Characterization and biological activity of antifungal compounds present in *Breonadia salicina* (Rubiaceae) leaves

Salome Mamokone Mahlo (26289182)

M.Sc (Botany) (University of Limpopo)

Submitted in fulfillment of the requirements for the degree of

Philosophiae Doctor (PhD)

in the

Phytomedicine Programme, Department of Paraclinical Sciences

Faculty of Veterinary Sciences

Promoter: Prof J.N. Eloff

Co-Promoter: Dr L.J. McGaw

Date of Submission: October 2009

DECLARATION

I **Salome Mamokone Mahlo,** hereby declare that this thesis submitted to the University of Pretoria for the degree of Doctor Philosophiae is the result of my own investigations in execution and has never been submitted at any other university or research institution. Any help I received is acknowledged in the thesis.

…….

Salome Mamokone Mahlo

…….

Prof J.N Eloff

Promoter

…….

Dr L.J McGaw

Co-promoter

DEDICATION

This work is dedicated to my daughter Dunisani Maashaba Mahlo and my parents Wilson and Maria Mahlo for their support throughout my studies.

ACKNOWLEDGEMENTS

Supervisor Prof J. N Eloff for the advice, support and encouragement he gave me throughout my project. His office was always open to discuss my work, and his inputs and criticism were very helpful, especially during discussion of scientific papers. Thanks for your negative and positive comments, they have made me grow from strength to strength and realise that if I want to be a good scientist, I really need to work harder.

Co-supervisor Dr LJ. McGaw, thanks for being a good listener and proof reading my thesis. There were time where things were falling apart and you gave me strength and make me understand the world of research.

Microbiology Department, thanks for allowing me to use your equipment. I am grateful to **Samuel Molekwa** and **John Tsatsi,** thanks for making sure that my glassware were always clean and when I needed something you made provision for.

Thank you **Marelize Potgieter** (University of Witwatersrand) for performing mass spectroscopy.

Dr Xolani Peter from Council for Scientific and Industrial Research (CSIR) **t**hanks for your excellent assistance with NMR, EIMS, structure elucidation and chemical characterization.

Tharien de Winnaar, for making sure that my oranges were available and also for ordering necessary equipment and materials.

National Research Foundation (NRF) and **University of Pretoria** for financial support.

I am thankful to my parents, **Maria and Wilson Mahlo** for their love, support, guidance and mostly believing in me throughout my study life and making sure that my dream becomes a reality. I would like to thank God for blessing me with such a wonderful mother, who is always there for me and especially for taking good care of my daughter while I was far away. Mom, you are so incredible, especially for raising my daughter as if it is your own child. “Mma ngwana o tshwara thipa ka bogale.”

I am grateful to my daughter **Dunisani Maashaba Mahlo,** for being so strong and giving me strength throughout my project even when things were falling apart. My angel, you are the pillow of my strength. I thank God for blessing me with such a lovely child.

Brothers (Frans, Peter, Jimmy, Godfrey and Prince) and Sisters (Millicent and Mahlatse) thanks for the support you gave me throughout my studies.

A very special thanks goes to my fiancé **Dr Hasani Chauke,** who have been there for me throughout my project. I am very grateful to you for taking good care of our child especially in the most difficult time, while she was very sick. Thanks for the word of encouragement that you gave me and I really appreciate your contribution towards my success.

Lastly, thanks to **God Almighty,** for his grace, wisdom and comfort throughout my studies. There were times where things were very tough and God was there to protect and give me strength to overcome heavy storms and strong waves. This was indeed a very long journey and I praise you God always.

Psalm 23:4 Even though I walk through the valley of the shadow of death, I will fear no evil, for you are with me; your rod and your staff, they comfort me.

