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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Submitted in fulfilment of the requirements for the degree of Doctorae Philosophiae (PhD)

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Pretoria
2009

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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Sign: ----------
Prof V. Naidoo (Co-supervisor)

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).

Acknowledgements

I am most thankful to Prof J.N. Eloff for his dedicated mentorship towards achieving this goal of getting a PhD. Your positive comments and encouragements are highly appreciated. You also stand by me during the passing away of my wife (Halimatu Sadiya). Upon your recommendation I received a PhD bursary from the South African National Research Foundation (SA-NRF). I will forever, remain indebted to Kobus for his noteworthy contribution to my academic development and his kindness to me and my entire family.

The steadfast supervision provided by Prof V. Naidoo in the design, execution and documentation of the animal studies is greatly appreciated. His encouragements and keen interest led to the realization and successful completion of this study.

I will like to extend special thanks to Dr L.J. McGaw who did the proof reading of most of the drafted manuscripts from this thesis. Her patience, kindness and willingness at all times to offer a helping hand are gratefully acknowledged.

My sincere thanks also goes to Dr S.B. Babatunde and Prof N. Duncan for assisting with structure elucidation of isolated compounds and histopathological sections and interpretations, respectively.

I am also grateful to Antonette van Wyk (Poultry Reference Centre UP), Elsabe Myburg (Veterinary Clinical Pathology Section, UP) for their wonderful technical assistance.

My profound gratitude goes to Ella Bischoff, Lita Pauw, Tharien De Winnar, and Denise Marais for your wonderful planning and execution of all administrative duties relating to my research work. You ensure that all necessary funds, equipment and materials that could facilitate my welfare, comfort and academic pursuit are made available on time. “Baie dankie”

I am particularly thankful to Dr V. Bagla (colleague and friend) for teaching me techniques on cell culture and Dr E. Iwalewa for useful discussions that helped in my research work.

I fully appreciate the kind help provided by Bellona Sakong, in ensuring that all consumables used in the course of my research are adequately provided in the Phytomedicine laboratory, UP. “Ngiyabonga”

The patience and understanding of my mother, that of my late wife, Halimatu Sadiya, my daughters, Aishatu and Khadija, as well as that of the rest of my family and friends are highly appreciated.

I reserved special thanks to the families of Prof. D.A.Y. Adawa, that of Drs. Idris Abubakar Misau, Rabiu Nasiru Charanci, Haruna Jibrin, Sodangi A Luka and Mrs Hassana Muazu for being special parents to Aishatu and Khadija (my daughters).

I shall remain ever grateful to Profs. Y.O. Aliu and M. Mamman for their advice when I almost gave up coming to back to Pretoria after I lost my beloved wife.

I extend special thanks to the entire staff of the Department of Veterinary Physiology and Pharmacology, Ahmadu Bello University (A.B.U.), Zaria for sharing my teaching responsibilities during the period of my study fellowship, authorities of (A.B.U.) for granting me the study fellowship, University of Pretoria for partially financing my studentship through the University postgraduate bursary and also funding my PhD research.

Above all, I remain eternally grateful to GOD (The Exalted, The Almighty) for His mercy.

Conference presentations

Suleiman, M.M., McGaw, L.J., Eloff, J.N., "Screening for antifungal activity in selected South African traditional medicinal plants" Presented during the 10th Annual International Plant Use Forum (IPUF) Conference at University of Johannesburg, Johannesburg, South Africa, July, 2007.

Suleiman, M.M., McGaw, L.J., Bagla, V.P., Eloff, J.N., "Screening for antibacterial activity in selected South African traditional medicinal plants" Presented during the 10th Annual International Plant Use Forum (IPUF) Conference at University of Johannesburg, Johannesburg, South Africa, July, 2007.

Suleiman, M.M., McGaw, L.J., Picard, J., Naidoo, V., Eloff, J.N., "Detection of antimicrobial compounds by direct bioautography in seven South African plants" Presented during the 4th World Conference on Medicinal and Aromatic Plants (WOCMAP) Conference at Cape Town International Conference Centre, Cape Town, South Africa, November, 2008.

Manuscripts published and submitted

Suleiman, M.M., McGaw, L.J., Naidoo, V., Eloff, J.N., 2010. Detection of antimicrobial compounds by bioautography of different extracts of leaves of selected South African tree species. African Journal of Traditional, Complementary and Alternative Medicines, 7, 64-78.

Suleiman, M.M., McGaw, L.J., Naidoo, V., Eloff, J.N., 2010. Evaluation of several tree species for activity against the animal fungal pathogen *Aspergillus fumigatus*. South African Journal of Botany, 76, 64-71.

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Suleiman, M.M., Naidoo, V., Eloff, J.N., 2010. Preliminary screening of some fractions of *Loxostylis alata* (Anacardiaceae) for antimicrobial and antioxidant activities. Prepared for submission to the African Journal of Biotechnology for publication.

Suleiman, M.M., Naidoo, V., Samuel, B.B., Elgorashi, E.E., Eloff, J.N., 2010. Biological activities of two antifungal compounds isolated from *Loxostylis alata* (Anacardiaceae).Biological activities of compounds isolated from *Loxostylis alata* (Anacardiaceae). Prepared for submission to the South African Journal of Botany for publication.

Suleiman, M.M., Naidoo, V., Bisschop, S.P.R., Eloff, J.N., 2010. A leaf extract of *Loxostylis alata* (Anacardiaceae) prevented experimental aspergillosis in chicks. Prepared for submission to Onderstepoort Journal of Veterinary Research for publication.

List of abbreviations used

ABTS	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)
Alb	Albumin
AF	*Aspergillus fumigatus*
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	β-sitosterol
CA	*Candida albicans*
Ca	Calcium
CCl_4	Carbon tetrachloride
CEF	Chloroform: ethyl acetate: formic acid (5:4:1)
CH	*Commiphora harveyi*
CN	*Cryptococcus neoformans*
^{13}CNMR	Carbon 13 Nuclear magnetic resonance
COX	Cyclo-oxygenase
CV	*Combretum vendae*
DCM	Dichloromethane
DMSO	Dimethylsulphoxide
EC	*Escherichia coli*
EC_{50}	Effective concentration 50
EF	*Enterococcus faecalis*
EMW	Ethyl acetate: methanol: water (40:5.4:4)
GGT	γ-glutamyltransferase
Glob	Globulin
H NMR	Proton Nuclear Magnetic Resonance
Hb	Haemoglobin concentration
H&E	Haematoxylin & Eosin

HT	Haematocrit
IC_{50}	Inhibitory concentration
INT	*p*-iodonitrotetrazolium violet
IPUF	Indigenous Plant Use Forum
KA	*Khaya anthotheca*
KW	*Kirkia wilmsii*
LA	*Loxostylis alata*
LC_{50}	Lethal concentration 50
LP	Lupeol
MCH	Mean cell haemoglobin
MCHC	Mean cell haemoglobin concentration
MCV	Mean cell volume
MIC	Minimum inhibitory concentration
MC	*Microsporum canis*
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	*Ochna natalitia*
OECD	Organisation for Economic Co-operation and Development
PA	*Pseudomonas aeruginosa*
PL	*Protorhus longifolia*
PM	Post mortem
RCC	Red cell count
RDW	Red cell distribution width
R_f	Retardation factor
ROS	Reactive oxygen species
SA	*Staphylococcus aureus*
SD	Sabouraud dextrose
SEM	Standard error of the mean
SIP	Serum inorganic phosphate
SS	*Sporothrix schenckii*

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*, *Microsporum 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 haemagluttination 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_{50}, 3.58 ± 0.23 µg/ml) and *K. wilmsii* (EC_{50}, 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_{50} 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_{50} of 0.06 ± 0.01 µg/ml, 0.19 ± 0.00 µg/ml, respectively. Lower EC_{50} 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_4), chloroform (CC), hexane and water fractions was determined by bioautography. The CCl_4 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. *Microsporum 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*, *Microsporum canis* and *Candida albicans*. A bioassay guided fractionation of the crude extract yielded two antimicrobial compounds namely, Lupeol and β-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 ≤ 100 µg/ml against 8 out of 10 of the tested pathogens. However, β-sitosterol had activity against only *S. aureus* and *E. coli* with MICs values of 90 and 110 µg/ml, respectively. In addition β-sitosterol had selective inhibition of COX-1 (IC_{50} = 55.3 ± 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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Chapter 1
Introduction

Long before mankind discovered the existence of microbes as the major cause of disease, the idea that certain plants had healing potential was well accepted. Since antiquity, man has used plants to treat common infectious diseases and some of these traditional medicines are still included as part of the habitual treatment of various maladies (Buwa, and van Staden, 2006). For example, the use of bearberry (*Arctostaphylos uva-ursi*) and cranberry juice (*Vaccinium macrocarpon*) to treat urinary tract infections is reported in different manuals of phytotherapy, while species such as lemon balm (*Melissa officinalis*), garlic (*Allium sativum*) and tee tree (*Melaleuca alternifolia*) are described as broad-spectrum antimicrobial agents (Heinrich et al., 2004). Archaeological records suggest that the earliest human use of plants for medicinal purposes date back to about 60,000 years ago (Fabricant and Farnsworth, 2001). The famous physician of the middle ages, Philippus Aureolus Theophrastus Bombast von Hohenheim, commonly known as Paracelsus, who lived from 1490 to 1540 probably applied the term “laudanum” (something to be praised) to several different preparations, all of which contained opium as the basic constituent. The laudanum of the early London pharmacopoeias contained opium, wine, and other ingredients. One of first documented evidence of medicinal plant use was that of a French surgeon, Ambrose (1517-1590), who treated gunshot wounds with a mixture of chamomile, melilot flowers, lavender, rosemary, sage, thyme and the extract from red roses boiled in white wine (Macht, 1915).

For centuries people have used plants for healing purposes (Raskin et al., 2002). Plant products, as parts of foods or botanical potions and powders have been used with varying success to cure and prevent diseases throughout history. The strong historic bond between plants and human health began to unwind in 1897, when Friedrich Bayer and his co-workers introduced synthetic acetyl salicylic acid (aspirin) to the world. Aspirin is a safer synthetic analogue of salicylic acid, an active ingredient of willow bark, and was discovered independently by residents of both the New and Old worlds as a remedy for aches and fevers (Pierpoint, 1994). This was the start of the modern pharmaceutical industry that made medicines much more accessible to the general public. This wide scale of use came with the major pitfall of drug resistance. Nonetheless, in the global context, natural products and their derivatives form about 50% of drugs in clinical use with about 25% derived from higher plants (Farnsworth, 1984; O’Neill and Lewis, 1993; Harborne, 1998). Many conventional therapeutic agents are obtained from plant sources. Examples include salicylic acid (from willow bark), digoxin (from foxglove), quinine (from cinchona bark), and morphine (from the opium poppy) (Vickers and Zollman, 1999). With the emergence of extreme drug resistance from the modern pharmaceutical industry new drugs molecules need to be found.

The plant kingdom is a reservoir of varied chemicals and so far only a small fraction of plants have been assayed for medicinal activity. It has been variously estimated that there are about 250,000 species of plants on Earth (Cowan, 1999). More importantly, with herbal remedies continuing to be widely used in management of human disease, this represents major ethnobotanical leads in the discovery of new medicines. In South Africa and in many African homes, medicinal plants are sold or prescribed by traditional medical practitioners (Fyhrquist et al., 2002). Southern Africa is exceptionally rich in plant diversity with some 24,000 species of flowering plants almost one tenth of the worlds higher plants of which 80% are endemic. This includes 10 plant families and 29% of the total genera of the world (Goldblatt, 1978). It is estimated that 27 million South Africans depend on traditional medicines from as many as 1020 plant species (Dauskardt, 1990; Mander, 1998). The trade equates to approximately 20,000 tonnes of plant material sold annually as drugs in South Africa (Mander, 1998).

The antiseptic qualities of aromatic and medicinal plants and their extracts have been recognized since antiquity, while attempts to characterize these properties in the laboratory date back to the early 1900s (Martindale 1910; Hoffman and Evans 1911). Plant volatile oils are generally isolated from non-woody plant material by distillation methods, usually steam or hydrodistillation, and are variable mixtures of principally terpenoids, and a variety of low molecular weight aliphatic hydrocarbons, coumarins and homologues of phenylpropanoids. Terpenes are amongst the chemicals responsible for the medicinal, culinary and fragrant uses of aromatic and medicinal plants (Dorman and Deans, 2000). Although plant secondary products have historically been defined as chemicals that do not appear to have a vital biochemical role in the process of building and maintaining plant cells, recent research has shown a pivotal role of many of these chemicals in ecophysiology of plants (Briskin, 2000). Accordingly secondary products have both a defensive role against herbivory, pathogen attack, and interplay competition and an attractant role toward beneficial organisms such as pollinators or symbionts (Wink and Schimmer, 1999).

Plants produce a huge variety of secondary compounds as natural protection against microbial and insect attack. Some of these compounds are also toxic to animals, but others may not be toxic. Indeed, many of these compounds have been used in the form of whole plants or plant extracts for food or medical applications in man (Wallace, 2004).

The diversity of plants growing worldwide, along with their known ethnopharmacological uses offer an enormous possibility of finding novel chemical agents with efficacious antifungal properties. These plants include amongst others *Terminalia australis* which is used to treat aspergillosis and candidosis (Carparo et al., 2003), and *Acacia caven* for treating vaginal mycoses (Hilgert, 2001). The aqueous and alcoholic

extracts of *Sebastiana commersoniana* have shown antibacterial and antifungal activities (Penna et al., 2001). Tannins isolated from *Terminalia trifolora* demonstrated a powerful antifungal action in clinical studies (Latte Kolodziej, 2000). In a similar study, Masoko et al (2005), showed the efficacy of six *Terminalia* species found in South Africa against *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporum canis* and *Sporothrix schenkii*. Quite a number of plants in Tanzania are used in traditional medicine and have shown good results when tested against *Candida* infections of human (Runkoro et al., 2006). The volatile oils of black pepper *Piper nigrum* L. (Piperaceae)], clove [*Syzygium aromaticum* (L.) Merr. & Perry (Myrtaceae)], geranium [*Pelargonium graveolens* L'Herit (Geraniaceae)], nutmeg [*Myristica fragrans* Houtt. (Myristicaceae), oregano [*Origanum vulgare* ssp. *hirtum* (Link) Letsw. (Lamiaceae)] and thyme [*Thymus vulgaris* L. (Lamiaceae)] were assessed for antibacterial activity against 25 different genera of bacteria. These included animal and plant pathogens, food poisoning and spoilage bacteria. The volatile oils exhibited varying levels of inhibitory effects against all the organisms (Dorman and Deans, 2000).

1.1. Hypothesis

Plants contain antimicrobial agents which are active against animal and human pathogenic bacteria and fungi including *Aspergillus fumigatus*. They also contain antioxidant and anti-inflammatory compounds that could be use to ameliorate or abolish the severity of diseases cause by antimicrobial agents. These active compounds can be isolated and characterized to yield potential drugs with novel structures or activities for use in treating and protecting humans and animals against pathogenic bacteria and fungi, and also against certain inflammatory diseases. Moreover, the plant extracts or compounds can also be used to enhance animal productivity.

1.2. Justification of the study

Results generated from this study will add to the ever increasing database knowledge on plant medicines and contribute to the safe and efficacious use of plant drugs in both rural and urban set ups. Natural plants that are not effective will not form part of our disease management system in future. Because aspergillosis is an important poultry disease searching for plant extracts with good activity against *Aspergillus fumigatus* may lead to a commercially useful therapeutic agent.

Organised ethnomedical research and development through cultivation of medicinal herbs and the creation of local trade and industry can bring more jobs and income to rural inhabitants (McCorkle, 1995). The use of alternative drugs has been recommended as a measure to avoid the development of resistant strains of

microorganisms and to increase the possibility of reducing the cost of controlling the disease (Cowan, 1999).

The high incidence of fatal fungal and bacterial infections occurring in association with increased use of immunosuppressive drugs stimulated the desire to explore an additional chemotherapeutic weapon in the fight against microbial diseases (Frank et al., 2003). A practical solution to this problem is to develop acceptably effective drugs from reasonably inexpensive and locally available raw materials. An obvious way of achieving this goal is through the study of traditional medical herbs, selecting from those that show promising results for development into effective and safer drugs (Ibrahim et al., 1983). Another approach is to use data for plant species randomly screened for biological activity such as the Phytomedicine Programme database of activities against leaf extracts of close to 500 tree species. (Phytomedicine Programme website www.up.ac.za/phyto).

1.3. Summary and problem statements

- Aspergillosis in poultry and domestic animals is of economic importance, while it is a major health problem in humans. Diseases cause by fungal organisms particularly in immunocompromised patients remain a major cause of concern in Africa and the rest of the world.

- Resistance developed by microbial organisms against commonly and licensed commercial antimicrobials render many infectious diseases difficult to treat and control (Van der Waaij, 1987).

- Although there appear to be an array of drugs for the treatment of systemic and superficial mycoses, none of them is ideal in terms of efficacy, safety or antifungal spectrum (Di Domenico 1998; Ablordeppey et al., 1999).

1.4. Aim and objectives

The aim of this study is to find a plant extract or isolated compound that could be used to combat aspergillosis in poultry. The following objectives can be identified to attain this aim:

- Evaluate the antibacterial and antifungal activity selected South African plant species against a range of pathogenic bacterial and fungal species, in order to select the species with the best antimicrobial activity for further investigation.

- Isolate and characterize the compounds active against *Aspergillus fumigatus* from extracts of selected plant species.

- Determine the in *vitro* antimicrobial, antioxidant and anti-inflammatory activities and cytotoxicity of the extracts and isolated compound(s) of selected plant species.

- Evaluate the efficacy of the isolated compound(s) or crude extracts *in vivo* in a poultry model.

Chapter 2
Literature Review

2.1. Importance of poultry

Poultry is by far the largest livestock group and is estimated to consist of about 14 000 million birds, consisting mainly chickens, ducks and turkeys (FAO 1999). In total, poultry products (egg and meat) constitute 30% of all animal protein consumed worldwide. Within the last 10 years, this proportion has increased from 20% to 30% of all animal protein and is predicted to increase to 40% before the year 2015 (FAO 1999). Poultry provides a vital supply of food for the world's population. All over the globe, poultry meat and eggs are preferred to other kinds of animal food products for a variety of reasons. Mack et al., (2005) estimated that 25 percent of the world's meat supply is derived from poultry, i.e. chicken, turkey, duck, geese, domesticated quail, etc. and the proportion is increasing steadily. The trend has been more noticeable in developing countries in recent years. World poultry meat output increased nearly eight-fold in 1961-2001, while the output in middle-income countries even rose more than twelve-fold. The biggest global poultry meat producers are the United States, the EU, China, Brazil, Mexico, Canada and Japan. Among middle-income countries, China was the major producer in 2001, followed by Brazil, Mexico, Argentina, Iran, Russia, Egypt and Poland. In 1961, middle-income countries produced 34 percent of world poultry meat, high-income countries 61 percent, and low-income countries the remaining 5 percent. By the mid-1990s, middle-income country production had reached a level of 47 percent of the output of high-income countries. By 2001, middle-income countries accounted for the major share of world poultry production (52 percent) compared with 42 percent in high-income countries and less than 6 percent in low-income countries (Regmi, 2001).

In 1995, African livestock population poultry was the most numerous species of farm animal (Anonymous, 1996). More than 80% of poultry are kept in rural areas (Sonaiya and Olori, 1997). Throughout the African continent all ethnic groups are involved in poultry production (Guéye, 1998). In addition to providing farmers with eggs and meat for their home consumption, poultry or their products are kept for sale (or barter) thereby generating a source of income for the family. Some communities, indigenous fowl have a symbolic importance within many social activities (e.g. special banquets for distinguished guests) and/or religious ceremonies. For example cocks are offered to the deities (Bell and Abdou, 1995). In the Western Middle-Belt region of Nigeria, Atteh (1989) reported the reasons for keeping village fowls as being 11% for income alone, 28% for consumption alone, 45% for income and consumption, 3% for ceremonies, 11% for income and ceremonies, 3% for consumption and ceremonies and 1% for recreational purposes. In the Keita

region of Niger home consumption accounted for 47%, sales for 38% and gifts for 16% (Bell and Abdou, 1995). In the traditional society of the Mamprusi tribe in Northern Ghana, the uses of poultry were 35% for sacrifice, 28% for sale, 15% for consumption, 13% for gifts and 10% for breeding stock, while 71% of the eggs were set aside for hatching, 18% for sale, 5% for consumption and 5% for gifts. (Veluw, 1987) Additionally, indigenous fowl play an important role in traditional ritual prayers to appease the gods (Lul, 1990; Ngou Ngoupayou, 1995).

2.2. Poultry production

World production of poultry meat (all domestic birds) and eggs was 28.0 and 26.5 million metric tonnes respectively in 1979. On a continent basis, Africa, Oceania and South America contribute proportionally considerably less to world production than the other continents (Biggs, 1982). Africa, Asia and South America produce considerably less per person than Europe, North and Central America and Oceania. With the exception of Europe the major part of both poultry meat and egg production for each continent is provided by one to three countries. The difference in production *per capita* reflects each continent's development stage. Production *per capita* 6.5 times greater for poultry meat in developed countries compared with developing countries and 5.4 times greater for egg production (FAO, 1999).

In South Africa, the poultry industry contributes greatly to the Agricultural sector. It is estimated that the industry contributes approximately 16% of the total gross value of agriculture in South Africa. The broiler industry in South Africa currently produces on average 13.8 million broilers per week, growing steadily from 1990 when only 7.6 million broilers per week were produced (Global Agricultural Information Network report SF7042, 2007). In 2007, about 950 million and 23 million broilers and layer chickens, respectively were produced in South Africa (SAPA, 2008). In addition, SAPA (2008) reported the production of 360,000 eggs per week in the year 2007. Latest estimates suggest that up to 7,000 people are employed by the poultry industry in South Africa, making the industry and important rural employer (SAPA, 2008).

2.3. Constrains to poultry production

Some problems associated with poultry production include disease control, protection against various predators, better feeding, genetic improvements, marketing, training and management, access to production inputs, infrastructure and capital, farmer organization, and, foremost, conducive institutions and governmental policies (Mack et al., 2005). The introduction of modern intensive production methods, new breeds and improved preventive disease control and bio-security measures has rapidly change poultry production in the past few decades. The progress in industrial poultry production methods has however had

little effect on subsistence poultry production methods in rural and peri-urban areas, where inputs into disease control remain minimal especially in developing countries (Hoffmann, 1998). There is therefore the need to find more alternative approaches towards disease control and prevention. Important infectious poultry diseases are cause by bacteria, viruses, parasites and fungi. Advances and discoveries in the control and treatment of other infectious poultry diseases have been outstanding in recent years. However, less progress has been made in the control of fungal infections in poultry (Chute and Richard, 1997). Fungal diseases especially aspergillosis cause by *Aspergillus fumigatus* has an impact on health of birds and hence lowers their ability to produce enough meat and egg for the populace. Because of negative associations with the use of antimicrobial feed additives in animal production, there is an incentive to develop plant product based products that can replace antbiotic feed additives.

2.4. Aspergillosis in humans and animals

Denning, et al., 1991 and Latgé, 2001 are excellent references for this topic and some of the material in this section were found there.

Aspergillus fumigatus has become the most prevalent airborne fungal pathogen, causing severe and usually fatal infections in immunocompromised hosts in developed countries (Andriole, 1993). In addition the prevalence of the disease has increased fourfold in the last 12 years. In 1992, invasive *Aspergillus* (IA) was responsible for approximately 30% of fungal infections in patients dying of cancer, and it is estimated that IA occurs in 10 to 25% of all leukaemia patients, in whom the mortality rate is 80 to 90%, even when treated (Bodey et al., 1992). IA is now a major cause of death at leukaemia treatment centres and bone marrow transplantation (BMT) and solid-organ transplantation units (Patel and Paya, 1997).

While in the immunocompetent host, *A. fumigatus* is seldom pathogenic, the downregulation of the immune system induced by immunosuppressive therapies and congenital defects triggers the development of aspergillosis in most human infections (Latgé, 2001). In recent years, aspergillosis has emerged as a significant disease in humans that are immunocompromised by acquired immunodeficiency syndrome, neoplasia, or chemotherapy (Denning, et al., 1991). In humans, aspergillosis resulting from exposure to *A. fumigatus* can be primarily a manifestation of hypersensitivity responses (Disch et al., 1995), damage to host tissues by invasive colonization (Denning, et al., 1991), or a complexity of allergy and infection (Awadhiya et al., 1981).

For most patients, the main portal of entry and site of infection for *A. fumigatus* is the respiratory tract, although other sites of infections have been described in the normal or immunocompromised host, such as

the skin, peritoneum, kidneys, bones, eyes, and gastrointestinal tract, nonrespiratory infections are infrequent (Prescott et al., 1992). Pulmonary diseases caused by *A. fumigatus* can be classified according to the site of the disease within the respiratory tract and the extent of mycelial colonization or invasion, both of which are influenced by the immunological status of the host (Dixon, and Walsh, 1992). Allergic diseases, including asthma, allergic sinusitis, and alveolitis, are also common with infection due to *A. fumigatus*. They occur following repeated exposure to conidia or antigens of *Aspergillus* in the absence of mycelial colonization, and in most cases, removal of the patient from the environmental source results in clinical improvement. In contrast, allergic bronchopulmonary aspergillosis (ABPA), aspergilloma, and IA, syndromes involving mycelial growth of *A. fumigatus* in the body, usually require therapeutic intervention (Latġe, 1999).

Sinusitis and orbital cellulitis caused by *Aspergillus* species is known to exhibit a granulomatous response in cats (Wilkinson, et al., 1982). This reported case was similar to that observed in dogs (Lane et al., 1974), as a destructive lesion with lysis of the turbinate tissues in the absence of significant soft tissue proliferation. Immuno-incompetence, tissue damage by other microbial infections, trauma or neoplasia have been cited as predisposing factors in the initiation of aspergillosis in the dog (Lane, 1982), dolichocephalic breeds being predominantly affected.

It was also proposed that infection with feline leukaemia virus in cat, combined with prolonged antibiotic treatment regimes allowed aspergillosis to become established in the nasal chamber. Another animal that is affected by aspergillosis is the horse, specifically in the head region. Adult immunocompetent horses are usually affected as a result of epistaxis or neurologic deficits. *A. fumigatus* is the organism most commonly associated with this disease. Guttural pouch mycosis is predisposed by factors that include soft tissue trauma and environmental conditions that encourage conidial germination such as poor ventilation, high humidity, and warm temperatures. The mode of entry is presumed to be oropharyngeal during expiration and deglutination. The guttural pouch is a large air filled extension of the Eustachian tube that has openings into both the middle ear and oropharynx. This anatomic structure is unique to the order Perrisodactyl. There are numerous arteries, veins and nerves that pass through the guttural pouch. The guttural pouch helps to cool internal carotid blood before perfusing the brain (Tell, 2005). *A. fumigatus* has been recovered from the faeces of horses with a history of persistent diarrhoea and given aetiological significance (Lundvall and Romberg, 1960).

Aspergillus fumigatus is associated with sporadic bovine mycotic abortion in the northern hemisphere especially in cows that are in their second or third trimester of pregnancy. The incidence of this disease is

highest during the winter months when gravid cows are confined to sheds and fed hay or silage that are heavily contaminated with *Aspergillus* spores.(Tell, 2005).

2.5. Aspergillosis in poultry

The poultry industry faces heavy economic losses (mortality and morbidity) due to *Aspergillus fumigatus* infection and its mycotoxins. The financial losses due to aspergillosis are enormous with about US$11 million reported as an average annual loss in the United States of America (Kunkle, 2003). *Aspergillus fumigatus* has been recognized as pathogenic to birds for more than a century (Wright et al., 1962) and in addition to having a worldwide distribution it has been reported to be pathogenic in almost all farm birds and in numerous other wild species of birds (Akan et al., 2002; Chang Reissig et al., 2002).

Aspergillosis is a fungal disease mainly located in the respiratory system and characterized with lesions in the internal organs, eyes and, in certain cases, the brain in poultry. *Aspergillus fumigatus* has been determined as the most pathogenic among the causative agents of aspergillosis with widespread resistant spores present in the natural environment (Richard, 1997). Poultry aspergillosis, mainly observed in the young of turkeys and chicken, is also reported in other poultry species, including ducks, geese, quails, ostriches, parrots, canaries, pigeons, flamingos and penguins (Richard, 1997). In the acute form of the disease, which develops within 24-48 h in young animals, the rate of mortality may vary from 70% to 90%, whereas in the sub-acute form of the disease which develops within 8-10 days in animals up to the age of 2 weeks various clinical phases are reported (Roy et al 1991). Pathological observations in all poultry species are essentially the same (Richard, 1997). Macroscopic findings differ with regard to the location of the disease. Lungs are the internal organs reported to be most affected. Lesions may range from miliary to larger granulomatous foci (Richard, 1997) which are grey-yellow-white in colour, dry in consistency and protrusive with regard to the surface of the internal organ in which they are located (Richard, 1997). Thickening and dullness of the walls of the air sacs have been reported to develop because of the infection (Richard, 1997). Microscopic examination has revealed the presence of granulomatous foci and caseous necrosis with a surrounding region of proliferation including giant cells, macrophages, hetereophils and lymphocytes and an outer capsule of connective tissue. Fungal hyphae with or without septa and fungal spores located within regions of necrosis may easily be observed microscopically by using special staining methods (Richard, 1997).

As a disease, aspergillosis affects birds in captive or free-ranging environments, young and mature, immunocompetent or immunosuppressed. Predisposing factors include species predilection, environmental

conditions (limited air exchange, exposure to allergens resulting in mucosal irritation, and extreme temperature and humidity), immunosuppression secondary to intensive production, physical exertion (migration), and administration of exogenous corticosteroids. Aspergillosis leads to consequential economic losses related to low productivity, mortality and carcass condemnations at slaughter inspection (Morris & Fletcher, 1988; Richard, 1997). Two forms of the disease are regularly reported in turkey poults. The first form is an acute aspergillosis leading to severe outbreaks in very young birds with clinical signs of dyspnoea, gasping and inappetence. The chronic form of aspergillosis most commonly occurs in 13-week-old to 18-weekold turkeys, late in the growing cycle and associated with respiratory distress as clinical signs.

Aspergillus fumigatus is also considered an opportunistic pathogen that causes disease in immunocompromised birds or in birds exposed to overwhelming numbers of fungal spores. While In most cases, the primary site of development is the respiratory tract (air sacs and lungs) blood dissemination frequently occurs, leading to macroscopic lesions in a wide range of organs or tissues. In spontaneous cases, lesions range from miliary to larger granulomatous foci (Singh et al., 1994), which are white in colour and protrusive to the surface of the internal organ. Thickening of the walls of the air sacs is frequently reported (Perelman and Kuttin, 1992; Richard, 1997). Lesions in avian species are commonly confined to the lungs (Figure 2.1.) and air sacs, although infections of oral mucosa, trachea, brain, eye, skin, bone, liver, kidney (Richard, 1991), and nasal passages (Fitzgerald and Moisan 1995) have also been described. Typical lesions are characterized by granulomatous inflammation with necrosis, haemorrhage, and intralesional fungal elements that are locally invasive.

Figure 2.1. Nodular form of aspergillosis in the lungs. Note the presence of yellowish dense nodules on the lungs.

2.6. Mycotoxicoses

Mycotoxicoses is a toxic syndrome resulting from the intake of mould contaminated feed, which has toxic metabolites of mould called mycotoxins. *Aspergillus fumigatus* is one species that produces potent toxic metabolites like aflatoxin, gliotoxin and ochratoxin. Mycotoxicoses have interactions with bacterial infections i.e. the increased intake of aflatoxin in feed will increase susceptibility of chicken to bacteria due to immune suppression in the host animal by the toxin infection. Likewise there will be synergistic effects when more than one type of toxin is present in the feed. Mycotoxicoses are of public health importance since they and their metabolites are present in poultry fed with contaminated feed and are harmful to human beings (Raja and Lakshmana, 1991). Consumption of aflatoxin by poultry can interfere with resistance to various infections (Chute and Richard, 1997).

Gliotoxin is one of the several toxins produced by various isolates involved in an outbreak of aspergillosis of turkeys (Richard, 1990). Gliotoxin is immunosuppressive, cytotoxic and inhibits transformation of turkey peripheral blood lymphocytes (Richard et al., 1994). Ochratoxins are metabolites of both *Aspergillus* and *Fusarium* species which are chemically described as 3,4-dihydromethylisocoumarin derivatives (Cole and Cox, 1981). These compounds are known for their nephrotoxic effects (renal damage) in poultry (Lanza et al., 1980; Manning and Wyatt, 1984).

2.7. Treatment of aspergillosis in birds

The two most important antifungal drugs currently available for the treatment of aspergillosis in birds are amphotericin B and itraconazole. Amphotericin B is the only fungicidal drug currently available and remains the gold standard with which other drugs are compared (Lyman and Walsh, 1992). Amphotericin B has

been used to treat birds both topically and systemically (Orosz, 2000). Itraconazole is a lipophilic triazole that is used for the treatment of aspergillosis. Few, if any, drugs have proven to be effective against *A. fumigatus.* Drugs such as nystatin and amphotericin B have been used in poultry without reproducible results (Chute, 1984).

Although there appear to be an array of drugs for the treatment of systemic and superficial mycoses, none of them is ideal in terms of efficacy, safety or antifungal spectrum (Di Domenico, 1998; Ablordeppey et al., 1999). Many of the drugs have undesirable effects or are very toxic (amphotericin B), produce recurrence, have drug–drug interactions (azoles) or lead to the development of resistance (fluconazole, 5-flucytosine) (White et al., 1998). Numerous useful drugs from higher plants have been discovered by random selection followed by chemical screening or through ethnobotanical (Fabricant and Farnsworth, 2001). The diversity of plants growing in South Africa, offer an enormous possibility of finding novel structures or extracts with antifungal properties.

2.8. Antifungal drugs in common use and their mode of actions

Reviews by Ghannoum and Rice, 1999, Odds et al., 2003, and Parks and Casey, 1996 on the mechanism of actions of antifungal drugs are excellent and some of the materials presented under this heading are obtained from those articles.

2.8.1. Drugs interfering with microtubular function

The earliest inhibitory agent specific to fungal species was griseofulvin (Odds et al., 2003). Griseofulvin is an antifungal agent isolated from the fungus *Penicillium griseofulvum*. The drug is believed to interfere with microtubule assembly and mitosis in the fungal cell. The selective toxicity of griseofulvin for fungi is only moderate (liver toxicity is recognized as an occasional hazard) and its spectrum of action is restricted mainly to the dermatophyte fungi – causes of ringworm and athlete's foot. It is taken up selectively by newly formed skin and concentrated in the keratin (Rang et al., 2003).

2.8.2. Agents affecting fungal sterols

The three major groups of antifungal agents in clinical use, azoles, polyenes, and allylamine/thiocarbamates, all owe their antifungal activities to inhibition of synthesis of or direct interaction with ergosterol. Ergosterol is the predominant component of the fungal cell membrane (Figure 2.2.) (Parks and Casey, 1996).

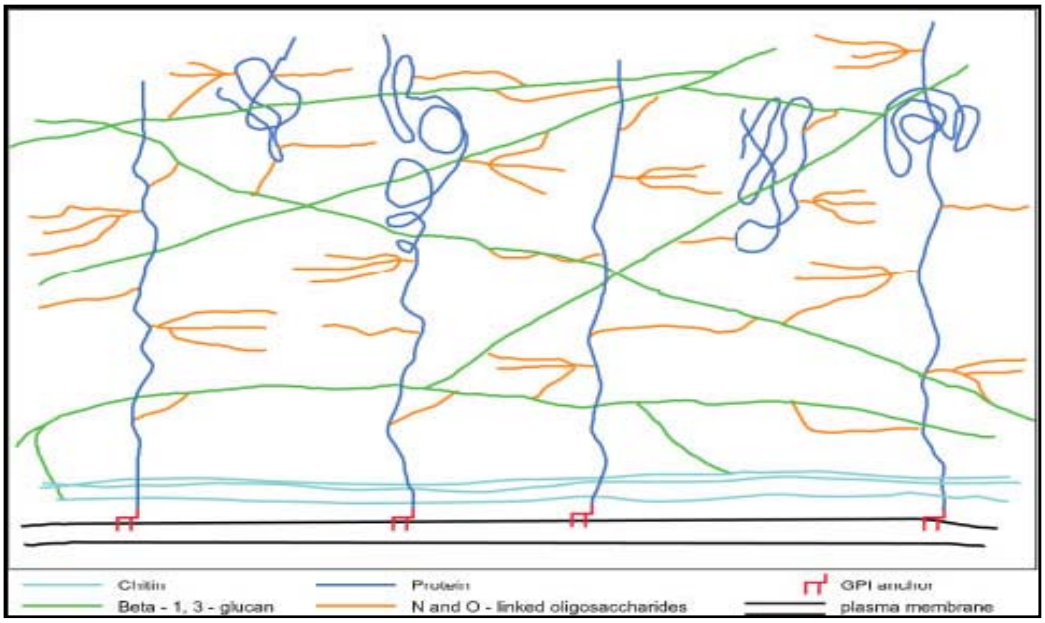


Figure 2.2. Representation of the fungal cell wall. The major components of the fungal cell wall are chitin, glucans and glycoproteins. Most of the chitin is considered to be located near to the plasma membrane. The beta-1, 3-glucan extends throughout the cell wall. The glycoproteins are extensively modified with N- and O-linked oligosaccharides. Many of the glycoproteins have GPI anchors, which tether them to the plasma membrane while other glycoproteins are secreted into the cell wall space. The proteins, glucans and chitin components are integrated into the wall by cross linking the chitin, glucans, protein-associated oligosaccharides and GPI anchors together. (Adapted from Bowman and Free, 2006).

2.8.2.1. Azoles

The first reports of the antifungal properties of azoles were published in the late 1960s (Holt, 1980; Sheehan et al., 1999) These original compounds, such as miconazole and econazole, and those that followed, such as ketoconazole, fluconazole, and itraconazole, proved to be important drugs for combating human fungal infections. The clinical efficacy and safety of fluconazole in particular has resulted in widespread use.

Ergosterol serves as a bioregulator of membrane fluidity and asymmetry and consequently of membrane integrity in fungal cells (Nozawa and Morita, 1986). Integrity of the cell membrane requires that inserted sterols lack C-4 methyl groups. The primary target of azoles is the heme protein, which co-catalyzes cytochrome P-450-dependent 14α-demethylation of lanosterol (Hitchcock et al., 1990). Inhibition of 14 α -demethylase leads to depletion of ergosterol and accumulation of sterol precursors, including 14 α -

methylated sterols (lanosterol, 4,14-dimethylzymosterol, and 24-methylenedihydrolanosterol), resulting in the formation of a plasma membrane with altered structure and function. The more recent triazole derivatives, such as fluconazole, itraconazole, and voriconazole (a triazole in development), owe their antifungal activity at least in part to inhibition of cytochrome P-450-dependent 14 α -sterol demethylase (Sanati et al 1997).

Mammalian cholesterol synthesis is also blocked by azoles at the stage of 14 α-demethylation; however, the dose required to effect the same degree of inhibition is much higher than that required for fungi (Hitchcock, 1990). The main hazard of ketaconazole is liver toxicity, which is rare but can prove fatal. Other azoles derivatives produce unwanted effects that are mild and these include gastrointestinal disturbances, nausea, allergic skin reactions and dizziness (Rang et al., 2003).

