Characterization of antimicrobial compounds from *Combretum paniculatum,* a plant with proven anti-HIV replication activity

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DECLARATION

The work described in this thesis was conducted in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria between July 2003 and June 2006, and the Department of Molecular Natural Products Research, Hans-Knöll Institute (HKI), Jena, Germany from June 2005 to August 2005, under the supervision of Prof J.N. Eloff, Prof M. van Vuuren, Dr I. Sattler and Dr L.J. McGaw.

The data included in this thesis are the results of my investigations, and I hereby declare that this work has been written by me and that it is a record of my research work. References made to published literature have been duly acknowledged.

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I declare the above statement to be true.

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SUMMARY

There is an urgent need to discover new antimicrobial and antiviral compounds owing to increasing problems of resistance to drugs encountered in many pathogenic organisms. There are also problems with currently used drugs in terms of side effects and expense. Plants have been used for many generations for healing purposes, and screening of extracts of these plants has often yielded positive results. In particular, plants with antimicrobial properties are the subject of much investigation. This study focuses on isolating the compounds responsible for biological activity in one such medicinal plant, *Combretum paniculatum*, extracts of which have been shown to possess antimicrobial activity.

Members of the genus *Combretum* are widely used for medicinal purposes by many groups in Africa, to treat various conditions. Other researchers have discovered antifungal, antibacterial, anti-inflammatory and molluscicidal effects of these plants. One species of this genus, *C. paniculatum*, has been reported in the literature to have antiviral activity against HIV-2 with a promising selectivity index. It is important to exclude highly toxic effects of potential antimicrobial preparations. *C. paniculatum* extracts also displayed good antibacterial activity and some anti-inflammatory activity in other studies. Although many active compounds, especially antibacterial and antifungal, have been isolated from other *Combretum* species, little is known about the identity of compounds responsible for activity in *C. paniculatum.*

In the initial stages of this project, the crude extracts of leaves of *C. paniculatum* were investigated for antiviral and cytotoxic activity. It was found that the acetone and water extracts of *C. paniculatum* leaves reduced the cytopathic effect of feline herpesvirus type 1 by 3.0 $\log_{10}$, a very promising result. Investigations were carried out to determine the best solvent to use for extracting the active components. It was found that acetone was the best extractant in terms of the number of compounds extracted from the plant after analysis using thin layer chromatography (TLC) and the number of bioactive compounds using bioautography against bacteria. Water extracted a large quantity of material.

Different plant parts, namely stem bark, root bark and leaves, were screened for antiviral and antibacterial activity and the leaves and stem bark showed good activity. The test organisms were feline herpesvirus type 1 (FHV-1) for antiviral testing, and a range of Gram-positive and Gram-negative bacteria for antibacterial activity. Cytotoxicity against African green monkey kidney (Vero) cells was observed only at a relatively high concentration of 0.28 mg/ml. Based on availability and sustainability, the leaves were chosen for further work especially since leaves were used in the published data.

Isolation of active compounds from a 70% acetone extract of a large quantity of *C. paniculatum* leaf material was carried out using bioassay-guided fractionation. The bioassay used to select the active fractions for further fractionation was an antibacterial assay since it is easier and more rapid to detect antibacterial activity than antiviral activity. Various techniques including column chromatography and high performance liquid chromatography (HPLC) were used to fractionate the extract to result in pure compounds. The isolated compounds were structurally elucidated by nuclear magnetic resonance spectroscopy (NMR) and mass spectrometry (MS) analysis. Nine compounds were identified as cholest-5-en-3-ol, 2-phyten-1-ol, isoquercitrin, p-coumaric acid, 2, 3, 8-tri-O-methylellagic acid, beta-sitosterol, gallocatechin, apigenin and apigenin-7-glucoside.

