Isolation, chemical characterization and clinical application of an antibacterial compound from *Terminalia sericea*

By

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Preface

I hereby confirm that this is my own work and that it has not previously been submitted to any other institution.

Johann Kruger

Date: 23 August 2004

Acknowledgements

I would like to thank my Heavenly Father for the opportunity, blessing and grace to complete this, the most important task I have ever set out to achieve. I would like to thank my promoter Professor Kobus Eloff for his continued support, endless patience and vast source of knowledge in attempting to make a scientist of me. My thanks also go out to Dr David Katerere for being supportive and for assisting me with his expertise. Thank you very much to Dr Lyndie McGaw and Mrs Lita Pauw for their input and the rest of the laboratory personnel that helped to make this work possible.

Conference Presentations

Indigenous Plant Use Forum – Nelspruit - 28 July 2000

Kruger, J.P., Eloff, J.N Isolation and partial characterization of antimicrobial components of representative *Terminalia* species

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SA Association of Botanists – RAU – Johannesburg - 16 January 2001

Kruger, J.P., Eloff, J.N. Antibacterial effects of *Terminalia* species extracts

Indigenous Plant Use Forum – Knysna - 27 June 2002

Kruger, J.P., Eloff, J.N. Animal model for testing *Terminalia sericea* extracts

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International Society for Ethnopharmacology – Pretoria – 12 January 2003

JP Kruger, JN Eloff and DRP Katerere. Antibacterial compounds present in *Terminalia sericea* leaves heal bacterial infections on rats. South African Journal of Botany vol 69: pp.235.

Manuscripts prepared for publication

i) Selective extraction of antibacterial compounds from three *Terminalia* (Combretaceae) species – to be submitted to Pharmaceutical Biology

ii) An evaluation of the antibacterial activity of seven different *Terminalia* species – to be submitted to Journal of Ethnopharmacology

iii) Crude extracts and isolated antibacterial compounds from *Terminalia sericea* leaves heal *Staphylococcus aureus* infections in rats – to be submitted to Phytomedicine.

Opsomming

Die proses om antibakteriese aktiwiteit in *Terminalia sericea* te bepaal het begin met n deeglike literatuurstudie oor die gebruik van plante vir medisinale doeleindes op 'n historiese grondslag sowel as 'n studie oor die huidige gebruik van verwante plante vir die doel. Die volgende stap was die identifisering van n ekstraheërmiddel vir die gedroogde blare. Drie plante uit drie verskillende groepe *Terminalia*'s nl *T.sericea*, *T. prunoides* en *T. phaneroplebia* is gekies vir die doel en twee Gram-positiewe organismes, *Staphylococcus aureus* en *Pseudomonas aeuroginosa* en twee Gram-negatiewe organismes, *Escherichia coli* en *Enterococcus faecalis* is gekies om die antibakteriele aktiwiteit te bepaal, gebaseer op soortgelyke studies wêreldwyd. Asetoon is uiteindelik as die beste ekstraksiemiddel gekies, gebaseer op die konstante goeie vertoning daarvan ten opsigte van; die massa geëkstraheer, die lae mimimum inhiberende konsentrasie, die hoë totale aktiwiteit en die lae toksisiteit teenoor die toetsorganismes sowel as die maklike verwydering daarvan na ekstraksie.

Die tweede fase was om sewe *Terminalia* spesies nl. *T. sericea, T. prunoides, T. phanerophlebia, T. gazenzis, T. sambesiaca, T. mollis* and *T. brachystema* te ondersoek. Die plante is almal geevalueer ten opsigte van die massa geëkstraheer, MIK teenoor die vier toetsorganismes, die totale aktiwiteit en die bio-outogramme ten einde n seleksie te maak van die plant wat finaal gebruik sou word.

Terminalia sericea is gekies onder andere as gevolg van die wye beskikbaarheid in die omgewing en die wye verspreiding daarvan onder die plaaslike bevolking wat dit moontlik medisinaal sou kon gebruik. Die plantmateriaal is daaropvolgens aan groepskeiding onderwerp met vloeistof-vloeistof skeiding as 'n eerste stap waar daar

vasgestel is dat die chloroform fraksie oor die hoogste antibakteriese aktiwiteit beskik. Die proses is egter nie verder gevoer nie, omdat dit nie kostedoeltreffend ten opsigte van materiaal en tyd was nie. Instede daarvan is vakuum-vloeistof kolom chromatografie gebruik vir die voorlopige skeiding. Verdere silika gel kolomchromatografie is gebruik om na 'n suiwer komponent wat oor antibakteriele aktiwiteit beskik te soek. So 'n komponent is uiteindelik geisoleer en na KMR en massaspektroskopiese analise as terminoiese suur geidentifiseer. Hierdie verbinding is vroëer uit wortels van *Terminalia* geisoleer, maar die antibakteriese aktiwiteit met 'n MIK van 0.33 mg/ml teenoor die toetsorganismes was nie vroëer bekend nie.

Die laaste fase in die ondersoek is gedoen deur 'n proefdiermodel te ontwikkel en *in vivo* ondersoeke na terminoiese suur sowel as 'n asetoon blaarekstrak te doen. Die evaluasie was gebaseer op die effek wat terminoiese suur en die kru bestandeel op infeksies deur *Staphylococcus aureus* in wondletsels wat op proefrotte aangebring is, te evalueer. Eritreem, eksudaat wat die wond uitskei sowel as die verkleining in wondgrootte is daagliks gemeet teenoor 'n positiewe kontrole (gentamisien) en 'n negatiewe kontrole (die onbehandelde wond). Dit is bewys dat daar 'n beduidende *in vivo* antibakteriele aktiwiteit bestaan vir beide die terminoiese suur wat vir die eerste keer uit *T. sericea* geisoleer is en die asetoon ekstrak teenoor 'n algemene organisme wat topikale wonde veroorsaak nl *S. aureus* by wyse van *in vitro* en *in vivo* studies. Dit ondersteun die etnobotaniese gebruik van die plant vir die behandeling van topikale infeksies. Dit laat die moontlikheid daar om 'n volhoubare bron van 'n antibakteriese middel daar te stel vir die gebruik deur inheemse mense om oppervlakkige nie-gekompliseerde infeksies te behandel.

Summary

The process of determining the antibacterial activity of *Terminalia sericea* started with a literature review on the use of plants for medicinal purposes on a historical as well as current use. The next step was to identify the best extractant for the extraction of antibacterial compounds from the dried leaves. Three plants from three different sections of *Terminalia* were selected namely *T. sericea*, *T. prunoides* and *T. phanerophlebia* to determine their antibacterial activity against the four most important nosocomial pathogens that are used worldwide namely two Gram positive, *Staphylococcus aureus* and *Pseudomonas aeruginosa* as well as the two Gram negative, *Escherichia coli* and *Enterococcus faecalis*. Acetone was eventually chosen as best extractant based on its ability to extract relatively high masses, as well as the relatively low minimum inhibitory concentration, its high total activity and its low toxicity against the test organisms as well as the relative ease with which it can be removed after the extraction process.

The next phase was to determine which *Terminalia* species was the best to use for isolating antibacterial compounds. Seven different *Terminalia* species occurring in southern Africa which were representative of each of the three sections of the *Terminalia* genus were selected. These were *T. sericea, T. prunoides, T. phanerophlebia, T. gazenzis, T. sambesiaca, T. mollis* and *T. brachystemma*. The plants were all evaluated for the mass extracted from the dried plant material for the MIC of the acetone extract against the four test organisms, and their total antibacterial activity in order to select a single plant that could be investigated further. *Terminalia sericea* was selected in the end because of its relative high antibacterial activity as well as the fact that it is widely

distributed and could potentially be of practical use for the indigenous people as an antibacterial agent. The plant material (leaves) were dried and ground to a fine powder and then put through a group separation process first with liquid-liquid separation which indicated that the chloroform fraction contained the most antibacterial compounds. This process was abandoned because it proved to be a labour intensive process for scaling up. Vacuum-liquid chromatography was applied to the acetone extracts. Three promising fractions were fractionated by silica gel column chromatography. The search was for a pure compound that had high antibacterial activity. Such a compound was found in fraction JK3-2 and after NMR and mass spectroscopy identified as terminoic acid with a MIC of 0.33 mg/ml.

The last phase of the study was to develop and animal test model for the *in vivo* evaluation of the compound. A 20% emulsified cream base was prepared of the acetone crude extract as well as a 1% cream base of the terminoic acid. The test animal's backs were shaven and then four test sites were scarified after which the wounds were infected with a known pathogen (Cowan A strain of *Staphylococcus aureus*). The sites were left to incubate for 8 hours after which a daily treatment were applied of each of the positive control (gentamycin cream), the crude extract as well as terminoic acid which were formulated into a cream and lastly the negative control which received no treatment at all. The parameters which were evaluated were 1) the wound size 2) the exudate that was formed and 3) the erythema that was present. The last two parameters were measured on an arbitrary scale of 1-5 with one being the lowest level and five being the highest level of erythema/exudate visible on the wound.

The results proved that terminoic acid as well as the crude extract had a significantly higher antibacterial effect compared to the commercial gentamycin cream at the concentrations used against *S. aureus* on the three parameters evaluated. Results support the ethnobotanical use of *Terminalia sericea* for the topical treatment of wounds. The results may lead to the practical, sustainable use of the leaf-extract of a topical antibiotic in indigenous areas.

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Glossary of abbreviations

ATCC	= American Type Culture Collection
BEA	= benzene: ethanol: ammonia (36:5.4:4)
CEF	= chloroform: ethyl acetate: formic acid (5:4:1)
E. coli	= *Escherichia coli* (ATCC 27853)
E. faecalis	= *Enterococcus faecalis* (ATCC 29212)
EMW	= ethylacetate: methanol: water (40:5:4.4)
^{1}H	= proton
INT	= *p*-iodonitrotetrazolium violet
MIC	= Minimum Inhibitory Concentration
NMR	= Nuclear Magnetic Resonance (spectroscopy)
P. aeruginosa	= *Pseudomonas aeruginosa* (ATCC 25922)
S. aureus	= *Staphylococcus aureus* (ATCC 29213)
TA	= Total Activity
TLC	= Thin Layer Chromatography
UV	= Ultraviolet light
Vanillin SR	= vanillin spray reagent

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Chapter 1 Introduction and aim of study

Chapter 1

Introduction and aim of study

1.1 Antibiotics and antibacterial resistance

The discovery of antibiotics must certainly be one of the most important events in the history of medicine. Although the discovery of antibiotics only occurred within the lifespan of modern man, there is anthropological evidence that Nubian man more than a thousand years ago used tetracycline. The survival of the Nubian nation up to the 14th century is partially ascribed to the use of antibiotics such as tetracycline (Levy, 1984).

The development of anti-infective therapy occurred in three phases. The first was from the early 1600's to the 1900's and involved the use of *Cinchona* bark for the treatment of malaria. Quinine was isolated as the active ingredient in the 1820's. The 1900's marked the development of the synthetic era and saw the advent of dyes to treat bacterial infections, the first being pyocyanase, a blue pigment produced by *Pseudomonas aeruginosa* that reduced the growth of other bacteria *in vitro*. However, when used *in vivo*, it proved to be toxic and unstable (Edwards, 1980).

The most important event in the history of antibiotics was the discovery of penicillin by Alexander Fleming in 1929. The real potential for its use was only recognized with the

advent of the Second World War, which speeded up the commercial use of the product in the treatment of septic wounds. The subsequent wide distribution of the drug in over-the-counter preparations and even in cosmetic use led to the rapid development of widespread bacterial resistance (Levy, 1984).

Finding healing power in plants is an ancient idea. There is evidence that Neanderthal man 60,000 years ago used plants such as hollyhock for medicinal purposes. Since the advent of antibiotics in the 1950's however, the use of plant materials as antimicrobials has been virtually non-existent in First World countries (Cowan, 1999).

It has become apparent in recent times that increasing numbers of pathogenic bacteria are becoming resistant to currently available antibiotics (Berkowitz, 1995). This resistance results from the rate at which bacteria multiply and the ease with which they can change their genetic material or acquire new genes. Resistance to penicillin in some strains of staphylococci was recognised almost immediately after the discovery of the drug by Alexander Fleming (Anon, 1939). Today, resistance occurs in as many as 80% of all strains of *Staphylococcus aureus*.

A difference of opinion exists over the actual degree of antibiotic resistance. This can be ascribed to a general lack of national or international monitoring systems for antibiotic susceptibility testing conforming to a single set of standard operating procedures with appropriate quality controls. Confusion also exists around the terminology amongst those who report the results of the susceptibility testing (Walker and Thornsberry, 1998).

The basis of resistance can be classified as follows:

- Inherent (natural) resistance: bacteria may be inherently resistant to antibiotics. For instance, a Streptomycete may have a gene responsible for resistance to an antibiotic. In a further example, Gram-negative bacteria have an outer membrane structure that establishes a permeability barrier against certain antibiotics.
- Acquired resistance: bacteria can develop resistance to antibiotics by changing the bacterial genome. Acquired resistance is driven by two genetic processes in bacteria, mutation and selection, sometimes referred to as

  i) Vertical evolution, which is strictly a matter of Darwinian evolution driven by principles of natural selection: a spontaneous mutation in the bacterial chromosome imparts resistance to a member of the bacterial population.

  ii) Horizontal evolution, which is the acquisition of genes for resistance from another organism. Some bacteria develop genetic resistance through a process of mutation and natural selection and then donate these genes to some other bacterium through one of several processes for genetic exchange that exist in bacteria.

Bacteria are able to exchange genes in nature by three processes:

- Conjugation - involves cell-to-cell contact as DNA crosses a sex pilus from donor to recipient.
- Transduction - a virus transfers the genes between mating bacteria.
- Transformation - DNA is acquired directly from the environment, having been released from another cell.

Genetic recombination can follow the transfer of DNA from one cell to another, leading to emergence of a new genotype (recombinant). It is common for the DNA to be transferred as plasmids between mating bacteria. Since bacteria usually carry their genes for drug resistance on plasmids (called resistance transfer factors or RTF's), they are able to spread drug resistance to other strains and species during genetic exchange processes.

The combined effects of fast growth, high concentration of cells, genetic processes of mutation and selection and the ability to exchange genes, account for the extraordinary rates of adaptation and evolution that can be observed in bacteria. For these reasons, bacterial adaptation (resistance) to the antibiotic environment occurs very rapidly in evolutionary time; bacteria evolve fast!

1.2 Background to Phytomedicine

1.2.1 Ethnopharmacological overview

1.2.1.1 Introduction

With the present renewed interest in natural medicine, pharmaceutical companies are increasing their commitment in this field. In the USA and Europe, in particular, widespread screening and analysis of plants for biological activity is carried out with plants mainly sourced from Africa (Alexander *et al.,* 1992). Plants are used for many different ailments as described by Leder (1997)(Table 1.1)

Table 1.1. List of ailments that can be treated with some medicinal plants and the plant parts used (Leder, 1997)

Ailment	Medicinal Plant	Plant part used
Backache	*Pleiotaxis antunesii*	leaves
Birth, heavy bleeding while giving	*Peltophorum africanum*	roots
Birth, induce	*Dichapetalum rhodesicum*	roots
Bruise	*Psydrax livida*	roots
	Ricinus communis	
Burns	*Ochna pulchra*	bark
Chest pain	*Ancylanthos bainesii*	roots
Colds	*Becium filamentosum*	whole herb
	Hibiscus sabdariffa	calyx
	Tephrosia cephalanta var decumbens	roots
Contraceptive	*Dichapetalum rhodesicum*	roots
	Grewia falcistipula	roots
	Melhania burchellii	roots
Cough	*Ancylanthos bainesii*	roots
	Combretum zeyheri	leaves
	Guibourtia coleosperma	young leaves
	Peltophorum africanum	roots
	Pterocarpus angolensis	red plant sap
	Rhus tenuinervis	roots
	Strychnos pungens	leaves
	Tephrosia lupinifolia	roots
	Tephrosia cf. Oxygona	roots
	Tephrosia cf. Purpurea	roots
	Terminalia sericea	leaves
	Ziziphus mucronata subsp mucronata	roots
Diarrhoea	*Combretum platypetalum subsp. baumii*	roots
	Dialium englerianum	inner bark
	Dichapetalum cymosum	roots
Ailment	**Medicinal Plant**	**Plant part used**

	Ozoroa insignis subsp. Latifolia	roots
	Schinziophyton rautanenii	bark
	Terminalia sericea	roots, leaves
	Ziziphus mucronata subsp mucronata	roots
Eyes, sore	*Grewia avellana*	roots
Ears, suppurating	*Sansevieria pearsonii*	sap, rhizome
Fever	*Becium filamentosum*	whole herb
	Psydrax livida	roots
	Tephrosia lupinifolia	roots
Headache	*Ancylanthos bainesii*	roots
Infertility	*Clerodendron dekindtii*	roots
	Combretum albopunctatum	roots
Liver problems	*Dichapetalum cymosum*	roots
	Peltophorum africanum	bark
Malaria	*Combretum psidioides*	roots
Mental disorientation	*Otoptera burchellii*	roots
Sickness during Pregnancy	*Peltophorum africanum*	roots
	Schinziophyton rautanenii	bark
Sinusitis	*Otoptera burchellii*	roots
Skin inflammations	*Lonchocarpus nelsii subsp. Nelsii*	frass of larvae
Spleen complaints	*Solanum delagoense*	roots, sap of fruits
Stomach pain	*Burkea africana*	roots
	Combretum zeyheri	roots
	Grewia avellana	roots
	Schinziophyton rautanenii	bark
	Strychnos cocculoides	bark
	Terminalia sericea	roots
Stomach ulcers	*Ziziphus mucronata subsp. Mucronata*	roots
Tuberculosis	*Acacia erioloba*	roots
	Peltophorum africanum	roots
	Ziziphus mucronata subsp. Mucronata	roots
Tooth ache	*Acacia erioloba*	roots
	Annona stenophylla subsp. Nana	roots
	Burkea africana	roots
	Psydrax livida	roots
	Securidaca longepedunculata	roots
Urine, blood in the	*Pleiotaxis antunesii*	leaves
Vomiting	*Ziziphus mucronata subsp. Mucronata*	roots
Vomiting, induce	*Strychnos cocculoides*	unripe fruits
Wounds	*Psydrax livida*	roots
	Strychnos cocculoides	leaves
	Terminalia sericea	roots
Plants used as general Treatments and Tonics:		
For babies	*Baphia massaiensis subsp. Obovata var. obovata*	roots
	Ochna pulchra	young leaves
	Ozoroa paniculosa var. paniculosa	roots
For infants	*Bauhinia urbaniana*	roots
	Dichapetalum rhodesicum	roots
	Hoffmannseggia burchellii	roots
For adults	*Bauhinia urbaniana*	roots
	Combretum psidioides	roots
	Lapeirousia otaviensis	Tuber

South African scientists have also started focussing attention on this important indigenous resource, both in the authentication of traditional medicinal plant use, and in the discovery of compounds with therapeutic/clinical potential. Several members of the Combretaceae have been used for treating bacterial diseases in southern Africa. A number of studies have been conducted on plants of the Combretaceae to verify antibacterial and other biological activities (Eloff, 1999b; Martini and Eloff, 2000, Katerere, 2001,Martini *et al*, 2004).

1.2.1.2 Overview of research on methodology and antibacterial activity

Although most of the work done to date was on *Combretum* species, an outline of the in Combretaceae research activities in the Phytomedicine Programme, University of Pretoria, is given to provide background information to the selection of the topic and methodology employed in this PhD project.

i) Selection of plants to investigate

An analysis was made of approaches to be followed in selecting plants for research and gene banking. Plants used as phytomedicines in Africa were also analyzed, and of these, the Combretaceae constituted a major group (Eloff 1999a).

ii) Selection of optimal extraction procedure

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

iii). 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 developed (Eloff, 1998b).

iv) Development of a novel way to determine antibacterial activity

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

v) Antibacterial activity of *Combretum erythrophyllum*

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

vi) Antibacterial activity and stability of members of the Combretaceae

Acetone leaf extracts of 27 species of *Combretum, Terminalia, Pteleopsis* and *Quisqualis* had antibacterial activity ranging from 0.1 – 6 mg/ml (Eloff, 1999b). Storing extracts for 6 weeks at room temperature did not affect the MIC values.

vii) Stability of antibacterial activity in *C. erythrophyllum*

Leaves of *C. erythrophyllum* collected from the same area and stored in herbaria for up to 92 years did not lose any antibacterial activity (Eloff, 1999c)

viii) A proposal for expressing antibacterial activity

MIC values do not give sufficient indication of the activity present in a plant. A proposal was made that "total activity" should be determined by dividing the quantity in mg extracted from 1 g of plant material by the MIC in mg/ml. The resultant value in ml/g gives the highest volume to which a plant extract can be diluted and still inhibit the growth of the test organism (Eloff, 2000).

ix) Other biological activities of *Combretum* species

Breytenbach and Malan (1989) isolated three antimicrobial compounds from *C. zeyheri* and Alexander *et al.* (1992), found antimicrobial activity in 6 species of *Combretum*. The anti-inflammatory, anthelminthic and antischistosomal activity of 20 *Combretum* species was determined. There was very little antischistosomial activity, low to medium anthelminthic activity and medium to strong anti-inflammatory activity in extracts of the different species (McGaw *et al.* 2001).

x) The stability and relationship between antibacterial and anti-inflammatory activity of southern African *Combretum* species

Both antibacterial and anti-inflammatory activities were stable in stored extracts and there was a reasonable correlation between antibacterial and anti-inflammatory activity,

indicating that similar compounds may be responsible for the biological activities (Eloff *et al,* 2001).

xi) Extraction of antibacterial compounds from *Combretum microphyllum*

Several extractants were tested to determine if any extractant selectively extracted antibacterial compounds. The three most promising extractants were di-isopropyl ether, ethanol, ethyl ether, acetone and ethyl acetate. The activity towards Gram-negative and Gram-positive bacteria was similar (Kotze and Eloff, 2002).

xii) Isolation of antibacterial compounds from *C. erythrophyllum*

For her PhD study Nataly Martini isolated and characterized seven antibacterial compounds from *C. erythrophyllum*. Four were flavanols: kaempferol, rhamnocitrin, rhamnazin, quercitin 5, 3'-dimethylether and three were flavones: apigenin, genkwanin and 5-hydroxy-7, 4'-dimethoxyflavone(Martini et al 2004 a). All test compounds had good activity against *Vibrio cholerae* and *E. faecalis*, with MIC values in the range of 25-50 µg/ml. Rhamnocitrin and quercetin-5, 3'-dimethylether also showed good activity (25 µg/ml) against *Micrococcus luteus* and *Shigella sonei.* Toxicity testing of the isolated compounds 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 relating to some of these compounds (Martini, 2000, Martini *et a*l 2004 b).