2.8.2.2. Polyenes

Amphotericin B was licensed in 1959, and is licensed for the treatment of "progressive and potentially life threatening fungal infections: aspergillosis, cryptococcosis (torulosis), North American blastomycosis, systemic candidiasis, histoplasmosis, zygomycosis including mucormycosis due to susceptible species of the genera *Absidia, Mucor* and *Rhizopus,* and infections due to related susceptible species of *Conidiobolus* and *Basidiobolus,* and sporotrichosis (Dismukes, 2000.). Amphotericin B has for many years been the only antifungal polyene that can be administered systemically to treat a visceral infection (Odds et al., 2003). The polyene antifungal agents such as amphotericin B represented the standard of therapy for systemic fungal infections (Sugar, 1986). There is an association between polyene susceptibility and the presence of sterols in the plasma membrane of the cells. All organisms susceptible to polyenes, contain sterols in their outer membrane, while resistant organisms do not (Norman et al., 1972). It was suggested that this effect is due to a physicochemical interaction between added sterols and the polyenes, which prevents the drug from binding with the cellular sterols. It has been proposed that the interaction of the polyene antifungal with membrane sterol results in the production of aqueous pores leading to altered permeability, leakage of vital cytoplasmic components, and death of the organism (Kerridge, 1985). The fatty acyl composition of the phospholipids has also been implicated in polyene susceptibility of yeast (Rao et al., 1985). In addition, killing of *C. albicans* has been attributed to oxidative damage caused by polyenes (Titsworth and Grunberg, 1973). Amphotericin B incorporated into liposomes, may participate in a selective transfer mechanism, which involves its transfer from the "donor" liposome to the ergosterol-containing "target" in the fungal cell membrane aided by the fungal and/or host phospholipases (Juliano et al., 1987).
The precise way in which this fungicidal effect occurs still remains unclear. The ergosterol molecule of fungi has a cylindrical three-dimensional structure, unlike cholesterol, the major sterol in mammalian

membranes, which has a sigmoid shape. This conformational difference is probably sufficient to explain the greater binding affinity of amphotericin B for ergosterol over cholesterol (Figure 2.3.). This difference and the higher ratio of ergosterol:phospholipid in fungi is the basis for the antifungal selectivity of amphotericin B (Odds et al., 2003). The commonest and most serious unwanted effect of Amphotericin B is renal toxicity. Other effects that are not desirable produce by the drug include impaired hepatic function, thrombocytopenia, and anaphylactic reactions (Rang et al., 2003)

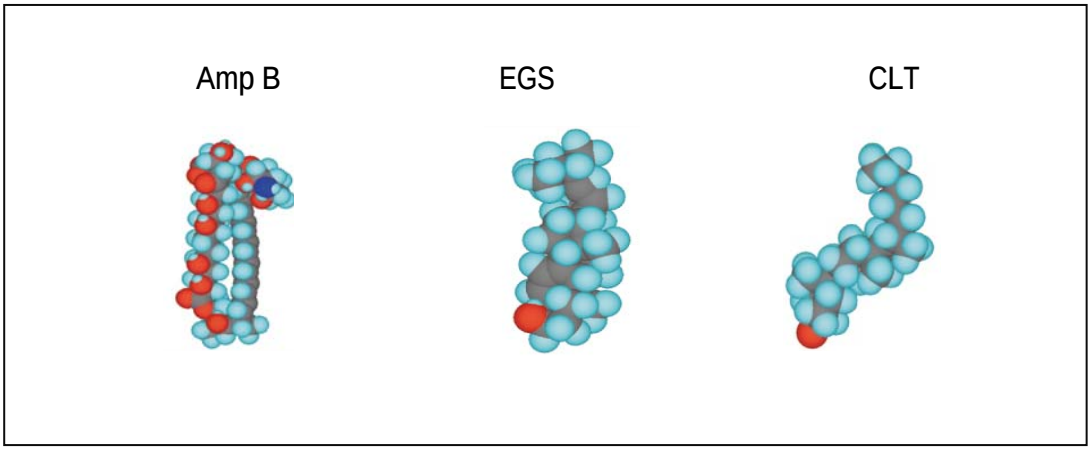


Figure 2.3. The polyene antifungal agent, amphotericin B (Amp B), ergosterol (EGS) and cholesterol (CLT) visualised in three dimensions. Ergosterol, the sterol found in fungal cell membranes, retains a cylindrical shape in all rotations and binds better to the hydrophobic (right-hand) side of the amphotericin B molecule than does cholesterol, with its sigmoid structure. Cholesterol is the membrane sterol found in mammalian cells; the differential binding affinity of amphotericin B for the two sterols is the basis of its selective antifungal action.

2.8.2.3. Allylamines

Terbinafine and naftifine are example of this class of antifungals. They have been developed as a new class of ergosterol biosynthetic inhibitors that are functionally as well as chemically distinct from the other major classes of ergosterol-inhibiting antifungal agents (Ryder et al., 1984; Ryder and Favre, 1997). Terbinafine is highly effective against dermatophytes *in vivo* and *in vitro*. Ryder and Favre, (1997), reported further that terbinafine has good activity against at least some azole-resistant *C. albicans* strains. It also had activity high activity against *Cryptococcus neoformans*.

Allylamines act by inhibiting early steps of ergosterol biosynthesis. This inhibition coincides with accumulation of the sterol precursor squalene and the absence of any other sterol intermediate (Kerridge, 1980), suggesting that allylamine inhibition of sterol synthesis occurs at the point of squalene epoxidation, a reaction catalyzed by squalene epoxidase. Studies with isolated squalene epoxidase indicate that it is the target for allylamine activity (Ryder and Favre, 1997). Fungal cell death is related primarily to the accumulation of squalene rather than to ergosterol deficiency (Ryder and Favre, 1997). High levels of

squalene may increase membrane permeability (Lanyi et al., 1974), leading to disruption of cellular organization.

2.8.3. Compounds active against fungal cell walls

The fungal cell wall contains compounds, such as mannan, chitin, and α- and β-glucans, which are unique to the fungal kingdom. Since these components are not found elsewhere in nature, they have been identified as possible targets that provide selective toxicity advantages (Hector, 1993). The cell wall of *Candida albicans* is a multilayered structure composed of chitin, β -glucan and mannoprotein, with the last two constituents making up to 80% of the wall mass (Poulain et al., 1978; Cassone et al., 1979). The outer layers are composed of mannan, mannoprotein, and β -(1,6)-glucan, while the inner layers are predominantly β -(1,3)-glucan and chitin with some mannoprotein (Surarit et al., 1988.)

2.8.3.1. Inhibitors of glucan synthesis

The echinocandins are specific inhibitors of fungal 3 β -glucan synthase. They are natural products discovered in the 1970s (Tkacz, 1992).fungal secondary metabolites comprising a cyclic hexapeptide core with a lipid side chain responsible for antifungal activity Echinocandins, which are lipopeptides, have fungicidal activity both *in vitro* and *in vivo* against *Candida* and *Aspergillus* species (Walsh, et al., 1991). β -Glucan inhibitors act as specific noncompetitive inhibitors of β -(1,3)-glucan synthetase, a large (210-kDa) integral membrane heterodimeric protein (Hector, 1993). Treatment of fungi with these compounds inhibits the synthesis of the structural glucan component without affecting nucleic acid or mannan synthesis (Mizoguchi et al., 1977). Inhibitors of glucan synthesis also have secondary effects on other components of intact cells including a reduction in the ergosterol and lanosterol content and an increase in the chitin content of the cell wall (Pfaller et al., 1989). Inhibition of β -(1,3)-glucan synthetase results in cytological and ultrastructural changes in fungi characterized by growth as pseudohyphae, thickened cell wall, and buds failing to separate from mother cells. Cells also become osmotically sensitive (Traxler et al., 1977), with lysis being restricted largely to the growing tips of budding cells (Bozzola et al., 1984).

2.8.3.2. Chitin synthesis inhibitors

Chitin is a linear homopolymer of β-(1,4)-linked *N*-acetylglucosamine (GlcNAc) residues. It is synthesized on the cytoplasmic surface of the plasma membrane, extruded perpendicularly to the cell surface as microfibrils, and crystallized outside the cell through extensive hydrogen bonding as a-chitin (the poly GlcNAc chains run antiparallel). The polymerization of GlcNAc is catalyzed by chitin synthases, membrane-

bound enzymes found in cell homogenates largely as zymogens (Bulawa, 1993). Chitin synthesis is inhibited competitively by polyoxins and nikkomycins. They are nucleoside-peptide antibiotics and were isolated from two different *Streptomyces* species: *S. tendae* (nikkomycin) and *S. cacaoi var. asoensis* (polyoxin). They act as analogs of the substrate UDP-GlcNAc, inhibiting chitin synthase. The effect on the fungus is inhibition of septation and osmotic lysis (Becker et al., 1983). The nucleoside-peptide inhibitors are taken up by a dipeptide permease, and thus, peptides in body fluids antagonize their transport. *C. albicans* and other medically important fungi are resistant to polyoxins owing to their poor transport across the cell membrane (Yadan et al., 1984).

2.8.3.3. Compounds inhibiting nucleic acids

2.8.3.3.1. 5-Fluorocytosine

5-Fluorocytosine is a fluorinated pyrimidine with inhibitory activity against many types of yeast, including *Candida* and *Cryptococcus neoformans*. The majority of the candidal isolates studied were susceptible to 5-Fluorocytosine. 5-Fluorocytosine enters fungal cells aided by a permease enzyme. Once inside, it is converted to 5-fluorouracil (5FU) by the enzyme cytosine deaminase. Subsequently, 5FU is converted by UMP pyrophosphorylase into 5-fluorouridylic acid (FUMP), which is phosphorylated further and incorporated into RNA, resulting in disruption of protein synthesis (Polak and Scholer, 1975). 5FU also is converted to 5-fluorodeoxyuridine monophosphate, a potent inhibitor of thymidylate synthase, an enzyme involved in DNA synthesis and nuclear division (Diasio et al., 1978). Thus, 5FC acts by interfering with pyrimidine metabolism, as well as RNA, DNA, and protein synthesis in the fungal cell. The summary of the mechanism of action of commonly used antifungal agents is presented on figure 2.4.

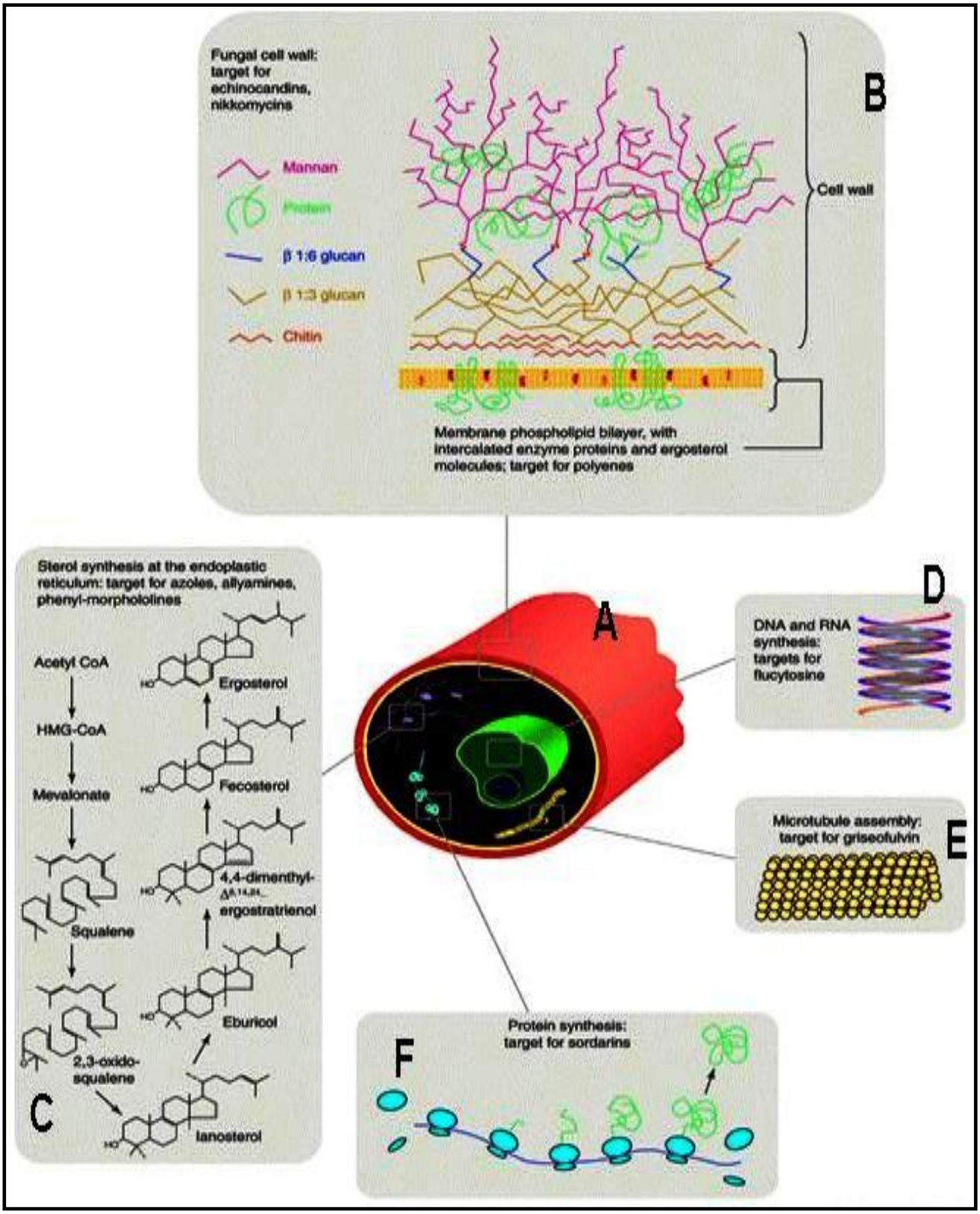


Figure 2.4. Diagrammatic representations showing the mechanism of action of commonly used antifungal agents. Adapted from Odds et al (2003). A= cross section of the fungal cell; B= membrane phospholipids bilayer: a target for polyenes antifungals, while the cell wall serve as target for echinocandins and nikkomycins; C= biochemical pathway for sterol synthesis at the endoplasmic reticulum: a target for azoles and allyamines; D= lucytosine inhibits the synthesis of nuclear membrane (DNA and RNA); E= microtubular assembly in the fungal cell is inhibited by griseofulvin; F= The sodarins target and inhibit protein synthesis in the fungal cell.

2.9. Plants as antimicrobial agents

The antimicrobial qualities of aromatic and medicinal plants and their extracts have been recognized since antiquity, while attempts to characterize these properties in the laboratory date back to the early 1900s (Martindale 1910; Hoffman and Evans 1911). Plant volatile oils are generally isolated from non-woody plant material by distillation methods, usually steam or hydro distillation, and are variable mixtures of principally terpenoids, and a variety of low molecular weight aliphatic hydrocarbons, coumarins and homologues of phenylpropanoids. Terpenes are amongst the chemicals responsible for the medicinal, culinary and fragrant uses of aromatic and medicinal plants (Dorman and Deans, 2000). Although plant secondary products have historically been defined as chemicals that do not appear to have a vital biochemical role in the process of building and maintaining plant cells, recent research has revealed a pivotal role of many of these chemicals in ecophysiology of plants (Briskin, 2000). Accordingly secondary products may have both a defensive role against herbivory, pathogen attack, and interplay competition and an attractant role toward beneficial organisms such as pollinators or symbionts (Wink and Schimmer, 1999).
Plants produce a huge variety of secondary compounds as natural protection against microbial and insect attack. Some of these compounds are also toxic to animals, but others may not be toxic. Indeed, many of these compounds have been used in the form of whole plants or plant extracts for food or medical applications in man (Wallace, 2004). Plant secondary products can have a variety of functions in plants. For example, certain plant secondary products are produce to defend the plant against microbial attacks by destroying microbial cells. Those compounds if not toxic to mammalian cells could prove useful as antimicrobial medicines in animals and humans (Briskin, 2000).

The diversity of plants growing in South Africa, along with their known ethnopharmacological uses offer a good possibility of finding novel chemical agents with efficacious antifungal properties. Tannins isolated from *Terminalia trifolora* demonstrated a powerful antifungal action in clinical studies (Latte and Kolodziej, 2000). In a similar study, six *Terminalia* species found in South Africa had activity against *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporum canis* and *Sporothrix schenckii* (Masoko et al., 2005). Quite a number of plants in Tanzania are used in traditional medicine and had good results when tested against *Candida* infections of human (Runkoro et al., 2006).

2.9.1. Secondary plant compounds with antimicrobial properties

2.9.1.1. Phenols and phenolic acids

Simple substituted phenolic ring compounds like Cinnamic and caffeic acids are bioactive phytochemicals and represent wide range of phenylpropane-derived compounds (Figure 2.5) which are in the highest oxidation state. Herbs such as tarragon and thyme both contain caffeic acid, which is effective against fungi (Duke, 1985). Hydroxylated phenols like catechol and pyrogallol possessed activity against microorganisms. Chemical hydroxylation of this compounds results in increase in their relative toxicities to microorganisms (Geissman, 1963). Enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins is believed to be responsible for their toxicities to microorganism (Mason and Wasserman, 1987). Eugenol, found in clove oil is a phenolic compound possessing a C3 side chain at a lower level of oxidation and contains no oxygen, this property, to a large extend imparts on the compound bacteriostatic activity against both bacteria (Thomson, 1978) and fungi (Duke, 1985).

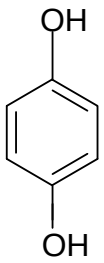


Figure 2.5. Hydroquinone (the most widely distributed phenol in plants)

2.9.1.2. Quinones

Quinones are found abundantly in nature and are highly reactive (Figure 2.6). Being coloured they are responsible for the browning colour in cut or injured fruits and vegetables and serve as intermediaries in the synthesis of melanin in the human skin (Schmidt, 1988). Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes. Quinones may also render substrates unavailable to the microorganism. Kazmi et al (1994) described an anthraquinone from *Cassia italica*, a Pakistani tree, which was bacteriostatic for *Bacillus anthracis*, *Corynebacterium pseudodiphthericum*, and *Pseudomonas aeruginosa* and bactericidal for *Pseudomonas pseudomalliae*.

Hypericin, an anthraquinone from St. John's wort (*Hypericum perforatum*) was reported to possess general antimicrobial properties (Duke, 1985).

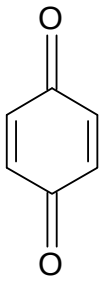


Figure 2.6. Quinones

2.9.1.3. Flavones, flavonoids, and flavonols

Flavones (Figure 2.7.) are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones).The addition of a 3-hydroxyl group yields a flavonol (Fessenden and Fessenden, 1982). Flavonoids are also hydroxylated phenolic substances but occur as a C6-C3 unit linked to an aromatic ring. Since they are known to be synthesized by plants in response to microbial infection (Dixon et al., 1983), it is not surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya et al., 1996). Catechins, the most reduced form of the C3 unit in flavonoids compounds, exerted antimicrobial activity (Toda, 1989) and that they contain a mixture of catechin compounds. These compounds inhibited *in vitro Vibrio cholerae* O1 (Borris, 1996), *Streptococcus mutans* (Batista et al., 1994.), *Shigella* (Vijaya et al., 1995, and other bacteria and microorganisms (Thomson, 1978).

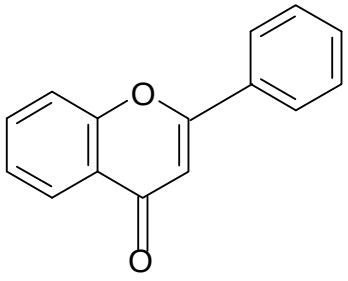


Figure 2.7. Flavone

2.9.1.4. Tannins

"Tannin" is a general descriptive name for a group of polymeric phenolic substances (Figure 2.8) capable of tanning leather or precipitating gelatin from solution, a property known as astringency. Their molecular weights range from 500 to 3,000 (Haslam, 1996), and they are found in almost every plant part: bark, wood, leaves, fruits, and roots (Scalbert, 1991). They are divided into two groups, hydrolyzable and condensed tannins. Hydrolyzable tannins are based on gallic acid, usually as multiple esters with D-glucose; while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers. One of their molecular actions is to complex with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Haslam, 1996). Their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins, etc. Condensed tannins have been determined to bind cell walls of ruminal bacteria, preventing growth and protease activity (Jones et al., 1994). Tannins are considered for the partial antibiotic activity of methanol extracts of the bark of *Terminalia alata* found in Nepal (Taylor et al., 1996).

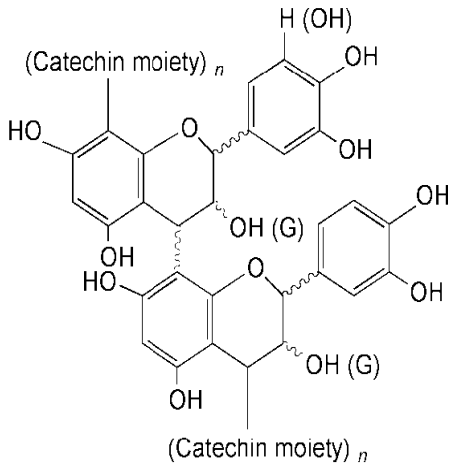


Figure 2.8. Tannins

2.9.1.5. Coumarins

Coumarins are phenolic substances made of fused benzene and a-pyrone rings (Figure 2.9). Coumarin was found *in vitro* to inhibit *Candida albicans*. As a group, coumarins have been found to stimulate macrophages (Casley-Smith and Casley-Smith, 1997), which could have an indirect negative effect on infections. Phytoalexins, which are hydroxylated derivatives of coumarins, are produced in carrots in response to fungal infection and can be presumed to have antifungal activity (Hoult and Paya, 1996).

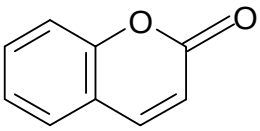


Figure 2.9. Coumarins

2.9.1.6. Terpenoids and Essential Oils

Terpenes are the secondary metabolites that impart the fragrance of plants (Figure 2.10). They are also referred to as essential oils. They occur as monoterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), and tetraterpenes (C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When the compounds contain additional elements, usually oxygen, they are termed terpenoids. Terpenoids are synthesized from mevalonate units. Examples of common terpenoids are mentol and camphor (monoterpenes), farnesol and artemisinine (sesquiterpenoids). Artemisinine and its derivative α-arteether, also known by the name qinghaosu, find current use as antimalarials (Vishwakarma, 1990). Terpenes or terpenoids are active against bacteria (Ahmed et al., 1993), fungi (Ayafor et al., 1994), viruses (Fujioka and Kashiwada, 1994), and protozoa (Ghoshal et al., 1996). The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. Two diterpenes isolated by Batista et al (1994) were found to be active against *Staphylococcus aureus*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, and *Candida* spp.

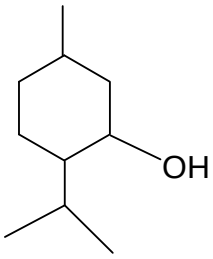


Figure 2.10. Terpenoids

2.9.1.7. Alkaloids

Alkaloids are heterocyclic nitrogen compounds. The first medically useful example of an alkaloid was morphine, isolated in 1805 from the unripe seed capsule of the oriental poppy plant (*Papaver somniferum*) (Fessenden and Fessenden, 1982); the name morphine comes from the Greek Morpheus, god of dreams. Diterpenoid alkaloids, commonly isolated from the plants of the Ranunculaceae are commonly found to have antimicrobial properties (Omulokoli et al., 1997). Alkaloids have also been found to have microbiocidal

effects (Ghoshal et al., 1996). Berberine (Figure 2.11) is an important representative of the alkaloid group and is potentially effective against some haemoprotozoan parasites (Freiburghaus et al., 1996, Omulokoli et al., 1997). The mechanism of action of highly aromatic planar quaternary alkaloids such as berberine and harmane is attributed to their ability to intercalate with DNA (Phillipson and O'Neill. 1987). Zhao et al (1998) reported the isolation of 6 alkaloids that are active against the plant pathogenic fungi *Cladosporium cucumerinum*.

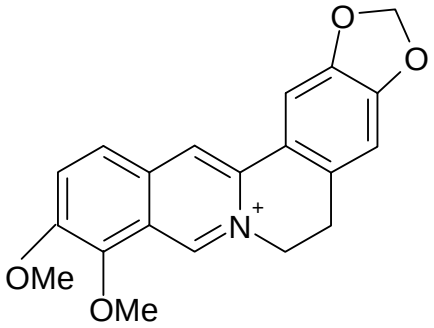


Figure 2.11. Alkaloid (berberine)

2.9.1.8. Lectins and Polypeptides

Peptides which are inhibitory to microorganisms were first reported in 1942 (Balls et al., 1942). They are often positively charged and contain disulfide bonds (Zhang and Lewis, 1997). Their mechanism of action may be the formation of ion channels in the microbial membrane (Zhang and Lewis, 1997) or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors (Shah et al., 1997).Inhibition of bacteria and fungi by these macromolecules, such as that from the herbaceous *Amaranthus*, has long been known (De Bolle, 1996). Thionins are peptides commonly found in barley and wheat and consist of 47 amino acid residues (Colilla et al., 1990). They are toxic to yeasts and gram-negative and gram-positive bacteria (Fernandes de Caleya et al., 1972). Thionins AX1 and AX2 from sugar beet are active against fungi but not bacteria (Kragh et al., 1995).

2.10. Fungal diseases and Inflammation

Pathogenic fungi (dermatophytic, subcutaneous, and systemic) have the ability to produce eicosanoids (prostaglandins and leukotrienes) both from host derived arachidonic acid. Host-derived eicosanoids have been previously demonstrated to enhance fungal colonization and the development of inflammation during fungal infections (Noverr et al., 2002). Eicosanoids are potent regulators of host immune responses (Peters-Golden, 1997); they inhibit Th1 type immune responses, chemokine production, phagocytosis, and lymphocyte proliferation (Kunkel et al., 1988; Betz and Fox, 1991; Matsuoka et al., 2000). Leukotrienes are potent leukocyte chemotactic factors (Jonsson, and Dahlen 1999). Moreover, eicosanoids can also

promote Th2 type responses and tissue eosinophilia (Demeure et al., 1997). Fungal diseases cause a shift from Th1 toward Th2 type responses (Romani and Kaufmann, 1998). Host cells are one source of eicosanoids during fungal infection; however, another potential source of eicosanoids is the fungal pathogen itself. The pathogenic fungi *Cryptococcus neoformans* and *Candida albicans* produce prostaglandins de novo from simple exogenous arachidonic acid (Noverr et al., 2002). Phospholipase A_2 and phospholipase B have been identified in a large number of eukaryotic microbes including *A. fumigatus* (Ghannoum, 2000). Phospholipases A_2 and B in fungi cleave the fatty acid side chains of phospholipids and have been implicated in virulence in a number of parasitic and fungal species, presumably via destruction of host cell membranes and subsequent lysis (Noverr et al., 2003). It therefore clearly indicates that enhanced prostaglandin production during fungal infection could be an important factor in promoting fungal colonization and chronic infection.

2.11. Resistance to antifungal agents

Antifungal resistance is a broad concept describing failure of a fungal infection to respond to antifungal therapy (Alexander and Perfect, 1997). Antifungal resistance has been traditionally classified as either primary (intrinsic) i.e., present before exposure to antifungals-or secondary (acquired) i.e., that which develops after exposure to antifungals owing to stable or transient genotypic alterations. A schematic representation of resistance to antifungal agents is presented in Figure 2.12. A third type of antifungal resistance could be described as “clinical resistance”, which encompasses progression or relapse of an infection by a fungal isolate that seems, in laboratory testing, to be fully susceptible to the antifungal used for the treatment of infection. Clinical resistance of fungi is typically seen in patients with persistent or profound immune defects (e.g., AIDS). In some cases, suboptimum drug concentrations in the blood caused by drug interactions might contribute to clinical resistance (Kontoyiannis and Lewis, 2002).

2.11.1. Resistance to azoles

Resistant fungal strains either exhibit a modification in the quality or quantity of target enzyme, reduced access to the target, or some combination of these mechanisms. Modification in the quantity or quality of 14α-demethylase expression is responsible for resistance to azole antifungal agents. Resistance to azoles can also occur by mutations that modify the target molecule or by over expression of membrane efflux pumps that export the antifungals from the cell. Combinations of both mechanisms have been detected in some *C. albicans* isolates.

2.11.2. Resistance to Polyenes

Alterations in the membrane ergosterol content secondary to mutations in the ergosterol biosynthetic pathway seem to be the most characterized mechanism of polyene resistance of *Candida* and the genetically similar non-pathogenic model yeast *Saccharomyces cerevisiae* (Woods and Ahmed, 1968). Alterations in the cell wall composition have also been described as a mechanism of broad-spectrum polyene resistance in a laboratory strain of *A. flavus* (Kontoyiannis and Lewis, 2002). Resistance to polyenes may result from reduced ergosterol content in the fungal cell membrane. Furthermore, resistance may occur from replacement, by fungi, of ergosterol with sterols with low affinity for polyenes (Rogers, 2002).

2.11.3. Resistance to Allylamines

Allylamine resistant fungi are as yet not well described; comparisons of resistance mechanisms are rare (Ghannoum and Rice, 1999). However, the different sites of action of the azoles, polyenes, and allylamine resemble the sequential actions on cell wall synthesis exhibited by different antibacterial agents (Kahan et al., 1974), penicillin (which acts at an intermediate step), and vancomycin (which acts at the final step in cross-linking). As in the study of cell wall synthesis in bacteria, some of the mechanisms of action of antifungal agents have been elucidated by analyzing the accumulation of specific precursors after exposure to the antibiotic. Since all of the antibiotics act at different steps of the same process, it is perhaps not surprising that specific mutations will result in cross-resistance to several of the compounds. One striking example is depicted by *Candida glabrata* isolate, which was initially susceptible *in vitro* to fluconazole, voriconazole and posoconazole, developed cross-resistance to all currently available triazole antifungals after a course of fluconazole therapy and with no known prior exposure to expanded-spectrum triazoles (Magill et al., 2006).

2.11.4. Resistance to echinocandins (Inhibitors of glucan synthesis)

Kurtz and Douglas 1997 reported the isolation of resistant mutants of *Saccharomyces cerevisiae*. The target of lipopeptides, including echinocandins, is glucan synthase (a heterodimeric enzyme), which in *S. cerevisiae* is encoded by *FKS1* and *RHO1. S. cerevisiae* also contains another gene, *FKS2*, which is highly homologous to *FKS1*. Mutations in the *FKS1* gene confer high-level *in vitro* resistance to lipopeptides. Low-level resistance is associated with mutations in another cell wall synthesis gene, *GNS1* that encodes an enzyme involved in fatty acid elongation. Mutations in *FKS2* gene do not confer resistance. Studies with *C. albicans* mutants indicated that resistance to pneumocandin in *S. cerevisiae* and *C. albicans* is very much alike.

2.11.5. Resistance to 5-fluorocytosine (Inhibitor of nucleic acid synthesis)

Resistance to 5-fluorocytosine may result from decreased uptake (loss of permease activity) or loss of enzymatic activity responsible for conversion to 5-fluorouridylic acid (FUMP). FUMP is phosphorylated in fungi and further incorporated into RNA, resulting in disruption of protein synthesis. Blocking the formation of FUMP by loss of cytosine deaminase activity or by loss of uracil phosphoribosyltransfnerase (UPRTase) activity is sufficient to confer 5-fluorocytosine resistance. Resistance in the large majority of both clinical and laboratory strains of 5-fluorocytosine-resistant *C. albicans* and *Cryptococcus neoformans* is attributable to mutational loss of one of the pyrimidine salvage enzymes (Normark and Schonebeck, 1972). Decreased UPRTase activity was associated with resistance in a gene dosage-dependent manner in *C. albicans* (Whelan and Kerridge, 1984).

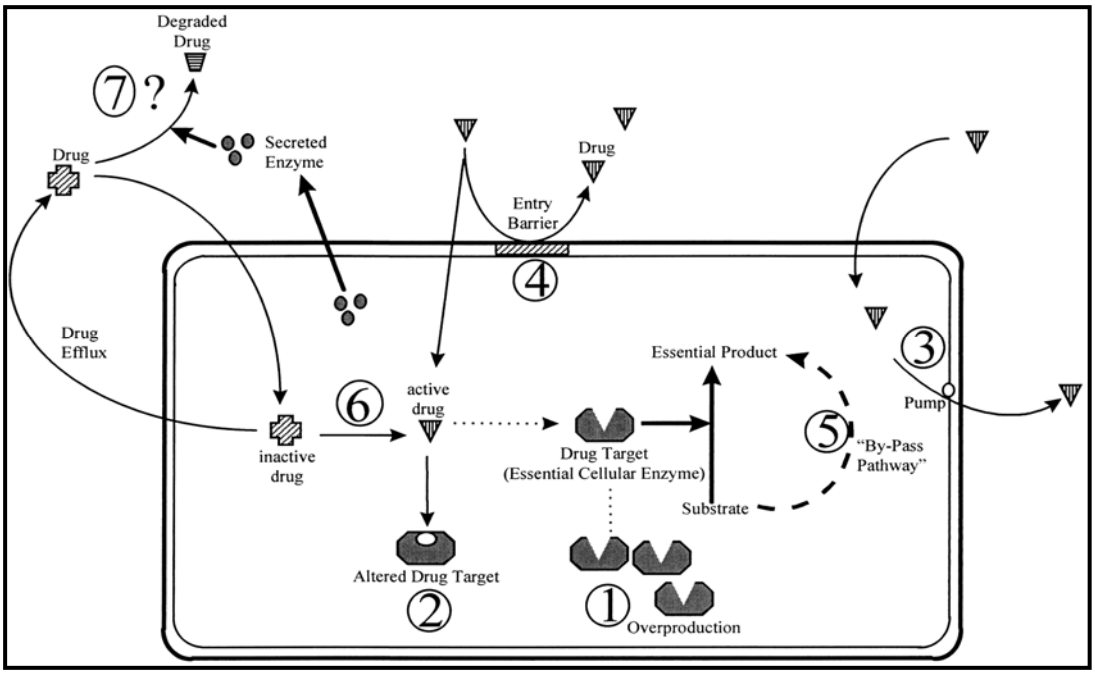


Figure 2.12. Mechanisms by which microbial cells might develop resistance. 1. The target enzyme is overproduced, so that the drug does not inhibit the biochemical reaction completely. 2. The drug target is altered so that the drug cannot bind to the target. 3. The drug is pumped out by an efflux pump. 4. The entry of the drug is prevented at the cell membrane/cell wall level. 5. The cell has a bypass pathway that compensates for the loss-of-function inhibition due to the drug activity. 6. Some fungal "enzymes" that convert an inactive drug to its active form are inhibited. 7. The cell secretes some enzymes to the extracellular medium, which degrade the drug (Ghannoum, and Rice, 1999).

2.12. Plant species used in the study

Seven plant species that had good activity against the pathogenic fungus *Cryptococcus neoformans* in the tree screening database of the Phytomedicine Programme were selected for further evaluation of their potential value against *Aspergillus fumigatus* and other economically important animal fungi. The species selected are: *Loxostylis alata*, *Protorhus longifolia*, *Kirkia wilmsii*, *Khaya anthotheca*, *Commiphora harveyi*,

Combretum vendae and *Ochna natalitia* (Table 2.1). Most of these species have also been used used traditionally to treat diseases associated with infectious and non-infectious agents (Coates-Palgrave, 2002).

2.13. *Loxostylis alata* A. Spreng. ex Rchb. Anacardiaceae

The name *Loxostylis* is derived from the Greek *loxos* meaning 'crooked' or 'oblique', and the Latin *stylis* for style, a reference to the lateral attachment of the style to the ovary. The common name tarwood presumably refers to the oily residue from the fruits. Tarwood is an evergreen, ornamental tree that grows to a height of 5 metres which is grown in cultivation in a wide range of ecological habitats, while naturally it occurs in rocky and forest areas along river banks. The leaves are alternate and compound with 2 to 5 pairs of leaflets, including a terminal leaflet. Typical of the species is the conspicuous winged rachis (midrib). The specific name is based on the Latin *alatus* meaning 'winged'. Young leaves are red. The bark is pale grey and has vertical shallow fissures with latex present. The flowers are male or female on different trees and produced from November to February. The male flowers are white and pleasantly scented and the female flowers are greenish white. The petals of the female flowers fall soon, but their sepals enlarge substantially and turn pink-red, covering the developing fruit and creating a very attractive display. The fruits of *L. alata* are small, fleshy and measure about 4 mm in diameter, usually they are found embedded in the brightly coloured sepals. The seed skin contains a black sticky substance like tar; it is difficult to wash when touched (Coates-Palgrave, 2002).

The bark and leaves are widely used in South Africa as traditional medicine to relieve pain during childbirth particularly among the Zulu tribe (Pooley, 1993). Figure 2.13 shows the leaves and stem of *Loxostylis alata*.

Figure 2.13. The leaves of *L. alata* growing in its natural habitat (Photographed at the University of Pretoria Botanical Garden, June 2008).

2.14. *Protorhus longifolia* (Bernh.) Engl. Anacardiaceae

Protorhus longifolia has the common names harpuisboom, red beech, rooiblaar. The plant grows up to a height of 15 metres and occurs in forest areas, open woodland and on river banks. The bark is brown, smooth to rough and has milky latex on it. Domestically, the wood provides general purpose timber, which is used for making furniture. Exudate from the bark is used as gum to fix blades of assegais into their handles, and also as a depilatory (Coates-Palgrave, 2002).

Traditionally, in South Africa the bark is injected to cure hemiplegic paralysis believed to be cause by witchcraft (Gerstner, 1941). Decoctions prepared from the plant are used as emetics to relieved heartburn and bleeding from the stomach (Watt and Breyer-Brandwijk, 1962; Pujol, 1990). The bark is also used traditionally to treat of diarrhoea and heartwater (Donald and Cocks, 2001). Figure 2.14 shows the leaves of *Protorhus longifolia*.

Figure 2.14. The leaves and stem of *P. longifolia* in their natural habitat (Photographed at the University of Pretoria Botanical Garden, South Africa in June 2008).

2.15. *Kirkia wilmsii* (Engl.) Kirkiaceae

Kirkia wilmsii occurs in mountain slope and rocky hills. The bark is grey in colour and smooth, the branchlets are marked with leaf scars. The plant is deciduous, medium to large tree with a rounded crown with beautiful autumn colours from April to May. The trunk is known to branch near the base. Smooth grey bark, often having irregular patching. The flowers appear from spring to summer in sprays of greenish white to greenish cream. The leaves have brilliant autumn colours and are crowded at the end of the branchlets. The flowers are small (about 4-7 cm long) and are greenish white in colour. Fruits formed small capsule, narrowly ovoid and split into 4 valves which remain joined at the apex. The roots of this plant sometimes

produce shoots as the sprawl among rocks. The wood is light, coarse and provides a good strong fibre (Coates-Palgrave, 2002).

The leaves are used traditionally to treat malaria and feverish conditions (Clarkson et al., 2004). Figure 2.15 shows the leaves and stem of *Kirkia wilmsii*.

Figure 2.15. The leaves and stem of *K. wilmsii* in their natural habitat (Photographed at the University of Pretoria Botanical Garden, South Africa in October 2008).

2.16. *Khaya anthotheca* (Welm.) C.D.C. Meliaceae

The plant is commonly called east African mahogany. *Khaya anthotheca* is a large evergreen tree up to 60 m tall (up to 30 m in the garden) with an elongated or rounded, much-branched crown; the trunk is buttressed in old specimens. The bark on the young branches is smooth and greyish brown but smooth to sometimes mottled grey and brown, flaking on the older branches and stems. Leaves are alternate, evenly compound with 3–7 pairs of leaflets, 150–300 mm long and dark glossy green, base broadly tapering to round and slightly asymmetric, smooth and glossy, veins distinct on the lower surface, margin smooth. Flowers appear in branched sprays at the tips of branches, are white and sweetly scented, up to 10 mm in diameter, the male and female flowers are separate but on the same tree, and the stamens join to form a tube up to 6 mm long. The flowering period is from September to December. The fruit is a hard, woody, oval, splitting capsule up to 60 mm in diameter, with 4 or 5 valves. Fruiting occurs from March to September (Estherhuyse et al., 2001).

