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CHARACTERIZATION OF COMPOUNDS FROM *CURTISIA DENTATA* (CORNACEAE) ACTIVE AGAINST *CANDIDA ALBICANS*

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DECLARATION

I declare that the thesis hereby submitted to the University of Pretoria for the degree Philosophiae Doctor has not previously been submitted by me for a degree at this or any other university, that it is my own work in design and in execution, and that all material contained herein has been duly acknowledged.

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'Moreko ga itekole, ebile motho ke motho ka bangwe batho (Sepedi proverb). In short, no man is an island'. THIS PROJECT IS DEDICATED TO MY FAMILY (My wife Grace, my daughters Pontsho and Bonnie, I love you. This is for you).

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LIST OF ABBREVIATIONS

Af	*Aspergillus fumigatus*
AMB	Amphotericin B
BEA	Benzene, ethyl acetate, ammonia (90:10:1)
Ca	*Candida albicans*
ACN	acetone
CD	*Curtisia dentata*
CEF	Chloroform: ethyl acetate: formic acid (5:4:1)
Cn	*Cryptococcus neoformans*
CZ	*Cussonia zuluensis*
DCM	dichloromethane
DE	Dichloromethane: ethyl acetate (4:1)
DEPT	Distortionless enhancement by polarization transfer
DMSO	Dimethylsulfoxide
Ec	*Escherichia coli*
Ef	*Enterococcus faecalis*
EMW	Ethyl acetate: methanol: water (40:5.4:4)
IPUF	Indigenous Plant Use Forum
KA	*Kigelia africana*
Mc	*Microsporum canis*
MIC	Minimal inhibitory concentration
MS	Mass spectrometry
NMR	Nuclear magnetic resonance
Pa	*Pseudomonas aeruginosa*
Rf	Retardation factor
Sa	*Staphylococcus aureus*
Ss	*Sporothrix schenckii*
TE	*Trichilia emetica*
TLC	Thin layer chromatography
TP	*Terminalia phanerophlebia*
TS	*Terminalia sambesiaca*
UPPP	University of Pretoria's Phytomedicine Programme
UV	Ultraviolet
VR	*Vepris reflexa*

PAPERS PREPARED FROM THIS THESIS

LJ Shai, LJ McGaw, P Masoko and JN Eloff. Evaluation of seven South African plant species with activity against *Candida albicans* (Manuscript).

LJ Shai, LJ McGaw, MA Aderogba, LK Mdee and JN Eloff, Antimicrobial Activity of Four Pentacyclic Triterpenoids from *Curtisia dentata* (Manuscript)

L.J. Shai, E.S. Bizimenyera, L.J. McGaw and J.N. Eloff
Lupeol, ursolic acid, betulinic acid and extracts of *Curtisia dentata* inhibit motility of *Trichostrongylus colubriformis, Haemonchus contortus* and *Caenorhabditis elegans* (Manuscript)

L.J. Shai, L.J. McGaw, J.N. Eloff. Extracts of the leaves and twigs of *Curtisia dentata* are more active against *Candida albicans* than the stem bark extract (Manuscript)

CONFERENCE PRESENTATIONS

2005

Paper: L.J. Shai, L.J. McGaw, M. Aderogba and J.N. Eloff. Anti-*Candida* activity of *Curtisia dentata* extracts. **Indigenous Plant Use Forum (IPUF), Rhodes University, Grahamstown (South Africa).**

2006

Paper: L.J. Shai, L.J. McGaw, L.K. Mdee, M. Aderogba and J.N. Eloff. Antifungal constituents from *Curtisia dentata* leaves. **Indigenous Plant Use forum (IPUF), University of Botswana, Gaborone (Botswana).**

Poster: L.J. Shai, L.J. McGaw, L.K. Mdee, M. Aderogba and J.N. Eloff. Antibacterial triterpenes isolated from *Curtisia dentata.* **27th African Health Sciences Congress (AHSC), Durban, South Africa.**

Poster: L.J. Shai., E. Bizimenyera, L.J. McGaw and J.N. Eloff. *Curtisia dentata* extracts, betulinic acid, lupeol and ursolic acid have anthelminthic activity against

Trichostrongylus colubriformis and *Hemonchus contortus in vitro.* . **27th African Health Sciences Congress (AHSC), Durban, South Africa.**

