Characterization of antifungal compounds isolated from *Combretum* and *Terminalia* species (Combretaceae)

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DECLARATION

I, **PETER MASOKO**, hereby do declare that this thesis submitted for the award of the degree of **PHILOSOPHIAE DOCTOR (PhD**) of University of Pretoria is my independent work and it has previously not been submitted for a degree or any other examination at this of any other university.

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Peter Masoko

_____ day of _____ 2006

DEDICATION

This work is dedicated first of all, to my parents who were my first teachers, my younger brothers Kegomoditswe, Kabelo, Mojalefa and sister Refilwe. Secondly my grandmother Mosepele Shongwane and my late uncle Mosalagae.

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"The light shines in the darkness and the darkness has never put it out" John 1.5

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Most of the chapters in this thesis have been written in the form of a manuscript for publication and will be submitted.

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ATCC	American type culture collection
BEA	Benzene/Ethanol/Ammonium hydroxide (90/10/1 v/v/v)
C_{18} column	18-Carbon reverse phase siliga gel column
CEF	Chloroform/Ethylacetate/Formic acid (5/4/1 v/v/v)
CsA	Cycosporin A
DEPT	Distortionless enhancement by polarization transfer
DAC	Dicationic aromatic compounds
DCM	Dichloromethane
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribose nucleic acid
DPPH	2, 2,diphenyl-1-picrylhydrazyl
EF3	Elongation factor
ELISA	Enzyme linked immunosorbent assay
EMW	Ethylacetate/Methanol/Water (40/5.4/4 v/v/v)
GGT	Geranylgeranytransferase
GS	Glucan synthase
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HPLC	High performance liquid chromatography
INT	Iodonitro-tetrazolium salts
LC_{50}	Lethal concentration for 50% of the cells
LPO	Lactoperoxidase
LNBG	Lowveld National Botanical Garden
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye
NaCl	Sodium chloride
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCCLS	National Committee for Clinical Laboratory Standards
NMR (^{13}C and ^{1}H)	Nuclear magnetic resonance (carbon 13 and proton)
PBS	Phosphate buffer saline
R_f	Retardation factor

rpm	revolutions per minute
SEE	Serial exhaustive extraction
TLC	Thin layer chromatography
UP	University of Pretoria
UV	Ultra violet radiation
v/v	volume per volume
VLC	Vacuum liquid chromatography
WHO	World Health Organisation

SUMMARY

Several investigations into the antimicrobial activity of members of the Combretaceae have been undertaken in recent years. Although the antibacterial properties of various species of *Combretum, Terminalia* and *Pteleopsis* have been investigated in depth, this is not the case for their antifungal properties. Due to the increasing importance of fungal infections the aim is to address this by focusing on antifungal activities of Combretaceae species. This was done by focusing on the following objectives:

1. Developing minimum inhibitory concentration (MIC) and bioautography procedures for fungi to be used in the laboratory in order to screen *Combretum* and *Terminalia* species for antifungal activity.
2. Selecting three or four species for further investigation based on antifungal activity and availability.
3. Isolating the antifungal compounds from one or more of the selected species.
4. Determining the chemical structure and *in vitro* biological activity of the antifungal compound.
5. Developing and applying a protocol and determining *in vivo* antifungal activity of *Combretum* and *Terminalia* extracts and isolated compounds in rats infected with different fungal pathogens.

Leaves of 24 *Combretum* and 6 *Terminalia* species were collected in the Lowveld National Botanical Gardens (LNBG) in Nelspruit. After the dried plants were milled to a fine powder, they were extracted with hexane, dichloromethane, acetone and methanol. Chemical constituents of the 120 extracts were analyzed by thin layer chromatography (TLC). The TLC plates were developed with one of the three eluent systems developed in our laboratory that separate components of Combretaceae extracts well i.e.: Ethyl acetate/methanol/water (40:5.4:5) [EMW] (polar/neutral), Chloroform/ethyl acetate/formic acid (5:4:1) [CEF] (intermediate polarity/acidic) and Benzene/ethanol/ammonia hydroxide (90:10:1) [BEA] (non-polar/basic). To detect the separated compounds, vanillin-sulphuric acid-methanol was sprayed on the chromatograms and heated at 110 ^{o}C to optimal colour development. Methanol was the best extractant, extracting a greater quantity of plant material than any of the other solvents. There was similarity in the chemical composition of the non-polar compounds of extracts using extractants of varying polarity

Qualitative analysis of antioxidant activity, the 2, 2,diphenyl-1-picrylhydrazyl (DPPH) assay on TLC plates was used as a screen test for the radical scavenging ability of the compounds present in the different 120 extracts. TLC-DPPH screening method indicated the presence of

antioxidant compounds in some of the extracts tested, with *C. woodii* and *C. hereroense* showing the most prominent antioxidant activity. Methanol and acetone extracted the most antioxidant compounds based on DPPH TLC. I*n vitro* studies coupled with the phytochemical analysis confirm that the extracts had antioxidant activity.