(xiii) Isolation of antibacterial compounds from *C. woodii*

For his M.Sc study James Famakin isolated the stilbene 2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxybibenzyl (combretastatin B5) from the leaves of *C. woodii*. It showed significant activity against *S. aureus* with an MIC of 16 µg/ml, some activity against *P. aeruginosa* (MIC of 125 µg/ml) and *E. faecalis* (MIC of 125 µg/ml), and slight activity against *E. coli*. This is the first report of the antimicrobial activity of combretastatin B5 (Famakin, 2002).

xiv) Isolation of antibacterial compounds from *C. apiculatum*

For his M.Sc study Andrew Serage elucidated the structures of two flavanones (alpinetin and pinocembrin) and one chalcone (flavokawain) from the leaves of *C. apiculatum* subsp. *apiculatum*. All the compounds had substantial activity against the bacterial pathogens tested (Serage, 2003).

More students are working on isolating antibacterial, antioxidant and antifungal compounds from other *Combretum* species. Jerry Angeh has isolated and elucidated seven compounds including two new compounds from *C. imberbe* and *C. padoides* as part of his ongoiing PhD study. The promising preliminary results on *Terminalia* species motivated this study. The techniques used are described in previous publications and theses.

1.2.1.3 *Terminalia* species and antimicrobial activity

Some *Terminalia* and *Combretum* species are used for a variety of complaints, ranging from the treatment of infertility and sexual impotence to the management of stomach disorders, including diarrhoea and helminthiasis, to haematuria (Gelfand *et al.,* 1985, Table 1.1). The genus *Terminalia* contains about 250 species, of which only a handful have been investigated phytochemically and/or for pharmacological efficacy (Katerere, 2001). Table 1.2 describes the traditional medical uses of some *Terminalia* species in southern Africa.

Table 1.2. Some uses of *Terminalia* spp. in southern African traditional medicine as reported in the PhD thesis of Katerere, 2001.

PLANT SPECIES	MEDICINAL USES
Terminalia brachystema	Bile emesis, Constipation, Haematuria
Terminalia sericea	Abscesses and wounds, Abdominal pain, Anti-emetic, Anthelmintic, Bilharziasis, Diarrhoea, Dilatation of birth canal, Depressed fontanelle in children, Epistaxis, General malaise, Gonorrhoea, Infertility, Prevention of abortion, Sore throat, Tonic
Terminalia mollis	Bile emesis

Sato *et al.* (1997) investigated the antimicrobial activity of six East African medicinal plants including *Terminalia spinosa* against 105 strains of bacteria from seven genera. The study showed that *Terminalia spinosa* possesses limited antibacterial activity (MIC

< 8 mg/ml) against *Staphylococcus aureus, Enterococci spp., Pseudomonas aeruginosa* and species of the Enterobacteriaceae.

Methicillin-resistant strains of *S. aureus* (MRSA) are resistant to many aminoglycoside antibiotics, and essentially to all beta-lactam antibiotics. Ethanol extracts of the fruiting bodies of *Terminalia chebula* contained two compounds with antimicrobial activity against MRSA (Sato *et al.,* 1997).

The bark of *Terminalia alata* is active against eight out of eleven selected bacterial and fungal species, as well as against fungal spores (Taylor *et al.,* 1996). The ethanolic extracts of twelve plants selected through an ethnomedical survey (the roots of the plants are used to treat venereal diseases) in Guinea-Bissau were investigated for their *in vivo* antibacterial properties against ten bacterial species and *Candida albicans* using agar diffusion and dilution methods. *Terminalia macroptera* extracts showed activity against the bacteria as well as some activity against *C. albicans.* An interesting profile of activity against most of the enteropathogenic microorganisms was also shown (Taylor *et al.,* 1996).

The activity of a crude extract formulation of *Terminalia chebula* and other plants was evaluated in experimental amoebic liver abscess in golden hamsters (Sohni and Bhatt, 1996). The formulation had a maximum cure rate of 73% at a dose of 800 mg/kg/day, showing a reduction in the average degree of infection (ADI) of 1.3 compared to 4.2 for the sham-treated controls (Sohni and Bhatt, 1996). In immunomodulation studies,

humoral immunity was enhanced as evidenced by the haemagglutination titre. The T-cell counts remained unaffected in the animals treated with the formulation but cell-mediated immune response was stimulated as observed in the leukocyte migration inhibition tests (Sohni and Bhatt, 1996). Gallic acid, chebulagic acid and chebulinic acid isolated from *Terminalia chebula* were found to have moderate activity against cultured tumor cells *in vitro* (Lee *et al.,* 1995). Gallic acid and its ethyl derivative are also responsible for the potent antimicrobial activity which this plant exhibits even against methicillin-resistant *Staphylococcus aureus* (Sato *et al.,* 1997).

A bioactivity-guided fractionation of an extract of *Terminalia bellerica* fruit rind led to the isolation of two new lignans named termilignan (1) and thannilignan (2), together with 7-hydroxy-3', 4'-(methylenedioxy) flavan (3) and anolignan B (4). All four compounds possessed demonstrable anti-HIV-1, anti-malarial and anti-fungal activity *in vitro.*

In another study by Sohni *et al.* (1995) on the crude drug formulation of plants including *Terminalia chebula,* the dried and pulverized plants were extracted with ethanol together and individually. The *in vitro* amoebicidal activity was studied to determine the minimum inhibitory concentration (MIC) values of all the constituent extracts as well as the whole formulation. The formulation had a MIC of 1000 μg/ml as compared to the 10 μg/ml of the reference compound metronidazole. In experimental caecal amoebiasis in rats the formulation had a curative rate of 89% with the average degree of infection (ADI) reduced to 0.4 in a group dosed with 500 mg/kg/day compared with ADI of 3.8 for the sham-treated control rats. Metronidazole had a cure rate of 89% (ADI = 0.4).

Extracts of *T. bellerica*, *T. chebula*, *T. horrida* and 38 other plants were screened for their inhibitory effects on Human Immunodeficiency Virus-1 transcriptase (El-Mekkawy *et al.,* 1995). The extracts showed significant inhibitory activity with IC_{50} values less than or equivalent to 50 µg/ml. Extracts of *Terminalia spinosa* and other plants showed activity against various *Candida* species at MIF's (minimum fungicidal concentrations) of 0.06 to > 8 mg/ml and MIC's of 0.006 to 8 mg/ml. A methanol extract of the dried fruit of *Terminalia pallida* was evaluated and found to have a broad spectrum of antimicrobial activity (Gupta *et al.,* 2001).

Terminalia arjuna is widely used in India by Ayurverdic doctors for managing cardiovascular disorders. The tannins isolated from the plant were found to exert a cholinergic hypotensive effect in rats (Tanaka *et al.,* 1986). The compounds 3β-hydroxyolean-12-ene and related oleane-type triterpenoids were isolated from the leaves (Chauhan *et al.,* 1997). The bark powder apparently has hypolipidaemic effects (Khanna *et al.,* 1996) and Petit and colleagues (1998) found the bark constituents to be gallic acid, ethyl gallate and luteolin, which were found to be inhibitors of cancer cell growth.

Young branches of *Terminalia spinosa* have been found to have anti-fungal activity (Fabry *et al.,* 1997), as well as activity against *Helicobacter pylori* and *Candida* species. *Terminalia macroptera* root extracts showed some activity against *Candida albicans* and enteropathogenic bacteria, with the best results obtained against clinical strains of

Shigella dysenteriae and *Vibrio cholera* (Silva *et al.,* 1996). Ellagitannins were found to be the main constituents in the active fractions. In general, biological activity arising from condensed tannins is not reproducible *in vivo* but some ellagitannins can enter the bloodstream and have shown biological activity which may have potential for use in chemotherapy (Nash, 2000). Tannins isolated from the methanol extract of *Terminalia citrina* have been tested for antimicrobial activity (Burpadaja and Bunchoo, 1995). *Terminalia avicennoides* has been found to possess activity against *Vibrio cholera* (Alkinside and Olukoya, 1995).

Plants of the genus *Terminalia* are used in African traditional medicine for a variety of conditions that may be of infective nature e.g. bilious vomiting, diarrhoea, billharzia, sore throat, gonorrhoea, hepatitis and malaria. The use of many of the traditional medicinal plants discussed in this section has in many cases been scientifically validated for infectious ailments. The antimicrobial action of plant extracts, including anti-vibrio and anti-amoebic activity, has been demonstrated in various studies (Eloff, 1999b; Sato *et al.,* 1997; Silva *et al.,* 1996).

1.2.2 Botanical overview

This section deals with the botanical background of the plants investigated in this study. The Combretaceae family belongs to the order Myrtales and is divided into two sub-families: Combretoidaea and Strephonematoidaea. Combretoidaea, which is of interest in this study, is divided into three sub-tribes, Combretinae, Pteleosidinaea and Terminaliinae (which consists of 8 genera).

The African Combretaceae comprise 19 genera but the two largest, *Combretum* and *Terminalia*, occur in most parts of Africa where they are often numerically the dominant group (Carr, 1988). They may be climbers, shrubs or trees, and are readily characterized by fruits with wing-shaped appendages. Although traditional healers throughout Africa have used species of the Combretaceae for the treatment of a wide range of disorders, only about 25 of the approximately 99 African species of Combretum have been subjected to any form of scientific study (Rogers and Verotta, 1988).

According to Lawrence (1951), the Combretaceae family has, within its 18 genera, some 500 species distributed throughout the tropics and subtropics. Carr (1988) classified the *Terminalia* species occurring in southern Africa into three sections based on external morphology as shown in Table 1.3. Thirty-three species of the Combretaceae occur in southern Africa including eleven *Terminalia* species (Carr, 1988). In this study, three species (representing each section) were initially chosen for extractant suitability studies. Subsequently, seven species were selected for preliminary antibacterial activity investigation and these are described in detail below.

1.2.2.1 Terminalia sericea

Common names: Vaalboom, (Afrikaans) Silver cluster-leaf, (English) Mokonona (Shona)

SA National tree number; 551

Distribution: Most widely distributed of all the *Terminalia* species in southern Africa. Found in the northern parts of Gauteng, mainly in the Limpopo province and northern

parts of the Northern Cape in South Africa, as well as the whole of Zimbabwe and Botswana and western Namibia.

Table 1.3. The three sections of *Terminalia* (Carr, 1988) with the species represented in each section

***Section Abbreviate** Exell*
Terminalia prunoides Exell
Terminalia randii Bak. F.
Terminalia stuhlmanii Engl.
***Section Psidioides** Exell*
Terminalia brachystema Welw. Ex Hierr
Terminalia sericea Burch. Ex DC
Terminalia trichopoda Diels
***Section Platycarpae** Eng. & Diels emend* Exell
Terminalia gazenzis Bak. f.
Terminalia phanerophlebia Eng. & Diels
Terminalia mollis Laws
Terminalia sambesiaca Eng. & Diels
Terminalia stenostachya Eng. & Diels

Habitat: Restricted to sand or sandy soils at altitudes ranging from sea level to 1600 m. It occurs in open and tree savanna, woodland situations and as a riverine growth in

semi-desert areas.

General: Deciduous species, often seen as a shrub or sapling 2-4 m high. The usual maximum height is 7–8 m but it can reach heights of 12 m. Photographs of a *T. sericea* tree are shown in Figure 1.1.

Bark: Dark to medium grey with pronounced fissuring.

Foliage: Leaves are clustered on whorls of up to 10, have a long even taper to the base and appear blue-grey with silvery tinges. The underside of the leaf has a raised main vein with a pale brownish-green colour.

Fruits: Ripens in June and is a single winged samara, elliptic in outline with a constricted taper to the base and a slight attenuation near the apex, which is emarginate.

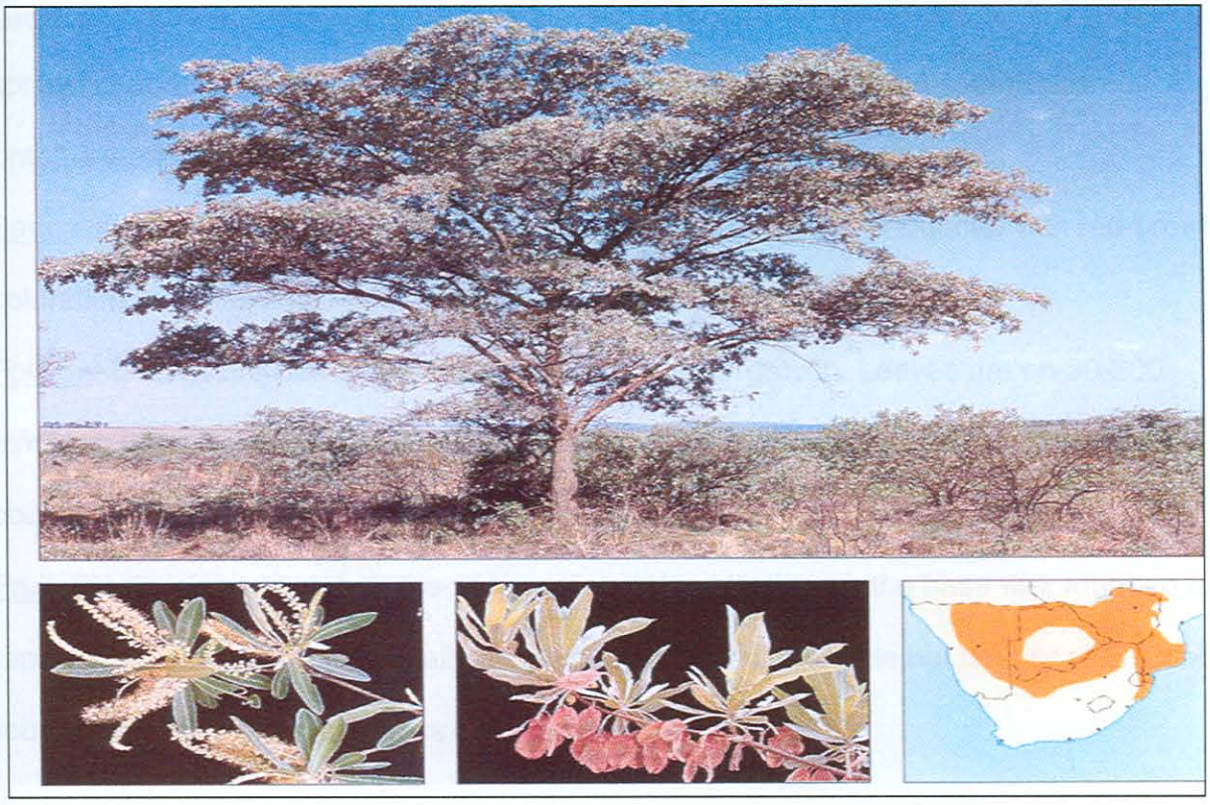

Fig. 1.1. A *Terminalia sericea* tree, with close-up view of foliage and flowers van Wyk *et al* 2002

1.2.2.2 Terminalia brachystema

Common names: Bastervaalboom (Afrikaans), Bastard Clusterleaf (English), Muogonono (Shona).

SA National tree number: 548

Distribution: Sporadic occurrences in the central part of the Limpopo province with the main distribution along the Caprivi-strip and the area adjacent the Zambezi river.

Habitat: Savannah and woodland areas, on the Kalahari and other sand at altitudes of 800-1200 m.

General: Shrub or tree usually up to 4–5 m but in favourable conditions up to 7 m with a spread of up to 9 m. The species has been split by Wickens into the subspecies *brachystema* and *sessiliflora* (not occurring in the southern African area).

Bark: Dark to medium grey or grey-brown. Fissures are fairly pronounced with red-brown coloration in the hollows.

Foliage: Leaves are borne only on the last 12 months' growth. Leaves are on 30-200 mm long laterals on which there can be one or more whorls having up to eight leaves. It could have sessile or petiolate leaves.

Fruit: Single winged samara, the wing outline being elliptic with the base well rounded to tapered and the apex occasionally acuminate and even attenuate but usually rather well rounded and in most such cases emarginate.

1.2.2.3 Terminalia gazenzis

Common name: Fringe-leafed Terminalia (English).

SA National tree number (Zimbabwe) 786

Distribution: Central-southern Zimbabwe and odd clusters in other parts of Zimbabwe, Malawi and Mozambique.

Habitat: Found in savanna, woodland and forest margins at medium altitudes of up to 1200 m.

General: It is a tree of up to 10–20 m, wide spreading with a flattish to deeply rounded crown.

Bark: Usually single stemmed with a dark brown bark, somewhat fissured near the base but further up a light brown to dark biscuit colour.

Foliage: Four to five or even up to twelve leaves per whorl. The lamina is obovate with a gradual taper to the base and the apex is rounded. It usually has an inconspicuous apicula but is sometimes emarginate.

Fruit: Single winged samara with an outline that is elliptic to oblong elliptic, often with appreciable irregularities.

1.2.2 4 Terminalia mollis

Common name: Large-leafed Terminalia (English).

National tree number: (Zimbabwe) 787

Distribution: Various geographical areas in Zimbabwe, as well as in Tanzania, Zaïre, Angola, Kenya and West Africa.

Habitat: Found in savanna or woodland situations in association with mopane trees.

General: A robust-looking tree, usually up to 12 m with a well-rounded or rather flatly rounded crown which can exceed the height.

Bark: Medium grey, coarsely longitudinally fissured with light grey coloration in the fissures.

Foliage: Leaves are borne on the previous year's growth as well as on current extensions and are orientated 360 degrees around the stems in groups, to form in effect a whorl. Leaves are petiolate and broadly elliptic, tapering or rounding at the base with the apex rounded to bluntly acuminate and often with a smaller apicula.

Fruit: Single-winged and elliptic in outline though there can be considerable irregularities in the wing margins. Fruit is borne in large numbers and, being at the terminals, is prominently displayed.

1.2.2.5 Terminalia phanerophlebia

Common names: Lebombo cluster leaf (English), Lebombo trosblaar (Afrikaans).

SA National tree number: 549

Distribution: In the southern parts of the Kruger National Park and the northern parts of Kwa Zulu-Natal in the Republic of South Africa as well as parts of Swaziland.

Habitat: Found in mixed savanna, along watercourses and on slab rock hillslopes at altitudes usually not exceeding 700 m but sometimes as high as 1200 m.

General: A shrub of up to 1.5-2 m, but sometimes as high as 6 m. May be single-stemmed but are often multiple-stemmed. Irregular crown, which may exceed the height.

Bark: Darkish grey with pronounced, wavy, longitudinal fissures, which may result in an elongated diamond pattern.

Foliage: Leaves are in whorls of up to 20 where there are no extensions. The lamina, sometimes broadly elliptic, is usually more obovate with a rounded to straight to attenuate taper to the base where it may also be decurrent along the petiole.

Fruit: Single-winged samara, elliptic to broadly elliptic in outline.

1.2.2.6 Terminalia prunoides

Common names: Lowveld cluster-leaf (English), Sterkbos (Afrikaans), Bakone (Shona).

SA National tree number: 550

Distribution: Right across the width of southern Africa - from the northern parts of Namibia through the northern parts of Botswana, including the Caprivi Strip down to the northern parts of South Africa.

Habitat: Found in many different types of woodland, tree savanna and scrub savanna at medium to low altitudes.

General: Seen most frequently as a many-stemmed, rather rounded shrub, 3-5 m in height with a spread of up to 7 m, but may be a single-stemmed tree of 15 m in height.

Bark: Light grey with a shallow fissure and a gingery coloration at the base of the fissure. Some criss-crossing of the fissures tend to form an elongated diamond pattern.

Foliage: Leaves are produced in whorls of 6-8. Leaves are petiolate, the lamina being elliptic to obovate with a straight to rounded taper to the base and the apex bluntly acuminate and slight attenuate, to rounded and sometimes emarginate.

Fruit: A samara with a single rounded wing, obovate in outline with wing margins tapering to the base and the apex bluntly acuminate to rounded, often with slight attenuation and then, as a rule, emarginate.

1.2.2.7 Terminalia sambesiaca

Common names: River Terminalia (English).

National tree number: (Zimbabwe): 790

Distribution: Northern parts of Zimbabwe around the Kariba region bordering Zambia.

Habitat: Found in riverine fringing forests and rocky hill slopes at altitudes from 800-1250 m.

General: Usually a single-stemmed tree of up to 20 m in height.

Bark: Smooth, somewhat fissured, grey, overmarked with longitudinally orientated, light red-brown, slightly raised ridges.

Foliage: Leaves are clustered in whorls of 3-6. The lamina is obovate to elliptic, either tapering to or rounding at the base and acuminate or rounded at the apex, which almost invariably has a pronounced apicula.

Fruit: Hang in bunches from old inflorescence branches. Has a single surrounding wing elliptic in outline with a blunt taper to the base, the apex rounded and usually emarginate.

All the above information was derived from Carr (1988)

1.2.3 Phytochemical overview

This section deals with the chemistry of biologically active compounds isolated from *Terminalia* species in general, and from the species specifically under investigation, by previous researchers. A range of phytochemical compounds has been isolated from *Terminalia* and *Combretum* species (e.g. combretastatins from *Combretum* species and flavonoids and terpenoids from *Terminalia* species). The main biologically active compounds will be described under the headings that follow.

1.2.3.1 Terpenoids

Plant fragrances are carried in the so-called *quinta essentia* or essential oil fraction (Cowan, 1999). These oils are secondary metabolites and are highly enriched in compounds based on an isoprene structure, called terpenes. Terpenes have a general chemical structure of $C_{10}H_{16}$. They are classified as monoterpenes, diterpenes, triterpenes and tetraterpenes (C_{10}, C_{20}, C_{30} and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes C_{15}). When the compounds contain additional elements, usually oxygen, they are termed terpenoids (Cowan, 1999).

Terpenoids are synthesized from acetate units and as such, share their origin with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are menthol and camphor (monoterpenes). Terpenoids are active against bacteria and fungi (Taylor *et al.,* 1996). The mechanism of action of terpenes is not fully understood but is thought to involve membrane disruption of the lipophilic compounds. A rich variety of triterpenoid acids have been isolated from

C. molle and *C. imberbe* (Rogers and Verotta, 1996). Katerere (2001) isolated oleanane compounds from *T. stuhlmani* that are glycosides of imberbic acid, originally isolated from *C. imberbe.* These compounds establish a chemotaxonomic link between the two genera. An example of a terpenoid is shown in Figure 1.2.

