electron spray impact
GI	Growth inhibition
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
IC	Inhibition concentration
IUPF	Indigenous Plant use Forum
MIC	Minimum inhibitory concentration
MS	Mass spectrometer
NMR	Nuclear magnetic resonance
NOESY	Nuclear overhauser enhancement spectroscopy
n-phase	normal phase
NCCLS	National committee for clinical laboratory standards
TDH	Threonine dehydrogenase
B1	<i>Bacillus subtilis</i> ATTC 6633 (IMET) NA
B2	<i>Bacillus subtilis</i> ATTC 6633(IMET) AS
B3	<i>Staphylococcus aureus</i> (IMET 10760) SG 511
B4	<i>Escherichia coli</i> SG 458
B9	<i>Pseudomonas aeruginosa</i> K799/61
M2	<i>Mycobacterium smegmatis</i> SG 987 (HK10056)
M4	<i>Mycobacterium vaccae</i> IMET 10670
H4	<i>Sporobolomyces salmonicolor</i> SBUG 549
H8	<i>Candida albicans</i> BMSY 212
P1	<i>Penicillium notatum</i> JP 36
HKI	Hans-Knoll Institute

VLC	Vacuum liquid chromatography
TLC	Thin layer chromatography
HPLC	High performance liquid chromatography
EMW	Ethyl acetate, methanol, water (40:5.4:4)
BEA	Benzene, ethyl acetate, ammonia (90:10:1)
CEF	Chloroform, ethyl acetate, formic acid (5:4:1)
INT	p-iodonitrotetrazolium violet
C.I	<i>Combretum imberbe</i>
C.P	<i>Combretum padoides</i>
C.Cs.C	<i>Combretum celastroides</i> ssp. <i>celastroides</i>
C.Cs.O	<i>Combretum celastroides</i> ssp. <i>orientale</i>
TDH	Threonine dehydrogenase
EtOAc	Ethyl acetate
R _f	Retention factor
SA	<i>Staphylococcus aureus</i>
EF	<i>Enterococcus faecalis</i>
EC	<i>Echerichia coli</i>
PA	<i>Pseudomonas aeruginosa</i>
ssp	Subspecies

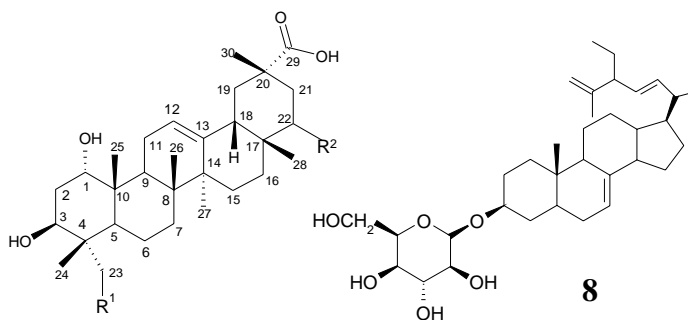
ABSTRACT

Combretum imberbe (leadwood, hardekool) has been used for medicinal purpose and several studies have been carried out to investigate the chemical compounds present in the bark of this plant. Preliminary experiments indicated that the leaves of this plant contain antibacterial compounds that do not occur in other *Combretum* species. Leaves of *C. imberbe* and the closely related *C. padoides* belonging to the *Combretum* section, Hypocrateropsis of the African Combretaceae were extracted and fractionated by bioassay-guided fractionation yielding 5 compounds.

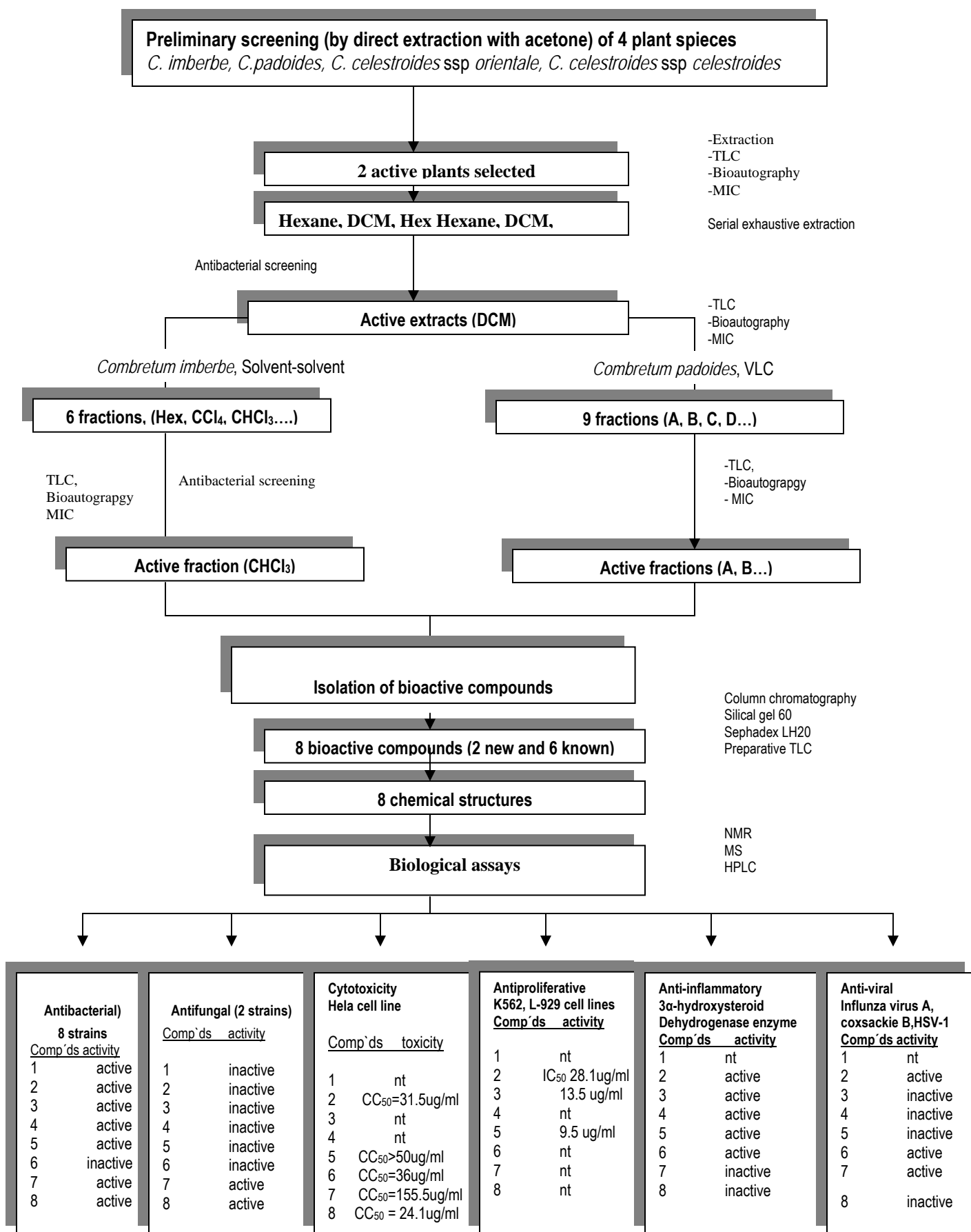


Compounds	R ₁	R ₂	R ₃	R ₄
1	OH	H	H	COOH
2	H	H	H	COOH
3	H	H	=O	CH ₂ OH
4	H	H	OH	COOH
5	2,4-Di-Ac-O-Rh	OH	H	H

Two new antibacterial pentacyclic triterpenoids (1 α , 24 β -dihydroxyl-12-oleanen-29-oic acid-3 β -O- α -2, 4-diacetylamnopyranoside and 1 α , 3 β -dihydroxyl-12-oleanen-29-oic acid-23 β -O- α -4-acetylamnopyranoside) **5** and **6** along with six known triterpenoids **1-4** (1 α , 3 β -dihydroxyoleanen-12-29-oic, 3-hydroxyl-12-olean-30-oic, 3, 30-dihydroxyl-12-oleanen-22-one, 1,3, 24-trihydroxyl-12-olean-29-oic acid **7** (1 α , 22 β -dihydroxyl-12-oleanen-30-oic acid) and **8** (24-ethylcholesta-7, 22,25-trien-3-ol-O- β -D-glucopyranoside) were isolated with the aid of closed and open column chromatography.



Compound	R ¹	R ²
6	4-Ac-O-Rh	H
7	H	OH



Activity not tested (nt), Compounds (comp'd)

All eight compounds had moderate (MIC of 60 µg/ml) to strong (10 µg/ml) antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis*, and *Mycobacterium vaccae* with **2**, **5** and **7** being most active. Compound **2** and **3** also had strong anti-inflammatory activity against 3 α -hydroxysteroid dehydrogenase enzyme with an IC₅₀ of 10 µg/ml and 7.8 µg/ml as well as moderate cytotoxicity (CC₅₀ = 17.6 µg/ml and CC₅₀ = 10.5 µg/ml) against Hella cell lines. Compound **2** and **5** also had moderate anti-proliferative (GI₅₀ = 16.5 µg/ml, 13.2 µg/ml, 8.7 µg/ml) activity against K-562, L-929 cell lines.

The structures of the compounds were elucidated on the basis of 1-dimensional (1D) and 2-dimensional (2D) Nuclear Magnetic Resonance (NMR) experiments, as well as Electron Impact (EI) and Electron Spray Impact (ESI) mass spectrometric techniques.

The results of this study have added new compounds to the global database of phytochemicals, expanded our knowledge on the phytochemistry of *Combretum*, confirmed the rationale of the ethnomedicinal use of *C. imberbe*, and opened up potential new applications of extracts.

PAPERS PREPARED FROM THIS THESIS

Angeh, J. E., Eloff, J. N., Swan, G.E., 2005. Comparing two extraction methods in isolating antibacterial compounds from *Combretum* section Hypocrateropsis (Manuscript).

Angeh, J. E., Eloff, J. N., Swan, G.E., Huang S., Sattler I., 2005. Activity guided isolation of a new anti-microbial triterpenoid from *Combretum imberbe*. (Manuscript).

Angeh, J. E., Eloff, J. N., Swan, G.E., Huang S., Sattler I., 2005. Novel anti-microbial triterpenoid from *Combretum padoides* (Manuscript).

Angeh, J. E., Eloff, J. N., 2005. Synergism in isolated natural compounds (Manuscript).

CONFERENCES AND PROCEEDINGS

2003

Indigenous Plant Forum (IPUF), Kloof Avenue Rustenburg (South Africa)

Poster: JE Angeh, GE Swan, JN Eloff. Comparing two extraction methods in isolating antibacterial compounds from *Combretum Section Hypocrateropsis*.

Faculty day, Faculty of Veterinary Science, University of Pretoria.

Poster: JE Angeh, GE Swan, JN Eloff. The significance of serial exhaustive extraction in isolating antibacterial compounds from *Combretum imberbe*.

2004

Indigenous Plant Forum (IPUF), Clanwilliam (South Africa).

Paper: JE Angeh, GE Swan, JN Eloff, S. Huang, I. Sattler. Bioassay-guided isolation of pentacyclic triterpenoids from *Combretum imberbe*.

Faculty day, Faculty of Veterinary Science, University of Pretoria.

Paper: JE Angeh, GE Swan, JN Eloff, S. Huang, I. Sattler. Novel biologically active triterpenes from *Combretum imberbe* and *Combretum padoides*

2005

Annual conference of Society for Medicinal Plants Research, Florence, Italy

Paper: J. Angeh, J. Eloff, G. Swan, S. Huangi I, Sattler. Novel biologically active triterpenoids from the African Combretaceae

Indigenous Plant Use Forum (IPUF), Gramhamstown

Paper: J. Angeh, J. Eloff. Synegetic effect of triterpenoids isolated from *Combretum* species.

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CHAPTER 1

INTRODUCTION

1.1 Introduction

Natural products and their derivatives represent more than 50% of the drugs in clinical use in the world (Cowan, 1999, Sofowora, 1984). One of the paramount reasons for pursuing natural products chemistry resides in the actual or potential pharmacological activity to be found in alkaloids, terpenoids, coumarins, flavonoids, lignans and the like. Since the advent of antibiotics in the 1950's, the use of plant derivatives as a source of antimicrobials has been virtually non-existent (Cowan, 1999). Antimicrobial plant extracts have been recognised as a future source of new antimicrobials in the event of the current downturn in the pace at which these are being derived from micro-organisms. The public is also becoming more aware of problems with overprescription and misuse of traditional antibiotics (Cowan, 1999).

Resistance to anti-microbial agents is recognized at present as a major global public health problem. Infective diseases account for approximately one-half of all deaths in countries in tropical regions. In industrialized nations, despite the progress made in the understanding of microorganisms and their control, incidents of epidemics due to drug resistant microorganisms and the emergence of hitherto unknown disease-causing microbes, pose enormous public health concerns (Iwu *et al*, 1999). The number of resistant strains of microbial pathogens is also growing since penicillin resistance and multiresistant pneumococci caused a major problem in South African hospitals in 1977 (Berkowitz, 1995). Berkowitz, (1995) referred to the emergence of drug resistant bacteria as a medical catastrophe. Leggiadro (1995) stated that effective regimens might not be available to treat some enterococci isolates and that it is critically important to develop new anti-microbial compounds for these and other organisms before we enter the post-antibiotic era.

The cost of drugs is a sizable proportion of total health expenditure in most developing countries. In some of these countries, drug related expenses account for up to 30-50% of the total cost of health care (Sofowora, 1984). This situation is becoming increasingly unbearable to many nations including South Africa. The World Health Organization (WHO), have observed that up to 80% of the rural populace in the developing countries depend on herbal or alternative medicine and requested member countries to explore safe indigenous medicines for their national health care (Sofowora, 1984).

Plants have served as a source of new pharmaceutical products and inexpensive starting materials for the synthesis of some known drugs. Components with medicinal properties from plants play an important role in conventional Western medicine. In 1984, at least 25% of the Western medicine issued in the US and Canada were derived from or modelled after plant natural products and 119 secondary metabolites were used globally as drugs (Farnsworth, 1994). It has been estimated that 14-28% of higher plant species are used medically. Only 15% of all angiosperms have been investigated chemically and 74% of pharmaceutically active plant derived components were discovered after following up on ethnomedical use of the plant (Farnsworth, 1991).

The traditions of collecting, processing and applying plant and plant-based medications have been handed down from generation to generation. In many African countries, traditional medicines, with medicinal plants as their most important components, are sold in marketplaces or prescribed by traditional healers (without accurate dose value) in their homes (Herdberg and Staugard, 1989). Because of this strong dependence on plants as medicines, it is important to study their safety and efficacy (Farnsworth, 1994). The value of ethno-medicine and traditional pharmacology is nowadays gaining increasing recognition in modern medicine because the search for new potential medicinal plants is frequently based on an ethno-medicinal basis. In the ethno-pharmacological approach, local knowledge about the potential uses of the plants is very useful as compared to the random approach where indigenous knowledge is not taken into consideration.

Compounds inhibiting microorganisms, such as benzoin and emetine have been isolated from plants (Cox, 1994). It is possible that anti-microbial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in the treatment of resistant microbial strains. For this reason, it is therefore important to investigate plants as alternative sources of anti-microbial compounds.

Preliminary work done on the southern African members of the section *Hypocrateropsis* (Eloff, 1999a) indicated that most of the members of this section had substantial antibacterial activity against Gram-positive and Gram-negative bacteria. Bioautography studies indicated that members of the section *Hypocrateropsis* have different antibacterial compounds from members of other sections. Substantial antibacterial activity of some species further motivated this study.

1.2 Hypothesis

Combretum species from the section Hypocrateropsis contain antibacterial compounds that can be isolated and tested for in *vivo* activity.

1.3 Aim of research

To isolate the main antibacterial compounds present in *Combretum* section Hypocrateropsis in a bioassay guided process, and to characterize these compounds chemically and biologically.

1.3.1 Objectives

- Select the most active plant species
- Select and evaluate the best fractionation procedure for isolation
- Isolate antibacterial compounds
- Determine the chemical structure of isolated compounds
- Determine the biological activity of isolated compounds
- Determine the possible synergistic effects of isolated compounds
- Evaluate how well phytochemistry agrees with taxonomy based on morphology.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Plants have served as a source of new pharmaceutical products and inexpensive starting materials for the synthesis of many known drugs. Natural products and their derivatives represent more than 50% of the drugs in clinical use in the world (Cowan, 1999, Sofowora, 1984) (Table 2-2). Although the first chemical substance to be isolated from plants was benzoic acid in 1560, the search for useful drugs of known structures did not begin until 1804 when morphine was separated from *Papaver somniferum L. (Pium)*. Since then many drugs from higher plants have been discovered, but less than 100 with defined structures are in common use. Less than half of these (Table 2-1) are accepted as useful drugs in industrialized countries (Farnsworth, 1984). Considering the great number of chemicals that have been derived from plants as medicine, scientific evaluation of plants used traditionally for the treatment of bacterial infection seems to be a logical step of exploiting the anti-microbial compounds, which may be present in plants. Plant-based anti-microbials represent a vast untapped source of medicines With enormous therapeutic potential (Cowan, 1999). They are supposedly effective in treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic anti-microbials (Iwu *et al.*, 1999)

Table 2-1: Plant-derived drugs widely employed in Western medicine (Adapted from Farnsworth, 1984)

Acetyldigoxin	Ephedrine*	Pseudoephedrine*	Xanthotoxin
Aescin	Hyoscyamine	Quinidine	
Ajmalicine	Khellin	Quinine	
Allantoin*	Lanatoside	Reserpine	
Atropine	Leurocristine	Rescinnamine	
Bromelain	Lobeline	Scillarens A & B	
Caffeine	Morphine	Scopolamine	
Codeine	Narcotine	Sennosides A & B	
Colchicine	Ouabain	Sparteine	
Danthron*	Papain	Strychnine	
Deserpidine	Papaverine*	Tetrahydrocannabinol	
Digitoxin	Physostigmine	Theobromine*	
Digoxin	Picrotoxin	Theophylline*	
L-Dopa*	Pilocarpine	Tubocurarine	
Emetine	Protobertrines A & B	Vincalokoblastine	

* Produced industrially by synthesis

2.2 Antibiotic resistance

Resistance to anti-microbial agents is recognized at present as a major global public health problem. Infective diseases account for approximately one-half of all deaths in tropical countries. In industrialized nations, despite the progress made in the understanding of microorganisms and their control, incidents of epidemics due to drug resistant microorganisms and the emergence of hitherto unknown disease-causing microbes, pose enormous public health concerns (Iwu *et al.*, 1999).

Almost since the beginning of the antibiotic era, bacterial resistance has been seen as the major obstacle to successful treatment (Iwu *et al.*, 1999). Microbial resistance to antibiotics in the clinic emerged soon after their first use in the treatment of infectious disease, and continue to pose a significant challenge for the health care sector. Resistance has now firmly emerged as a problem in the wider community. At the end of the 1960s the Surgeon General of the United States stated that: “we could close the book on infectious diseases.” At the time he uttered these words the emergence of resistance did not seem to affect therapeutic options. Although *S. aureus* had become resistant to benzylpenicillin and showing resistance to thethincillin, it remained sensitive to gentamicin and infections could therefore still be treated. At the start of the next century, things looked very different. Already at least three bacterial species, capable of causing life-threatening illness (*Enterococcus faecalis*, *Mycobacterium tuberculosis* and *Pseudomonas aeruginosa*), had become resistant to every one of the 100 antibiotics, available except for vancomycin (Iwu, 1999). Vancomycin is the antibiotic of last resort for treatment of resistant infections and within the past year scientists have found strains of *Streptococcus pneumoniae* and *S. aureus* to be resistant to this antibiotic. This is attested by the spread, with associated deaths, of infection by methicillin-resistant *Staphylococcus aureus* and the increased prevalence of drug-resistant *S. pneumoniae* in patients suffering from pneumonia. Anti-microbial resistance is driven by inescapable evolutionary pressures and is therefore predictable and inevitable. The emergence in the past year of vancomycin-resistant *S. aureus* an event that has been anticipated for the past decade with great dread, punctuates this assertion. Hardly any group of antibiotics has been introduced to which some bacterium has not developed resistance (Iwu *et al.*, 1999).

Recent reports have shown a marked increase in antibiotic resistance of food-poisoning bacteria due to non-rational and excessive use of antibiotics as therapeutic agents or as growth promoters in livestock. Another factor of resistance potentially lies in the use of antibiotic resistant genes as selection markers in genetically modified organisms (GMOs) (<http://www.biosafety.ihe.de> 1999). The main safety issue of

concern is the release of these resistant genes to sensitive organisms when these GMOs are introduced into the environment.

Due to emergence of drug resistant bacteria, the search for new antibacterial compounds with improved activity is necessary (Harold and Heath 1992a). Many indigenous plants are used in treating bacterial related diseases. Only a small fraction of these indigenous plants has been investigated (Carr and Rogers 1987).

Understanding of the mechanism of action of resistance development remains the foundation of new cycles of antibiotic discovery. Such events demonstrate that antibiotic management and new discovery must continue in the face of these pressures.

2.3 Natural products in drug discovery

Medicinal plants use is widespread (Farnsworth, 1991). The production of medicines and the pharmacological treatment of diseases began with the use of herbs (Tyler, 1997). Life saving and essential drugs from medicinal plants such as morphine, digoxin, aspirin, emetine, and ephedrine were introduced into modern therapeutics several centuries ago. However, plants have been used as drugs for over millenia by human beings. Plants historically have served as models in drug development for some major reasons: The first being that each plant is a unique chemical factory capable of synthesizing large numbers of highly complex and unusual chemical substances. In the United States of America alone, about 25% of popularity in the use of plant-derived preparations (Farnsworth and Morris, 1976). It has also been estimated by the World Health Organization (WHO) that about 80% of the population of the developing countries rely exclusively on plants to meet their health care needs (Farnsworth et al., 1985).

The second reason involves biologically active substances derived from plants have served as templates for synthesis of pharmaceuticals. Such compounds may have poor pharmacological and toxicological profiles. While the reason concerns the fact that highly active secondary plant constituents have been instrumental as pharmacological tools to evaluate physiological processes (Farnsworth, 1984). There are numerous illustrations of plant-derived drugs.

Despite the expense involved in the development of a drug today, at least US\$230 million and a time span between 10 - 20 years (Farnsworth, 1984), nature remains the most reliable and most important source of novel drug molecules. Nature provides 80% of all pharmacological and therapeutic lead compounds and the NCI estimates that over 60% of the compounds currently in pre-clinical and clinical development in its

laboratories are of natural origin. Thus higher plants remain an important and reliable source of potentially useful chemical compounds not only for direct use drugs, but also as unique prototypes for synthetic analogues and as tools that can be used for a better understanding of biological processes (Farnsworth, 1984).

Literally thousands of phytochemicals with inhibiting effects on microorganisms have shown *in-vitro* activity. One may argue that these compounds have not been tested *in vivo* and therefore activity cannot be claimed, but one must take into consideration that many, if not all, of these plants have been used for centuries by various cultures in the treatment of diseases. Another argument could possibly be that at very high concentrations, any compound is likely to inhibit the growth of microorganisms. Firstly, if this is the case, the high concentrations required would no doubt have serious side effects on the patient unfortunate enough to contract an illness. Secondly, these compounds are compared with those of standard antibiotics already available in the market. This means that the concentrations used must compare favourably to those that have already passed the test. A summary of useful anti-microbial phytochemicals is given in **Table 2-2** (Cowan, 1999).

Asiaticode, an anti-microbial compound isolated from *Centella asiatica* (used traditionally in skin diseases and leprosy), has been studied in normal as well as delayed-type wound healing. The results indicated significant wound healing in both models. Another compound, cryptolepine, isolated from *Cryptolepis sanguinolenta* and active against *Campylobacter* species, has been used traditionally in Guinea Bissau in the treatment of hepatitis and in Ghana for the treatment of urinary and upper respiratory tract infections and malaria.

Table 2-2: Plants containing chemotherapeutic activity

Common name	Scientific name	Compounds	Class	Activity	RT ^a
Alfalfa	<i>Medicago sativa</i>	?		Gram-positive	2.3
All specie	<i>Pimenta dioica</i>	Eugenol	Essential oil	General	2.5
Aloe	<i>Aloe barbandensis, Aloe vera</i>	Latex	Complex mixture	Corynebacterium	2.7
Apple	<i>Malus sylvestris</i>	?		Salmonella	
Ashawagandha	<i>Withania somnifera</i>	Phloretin, Withafarin A	Flavonoid derivatives	General Bacteria, fungi, <i>S. aureus</i>	3
Aveloz	<i>Euphorbia tirucalli</i>	?	Flavonoid derivatives	General	1
Bael tree	<i>Aegle marmelos</i>	Essential oils	Terpenoids	Fungi	
Balsam pear	<i>Mormordica charantia</i>	?	Terpenoids	General	1
Barberry	<i>Berberis vulgaris</i>	Berberine	Alkaloids	Bacteria, Protozoa	2
Basil	<i>Ocimum basilicum</i>	Essential oils	Terpenoids	Salmonella, bacteria	2.5
Bay	<i>Laurus nobilis</i>	Essential oils	Terpenoids	Bacteria, fungi	0.7

Common name	Scientific name	Compounds	Class	Activity	RT ^d
Betel pepper	<i>Piper betel</i>	Catechols, eugenol	Essential oils	General	1
Black pepper	<i>Piper nigrum</i>	Alkaloid	Alkaloids	Fungi, lactosbacillus, E. coli	1
Blueberry	<i>Vaccinium spp.</i>	Fructose	Monosaccharide	E. coli	
Brazilian pepper	<i>Schenus terebinthifolius</i>	Terebinthone	Terpenoids	General	
Buchu	<i>Barosma setulina</i>	Essential oils	Terpenoids	General	2
Burdock	<i>Articum lappa</i>	?	Polyacetylene, tannin	Bacteria, fungi, virus	1
Buttercup	<i>Ranunculus bulbosus</i>	Protoanemonin	Lactone	General	2
Caraway	<i>Carum carvi</i>	?	Coumarins polyphenols	Bacteria, fungi, virus	1
Cascara sagrada	<i>Rhamnus purshiana</i>	Tannins	Coumarins polyphenols	Bacteria, fungi, virus	
Cashew	<i>Anacardium pulsatilla</i>	Salicylic acids	Anthraquinone, polyphenols	P.acnes, bacteria, fungi	
Castor bean	<i>Ricinus communis</i>	?		General	0*
Chamomile	<i>Matricaria chamomilla</i>	Anthemiac acid	Phenolics acid	<i>M. tuberculosis, S. typhimurium</i>	
Chapparal	<i>Larrea tridentate</i>	?	Coumarins	<i>S. aureus, virus</i>	2
Chili pepper	<i>Capsicum annum</i>	Capsaicin	Terpenoids	Bacteria	2
Clove	<i>Syzygium aromaticum</i>	Eugenol	Terpenoids	General	1.7
Coca	<i>Erythroxylum coca</i>	Cocaine	Alkaloid	Gram-negative & positive cocci	1.7
Cockle	<i>Agrostemma githago</i>	?		General	1
Coltsfoot	<i>Tussilago farfarva</i>	?		General	2
Coriander, cilantro	<i>Coriandrum sativum</i>	?		Bacteria, fungi	
Cranberry	<i>Vaccinium spp</i>	Fructose	Monosaccharides	Bacteria	
Dandelion	<i>Taraxacum officinale</i>	?	?	<i>C. Albicans</i>	
Dill	<i>Anethum graveolens</i>	Essential oil	Terpenoid		
Echinacea	<i>Echinaceae angustifolia</i>	?		General	
Eucalyptus	<i>Eucalyptus globules</i>	Tannin	Polyphenol	Bacterial, virus	1.5
Fava bean	<i>Vicia faba</i>	Fabatin	Thionin	Bacteria	
Gamboge	<i>Garcinia hanbyryi</i>		Resin	General	0.5
Garlic	<i>Allium sativum</i>	Allicin, ajoene	Sulfoxide, sulphated		
Glory lily	<i>Gloriosa superba</i>	Colchicine	Alkaloid	General	0*
Goldenseal	<i>Hydrastis canadensis</i>	Berberine, hydrastin	Alkaloid	Bacteria, Giardia duodenale	2
Gotu cola	<i>Centella asiatica</i>	Asiatocostide	Terpenoid	<i>M. leprae</i>	1.7
Grapefruit tea	<i>Citrus paradisa</i>		Terpenoid	Fungi	
Green tea	<i>Camellia sinensis</i>	Catechin	Flavonoids	General, shigella,	
Ginseng	<i>Panax notoginseng</i>		Saponins	Schenchi	2.7
Harmel, rue	<i>Peganum harmala</i>	?		Bacteria, fungi	1
Hemp	<i>Cannabis sativa</i>	Beta-resercyclic acid	Organic acid	Bacteria, virus	
Henna	<i>Lawsiana intermis</i>	Garlic acid	Phenolics	<i>S. aureus</i>	1.5
Hops	<i>Humulus lupulus</i>	Lupulone	Phenolic acid	General	3
Hyssop	<i>Hyssopus officinalis</i>		Terpenoid	Viruses	
(Japanese) herb	<i>Rabdosia trichocarpa</i>	Trichorabdal A	Terpene	<i>Helicobacter pylori</i>	
Lantana	<i>Lantana camara</i>	?		General	

Common name	Scientific name	Compounds	Class	Activity	RT ^d
Lavender-cotton	<i>Santolina</i>	?		Gram-positive bacte	
Legume	<i>Milletia thonningii</i>	Alpinumisoflavone	Flavones		
Lemon balm	<i>Melissa officinalis</i>	Tannins	Polyphenols	Viruses	
Lemon verbena	<i>Aloysia</i>	Essential oil	Terpenoid		1.5
Licorice	<i>Glycyrrhiza glabra</i>	Glabrol	Phenolic alcohol	<i>M. tuberculosis</i>	
Officinalis	<i>Thevetia peruviana</i>	?		<i>S. aureus</i>	2
Mace, nutmeg	<i>Myristica fragrans</i>	?		General	1.5
Marigold	<i>Calendula</i>	?		Bacteria	2.5
Mesquite	<i>Prosopis juliflora</i>	?		General	1.5
Mountain tobacco	<i>Arnica montana</i>	Helamins	Lactones	General	2
Oak	<i>Quercus rubra</i>	Tannins	Polyphenol		
Olive oil	<i>Olea europaea</i>	Hexanal	Aldehyde	General	
Onion	<i>Allium cepa</i>	Allicin	Sulfoxide	Bacteria, <i>Candida</i>	
Oregon grape	<i>Mahonia aquifolia</i>	Berberine	Alkaloid		2
Pao d'arco	<i>Tabebuia</i>	Sesquiterpenes	Terpenoids	Fungi	1
Papaya	<i>Carica papaya</i>	Latex	Mix of terpenoids , alkaloid		
Pasque-flower	<i>Anemone pulsatilla</i>	Anemonins	Lactone	Bacteria	0.5
Perpermint	<i>Mentha piperita</i>	Menthol	Terpenoids	Bacteria	
Periwinkle	<i>Vica minor</i>	Reserpine	Alkalods	General	1.5
Poppy	<i>Papaver somniferum</i>	Opium	Alkaloids, others	General	0.5
Potato	<i>Solanum tuberosum</i>	?		Bacteria, fungi	2
Quinine	<i>Chincona sp.</i>	Quinine	Alkaloids		2
Rosemary	<i>Rosemarinus officinalis</i>	Oil	Terpenoid	General	
Savory	<i>Satureja montana</i>	Carvacrol	Terpenoid	General	2
Thyme	<i>Thymus vulgaris</i>	Caffeic acids, thymol	Terpenoids, phenolic acid	Viruses, bacteria, fungi	
Tree bard	<i>Podocarpus nagi</i>	Totarol	Flavonoids	Gram-positive bacteria	
Yellow dock	<i>Rumex crispus</i>	?		<i>S.aureus, E. Coli, Samonella</i>	

^d Relative toxicity: 0=no toxic activity, < 1= toxic, > 3= very toxic, RT= Relative Toxicity^d. Adapted from (Cowan, 1999)

2.4 Plants as a potential source of antibiotics

The use of medicinal plants is widespread (Farnsworth, 1994). The production of medicines and the pharmacological treatment of diseases began with the use of herbs (Tyler, 1997). Life saving and essential drugs from medicinal plants such as morphine, digoxin, aspirin, emetine, and ephedrine were introduced into modern therapeutics several centuries ago. However, plants have been used as drugs for over millenia by human beings.

Other than for purposes of scientific inquiry, plants historically have served as models in drug development for three reasons: (a) Each plant is a unique chemical factory capable of synthesizing large numbers of highly complex and unusual chemical substances. In the United States of America alone, about 25% of prescription drugs contain active principles that are still extracted from higher plants and there is increasing popularity in the use of plant-derived preparations (Farnsworth and Morris, 1976). It has also been estimated by the World Health Organization (WHO) that about 80% of the population of the developing countries rely exclusively on plants to meet their health care needs (Farnsworth *et al.*, 1985). (b) The biologically active substances derived from plants have served as templates for synthesis of pharmaceuticals. Such compounds may have poor pharmacological and toxicological profiles. (c) Many highly active secondary plant constituents have been instrumental as pharmacological tools to evaluate physiological processes (Farnsworth, 1984).

There are numerous illustrations of plant-derived drugs. Some selected examples are presented in **Table 2-3**: The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha* and related species has been used for many years as an amoebicidal drug as well as for the treatment of abscesses resulting from *Escherichia histolytica* infections. Another important drug of plant origin with a long history of use is quinine. This alkaloid occurs in the bark of the cinchona tree. Apart from its usefulness in the treatment of malaria, it can be used to relieve nocturnal leg cramps (Iwu *et al.*, 1999).

Similarly, higher plants have also played important roles in cancer therapies. Recent examples include, combretastatins from *Combretum cafrum* (Pettit and Shigh, 1987). In the last two decades a series of stilbenes and dihydrostilbenes (the combretastatins) with potent cytotoxic activity, and acidic triterpenoids and their glycosides with molluscicidal, antifungal, antimicrobial activity, have been isolated from species of *Combretum* (Rogers, 1989b). Other antineoplastic agents include taxol and several derivatives of camptothecin from *Taxus brevifolia* and *Camptotheca acuminata*, respectively.

Table 2-3: Some plant-derived preparations for medicinal use

Active compound	Origin	Application
Ephedrine		Bronchodilator
Ergotamine	<i>Ergot spp</i>	Migraine remedy
Hyoscyamine		Anticholinergic
Ipratropium		Bronchodilator
Morphine	<i>Papaver somniferum</i>	Analgesic
Physostigmine	<i>Physostigma venenosum</i>	Cholinesterase inhibitor

Active compound	Origin	Application
Pilocarpine	<i>Pilocarpus jaborandi</i>	Glaucoma remedy
Quinidine	<i>Cinchona pubescens</i>	Anti arrhythmic
Quinine	<i>Cinchona pubescens</i>	Antimalarial
Reserpine	<i>Rauwolfia serpentine</i>	Antihypertensive
Salicin	Salix spp	Anti-inflammatory
Scopolamine	<i>Datura stramonium</i>	Antispasmodic
Sennoside A+ B	<i>Cassia angustifolia</i>	Laxative
Theophylline	<i>Camellia sinensis</i>	Bronchodilator
Vinblastine	<i>Catharantus roseus</i>	Antineoplastic

2.5 Plants and antibacterial production

An antibiotic has been defined as a chemical compound derived from or produced by living organisms, which is capable, in small concentrations of inhibiting the growth of micro-organisms (Evans, 1989). This definition limited antibiotics to substances produced by microorganisms but the definition could now be extended to include similar substances present in higher plants. Plants have many ways of generating antibacterial compounds to protect them against pathogens (Kuc, 1990). External plant surfaces are often protected by biopolymers e.g. waxes, and fatty acid esters such as cutin and suberin. In addition, external tissues can be rich in phenolic compounds, alkaloids, diterpenoids, steroid glycoalkaloids and other compounds, which inhibit the development of fungi and bacteria (Kuc, 1985). Cell walls of at least some monocotyledons also contain antimicrobial proteins, referred to as thionins (Carr and Klessig 1989).

Plant cells containing sequestered glycosides release them when ruptured by injury or infection. These glycosides may have antimicrobial activity against the invading pathogens or may be hydrolyzed by glycosidases to yield more active aglycones. In the case of phenolic compounds, these may be oxidized to highly reactive, antimicrobial quinones and free radicals (Kuc, 1985; Dean and Kuc, 1987). Thus, damage to a few cells may rapidly create an extremely hostile environment for a developing pathogen. This rapid, but restricted disruption of a few cells after infection can also result in the biosynthesis and accumulation of phytoalexins, which are low molecular weight anti-microbial compounds, which accumulate at sites of infection (Kuc, 1985; Carr and Klessig, 1989; Dean and Kuc, 1987). Some phytoalexins are synthesized by the malonate pathway others by the mevalonate, or shikimate pathways, whereas still others require participation of two or all three of the pathways. Phytoalexins are degraded by some pathogens and by the plant; thus they are transient constituents and their accumulation is a reflection of both synthesis and degradation rates.

Biopolymers are also often associated with the phytoalexin accumulation at the site of injury or infection. These biopolymers include: lignin, a polymer of oxidized phenolic compounds; callose, a polymer of β -1, 3-linked glucopyranose; hydroxyproline-rich glycoproteins, and suberin. They provide both mechanical and chemical restriction of development of pathogens (Kuc 1985; Carr and Klessig, 1989; Rao and Kuc, 1990). The macromolecule produced after infection or certain forms of physiological stress includes enzymes, which can hydrolyse the walls of some pathogens including chitinases, β -1,3-glucanases and proteases (Carr and Klessig, 1989). Unlike the phytoalexins and structural biopolymers, the amounts of these enzymes increase systemically in infected plants even in response to localized infection. These enzymes are part of a group of stress or infection-related proteins commonly referred to as pathogenesis-related (PR) proteins. The function of many of these proteins is unknown. Some may be defense compounds while others may regulate the response to infection (Carr and Klessig, 1989; Boller, 1987; Rao and Kuc, 1990).

Another group of systemically produced biopolymer defense compounds comprises the peroxidases and phenoloxidases (Hammerschmidt *et al.*, 1982; Rao and Kuc, 1990). Both can oxidize phenols to generate protective barriers to infection, including lignin. Phenolic oxidation products can also cross-link to carbohydrates and proteins in the cell walls of plants and fungi to restrict further microbial development (Stermer and Hammerschmidt, 1987). Peroxidases also generate hydrogen peroxide, which is strongly antimicrobial. Associated with peroxidative reactions after infection is the transient localized accumulation of hydroxyl radicals and super oxide anion, both of which are highly reactive and toxic to cells.

Plants therefore have several mechanisms to counter anti-microbial attack. Some of the anti-microbial compounds in plants may be exploited for use against bacterial diseases in man. Plants have developed an arsenal of weapons to survive attacks by microbial invasions. These include both physical barriers as well as chemical ones, i.e. the presence or accumulation of anti-microbial metabolites. These are either produced in the plant (prohibitins) or induced after infection, the so-called phytoalexins. Since phytoalexins can also be induced by abiotic factors such as UV irradiation, they have been defined as 'antibiotics formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors (Grayer *et al.*, 1994).

When an infection or damage to a plant takes place, a number of processes are activated and some of the compounds produced become activated immediately whereas phytoalexins take two three days to be produced. Sometimes it is difficult to determine whether the compounds are phytoalexins or prohibitins and moreover, the same compound may be a preformed anti-microbial in one species and a phytoalexin in

another (Grayer *et al.*, 1994). Since the advent of antibiotics in 1950s, the use of plant derivatives as anti-microbials has been virtually non-existent but that pace is rapidly on the increase as we begin to realize the need for new and effective treatments. The worldwide spending on finding new anti-infective agents is expected to increase 60% as from 1993 and plant source are especially being investigated (Grayer *et al.*, 1994).

2.6 The Family Combretaceae

2.6.1 Taxonomy

The family Combretaceae belongs to the order Myrtales and consists of 600 species of trees and shrubs in 20 genera, which include *Anogeissus*, *Bucida*, *Combretum*, *Quisqualis*, *Terminalia* and *Thiloa*, and are found throughout the tropics and sub-tropics. The largest genus is *Combretum*, with about 370 species, while *Terminalia* the second largest, and has about 200 species. They occur in most parts of Africa and are often the dominant vegetation (Rogers, 1996). The other genera are much smaller, including *Calopyxes* and *Buchenavia* which have 22 species each and *Quisqualis*, *Anogeissus*, *Conocarpis*, and *Pteleopsis* each with 16, 14, 12 and 10 species, respectively (Rogers and Verotta, 1996). The sub generic classification for southern Africa *Combretaceae* according to Carr (1988) is indicated in (Fig. 2-1).

In general, the genus of *Combretum* has 4-5-winged, ridged, angled, sessile or stipitate fruit while *Terminalia* has 2-winged fruit. Hybridization is a common occurrence in both genera. This results in the formation of numerous subspecies that look, on visual inspection, quite different. For example *C. albopunctatum* and *C. apiculatum* are similar in many respects, and so are *C. psisiodes* and *C. molle*, and *T. mollis* as well as *T. stenostachya* (Carr, 1988).