LIST OF ABBREVIATIONS

Amph B	Amphotericin B
BEA	Benzene: ethanol: ammonia
CC	Column chromatography
CEF	Chloroform: ethyl acetate: formic acid
DCM	Dichloromethane
DEPT	Distortionless enhancement by polarization transfer
DPPH	2,2-diphenyl-1-picrylhydrazyl
EIMS	Electron impact mass spectrometry
EMW	Ethyl acetate: methanol: water
EtOAc	Ethyl acetate
INT	p-Iodonitrotetrazolium violet
IPUF	Indigenous Plant Use Forum
LC_{50}	Lethal Concentration for 50% of the cells
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MS	Mass spectrometer
MTT	(3-(4,5-dimethylthiazol)-2,5-diphenyl tetrazolium bromide
NMR	Nuclear magnetic resonance
R_f	Retention factor
SDA	Sabouraud Dextrose Agar
TLC	Thin Layer Chromatography

ABSTRACT

The aim of this study was to investigate plant species to develop a product with the potential of protecting plants or plant products against plant fungal pathogens. Hexane, dichloromethane, acetone, and methanol leaf extracts of six plant species (*Bucida buceras*, *Breonadia salicina*, *Harpephyllum caffrum*, *Olinia ventosa*, *Vangueria infausta* and *Xylotheca kraussiana*) were evaluated for antifungal activity against seven plant fungal pathogens (*Aspergillus niger*, *A. parasiticus*, *Colletotrichum gloeosporioides*, *Penicillium janthinellum*, *P. expansum*, *Trichoderma harzianum* and *Fusarium oxysporum*). These plant species were selected from more than 400 plant species evaluated in the Phytomedicine Programme that had good activity against two animal fungal pathogens. All the leaf extracts were active against at least one or more of the phytopathogenic fungi in a serial microdilution assay. Of the six plant species, *B. buceras* had the best antifungal activity against four of the fungi, with MIC values as low as 0.02 mg/ml and 0.08 mg/ml against *Penicillium expansum*, *P. janthinellum*, *Trichoderma harzianum* and *Fusarium oxysporum*.

The number of active compounds in the plant extracts was determined using bioautography with the above-mentioned plant pathogens. No active compounds were observed in some plant extracts against the fungal plant pathogens indicating possible synergism between metabolites responsible for the antifungal activity of the extract. *B. salicina* and *O. ventosa* were the most promising plant species, with at least three antifungal compounds.

The antioxidant activities of plant extracts were determined using the qualitative method by spraying TLC chromatograms developed in three eluent systems BEA, CEF and EMW with 1, l-diphenyl -2 picrylhydrazyl (DPPH). The plant extracts of five of these species did not have a strong antioxidant activity. The methanol extract of *X. kraussiana* was the most active radical scavenger in the DPPH assay amongst the six medicinal plants screened.

Based on good activity against *Aspergillus niger* and *A. parasiticus*, leaf extracts of the six plant species were also tested for antifungal activity against *A. fumigatus*, a very important animal fungal pathogen. The acetone extracts of *B. buceras*, *B. salicina*, *V. infausta* and *X. kraussina* had good antifungal activity against the animal pathogens, with MIC values ranging between 0.02 and 0.08 mg/ml. This indicates that crude extracts of these species may be more

valuable in combating *Aspergillus* infections in animals than in humans. Based on the results discussed above, *B. salicina* was selected for in-depth study.

Serial exhaustive extraction was used to extract plant material with solvents of increasing polarities namely, hexane, chloroform, acetone and MeOH. Amongst the four extractants, MeOH extracted the largest quantity of plant material 12.3% (61.5g), followed by acetone 5.6% (27.8 g), hexane 2.6% (12.8 g) and chloroform 2.1% (10.3 g). The chloroform fraction was selected for further work because it had the best antifungal activity against *A. niger*, *C. gloeosporioides*, *P. janthinellum* and *T. harzianum* and the bioautography assay showed the presence of several antifungal compounds in the chloroform fraction.