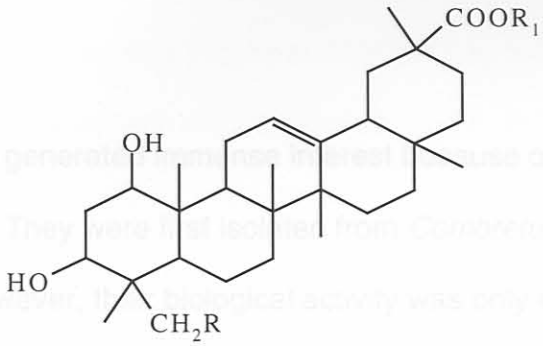

Fig. 1.2. Structure of a terpenoid - an example of a 1,3-hydroxylated pentacyclic triterpenoid

1.2.3.2 Tannins

Tannins are polyphenolic compounds of high molecular weight. They have the ability to precipitate proteins (Katerere, 2001), and they may also form complexes with starch, cellulose and minerals. They are synthethised via the shikimate pathway, which is the same pathway that results in the formation of isoflavones, coumarins, stilbenoids and other phenolic metabolites.

Gallic acid and its derivatives are common constituents of the Combretaceae. A number of elaborate tannins have been isolated mainly from *Terminalia* species, e.g. the

diphenoyl-gallagylglucose isolated from *T. oblongata,* and ellagitannin from the leaf of *T. calamansanai*, as well as derivatives from *T. catappa* and *T. chebula* (Tanaka *et al.,* 1986) These compounds have been shown to have anti-tumour activity in numerous studies (Pettit *et al.,* 1996).

1.2.3.3 Stilbenoids

These compounds have generated immense interest because of their biological potency and structural simplicity. They were first isolated from *Combretum* species by Letcher and Nhamo in 1971. However, their biological activity was only discovered by Pettit and colleagues in the early 1980's who 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 named these compounds "combretastatins", and they have since been isolated from *C. kraussi, C. molle, C. psidiodes*, *C. apiculatum* and *C. woodii* (Malan and Swinny, 1993; Schwikkard *et al.,* 2000; Katerere, 2001; Famakin, 2001). They have been designated A, B 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 former, meanwhile, has an ethane-type bond structure and is closer to the dihydrostilbenes.

1.2.3.4 Flavonoids

Flavonoids are conjugated aromatic phenols which may occur in glycosidic combinations or as aglycones (Harborne, 1973). At least nine classes of flavonoids are recognised, all of which are biosynthetically related and originate from the amino acid, phenylalanine. They are one of the most diverse and widespread groups of natural constituents, and are of great interest to phytochemists (Agrawal, 1989). They may represent an interesting new therapeutic approach with low toxicity and a wide variety of chemical structures. Flavonoids are also widely used taxonomic markers. The flavonoid, luteolin, has been isolated from *Terminalia arjuna* and has shown to be active against murine P388 lymphocytic leukemia and human cancer cell line as well as inhibiting the growth of *Neisseria gonorrhoea* (Pettit *et al.,* 1996). Luteolin has been shown to be both an antitumour promotor and a mutagen.

1.3 Aim of the study

The hypothesis is that *Terminalia* species occurring in South Africa contain useful antibacterial compounds because in a preliminary investigation *T. sericea* possessed the third highest total activity of 27 Combretaceae species examined (Eloff, 1999b).

It is the aim of this study therefore

(1) to investigate the presence of antibacterial compounds in the leaves of different *Terminalia* species found in southern Africa and to determine the antibacterial activity using *in vitro* methods.

(2) to isolate and identify one or more of the antibacterial compounds, and

(3) to determine if a preparation of the isolated compound can be used to treat animals infected with a bacterial pathogen.

Chapter 2

Selection of the best extractant

2.1 Problem statement and aim of the exercise

There has been a substantial increase in the number of papers where authors have screened plants for antimicrobial properties. It is clear that researchers use many different extractants, for example 80% ethanol (Vlietinck *et al.,* 1995), methanol (Taylor, *et al.,* 1995), petroleum ether, chloroform, ethanol and water (Salie *et al.,* 1996). After reviewing the re-awakening of pharmacognosy, Cordell (1993) concluded, "There is clearly substantial room for improvement in the extraction methodologies given that there are a variety of techniques that could be used to prepare extracts". Farnsworth (1996) stated that the biggest problem in drug development with plants is answering a very simple question: what kind of extract should we test?

There are many ways in which to extract the active components from plants.
It was decided in this study to evaluate a number of extractants with different polarities (and selectivity) to determine the extractant that would firstly give a high yield of extracted residue, and secondly extract the active components. If an extractant could be found that extracted mainly biologically active compounds, it would simplify the isolation

of these compounds to a large degree. A number of possible solvents, along with their polarities and grouping based on selectivity, are listed in Table 2.1.

Table 2.1 Different solvents with their distinctive polarities and selectivity groups (Snyder and Kirkland, 1979)

SOLVENT	POLARITY	GROUP
Hexane	0.1	I
Isopropylether	2.4	I
Diethyl ether	2.8	I
Ethylacetate	4.4	IV a
Tetrahydrofuran	4.0	III
Dichloromethane	3.1	V
Acetone	5.1	VI a
Methanol	5.1	II
Ethanol	4.3	II
Water	10.2	VIII

Following published investigation on several *Terminalia* species, it was decided to investigate the antibacterial activities of *Terminalia* species found in southern Africa. This would serve the purpose of scientifically verifying the use of extracts for their wound-healing properties by rural people, personal communication (traditional healer in Pretoria city central). An additional aspect is that proven antibacterial activity of these

selected plants would have clear advantages for rural people reliant on medicinal plants for cost-effective treatment of various bacterially induced afflictions. We decided to concentrate on leaves and not other plant parts for sustainable utilisation reasons. Initially only three species (based on their availability) representative of the three sections of *Terminalia* (Table 3; Carr, 1988), i.e. *T. sericea (*Section Psidioides), *T. phanerophlebia* (Section Platycarpae) and *T. prunoides* (Section Abbreviate) were selected for the evaluation of the best extractant.

2.2 Materials and Methods

2.2.1 Collection and processing of plant material

The study was performed on the leaf material of *T. sericea*, *T. phanerophlebia* and *T. prunoides*. Plant material was collected from the Lowveld National Botanical Garden in Nelspruit. The permanent label on the tree assisted in the identification. The collection origin and voucher specimens are deposited in the Lowveld National Botanical Garden Herbarium in Nelspruit.

2.2.2 Extraction of plant material

Leaves were dried in the shade at room temperature. Stems and thick veins were removed and the remainder of the leaf material ground to a fine powder using a Jankel and Kunkel Model A10 mill. Extraction was carried out on different samples (1g) with 10 different solvents (10µl) of varying polarities as described in Table 2.1. Extracts were dried in a cold air stream and the mass of residue resulting from extraction with each solvent for the three plant species was noted.

2.2.3 Analysis of extracts by thin layer chromatography

Thin layer chromatography (TLC) was used to separate the components of each extract. The efficacy of the eluant used to separate the components by TLC depends on the polarity of the components and the selectivity of the eluents (Kirkland, 1979). Table 2.2 depicts the various eluting solvent systems developed in the Program for Phytomedicine at the University of Pretoria, employed in TLC studies.

Table 2.2. Three different eluant systems used to analyze plant extracts

ELUANT	COMPOSITION	SEPARATION CHARACTERIZTICS
BEA	Benzene:ethanol:ammonia 9:1:0.1 (v/v/v)	Separates low polarity components
CEF	Chloroform:ethyl acetate:formic acid 10:8:2 (v/v/v)	Separates intermediate polarity components
EMW	Ethyl acetate:methanol:water 20:2.7:2 (v/v/v)	Separates high polarity components

TLC was an integral part of the process of identification and isolation of active substance(s) with antibacterial activity in the three *Terminalia* species. The dried extracts of the ten different solvents were reconstituted to a concentration of 10 mg/ml in acetone. Acetone was the solvent of choice owing to its wide extraction capacity and low toxicity towards the test organisms in the bioassay procedures (Eloff, 1998b).

Approximately 100 µg aliquots (5 µl of a 20 mg/ml solution) of each of the extracts were loaded in 1 cm bands on three 20 x 10cm F_{254} TLC plates (Merck, 0.25 mm thick) and each of these was developed with CEF, EMW or BEA (Table 2.2). The extracts were applied approximately 1 cm from the bottom of the plates with a micropipette and allowed to develop for 8 to 9 cm in a tank containing eluant. The atmosphere in the tank was saturated by placing filter paper wetted with the eluant against the walls of the tanks, which were then sealed with lids.

Once developed, the separated compounds were investigated under a Camac Universal TL-600 UV light at 360 nm and 254 nm and the fluorescing (360nm), or quenching(254nm) compounds marked. The chromatograms were then sprayed with vanillin spray reagent (0.59 g vanillin dissolved in 100 ml sulphuric acid: ethanol [4:1] according to Stahl, 1969) and *p*-anisaldehyde spray reagent (5 ml *p*-anisaldehyde dissolved in 90 ml ethanol and 5 ml concentrated sulphuric acid (Carr and Rogers, 1986). The plates were heated in an oven at 105 °C for several minutes until the coloured bands showed clearly on the plates. On each of the plates, from 1 to 12

different compounds could be seen appearing as different coloured bands across the length of the run.

2.2.4 Antibacterial assay

Minimum inhibitory concentration (MIC) values were determined by a microplate serial dilution method (Eloff, 1998c). The test organisms were the Gram-positive *Staphylococcus aureus* (ATCC 29213) and *Pseudomonas aeruginosa* (ATCC 25922), and the Gram-negative *Escherichia coli* (ATCC 27853) and *Enterococcus faecalis* (ATCC 29212). These are the reference strains recommended by the National Committee for Clinical Laboratory Standards (Villanova, Pennsylvania, USA). These bacteria are responsible for most nosocomial infections. The cultures were grown at 37°C in Mueller-Hinton broth (Merck), maintained at 4 °C, and were regularly subcultured using a 1% inoculum. Approximately every six months, fresh cultures were obtained from Dr. F. Huygens in the Department of Medical Microbiology of the University of Pretoria.

The MIC assay used subsequently as well is described here:
Dried plant extracts were reconstituted to 10 mg/ml in acetone. Four 96-well microtitre plates were labeled with sample codes in landscape mode. Using a Socorex multichannel pipette, the plant extracts (100 µl) were serially diluted 50% with water in 96-well microtitre plates. An aliquot (100 µl) of each test bacterial species was added to the plant extracts in the wells and mixed. MH broth cultures of preferably 24 hours, but not more than 10 days old, were used in the assay, following the recommendation of

Eloff (1998b). The inoculation of the wells as well as the subculturing of the bacteria was undertaken in a laminar flow cabinet in order to minimize contamination. The four microplates (one for each of four test organisms) were stacked upon each other, covered by an empty plate to serve as a lid and sealed in a plastic bag. The plates were then incubated at 37° C in an incubating oven.

After incubating overnight for approximately 18 hours, the plates were removed from the incubator and 40 µl of a 0.2 mg/ml solution of INT (ρ-iodonitrotetrazolium violet) were added to each well with a multichannel micropipette. The plates were incubated for a further half an hour at 37° C before the first evaluation of colour development was made. The coloured tetrazolium salt (INT) acts as an electron acceptor and is reduced to a colourless formazan product by biologically active organisms (Eloff, 1998c), so where bacterial growth is inhibited, the solution in the well will remain clear after incubation with INT. In this study, the wells with decreased colour development were regarded as showing growth inhibition, and were used to calculate the MIC values. The microplates were again evaluated after 60 minutes and 120 minutes to confirm the results. The microtitre plate containing *Enterococcus faecalis* took up to 8 hours of incubation to show bacterial growth (or the lack thereof), as reflected by colour development. The minimum inhibitory concentration (MIC) values of each plant extract for each bacterial species were calculated from the original concentration of the extracts.

2.3 Results and Discussion

2.3.1 Extraction of raw material

The mass that each solvent extracted from 500 mg leaf material is recorded in Table 2.3.

Table 2.3. The mass (mg) of residue extracted by each of the ten different extractants from 500 mg of dried leaves of *T. sericea*, *T. phanerophlebia* and *T. prunoides*

EXTRACTANT	MASS RESIDUE EXTRACTED (mg)			AVERAGE (mg)
	T. sericea	*T. phanerophlebia*	*T. prunoides*	
Hexane	8	8	9	8.3
Isopropylether	8	10	10	9.3
Ethylether	5	11	19	11.6
Ethyl acetate	8	16	20	14.6
Tetrahydrofuran	24	26	132	60.6
Dichloromethane	6	13	34	17.6
Acetone	11	14	97	40.6
Methanol	10	11	96	39
Ethanol	13	19	126	52.6
Water	4	6	5	5
AVERAGE	9.7	13.4	54.8	25.9

Tetrahydrofuran (60.6 mg), methanol (39 mg), acetone (40.6 mg) and ethanol (52.6 mg) extracted the most material in terms of mass from the three plants. These extractants were situated in the intermediate to polar region of solvents. The highest average mass was extracted from *T. prunoides*.

2.3.2 TLC analysis of extracts

The reason for using different extractants was to determine if any of the extractants would preferentially extract antibacterial compounds to facilitate subsequent isolates of these compounds. In this section the complexity of the extracts were evaluated by TLC. An extract with few compounds and high antibacterial activity would be a logical choice for isolating the antibacterial compounds. On the other hand, the higher the number of compounds extracted by different extractants, the better the chance that bioactive compounds will be extracted. Each of the extracts was analyzed by TLC using the three solvent systems. The average number of bands separated with the different extracts varied from 2.5 (water) to 7.2 (dichloromethane) (Table 2.4)

Dichloromethane, isopropylether and acetone extracted the highest number of compounds that reacted with the vanillin spray reagent on the TLC plates, based on the number of visible bands on the TLC. BEA as an eluant showed the best results. Because BEA separates non-polar compounds well, this indicates the character of the compounds present in *Terminalia* extracts. Most compounds had R_f values in the mid- to more polar range, correlating with the polarity of the solvents that extracted a higher mass of residue from the plants. (Table 2.4)

Table 2.4. Average number of compounds visible on chromatograms of 10 *Terminalia* leaf extracts using three different eluents (chromatograms treated with vanillin spray reagent)

EXTRACTANT	AVERAGE NUMBER OF COMPOUNDS			
	BEA (non-polar)	CEF (intermediate)	EMW (polar)	AVERAGE
Hexane	4.7	4.3	2.8	3.9
Isopropylether	8.4	7.8	5.0	7.0
Ethylether	7.4	6.0	5.0	6.1
Ethyl acetate	6.7	6.7	4.3	5.9
Tetrahydrofuran	5.2	6.5	3.0	4.9
Dichloromethane	8.4	8.2	5.0	7.2
Acetone	8.2	7.5	4.5	6.7
Methanol	7.7	6.7	5.0	6.4
Ethanol	6.5	6.2	3.8	5.5
Water	2.7	2.5	2.3	2.5

2.3.3 Antibacterial assay

The average MIC of all four test organisms were evaluated of all 10 extractants as was done by Kotze and Eloff (2001). The results were similar for acetone although the results shown in table 2.5 were the average for all four organisms, ranging from 0.58 for water to 1.14 mg/ml for acetone.

Table 2.5. Average MIC values in mg/ml of each of the extractants against the four test organisms

Extractant	Average MIC value mg/ml
Hexane	0.78
Isopropyl ether	0.74
Ethyl ether	1.08
Ethyl acetate	0.91
Tetrahydrofurane	0.69
Dichloromethane	1.10
Acetone	1.14
Methanol	0.70
Ethanol	0.89
Water	0.58

The average total activity values of the various extracts against the four test bacteria varied from 11 to 126 ml for the different extractants (Table 2.6). *T. phanerophlebia* had the highest total activity for the different extractants (75 ml) compared to 51 and 50 ml for *T. prunoides* and *T. sericea* respectively.

Table 2.6. Total activity values (ml/g) of each of the plant extracts evaluated against Ps= *Pseudomonas aeruginosa, Ec= Escherichia coli, En= Enterococcus faecalis* and St= *Staphylococcus aureus*

PLANT	*T. sericea*				*T. phanerophlebia*				*T. prunoides*				
Organism	***Ps***	***Ec***	***St***	***En***	***Ps***	***Ec***	***St***	***En***	***Ps***	***Ec***	***St***	***En***	**Ave/ Extr**
Extractant													
Hexane	200	15	44	44	5	5	5	42	200	15	44	44	56
Isopropyl alcohol	32	4	16	32	6	12	48	100	32	4	16	32	28
Ethyl ether	125	4	14	28	6	48	48	100	125	4	14	28	45
Ethyl acetate	80	47	5	19	8	64	267	123	80	47	5	19	64
Tetrahydrofuran	200	141	14	57	26	51	51	104	240	141	14	57	91
Dichloromethane	50	2	13	13	8	65	130	260	50	2	13	13	51
Acetone	52	2	6	26	8	33	280	280	52	2	6	26	64
Methanol	90	3	11	45	15	61	122	122	90	3	11	45	52
Ethanol	433	4	16	65	34	34	271	136	433	4	16	65	126
Water	11	11	11	21	7	7	7	7	11	11	11	21	11
Average TA	127	23	15	35	12	38	123	127	131	23	15	35	59
Average TA/Plant	50				75				51				

TA = total activity =(mass extracted in mg from 1g / MIC in mg ml^{-1})

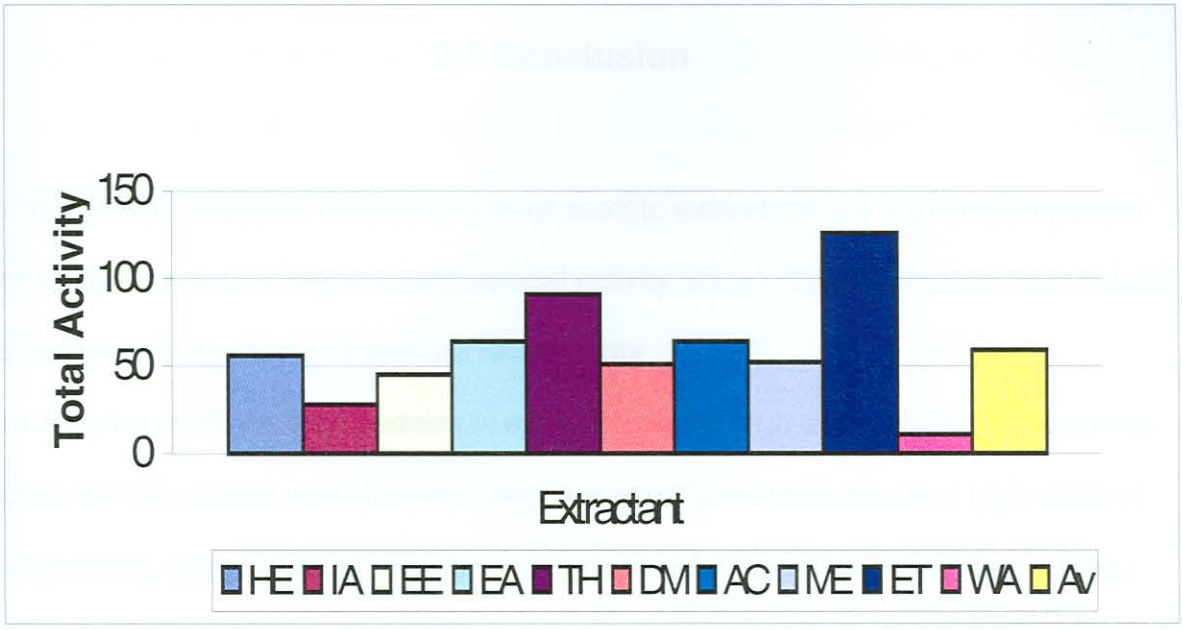

Fig.2.1. The average value of the total activity of each of the extractants evaluated against *Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis* and *Staphylococcus aureus.* HE= Hexane, IA= Isopropyl alcohol, EE= Ethylether, EA= Ethyl acetate, TH=Tetrahydrofuran, DM=Dichloromethane, AC=Acetone, ME=Methanol, ET= Ethanol, WA= Water and Ave= Average

2.4 Conclusion

Tetrahydrofuran, acetone, ethanol and ethyl acetate extracts of the *Terminalia* species under study showed the highest antibacterial activity, thus indicating that the best results were shown by extractants of intermediate polarity.

There is some evidence that *Terminalia* extracts contain high concentrations of tannins. Because tannins adsorb onto proteins, they are usually not considered of high value in bioprospecting. Some authors recommend that extracts should be detannified before bioassay to remove tannins. Tannins are highly soluble in polar solvents. Intermediate polarity extracts had the highest antibacterial activity in this experiment, indicating that tannins are probably not responsible for the antibacterial activity in the leaves of the *Terminalia* species.

The average total activity of the three plants compared relatively well with each other with *T. phanerophlebia* showing the highest total activity against *S. aureus* (267 ml for ethyl acetate, 280 ml for acetone and 271 for ml ethanol) whereas the total activity values for *T. prunoides* (5-44 ml) and *T. sericea* (5-44ml) were not as promising against the same bacterial species.

In considering various solvents as extractants, tetrahydrofuran (60.6 mg) yielded the largest residue mass, followed by acetone (40.6 mg) and ethanol (52.6 mg). Ethyl ether and ethyl acetate extracts showed the best results concerning clear separation of components by TLC, while dichloromethane and acetone also provided good results. On

counting the number of compounds visible on the TLC chromatograms, both with vanillin- and anisaldehyde spray reagents, dichloromethane (Average 7.2) showed the best results, with acetone also producing good results. On determining the total activity, the ethanol extract (126 ml) had the highest total activity with tetrahydrofuran (Average 91 ml), acetone (64 ml) and ethyl acetate (64 ml) also displaying promising activity.

In conclusion, because of the qualities of reasonable extraction yield, clear separation and high number of components in TLC, and good total activity against various test bacteria, acetone was a good general extractant. A similar study was done by Kotze and Eloff (2001) on *Combretum microphyllum* and obtained results that compared well with the study done here with MIC values against *S. aureus* of 0.01 mg/ml with acetone as extractant. They found MIC values against *E. faecalis* (0.11 mg/ml), *P. aeruginosa* (0.23 mg/ml) and *E. coli* (0.03 mg/ml). This also supports the results of Eloff (1998a) who investigated different solvents with respect to the diversity of compounds extracted, the number of inhibitors extracted, the rate of extraction, the ease of removal of solvent and the potential biological hazard posed by the solvent. Consequently acetone was selected as the best extractant for the large-scale isolation of antibacterial compounds.