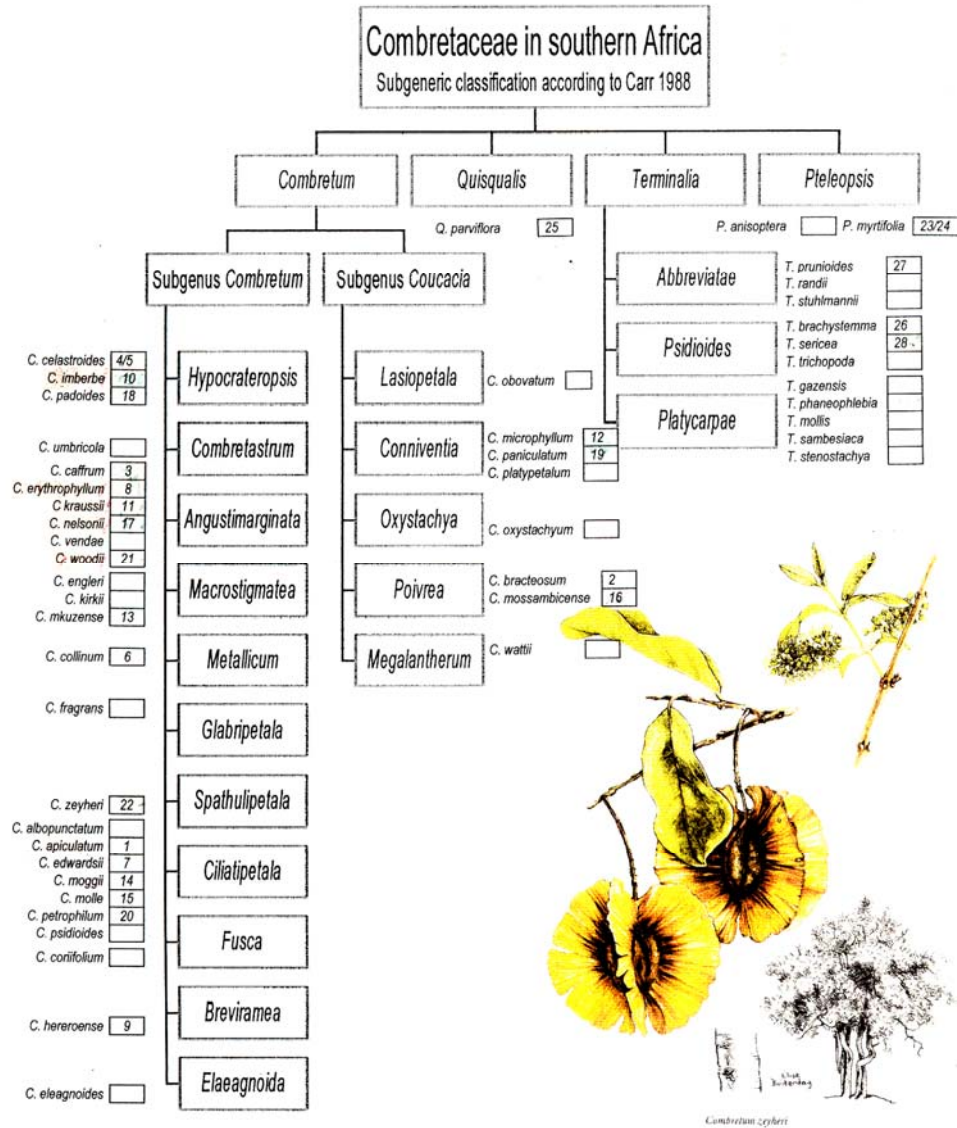


Figure 2-1: The sub generic classification for South Africa Combretaceae according to Carr (1988)

2.6.2 Taxonomy of the section *Hypocrateropsis*

The section *Hypocrateropsis* is made up of 4 species as described below:

2.6.2.1 *Combretum imberbe* Engl. & Diels (*mutsviri*) (*Leadwood*)

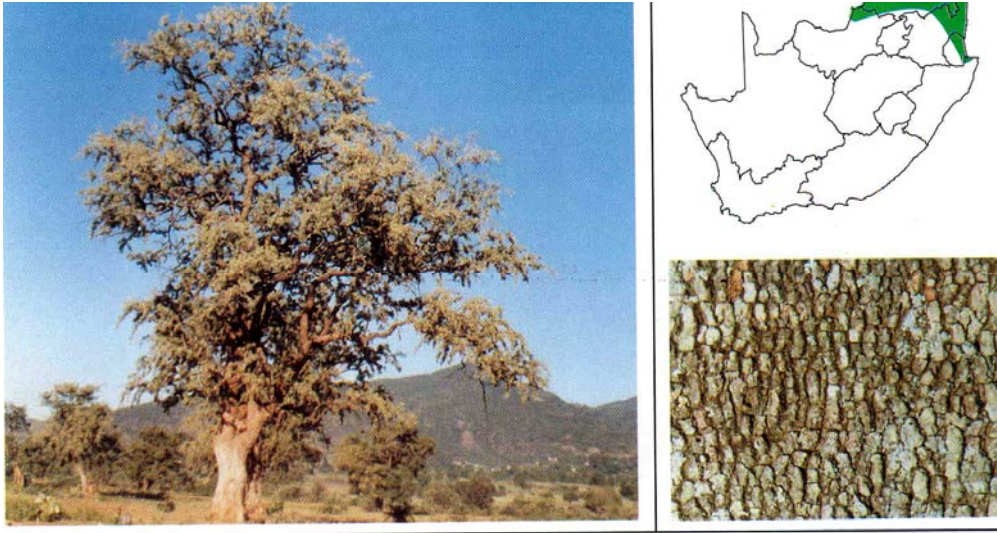


Figure 2-2: *C. imberbe* Leadwood/hardekool (Steyn, 1994)

Combretum imberbe (Fig. 2-2) is sometimes a shrub, but most frequently a small to large tree 7-15 m in height, with a grayish appearance. It occurs at medium to low altitudes, in mixed woodland and bushveld, often along rivers or dry watercourses, particularly on alluvial soils. **Bark:** Sometimes pale grey, smoothish and cracked in rectangular flakes, but mostly black and rough with characteristic deep longitudinal furrows and irregular transverse cracks, producing a mesh rather like crocodile skin. **Leaves:** occurs on short, opposite, often spine-tipped twigs; small, obovate to oblong, 2, 5-8x1-3 cm, usually 4 x 2 cm, grey-green, hairless, with silvery, microscopic scales densely covering both surfaces, thinly textured to rather leathery, 4-7 pairs of lateral veins visible on both surfaces; apex broadly tapering to round, often with a fine hair-like tip; base broadly to narrowing tapering; and petiole 4-10 mm long. **Flowers:** Cream to yellow, sweetly scented, in rather slender spikes 4-8 cm long, in the axils of the leaves or sometimes forming a terminal head, or panicle (Nov.-Mar.). **Fruits:** 4-winged, seldom exceeding 1,5 x 1,5 cm, somewhat round or often D-shaped in outline, apical peg distinct, densely covered with silvery scales, characteristically pale yellow drying to pale straw-coloured, giving the tree a distinct appearance (Feb. onward).

The heartwood is dark and extremely hard, heavy and durable. It is difficult to work and rapidly blunts and breaks tools, so it is not suitable for furniture; however, it bunts well and has been used for ornaments. It makes good fencing standards, railway sleepers and mine props. The wood is so hard that blades for hoes

were made from it before metal became available. The wood burns slowly; the ash has high lime content and has been used as toothpaste and is also suitable for whitewash (Carr, 1988)

2.6.2.2 *Combretum padoides* Engl. & Diel

Combretum padoides (Fig. 2-3) is a shrub, often thicket-forming, using its long trailing branches to scramble into adjacent trees, or a small tree 3-5 m in height; occurring at low altitudes in hot dry areas, in riverine fringes, on rocky hills and along escarpments in mixed woodland and bushedveld. **Bark:** light brownish grey, smoothish, flaking. **Leaves:** elliptic to narrowly so, 3-10 x 3-4, 5 cm, thinly textured, dull green, 6-8 pairs of lateral veins, yellowish and conspicuous with hairs along the veins below, net veining indistinct, with scales on the undersurface giving the appearance of skin that have been stippled or dotted with a white ballpoint pen; apex tapering to a point; base tapering to round; margin entire, wavy; petiole slender, up to 10 mm long. **Flowers:** creaming yellow, disc in the center hairy, in simple or branched, axillary or terminal, rather loose spikes up to 10 cm long, often in profusion (Dec.-Feb). **Fruits:** 4-winged, 1-1,5 cm long, pale yellow green drying to light brown, on very slender stalks about 3 mm long, often in profusion (Mar. -Jun).

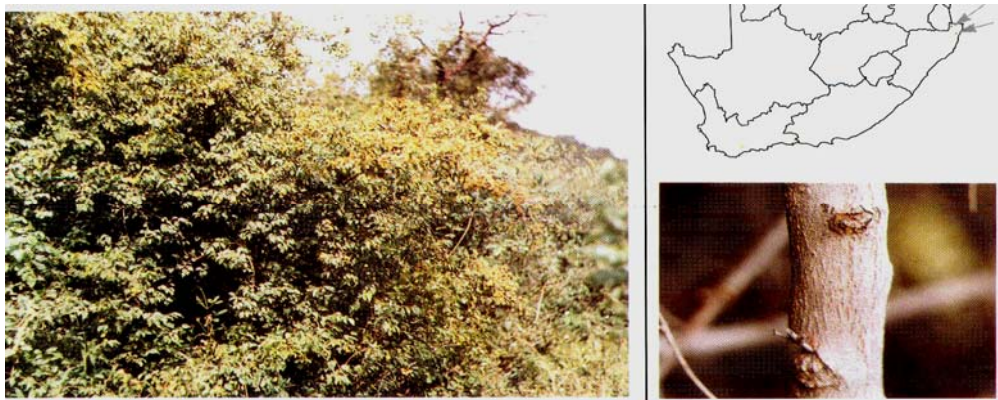


Figure 2-3: *C. padoides* (Steyn, 1997)

2.6.2.3 *Combretum celastroides* Welw Ex M.A. Lawson

A dense, straggling shrub about 4 m in height, or a tree up to 7 m; occurring in dry wood land, on rocky hillsides, frequently on Kalahari sand. **Bark:** creamy brown or grey with patches of lichen; rather smooth. **Leaves:** elliptic to broadly obovate, 2,5-14 x 1-8 cm, but usually about 4-5 cm long, thinly leathery, green, becoming beautiful deep red to plum-coloured in autumn, upper surface hairless with small dots visible against the light, undersurface with or without hairs (depending on the subspecies) but with scales that give

it the appearance of skin stippled or dotted with white boil point pen, 4-6 pairs with hair-tuft domatia-veining indistinct; apex bluntly acuminate; base tapering to rounded; margin entire; petiole up to 8 mm long. **Flowers:** greenish to yellow, disc in the center hairless, glossy; in rather sparse axillary spikes, usually about 5-8 cm long but sometimes reaching 12 cm (Dec.-Mar.). **Fruit:** 4-winged, 1-2 cm long, the wings becoming bright red while the body of the fruit remains greenish yellow, drying to golden brown with satiny sheen, with a distinct apical peg.

Ssp. *celastroides*: Occurring in Kalahari wood land and jesse-bush in Zimbabwe and extending into Botswana and the more arids area in Namibia; leaves usually 5-10 cm long with a finely velvety undersurface; flowers large, the disc measuring up to 4 mm diameter.

Ssp. *orientale* Excell (Fig. 2-4): Usually occurring on sand or silt at 50-500 m in coastal sand forest, lowveld savanna in the northern Limpopo and adjacent areas in Zimbabwe and Mozambique, and also in northern Kwazulu natal; Leaves about 5 x 2 cm, almost hairless except for hair-tuft domatia on the under surface; Flowers smaller, the disc measuring 2-2,5 mm in diameter. This ssp. is rather similar to *C. padoides*, but the later has a different habit, often with long trailing branches scrambling into adjacent trees, the fruits are smaller with apical peg very short to absent, and the disc is hairy.

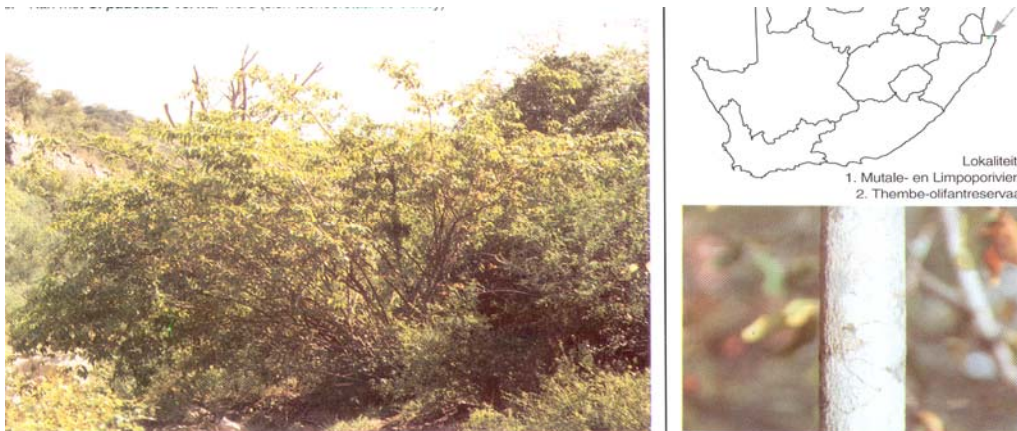


Figure 2-4: *C. celastroides* ssp. *orientale* (Steyn, 1994)

2.7 Ethnopharmacology of Combreteceae

Species of Combretaceae contain compounds with potential antimicrobial properties (Eloff, 1999a).

There is a large variation in the chemical composition and antibacterial activity among different genera and species in the Combretaceae. Seven species of Combretaceae used in traditional medicine in West Africa have been investigated for their antifungal activity against the pathogenic fungi. Phytochemical screening revealed that these plants are particularly rich in tannins and saponins, which might be responsible for their antifungal activity (Baba-Moussa *et al.*, 1999).

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

The ethnopharmacological use of *C. zeyheri* against diarrhoea and eye infections has been ascribed to its antibacterial activity towards Gram- positive microbes (Breytenbach and Malan, 1989).

C. erythrophyllum has been shown to possess many antibacterial compounds and some of these had activities higher than chloramphenicol and ampicillin (Martini and Eloff, 1998). Eloff (1999a) also found that all the leaf extracts from 27 Southern African members of the Combretaceae including *C. woodii* exhibited antibacterial activity against *S. aureus*, *E. coli*, *E. feacalis* and *P. aeruginosa*. The leaves of *C. molle* and *C. imberbe* have been shown to have molluscicidal activity against *Biomphalaria glabrata* snails (Rogers and Verotta, 1996).

2.8 Phytochemistry/chemistry and biological activity of Combretaceae

Phytochemistry, or plant chemistry, has developed in recent years as a distinct discipline, somewhere in between natural product organic chemistry and plant biochemistry and is closely related to both. It is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with the chemical structures of these substances, their biosynthesis, turnover and metabolism, distribution and their biological functions. The range and number of discrete molecular structures produced by plants is huge and such is the present rate of advancement of our knowledge of them that a major problem in phytochemical research is the collection of existing data on each particular class of compound. Because the number of known substances is so large, this section deals with a summary of the structural variation existing within each class of compound, outlining those compounds that are commonly occurring and illustrating the chemical variation within representative formulae. Classification is based on the presence of certain functional groups. Phytochemical work on *Combretum* and *Terminalia* start since the

early 1970s and extends well into the present. This section also outlines the phytochemical constituents that have been isolated in detail and considers the sources. The biological activities of some of these constituents are indicated as well.

2.8.1 Tannins

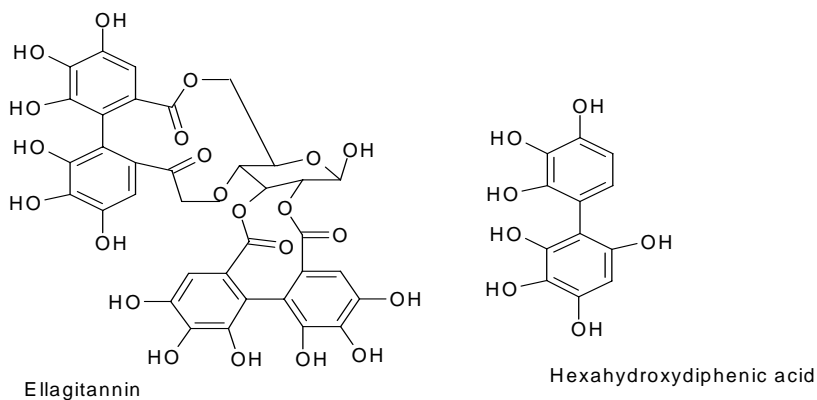
Plant polyphenols (vegetable tannins) are secondary metabolites widely distributed in the plant kingdom. They are based upon two broad structural themes: Hydrolysable tannins (HT) and Proanthocyanidins (PA) (often called condensed tannins)

2.8.1.1 *Classes of tannins*

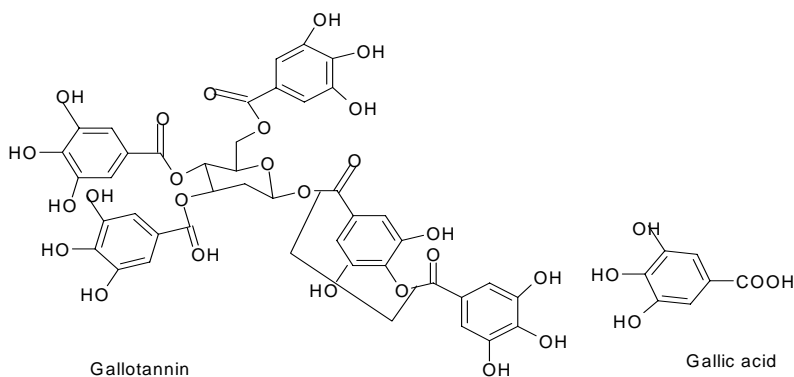
2.8.1.1.1 *Hydrolysable tannins (HTs)*

Hydrolysable tannins are molecules with a polyol (generally D-glucose) as a central core. The hydroxyl groups of these carbohydrates are partially or totally esterified with phenolic groups like gallic acid (gallotannins) or ellagic acid (ellagitannins). Hydrolysable tannins are usually present in low amounts in plants. Some authors define two additional classes of hydrolysable tannins: taragallotannins (gallic acid and quinic acid as the core) and caffetannins (caffeic acid and quinic acid)

Gallotannins: The phenolic groups that esterify with the core are sometimes constituted by dimers or higher oligomers of gallic acid (each single monomer is called galloyl). Each HT molecule is usually composed of a core of D-glucose and 6 to 9 galloyl groups. In nature, there is an abundance of mono and di-galloyl esters of glucose (MW about 900) that are not considered to be tannins. At least 3 hydroxyl groups of the glucose must be esterified to exhibit a sufficiently strong binding capacity to be classified as tannin. The most famous source of gallotannins is tannic acid obtained from the twig galls of *Rhus semialata*. It has a penta galloyl-D-glucose core and five more units of galloyl linked to one of the galloyl of the core (Harbone, 1994).



Ellagitannins: The phenolic groups consist of hexahydroxydiphenic acid, which spontaneously dehydrates to the lactone form, ellagic acid. Molecular weight range: 2000-5000.

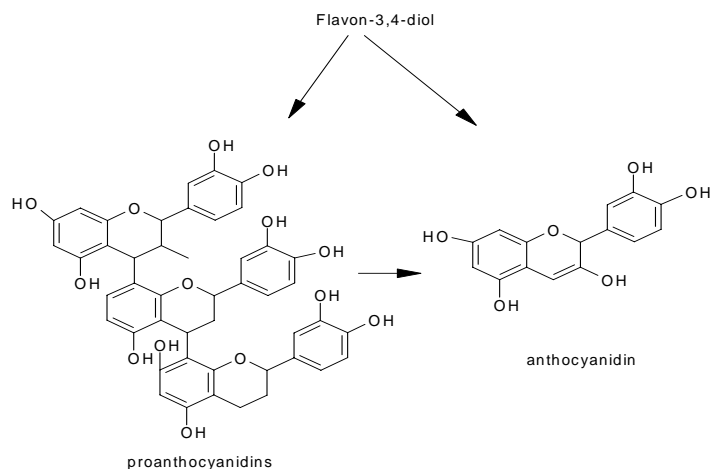


Hydrolysed by mild acids or mild bases to yield carbohydrate and phenolic acids. Under the same conditions, proanthocyanidins (condensed tannins) do not hydrolyze. Hydrolysable tannins are also hydrolyzed by hot water or enzymes (i.e. tannase).

2.8.1.1.2 Proanthocyanidins (condensed tannins)

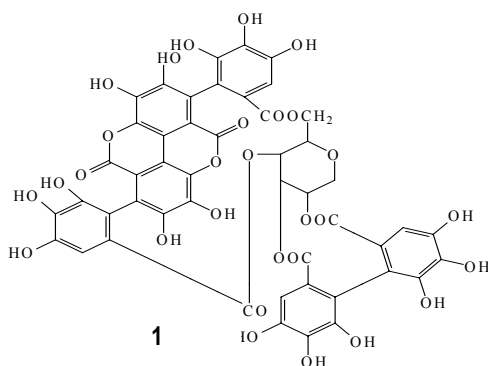
Proanthocyanidins are more widely distributed than HTs. They are oligomers or polymers of flavonoid units (i.e. flavan-3-ol) linked by carbon-carbon bonds not susceptible to cleavage by hydrolysis. Proanthocyanidins are more often called condensed tannins due to their condensed chemical structure.

However, HTs also undergo condensation reaction. The term, condensed tannins, is therefore potentially confusing. The term, proanthocyanidins, is derived from the acid catalyzed oxidation reaction that produces red anthocyanidins upon heating PAs in acidic alcohol solutions. The most common anthocyanidins produced are cyanidin (flavan-3-ol, from procyanidin) and delphinidin (from prodelphinidin). Proanthocyanidins may contain from 2 to 50 or greater flavonoid units; PA polymers have complex structures because the flavonoid units can differ for some substituent and because of the variable sites for interflavan bonds. Anthocyanidin pigments are responsible for the wide array of pink, scarlet, red, mauve, violet, and blue colors in flowers, leaves, fruits, fruit juices, and wines. Proanthocyanidin is the preferred name for condensed tannins (or flavolans), a series of flavan-3-ol oligomers that are usually based on a C-C link from the 8-position of one flavan unit to the 4-position of a second unit. As with the monomeric leucoanthocyanidins, they produce colored anthocyanidins on heating with mineral acid, but they have the additional property of binding to protein. The best-known proanthocyanidins are procyanidins, based on catechin and/or epicatechin units, and oligomers up to the hexamer have now been found in plants. Two common procyanidin dimers are described as epicatechin- (4, 8)-catechin and *ent*-epicatechin- (4, 8)-epicatechin respectively. A considerable number of doubly linked proanthocyanidins are known, where there is a second linkage through C-2 to O-7. The naming of such compounds can be accommodated in the same general way, e.g. one such compound is epicatechin- (2, 7, 4, 8)-epicatechin. Many Oligomeric proanthocyanidins with molecular sizes greater than the hexamer have been isolated from plants but their stereochemistries have yet to be determined (Harbone, 1994).



2.8.2 Tannins chemistry of Combretaceae

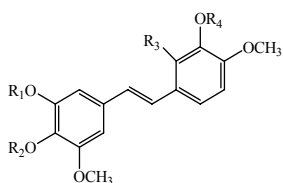
Gallic acid and its derivatives are commonly occurring constituents of the Combretaceae. A number of elaborate tannins have been isolated mainly from *Terminalia* e.g. the diphenoyl-gallagylglucose (1) isolated from *T. oblongata* and an ellagitannin from the leaf of *T. calamansanai* as well as derivatives from *T. catappa* and *T. chebula* (Pettit *et al.*, 1996).



These compounds as referred to by Keterere, 2001, have been shown to have anti-tumour activity in numerous studies. However the pharmacological activity induced by tannins (with the recent exception of ellagitannins) is, in general, non-specific and thus of little clinical potential (Pettit *et al.*, 1996).

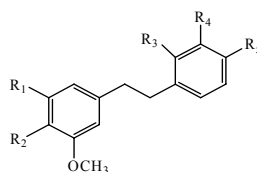
2.8.3 Stilbenoids

These compounds, which are biogenetically related, have generated immense interest because of their biological potency and structural simplicity. They were first isolated from *Combretum* species by Letcher and Nhamo (1973). Later in the 1980, Pettit and colleagues isolated stilbenes from a methylene chloride–methanol extract of the leaves, fruit and stemwood of *C. caffrum* (Cape bush willow tree) (Pettit *et al.*, 1982). They called these compounds combretastatins (2)-(25) which have since been isolated from *C. kraussii*, *C. molle*, *C. psidiodes* and *C. apiculatum* (Malan *et al.*, 1993). (See 4 and Table 2). Combretastatins have been designated as A, B, C and D according to their chemical structures. Combretastatins A and B are almost identical but differ in that the former has an ethylene bridge joining the two benzyl groups and is chemically identified as a stilbene. The latter, meanwhile, has an ethane-type bond and is based on the dihydrostilbenes (also called bibenzyls).



(2)- (6)

Primary structure of Combretastatins A



(7)-(25)

Primary structure of Combretastatins B

The work by Letcher and Nhamo (1973) as referred to by Keterere, 2001 was mainly concerned with the chemical analysis (including organic synthesis) of constituents of *C. apiculatum*, *C. psidiodes*, *C. molle*. Letcher and Nhamo (1973) also identified several unknown substituted phenanthrenes and 9, 10-dihydrophenanthrenes (26)-(44) (See **Table 2-6**) in addition to isolating a few stilbenes. Some of these compounds have since been also isolated from related species (Malan and Swinny, 1993). Combretastatin B5 has been isolated as the main antibacterial compound present in high concentration in leaves of *Combretum woodii* (Eloff *et al.*, 2005).

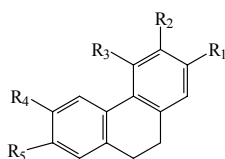
Table 2-4: Derivatives of stilbenes (Combrestatins) isolated from Combretaceae

	R ₁	R ₂	R ₃	R ₄		Plant source
2	CH ₃	CH ₃	OH	H	C-A1	<i>C. caffrum</i> <i>C. kraussii</i>
3	-CH ₂ -	-CH ₂ -	OH	H	C-A2	<i>C. caffrum</i>
4	H	CH ₃	H	OH	C-A3	<i>C. caffrum</i>
5	H	CH ₃	H	CH ₃	C-A6	<i>C. caffrum</i>
6	CH ₃	CH ₃	OH	OGlc		<i>C. kraussii</i>

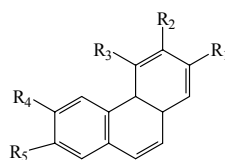
Table 2-5: Derivatives of dihydrostilbenes (Combrestatins) isolated from Combretaceae

	R ₁	R ₂	R ₃	R ₄	R ₅		Plant
7	OCH ₃	OCH ₃	OH	OH	CH ₃	C-B1	<i>C. caffrum</i> <i>C. kraussii</i>
8	O-CH ₂ -O	O-CH ₂ -O	OH	OH	CH ₃	C-B2	<i>C. caffrum</i>
9	OCH ₃	OCH ₃	H	OH	H	C-B3	<i>C. caffrum</i>
10	OCH ₃	H	H	OH	H	C-B4	<i>C. caffrum</i>
11	OCH ₃	H	H	OH	CH ₃		<i>C. caffrum</i>
12	OCH ₃	H	H	H	H		<i>C. caffrum</i>
13	OCH ₃	OCH ₃	H	H	H		<i>C. psidiodes</i> , <i>C. caffrum</i>

	R ₁	R ₂	R ₃	R ₄	R ₅	Plant
14	OH	H	-	H	H	<i>C. apiculatum</i>
15	OH	H	H	OH	CH ₃	<i>C. apiculatum</i>
16	OH	OCH ₃	H	OCH ₃	H	<i>C. apiculatum</i>
17	OH	OH	H	OCH ₃	CH ₃	<i>C. apiculatum</i>
18	OH	OCH ₃	O-Glc	OH	H	<i>C. apiculatum</i>
19	OH	OH	OH	H	CH ₃	<i>C. apiculatum</i>
20	OH	OCH ₃	-Glc	H	H	<i>C. apiculatum</i> <i>C. molle</i>
21	OCH ₃	OH	H	H	H	<i>C. apiculatum</i> , <i>C. psidiodes</i>
22	OCH ₃	OCH ₃	CH ₃	OH	O-Glc	<i>C. kraussi</i>
23	OCH ₃	OH	CH ₃	OH	OH	C-B5 <i>C. kraussi</i>
24	OCH ₃	OCH ₃	CH ₃	OH	O-Glc	<i>C. kraussi</i>
25	OCH ₃	OH	CH ₃	OH	O-Glc	<i>C. kraussi</i>



26-44



26-44

Table 2-6: Derivatives of phenanthrenes isolated from Combretaceae

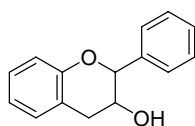
	R ₁	R ₂	R ₃	R ₄	R ₅	Plant
26	OCH ₃	OCH ₃	OH	OH	OH	<i>C. apiculatum</i>
27	OH	OCH ₃	OCH ₃	OH	OCH ₃	<i>C. apiculatum</i>
28	OCH ₃	OCH ₃	OH	OCH ₃	OH	<i>C. apiculatum</i> , <i>C. molle</i>
29	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OH	<i>C. caffrum</i>
30	OH	OH	OCH ₃	OCH ₃	OH	<i>C. apiculatum</i>
31	OCH ₃	OH	OCH ₃	OH	OH	<i>C. apiculatum</i>
32	OCH ₃	OH	OCH ₃	OCH ₃	OH	<i>C. apiculatum</i>
33	OCH ₃	OH	OCH ₃	OCH ₃	OCH ₃	<i>C. apiculatum</i>
34	OCH ₃	H	OH	OCH ₃	OH	<i>C. apiculatum</i>
35	OCH ₃	OCH ₃	OH	OCH ₃	OH	<i>C. apiculatum</i> , <i>C. molle</i>
36	OH	OCH ₃	OCH ₃	OH	OCH ₃	<i>C. apiculatum</i> <i>C. molle</i>
37	OCH ₃	OCH ₃	OH	OH	OH	<i>C. apiculatum</i> , <i>C. molle</i>
38	OH	OCH ₃	OCH ₃	OH	OCH ₃	<i>C. apiculatum</i> , <i>C. molle</i>
39	OCH ₃	OCH ₃	OH	OH	OH	<i>C. apiculatum</i> , <i>C. molle</i>
40	OCH ₃	OCH ₃	OCH ₃	OH	OH	<i>C. apiculatum</i> , <i>C. caffrum</i> ,

	R ₁	R ₂	R ₃	R ₄	R ₅	Plant
41	OH	OCH ₃	OCH ₃	OCH ₃	OCH ₃	<i>C. caffrum</i>
42	OH	OCH ₃	OCH ₃	OCH ₃	OH	<i>C. caffrum, C. apiculatum, C. psidiodes</i>
43	OCH ₃	OCH ₃	OCH ₃	OCH ₃	OH	<i>C. apiculatum, C. psidiodes, C. caffrum</i>
44	OCH ₃	OH	OCH ₃	OCH ₃	OCH ₃	<i>C. apiculatum</i>

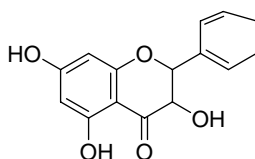
2.8.4 Flavonoids

Flavonoids are a group of polyphenolic compounds, which are widely distributed through out the plant kingdom. To date about 3000 varieties of flavonoids are known. Many have low toxicity in mammals and some of them are widely used in medicine for maintenance of capillary integrity. Flavonoids exhibit several biological effects such as anti-inflammatory, anti-hepatotoxic and anti-ulcer actions. They also inhibit enzymes such as aldose reductase and xanthine oxidase. They are potent antioxidants and have free radical scavenging abilities. Many have anti-allergic, antiviral actions and some of them provide protection against cardiovascular mortality. They have been shown to inhibit the growth of various cancer cell lines *in vitro*, and reduce tumour development in experimental animals (Narayana, *et al.*, 2000)

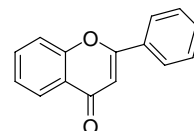
Structure and classification of flavonoids: Flavonoids occur as aglycones, glycosides and methylated derivatives. The flavonoid aglycone consists of a benzene ring (A) condensed with a six-membered ring (C), which in the 2-position carries a phenyl ring (B) as a substituent. Six-membered ring condensed with the benzene ring is either a pyrone (flavonols and flavonones) or its dihydroderivative (flavanols and flavanones). The position of the benzenoid substituent divides the flavonoid class into flavonoids (2-position) and isoflavonoids (3-position). Flavonols differ from flavonones by hydroxyl group at the 3-position and a C2-C3 double bond. Flavonoids are often hydroxylated in positions 3, 5, 7, 2', 3', 4', 5'. Methyl ethers and acetyl esters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucorhamnose, galactose or arabinose. The most common flavonoids are listed in **Table 2-7**.



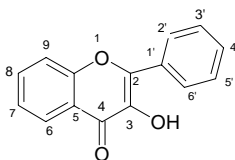
Flavan-3-ol



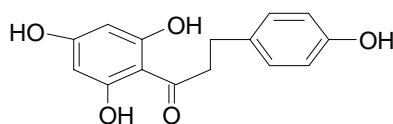
Flavanolols



Flavone



Flavonol



Chalcone

Table 2-7; Nomenclature of the subclasses of flavonoids (Narayana *et al.*, 2000),

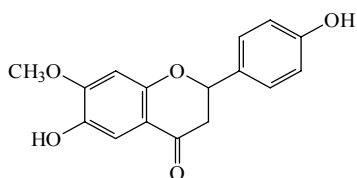
	3	5	7	2'	3'	4'	5'
Flavonols:							
Kaempferol	OH	OH	OH	H	H	OH	H
Morin	OH	OH	OH	OH	H	OH	H
Rutin	O-R ¹	OH	OH	H	OH	OH	OH
Myricetin	OH	OH	OH	H	OH	OH	OH
Quercetin	OH	OH	OH	H	OH	OH	H
Quercetrin	O-Rh	OH	OH	H	OH	OH	H
Myricitrin	O-Rh	OH	OH	H	OH	OH	OH
Spirenoside	OH	OH	OH	H	OH	O-Glu	H
Galangin	OH	OH	OH	H	H	H	H
Robinin	O-R ¹	OH	OH	H	H	OH	H
Kaempferide	OH	OH	OH	H	H	O-Me	H
Fisetin	OH	H	OH	H	OH	OH	H
Rhamnetin	OH	OH	O-Me	H	OH	OH	H
Flavonones:							
Hesperitin	H	OH	OH	H	OH	O-Me	H
Naringin	H	OH	O-R	H	H	OH	H
Naringenin	H	OH	OH	H	H	OH	H
Eriodictyol	H	OH	OH	H	OH	OH	H
Hesperidin	H	OH	O-Me	H	OH	O-Me	H
Pinocembrin	H	OH	OH	H	H	H	H
Likvirin	H	H	OH	H	H	O-Glu H	H
Flavones:							
Rpofolin	H	OH	O-R	H	H	OH	H
Apigenin	H	OH	OH	H	H	OH	H
Tangeretin	H	O-Me	O-Me	H	H	O-Me	H
Flavone	H	H	H	H	H	H	H
Baicalein	H	OH	OH	H	H	H	H
Luteolin	H	OH	OH	H	OH	OH	H
Chrysin	H	OH	OH	H	H	H	H
Techtochrysin	H	OH	O-Me	H	H	H	H
Diosmetin	H	OH	OH	H	OH	O-Me	H
Diosmin	H	OH	O-R ¹	H	OH	O-Me	H
Flavandols:							
Silibinin	OH	OH	OH	H	H	O-L-O -	H
Silymarin	OH	OH	OH	H	H	O-L-O -	H
Taxifolin	OH	OH	OH	H	OH	OH	H
Pinobanksin	OH	OH	OH	H	H	H	H
Flavan-3-ols:							
Catechin	OH	OH	OH	H	OH	OH	H
Isoflavones:							
Genistein	-	OH	OH	H	H	OH	H
Daidzin	-	H	O-Glu	H	H	OH	H

-O-Me = Methoxy -O-Glu = Glucosyl -O-R¹ = Alkoxy -O-L-O = Selaene

2.8.4.1 Flavonoids chemistry of Combretaceae

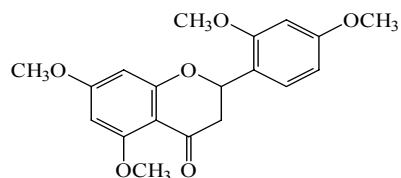
The flavonoids, luteolin, has been isolated from *Terminalia arjuna* and shown to be active against a murine P388 lymphocytic leukemia and human cancer cell line as well as inhibiting the growth of *Neisseria gonorrhoeae* (Pettit *et al.*, 1996). Luteolin has been shown to be both an anti-tumour promotor and mutagen.

Arjunolone (1) and arjunone (2), two flavanones, have also been isolated from *T. arjuna* which is widely used in India and surrounding countries for many medical conditions arising from cardiovascular insufficiency.



(1)

Arjunolone



(2)

Arjunone

A study of the leaves of the South American plant, *C. leprosum* showed two flavonoids, 3-O-methylquercetin and 3-O- α -L-rhamnopyranosylquercetin (quercetin). Seven antibacterial flavonoids were subsequently isolated by bioassay-guided fractionation, i.e. apigenin; genkwanin; 5-hydroxy-7, 4-dimethoxyflavone, rhamnocitrin; kaempferol; quercetin-5, 3-dimethylether; rhamnazin from *C. erythrophyllum* (Martini *et al.*, 2004)

2.8.5 Terpenoids

Terpenoids are all based on the isoprene molecule $\text{CH}_2=\text{C}(\text{CH}_3)-\text{CH}=\text{CH}_2$ and their carbon skeletons are built up from the union of two or more of these C_5 units. They are then classified according to whether they contain two (C_{10}), three (C_{15}), four (C_{20}), six (C_{30}) or eight (C_{40}) such units. They range from the essential oil components, volatile mono- and sesquiterpenes (C_{10} and C_{15}) through to the less volatile diterpenes (C_{20}) to the involatile triterpenoids and steroids (C_{30}) and carotenoids pigments (C_{40}). Chemically, terpenoids are generally lipid soluble and are located in the cytoplasm of the plant cell. Essential oils sometimes occur in special granular cells on the leaf surface, whilst carotenoids are especially associated with the chloroplast. Terpenoids are normally extracted from plant tissue with light petroleum ether or chloroform.

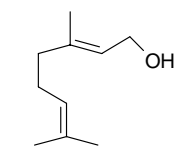
A considerable number of different functions have been ascribed to plant terpenoids. Their growth regulating properties are very well documented; two of the major classes of growth regulator are the sesquiterpenoids, abscisins and the diterpenoid-based gibberellins. The important contribution of carotenoids to plant colour is well known and it is almost certain that these C_{40} terpenoids are also involved as accessory pigments in photosynthesis. The mono- and sesquiterpenes provide plants with their distinctive smell. Also, certain nonvolatile terpenoids have been implicated as sex hormones among the fungi (Harbone, 1994).

2.8.5.1 Classes of terpenes

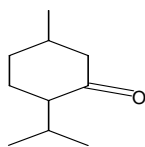
2.8.5.1.1 Terpene essential oils

Chemically, this group of terpenes can be divided into classes; the mono- and the sesquiterpenes. The monoterpenes can further be divided into three groups depending on whether they are acyclic (geraniol), monocyclic (limonene) or have functional groups alcohol (menthol), aldehyde or ketone.

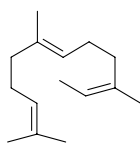
Like the monoterpenes, the sesquiterpenes fall chemically into groups according to the basic carbon skeleton; the common ones are acyclic (farnesol), monocyclic (γ -bisabolene) or bicyclic (beta-selinene, carotol).



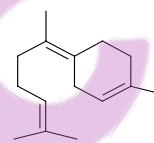
Geraniol
(Acyclic)



Menthone
(monocyclic)



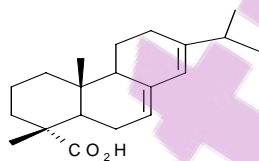
Farnesol



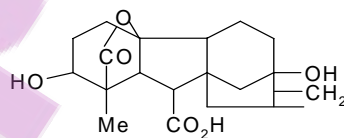
γ -bisabolene

2.8.5.1.2 Diterpenoids and Gibberellins

Diterpenoids comprise a chemically heterogeneous group of compounds, all with a carbon skeleton based on four isoprene units; most have a very limited distribution. Many classes of diterpenes are known, among them resin diterpenes, toxic diterpenes and gibberellins. The resin compounds are protective in nature and are exuded from wood trees or latex of plants. The toxic diterpenes occur in the foliage of the leaves of some plants and are responsible for the poisonous nature of the foliage. The gibberellins are a group of hormones, which generally stimulate growth and are known to be widespread in plants (Goodwin, 1981).