The solvent tolerance of the microorganisms was tested using the following solvents; DMSO, acetone, methanol and ethanol. In order to determine the maximum concentration at which different solvents would allow the test microorganisms to reach normal growth, different concentrations from 10 to 100% were used. Uninhibited growth was evaluated as no toxic effects of the solvent. Methanol and ethanol were found to be toxic. The growths of the fungi were not affected by DMSO and acetone concentrations up to 60%.

A serial microdilution assay was used to determine the minimum inhibitory concentration (MIC) values for plant extracts using tetrazolium violet reduction as an indicator of growth. This method had previously been used only for antibacterial activities. To apply it to measuring antifungal activities, a slight modification was made to suit fungal growth conditions. The following fungal pathogens were used: yeasts (*Candida albicans* and *Cryptococcus neoformans*), thermally dimorphic fungi (*Sporothrix schenckii*) and moulds (*Aspergillus fumigatus* and *Microsporum canis*). To determine MIC values, growth was checked after 24 and 48 hours to determine the end point. The MIC values of most of the extracts were in the order of 0.08 mg/ml and some had values as low as 0.02 – 0.04 mg/ml after 24 hours incubation.

TLC plates were loaded with 100 μg (5 μl of 20 mg/ml) of each of the extracts. The prepared plates were developed in the three different mobile systems used: CEF, BEA and EMW. The chromatograms were dried for a week at room temperature under a stream of air to remove the remaining solvent. The TLC plates developed were inoculated with a fine spray of the concentrated suspension containing approximately 10^9 organisms per ml of actively growing fungi e.g. conidia for *A. fumigatus* and yeast cells (blastocysts) for the other fungi in a Biosafety Class II cabinet (Labotec, SA) cupboard. The plates were sprayed until they were just wet, and after drying were sprayed with a 2 mg/ml solution of INT. White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested fungi.

During this study we experienced a number of difficulties. Firstly I found that preparing cultures some days before spraying them makes it difficult to get good results, possibly due to quick mycelial overgrowth and blockage of the spray gun with mycelia. The new method

was developed. This procedure led to reduced overgrowth of the mycelia. In the study of biologically active compounds from extracts, it was indicated that the extracts had antifungal compounds.

Fractionation and bioassay-guided isolation of the antifungal compounds was undertaken on the crude extracts of *C. nelsonii,* based on very low MIC's of the crude extracts on all tested pathogens, it had several compound which are active against all pathogens, lastly it is one of the *Combretum* species which have never being worked on. Antifungal compound was successfully isolated from the leaves of *C. nelsonii*. The structure was elucidated.

After structure elucidation bioassays of isolated active compounds was done to confirm that the compound isolated is the one expected, and how active the compound is, on its own. The compound was very active against all tested pathogens.

Cytotoxicity of the acetone extracts of *C. imberbe*, *C. nelsonii*, *C. albopunctactum* and *T. sericea* were evaluated using Brine shrimp (*Artemia salina*) assay and tetrazolium-based colorimetric assay (MTT assay) on Vero monkey kidney cells. These four extracts were chosen because of the good *in vitro* antifungal activity of crude extracts and there was intention of using them in *in vivo* studies in animal models. The results on brine shrimps indicated that the four leaf extracts have LC_{50} values above 20 µg/ml, the recommended cut-off point for detecting cytotoxic activity. Using MTT assay it was found that the four extracts did not suppress mitochondrial respiration in monkey kidney cells. Only *C. imberbe* was closer to the cut-off value (200 µg/ml), which was used by other authors. In searching for cytotoxic activity to the criteria of the American National Cancer Institute, the LC_{50} limit to consider a crude extract promising for further purification is lower than 30 µg/ml.

In vivo antifungal activity was investigated on the wound irritancy and efficacy of the four most promising, *Combretum nelsonii*, *Combretum imberbe*, *Combretum albopunctactum* and *Terminalia sericea* extracts applied topically to skin wounds in fungal infected skin wound of rat model. Wound irritancy and wound healing were evaluated by macroscopical, physical and histological methods. Aspects evaluated include wound healing, erythema, exudate formation and possible toxic effects of the extracts. Twenty rats were used in two pilot studies (Exploratory studies and Infection with different pathogens). During the pilot studies rats were not irritated by treatment of infection. The wound healed within three weeks. Only one rat was terminated due to weight loss and it was found that nasal discharge was due to external factors, which were not related to the experiment.

The clinical treatment of skin infected with pathogens continues to be a major problem especially in immuno-compromised patients. Therapeutic agents selected for the treatment of infected wounds had ideally shown antifungal activity on *in vitro* studies. I investigated whether these agents would improve phases of wound healing without producing deleterious side effects. All the parameters showed that the crude extracts and amphotericin B were effective in decreasing formation of the exudate, increasing crust formation and that they have antifungal activities used in *in vivo* studies. Acetone extract of leaves of *C. nelsonii*, *C. albopunctactum*, *C. imberbe* and *T. sericea* possessed remarkable growth inhibitory activities against fungal pathogens. Acetone extracts of leaves and isolated compound demonstrated wound healing properties comparable with that of antibiotic powder (amphotericin B).

The results of this study in general indicate that the *Terminalia* and *Combretum* species possess substantial antifungal properties. This explains the use of these plants in folk medicine for the treatment of various diseases related to fungal infections.

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