Chapter 3

Selection of the best *Terminalia* species for isolation of antibacterial compounds

3.1 Problem statement and aim of the exercise

Rogers and Verotta (1996) stated that of the 99 African species of *Combretum,* only 25 species have been subjected to any form of scientific study. The same situation holds for *Terminalia* spp. Of the 11 species occurring in South Africa, only *T.sericea* has been investigated superficially to date. In the previous chapter, it could be seen that there were large differences in specificity of the different extracts to the different bacterial species. *T. phanerophlebia*, for example, had the highest average total antibacterial activity but *T. prunoides* provided the highest yield in terms of mass extracted. It was therefore decided to expand the research to seven *Terminalia* species found in southern Africa. Each species would be subjected to an *in vitro* investigation for their potential antibacterial activity. From these results the best *Terminalia* species to use for isolating the antibacterial compounds would be determined.

3.2 Material and Methods

3.2.1 Collection and processing of plant material

Leaf material of *T. sericea, T. prunoides, T. phanerophlebia, T. gazenzis, T. sambesiaca, T. mollis* and *T. brachystema* was collected from the Lowveld Botanical Gardens in Nelspruit during autumn. Voucher specimens were deposited in the Garden's herbarium. The leaves were dried at room temperature and after the stems were removed, ground to a fine powder with a Jankel and Kunkel model 10A mill. The powder was stored in a closed glass container at room temperature in the dark to limit photo-oxidative changes.

3.2.2 Extraction and TLC analysis

One gram of each powdered plant was extracted in 10 ml of acetone based on the results presented in Chapter 2. The amount extracted was increased from the 500 mg used in the previous chapter because of the relatively low antibacterial activity and low quantity extracted in the previous experiment. TLC was performed on the extracts using CEF as eluant. The TLC plate was sprayed with vanillin after development. Gallic acid, which has been isolated from *Terminalia arjuna* and shown to have antibacterial activity (Sato *et al.,* 1997) was used as a standard.

3.2.3 Antibacterial assay

The antibacterial activity of the seven *Terminalia* species was evaluated using bioautography (Buege and Kline, 1972) against four test organisms, namely *S. aureus, E. faecalis, E. coli* and *P. aeruginosa.*

Plant extract components were separated using TLC as described in section 2.2.3. TLC plates were developed using the eluents CEF, EMW and BEA, one for each of the four test organisms. The fluorescent components on each chromatogram were visualised under UV light. Areas of fluorescence (360 nm) or fluorescent quenching (254 nm) were marked and the chromatogram was scanned for future reference.

The plates were left overnight in front of an air stream in a fume cabinet to completely remove residual solvent that might be lethal to the bacteria in the bioautography experiments. The plates were sprayed the next morning with 48-hour-old bacterial cultures. Approximately 20 ml of the culture was centrifuged at 3500 rpm for 10 minutes. The clear supernatant was decanted and discarded. The bacterial pellet was retained and, if it was smaller than 2 to 3 mm in diameter, the process was repeated to obtain a clearly visible pellet. This pellet was resuspended in 20 ml sterile MH broth using a vortex shaker. The resulting mixture was poured into a glass spray-tube that had previously been tested with clean water to verify the spray pattern. The marked TLC plates were sprayed until clearly wet with bacteria and then allowed to dry for 5 to 10 minutes to remove excess moisture. Each plate was then placed in a closed container on a raised surface with enough water below the TLC plate (but not touching it) to

provide 100% relative humidity. The container was placed in an incubating oven and left overnight at 37^{0}C. The next morning the plates were sprayed with a 2 mg/ml solution of INT (Sigma) in water and placed back in the incubating container in the oven. The plates were examined after 30, 60 and 120 minutes before being dried and scanned to provide a permanent record. Clear zones against a pink background indicated regions of bacterial growth inhibition, as the tetrazolium salt (INT) was reduced to a pink colour only where bacteria were not actively growing.

MIC values were also determined for each of the *Terminalia* species against each of the four test bacteria.

3.3 Results and Discussion

3.3.1 TLC analysis

The chromatogram in Fig. 3.1 indicates the similarities in compounds extracted from the seven different *Terminalia* species under investigation. The complexity of the plants can be seen from the number of components in each of the plant extracts. Similar compounds are visible in most of the extracts.

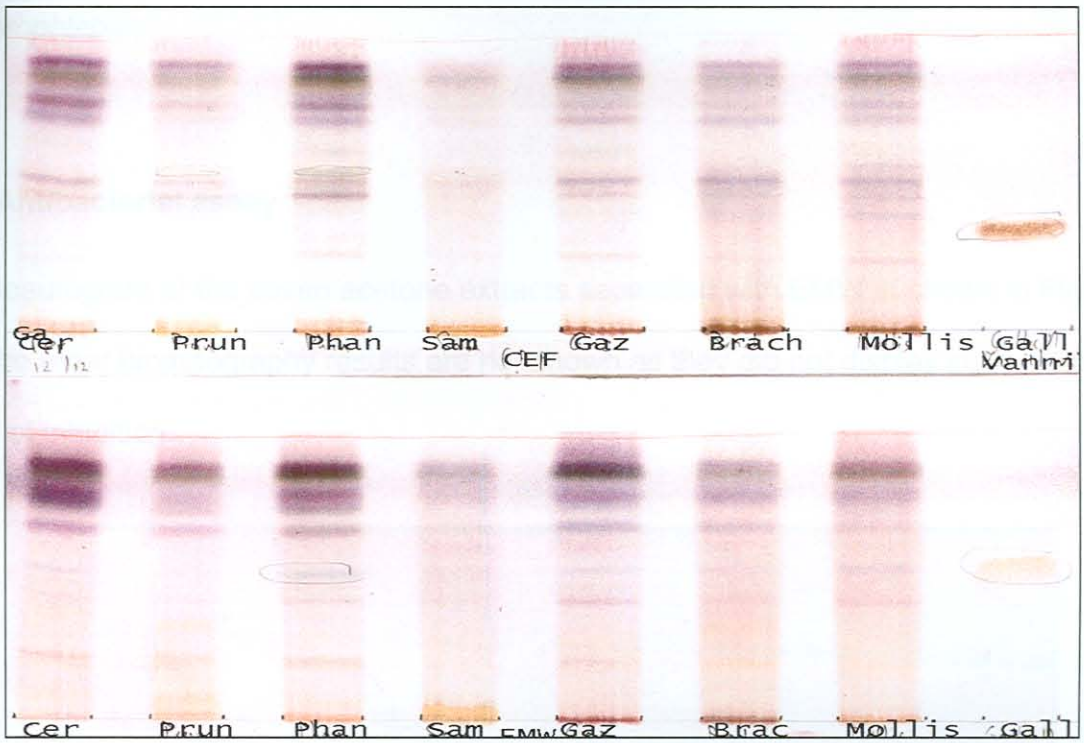

Fig. 3.1. Chromatogram of seven *Terminalia* species developed with CEF as eluant [top] and EMW [bottom] and sprayed with vanillin spray reagent where, Cer = *T. cerisea,* Prun = *T. prunoides,* Phan = *T. phanerophlebia,* Sam = *T. sambesiaca,* Gaz = *T. gazenzis,* Brac = *T. brachystema,* Mollis = *T. mollis,* Gal = Gallic acid.

T. sericea and *T. brachystema* are of the same Psidiodes section, *T. prunoides* belongs to the Abbreviate section and the rest are from the Platycarpae section. It does not appear that a differentiation on the basis of sections can be done using TLC as a technique other than the fact that *T. prunoides* has a slightly different chromatographic

profile from the rest. Gallic acid seems to be present in all the plants, most visibly in *T. phanerophlebia*.

3.3.2 Antibacterial assay

The bioautogram of the seven acetone extracts separated with EMW is shown in Figure 3.2. The other bioautography results are not shown as they did not display such clear zones of inhibition.

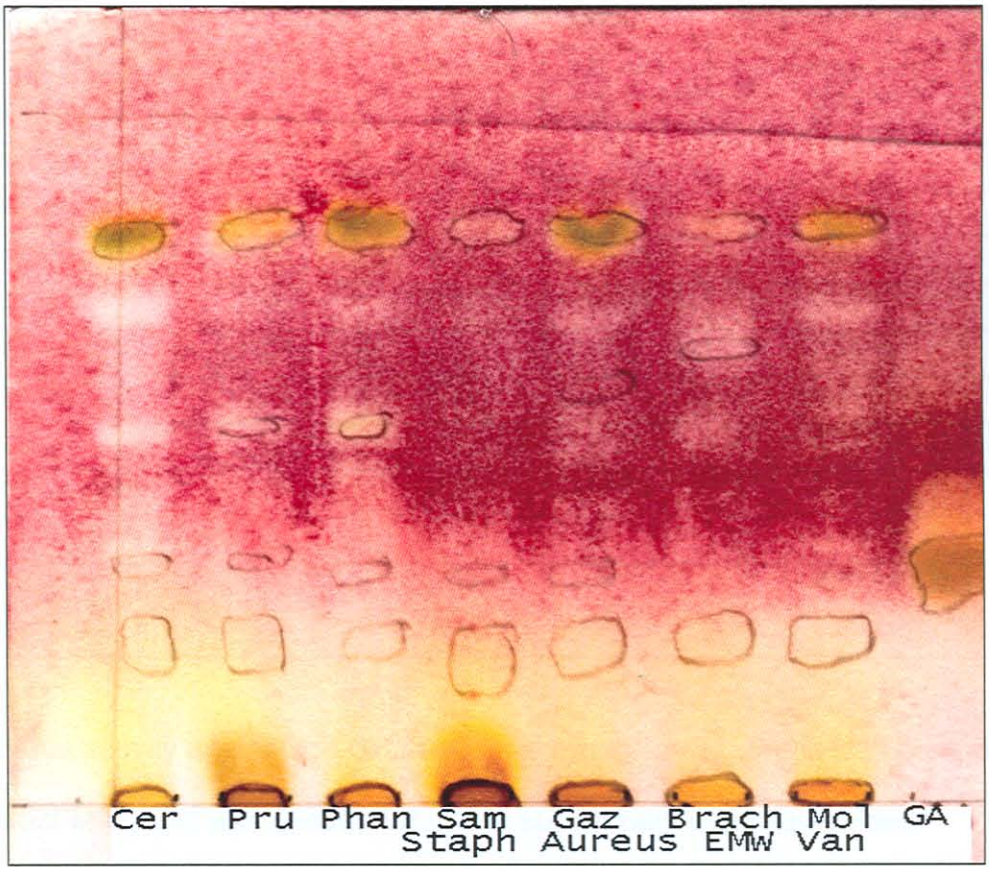

Fig. 3.2. Bioautogram of acetone extracts of seven *Terminalia* species separated with EMW and sprayed with *S. aureus*. Areas circled indicate fluorescent compounds.

MIC values for each plant extract against each bacterial species are shown in Table 3.1. The results are expanded further in Table 3.2 where the mass extracted, the acetone soluble fraction of the extract and the total antibacterial activity for each *Terminalia* species are recorded.

Table 3.1. MIC values (mg/ml) of the seven *Terminalia* species tested against *Pseudomonas aeruginosa, Escherichia coli, Enterococcus faecalis* and *Staphylococcus aureus*

EXTRACT	MIC (mg/ml)				
	S. aureus	*E. faecalis*	*E. coli*	*P. aeruginosa*	AVERAGE
T. sericea	0.16	0.63	0.31	0.16	0.32
T. prunoides	0.13	2.13	2.13	2.13	1.63
T. phanerophlebia	0.11	3.38	0.21	0.21	0.98
T. sambesiaca	0.20	3.25	0.20	0.41	1.02
T. gazenzis	0.06	0.25	0.25	0.25	0.2
T. brachystema	0.28	1.13	0.14	0.56	0.53
T. mollis	0.34	1.38	0.34	0.09	0.54
Average/organism	0.18	1.74	0.51	0.54	0.75
Gallic acid	0.31	2.50	0.31	0.31	0.86
Gentamycin*	0.16	1.00	0.16	0.63	0.49

The antibacterial activity against the Gram-positive organism *S. aureus* was generally the best (lowest MIC) and was chosen for use in subsequent bioassay-guided fractionation for the discovery of antibacterial compounds.

Table 3.2. Mass (mg) extracted with acetone from 500 g leaf material with the total mass soluble in acetone, the MIC values against four bacteria, and the total activity of the seven different *Terminalia* species

PLANT/COMPOUND	TOT EXT (mg)	TOT AC SOL (mg)	MIC (mg/ml)	TOTAL ACTIVITY
T. sericea	56	10	2.2	4.60
T. prunoides	99	34	4.7	7.20
T. phanerophlebia	53	27	1.4	19.80
T. sambesiaca	126	52	1.5	33.80
T. gazenzis	53	16	0.7	23.80
T. brachystema	89	36	1.4	19.30
T. mollis	63	22	1.1	20.00
Gallic acid			0.09	
Gentamycin			0.03	

Tot Extr = Total mass extracted in mg

Tot Ac sol = Total mass soluble in acetone (mg)

MIC = Average minimum inhibitory concentration

The total activity was determined by dividing the quantity (in mg) dissolved as acetone soluble by the MIC value. It became clear from this that redisolving in acetone was a problem. *T. sambesiaca* not only had the highest yield in terms of mass of compound extracted that was soluble in acetone but also had the highest total activity, while *T. gazenzis* had the lowest MIC of the test plant species.

3.4 Conclusion

In his study Eloff (1999b) acetone leaf extracts of *Terminalia sericea* had MIC values ranging from 0.1 – 6 mg/ml for the four test organisms compared to the average values of 0.16 to 0.63 mg/ml for *T. sericea*. The average values for the other species were 0.7 mg/ml (*T. gazenzis*) and 4.7 mg/*ml (T. prunoides*) in this study.

Eloff (1999b) evaluated the total activity and stability of 27 members of the Combretaceae. The total activity values obtained from the plants in this experiment were much lower than those obtained with previous work on other Combretaceae species (Eloff, 1999b) but were still worth investigating. The activity of the plants was tested against *S. aureus* because of the relative ease of performing bioautography with this organism.

In the bioautography experiments, *T. sericea* showed two distinctive areas of bacterial growth inhibition worthy of further investigation, the R_f values of 0.55 and 0.75 in a CEF/vanillin system, in addition to substantial antibacterial activity in the polar components (Fig 3.2). Gallic acid had a R_f value of 0.57 and appears not to be the compound that has the major antibacterial activity in this case.

There was substantial antibacterial activity in the polar fractions, which correlated with the results from Chapter 2 where ethanol, a relatively polar solvent extracts had the highest total antibacterial activity but the water extract had very low antibacterial activity. The average total activity of *T. sericea* against all four test organisms was 50 ml, compared to 51 ml for *T. prunoides* and 75 ml for *T. phanerophlebia*.

Chapter 4.

Isolation and characterization of antibacterial compounds from *Terminalia sericea* leaves

4.1 Problem statement and aim

After having found the best extractant and selected *T.sericea* as the plant of choice, the aim in this chapter was to isolate the antibacterial compound(s) using bioassay-guided fractionation and to characterize the isolated compounds.

4.2 Materials and Methods

4.2.1 Extraction

The plant material was extracted by placing 500 g finely powdered leaf material in c. 2 *L* of acetone to fully immerse and wet the plant powder in a 2.5 *L* glass container with a closeable lid. The container was then vigorously shaken for 30 minutes on a Labotec shaking machine. The mixture was left to settle and the clear liquid decanted and filtered through a Büchner funnel into a clean container. The process was repeated three times on the same plant material and the filtrate combined. The extract was reduced to dryness using a rotary evaporator (Büchi, Germany) under reduced pressure at 50°C.

4.2.2 Group separation

The solid and liquid phases prior to liquid-liquid extraction was separated by vacuum filtration and centrifugation to ensure that no particulate matter was introduced in the separatory funnels.

Separation was undertaken with immiscible solvents to fractionate compounds with different polarities. The method employed was that developed by the National Cancer Institute and applied in the analysis of *Anthocleista grandiflora* by Eloff (1998a).

The group separation process followed is described below and illustrated in Figure 4.1.

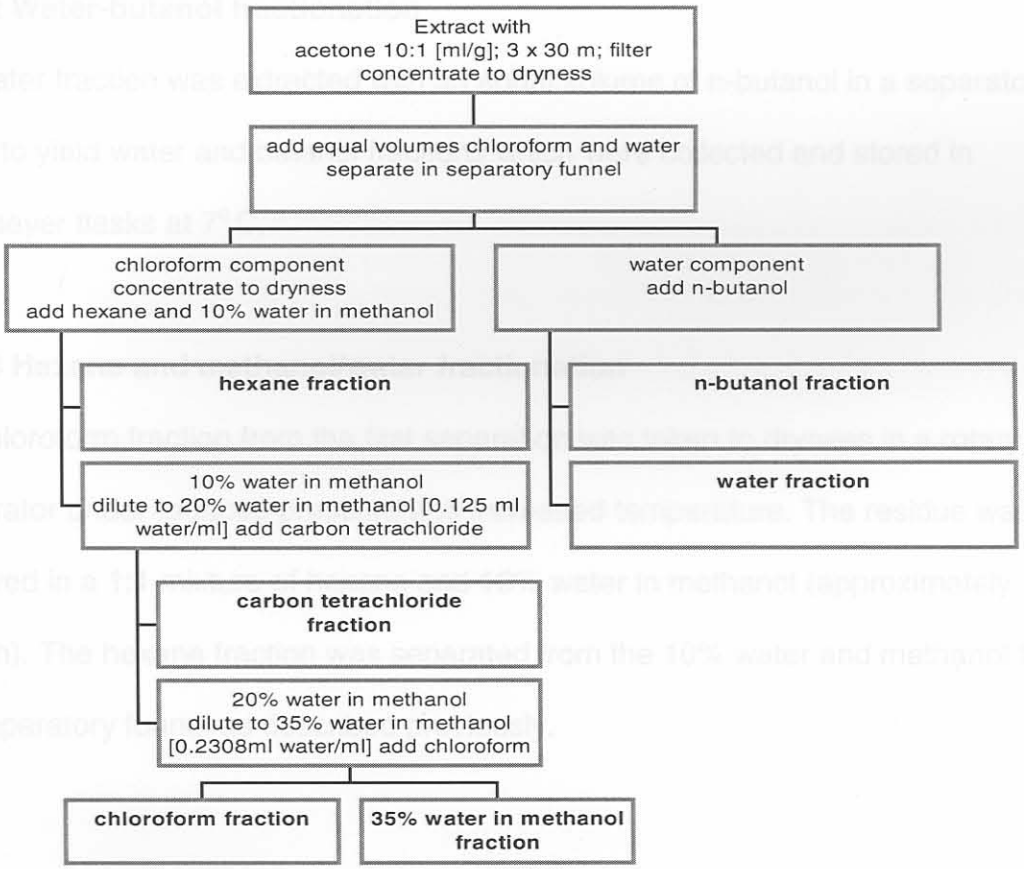

Fig. 4.1. Solvent-solvent fractionation process

4.2.2.1. Chloroform-water fractionation

Equal parts of chloroform (added first) and water was added to the 24.35 g of dried acetone extract. The minimum volume (about 100 ml) necessary to dissolve the extract was used, and after thorough mixing, the contents were poured into a separation funnel. Care was taken not to shake the contents excessively to avoid emulsions forming. The components were left to separate, resulting in the water fraction on top and the heavier chloroform fraction at the bottom. The fractions were collected separately in Erlenmeyer flasks that were then sealed with aluminium foil.

4.2.2.2 Water-butanol fractionation

The water fraction was extracted with an equal volume of n-butanol in a separatory funnel to yield water and butanol fractions which were collected and stored in Erlenmeyer flasks at 7° C.

4.2.2.3 Hexane and methanol/water fractionation

The chloroform fraction from the first separation was taken to dryness in a rotary evaporator under reduced pressure and increased temperature. The residue was then dissolved in a 1:1 mixture of hexane and 10% water in methanol (approximately 100 ml of each). The hexane fraction was separated from the 10% water and methanol fraction in a separatory funnel as described previously.

4.2.2.4 Carbon tetrachloride and methanol/water fractionation

The 10% water in methanol fraction was placed in a separatory funnel and further diluted to 20% water in methanol by adding 0.125 ml of water for every ml of the 10% water in methanol fraction. An equal volume of carbon tetrachloride was then added to obtain a carbon tetrachloride fraction and a methanol/water fraction after separation.

4.2.2.5 Chloroform and methanol/water fractionation

The 20% water in methanol fraction was further diluted to 35% water in methanol by adding 0.23 ml of water for every ml of the 20% water in methanol fraction. An equal volume of chloroform was added to the mixture, which was then partitioned to obtain a chloroform fraction and a 35% water in methanol fraction.

In all of the above separation procedures, the upper phase was re-extracted with fresh lower phase solvent two or three times, using about 10% of the original lower-phase volume, to ensure adequate separation. The fractions resulting from the solvent-solvent extraction process were concentrated by rotary evaporation. Assaying the components was the next important step in identifying the antibacterial active fractions. Following this, the extracts were subjected to TLC as described in 2.2.3 and bioautography to localize the antibacterial activity.

4.2.3 Vacuum liquid chromatography (VLC)

I decided to investigate VLC for large-scale preliminary fractionation of extracts as scaling up solvent-solvent extraction is labour-intensive and requires large quantities of solvents. The VLC served as a crude fractionation process beginning the search for single or "pure" components.

Antibacterial activity would be established by bioautography and MIC determination. A schematic summary of the procedure to be followed is shown in figure 4.2

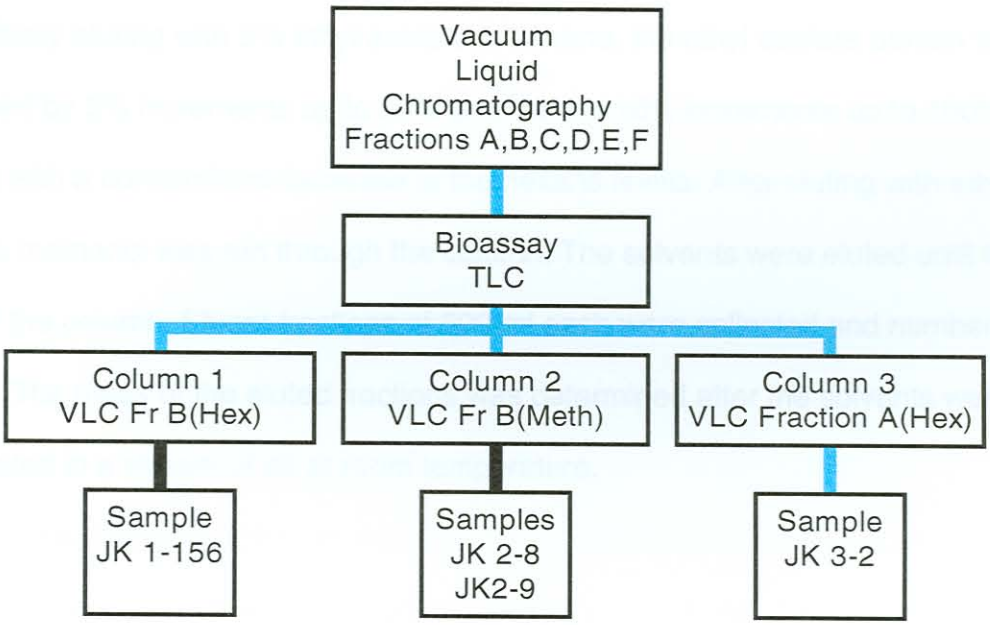

Fig. 4.2. Schematic summary of the fractionation process followed to obtain the final fractions for structure elucidation

One kilogram of finely ground *T.sericea* leaves was extracted in 2.5 L acetone by maceration overnight. The extract was dried in a Büchi rotary evaporator under reduced pressure to yield 149.75 g of residue. One hundred grams of this crude material was mixed with as small an amount as possible of silica gel (Merck) to form a dry slurry, which was then loaded onto a VLC column (diameter 9.5 cm and length 20 cm) filled with silica gel as stationary phase.