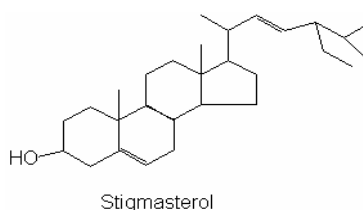
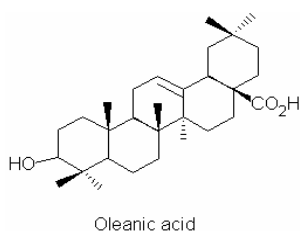
Abietic acid (resin)



Gibberellic acid

2.8.5.1.3 Triterpenoids and Steroids

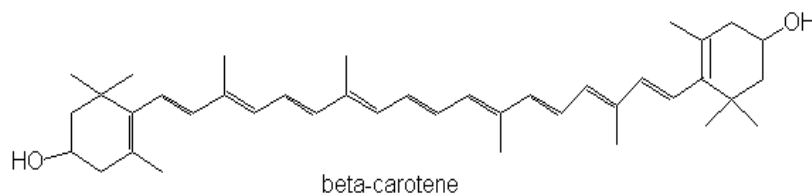
Triterpenoids are compounds with a carbon skeleton based on six isoprene units. They are relatively complex cyclic structures, most being either alcohols, aldehydes or carboxylic acids. They are colourless, crystalline, often with a high melting point, optically active substances that are generally difficult to characterize because of their lack of chemical reactivity. Triterpenoids can be divided into at least four groups of compounds: true terpenes, steroids, saponins and cardiac glycosides. The latter two groups are terpenes or steroids that occur mainly as glycosides. Many triterpenes are known in plants and new ones are regularly being discovered and characterized. So far only a few are known to be widely distributed. This is true of the pentacyclic triterpenes alpha and beta-amyrin and the derived acids, ursolic and oleanic acids. These and related compounds occur especially in the waxy coating of leaves and fruits such as apple and pear have a protective function of repelling insect and microbial attack. Steroids are triterpenes, which are based on the cyclopentane perhydrophenanthrene ring system. At one time, steroids were considered to be mainly animal substances (sex hormones) but in recent years such compounds have been detected in plant tissues e.g stigmasterol is a phytosterol. Saponins are glycosides of both triterpenes and steroids and have been detected in several families of plants. They are surface-active agents with soap-like properties and can be detected by their ability to cause foaming and to haemolyse blood cells. The glycoside patterns of the saponins are often complex; many have as many as five sugar units attached and glucuronic acid is a common component (Goodwin, 1981).



2.8.5.1.4 Tetraterpenoids (Carotenoids)

Carotenoids that are C₄₀ tetraterpenoids are a widely distributed group of lipid-soluble pigments, found in all kinds of plants. In plants, carotenoids have two principal functions: as accessory pigments in photosynthesis and as coloring matters in flowers and fruits. In flowers, they mostly occur as yellow colours, while in fruits, they may, in addition, be orange or red. Well-known carotenoids are either simple unsaturated hydrocarbon based on lycopene or their oxygenated derivatives known as as xanthophylls. The chemical structure of lycopene consists of a long chain of eight isoprene units joined head to tail to form a completely conjugated system of alternate double bonds which is the chromophore giving it colour. Cyclization of lycopene at one

end gives γ -carotene while cyclization at both ends provides the bicyclic hydrocarbon beta-carotene. Beta-carotene is the most common of all these pigments.

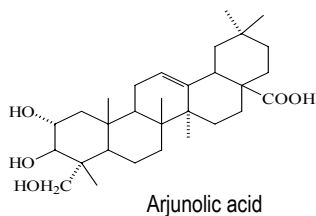


2.8.5.2 Terpenoids chemistry of Combretaceae

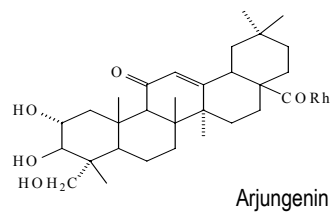
Triterpenoids are the most ubiquitous non-steroidal secondary metabolites in terrestrial and marine flora and fauna (Mahato and Nandy, 1991). Their medicinal use is rather limited but there remains some potential for application.

The Combretaceae as referred to by Keterere, 2001, has yielded a number of different pentacyclic triterpenoid structures, including oleanolic and ursanoic acids, friedelins, cycloartanes and dammaranes. The fruit of *C. molle*, when extracted with acetone, yielded arjunolic acid (1) arjungenin (2) and arjunglucoside (3), pentacyclic triterpenoids previously found in *Terminalia arjuna* (Panzini, 1993).

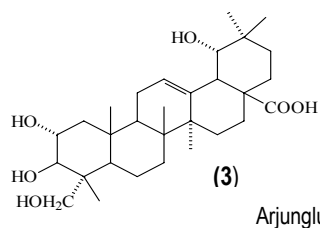
The ether extract of *C. imberbe* leaves has yielded a complex mix of triterpenoids and their glycosides (Roger, 1988). Rogers and Subramony (1988) isolated friedelin, epifriedelin and betulinic acid from the bark of *C. imberbe* and an oleanene-based pentacyclic triterpene with its glycosides from the leaves (4)-(5).



(1)

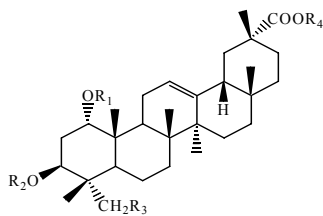


(2)



(3)

The latter was characterized as $1\alpha, 3\beta$ -dihydroxyolean-12-en-29-oic acid, and named imberbic acid.



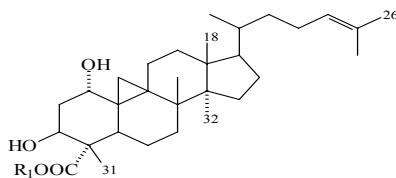
Compound	R 1	R 2	R 3	R 4
4	H	H	O-Rh	H
5	Ac	H	O-Rh	H

23-hydroxyimberbic acid 23-O- α -L-rhamnopyranoside (**4**)

23-hydroxyimberbic acid 23-O- α -L-rhamnopyranoside-1-acetate (**5**)

The ether extract of *C. edwardsii* leaves yielded the xyloside and arabinoside of mollic acid, previously isolated from *C. molle* and also reported to be present in a South American species *C. leprosum* (Rogers, 1988)

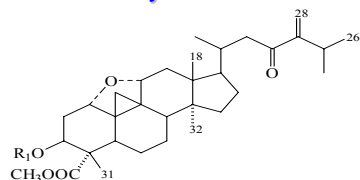
The isolation of oleanene- type pentacyclic triterpenoids containing 29-carboxy-1 α -hydroxy groups from *C. molle*, *C. edwardsii*, *C. eleagnoides*, *C. imberbe*, *C. apiculatum*, *C. kraussi* and *C. padoides* confirms chemotaxonomically significant bifurcation in triterpenoid synthesis in the *Combretum* species (Rogers and Verotta, 1996). Both imberbic acid and mollic acid have anti-inflammatory and molluscicidal activity (Panzini *et al*, 1993). Molic acid (**6**)-(7) has been isolated from *C. molle*. Jessic acid (**8**)-(9) was previously isolated from *C. eleagnoides*. It was later found in the acetone fraction of *C. molle* fruit as the 3-O- β -D-xylopyranoside.



Compound	R 1
6	H
7	Me

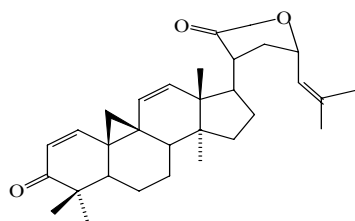
Derivatives of jessic acid, a cycloartane triterpenoid, have been found; the most chemically interesting being methyl jessate 1 α , 11 α -oxide (**8**) isolated from the hexane fraction of dry leaves and which shows a boat-like oxide bridge.

Combretum erythrophyllum as referred to by Kerere, 2001, has yielded some unusual cycloartane dienone lactones (10) and (11) from the leaves (Rogers, 1996).

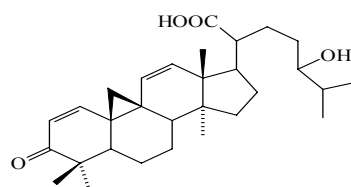


Compound	R 1
8	H
9	Ac

From this survey of phytochemical constituents it may be concluded that terpenoids not only appear to be taxonomic markers to establish biogenetic relationships, but also are the most commonly occurring compounds in the Combretaceae.



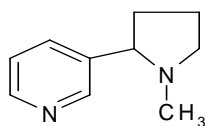
(10)



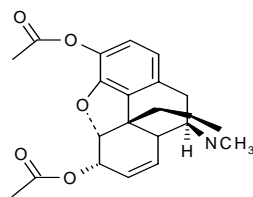
(11)

2.8.6 Alkaloids

Alkaloids are the largest group of secondary plant compounds (more than 12,000) and many exhibit marked physiological and pharmacological properties. Alkaloids are a heterogeneous group of compounds, which defy any adequate definition. In general, alkaloids are basic compounds containing one or more heterocyclic nitrogen atoms (e.g. nicotine).



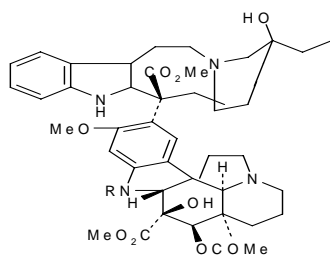
nicotine



heroin

Heroin is a synthetic derivative of morphine and was originally considered a wonder drug.

2.8.6.1.3 Tryptophan derivatives

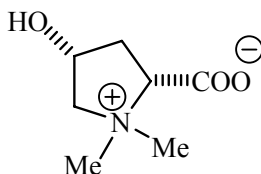


Vinca alkaloids vinblastine (R=Me) and vincristine (R=CHO)

The tryptophan derivatives include the vinca alkaloids vinblastine (R=me) and the related compound vincristine (R=CHO), both of which are potent chemotherapeutic agents. The Madagascar periwinkle (*Catharanthus roseus*) is a natural source of these compounds (Harbone, 1994).

2.8.6.2 Combretaceae alkaloids chemistry

No other alkaloids are known to have been isolated from the Combretaceae, apart from the simple betaine (1) isolated from *C. micranthum* and simple indole alkaloids from the species of a related genus, *Guiera senegalensis*. This may be attributed to the inability of the separation methods to detect polar constituents.



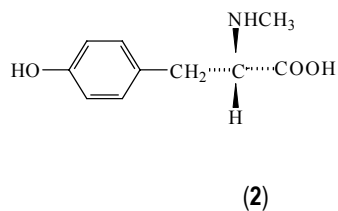
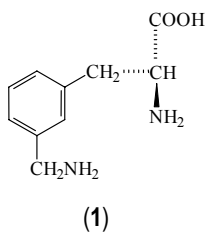
(1)

Hydroxyproline betaine, an alkaloid from the leaves of *C. micranthum*

2.8.7 Other constituents

The fruit of *C. zeyheri* as referred to Kerere, 2001, has shown the presence of the amino acids, L-3-(3-hydroxymethylphenyl) alanine (1), its glucoside and L-N-methyltyrosine β-D-glucopyranoside (2) (Panzini *et al.*, 1993).

The hexane extract of *C. apiculatum* fruit produced a compound identified as the anti-oxidant, 2, 6-di-*tert*-butyl-*p*-cresol (BHT), also isolated from *C. zeyheri* leaves.



This compound is toxic to brine shrimp but there are doubts about its authenticity as a plant metabolite, though it is reported to have been isolated from the family Annonaceae (Panzini *et al.*, 1993).

2.9 Methods developed and results obtained in the phytomedicine programme

2.9.1 Bioautography and MIC methods

The bioautography is a fast and quick guide toward bioassay-guided isolation and fractionation of antibacterial compounds and fractions respectively. In this approach, the activity of plant extracts on bacteria is determined on chromatography plates, in accordance with the bioautography procedure of (Begue and Kline 1972). This method is explained in **section 4.2.2** (page 51).

In an attempt to quantify the activity of the extracts, the microplate dilution method is used to determine the Minimum Inhibitory Concentration (MIC) (Eloff 1998c). This method makes use of a 2-fold serial dilution of extracts beyond the level where no inhibition of growth is observed. This method is explained in **section 4.2.3** (page 52)

2.9.2 Overview of activities in Combretaceae research in the phytomedicine group

2.9.2.1 Selection of plants

An analysis was made of approaches to be followed towards selecting plants for research and gene banking. Plants used as phytomedicines in Africa and were also analyzed and the Combretaceae made up a major group (Eloff, 1998a).

2.9.2.2 Selection of best extraction procedure

Several extractants were tested and evaluated on many different parameters. Acetone was found to be the best extractant (Eloff, 1998b).

2.9.2.3 Selection of best purification procedures

The solvent-solvent fractionation procedure used by the USA National Cancer Institute was tested and refined and several TLC separation procedures were also developed. (Eloff, 1998c)

2.9.2.4 Developing a novel way of determining antibacterial activity

It could be shown that the traditional agar diffusion assays for determining activity of plant extracts did not work. A new serial dilution microplate assay using INT was developed. (Eloff, 1998c)

2.9.2.5 Antibacterial activity of *C. erythrophyllum*

Using the techniques developed above it was shown that *C. erythrophyllum* contains at least 14 antibacterial compounds (Martini and Eloff 1998). Extracts had MIC values as low as 50 µg/ml.

2.9.2.6 Antibacterial activity and stability of 27 members of Combretaceae

Acetone leaf extracts of 27 species of *Combretum*, *Terminalia*, *Pteleopsis* and *Quisqualis* all had antibacterial activity ranging from 0.1 –6 mg/ml. Storing extracts for 6 weeks at room temperature did not affect MIC values.

2.9.2.7 Stability of antibacterial activity in *C. erythrophyllum*

Leaves of *C. erythrophyllum* stored in herbaria for up to 92 years did not lose any antibacterial activity (Eloff, 1999b).

2.9.2.8 A proposal for expressing antibacterial activity

MIC values do not give any indication of the activity present in a plant. A proposal was made that “total activity” should be determined by dividing the quantity extracted from 1 g of plant material in mg by the MIC in mg/ml. The resultant value in ml/g gives the highest dilution to which a plant extract can be diluted and still inhibited the growth of the test organism. The same proposal can be used to evaluate the efficacy of different steps in a bioassay guided isolation (Eloff, 2004).

2.9.2.9 Isolation of antibacterial compound from *C. woodii*

C. woodii acetone leave extracts had a better MIC values than ampicillin and chloramphenicol. The antibacterial compound combretastatin B5 was isolated and characterized. This compound had an MIC of 16 ug/ml against *S. aureus* and was present at a concentration of 5-10 mg/g in leaf extract (Eloff *et al.*, 2005)

2.9.2.2.10. Isolation of antibacterial compounds from *C. erythrophyllum*

For her PhD study Martini (2002) isolated and characterized seven antibacterial compounds. Four were flavanols: kaemferol, rhamnocitrin, rhamnazin, quercetin 5, 3 -dimethyl ether] and three flavones apigenin, genkwanin and 5-hydroxy-7, 4'-dimethoxyflavone (Martini *et al.*, 2004).

All test compounds had good activity against *Vibrio cholerae* and *Enterococcus faecalis*, with MIC values in the range of 25-50 µg/ml. Rhamnocitrin and quercetin-5,3-dimethylether showed additional good activity (25 µg/ml) against *Micrococcus luteus* and *Shigella sonnei*. Toxicity testing showed little or no toxicity towards human lymphocytes with the exception of 5-hydroxy-7, 4-dimethoxyflavone. This compound is potentially toxic to human cells and exhibited the poorest antioxidant activity. Both rhamnocitrin and rhamnazin exhibited strong antioxidant activity with potential anti-inflammatory activity. Although these flavonoids are known, this was the first report of biological activity with some of these compounds (Martini *et al.*, 2004).

2.9.2.11 Other biological activities of *Combretum* species

The anti-inflammatory, anthelmintic and antischistosomal activity of 20 *Combretum* species was determined. There was very little antischistosomal activity, low to medium anthelmintic activity and medium to strong anti-inflammatory activity in extracts of the different species (McGaw *et al.* 2001)

2.9.3 Other work done on *C. imberbe* and *C. padoides*

Rogers and Subramony (1988) have isolated pentacyclic triterpene acids from the leave of *C. imberbe*. Related glycoside have also been isolated, all based on the olean-12-en-29-oate aglycone which has been given the trivial name imberbic acid (Roger, 1988).

Katerere *et al.*, (2002) isolated novel glycoside derivatives of hydroxyimberbic acid from the leaves of *C. imberbe*. These compounds had antibacterial activity against few strains of nosocomial organisms.

Rogers (1989b) isolated triterpenoid desmosides and 25 (27)-dehydroporiferasterol from the leaves of *C. padoides*. The biological activities of these compounds were never investigated.

Eloff (1998a) investigated the antibacterial activity of some 27 members of Combretaceae found *C. imberbe* and *C. padoides* among ther to have many antibacterial compounds and good antibacterial activity after quantification.

CHAPTER 3

PLANT COLLECTION, EXTRACTION AND ANALYSIS

3.1 Introduction

Fresh or dried plant material can be used as a source for the extraction of secondary plant components.

Most scientists have opted to use dry material for several reasons:

Traditional healers frequently use dry plant material.

The time delay between collecting plant material and processing it makes it difficult to work with fresh material.

There are fewer problems associated with the large-scale extraction of dry plant material. Freshly harvested and dried material is more commonly used since old dried material stored for a period of time may undergo some qualitative changes.

In this chapter dry leaves were used for direct extraction with acetone for the preliminary screening step. Serial exhaustive extraction using hexane, dichloromethane (DCM), acetone, and methanol as successive extractants was employed for the extraction leading to isolation of antibacterial compounds.

3.2 Material and Methods

The standard procedures for extracting and testing for antibacterial activity to be used in the present study [sections 3.2.3.1, 2.2.3.2 and 4.2.2 (page 51)] have been developed to such a degree in the University of Pretoria Phytomedicine laboratory that few problems were encountered. Nevertheless, there is such a difference in anti-microbial activity and chemical composition between different plant species, that much innovation and modification were required in the isolation.

3.2.1 Experimental design for bioassays

Four treatments were used in bioassays.

Group 1: Test Group: Consisted of the organism (ATCC strains of *Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*) plus different concentrations of the extracts (this group determined if the extracts are effective as antibacterial agents).

Group 2: Positive control: Organisms plus a known antibiotic (This ensured that utilized organisms were susceptible to common chemotherapeutics and were not resistant strains).

Group 3: Pure cultures: Only the organism in the absence of antibiotic or plant extract. This was to ensure that the organism was growing properly under the defined laboratory conditions. This was necessary to distinguish poor growth from inhibition of growth.

Group 4: Negative controls: Organisms plus the pure extraction solvent (this was necessary to prove that the extraction solvent had no inhibitory action of its' own). All determinations where quantitative data are important were carried out in duplicate.

3.2.2 Plant collection

C. imberbe, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides* leaves were collected from the National Botanic Garden in Nelspruit, South Africa in September of 2002. The plant label identified the trees and Prof. JN Eloff of the University of Pretoria, confirmed the identity. The origin of each tree was recorded in the database of the botanical garden and the Garden's Herbarium, contain voucher specimens

3.2.3 Plant preparation and extraction

Leaves were carefully examined and old, insect damaged, fungus-infested leaves and twigs were removed. Healthy leaves were spread out and dried in the laboratory at room temperature until they broke easily by hand. Leaf material was ground to a fine powder using a Jankel and Kunkel Model A10 mill once dried completely. Large quantity was ground using a Wiley mill to fine powder of c1 mm diameter.

3.2.3.1 Direct extraction with acetone

Direct extraction with acetone following the method of (Eloff, 1998) was used as an extraction method for the purpose of preliminary screening of the Hypocrateropsis species. Acetone will extract a wider variety of

constituents (Elff, 1998) making it the best extractant to be used in the extraction of species of the Combretaceae (Eloff, 1998).

In this method, finely ground plant material (0.5 g) was extracted with 5 ml of technical grade (Merck) acetone in a centrifuge tube while shaking. The extracts were decanted into pre-weighed glass vials after centrifuging at c. 3000-x g for 5 minutes. The process was repeated three times on the same plant material but using fresh solvent. The solvent was removed by placing the extracts in front of a stream of air in a fume hood at room temperature. The extracted residues were weighed and re-dissolved in acetone to yield 10 mg/ml solutions ready for further analysis

3.2.3.2 Serial exhaustive extraction

A number of factors were taken into consideration in choosing solvents that were to be used in the serial exhaustive extraction. The choice of solvent also depended on what was planned with the extract. The effect of solvent on subsequent bioassay was an important factor. From previously published work, where authors screened plant material for anti-microbial properties, various extractants, from 80% ethanol, methanol (Taylor *et al.*, 1995), petroleum ether, chloroform, ethanol and water were used. Eloff (1998) found that acetone extracted a greater number of inhibitors (14) than other solvents used. The defatting process by hexane is of importance in the isolation process since nonpolar compounds were extracted rapidly in this process. Therefore serial exhaustive extraction was used with hexane as a starting solvent, followed by dichloromethane (DCM), acetone and methanol as extractants. The process was repeated three times for each solvent. The polarity of solvents gradually increased and ranged from a non-polar solvent (hexane) to a more polar solvent (methanol). This was to ensure that a wide polarity range of compounds could be extracted in the process.

Extraction was initially performed on a Labotec Model 20.2 shaking apparatus with a 10 ml: 1 g solvent to dry weight ratio. With large quantities of plant material, the ratio was raised on a proportional scale. Dried leaves (500 g) of the various plants were exhaustively extracted in serial manner with solvents of increasing polarity. Finely ground plant material (500 g) was initially extracted with 5000 ml of hexane. The solvent was allowed to extract for 1 hour while shaking before being decanted. The same quantity of solvent was added to the marc and shaken for an hour again. The process was repeated six times. The marc was allowed to dry and the process of extraction was repeated with dichloromethane, acetone and finally methanol. The extracts were filtered through Whatman (no. 2) filter paper using a Büchner funnel, and solvent was removed by vacuum distillation in a Büchi rotary evaporator at 60°C. Once concentrated to a small volume,

the extracts were placed in pre-weighed beakers and allowed to dry completely in front of a cool stream of air. The mass extracted with each solvent was measured. To determine the TLC chemical profile, 20 mg of each extract was weighed into a pill vial and made up to a concentration of 5 or 10 mg/ml by re-dissolving in acetone.

3.2.4 Analysis of plant extracts for preliminary screening

The chemical profile of extracts was determined by TLC using aluminum backed thin layer chromatography plates [ALIGRAM^R SIL g/UV 254 – MACHERY – NAGEL]. In each case 50 µg was chromatographed. The following three solvent systems were used to develop the plates: Ethyl acetate/methanol/water (40:5.4:4) [EMW] (polar), Chloroform/ethyl acetate/formic acid: 5:4:1 [CEF (intermediate polarity/acidic) Benzene/ethanol/ammonium hydroxide: 90:10:1 [BEA] (non-polar/basic). The solvent systems have been optimized to separate components of each extracts of Combretaceae members (Eloff, 1998c). Development of the chromatogram was done in closed tanks in which the atmosphere had been saturated with eluent vapour by wetting a filter paper lining. Samples were applied rapidly and developed without delay to minimize the possibility of oxidation or photo-oxidation of constituents. The separated components were visualized under visible and ultraviolet light [254 and 360 nm, Camac Universal UV lamp TL-600]. The TLC plates were subsequently sprayed with vanillin sulphuric acid spray reagent (2 mg of vanillin in 28 ml of methanol plus 1ml of concentrated sulphuric acid) and heated for 4-5 minutes at 100°C to allow for development of colour.

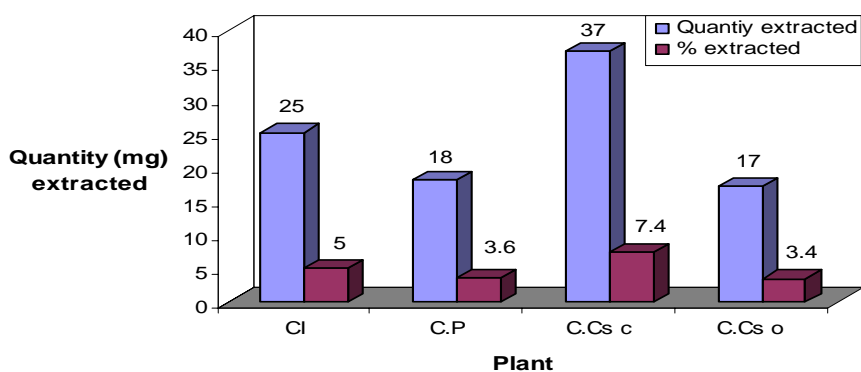
For the isolation work done at Hans-Knöll Institute (HKI) Jena, Germany, the solvent systems used for the analysis were different; the most frequently used solvent systems were chloroform: methanol (CM) 20:1; chloroform: methanol (CM) 9:1; chloroform: methanol (CM) 4:1 and cyclohexane:chloroform (CyC) 9: 1.

3.3 Results

3.3.1 Extraction

3.3.1.1 Direct extraction with acetone

Direct extraction with acetone was used to prepare extracts from the 4 plant species for TLC analysis and subsequent Bioautography and MIC for preliminary screening purposes. The quantity of material extracted, and the percentage extracted from *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides*, differed substantially (Fig. 3-1 and 3-2).



C. imberbe (C.I), *C. padoides* (C.P), *C. celastroides* ssp. *celastroides* (C.Cs.c) and *C. celastroides* ssp. *orientale* (C.Cs.O).

Figure 3-1: Quantity (g) and percentage of material extracted from 500 g of *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale*, *C. celastroides* ssp. *celastroides* in a direct extraction with acetone process.

3.3.1.2 Serial exhaustive extraction

Based on the Bioautography results of the preliminary screening to be discussed later *C. imberbe* and *C. padoides* were selected for isolation purposes. Extracts of these two plants had many antibacterial compounds on their bioautogram compared to *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides*, which had a single active spot each in the highly non-polar region of the bioautogram. This indicated that the compound responsible for activity may be a fatty acid. The MIC results of these two

species were also very low giving an indication of poor activity of the single less polar compound (discussed latter). Consequently *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides* were not investigated further. Serial exhaustive extraction was then carried out on *C. imberbe* and *C. padoides*. The result of the serial exhaustive extraction for *C. imberbe* and *C. padoides* are summarised in **Table 3-1** and **Table 3-2** respectively.

Table 3-1: Quantity in g extracted at each batch (A, B, C, D, E, and F) in a serial exhaustive extraction from 500 g of *C. imberbe* leaves

Extractant	Quantity extracted in each batch						Total
	A	B	C	D	E	F	
Hexane	6.5	2.7	1	0.6	0.26	0.2	11.26
DCM	10.1	4.1	2.4	1.4	0.6	0.39	18.99
Acetone	5.6	2.6	1.1	1	0.5	0.3	11.1
Methanol	29.9	9	4.9	2	1.1	0.3	55.3

Table 3-2: Quantity in g extracted at each batch (A, B, C, D, E and F) in a serial exhaustive extraction from 500 g of *C. padoides* leaves.

Extractant	Quantity extracted in each batch						Total
	A	B	C	D	E	F	
Hexane	6.6	3.6	1	0.5	0.2	0.18	12.08
DCM	5.4	3.2	1.6	0.8	0.5	0.3	11.8
Acetone	10.1	4.5	2.2	1	0.5	0.4	19.6
Methanol	57.1	27.4	7.4	3	1.6	0.7	97.2

Methanol extracted the highest quantity of material from *C. imberbe* (55.3 g, 11.06 %) and *C. padoides* (97.2 g, 19.4 %) (**Table 3-1**) and (**Table 3-2**). Acetone extracted the lowest quantity of material for *C. imberbe* (11.1 g, 2.2 %) and the lowest quantity of material was extracted from *C. padoides* by DCM (11.8 g, 2.36 %).

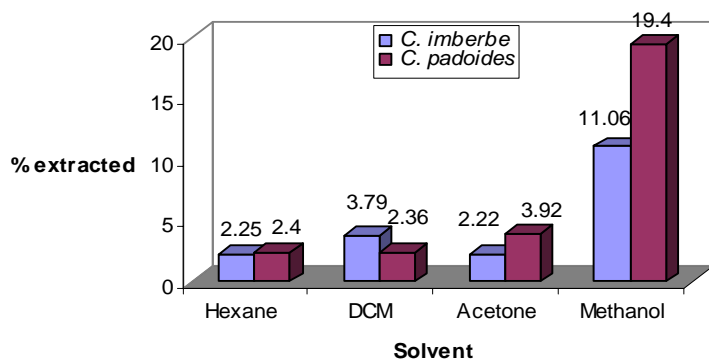


Figure 3-2: Percentage of material extracted from *C. imberbe* and *C. padoides* by each solvent in the serial exhaustive extraction process.

3.3.2 TLC analysis of plant extracts for preliminary screening

Extracts of the four plant species were analyzed by TLC and viewed under UV 365 nm (left) and also sprayed with vanillin sulphuric acid spray reagent (right). The chemical profile of the chromatograms is indicated in (Fig. 3-3). All chromophoric compounds were not vanillin active.

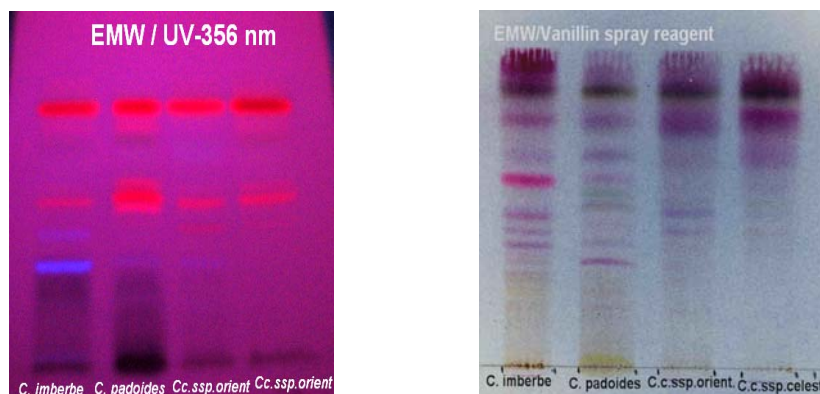


Figure 3-3: TLC chromatograms (viewed under UV 365 nm and vanillin sulphuric acid spray reagent) of extracts from direct extraction with acetone in preliminary screening process

Table 3-3: Total number of UV and vanillin spray reagent active compounds resulting from direct extraction with acetone of *C. imberbe* and *C. padoides*

Plant species	<i>C. imberbe</i>	<i>C. padoides</i>	<i>C. celestroides</i> ssp. <i>orientale</i>	<i>C. celestroides</i> ssp. <i>celestroides</i>
No of UV (254) active compounds	4	4	2	1
No of UV (356) active compounds	5	6	4	3
No of Vanillin active compounds	11	10	8	9
Total No of compounds	20	20	14	13

C. imberbe and *C. padoides* had the highest number of vanillin and UV active compounds (20 each), and *C. celestroides* ssp *celestroides* had the lowest number of vanillin and UV active compounds (Table 3-3).

3.3.3 TLC analysis of plant extracts from serial exhaustive extraction.

Five µl of 10 mg/ml plant extract (50 µg) were spotted on the TLC plate and developed with three solvent systems (EMW, CEF and BEA). Separation was more effective in EMW solvent system (Fig. 3-4, Fig. 3-5 and Fig. 3-6 respectively).

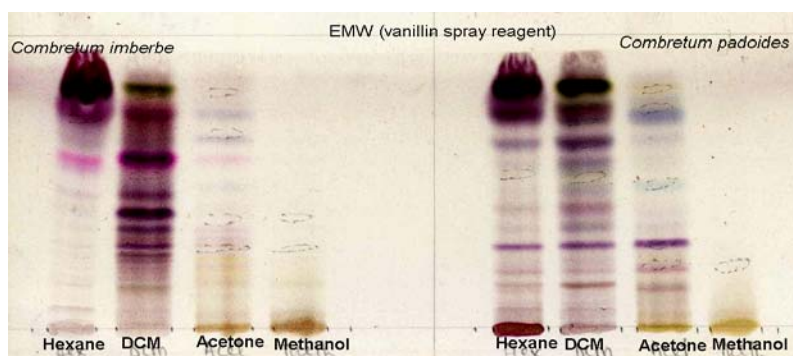


Figure 3-4: TLC chromatogram (viewed after spraying with vanillin sulphuric acid spray reagent) of serial exhaustive extraction extracts of *C. imberbe* and *C. padoides* developed with EMW. Areas on the chromatogram cycled indicate UV active compounds

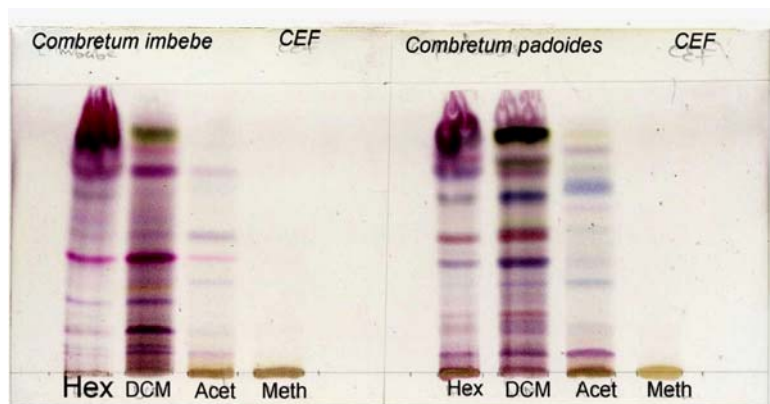


Figure 3-5: TLC chromatogram (viewed after spraying with vanillin sulphuric acid spray reagent) of serial exhaustive extraction extracts of *C. imberbe* and *C. padoides* developed with CEF. Areas on the chromatogram cycled indicate UV active compounds.

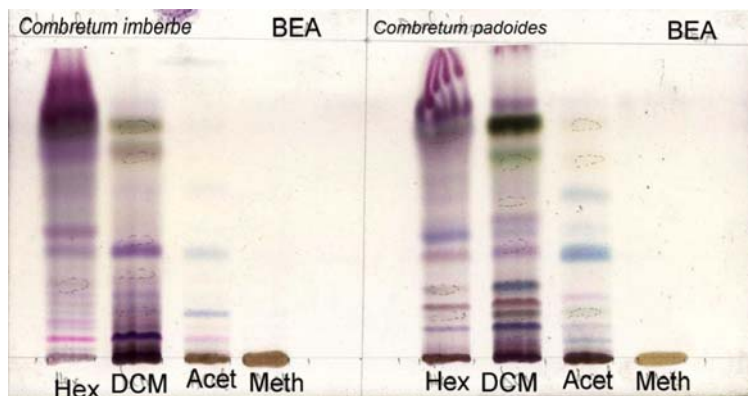


Figure 3-6: TLC chromatogram (viewed after spraying with vanillin sulphuric acid spray reagent) of serial exhaustive extraction extracts of *C. imberbe* and *C. padoides* developed with BEA. Areas on the chromatogram cycled indicate UV active compounds.

In the serial exhaustive extraction, DCM had extracted the highest number of vanillin and UV active compounds for *C. imberbe* (14) and for *C. padoides* (15). In the same process, methanol had extracted the lowest number of vanillin and UV active compounds for *C. imberbe* (5) and for *C. padoides* (3) (Table 3-4).

Table 3-4: Total number of UV and vanillin sulphuric acid spray reagent active compounds resulting from the serial exhaustive extraction extracts of *C. imberbe* and *C. padoides* in EMW solvent system.

Combretum imberbe

Solvents	Hexane	DCM	Acetone	Methanol
Aproximate No of UV (254) active compounds	0	1	0	0
Aproximate No of UV (356) active compounds	0	1	3	2
Aproximate No of Vanillin active compounds	11	13	9	3
Aproximate total No of active compounds	11	14	13	5
<i>Combretum padoides</i>	Hexane	DCM	Acetone	Methanol
Aproximate No of UV (254) active compounds	1	0	0	0
Aproximate No of UV (356) active compounds	1	1	3	1
Aproximate No of Vanillin active compounds		14	10	2
Aproximate total no of active compounds	14	15	13	3

3.4 Discussion and conclusion

In the direct extraction experiment, acetone extracted a broad range of compounds thus, allowing an effective preliminary screening for bioactive compounds (Eloff, 1998). Other solvents such as hexane, and methanol might extract a wide range of compounds but acetone will always extract compounds with a wider range of polarities. In this process, acetone extracted the highest quantity of extract from *C. celastroides* ssp. *orientale* (7.4%), followed by *C. imberbe* (5%). The lowest quantity extracted was from *C. celastroides* ssp. *celastroides* (3.4%) and (3.6%) from *C. padoides*. The TLC chemical profile of the 4 plant species indicated differences in the number of compounds extracted. The same numbers of compounds (20) were extracted from *C. imberbe* and *C. padoides* followed by *C. celastroides* ssp. *orientale* (14) and *C. celastroides* ssp. *celastroides* (13) (Table 3-3). Although many compounds were extracted from *C. imberbe* and *C. padoides* the numbers of compounds extracted were not directly related to the quantity extracted. A greater quantity of material was extracted from *C. celastroides* ssp. *orientale* but fewer compounds were visible compared to *C. imberbe* and *C. celastroides* ssp. *celastroides*. This could be as a result of the fact that some of the compounds extracted might have been glycosides that do not move from the origin with the system used.

In the serial exhaustive extraction (SEE), methanol extracted the highest quantity of material, 8% from *C. imberbe* and 10.54% from *C. padoides*. DCM extracted the lowest quantity (2.2 %) from *C. imberbe* while the lowest quantity (1.84%) extracted from *C. padoides* was by hexane. The TLC chemical profile of both plants (*C. imberbe* and *C. padoides*) indicated that DCM extracted the highest number of visible compounds (14 and 15 respectively). Most of the compounds present in the hexane were non-polar while those in DCM and acetone extracts were of varying polarity. This was seen from their effective separation in non-polar BEA and intermediate polarity CEF solvent systems respectively. Compounds that appeared in the chemical profile of methanol were seen to be mostly polar since most of them showed up at the lower part of the chromatogram developed with the polar solvent system (EMW). EMW is a polar solvent system which could effectively separate polar compounds, compounds seated at the lower part of a chromatogram developed with EMW most therefore be of higher polarity.

In this process, (serial exhaustive extraction) methanol extracted the largest quantity of material in both plants but the least number of compounds (3) showed up in this extract (**Table 3-4**). More compounds showed up in DCM but less material was extracted compared to methanol. DCM could be a useful solvent to extract selectively material from *C. imberbe* and *C. padoides*.

3.5 Summary

Leaf material of *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides* was collected from the National Botanic Garden in Nelspruit (South Africa), dried under standard conditions and ground to powder. The material was extracted directly with acetone as well as exhaustively with hexane, DCM, acetone and methanol in a serial fashion. This was necessary for the isolation process since hexane will first of all defat the plant material by extracting non-polar compound paving the way for the subsequent solvents to extract intermediate polarity antibacterial compounds. The quantity of material extracted from each plant species varied with the different solvents used and also when extracted with the same solvent.

Different numbers of chemical components were detected in the different plant species analysed by TLC. From these results, it appears that the DCM extract resulting from the serial exhaustive extraction could be the best extract to be used for isolation of phytochemicals since it contained the highest number of visible compounds than any other extract. The antibacterial activity of the different extracts is the most important factor and this is addressed in chapter 4.

CHAPTER 4

BIOLOGICAL ASSAYS FOR PRELIMINARY SCREENING

4.1 Introduction

Natural products isolated from higher plants and microorganisms have provided novel, clinically active drugs. The key to the success of discovering naturally occurring therapeutic agents rests on bioassay-guided fractionation and purification procedures. The route involved in isolating bioactive compounds is an important factor towards rapid attainment of biologically active natural compounds. Much time is involved in isolating a compound first before determining whether the compound is active or not. In this chapter, the importance of bioassay-guided isolation of antibacterial compounds is highlighted. The use of bioautography and the minimum inhibitory concentration (MIC) assay are the most important in preliminary screening.

4.2 Material and Methods

4.2.1 Antibacterial assay

4.2.1.1 Test organisms

The following test organisms were used for the bioautography and MIC assay of the plant extracts *Staphylococcus aureus* (Gram-positive) [American Type Culture Collection ATCC number [29213], *Pseudomonas aeruginosa* (Gram-negative) [ATCC 27853], *Escherichia coli* (Gram-negative) [ATCC 25922] and *Enterococcus faecalis* (Gram-positive) [ATCC 29212]. All these organisms are important nosocomial pathogens widely used in screening tests and are reference isolates recommended by the National Committee for Clinical Laboratory Standards, USA [NCCLS, 1992]

4.2.2 Bioautography of preliminary screening

Bioautography is a rapid aid in the bioassay-guided isolation and fractionation of antibacterial compounds and fractions. In this approach, the activity of plant extracts against bacteria is determined on chromatograms, in accordance with the bioautography procedure of Begue and Kline (1972)

Developed chromatography plates of 50 µg extracts of *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides* were dried overnight, sprayed with a suspension of actively growing cells of Gram-positive or Gram-negative bacteria, and incubated at 37°C in a chamber at 100% relative humidity for 18 hours. Plates are sprayed with 0.2 mg/ml p-iodonitrotetrazolium violet. Clear zones on the chromatogram indicate inhibition of growth after incubating for 1 hour at 37°C. This method was chosen for its simplicity, low cost, accuracy and rapid result that makes it ideal for bioassay-guided isolation (Eloff, 1998a).

p-iodonitrotetrazolium violet (INT) reaction

The INT reaction is based on the transfer of electrons from NADH; a product of for example the threonine dehydrogenase [TDH] catalyzed reaction, to the tetrazolium dye [p-iodonitrotetrazolium violet]. Threonine dehydrogenase [TDH] from bacteria catalyses the NAD-dependent oxidation of threonine to form 2- amino-3-ketobutyrate and NADH. During the active growth of bacteria, an electron is transferred from NADH [which is transparent in the visible range] to p-iodonitrotetrazolium violet, a formazan dye which is purple-red in colour. Therefore, the clear zones (s) on the chromatogram indicate areas of inhibition [zones where no active growth of bacteria has taken place].

