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

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

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PREFACE

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

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

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

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

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

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

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ABSTRACT

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

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

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

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

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

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

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

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

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

OPSOMMING

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

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

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

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

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

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

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

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

LIST OF ABBREVIATIONS

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

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

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

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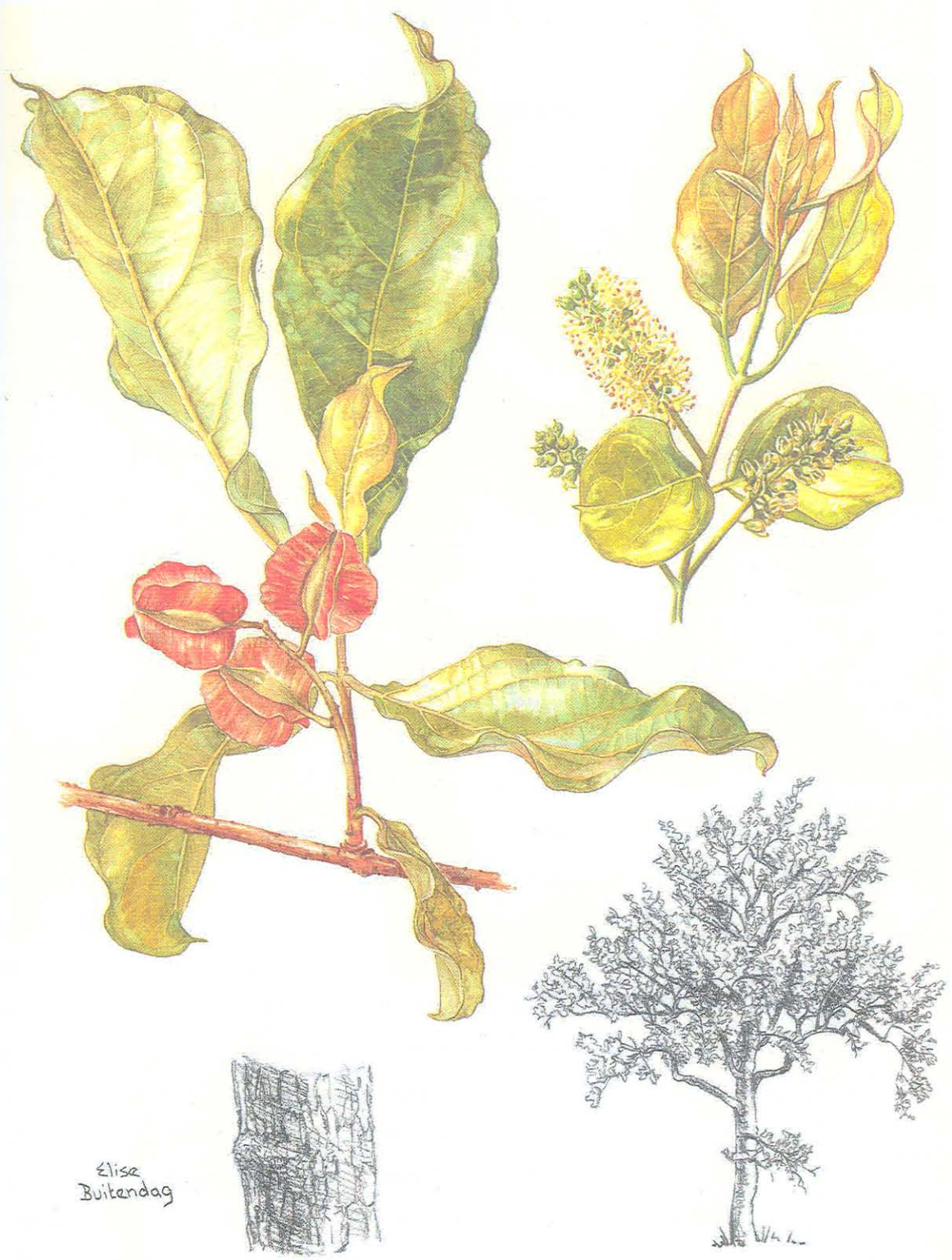

Combretum apiculatum subsp. *apiculatum*

Painting of *C. apiculatum* from Carr 1998