Column chromatography was used in a bio-assay guided fractionation and led to isolation of four compounds. The antimicrobial activity was determined against seven plant pathogenic fungi and three bacteria, including the Gram-positive *Staphylococcus aureus* (ATCC 29213) and the Gram-negative *Escherichia coli* (ATCC 25922) and *Pseudomonas aureus* (ATCC 27853). The isolated compounds had good antifungal activity against *A. parasiticus* with an MIC of 10 µg/ml, while in other cases it ranged from 20 to 250 µg/ml. Amongst the four compounds tested, only three had a clear band, indicating that the growth of the pathogenic fungi was inhibited in the bioautography assay.

Nuclear magnetic resonance spectroscopy (NMR) and mass spectroscopy (MS) were used for identification of isolated compounds. Only one compound was identified as the triterpenoid ursolic acid. Ursolic acid has been isolated from several plant species and has antifungal activity against *Candida albicans* (Shai et al. 2008). This is the first report on the isolation of antifungal compounds from leaves of *Breonadia salicina*. The other compounds isolated appeared to be mixtures of fatty acids based on mass spectroscopy and the structures were not elucidated.

The cytotoxicity of acetone extracts and the four isolated compounds were determined against Vero cells using a tetrazolium-based colorimetric (MTT) assay. The acetone extract was selected based on good *in vitro* antifungal activity and was used in an *in vivo* fruit experiment. The acetone extract was less toxic toward the Vero cells with an LC_{50} of 82 µg/ml than

ursolic acid and compound 4 which had LC_{50} values of 25 and 36 µg/ml respectively. Compounds 2 and 3 had low toxicity against the cells with LC_{50} values greater than 200 µg/ml.

The potential use of the extract or isolated compound(s) against three plant fungal pathogens *Penicillium expansum* and *P. janthinellum* as well as *P. digitatum* (isolated from infected oranges) were tested after treating the oranges with the extract and ursolic acid. The model used gave good reproducible results. The concentration that inhibited growth correlated reasonably well with MIC values determined by serial microplate dilution. There were substantial differences in the susceptibility of the different isolates tested. The activity of ursolic acid was in the same order as that of the crude acetone leaf extract of *B. salicina*. The LC_{50} of the extract varied from 1 to 1.8 mg/ml.

Penicillium digitatum was more resistant to amphotericin B in comparison to other *Penicillium* species. It has been reported that the fungus was resistant to the three fungicides: sodium *o*-phenylphenate (*o*-phenylphenol), imazalil, and thiabendazole used commercially in the fruit industry to reduce postharvest decay (Holmes and Eckert 1999).

The toxicity of the extract to Vero cells was in the order of 10 times lower than the LC_{50} of the extracts to the fungal pathogens. Although much work still has to be done, there is good potential that a commercial product can be developed from an acetone leaf extract of *B. salicina* leaves, especially if the activity of this extract can be improved by removing inactive compounds.

The results confirm the traditional use of *B. salicina* and demonstrate the potential value of developing biopesticides from plants.

CONFERENCES AND WORKSHOP

2007

Presented at Indigenous Plant Use Forum (IPUF), held at University of Johannesburg.

Paper: S.M Mahlo, McGaw L.J., Eloff J.N. Plant antifungal extracts active against plant pathogenic fungi.

2008

Presented at Royal Society/NRF joint programme, "Scientific validation of South Africa plants with medicinal value" held at Onderstepoort, University of Pretoria.

Paper: S.M Mahlo, McGaw L.J., Eloff J.N. Plant antifungal extracts active against plant pathogenic fungi.

2008

Presented at World Conference on Medicinal and Aromatic Plants (WOCMAP) IV, held at Cape Town International conference centre 2008.

Paper: S.M Mahlo, McGaw L.J., Eloff J.N. Plant antifungal extracts active against plant pathogenic fungi and isolation of antifungal compounds from *B. salicina*.

TABLE OF CONTENTS

Table of Contents

LIST OF FIGURES

LIST OF TABLES