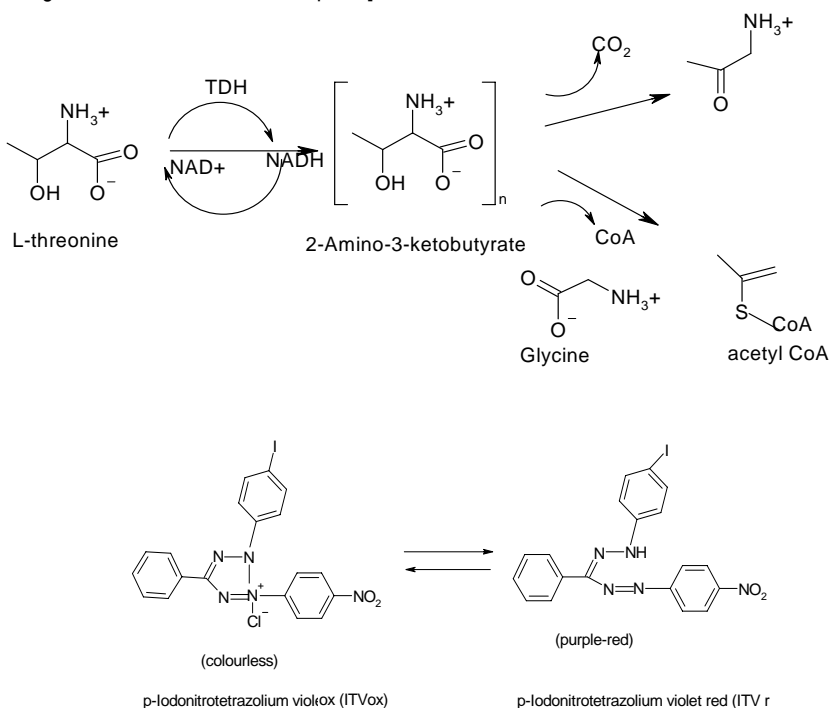


Figure 4-1: INT, coupling reagent for the colorimetric assay (reaction pathway for the assay of TDH)

4.2.3 Microplate dilution assay for preliminary screening

Agar diffusion assay is used widely to determine the antibacterial activity of plant extracts. The technique works well with defined inhibitors (Hewit and Vincent, 1989). However, when examining extracts containing unknown components, there are problems leading to false positive and false negative results (Eloff, 1998).

In this study, with the aim of quantifying the activity of the extracts, the microplate dilution method was used to determine the Minimum Inhibitory Concentration (MIC) values of the extracts against each test bacterial species (Eloff, 1998). This was determined by 2-fold serial dilution of extracts beyond the level where no inhibition of growth of *S. aureus* ATCC 29213, *P. aeruginosa* ATCC 27853, *E. faecalis* ATCC 29212 and *E. coli* ATCC 25922 was observed (Eloff, 1998). Plant extracts or fractions were reconstituted to 10 mg/ml with acetone and 100 µl of the fractions were serially diluted 50% with water in 96-well microplates. Muller-Hinton [MH] broth culture was inoculated (1%) with the test bacteria and was incubated at 37°C overnight, and 100 µl of the resulting culture were added to each well. Neomycin was used as a reference antibiotic and two wells were used as sterility and growth controls respectively with the sterility control containing only Oxoid MH broth, while the negative growth control contained both MH broth as well as test organism. The microplates were sealed and incubated at 37 °C at 100% relative humidity for 18 hours. As an indicator of bacterial growth, 40 µl of 0.2 mg/ml solution of p-iodonitrotetrazolium violet [INT] dissolved in water were added to the microplate wells and incubated at 37°C for 30 minutes. The MIC was recorded as the lowest concentration of plant extract at which bacterial growth was inhibited. The colourless tetrazolium salt acted as an electron acceptor and was reduced to a red coloured formazan product by biologically active organisms (Fig. 4-1).

4.2.4 Determination of total activity

Total activity is a measure of the amount extracted from a plant in relation to the MIC of the extract, fraction or compound isolated. There are several reasons for screening studies: to find new lead biologically active compounds for developing pharmaceuticals, and to confirm the ethnomedicinal use of plants to develop phytomedicines for use as herbal medicine. In many screening studies, activities are reported non-quantitatively. Even if extracts data are expressed in quantitative terms such as antibacterial activity in MIC, it is usually not possible to compare different plants with the result presented. To compare plants however, the quantity extracted from the plant should be brought into the equation (Eloff, 2004). In mathematical terms it can be expressed as:

$$\text{Total activity (ml)} = \frac{\text{Amount extracted from 1 g (mg)}}{\text{MIC (mg/ml)}}$$

The units are adjusted to ml and indicate the degree to which the active extracts, fractions or compounds in one gram of plant material can be diluted and still inhibit the growth of the test organisms (Eloff, 2004).

The same approach can be used to determine the total activity of different fractions to determine if any activity has been lost (Eloff, 2004). In this case the values are expressed in ml/fraction and indicate to what volume the fraction can be diluted and still kill the bacterial. In this work, to compare the activity of extracts or fractions, the total activity of each extract was determined using the formula stated above.

4.2 Results

4.3.1 Antibacterial assays

4.3.1.1 Bioautography



Figure 4-2: Bioautograms of acetone extracts of *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides*. TLC developed in EMW and sprayed with actively growing *S. aureus* cultures and later sprayed with INT. White areas indicate zones of growth inhibition.

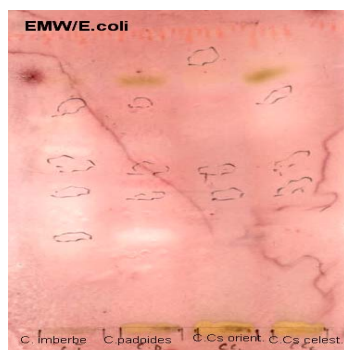


Figure 4-3 Bioautograms of acetone extracts of *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides*. TLC developed in EMW and sprayed with actively growing *E.coli* cultures and later sprayed with INT. White areas indicate zones of growth inhibition

In this screening, when chromatograms were developed with EMW (Fig. 4-2), the *C. celastroides* extracts exhibited only a limited activity. They all indicated one active compound each in the non-polar region of the chromatogram. *C. imberbe* indicated the highest number of active compounds followed by *C. padoides* (Fig. 4-2). This solvent system yielded good separation of compounds against *S. aureus* as indicated on the bioautogram. When chromatograms were developed with the less polar acidic solvent system BEA, all active compounds remained on the base of the chromatogram without any proper separation. With BEA, more compounds seem to show activity against *E. coli* than with EMW solvent system (Fig. 4-5). This indicates the importance of the right choice of solvent system when developing chromatograms for antibacterial activity in a bioassay-guided process. However, this system did not give a good separation for *S. aureus* since most of the active compounds were so polar that they hardly moved from the origin (Fig. 4-4).

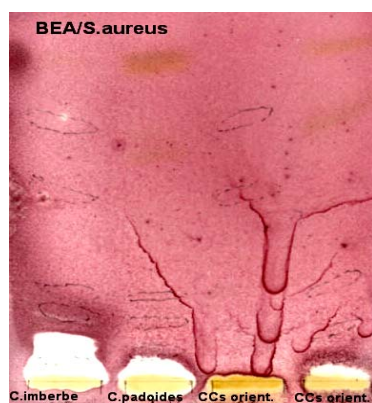


Figure 4-4: Bioautograms of acetone extracts of *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides*. TLC developed in BEA and sprayed with actively growing *S. aureus* cultures and later sprayed with INT. White areas indicate zones of growth inhibition.

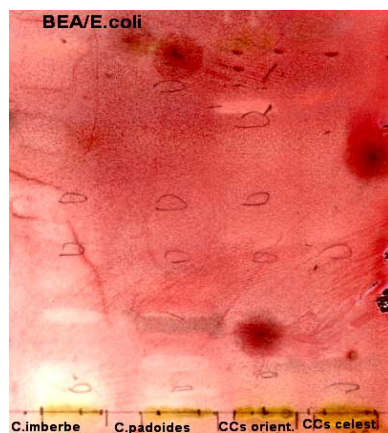


Figure 4-5: Bioautograms of acetone extracts of *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides*. TLC developed in BEA and sprayed with actively growing *E. coli* cultures and later sprayed with INT. White areas indicate zones of growth inhibition.

Table 4-1: Bioautography (TLC in EMW) and Minimum Inhibitory Concentration (MIC) results of preliminary screening of *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides* acetone extracts against *S. aureus* (SA), *E. faecalis* (EF), *E. coli* (EA) and *P. aeruginosa* (PA) .

Extracts	No of bioactive compounds on bioautogram				MIC mg/ml			
	SA	EF	PA	EC	SA	EF	PA	EC
<i>C. imberbe</i>	8	7	0	4	0.625	0.625	2.5	2.5
			Total activity (ml)		416	416	<5.2	< 5.2
<i>C. padoides</i>	7	6	0	2	0.625	0.625	2.5	1.25
			Total activity(ml)		250	250	7.2	14.4
<i>C. celastroides</i> ssp. <i>celastroides</i>	1	0	0	0	1.25	1.25	2.5	2.5
			Total activity (ml)		28	28	<14	<14
<i>C. celastroides</i> ssp. <i>orientale</i>	1	0	0	0	1.25	1.25	1.25	2.5
			Total activity (ml)		28	28	28	< 14

4.3.1.2 Bioautography of serial exhaustive extraction extracts

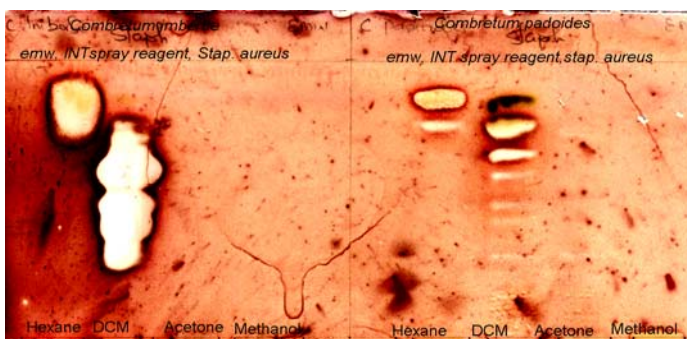


Figure 4-6: Bioautography of serial exhaustive extraction extracts on *S. aureus* sprayed with INT spray reagent. White areas indicate zones of inhibition.

In the serial exhaustive extraction screening, acetone and methanol did not extract any visible antibacterial compounds (Fig. 4-6). *S. aureus* was used as the indicator organism in the bioassay guided pathway as indicated in the bioautogram. The activities of other pathogenic organisms are indicated in Table 4-1. DCM extracted most of the active compounds in both *C. imberbe* and *C. padoides*. The active compounds extracted by DCM were of varying polarity. Hexane extracted fewer active compounds, most of which were present in the non-polar region of the bioautogram (Fig. 4-7) as could be expected.

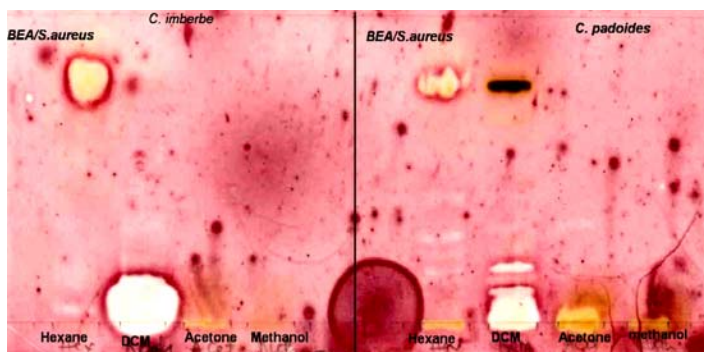


Figure 4-7: Bioautography (BEA solvent system) of serial exhaustive extraction extracts against *S. aureus* sprayed with INT spray reagent. White areas indicate zones of inhibition.

Most of the active compounds occurred in the DCM extracts of both *C. imberbe* and *C. padoides*. The hexane extracts of both plants species indicated just a few antibacterial compounds and no active

compound was seen from acetone and methanol extracts (Fig. 4-6). Extracts of both *C. imberbe* and *C. padoides* had minimal activity against *E. coli* (Fig. 4-8). The activity profile appeared to be the same when chromatograms were developed with BEA but as expected from previous results all the active compounds were found at the base of the bioautogram (Fig. 4-7)

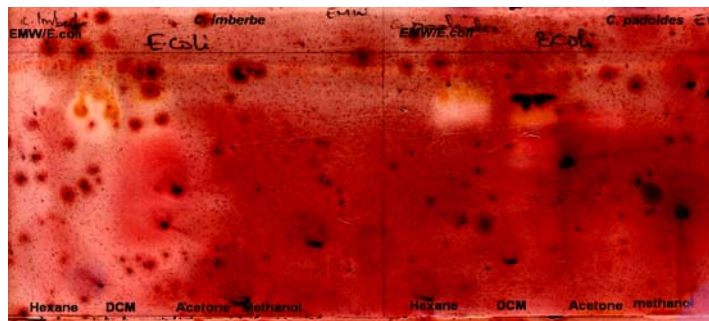


Figure 4-8: Bioautography of serial exhaustive extraction extracts against *E. coli*, using EMW solvent system and sprayed with INT spray reagent. White areas indicate zones of inhibition.

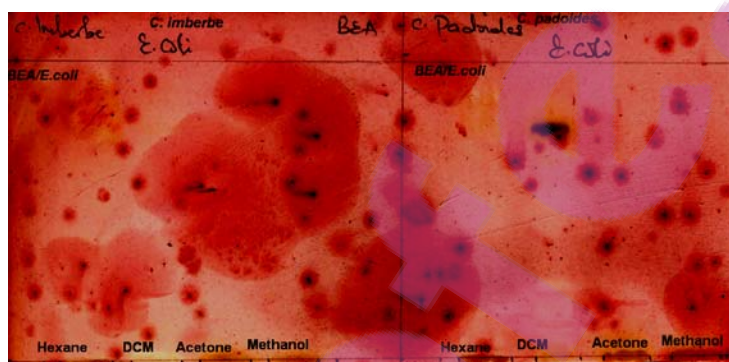


Figure 4-9: Bioautography of serial exhaustive extraction extracts against *E. coli*, using BEA solvent system and sprayed with INT spray reagent. White areas indicate zones of inhibition.

4.3.2 Minimum inhibitory concentration of extracts from serial exhaustive extraction

The hexane and DCM extracts of *C. imberbe* and *C. padoides* were the only extracts that indicated promising activity in the bioautogram results. The microtitre plate assay (Fig. 4-10 and 4-11) was then used to quantify the level of activity of these extracts by determining their minimum inhibitory concentration (MIC) values. In both species (*C. imberbe* and *C. padoides*), the DCM extract indicated a much lower MIC (an

indication of good antibacterial activity) on both Gram-positive and Gram-negative pathogens than the hexane extracts. The MIC values obtained against the four pathogenic organisms are indicated in (Table 4-2). Five mg/ml of each extract was used and the total activities against bacterial are indicated Table 4-2.

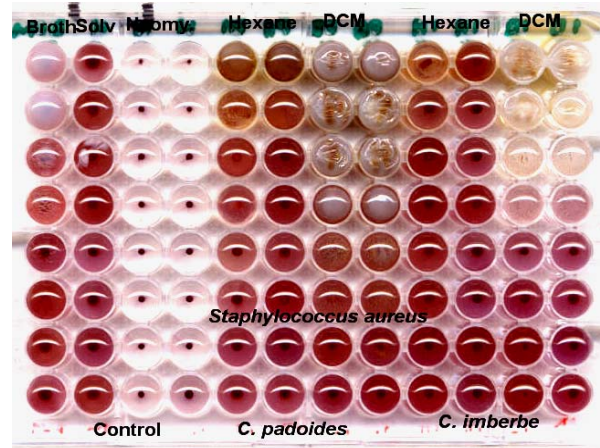


Figure 4-10: MIC of the hexane and DCM extracts of *C. imberbe* and *C. padoides* on *S. aureus* on microtitre plates. White wells indicate inhibition and purple wells indicate bacterial growth.

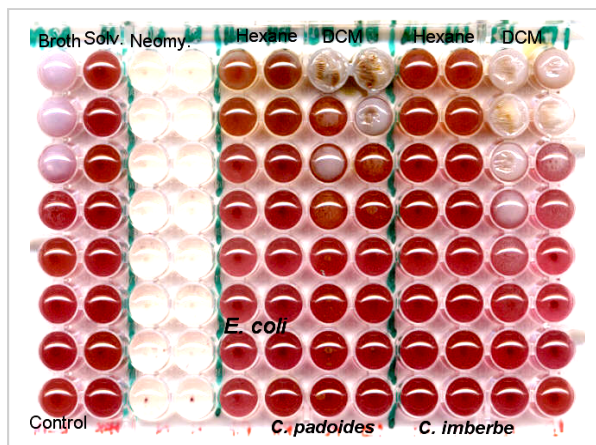


Figure 4-11: MIC of the hexane and DCM extracts of *C. imberbe* and *C. padoides* against *E. coli*

4.2.3 Total activity of extracts

The total activity of the DCM extract was calculated for each organism (**Section 4.2.4**). The DCM extract of *C. imberbe* had a higher total activity (489.7 ml) than that of *C. padoides* (151 g/mg/ml) against *S. aureus* (**Table 4-2**).

Table 4-2: Bioautography, MIC and total activity (DCM extracts only) of serial exhaustive extracts (*S. aureus* (SA), *E. faecalis* (EF), *E. coli* (EC) and *P. aeruginosa* (PA))

Extracts	No of BAC on bioautogram				MIC (mg/ml)			
	SA	EF	PC	EC	SA	EF	PA	EC
<i>C. imberbe</i> extracts								
Hexane	3	0	0	0	> 2.5	>2.5	> 2.5	> 2.5
DCM	8	3	0	0	0.039	0.156	0.313	0.156
Acetone	0	0	0	0	nt	nt	nt	nt
Methanol	0	0	0	0	nt	nt	nt	nt
	Total activity DCM extract (ml)				489.7	122.4	61	122.4
<i>C. padoides</i> extracts								
Hexane	3	0	3	1	>2.5	>2.5	> 2.5	> 2.5
DCM	8	0	3	1	0.078	0.019	0.078	0.313
Acetone	0	0	0	0	nt	nt	nt	Nt
Methanol	0	0	0	0	nt	nt	nt	nt
	Total activity DCM extract (ml)				151	621	151	37.7

(BAC) Biologically active compounds, (nt) Indicates that MIC was not carried on the corresponding extract because the extract did not exhibited activity in the bioautography.

Against *E. faecalis*, the DCM extract of *C. padoides* had a higher total activity (621 g/mg/ml) than that of *C. imberbe* (122.4 ml). It is obvious that the total activity for each extract depends on the organism in question. The higher the total activity, the better the activity of the whole plant extracts. There were also large differences between the total activity of the DCM extracts towards the different test organisms.

4.4 Discussion and conclusion

The lowest MIC indicating highest activity was recorded for the DCM extracts of *C. imberbe* and *C. padoides* against *S. aureus* (0.039 and 0.078 mg/ml, respectively and *E. faecalis* (0.156 and 0.019 mg/ml

respectively). The DCM extract of *C. padoides* also indicated very good activity against *P. aeruginosa*, with an MIC of 0.078 mg/ml. Comparing the activity of the two plant species according to total activity, the DCM extract of *C. padoides* recorded the highest total activity (621 ml) as compared to 122.4 ml for *C. imberbe* against *E. faecalis*. The DCM extract of *C. imberbe* had a higher (489.7 ml) total activity than the DCM extract of *C. padoides* (against *S. aureus*), 151 ml/g. It is therefore evident that the activity of an extract varies with respect to different test organisms. The high activity and more antibacterial compounds present in the DCM extracts of *C. imberbe* and *C. padoides* provided the rationale for further work on these extracts to be discussed in Chapter 4.

4.5 Summary

In this chapter, bioautogram and MIC values of *C. imberbe*, *C. padoides*, *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides* were determined for preliminary screening purposes. According to the results of the acetone extracts, *C. imberbe* (8 antibacterial compounds, MIC of 0.68 mg/ml) and *C. padoides* (7 antibacterial compounds, MIC of 0.625 mg/ml) were selected for further work. *C. celastroides* ssp. *orientale* and *C. celastroides* ssp. *celastroides* were eliminated due to their low number of antibacterial compounds (1 each) and high MIC (> 2.5 mg/ml) values as well as low total activity values (Table 4-T1). Bioautography and MIC were also carried out on the serial exhaustive extraction extracts of *C. imberbe* and *C. padoides* to find out which extractant extracted most of the antibacterial compounds.

Most of the active compounds were found in the DCM extract of each of the plant species. The DCM extracts of *C. imberbe* had about 8 antibacterial compounds with a MIC of 0.039 mg/ml against *S. aureus* as compared to 3 antibacterial compounds in the hexane extract (MIC > 2.5 mg/ml). The acetone and methanol extracts did not have any antibacterial compounds. DCM extracts of *C. padoides* had about 8 antibacterial compounds with an MIC of 0.078 mg/ml against *S. aureus* and 0.019 mg/ml against *E. coli* as compared to 3 antibacterial compounds in the hexane extract (MIC > 2.5 mg/ml). The acetone and methanol extracts did not have any antibacterial compounds. DCM extract of *C. imberbe* had a higher total activity against *S. aureus* than *C. padoides*. The DCM extract of *C. padoides* on the other hand indicated a higher total activity against *E. faecalis* than that of *C. imberbe*. Therefore the total activity depended on the organism used.

CHAPTER 5

PRELIMINARY SEPARATION AND ISOLATION OF BIOACTIVE COMPOUNDS

5.1 Introduction

Separation techniques leading to the isolation of bioactive compounds are important in the natural product isolation process. The complexity of a plant extract can be simplified through different separation techniques. In this chapter the use of solvent-solvent fractionation and vacuum liquid chromatography (VLC) processes as important techniques in the preliminary separation and isolation of natural product compounds were compared. Separation and isolation of the bioactive compounds was performed according to **Fig. 5-1**.

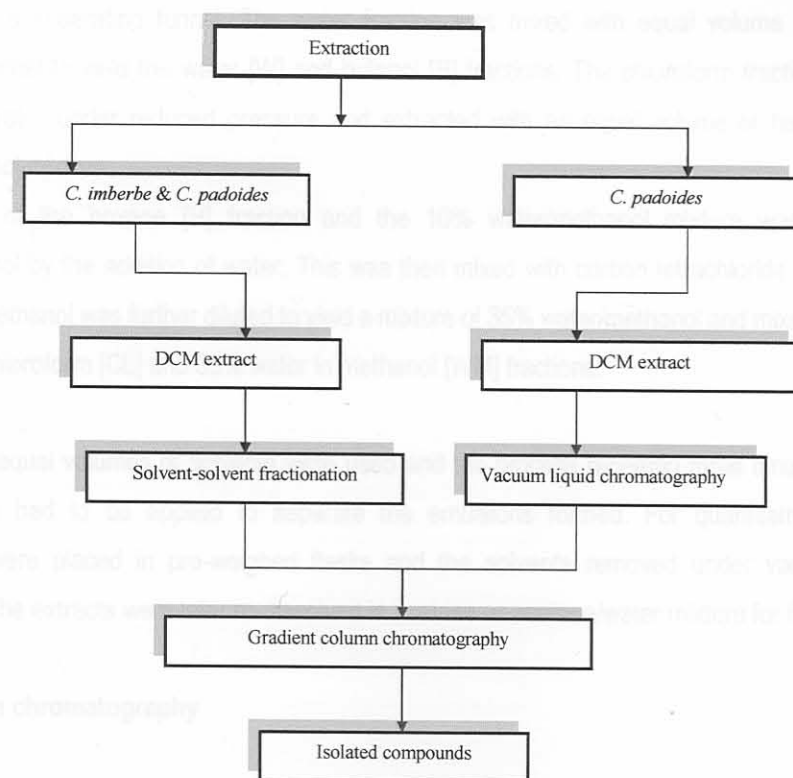


Figure 5-1: Diagrammatic summary of the isolation routes

In order to determine the fraction(s) containing antibacterial compounds to quantify the activity of the solvent-solvent and VLC fractions, bio-autography and the MIC of the fractions were determined. The bioautography method was based on Begue and Kline (1972) and the MIC method was in accordance with Eloff, (1998) as described in sections 4.2.2 (page 51) and 4.2.3 (page 52).

5.2 Materials and methods

5.2.1 Solvent-solvent fractionation

The purpose of solvent-solvent partitioning is to simplify extracts by fractionating the chemical compounds into broad groups based on their solubility (Suffness and Dous, 1979). The procedure is summarised in Fig. 5.2. Three g of *C. imberbe* and 10 g *C. padoides* DCM extracts obtained from serial exhaustive extraction were used. These extracts were dissolved in 1:1 mixture of chloroform and water and the two phases were separated in a separating funnel. The water fraction was mixed with equal volume of n-butanol in a separating funnel to yield the water [W] and butanol [B] fractions. The chloroform fraction was dried in a rotary evaporator under reduced pressure and extracted with an equal volume of hexane and a 10% water/methanol mixture.

The yielded of the hexane [H] fraction and the 10% water/methanol mixture was diluted to 20% water/methanol by the addition of water. This was then mixed with carbon tetrachloride [CT] fraction. The 20% water/methanol was further diluted to yield a mixture of 35% water/methanol and mixed with chloroform to yield the chloroform [CL] and 35% water in methanol [WM] fractions.

In all cases, equal volumes of solvents were used and the process repeated three times. In some cases centrifugation had to be applied to separate the emulsions formed. For quantitative determination, extractants were placed in pre-weighed flasks and the solvents removed under vacuum in a rotary evaporator. The extracts were later re-dissolved in acetone or acetone/water mixture for further analysis.

5.2.2 Column chromatography

5.2.2.1 Vacuum liquid chromatography

In an attempt to compare solvent-solvent fractionation and vacuum liquid chromatography (VLC) routes in isolating antibacterial compounds, 10 g of the DCM extract of *C. imberbe* was fractionated on a 10 x 30 cm VLC column. The extracts were separated by TLC using 3 solvent systems [hexane:ethylacetate (1:4), (3:2),

(1:1)] to select the best system necessary to start the VLC gradient elution. Hexane:ethylacetate (3:2) indicated the best separation required to start the gradient elution. One thousand grams silica gel 60 was placed into a 10 x 30 cm diameter column in which the mobile phase was sucked through by reduced pressure. A separation funnel was connected to the column via a T-piece adaptor, with the side arm connected to the vacuum line; fractions were collected as the column was developed. Eluents were applied in a stepwise gradient (**Table 5-1**).

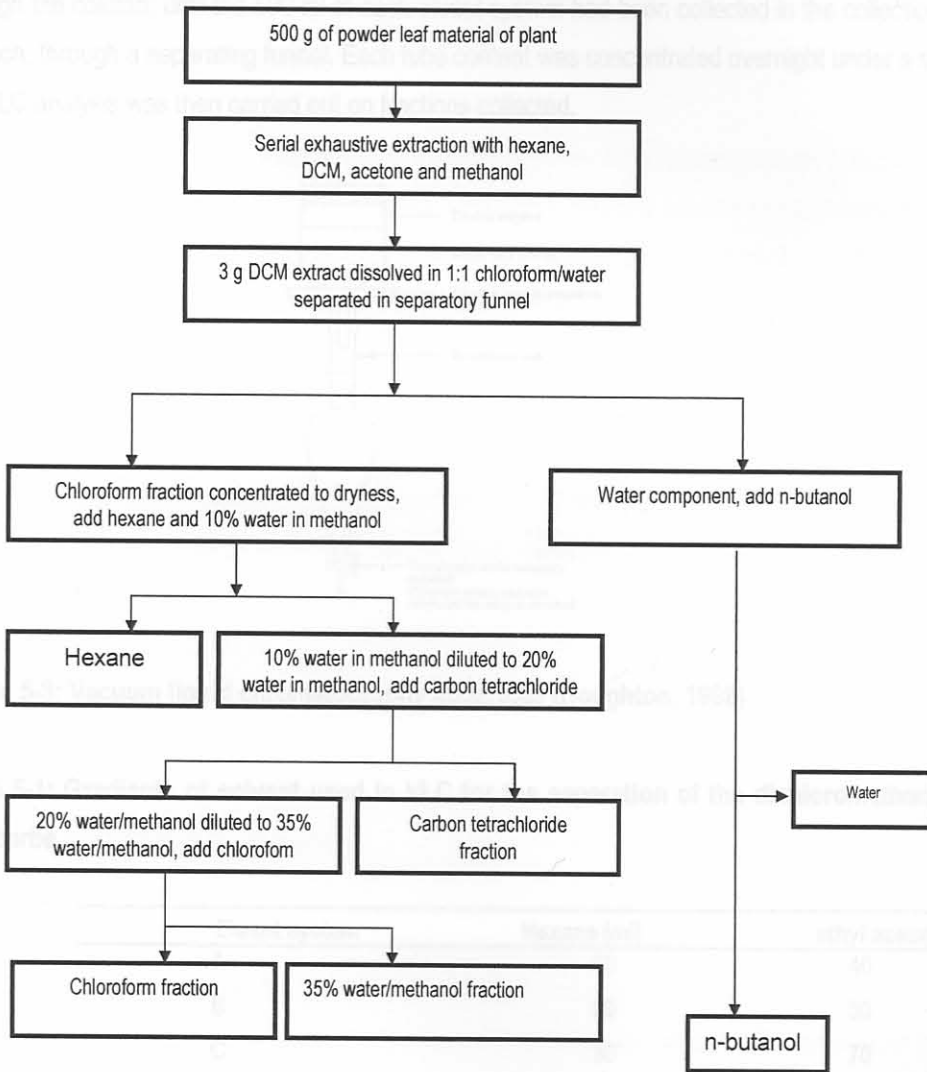


Figure 5-2: The protocol used for the solvent-solvent fractionation of the components in the DCM extract of *C. imberbe* and *C. padoides*.

The dry method for packing of chromatographic columns was used. Approximately 1000 g of silica gel 60 was poured slowly into the column, on top of a small amount of cotton wool. Silica gel (5 g) was mixed with 10 g of dichloromethane extract (dissolved in a small amount of DCM) and allowed to dry. The dry sample was then placed neatly on top of the silica in the column. Filter paper was neatly placed on top of the sample to prevent disturbance at the surface during solvent introduction. With the stepwise addition of each eluent system (500 ml) onto the column, the vacuum was switched on. The solvent was allowed to run through the column; until the 500 ml of each eluent system had been collected in the collection tubes of 50 ml each, through a separating funnel. Each tube content was concentrated overnight under a stream of cold air. TLC analysis was then carried out on fractions collected.

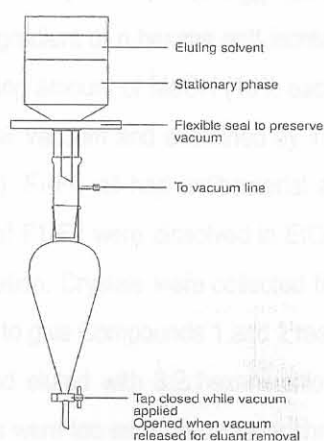


Figure 5-3: Vacuum liquid chromatography apparatus (Houghton, 1998)

Table 5-1: Gradients of solvent used in VLC for the separation of the dichloromethane extract of *C.imberbe*

Eluent system	Hexane (ml)	ethyl acetate (ml)
A	60	40
B	50	50
C	30	70
D	10	90
E	0	100
	Ethyl acetate (ml)	methanol (ml)
F	90	10
G	80	20
H	60	40

The solvents used were hexane, ethyl acetate and methanol. The gradient began with 3:2 (hexane: ethyl acetate)

5.2.2.2 Gravity gradient column chromatography

The DCM extract of *C. imberbe* (16.5 g), having the highest antibacterial activity was subjected to liquid-liquid fractionation process that afforded six fractions [chloroform (6.5 g), water (0.125 g), n-butanol (0.476 g), hexane (0.8), CCl₂ (1.356 g), 35% H₂O in MeOH fractions].

The high activity antibacterial fraction [chloroform (6.5 g)] was chromatographed on a 2x30 cm silica gel 60 open column using a stepwise gradient of n-hexane and increasing amount of ethyl acetate (EtOAc) (20% each step); EtOAc with increasing amount of MeOH (10% each step); and finally at 40% MeOH. Collected fractions were evaporated under vacuum and examined by TLC. Homogeneous fractions were pooled to give 12 major fractions (F₁-F₁₂). F₁-F₁₂ all had antibacterial activity according to bioautography (Section 5.3.3). The dried components of F₁-F₂ were dissolved in EtOAc, allowed to evaporate under a stream of cool air to encourage crystallisation. Crystals were collected from F₂ and F₆ and washed with hexane and 1:1 hexane: chloroform mixture to give Compounds 1 and 2 respectively. F₁ (0.78 g) was separated on a 1x 20 cm silica gel 60 column and eluted with 3:2 hexane:chloroform to yield 7 mg of Compound 3. The quantities of the others fractions were too small to consider chromatographic processing.

The DCM (3 g) extract that was the most active extract from *C. padoides* according to bioautography (8 antibacterial compounds) and MIC (0.08 mg/ml), was subjected to liquid-liquid fractionation process that afforded six fractions chloroform (1.69 g), water (0.28 g), n-butanol (0.78 g), hexane (0.321 g), CCl₄ (0.283 g), 35% H₂O in MeOH (1.25 g) fractions.

The high activity antibacterial fraction (chloroform 1.69 g) (**Table 5-2**) according to bioautography (8 antibacterial compounds) and MIC (0.037 mg/ml) results was chromatographed on a 2x30 cm silica gel 60 open column using a stepwise gradient of n-hexane and increasing amount of ethyl acetate (EtOAc) (20% each step), with increasing amount of MeOH (10% each step) and ending up at 50% MeOH. Collected fractions were evaporated under vacuum or cold air stream and examined by TLC. Homogeneous fractions were pooled to give 13 major fractions (A-K). Re-grouped fractions resulted in seven more distinct fractions (F₁-F₇) and were all found to have antibacterial activity.

Crystals were observed in tube containing fraction F4 and were carefully washed with hexane and acetone to yield 7 mg of Compound **8** with R_f value of 0.5 in EMW at room temperature. Precipitated sediment was filtered out in large quantity from fraction F6 and washed with 100% hexane and a combination of hexane and chloroform with 10% increase amount of chloroform at each wash. The result of this gave 53 mg of Compound **7** with R_f value of 0.3 in EMW and appeared to be the major antibacterial compound in the extract of *C. padoides*.

5.2.2.3 Sephadex LH-20

One of the commonest problems in phytochemical and biochemical research is to separate the many components, frequently macromolecules, in the plant cell extracts. Methods for separating the components of a mixture exploit differences in size, electrical charge and solubility in different solvents of the molecules in the extract. Two examples are; electrophoresis, which separates such macromolecules as proteins and DNA by their charge (and sometimes size as well), and gel filtration using e.g., Sephadex LH-20 that separates mainly by virtue of size. The porosity of the gel can be selected to exclude all molecules above a certain size. Known as size exclusion chromatography, Sephadex and Sepharose are trade names for gels that are available commercially in a broad range of porosities. In this work, Sephadex LH-20 was used for the separation of very polar fractions of the extracts. A column was filled with semi-solid beads of a Sephadex gel that admits ions and small molecules into their interior but not large ones. When a mixture of molecules dissolved in a solvent is applied to the top of the column, the smaller molecules are retarded more than larger molecules. Consequently, the large molecules move more rapidly through the column, and in this way the mixture components can be separated.

Fractions F₉-F₁₂ were combined to give (F₁₃) that was dried in a rotary evaporator to yield 3.2 g of the combined fractions. The combined fraction F₁₃ was filtered and then applied to the Sephadex L-H20 (3x120) cm column and eluted with methanol as eluent to yield six sub-fractions F_{13.1}-F_{13.6}. Sub-fractions F_{13.4} and F_{13.5} were combined (0.3 g) and subjected to a silica gel 60 2x30 cm closed column and eluted with a mixture of chloroform and methanol (9:1, 1200 ml) to yield pure Compounds **4** and **5**. From *C. padoides*, 150 mg of fraction F7 was further chromatographed on a 3 x 120 cm Sephadex LH-20 and eluted with methanol. This yielded 5 mg of Compound **6** with R_f value of 0.22 in EMW. This appeared to be a minor compound as indicated by its low concentration on the TLC chromatogram compared to other compounds (Fig. 5-17).

5.2.3 Conventional Preparative TLC

Preparative TLC was used to purify limited quantities of (< 50 mg) semi-pure fractions of *C. imberbe* with about 2 or 3 compounds on preparative TLC. Preparative TLC is one of the simplest and cheapest methods available for the isolation of a component or compounds from a mixture, only small amounts can be obtained from each fractionation procedure. Fraction A (30 mg) of *C. imberbe* resulting from the gravity based chromatographic column (F2) was applied in a form of a band on a TLC plate. The plates used in this method were 0.5-1 mm thick (analytically TLC uses plates of 0.25 mm thickness). This allowed a greater amount of sample to be loaded on to the plate. The plates were developed in a solvent (EMW) system known to separate the components. A Non-destructive detection method was used in detecting the separated compounds. The most common method of visualizing developed chromatograms was the use UV light, to detect all quenching compounds. However, there are a large number of naturally occurring compounds that do not fluoresce or quench, so other detection methods were used. A simple method for non-water-soluble compounds is to spray the layer with a fine mist of water so that it was wet enough to become transparent. Non-water-soluble compounds appeared as dark areas with transmitted light and as lighter areas when the plate is viewed in reflected light. Chromatograms were also sprayed with vanillin sulphuric acid to view vanillin active compounds. At least 90% of the plate was covered while the exposed part was sprayed. The active fraction was scraped off the TLC plates and eluted from the silica with ethanol. The active compound in ethanol was filtered through Millipore filters (0.45 μm and 0.22 μm) to remove the silica and this yielded more of Compound 2.

5.2.4 Analysis and Grouping of fractions

Following vacuum liquid chromatography (VLC) or gravity-based chromatography, test tubes were placed under a stream of air to facilitate concentration of the fractions for TLC analysis and bioassays. After about 50% of the volume of the eluent had been evaporated, 5 ml was collected from each test tube into a pre-weighed pill vial and allowed under a stream of air to dry rapidly. The mass of each was measured and the concentration (10 mg/ml) determined. Fractions were analyzed by TLC as described in Section 3.2.4

For the isolation work done at Hans-Knoll institute (HKI), fractions (5 ml each) collected from the fraction collector machine were analyzed directly by TLC without further concentration. The solvent systems used in analysis were, chloroform: methanol (C:M) 9:1, and (C:M) 20:1. Combined fractions were concentrated immediately using a rotary evaporator at 40°C

5.2.4.1 Combination of fractions

From TLC results, fractions were combined according to the similarity of their chemical profile. Combined fractions (A, B, C, D, E, F and G) were placed under air current a slowly blowing fan to facilitate drying and crystallization. Once dry, the fractions were weighed to calculate the total mass fractionated and the crystallized fractions were washed with a combination of solvents to obtain pure compounds. Active fractions, Sections 5.2.1 and 4.2.2 were further chromatographed 3 x 35 cm silica gel column to obtain the active compounds.

5.2.5 Dereplication

A system was established to identify isolated compounds from the crude extract. The dereplication method relies on the R_f value, UV active and vanillin sulphuric acid active colours and the activity of the compounds as indicated on the bioautogram. These parameters of the pure compounds were compared with that of crude extract to confirm the identity of the isolated compounds.

5.3 Results and discussions

5.3.1 Solvent-solvent fractionation of the DCM extracts.

The main aim of the solvent - solvent fractionation process was to determine the extent to which the extracts can be fractionated without losing antibacterial activity. Different solvents of varying polarities were used to simplify the complex DCM extracts of *C. imberbe* and *C. padoides* hence facilitating the isolation of antibacterial compounds. The quantity in each solvent-solvent fraction was calculated after drying off the solvent in a pre-weighed glass flask. The highest percentage of the DCM extract from *C. imberbe* was in the chloroform (65.5%) and carbon tetrachloride fractions (13.6%). Water and 35% water in methanol fractions yielded the lowest percentages (1.33% and 1.25%). This result was similar to an earlier study carried out on *C. woodii* in our laboratory (Eloff *et al.*, 2005a). With the *C. padoides* DCM extract, the highest percentage fraction was in hexane (32.15%) and chloroform (28.23%). The lowest mass was in water (2.8%) and n-butanol (7.86%). This result differed from that obtained with *C. imberbe* (Table. 5-2). The leaves of *C. imberbe* and *C. padoides* were harvested in November during the high rainfall period that is when the leaves were young, fresh and potentially with a high content of chlorophyll that will most likely appear in the hexane fraction, giving it a higher percentage.