The compounds were subjected to various bioassays to evaluate their biological activity. The isolated compounds had a broad-spectrum antibacterial activity against Gram-positive and Gram-negative pathogens, as well as some antifungal and antimycobacterial activity. Cholest-5-en-3-ol, 2-phyten-1-ol, gallocatechin and apigenin were active against *Escherichia coli* (Gram-negative) and *Mycobacterium vaccae*, and against the fungi *Sporobolomyces salmonicolor* and *Penicillium notatum*. Cholest-5-en-3-ol and 2-phyten-1-ol were also active against *Bacillus subtilis* (Gram-positive). None of the compounds showed substantial antiviral activity against coxsackievirus strain B3 Nancy, influenzavirus type A strain Hong Kong and herpes simplex virus type 1 strain K1. The compounds were generally moderately cytotoxic to the HeLa cell line but were less toxic to the Madin-Darby Canine Kidney (MDCK) and Vero cell lines.

The results obtained confirm the ethnobotanical use of *C. paniculatum*. Nine compounds with various biological activities were isolated from the leaf extract. The constituents responsible for antiviral activity still remain to be isolated and further work should be carried out making use of antiviral assay-guided isolation. These compounds may be present in low concentrations in *C. paniculatum*. Synergistic effects of isolated compounds on biological activity, particularly antiviral activity, could be investigated. The results reported here confirm that the presence of antibacterial activity in plant extracts is not an indicator of antiviral activity. Although the crude extracts of *C. paniculatum* had both antibacterial and antiviral activity, different compounds are responsible for antibacterial and antiviral activity respectively.

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List of Abbreviations

1D	1- dimensional
2D	2-dimensional
AIDS	Acquired Immune Deficiency Syndrome
APUA	Alliance for the Prudent Use of Antibiotics
ATCC	American Tissue Culture Center
BEA	Benzene, ethyl acetate, acetone
BuOH	Butanol
Bs	*Bacillus subtilis* ATTC 6633
Ca	*Candida albicans* BMSY 212
CAP	Community Acquired Pneumonia
CC	Cytotoxic concentration
CEF	Chloroform, ethyl acetate, formic acid
CFIDS	Chronic Fatigue Immune Deficiency Syndrome
CMV	Cytomegalovirus
CNS	Central Nervous System
COSY	Correlation Spectroscopy
CPE	Cytopathic effect
CRFK	Crandell Feline Kidney cells
DCM	Dichloromethane
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl hydrazyl
DS	Double-stranded
EBV	Epstein-Barr Virus
Ec	*Escherichia coli* SG 458
EC_{50}	Effective Concentration 50
EDTA	Ethylene Diamine Tetraacetic Acid
EMW	Ethyl acetate, methanol, water
FAAIR	Facts about Antibiotics in Animals and their Impact on Resistance

FAWE	Formic acid, acetic acid, water and ethyl acetate
GMK	Green Monkey Kidney cells (Vero)
HIV	Human immunodeficiency virus
HKI	Hans-Knöll Institute
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
HSV	Herpes Simplex Virus
IC_{50}	Inhibitory Concentration 50
ICTV	International Committee on Taxonomy of Viruses
ICU	Intensive Care Unit
INT	p-Iodonitrotetrazolium chloride
MDCK	Madin-Darby Canine Kidney cells
ME	Myalgic Encephalomyelopathy
MEM	Minimum Essential Medium
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass Spectrometry
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
Mv	*Mycobacterium vaccae* IMET 10670
MW	Molecular Weight
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
Pa	*Pseudomonas aeruginosa* K 799/61
PBS	Phosphate Buffer Solution
PEP	Pyruvate
Pn	*Penicillium notatum* JP 36
RNA	Ribonucleic acid
RSV	Respiratory Syncytial Virus
Sa	*Staphylococcus aureus* SG 511
SAR	Structure Activity Relationship
SARS	Severe Acute Respiratory Syndrome

Ss	*Sporobolomyces salmonicolor* SBUG 549
TA	Total activity
$TCID_{50}$	Tissue Culture Infectious Dose 50
TLC	Thin Layer Chromatography
THF	Tetrahydrofuran
UV	Ultraviolet
VREF	Vancomycin-resistant *Enterococcus faecalis*