

# The *in vitro* and *in vivo* biological activities of antifungal compounds isolated from *Loxostylis alata* A.Spreng. ex Rchb. leaf extracts

By

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## Declaration

I declare that the experimental work described in this thesis is my original work (except where the input of others is acknowledged), conducted in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Sciences, University of Pretoria, and has not been submitted in any other form to any University or academic institution. I Mohammed Musa Suleiman declare the above statement to be true.

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## Dedication

This work is dedicated in loving memory to Halimatu Sadiya. May her gentle soul rest in perfect peace. Amen. Every soul will taste death, and then to US will you be returned (Quran 29:57).

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## List of abbreviations used

ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)
Alb	Albumin
AF	<i>Aspergillus fumigatus</i>
A/G	Albumin/globulin ratio
ALT	Alanine amino transferase
Amp B	Amphotericin B
ANOVA	Analysis of variance
AST	Aspartate amino transferase
ATCC	American Type Culture Collection
AUCC	Animal Use and Care Committee
BEA	Benzene, ethyl acetate, ammonia (90:10:1)
BS	$\beta$ -sitosterol
CA	<i>Candida albicans</i>
Ca	Calcium
CCl <sub>4</sub>	Carbon tetrachloride
CEF	Chloroform: ethyl acetate: formic acid (5:4:1)
CH	<i>Commiphora harveyi</i>
CN	<i>Cryptococcus neoformans</i>
<sup>13</sup> CNMR	Carbon 13 Nuclear magnetic resonance
COX	Cyclo-oxygenase
CV	<i>Combretum vendae</i>
DCM	Dichloromethane
DMSO	Dimethylsulphoxide
EC	<i>Escherichia coli</i>
EC <sub>50</sub>	Effective concentration 50
EF	<i>Enterococcus faecalis</i>
EMW	Ethyl acetate: methanol: water (40:5.4:4)
GGT	$\gamma$ -glutamyltransferase
Glob	Globulin
H NMR	Proton Nuclear Magnetic Resonance
Hb	Haemoglobin concentration
H&E	Haematoxylin & Eosin

HT	Haematocrit
IC <sub>50</sub>	Inhibitory concentration
INT	<i>p</i> -iodonitrotetrazolium violet
IPUF	Indigenous Plant Use Forum
KA	<i>Khaya anthotheca</i>
KW	<i>Kirkia wilmsii</i>
LA	<i>Loxostylis alata</i>
LC <sub>50</sub>	Lethal concentration 50
LP	Lupeol
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
MIC	Minimum inhibitory concentration
MC	<i>Microsporium canis</i>
MTD	Maximum tolerated dose
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium
MS	Mass spectrometry
MH	Mueller Hinton
NSAIDS	Nonsteroidal anti-inflammatory drugs
4-NQO	4-nitroquinoline-1-oxide
ON	<i>Ochna natalitia</i>
OECD	Organisation for Economic Co-operation and Development
PA	<i>Pseudomonas aeruginosa</i>
PL	<i>Protorhus longifolia</i>
PM	Post mortem
RCC	Red cell count
RDW	Red cell distribution width
R <sub>f</sub>	Retardation factor
ROS	Reactive oxygen species
SA	<i>Staphylococcus aureus</i>
SD	Sabouraud dextrose
SEM	Standard error of the mean
SIP	Serum inorganic phosphate
SS	<i>Sporothrix schenckii</i>



TA	Total activity
TEAC	Trolox equivalent antioxidant capacity
TLC	Thin layer chromatography
TSP	Total serum protein
UP	University of Pretoria
WCC	White cell count

## Abstract

The main aim of this study was to find a plant extract or isolated compound that could be used to combat aspergillosis in animals. *Aspergillus fumigatus* is one of the most common pathogenic fungal species in humans and animals. *A. fumigatus* is also an economically important fungus in the poultry industry. Current treatment of the disease is hampered by drug resistance of the organism to conventional antifungals and also its widespread toxicity to the animals.

Seven tree species that had good antifungal activity against *Cryptococcus neoformans* in the Phytomedicine Programme database were selected for further work. These tree species were: *Combretum vendae* A.E. van Wyk (Combretaceae), *Commiphora harveyi* (Engl.) Engl. (Burseraceae), *Khaya anthotheca* (Welm.) C.DC (Meliaceae), *Kirkia wilmsii* Engl. (Kirkiaceae), *Loxostylis alata* A. Spreng. ex Rchb. (Anacardiaceae), *Ochna natalitia* (Meisn.) Walp. (Ochnaceae) and *Protorhus longifolia* (Bernh. Ex C. Krauss) Engl. (Anacardiaceae). The antimicrobial activity of leaf extracts of the selected plant species were determined against four important nosocomial bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*) and five important animal fungi (*Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporium canis* and *Sporothrix schenckii*) using a serial microplate dilution method. The minimal inhibitory concentrations (MIC), of an acetone extract of *Loxostylis alata* was the lowest against *Aspergillus fumigatus* with an MIC value of 0.05 mg/ml. The number of antifungal compounds in extracts was determined by bioautography. The acetone extract of *L. alata* had the most active zones (10).