The wood weathers well and is resistant to borers and termites. It is moderately resistant to fungal decay. The timber saws well but is inclined to be tough, and sharp equipment is therefore needed. The wood is dark, hard, reddish brown and durable, suitable for furniture, flooring, panelling, and excellent for boat

building, moderately heavy the bark is bitter, similar to quinine, and is used for colds. Oil from the seed is rubbed into the scalp to kill insects (Estherhuyse et al., 2001). Figure 2.16 shows the leaves and stem of *K. anthotheca*.

Figure 2.16. The leaves of *K. anthotheca* growing in its natural habitat (Photographed at the University of Pretoria Botanical Garden, South Africa in June 2008).

2.17. *Commiphora harveyi* (Engl.) Engl. Burseraceae

The tree is commonly known as copper-stem corkwood. It is a small squat deciduous tree measuring about 4-18 metres in height and it occurs on stony hill slopes and rocky river valleys of coastal forest and bushveld. The leaves are usually pinnate with two to three pairs of opposite leaflets. The bark is bronze papery pieces or discs and dark green underneath. Fruits are ovoid to spherical and measure about 10 mm in diameter, becoming red, pseudo-aril orange or red, 4-lobed, with 2 lobes partially covering the stone and 2 longer lobes almost reaching its apex in the months of January to March (Coates-Palgrave, 2002). The soft, white wood has been used in making spoons and small stools which are often sold to tourist. Traditionally the bark is used as disinfectant for wounds, anthelmintic and treatment of snake bite (Watt and Breyer-Brandwijk, 1962). Figure 2.17 shows the leaves and stem of *Commiphora harveyi*.

Figure 2.17. The leaves of *C. harveyi* growing in its natural habitat (Photographed at the University of Pretoria Botanical Garden, South Africa in June 2008).

2.18. *Combretum vendae* (A.E. van Wyk) Combretaceae

The plant is commonly referred to as the Venda bushwillow. It is usually a shrub growing up to 1.5-3 metres high, occurring on the higher slopes of the Soutpansberg on rocky and deep soils. The bark is grey, smooth and densely covered with white or greyish hairs. Leaves are broadly elliptic to obovate and are medium green above. Flowers are light yellow or cream colour. The fruits are 4-winged, occasionally 3-winged and ellipsoidal, green flushed pink or red, becoming dark wine-red, on a stalk 4-7 mm long around the month of March to August.

The leaves, bark, root are used for treating leprosy and as ophthalmic remedy, and blood purification (Watt and Breyer-Brandwijk, 1962). Figure 2.18 shows the leaves and stem of *Combretum vendae*.

Figure 2.18. The leaves of *C. vendae* growing in its natural habitat (Photographed at the University of Pretoria Botanical Garden, South Africa in June 2008).

2.19. Ochna natalitia (Meisn.) (Walp.) Ochnaceae

The common names for this plant are Cape plane, Transvaal boxwood, Rooihout and Ysterhout.
The plant is a small to medium sized shrub measuring up to 10 meters in height, it occurs in bushveld and grassland, frequently in shallow soil among rocks. The bark is grey-brown or brown, finely fissured to rough. Young leaves have attractive coppery red colour and green when matured with numerous lateral veins that are close together. Flowers are yellow to golden yellow and have a diameter of 1.5-3 cm. The fruits occur in 2-3 druplets, ovoid and 5-10 mm long. The fruits are attached near base and are black in colour becoming red or spreading on recurved stalks (Coates-Palgrave, 2002).

Infusions of the root are taken traditionally to cure barrenness (Palmer and Pitman, 1972). The name *isithundu* refers to medicine used to bring prosperity (Doke and Vilakazi, 1972). Figure 2.19 shows the leaves and stem of *Ochna natalitia*.

Figure 2.19. The leaves of *O. natalitia* growing in its natural habitat (Photographed at the University of Pretoria Botanical Garden, South Africa in October 2008).

Table 2.1. Summary on the data and traditional uses of the plants selected for this study.

Botanical name	Voucher specimen number	Family	Part used	Claimed medicinal uses	Reference
Combretum vendae (A.E. van Wyk)	PRU96507	Combretaceae	leaves, bark, root	Leprosy, ophthalmic remedy, and blood purification	Watt and Breyer-Brandwijk, 1962
Commiphora harveyi (Engl.) Engl.	PRU96506	Burseraceae	Bark	Used as disinfectant for wounds, anthelmintic and treatment of snake bite	Watt and Breyer-Brandwijk, 1962
Khaya anthotheca (Welm.)	PRU96509	Meliaceae	Bark	Skin diseases, black quarter, helminthiasis	Watt and Breyer-Brandwijk, 1962, Nfi et al., 2001
Kirkia wilmsii (Engl.)	PRU96503	Kirkiaceae	Leaves	Treatment of malaria and feverish conditions.	Clarkson et al., 2004
Loxostylis alata A.Spreng. ex Rchb.	PRU96508	Anacardiaceae	leaves, bark	Stimulation of immune system, relieve of pain during childbirth	Pooley, 1993; Pell, 2004
Ochna natalitia (Meisn.) (Walp.)	PRU96504	Ochnaceae	leaves, root	Infusions and decoctions for headache, and respiratory diseases	Watt and Breyer-Brandwijk, 1962
Protorhus longifolia (Bernh.) Engl.	PRU96505	Anacardiaceae	Bark	Treatment of diarrhoea and heartwater	Donald and Cocks, 2001

2.20. Conclusion

The importance of poultry as a source of protein and employment of the rural populations in Africa warrants serious research into factors that could jeopardize production. The role *Aspergillus fumigatus* plays in the production process cannot be underestimated. Aspergillosis poses a great health risk to the poultry industry. Currently used antifungals against aspergillosis either proved inefficient or are not safe for use. There is therefore the need to find effective, safer and cheaper remedy against aspergillosis in poultry. A practical solution to this problem is to develop effective drugs or extracts from reasonably inexpensive and locally available raw materials. The most obvious way of achieving this goal is through the study of plants, selecting from those that have promising *in vitro* activity for development into effective and safer drugs.

Plant extracts with antioxidant activity may enhance the immune status of target animals and hence decrease their susceptibility to aspergillosis, which can lead to increased production. Inflammation provides an enabling condition for fungi to strive well, the use of alternative anti-inflammatory compounds from plants that are more effective and cheaper could arrest the development of aspergillosis in infected poultry. It is with this hypothesis that the extract of *L. alata* that had good activity against *Aspergillus fumigatus in vitro* and also had antioxidant and anti-inflammatory properties both in an *in vitro* study was selected for in depth study. This could eventually lead to an *in vivo* trial so as to find safer and more efficacious antifungal, antioxidant and anti-inflammatory agent for use in target animal species.

Chapter 3
Evaluation of several tree species for activity against the animal fungal pathogen *Aspergillus fumigatus*

M.M. Suleiman, L.J. McGaw, V. Naidoo, J.N. Eloff

PREFACE

The plant species that were selected in the previous chapter had to be evaluated to determine which species should be used for in depth investigation. The minimum inhibitory concentration (MIC) of the extract was determined to confirmed earlier investigation. We evaluate the effects of the extract further on other important animal fungal pathogens. The text in this chapter was submitted and accepted as manuscript for publication to South African Journal of Botany, Vol. 76, pages 64-71 to enable wider readership of the results obtained.

Abstract

Aspergillus fumigatus causes severe problems in poultry production systems. Seven South African tree species were selected from the database of the Phytomedicine Programme based on its antifungal activity against the fungus *Cryptococcus neoformans*. The acetone leaf extracts of the plants had minimum inhibitory concentrations (MICs) of 0.1 mg/ml and lower in the preliminary screening. The antibacterial and antifungal activities of hexane, dichloromethane, acetone and methanol extracts of the leaves were determined using a two-fold serial microdilution method against a range of commonly encountered animal pathogenic fungi (*Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporum canis* and *Sporothrix schenckii*) and four nosocomial bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*). The plant species investigated 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.) Engl. (Anacardiaceae). All the extracts had activity against at least one of the test organisms over an incubation period of 24 or 48 h. The MIC values of the hexane extracts of *Ochna natalitia*, *Khaya anthotheca* and *Combretum vendae* against one or more of the tested pathogens were as low as 0.08 mg/ml. Similarly the hexane and acetone extracts of *Commiphora harveyi*, acetone extract of *Protorhus longifolia* and dichloromethane extract of *Combretum vendae* depicted MICs as low as 0.08 mg/ml against at least one of the tested bacteria. Furthermore, the acetone extracts of *Loxostylis alata*, *Kirkia wilmsii*, *Ochna natalitia* and *Combretum vendae* had antifungal activities with MIC values ranging from 0.04-0.08 mg/ml against at least one of the tested fungi. The average MIC values of the plant extracts against bacteria ranged from 0.17-

2.11 mg/ml, while the range was 0.23-1.98 mg/ml for fungi. The Gram-positive organisms (*S. aureus* and *E. faecalis*) were more susceptible to the plant extracts than the Gram-negative organisms (*E. coli and P. aeruginosa*). *E. faecalis* was the most susceptible microbe and *Combretum vendae* extracts were the most active against nearly all the bacteria tested. The acetone extract of *Loxostylis alata* was the most active against fungal pathogens, with activity against at least 3 fungal organisms. *Loxostylis alata* was selected for further work to isolate compounds active against *Aspergillus fumigatus* and other fungal pathogens.

Keywords: Antibacterial; Antifungal; Medicinal plants; Minimum inhibitory concentration; Microdilution assay

3.1. Introduction

With the development of relatively effective and safe antibiotics in the 1940's, medical treatment had been revolutionalised leading to a drastic drop in morbidity and mortality previously induced by microbial diseases (Rang et al., 2003). Unfortunately, this development was rapidly hampered by the emergence of drug-resistant microbes (Walsh, 2000). This resistance has resulted in an increased incidence of infectious diseases with some pathogens (Kunin, 1993; Archibald et al., 1997; Sahm et al., 1999). With the evolutionary process that enables microbes to adapt genetically to changes in their environment, the unwise use of antibiotics inevitably selects for resistant microbes (Clardy et al., 2006). As a result new drugs have to be consistently developed to counteract the development of resistance and to possibly reduce the cost of controlling the disease. (Cowan, 1999).

In addition to the pathogenic bacteria, opportunistic fungal infections are becoming more important especially due to the immune deficiency induced by HIV-AIDS (Groll et al., 1996). Invasive pulmonary aspergillosis (IPA) is a serious fungal infection of immunocompromised patients usually caused by *Aspergillus fumigatus* with ever increasing incidence (Stevens, 1990; Denning, 1998). In contrast, however, there are only a limited number of antimicrobial drugs which are active against fungal pathogens (Denning, 1998). Although conventional antifungals remain the standard therapy for many invasive or life-threatening mycoses, these drugs are associated with significant toxicity (Dismukes, 2000). Against this backdrop, there is the need to develop cheaper, safer and effective antifungal drugs that could be used to control opportunistic fungal infections.

Plants have an almost limitless ability to synthesize secondary chemical substances, which play a pivotal role in their ecophysiology (Briskin, 2000). Accordingly, secondary products may have both a defensive role against herbivores, pathogen attack, and interplay competition and an attractant role toward beneficial organisms such as pollinators or symbionts (Wink and Schimmer, 1999). Some of the secondary-derived compounds may therefore have beneficial effects in the treatment of microbial infections in animals and humans (Cowan, 1999; Kuete et al., 2007). Recently, the interest in these metabolites has increased following searches for new antimicrobial agents from plant sources (Hostettmann et al., 2000).

Southern Africa is exceptionally rich in plant and animal diversity. It has the richest temperate flora in the world, with a floristic diversity of about 24 000 species and intraspecific taxa in 368 families. Only 2.5% of the world's land surface area and contains more than 10% of the world's vascular plant flora (Germishuizen and Meyer, 2003). Southern Africa also contains a major proportion of the 50 500 taxa present in sub-Saharan Africa (Klopper et al., 2006).

From data of approximately 350 plant species tested for biological activity obtained in an ongoing tree screening project of the Phytomedicine Programme, seven plant species (Table 3.1) with minimum inhibitory concentrations of 0.16 mg/ml and lower against *Cryptococcus neoformans* were selected for evaluation of their potential action against other important pathogenic bacteria and fungi. Although the selection was based on previously reported activity against the fungus *Cryptococcus neoformans* (L. Pauw and J.N. Eloff, unpublished data), these plant species are also used by indigenous healers for different disease conditions (van Wyk et al., 2000) and a summary of their traditional usage is presented in Table 3.1. Leaves of the selected tree species were screened for activity against five important fungal pathogens (*Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporum canis* and *Sporothrix schenckii*). Additionally, we also investigated the antibacterial activity of against four important nosocomial bacteria (*Staphylococcus aureus*, *Enterococcus faecalis*, *Escherichia coli* and *Pseudomonas aeruginosa*). The focus was however, on Aspergillus *fumigatus* one of the most common pathogenic fungal species in humans and animals (Rippon, 1982). It also plays role in the economically important disease aspergillosis in poultry. The aim of this publication was therefore to select the tree species with the best potential for developing a commercially useful antifungal product. To promote the sustainable use of plants only tree leaves were considered in this study.

Table 3.1. Botanical names and traditional use of the plants studied

Botanical name	Family	Voucher specimen number	Traditional medicinal uses	Reference
Combretum vendae A.E. van Wyk	Combretaceae	PRU96507	Leprosy, ophthalmic remedy, and blood purification	Watt and Breyer-Brandwijk, 1962
Commiphora harveyi (Engl.) Engl.	Burseraceae	PRU96506	Used as disinfectant for wounds, anthelmintic and treatment of snake bite	Watt and Breyer-Brandwijk, 1962
Khaya anthotheca (Welm.) C.DC	Meliaceae	PRU96509	Skin diseases, black quarter, helminthosis	Watt and Breyer-Brandwijk, 1962, Nfi et al., 2001
Kirkia wilmsii Engl.	Kirkiaceae	PRU96503	Treatment of malaria and feverish conditions.	Clarkson et al., 2004
Loxostylis alata A. Spreng. ex Rchb.	Anacardiaceae	PRU96508	Stimulation of immune system, relief of pain during child birth	Pooley, 1993; Pell, 2004
Ochna natalitia (Meisn.) Walp.	Ochnaceae	PRU96504	Infusions and decoctions for headache, and respiratory diseases	Watt and Breyer-Brandwijk, 1962
Protorhus longifolia (Bernh. ex C.krauss) Engl.	Anacardiaceae	PRU96505	Treatment of diarrhoea and heartwater	Dold and Cocks, 2001

3.2. Materials and methods

3.2.1. Plant collection

Commiphora harveyi (Engl.) Engl. (Burseraceae), *Combretum vendae* (A.E. van Wyk) (Combretaceae), *Khaya anthotheca* (Welm.) C.DC (Meliaceae), *Loxostylis alata* A. Spreng. ex Rchb. (Anacardiaceae) and *Protorhus longifolia* (Bernh.) Engl. (Anacardiaceae) leaves were collected at the Manie van der Schijff Botanical Garden of the University of Pretoria, South Africa. *Kirkia wilmsii* Engl. (Kirkiaceae) and *Ochna natalitia* (Meisn.) Walp. (Ochnaceae) were collected at the Lowveld National Botanical Garden in Nelspruit, South Africa. All plant leaves were collected in summer (November 2006) between 9:30 am and 12:30 pm. Samples of the plants were identified and authenticated by Lorraine Middleton, the herbarium curator, and Magda Nel at the Botanical Garden of the University of Pretoria. Voucher specimens of the plants were deposited at the Schweikert Herbarium of the Department of Plant Sciences, University of Pretoria, South Africa. The botanical names of the plants, tree reference numbers, and the plant parts used are presented in Table 3.1.

3.2.2. Plant storage

Immediately after collection and transportation to the laboratory, leaves were separated from stems and dried at room temperature under natural ventilation. The dried plant leaves were milled to a fine powder in a Macsalab mill (Model 200 LAB), Eriez®, Bramley, and stored at room temperature in closed containers in the dark until used.

3.2.3. Plant extraction

Plant samples from each species were individually extracted by weighing four aliquots of 1 g of finely ground plant material and extracting each aliquot with 10 ml of acetone, hexane, dichloromethane (DCM) or methanol (technical grade-Merck) respectively in centrifuge tubes. Tubes were vigorously shaken for 1 h using a Labotec model 20.2 shaking machine at a moderate speed. Extracting at lower speed for a longer period allows the solvent to penetrate more into the plant tissues, allowing the extraction of more of the compounds contained in the plant species (Silva et al., 1998). After centrifuging at 3500 x *g* for 10 min, the supernatant was decanted into pre-weighed labelled glass vials. The whole process was repeated three times on the marc to exhaustively extract the plant material. The solvent was removed under a stream of air in a fume cupboard at room temperature to quantify the extraction.

3.2.4. Microorganisms and medium

The bacterial organisms used in this study were obtained from the Department of Microbiology at the Medical Campus, University of Pretoria. They included the Gram-positive bacteria, *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212), and the Gram-negative bacteria, *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 27853). All bacterial cultures were maintained on Mueller Hinton (MH) agar and subcultured before use in MH broth (Oxoid, Basingstoke, UK). The five fungal organisms that were used included *Aspergillus fumigatus, Microsporum canis*, *Candida albicans*, *Cryptococcus neoformans* and *Sporothrix schenckii*. All fungal organisms were isolated from animal clinical cases prior to treatment, by the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. These fungi are important disease-causing pathogens of animals and man. Sabouraud dextrose (SD) agar (Oxoid, Basingstoke, UK) was used as the maintenance growth medium for the fungi.

3.2.5. Antimicrobial sensitivity test

The minimum inhibitory concentration (MIC) is defined as the lowest concentration of extract that inhibits visible growth of the micro-organism. For the different microbial species, the MIC was determined using the serial microdilution assay (Eloff, 1998a).

3.2.5.1. Bacterial organisms

Different plant extracts (hexane, acetone, dichloromethane and methanol) were dissolved in acetone to a concentration of 10 mg/ml. Acetone was non-toxic to the micro-organisms at the concentrations used in this assay (Eloff, 1998b; Masoko et al, 2005). One hundred µl of each plant extract were serially diluted 2-fold with sterile distilled water in 96-well microtitre plates. One millilitre of concentrated bacterial culture grown at 37 ºC for 3 days was transferred to 100 ml of fresh MH broth and 100 µl of the resultant culture was added to each well. Densities of bacterial cultures used for the screening were as follows: *S. aureus*, 2.6×10^{12} cfu/ml; *E. faecalis*, 1.5×10^{10} cfu/ml; *P. aeruginosa*, 5.2×10^{13} cfu/ml; *E. coli*, 3.0×10^{11} cfu/ml. Gentamicin at 0.1 mg/ml (Virbac®) and acetone were used as positive and negative control agents, respectively. After incubation overnight at 37 °C, *p*-iodonitrotetrazolium violet (INT, Sigma) at a concentration of 0.2 mg/ml was used as an indicator of bacterial growth. Forty µl of INT was added to each of the microtitre wells. Thereafter, the plates were incubated at 37 °C and the MIC was assessed 1 and 2 h after the addition of INT. Bacterial cultures react with INT and give red or purple colouration within 10-60 min (Eloff, 1998a).

3.2.5.2. Fungal organisms

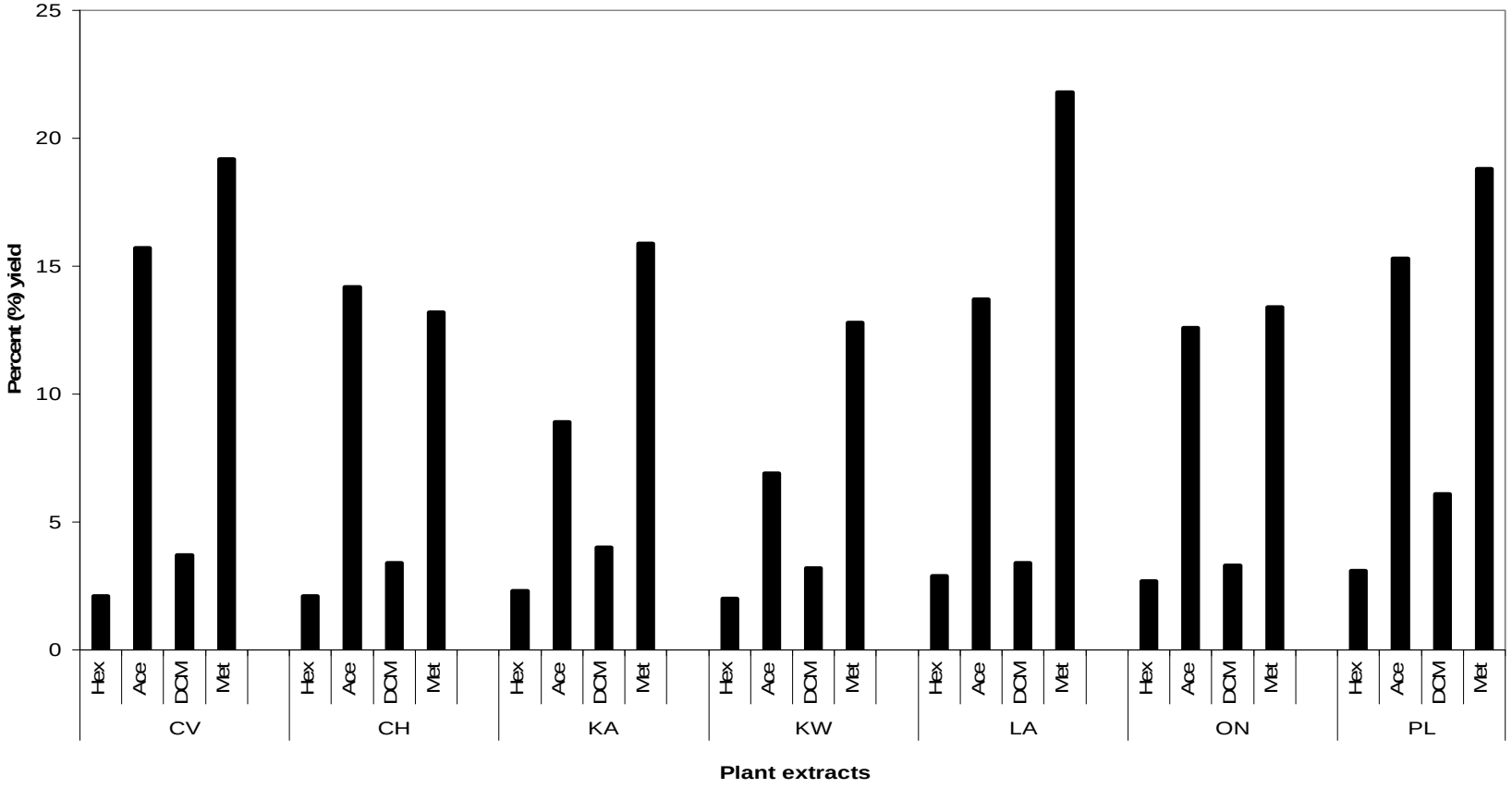
Fungal cultures were transferred from agar culture plates to fresh SD broth and 100 µl of the broth was added to each well. Densities of fungal cultures used for the screening were as follows: *A. fumigatus*, 8.1 × 10^4 cfu/ml; *C. albicans*, 2.5 × 10^4 cfu/ml; *C. neoformans*, 2.6 × 10^4 cfu/ml; *M. canis*, 1.5 × 10^5 cfu/ml; *S. schenckii*, 1.4 × 10^5 cfu/ml. Amphotericin B and acetone were used as positive and negative control substances, respectively. Forty µl of *p*-iodonitrotetrazolium violet INT (0.2 mg/ml) was added to each of the microtitre wells to serve as an indicator of fungal growth. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of extract to inhibit bacterial growth in the microtitre plate as indicated by a reduction in the red colour of the INT formazan (Masoko et al., 2005) and was assessed after 24 and 48 h incubation period.

3.3. Results and discussion

3.3.1. Mass extracted

The amounts of dried plant material extracted by each of the solvents (hexane, acetone, dichloromethane and methanol) used in this study are presented in Figure 3.1. Methanol extracted the highest quantity of plant material. The highest quantity was extracted from *Loxostylis alata* (218 mg) representing 21.8%. The masses extracted by acetone and dichloromethane extracts were generally second and third highest, respectively, in all the plants except *Commiphora harveyi*. The result was comparable to what was reported by Kotze and Eloff (2002) where methanol was the best extractant for *Combretum erythrophyllum* found in South Africa. In contrast, acetone extracted more plant material from *Commiphora harveyi* (142 mg) representing 14.2%. In a study with 27 members of the Combretaceae family, acetone extracted more plant material than the other solvents used (Eloff, 1999). The lowest amount of extract was obtained from the hexane extraction of *Kirkia wilmsii* (20 mg) representing 2% of extractable material. In traditional medical practice, water is used as the major extractant. The implication of this is that potential active compounds that are not hydrophilic may not be extracted and a plant disregarded as not being active biologically, especially if the polar extracts are not active in the bioassay. Such a problem was circumvented in our study by extracting the plant leaves in parallel with solvents of low to high polarity. Acetone was used to re-dissolve the extracts of hexane, acetone, dichloromethane and methanol prior to bioassay.

Figure 3.1. Percentage yield extracted by different solvents from leaves of different South African plant species



Hexane (Hex); acetone (Ace), dichloromethane (DCM) and methanol (Met).
Loxostylis alata (LA), *Kirkia wilmsii* (KW), *Ochna natalitia* (ON), *Khaya anthotheca* (KA), *Combretum vendae* (CV), *Commiphora harveyi* (CH) and *Protorhus longifolia* (PL)

3.3.2. Minimum inhibitory concentration

3.3.2.1. Bacterial species

The plant extracts differed greatly in their activity against the test bacteria and the best bacterial inhibition was observed with MIC = 0.04 mg/ml by the hexane extract of *Khaya anthotheca* against *S. aureus*. There are no validated criteria for the MIC end points for *in vitro* testing of plant extracts. However, an attempt was made to grade MIC of plant extracts/compounds by Holetz et al (2002). He proposed: good antimicrobial activity = MIC less than 0.1 mg/ml; moderate antimicrobial activity = MIC of 0.1 to 0.5 mg/ml; weak antimicrobial activity = MIC of 0.5 to 1 mg/ml; MIC of greater than 1 mg/ml was considered inactive.

Among the tested extracts, the hexane extracts of *Combretum vendae*, *Commiphora harveyi*, *Khaya anthotheca*, *Ochna natalitia* and *Loxostylis alata*, the acetone extracts of *Commiphora harveyi*, *Loxostylis alata* and *Protorhus longifolia*, and the dichloromethane extracts of *Combretum vendae*, *Commiphora harveyi* and *Loxostylis alata* had the best antibacterial activity against at least one of the tested pathogens. The MIC values of these extracts were the lowest, ranging from 0.04 to 0.01 mg/ml (Table 3.2). The extracts of *Loxostylis alata* had very promising results with good antibacterial activity in 3 out of 4 of the extracts tested. The hexane, acetone and dichloromethane extracts of *Loxostylis alata* had MIC values as low as 0.08 mg/ml against *S. aureus*, *E. faecalis* and *E. coli*. The reference antibiotic (gentamicin) had an MIC of 0.025 mg/ml against the mentioned pathogen. Perhaps when the active compound(s) are isolated in pure forms from the crude extracts they might have increased antimicrobial action. The action of most of the extracts appeared to be bacteriostatic, as growth of the bacteria and resulting red colour formation appeared to resume after the 24-h incubation period with INT (Table 3.2).

The Gram-negative organisms (*E. coli* and *P. aeruginosa*) were more resistant to the extracts than the Gram-positive organisms (*S. aureus* and *E. faecalis*) as indicated by their high MIC values. Gram-negative bacteria are relatively resistant to plant extracts owing to the presence of an outer membrane which is known to present a barrier to penetration of numerous antimicrobial molecules, and the periplasmic space contains enzymes which are capable of breaking down foreign molecules introduced from outside (Nikaido, 1996). *S. aureus* exhibited the highest susceptibility to the plant extracts used in other studies conducted (Stickler and King, 1992; Martínez et al., 1996; Chariandy et al., 1999). Similar results were obtained in this study.

Table 3.2. Minimum inhibitory concentrations (average of triplicate determinations) of four different extracts from seven South African plants tested against bacteria. MIC assessment was done 1 and 2 h after INT (indicator of bacterial growth) was added to the bacterial cultures.

Microorganism	Time (h)	MIC (mg/ml)															
		Combretum vendae				*Commiphora harveyi*				*Khaya anthotheca*				*Kirkia wilmsii*			
		H	A	D	M	H	A	D	M	H	A	D	M	H	A	D	M
P. aeruginosa	1	0.31	0.16	0.16	1.25	0.31	0.63	0.63	2.50	0.31	0.16	0.16	1.25	0.16	0.24	0.31	0.63
	2	0.63	0.31	0.16	1.25	0.63	1.25	0.63	2.50	0.63	0.31	0.16	1.25	0.31	0.47	0.63	2.50
S. aureus	1	0.31	0.63	0.31	1.25	0.31	0.63	0.08	2.50	0.04	0.16	0.08	1.25	0.63	0.31	0.63	1.25
	2	0.31	1.25	0.63	2.50	0.31	0.63	0.16	2.50	0.08	0.16	0.08	1.25	1.25	0.31	0.63	1.25
E. coli	1	0.78	0.63	0.31	1.25	2.50	1.25	1.25	2.50	2.50	1.25	1.25	2.50	0.16	0.31	0.63	1.25
	2	1.25	0.63	0.63	2.50	2.50	2.50	1.25	2.50	2.50	2.50	0.63	2.50	0.16	0.31	1.25	2.50
E. faecalis	1	0.08	0.12	0.08	0.16	0.06	0.08	0.08	0.63	0.16	0.16	0.16	1.25	0.31	0.31	0.31	1.25
	2	0.16	0.12	0.08	0.31	0.31	0.24	0.16	1.25	0.16	0.31	0.16	2.50	0.31	0.31	0.31	1.25
Average		0.48	0.48	0.30	1.31	0.87	0.90	0.53	2.11	0.80	0.63	0.34	1.72	0.41	0.32	0.59	1.49

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

Table 3.2. cont...

Microorganism	Time (h)	MIC (mg/ml)												Gentamicin
		Loxostylis alata				*Ochna natalitia*				*Protorhus longifolia*				
		H	A	D	M	H	A	D	M	H	A	D	M	
P. aeruginosa	1	0.31	0.31	0.47	0.31	0.16	0.31	0.31	1.25	0.16	0.08	0.16	2.50	0.015
	2	0.63	0.31	0.47	0.31	0.31	1.25	0.63	1.25	0.63	0.16	0.31	2.50	0.06
S. aureus	1	0.08	0.06	0.63	2.50	0.16	0.31	0.16	1.25	0.63	0.63	0.63	1.25	0.007
	2	0.1	0.06	1.25	2.50	0.16	1.25	0.63	2.50	0.63	0.63	0.31	1.25	0.025
E. coli	1	0.08	0.06	0.63	1.25	0.31	0.63	2.50	2.50	0.63	0.31	0.31	1.25	0.025
	2	0.16	0.08	0.08	1.25	0.63	0.63	1.25	2.50	0.63	0.31	0.63	2.50	0.05
E. faecalis	1	1.25	0.16	0.16	0.16	0.08	0.16	0.24	0.63	0.31	0.63	0.63	1.25	0.003
	2	2.5	0.31	0.63	0.63	0.08	0.16	0.31	1.25	1.25	1.25	0.63	2.50	0.006
Average		0.64	0.17	0.54	1.11	0.24	0.59	0.75	1.64	0.61	0.50	0.45	1.88	

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

3.3.2.2. Fungal species

In the present investigation, the 28 extracts screened had activity against at least one of the test organisms (Table 3.3). The acetone extracts of *Loxostylis alata*, *Kirkia wilmsii*, *Ochna natalitia*, and *Combretum vendae* had high antifungal activity with MIC values ranging from 0.04-0.8 mg/ml against one or more of the tested micro-organisms. In a similar study, a member of the Anacardiaceae family, *Sclerocarya birrea* exhibited very good antifungal activity against some selected fungal pathogens (Hamza et al., 2006). The hexane extracts of *Combretum vendae*, *Khaya anthotheca* and *Ochna natalitia* had MIC values ranging from 0.05-0.09 mg/ml. The hexane extract of *Combretum vendae* had the lowest average MIC of 0.23 mg/ml against all the tested pathogens. As in the bacterial assays, most of the methanol extracts were relatively inactive against all the tested pathogens. However, the methanol extract of the stem bark of *Khaya anthotheca* was reported by Hamza et al (2006) to be very active against *Candida krusei* but inactive against other pathogenic yeast namely *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, *Candida tropicalis* and *Cryptococcus neoformans*. The highest average MIC value of 1.73 mg/ml was obtained with the methanol extract of *Ochna natalitia*. Plant extracts with MIC of 0.78 mg/ml against *Candida albicans* are regarded to have good activity (Buwa and van Staden, 2006). Similarly, Hamza et al (2006) reported that extracts having MIC of 0.5 mg/ml or less as being strong inhibitors of fungal growth. Their report was based on classification of MIC earlier reported by Aligiannis et al. (2001) who proposed that plant extracts having MIC of 0.5 mg/ml as strong inhibitors; moderate inhibitors have MIC between 0.6 and 1.5 mg/ml. Extracts having MIC above 1.6 mg/ml are considered weak inhibitors.

M. canis had the highest susceptibility to the extracts, being sensitive to 5 of the tested extracts at concentrations as low as 0.05-0.08 mg/ml, while *C. albicans* had the lowest sensitivity to the plant extracts. *C. neoformans*, *S. schenckii* and *A. fumigatus* were sensitive to 3, 2 and 1 of the tested extracts respectively, with MIC values lower than 0.1 mg/ml. No growth inhibition was detected in the negative control wells. The antifungal activities of the plant extracts screened were not as effective as that of amphotericin B which is the reference compound (Table 3.3). The positive control (amphotericin B) had MIC values of 0.01-0.0003 mg/ml against the tested fungi. Similarly, as was mentioned for antibacterial screening, the action of most of the extracts on fungi appears to be fungistatic, as growth of the organisms and resulting red colour formation appeared to resume after the 48-h incubation period with INT (Table 3.3).

Table 3.3. Minimum inhibitory concentrations (average of triplicate determinations) of four different extracts from seven South African plants tested against some animal pathogenic fungi.

Microorganism	Time (h)	MIC (mg/ml)															
		Combretum vendae				*Commiphora harveyi*				*Khaya anthotheca*				*Kirkia wilmsii*			
		H	A	D	M	H	A	D	M	H	A	D	M	H	A	D	M
A. fumigatus	24	0.31	0.26	0.31	0.63	1.88	2.5	1.25	2.50	0.31	0.31	0.31	0.13	0.16	0.13	0.21	1.04
	48	0.42	0.37	0.31	0.63	2.5	2.5	1.67	2.50	1.25	1.67	1.25	2.08	1.25	1.25	2.50	2.50
C. albicans	24	0.21	0.26	0.26	0.63	0.52	0.16	0.32	1.25	0.63	0.83	0.63	1.67	0.31	0.83	1.25	1.25
	48	0.31	0.31	0.63	1.25	0.52	0.13	0.37	2.08	2.08	1.46	1.67	2.50	0.83	1.25	1.67	2.50
C. neoformans	24	0.08	0.31	0.31	1.25	2.08	0.21	1.25	0.84	0.05	0.16	0.31	1.04	1.25	0.07	0.63	0.31
	48	0.16	0.63	1.67	2.50	2.08	0.63	2.50	0.84	0.11	0.31	0.63	2.50	2.50	0.13	2.08	1.25
M. canis	24	0.16	0.08	0.11	0.31	0.11	0.16	0.63	1.25	0.21	0.26	0.63	1.04	1.04	0.08	0.84	1.25
	48	0.31	0.21	0.11	1.25	0.16	0.16	1.67	2.50	0.31	0.37	1.88	2.50	2.50	0.37	1.04	2.50
S. schenckii	24	0.13	0.31	0.63	1.11	0.63	0.31	1.04	2.50	0.16	0.11	0.63	0.94	1.25	0.26	1.25	0.31
	48	0.16	0.63	0.63	2.5	1.67	0.84	2.08	2.50	0.26	0.16	1.25	1.04	2.50	0.63	2.50	2.50
Average		0.23	0.34	0.50	1.21	1.22	0.76	1.28	1.88	0.54	0.56	0.92	1.54	1.50	0.50	1.40	1.54

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

Table 3.3 cont...

Microorganism	Time (h)	MIC (mg/ml)												Amphotericin B (mg/ml)
		Loxostylis alata				*Ochna natalitia*				*Protorhus longifolia*				
		H	A	D	M	H	A	D	M	H	A	D	M	
A. fumigatus	24	1.25	0.05	0.52	0.42	2.08	1.25	1.25	2.50	1.67	0.31	0.63	1.25	0.005
	48	2.08	0.16	1.25	2.50	2.50	2.50	2.08	2.50	2.50	2.50	1.67	2.50	0.01
C. albicans	24	2.50	0.31	1.25	1.67	0.31	1.25	1.25	1.25	2.50	1.67	0.52	1.25	0.025
	48	2.50	1.04	2.50	1.67	0.31	1.25	2.08	2.50	2.08	1.67	0.52	1.25	0.005
C. neoformans	24	1.67	0.21	0.63	1.67	0.84	0.31	0.42	1.67	2.08	0.21	0.52	0.31	0.00063
	48	2.50	0.52	1.25	2.50	1.25	1.25	0.62	2.50	2.50	0.31	0.63	0.63	0.025
M. canis	24	1.04	0.07	1.04	0.63	0.08	0.07	0.11	0.13	0.31	0.63	0.94	0.31	0.00031
	48	2.50	0.07	1.25	1.25	0.52	0.09	0.52	0.52	1.04	1.67	1.67	2.50	0.00063
S. schenckii	24	1.25	0.04	0.13	1.25	0.08	0.26	0.42	1.25	1.04	1.25	0.84	1.25	0.00063
	48	2.50	0.08	0.31	2.5	0.21	0.31	1.25	2.50	2.50	1.25	0.84	2.50	0.005
Average		1.98	0.26	1.01	1.61	0.82	0.85	1.00	1.73	1.82	1.15	0.88	1.38	

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

3.3.3. Total activity

For ethnopharmacological research to be locally relevant, not only the MIC is important, but also the quantity extracted from each plant species. Total activity is calculated by dividing the quantity extracted from one gram of plant material with the MIC value in mg/ml (Eloff, 2001). This value indicates the volume to which the active constituent present in one gram of the plant material can be diluted and still inhibit the growth of the test organism.

For bacterial organisms, the total activity of the plants ranged from 5 to 2283 ml/g (Table 3.4). The highest total activity of 2283 mg/ml was produced by the acetone extract of *Loxostylis alata* against *S. aureus* and *E. faecalis*. It therefore means that 1 gram of *Loxostylis alata* acetone extract can be diluted in 2283 ml of the solvent used and still inhibit the growth of the organisms. Similarly, the total activities of the plant extracts against fungi ranged from 5-3425 ml/g (Table 3.5). *Loxostylis alata* was the most active, with the acetone extract having a total activity of 3425 ml/g against *Microsporum canis* over an incubation period of 24 h. This is a step towards the rational use of plants in traditional primary health care and could be of benefit in enabling rural use of the plants as information regarding the usefulness of the plant could be handed to rural people. Higher values of total activity indicate increased usefulness and potential economic value.