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Paper: Shai L.J., McGaw L.J., Picard J. and Eloff J.N**.** *In vivo* wound healing activity of *Curtisia dentata* extracts and isolated compounds. **Indigenous Plant Use forum (IPUF), University of Johannesburg, South Africa.**

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ABSTRACT

The main aim of the study was to isolate compounds active against *Candida albicans* from the most active species from a pool of several trees. Seven tree species with good antifungal activity were selected from the Phytomedicine Programme database. The selected plant species investigated were screened for growth inhibitory activity against *Candida albicans* using bioautography and serial microplate dilution methods. These tree species were: *Cussonia zuluensis, Vepris reflexa, Curtisia dentata, Trichilia emetica, Terminalia phanerophlebia, Terminalia sambesiaca* and *Kigelia africana.* Using the serial microplate dilution method for the determination of minimal inhibitory concentrations, *Terminalia phanerophlebia* and *T. sambesiaca* were active against *Candida albicans* with MIC values as low 0.02 mg/ml. The acetone and dichloromethane extracts of all plant leaves were active against *C. albicans* with MICs varying from 0.02-2.5 mg/ml. Based on bioautography, the acetone extract of the leaves of *Curtisia dentata* had more active (5) compounds against *C. albicans* than any of the tree species investigated.

The dichloromethane, acetone and hexane extracts of the seven tree species were further screened for antifungal activity using other fungal test organisms. The fungal species used were *Aspergillus fumigatus, Microsporum canis, Sporothrix schenckii* and *Cryptococcus neoformans.* Extracts of *Curtisia dentata, Terminalia sambesiaca* and *Terminalia phanerophlebia* had the highest activities against these fungal test organisms with minimal inhibitory concentration (MIC) values as low as 0.02 mg/ml. *Cussonia zuluensis* was the least active with high MIC values (>250 µg/ml in some cases) and the lowest number (1) of active chemical components on bioautograms. The highest number of active compounds (5) against *C. albicans* on bioautograms was observed in the acetone extracts of *C. dentata.* The plant species were further investigated for presence of antibacterial compounds, using *Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeruginosa* as test bacterial organisms. Compounds with similar R_f values in the acetone extract of *C. dentata* were active against both bacterial and fungal test organisms, suggesting that the growth inhibitory activity of *C. dentata* extracts was non-selective. *C. dentata* was chosen for isolation of compounds due to 1) the highest number of active compounds on bioautogram against *C. albicans,* 2) the MIC values (0.12-0.6 mg/ml) against *C. albicans.*

Acetone extracts of the leaves, stem bark and twigs of *Curtisia dentata* were compared for antibacterial and antifungal activity using the serial microplate dilution and bioautography methods in order to select the plant part to isolate compounds from. The TLC fingerprints of the twigs and leaves were largely similar. A non-polar compound and two medium polarity compounds, present in the leaves and twigs, were missing in the stem bark extract. Bioautography indicated that the leaves contained more antibacterial and antifungal compounds than the stem bark extracts. Extracts of the leaves were 5-fold more active than the stem bark extracts against *Candida albicans,* with total activities of 1072 and 190 ml/g, respectively. Against bacterial test organisms extracts of the leaves, stem bark and twigs resulted in comparable activities. These findings encourage the interchangeable usage of the stem bark, leaves and twigs of this plant, which may lead to sustainable harvesting of the species. This approach may conserve this and other threatened or endangered plant species.

The leaves of *Curtisia dentata* (Cornaceae) were serially extracted with solvents of varying polarities, starting with hexane, then dichloromethane, followed by acetone with methanol completing the fractionation. The dichloromethane (DCM) and acetone bulk fractions of *Curtisia dentata* contained the highest number of active compounds and resulted in low MIC values. The hexane and the methanol bulk fractions were the least active. In the hexane bulk fraction, bioautography revealed the presence of one active compound. The DCM bulk fraction showed cytotoxicity against Vero cells similar to the positive control, berberine with an LC_{50} value of 10 µg/ml. The acetone and dichloromethane fractions resulted in total activity values of 3312 and 4240 ml, respectively. However, these fractions were cytotoxic to the Vero cells with LC_{50} values of 24.4 µg/ml for acetone fraction and 6.6 µg/ml for the dichloromethane fraction. The cytotoxicity data may serve to discourage the use of these extracts to treat candidosis. However, preparations of these fractions may be used topically on wounds to combat infections. The application of these extracts on rat wound model did not result in any observable pathologies.