The column was eluted stepwise under vacuum with solvents of increasing polarity, ranging from a mixture of hexane and ethyl acetate, to pure ethyl acetate. To elaborate, after initially eluting with 5% ethyl acetate in hexane, the ethyl acetate portion was increased by 5% increments up to 50% and then in 10% increments up to 100% ethyl acetate with a concomitant decrease in the hexane levels. After eluting with ethyl acetate, methanol was run through the column. The solvents were eluted until it ran clear of the column. Eluant fractions of 500 ml each were collected and numbered from 1 to 37. The mass of the eluted fractions was determined after the solvents were evaporated in a stream of air at room temperature.

4.2.4 TLC and bioautography on VLC fractions

The fractions obtained from the VLC were analyzed by TLC, and fractions with a similar profile were combined. Aliquots of these fractions were reconstituted in acetone and analyzed for antibacterial activity using bioautography only as the initial search was for

single compounds with antibacterial activity. MIC would be determined once pure compounds were isolated. Machery-Nagel silica gel TLC plates were used to analyze the VLC fractions. The non-polar fractions (1 – 6) were separated with BEA, the samples with intermediate polarity (7 – 12) with CEF, and the most polar fractions (13 – 35) with EMW. Five µl of each sample was loaded on the TLC plates using the method described earlier. After development, the TLC plates were examined under UV light before spraying with vanillin spray reagent in an effort to detect the maximum number of compounds in each fraction.

Bioautography (as described in section 3.2.3) was performed using *Staphylococcus aureus* as test organism to indicate the antibacterial activity of the separated components. (Fig.4.3). The solvent system used was BEA, and the method as described, was followed. Antibacterial activity would be investigated by means of bioautography and MIC determination. A schematic summary of the procedure to be followed is shown in figure 4.2 as described previously.

Owing to the similarity of their composition determined by TLC analysis, fractions 1 and 2, fractions 3 to 13, fractions 15 to 20, fractions 24 and 25, fractions 26 to 29 as well as fractions 30 to 35 were combined in fractions A to F respectively. The masses of the combined fractions were determined to explore whether the selected fractions would be suitable for further investigation by column chromatography. Based on the results found with VLC I continued with these fractions only. The fractions obtained with solvent-solvent fractionation were not further investigated.

4.2.5 Column chromatography

Column chromatography was initially performed on sample B (VLC fractions 3 – 13) due to its high antibacterial activity and because of the apparently simple nature of the components as seen in TLC. Most of sample B was dissolved in methanol, and a small insoluble portion that remained was dissolved in hexane. Of the original 100 g "crude" extract, 52.6 g was used for the column. It was dissolved in 100 ml equal parts of hexane and chloroform in an ultrasonic bath until a soft paste was formed. The column was wet packed by suspending c. 250 g silica in hexane and pouring it into the column and leaving it to settle in a vertical position to prevent cracks and unevenness in the column. A problem arose that the paste was too sticky for the eluant to move it through the column and alternative ways and means were to be investigated to overcome the problem. The fraction was removed and dissolved in methanol and separated in column 2 and the rest that did not dissolve was dissolved in hexane and separated in column 1.

4.2.5.1 Column 1

This column was run using the hexane-soluble portion of sample B (0.101 gram). The column was coupled to an automatic fraction sampler (Isco Foxy Jr.) and standardised to 600 drops per fraction (c. 2ml/min) and 167 fractions were collected. The column was developed starting of with a 100% hexane as eluant (non-polar) and 5 % increments of dichloromethane (DCM) (more polar) was added while the hexane was incrementally lowered. The eluant mixture was run through the column each time until it was clear before the next increment would be used.

4.2.5.2 Column 2

Column 2 was run using the methanol-soluble component of sample B (1.495gram) in the same manner as that of column 1 and 187 fractions was collected.

Once single compounds were detected by TLC (Fig. 4.5) in various solvent systems, MIC values were determined for the isolated compounds. Bioautography was also performed using *S. aureus* as the test for bacterial species (Fig 4.3).

4.2.5.3 Column 3

Sample A (VLC fractions 1 – 2) was used to run a third column. Sample A was dissolved in 100 ml of equal parts of chloroform and hexane. The column was eluted using an initial mixture of 20% dichloromethane (DCM) and 80% hexane, gradually changing the concentration in 5% increments to 25% DCM: 75% hexane mixture etc. The column was finally eluted with a 30:70 DCM:hexane mixture each time until the eluant ran clear. The column was coupled to an automatic fraction sampler (Isco Foxy Jr.) and standardised to 600 drops per fraction (c. 2 ml/min). Fractions 1 – 26 were collected from the 15:85 ratio of DCM:hexane eluant mixture. From fraction 27 onwards the 20:80 mixture of DCM:hexane was used.

TLC using BEA, CEF and EMW was performed on every fifth sample to screen the compounds in the different fractions (Fig.4.6). The TLC plates were run in three stages (run for a third of the length of the plate, left to dry, run for another third of the plate and

the process repeated for the last third). This method was employed to enhance the separation of polar components on the plate.

Fractions 2 and 3 were combined to produce a sample containing a compound that appeared to be pure. The microplate dilution assay to obtain a MIC value was performed on this isolated compound which was named JK 3-2. The original crude acetone-dissolved fraction was also tested for its antibacterial activity (microplate dilution assay) in order to investigate the practical use of an acetone extract of the leaf material for use by rural people. A standard reference aminoglycoside antibiotic, neomycin, was used as a positive control to compare the MIC values obtained.

4.2.6 Chemical characterization of isolated compounds

Nuclear magnetic resonance (NMR) spectroscopy is a useful tool in elucidating chemical structures. ^{1}H- and ^{13}C- NMR spectra were obtained at the Medical University of South Africa (Medunsa) on a 300 MHz Varian NMR Machine (Oxford Instruments). Mass Spectrometry (MS) was performed on a VG70SEQ instrument at the Cape Technikon.

Samples JK 1-156, JK 2 – 8, JK 2 – 9, JK 2 – 12 were not pure enough according to NMR spectra for structure elucidation. Only sample JK 3 – 2 was found to be sufficiently pure to enable the elucidation of its structure (Fig 4.5).

4.3 Results and Discussion

4.3.1 Extraction and MIC values

One kilogram of ground *T. sericea* leaves yielded 149.75g of acetone extract. The 15% yield compares well with other data. (Eloff, 1998a)

From this a 100 mg was separated by solvent-solvent fractionation and the MIC values were determined as in table 4.1.

Table 4.1. MIC's of the solvent fractions in mg/ml

Solvent	Mass(mg)	MIC	TA
Butanol	19	0.12	158.
Water	44	1.00	44
Hexane	4	0.08	50
Carbon tetrachloride	5	0.18	278
Chloroform	14	0.50	28
35%Wat/MeOH	5	0.18	28

Hexane had the highest antibacterial activity and lowest MIC thus would be the best fraction for searching for single compounds with high antibacterial activity. Although it extracted the least mass (4 g), it had a high total activity. The largest mass was collected by the water fraction but it had the lowest antibacterial and second lowest total activity. The carbon tetrachloride fraction had the highest total activity. To determine if the same antibacterial compounds occur in the same fractions, bioautography was carried our on the fractions.

4.3.2 Bioautography

Bioautograms of the solvent extraction fractions are depicted in Figure 4.3.

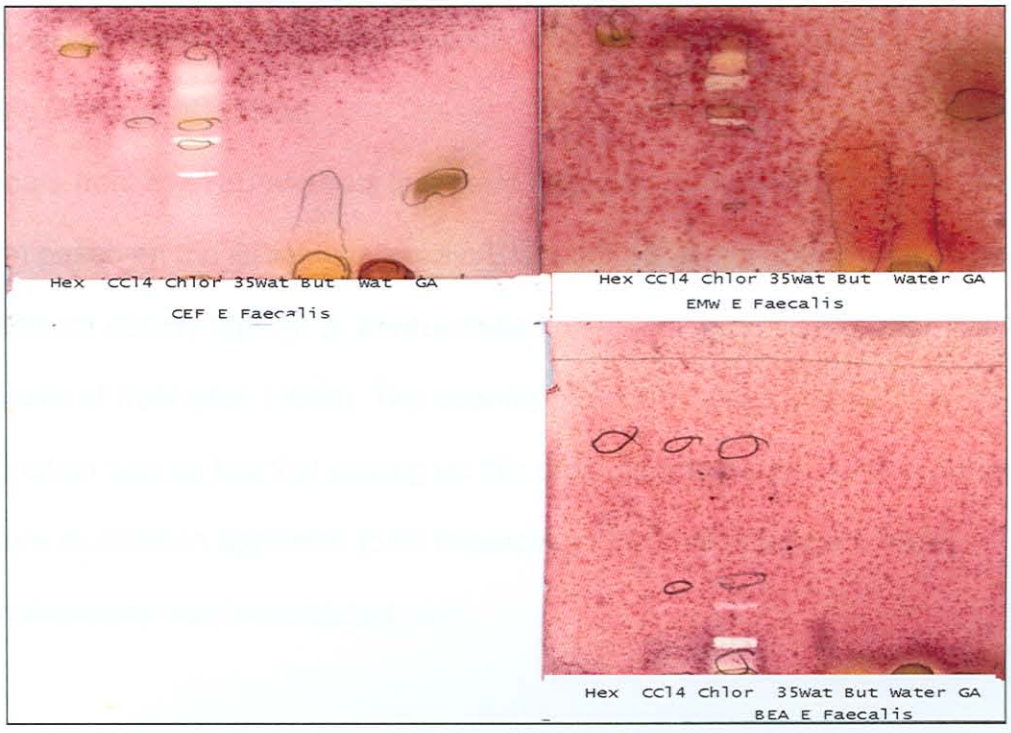

Fig. 4.3. Bioautograms of the solvent-solvent extraction fractions after TLC using different solvent systems (CEF, EMW and BEA) and spraying with *E. faecalis;* encircled areas identify fluorescent compounds where Hex = Hexane, CCL4= carbon tetrachloride, Chlor= chloroform, 35Wat = 35% water in methanol, But= butanol, Water = water fractions and GA= gallic acid.

It was surprising that the hexane fraction, which had the lowest MIC value (Table 4.1) did not have any antibacterial bands in the bio-autography. (Fig.4.3). The compound responsible for the low MIC may have been sufficiently volatile to have evaporated from the chromatogram during the drying stage prior to spraying with the test organism.

The chloroform fraction showed the highest number of antibacterial compounds (at least 3) with R_f values of 0.57, 0.71 and 0.85 and would therefore a promising fraction to continue with bioactive compound isolation and identification. Gallic acid was used as a reference compound as it is an anti-cancer and antibacterial compound found in *T. chebula* (Pettit *et al.*, (1988).

It appears from the TLC analysis that gallic acid may be present in the chloroform fraction based on the R_f value in vanillin/EMW but it was not one of the compounds with antibacterial activity against *S. aureus* based on autobiography (Fig.3.2). This questions the results of Petit *et al.* (1988). The quantity of material produced by liquid-liquid fractionation was so low that scaling up this procedure to isolate sufficient quantities for structure elucidation appeared to be impractical. The use of vacuum liquid chromatography was investigated next.

4.3.3 Vacuum Liquid Chromatography (VLC)

The mass of the different fractions obtained from the vacuum liquid chromatography procedure using the eluants ethyl acetate and hexane in a decreasing ratio varied from 0.183 to 1.342 g. (Table 4.2)

Table 4.2 Mass (g) of residue from different fractions of the ethyl acetate/hexane vacuum liquid chromatography fractionation process

FRACTION NUMBER	ETHYL AC/HEXANE RATIO	MASS (g)
1,2,3	0/100	0.632
4	5/95	0.599
5	10/90	0.291
6	15/85	0.183
7	20/80	0.605
8	25/75	1.392
9	30/70	0.679
10	35/65	0.685
11	40/60	0.787
12	45/55	0.438
13	50/50	0.29
14	60/40	0.171
15	70/30	0.159
16	80/20	0.25
17	90./100	10.075
18	100	0.58
Total		17.816

Table 4.3. Mass (g) obtained from fractions after VLC column eluted with ethyl acetate/methanol

FRACTION NUMBER	ETHYL ACETATE/ METHANOL RATIO	MASS (g)
19	100/0	0.394
20	95/5	0.209
21	90/10	0.167
22	85/15	1.131
23	80/20	1.832
24	75/25	2.006
25	70/30	1.731
26	65/35	1.479
27	60/40	0.871
28	55/45	0.709
29	50/50	0.554
30	45/55	0.391
31	40/60	0.369
32	30/70	0.333
33	20/80	0.249
34	10/90	0.224
35	0/100 – 1	0.091
36	0/100 – 2	0.272
37	0/100 – 3	0.119
Total mass		13.131

The mass of residue resulting from subsequent elution with ethyl acetate and methanol is shown in Table 4.2.The total mass recovered (Table 4.2 and 4.3) was 30.947 g of the original c 5225 g that was placed on the columns. Therefore only 58.8 % was recovered which means that 41.2% was possibly retained in the column or was volatile enough to have been lost during drying.

4.3.4 TLC and bioautography on VLC fractions

The fractions analyzed by TLC plates using CEF or EMW as eluants revealed up to three compounds present at high concentrations. No further compounds could be seen under UV light at 254 or 360 nm. Fractions 4 and 5 (eluted with BEA) yielded only one compound each. Fractions 1-3 contained several components. Fractions 4 to 22 showed only one component clearly visible under UV light at 254 nm. Fractions 23 – 35 also showed one component only whereas fractions 30 - 35 displayed multiple components. Bioautography revealed that there were quite a number of compounds with antibacterial activity against *S. aureus* (Figure 4.4). The most active components appeared to have intermediate polarity because they were moved from the origin by the highly non-polar eluant BEA.

Upon partial drying of the VLC fractions, crystals formed in the test tubes of fractions 4 and 5. The crystallization indicated the presence of a relatively pure compound in each of these fractions. In an attempt to further purify the crystals that formed, it was rinsed with hexane to remove possibly highly non-polar contaminants. The hexane rinse containing non-polar minor components was labelled A, e.g. 29A and 34A in Fig 4.4. The remaining crystals were labelled B e.g. 29B.

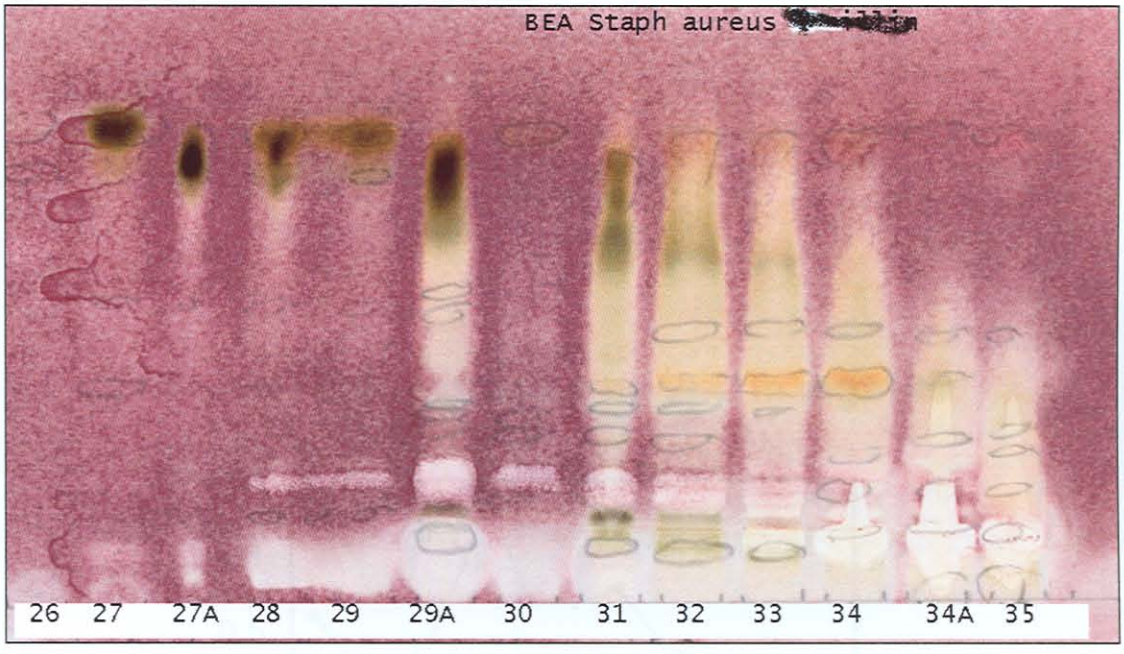

Fig. 4.4. Bioautography plate showing clear antibacterial zones of fractions collected after VLC using *S. aureus* as the test organism and BEA as the eluant. Encircled areas indicate fluorescent compounds.

Based on the TLC analysis, fractions were pooled to group fractions with similar chemical composition. It was decided to combine fractions 1 and 2(A), fractions 3 to 13(B), 15 to 20(C), 24 and 25(D), 26 to 29(E), as well as 30 to 35(F) because of the

similarity in their appearance on the TLC plate. (A indicates a hexane soluble fraction). Pooled fractions that were the most promising to use for isolating antibacterial compounds were subjected to further column chromatography using longer and narrower columns.

Table 4.4. Mass (mg) of pooled fractions resulting from Vacuum Liquid Chromatography

SAMPLE	COMBINED VLC FRACTIONS	TOTAL MASS (mg)
A	1 – 2	30
B	3 – 13	100
C	15 – 20	32
D	24 – 25	14
E	26 – 29	33
F	30 – 35	3

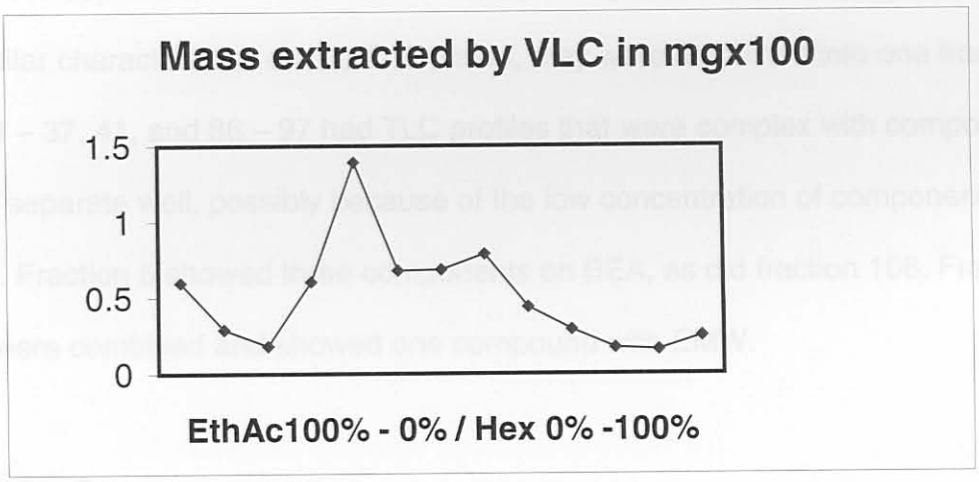

Fig.4.5 Mass (in mg x 100) of different fractions separated with gradients of ethylacetate/hexane ranging from a 100% ethylacetate in 0% hexane up to 0% ethylacetate in 100% hexane

4.3.5 Column chromatography

As discussed in section 4.2.5, the viscosity of the pooled VLC fractions was so high that it caused problems in the chromatography. Sample B of the VLC column was subsequently split into a hexane soluble fraction and fractionated in column 1. The balance that did not dissolve in hexane was dissolved in methanol and fractionated in column 2. Sample A of the VLC extraction was fractionated in column 3. These samples were selected because of the relatively simple chemical profile as well as the antibacterial activity it possessed.

4.3.5.1 Column 1

Selected fractions were analysed by TLC and, if found to possess only a single constituent, the adjacent fractions were also analyzed by TLC. Where adjacent fractions showed similar characteristics on the TLC plates, they were combined into one fraction. Samples 34 – 37, 41, and 86 – 97 had TLC profiles that were complex with compounds that did not separate well, possibly because of the low concentration of components in the fraction. Fraction 5 showed three components on BEA, as did fraction 108. Fractions 156 – 161 were combined and showed one compound with EMW.

4.3.5.2 Column 2

Fractions 00 – 7 were combined on the basis of their similarity on the CEF chromatogram. Fractions 8 – 14 were also combined, as were fractions 23 – 29, 30 – 41, 58 – 72 and 81 – 105. Fractions 106 – 181 were very polar and difficult to elute. A 20% methanol in chloroform mixture was used but even that showed poor results so it

was decided not to continue with these fractions, as their highly polar characteristics would have made them difficult to work with. The 10% water: 90% ethanol mixture as eluant showed some components. The TLC profile of fractions 81 – 105 is presented in Figure 4.5.