This study investigated the *in vitro* antimicrobial activity of selected plant species, and has supplied preliminary evidence of the efficacy of these plant species for the traditional treatment of various bacterially-related diseases. However, *in vivo* data is necessary in determining the potential usefulness of these plants for treatment of infectious diseases. One of the inherent problems associated with *in vitro* testing is the absence of body metabolic processes. More importantly, factors such as absorption and metabolism may be responsible for discrepancies between *in vitro* and *in vivo* activity (Houghton et al., 2007). However, *in vitro* activity may serve as a lead towards the discovery of plant chemical agents that are potentially active *in vivo*.

In terms of conservation, the results revealed that leaf material of these plants is useful for antimicrobial uses because this material can be used without any detrimental effect on the plant (Holetz et al., 2002).

Table 3.4. Total activity in ml/g of seven South African plants screened for antibacterial activity.

Microorganis m	Time (h)	Total activity (ml/g)															
		Combretum vendae				*Commiphora harveyi*				*Khaya anthotheca*				*Kirkia wilmsii*			
		H	A	D	M	H	A	D	M	H	A	D	M	H	A	D	M
P. aeruginosa	1 2	68 33	981 506	23 1 23 1	154 154	68 33	225 114	54 54	5 5	74 37	55 6 28 7	25 0 25 0	12 7 12 7	12 5 65	28 8 14 7	10 3 51	20 3 51
S. aureus	1 2	68 68	249 126	11 9 30	154 77	68 68	225 225	42 5 21 3	5 5	57 5 28 8	55 6 55 6	50 0 50 0	12 7 12 7	32 16	22 3 22 3	51 51	10 2 10 2
E. coli	1 2	27 17	249 249	11 9 59	154 77	8 8	114 57	27 27	5 5	9 9	71 36	32 63	64 64	12 5 12 5	22 3 22 3	51 26	10 2 51
E. faecalis	1 2	26 3 13 1	130 8 130 8	46 3 46 3	120 0 619	35 0 68	177 5 592	42 5 21 3	2 1 1 1	14 4 14 4	55 6 28 7	25 0 25 0	12 7 64	65 65	22 3 22 3	10 3 10 3	10 2 10 2
Average		84	622	21 4	323	84	416	18 0	8	16 0	36 3	26 2	10 3	77	22 1	67	10 2

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

Table 3.4 cont...

Microorganism	Time (h)	Total activity (ml/g)											
		Loxostylis alata				*Ochna natalitia*				*Protorhus longifolia*			
		H	A	D	M	H	A	D	M	H	A	D	M
P. aeruginosa	1	94	442	72	703	169	406	106	107	194	1913	381	75
	2	46	442	72	703	87	101	52	107	49	956	197	75
S. aureus	1	363	2283	54	87	169	406	206	107	49	243	97	150
	2	290	2283	27	87	169	101	52	54	49	243	197	150
E. coli	1	363	2283	54	174	87	200	13	54	49	494	197	150
	2	181	1713	425	174	43	200	26	54	49	494	97	75
E. faecalis	1	23	856	213	1363	338	788	138	213	100	243	97	150
	2	12	442	54	346	338	788	106	107	25	122	97	75
Average		171	1343	121	455	175	374	88	100	71	588	170	113

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

Table 3.5. Total activity in ml/g of seven South African plants screened for antifungal activity.

Microorganism	Time (h)	Total activity (ml/g)															
		Combretum vendae				*Commiphora harveyi*				*Khaya anthotheca*				*Kirkia wilmsii*			
		H	A	D	M	H	A	D	M	H	A	D	M	H	A	D	M
A. fumigatus	24	68	604	119	305	11	57	27	5	74	287	129	1223	125	531	152	123
	48	50	424	119	305	8	57	20	5	18	53	32	76	16	55	13	51
C. albicans	24	100	604	142	305	40	888	106	11	37	107	63	95	65	83	26	102
	48	68	506	59	154	40	1092	92	6	11	61	24	64	24	55	19	51
C. neoformans	24	263	506	119	154	10	676	27	16	460	556	129	153	16	986	51	413
	48	131	249	15	77	10	225	14	16	209	287	63	64	8	531	15	102
M. canis	24	131	1963	119	619	191	888	54	11	110	342	63	153	19	863	38	102
	48	68	748	30	154	131	888	20	5	74	241	21	64	8	186	31	51
S. schenckii	24	162	506	33	173	33	458	33	5	144	809	63	169	16	265	26	413
	48	131	249	15	77	13	169	16	5	88	556	32	153	8	110	13	51
Average		117	636	77	232	49	540	41	9	123	330	62	221	30	366	38	146

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

Table 3.5. cont...

Microorganism	Time (h)	Total activity (ml/g)											
		Loxostylis alata				*Ochna natalitia*				*Protorhus longifolia*			
		H	A	D	M	H	A	D	M	H	A	D	M
A. fumigatus	24	23	2740	65	519	13	101	26	54	19	494	97	150
	48	14	856	27	87	11	50	16	54	12	61	37	75
C. albicans	24	12	442	27	131	87	101	26	107	12	92	117	150
	48	12	132	14	131	87	101	16	54	15	92	117	150
C. neoformans	24	17	652	54	131	32	406	79	80	15	729	117	606
	48	12	263	27	87	22	101	53	54	12	494	97	298
M. canis	24	28	1957	33	346	338	1800	300	1031	100	243	65	606
	48	12	1957	27	174	52	1400	63	258	30	92	37	75
S. schenckii	24	23	3425	27	174	338	485	79	107	30	122	73	150
	48	12	1713	14	87	129	406	26	54	12	122	73	75
Average		16	1414	32	187	111	495	68	185	26	254	83	234

H= hexane extract; A= acetone extract; D= dichloromethane extract; M= methanol extract

3.4. Conclusion

The plant extracts tested had varying levels of activity against bacteria. MIC data revealed that the hexane extract of *Khaya anthotheca* had the highest antibacterial activity with lowest MIC against *Staphylococcus aureus*, while the acetone extract of *Loxostylis alata* exhibited the most antifungal activity with lowest MIC against *Sporothrix schenckii*. From this study, we can infer that the South African flora offers a good potential as a source of antimicrobial agents. The bioassay-guided fractionation procedure to isolate and characterise active compounds from *Loxostylis alata* and other active plants is currently being undertaken.

Postscript

All the plant species possess varying degree of antibacterial and antifungal activities. We therefore went further to determine the number of antimicrobial compounds present in each extract. That will assist in further designing a strategy for isolating the active plant components.

Chapter 4
Detection of antimicrobial compounds by direct bioautography of different extracts of leaves of selected South African plant species

M.M. Suleiman, L.J. McGaw, V. Naidoo and J.N. Eloff

Preface

In addition to the antifungal activity one of the most important aspects to consider in selecting the species to work on is how many antifungal compounds are present in extracts that have promising. In some cases the antifungal activity is due to the presence of a mixture of compounds because no activity is found after the compounds are separated and evaluated by bioautography. This makes it impossible to isolated antifungal compounds. Bioautography is therefore a very important step in selecting the best species to work on. The text in this chapter was submitted and accepted as manuscript for publication to African Journal of Traditional, Complementary and Alternative Medicines, Vol. 7, pages 64-78.

Abstract

The hexane, acetone, dichloromethane and methanol extracts of *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) were screened for their antimicrobial activity. The test organisms included bacteria (*Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Staphylococcus aureus*), and fungi (*Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporum canis* and *Sporothrix schenckii*). A simple bioautographic procedure, involving spraying suspensions of the bacteria or fungi on thin layer chromatography (TLC) plates developed in solvents of varying polarities was used to detect antibacterial and antifungal activity of the extracts. All the extracts had varying levels of antimicrobial activity against at least one of the test microorganisms. This activity was denoted by white spots against a red-purple background on the TLC plates after spraying with a tetrazolium salt indicator. Twenty seven TLC plates; 9 for each solvent system and 3 different solvent systems per organism were tested in the bioautographic procedure. Of the bacteria tested, *S. aureus* also appeared to be the most susceptible organism, being inhibited by almost all the compounds separated on the TLC plates from all the tested plants. Similarly, *C. neoformans* depicted the highest susceptibility among fungal organisms.
Loxostylis alata appeared to be the most active plant with the highest number of inhibition zones when compared with other plants tested against both bacteria and fungi.

Keywords: Bioautography; Medicinal plants; South Africa; Plant extracts; R_f values

4.1. Introduction

Despite the existence of conventional antimicrobial agents, resistant or multi-resistant strains of pathogenic microorganisms are continuously appearing, imposing the need for a thorough search for and development of new drugs (Silver and Bostian, 1993). Fungi and bacteria cause important human and animal diseases, especially in tropical and subtropical regions, and commonly occur in immunocompromised or immunodeficient patients. Over the last decade, there has been a renewed interest in plants; and the pharmaceutical industry considers plants as a viable option for the discovery of new leads (Soejarto, 1996). In fact, it is also estimated that natural products are implicated in the development of 44% of all new drugs, generally as leads for the preparation of semi-synthetic derivatives (Hostettmann et al., 2000 and 2001).

In an effort to discover new lead compounds, many research groups screen plant extracts to detect secondary metabolites with relevant biological activities. In this regard, several bioassays were developed for screening purposes (Hostettmann 1991).

Once the technique has been mastered, bioautography can be considered a highly efficacious assay for the detection of antimicrobial compounds because it allows localization of activity even in a complex matrix, and therefore permits a target-directed isolation of the active constituents (Rahalison et al., 1991). Bioautography has enabled rapid progress for quick detection of new antimicrobial compounds from plants and other natural products. This technique allows the localization of antimicrobial activity directly on a chromatographic plate where the organism is applied (Navarro et al 1998). The method is fast, cheap, and permits a better bioassay-directed fractionation of bioactive compounds (Hamburger and Cordell, 1987). Bioautography is particularly important to avoid the time-consuming isolation of known substances or inactive ones. TLC bioautographic methods combine chromatographic separation and *in situ* activity determination facilitating the localization and target-directed isolation of active constituents in a mixture (Shahverdi et al., 2007). A number of bioautographic assays have been developed, which can be divided into three groups (Rios et al., 1988). These include direct bioautography; where the microorganisms grow directly on thin-layer chromatography (TLC) plates, contact bioautography; where the antimicrobial compounds are transferred from the TLC plate to an inoculated agar plate through direct contact, and agar overlay or immersion bioautography; where a seeded agar medium is applied onto the TLC plate. The latter technique can be considered as a hybrid of direct and contact bioautography (Islam et al., 2003).

The purpose of our research was to determine the antimicrobial activity of hexane, dichloromethane, acetone and methanol extracts of seven South African tree leaves by direct bioautography. In the ongoing tree screening project of the Phytomedicine Programme, University of Pretoria (www.up.ac.za/phyto), plant species that had excellent activity against *Candida albicans* depicted by low minimum inhibitory concentrations (≤ 0.08 mg/ml) in a broth microdilution assay (Eloff, 1998a) were selected for evaluation of their potential antimicrobial activity against other animal fungal and bacterial organisms. The localization of active compounds contained in these extracts will assist in directing methods for their isolation.

4.2. Materials and methods

4.2.1. Plant collection

Combretum vendae, *Commiphora harveyi*, *Khaya anthotheca* and *Loxostylis alata* leaves were collected at the Manie van der Schijff Botanical Garden of the University of Pretoria, South Africa. *Kirkia wilmsii* and *Ochna natalitia* were collected at the Lowveld National Botanical Garden in Nelspruit, South Africa. All plant leaves were collected in summer (November 2006) between 9:30 am and 12:30 pm. Samples of the plants were identified and authenticated by Ms Lorraine Middleton and Magda Nel of the University of Pretoria. Voucher specimens of the plants were deposited at the Schweikert Herbarium of the Department of Plant Sciences, University of Pretoria, South Africa.

4.2.2. Plant storage

Immediately after collection and transportation to our laboratory, leaves were separated from stems and dried at room temperature with good ventilation. The dried plants were milled to a fine powder in a Macsalab mill (Model 200 LAB), Eriez®, Bramley, and stored at room temperature in closed containers in the dark until use.

4.2.3. Plant extraction

Plant samples from each species were individually extracted by weighing four aliquots of 1 g of finely ground plant material and extracting with 10 ml of either acetone, hexane, dichloromethane (DCM) or methanol (technical grade-Merck) in centrifuge tubes. Tubes were vigorously shaken for 1 hour in a Labotec model 20.2 shaking machine at a moderate speed. Extracting plant powdered material at low speed for a longer period allows greater penetration of the solvent into the plant tissues which allows more of the plant compounds to be extracted (Silva et al., 1998). After centrifuging at 3500 x g for 10 min, the supernatant was decanted into pre-weighed labelled containers. The whole process was repeated three

times to exhaustively extract the plant material and the extracts were combined. The solvent was removed under a stream of air in a fume cupboard at room temperature to quantify the extraction.

4.2.4. Microorganisms and medium

The fungal organisms used in this study were moulds (*Aspergillus fumigatus* and *Microsporum canis*), yeast (*Candida albicans* and *Cryptococcus neoformans*) and a thermally dimorphic fungus (*Sporothrix schenckii*). All fungal organisms were isolated from clinical cases that were not treated prior to sampling in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. *A. fumigatus* was isolated from a chicken, *C. albicans* from a Goldian finch, *C. neoformans* from a cheetah, *M. canis* from a cat suffering from dermatophytosis and *Sporothrix schenckii* from a horse with cutaneous lymphangitis. These fungi represent the most common and important disease-causing fungi of animals (Masoko et al., 2005). Sabouraud dextrose (SD) agar (Oxoid, Basingstoke, UK) was used as the maintenance growth medium for all the fungal strains used, and the fungi were cultured in SD broth.

The bacteria used were the Gram-positive bacteria: *Staphylococcus aureus* (ATCC 29213) and *Enterococcus faecalis* (ATCC 29212), and the Gram-negative bacteria: *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 27853). All bacterial cultures were maintained on Mueller Hinton (MH) agar (Oxoid, Basingstoke, UK) at 4°C and cultured in MH broth at 37°C.

4.2.5. Phytochemical analysis

Chemical constituents of the extracts were separated on aluminium-backed thin layer chromatography (TLC) plates (Merck, silica gel 60 F_{254}). The TLC plates were developed under saturated conditions with one of the three eluent systems developed in our laboratory, 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); benzene/ethanol/ammonia hydroxide (18:2:0.2): [BEA] (non-polar/basic) (Kotze and Eloff, 2002). Separated chemical compounds were detected using acidified vanillin (0.1 g vanillin: 28 ml methanol:1ml sulphuric acid) as a spray. After spraying, the chromatograms were heated at 110°C in an incubator to allow for optimal colour development.

4.2.6. Bioautography

Ten µl (10 mg/ml) of each extract were loaded onto TLC plates and eluted using the three different mobile solvent systems (CEF, BEA and EMW). The developed plates were dried under a stream of fast moving air for 5 days to remove traces of solvent on the plates. One week old cultures of fungal organisms grown on SD agar were each transferred into 250 ml of freshly prepared SD broth using a sterile swab. Densities of fungal cultures used for *A. fumigatus*, *C. albicans*, *C. neoformans*, *M. canis* and *S. schenckii* were approximately 8 $\times 10^6$, 3 $\times 10^6$, 3 $\times 10^6$, 2 $\times 10^5$ and 1 $\times 10^5$ cells/ml respectively, In the case of bacteria, overnight cultures grown on MH broth were used and the densities of bacterial organism used for *E. faecalis*, *E. coli*, *P. aeruginosa* and *S. aureus* were approximately 2 $\times 10^{10}$, 3 $\times 10^{11}$, 5 $\times 10^{13}$ and 3 $\times 10^{12}$ cfu/ml, respectively. The prepared chromatograms were sprayed with the fungal or bacterial suspension until wet. This process was carried out in a biosafety Class II cabinet (Labotec, SA) for fungi, and Laminar flow cabinet (Labotec, SA) for bacteria. Thereafter, the plates were incubated overnight at 35°C and 100% relative humidity in the dark and then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (Sigma®) (INT) (Begue and Klein, 1972) and further incubated overnight or longer in the case of *S. schenckii* and *M. canis*. White areas or spots 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 organisms.

4.3. Results and discussions

Phytochemical screening revealed the presence of varied chemical components in the different extracts of the plants. This is notable from the different colour changes depicted by individual compounds due to their reaction with the spray reagent used (vanillin/sulphuric acid) (Figure 4.1).

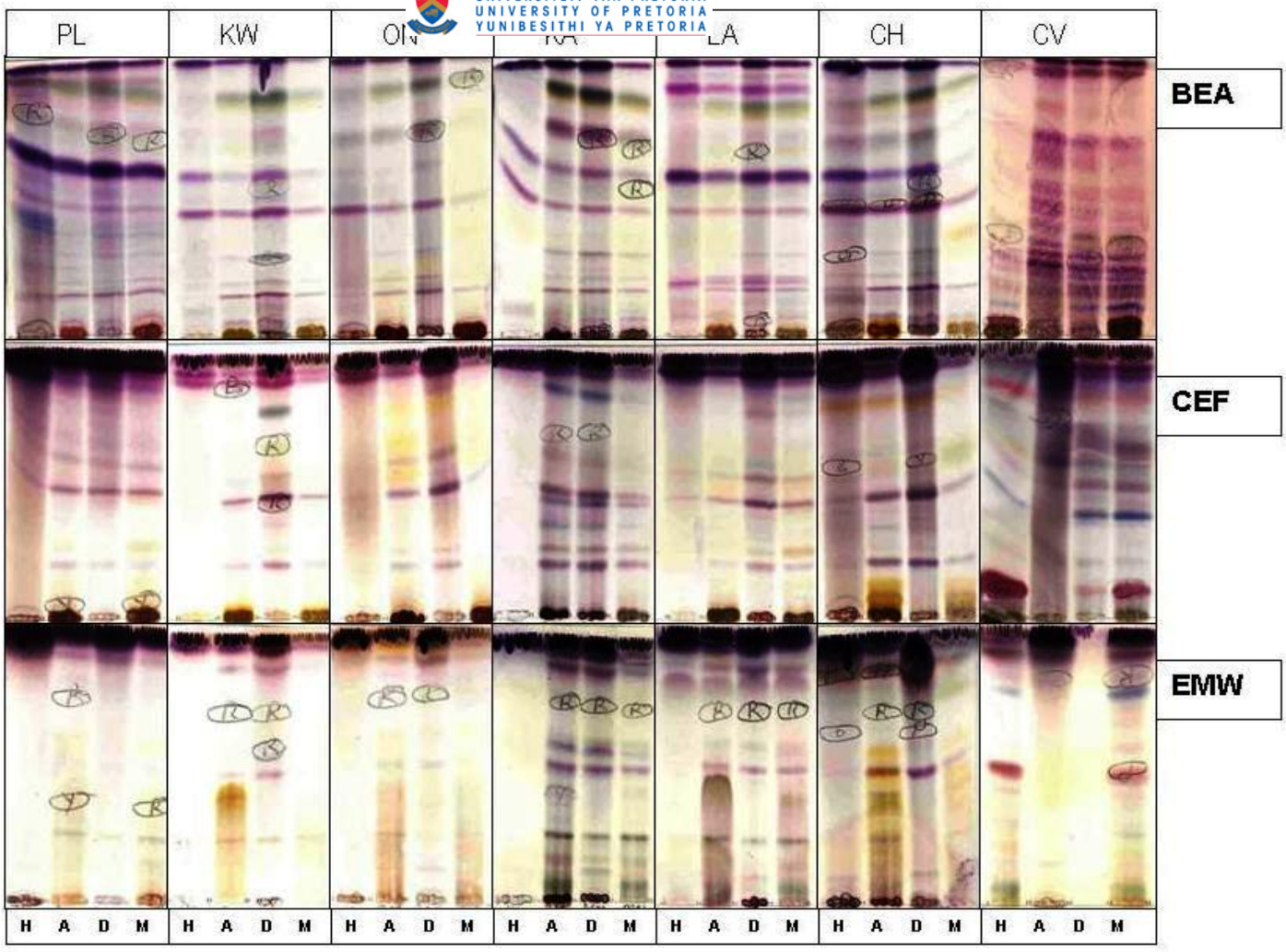


Figure 4.1. Chromatograms of extracts of hexane (H), acetone (A), dichloromethane (D) and methanol (M) developed in Benzene/Ethanol/Ammonia hydroxide: 90:10:1 [BEA] (non-polar/basic), Chloroform/Ethyl acetate/Formic acid: 5:4:1 [CEF] (intermediate polar/acidic) and Ethyl acetate/Methanol/Water: 40:5.4:4 [EMW] (polar/neutral) and sprayed with vanillin in concentrated sulphuric acid. PL = *P. longifolia*, KW = *Kirkia wilmsii*, ON = *Ochna natalitia*, KA= *Khaya anthotheca*, LA = *Loxostylis alata*, CH = *Commiphora harveyi*, CV = *Combretum vendae*.

For example, terpenes exhibit red or blue colouration on the chromatograms when sprayed with vanillin/sulphuric (Gibbons and Gray, 1998). Similarities exist between chemical compositions of the components of extracts separated using the same solvent system (Figure 4.1). Dellar et al. (1994) reported the isolation of antifungal sesquiterpenes aristolen-2-one and prostatherol from 2 species of *Prostanthera* (Labiatae). Studies into the effects of terpenoids on isolated bacterial membranes revealed their site of action to be at the phospholipid bilayer. They affect bacterial processes that include the inhibition of electron transport, protein translocation, phosphorylation steps and other enzyme-dependent reactions (Knobloch *et al*. 1986). Perhaps similar mechanisms of action were responsible for the antimicrobial actions of the plant extracts under study.

The appearance of white areas against a purple-red background on the chromatograms denotes inhibition of growth of the bacteria (Figure 3.2) or fungi (Figure 4.3) due to presence of compound(s) that inhibit their growth. Actively growing microorganisms have the ability to reduce INT to a purple-red colour (Begue and Klein, 1972). In the presence of active plant compounds on the chromatograms, the growth of the organism is inhibited.

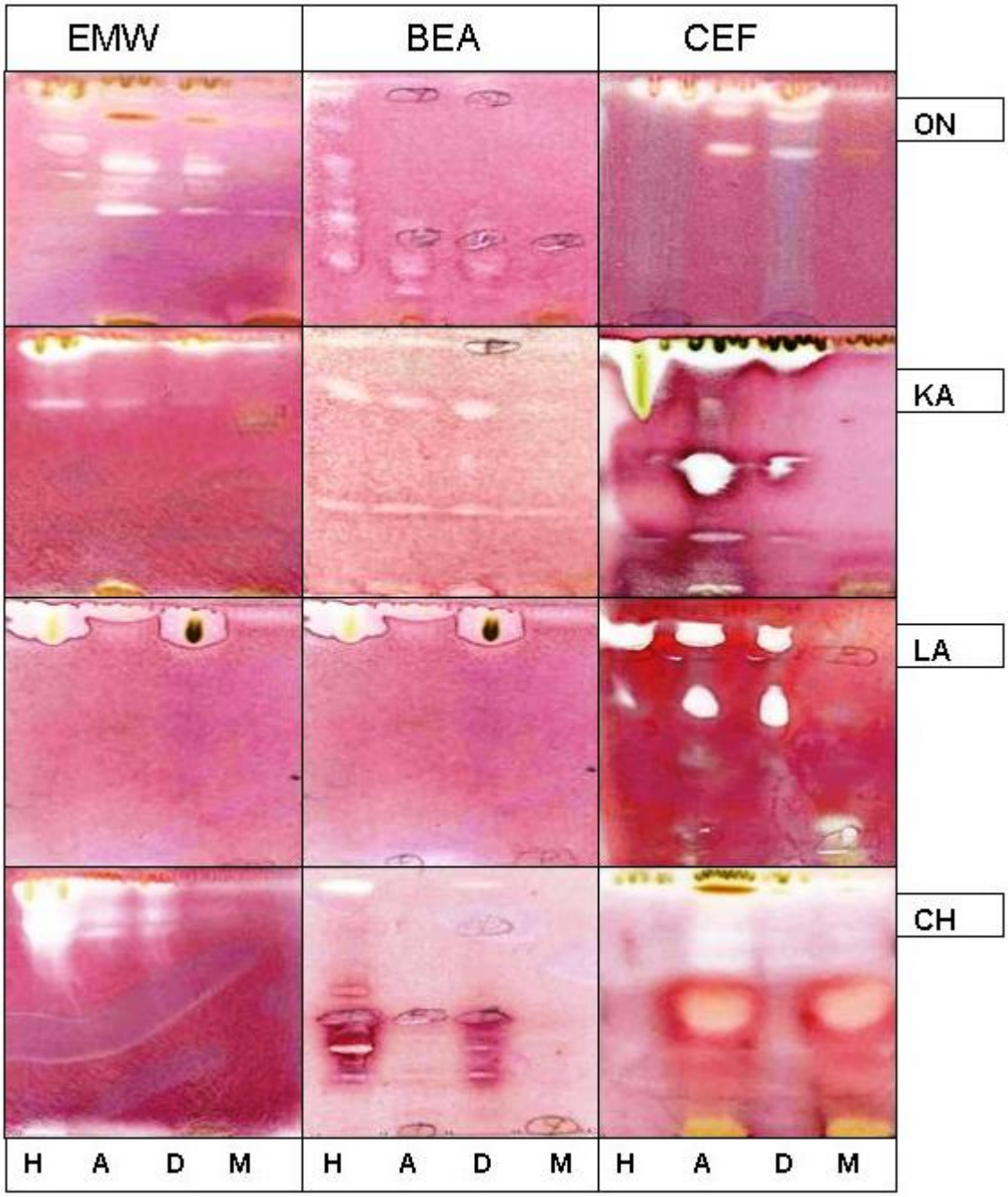


Figure 4.2. Hexane (H), acetone (A), dichloromethane (D), and methanol (M) extracts of *Ochna natalitia* (ON), *Khaya anthotheca* (KA) *Loxostylis alata* (LA) and *Commiphora harveyi* (CH) separated on TLC plates using EMW, BEA and CEF, sprayed with bacterial organisms and 24 hrs later by INT. White areas indicate inhibition of bacterial growth by compounds of the plant extract after 60 minutes of incubation at 37°C. ON and KA were sprayed with *E. coli* while LA and CH were sprayed with *S. aureus*.

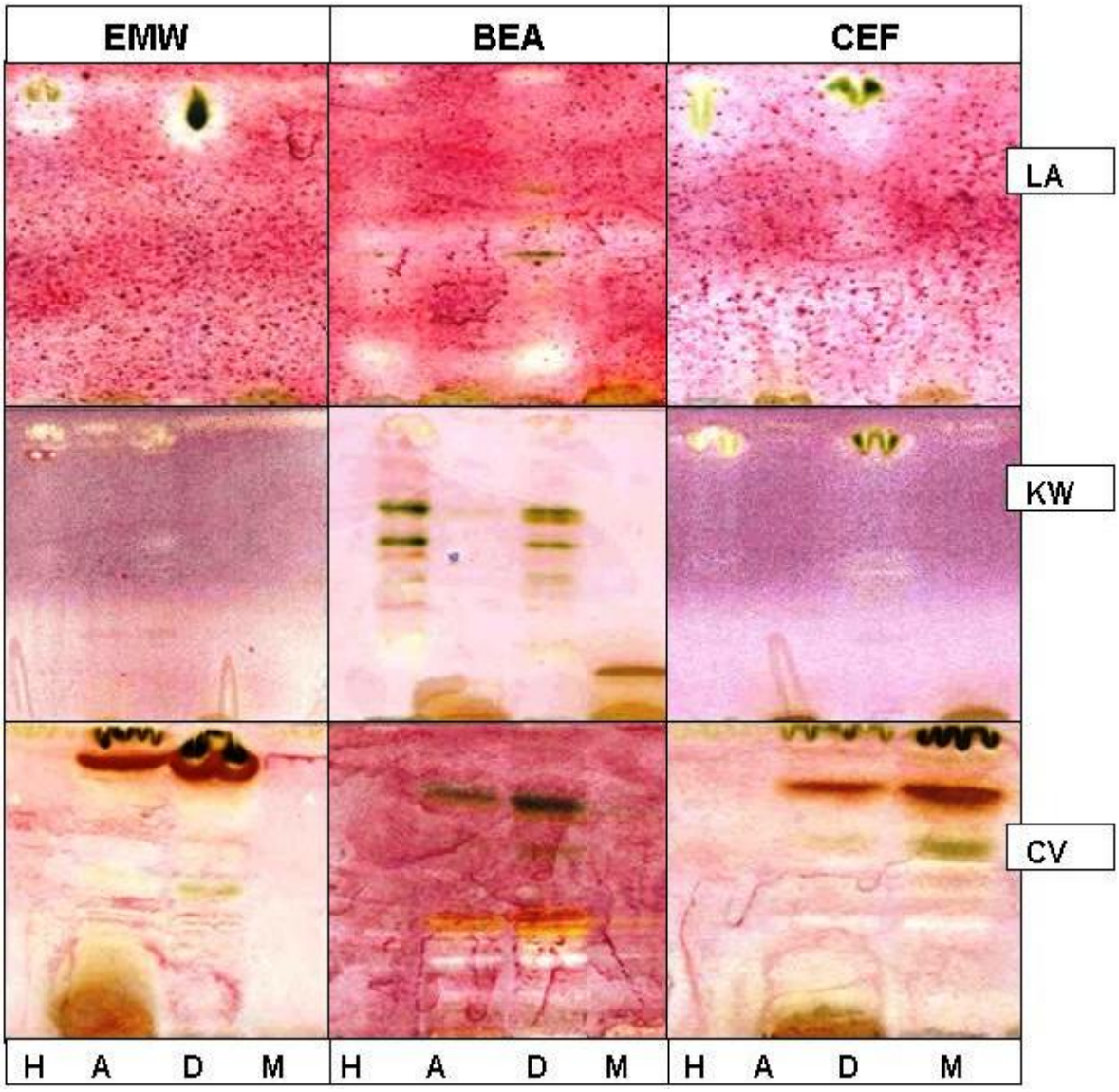


Figure 4.3. Hexane (H), acetone (A), dichloromethane (D), and methanol (M) extracts of *Loxostylis alata* (LA), *Kirkia wilmsii* (KW) and *Combretum vendae* (CV) separated on TLC plates using EMW, BEA and CEF, sprayed with fungal organisms and 24 hours later by INT. White areas indicate inhibition of fungal growth by compounds of the plant extract after 24 hrs of incubation at 37 °C. LA, KW and CV were sprayed with *A. fumigatus*, *C. albicans* and *S.schenkii*, respectively.

An important factor in quantifying the movement of a compound on a stationary phase e.g. silica with a certain solvent system is the R_f (relative front) value and is the ratio of the distance moved by the compound from its origin to the movement of the solvent from the origin. R_f values are less than 1 as they are ratios (Gibbons and Gray, 1998). As expected, compounds that had relatively high R_f values in polar solvents e.g. EMW, depicted low R_f values in non-polar solvents like BEA. As a consequence of development, compounds of a mixture will separate according to their relative polarities on TLC. Generally, based on the R_f values, the same compounds may be active against both fungi and bacteria. This suggests that the antimicrobial activity of compounds is not selective. The R_f values of the extracts eluted with

different solvents against *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *A. fumigatus*, *S. schenckii*, *M. canis*, *C. neoformans* and *C. albicans* are presented on Tables 4.1, 4.2, 4.3, 4.4, 4.5, 4.6, 4.7, 4.8 and 4.9, respectively. In some cases, no inhibition of microbial growth was observed. The absence of activity could be due to evaporation of the active compounds, photo-oxidation or due to very little amount of the active compound (Masoko and Eloff, 2005).

Table 4.1. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *S. aureus*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Hexane	0.92, 0.83, 0.47, 0.26	4	21
		Acetone	0.13	1	
		DCM	0.92, 0.83, 0.47, 0.26, 0.13	5	
	CEF	Hexane	0.53, 0.51	2	
		Acetone	0.1	1	
		DCM	0.53, 0.51, 0.2	3	
	EMW	Hexane	0.31, 0.2,	2	
		Acetone	0.83	1	
		DCM	0.31,0.2	2	
P. longifolia	BEA	Hexane	0.172	1	8
		Acetone	0.27	1	
		DCM	0.26	1	
	CEF	Hexane	0.31	1	
		Acetone	0.34	1	
	EMW	Hexane	0.72, 0.81	2	
		Acetone	0.63	1	
K. wilmsii	BEA	Acetone	0.23	1	5
		Methanol	0.62	1	
	CEF	Acetone	0.23	1	
		Acetone	0.54, 0.08	2	
		Methanol	0.08	1	
C. harveyi	BEA	Hexane	0.95, 0.32	2	11
		DCM	0.95, 0.32, 0.21	3	
	CEF	Hexane	0.41	1	
		DCM	0.41	1	
		Methanol	0.32	1	
	EMW	Hexane	0.31	1	
		DCM	0.31	1	
		Methanol	0.72	1	
O. natalitia	BEA	DCM	0.1	1	5
	CEF	Hexane	0.65	1	
		Acetone	0.76	1	
		DCM	0.97	1	
	EMW	Acetone	0.97	1	
K. anthotheca	BEA	Hexane	0.94	1	4
		DCM	0.94	1	
	CEF	Hexane	0.2	1	
		DCM	0.2	1	
C. vendae	BEA	Hexane	0.21	1	8
		Acetone	0.21	1	
		DCM	0.12	1	
		Methanol	0.32	1	
	CEF	Hexane	0.74	1	
		Acetone	0.41	1	
		DCM	0.65	1	
		Methanol	0.41	1	

Table 4.2. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *E. faecalis*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Hexane	0.78, 0.82	2	6
		DCM	0.82	1	
		Acetone	0.16	1	
		DCM	0.67	1	
	EMW	Acetone	0.45	1	
	BEA	Acetone	0.56	1	
P. longifolia	CEF	Acetone	0.98	1	2
K. wilmsii	BEA	Hexane	0.34	1	3
		Acetone	0.56	1	
		Methanol	0.67	1	
O. natalitia	CEF	Hexane	0.91	1	3
		Acetone	0.91, 0.57	2	
K. anthotheca	BEA	Acetone	0.24	1	3
	CEF	Hexane	0.94	1	
		Acetone	0.76	1	
C. vendae	BEA	Hexane	0.56, 0.67	2	3
	CEF	Hexane	0.97	1	

Table 4.3. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *E. coli*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Hexane	0.62	1	5
		DCM	0.62, 0.21	2	
	CEF	DCM	0.67	1	
	EMW	DCM	0.98	1	
P. longifolia	BEA	Acetone	0.76	1	6
		DCM	0.95	1	
	CEF	Acetone	0.33	1	
		DCM	0.33	1	
		Hexane	0.23	1	
	EMW	DCM	0.23	1	
K. wilmsii	CEF	Methanol	0.87	1	2
		Acetone	0.24	1	
O. natalitia	BEA	DCM	0.95	1	3
		Hexane	0.95	1	
	EMW	DCM	0.27	1	
K. anthotheca	CEF	Hexane	0.93	1	5
		Acetone	0.93, 0.67	2	
		DCM	0.93	1	
	EMW	Methanol	0.34	1	
C. vendae	BEA	Hexane	0.40, 0.2	2	8
		Acetone	0.13	1	
		DCM	0.26	1	
	CEF	Hexane	0.87	1	
		Acetone	0.98	1	
		DCM	0.98	1	
		Methanol	0.56	1	

Table 4.4. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *P. aeruginosa*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	EMW	Acetone	0.89	1	1
		Hexane	0.90	1	
P. longifolia	CEF	Acetone	0.90	1	3
		DCM	0.90	1	
		Hexane	0.1	1	
	BEA	DCM	0.1	1	
		Hexane	0.67	1	
K. wilmsii	CEF	Acetone	0.67	1	8
		DCM	0.67	1	
		Hexane	0.85	1	
	EMW	Acetone	0.85	1	
		DCM	0.85	1	
O. natalitia	CEF	Methanol	0.56	1	1
	BEA	DCM	0.27	1	
K. anthotheca		Hexane	0.93	1	3
	CEF	Acetone	0.42	1	
		Hexane	0.67	1	
C. vendae	CEF	Acetone	0.54	1	3
		DCM	0.45	1	

Table 4.5. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *A. fumigatus*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Hexane	0.13, 0.54, 0.92	3	10
		DCM	0.13, 0.54, 0.92	3	
	CEF	Hexane	0.63	1	
		DCM	0.63	1	
	EMW	Hexane	0.81	1	
		DCM	0.81	1	
P. longifolia	BEA	DCM	0.2	1	3
	CEF	DCM	0.83	1	
	EMW	DCM	0.90	1	
C. harveyi	BEA	Hexane	0.33	1	3
		DCM	0.33, 0.46	2	
K. anthotheca	BEA	Hexane	0.26	1	8
		Acetone	0.21	1	
		DCM	0.21	1	
	CEF	Hexane	0.56	1	
		DCM	0.53	1	
	EMW	Hexane	0.93	1	
		Acetone	0.93	1	
		DCM	0.93	1	
C. vendae	CEF	Hexane	0.94	1	2
	EMW	Acetone	0.81	1	

Table 4.6. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *S. schenckii*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	DCM	0.89, 0.43	2	7
		Methanol	0.43	1	
	CEF	Hexane	0.58	1	
		Acetone	0.58	1	
		DCM	0.58	1	
	EMW	Hexane	0.92	1	
P. longifolia	BEA	DCM	0.62, 0.46	2	3
		Methanol	0.46	1	
		Acetone	0.31	1	
K. wilmsii	EMW	Methanol	0.31	1	2
		Hexane	0.96, 0.55, 0.44	3	
C. harveyi	BEA	Methanol	0.96	1	8
		Hexane	0.84	1	
	CEF	Acetone	0.68	1	
		Hexane	0.94	1	
	EMW	Methanol	0.12	1	
O. natalitia	EMW	Acetone	0.44	1	1
		Hexane	0.96	1	
K. anthotheca	CEF	Acetone	0.96	1	3
		DCM	0.96	1	
C. vendae	BEA	DCM	0.89	1	6
		Methanol	0.89	1	
		Acetone	0.58, 0.32	2	
	CEF	DCM	0.58, 0.32	2	

Table 4.7. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *M. canis*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Acetone	0.31, 0.79	2	6
		DCM	0.31	1	
		Hexane	0.27, 0.42	2	
	EMW	Acetone	0.94	1	
		Hexane	0.87	1	
P. longifolia	CEF	Acetone	0.87, 0.41	2	5
		DCM	0.41	1	
	EMW	Acetone	0.91	1	
		DCM	0.77	1	
K. wilmsii	BEA				2
	CEF	Acetone	0.57	1	
O. natalitia	BEA	Acetone	0.73	1	1
K. anthotheca	BEA	Hexane	0.91	1	3
		Acetone	0.63	1	
	EMW	Hexane	0.29	1	
C. vendae	BEA	Hexane	0.33	1	6
		Acetone	0.29, 0.33	2	
		DCM	0.33	1	
	CEF	Acetone	0.67	1	
		DCM	0.67	1	

Table 4.8. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *C. neoformans*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Hexane	0.23, 0.38	2	11
		Acetone	0.92	1	
		DCM	0.21	1	
	CEF	Hexane	0.90	1	
		Acetone	0.90	1	
		DCM	0.90	1	
		Methanol	0.90	1	
	EMW	Hexane	0.77	1	
		Acetone	0.26	1	
		DCM	0.85	1	
P. longifolia	CEF	Hexane	0.26, 0.41	2	8
		Acetone	0.26, 0.41, 0.52	3	
		DCM	0.26	1	
	EMW	Hexane	0.98	1	
		Acetone	0.98	1	
K. wilmsii	BEA	Hexane	0.84	1	5
		Acetone	0.14	1	
		DCM	0.96	1	
	CEF	Acetone	0.72	1	
	EMW	DCM	0.16	1	
C. harveyi	BEA	Hexane	0.93	1	5
		Acetone	0.18	1	
	CEF	Acetone	0.90	1	
		DCM	0.90	1	
		Methanol	0.90	1	
O. natalitia	BEA	Hexane	0.97	1	2
	EMW	Hexane	0.13	1	
K. anthotheca	BEA	Hexane	0.92	1	4
	CEF	Hexane	0.94	1	
		Acetone	0.94	1	
	EMW	Hexane	0.21	1	
C. vendae	BEA	Hexane	0.96	1	5
		Acetone	0.96	1	
		DCM	0.13	1	
	CEF	DCM	0.67	1	
	EMW	Acetone	0.12	1	

Table 4.9. Inhibition of growth on bioautographic TLC plates by 24 extracts of 7 South African tree leaves against *C. albicans*.

