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

By

Johannes Petrus Kruger

B.Pharm, M.Pharm (PU for CHE)

Dissertation submitted to the Faculty of Health Sciences, Department of Pharmacology, University of Pretoria, PRETORIA in fulfilment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Promotor: Dr JN Eloff

Co-Promotor: Dr DR Katerere

Date of submission; 23 August 2004

Contents

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

.

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

Published abstract presentation

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.

List of Figures

Chapter 1

Chapter 2

Chapter 3

Chapter 4

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

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

CONTENTS

Chapter 1 Introduction and aim of study