Table 5-2: Quantity (g) and percentage of initial mass (10 g) of *C. imberbe* and *C. padoides* DCM extracts, extracted by different solvents in a solvent-solvent fractionation process

Fractions from DCM	Mass from		Mass from	
	<i>C. imberbe</i>	% of initial mass	<i>C. padoides</i>	% of initial mass
Hexane (H)	0.8	8,05	3.211	32,11
Water (W)	0.125	1,25	0.28	2,8
n-butane (B)	476	4,76	0.786	7,86
35% WM	0.133	1,33	1.256	12,56
CCL ₄	1.356	13,56	2.832	28,32
CHCL ₃	6.551	65,51	1.698	16,98

5.3.1.1 Complexity of fractions

TLC analysis was used to ascertain the complexity of the fractions. The analysis was done in three solvent systems (see Section 3.2.4) developed in our laboratory for the separation of components of Combretaceae. The hexane fraction had the largest concentration of non-polar compounds, which were very well separated by BEA. The carbon tetrachloride fraction also had many nonpolar compounds. As expected very few non-polar compounds showed up in the chloroform, 35% water in methanol, butanol and water fractions, because these compounds were already separated into hexane and carbon tetrachloride fractions. The 35% water in methanol and chloroform fractions had the most complex mixture of compounds. But the complexity was far less than that of the crude DCM extracts that is made up of a mixture of mostly intermediate polarity compounds. Most of the compounds that appeared in these fractions seemed to be of intermediate polarity separated effectively in EMW and most effectively in CEF (intermediate polarity solvent system). A few compounds were present in the water fraction. It was also observed that the carbon tetrachloride fraction resulting from *C. padoides* had a highly complex mixture of components of intermediate polarity. Generally, the solvent-solvent fractionation process assists in reducing the complexity of a crude extract that makes it easier to isolate the pure compounds using gradient column chromatography. In this study, solvent-solvent fractionation was more successful than the VLC process.

Vacuum liquid chromatography was carried out in the study with a similar quantity of DCM extract (10 g) of *C. imberbe* in order to compare the isolation process with that of the solvent-solvent fractionation. This approach was taken only with the *C. imberbe* DCM fraction. In the VLC process, 100 fractions (50 mL each) were collected and grouped according to their similarity in TLC chemical profile into 7 fractions A, B, C, D, E, F and G (Fig. 5-7).

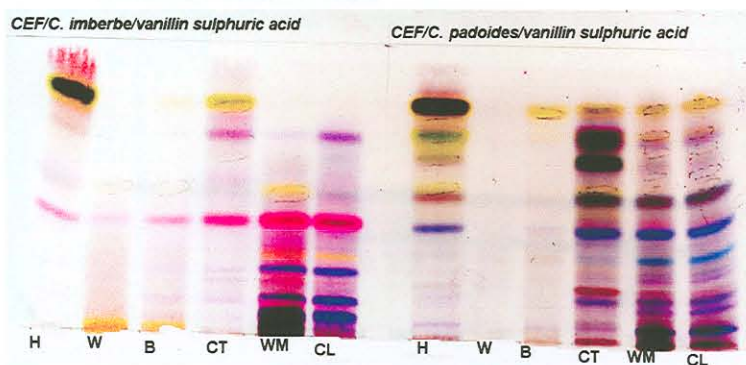


Figure 5-4: Separation of components present in the different fractions obtained by solvent-solvent extraction with CEF and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, water, n-butanol, carbon tetrachloride, 35% water in methanol and chloroforms fractions. In each case 50 μ g of 5 mg/ml stock solution was chromatographed.

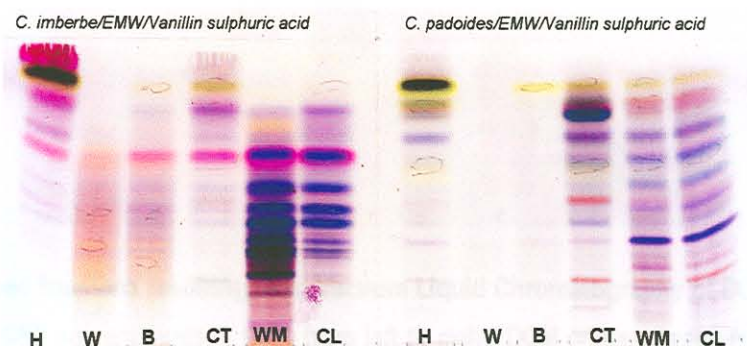


Figure 5-5: Separation of components present in the different fractions obtained by solvent-solvent extraction by EMW and sprayed with vanillin-sulphuric acid. Lanes from left to right: hexane, water, n-butane, carbon tetrachloride, 35% water in methanol and chloroform fractions. In each case 50 μ g of 5 mg/ml stock solution was chromatographed.

5.3.2 Vacuum liquid chromatography

Vacuum liquid chromatography was carried out in this study with a similar quantity of DCM extract (10 g) of *C. imberbe* in order to compare the isolation process with that of the solvent-solvent fractionation. This approach was taken only with the *C. imberbe* DCM fraction. In the VLC process, 198 test tubes (50 ml each) were collected and grouped according to their similarity in TLC chemical profile into 7 fractions A, B, C, D, E, F and G (**Fig. 5-7**)

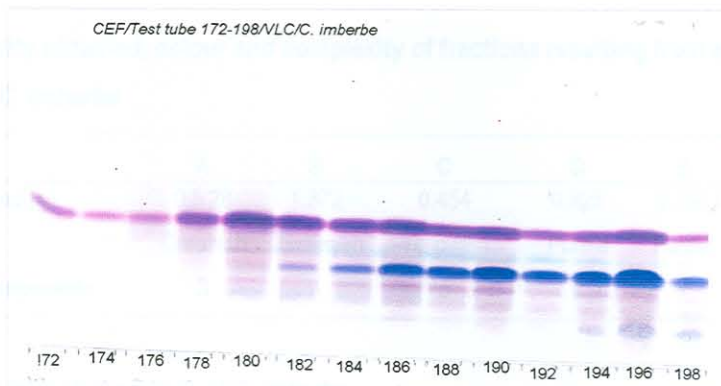


Figure 5-6: Chromatogram run in CEF indicating number of test tubes collected in the VLC process

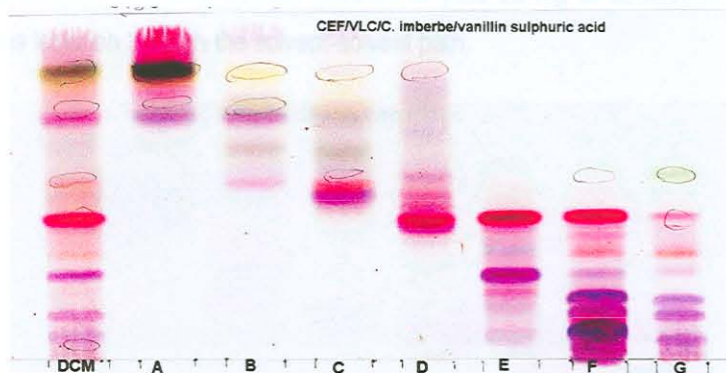


Figure 5-7: Grouped fractions resulting from Vacuum Liquid Chromatography of DCM extract of *C. imberbe* ran with CEF solvent system. Lane from left to right: DCM crude extract, fractions A, B, C, D, E, F and G

To indicate the extent of fractionation in the VLC, TLC of grouped fractions (A-G) was run along with the DCM crude extract, Fig 5-7. Fractions A, B, C and D were consisted mostly of non-polar and intermediate polarity compounds. While fractions E, F and G were made up of intermediate polarity and polar compounds. The quantity fractionated and the complexity of each fraction resulting from the VLC process is indicated in Table 5-3. Fractions E (2.294 g), F (1.564 g) and G (1.777 g) consisted of mostly polar compounds in higher quantities as compared to fractions A (0.528 g), B (1.372), C (0.453 g) and D (0.825 g) which contained less polar compounds in low quantities. Based on the TLC chemical profile of the fractions, the more polar fractions (E-G) were more complex than the less polar fractions A-D.

Table 5-3: Quantity obtained, colour and complexity of fractions resulting from a VLC process of the DCM extract of *C. imberbe*

Fractions	A	B	C	D	E	F	G
Quantity obtained (g)	0.5 28	1.372	0.454	0.825	2.294	1.564	1.777
Colour	Greenish	Greenish	Yellowish	Purple	Purple	Brown	Dark brown
No of visible compounds	3	4	5	8	7	8	8

5.3.2.1 Fractions from VLC process of *C. imberbe*

Fractions E, F and G were combined (5.635 g) and 2.5 g separated on 2 x 35 cm silica gel 60 under a 20% gradual increase of hexane and ethyl acetate gradient to yield 22 mg of compound 1 which had been isolated in a previous isolation through the solvent-solvent path.

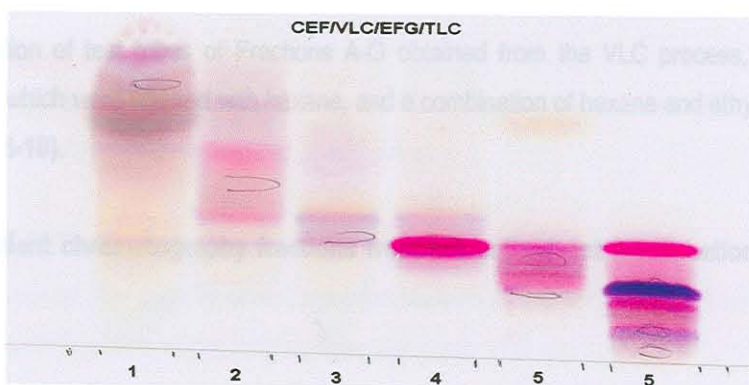


Figure 5-8: Chromatogram from the combined fractions E, F and G ran on CEF solvent system
Fractions 1, 1a, 1b, 2, 2a, 3a and 4 were combined to yield 22 mg of Compound 1

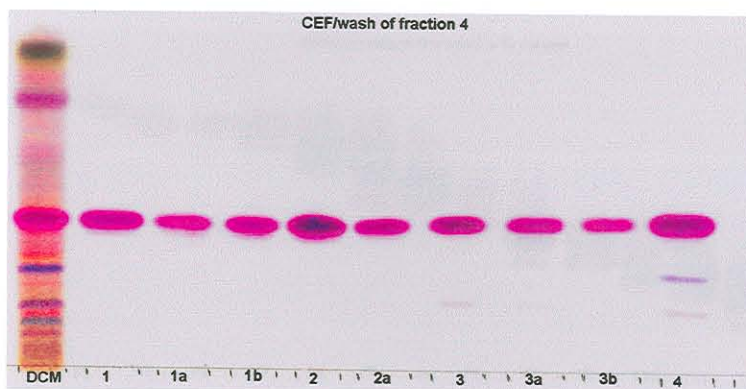


Figure 5-9: Chromatogram of fractions resulting from the wash of the crystallized test tube of Fraction 4 run along the crude DCM extract. (a and b indicates fractions from other separations).

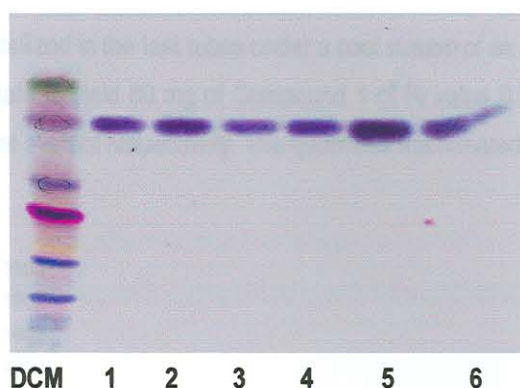


Figure 5-10: Chromatogram of fractions resulting from the wash of crystallized test tubes of fraction A and B run along the crude DCM extract. (1, 2,3,4,5, and 6 indicate fractions from other separations).

Based on observation of test tubes of Fractions A-G obtained from the VLC process, tubes A and B contained crystals, which were washed with hexane, and a combination of hexane and ethyl acetate to yield Compound 2 (Fig. 5-10).

5.3.3 Gravity gradient chromatography fractions from solvent-solvent fractionation process of *C. imberbe*

The antibacterial chloroform fraction (6.55 g) obtained by solvent-solvent fractionation was chromatographed over silica gel 60 3 x 35 cm column and eluted with 20%, 40%, 60% 80%, 100% ethyl acetate in hexane and 10%, 20%, 30% and 40% methanol in ethyl acetate to yield 12 major fractions (F1-F12).

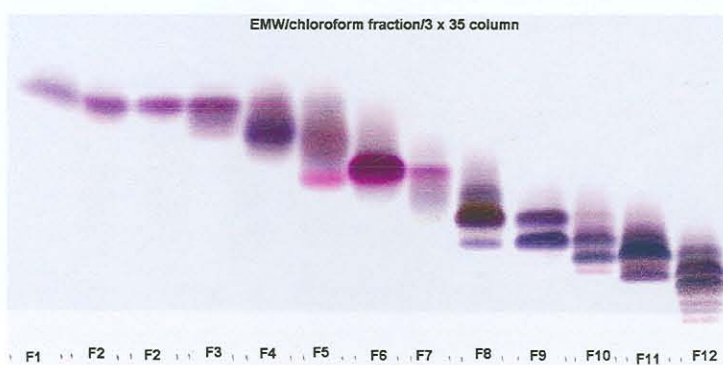


Figure 5-11: Separation of components present in 50 μ g of 12 different fractions resulting from 3 x 35 cm silica gel 60 column using EMW as eluent and vanillin-sulphuric acid spray reagent.

Fractions F2 and F6 crystallized in the test tubes under a cool stream of air and were washed with a mixture of hexane and ethyl acetate to yield 80 mg of Compound **1** of R_f value 0.81 and 7 mg of Compound **2** R_f value 0.57 in EMW solvent system respectively. The quantities fractionated are indicated in **Fig 5-12**.

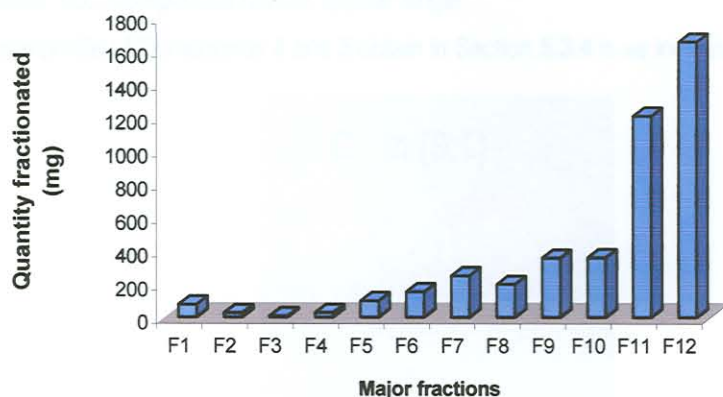


Figure 5-12: Quantities obtained from 6.55 g of chloroform fraction with the solvent-solvent fractionation process of the DCM extract of *C. imberbe*.

It was observed that the quantity fractionated from 6.55 g of the chloroform fraction increased with the polarity of the compounds. The quantity of non-polar fractions F₁-F₇ extracted ranged from 7 mg to 251 mg and quantity of the polar fractions varied ranging from 199 mg to 1657 mg.

5.3.4 Sephadex LH-20

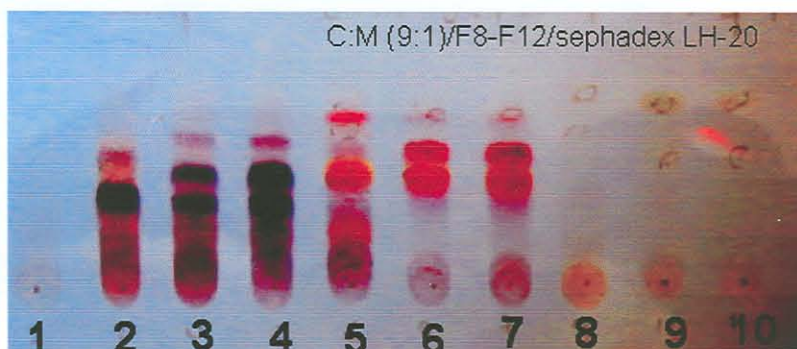


Figure 5-13: Chromatogram of the separation of 2.5 g of F₂-F₁₂ combined fraction on Sephadex LH-20 separated with chloroform/methanol (9:1) solvent system and sprayed with vanillin sulphuric acid spray reagent.

5.3.5 Conventional preparative TLC

The conventional preparative TLC method applied was successful. Bands appeared to be single after spraying with vanillin sulphuric acid reagent on the preparative chromatogram but when collected and then analyzed on normal TLC, compounds did not appear single.

The TLC chemical profile of Compounds 4 and 5 obtain in Section 5.3.4 is as indicated in **Fig. 5-14**

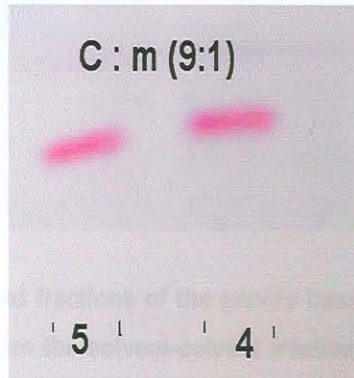


Figure 5-14: Compounds 4 and 5 isolated from *C. imberbe* by Sephadex LH-20 column separated by TLC (c:m, 9:1)

5.3.6 Gravity gradient chromatography

5.3.6.1 Fractions from solvent-solvent fractionation process of *Combretum padoides*

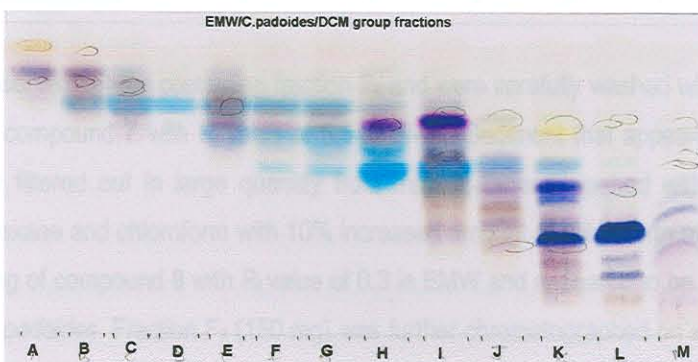


Figure 5-15: Gravity based separation of components of the chloroform fraction resulting from the solvent-solvent fractionation of the DCM fraction of *C. padoides* separated in EMW and sprayed with vanillin sulphuric acid.

TLC analysis of the fraction A-M indicated that some of the fractions still had similar chemical profiles. The fractions were again re-grouped in to seven major fractions (F1-F7) as indicated in **Fig. 5-16**

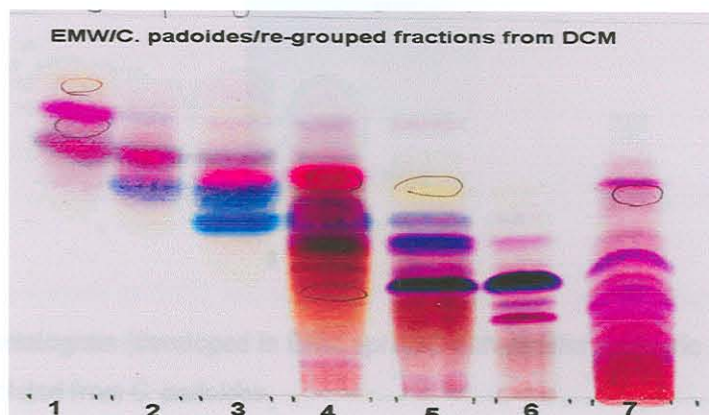


Figure 5-16: Separation of re-grouped fractions of the gravity based separation of components of the chloroform fraction resulting from the solvent-solvent fractionation of the DCM fraction of *C. padoides*

Table 5-4: Quantity of fractions derived through gravity gradient column chromatography of the chloroform fraction obtained from solvent-solvent fractionation of the DCM extract of *C. padoides*.

Fractions	F ₁	F ₂	F ₃	F ₄	F ₅	F ₆	F ₇
Quantity fractionated (mg)	19	74	52	453	153	153	156
No of vanillin active compounds	3	4	6	8	7	5	8

Crystals were observed in tube containing fraction F₄ and were carefully washed with hexane and acetone to yield 7 mg of compound **7** with R_f value of 0.5 in EMW. Sediment that appeared to be a precipitated compound, were filtered out in large quantity from fraction F₆ and washed with 100% hexane and a combination of hexane and chloroform with 10% increased amount of chloroform at each wash. The result of this gave 53 mg of compound **8** with R_f value of 0.3 in EMW and appeared to be the major compound in the extract of *C. padoides*. Fraction F₇ (150 mg) was further chromatographed on a 3 x 120 cm Sephadex LH-20 eluted with methanol. This resulted in 5 mg Compound **6** with R_f value of 0.22 in EMW and appeared to be minor compound as indicated by its low concentration on the chromatogram compared to other compounds (**Fig. 5-17**).



Figure 5-17: Chromatogram (developed in EMW sprayed with vanillin sulphuric acid spray reagent) of compounds isolated from *C. padoides*.

5.3.7 Bioautography and MIC

The bioautography result of the solvent-solvent fractionation process of *C. imberbe* indicated in Fig. 5-18.



Figure 5-18: Bioautogram of DCM extracts of *C. imberbe* (left) and *C. padoides* (right) leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in EMW and sprayed with *S. aureus* culture incubated overnight and then sprayed with INT. Growth inhibition is indicated by colourless zones on TLC plates. Lanes from left to right: hexane, water, n-butanol, carbon tetrachloride, 35% water in methanol, chloroform fractions.



Figure 5-19: Bioautogram of DCM extracts of *C. imberbe* (left) and *C. padoides* (right) leaves separated into different fractions by solvent-solvent extraction. TLC plate developed in BEA and sprayed with *S. aureus*. Lanes from left to right: hexane, water, n-butanol, carbon tetrachloride, 35% water in methanol, chloroform fractions.

In *C. imberbe*, most of the compounds in the 35% water in methanol (15) and chloroform (11) fractions showed antibacterial activity against *S. aureus*. This is indicated by the white areas present on the bioautogram (**Fig. 5-18**). Few compounds in the carbon tetrachloride and hexane fractions had antibacterial activity. The EMW solvent led to a better separation of antibacterial compounds compared to the BEA solvent system where most of the antibacterial compounds (**Fig. 5-19**) were found at the base of the bioautogram. The EMW solvent system gave a good separation of components from *C. padoides*. Most of the antibacterial compounds were found in the 35% water in methanol (7), chloroform (8) and carbon tetrachloride (6) fractions. Few active compounds (2) were observed in the hexane fraction (**Table 5-5**). Both the 35% water in methanol and chloroform fractions from *C. imberbe* had a good activity against *S. aureus*, with an MIC 0.03 mg/ml and an MIC of 0.02 mg/ml against *E. faecalis*. Activity against the Gram-negative bacteria (MIC > 2.5 mg/ml) was poor. Fractions that were not active on the bioautogram were not tested for MIC.

Similar fractions from *C. padoides* also had similar activity. The 35% water in methanol, chloroform fractions from *C. padoides* had a good activity against *S. aureus* (0.08 mg/ml each) and *E. faecalis* (0.02 mg/ml each). The two fractions did not have an activity against the Gram-negative pathogens (MIC > 2.5 mg/ml each). From the bioautography and MIC results, the chloroform and 35% water in methanol fractions were the most active fractions that could be used for further investigation. From the bioautogram, the active compounds found in these two fractions were similar; therefore, the chloroform fraction from each of the plant species was chosen for further analysis because of its higher number of active compounds and higher activity.

Table 5-5: Number of antibacterial compounds and MIC values of fractions resulting from the solvent-solvent fractionation process of the DCM fraction of *C. imberbe* and *C. padoides*

Fractions	Quantity (g) fractionated	Approximate no of antibacterial Compounds	SA	MIC (mg/ml)		
				EF	EC	PA
<i>C. imberbe</i>						
Hexane	0.8	3	nt	nt	nt	nt
Water	0.1	0	nt	nt	nt	nt
n-butane	0.5	1	nt	nt	nt	nt
35% W/M	0.1	15	0.01	0.02	> 2.5	> 2.5
CCL4	1.4	2	nt	nt	nt	nt
Chloroform	6.6	11	0.01	0.02	> 2.5	> 2.5
<i>C. padoides</i>						
Hexane	3.2	2	nt	nt	nt	nt
Water	0.3	0	nt	nt	nt	nt
n-butane	0.8	0	nt	nt	nt	nt
35% W/M	1.3	7	0.08	0.02	> 2.5	> 2.5
CCL4	2.8	6	nt	nt	nt	nt
Chloroform	1.7	8	0.08	0.02	> 2.5	> 2.5

S. aureus (SA), *E. faecalis* (EF), *E. coli* (EC), *P. aeruginosa* (PA), MIC test not done because of no activity of these (nt) fractions on bioautography.

The chloroform fraction (6.55 g) of *C. imberbe* was chromatographed by gravity-based silica gel 60 column to give 12 fractions. All the fractions F₁-F₁₂ had activity against *S. aureus* (Fig. 5-20). Since all the fractions had activity on bioautogram, most of the fractions were used for further work in isolating the individual compounds responsible for the activity.



Figure 5-20: Bioautogram of the group separation (silica gel 60) of 6.55 g of the chloroform resulting from the solvent-solvent separation of DCM extract of *C. imberbe* on EMW.

5.3.8 Bioautography and MIC of VLC fractions

The bioautography result of the VLC fractions is indicated in **Fig. 5-21**. The VLC process resulted in seven major fractions (A-G) with quantities fractionated as 0.5 g (A), 1.4 g (B), 0.5 g (C), 0.8 g (D), 2.3 g (E), 1.6 g (F) and 1.8 g (G). All the fractions indicated at least one compound active against *S. aureus* (**Fig. 5-21**).

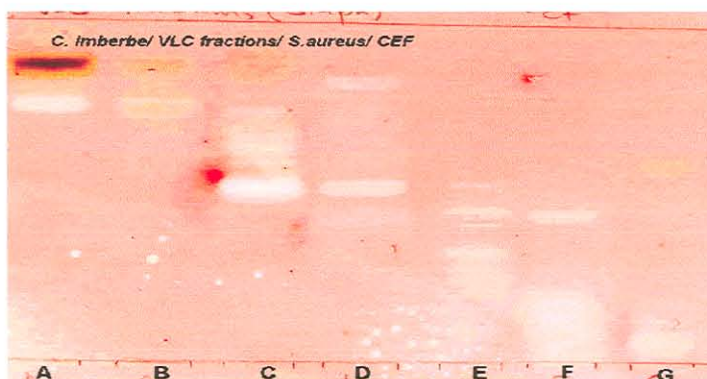


Figure 5-21: Bioautogram of the DCM extract of *C. imberbe* separated into different fractions in a Vacuum Liquid Chromatography process. TLC plate developed in CEF (as best separating system) and sprayed with *S. aureus*. Lanes from left to right: fractions A, B, C, D, E, F and G.

Most of the fractions from the VLC, fractions (B-G) also indicated a good activity against both Gram-negative and Gram-positive bacteria. Fraction E and G indicated the best activity (0.02 mg/ml) against *E. faecalis* and *E. coli* respectively.

Table 5-6: Quantity obtained, no of compounds, and MIC of VLC fractions of DCM extract of *C. imberbe*.

Fractions	Colour	Quantity (g)	No of compounds	MIC (mg/ml)			
				SA	EF	EC	PA
A	Greenish	0.528	5	0.08	0.06	> 2.5	> 2.5
B	Greenish	1.372	6	0.16	> 10	0.3	0.3
C	Yellowish	0.454	7	0.08	0.3	0.3	0.3
D	Purple	0.825	7	0.16	0.3	0.6	0.6
E	Purple	2.294	7	0.02	0.08	> 10	0.02
F	Brown	1.564	9	0.02	0.02	> 10	0.3
G	Brown	1.777	10	0.04	0.02	> 10	0.3

S. aureus (SA), *E. faecalis* (EF), *E. coli* (EC), *P. aeruginosa* (PA)

5.3.9 Dereplication of compounds

The available compounds (1,3,4,5) isolated from *C. imberbe* were separated along with the crude DCM extract. **Fig. 5-22** indicates that compound (1) $R_f = 0.597$, (3) $R_f = 0.746$, (4) $R_f = 0.417$ and (5) $R_f = 0.433$ were found on the crude with the same R_f values. **Table 5-7** indicates the R_f values of isolated compounds in different solvent systems.

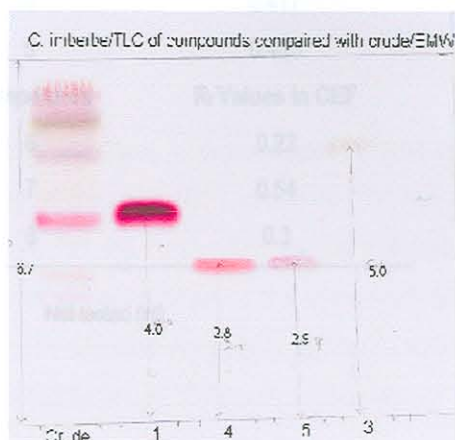


Figure 5-22: Chromatogram of the crude extract of *C. imberbe* compared with isolated compounds to dereplicate isolated compounds from the crude extract. Lanes from left to right: Crude extract of *C. imberbe*, compounds 1, 4, 5 and 3.

This is an indication that the isolated compounds were not denatured during the isolation process. The same dereplication process was carried out with compounds isolated from *C. padoides* and compounds (6) $R_f = 0.22$, (7) $R_f = 0.54$ and (8) $R_f = 0.30$ all on CEF solvent system could be found with similar R_f values on the crude extract. This again is an indication that oxidation or any chemical or physical processes had not changed the compounds during the isolation (**Fig. 5-23**).

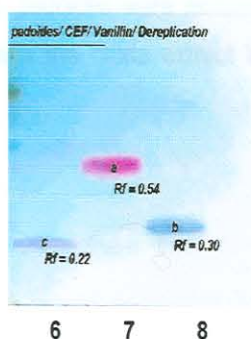


Figure 5-23: Identification of isolated compounds from the crude extract of *C. padoides*

Table 5-7: Dereplication, R_f values of isolated compounds

Compounds	R _f Value in EMW
1	0.597
2	nt
3	746
4	0.417
5	0.433
Compounds	R _f Values in CEF
6	0.22
7	0.54
8	0.3

Not tested (nt)

5.4 Summary

Fractions resulting from the solvent-solvent fraction process of the DCM extract of *C. imberbe* and *C. padoides* had many antibacterial compounds based on bioautography. The 35% water in methanol and chloroform extracts from both plant species had higher number of antibacterial compounds with MIC values of 0.02 mg/ml against both *S. aureus* and *E. faecalis* for *C. imberbe* fractions. MIC values of 0.08 mg/ml and 0.02 mg/ml against *S. aureus* and *E. faecalis* were observed from the same fractions from *C. padoides* respectively. Isolation through the solvent-solvent fraction process was more successful than the VLC process. Five antibacterial compounds were isolated from *C. imberbe* through the solvent-solvent process as compared to two compounds isolated from the same species through the VLC process.

Isolation of compounds from *C. padoides* was through the solvent-solvent fraction approach only. Three antibacterial compounds from *C. padoides* were isolated through this process. A comparison of the R_f values of isolated compounds with the crude extract indicated that the compounds isolated were not artifacts of the isolation process.

CHAPTER 6

INSTRUMENTAL ANALYSIS AND STRUCTURAL ELUCIDATION OF ISOLATED COMPOUNDS

6.1 Introduction

Identification of compounds usually involves a combination of different techniques including nuclear magnetic resonance (NMR), mass spectrometry (MS), ultraviolet (UV) and infrared (IR) spectrometry. Other ways of confirming the identification of the compounds include calculation of the R_f values in different solvent systems and determination of melting point. In this study NMR and MS techniques were used as tools for identifying the structure of compounds. Retardation factor (R_f) values were calculated in most cases using CEF solvent system as the mobile phase. UV spectrometry was not required as structure elucidation was completed in all cases without the use of this technique.

6.2 Nuclear magnetic resonance spectroscopy [NMR]

For structural elucidation purposes, compounds isolated were subjected to instrumental analysis. The Nuclear Magnetic Resonance (NMR) [both ^1H and ^{13}C] spectra was determined with the assistance of the regional analytical center at Rand Afrikaans University, Johannesburg, and also at the Department of Chemistry and Biochemistry, Medical University of Southern Africa using Varian Unity Inova 300 and 600 MHz NMR system. Some analyses were at the Hans-Knoll institute, Jena, Germany using a DPX-300, "DNMR, DPX-500 (Bruker, Germany).

6.2.1 NMR sample preparation

Isolated compounds were dried, weighed (5-10 mg) and dissolved in a maximum of 2 ml of deuterated solvents [Merck] used for NMR. Pyridine and chloroform were used as solvents of choice, because the compounds under investigation were soluble in them. The solutions [free of insoluble impurities] were then pipetted into NMR tubes [commonly of 5 mm diameter to a depth of 2-3 cm] using a clean Pasteur pipette and taken to either of the above universities for proton (^1H), carbon 13 (^{13}C), distortionless enhancement through polarization transfer (DEPT), correlated spectroscopy (COSY), heteronuclear multiple quantum

coherence (HMQC), heteronuclear multiple bond connectivity (HMBC) and mass spectrometer (MS) analysis. For NMR analysis, the tubes were lowered into a probe placed between the poles of a magnet. The probe had a transmitter and receiver coils connected to it. The magnet was then adjusted to give the highest level of homogeneity and the tube was spun. The spectrum was then taken using the instrument controls.

6.2.2 One-dimensional spectroscopy

6.2.2.1 ¹H-NMR

Proton magnetic resonance (PMR/¹H NMR) was widely employed. The spectrum appears predominantly in the range 0–10 ppm downfield from the reference signal of trimethylsilane. Proton NMR gives a measure of the absorptions of the different proton signals from a compound. The integral of the signal is proportional to the number of protons it represents, and the nature of the hydrogen is established by the chemical shift. The absorption of a signal is generally proportional to the number of protons coming into resonance frequency of the signal with the result that the area under the absorption peak is proportional to the number of protons being detected. A nucleus in a region of high electron density experiences a chemical field proportionately weaker than those in a region of low electron density, and a higher field has to be applied to bring it into resonance. Such nuclei are said to be shielded by the electrons. A high electron density shields a nucleus and causes resonance to occur at relatively high field [with low delta value]. Likewise, a low electron density causes resonance to occur at relatively low field [i.e. with high chemical shift value] and the nucleus is said to be deshielded (Friebolin, 1998).

6.2.2.2 ¹³C-NMR

¹³C-NMR was used to determine the precise frequency at which each carbon comes into resonance and is determined not only by the applied field β_0 , but also by minute differences in the magnetic environment experienced by each nucleus. These minute differences are caused largely by the variation in electrons in the neighborhood of each nucleus, with the result that each chemically distinct carbon atom in a structure, when it happens to be a ¹³C, will come into resonance at a slightly different frequency from all the others. Each upward line in a ¹³C spectrum corresponds to one carbon atom.

6.2.2.3 DEPT

Distortionless enhancement through polarization transfer [DEPT] is a technique that allows a separate spectrum to be obtained for the ^{13}C of CH_3 , CH_2 , and CH . So called because the impulse sequence used forces part of the higher sensitivity associated with proton detection on to ^{13}C , a process that enhances the ^{13}C signal intensity by polarization transfer from ^1H to ^{13}C .

6.2.3 Two-dimensional spectroscopy

Two-dimensional (2-D) spectroscopy is a more recent innovation. The spectrum contains signals dispersed according to two characteristic frequencies rather than one so that the numbers of distinct signals that can be resolved are more than in a normal 1D spectrum. In 'resolved' 2-D experiments, chemical shifts and hetero or homonuclear spin couplings are separated into two dimensions. The 'correlation' experiments meanwhile differ from resolved experiments in that they contain a mixing period during which coherence is transferred or evolves in the spin system. The result is a 2-D spectrum exhibiting connectivities, i.e. cross peaks between signals from coupled spins. The most common method of presenting this data is by contour plot as it is able to cope with crowded spectra and also allows easier determination of the frequency coordinates of peaks (Friebolin, 1998).

6.2.3.1 Homonuclear correlations

6.2.3.1.1 ^1H - ^1H Correlation Spectroscopy (COSY)

Correlated Spectroscopy (COSY) is a two-dimensional experiment that indicates all the spin-spin coupled protons in one spectrum. In a COSY spectrum, two essentially identical chemical shift axes are plotted orthogonally (although the resolution on each chemical shift axes is normally different). All peaks that are mutually spin-spin coupled are shown by cross peaks, which are symmetrically placed about the diagonal. By introducing extra delays in the pulse sequence, COSY spectra can be obtained to emphasize long-range coupling. In such long range COSY or delayed COSY spectra, this may be used to uncover coupling of the order of 1Hz and thereby giving connectivities over 4 or 5 bonds.

6.2.3.2 Heteronuclear correlations

Heteronuclear correlation spectroscopy is analogous to COSY. However, a different experimental regime is required since two types of nuclei with different Larmor frequencies are involved (Neri and Tringali 2001). It differs from COSY only in that the second pulse is applied simultaneously to both nuclei. It can be used to work out one-bond correlations or longer distance correlations and thus aids in building up a picture.

6.2.3.2.1 Heteronuclear Multiple Quantum Coherence (HMQC) (^1H - ^{13}C COSY)

This ^1H - ^{13}C COSY spectrum allows the use of the carbon spectrum to unambiguously assign often severely overlapping proton signals. It has the advantage that even in complicated molecules there is little overlapping of the correlation peaks, which combine the large chemical shifts of ^{13}C NMR and those of ^1H NMR spectroscopy.

6.2.3.2.2 Heteronuclear Multiple Bond Connectivity (HMBC) (Long range ^{13}C - ^1H COSY)

In this sequence, a time delay of about 50 ms in the pulse sequence is used to determine two and three bond coupling. It is crucial in constructing extended pieces of the molecular skeleton that can be joined together to complete structure elucidation. Since many ^1H - ^{13}C (two-bond) and ^1H -C-C- ^{13}C (three-bond) coupling constants are rather similar in value and lie in the range of 2-20 Hz, then ^{13}C chemical shifts are now correlated with the chemical shift of those protons separated from them by two and three bonds.

6.2.3.2.3 NOESY spectroscopy

The nuclear Overhauser effect (n.O.e) results from through-space interactions of magnetic nuclei. It is defined as a change in the integrated NMR absorption intensity of a nuclear spin when the NMR absorption of another spin is saturated. It allows moieties that are spatially proximal (but may be too far to couple through bonds) to be located and it makes a major contribution to the determination of stereochemistry (Neri and Tringali 2001).

6.3 Mass spectrometry

6.3.1 Sample preparation

Approximately 1 mg of each isolated compounds was dried, put in 2 ml vial and sent to either of the analytical centres mentioned in Section 6.2 for mass spectrometry analysis.

6.3.2 Mass spectrometry (MS)

Specific identification of molecules is more certain with the use of mass spectrometer (MS). In electron impact mass spectrometry (EIMS) the effluent which contains the separated and vaporized compounds, passes into the ion chamber of the mass spectrometer, which is under a high vacuum. A beam of electrons accelerated from a filament, which ionizes and fragments them, bombards the molecules. Initially, one electron is removed from each molecule to form a positively charged molecular ion (M^+). Breakage of bonds relative to bond strength occurs rapidly in the molecular ion to generate fragment ions. The various ions are accelerated into the analyzer portion of the mass spectrometer where they are sorted according to their mass to charge ratios (m/z values) that are equivalent to the molecular weights of the fragments. The ion signal is amplified by an electron multiplier and the mass spectrum is plotted from low to high mass. The m/z values are plotted against relative abundance of the ions. The most abundant ion (base peak) in the spectrum is assigned as 100%

6.3.2.1 Electron Impact Spray MS (EIS-MS)

For all the compounds isolated from the Hans Knöll Institute (HKI) in Jena Germany, ESIMS, triple quadrupole mass spectrometer Quattro (VG Biotech, England); EIMS, 70 eV direct inlet, high resolution with perfluorokerosine as a standard, MAT 95 XL (Finnigan, Germany) was used for the analysis. The EIS-MS is an MS technique performed to give a more precise molecular ion that could be of great assistance for molecular formula determination.

6.4 IR

For IR analysis, IFS55 spectrometer (Bruker, Germany) was used for the analysis.

6.5 Results and Discussions

6.5.1 Identification of compounds

Most of the compounds had the basic triterpenoids skeleton (**Fig 6-1**). The result of compounds 1-5 are as discussed in the following sections.

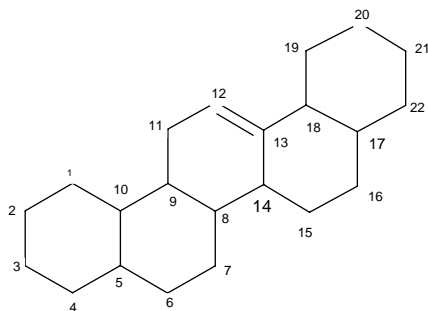


Figure 6-1: Skeletal unit of olean-12-ene type of pentacyclic triterpenoid isolated from *C. imberbe*

Compound **1** crystallized in most of the fractions and was isolated in large quantity. It has been previously isolated from the same species by Katerere *et al.*, 2002 and Rogers and Subramony, 1988, and appeared to be the major constituent in the leaves of *C. imberbe*. Compound **2**, **3** and **4** have been isolated from different plant species. Compound **5**, a glycoside, was isolated and reported here for the first time.