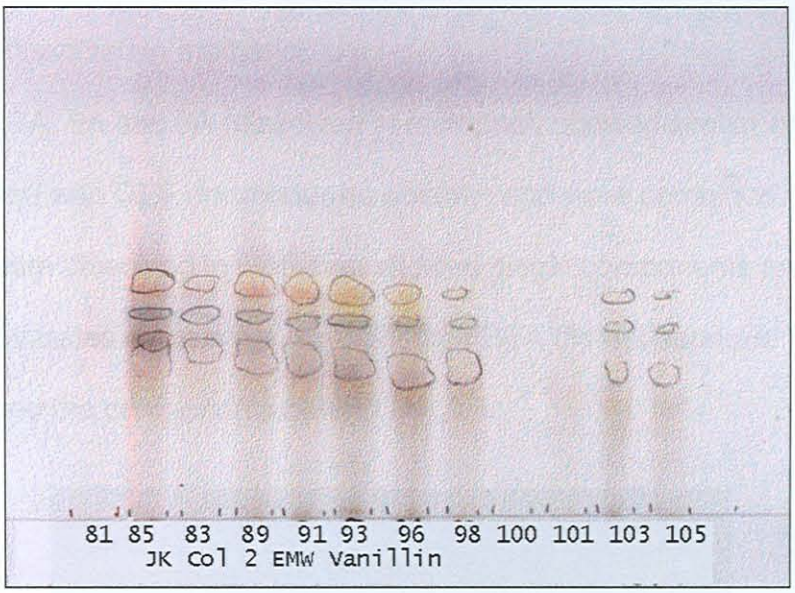

Fig. 4.6. TLC plate (sprayed with vanillin) presenting the complexity of fraction components eluted from fractions from column 2 using EMW as eluant. Encircled areas indicate fluorescent compounds under UV light

4.3.5.3 Column 3

Promising results were obtained with the third column separation in that some fractions showed only one component (for example fraction 52 which displayed a compound under 360 nm UV light). Fractions showing similar components were combined

In the TLC analysis of the initial column fractions, fractions 2 and 3 showed a single component (this was to be the fraction used for the final isolation and testing of compounds). Fraction 2 and 3 that were combined yielded a mass of 0.107g.

These fractions dissolved in hexane while an "A" fraction (the part that was insoluble in hexane) was dissolved in methanol.

Fractions 6A, 7A, 8A and 9A (dissolved in methanol) showed similar characteristics when separated with CEF (for moderate polarity) and were combined. Fractions 3A, 6A and 7A that were dissolved in methanol showed single components on TLC when eluted with EMW (separates more polar components). BEA did not separate fractions-33 – 37. EMW separated the components better.

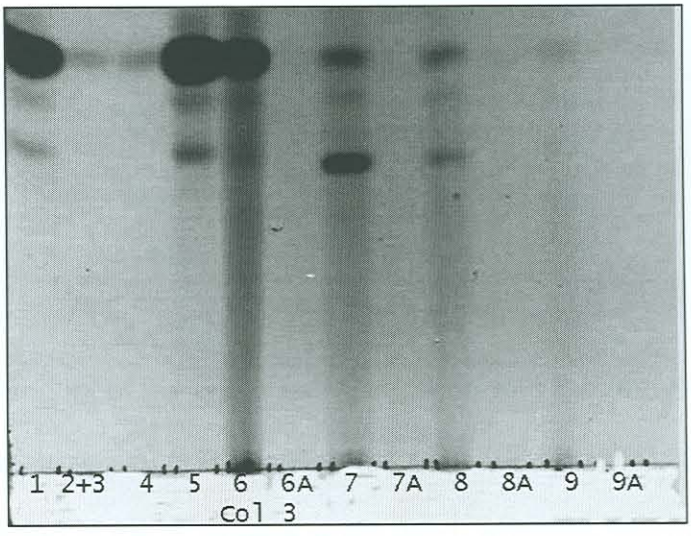

Fig. 4.7. Chromatogram (sprayed with vanillin) of fractions obtained from Column 3 by TLC with BEA as eluant and vanillin as spray reagent.

There was a single component present in fractions 2+3 (R_f value of 0.85). Much higher concentrations of a compound with a similar R_f value were present in fraction 4, but this fraction had a high concentration of contaminant. This compound was not the compound that inhibited growth of *E. faecalis.* (Fig. 4.3 c).

The MIC values obtained in the microplate dilution assay for the crude acetone extract and the isolated compound JK3-2 (Johann Kruger column 3, sample 2) are reported in Table 4.5.

Table 4.5. MIC values of the isolated compound and crude extract of *T.sericea*, compared to the reference antibiotic neomycin

COMPOUND	MIC (mg/ml)
JK3-2	0.33 mg/ml
Crude extract	1.56 mg/ml
Neomycin	0.003 mg/ml

The MIC of 1.56 mg/ml of the crude extract agreed with the results of Eloff (1999b) of 1.7 mg/ml for the same plant. The isolate (JK 3-2) at a MIC of 0.33 mg/ml showed reasonable inhibition of the *S. aureus* strain used, whereas neomycin had an MIC of 0.003 mg/ml.

Although inhibition of JK3-2 occurred at almost 100 times the concentration of the positive control, cost and availability factors indicate that both the crude extract and the isolated compound could possibly be useful as antibacterial remedies for use by rural

people. NMR spectra of apparently "pure" compounds – JK1-156, JK2-8, JK2-9 and JK2-12 indicated that these compounds more were complex than it appeared on the TLC plate and were not investigated further because only very small quantities were available. It is clear that there are more active compounds present in some of the other fractions (Fig. 4.7). This specific fraction was chosen because it appeared to be a single compound that could be isolated and identified. Further studies will have to be done on the other antibacterial compounds to determine their structure and characteristics.

4.3.5.4 Summary of column chromatography results

The origins of the different fractions obtained are presented in table 4.6.

Table 4.6 Sample identification and the fractions and columns from which it was taken.

Column from which sample was taken	Fractions from column that sample was taken from to be pooled	Sample ID
Column 1	Fractions 156 – 161	Sample JK 1-156
Column 2	Fraction 8	Sample JK 2 – 8
	Fractions 9 – 11	Sample JK 2 – 9
	Fraction 12	Sample JK 2 – 12
Column 3	Fraction 2 – 3	Sample JK 3 – 2

4.3.6 Chemical characterization of isolated compounds

4.3.6.1 Mass spectrometry data

Fast Atom Bombardment Mass Spectrometry (FABMS) gave a molecular ion at m/z 488 (2.5%) and a molecular formula of $C_{30}H_{48}O_5$ was deduced from this. Other prominent peaks were at *m/z* 470 (2%) $[M - H_2O]^+$, *m/z* 452 (6%) $[M - 2H_2O]^+$, *m/z* 442 (12.5%) $[M - HCOOH]^+$, *m/z* 248 (91%) $[M - C_{14}H_{24}0_3]^+$, *m/z* 249 (26.6%) $[M - C_{14}H_{23}O_3]^+$, *m/z* 203 (100%) $[M - C_{15}H_{23}O_5]^+$.

These fragments were strongly suggestive of a hydroxylated pentacyclic triterpene. There is an initial loss of water, then a Wagner-Meerwein rearrangement with a subsequent loss of another molecule of water (Geissman, 1959). The rearranged fragment then typically undergoes a retro-Diels-Alder (RDA) fragmentation (Djerassi *et al*, 1962; Karliner and Djerassi, 1966) (Fig. 4.9).

4.3.6.2 NMR data

The result of nuclear magnetic resonance spectroscopy experiment is presented in Fig.4.8

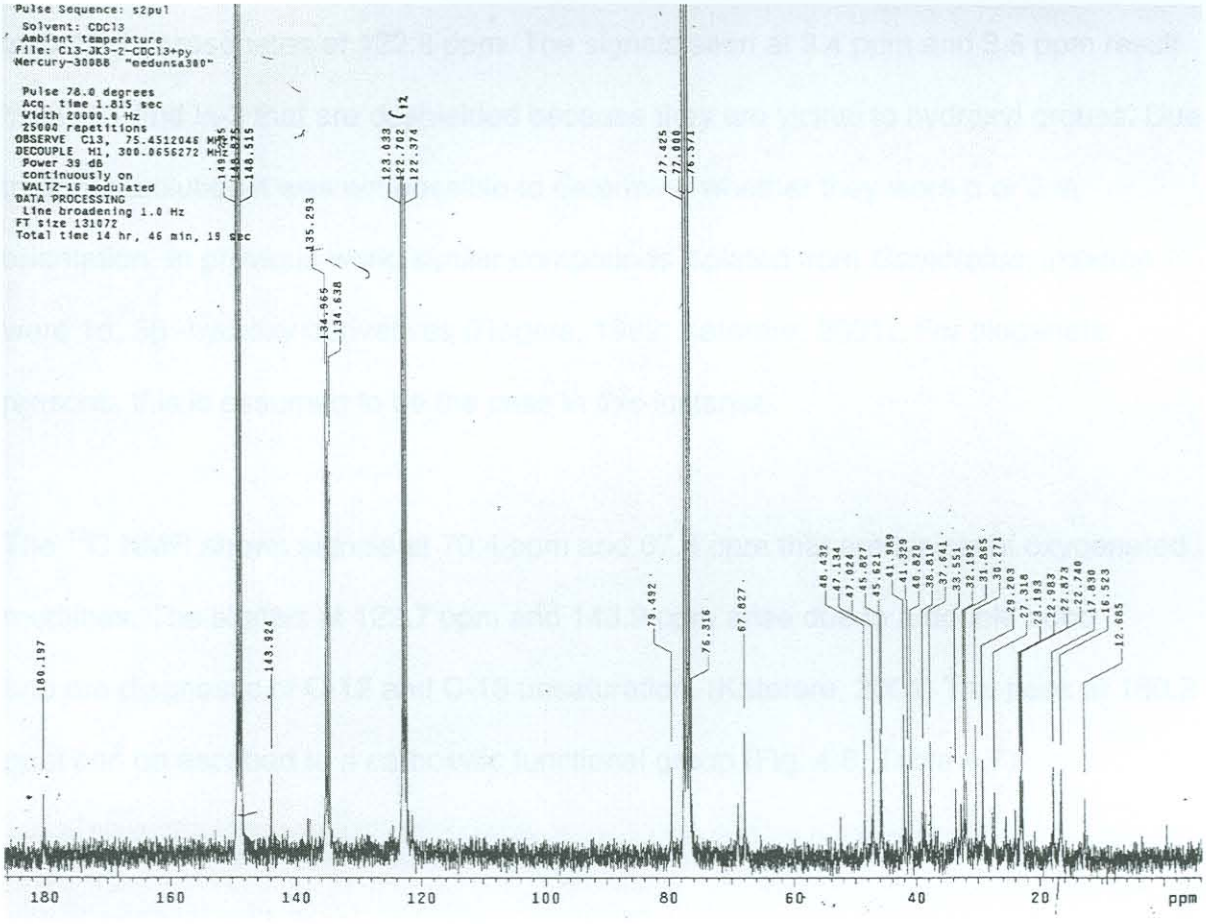

Fig. 4.8. An example of ^{1}H- and ^{13}C- NMR spectra of JK 3-2

The ^{1}H-NMR spectrum shows numerous peaks between 0.7 and 1.3 ppm. This is typical of methyl signals in a triterpenoid skeleton. There are seven methyl signals in this region. The carbon spectrum appears to confirm this.

A broad singlet occurs at 5.3 ppm and this is due to an olefinic proton. This is attached to C-12 that resonates at 122.8 ppm. The signals seen at 3.4 ppm and 3.6 ppm result from H-1 and H-3 that are deshielded because they are vicinal to hydroxyl groups. Due to poor resolution it was not possible to determine whether they were α or β in orientation. In previous work, similar compounds isolated from *Combretum imberbe* were 1α, 3β -hydroxy derivatives (Rogers, 1989; Katerere, 2001). For biogenetic reasons, this is assumed to be the case in this instance.

The ^{13}C NMR shows signals at 70.4 ppm and 67.8 ppm that are typical of oxygenated methines. The signals at 122.7 ppm and 143.9 ppm arise due to a double bond and are diagnostic of C-12 and C-13 unsaturation. (Katerere, 2001) The peak at 180.2 ppm can be ascribed to a carboxylic functional group (Fig. 4.8, Table 4.7).

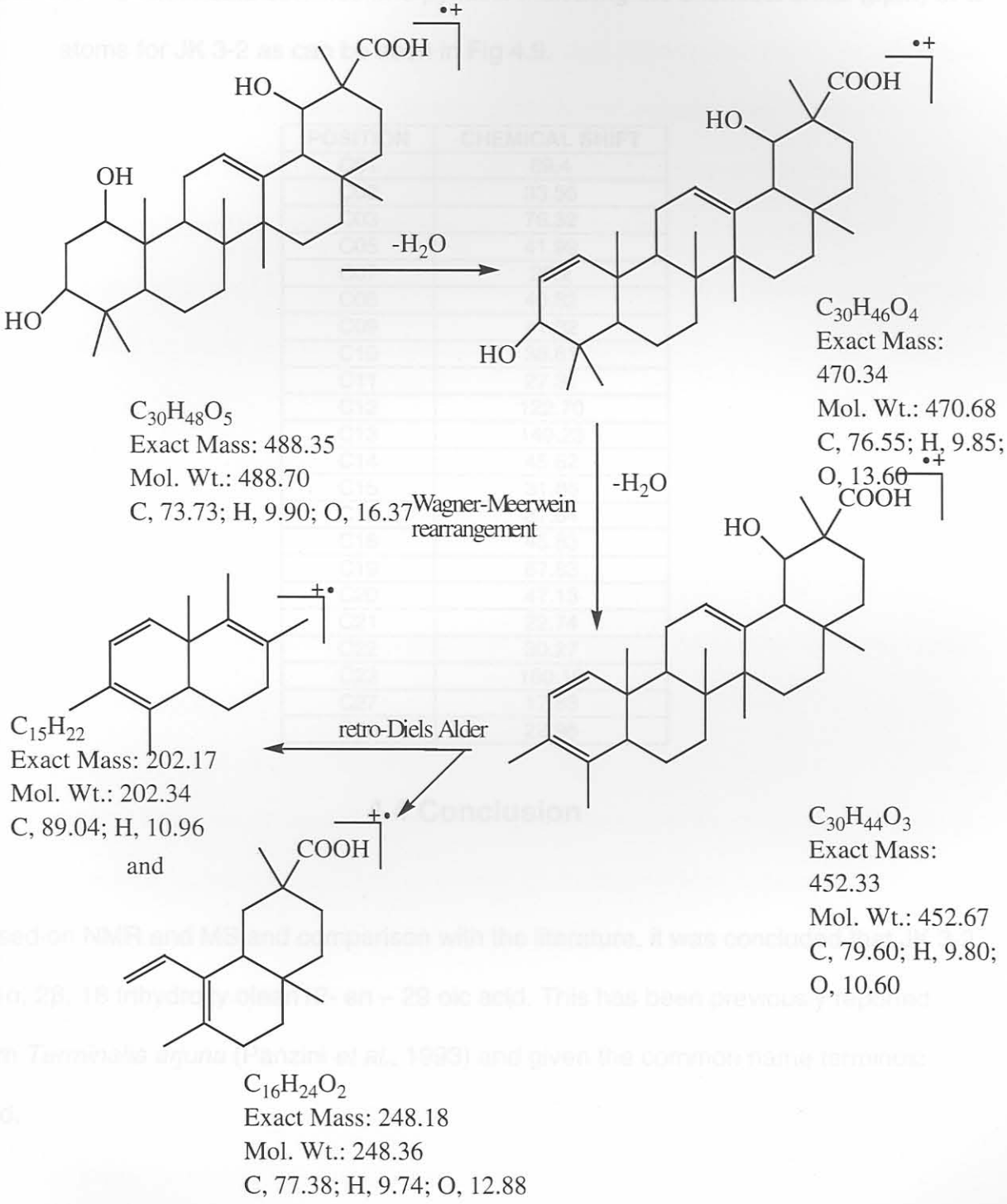

Fig. 4.9. The suggested fragmentation pattern of JK 3-2 (typical of a pentacyclic triterpenoid)

Table 4.7. ^{13}C NMR data obtained in d-pyridine indicating the chemical shifts (ppm) of C atoms for JK 3-2 as can be seen in Fig 4.9.

POSITION	CHEMICAL SHIFT
C01	69.4
C02	33.55
C03	76.32
C05	41.99
C07	29.2
C08	40.82
C09	41.32
C10	38.81
C11	27.31
C12	122.70
C13	149.23
C14	45.62
C15	31.86
C16	37.64
C18	45.83
C19	67.83
C20	47.13
C21	22.74
C22	30.27
C23	180.19
C27	17.83
C30	22.98

4.4 Conclusion

Based on NMR and MS and comparison with the literature, it was concluded that JK 3-2 is 1α, 2β, 18 trihydroxy olean12- en – 29 oic acid. This has been previously reported from *Terminalia arjuna* (Panzini *et al.,* 1993) and given the common name terminoic acid.

Triterpenoids have been attractive phytochemicals for research, not so much perhaps for their commercial or therapeutic importance, but for the relative ease with which they are available and amenable to purification (Mahato and Sen, 1997). NMR spectroscopy

is now almost invariably used for confirmation of a proposed structure by the assignment of a ^{13}C or ^{1}H spectrum, usually supported by NMR data from related model compounds. Triterpenoids, especially the pentacyclic triterpenoids that possess five fused rings and eight chiral centres, have attracted the attention of synthetic chemists for several decades (Mahato and Sen, 1997). Djerassi *et al.* reported in 1963 the mass fragmentation patterns of pentacyclic triterpenoids belonging to the oleanane and ursane classes as well as some rearranged oleananes and ursanes.

Triterpenoids are lipid-soluble secondary metabolites, which are biosynthetically derived from isoprenoid units. There are at least five classes of importance in the growth, metabolism and ecology of plants (Harborne, 1973). Triterpenoids are the most ubiquitous non-steroidal secondary metabolites in terrestrial and marine flora and fauna (Mahato *et al.,* 1992).

This is the first time that an olean12- en – 29-oic acid has been reported from *T. sericea.* The triterpenoids isolated from *Combretum* spp. of southern Africa appear to confirm close biogenetic, chemotaxonomic relationships. (Rogers and Subramony, 1988). Katerere (2001) also proposed that the presence of 1α, 2β-dihydroxy pentacyclic triterpenoidal acids in *T. stuhlmanii* similar to those from some *Combretum* species confirms a close relationship with *Terminalia* species. This study provides further chemical evidence of the taxonomic proximity of the two genera.

Chapter 5

Biological activity of the extracts in test animals

5.1 Problem statement and aim of the exercise

As seen in the results thus far, *Terminalia sericea* had reasonable antibacterial activity against *Staphylococcus aureus*, so it was decided to test both the crude extract as discussed in 4.2.3 and terminoic acid isolated from the leaves of *T. sericea* in an animal model. A method had to be developed for efficacy testing against skin infections caused by *S. aureus.*

5.1.1 Introduction

5.1.1.1 Development of the animal model: criteria for investigation

Animal models may be developed in several ways to study antimicrobial agents (Hobson *et al.,* 1968), Sanford, M. *et al* (1967). A few of the methods used thus far are discussed below.

5.1.1.1.1 Expanded flora test

This method can be used to evaluate broad-spectrum antimicrobial activity against large numbers of Gram-positive and Gram-negative organisms introduced by pre-treatment

occlusion i.e. by closing the treated area to inhibit infection by external pathogens. The expansion of flora that normally occurs on the skin of the animal when an occlusive wrap is applied to the skin is inhibited by the introduced antibiotic. A plastic wrap is applied for 48 hours prior to the application of an antimicrobial agent. To be considered effective, a test material should destroy 99 percent of microorganisms. Bacterial counts will be low only if the antibiotic is active against both Gram-positive and Gram-negative organisms.

5.1.1.1.2 Reduction of expanded flora produced by occlusive wrapping of the site

The inhibition of the expanded flora can be observed by application of an antimicrobial agent after a sufficient time-period of occlusion with the wrap. Prevention of expansion of the flora can also be used to test antibacterial activity. The antibacterial agent is applied to the wound or test site and after continued occlusion, the inhibition of expansion of the flora is observed. The antimicrobial agent should eliminate the organism(s) from most lesions cultured within 18 hours after the agent is applied.

5.1.1.1.3 Persistence test

This test determines the reservoir effect of the antimicrobial agent, or its ability to bind to the stratum corneum to give a prolonged effect. In this test, the antimicrobial agent is usually applied three times a day for 3 days. After three days, these areas are occluded for 24 hours and then sampled for bacteria. Occlusion allows bacterial growth in the presence of the antimicrobial agent being tested. The persistence of antimicrobial activity after application on the skin extends the time over which an antibiotic can exert

its effect on the bacterial cell. This test is one way of measuring this characteristic on an animal model.

5.1.1.1.4 Occlusion test

This test primarily estimates the bacteriostatic activity of an antimicrobial agent against Gram-positive microorganisms found on normal skin, showing how well the agent prevents a small number of bacteria from rapidly proliferating. Lesions are produced either by stripping with cellophane tape or by application of ammonium hydroxide, a skin irritant that increases susceptibility to infections, followed by inoculation with pathogenic organisms (usually *staphylococci*) and are then covered by an occlusive wrap. Lesions may be treated with a test compound after inoculation and then inhibition of growth is observed or rapidity of healing is judged.

We decided to use the occlusion test for our *in vivo* experiments. To limit the use of animals as well as to prevent inter animal differential resistance we decided to use each animal as its own positive and negative control as described below.

5.2 Materials and Methods

5.2.1 *In vitro* testing

The sensitivity of four different strains of *S. aureus* was tested as an initial step in order to find a strain that was sensitive to the gentamycin (Garamycin^{R}) standard cream that

was to be used as a positive control. The National Committee for Clinical Laboratory Standards (NCCLS) strains ATCC 25923 (Cowan A), ATCC 25913 and two other available local laboratory strains were tested. The Cowan A strain was selected because it showed the greatest sensitivity in an *in vitro* test by Dr Maryke Henton from the microbiology laboratory at the Agricultural Research Council at Onderstepoort.

The two *T. sericea* test preparations (crude extract (4.2.3) and terminoic acid) as well as a gentamycin cream and gentamycin in an injectable form (Fermentycin^{R}) were evaluated. The cream as well as the injectable form was evaluated to have an alternative dosage form available if one should be unsuitable. Mueller-Hinton agar was used according to the NCCLS guidelines for a disk diffusion method. A form of an *in vivo* antibiogram test, based on NCCLS guidelines was employed. A lawn of *S. aureus* was prepared on the Mueller-Hinton agar. The various preparations (gentamycin cream and injectable form, *T. sericea* crude extract, and terminoic acid) were each placed in a 6 mm well. Amounts of 0.1 ml of each preparation were used in the test. The sensitivity or resistance of *S. aureus* to the preparations was then evaluated. The test was purely meant as a screening test and was performed by Dr Maryke Henton. In all cases the test organisms were sensitive to the different formulations. We consequently decided to use a cream as carrier base for the test material as the positive commercial control was in a cream, as well as the fact that creams are the most practical application for applying topical treatments. A 1% concentration of terminoic acid and a 20% concentration of the crude extract in emulsifying cream (British Pharmacopoeia) were prepared. The test substances were formulated into topical creams by blending using a mortar and pestle.

The positive control was a commercial 0.1% gentamycin cream (Garamycin^{R}) by Schering-Plough (SA).