The DCM and acetone bulk fractions each contained 4 compounds active against *Candida albicans.* Only the dichloromethane extract was fractionated as these extracts contained almost similar active compounds. Column chromatography using silica as the stationary phase afforded four compounds from the DCM extract. These compounds were identified using nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) as lupeol (**CI**), betulinic acid (**CII**), ursolic acid (**CIII**) and

hydroxyl-ursolic acid (**CIV**). These compounds have been isolated from several plant species and have been to be found active against several pathogens including the human immunodeficiency virus (HIV). This is the first report of the isolation of these compounds from *Curtisia dentata.* The antibacterial activity of these compounds have been reported. The anti-*Candida* activity of ursolic oleanolic and ursolic acid has been reported with MIC values exceeding 128 µg/ml (Hiriuchi *et al.,* 2007). However, the anti-*Candida* activity of betulinic acid and lupeol has not been reported.

The four isolated compounds were tested for activity against several fungal (*Candida albicans, C. spicata, C. guillermondi, Aspergillus fumigatus, Sporothrix shenckii, Cryptococcus neoformans* and *microsporaum canis*) and bacterial (*Escherichia coli, Staphylococcus aureus, Enterococcus faecalis* and *Pseudomonas aeruginosa*) species. Ursolic acid and hydroxyursolic acid were the most active with MIC values. Hydroxyursolic acid resulted in an MIC value as low as 8 µg/ml against *M. canis. A. fumigatus* was the most resistant microorganism while *M. canis* and *S. schenckii* were the most sensitive. *C. albicans* was moderately sensitive to the compounds with MIC values ranging from 16 µg/ml for betulinic acid to over 250 µg/ml for lupeol.

Compounds isolated in sufficient quantities, namely, lupeol and betulinic acid, were investigated for cytotoxicity against Vero cells. It appeared that lupeol was less toxic than betulinic acid, with LC_{50} values of 89.5 and 10.9 µg/ml, respectively. The cytotoxicity of betulinic acid was comparable to that induced by the positive control, berberine with an LC_{50} of 10 µg/ml.

Lupeol was the least active of the isolated compounds. Betulinic acid and lupeol, together with the water and acetone extracts were tested in an *in vivo* rat model to determine antifungal and wound healing activities. The rats were immunocompromised prior to the surgical and treatment procedures. Treatments with any of the formulations did not affect wound healing activity. The rate of wound healing was comparable to both the positive (amphotericin B) and negative (cream only) controls. It was however difficult to judge and score antifungal activity. The model developed to evaluate skin infections will have to be improved to allow for testing for anti-*Candida* activity *in vivo*.

Some antifungal compounds, such as azoles, are known to also have anthelminthic activity. The isolated compounds, which had antifungal activity, were tested for anthelminthic activity against both parasitic and free-living nematodes. Furthermore, other publications demonstrated that betulinic acid had anthelminthic activity against

C. elegans. Lupeol, ursolic acid and betulinic acid, together with the DCM and acetone extracts were investigated for anthelminthic activity against both free living and parasitic nematodes. The acetone and dichloromethane extracts were active against all nematodes to concentrations as low as 160 µg/ml. Betulinic acid and lupeol were active against the parasitic nematodes at high concentrations of 1000 and 200 µg/ml. All compounds were active against the free-living *Caenorhabditis elegans* with concentrations as low as 8 µg/ml. Betulinic acid was less active than lupeol and ursolic acid against *C. elegans.* The acetone and dichloromethane extracts were also active against *C. elegans* with a concentration of 0.31 mg/ml resulting in almost 80% inhibition of larval motility. It would appear that the anthelminthic activity against both parasitic and free-living nematodes occurred at high concentrations of the compounds or extracts. Extracts of various medicinal plant species may provide the solutions to ill-health of small ruminants caused by parasitic nematodes in poor communities of southern Africa.

The extracts of *Curtisia dentata* and isolated compounds have anti-*Candida* activity *in vitro.* Their usage is hampered by associated toxicity. The cytotoxicity of the compounds and extracts was only demonstrated with Vero cells (monkey line). Experiments with several human cell lines may indicate the safety of these compound and extracts when used as treatment against *Candida* infections. No toxic effects were noted when extracts and isolated compounds were tested in an animal experiment indicating that extracts may be safe in a topical application. The extract from 1 g of leaf material can be diluted to more than a litre and still inhibit the growth of *C. albicans.*