From previous work done; pentacyclic triterpenes have been isolated from *C. imberbe* and related species of Combretaceae (Roger, 1989b; Rogers and Subramony, 1988 and Rogers and Verotta, 1996). This was of great assistance in the preliminary analysis of NMR data, which indicated that the isoprenoids were triterpenoid compounds in nature. IR analysis indicated the presence of olefinic (ca 1652.84 cm^{-1}), hydroxyl (ca.3440 cm^{-1}), carboxylic acid OH (ca.2923.37 cm^{-1}), methyl (ca.1456 cm^{-1}) and carboxylic acid C=O (ca.17717 cm^{-1}) in most of the compounds.

6.5.1.1 Compound 1

Compound **1** crystallized as a white solid from the first silica gel 60 column after elution with 100% ethyl acetate. NMR spectra were suggestive of an olean-12- skeleton that has previously been isolated from this plant with a carboxylic function, a trisubstituted double bond, carbon bearing OH bonds and an AB system.

This pattern was observed in all other compounds isolated in this study, suggesting a similarity in the chemical structure in the main skeletal unit (**Fig. 6-1**) modified by the various constituents.

MS analysis of Compound **1** (**Fig. 6-3**) gave a molecular formula of $C_{30}H_{48}O_4$ (m/z 472) and characteristic peak at m/z 248 ($M-C_{14}H_{24}O_2$)⁺, m/z 454 ($M-H_2O$)⁺, m/z 187 ($M-C_{16}H_{27}O_4$)⁺, m/z 130 ($C_{10}H_{10}$)⁺, and m/z 93 (C_7H_9)⁺ in an EI mass spectrum due to a retro-Diels-Alder fragmentation of an oleanene skeleton with a free carboxylic function either in ring D or E. There was an initial loss of water, then a Wagner-Meerwein rearrangement with subsequent loss of another water (**Fig. 6-2**). The rearranged fragment then undergoes retro-Diels fragmentation. This structural type was further supported by the indication of seven degree of unsaturation and the ¹H NMR spectrum. It contained resonance for seven skeletal methyl groups and a broad triplet at δ_H 5.21 for olefinic proton (H-12), carboxylic acid functionality (δ_c 182.0) and two hydroxyl groups (δ_H 3.6, δ_c 72.38 and δ_H 3.71, δ_c 73.35). The assignment presented resulted from a combination of ¹H-¹H COSY, HMQC and HMBC experiments. The free carboxylic acid group was assigned to C-30, since it showed an HMBC correlation to H₃-29 and the nature of the fragmentation pattern suggested the presence of the two hydroxyl groups in ring A. Using this information along with the ¹H-¹H COSY, HMQC and HMBC data the partial structure of Compound **1** which was suggestive of a 1,3-dihydroxy-12-oleanen-29-oic acid, corresponding to 1 α , 3 β -dihydroxyoleanen-12-29-oic previously isolated from the same species by Rogers and Subramony 1988 after perfect comparison of the experimental data.

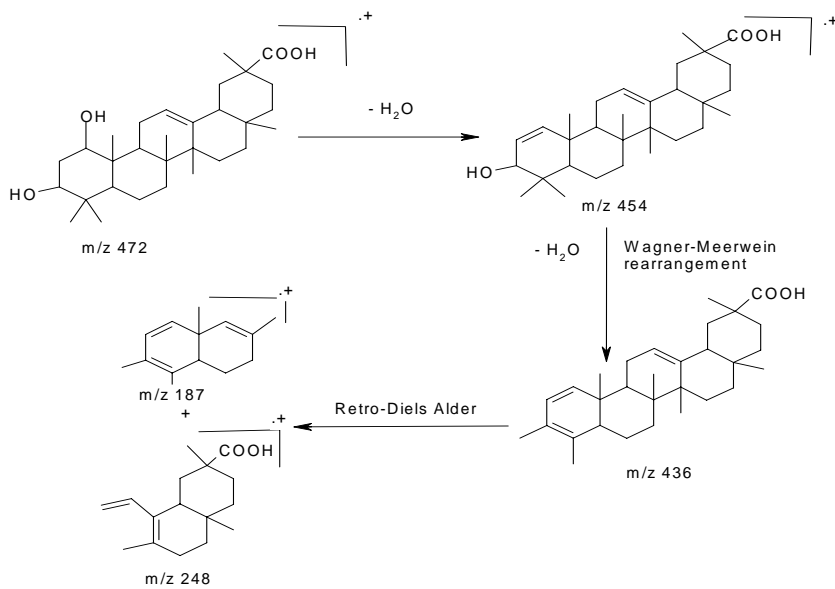


Figure 6-2: Fragmentation pattern of compound 1

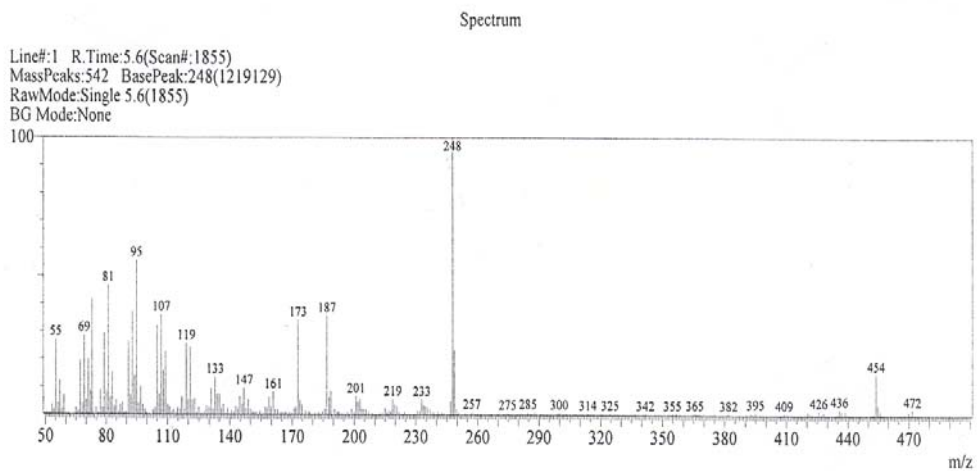


Figure 6-3: EI-MS spectrum of compound 1

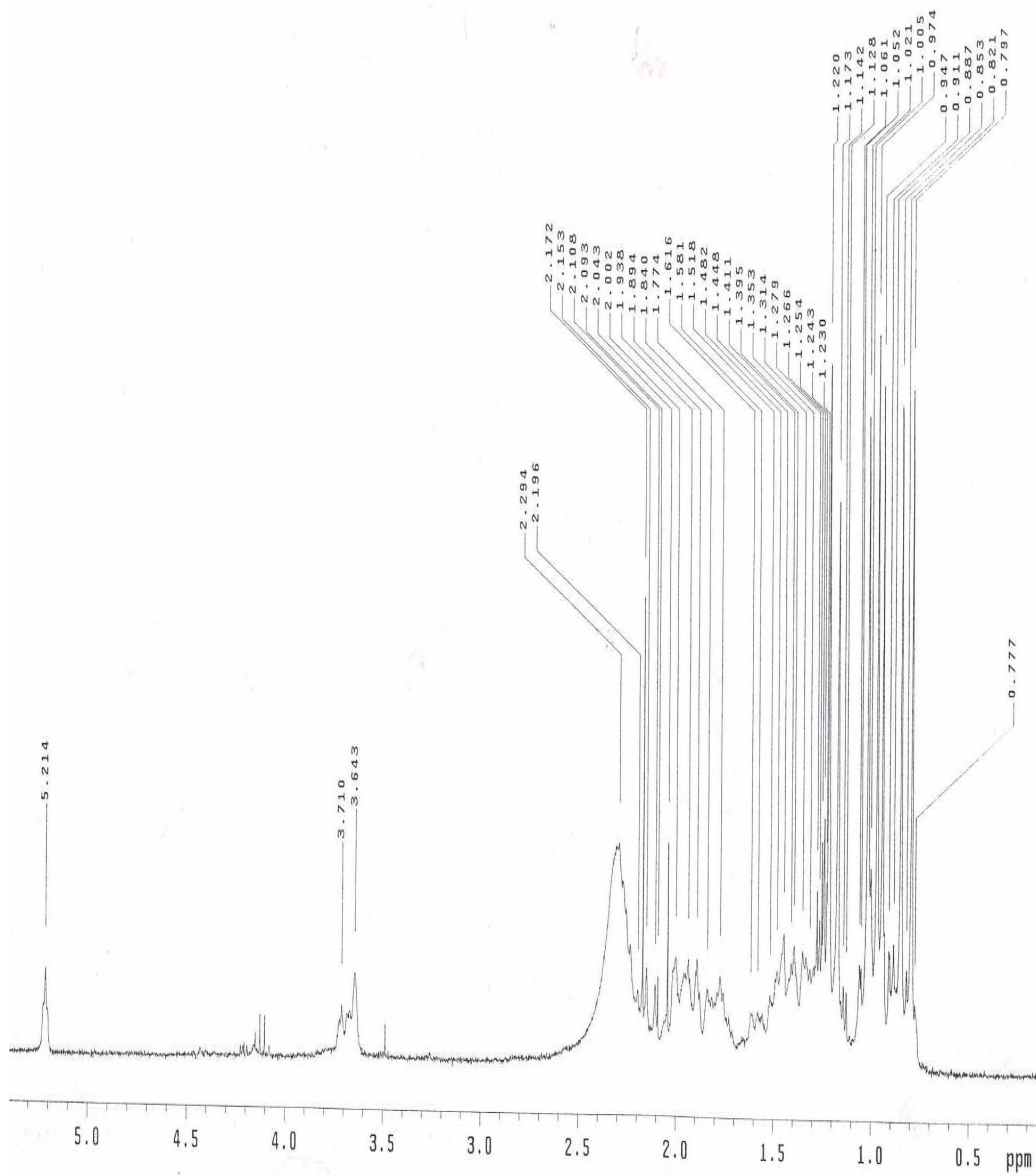


Figure 6-4: H NMR spectrum of Compound 1

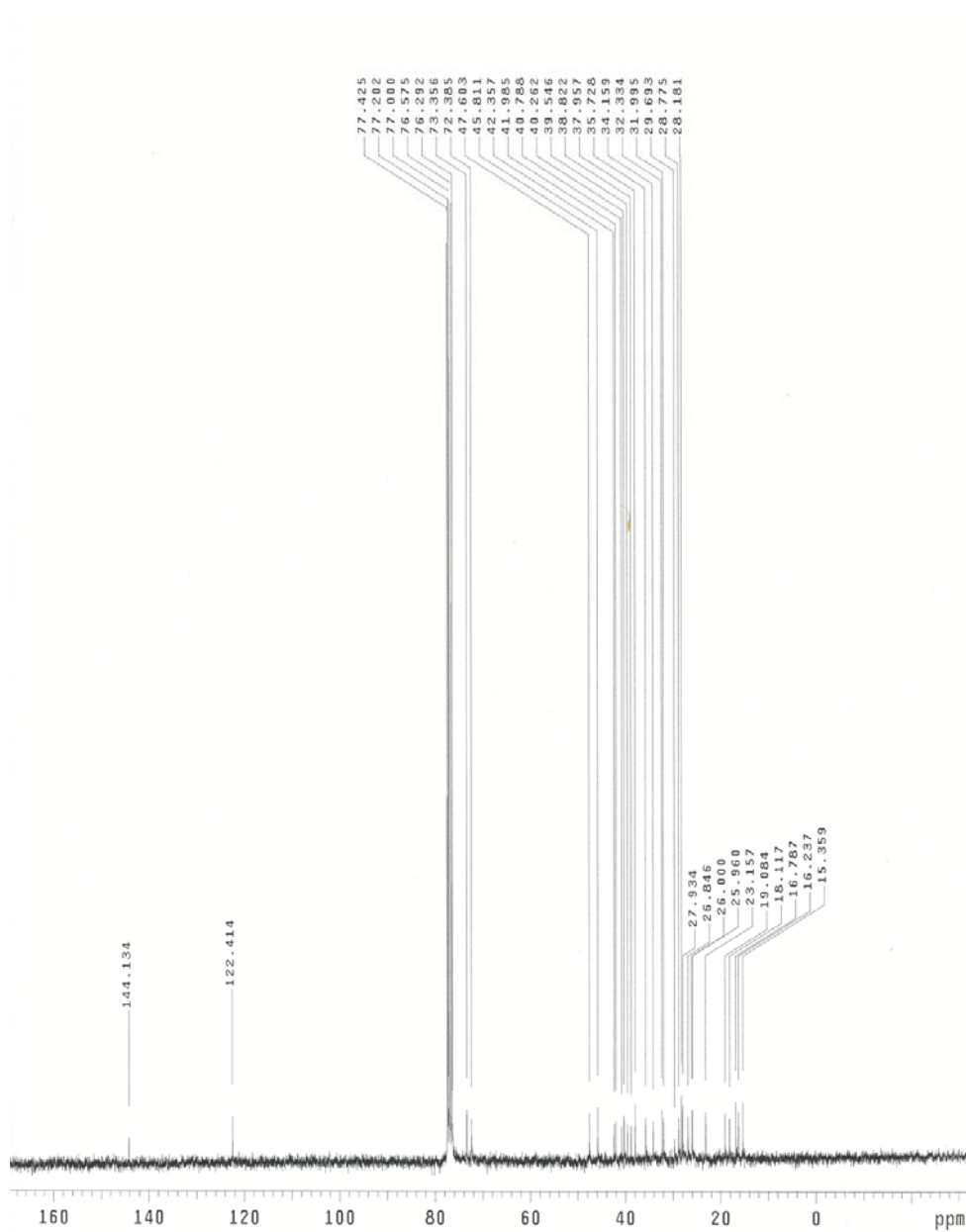


Figure 6-5: ^{13}C NMR spectrum of Compound 1

6.5.1.2 Compound 2

The ^{13}C (Table 6-1), ^1H and mass spectral data of Compound 2, 3 and 4 showed a strong resemblance to those of Compound 1. The only difference between these three molecules could be observed in Ring A and E. Compound 2 and 3 had a similar molecular formula $\text{C}_{30}\text{H}_{48}\text{O}_3$ (m/z 456) by mass spectrometry, revealing the presence of seven degrees of unsaturations within the molecule. The ^{13}C NMR spectrum of compounds 1-4 is shown on Table 5-6. The differences in their structures were observed in Ring E where Compound 2 the ^{13}C (Fig. 6-6) had resonance at δ_c 182.87 at C-30 indicating the acid functionality and Compound 3 which had ^{13}C resonance at δ_c 210 to indicated a carbonyl functionality at C-24 and two hydroxyl groups (δ_c 79.05, δ 68.15) at C-30 and C-3 as observed in the HMBC correlations. The ^{13}C NMR resonances at δ_c 182.6, 143.5, 122.5, 79.0, HMBC correlations together with their literature comparison suggest Compound 2 to be another oleanene and identified as 3-hydroxyl-12-olean-30-oic (Mukherjee, *et al.*, 1994).

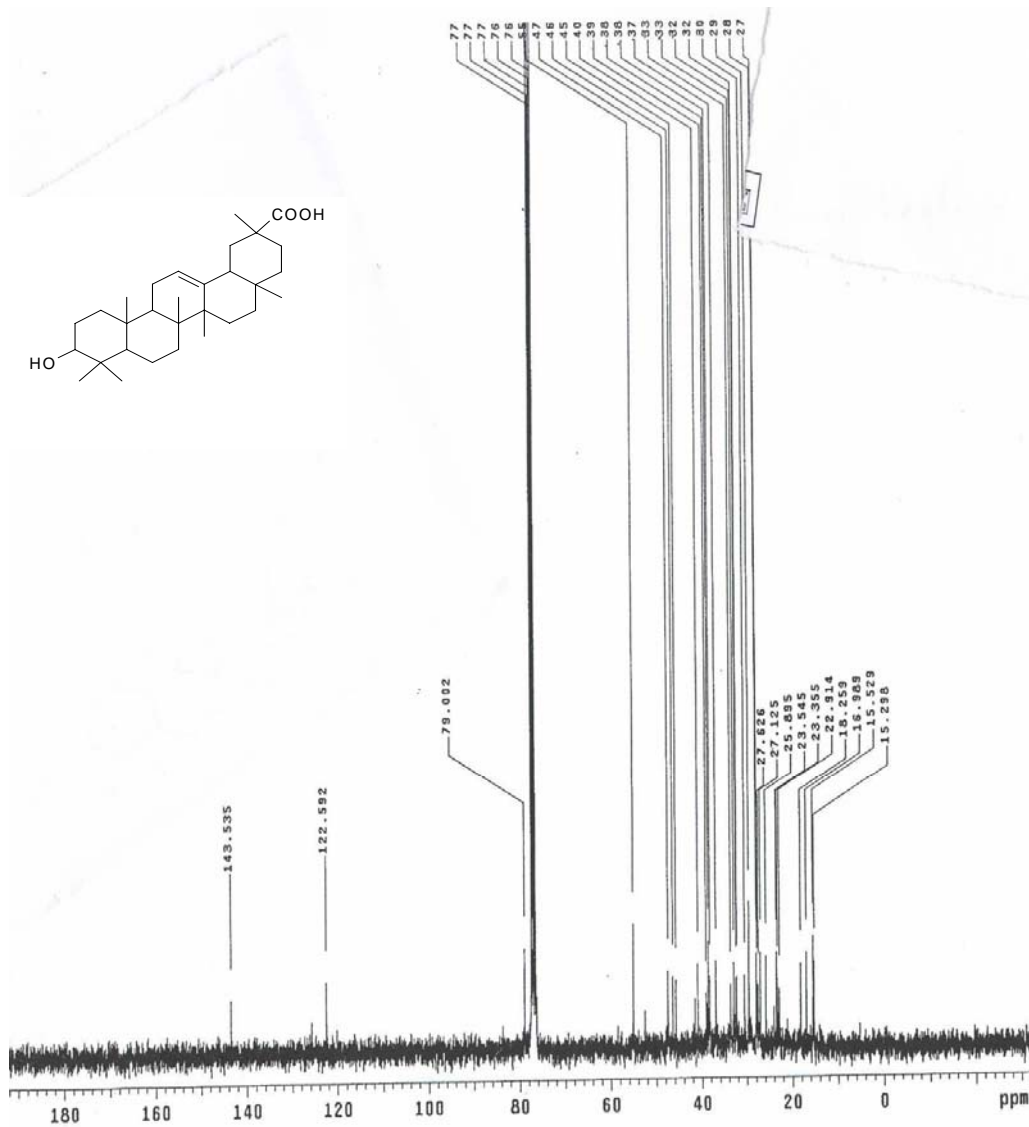


Figure 6-6: ¹³C NMR spectrum of Compound 2

6.5.1.3 Compound 3

Compound 3 was isolated as a white powder. EI-MS mass analysis indicated the presence of a molecular ion at m/z 457, and characteristic peaks at 439 ($M-H_2O$), 190 and 247 (Fig. 6-8), which corresponds to the

molecular formula $C_{30}H_{48}O_3$. The ^{13}C NMR resonances at $\delta_c 144.8$, $\delta_c 122.6$, $\delta_c 79.0$, $\delta_c 68.1$, HMMC correlations together with literature structure comparison (De Sousa *et al.*, 1990) suggested Compound 3 to be 3, 30-dihydroxyl-12-oleanen-22-one. The proton spectra of Compound 3 (Fig. 6-9) determine at the Rand Afrikaans University is different from those determined in MEDUNSA and HKI Germany.

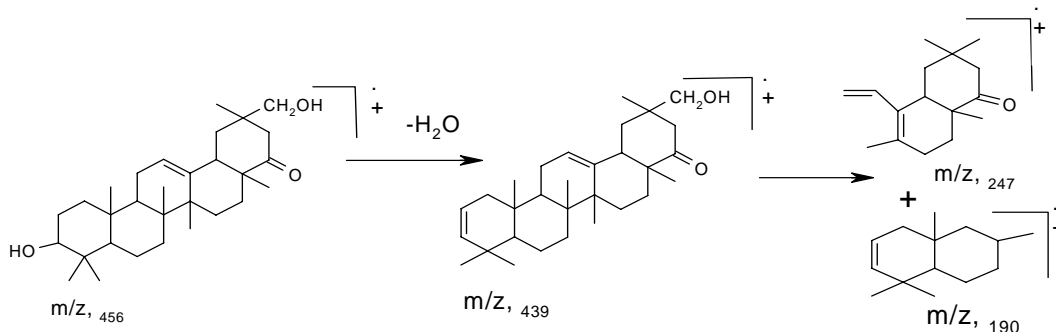


Figure 6-7: Fragmentation pattern of compound 3

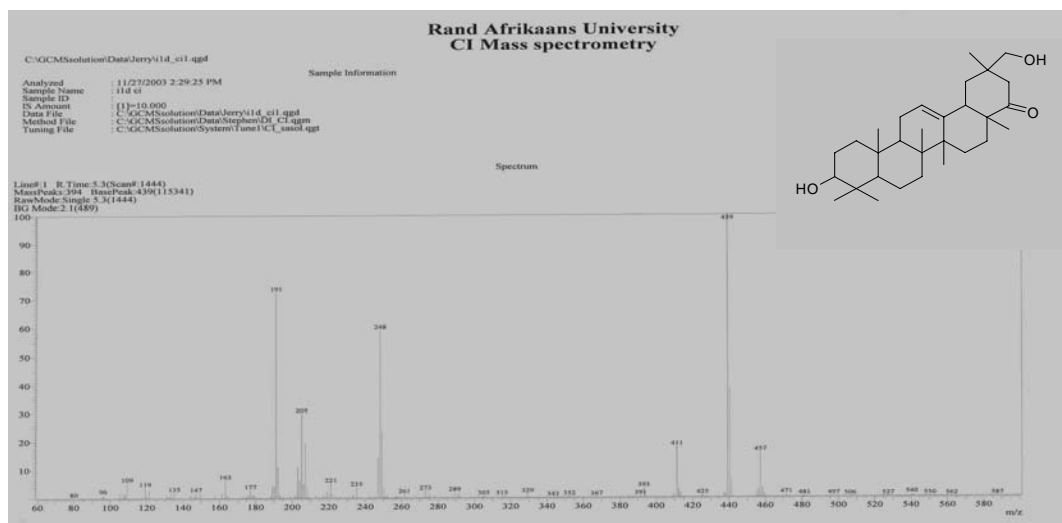


Figure 6-8: Electron impact mass spectrum of Compound 3 indicating the m/e of each fragment

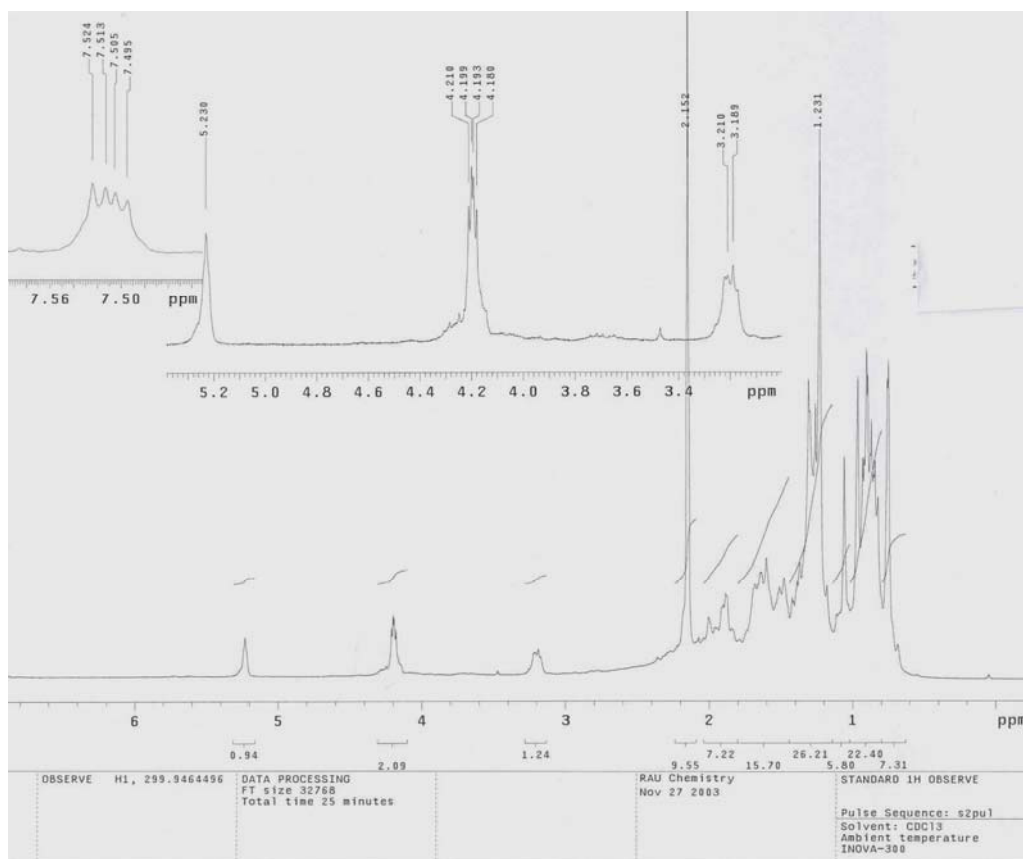


Figure 6-9: ^1H NMR spectrum of Compound 3

6.5.1.4 Compound 4

Compound 4 was isolated as white crystals and had $[\text{M}+\text{Na}]^+$ ion peak at m/z 511 in the positive ion ESI-MS suggesting a molecular formula of $\text{C}_{30}\text{H}_{48}\text{O}_5$. Comparing the ^{13}C of Compound 4 with Compound 1, there was a very strong resemblance. The only difference was observed in the number of hydroxyl groups present in each compound. Compound 4 showed the presence of three hydroxyl groups (Fig. 6-10) at δ_c 66.85, δ_H 4.23 δ_c 73.71, δ_H 3.77 δ_c 74.60, δ_H 3.57 along with signal at δ_c 182.69 indicating a carboxylic acid group and at δ 149.19, 126.70 indicating the presence of the olefinic carbon C-12 and C-13. DEPT analysis (Fig. 6-11) also showed that Compound 4 had seven methyl (CH_3 -) groups, eight methylene groups ($-\text{CH}_2-$) and seven methine groups ($-\text{CH}-$). The ^{13}C and ^1H NMR, DEPT as well as literature comparison suggest Compound 4 to be another oleanene, (1, 3, 24-trihydroxyl-12-olean-29-oic acid) that has been reported by Duan and Takaihi (2001) previously.

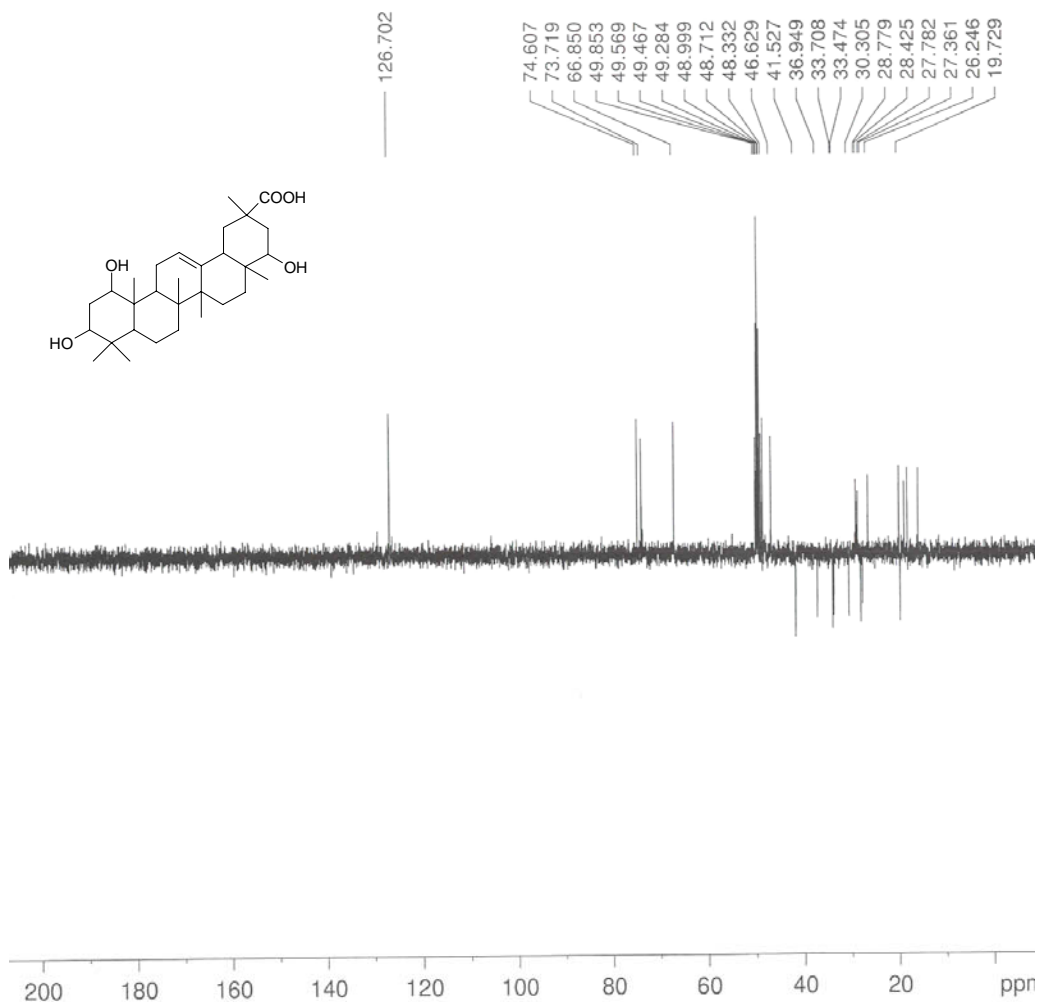


Figure 6-11: DEPT NMR spectrum of Compound 4 differentiating CH₃-, CH₂-, and CH- carbons

6.5.1.5 Compound 5

Compound **5** was isolated as white crystals and has a molecular formula of C₄₀H₆₂O₁₁ as determined by ¹H, ¹³C NMR, DEPT (**Fig. 6-13**) data and a [M+Na]⁺ ion peak at *m/z* 718.0 in the positive ESIMS. The IR spectrum indicated similar structural features as in compounds **1-4** (presence of olefinic (*ca* 1652.84cm⁻¹), hydroxyl (*ca*.3440 cm⁻¹), carboxylic acid OH (*ca*.2923.37 cm⁻¹), methyl (*ca*.1456 cm⁻¹) and carboxylic acid

C=O (*ca.*17717 cm^{-1}). ^1H NMR signals (**Fig. 6-12**) at δ_{H} 5.25 were indicating a vinylic ($\text{R}_2\text{C}=\text{CHR}$) moiety and those at δ_{H} 3.6 and 3.7 hydroxylated methines in the aglycone. The signals at δ_{H} 4.70 and δ_{C} 98.62 indicate an anomeric CH group (acetal) of an oxygen-linked sugar moiety. The ^{13}C NMR data of **5** (**Table 6-2**) were almost identical to those of 1α , 3β , 23-trihydroxy-12-oleanen-29-oic acid (Rogers and Subranomy, 1988) except for an additional sugar moiety and the missing hydroxyl group at C-3 that had been replaced with the ether linked sugar moiety, as indicated by δ_{C} 67.0. The location of the new sugar moiety was confirmed by HMBC correlations between H-3 and the anomeric carbon and between the anomeric proton and C-3. Two acetylated methines were indicated at δ_{C} 75.4, δ_{H} 4.90 (C-4') and δ_{C} 73.9, δ_{H} 5.11 (C-2') through correlations to two acetyl groups. All ^1H and ^{13}C NMR data were assigned through HMQC, HMBC (Table 6-3) and COSY experiments.

According to the coupling constants and correlation peaks in NOESY spectrum, **5** showed the same relative stereochemistry in the triterpenoid skeleton as 1α , 3β , 23-trihydroxy-12-oleanen-29-oic acid and the anomeric proton located at β position in sugar moiety. The relative configuration of the chiral centres at C-1, C-3, C-4, C-8, C-15, C-20 and C-10 was determined by detailed NOESY analysis. Correlations between H-1 (δ_{H} 3.55) and H-3 (δ_{H} 4.19), H₃-25 (δ_{H} 1.00) and H₃-26 (δ_{H} 1.05) and H₃-28 (δ_{H} 0.93) and H₃-23 (δ_{H} 0.7) indicated these protons to be oriented on the same side of the ring system. On the other hand, NOESY correlations between H-9 (δ_{H} 2.52) and H-27 (δ_{H} 1.30) and none of the above protons indicated these two protons to be located on the other ring plane. Thus, compound **5** was elucidated as 1α , 23 β -dihydroxy-12-oleanen-29-oic-acid-3 β -O- α -2,4-diacetylramnopyranoside. This compound is reported here for the first time.

Table 6-1. ¹³C NMR data of compounds 1-4 in CH₃OH.

Carbon	1	2	3	4
C-1	73.3	39.2	39.0	74.6
C-2	35.8	27.1	27.2	36.9
C-3	72.3	79.0	68.1	73.7
C-4	40.2	39.2	39.0	40.2
C-5	47.6	55.1	55.2	48.4
C-6	18.1	18.2	18.2	18.6
C-7	31.9	32.3	32.4	33.1
C-8	39.5	40.9	40.0	41.5
C-9	38.8	52.5	53.5	36.9
C-10	42.3	41.5	42.0	42.8
C-11	23.1	23.3	23.6	26.4
C-12	122.4	122.5	122.6	126.7
C-13	144.1	143.5	144.8	149.1
C-14	41.9	45.8	46.5	43.2
C-15	26.6	27.6	28.3	27.8
C-16	27.4	27.9	28.1	28.4
C-17	32.8	32.5	33.2	33.4
C-18	46.7	46.4	46.7	46.6
C-19	41.6	39.2	42.2	42.8
C-20	42.8	46.4	42.5	43.2
C-21	29.8	30.6	31.1	30.3
C-22	36.8	37.0	205.0	66.8
C-23	28.1	28.0	28.1	36.9
C-24	18.1	16.9	17.5	17.9
C-25	16.2	15.5	16.6	19.5
C-26	16.7	15.2	17.4	17.9
C-27	26.0	25.8	26.2	26.2
C-28	28.7	29.6	28.7	28.7
C-29	19.0	22.9	21.4	19.7
C-30	181.4	181.3	79.0	182.6

All measured in *α*-CH₃OH relative to TMS, - No carban at that position.

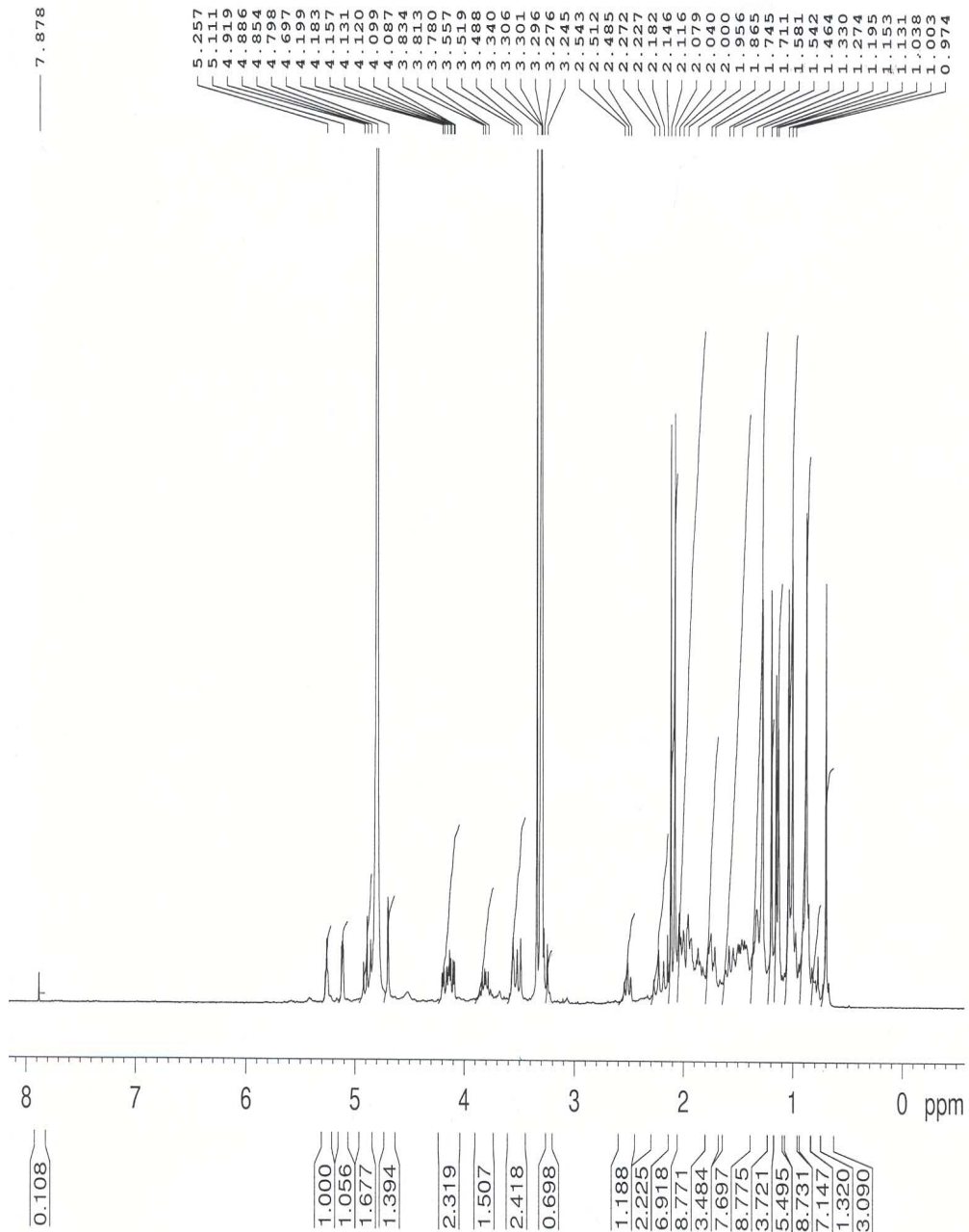


Figure 6-12: ¹H NMR spectrum of compound 5

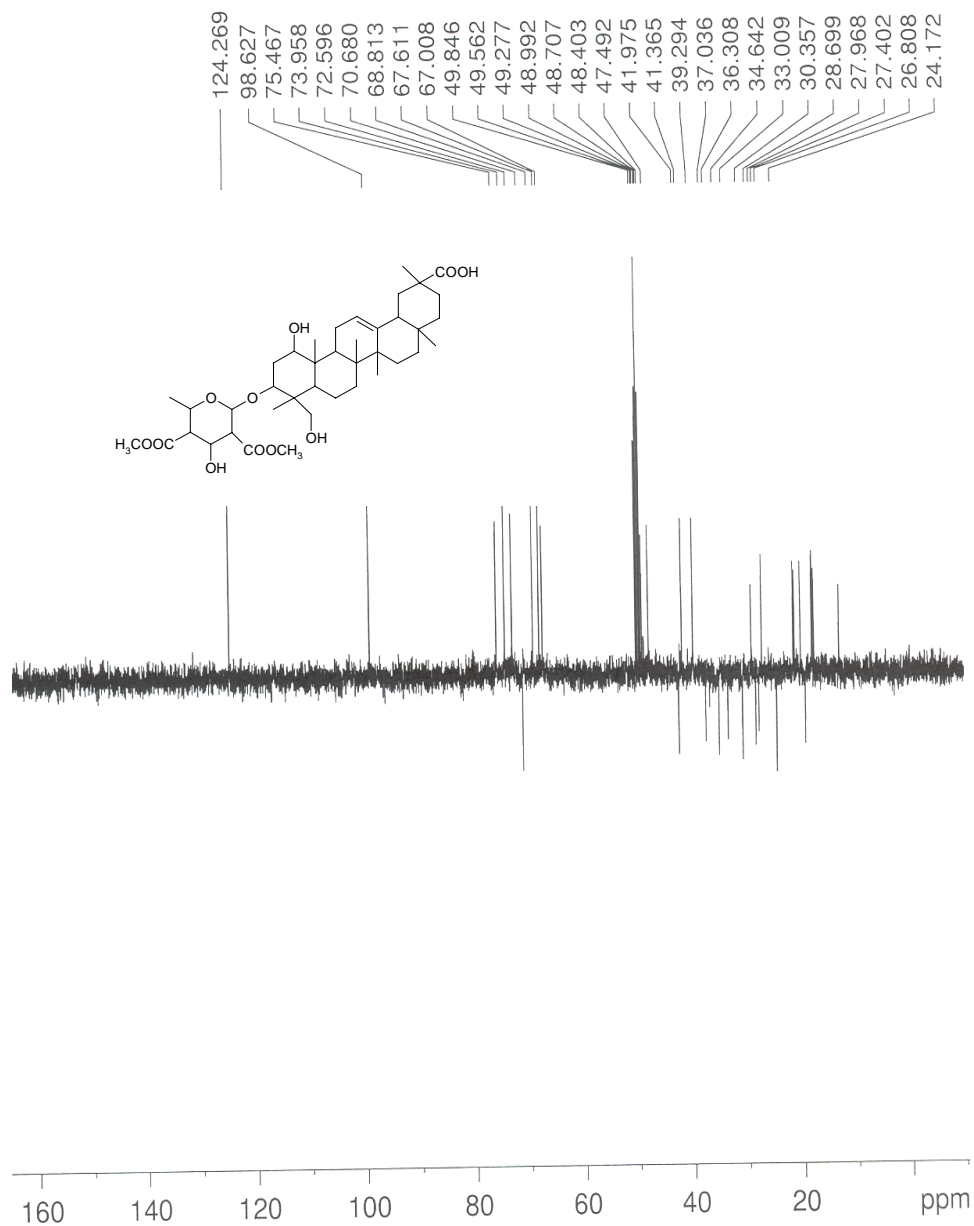


Figure 6-13: DEPT spectrum of Compound 5 indicating the presence of CH₃-, CH₂- and CH- carbons in the compound

Table 6-2: ^{13}C (75.4 MHz) and ^1H (300 MHz) NMR data of 5 in CD_3OD .