5.2.2 *In vivo* testing

Rats were used for testing the antimicrobial effect of the *Terminalia* extracts. After promising results with the *in vitro* testing of antibacterial compounds from various *Terminalia* species and *Terminalia sericea* in particular (Eloff, 1999b; Chapter 4), it was decided to conduct an *in vivo* evaluation of the isolated compound as well as a crude extract of the plant. After a few different procedures were tested in preliminary experiments, a procedure was developed. The ethics committee of the Onderstepoort Veterinary Institute (OVI) approved the method and the procedure was carried out under the supervision of veterinarian, Dr. Johan Joubert – head of Toxicology.

The test method was applied to 11 rats supplied, fed and maintained at the Onderstepoort Veterinary Institute of the Agricultural Research Council's (ARC) Department of Toxicology.

The rat hair on the test area was removed by first cutting it with scissors and then shaving the skin with a blade. The area was then sterilised by cleaning it with 70% alcohol. The rats were sedated with a benzodiazepine derivative (Comelin^{R}) and left for 15 minutes for the drug to take effect. Lesions were produced by cutting four roughly circular areas (marked A = crude, B = terminoic acid, C = negative control (no treatment) and D = Positive control; gentamycin) of skin on the back of the rat on both sides with a pair of scissors and introducing the test organism (*Staphylococcus aureus*) onto the test area. The area was covered with an occlusive wrapping (Transpore^{R}) and left to

incubate for 48 hours. After 48 hours, the antimicrobial agents and gentamycin were introduced. The crude extract, terminoic acid (sample JK 3-2) and gentamycin control were applied to the marked sites A, B and C respectively, while D was left to serve as a negative control. Each animal therefore served as a control in itself by having two test sites for the crude and isolated compound, one for a positive control with gentamycin and one site as a negative control. Figure 5.1 shows a photograph of a rat prepared for an experiment. The wound sites were covered after initial digital images were recorded.

The resulting inhibition of growth or healing was quantified on the basis of erythema (red discoloration of wound), exudate (puss) formation and physical size of the lesion on a daily basis for 5 days. Factors such as muscle necrosis, foreign body, skin contamination and variation in number of organisms were taken into consideration as controls. Measuring the size of the lesion or the degree of healing of the lesion was used as one way to determine the antimicrobial activity. On each day, at 08:00 from day 1 to day 5, each infected site on each rat was inspected. The dressing was removed and the different parameters were measured and tabled. Thereafter, the test samples, positive control and new dressings were re-applied to each test rat and the animals were then replaced in their cages.

An arbitrary figure was allocated, as it was difficult to measure the degree of erythema as well as the quantification of the exudate that formed. Subsequently a scale from 1 to 5 was used with one being the lowest degree of erythema or exudate formed and 5 the highest degree of erythema or exudate formed. Quantification was by comparing initial erythema and exudate after incubation and before each treatment.

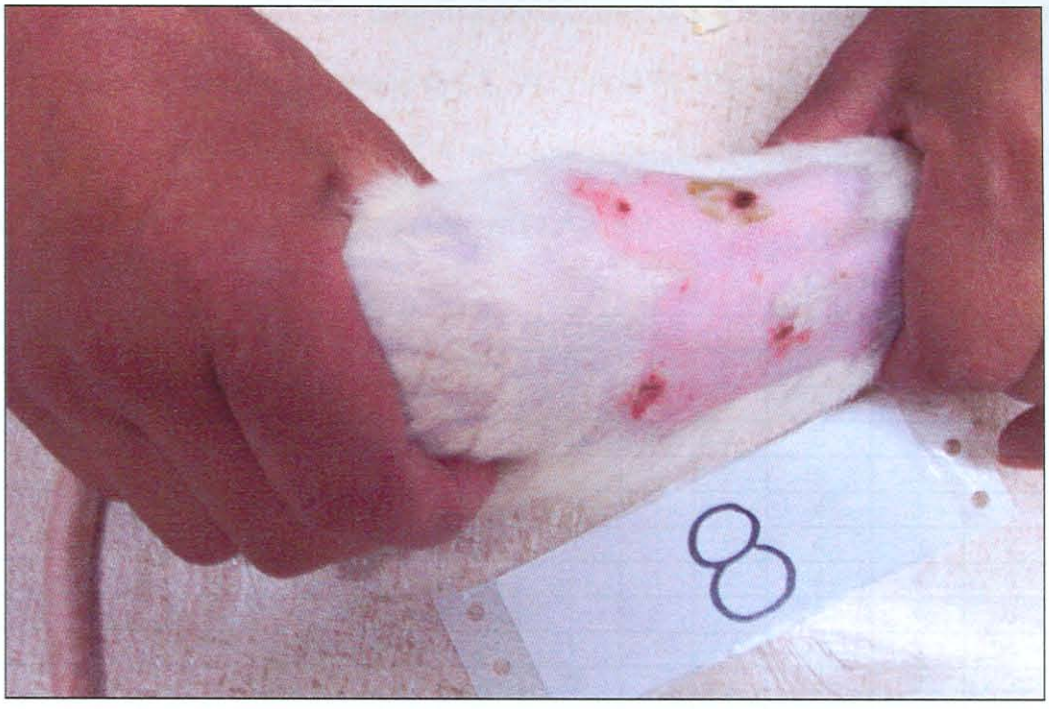

Fig. 5.1. Photograph of back of rat during experiment indicating treatment areas

5.3 Results

In the initial in vitro experiments on the sensitivity of *S. aureus* to gentamycin preparations, *T. sericea* crude extract and terminoic acid, the *S. aureus* strain used was sensitive to all the treatments and formulations.

The results of the *in vivo* rat model experiment are recorded in Table 5.2 and in Figures 5.1 to 5.5.

Table 5.1. Different parameters measured on the 11 rats on the different test sites (A = crude, B = Terminoic acid, C = negative control and D = Positive control (gentamycin)) on 5 consecutive days of the week (M, T, W, T and F). The exudate and erythema were measured on an arbitrary scale of 1-5 with one being the best rate of healing and five the worst while the lesion diameter was measured in mm

DAY	RAT NO	EXUDATE/5				ERYTHEMA/5				LESION SIZE (mm)			
		A	B	C	D	A	B	C	D	A	B	C	D
M	3	2	3	1	2	4	2	2	2	5	3	3	2
T		3	3	3	2	4	2	2	2	5	3	3	2
W		2	3	1	2	4	2	2	2	4	3	2	2
T		1	1	1	2	2	1	1	1	4	3	2	2
F		0	0	0	0	1	0	1	0	4	3	2	2
M	4	3	1	1	1	1	1	1	2	4	4	4	4
T		2	1	3	1	2	1	2	1	5	4	6	7
W		1	1	1	1	2	1	1	1	4	3	4	5
T		1	1	1	1	1	1	1	1	3	2	3	4
F		0	0	0	1	0	0	0	1	3	2	3	4
M	5	3	1	5	3	4	1	5	3	3	2	6	3
T		3	1	5	2	3	1	5	2	4	3	7	2
W		2	1	3	2	2	1	4	2	3	3	6	2
T		1	0	2	1	1	1	1	1	3	2	4	2
F		0	0	1	1	1	1	1	1	3	2	4	2
M	6	2	3	1	5	3	3	2	4	3	5	3	7
T		3	2	1	3	3	2	2	3	5	5	3	4
W		2	1	1	2	2	2	2	2	4	3	2	3
T		1	0	0	0	1	1	1	1	3	2	2	2
F		0	0	0	0	0	0	1	1	2	2	2	2
M	7	2	3	2	4	2	2	2	3	3	3	3	5
T		1	2	2	4	1	1	1	4	2	2	3	6
W		0	0	2	3	0	0	1	3	0	0	3	6
T		0	0	2	3	0	0	1	3	0	0	3	6
F		0	0	1	2	0	0	1	2	0	0	2	4
M	8	4	3	2	2	2	2	3	2	4	4	4	5
T		3	2	2	2	1	2	3	2	4	3	2	3
W		1	1	2	2	1	1	3	2	4	3	2	3
T		1	1	2	1	1	0	2	2	4	3	2	3
F		0	0	1	1	0	0	2	2	3	2	2	2

DAY	RAT NO	EXUDATE/5				ERYTHEMA/5				LESION SIZE (MM)			
		A	B	C	D	A	B	C	D	A	B	C	D
M	9	1	3	4	5	1	3	3	3	3	4	5	7
T		2	2	4	4	1	2	4	2	5	4	6	5
W		1	1	4	3	1	1	3	3	5	3	6	4
T		0	0	4	2	0	0	3	2	5	3	5	4
F		0	0	3	2	0	0	2	2	4	3	4	4
M	10	4	3	3	3	3	4	4	4	5	3	4	4
T		3	2	2	2	2	3	3	3	5	3	5	6
W		2	2	2	1	1	2	2	2	3	3	4	5
T		1	1	2	1	1	1	1	2	3	3	3	4
F		1	1	1	1	0	1	1	1	2	3	3	4
M	11	1	4	4	3	2	1	2	4	3	6	5	4
T		1	2	4	2	2	1	3	3	3	4	4	3
W		1	1	3	2	1	1	2	2	2	3	4	3
T		1	1	2	1	0	0	2	1	2	2	2	3
F		1	2	1	1	0	1	2	0	2	5	3	2
M	12	2	4	4	2	1	2	4	4	2	5	5	5
T		1	3	4	2	1	3	4	3	5	6	5	3
W		1	3	4	1	1	3	4	2	2	5	3	3
T		1	2	2	1	0	1	3	1	2	3	2	3
F		0	1	2	0	0	1	2	0	2	3	2	3
M	13	1	1	3	1	2	2	3	2	2	2	4	2
T		1	1	2	1	1	1	2	2	2	2	4	2
W		1	1	1	1	0	0	1	1	2	2	3	2
T		1	1	1	1	0	0	1	1	2	2	2	2
F		0	0	1	1	0	0	1	0	2	2	2	2
Av		1.29	1.4	2.1	1.8	1.27	1.2	2.15	1.96	3.14	2.96	3.49	3.54

Table 5.2. Average effect of four treatments on lesion size, erythema and exudate (From Table 5.1) Same letters indicate where no significant differences were found. P=0.05.

Treatment	Lesion size	Erythema	Exudate
Crude extract	3.14 a	1.27 d	1.29 h
Terminoic acid	2.96 b	1.20 e	1.40 l
Negative control	3.49 a, b	2.15 f	2.10 j
Gentamycin	3.54 c	1.96 g	1.80 k

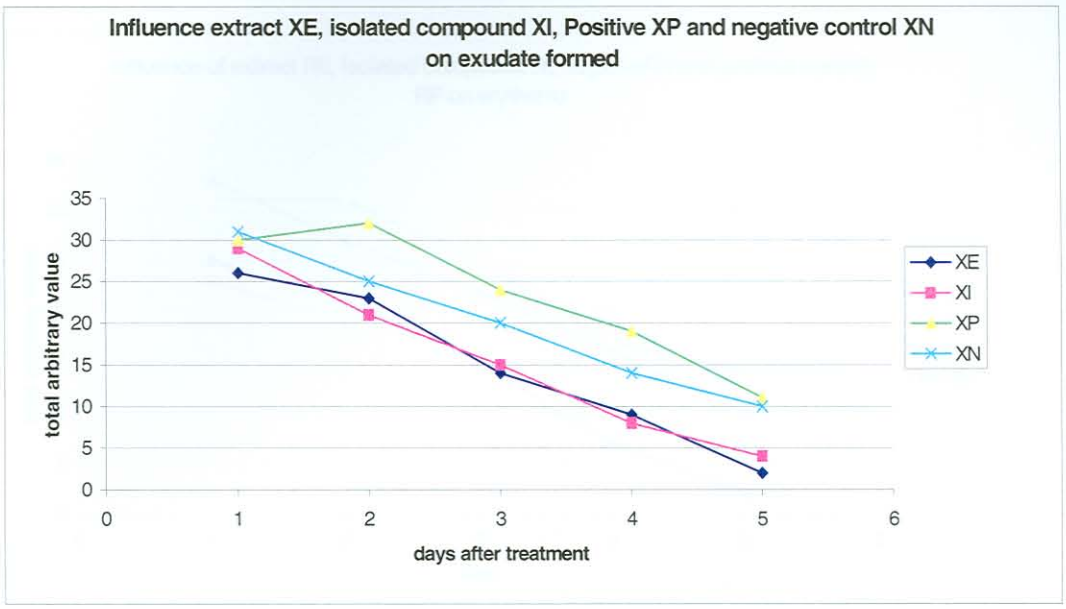

Fig. 5.2. The influence of the crude extract XE, the isolated terminoic acid XI, the positive control XP and the negative control XN on the exudate formed

5.3.1 Effects of extracts on exudate

On average there was more exudate formed from the control (2.1), followed by the gentamycin treatment (1.8) then terminoic acid (1.4) and then the crude extract (1.29) The difference over a time-period is presented in Fig 5.2. The crude extract and terminoic acid appeared to decrease the exudate formation, but the gentamycin treatment initially led to more exudate formation than the negative control.

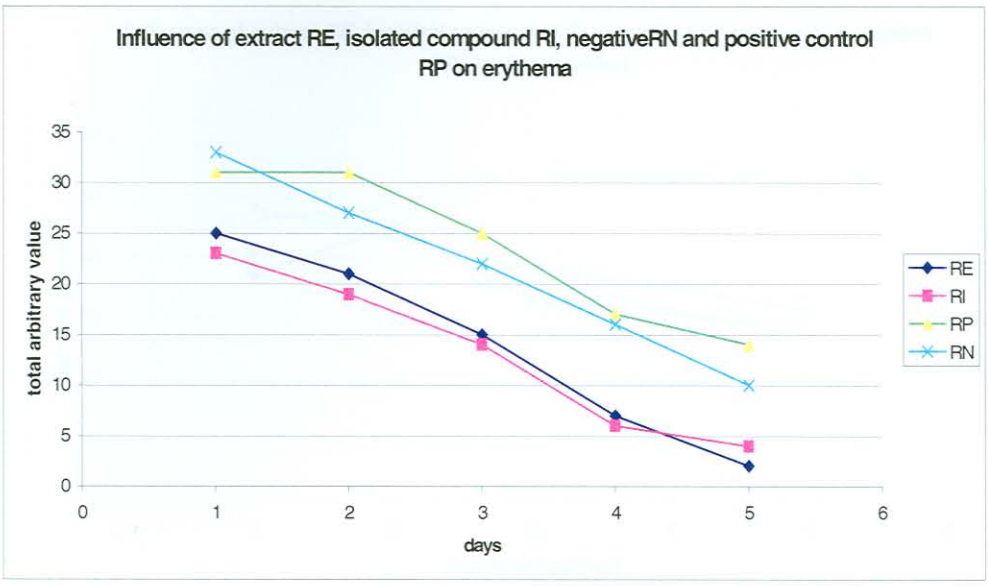

Fig. 5.3. The influence of the crude extract RE, isolated terminoic acid RI, positive RP as well as negative RN controls on the wound erythema

5.3.2 Effects of extracts on wound erythema

Terminoic acid and the crude extract proved to be better in reducing the erythema of the wounds, which is indicative of the infected state of the wound, than gentamycin as positive control and the negative control. These results agreed well with the results on exudate formation.

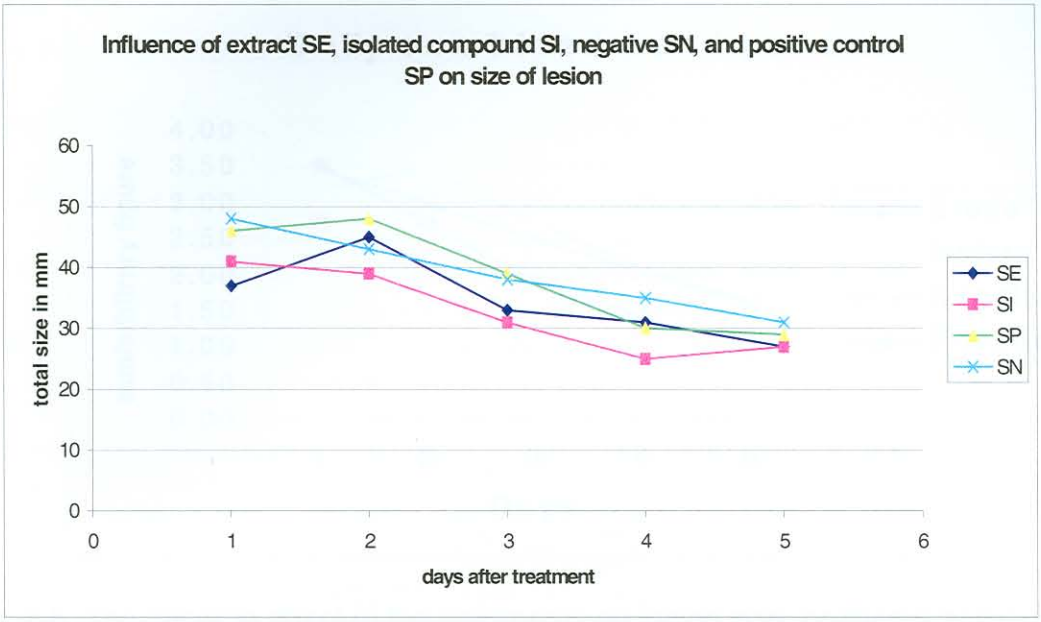

Fig. 5.4. The reduction in total diameter (mm) over a 5-day period of the different test sites using the crude extract, the isolated extract and gentamycin for the different animals.

5.3.3 Effects of extracts on wound size

The four treatments led to a similar result on wound size than the more subjective results obtained with erythema and exudate formation.

It is clear from Figure 5.5 that the crude and the isolated extract were more effective than non-treatment or gentamycin in reducing the size of the lesions.

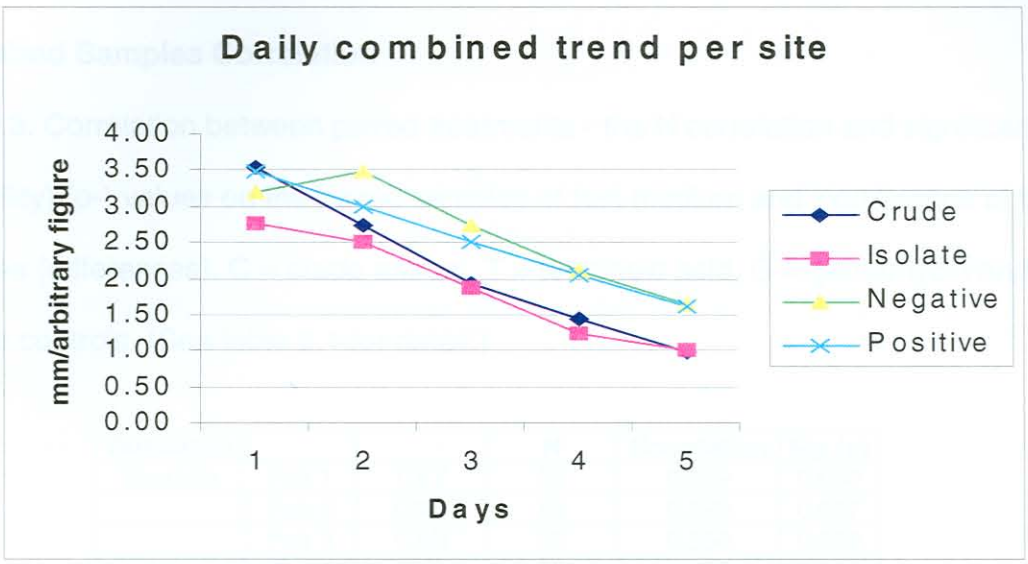

Fig. 5.5. The average effect of the treatments on lesion size, erythema and exudate formed on the test sites of the 11 rats over a five-day period.

5.3.4 Combined effects of extracts and controls

The combined effect of the three parameters on the sites (Figure 5.5) showed that the wound healing ability of the crude compound and terminoic acid was superior to that of the positive and negative controls.

To determine if the differences observed are significant, the data was analyzed using the student-t test with the help of Dr Annemarie Kruger of the Potchefstroom University for CHE. On each outcome (wound size, exudate and erythema), each test application was compared to each other (e.g. Treatment 1(crude extract) with Treatment 2 (terminoic acid) and Treatment 1 with Treatment 3(gentamycin) etc so a total of 6 correlation's were evaluated for each outcome to derive at the mean and p-values on paired and sample statistics.

5.3.6 Paired Samples Correlation

Table 5.3. Correlation between paired treatments - the N correlation and significance (probability) (p-) values on the paired samples of test medium and significance of the outcomes (differences). C = crude extract, T = terminoic acid, G = gentamycin and N = negative controls. (See table 5.1 for detail.)

Outcomes			N	Correlation	Sig.(p)
Exudate	Pair 1	C&T	55	0.609	0.000
	Pair 2	C&G	55	0.299	0.027
	Pair 3	C&N	55	0.350	0.009
	Pair 4	T&G	55	0.375	0.005
	Pair 5	T&N	55	0.474	0.000
	Pair 6	G&N	55	0.427	0.001
Erythema	Pair 1	C&T	55	0.572	0.000
	Pair 2	C&G	55	0.390	0.003
	Pair 3	C&N	55	0.396	0.003
	Pair 4	T&G	55	0.522	0.000
	Pair 5	T&N	55	0.487	0.000
	Pair 6	G&N	55	0.428	0.001
Lesion size	Pair 1	C&T	55	0.507	0.000
	Pair 2	C&G	55	0.344	0.010
	Pair 3	C&N	55	-0.003	0.980
	Pair 4	T&G	55	0.324	0.016
	Pair 5	T&N	55	0.128	0.352
	Pair 6	G&N	55	0.302	0.025

The significance values represent the p-values where > or = 0.05 means a statistically significant value and > or = 0.01 means a highly significant difference (Student t-test).

5.4 Discussion

5.4.1 Exudate

The average exudate formation of the crude extract treatment (1.29)(p=0.000) was highly significantly lower than terminoic acid (1.40) (p=0.000), gentamycin (1.8)(p=0.009) and the control (2.1) (p=0.005) treatment. Furthermore the exudate formed in the crude extract was significantly lower than the exudate formed with terminoic acid (1.4) (p=0.022). The average exudate formed with the gentamycin was highly significantly higher then the control treatment. The crude extract (20%) therefore produced less exudate than terminoic acid (10%) and gentamycin (1%). Gentamycin produced more exudate than the control treatment. There was no significant difference in the lesion size between crude extract and negative control and also between terminoic acid and negative control.

The production of exudate may be related to inflammation. The crude extract especially and also terminoic acid may have anti-inflammatory activity which would partially explain the decreased exudate. It is not easy to explain why the gentamycin produced more exudate than the control treatment, unless a component in the gentamycin formulation had an irritant effect. It may very well be that an aspect related to the cream may have had an effect because the negative control was not treated with a cream. Nevertheless the crude and terminoic acid formulated with the cream did not increase exudate formation.