Plant	Solvent system	Extracts	R_f values	Active bands	Total
L. alata	BEA	Hexane	0.87, 0.21	2	6
		DCM	0.21	1	
	CEF	Hexane	0.93	1	
		Acetone	0.93	1	
	EMW	Hexane	0.26	1	
P. longifolia	BEA	Methanol	0.21	1	3
	CEF	Acetone	0.76	1	
	EMW	Hexane	0.42	1	
K. wilmsii	BEA	Hexane	0.93	1	2
	CEF	Hexane	0.74	1	
C. harveyi	BEA	Hexane	0.54	1	6
		Acetone	0.54	1	
		DCM	0.30	1	
	CEF	Hexane	0.93	1	
		Acetone	0.93	1	
		DCM	0.93	1	
O. natalitia	BEA	Methanol	0.34	1	3
	CEF	Acetone	0.94	1	
	EMW	Acetone	0.56	1	
K. anthotheca	BEA	Hexane	0.59	1	5
		Acetone	0.59	1	
		DCM	0.59	1	
	CEF	Hexane	0.93	1	
		Acetone	0.93	1	
C. vendae	BEA	Hexane	0.34	1	8
		Acetone	0.47	1	
		DCM	0.59	1	
	CEF	Hexane	0.71	1	
		Acetone	0.63	1	
		DCM	0.29	1	
		Methanol	0.88, 0.29	2	

Most of the antimicrobial agents detected in this study were present in extracts of non-polar solvents. These findings agreed with previously published results (Masoko and Eloff, 2005, 2006) that the substances responsible for the antimicrobial activity were strongly non-polar in nature. However, the acetone and methanol fractions of *Punica granatum* and *Delonix regia* in contrast to the benzene fraction had good activity against methicillin resistant *S. aureus* (Aqil et al., 2005). Bioautography therefore, allows the determination of the polarity of the active compounds.

Loxostylis alata appeared to be the most active plant; having the highest number of inhibition zones of 35 and 40 against bacteria and fungi, respectively, while *O. natalitia* was the less active plant with lowest number of inhibition zones of 18 and 11 against bacteria and fungi, respectively. *C. vendae*, *P. longifolia*, *K. anthotheca*, *C. harveyi, K. wilmsii* and *O. natalitia* had inhibition zones of 22, 19, 15, 11, 18 and 12, respectively, against bacteria, and 27, 22, 38, 22, 11 and 7, respectively, against fungi. In another study, the methanol extract of the stem bark of *Khaya anthotheca* was reported to be very active against the fungus *Candida krusei* Hamza et al., 2006. Some pentacyclic triterpenes isolated from *Combretum imberbe* and *Terminalia stuhlmannii* (Combretaceae) have antimicrobial properties against bacteria and fungi (Katerere et al., 2003). Perhaps similar chemical constituents might be responsible for the antimicrobial action of *Combretum vendae* a member of the same family. In a similar manner, some triterpenoid and phenolic compounds isolated from *Commiphora opobalsamum* (Burseraceae) possess antimicrobial activity (Abbas et al., 2007). Of the bacteria tested, *S. aureus* also appeared to be the most susceptible organism. The growth of *S. aureus* was inhibited most by the compounds of the tested plants. Similarly, *C. neoformans* depicted the highest susceptibility among fungal organisms. Some of the compounds are active against both bacteria and fungi, while others are selective in their activity. It is possible that compounds that have activity against all the tested organisms possess a broad antimicrobial action or they may likely be general metabolic toxins. That is actually a subject of another investigation and we are following it up.

The absence of bioactivity in some of the plant extracts does not preclude the fact that they are not active, as synergistic or additive interactions of plant extracts or phytocompounds is the basis of activity of several herbal formulations (Ahmad and Aqil, 2007). Moreover, the non-activity could also be explained by a weak selectivity of the extract components against the microorganisms chosen for this study, or the very low concentration of the active compounds in the crude extract under the tested conditions (Schmourlo et al., 2004).

4.4. Conclusion

The South African flora offers great potential in the search for natural compounds with antimicrobial activity. The bioassay-guided fractionation to isolate and characterise active compounds against bacteria and fungi from those plant extracts that had the highest activity is currently being undertaken in our laboratory. In addition, we are also carrying out toxicological and other pharmacological evaluations on the active plant extracts.

Postscript

All the plant species possess varying degree of antibacterial and antifungal activities and we therefore went further to examine their antioxidant and antiplatelet activities with a view of finding pharmacologically activity in the plant species under investigation.

Chapter 5
Evaluation of selected South African plant species for antioxidant, antiplatelet and cytotoxic activity

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Preface

Aspergillosis and most fungal infections are associated with immune depression and causing inflammation. Antioxidants essentially reverse several conditions associated with immune deficiencies and inflammatory diseases. Plant extracts that have antifungal activity in this study will be evaluated for antioxidant activity as an indicator of their ability to boost the immune system. Since acetone extract both polar and intermediate polar compounds where antioxidants are found, it was used as an extractant. We further evaluated the safety of the extracts as a step towards the use of active plant extracts in clinical practice. The text in this chapter was submitted and accepted as manuscript for publication to Pharmaceutical Biology to indicate the results obtained.

Abstract

The antioxidant, antiplatelet and cytotoxic effects of seven South African plant extracts, namely *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* (Anacardiaceae) were evaluated using established *in vitro* assays. All the extracts had comparably low toxicity except for the extract of *C. harveyi* that had a high haemagluttination assay titre value, which indicates toxicity. The extracts of *P. longifolia, K. wilmsii, O. natalitia, L. alata, C. harveyi* and *C. vendae* had antioxidant properties in the qualitative assay using DPPH. In the quantification of antioxidant activity using ABTS, only the extracts of *P. longifolia, L. alata, and C. vendae* had antioxidant activity with TEAC values of 1.39, 1.94 and 2.08 respectively. In the quantitative DPPH assay, *L. alata* (EC_{50}, 3.6 ± 0.2 µg/ml) and *K. wilmsii* (EC_{50}, 3.6 ± 0.4 µg/ml) did not differ significantly ($p \leq 0.05$) from the control. *K. anthotheca* had a higher EC_{50} (176± 27 µg/ml) value, 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 antiplatelet activity that were significantly ($p \leq 0.05$) better than the control (aspirin) with EC_{50} of 0.06 ± 0.01 µg/ml, 0.19 ± 0.00 µg/ml, respectively. Lower EC_{50} values in the antioxidant and antiplatelet studies indicate superior activity of the plant extract against oxidation and platelet aggregation.

Keywords: South African plants, antioxidant assay, antiplatelet activity, cytotoxicity effect.

5.1. Introduction

Bioactive compounds commonly found in plants have possible health benefits partly due to their antioxidative properties (Cao and Cao, 1999). It is well known that reactive oxygen species (ROS) are involved in a diversity of important processes in medicine, including among others: inflammation, atherosclerosis, cancer and reperfusion injury (Kehrer, 1993). One of the major fundamental tissue-destructive mechanisms is oxidative stress through an excessive release of reactive oxygen metabolites (ROM) (McCord, 2000). Although the generation by phagocytes (and to a lesser extent by eosinophils, lymphocytes and fibroblasts) is essential for an effective host defence against bacterial infection, continuous overproduction during inflammatory processes may also cause extensive tissue destruction (Weiss, 1989). One way by which a substance can interfere with these processes is by acting as an antioxidant or free radical scavenger. Antioxidants abate inflammation and protect tissues from oxidative damage cause by free radicals. Inflammation has been described as the release of chemicals from tissues and migrating cells. Agents such as prostaglandins, leukotrienes, histamine, bradykinin, platelet-activating factor (PAF), 1 and interleukin 1 (IL 1) play some part in the inflammatory processes. In addition, prostaglandins and other inflammatory mediators produce oxidant products that play an important role in causing tissue oxidation (Pekoe et al., 1982). Many anti-inflammatory drugs are able to react with oxidants *in vitro*, so considerable interest has been expressed in the possibility that oxidant scavenging contributes to the action of these drugs *in vivo* (Halliwell et al., 1988). Despite the presence of wide arrays of anti-inflammatory drugs (steroidal and nonsteroidal), they still present a wide range of side effects for which the major reason is nonselective inhibition of cyclooxygenase I (COX I) and cyclooxygenase II (COX II) (Vane and Botling, 1995). Prostaglandins, thromboxanes and platelet-activating factor (PAF) contribute to platelet aggregation and, moreover, platelets have an important role in acute inflammation by releasing arachidonic acid (AA) metabolites and PAF (Holmsen et al., 1977; Vincent et al., 1977). Furthermore, thromboxane A_2 (TXA_2), an AA product formed via the COX pathway in platelets has been reported to be a potent vasoconstrictor and pro-aggregatory agent (Page et al., 1984). COX inhibitors, such as aspirin, are known to inhibit platelet aggregation (Saeedu et al., 1997).

A large number of naturally occurring compounds such as flavonoids, catechins, lignans, and phenolic acids contained in plants and herb remedies have antioxidant properties (Dall'Acqua et al., 2008). These reasons have recently prompted research into natural antioxidants. The aim of this study was to test the antioxidant, anti-platelet and cytotoxicity effects of acetone extracts of leaves of seven South African tree species which were selected based on their antifungal activity in an effort to develop a product that could protect poultry against aspergillosis. .

5.2. Materials and methods

5.2.1. Plant collection

The leaves of *Commiphora harveyi* (Engl.) Engl. (Burseraceae), *Combretum vendae* A.E. van Wyk (Combretaceae), *Khaya anthotheca* (Welm.) C.DC (Meliaceae), *Loxostylis alata* A. Spreng. ex Rchb. (Anacardiaceae) and *Protorhus longifolia* (Bernh. Ex C. Krauss) Engl. (Anacardiaceae) were collected at the Manie van der Schijff Botanical Garden of the University of Pretoria, South Africa. *Kirkia wilmsii* Engl. (Kirkiaceae) and *Ochna natalitia* (Meisn.) Walp. (Ochnaceae) were collected at the Lowveld National Botanical Garden in Nelspruit, South Africa. All plants were collected in November 2006 and were identified and authenticated by Lorraine Middleton and Magda Nel of the University of Pretoria. Voucher specimens of the plants were deposited at the Schweikert Herbarium of the Department of Plant Sciences, University of Pretoria, South Africa.

5.2.2. Plant storage

Immediately after collection and transportation to our laboratory, leaves were separated from stems and dried at room temperature with good ventilation. The dried plants were milled to a fine powder in a Macsalab mill (Model 200 LAB), Eriez®, Bramley, and stored at room temperature in closed containers in the dark until used.

5.2.3. Plant extraction

Plant samples from each species were individually extracted by weighing four aliquots of 1 g of finely ground plant material and extracting with 10 ml of acetone, hexane, dichloromethane (DCM) or methanol (technical grade-Merck) in polyester centrifuge tubes. Tubes were vigorously shaken for 1 h using a Labotec model 20.2 shaking machine at a moderate speed. Extracting at lower speed for a longer period allows the solvent to penetrate more into the plant tissues; allowing the extraction of more of the compounds contained in the plant species (Silva et al., 1998). After centrifuging at 3500 x *g* for 10 min, the supernatant was decanted into pre-weighed labelled containers. The whole process was repeated three times to exhaustively extract the plant material and the extracts were combined. The solvent was removed under a stream of air in a fume cupboard at room temperature to quantify the extraction.

5.2.4. Evaluation of antioxidant activity

Qualitative antioxidant screening to determine the number of antioxidant compounds was employed using 2,2-diphenyl-1-picryl hydrazyl (DPPH) (Takao et al., 1994). TLC plates loaded with 100 µg of each extract were developed in chloroform/ethyl acetate/formic acid (5:4:2) [CEF] solvent system and sprayed with 0.2% DPPH in methanol. Compounds with antioxidant activity were visualized as yellow bands against a purple background (Bors et al., 1992). Quantification of antioxidant activity was by spectrophotometric means using two radicals, ABTS and DPPH.

In the ABTS method, the Trolox (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) equivalent antioxidant capacity (TEAC) assay (Re et al., 1999) was determined. This was based on the scavenging of the ABTS radical into a colourless product by antioxidant substances. The blue/green chromophore $ABTS^{+}$ was produced through the reaction between ABTS and potassium persulfate. The absorbance was read at 734 nm using a Versamax microplate reader (Molecular Devices). Trolox is a Vitamin-E analogue and was used as a standard in this assay. Percentage change in absorbency was calculated by the formula below:

Initial absorbency of $ABTS^{+}$ - New Initial absorbency of $ABTS^{+}$/Initial absorbency of $ABTS^{+}$ × 100

The curves were plotted with the dependent variable being the percentage change in absorbency and the independent variable being the different concentrations at which test substances were analysed. Mathematical comparison of antioxidant activity of different plant extracts was done by dividing the slope obtained for extract to that of Trolox to get the Trolox equivalent antioxidant capacity (TEAC). An extract with a TEAC value of 1 indicates an equivalent antioxidant value of Trolox. Decrease or increase of antioxidant activity is depicted by a lower or higher value of TEAC, respectively.

The DPPH free radical assay was conducted as described by Mensor et al (2001). Briefly, 10 µL of 0.3 mM DPPH in ethanol was added to 25 µL of each concentration of extract tested and allowed to react at room temperature in the dark for 30 minutes. Appropriate blank and negative control solutions were prepared for each test. L-ascorbic acid (Vitamin C) was used as positive control. The decrease in absorbance was measured at 518 nm. Values obtained were converted to percentage antioxidant activity (AA %) using the formula:

$$AA\% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100]/Abs_{control}\}$$

Where Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and $Abs_{control}$ is the absorbance of the control. The EC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was calculated from the linear regression of plots of concentration of the test extracts (µg/ml) against the mean percentage of the antioxidant activity obtained from three replicate assays using Microsoft Office Excel. EC_{50} values obtained from the regression lines had coefficient of determination $r^2 \geq 95\%$. A lower EC_{50} value indicates high antioxidant activity.

5.2.5. *In vitro* platelet aggregation assay

The modified method of Fratantoni and Poindexter (1990) was used to determine the inhibitory effect of the plant extracts on platelet aggregation. Briefly, fresh equine (*Equus caballus*) blood was collected from healthy representative breeds in the Equine Research Centre, Faculty of Veterinary Sciences, University of Pretoria by Mrs. Stellest de Villiers into sterile 5 ml glass tubes containing 3.8% trisodium citrate solution as anticoagulant at a ratio of 1:9 volume of anticoagulant to blood.

The platelet rich plasma (PRP) and platelet poor plasma (PPP) were separated by centrifugation at 160 g for 10 min and 1600 g for 15-30 min at 20-25ºC, respectively, using a Beckman(R) GS-15R centrifuge. PRP was later centrifuged at 1000 g for 15 min at 20-25ºC to sediment the platelets. The platelet count of the PRP was adjusted to 300,000/pl by adding PPP. Both PRP and PPP were then stored at room temperature. The cell suspension was adjusted to approximately 3.0×10^8 platelets per ml using phosphate buffered saline (PBS). Different concentrations of the extracts and aspirin as the reference drug were added and incubated at 37ºC for 3 min. After incubation, platelet aggregation was induced by the addition of 50 µl of adrenaline. The degree of platelet aggregation was determined spectophotometrically at 600 nm after 30 min. Percentage platelet aggregation inhibition was calculated using the following equation.

$$X\ (\%) = A\text{-}B/A \times 100$$

Where A= maximal aggregation of the control and B= maximal aggregation of sample-treated PRP.

The EC_{50} values were calculated by linear regression of plots using Microsoft Office Excel. The abscissa represents the concentration of tested plant extracts and the ordinate the average percent of antioxidant activity from three separate tests.

5.2.6. Cytotoxicity assay

5.2.6.1. MTT assay

The plant extracts were tested for cytotoxicity against the Vero monkey kidney cell line. The cells were maintained in minimal essential medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). To prepare the cells for the assay, cell suspensions were prepared from confluent monolayer cultures and plated at a density of 0.5×10^3 cells into each well of a 96-well microtitre plate. After overnight incubation at 37ºC in a 5% CO_2 incubator, the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. Stock solutions of the plant extracts were prepared by reconstitution to a concentration of 100 mg/ml in DMSO. Serial 10-fold dilutions of each extract were prepared in growth medium (1-1000 µg /ml). The method described by Mosmann (1983) was used to determine the viability of cell growth after 120 h incubation with plant extracts. 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) was used as an indicator for cell growth. The absorbance was measured at 570 nm. Berberine chloride (Sigma) was used as a positive control. Tests were carried out in quadruplicate and each experiment was done in triplicate.

5.2.6.2. Haemagglutination assay

Fresh equine (*Equus caballus*) blood was collected from a representative breed in the Equine Research Centre, Faculty of Veterinary Sciences, University of Pretoria into sterile 5 ml glass tubes containing 3.8% trisodium citrate solution. Erythrocytes were fixed with formalin and prepared according to method of Sadique et al. (1989) as modified by Iwalewa et al. (2005). Briefly, 20 ml of the mixed blood was centrifuged at 4000 rpm for 10 min, using a Beckman(R) GS-15R centrifuge. The packed red blood cells were washed with 10 mM phosphate buffer saline (PBS) pH 7.2 until a clear supernatant was obtained. The washed packed RBCs were suspended in 5% (v/v) formaldehyde-phosphate buffer saline (1:12.3 v/v) solution. The mixture was left at room temperature for 24 h. The final fixed RBC were washed and centrifuged with PBS 3 times, and preserved with 1 ml (50 mg/ml) gentamicin containing 0.1% methyl paraben to prevent microbial growth and stored at 4ºC.

PBS (100 µL) was added to wells of 96-well microtitre plates. The first row was used as a control without extracts. The extracts (100 µl) were added into the first well of the second row, and a 2-fold serial dilution was made until the last well (well 12). Then 50 µl of equine RBC was added to all the wells. They were incubated at room temperature for 1 h. The presence of buttons in the centre of the well indicated no agglutination and the haemagglutination titre values of the extracts were read as the reciprocal of the last dilution having agglutination

5.2.7. Statistical analysis

Antioxidant and antiplatelet experiments were done in triplicate. The results are presented as mean ± standard error of mean (SEM). A one-way analysis of variance (ANOVA) was used for comparison of means using Microsoft Office Excel 2003. A difference was considered statistically significant when $p \leq 0.05$.

5.3. Results

The extraction yields of acetone extracts of plants used in this study are presented in Table 1. *C. vendae* gave the highest yield of 15.7%, while *K. wilmsii* possess the lowest yield (6.9 %). The TLC DPPH method of qualitative antioxidant detection showed that the acetone extracts of *P. longifolia, K. wilmsii, O. natalitia, L. alata, C. harveyi and C. vendae* displayed antioxidant compounds due to their DPPH free radical scavenging activity. Antioxidant compounds were seen as yellow bands against a purple background. The *L. alata* extract had the most antioxidant compounds. The acetone extract of *K. anthotheca*, however, did not show any antioxidant compound on the TLC plate (Figure 5.1).

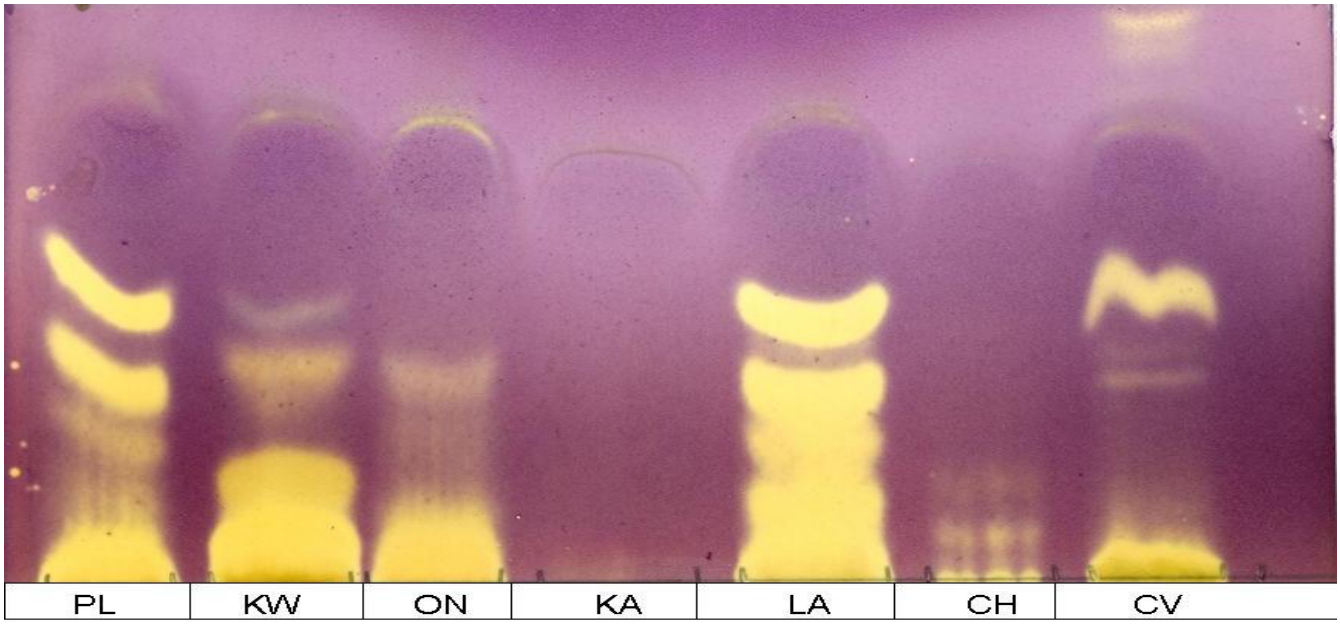


Figure 5.1. Chromatogram of 100 µg acetone extracts of the leaves of *P. longifolia* (PL), *K. wilmsii* (KW), *O. natalitia* (ON), *K. anthotheca* (KA), *L. alata* (LA), *C. harveyi* (CH) *and C. vendae* (CV), separated with CEF mobile phase and sprayed with 0.2% DPPH. Antioxidant compounds are indicated by yellow areas.

In the TEAC antioxidant assay, extracts of *P. longifolia, L. alata,* and *C. vendae* had superior free radical scavenging activity compared to the other extracts and the standard antioxidant (Trolox) used in this study

with respective TEAC value of 1.39, 1.94, and 2.08 (Table 5.1). The results corroborate that of the qualitative assay where the extracts showed the presence of antioxidant compounds. Extracts of *O. natalitia*, *K. wilmsii*, *C. harveyi* and *K. anthotheca* had lower TEAC values of 0.79, 0.67, 0.15 and 0.10, respectively.

In the quantitative DPPH assay (Table 1), L-ascorbic acid had the lowest EC_{50} (1.59 ± 0.80 µg/ml) value than all the extracts (I.e. the highest antioxidant activity), however, L-ascorbic acid no significant ($p \leq 0.05$) difference in its free radical scavenging effect when compared with *L. alata* (EC_{50}, 3.58 ± 0.23 µg/ml) and *K. wilmsii* (EC_{50}, 3.57 ± 0.41 µg/ml). *K. anthotheca* had a higher EC_{50} (176.40 ± 26.56 µg/ml) value, and differed significantly ($p \leq 0.05$) from all the other extracts and L-ascorbic acid. Extracts of *P. longifolia*, *C. vendae*, *C. harveyi*, and *O. natalitia* had EC_{50} of 6.57 ± 0.23, 4.41 ± 0.14, 10.47 ± 1.96 and 7.50 ± 0.13, respectively. The lower the EC_{50} of a substance, the more effective is its free radical scavenging effect.

Table 5.1. Antioxidant and antiplatelet activity of acetone extracts of seven South African plants.

Plant	Voucher specimen number	Extract yield (%)	Antioxidant values		Antiplatelet activity (EC_{50} ± SEM, µg/ml
			TEAC	DPPH (EC_{50} ± SEM, µg/ml)	
C. vendae	PRU96507	15.7	2.08	4.41 ± 0.14 [b]	0.06 ± 0.01 [a]
C. harveyi	PRU96506	14.2	0.15	10.47 ± 1.96 [b]	0.19 ± 0.00 [a]
K. anthotheca	PRU96509	8.9	0.10	176.40 ± 26.56[c]	0.97 ± 0.03
K. wilmsii	PRU96503	6.9	0.67	3.57 ± 0.41[a]	0.22 ± 0.02
L. alata	PRU96508	13.7	1.94	3.58 ± 0.23 [a]	0.35 ± 0.03
O. natalitia	PRU96504	12.6	0.79	7.50 ± 0.13[b]	0.39 ± 0.03
P. longifolia	PRU96505	15.3	1.39	6.57 ± 0.23[b]	0.23 ± 0.02
L-ascorbic acid	-	N/A	N/A	1.59 ± 0.80 [a]	N/A
Aspirin	-	N/A	N/A	N/A	0.04 ± 0.00 [a]

N/A = Not available

Means within the same column and with different superscript letters (a, b or c) differ significantly ($p \leq 0.05$)

The antiplatelet actions of *C. vendae* and *C. harveyi* did not differ significantly ($p \leq 0.05$) from that of aspirin (standard agent) (Table 5.1). The low EC_{50} by *C. vendae* (0.06 ± 0.01 µg/ml) and *C. harveyi* (0.19 ± 0.00 µg/ml) depicts good antiplatelet activity when compared with that of aspirin (0.04 ± 0.00 µg/ml).

The cytotoxic activities of the extracts in the Vero Monkey kidney cell line assay are provided in Figure 5.2. In the assay the extracts of *O. natalitia, K. anthotheca, L. alata, C. harveyi and C. vendae* at the highest concentration used were relatively toxic; they caused complete death of the Vero cells. However, at lower concentrations all the extracts had relatively lower toxicity when compared with the reference agent berberine (cytotoxic agent). Over 90% of Vero cells treated with berberine at the concentration of 10 µg/ml were not viable. The effects of the extracts on fixed equine erythrocytes are presented in Table 5.2. All the plant extracts exhibited very low HA titre values with a wide range of concentrations at which agglutination occurred on. However, the acetone extract of *C. harveyi* had a very high HA titre value and a very low concentration at which agglutination occurred.

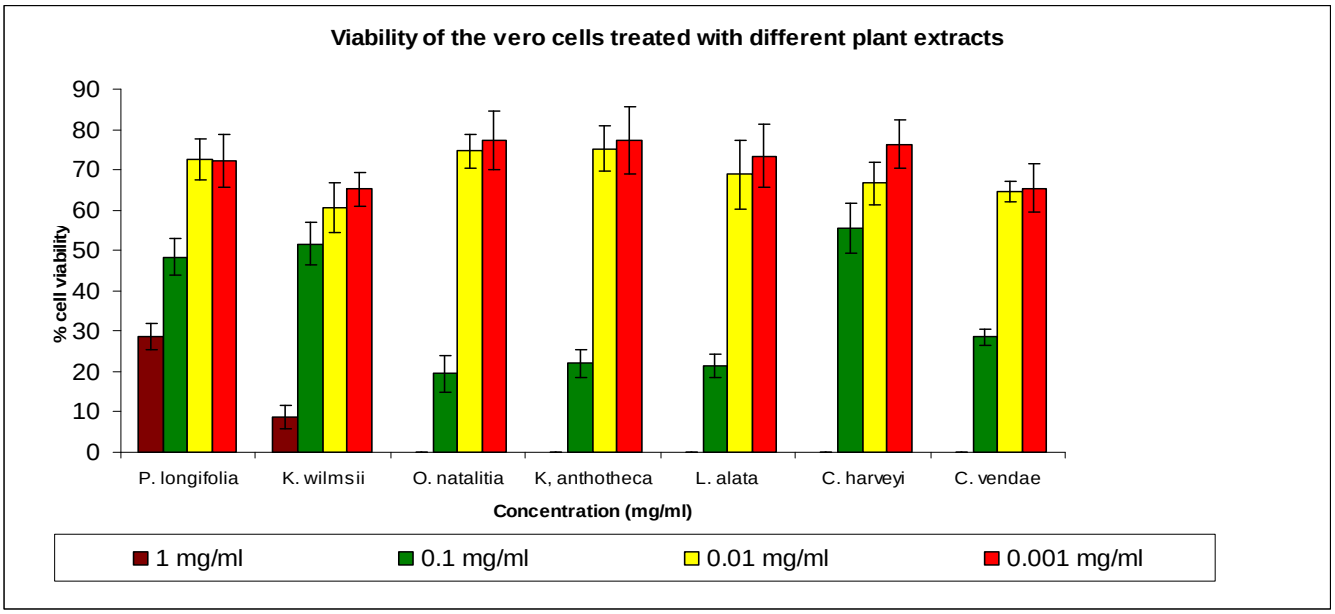


Figure 5.2. Viability of Vero monkey kidney cell line treated with different concentrations of acetone extracts of seven South African plants. Values are mean ± S.E.M.

Table 5.2. Cytotoxic effect of acetone extracts of seven South African plants on formaldehyde-fixed equine erythrocytes.

Plant extract	Concentration value where agglutination occurred (mg/ml)	Haemagglutination assay titre value
C. vendae	1.25	0.80
C. harveyi	0.31	3.23
K. anthotheca	1.25	0.80
K. wilmsii	1.25	0.80
L. alata	1.25	0.80
O. natalitia	2.5	0.40
P. longifolia	1.25	0.80

5.4. Discussion

In this study, we have applied established *in vitro* assays in order to evaluate the antioxidant, antiplatelet and cytotoxic action of extracts of leaves of seven South African tree species. Extracts of these species had promising antifungal and bacterial activities in previous studies. This study is the first to report the antioxidant, antiplatelet and cytotoxicity activities of these plants. These natural products were able to scavenge free radicals in a concentration-dependent fashion in two separate antioxidant assays. DPPH is a free radical, stable at room temperature, which produces a violet solution in ethanol. It is reduced in the presence of an antioxidant molecule, giving rise to uncoloured solutions. The use of DPPH provides an easy and rapid way to evaluate antioxidants (Mensor et al., 2001). The extracts that had antioxidant activity separated poorly from the origin when applied and eluted on TLC. This could have been the result of overloading the TLC plates or due to presence of polyphenolic compounds (Naidoo et al., 2006). Polyphenols do not move well from the origin due to their high polarity leading to tight binding with normal phase silica (Davidson, 1964).

The $ABTS^{\cdot+}$ assay described here involves the direct production of the blue/green $ABTS^{\cdot+}$ chromophore through the reaction between ABTS and potassium sulphate, which has absorption maxima at wavelengths 645 nm, 734 nm and 815 nm (Miller et al., 1993; Re et al., 1999).

Both Trolox and L-ascorbic acid have been used as standards in quantification of antioxidant activity (Fukomoto and Mazza, 2000). The EC_{50} of the *P. longifolia, K. wilmsii, O. natalitia, L. alata, C. harveyi and C. vendae* were lower than that of *Ginkgo biloba* extract (EGb 761) whose EC_{50} is 40.72 µg/ml (Mensor et al., 2001; Bridi et al., 2001; Aderogba et al., 2004). The extract of *Ginkgo biloba* (EGb 761) has been widely

employed for its significant benefit as an antioxidant for the prevention of neurodegenerative disorders (Bridi et al., 2001). Similarly, these extracts scavenge free radicals in the DPPH TLC assay.

It has been suggested that more than one method of antioxidant testing should be used to obtain detailed knowledge of antioxidant activity of test substances, and in addition, the extrapolation of *in vitro* data to *in vivo* situations is often difficult (Aruoma, 2003). The TEAC assay is used commonly for screening compounds, food products and extracts for antioxidant activity, and is particularly useful in providing a ranking order of antioxidants (van den Berg et al., 1999) despite the limitations it carries.

The agonist adrenaline (epinephrine) induced platelet aggregation in equine platelets. The extracts of *P. longifolia, K. wilmsii, O. natalitia, K. anthotheca, L. alata, C. harveyi*, and *C. vendae* inhibited platelet aggregation induced by this agonist with different potencies. However, only the extract of *C. vendae* had a statistically significant ($p<0.05$) platelet inhibitory effect.

Platelet activation is usually accompanied by a rise in cytosolic Ca^{2+} levels and this occurs through stimulation of the enzymes that are not fully functional at the low Ca^{2+} concentration present in the resting platelets (Berridge, 1993; Heemskerk and Sage, 1994). In platelets, either the stimulation of phospholipase C (PLC) or the activation of inhibitory G-protein (Gi)-linked receptors elevates the cytosolic Ca^{2+} levels (Puri *et al.,* 1995). This takes place through the release of Ca^{2+} from internal stores or through the entry of Ca^{2+} across the plasma membrane from external medium (Berven *et al.,* 1995; Obberghen-Schilling and Pouyssegur, 1993).

An alternate pathway of increasing the Ca^{2+} influx is through activation of the Gi-linked pathway. Agonist like adrenaline is known to inhibit adenylyl cyclase activity in platelets, leading to a decrease in intracellular cyclic adenosine monophosphate (cAMP) levels (Puri *et al.,* 1995). Multiple studies revealed that agents that decrease cAMP levels stimulate platelet aggregation (Niewland *et al.,* 1994; Siess *et al.,* 1993). This occurs through activation of α_2-adrenergic receptors (Siess and Lapetina, 1989). α_2-Adrenoceptors in platelets are known to be coupled to the guanine-nucleotide-binding protein G., which mediates inhibition of adenylate cyclase. This mainly takes place either through an increase in Ca^{2+} influx (Shah *et al.,* 1996) or as a result of activation of some other proteins (Musgrave and Seifert, 1995). It is possible that the extracts in this study may contain components which block these Ca^{2+}channels or act via an unknown mechanism to cause increase level of intracellular cAMP in platelets, as demonstrated through inhibition of platelet aggregation.

Anti-inflammatory drugs could affect oxidant damage in several ways. First, they might directly scavenge such reactive oxidants as ·OH and HOCl. Most, if not all, anti-inflammatory drugs are capable of reacting quickly with OH. Hence drugs with good anti-inflammatory activity could be of use as free radical scavengers (Halliwell et al., 1988).

Platelet aggregation plays a pathophysiological role in a variety of thromboembolic disorders. Therefore, prevention of platelet aggregation by drugs should provide effective prophylactic and/or therapeutic treatments for such diseases (Hsiao et al., 2003).

Studies have demonstrated that botany and medicine are related. Free radicals and lipid peroxidation have been suggested as potentially important causative agents of several diseases in animals and humans (Nair et al., 2003). It will be worthwhile to explore and find safer and efficacious remedies from natural sources, particularly from the relatively undiscovered and unexplored rich flora of South Africa.

The antioxidant and antiplatelet effects of these extracts, and the additional benefit of their low cytotoxicity, provide strong motivation for the development of these plants as possible drugs for the control of diseases in animals and humans. Most considerably, these properties substantiate the use of plant screening exercises for detecting disease control agents.

In conclusion, these plant species appeared to be potential sources of antioxidant and anti-inflammatory agents. Moreover, they are also good candidates as antifungal agents. From these results *L. alata* was selected for bioassay-guided fractionation procedure to isolate and characterise antifungal compounds in a continuation of this study.

Postscript

Loxostylis alata had promising pharmacological action and was therefore selected for further research work. In the next chapter the possibility of using different extractants to facilitate the isolation of the antimicrobial and anti oxidative compounds will be discussed.

Chapter 6
Preliminary screening of some fractions of *Loxostylis alata* (Anacardiaceae) for antimicrobial and antioxidant activities

M.M. Suleiman, V. Naidoo, J. N. Eloff

Preface

To simplify the isolation of the antifungal compounds from the *L. alata* fractions the extract was first fractionated into different fractions based on polarity in a mild solvent-solvent fractionation process. The pharmacological activities of the different fractions were determined to facilitate the isolation of the antifungal compounds. Because stimulation of the immune system could also aid in an organism combating the infections, antioxidant activity of the different fractions was also determined.

The text presented in this chapter was prepared for submission to the African Journal of Biotechnology for publication.

Abstract

An acetone extract of *Loxostylis alata* was separated into six fractions based on polarity by a solvent-solvent fractionation procedure and the different fractions were screened for antimicrobial and antioxidant activities. The number of antimicrobial compounds in the carbon tetrachloride (CCl_4), chloroform (CC), aqueous methanol (AM) and butanol (BT) fractions was determined by bioautography. Each fraction was loaded onto TLC plates and eluted using the two different mobile solvent systems, namely chloroform/ethyl acetate/formic acid (5:4:1): [CEF] and ethyl acetate/methanol/water (40:5.4:5) [EMW]. Relative front (R_f) values of active compounds against bacteria ranged from 0.4-0.9 in CEF and EMW solvent system. Similarly, the R_f values of compounds active against fungi are in the range of 0.7-0.9. Hexane, carbon tetrachloride, chloroform, aqueous methanol and butanol fractions showed areas of inhibition against bacterial organisms, while only hexane and carbon tetrachloride fractions depicted areas of fungal growth inhibition on their chromatograms. The CCl_4 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. *Microsporum canis* was the most sensitive organism with the lowest average MIC of 0.16 mg/ml. Qualitative antioxidation using DPPH and qualitative assay using both ABTS and DPPH radicals revealed the presence of three antioxidant compounds in the AM and BT fractions, while the water fraction had only one antioxidant compound. However, the concentration of the antioxidant compounds is more in the AM and BT fractions as revealed by their pronounced colour intensity on the TLC plates. The water fraction had more free radical

scavenging effect against DPPH with EC_{50} value of 0.62 ± 0.03 µg/ml. The lower the EC_{50} value of an extract, the more effective its antioxidant activity. In a similar manner, TEAC value of water fraction was 2.97, which further indicates superior free radical scavenging effect of the fraction against ABTS radical. The greatest reduction of reactive oxygen species (ROS) production induced by diclofenac was also achieved by the water fraction which on the overall shows that the water fraction contains more antioxidants.

Keywords: Medicinal plant, antibacterial, antifungal, serial microdilution, minimum inhibitory concentration, antioxidants

6.1. Introduction

The plant kingdom represents an enormous reservoir of biologically active molecules and so far only a small fraction of plants have been assayed for medical activity. It is estimated that there are about 250,000 species of plants on Earth (Verpoorte, 1998). Such a wealth of identified species, which have not been thoroughly investigated, constitutes an enormous potential source of plant-derived chemicals useful to man (Cowan, 1999). Plants have a long history of use on the African continent for the treatment of various diseases and complaints. In certain African countries, up to 90% of the population still relies exclusively on plants as a source of medicines (Hostettmann et al., 2000). In the global context, natural products and their derivatives form about 50% of drugs in clinical use with about 25% coming from higher plants (Farnsworth and Morris, 1976; Buwa and Staden. 2006; O'Neill and Lewis, 1993).

In a tree screening project of the Phytomedicine Programme (University of Pretoria), plant species with activity against the animal pathogenic fungus *Cryptococcus neoformans* were selected for further testing against *Aspergillus fumigatus* which is an important fungus in the poultry industry The crude extract of *Loxostylis alata* was one of the plants tested and found to have activity against the tested pathogen.

Loxostylis alata A.Spreng. ex Rchb belongs to the family Anacardiaceae (Coates-Palgrave, 2002). In South Africa, the bark and leaves of *L. alata* are used in traditional medicine during childbirth (Pooley, 1993) and also to stimulate the immune system (Pell, 2004). Compounds like 3-(8Z-pentadecenyl) phenol (ginkgol) and 6-(8Z-pentadecenyl) salicylic acid (ginkgolic acid) were isolated from the leaves of *L. alata* (Drewes, et al., 1998).

