

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

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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.

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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.

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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
LC₅₀	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 *Xylothea 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, 1-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. kraussiana* 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 LC₅₀ of 82 µg/ml than

ursolic acid and compound 4 which had LC₅₀ values of 25 and 36 µg/ml respectively. Compounds 2 and 3 had low toxicity against the cells with LC₅₀ 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 LC₅₀ 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 LC₅₀ 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.

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