The antioxidant, antiplatelet and cytotoxic effects of the seven plant species were evaluated using established *in vitro* assays. All the extracts had comparably low toxicity except for the extract of *C. harveyi* that had high haemagglutination assay titre value, which indicates toxicity. The extracts of *P. longifolia*, *K. wilmsii*, *O. natalitia*, *L. alata*, *C. harveyi* and *C. vendae* contained antioxidant compounds in the qualitative assay using DPPH. In the quantification of antioxidation using ABTS, only the extracts of *P. longifolia*, *L. alata*, and *C. vendae* had substantial antioxidant activity with respective TEAC value of 1.39, 1.94 and 2.08. Similarly, in the quantitative DPPH assay, *L. alata* (EC<sub>50</sub>, 3.58 ± 0.23 µg/ml) and *K. wilmsii* (EC<sub>50</sub>, 3.57 ± 0.41 µg/ml) did not differ significantly ( $p \leq 0.05$ ) from the positive control (L-ascorbic acid). *K. anthotheca* had a much lower antioxidant activity (EC<sub>50</sub> 176.40 ± 26.56 µg/ml), and differed significantly ( $p \leq 0.05$ ) from all the other extracts and control. In addition, the extract of *C. vendae* and *C. harveyi* had significant ( $p \leq 0.05$ ) antiplatelet activity and did not differ from the control (aspirin) with EC<sub>50</sub> of 0.06 ± 0.01 µg/ml, 0.19 ± 0.00 µg/ml, respectively. Lower EC<sub>50</sub> values in the antioxidant and antiplatelet studies are indicative of

superior activity of the plant extract against oxidation and platelet aggregation. Based on the results obtained *L. alata* was selected for further examination.

To simplify the isolation of the antifungal compounds from the *L. alata* fractions the acetone extract was first separated into six different fractions based on polarity in a mild solvent-solvent fractionation process. The fractions were aqueous methanol, butanol, carbon tetrachloride, chloroform, hexane and water fractions. The antimicrobial activities of the fractions as well as other relevant pharmacological tests on the different fractions were carried out.

The number of antimicrobial compounds present in the aqueous methanol (AM), butanol (BT), carbon tetrachloride (CCl<sub>4</sub>), chloroform (CC), hexane and water fractions was determined by bioautography. The CCl<sub>4</sub> extract was active against six out of the 9 microbial strains used and was particularly active against *S. aureus*, *E. faecalis*, *A. fumigatus*, *C. albicans*, *C. neoformans* and *M. canis* with MIC of 0.04, 0.04, 0.1, 0.1, 0.06 and 0.03 mg/ml, respectively. *Microsporium canis* was the most sensitive organism with the lowest average MIC of 0.16 mg/ml. Qualitative antioxidation using DPPH and quantitative assay using both ABTS and DPPH radicals revealed the presence of several antioxidant compounds in the AM, BT and water fractions of *Loxostylis alata*. This supported the usefulness of *L. alata* in treating fungal diseases, as aspergillosis and most fungal infections are associated with immune depression of the host. Antioxidants may reverse several conditions associated with immune deficiencies, resulting in increased levels of interleukin-2, elevated numbers of total lymphocytes and T-cell subsets.

*Loxostylis alata* is used in southern African traditional medicine to control labour pain and to boost the immune system. Extracts and compounds isolated from leaves of *Loxostylis alata* were therefore also evaluated for their *in vitro* antimicrobial, anti-inflammatory (cyclooxygenase-1 and -2) activities and evaluated for their potential toxic effects using 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) and *Salmonella typhimurium* tester strains TA98 and TA100. Antimicrobial activity was evaluated using a serial microdilution assay. The bacterial strains used were *Staphylococcus aureus* (ATCC29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). The fungal strains used were *Cryptococcus neoformans*, *Sporothrix schenckii*, *Aspergillus fumigatus*, *Microsporium canis* and *Candida albicans*. A bioassay guided fractionation of the crude extract yielded two antimicrobial compounds namely, Lupeol and  $\beta$ -sitosterol. Lupeol had the most pronounced zone of inhibition against *S. aureus* and *A. fumigatus*. When MICs of the 2 compounds were determined, only lupeol had relatively good activity with MICs values  $\leq 100$   $\mu$ g/ml against 8 out of 10 of the tested pathogens. However,  $\beta$ -sitosterol had activity against only *S. aureus* and *E. coli* with MICs values of 90 and 110  $\mu$ g/ml, respectively. In addition  $\beta$ -sitosterol had selective inhibition of COX-1 ( $IC_{50} = 55.3 \pm 2$ ) None of

the compounds isolated were toxic in the *Salmonella typhimurium*/microsome assay and MTT cytotoxicity test. The isolation of these two compounds is reported for the first time from *Loxostylis alata*.

It was disappointing that the two antifungal compounds isolated from *L. alata* had such a low activity against *Aspergillus fumigatus*. This inhibits the development of a single compound that can be used therapeutically. Because the crude extract had very good activity we decided to investigate the safety and potential use of this extract in target animal species. At a dose of 300 mg/kg, the chicks had some signs of intoxication, but not at a dose of 200 mg/kg.

Aspergillosis was induced experimentally, in broiler chicks. The degree of infection was assessed by comparing degree and severity of clinical signs, lesion scores and fungal re-isolation from treated chicks with those from infected chicks not treated with the extract. The extract at a dose of 100 and 200 mg/kg reduced significantly ( $p \leq 0.05$ ) the lesions due to aspergillosis and the amount of *Aspergillus fumigatus* isolated from infected chicks in an excellent dose related response. The crude extract of *L. alata* leaves was as active as the commercially used ketoconazole against avian aspergillosis. It appears likely that the crude acetone extract could be produced at a much lower cost than ketoconazole or other chemical antimicrobial products. If these results can be confirmed in larger studies and if the crude extract does not have a negative effect on the production of the poultry the crude extract of *L. alata* may prove to be a viable and cost effective alternative to using current antimicrobial products. This study proves that it may be worthwhile to invest human and financial resources in searching for plant related products than can increase animal health and productivity.

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