The aim of the present study was to further assess the antimicrobial properties of fractions of *L. alata* against some important animal pathogenic fungi and bacteria, and also to test the antioxidant activity of the fractions. This is done with a view of isolating compound(s) that are active.

6.2. Materials and methods

6.2.1. Plant collection and processing

Loxostylis alata leaves were collected at the Manie van der Schijff Botanical Garden of the University of Pretoria, South Africa. The plant leaves were collected in April 2007. Samples of the plant were identified and authenticated by Lorraine Middleton and Magda Nel of the University of Pretoria. Voucher specimen of the plant with number; PRU PRU96508 was deposited at the Schweikert Herbarium of the Department of

Plant Sciences, University of Pretoria, South Africa. The dried leaves were milled to a fine powder in a Macsalab mill (Model 200 LAB), Eriez®, Bramley, and stored at room temperature in closed containers in the dark until used. Five hundred grams of finely ground plant material was extracted with 5 litres of acetone (technical grade-Merck) in a macerating bottle. The bottle was shaken for 1 hour in Labotec model 20.2, shaking machine at low speed. Lower speed extraction method was employed for about 24 hours in other to allow the solvent penetrate deeper into the plant tissues so as to extract more of the plant compounds (Silva et al., 1998). After allowing the mixture to settle for 15 min the supernatant was filtered into clean, labelled containers. The extraction process was repeated three times to exhaustively extract the same plant material, and the fractions were combined. The solvent was removed under reduced pressure using a rotary evaporator (Büchi Rotavapor R-114, Switzerland). Seventy grams of the extract was fractionated using solvents of varying polarities (Suffness and Douros. 1979).

6.2.2. Microorganisms and medium

Fungal organisms used were *Aspergillus fumigatus*, *Candida albicans*, *Cryptococcus neoformans*, *Microsporum canis* and *Sporothrix schenckii*. All fungal organisms were isolated from animal clinical cases that were not treated prior to sampling in the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. Sabouraud dextrose (SD) agar (Oxoid, Basingstoke, UK) was used as the maintenance growth medium for all the fungal strains used, and the fungi were cultured in SD broth. The bacteria used were the Gram-positive bacteria: *Enterococcus faecalis* (ATCC 29212), and *Staphylococcus aureus* (ATCC 29213), and the Gram-negative bacteria: *Escherichia coli* (ATCC 27853) and *Pseudomonas aeruginosa* (ATCC 27853). All bacterial cultures were maintained on Mueller Hinton (MH) agar (Oxoid, Basingstoke, UK) at 4°C and cultured in MH broth at 37 °C.

6.2.3. Minimum inhibitory concentration (MIC) determination

The MIC was determined using a serial microdilution assay (Eloff, 1998a). The fractions were individually dissolved in acetone to a concentration of 10 mg/ml. One hundred µl of each plant extract were serially diluted 2-fold with autoclaved distilled water in 96-well microtitre plates. Two millilitres of concentrated fungal or bacterial cultures grown overnight at 37 °C were transferred to 100 ml of fresh SD or MH broths for fungi and bacteria, respectively, and 100 µl of the resultant culture was added to each well. Densities of bacterial cultures used for the screening were as follows: *S. aureus*, 2.6×10^{12} cfu/ml; *E. faecalis*, 1.5×10^{10} cfu/ml; *P. aeruginosa*, 5.2×10^{13} cfu/ml; *E. coli*, 3.0×10^{11} cfu/ml and that of fungi were *A. fumigatus*, 8.1×10^{4} cfu/ml; *C. albicans*, 2.5×10^{4} cfu/ml; *C. neoformans*, 2.6×10^{4} cfu/ml; *M. canis*, 1.5×10^{5} cfu/ml; *S. schenckii*, 1.4×10^{5} cfu/ml. Amphotericin B and Gentamicin (Virbac®) were used as positive controls for

fungi and bacteria, respectively. Acetone was used as the negative control agent in all assays. After incubating bacteria overnight at 37°C, *p*-iodonitrotetrazolium violet (INT) at a concentration of 0.2 mg/ml was used as an indicator of bacterial growth. Forty µl of INT was added to each of the microtitre wells. Thereafter, the plates were incubated at 37°C for 1 hour. For the fungal assays, INT was added immediately after the serial dilution and plates were incubated as described earlier. MIC values were assessed after 1 and 2 hour periods for bacteria and 24 and 48 hours for fungi. The minimum inhibitory concentration (MIC) is defined as the lowest concentration of fraction that inhibits bacterial growth as indicated by INT formazan formation.

6.2.4. Bioautography

Ten µl (10 mg/ml) of each fraction were loaded onto TLC plates and eluted using the two different mobile solvent systems, namely chloroform/ethyl acetate/formic acid (5:4:1): [CEF] and ethyl acetate/methanol/water (40:5.4:5) [EMW] (Kotze and Eloff, 2002). The developed plates were dried under a stream of fast moving air for 5 days to remove traces of solvent on the plates. One week old cultures of fungal organisms grown on SD agar were each transferred into 250 ml of freshly prepared SD broth using a sterile swab so as to contain approximately 1 x 10^9 organisms per ml of actively growing fungi. In the case of bacteria, overnight cultures grown on MH broth were used. The prepared chromatograms were sprayed with the fungal or bacterial suspension until wet. This process was carried out using a biosafety Class II cabinet (Labotec, SA) for fungi, and a laminar flow cabinet (Labotec, SA) for bacteria. Thereafter, the plates were incubated overnight at 35°C and 100% relative humidity in the dark and then sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium violet (INT) (Begue and Klein. 1972) and further incubated overnight or longer in the case of *S. schenckii* and *M. canis*. White areas or spots 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 organisms.

6.2.5. Evaluation of antioxidant activity

Qualitative antioxidant screening was by using 2, 2-diphenyl-1-picryl hydrazyl (DPPH) (Takao et al., 1994). TLC plate loaded with 100 µg of each of the 6 fractions of *L. alata* was developed in CEF solvent system and sprayed with 0.2% DPPH in methanol. Compounds with antioxidant activity were visualized as yellow bands against a purple background (Bors et al., 1992).

The antioxidant activity was quantified by spectrophotometry using two radicals, ABTS and DPPH. In the ABTS method, the TROLOX (6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid) equivalent antioxidant capacity (TEAC) assay (Re et al., 1999) was determined. This was based on the scavenging of

the ABTS radical into a colourless product by antioxidant substances. The blue/green chromophore $ABTS^{+}$ was produced through the reaction between ABTS and potassium sulphate. The absorbance was read at 734 nm using a Versamax microplate reader (Molecular Devices). TROLOX is a Vitamin-E analogue and was used as a standard in this assay. An extract with a TEAC value of 1 indicates an equivalent antioxidant value of TROLOX. Decrease or increase in antioxidant activity is depicted by a lower or upper value of TEAC, respectively. The DPPH free radical assay (Mensor et al., 2001) was also employed to quantified antioxidant compounds in the fractions. Briefly, 10 µl of 0.3 mM DPPH in ethanol was added to 25 µl of each concentration of extract tested and allowed to react at room temperature in the dark for 30 minutes. Appropriate blank and negative control solutions were prepared for each test. L-ascorbic acid (Vitamin C) was used as positive control. The decrease in absorbance was measured at 518 nm. Values obtained were converted to percentage antioxidant activity (AA %) using the formula:

$$AA\ \% = 100 - \{[(Abs_{sample} - Abs_{blank}) \times 100] / Abs_{control}\}$$

Where Abs_{sample} is the absorbance of the sample, Abs_{blank} is the absorbance of the blank and $Abs_{control}$ is the absorbance of the control. The EC_{50} value, defined as the concentration of the sample leading to 50% reduction of the initial DPPH concentration, was calculated from the linear regression of plots of concentration of the test fractions (µg/ml) against the mean percentage of the antioxidant activity obtained from three replicate assays using Microsoft Office Excel. EC_{50} values obtained from the regression lines had coefficient of determination $r^2 \geq 95$ %. A lower EC_{50} value indicates high antioxidant activity.

6.2.6. ROS studies

Vero monkey kidney cells were grown in minimal essential medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1% gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). The cells were incubated for 12 hours with diclofenac (50 µM) per well in the presence or absence of different concentrations of the fractions. Diclofenac is a potent stimulant for ROS production in kidney cells (Naidoo and Swan, 2009). Thereafter, the cell cultures were incubated with dichlorofluorescin diacetate (DCFH-DA) as an indicator for intracellular ROS production, for 30 min and washed with PBS. The absorbance was measured at 504 nm (Somogyi et al., 2007). The degree of ROS production was evaluated as a percentage of ROS production of treated to untreated wells on the same plate. Tests were carried out in quadruplicate and each experiment was done in triplicate.

6.3. Results and discussion

6.3.1. Quantitative yield obtained after solvent-solvent fractionation

The acetone extract of *L. alata* was resolved into 6 different fractions using chloroform, carbon tetrachloride, hexane, aqueous methanol, butanol and water (Suffness and Douros, 1979). A schematic representation of the solvent-solvent resolution is shown in Figure 6.1. The butanol fraction had the greatest quantity of material from the crude acetone extract with a percentage yield of 47%, while chloroform extracted the least material with a yield of 1.30%. The recovery of the fractions from the original crude extract was 85%. It therefore indicates that 15% of the total mass of the crude extract was lost during fractionation. In some cases a pellicle is formed between different phases and the pellicle was discarded probably explaining the loss.

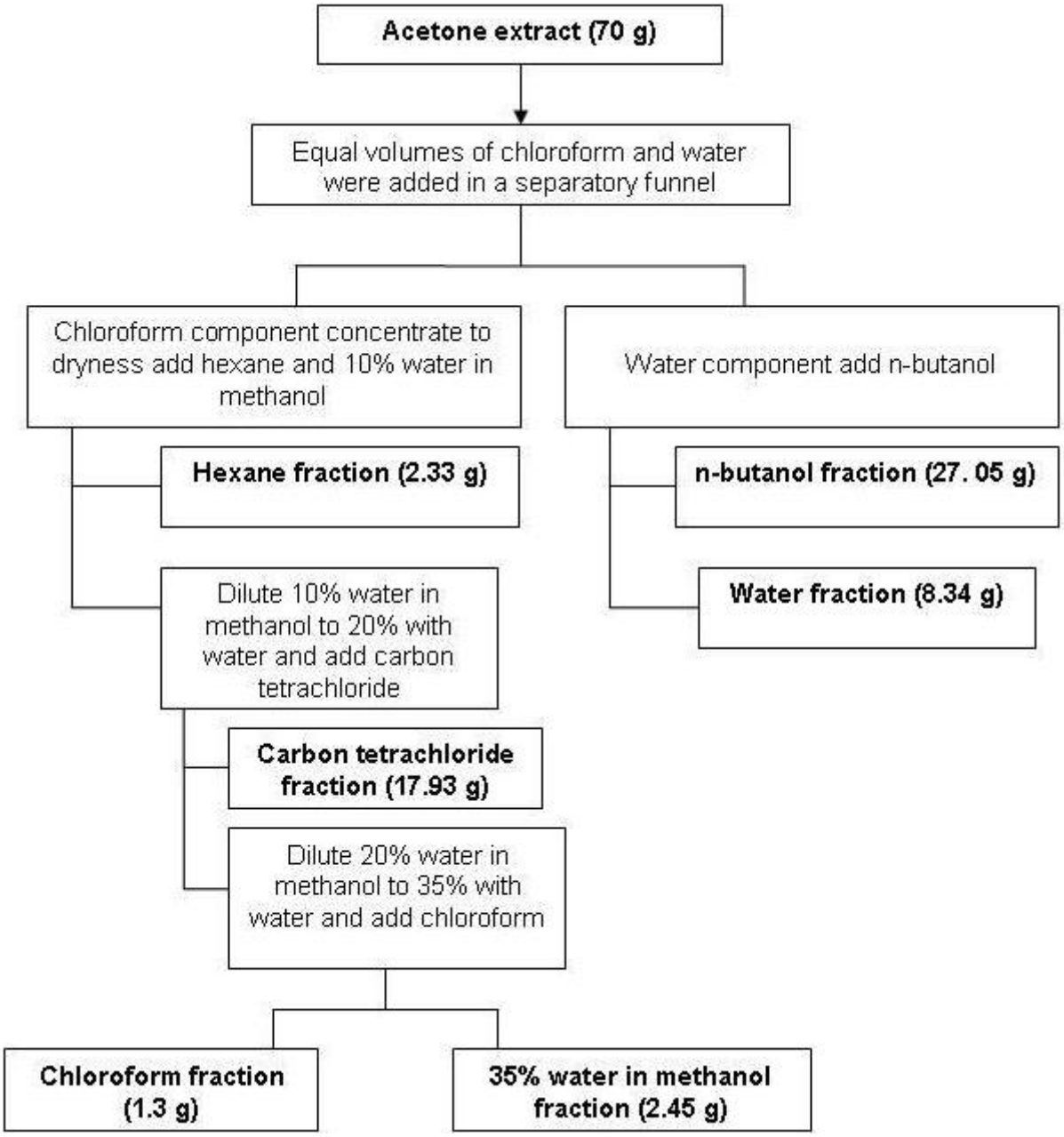


Figure 6.1. Stepwise procedure for the fractionation of *L. alata* acetone extract. Text in bold represents final collected fraction.

6.3.2. Bioautography

The bioautographic results against *S. aureus* and *A. fumigatus* are shown in Figure 6.2. Hexane, carbon tetrachloride, chloroform, aqueous methanol and butanol fractions showed areas of inhibition against bacterial organisms, while only hexane and carbon tetrachloride fractions had areas of fungal growth inhibition on their chromatograms. R_f values of active compounds against bacteria ranged from 0.4-0.9 in CEF and EMW solvent system. Similarly, the R_f values of compounds active against fungi are in the range of 0.7-0.9. Compounds that are active against bacteria in the hexane and carbon tetrachloride fractions also had activity against fungi as shown by their same R_f values. Inhibition of microbial growth was only seen clearly on TLC plates separated by CEF and EMW.

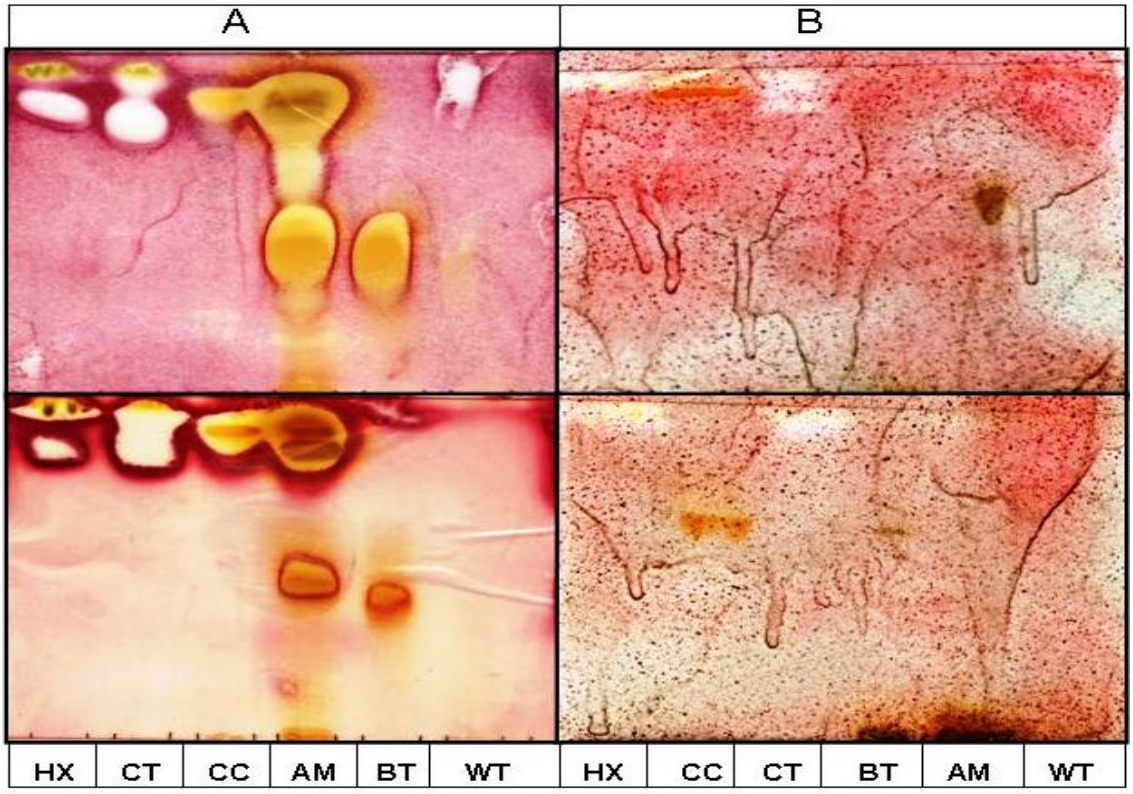


Figure 6.2. Hexane (HX), carbon tetrachloride (CT), chloroform (CC), aqueous methanol (AM), butanol (BT) and water (WT) fractions of *L. alata* separated on TLC plates using CEF and EMW and sprayed with *S. aureus* (A) and *A. fumigatus* (B) and 24 hours later by INT. White areas indicate inhibition of microbial growth after 60 minutes of incubation at 37°C.

6.3.3. Minimum inhibitory concentration of fractions

The MICs results are presented in Table 6.1. The chloroform ($CHCl_3$), and carbon tetrachloride (CCl_4) fractions from *L. alata* leaf had interesting activity against both bacteria and fungi. The CCl_4 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. Similarly, the $CHCl_3$ extract had activity against *E. faecalis*, *C. neoformans* and *M. canis* with respective MIC values of 0.08, 0.06 and 0.1 mg/ml. The CCl_4 extract was the most active, with an average MIC of 0.12 mg/ml against all the tested pathogens. *M. canis* was the most sensitive organism with lowest average MIC of 0.16 mg/ml, while *C. albicans* was the least affected with an MIC value of 1.53 mg/ml. The water extract of *L. alata* possessed the least activity against the tested pathogens with average MIC of 1.63 mg/ml. Plant drugs prepared using water as solvent do not usually extract the more lipophilic compounds (Buwa and Staden, 2006). Perhaps the antimicrobial activity of *L. alata* resides in the more lipid-soluble components as was presented in the results of this study. The non-polar fractions possessed the highest antimicrobial effect. Interestingly, the Gram-positive bacteria were more sensitive to the fractions than the Gram-negative bacteria. Gram-negative bacteria are relatively resistant to plant fractions owing to the presence of an outer membrane which is known to present a barrier to penetration of numerous antibiotic molecules, and the periplasmic space contains enzymes, which are capable of breaking down foreign molecules introduced from outside (Nikaido, 1996). The antibacterial and antifungal properties of the fractions of *L. alata* were not as effective as that of the reference drugs gentamicin and amphotericin B (Amp B). However, the reference drugs are pure compounds, which perhaps may be responsible for their high activity. The active compounds when isolated in their pure forms may prove to have comparable or higher activities than the reference compounds.

6.3.4. Total activity and evidence for synergism

The total activity (TA) of the fractions was also calculated (Table 6.1), Total activity is calculated by dividing the quantity present in the extract in mg with the MIC value in mg/ml (Eloff, 2004). This value indicates the volume to which the active constituents present in the fraction can be diluted and still inhibit the growth of the test organism. The TA of the fractions of *L. alata* ranged from 984-597 667 ml. The CCl_4 fraction was the most active with TA value of 597 667 ml against *M. canis*. It therefore implies that if one gram of the CCL_4 fraction is dissolved in 597 667 ml of acetone, the solution obtained will still inhibit the growth of *M. canis*. Furthermore, TA calculation will detect at each step loss in biological activity.

More importantly, this will allow the detection of synergism between the plant compounds if any exist (Eloff, 2004). The crude acetone extract of *L. alata* had TA values of 1 166 666, 437 500, 437 500, 225 807, 1 400 000, 225 807, 333 333, 1 000000 and 1750000 ml against *S. aureus, E. faecalis, E. coli, P. aeruginosa, A. fumigatus, C. albicans, C. neoformans, M. canis* and *S. schenckii,* respectively. The results herein indicated a combined TA of the fractions against *S. aureus*, *E. faecalis*, *E. coli*, *P. aeruginosa*, *A. fumigatus*, *C. albicans*, *C. neoformans*, *M. canis* and *S. schenckii* to be 509718, 522528, 95027, 109530, 205856, 198982, 355034, 770873 and 160449, respectively. There were lost in total activity in the fractions compared to the crude extract against *S. aureus*, *E. coli*, *P. aeruginosa*, *A. fumigatus*, *C. albicans*, *M. canis* and *S. schenckii* of 56%, 92%, 51%, 85%, 12%, 23% and 91%, respectively. Against *E. faecalis* and *C. neoformans* however, there was a slight increase in activity. Because the method used to determine the MIC was based on a two fold serial dilution changes in the activity of c. 50% are probably not significant and may be due to methodological variation.

The results indicate a substantial loss of activity against especially *E. coli, A. fumigatus* and *S. schenckii*. In comparing the mass recovered in the different fractions compared to the 70 g of the crude, it represented a loss of about 15%. The loss of > 85% of activity against *E. coli, A. fumigatus* and *S. schenckii* is strong evidence for the existence of synergism in the crude acetone extract of *L. alata* against these pathogens.

It also indicates that it may be more efficient to use a crude extract rather than one of the fractions obtained by solvent-solvent fractionation because it would be much cheaper to produce and extract with a higher activity.

Table 6.1. Minimum inhibitory concentrations (MIC) and total activity (TA) of fractions of *Loxostylis alata* against some pathogenic bacteria and fungi (average of three determinations).

Fractions	Mass (mg)	Microorganism																	
		MIC (mg/ml)									TA (ml)								
		SA	EF	EC	PA	AF	CA	CN	MC	SS	SA	EF	EC	PA	AF	CA	CN	MC	SS
$CHCl_3$	1300	0.63	0.08	0.31	0.63	0.94	0.63	0.06	0.1	0.63	2063	16250	4194	2063	1383	2063	21667	13000	2063
CCl_4	17930	0.04	0.04	0.31	0.24	0.1	0.1	0.06	0.03	0.2	448250	448250	57839	74708	179300	179300	298833	597667	89650
Hexane	2330	0.08	0.11	0.63	0.94	0.24	0.94	0.4	0.34	0.71	29125	21182	3698	2479	9708	2479	5825	6853	3282
Butanol	27050	1.25	1.25	1.25	1.25	2.5	2.5	1.25	0.2	0.47	21640	21640	21640	21640	10820	10820	21640	135250	57553
Aq. MeOH	2460	1.25	1.25	2.5	1.25	1.88	2.5	1.4	0.18	0.71	1968	1968	984	1968	1309	984	1757	13667	3465
Water	8340	1.25	0.63	1.25	1.25	2.5	2.5	1.57	1.88	1.88	6672	13238	6672	6672	3336	3336	5312	4436	4436
Amp. B	-	NT	NT	NT	NT	0.00063	0.00031	0.00016	0.00016	0.00016	-	-	-	-	-	-	-	-	-
Gentamicin		0.0063	0.0031	0.015	0.015	NT	NT	NT	NT	NT									
Average MIC	-	0.75	0.56	1.04	0.93	1.36	1.53	0.79	0.46	0.77									
Sum of TA	-	-	-	-	-	-	-		-	-	509718	522528	95027	109530	205856	198982	355034	770873	160449
Crude	70000	0.06	0.16	0.06	0.31	0.05	0..31	0.21	0.07	0.04	1166666	437500	1166666	225807	1400000	225807	333333	1000000	1750000
% loss in activity												56	-19	91	51	85	-12	7	91

Aq. MeOH = Aqueous methanol
S. aureus (SA); *E. faecalis* (EF); *E. coli* (EC); *P. aeruginosa* (PA); *A. fumigatus* (AF); *C. albicans* (CA); *C. neoformans* (CN); *M. canis* (MC); *S. schenckii* (SS)
NT = not tested
Amp. B= Amphotericin B

6.3.5. Qualitative analysis of antioxidant compounds in different fractions

Antioxidants are substances that assist greatly to stimulate the immune system to combat microbial infection (Knight, 2000). In view of that and based on ethnobotanical information that *L. alata* is used to stimulate immune response (Pell, 2004), *the* antioxidant activity of the different fractions was also determined. Antioxidants when present at low concentrations compared with that of an oxidizable substrate (carbohydrate, lipid, DNA or protein), significantly delay or prevent the oxidation of that substrate (Halliwell, 1990). Oxygen radicals and lipid peroxides are implicated in the aetiology of many diseases (Halliwell et al., 1988). The potential value of antioxidants has prompted investigators to search for compounds with potent antioxidant activity. The use of the DPPH radical as TLC spray reagent proposed for the first time in 1994 (Takao et al., 1994) for screening antioxidants in marine bacteria, appears to be also well suited for the detection of antioxidants in crude plant fractions or pure compounds isolated from plant material.

In the qualitative assay, the aqueous methanol, butanol and water fractions showed antioxidant compounds with R_f values ranging from 0.1-0.6 (Figure 6.3). The aqueous methanol fraction had three major antioxidative compounds but not one of the had the same R_f value as the antifungal compounds found by bioautography. This is not surprising because antioxidant compounds are usually polar in nature and in the experience of the Phytomedicine Programme antimicrobial compounds are usually relatively non-polar. These results also indicate the potential of using a crude extract rather than the separated fractions because the crude would contain both antimicrobial and anti oxidant activities.

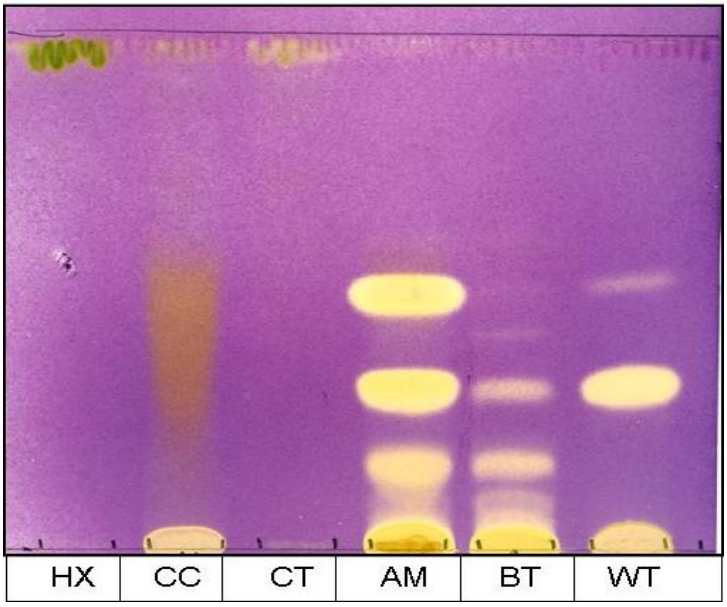


Figure 6.3. Chromatogram of hexane (HX), chloroform (CC), carbon tetrachloride (CT), aqueous methanol (AM), butanol (BT) and water (WT) fractions of *Loxostylis alata* separated with CEF mobile phase and sprayed with 0.2% DPPH. Antioxidant compounds are indicated by yellow areas over purplish background.

6.3.6. Determining qualitative antioxidant activity

Qualitative antioxidant assays had a good correlation with the qualitative assay, with the aqueous methanol, butanol and water fractions having significant ($p \leq 0.05$) antioxidant activity in the DPPH assay (Table 6.2). The aqueous methanol, butanol and water fractions had respective EC_{50} values of 1.82 ± 0.03, 1.05 ± 0.06 and 0.62 ± 0.03. The chloroform, carbon tetrachloride and hexane fractions had EC_{50} values of 2.76 ± 0.18, 3.42 ± 0.09 and 12.42 ± 0.13 µg/ml, respectively. The lower the EC_{50} value of an extract, the more effective its antioxidant activity. Similarly, the TEAC values of aqueous methanol, butanol, water, chloroform, carbon tetrachloride and hexane fractions were 1.55, 2.21, 2.97, 0.45, 0.12 and 0.56, respectively. A TEAC value greater than 1 is indicative of good antioxidant activity, as it is higher than that of the reference compound. Polyphenols, although not the only compounds, are the major plant secondary metabolites with antioxidant activity. The antioxidant activity of phenolic compounds is reported to be mainly due to their redox properties (Galato et al., 2001), which can play an important role in adsorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides. Perhaps similar compounds are present in the aqueous methanol, butanol and water fractions of *L. alata*, which could be responsible for their antioxidant action.

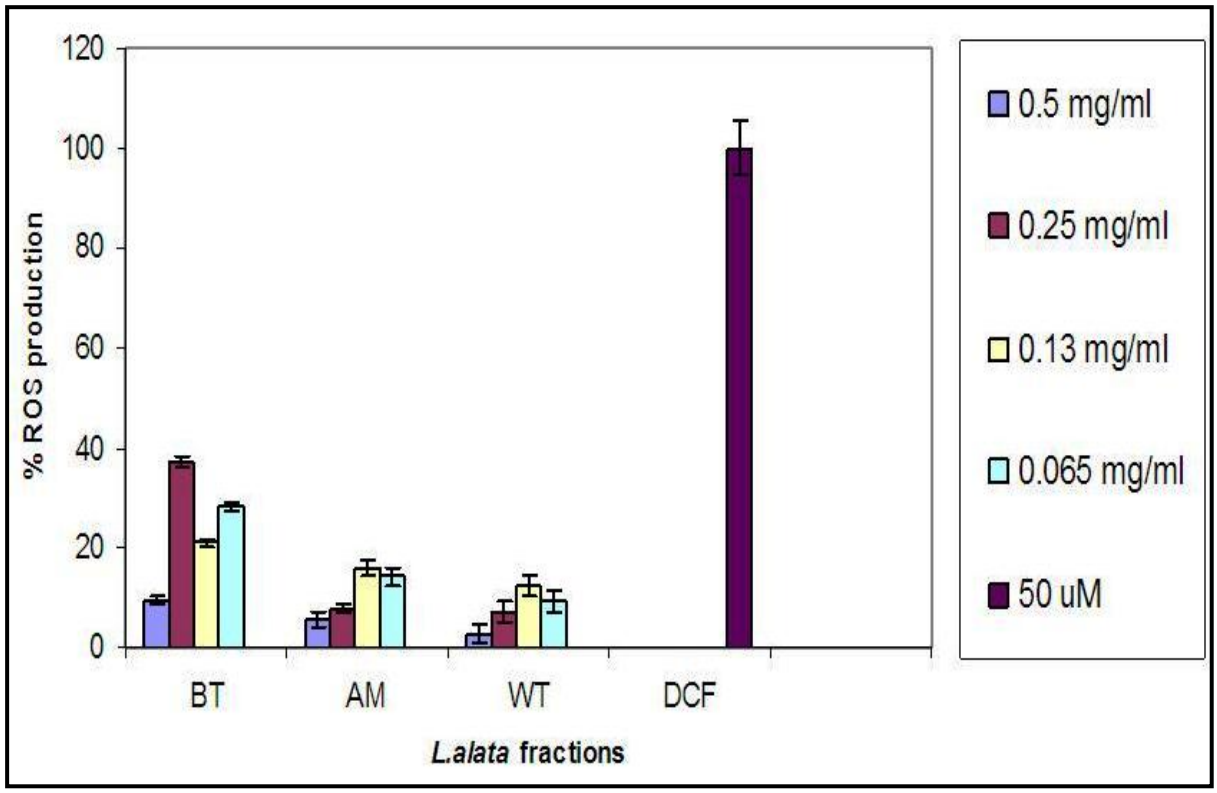
Table 6.2. Fraction yield and antioxidant activity of fractions of *L. alata*.

Fraction	Fraction yield (%)	Antioxidant values		
		TEAC	DPPH (EC_{50} ± SEM, µg/ml)	DPPH ascorbic acid equivalent*
Chloroform	1.30	0.45	2.76 ± 0.18 [b]	0.34
Carbon tetrachloride	25.61	0.12	3.42 ± 0.09 [b]	0.27
Hexane	3.33	0.56	12.42 ± 0.13 [c]	0.075
Butanol	38.64	2.21	1.05 ± 0.06 [a]	0.90
Aqueous methanol	3.57	1.55	1.82 ± 0.03 [a]	0.52
Water	11.92	2.97	0.62 ± 0.03 [a]	1.52
Trolox	-	1.0	-	-
L-ascorbic acid	-	-	0.94 ± 0.11[a]	1.0

Means within the same column and with different superscript letters differ significantly ($p \leq 0.05$)
* calculated by dividing ascorbic value with value for fraction

6.3.7. Reactive oxygen species inhibition by fractions

Diclofenac inhibits the transport of malate and glutamate into mitochondria via the malate–aspartate shuttle, the most important mitochondrial metabolite transport system in the kidney, liver, and heart (Ng et al., 2006). Another plausible mechanism explaining its toxic effect is its ability to cause production of ROS (Naidoo and Swan, 2009). ROS production by Vero monkey kidney cells was greatly reduced by incorporating the fractions of *L. alata* (Figure 6.4). The greatest reduction of ROS production was also achieved by the water fraction. Reduction of ROS production by the fractions, however, suggests that they can either reduce diclofenac-induced ROS production or neutralize ROS when produced. Since diclofenac also interfered with uric acid (an important antioxidant) transport in the kidneys (Naidoo and Swan, 2009). It will be reasonable to assume also that the fractions have an important role in preventing the interference of uric acid transport in the kidneys, hence another important mechanism of their antioxidant action.



But = Butanol; Aq Met = Aqueous methanol

Figure 6.4. Inhibition of ROS production in Vero monkey kidney cell by *L. alata* fractions.

6.4. Conclusion

The presence of antimicrobial and antioxidant activities in the fractions of *Loxostylis alata* suggests that this plant may be a source of bioactive substances with multifaceted activity. The presence of antioxidant compounds in this plant may be responsible for its immunostimulant action, which is employed in traditional medicine (Pooley, 1993). Although the attempt to increase the antifungal by solvent-solvent fractionation activity did not yield good results, in terms of antifungal activity, it did facilitate efforts to isolate the antifungal compound from the crude extract.

Postscripts

The carbon tetrachloride fraction of *Loxostylis alata* had a higher activity than all the other fractions against *Aspergillus fumigatus* and other tested pathogens. The fraction was therefore chosen and subjected to column chromatography to isolate the active compound(s) present. Knowledge of the compound(s) that are active may assist in standardizing and formulating safer and effective dosages.

Chapter 7
Biological activities of two antifungal compounds isolated from *Loxostylis alata* (Anacardiaceae)

M.M. Suleiman, E.E. Elgorashi, B.B. Samuel, V. Naidoo, J.N. Eloff

Preface

The carbon tetrachloride fraction of *Loxostylis alata* was subjected to column chromatography to isolate the active compound(s) present. We further characterize the isolated compounds both chemically and biologically. Characterisation of the compounds will give an insight into ways to design and enhance the action of the isolated compounds. This chapter was prepared for submission to the **South African Journal of Botany** for publication.

Abstract

Loxostylis alata (Spreng.) f. ex Reichb 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 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 the micro-dilution and bioautographic assays. The bacterial strains used were *Staphylococcus aureus* (ATCC29213), *Enterococcus faecalis* (ATCC 29212), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). While the fungal strains used were *Cryptococcus neoformans*, *Sporothrix schenckii*, *Aspergillus fumigatus*, *Microsporum canis* and *Candida albicans*. A bioassay guided fractionation of the crude extract yielded two antimicrobial compounds namely, Lupeol **1** and β-sitosterol **2.** In addition β-sitosterol exhibited selective inhibition of COX-1 (IC_{50} = 55.3 ± 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*.

Keywords: *Loxostylis alata*; Antimicrobial activity; Anti-inflammatory activity; Cytotoxicity; Genotoxicity

7.1. Introduction

About 60% of the world's population relies almost entirely on herbal medicines to treat different ailments (Rates, 2001). Plant derived drugs have for ages been regarded as an essential source of therapeutically effective medicines and still remain enormously important with about 25% of the drugs prescribed worldwide being herbal formulations (Rates, 2001). Of the 252 drugs considered as basic and essential by the WHO, 11% are exclusively of plant origin and a significant number are synthetic drugs obtained from natural precursors (Rates, 2001). In 1997, the world market for phytomedicine products was estimated at US$10 billion (Soldati, 1997). Recent development of natural products into chemotherapeutic armamentarium include the antimalarial drug artemisinin and the anticancer agents taxol, docetaxel and camptothecin In some cases, the use of natural products has been the single most successful strategy for the discovery of new medicines (Harvey and Waterman, 1998). Where natural compounds do not have pharmacological activity, they may serve as lead compounds, allowing the design and rational planning of new drugs (Hamburger and Hostettmann, 1991).

As plants produce an array of diverse chemical compounds, the separation and determination of their active compounds will provide an insight into their pharmacological, pharmacokinetic and toxicological properties (Sun and Sheng, 1998).

Loxostylis alata A.Spreng. ex Rchb is a member of the family Anacardiaceae (Coates-Palgrave, 2002). The bark and leaves of *Loxostylis alata* are used in South African traditional medicine during childbirth to relieve pain during labour (Pooley 1993) and also to stimulate the immune system (Pell, 2004). Ginkgol (3-(8Z-pentadecenyl) phenol) and ginkgolic acid (6-(8Z-pentadecenyl) salicylic acid) were previously isolated from the plant (Drewes *et al*., 1998). To date no studies have been carried out on the species to determine its pharmacologically active constituents.

In a study of seven South African plant species active against the fugal pathogen *Cryptococcus neoformans*, *Loxostylis alata* had the highest activity. The primary objective of this study was to isolate the compounds active *Aspergillus fumigatus* (an important fungus in the poultry industry) from *Loxostylis alata* leaf extracts.. The isolated compounds were investigated further for their anti-inflammatory (Cyclooxygenase-1 and -2), mutagenic (*Salmonella*/microsome assay) and cytotoxic (MTT assay) activities to evaluate other activities and the safety.

7.2. Materials and methods

7.2.1. Plant collection

Leaves of *Loxostylis alata* A.Spreng. ex Rchb were collected at the Marie van der Schijff Botanical Garden of the University of Pretoria, South Africa. Samples of the plant were identified and authenticated by Lorraine Middleton and Magda Nel of the Botanical Garden of the University of Pretoria. Voucher specimen of the plant (number; PRU PRU96508) was deposited at the Schweikert Herbarium of the Department of Plant Science, University of Pretoria, South Africa.

7.2.2. Extraction, isolation and identification of constituents

Leaves of *Loxostylis alata* were dried at room temperature, milled to a fine powder and stored at room temperature in closed containers in the dark until used. The ground plant material (500 g) was extracted with acetone (5 litres × 3). The solvent of the combined extracts was removed *in vacuo*.
The same compounds that showed activity against *A. fumigatus* on bioautogram were also active against *S. aureus* because of their similar R_f values (Figures 7.2 and 7.4). Since it is difficult to work with *A. fumigatus* which is a spore forming organism and carries greater health risk to the researcher and the environment, we decided to use *S. aureus* as a model for isolating the active compounds. The acetone extract (70 g) was subjected to solvent fractionation using carbon tetrachloride, hexane, chloroform, aqueous methanol, butanol and water (Stuffiness and Douros, 1979).Column chromatography (37 × 5 cm, silica gel 60) of the CCl_4 fraction (10 g) using a hexane: ethyl acetate step gradient followed by ethyl acetate: methanol step gradient was performed. Initially, 100% hexane was used, and then reduced to 0% hexane by the addition of 10% ethyl acetate in succession. This followed by ethyl acetate: methanol gradient where ethyl acetate was reduced to 0% by the addition of 10% methanol in success increments. Thirteen fractions were collected and each tested for activity against *S. aureus* using the bioautographic method (Begue and Klein, 1972). Based on the bioautography profile, fractions containing active compounds with the same R_f value were combined and were further fractionated by column chromatography eluted isocratically with hexane: ethylacetate (7:3) to yield the pure compounds. Structures of the two isolated compounds were elucidated using ^{1}H NMR and ^{13}C NMR spectral analysis using Variant Unit Innova 300 MHz system (Oxford instruments) and Brüker DRX instrument at the Medical University of South Africa (MEDUNSA). The spectra were confirmed by Mass spectroscopy (MS) at the Department of Chemistry, University of Botswana, Gaborone.