Position		^{13}C (δ_c)	^1H (δ_H , mult., J in Hz)
C-1	CH	72.6	3.55 <i>t</i> br
C-2	CH ₂	34.6	1.74 <i>m</i> , 2.0, <i>m</i>
C-3	CH	67.0	4.17 <i>dd</i> (9.8, 4.5)
C-4	C	43.2	-
C-5	CH	41.3	1.75 <i>m</i>
C-6	CH ₂	18.9	1.45 <i>m</i> , 1.54 <i>m</i>
C-7	CH ₂	33.0	1.30 <i>m</i> , 1.60 <i>m</i>
C-8	C	40.7	-
C-9	CH	39.2	2.52 <i>dd</i> (9.3, 9.3)
C-10	C	41.8	-
C-11	CH ₂	24.1	1.95 <i>m</i>
C-12	CH	124.3	5.25 <i>s</i>
C-13	C	145.3	-
C-14	C	43.5	-
C-15	CH ₂	27.4	1.86 <i>m</i>
C-16	CH ₂	27.9	2.05 <i>m</i>
C-17	C	33.5	-
C-18	CH	47.5	2.05 <i>m</i>
C-19	CH ₂	41.9	1.30 <i>m</i> , 2.23 <i>m</i>
C-20	C	43.6	-
C-21	CH ₂	30.4	1.34 <i>m</i> , 1.86 <i>m</i>
C-22	CH ₂	37.0	1.32 <i>m</i> , 1.49 <i>m</i>
C-23	CH ₃	12.6	0.70 <i>s</i>
C-24	CH ₂	70.6	3.26 <i>d</i> (9.3), 3.50 <i>d</i> (9.3)
C-25	CH ₃	17.3	1.00 <i>s</i>
C-26	CH ₃	17.7	1.05 <i>s</i>
C-27	CH ₃	26.8	1.27 <i>s</i>
C-28	CH ₃	28.6	0.88 <i>s</i>
C-29	CH ₃	19.7	1.20 <i>s</i>
C-30	C	182.8	-
C-1'	CH	98.6	4.60 <i>s</i> br
C-2'	CH	73.9	5.11 <i>m</i>
C-3'	CH	68.8	4.11 <i>dd</i> (3.5, 9.8)
C-4'	CH	75.4	4.90, <i>dd</i> (9.8, 9.8)

C-5`	CH	67.6	3.81 <i>m</i>
C-6`	CH ₃	17.6	1.14 <i>d</i> (6.4)
C-1"	C=O	172.2	-
C-2"	CH ₃	21.0	2.12 <i>s</i>
C-3"	C=O	172.2	-
C-4"	CH ₃	20.8	2.08 <i>s</i>

All measured in *d*-CH₃OH relative to TMS, No carbon at that position (-)

Table 6-3. HMBC correlations for methyl groups in compound 5.

Position	δ_H	δ_C	2J	3J
23	0.7	12.6	43.2 (C-4)	67.0 (C-3), 41.3 (5), 70.6 (24)
25	1.0	17.3	41.8 (C-10),	72.6 (C-1), 41.3 (C-5), 39.2 (C-9)
26	1.05	17.7	40.7 (C-8)	39.2 (C-9), 33.0 (C-7), 43.5 (C18)
27	1.30	26.8	43.5 (C-14)	40.7 (C-8), 27.4 (C15), 145.3 (C-13)
28	0.90	28.6	33.5 (C-17)	47.5 (C-18), 27.9 (C-16), 37.0 (C-22)
29	1.20	19.7	43.6 (C-20)	182.8 (C-30), 30.4 (C-21), 41.9 (C-19)
Rh'-6	1.5	17.6	67.6 Rh'-5)	75.4 (Rh'-4)

6.5.1.6 Compounds 6

Compound **6** (150 mg) that was isolated as white crystals with a molecular ion peak at 694.8 [M+NH₄]⁺ in the ESIMS. The corresponding molecular weight of 676 g/mol suggested a molecular formula of C₃₈H₆₀O₁₀ containing nine degrees of unsaturation. IR analysis indicated the presence of olefinic (1653 cm⁻¹), hydroxyl (3440 cm⁻¹), methyl (1456 cm⁻¹) and carboxylic acid (hydroxyl at 2923 cm⁻¹ and 1772 cm⁻¹) moieties. The triterpenoid skeleton was easily deduced from ¹H NMR data (**Fig. 6-13**) by the appearance of vinylic (R₂C=CHR, δ_H 5.24) and hydroxylated methine (RCHOH, δ_H 3.55) .s (**Table 6-5**). There was also a crowded signal indicating the presence of -CH₃, -CH₂, and -CH protons (δ_H 0.5- δ_H 2.0). The ¹³C NMR spectrum (**Fig. 6-15**) confirmed these findings, additionally it showed the typical carboxyl at δ_C 183.1 of the -CO₂H constituent. Altogether, eight methyl groups, ten methylenes, eleven methines and nine quaternary carbons were revealed by DEPT data. The ¹H (**Fig. 6-14**) and ¹³C NMR data of compound **6** were similar to those of **7** except for differences in signals of rings A and E relating to different hydroxylation patterns. In **5**, the methyl group at C-23 carries an ether linked substitution as indicated by the new methylene group at δ_C 70.3/ δ_H 3.25 dd, 3.50 dd. In ring E, C-22 is a regular methylene and not oxgenated like in compound **7** (δ_C 31.1).

According to HMBC (**Fig. 6-16**) the carboxylic acid carbonyl is located at C-20 and one of the hydroxyl groups was located at C-1 (δ_c 72.6) as earlier stated by HMQC. Assisted by the ^1H - ^1H COSY, the spin system of the complete rhamnose-skeleton (Rh') was traced. Using HMQC and ^{13}C spectra, the proton appearing as a singlet at δ_H 4.69 was assigned as anomeric proton of the sugar unit. The proton at δ_H 4.93 (Rh'-4) was assigned to be located next to an acetyl substituent of the sugar moiety. C-23 was unequivocally assigned as site of glycosylation by a HMBC correlation between H-Rh-1 (δ_H 4.69) and the oxymethylene C-23. This position has been shown as a preferred site of glycosylation in similar compounds from the related *C. imberbe* (Roger and Subramony, 1988). The relative configurations of the chiral centres at C-1, C-3, C-4, C-8, C-10, C-14, C-17, and C-20 were determined by NOESY analysis: correlations between H-1 (δ_H 3.55) and H-27 (δ_H 1.26), H-24 (δ_H 0.70) and H-25 (δ_H 1.00), H-18 (δ_H 2.01) and H-28 (δ_H 0.87), H-18 (δ_H 2.01) and H-30 (δ_H 1.19), H-Rh'-2 (δ_H 3.90) and H-Rh'-4 (δ_H 4.93), H-Rh'-1 (δ_H 3.55) and H-Rh'-4 (δ_H 4.93) indicated that these protons were oriented in the same direction. On the other hand, the NOESY correlations between H-23 (δ_H 3.50) and H-27 (δ_H 1.26), H-Rh'-6 (δ_H 1.13) and H-Rh'-3 (δ_H 4.14) indicated these to orient in the opposite direction. On the basis of the above analysis, compound **6** was thus elucidated as 1 α ,3 β -dihydroxy-12-oleanen-29-oic-acid-23 β -O- α -4-acetylramnopyranoside. This compound has not been reported previously. A similar compound has been isolated from *C. imberbe* by Katerere *et al.*, 2003 with the acetyl rhamnosides attached at position 4 of the triterpene aglycone.

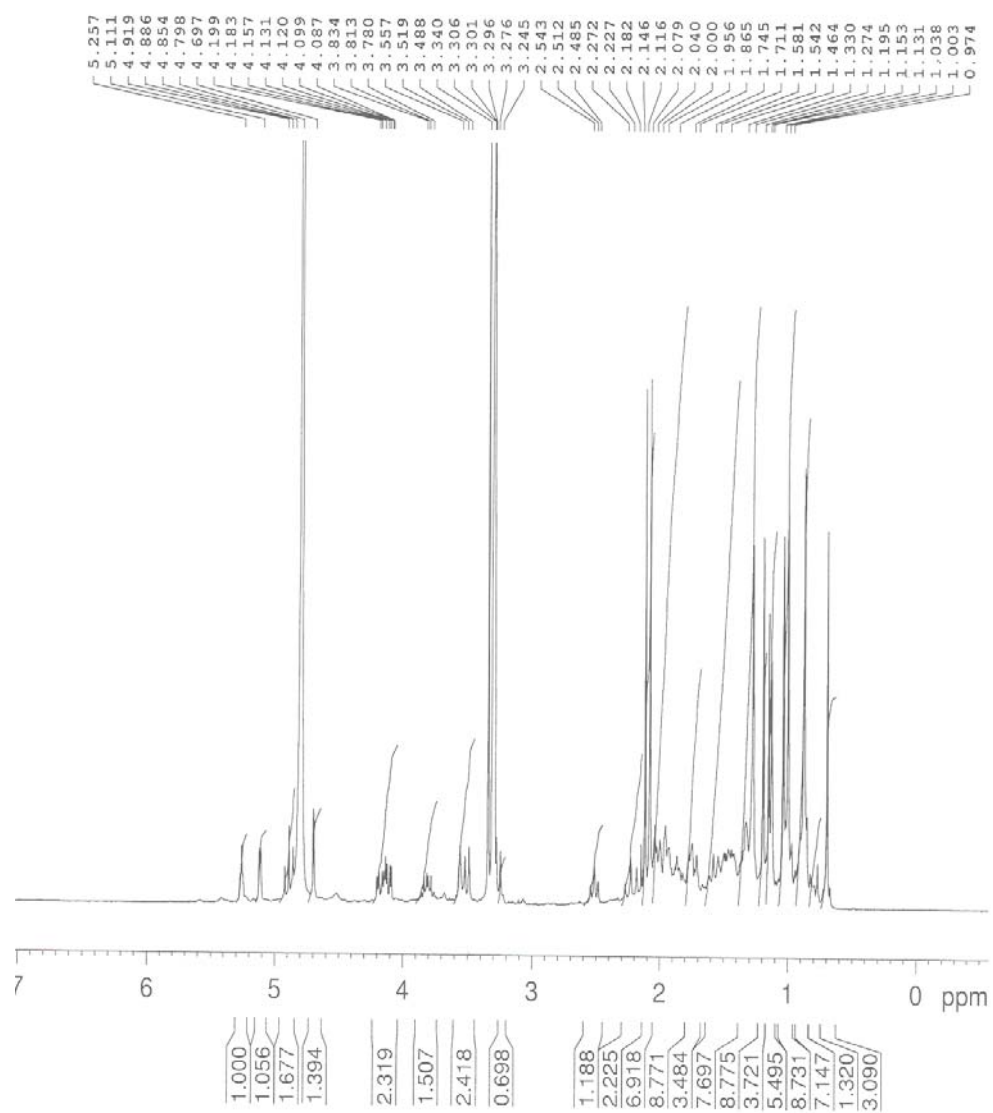


Figure 6-14: ¹H NMR spectrum of compound 6

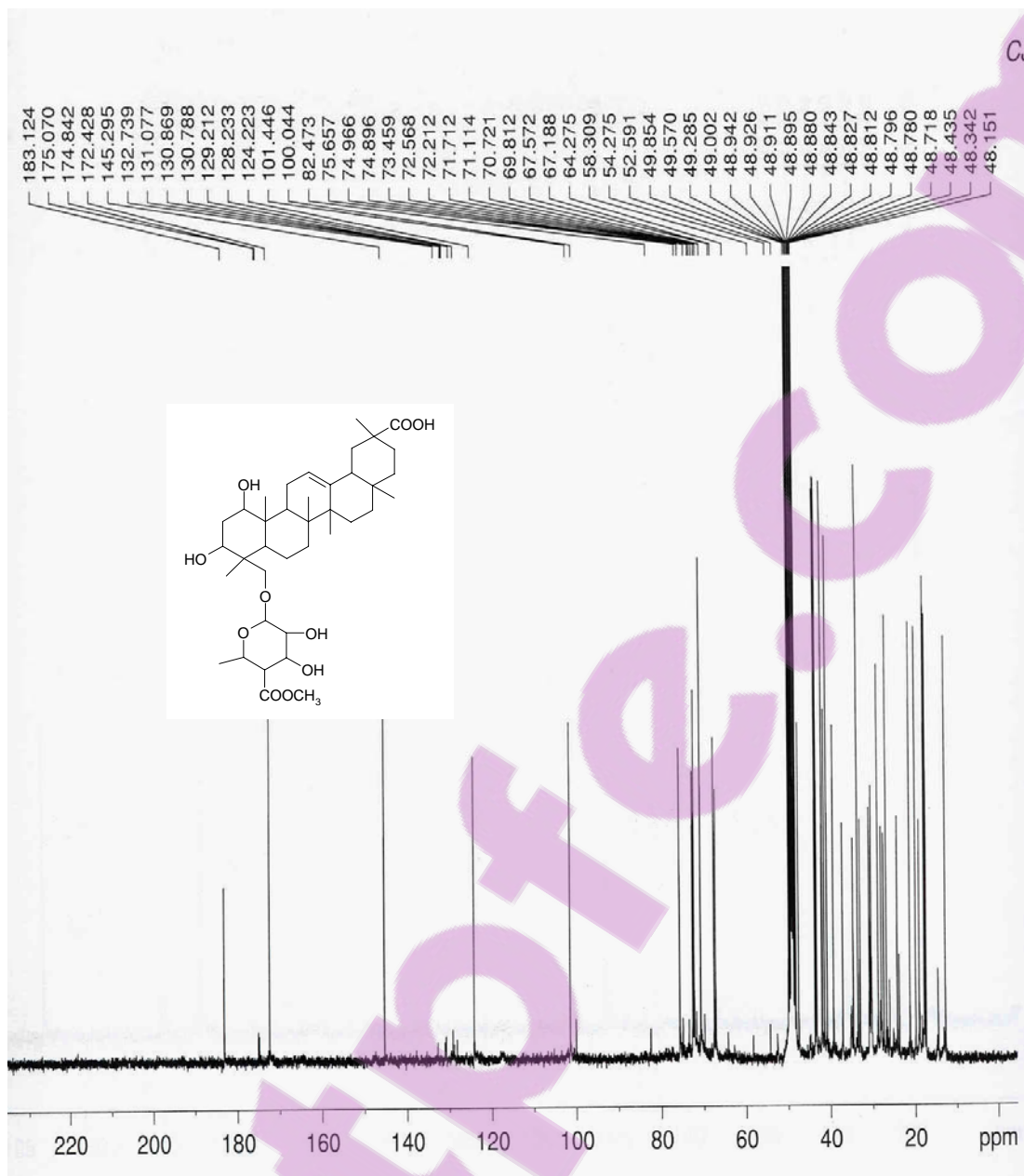


Figure 6-15: ¹³C NMR spectrum of Compound 6

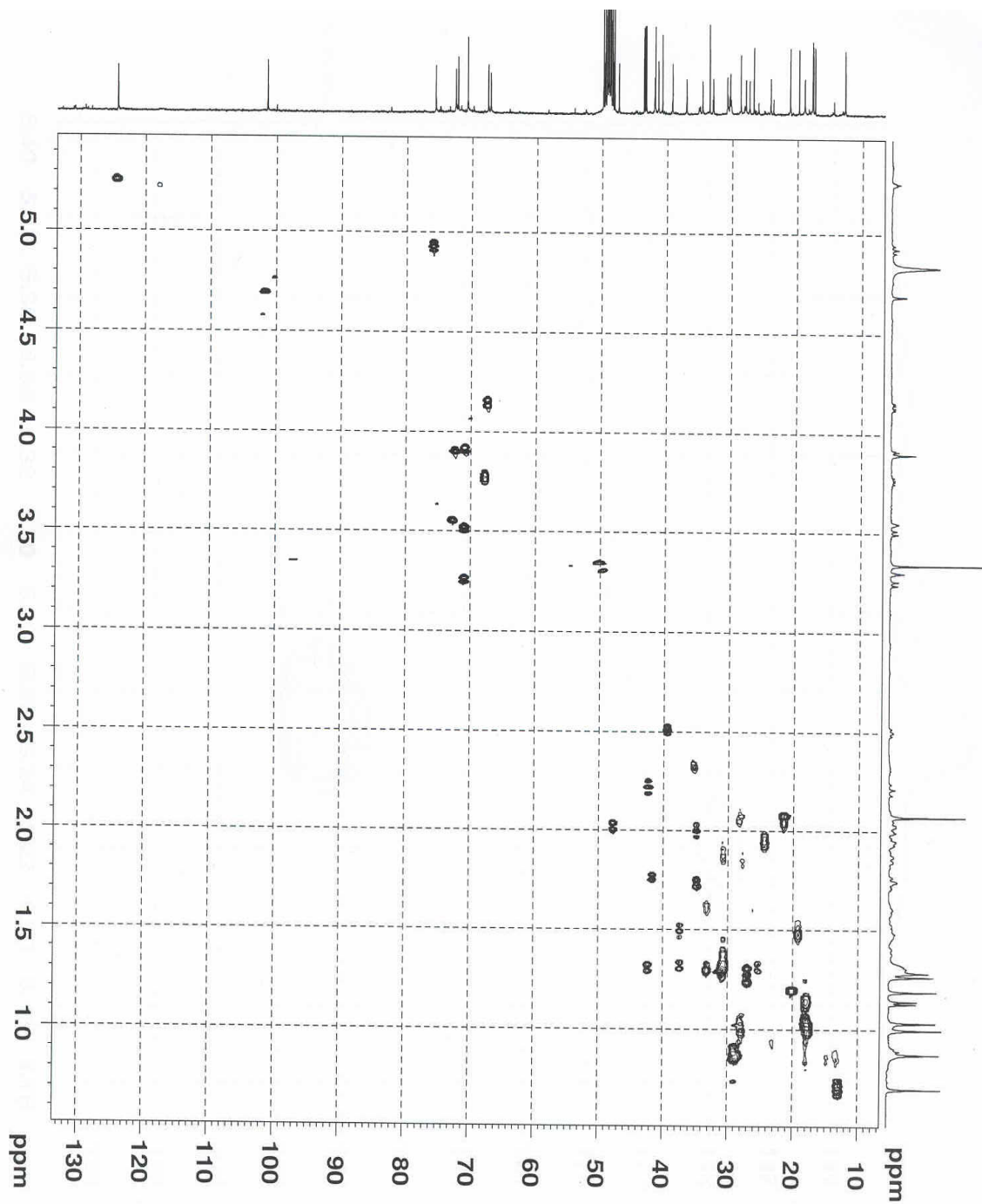


Figure 6-16: HMBC spectrum of Compound 6.

6.5.1.7 Compound 7

MS analysis of Compound 7 indicated a molecular ion peak ($M+Na^+$) of m/z 495.8 and suggested a molecular formula of $C_{30}H_{48}O_4$ according to ESI-MS, indicating seven degrees of unsaturation. This structural type was further supported by the 1H NMR spectrum (**Fig. 6-17**), which contained resonance for seven skeletal methyl groups, ten methylene groups, five methine groups, eight quaternary carbons (from DEPT, **Fig. 6-18** and **Fig. 6-19**) and a broad triplet at δ_H 5.25 for an olefinic proton (H-12), a carboxylic acid functionality (δ_C 182.90) and two-hydroxyl groups (δ_H 3.6, δ_C 74.01 and δ_H 3.75, δ_C 72.97). The final attribution of the 1H , ^{13}C (**Table 6-4**), DEPT, and EI-MS of Compound 7 were in complete agreement with literature data of 1α , 22β dihydroxy-12-oleanen-30-oic acid that has previously been isolated (Nakano K, 1997). Compound 7 was therefore elucidated as 1α , 22β -dihydroxy-12-oleanen-30-oic acid.

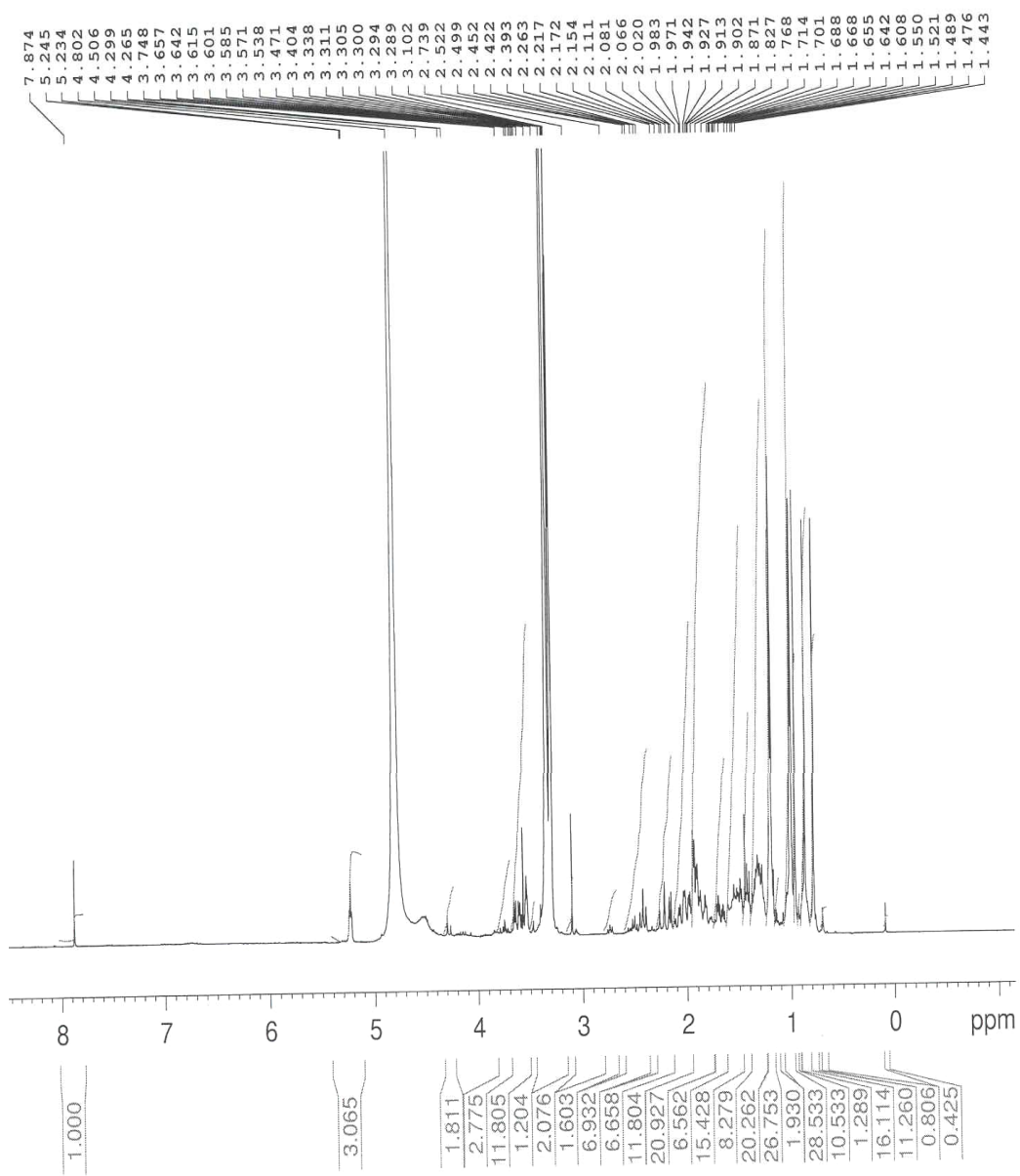


Figure 6-17: ¹H NMR of compound 7

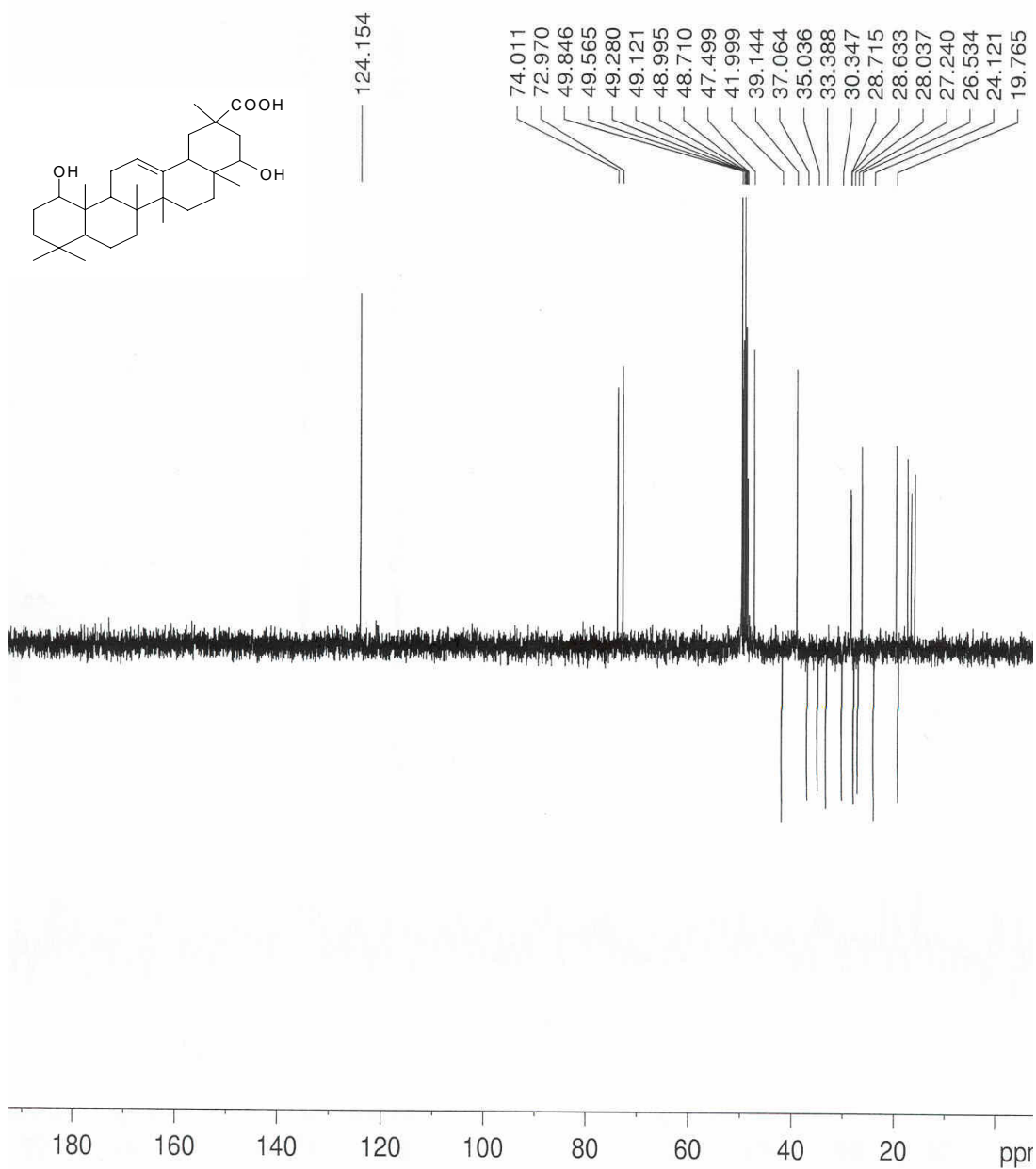


Figure 6-18: DEPT spectrum of Compound 7

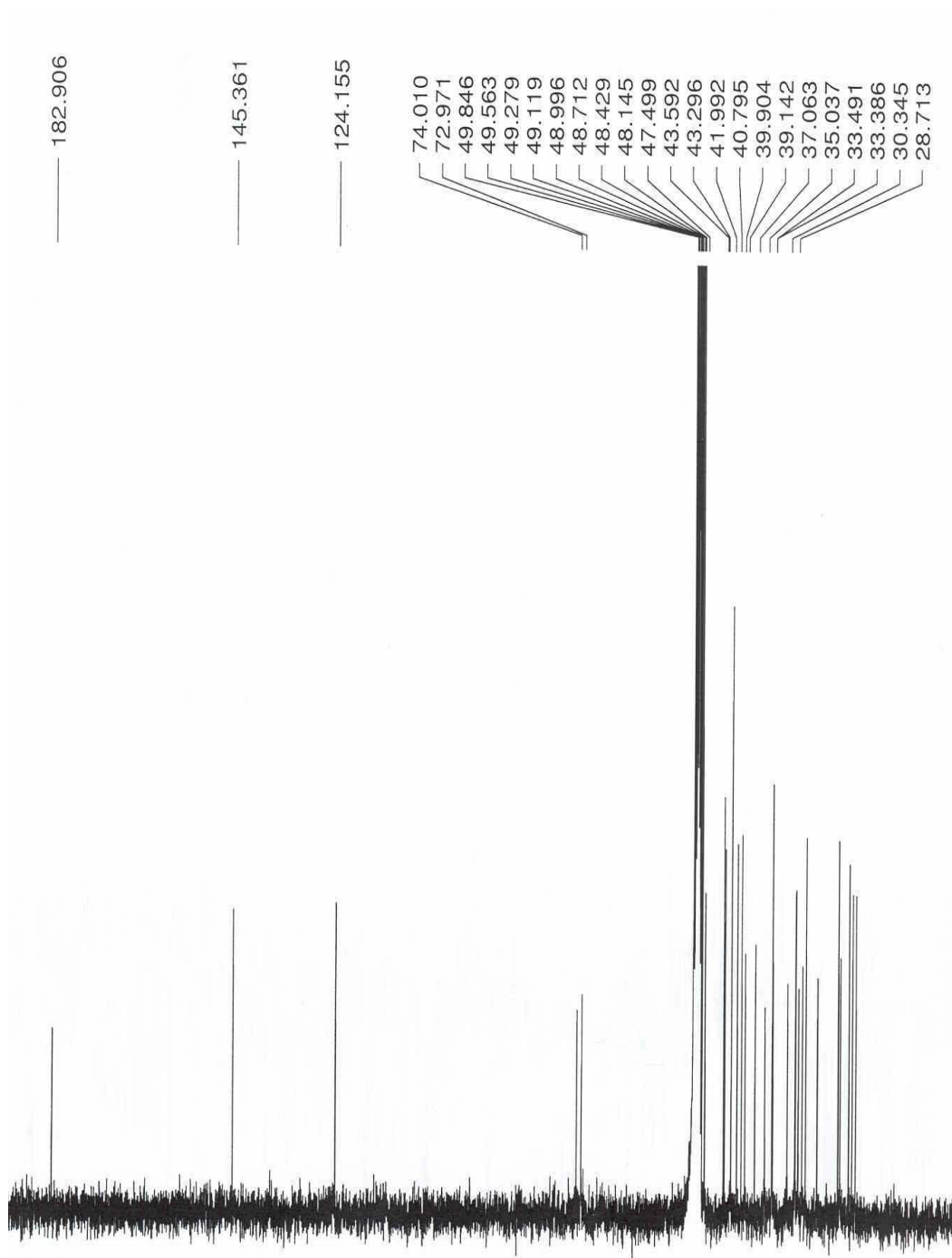


Figure 6-19: ^{13}C NMR of Compound 7

6.5.1.8 Compound 8

Compound **8** was isolated as a cream powder with a molecular ion peak $[M+NH_4^+]$ at 591.0 based on ESI-MS suggesting a molecular formula of $C_{35}H_{36}O_6$ and indicating eight degrees of unsaturation. Its 1H NMR spectrum (**Fig. 6-20**) revealed the presence of four hydroxyl groups at δ_H 4.15, 4 δ_H .45, δ_H 4.65, and δ_H 4.85, olefinic protons at δ_H 5.2, and δ_H 5.3; and an ester linkage proton at 4.22. Five methyls groups, eleven methylenes groups, sixteen methine groups, and three quaternary carbons were revealed by DEPT (**Fig. 6-21**) The ^{13}C NMR indicated the presence of an ester linkage carbon at δ_c 100.8; four hydroxyl groups at δ_c 61.22, δ_c 70.24, δ_c 73.57 and δ_c 76.78; five signals at δ_c 110.86, δ_c 121.29, δ_c 129.66, δ_c 136.90 indicating olefinic carbons. The structure of Compound **8** was established as 24-ethylcholesta-7, 22,25-trien-3-ol-O- β -D-glucopyranoside based on above data was in perfect agreement with literature of the same compound that has been isolated previously from *Clerodendron inerme* (Atta.ur-Rahman *et al.*, 1997).

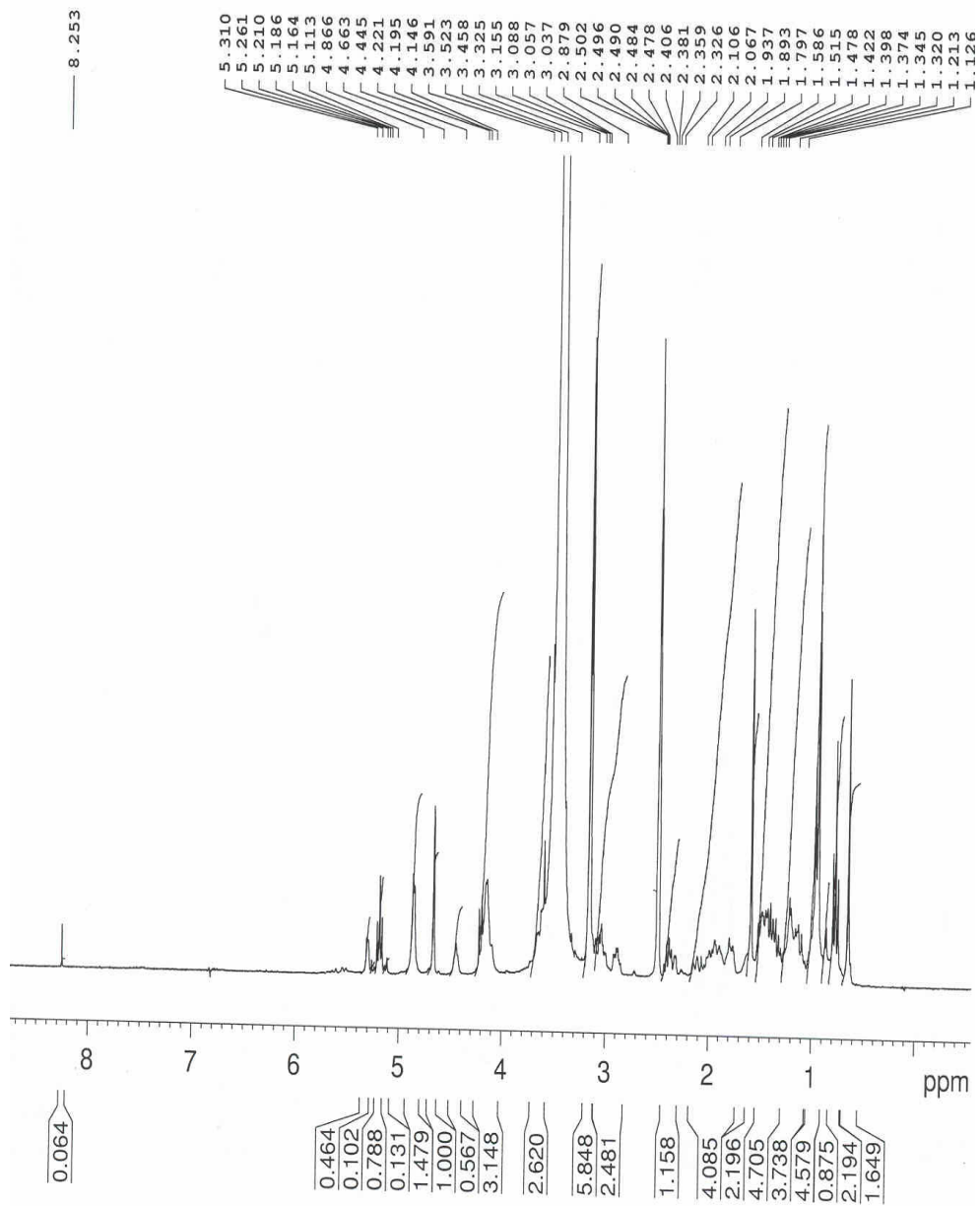


Figure 6-20: ¹H-NMR of Compound 8

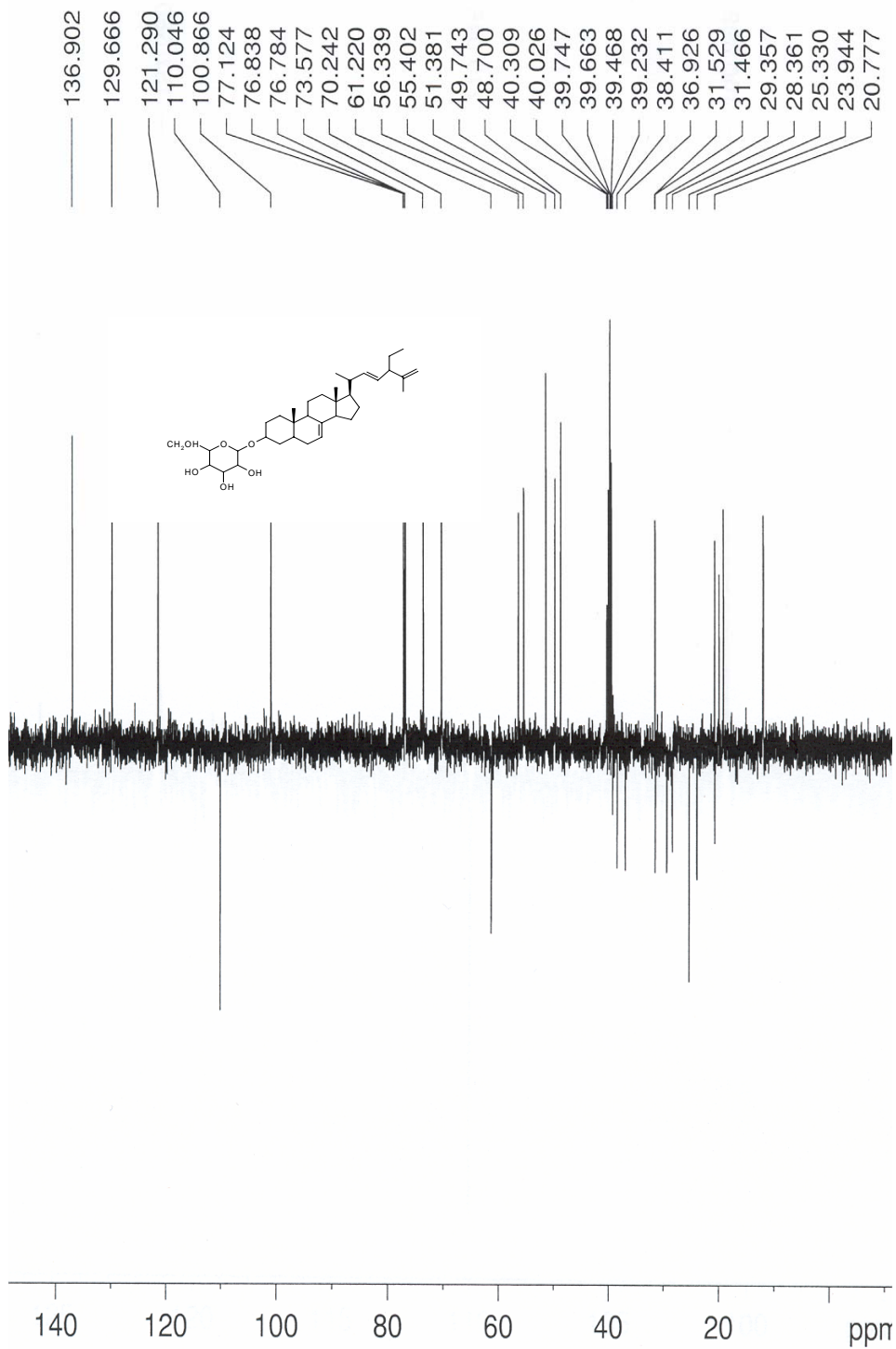


Figure 6-21: DEPT spectrum of Compound 8.

Table 6-4: ^{13}C (75.4 MHz) and ^1H (300 MHz) NMR data of 7 and 8 in CD_3OD .