The t-test between the crude extract and terminoic acid showed a highly significant difference (p= 0.000), whereas the t-test between the crude and negative was significant (p= 0.027) and between the crude and gentamycin highly significant (p= 0.009). The t-test between the terminoic acid and the positive (p= 0.000) and negative controls (p= 0.005) also proved to be highly significant.

The conclusion can thus be drawn that both the crude extract and terminoic acid were more effective than the positive and negative controls in decreasing formation of the exudate.

5.4.2 Erythema

The average exudate formation for terminoic acid (1.2) was highly significantly lower than values for gentamycin (1.96) (p=0.005) and the control (2.15) (p=0.005) while the crude was lower than terminoic acid treatment (1.2).

The t-test showed a highly significant difference between the crude extract and terminoic acid and the positive and negative controls (p = 0.000 - 0.003). The crude extract and terminoic acid differed significantly enough from the positive and negative controls to be regarded effective in the control of wound erythema.

5.4.3 Lesion size

The interpretation of the results of the lesion size proved to be more complex with highly significant differences occurring between crude (3.14 mm) and terminoic acid (2.96 mm) (p= 0.000) and significant differences between the crude (3.14 mm) and negative (3.54mm) (p= 0.010) to non-significant difference between terminoic acid and negative

control (p= 0.352) and a less significant difference between gentamycin and negative controls (p= 0.025). The explanation may be that the initial lesion sizes differed.

Table 5.4

The average lesion sizes at the start of the experiments.

Lesion	Average lesion size	Average lesion size(start)	Average lesion size (end)
Extract A	3.14	5	2
Terminoic acid B	2.96	3	2
Gentamycin C	3.49	3	2
Negative control D	3.54	2	2

The explanation for the averages in gentamycin and negative controls being higher than the initial values is that the parameter values increased on day 2 and only then started to decrease.

Overall, the statistical analysis verifies the conclusion that the crude *T. sericea* extract and isolated terminoic acid were more effective *in vivo* antibacterial compounds than the commercial gentamycin at the concentrations used.

5.5 Toxicology report

Dr. Johan Joubert, the Head of the Toxicology Department at the Onderstepoort Agricultural Research Institute carried out a post-mortem examination on the rats at the end of the *Terminalia* experiment.

The results indicated that rats numbers one and two used in the tests, number one had no subcutaneous lesions and internally had three pin-point sized abscesses in the lungs.

Rats two to thirteen showed no subcutaneous lesions, not even under the experimental skin lesions. Rat three had three pinpoint sized abscesses in the right lung. Rats two, four, five, nine, ten, eleven and twelve showed no internal abscesses or lesions. A single pea sized abscess was seen in the liver of rats six, seven, eight and thirteen.

Not one of the rats developed lesions or abscesses subcutaneously in the vicinity of the treated areas. This indicates that the liver and lung abscesses seen in a few of them most probably resulted from an infection in their brooder cages.

One can thus conclude that terminoic acid and the crude extract, as well as the controls had no toxic effects on the test animals and their antibacterial efficacy and possibly their anti-inflammatory effects, outweighs the possible toxic effects they might have.

A possible mistake with this study was that it was not blinded. I should have used codes for the different treatments assigned by someone else to ensure that unintentional bias did not occur when scoring the results.

Chapter 6

Discussion and Conclusion

6.1 Selection of plant

In a preliminary experiments it could be shown that leaf extracts of *Terminalia* species have antibacterial activity against several humans pathogenic bacteria (Eloff, 1999b). Because *Terminalia* species grow widely in South Africa and the research group of the Phytomedicine Programme at the University of Pretoria has developed substantial expertise in isolation and characterization of antibacterial compounds from members of the Combretaceae the aim of this study was to isolate antibacterial compounds from a *Terminalia* species and to determine whether such a compound can be used to treat animals or humans against infections. Hopefully this may lead to an inexpensive treatment of infections in humans in poor rural populations.

6.2 Extraction

The first step in the process was to determine the best extractant. Several extractants of varying polarity and selectivity were used to determine the quantity extracted, the chemical fingerprint of the extract and the antibacterial activity of the different extracts were determined. The results were in the main similar to the results obtained with

Combretum microphyllum (Kotze and Eloff, 2001). Tetrahydrofuran (91), acetone (64) and ethanol (126) had the highest total antibacterial activity (Table 2.6). Tetrahydrofuran (60.6 mg), methanol (39 mg), acetone (40.6 mg) and ethanol (52.6 mg) had the highest yield in terms of mass extracted from the 500g of leaf material that was started of with. Despite not scoring the highest, acetone was selected as extractant because of its miscibility with water, its non-toxicity to the microorganisms, its ease of removal and its safety in laboratory use as noted in earlier work on *Combretum erytrophyllum.* (Eloff, 1999b).

Seven *Terminalia* species occurring in southern Africa were analyzed to determine which was the most promising to use for isolating an antibacterial compound.
A second aspect was to confirm antibacterial activity with other members of the *Combretaceae.* The minimum inhibitory concentration (MIC) values for antibacterial activity of *T. sericea (*0.16 mg/ml*), T. prunoides (*0.13 mg/ml*), T. phanerophlebia (*0.11 mg/m*l)* and *T. gazenzis* (0.06 mg/ml) was as low as that of the positive control, gentamicin (0.16 mg/ml), against *S. aureus.* Against *E. coli, T. sericea (*0.31 mg/ml*), T. phanerophlebia (*0.21 mg/ml*), T. sambesiaca (*1.02 mg/ml*)* and *T. brachystema* (0.56 mg/ml) had the lowest MIC values. The results against the Gram-negative organisms showed *T. gazenzis* (0.25 and 0.25 mg/ml*)* and *T. sericea* (0.63 and 0.16 mg/ml) had the best antibacterial effect.

The *T. sericea* extract had on average the second best antibacterial activity and was chosen as the target plant for further investigation. This plant also had the advantage

that it is the most widely distributed in South Africa of the plants tested in this study. Development of an active product could benefit rural people in need of a cheap, effective antibacterial agent.

6.3 Isolation of terminoic acid

In the approach to isolate the active compound, a series of fractionating and isolating procedures was employed. The plant material was first extracted with acetone, and fractionated, before being subjected to VLC crude separation, and finally column chromatography. During these processes, the chemical composition and qualitative antibacterial activity was determined by TLC and bioautography on a continuous basis in order to search for single compounds and to confirm antibacterial activity. As a result of this, one sample, JK 3-2, was identified as a single, pure compound with antibacterial activity and was submitted to NMR and MS analyzes.

The NMR and MS spectra confirmed the identity as the triterpenoic acid, 1α, 2β-, 18 trihydroxy-olean 12-en-29-oic acid with a molecular mass of 488 and molecular formula of $C_{30}H_{48}O_5$ and the common name terminoic acid. This is the first time that this compound (terminoic acid) has been isolated from *T. sericea,* but it has previously been identified from *T. arjuna*.

It is likely that the most active compound was not isolated from *T.sericea* as can be seen from Fig. 4.6 where the chromatogram revealed higher levels of activity that was not pursued because of the complexity of the relevant fractions.

6.4 Animal study

An important final step was to perform an *in vivo* evaluation of the active compound and a crude extract. A system was developed in which two treatments as well as a positive and a negative control were applied to the same animal on infected skin lesions of rats. The results proved that both the 20% crude extract preparation as well as 1% terminoic acid had better clinical activity against *S. aureus* than the positive control, commercial 0.1% gentamycin cream on the three parameters investigated *i.e.*, formation of exudate, erythema and the size of the lesion.

6.5 Conclusion

If one looks at the original aim of the study, the third objective was to determine if the isolated compound can be used to treat superficial skin infections caused by *Staphylococcus aureus* pathogens, the objective certainly was met in the animal study and could be extrapolated for use in humans.

The plant leaves could be used with a simplified method of extraction like ethanol or just the macerated leave extract applied as a paste to the wound. This confirmed the hearsay evidence of the traditional healer that plant leaves were used in circumcision

ceremonies. The mechanism of action would still have to be cleared up as well as toxicity studies and systemic side effects. In the short term however the practical use would be limited to the crude extract to be incorporated in a cream and communicated to traditional healers to be used for mild, superficial septicaemia.

REFERENCES

Agrawal, P.K., (1989). Studies in organic chemistry 39. Carbon-13 NMR of Flavonoids. Elsevier, Amsterdam.

Alexander, D.M., Bhana, N., Bhika, K.H. and Rogers, C.B. (1992). *South African Journal of Science* 88, 342-344.

Alkinside, K.A. and Olukoya, D.K., (1995). Vibriocidal activities of some local herbs. *Journal of Diarrhoeal Disease Research* 13(2) 127-129.

Anon 1939 . Alexander Fleming resistance to bacteria

Berkowitz. F.E., (1995). Antibiotic resistance in bacteria. *Southern Medical Journal* 88, 797–804.

Begue, W.J. and Kline, R.M., (1972). The use of tetrazolium salts in bioautographic procedures. *Journal of Chromatography* 64, 182-184.

Breytenbach, J.C and Malan, S.F. (1989). Pharmacochemical properties of *Combretum zeyheri*. *South African Journal of Science* 85, 372–374.

Burpadaja, S. and Bunchoo, A. (1995) Antimicrobial activity of tannins from *Terminalia citrina. Planta Medica* 61(4), 365-366.

Carr, J.D. and Rogers, C.B., (1986). Chemosystematic studies of the genus Combretum (Combretaceae), I. A convenient method of identifying species of this genus by a comparison of the polar constituents extracted from leaf material. *South African Journal of Botany* 53, 173-176.

Http://www.bact.wisc.edu/Bact330/lecturebactres

Carr, J.D. (1988). *Combretaceae in Southern Africa.* Tree Society of Southern Africa, Johannesburg. pp. 182-222.

Chauhan S., Parkash S.and Kauschik R. (1997) Isolation of 3 beta-hydroxyolean-12 ene and related triterpenoids from the leaves of *Terminalia arjuna.* Indian Journal of Chemistry B 36, 3, 297 - 298

Cordell, G.A., (1993) Pharmacognosy – New roots for old science. In: Atta-ur-Rhaman (Ed), Studies in Natural Products Chemistry. Elsevier, Amsterdam, 13. Pp 629 – 675.

Cowan, M.M. (1999). Plant Products as Antimicrobial agents. *Clinical Microbiology Reviews*. 12(4), 564–582.

Djerassi, C., Budzekiewicz, H. and Wilson, J.M. (1963). Mass spectrometry in stuctural and stereochemical problems: unsaturated pentacyclic titerpenes. *Terahedron Letters* 7, 263-270.

Edwards, D. (1980). *Antimicrobial Drug Action*. MacMillan Press Ltd, London.

Eloff, J. N. (1998a). The presence of antibacterial compounds in *Anthocleista grandiflora* (Loganiaceae). *South African Journal of Botany* 64, 209-212.

Eloff, J.N (1998b). Which extractant should be used for the screening and isolation of antimicrobial components from plants? *Journal of Ethnopharmacology* 60, 1-8.

Eloff, J.N. (1998c). A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. *Planta Medica* 64, 711-714.

Eloff, J.N. (1999a). Conservation of Medicinal Plants: Selecting Medicinal Plants for Research and Gene Banking. Monographs in Systematic Botany from the Missouri Botanical Garden. In: Conservation of Plant Genes III: *Conservation and Utilization of African Plants*. Robert P. Adams and Janice E. Adams, eds., Missouri Botanical Garden Press, St. Louis, USA. pp. 209-222

Eloff, J.N. (1999b). The antibacterial activity of 27 southern African members of the Combretaceae. *South African Journal of Science* 95, 148-152.

Eloff, J.N. (1999c). It is possible to use herbarium specimens to screen for antibacterial components in some plants. *Journal of Ethnopharmacology* 67, 355-360.

Eloff, J.N. (2000). A proposal on expressing the antibacterial activity of plant extracts - a small first step in applying scientific knowledge to rural primary health care in South Africa. *South African Journal of Science* 96, 116-118.

Eloff, J.N., Jäger, A.K. and van Staden, J. (2001). The stability and the relationship between anti-inflammatory activity and antibacterial properties of southern African *Combretum* species. *South African Journal of Science.* 97, 291-293.

Eloff, J.N. (2004). Phytomedicine 11. 370 – 371

El-Mekkawy, S., Meselhy, M.R., Kusomoto, I.T., Kadota, S., Hattori, M., Fabry, W., Okemo, P. and Ansorg, R. (1995). Fungistatic and fungicidal activity of East African medicinal plants. *Mycoses* 39, 67-70.

Fabry, W., Okemo, P.O., and Ansorg, R., (1997). Antibacterial activity of East African medicinal plants. *Journal of Ethnopharmacology* 60, 79-84.

Famakin, J.O. (2002). Investigation of antibacterial compounds present in *Combretum woodii*. MSc thesis, Department of Pharmacology, University of Pretoria.

Farnsworth, N. R., (1966). Biological and phytochemical screening of plants. *Journal of Pharmaceutical Sciences*. 55, 225-275.

Gelfand, M., Mavi S., Drummond, R.B., and Ndemera, B., (1985). *The Traditional Medical Practitioner in Zimbabwe*. 1st Edition. Mambo Press, Gweru.

Geissman, T.A. (1959). *Principles of Organic Chemistry*. 3rd Edition. W.H Freeman and Company, San Francisco.

Gupta, M., Mazumder, U.K., Manikandan, S., Bhattacharya, S., Haldar, P.K. and Roy, S. (2001). *Fitoterapia* 73, 165-167.

Harborne, J.B. (1973). *Phytochemical Methods. A Guide to Modern techniques of Plant Analysis*. Chapman and Hall, London.

Hobson, W.B., Britt, L.G., Sherman, R.T. and Ledes, C.P. (1968). The use of topical antibiotics in the prevention of experimental wound infection. *Journal of Surgical research.* 8(6), 261–266.

Karliner, J. and Djerassi, C. (1996). Terpenoids. LVII. Mass spectral and nuclear resionance studies of pentacyclic triterpene hydrocarbons. *Journal of Organic Chemistry*. 31, 1945-1955.

Katerere, D.R. (2001). *Phytochemical and Pharmacological Studies of species of African Combretaceae*. PhD Thesis, University of Strathclyde, UK.

Khanna, A.K., Ramesh, C. and Kapoor, N.K. (1996). *Terminalia arjuna*: an Ayuverdic cardiotonic tonic regulates lipid metabolism in hyperlipidaemic rats. *Phytotherapy Research* 10, 663-665.

Kotze, M and Eloff. J.N. (2002). Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). South African Journal of Botany 68, 62-67.

Lawrence,L., 1951. Taxonomy of Vascular Plants. McMillan. New York

Leder, S., (1997). A description of today's use of medicinal plants in West Bushmanland.The Hidden Gifts of Nature. ded. Namibia.

Lee, S.H., Ryu, S.Y., Choi, S.U., No, Z.S., Kim, S.K. and Ahn, J.W. (1995). Hydrolyzable tannins and related compounds having cytotoxic activity from the fruits of *Terminalia chebula*. *Archives Pharmaceutical Research* 18(2),118-120.

Letcher, R.M. and Nhamo, L.R.M. (1971). Chemical constituents of the Combretaceae. Part IV. Phenathrene derivatives from the heartwood of *Combretum hereroense*. *Journal of the Chemical Society. Perkin Transactions* 1, 1179-1181.

Levy, S.B. (1984). The Antibiotic Paradox. How miracle drugs are destroying the miracle. Plenum Press. New York.

Mahato, S.B., Nandy, A.K. and Roy, G. (1992). Review article number 67.Triterpenoids. *Phytochemistry* 31(7), 2199–2249.

Mahato, S.B., Sen, S. (1996). Advances in Triterpenoid research 1990 – 1994. Review article. *Phytochemistry* 44(7), 1185–1235.

Malan, E. and Swinny E. (1993). Substituted bibenzyls, phenantrenes and 9,10-dihydrophenantrenes from the heartwood of *Combretum apiculatum*. *Phytochemistry* 34(4), 1139-1142.

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

Martini, N., (1998). The isolation of antimicrobial components from *Combretum erythrophyllum* (Burch.) Sond. M.Sc. thesis, Phamacology, University of Pretoria.

Martini, N., Katerere, D. R., and Eloff, J. N.(2004). Seven flavonoids with antibacterial activity isolated from *Combretum erytrophyllum. South African Journal of Botany* 70, 310-312.

Martini, N., Katerere, D. R., and Eloff, J. N.(2004). Biological activity of five antibacterial flavonoids isolated from *Combretum erytrophyllum* (Combretacea). *Journal of Ethnopharmacology* 93, 207-212.

McGaw, L.J., Rabe, T., Sparg, S., Jäger A.K., Eloff, J.N. and van Staden, J. (2001). An investigation into the biological activity of *Combretum* species. *Journal of Ethnopharmacology* 75, 45-50.

Nash, R.J., Watson, A.A., Winters, A.L., Fleet, G.W.J., Wormald, M.R., Dealler, S., Less, E., Asano, N. and Kizu, H. (1997). Novel Biologically-Active Alkaloids from British Plants in ; Wrigley S.,hayes M.,Thomas R and Chrystal E (eds) Phytochemical Diversity: A source of new industrial products. 1st edition. RSC. UK

Panzer, A., Joubert, A.M., Eloff, J.N., Albrecht, C.F., Erasmus, E. and Seegers, J. 2000 Chemical analyzes of Ukrain, a semisynthetic *Chelidonium majus* alkaloid derivative, fail to confirm its trimeric structure. *Cancer Letters* 160, 237-241.

Panzini, I., Pelizzoni, F., Verotta, L. and Rogers, C.B., 1993 Constituents of the fruit of South African *Combretum* species. South African Journal of Science 89 (7), 324-328.

Pettit, G.R., Cragg, G.M., Herald, D.L., Schmidt, J.M. and Lohavanijaya, P. (1982) Isolation and structure of combrestatin. *Canadian Journal of Chemistry*. 60, 1374-1376.

Pettit, G.R., Hoard, M.S., Doubeck, D.L., Schmidt, J.M., Petit, R.K., Tackett, L.P. and Chapuis, J-C. (1996). Antineoplastic agents 338. The cancer cell growth inhibitory constituents of *Terminalia arjuna* (Combretaceae). *Journal of Ethnopharmacology* 53, 57-63.

Rogers, C.B. and Verotta, L. (1996). Chemistry and biological properties of the African Combretaceae. In: Hostettmann, F., Chinyanganya, M., Maillard, M. and Wolfender, J.L. (eds.) *Chemistry, Biological and Pharmacological Properties of African Medicinal Plants*. University of Zimbabwe Publications. Harare, Zimbabwe.

Rogers, C.B. (1998). A convenient method of identifying species of this genus by a comparison of the polar constituents extracted from leaf material. *South African Journal of Botany* 53, 173-176.

Rogers, C.B. and Subramony, G., (1988). The structure of imberbic acid, a 1α-hydroxy pentacyclic triterpenoid from *Combretum imberbe* . *Phytochemistry* 27(2), 531-533.

Salie, F., Eagles, P.F.K., Leng, H.M.J., (1996). Preliminary antimicrobial screening of four South African Astraceae species. *Journal of Ethnopharmacology*. 52, 27-33

Sanford, M. and Rosenthal, M.D. (1967). Local and Systemic Therapy of *Pseudomonas* Septicaemia in Burned Mice. *Annals of Surgery* 165(1) 97 – 103.

Sato.Y., Oketani, H., Singyouchi, K., Ohtsubo, T., Kihara, M., Shibata, H., Higuti, T. (1997). Extraction and purification of effective antimicrobial contituents of *Terminalia chebula* Rets against methicillin-resistant *Staphylococcus aureus*. *Biological Pharmaceutical Bulletin.* 20(4) 401-404.

Schwikkard, S., Zhou, B-N., Glas, T.E., Sharp, J.L., Mattern, M.R., Johnson, R.K. and Kingston, D.G.I. (2001). Bioactive compounds from *Combretum erythrophyllum. Journal of Natural Products* 63, 457-560.

Serage, A. (2003). Isolation and characterization of antibacterial compounds present in *Combretum apiculatum* subsp *apiculatum*. MSc thesis, Programme for Phytomedicine, University of Pretoria.

Silva, O., Duarte, A., Cabrita, J., Pimentel, M., Diniz, A. and Gomes, E. (1996). Antimicrobial activity of Guinea-Bissau traditional remedies. *Journal of Ethnopharmacology* 50, 55–59.

Snyder, L.R. and Kirkland, J.J., (1979) Introduction to Liquid Chromatography. John Wiley, New York. USA

Sohni, Y.R., Kaimal, P., Bhatt, R.M. (1995). The antiamoebic effect of a crude drug formulation of herbal extracts against *Entamoeba histolytica in vitro* and *in vivo*. *Journal of Ethnopharmacology* 45, 43–52.

Sohni, Y.R., Bhatt, R.M. (1996). Activity of a crude extract formulation in experimental hepatic amoebiasis and in immunomodulation studies. *Journal of Ethnopharmacology* 54, 119-124.

Stahl, E., (1969) Thin Layer Chromatography, 2nd Edition, Springer-Velg, Berlin, Heidelberg, New York.

Tanaka, K and Toda, F. (1986). Stereoselective photocycloadditions of chalcone derivatives in a crystalline inclusion complex with 1,1,6,6-tetraphenylhexa-2,4-diyne-1,6-diol.*J.C.S.*(C), pp 593-594.

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

Taylor, R.S.L., Edel, F., Manandhar, N.P., Towers, G.H.N. (1996). Antimicrobial activities of southern Nepalese medicinal plants. *Journal of Ethnopharmacology* 50, 97–102.

Valsaraj, R., Pushpangadan, P., Smitt, U.W., Adsersen, A., Christensen, S.B., Sittie, A., Nyman, U., Nielsen, C., and Olsen, C.E. (1997). New anti HIV-1, antimalarial and anti fungal compounds from *Terminalia bellerica. Journal of Natural Products* 60(7), 739-742.

van Wyk, B.,van Wyk, P., van Wyk, B-E. 2000. Photographic guide to Trees of southern Africa. Briz Publications. South Africa.

Vlietinck, A.J., Van Hoof, L., Totté, J., Lasure, A., Van Den Berghe, D., Rwangabo, P.C. and Mvukiyumwami, J. (1995). Screening of a hundred Rwandese medicinal plants for anti-microbial and antiviral properties. *Journal of Ethnopharmacology* 46, 31-47.

Walker, R.D. and Thornsberry, C. (1998). Decrease in antibiotic susceptibility or increase in resistance? *Journal of Antimicrobial Chemotherapy*. 41, 1-4.