7.2.3. Antimicrobial activity

7.2.3.1. Fungal and bacterial cultures

Bacterial strains used for antibacterial testing were the Gram-positive *Staphylococcus aureus* (ATCC29213), *Enterococcus faecalis* (ATCC 29212), and the Gram-negative *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922). Pathogenic fungal isolates used were *Cryptococcus neoformans*, *Sporothrix schenckii*, *Aspergillus fumigatus*, *Microsporum canis* and *Candida albicans* (obtained from the Microbiology Unit, Department of Veterinary Tropical Diseases, University of Pretoria). Bacterial cells were inoculated into fresh Müller-Hinton (MH) broth (Fluka, Switzerland) and incubated at 37 °C for 14 h prior to the screening procedures. Fungal cultures were grown in Sabouraud dextrose (SD) broth at 37 °C and maintained on SD agar at 4 °C.

7.2.3.2. Bioautography

The antibacterial and antifungal bioautographic assays were carried out according to the method described by Begue and Kline (1972) with slight modification by Masoko and Eloff (2005). Briefly, thin layer chromatography (TLC) plates were loaded with 100 µg of each fraction or 10 µg of pure compound, and dried before developing in chloroform/ethyl acetate/formic acid (5:4:1): [CEF] and hexane/ethyl acetate (7:3) [HE] mobile phases for the fractions and the pure compounds, respectively. The solvent was allowed to evaporate from the plates under a stream of fast moving air for 2-5 days. Plates were then sprayed with concentrated cultures of bacteria or fungal species until completely moist. The moist plates were incubated at 37 °C for 24 h. Thereafter, the plates were then sprayed with 2 mg/ml of *p*-iodonitrotetrazolium violet (INT) and incubated for a further 1 h in case of bacteria and 24 h for fungi (Begue and Kline, 1972). White areas over a purple background on the TLC plate indicate the non-reduction of INT to coloured formazan and therefore an indication of microbial inhibition by the compounds present.

7.2.3.3. Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations of extracts, column fractions and isolated compounds against bacteria and fungi were determined using the serial microdilution assays (Eloff, 1998a; Masoko et al, 2005). In brief, two-fold serial dilutions of the samples were prepared in wells of 96-well microtitre plates. Bacterial or fungal culture (100 µl of an overnight culture) was then added to each well before incubation for 24 h for bacteria or 48 h in case of fungi at 37 °C. *p*-iodonitrotetrazolium chloride (INT, Sigma) was added to each well as indicator of bacterial or fungal growth. The minimum inhibitory concentration (MIC) was read as the concentration of sample that inhibited microbial growth, as indicated by a visible reduction in the red colour

of the INT formazan. In each assay, negative solvent controls, growth controls and a positive control were included. Gentamicin and amphotericin B (Sigma) were used as the antibacterial and antifungal controls, respectively. The samples were tested in triplicate and the assays were repeated twice to confirm results.

7.2.4. Cytotoxicity assay

The isolated compounds were tested for cytotoxicity against the Vero monkey kidney cell line. The cells were maintained in minimal essential medium (MEM, Highveld Biological, Johannesburg, South Africa) supplemented with 0.1% Gentamicin (Virbac) and 5% foetal calf serum (Adcock-Ingram). To prepare the cells for the assay, cell suspensions were prepared from confluent monolayer cultures and plated at a density of 0.5×10^3 cells into each well of a 96-well microtitre plate. After overnight incubation at 37 °C in a 5% CO_2 incubator, the subconfluent cells in the microtitre plate were used in the cytotoxicity assay. Stock solutions of the compounds were prepared by reconstitution to a concentration of 10 mg/ml in dimethylsulphoxide (DMSO). Serial 10-fold dilutions of each extract were prepared in growth medium (1-1000 µg /ml). The method described by Mosmann (1983) was used to determine the viability of cell growth after 5 days incubation with the compounds. MTT was used as an indicator for cell growth. The absorbance was measured at 570 nm. Berberine chloride (Sigma) and DMSO were used as positive and negative controls, respectively. Tests were carried out in quadruplicate and each experiment was done in triplicate. Furthermore, the selectivity index of each fraction was calculated as follows (Shai et al., 2008):

Selectivity index (SI) = Lethal concentration 50 (LC_{50})/Minimum inhibitory concentration (MIC)

This ratio gives the relative safety of each fraction.

7.2.5. Genotoxicity test

The potential mutagenic effects of the investigated plant compounds were detected using the Ames test. The Ames assay was performed with *Salmonella typhimurium* (TA98 and TA100) strains using the plate incorporation procedure described by Maron and Ames (1983). One hundred microliters of bacterial stock solution was incubated in 20 ml of Oxoid Nutrient broth for 16 h at 37 °C on an orbital shaker. The overnight culture (0.1 ml) was added to 2 ml top agar (containing traces of biotin and histidine) together with 0.1 ml test solution (plant extract, solvent control or positive control) and 0.5 ml phosphate buffer (for exposure without metabolic activation). The top agar mixture was poured over the surface of the agar plate and incubated for 48 h at 37 °C. After incubation, the number of revertant colonies (mutants) was counted. All cultures were made in triplicate (except the solvent control where five replicates were made) for each

assay. The assays were repeated twice. The positive control used was 4-nitroquinoline-1-oxide (4-NQO) at a concentration of 2 µg/ml.

7.2.6. Anti-inflammatory assay

Infection with *Aspergillus* organisms causes excessive production of prostaglandins by the host tissue. Prostaglandin production during fungal infection is an important factor in promoting fungal colonization, inflammation and chronic infection (Noverr et al., 2002). Inhibition of prostaglandin biosynthesis by the plant extract and isolated compounds was investigated using both the COX-1 and COX-2 assays (Jäger et al., 1996, Noreen et al. 1998). The COX-1 enzyme (from ram seminal vesicles, Sigma Aldrich) and COX-2 (human recombinant, Sigma-Aldrich) were activated with co-factor solution and pre-incubated on ice for 5 min. Sixty microliters of this enzyme/co-factor solution was added to 20 µl of crude extract of *Loxostylis alata* extract (20 µl of extract solution) or 20 µl of compound and pre-incubated for 5 min at room temperature. Twenty microliters of ^{14}C-arachidonic acid was added to the tested samples and incubated at 37 °C for 10 min. After incubation, the reaction was terminated by adding 10 µl of 2N HCl. Four microliters of a 0.2 mg/ml carrier solution of unlabelled prostaglandins was added. In each assay, four controls were run. Two were background in which the enzyme was inactivated with HCl before the addition of ^{14}C-arachidonic acid, and two were solvent blanks. Indomethacin was included in each test assay as a standard. Percentage inhibition of plant extracts was calculated by comparing the amount of radioactivity present in the sample to that in the solvent blank. IC_{50} was calculated from at least 5 concentrations. Results are presented as mean ± S.E.M. of two experiments carried out in duplicate.

7.3. Results and discussions

7.3.1. Biological activity of the extract

In a preliminary screening, the acetone extract of *Loxostylis alata* had promising activity against *Cryptococcus neoformans*. In this study, an investigation on the antifungal activity of the acetone extracts of *Loxostylis alata* against *Aspergillus fumigatus* confirmed earlier findings. The yield, MIC value together with the total activity of the crude extract and the different fractions resulted from solvent - solvent fractionation of the acetone extract (70 g) are presented in Table 7.1. Total activity (TA) is calculated by dividing the quantity extracted in mg with the MIC value in mg/ml. This value indicates the volume to which the active constituent present in one gram of the fraction can be diluted and still inhibit the growth of the test organism (Eloff, 2004). A higher value of total activity indicates increased usefulness and economic value of the plant material and is of benefit in enabling rural use of the plant. The CCl_4 fraction was the most active fraction with MIC and TA value of 0.08 mg/ml and 3201.79 ml/g, respectively. It therefore

means that 1 gram of CCl_4 fraction can be diluted in 3201.79 ml of the solvent used and still inhibit the growth of *A. fumigatus*. Similarly, the CCl_4 fraction showed greater zone of inhibition against all the tested microorganisms. Hexane and aqueous methanol fractions showed little zone of inhibition, while butanol and water fractions were not active when the TLC bioautographic method was used (Figure 7.1). In addition, column fractions of CCl_4 were separated on TLC plates using CEF and sprayed with *S. aureus*. All the fractions showed activity against the tested pathogen (Figure 7.2)

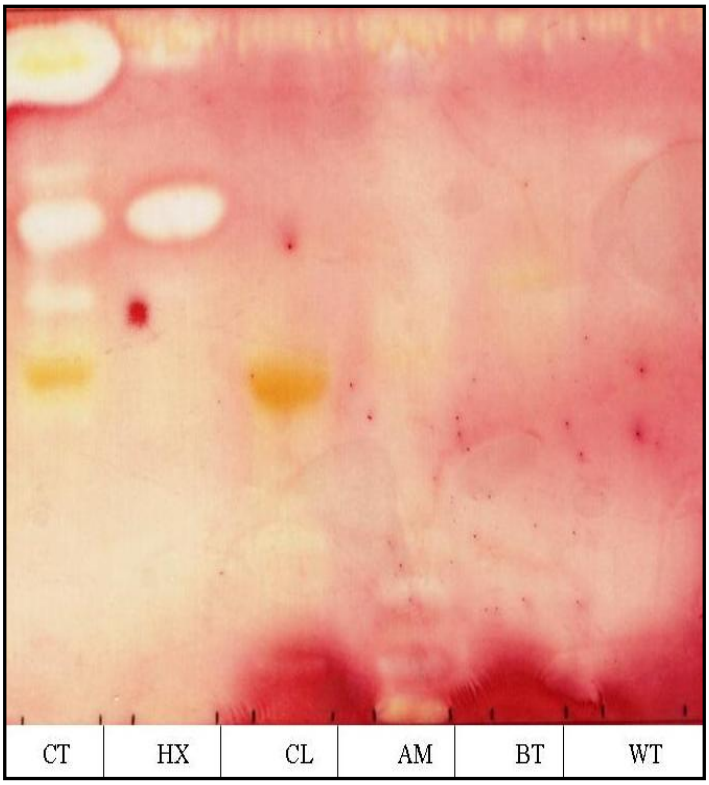


Figure 7.1. Carbon tetrachloride (CT), hexane (HX), chloroform (CL), aqueous methanol (AM), butanol (BT) and water (WT) fractions of *Loxostylis alata* separated on TLC plates using CEF and sprayed with *S. aureus* and 24 h later by INT. White areas indicate inhibition of microbial growth after 60 minutes of incubation at 37 ^{o}C.

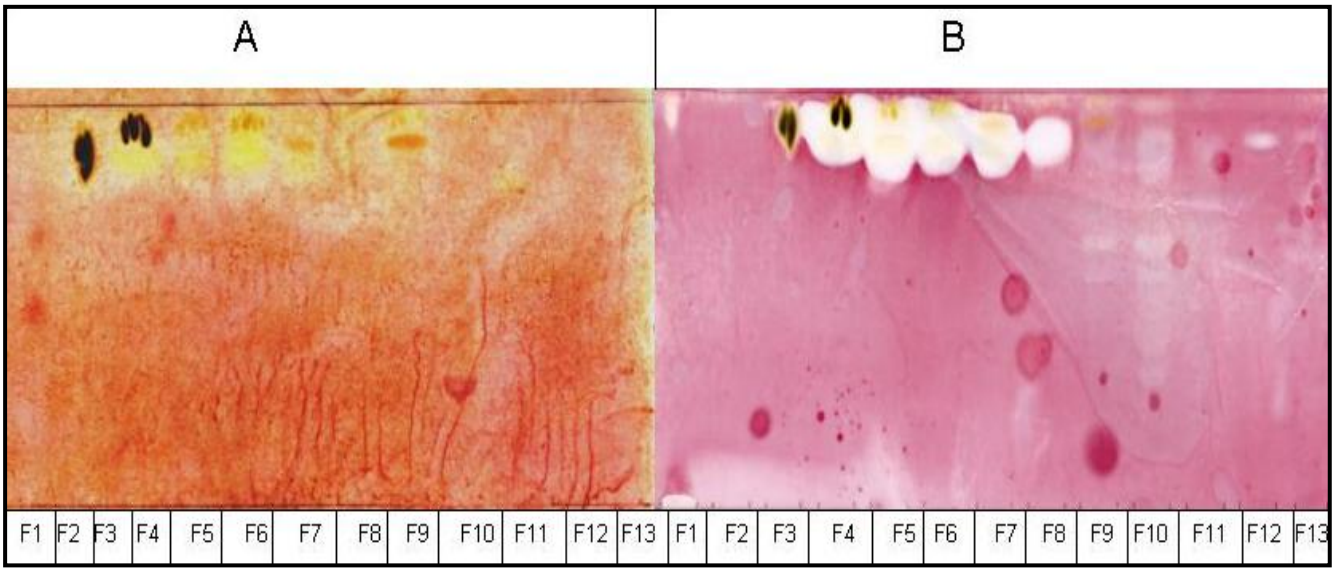


Figure 7.2. F1-F13 fractions of CCl_4 separated on TLC plates using CEF and sprayed with *A. fumigatus* (A) and *S. aureus* (B) and 24 h later by INT. White or yellow areas on the bioautogram indicate inhibition of microbial growth after 60 minutes of incubation at 37 °C.

Table 7.1. Minimal inhibitory concentrations and safety evaluation of various fractions of *Loxostylis alata* against *Aspergillus fumigatus*.

Fraction	Fraction yield (%)	MIC (mg/ml)	Total activity (ml/g)	Cytotoxicity (LC_{50} ± S.E.M in mg/ml)	Selectivity index
Hexane	3.33	0.24	138.69	0.41 ± 0.003	1.71
Carbon tetrachloride	25.61	0.08	3201.79	0.23 ±0.001	2.88
Chloroform	1.86	0.94	19.76	0.67 ±0.002	0.71
Aqueous methanol	3.50	1.88	18.62	NT	NA
Butanol	38.64	2.5	15.46	NT	NA
Water	11.91	2.5	47.66	NT	NA
Amphotericin B	-	0.091	NA	NT	NA
DMSO	-	NT	NA	NDT	NA

NT = not tested; NA= not available; NDT= no detectable toxicity

7.3.2. Chemistry of isolated compounds

Bioactivity guided fractionation of the CCl_4 fraction led to the isolation of two compounds **1** and **2**, which are lupeol and β-sitosterol, respectively (Figure 7.3.). The ^{1}H NMR and ^{13}C NMR spectral data of compound 1 exhibited characteristics spectra features of pentacyclic triterpene. The presence of olefinic protons (4.68 broad signal at H-29a, 4,56 broad signal at H-29b), 3.18 dd, with seven methyl signals are due to lupeol type triterpene. Signals were readily characterised by comparison with signals of lupeol from previous reports. Mass spectrum of the compound with M+426, and prominent signals at 218 and 207 confirmed that the compound is lupeol. (Sholichin et al., 1980; Mahato and Kundu, 1994; Tomosaka et al 2001; Imam et

al., 2007). Analysis and interpretation of the spectroscopic data obtained with previously reported data (Table 7.2) led to the proposed structure for the compound as lupeol (Figure 7.3 (1)) with a molecular formula $C_{30}H_{50}O$. Lupeol, although a compound commonly found in higher plants, is been reported for the first time in *L. alata*.

The characteristic signal of compound 2 is the chemical shift of the 4-6 olefinic signal (5.35) and multiplet at 3.55 due to H-3. This confirmed the isolated compound to be a 24-steroid derivative. Comparison of the carbon spectral data for compound 2 with previously compiled data (Table 7.3) led to the proposed structure of the compound to be β-sitosterol (Figure 7.3 (2)). Mass spectroscopy with molecular ion of 414 and prominent peaks at 396 and 105 served to confirmed the compound to be β-sitosterol with a molecular formula $C_{30}H_{50}O$ (Rubinstein et al., 1976; Chaurasia and Wichtl, 1987; Lopes et al., 1999).

Table 7.2. ^{13}C NMR spectra data for compound 1 (C1).

Carbon	C1	Lupeol (Sholichin et al. 1980)
1	38.7	38.7
2	27.5	27.5
3	79.4	79.0
4	38.9	38.9
5	55.6	55.3
6	18.3	18.3
7	34.3	34.3
8	40.9	40.9
9	50.5	50.5
10	37.2	37.2
11	21.0	21.0
12	25.2	25.2
13	38.4	38.1
14	42.9	42.9
15	27.5	27.5
16	35.1	35.6
17	43.0	43.0
18	48.0	48.0
19	48.3	48.3
20	151	150.9
21	29.9	29.9
22	40.0	40.0
23	28.0	28.0
24	15.3	15.3
25	16.1	16.1
26	16.0	16.0
27	14.6	14.6
28	18.0	18.0
29	19.3	19.3
30	109.3	109.3

Table 7.3. ^{13}C NMR spectra data for compound 2 (C2).

Carbon	C2	β-sitosterol (Shameel et al., 1996)
1	37.2	37.3
2	32.0	31.8
3	72.0	71.9
4	42.3	42.4
5	140.1	140.9
6	122	121.9
7	31.7	32.1
8	31.9	32.1
9	50.1	50.8
10	36.5	36.6
11	21.1	21.1
12	40.0	40.0
13	42.3	42.6
14	56.8	56.8
15	24.3	24.3
16	28.2	28.2
17	56.0	56.2
18	12.0	11.9
19	19.4	19.4
20	36.5	36.2
21	19.1	19.1
22	34.0	34.0
23	29.1	29.3
24	45.9	45.8
25	29.6	29.2
26	19.4	19.8
27	19.1	19.8
28	23.2	23.1
29	11.7	11.9

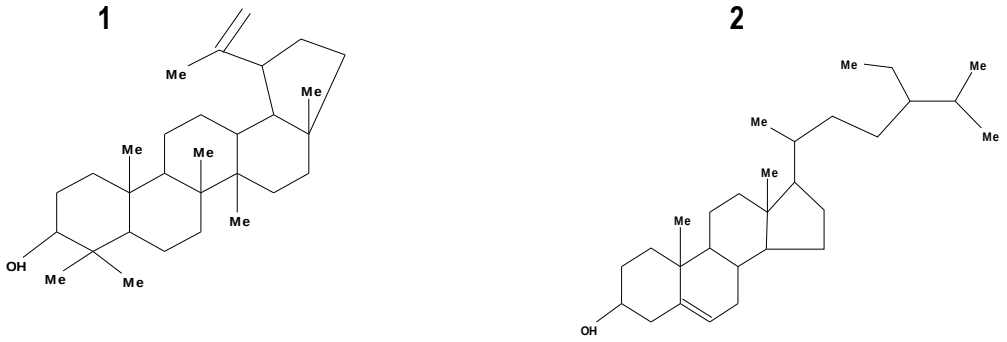


Figure 7.3. Structure of lupeol (**1**) and β-sitosterol (**2**) isolated from *Loxostylis alata* leaves.

7.3.3. Antimicrobial activity of isolated compounds

The two compounds isolated from CCl_4 fraction of *Loxostylis alata* were active against *S. aureus* and *E. coli* with R_f values in hexane: ethyl acetate (7:3) solvent system of 0.47 for lupeol and 0.81 for β-sitosterol. Figure 7.4 represents the biautogram of the 2 isolated compounds. Lupeol showed the most pronounced zone of inhibition against *S. aureus* and *A. fumigatus*. Similarly, when MICs of the 2 compounds were determined, only lupeol had relatively good activity with MIC ≤ 100 µg/ml against 8 out of 10 of the tested pathogens (Table 7.2). However, β-sitosterol had activity against *S. aureus* and *E. coli* with MICs of 90 and 110 µg/ml, respectively. In a similar study, lupeol had low activity against *Candida albicans* with MIC values of more than 250 µg/ml but had high activity against *Sporothrix schenckii* and *Microsporum canis* (MIC values of 12 and 16 µg/ml, respectively); this is in agreement with what Shai et al., 2008, reported. Lupeol and β-sitosterol, previously isolated from the stem bark of *Buchholzia coriacea*, had activity against some species of pathogenic bacteria and fungi (Ajaiyeoba et al., 2003). Similarly, β-sitosterol was found to have good inhibitory activity against the fungi *Aspergillus niger*, *C. cladosporioides*, and *Phytophthora sp*. (Lall et al., 2006). The isolated compounds from *L. alata* leaves are widely distributed in most plants and hence are not novel in character. We observed also that the activity of the compounds against *A. fumigatus* is lower than that produced by the crude extract of *L. alata*. Unless these compounds are destroyed during chemical elution by chromatographic means or bioautography, it strongly appears that synergism plays an important role in the plants activity against *A. fumigatus*. The antifungal compounds involved in synergism appear not to be active on their own.

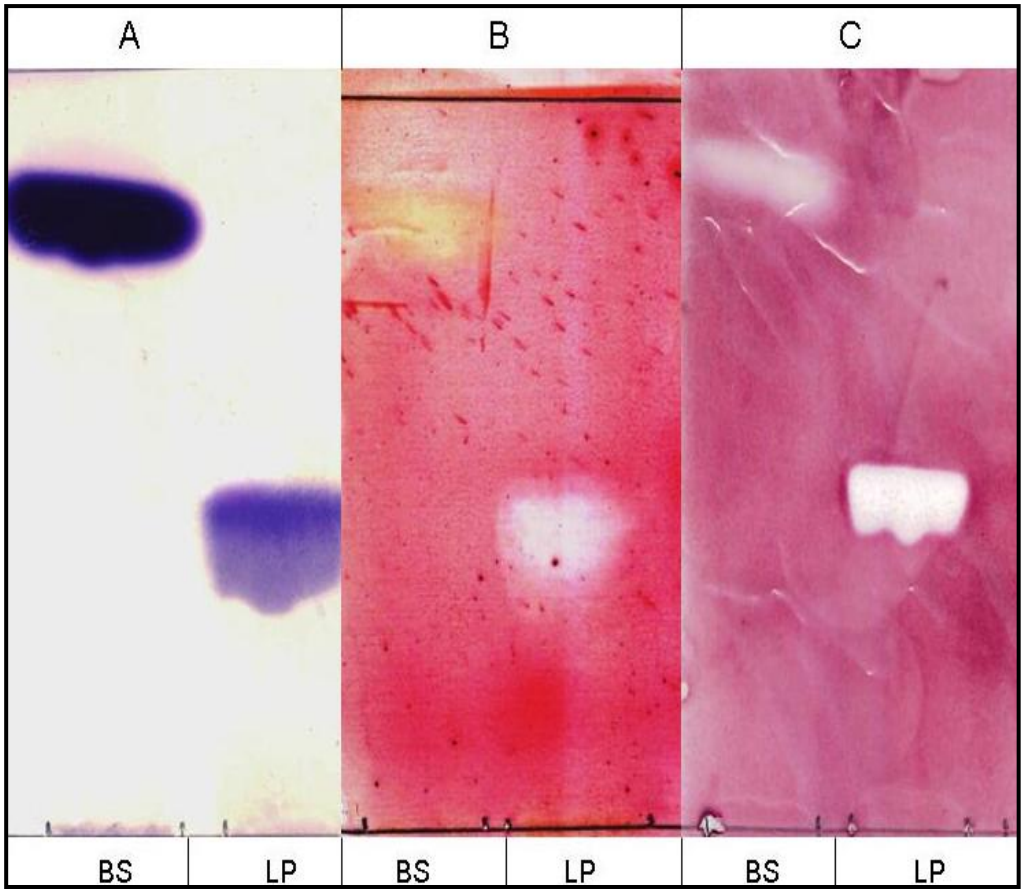


Figure 7.4. Isolated compounds of *Loxostylis alata* spotted on TLC plates and eluted in hexane: ethylacetate (7:3) solvent system. The compounds (spotted left to right) are β-sitosterol (BS) and lupeol (LP). The plates were sprayed with acidified vanillin (A), *A. fumigatus* (B) or *S. aureus* culture (C). White or yellow areas on plate B and C indicate inhibition of microbial growth after 60 minutes of incubation at 37 °C.

Table 7.4. Minimal inhibitory concentrations and safety evaluation of compounds isolated from *Loxostylis alata*.

Compound	MIC values against the tested pathogens (µg/ml)									**Cytotoxicity (LC_{50} ± S.E.M in µg/ml)**
	SA	EF	EC	PA	AF	CA	CN	MC	SS	
Lupeol	29	67	83	150	92	120	47	63	57	76.66 ± 4.1
β-sitosterol	90	>250	110	>250	>250	>250	>250	>250	>250	136.60 ± 7.2
Gentamicin	6.7	4.2	15.2	12.67	NA	NA	NA	NA	NA	NA
Amphotericin B	NA	NA	NA	NA	6.4	0.81	0.32	0.41	0.45	NA
Berberine	NA	NA	NA	NA	NA	NA	NA	NA	NA	6.36 ± 0.8

Staphylococcus aureus (SA), *Enterococcus faecalis* (EF), *Escherichia coli* (EC), *Pseudomonas aeruginosa* (PA), *Aspergillus fumigatus* (AF), *Candida albicans* (CA), *Cryptococcus neoformans* (CN), *Microsporum canis* (MC), *Sporothrix schenckii* (SS)
NA = not available

7.3.4. Anti-inflammatory assay

The crude extract of *Loxostylis alata* and lupeol inhibited COX-1 in a concentration dependent manner (IC_{50} = 92.5 ± 1.6 and 134.0 ± 5.1 μM, respectively). Indomethacin had an IC_{50} of 3.30 ± 0.006 and 122.5 μM against COX-1 and COX-2, respectively. Neither the crude extract nor lupeol or 2 had any inhibitory effect on COX-2. Although lupeol did not have activity against COX-2, it did produce a dose-dependant inhibition of carrageenan induced paw oedema in rats (Agarwal and Rangari, 2003). The *in vivo* action exhibited by lupeol may probably be ascribed to the fact that when administered into the body, it undergoes biotransformation to an active moiety that confers its anti-inflammatory action. In some cases a drug becomes pharmacologically active only after it has been metabolized in the body (Rang et al., 2003). Both COX -1 and -2 regulate the biosynthesis of prostaglandins from arachidonic acid. COX-1 is a constitutive form and has a clear physiological function while COX-2 is mainly induced by inflammatory mediators. It is the inhibition of prostaglandin synthesis by COX-1 and -2 that is responsible for the anti-inflammatory action of NSAIDs (Rang et al., 2003). In addition, COX-1 selective inhibitors have beneficial anti-thrombotic effect. Inhibition of COX-1 by both the crude extract and lupeol may exert beneficial anti-thrombotic effect and protect from heart diseases. Further supporting evidence is that the crude extract inhibited equine platelet aggregation (Suleiman et al., 2009). Moreover, lupeol could form a base for the development of new semi-synthetic drugs for the management of thrombolic disorders.

7.3.5. *In vitro* safety test

The cytotoxicity result of the two compounds is also summarized on Table 7.2. Compounds **1** and **2** were relatively non toxic with LC_{50} of 76.66 ± 4.13 and 136.60 ± 7.20 μg/ml, respectively as compared with the reference compound berberine with LC_{50} of 6.36 ± 0.81 μg/ml. β-sitosterol which occurs as an sterol in many plants is potentially useful in improving human health such as their action as anti-inflammatories, antipyretics, immunomodulators, and antineoplastics effects (Gupta et al. 1980; Bouic 2001).

Results obtained from the mutagenicity test of the 2 compounds using *Salmonella* TA98 and TA100 strains are expressed as mean ± S.E.M (Table 7.3) and are based on number of induced revertant colonies. Substances are considered active if the number of induced revertant colonies is twice the revertant colonies of the negative control (blank) (Maron and Ames., 1983). None of the compounds under investigation were mutagenic in the Salmonella/ microsome tester strains TA98 and TA 100. The lack of cytotoxic or mutagenic effects of these compounds does not guarantee their safe use as traditional medicines. Detailed laboratory and clinical evaluations are needed to justify their use as medicines (Debnath et al., 1991).

Table 7.5. Mutagenic activity expressed as the mean and standard error of mean of the number of revertants/plate in *Salmonella typhimurium* strains TA98 and TA100 exposed to extract and compounds of *Loxostylis alata*, at different concentration.

Treatment	Revertant/plate in *Salmonella typhimurium* strains					
	TA98 (µg/plate ± S.E.M)			TA100 (µg/plate ± S.E.M)		
	2000	200	20	200	20	2
Lupeol	29.30 ± 1.5	29.67 ± 3.6	27.33 ± 1.8	161.33 ± 7.9	167.00 ± 20.1	158.50 ± 8.5
B-sitosterol	22.30 ± 2.2	28.00 ± 2.0	26.67 ± 2.7	179.00 ± 5.9	170.70 ± 1.1	163.30 ± 1.1
Crude extract	NT	NT	NT	NT	NT	NT

Negative control for the *Salmonella typhimurium* test is DMSO (100 µl/plate; TA98: 19.30 ± 2.89; TA100: 152.60 ± 7.07), while the positive control is 4-nitroquinoline 1-oxide (4-NQO) (10 µg/plate; TA98: 170.33 ± 14.14; TA100: 960 ± 24.89). All values quoted are mean ± S.E.M

7.4. Conclusion

The extract of *Loxostylis alata* had varying degrees of antimicrobial and cyclooxygenase inhibitory activities. However, detailed toxicity studies using sophisticated means and also clinical trial in both laboratory and target animal species are required to justify their use in clinical practice. Moreover, there is also need for proper investigation into the mechanism underlying the antimicrobial actions of these compounds from *Loxostylis alata*.

Postscript

Since the extract of *Loxostylis alata* had very promising results in our earlier investigations, we decided to test its activity *in vivo* using target animal species with a view of finding out if what obtained *in vitro* will translate to in vivo activity. It is also a step towards establishing clinical usage of the plant extract.

Chapter 8
A leaf extract of *Loxostylis alata* (Anacardiaceae) prevents experimental aspergillosis in chicks

M.M. Suleiman, V. Naidoo, N. Duncan, S.P.R. Bisschop, J.N. Eloff

Preface

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 an animal experiment.

This chapter was prepared for submission to Onderstepoort Journal of Veterinary Research.

Abstract

A crude acetone extract of *Loxostylis alata* leaves was evaluated for toxicity and for antifungal activity in experimental aspergillosis of broiler chicks based on the excellent *in vitro* activity against this pathogen. At a dose of 300 mg/kg, the extract had some toxicity. Consequently, lower concentrations were used. Chicks were infected intraperitoneally with *A. fumigatus*. Antifungal activity was assessed by comparing the degree and severity of clinical signs, lesion scores, fungal re-isolation and a series of biochemical and haematological indices observed between from chicks treated and not treated with the extract. The extract at the dose of 100 and 200 mg/kg reduced the lesions due to aspergillosis and the amount of *Aspergillus* organism isolated from infected chicks significantly ($p \leq 0.05$) in a dose dependent fashion. The extract was as active as the positive control ketoconazole at a dosage of 60 mg/kg the highest allowable dose to prevent toxicity. The results indicate that a crude extract of *L. alata* leaves has potential as an antifungal agent to protect poultry against avian aspergillosis.

Keywords: *Loxostylis alata*; Aspergillosis; Broiler chicks; Safety evaluation; Lung tissues.

8.1. Introduction

The poultry industry which is an important component in world agricultural economy faces heavy economic losses due to many health hazards caused by the fungus *Aspergillus fumigatus*. Losses caused low productivity, mortality and carcass condemnations at slaughter (Morris and Fletcher, 1988; Richard, 1997; Kunkle, 2003). About US$11 million is the reported as average yearly lost due to aspergillosis alone in the USA (Kunkle, 2003). The disease affects mainly the respiratory tract of birds and has a worldwide distribution, having been reported in almost every farmed bird as well as in wild species (Akan et al., 2002; Chang Reissig et al., 2002).

As a disease, aspergillosis affects birds whether in captive or free-ranging environments, young and mature, and whether immunocompetent or immunosuppressed. However, young birds appear to be much more susceptible than adults. The lower respiratory tract is where *Aspergillus* spp. tends to initially colonize (Tell, 2005) but blood infection with subsequent dissemination to other organs frequently occurs, leading to macroscopic lesions in a wide range of organs or tissues. In spontaneous cases, lesions range from miliary to larger granulomatous foci (Singh et al., 1994). These white lesions are protrusive to the surface of the internal organ. Thickening of the walls of the air sacs frequently occurs (Perelman and Kuttin, 1992). Lesions in avian species are commonly confined to the lungs and air sacs, although infections also occurs in oral mucosa, trachea, brain, eye, skin, bone, liver, kidney (Richard, 1997), and nasal passages (Fitzgerald and Moisan 1995) have also been described. Typical lesions are characterized by granulomatous inflammation with necrosis, haemorrhage, and intralesional fungal elements that are locally invasive. The pathogenesis of aspergillosis appears to be complicated. In recent years, aspergillosis has also emerged as a significant disease in humans that are immunocompromised by HIV-AIDS, neoplasia, or chemotherapy (Denning et al., 1991).

Although there are commercial drugs available for the treatment of systemic and superficial mycoses (Uno et al., 1982; Lyman and Walsh, 1992; Orosz, 2000), none of them are ideal in terms of efficacy, safety and antifungal spectrum (Di Domenico 1998; Ablordeppey et al, 1999). There is therefore a need to explore new remedies to treat the disease.

In a preliminary study, the extracts of *Loxostylis alata* A. Spreng, ex Rchb. (common name tarwood) had good *in vitro* activity against *Aspergillus fumigatus* and other pathogenic animal fungi with minimum inhibitory concentration (MIC) as low as 0.07 mg/ml. Moreover, bioautography of *L. alata* extracts with *A. fumigatus* revealed good antifungal activity (results not shown). This study aims at evaluating the *in vivo* effect of *L. alata* leave extract against experimental aspergillosis in poultry.

8.2. Materials and methods

8.2.1. Plant collection, extraction and processing

Leaves were collected at the Manie van der Schijff Botanical Garden of the University of Pretoria, South Africa. Samples of the plant were identified and authenticated by Lorraine Middleton and Magda Nel of the University of Pretoria. Voucher specimen of the plant with number; PRU96508 was deposited at the Schweicker Herbarium, University of Pretoria, South Africa. After collection and transportation to the laboratory, leaves were separated from stems and dried at room temperature with good ventilation. The dried leaves were milled to a fine powder in a Macsalab mill (Model 200 LAB, Eriez®, Bramley) and stored at room temperature in closed containers in the dark until used. Five hundred grams of finely ground plant material was extracted with 5 litres of acetone. The extraction process was repeated three times to exhaustively extract the same plant material, and the extracts were combined. The solvent was removed *in vacuo* to yield 75 grams of dark greenish solid. The extract was dissolved in 0.2% aqueous dimethylsulphoxide (DMSO) at a concentration of 200 mg/ml.

8.2.2. Animals and management

Three day old broiler chicks were used to evaluate the safety of the extract in a toxicity trial (n=3) and its efficacy (n=8) on birds experimentally infected with *Aspergillus fumigatus*. The birds were purchased from a healthy breeding flock at Eagles Pride Hatchery, Pretoria. At the start of the study, the weight variation of the birds did not exceed 20% of the mean species average weight (National Institute of Environmental Health Sciences, 2001). Throughout the experiment, broiler chicks were kept at the Poultry Reference Centre, Faculty of Veterinary Science, University of Pretoria in an enclosed temperature-controlled house with adequate ventilation, an artificial light at the recommended light intervals source. Clean wood shaving was used as bedding on wood shavings (North, 1981). Feed intake and weight gain for each group were determined every other day. All experimental protocols described in this study were approved by the Animal Use and Care Committee of the University of Pretoria, South Africa (V036/08) in accordance with the international guidelines for use of animals in experimentation.

8.2.3. Safety evaluation of the extract

A modified version of the guidelines for Organisation for Economic Cooperation and Development (OECD) for determining the toxic nature of the chemical (Annex 2b; starting dose of 50 mg/kg) was used to determine the dose of the extract that will produce toxic signs and possibly death in treated birds. Toxic signs exhibited such as ruffled feathers, diarrhoea, depression, off-feed, etc rather than death was used as an end point in determining the safety of the extract. Chicks were randomly assigned into 4 groups of 3

birds each. The first group received the extract at the dose of 300 mg/kg recommended by OECD, while the second group was dosed with 0.2% aqueous dimethylsulphoxide at the dose of 0.2 ml/100 g body weight in water and served as control. All birds were monitored for 12 days. Due to the toxic effect of the starting dose (300 mg/kg), lower doses of 50 and 200 mg/kg of the extract were administered to groups 3 and 4, respectively. These chicks were also examined for signs of toxicity. The dose that did not produce any toxic sign was used as the maximum tolerated dose (MTD) for the chemotherapeutic trial. All treatments were given intraperitoneally with a 23½ G needle attached to a 1ml syringe (TERUMO Medical Corporation, Elkton, MD 21921, USA).

8.2.4. Experimental inoculum

The *Aspergillus fumigatus* used was isolated from an infected chick airsac on a broiler farm in Gauteng, South Africa by Dr J. Picard and maintained on Sabouroud dextrose agar 6.5% supplemented with 50 mg/ml gentamicin at the Department of Veterinary Tropical Diseases, University of Pretoria. Asexual spores (conidia) were obtained from 3 day-old culture by flooding the plates with sterile distilled water, pelleted by centrifugation at 3500 x g for 10 min washed in phosphate-buffered saline (0.15 M) and quantified by determining turbidity with a spectrophotometer (CECIL CE 1011, 1000 series) at a wavelength of 540 nm (Delap et al., 1989).

8.2.5. Chemotherapeutic trial

Five chicks were randomly selected from the control group and sacrificed at the beginning of the experiment, their lungs were aseptically removed and contamination by *A. fumigatus* was evaluated by placing lung sections onto Sabouraud dextrose agar. The plates were incubated at 37 °C and the presence of *A. fumigatus* colonies was checked every day for 1 week (Femenia et al., 2007). The chicks (n=8) except the neutral group (n=10) were inoculated by transcutaneous injection into the right caudal thoracic air sac with 100 µl spore suspension of a 3-day-old *A. fumigatus* culture containing 10^8 spores. Birds were observed at least twice a day for the appearance of clinical signs of aspergillosis (Femenia et al., 2007). Clinical sign of infection (dyspnoea) was evident 3 days post infection. Group 1 served as neutral control and was neither infected nor treated with any substance. Birds in groups 2, 3, and 4 were treated with the extract at 50, 100 and 200 mg/kg, respectively; while groups 5 and 6 were dosed with ketoconazole (60 mg/kg) the positive control and 0.2% DMSO in water (0.2 ml/100 g) the vehicle respectively. All birds were marked or tagged for identification and were fed non-medicated feed, and clean water was provided freely. All treatments were instituted 3 days post infection and were given intraperitoneally once daily for 3 consecutive days.

8.2.6. Biochemical and haematological analysis

At the end of toxicity and during chemotherapeutic trials, blood samples from all birds were collected from the wing vein or by jugular venipuncture in vacutainer tubes with or without heparin as anticoagulant. Blood samples were centrifuged at 1200 *x g* for 15 minutes in a refrigerated centrifuge (4 °C) to separate serum. Alanine aminotransferase (ALT), aspartate aminotransferase (AST) and γ-glutamyltransferase (GGT) were measured using Alfa Wassermann. Total protein, albumin, calcium and phosphorous concentrations were measured using NExCT™ Total Protein reagent, NExCT™ Albumin reagent, ACE™ Calcium-Arsenazo reagent and Alfa Wassermann Inorganic Phosphorus reagent, respectively. Serum globulin levels were deduced by subtracting the albumin levels from the total protein levels (Gildersleeve et al., 1983), and the albumin/globulin (A/G) ratio calculated. The analyses were performed using the ACE TM and NExT TM Clinical Chemistry Systems (ALFA Wassermann, Bayer Health Care, and Johannesburg). Heparinized blood samples were analysed for haemoglobin (Hb), red cell count (RCC), haematocrit (HT) , mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), mean corpuscular haemoglobin concentration (MCHC) and red cell distribution width (RDW) using ADVIA 2120 haematology analyser (ADVIA, , Bayer Health Care, and Johannesburg). White cell count (WCC), absolute neutrophil (total), absolute neutrophil (matured), absolute neutrophil (immatured), absolute lymphocyte, absolute monocyte, absolute eosinophil, absolute basophil analyses were done using manual counter.