Carbon numbers	7	8
1	72.9	36.9
2	33.4	31.4
3	35	77.0
4	43.2	38.4
5	45.2	140.5
6	19.3	121.2
7	33.3	29.3
8	40.7	31.5
9	39.9	49.7
10	43.5	36.3
11	24.1	21.1
12	124.1	38.4
13	145.3	41.8
14	43.2	56.3
15	30.3	23.9
16	27.2	28.4
17	28	55.3
18	39.1	11.9
19	41.9	19.1
20	43.6	40.4
21	30.3	19.9
22	74	136.8
23	16.1	129.6
24	16.8	51.3
25	19.7	147.7
26	17.5	110.0
27	26.5	20.7
28	28	25.3
29	19.3	12.0
30	182.9	-
Rh ¹ -1		100.0
Rh ¹ -2		76.7
Rh ¹ -3		76.7
Rh ¹ -4		70.1
Rh ¹ -5		76.8
Rh ¹ -6		61.1

Table 6-5: ^1H (300 MHz) and ^{13}C (75.4 MHz) NMR data of 6 (CD_3OD)

Position	Carbon type	^{13}C (δ_c)	^1H (δ_H) ^a
1	CH	72.6	3.55 brs
2	CH ₂	34.6	1.74 m, 1.93 m
3	CH	67.2	4.14 dd 13.2, 4.5
4	C	43.3	
5	CH	41.4	1.76 m
6	CH ₂	18.9	1.45 m
7	CH ₂	33.0	1.59 m; 1.28 m
8	C	40.8	
9	CH	39.3	2.50 dd 8.2, 8.2
10	C	42.0	
11	CH ₂	24.1	1.93-1.98 m
12	CH	124.3	5.24 s
13	C	145.3	
14	C	43.5	
15	CH ₂	27.4	1.86 dd 4.2, 8.0
16	CH ₂	28.0	2.07 m
17	C	33.6	
18	CH	47.5	2.01 brd
19	CH ₂	41.9	1.30 m; 2.21 dd 13.4, 13.4
20	C	43.6	
21	CH ₂	30.4	1.87 m
22	CH ₂	37.1	1.47 brt, 1.30 dd
23	CH ₂	70.7	3.50 d, 9.6; 3.25 d, 9.6
24	CH ₃	12.8	0.70 s
25	CH ₃	17.4	1.00 s
26	CH ₃	17.8	1.03 s
27	CH ₃	26.8	1.26 s
28	CH ₃	28.8	0.87 s
29	C	183.1	
30	CH ₃	19.8	1.19 s
Rh'-1 ^b	CH	101.5	4.69 brs
Rh'-2	CH	72.2	3.90 brs
Rh'-3	CH	70.7	3.91 dd cov

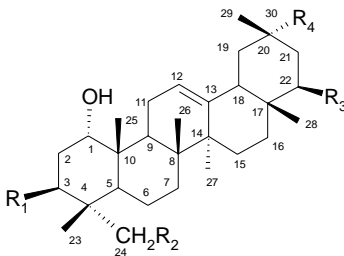
Rh'-4	CH	75.7	4.93 brt, 9.6
Rh'-5	CH	67.6	3.76 m
Rh'-6	CH ₃	17.7	1.13 d, 10.0
Ac-1 ^c	C	172.5	
Ac-2	CH ₃	21.2	2.07 s

^a multiplicity, *J* in Hz; ^b Rhamnopyranoside (Rh'); ^c acetyl (Ac)

It is important to mention that in related studies, triterpends have been isolated in closely related genera *Terminalia*. In some of these studies, a new *A-seco*-triterpene was isolated from the stem bark of *Terminalia glaucescens* Planchon and other known triterpenes, friedelin, β -sitosterol, stigmasterol, lupeol, betulinic acid, β -amyrin and long chain fatty acids were also isolated (Atta-ur-Rahman *et al.*, 2002). Also, a new triterpene, 2 α -hydroxymicromeric acid, and two known compounds, maslinic acid and 2 α -hydroxyursolic acid have been isolated from *Terminalia chebula leaves* (Chandan Singh, 1990). This may show an indication of a chemotaxonomic relationship between genera in Combretaceae.

6.6 Summary

A total of eight structures (Fig. 6-22 and Fig. 6-23) were elucidated with the aid of the NMR (¹H, ¹³C, HMBC, COSY, and HMQC), MS and IR data that were available. All compounds had an olean-12-ene skeleton, except Compound 8.



Compounds	R ₁	R ₂	R ₃	R ₄
1	OH	H	H	COOH
2	H	H	H	COOH
3	H	H	=O	CH ₂ OH
4	H	H	OH	COOH
5	2,4-Di-Ac-O-Rh	OH	H	H

Figure 6-22: Compounds 1-5 isolated from *C. imberbe*.

Compounds **1-5** were isolated from *C. imberbe*. Compound **1** was a known compound and elucidated as 1 α , 3 β -dihydroxy-12-oleanen-29-oic. Compound **2** very similar to compound **1** was elucidated as 3-hydroxyl-12-oleanene-29-oic. Compound **3**, isolated as a white powder had molecular formula similar to Compound **2** but appeared to have a different structure. It was elucidated as 3, 30-dihydroxyl-12-oleanen-22-one, see page 100. Compound **4** was isolated as white crystals and its structure elucidated as 1, 3, 24-trihydroxyl-12-olean-29-oic acid, see page 103.

Compound **5** and **6** were new compounds with optical activities of α_D^{26} : 36.6.Cc $\mu\text{g/ml}$ CH₃OH and α_D^{26} : 31.39 $^\circ$.Cc $\mu\text{g/ml}$. CH₃OH respectively. ¹H NMR, ¹³C NMR, ¹H COSY, HMBC, HMQC and NEOSY data were used to elucidate their structures as 1,3, 23-trihydroxy-12-oleanen-29-oic acid-3 β -O- α -L-2, 4 diacetylramnopyranoside and 1, 5-dihydroxyl-12-oleanen-29-oic acid 3 β -O- α -L-4-acetylramnopyranoside respectively. Compounds **6-8** (Fig. 6-23) were isolated from *C. padoides*. Compound **7** was found to be similar to Compound **6** but differ in the positions of their hydroxyl group. It was therefore elucidated as a 1, 22-dihydroxyl-12-oleanen-30-oic acid, see page 110.

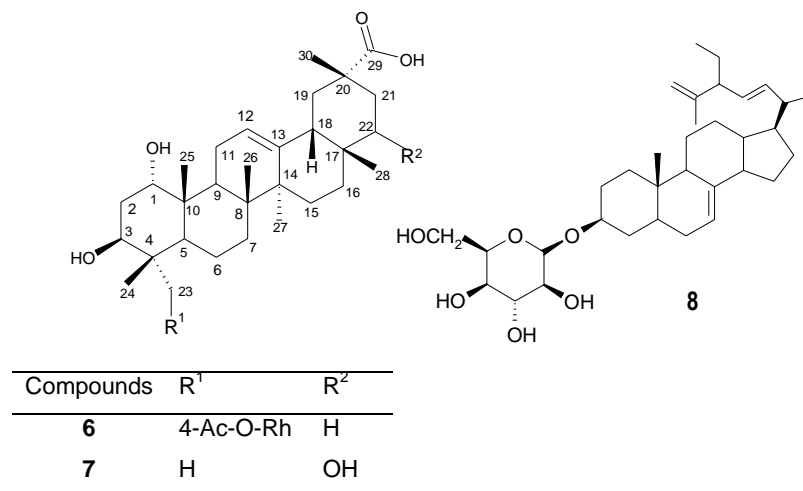


Figure 6-23: Compounds 6-8 isolated from *C. padoides*.

Compound **8** was a steroid glycoside and was much different in structure the other compounds isolated. Its structure was established as 24-ethylcholesta-7, 22, 25-trien-3-ol-O- β -D-glucopyranoside on the bases of ¹H NMR, ¹³C NMR, DEPT ESI-MS and literature comparison (Atta.Ur-Rahman *et al.*, 1997).

CHAPTER 7

BIOLOGICAL CHARACTERIZATION OF ISOLATED COMPOUNDS

7.1 Introduction

The biological activity of the isolated compounds were characterised for their individual and combined anti-microbial efficacy and individual anti-inflammatory action. In addition the antiproliferative and cytotoxic effects of the compounds were determined.

7.2 Material and methods

7.2.1 Anti-microbial activity

7.2.1.1 Antibacterial activity (Microplate dilution assay)

The MIC of the isolated compounds were determined using a serial microplate dilution assay (Eloff, 1998) as described in **Section 4.2.3**

7.2.1.2 Antibacterial and antifungal activity (Agar diffusion assay)

The agar diffusion method was used for the determination of antibacterial and antifungal activities of the micro-organisms listed in **Table 7-1**. Approximately 9 ml of Müller–Hinton agar for bacteria and Sabouraud Dextrose Agar for fungi (Oxoid, UK) were poured into Petri dishes (9 cm in diameter) and inoculated with the respective test organisms. Wells (4.0 mm) were punched out of the solid agar using pipette tips, and 1 ml of 50 µg/ml of the test compounds and control antibiotics were placed into each well. Petri dishes were incubated at 37 °C for 20 h and the average diameter of the inhibition zone surrounding the wells was measured.

Table 7-1: Bacteria and Fungi tested for efficacy in the Agar diffusion assay

Code	Bacteria	HKI Code
BNA	<i>Bacillus subtilis</i> ATTC 6633 (IMET) NA	B1
Bas	<i>Bacillus subtilis</i> ATTC 6633 (IMET) AS	B2
SA	<i>Staphylococcus aureus</i> (IMET 10760) SG 511	B3
EC	<i>Escherichia coli</i> SG 458	B4
PA	<i>Pseudomonas aeruginosa</i> K 799/61	B9
MS	<i>Mycobacterium smegmatis</i> SG 987 (HKI0056)	M2
MV	<i>Mycobacterium vaccae</i> IMET 10670	M4
Fungi		
SS	<i>Sporobolomyces salmonicolor</i> SBUG 549	H4
CA	<i>Candida albicans</i> BMSY 212	H8
PN	<i>Penicillium notatum</i>	P1

7.2.1.3 Antimicrobial effect of some combined compounds

After determining the MIC of isolated compounds, the compounds that were available in higher quantities were mixed in equal ratios and the MIC of the mixture determined to ascertain whether any synergistic activity of the combined compounds were present. From *C. imberbe*, Compound **1**, **4** and **5** were available in high enough quantity and 1 mg/ml of each compound combined as mixtures of **(1+4)**, **(1+5)** and **(1+4+5)**. Compounds **6**, **7** and **8** were used from *C. padoides* and combined as **(6+7)**, **(6+8)** and **(6+7+8)**. All combinations were in a 1:1 ratio. The MIC of all combined compounds was determined using 100 µl of each mixture as described in Section 4.2.3 and compared to the activity of the individual compounds. A decrease in the MIC of the most effective compound in the mixture was accepted as an indication synergistic activity.

7.2.2 Anti-inflammatory activity

This analysis was carried out by the Molecular Natural Product Research group of HKI, Jena, Germany.

The NAD (P)-linked enzyme, 3 α -hydroxysteroid dehydrogenase, has been purified to homogeneity from rat liver cytosol (Penning, 1983). This enzyme is known to reduce a variety of 3-ketosteroids, e.g., 5 α -dihydrotestosterone (5 α -androstane-17 β -one), 5 β -dihydrocortisone (5 β -pregnan-17 α , 21-diol-3, 11, 20-

trione), to the corresponding 3α -hydroxysteroids and, therefore, plays an important role in cortisone metabolism (Penning, 1983). A surprising property of the purified enzyme is that it is potently inhibited by the major classes of non-steroidal and steroidal anti-inflammatory drugs in rank order of their therapeutic potency (Penning, 1983). A high correlation exists between the logarithm of the concentration of drug required to produce 50% inhibition of the purified 3α -hydroxysteroid dehydrogenase ($\log IC_{50}$ value) with the dose required to produce an anti-inflammatory response in man. These observations led to the suggestion that the extent of inhibition of 3α -hydroxysteroid dehydrogenase could be used to predict anti-inflammatory drug potency (Penning, 1983).

7.2.2.1 Preparation of Cytosol

Adult male Sprague-Dawley rats (150-200 g) were killed by cervical dislocation. The livers were excised and homogenized in 3 volumes of 50 mM Tris-HCl of pH 8.6 containing 250 mM sucrose, 1 mM dithiothreitol, and 1 mM EDTA. Homogenates were centrifuged at 100,000xg for 30min; the supernatant (cytosol; i.e., source of 3α -hydroxysteroid dehydrogenase) was used for enzyme assays without further processing.

7.2.2.2 Preparation of Purified 3α -Hydroxysteroid Dehydrogenase

Homogeneous enzyme was prepared according to the method described by Penning, 1983. This enzyme had a final specific activity of 3.58 μmol of 5β -dihydrocortisone reduced/min/mg of protein.

7.2.2.3 Enzyme Assays

The reduction of 5β -dihydrocortisone was monitored by measuring the changes in the absorbance of the pyridine nucleotide at 340 nm. Each assay (1.0 ml) contained the following: 0.840 ml of H_2O , 0.100 ml of 1 M potassium phosphate buffer (pH 6.0), 20 μL of 9 M NADPH, 10 μL of 5 mM 5β -dihydrocortisone, and 30 μL of acetonitrile. The reactions were initiated by the addition of enzyme (30-50 μg of cytosolic protein or 0.6 μg of purified enzyme), and optical density change was followed over a period of 5 minutes. Control incubation experiments by addition of the cytosol in which either the 5β -dihydrocortisone or NADPH was absent, indicated that the presence of both substances was required before the cytosol would promote a change in absorbance at 340 nm.

7.2.2.4 Inhibition Studies

The % inhibition of seven isolated compounds was generated at three different concentrations (30 µg/ml, 3 µg/ml and 0.3 µg/ml). Increasing amounts of the isolated compound was added to the standard assay system, and the concentration of the compound required to reduce the rate of 5β -dihydrocortisone reductions by 50% (IC₅₀) was computed from the resulting In dose-response curves. **7.2.3**

7.2.3 Antiproliferative and cytotoxicity assay

This analysis was carried out by the Molecular Natural Product Research group of HKI, Jena, Germany.

Isolated compounds were assayed on cell lines K-562 (human chronic myeloid leukaemia) and L-929 (mouse fibroblast) for their antiproliferative effects (GI₅₀: concentration which inhibited cell growth by 50%), and against Hela for their cytotoxicity effects (CC₅₀: concentration at which numbers of cells are destroyed by 50%; used particularly in referring to the lysis of cells). The cells were incubated in 10 different concentrations of each of the target compounds. Fifteen cells of established suspended K-562 (DSM ACC 10) and Hela, and adherent L-929 (DSM ACC 2) cell lines were cultured in RPMI medium. The adherent cells of L-929 and Hela were harvested at the logarithmic growth phase after trypsinization, using 0.25 % trypsin in PBS containing 0.02% EDTA (Biochrom KG Kat.-Nr. L2163). The inherent cells of L-929 were harvested at the logarithmic growth phase after soft trypsinization, using 0.25% trypsin in PBS containing 0.02% EDTA (Biochrom KG L2163). For each experiment with L-929 and Hela approximately 10,000 cells were seeded with 0.1 ml RPMI 1640 (GIPCO BRL 21875-034), containing 25 µg/ml gentamicin sulfate (BioWhittaker 17- 528Z), but without HEPES, per well of the 96-well microplates (K-562: NUNC 163320, L-929, Hela: NUNC 167008). For the cytotoxic assay, the Hela cells were preinoculated for 48 hours without the test substances. The dilution of the compounds was carefully carried out on the monolayer of Hela cells after the pre-incubated time.

Cells of L-929, K-562 and Hela, with the presence the respective compounds, were incubated for 72 hours at 37°C in a humidified atmosphere and 5% CO₂. Suspension cultures of L-562 in microplates were analysed by an electronic cell analyser system CASY 1 (SCHARFE, Reutlingen, Germany) using an aperture of 150 µm. The 0.2 ml content of each well in the microplate was diluted 1:50 with CASYTON (SCHARFE). Every count/ml was automatically calculated from the arithmetic mean of three successive

counts of 0.4 ml each. From the dose response curves the GI₅₀ values were calculated with CASYSTAT. The GI₅₀ value was defined as the 50 % intersection line of the ln concentration-response curve, determined by the cell count/ml as compared to the control. The essential parameters for the estimation of growth inhibition and for change in diameter distribution curve were expressed as diagrams. The monolayer of the adherent L-929 and Hela cells were fixed by glutaraldehyde and stained with a solution of methylene blue. After gently washing, the stain was eluted by 0.2 ml of 0.33 N HCl in the wells. The optical densities were measured at 630 nm in a microplate reader.

7.3 Results and Discussion

7.3.1 Antibacterial activity (Microplate dilution assay)

The MIC result of the isolated compounds tested using the microplate dilution assay are presented in **Table 7-2**

Table 7-2: MIC of compounds isolated from *C. imberbe* and *C. padoides* [*S. aureus* (SA), *E. faecalis* (EF), and *E. coli* (EC), *P. aeruginosa* (PA)]

Compounds	MIC (µg/ml)			
	SA	EF	EC	PA
<i>C. imberbe</i>				
1	130	130	20	>300
2	90	20	>400	200
3	130	130	20	>300
4	60	> 300	20	>300
5	60	> 300	20	>300
<i>C. padoides</i>				
6	60	>300	60	>300
7	30	130	60	>300
8	>300	>300	60	>300
Gentamycin	5.8	5.8	5.8	5.8

All compounds, except for Compounds **2** and **8**, showed the highest antibacterial activity against *E. coli* and *S. aureus* and were all either poorly active or inactive against *P. aeruginosa*. Compound **2** was ineffective against *E. coli* and Compound **8** against *S. aureus*. Compound **2** was the only compound with high effectivity (20 µg/ml) against *E. faecalis*; all other compounds were either poorly active (>100 µg/ml) or inactive (>300 µg/ml). The high activity of Compounds **1**, **3**, **4** and **5** from *C. imberbe* against *E. coli* is in contrast to the observation that *E. coli* was resistant to other pentacyclic triterpenes isolated from the same plant species (Katerere *et al.*, 2002). Pentacyclic and tetracyclic triterpenes are known for their action as molluscides, particularly in their monodesmosidic form (Marston and Hostettmann, 1985). *C. molle* is known for its molluscidal constituent, mollic acid, which has been recommended for use in rural Africa to control schistosomiasis (Rogers, 1996). Arjunolic acid and arjungenin, arjunglucoside pentacyclic triterpenes have been isolated from *C. molle* (Panzini I., 1993).

Apart from this work, there are few data on the antimicrobial potential of the isoprenoid constituents of Combretaceae. Eloff (1998) reported preliminary data on crude extracts of *C. imberbe* and *C. padoides* against microbial culture. Martini and Eloff (1998) also showed that crude extracts of *C. erythrophyllum* are active against microbial cultures (*S. aureus* and *E. faecalis*) and indicated that their results potentially support the use of this plant in traditional medicine for relieving symptoms that appear to be caused by infective agents e.g. bloody diarrhoea, wounds and conjunctivitis (Gelfand *et al.*, 1985). The present results further confirm the activity of the constituents of *Combretum species* against bacteria and justify the potential use of *C. imberbe* in folk medicine, as well as expand our knowledge on the antibacterial activity of *C. imberbe* and *C. padoides*. Some of the compounds isolated are candidates for further work to evaluate their therapeutic potential.

7.3.2 Antimicrobial activity (Agar diffusion assay)

The agar diffusion method was used as an alternative method to evaluate the antibacterial effect of the isolated compounds as well as to test their antifungal activity. The results are presented in **Table 7-3**.

Table 7-3: Zone of inhibition of compounds isolated from *C. imberbe* and *C. padoides* against several bacterial and fungal organisms (*B. subtilis* ATTC 6633 (IMET) NA (B1), *B. subtilis* ATTC 6633 (IMET) NS (B2), *S. aureus* (IMET 10760) SG511 (B3), *E. coli* SG 458 (B4), *P. aeruginosa* K 799/61 (B9), *M. smegmatis* SG 987 (HKI0056) (M2), *M. vaccae* IMET 10670 (M4) H4 H8 & P1

Compounds	Zone of Inhibition (mm)									
	B1	B2	B3	B4	B9	M2	M4	H4	H8	P1
<i>C. imberbe</i>										
1	22		23/28p	0	14p		26	0	0	0
3	10	10	-	0	0	-	0	0	0	0
4	11	10	11	0	0	-	12p	0	0	0
5	21	21/26p	-	12p	17p	-	24	0	0	0
<i>C. padoides</i>										
6	14/17.5p	22/26.5p	-	0	12p	-	18	0	26p	0
7	11	10	11	0	0	-	12p	0	19p	0
8	0	0	'	0	0	-	0	0	0	0

p = partial inhibition (-) Test not done on these organisms because of low quantity of the compounds

Several compounds inhibited bacterial and fungal growth. The zones of inhibition for the active compounds were in the range 10-26 mm. Generally, in this assay all the isolated compounds had a better activity against bacteria than fungi. For the compounds isolated from *C. imberbe*, Compound 1 had a good inhibitory activity against *B. subtilis* (22 mm), *S. aureus* (23 mm and a partial inhibition of 26 mm), and *M. vaccae* (26 mm). Compound 5 also had a relatively good activity against *B. subtilis* (21 mm), *S. aureus* 21 mm and a partial inhibition of 26 mm and *M. vaccae* (24 mm). Earlier studies (Katerere *et al.*, 2002) have shown that pentacyclic triterpene from *C. imberbe* has activity against *M. fortuitum*.

Compounds isolated from *C. padoides* indicated a good activity as well. Compound 6 showed an inhibitory effect against *B. subtilis* (22 mm and a partial inhibition of 26.5 mm), *M. vaccae* (18 mm) and a partial inhibition of 26 mm against *C. albicans*.

It has been shown that extracts of some *C. species* (*C. glutinosum*, *C. hispidum*, *C. molle* and *C. nigricans*) have antifungal effect against dermatophytes as well *C. albicans* (Baba-Moussa *et al.*, 1999). This report proposed that tannins and saponins might be responsible for this activity that might explain the good activity of Compound 5 and 6 being isoprenoid glycosides.

All the organisms were resistant to Compound 8 that indicated that steroid glycosides might not have anti-microbial activity.

Comparison of the MIC and the zone inhibition results did not appear to indicate similarity in response against the same organisms. Despite high effectivity (low MIC) of Compounds 1, 3, 4, 6, 6 & 8 against *E. coli* on the microplate dilution assay, no zones of inhibition was observed in the Agar diffusion assay for all of these compounds, except Compound 5 that had a partial zone of inhibition. Compound 1 showed a wide zone of inhibition against *S. aureus* yet only had a moderate MIC, whereas Compounds 4 & 7, which had a lower MIC than Compound 1, had a smaller zone of inhibition. The difference could be in the rate of diffusion of the various compounds in agar medium. No activity was observed against *P aeruginosa* for all isolated compounds using the microplate dilution assay, whereas partial zones of inhibition were observed for Compounds 1, 5 and 6.

7.3.3 Combined antimicrobial effect of mixtures of isolated compounds

The synergistic and antagonistic effect of Compounds 1, 4 & 5 from *C. imberbe* and Compounds 6, 7 & 8 from *C. padoides* are shown in **Table 7-4**

The MIC values obtained with the individual compounds are consistent with the original microplate dilution assay using the isolated compounds. Synergistic activity were observed with compound mixtures 1+4+5 of *C. imberbe* against *S. aureus* and compound mixtures 6+7 and 6+8 of *C. padoides* against *S. aureus* and *E. coli*. The antibacterial effect of compound mixtures were regarded as synergistic if the MIC value achieved with the combination was less than lowest MIC value of any of the individual compounds. An antagonistic effect, where the MIC of the compound mixtures was larger more than two fold higher than the lowest MIC of the individual components, was noted with mixture 1+4 against all bacterial species; and mixtures 1+5 and 1+4+5 against *E. faecalis* and *E. coli*.

Table 7-4: MIC (µg/ml) values of individual and mixtures of isolated compounds

Compound	Minimum Inhibitory Concentration (µg/ml)		
	<i>S. aureus</i>	<i>E. faecalis</i>	<i>E. coli</i>
<i>C. imberbe</i>			
1	125	125	16
2	93	23	>250
3	125	125	16
4	63	>250	16
5	63	>250	16
1+4	>250	>250	31
1+5	63	>250	63
4+5	63	>250	63
1+4+5	31	>250	>250
<i>C. padoides</i>			
6	63	>250	63
7	31	125	63
8	>250	>250	63
6+7	16	125	31
6+8	31	>250	31
7+8	31	>250	63
6+7+8	31	>250	63

In contrast to single remedy product (Harris 2003) explains that combination preparation are difficult to evaluate scientifically. The synergistic effect of combinations of isolated compounds found in the current study is in contrast to the views by aromatherapists who maintain that isolated compounds do not exhibit synergy (Harris, 2003). Harris (2003) explained that this argument is not borne out by research, that it is possible to achieve synergism through mixing isolates; natural or synthetic. One such study (Didry et al., 1994) tested the effects of combination of components such as thymol, eugenol, carvacrol and cinnamaldehyde on oral bacteria. Synergistic effects were found between certain blends such as thymol and caevacrol, eugenol and thymol, eugenol and carvacrol. Delaquis (2002) also found that when the principal isolates of *Anethum graveolens* (limonene and carvone) were blended together and then enriched with either terpene, their antimicrobial effects were significantly more active than whole oil. It was concluded that

using the isolates as opposed to the whole oil would allow for preparations of constant chemical composition and confirmed synergy.

Antagonism between isolated components has previously been reported by (Fanaki, 1997) when testing a frequently used antimicrobial preparation of essential oil components (containing anethole, borneol, camphene, 1,8-cineole, fenchone and pinenes) against multi-resistant bacterial strains. The finding demonstrated that the combination was less effective than the combined activity of the isolates when tested individually.

It is important to understand that isolates when combined may produce synergistic effect or antagonistic effect depending on the ratio of their combination. Studies by Low *et al.*, 1974 on the interactions between citronellal and citronellol showed that as long as the naturally occurring ratio of these two substances was respected, a similar synergy will be obtained if the components were synthetically produced. Other factors that might not have been scientifically proven might also be involved.

7.3.4 Anti-inflammatory activity

The percentage inhibition of purified NAD (P)-linked 3 α -hydroxysteroid dehydrogenase of seven isolated compounds at three different concentrations (30 μ g/ml, 3 μ g/ml and 0.3 μ g/ml), IC₅₀ and HKI class are given in **Table 7-5**.

Table 7-5: Anti-inflammatory activity of compounds isolated from *C. imberbe* and *C. padoides*.

Compounds	% Inhibition 3 α -hydroxysteroid dehydrogenase			IC ₅₀	HKI Class
	30 μ g/ml	3 μ g/ml	0.3 μ g/ml		
<i>C. imberbe</i>					
1	88	84	0	0.3	3
3	85	36	0	0.3	3
4	59	10	0	7.8	1
5	63	17	13	9.5	1
<i>C. padoides</i>					
6	41	0	0	13	0
7	25	0	0	13.5	0
8	94	72	0	5.1	1

Not active (0), active (1), more active (2), highly active (3)

Inhibition of NAD (P) linked 3 α -hydroxysteroid dehydrogenase of rat liver cytosol is correlated with anti-inflammatory activity in man (Penning, 1983). Compounds **1** and **3** isolated from *C. imberbe* had an anti-inflammatory effect with IC₅₀ of 0.30 μ g/ml each. These compounds fall in Class 3 of the Hans-Knöll institut (HKI) standards, indicating very good anti-inflammatory activity. Compounds **4** and **5** from *C. imberbe* and Compound **8** from *C. padoides* had moderate anti-inflammatory effect and are considered as Class 1 anti-inflammatory compounds.

7.3.5 Anti-proliferative effect and Cytotoxicity

The antiproliferative and cytotoxic efficacy of compounds **1-8** was tested *in vitro* against (L-929, k-562) and (Hela) cell lines, respectively. The antiproliferative and cytotoxic data are shown in **Table 7-6**.

Table 7-6: Anti-proliferative and cytotoxic effect of compounds isolated from *C. imberbe* and *C. padoides*.

Compounds	Antiproliferative effect (μ g/ml)		Cytotoxic effect (μ g/ml)
	(L-929) GI ₅₀	(K-562)GI ₅₀	(Hela) CC ₅₀
<i>C. imberbe</i>			
1	32,9	28,1	34,9
2	-	-	-
3	9	8,7	10,5
4	> 50	> 50	> 50
5	16,5	13,5	17,5
<i>C. padoides</i>			
	L-929) GI ₅₀	(K-562)GI ₅₀	Hela) CC ₅₀
6	> 50	> 50	> 50
7	> 50	> 50	44,7
8	> 50	> 50	> 50

Antiproliferative activity not done with this compound because low quantity.

Most of the compounds particularly those isolated from *C. padoides* had slight anti-proliferative and cytotoxic effect. In general the compounds had a greater anti-proliferative than cytotoxic effect. Compounds **3** and **5** showed a strong antiproliferative activity against both L-929 and K-562 cell lines Compound **3** showed a moderate cytotoxic effect of CC₅₀ =10.5 μ g/ml against Hela cells.

There have been a very few reports on the anti-proliferative and cytotoxic effect of the constituents of Combretaceae. This study indicates that some members of the Combretaceae may have antiproliferative and cytotoxic constituents. This aspect may justify further research.

7.3.5 Structure activity relationship of isolated compounds

The level of antibacterial activity of the various isolated compounds was compared with the structure of each compound to determine the structural: activity relationship of each compound. Generally, the aglycones had a better antibacterial activity against *S. aureus* and *E. coli* than the glycosides (**Table 7-2**). It is also proposed that the antibacterial activity of the aglycones increase with respect to the numbers of OH groups present. This may explain why Compound **4** (0.060 mg/ml against *S. aureus*) with two OH groups has a lower MIC than Compound **3** with only one OH group.

Compounds **5** and **6** are glycosides with MIC of 0.016 mg/ml against *E. coli*, which is the same as the aglycone of these compounds hence suggesting that the sugar unit may not play any role in the activity of the compounds against *E. coli*. A very poor activity against all the organisms was observed with Compound **8** that is a glycoside with an aglycone that does not have any OH group. This further supports the observation that the presence of OH influences antibacterial effect. This is in contrast to earlier study which shows that the hydroxyl group position in the triterpene skeleton has an influence in the activity of triterpene and that the increase in the number of hydroxyl group decreases the antibacterial activity of a triterpene (Djoukeng *et al.*, 2005).

Structure activity relationships as observed in the current study may help natural product chemists to arrive at new biologically active derivatives through synthesis of compounds that might have pharmaceutical properties.

7.4 Summary

Most of the compounds isolated had a broad-spectrum antibacterial activity against Gram-positive and Gram-negative pathogens with an MIC range of 20 to 250 µg/ml for Gram-positive bacteria and 20 to 250 µg/ml for Gram-negative bacteria. In the agar well diffusion assay, Compound **1** had the highest activity against *M. viccae* IMET 10670 with inhibition zone of 26 mm. Compound **5** also had a good activity against *M. vaccae* with zone of inhibition of 22 mm. Compound **6** exhibited fungicidal activity (18 mm) against *S.*

salmonicolor and a partial activity of 26 mm against *C. albicans*. This confirms that components of Combretaceae have both antifungal and antibacterial activity.

There was synergistic effect as well as antagonistic effect amongst some mixtures of the isolated compounds. Compounds isolated from *C. padoides* exhibited significant synergistic effect while antagonism was observed mostly among compounds from *C. imberbe*. This study indicates that there may be synergistic as well as antagonistic effects in naturally isolated compounds. Because there was insufficient material to evaluate the statistical significance of the results, the conclusion should be considered tentative.

Compounds **1** and **3** exhibited very good anti-inflammatory effect against 3 α -hydroxysteroid dehydrogenase enzyme with IC₅₀ of 0.30 μ g/ml. Compounds **4**, **5** and **8** indicated a mild inhibitory effect.

Compound **3** had a moderate cytotoxic effect of CC₅₀ = 10, 5 μ g/ml against Hela and the rest of the compounds **1**, **2**, **4**, **5**, **6**, and **7** were generally non-toxic. Compound **3** showed a strong antiproliferative activity against both L-929 and K-562 cells with GI₅₀ of 9 μ g/ml, and 8.5 μ g/ml, and moderate antiproliferative activities of 16.5 μ g/ml, 13, 5 μ g/ml for compound **5** against both cell lines respectively.

CHAPTER 8

GENERAL CONCLUSION

8.1 Introduction

The increasing use of antibiotics and misuse by over prescribing and or poor patient compliance has led to the development of bacteria resistant to antibiotics. Medicinal plant research offers a good chance of discovering new prototype drugs (Malone, 1983). According to ethnobotanical literature the genus *Combretum* is used widely for a variety of conditions in African traditional medicine. Members of this genus have the following biological activities: antibacterial, antifungal, anti-inflammatory, diuretic, and molluscidal (Hutching *et al.* 1996). Some members of Combretaceae have been found to have compounds with antibacterial activity. Eloff (1999a) has previously reported that *Combretum imberbe* and *Combretum padoides* extracts had antibacterial activity. Therefore, the aim of this work was to isolate, chemically and biologically characterize antibacterial compounds present in *Combretum* section Hypocrateropsis in a bioassay guided process, with the objectives of:

- ✓ Selecting the most active plant specie(s)
- ✓ Selecting and evaluating the best fractionation procedure for isolation
- ✓ Isolating and determining the chemical structure of antibacterial compounds
- ✓ Determining the biological activities of isolated compounds
- ✓ Determining the effect of synergism on combinations of isolated compounds
- ✓ Evaluate how well phytochemistry agrees with taxonomy based on morphology.

The degree to which the above aim and objectives have been met is briefly outlined in the following sections:

8.2 Evaluation on the best preliminary fractionation procedure

In an attempt to isolate and characterize the antibacterial compounds in *C. imberbe* and *C. padoides*, two fractionation procedures were evaluated as preliminary processes. Initially, dried ground plant materials were extracted directly with acetone for screening purpose and bulk material was extracted serially and exhaustively with solvents of increasing polarity (hexane, DCM, acetone, methanol) for isolation purposes. DCM was the best extractant of antibacterial compounds according to bioautography and MIC analysis. The DCM extract was therefore used for the isolation of antibacterial compounds through a bioassay-guided

approach. The solvent-solvent fractionation method and vacuum liquid chromatography were used as preliminary fractionation procedures in order to evaluate the best approach toward quick and easy isolation of antibacterial compounds. The solvent-solvent fractionation was a better preliminary process because more antibacterial compounds were isolated through this approach compared to the vacuum liquid chromatography approach.

8.3 Isolation and chemical characterization of antibacterial compounds

Screening of the 4 plant species of the Hypocrateropsis section was done and species for further work were selected on the basis of number of antibacterial compounds as well the MIC of extracts. *Combretum imberbe* (8 antibacterial compounds, MIC 0.60 mg/ml against *S. aureus*) and *C. padoides* (7 antibacterial compounds, MIC 0.60 mg/ml against *S. aureus*) were selected for further work. Both screening and isolation processes were monitored by the TLC analysis using three different solvent systems developed in our laboratory. BEA separated non-polar compounds, CEF separated compounds of intermediate polarity and EMW separated polar compounds. EMW and CEF were the best mobile phases for separation of the active compounds. BEA was not a good system for TLC separation because most of the active compounds were of intermediate polarity. Vanillin sulphuric acid and *p*-anisaldehyde-sulphuric acid detection reagents were initially used. However, more compounds were visible with vanillin sulphuric acid than with the anisaldehyde spray reagent. Vanillin sulphuric acid spray reagent was therefore routinely used; it appears to be a good spray reagent for the detection of triterpenes. Vanillin-sulphuric acid spray reagent did not reveal many of the compounds that were seen under UV light.

Column chromatography using Silica gel 60 and Sephadex LH 20 was the main approach used for isolating compounds. Eight antibacterial compounds were isolated through the above process. NMR, MS and IR analysis were used to determine the chemical structures of the compounds. Five compounds were isolated from *C. imberbe* and elucidated as: 1,3-dihydroxyl-12-oleanen-29-oic acid (1), 3-hydroxyl-12-oleanen-29-oic acid (2), 3,30-dihydroxyl-12-oleanen-22-one (3), 1,3,22-trihydroxyl-12-oleanen-29-oic acid (4) and 1 α , 23 β -dihydroxyl-12-oleanen-29-oic acid-23 β -O- α -2,4-diacetylrhamnopyranoside (5). All of them were triterpenes with the olean-12-ene skeleton. Compound 5 was a triterpene glycoside, which has not been reported before. Three compounds were isolated from *C. padoides* and also elucidated as: {1 α , 23 β -dihydroxyl-12-oleanen-29-oic acid-3 β -O- α -4-acetylrhamnopyranoside (6), 1,22-dihydroxyl-12-oleanen-29-oic acid (7), and 24-ethylcholesta-7,22,25-trien-3-ol-O- β -D-glucopyranoside (8)}. Two 1 α , 23 β -dihydroxyl-12-oleanen-29-oic acid-3 β -O- α -4-acetylrhamnopyranoside (6), 1,22-dihydroxyl-12-oleanen-29-oic acid (7) had the olean-12-ene skeleton and the other 24-ethylcholesta-7,22,25-trien-3-ol-O- β -D-glucopyranoside (8)

was a steroid glycoside. Compound **6** from *C. padoides* was a new triterpene glycoside. Generally, the results of the study expand our knowledge on the phytochemistry of Combretaceae. It is evident that the above new structures will join the global natural product database and may serve as models for the synthesis of new antibacterial compounds.

8.4 Biological characterization of plant species and isolated compounds

In vitro anti-infective, anti-inflammatory, anti-proliferative and cytotoxic activities were tested for most of the compounds depending on the quantity of material that was available. Some of the compounds had a broad-spectrum antibacterial activity against both Gram-positive (*S. aureus* and *E. faecalis*) and Gram-negative organisms (*E. coli* and *P. aeruginosa*). The MIC of active compounds against the Gram-positive organisms ranged from 0.030 to > 0.25 mg/ml and 0.016 to > 0.25 mg/ml for the Gram-negative strains. The agar well diffusion method was carried out to determine the sensitivity of more bacteria and fungi strains against the compounds. Most of the compounds showed zones of inhibition ranging from 0 to 26 mm with Compound **1** showing the highest activity (26 mm) against *Mycobacterium vaccae*. Compound **6** was active against two fungi; *Sporobolomyces salmonicolor* (18 mm) and *Candida albicans* (26 mm). Limited data has been reported on the anti-microbial activity of components isolated from Combretaceae. This study confirms the first report of Katerere *et al.*, (2002) on the antibacterial activity of triterpenes isolated from African Combretaceae and also gives credence to the indigenous use of *C. imberbe* in tradition medicine against bacteria related diseases.

The anti-inflammatory activity of the compounds were tested against 3 α -hydroxysteroid dehydrogenase enzyme (Penning, 1983) and compounds **1** and **3** had good anti-inflammatory activity with IC₅₀ value of 0.30 μ g/ml each hence confirmed the anti-inflammatory activity of some *Combretum* species (Hutchings *et al.*, 1996).

The antiproliferative and cytotoxic efficacy of compounds **1-8** were tested *in vitro* against (L-929, k-562) and (Hela) cell lines respectively. Compounds **3** and **5** showed a strong antiproliferative activity against both L-929 and K-562 cell with GI₅₀ of 9 μ g/ml, 8, 5 μ g/ml for compound **3** and moderate antiproliferative activities of 16.5 μ g/ml, 13, 5 μ g/ml for compound **5**. Compound **3** showed a moderate cytotoxic effect of CC₅₀ = 10, 5 μ g/ml against Hela

Based on the MIC values of the new compounds (**5** and **6**) and compound **1** against *S. aureus* and *E. coli* further investigation into the biological activity of these compounds or derivatives against more resistant

strains may be fruitful. Generally, *C. imberbe* and *C. padoides* leaves contain many antibacterial compounds of which only few were isolated and characterized. Because this plant contains many compounds, further work may be carried out on isolation and characterization of other antibacterial compounds present in this species. These results validate the ethnobotanical use of many *C.* species for bacteria infections. In future work more antibacterial compounds that are present in low concentrations could be isolated by starting with large quantity of plant material.

8.5 Evaluation of how well phytochemistry agrees with taxonomy based on morphology

All compounds isolated from the two plant species are pentacyclic triterpenes. Classification based on morphology placed *C. imberbe* and *C. padoides* in the section Hypocrateropsis. TLC (presence of terpenes by vanillin sulphuric acid spray reagent) analysis has shown that members of the Hypocrateropsis section have similar chemical profile. The isolation result substantiates a biogenetic link between members of the Hypocrateropsis. The seven antibacterial flavanoids isolated from *C. erythrophyllum* a member of the section Angustimarginata (Martini *et al.*, 2004b). The main antibacterial compound isolated from *C. woodii* also a member of the Angustimarginata was a bibenzyl.

The chemical composition of antibacterial compounds appears to support the current taxonomical classification of the sections in the *Combretum* genus. Related studies have shown the presence of triterpenes in related genera of Combretaceae. The result of this work might indicate a chemotaxonomical correlation between genera of Combretaceae.

CHAPTER 9

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