8.2.7. Mycological and pathologic analyses

Birds that died during the toxicity trial were examined by examining the lungs, airsacs, liver, heart, kidneys, spleen and brain observed for gross lesions. The organs were later fixed in 10% formaldehyde. Sections of the organs were embedded in paraffin wax, sectioned at 4 µm and stained with haematoxylin-eosin stain for histopathological examination (Femenia et al., 2007). Birds that survived were euthanized in a CO_2 chamber and similarly examined.

The lungs, air sacs and liver of birds that died during the chemotherapeutic trial were specifically evaluated for aspergillosis related lesions. The lungs were aseptically removed and individually plated on SDA using sterile swabs. Plates were incubated for 48 hours at 37 °C. When fungal colonies developed, species identification was done by microscopic examination of conidiophores and conidia, in addition to the observation of colony morphology. *A. fumigatus* is characterized by green echinulate conidia, 2 to 3 mm in diameter, produced in chains from greenish phialids, 6 to 8 mm by 2 to 3 mm in size (de Hoog et al ., 2000). A portion of the lungs was also fixed for histopathology as for the toxicity study. The lungs were,

however, specifically stained with Grocott methenamine (hexamine) silver for further examination of *Aspergillus fumigatus* hyphae in the tissue section (Bancroft and Gamble, 2003).

8.2.8. Evaluation of healing effect

At the end of the chemotherapeutic trial, the organism was recovered from infected chicks was and the severity of infection was graded. Microbiological severity of infection evaluated by adding up the total area covered by individual colony of *Aspergillus fumigatus* on the SDA plate and was graded as follows: no growth: grade 0; colonies < 2 mm^2: grade 1; colonies 2 -10 mm^2: grade 2; colonies 10-20 mm^2: grade 3, colonies > 20 mm^2: grade 4. Lesion scores were based on gross examination. The grading system proposed by Delap et al. (1989) used. Briefly, the grading is done as follows: grade 0: negative (no lesion), grade 1: localized plaque, grade 2: discrete plaque-moderate, grade 3: discrete plaque that is extensive, grade 4: confluent plaques.

8.2.9. Statistical analysis

Data obtained from the re-isolation steps of *A. fumigatus* and the sum of area of growth in infected and treated groups were expressed as mean ± S.E.M. Difference between the groups was analyzed using one way analysis of variance (ANOVA). Results were considered significant if $p \leq 0.05$. A post-hoc Dunnett test was used to test for differences to the control for which ANOVA indicated a significant ($p \leq 0.05$) F-ratio.

8.3. Results

8.3.1. Safety evaluation of the extract

Chicks treated with 50 and 200 mg/kg of the extract did not exhibit any clinical sign of toxicity and remained apparently healthy throughout the experimental period. However, at the dose of 300 mg/kg (starting dose), depression, decrease feed intake, diarrhoea, marked weight loss were noticed. One chick died 4 days post treatment (p.t.). In addition, there was marked increased in weight in the DMSO (control) group compared with chicks treated with 50 and 200 mg/kg of the extract. The effect of the extract on weight changes of the birds is presented in Figure 8.1. The extract caused decreased weight gain when administered at 300 mg/kg. Results pertinent for the serum biochemical profile and haematological analysis conducted at the end of the toxicity trial are presented in Table 8.1 and 8.2, respectively. Only the globulin concentration of chicks treated with 300 mg/kg of the extract differ significantly ($p \leq 0.05$) from other groups. Haematologically, the white cell count (WCC), absolute neutrophil (total and immatured) and absolute lymphocyte of chicks treated with the extract at 200 mg/kg differed significantly ($p \leq 0.05$).

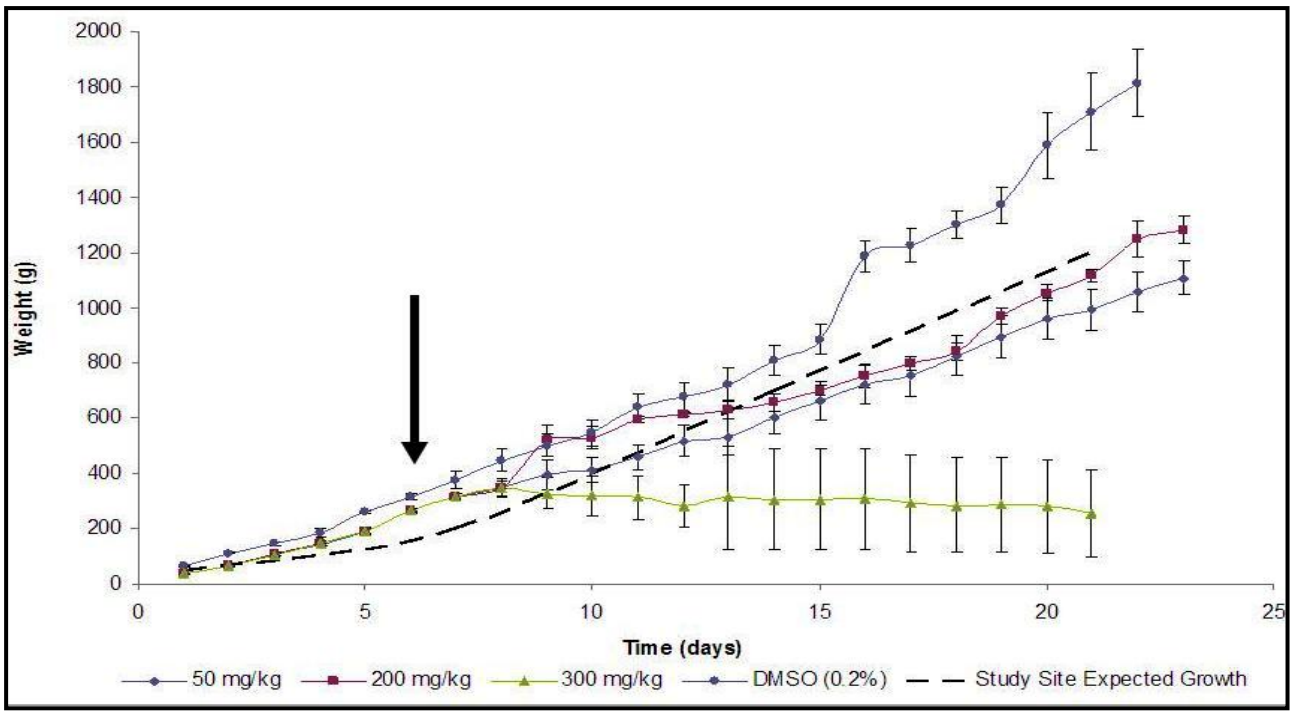


Figure 8.1. The effect of crude extract of *L. alata* on weight of broiler chicks. Arrow indicates time of extract administration.

Table 8.1. Biochemical indices of broiler-chicks given varying doses of crude extract of *Loxostylis alata* in the toxicity study.

Serum biochemistry	Dose administered (mg/kg)			
	Control (DMSO)	50	200	300*
Total serum protein (TSP)	28.53 ± 1.39	27. 13 ± 2.20	34. 43 ± 2.89	27.90 ± 0.35
Albumin (Alb)	16. 67 ± 0.58	15.03 ± 0.61	15.50 ± 1.53	15.50 ± 0.55
Globulin (Glob)	11.87 ± 0.83	12.10 ± 1.59	15.93 ± 3.88	6.70 ± 0.67[a]
Albumin/globulin ratio (A/G)	1.41 ± 0. 05	1.23 ± 0.12	0.96 ± 0.03	2.34 ± 0.01[a]
Calcium (Ca)	2.89 ± 0.03	2.58 ± 0.02	2.62 ± 0.04	2.60 ± 0.13
Serum inorganic phosphate (SIP)	2.45 ± 0.05	2.16 ± 0.07	1.87 ± 0.04	2.00 ± 0.12
Alanine amino transferase (ALT)	2.00 ± 0.82	1.33 ± 1.33	0.00 ± 0.00	2.00 ± 0.00
Aspartate amino transferase(AST)	176.67 ± 4.77	160.67 ± 13.61	148.00 ± 9.88	169.5 ± 9.53
γ-glutamyltransferase (GGT)	18.00 ± 0.47	23.00 ± 4.51	29.00 ± 1.53	23.00 ± 2.01

Means with superscript letter differ significantly ($p \leq 0.05$) from the control.
* Results were from 2 chicks, one of the chicks died 4-days p.t.

Table 8.2. Haematological indices of broiler-chicks given varying doses of crude extract of *Loxostylis alata* in the toxicity study.

Haematology	Dose administered (mg/kg)			
	Control (DMSO ml/kg)	50	200	300*
Haemoglobin (Hb)	143.33 ± 4.7	139.33 ± 0.88	144.67 ± 8.66	135.50 ± 1.50
Red cell count (RCC)	2.62 ± 0.12	2.50 ± 0.11	2.83 ± 0.09	2.40 ± 0.02
HT (haematocrit)	0.36 ± 0.01	0.34 ± 0.01	0.33 ± 0.03	0.30 ± 0.01
Mean cell volume (MCV)	140.00 ± 2.95	135.67 ± 1.77	126.33 ± 6.90	135.00 ± 1.00
Mean cell haemoglobin (MCH)	54.77 ± 0.84	54.17 ± 0.54	51.40 ± 3.22	55.10 ± 0.35
Mean cell haemoglobin concentration (MCHC)	39.27 ± 0.23	39.23 ± 0.84	40.87 ± 0.63	40.90 ± 0.05
Red cell distribution width (RDW)	13.47 ± 0.923	12.47 ± 0.67	14.77 ± 1.76	13.70 ± 1.15
White cell count (WCC)	3.73 ± 0.95	5.33 ± 1.09	19.80 ± 8.06[a]	4.40 ± 0.80
Absolute neutrophil (total)	1.31 ± 0.26	1.94 ± 0.47	10.31 ± 4.77[a]	2.30 ± 0.45
Absolute neutrophil (matured)	1.31 ± 0.26	1.94 ± 0.47	10.31 ± 4.77[a]	2.30 ± 0.45
Absolute neutrophil (immatured)	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
Absolute lymphocyte	1.99 ± 0.50	2.56 ± 0.49	6.25 ± 1.66[a]	1.50 ± 0.34
Absolute monocyte	0.35 ± 0.15	0.34 ± 0.05	1.83 ± 1.40	0.40 ± 0.16
Absolute eosinophil	0.00 ± 0.00	0.27 ± 0.11	0.88 ± 0.03	0.1 ± 0.03
Absolute basophil	0.09 ± 0.06	0.07 ± 0.04	0.42 ± 0.02	0.00 ± 0.00

Means with superscript letter differ significantly ($p \leq 0.05$) from the control.
Results were from 2 chicks, one of the chicks died 4-days p.t

Gross post-mortem results showed moderate ascites, hydropericardium, lung oedema and, soft and pliable kidneys in the chick that died from group treated with 300 mg/kg. The remaining two chicks that were sacrificed at the end of the experiment from the same group showed similar pathological signs. Birds treated with 50 and 200 mg/kg of the extract showed no gross pathological signs. Birds treated with 300 mg/kg had histological lesions i.e. scattered bile duct proliferation, focal periductular fibrosis and lymphocytes accumulation in the liver tissues (Figure 8.2). The heart and lungs had lympoplasmacytic pericarditis and moderate fibrinopurulent bronchitis (Figures 8.3 and 8.4), respectively. No changes were apparent in the organs of birds treated with 50 and 200 mg/kg of the extract.

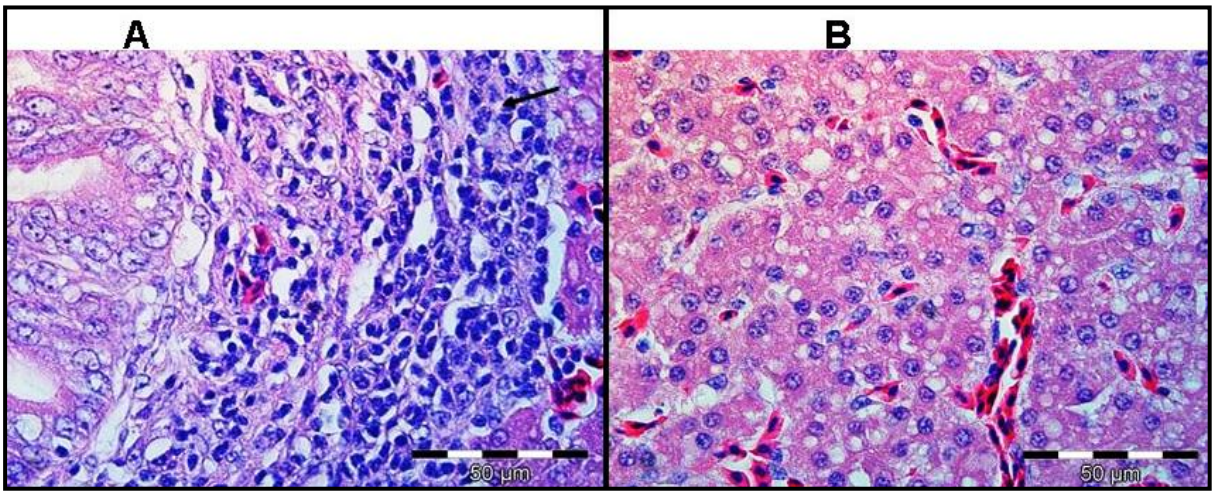


Figure 8.2. Liver tissue of birds treated with 300 mg/kg of extract (A) showing scattered bile duct proliferation, focal periductular fibrosis and lymphocytes accumulation (arrow). Normal liver tissue (B) is shown. H&E

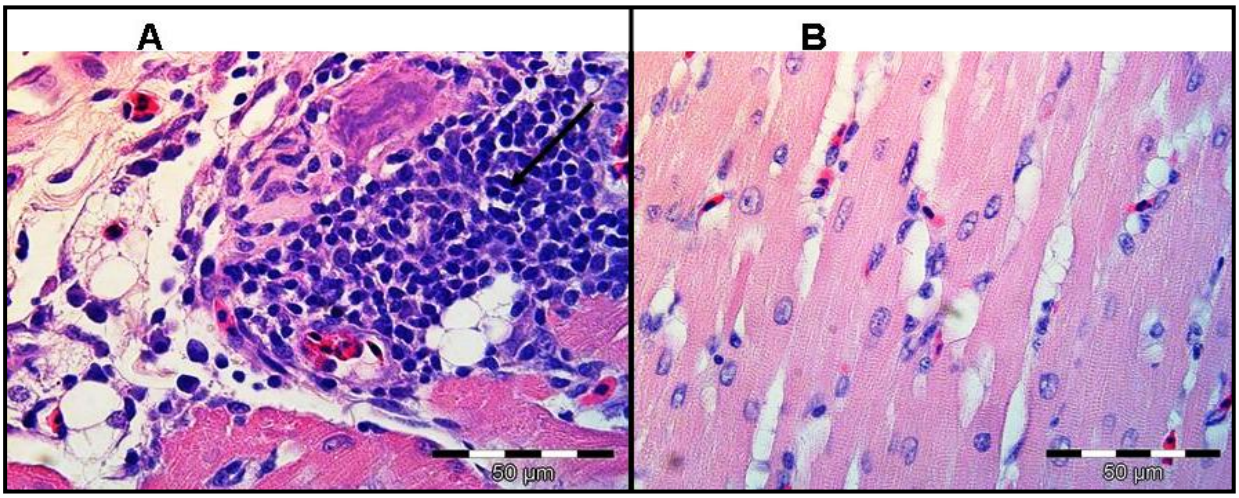


Figure 8.3. The heart tissue of birds treated with 300 mg/kg of extract (A) showing lympoplasmacytic pericarditis (arrow). Normal heart tissue (B) is shown. H&E

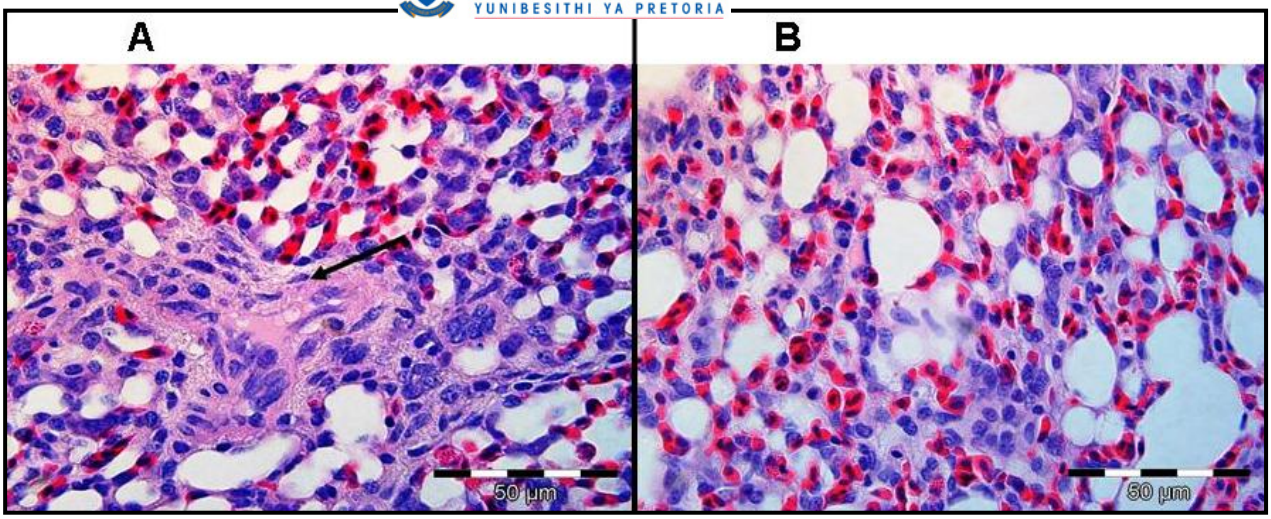


Figure 8.4. The lungs tissue of birds treated with 300 mg/kg of extract (A) showing moderate fibrinopurulent bronchitis (arrow). Normal lung tissue (B) is shown. H&E

8.3.2. Chemotherapeutic trial

8.3.2.1. Clinical signs and survivability

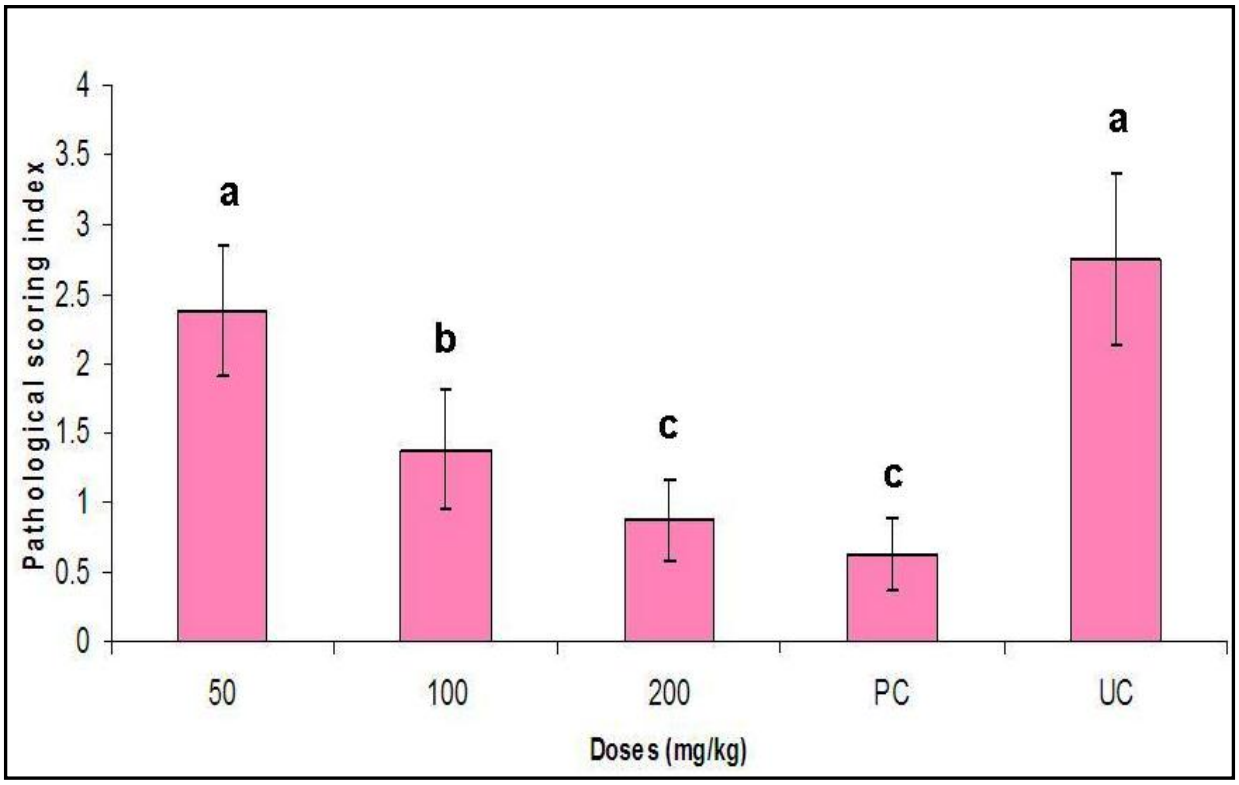
Chicks in the infected groups had clinical signs 3 days post infection (p.i), when one chick (out of eight) in the non-treated control group exhibited mild respiratory distress, ruffled feathers, weakness and diarrhoea. The birds in the non-infected and non-treated (negative) group remained apparently normal throughout the experimental period. The effect of the extract on survival of chicks infected with *Aspergillus fumigatus* is presented on Table 8.3. The extract produced a dose-dependant protection against death in chicks infected with *Aspergillus fumigatus*.

Table 8.3. The effect of the extract dose on survival of chicks infected with *Aspergillus fumigatus*.

Dose (mg/kg)	No. of chicks	No. survived	No. death	% Mortality
0	8	2	6	75
50	8	3	5	62.5
100	8	3	5	62.5
200	8	6	2	25
Ketaconazole (60 mg/ml)	8	7	1	12.5
Neutral	10	8	0	0

8.3.2.2. Macroscopic lesions

Gross lesions were detected at necropsy in all the infected-treated birds. Lesions consisted of small (1 to 3 mm) white nodules on the surface of the lungs and liver. Cloudiness of the thoracic air sacs was also noticed. However, the severity of the lesions was lower in chicks treated with the extract and also in the positive control group (ketaconazole treated chicks). The extract, therefore reduced the severity of the lesions in a dose-dependent fashion (Figure 8.5).

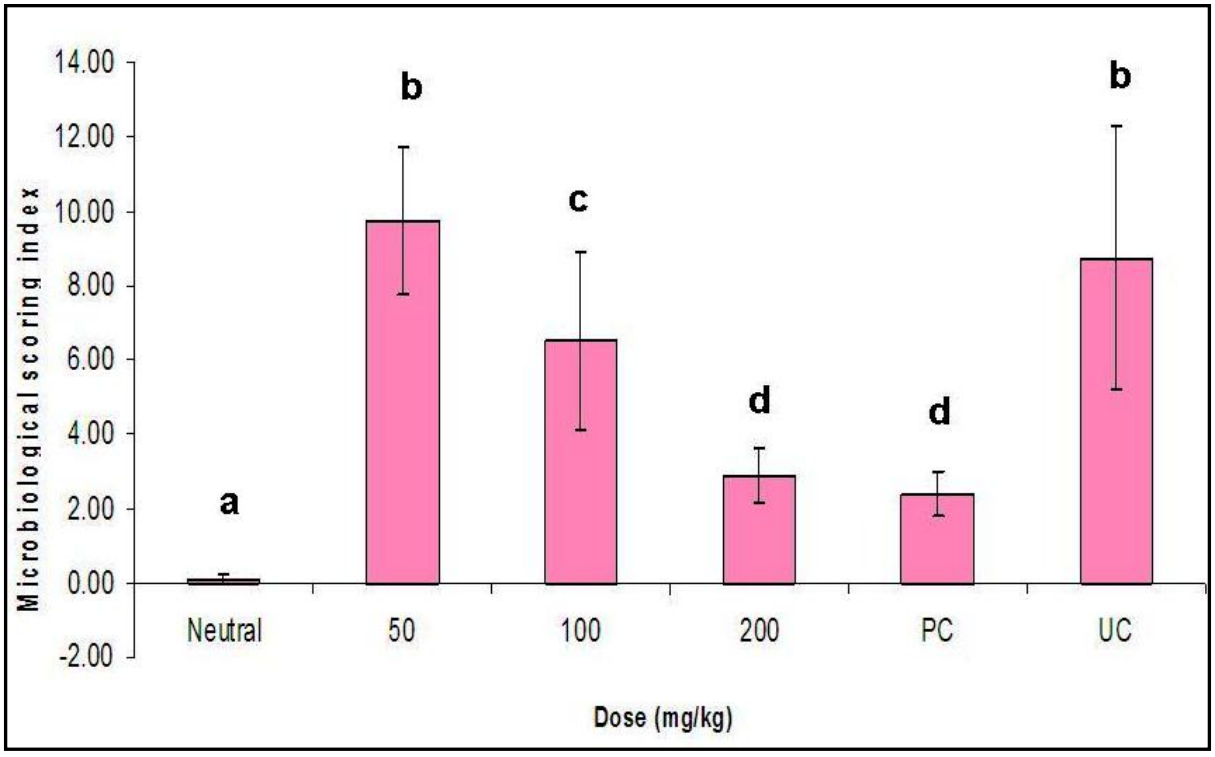


PC = positive control (ketoconazole); UC = untreated control (DMSO)

Figure 8.5. Effect of different doses of *L. alata* extract on pathological lesions in the lungs, airsac and liver of broiler chicks caused by *A. fumigatus*. Values are mean ± SEM. Means with different letter alphabet differ significantly with control and the other treatment groups ($p \leq 0.05$). No pathological lesion was recorded in the uninfected and untreated control chicks.

8.3.2.3. Mycological cultures

No *Aspergillus fumigatus* was isolated from the 5 chicks sacrificed at the start of chemotherapeutic trial. In the infected and treated groups varying amounts of *A. fumigatus* colonies were isolated from the airsac, lungs and liver tissue from days 3 to 7 days p.i. In addition, *A. fumigatus* was also isolated from the lung sample of one of the chicks in the neutral control group (Figure 8.6) indicating a subsequent contamination.



PC = positive control (ketaconazole); UC = untreated control (DMSO)

Figure 8.6. Re-isolation of *A. fumigatus* from the lung, airsac and liver of infected broiler chicks treated with different doses of *L. alata*. Values are mean ± SEM. Means with different letter alphabet differ significantly ($p \leq 0.05$) for the control and the other treatment groups.

8.3.2.4. Special histological evaluation of lungs

Sections of lungs from infected chicks showed the presence of fungal hyphae when special staining method was used. However, no fungal hyphae were noted in the lung samples of chicks in the neutral control group. Figure 8.7 showed the presence of small hyphae of *A. fumigatus* radiating in a pulmonary lobule.

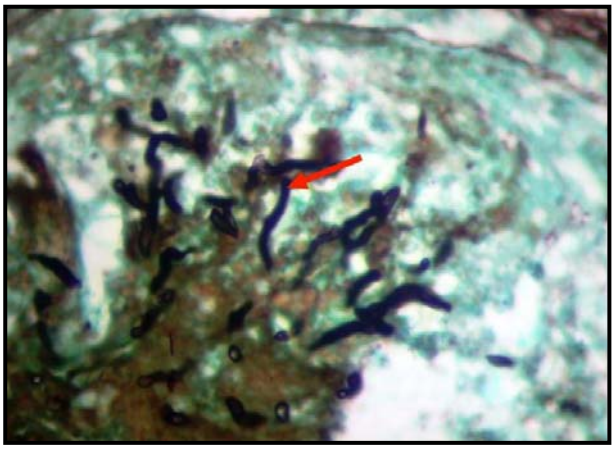


Figure 8.7. *Aspergillus fumigatus* hyphae (arrow) radiating in a pulmonary lobule of one of the infected chicks (X1000).

8.3.2.5. Biochemical and haematological profile

Results for the serum and blood analysis of chicks infected with *A. fumigatus* are presented in Tables 8.4 and 8.5, respectively. There was a significant ($p \leq 0.05$) increase in the concentration of aspartate amino transferase and globulin in chicks that received DMSO. Likewise, serum gamma glutamyl transferase (GGT) were markedly ($p \leq 0.05$) increased in chicks treated with ketaconazole and DMSO. Haematologically, the white cell count (WCC), absolute neutrophil (total and matured) and absolute monocytes in chicks treated with the extract at 200 mg/kg and ketaconazole do not differ significantly ($p \leq 0.05$), however, they increase markedly ($p \leq 0.05$) in untreated control groups.

Table 8.4. Biochemical indices of broiler-chicks infected with *A. fumigatus* and treated varying doses of crude extract of *Loxostylis alata*.

Serum biochemistry	Dose administered (mg/kg)					
	Neutral	50	100	200	PC (60)	UC
Total serum protein (TSP)	25.52 ± 0.48	26.67 ± 0.90	28.98 ± 0.74	30.07 ± 2.26	30.90 ± 2.03	36.05 ± 9.15
Albumin (Alb)	14.33 ± 0.26	14.43 ± 0.12	15.98 ± 0.62	15.33 ± 0.39	15.90 ± 0.17	15.60 ± 0.00
Globulin (Glob)	11.19 ± 0.30	12.23 ± 0.87	13.00 ± 0.35	14.74 ± 2.34	15.00 ± 1.93	24.50 ± 9.20[a]
Albumin/globulin ratio (A/G)	1.29 ± 0.03	1.19 ± 0.09	1.23 ± 0.06	1.13 ± 0.10	1.15 ± 0.12	0.61 ± 0.43[a]
Calcium (Ca)	3.29 ± 0.10	3.42 ± 0.10	3.53 ± 0.14	3.37 ± 0.16	3.40 ± 0.10	2.92 ± 0.07
Serum inorganic phosphate (SIP)	1.43 ± 0.08	1.59 ± 0.10	1.60 ± 0.10	1.66 ± 0.10	1.65 ± 0.11	2.38 ± 0.96
Alanine amino transferase (ALT)	3.00 ± 0.61	3.33 ± 1.67	2.80 ± 0.97	4.86 ± 2.58	1.71 ± 0.57	1.50 ± 1.50
Aspartate amino transferase(AST)	149.20 ± 4.08	153.00 ± 8.54	138.60 ± 7.26	155.00 ± 6.24	129.43 ± 9.86	171.50 ± 22.20[a]
γ-glutamyltransferase (GGT)	19.60 ± 0.50	22.67 ± 0.88	23.00 ± 2.45	24.14 ± 4.12	26.57 ± 3.09	36.50 ± 17.50[a]

Neutral group are chicks that were not infected with *A. fumigatus*, and were also not treated. Positive control (PC) (ketoconazole); Untreated control (UC).
Means with superscript letter differ significantly ($p \leq 0.05$) from the control.

Table 8.5. Haematological indices of broiler-chicks infected with *A. fumigatus* and treated varying doses of crude extract of *Loxostylis alata*.

Serum haematology	Dose administered (mg/kg)					
	Neutral	50	100	200	PC (60)	UC
Haemoglobin (Hb)	131.60 ± 1.93	134.33 ± 4.10	140.00 ± 2.92	133.14 ± 2.38	141.71 ± 5.97	138.00 ± 1.00
Red cell count (RCC)	2.43 ± 0.40	2.48 ± 0.11	2.60 ± 0.06	2.56 ± 09	2.70 ± 0.18	2.77 ± 0.10
HT (haematocrit)	0.34 ± 0.10	0.34 ± 01	0.35 ± 0.01	0.34 ± 01	0.36 ± 02	0.35 ± 0.01
Mean cell volume (MCV)	138.10 ± 1.06	139.00 ± 1.15	136.40 ± 3.75	134.43 ± 2.10	134.71 ± 3.31	125.50 ± 6.50
Mean cell haemoglobin (MCH)	54.14 ± 0.45	54.33 ± 0.94	53.80 ±0.86	52.29 ± 1.06	52.93 ± 1.40	50.00 ± 2.30
Mean cell haemoglobin concentration (MCHC)	39.17 ± 0.28	39.10 ± 0.53	39.52 ± 0.52	38.90 ± 0.35	39.33 ± 0.29	39.95 ± 0.25
(Red cell distribution width) RDW	12.83 ± 0.13	13.53 ± 0.38	13.40 ± 0.57	14.07 ± 0.41	13.93 ± 1.04	14.30 ± 1.40
White cell count (WCC)	15.54 ± 2.13	18.80 ± 1.50	16.60 ± 3. 18	28.29 ± 6.60^{a}	29.43 ± 10.32^{a}	49.00 ± 13.21^{b}
Absolute neutrophil (total)	4.71 ± 0.96	8.82 ± 1.04	6.71 ± 2.58	13.04 ± 4.52^{a}	17.79 ± 7.65^{a}	26.28 ± 16.36^{b}
Absolute neutrophil (mature)	4.71 ± 0. 96	8.82 ± 1.04	6.71 ± 2.58	13.04 ± 4.52^{a}	17.79 ± 7.65^{a}	26.28 ± 16.36^{b}
Absolute neutrophil (immature)	0.00 ± 00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00

Neutral group are chicks that were not infected with *A. fumigatus*, and were also not treated. Positive control (TC); Untreated control (UC)
Means with differing superscript letters in the same row are significantly different ($p \leq 0.05$).

8.4. Discussion and conclusions

Generally, aspergillosis in chickens is an acute disease causing high mortality in the first few days of life. The disease in some cases can be chronic, with lesions in the lungs, air sacs, and joints (Cutsem, 1983). Evidence of clinical signs associated with *A. fumigatus* infection was quite evident in this study, which is in agreement with was reported in literature.

Our data indicated that the crude extract of *L. alata* has certain level of activity against aspergillosis in broiler chicks and that these birds could serve as model for the study of agents intended for development into safer and effective anti-aspergillus agents. Plant drugs have been assumed to be safe. However, in recent times, there is evidence that many plants used as food or as medicine can be potentially toxic (Kassie et al., 1996; De Sã Ferrira and Ferrão Vargas, 1999). All drugs can produce harmful as well as beneficial effects, hence the need for toxicity testing of any substance intended for development into drug for human or animal use (Rang et al., 2003). Investigation of the acute toxicity is the first step in the toxicological investigations of an unknown substance (Lorke, 1983). The index of the acute toxicity is the lethal dose 50 (LD_{50}). However, the LD_{50} is not regarded as a biological constant, since differing results are obtained on repetition (Lorke, 1983). The OECD guidelines for testing chemicals (2000) for toxicity were adapted in testing the safety of the extract of *Loxostylis alata*. The method is not intended to allow the calculation of a precise LD_{50}, but does allow for the determination of defined exposure ranges where lethality is expected since death or appearance of toxic signs of a proportion of the animals is still the major endpoint of this test. The extract was toxic at the dose of 300 mg/kg based on its effects on different parameters measured and clinical signs noticed on the experimental chicks. Dose is an important factor in drug poisoning and most drugs cause clinical signs of poisoning when administered at relatively high dose (Rang et al., 2003). However, at lower doses (200 mg/kg and below) the extract had no apparent toxic effects. Although the chicks treated with 50 and 200 mg/kg had lower weight gain than those in the control group, the weight gain in all the chicks except those treated with 300 mg/kg are all within normal growth rate for broiler chickens (Naidoo et al., 2008; SAPA, 2008).

During preliminary chemotherapeutic trial, we were not able to establish an infection using 10^7 spore suspension of *A. fumigatus* when administered intraperitoneally similar to that reported by Femenia et al (2007). Infection in our study was established using 10^8 spores of *A. fumigatus* suspended in 0.2 ml of sterile distilled water administered intraperitoneally. However, Fadl Elmula et al. (1984) inoculated a lower number of spores (4×10^5 per chick) intraperitoneally to cause infection in 6-day old chicks. Disruption of the *alb1* gene in *A. fumigatus* leads both to virulence attenuation and to an immune response in the form of

increased complement binding and neutrophil-mediated phagocytosis. Hence the mutation led to increased recognition of the conidia by the immune system (Odds et al 2001). Perhaps a similar mechanism of virulence attenuation affected the isolate used for this study, which lowers the infectivity of the organism.

Clinical signs of aspergillosis observed in our study included ruffled feathers, gasping, dyspnoea, dullness, green watery diarrhoea and anorexia. These signs are in agreement and confirmed the clinical signs observed in previous studies (Akan et al., 2002). Although ascites and blindness were observed by Julian and Goryo (1990) and Akan et al (2002), respectively, we did not observe these clinical features in our study. Chicks in this study started dying 3 days p.i. with mortality reaching as up to 75% in the non-treated group. Gűműşsoy et al (2004) inoculated 3-week old quails with *A. fumigatus* intratracheally and recorded mortality of up to 100%. Furthermore, *A. fumigatus* was re-isolated from the lungs and airsacs of infected chicks starting 3 days p.i and up to 12 days p.i. Lesions were seen mainly in the lungs, liver and the airsac. Surprisingly, *A. fumigatus* was isolated from the lung sample of one chick in the neutral group, which was not infected and also not treated. Since systemic infection with *Aspergillus fumigatus* results naturally through the inhalation of spores, (Julian and Goryo, 1990) it is possible that the chicks in the neutral group were infected naturally from fungal spores inhaled in the air. However, since, we use a specific histological staining technique to confirm the presence of an infection, we are certain that the positive result was due to contamination during post mortem examination.

Increase AST and GGT activity as well as A/G ratio are indicative of a liver injury. This was supported by the isolation of the agent from liver. In addition, there was presence of lesions on the liver

Compared with the infected untreated group with a mortality rate of 75%, in the entire infected treated group, the mortality rate reduced and death was stopped on 11th day post-infection. Treatment with extract of *Loxostylis alata* at doses used confer a dose related success in combating infection due to *A. fumigatus* in broiler chicks and that compared favourably with the reference compound (ketoconazole). Compounds administered i.p. are absorbed primarily through the portal circulation and, therefore, must pass through the liver before reaching other organs. This factor can limit the amount of drug reaching its site of action (Lukas et al., 1971). Indeed, there are numerous examples in the literature of drugs that are less effective after i.p. administration than when given by other parenteral routes. Such examples include reserpine (Mueller and Shideman, 1968), phenelzine and phenipramine (Horita, 1961). It will therefore be reasonable to assume that when the extract is administered via another route that bypasses the portal circulation that could result in enhanced action of the extract.

In conclusion, broiler chicks are susceptible to the infection with *A. fumigatus* and the extract of *L. alata* appears to be beneficial in treating and curing it. Additional work is needed on extracts of this species to further establish its detailed safety and efficacy against *A. fumigatus* in broiler chicks and other target species.

Chapter 9
General discussions and conclusions

Aspergillus fumigatus is one of the most common pathogenic fungal species in humans and animals (Rippon, 1982). *A. fumigatus* also causes severe financial problems in the poultry industry. Current treatment of the disease is hampered by drug resistance of the organism to conventional antifungals and also their widespread toxicity to animals. The aim of this study was to find a plant extract or isolated compound that could be used to combat aspergillosis in animals. To attain this aim a number of objectives were identified i.e. to

- Evaluate the antibacterial and antifungal activity of selected South African plant species against a range of pathogenic bacterial and fungal species in order to select the species with the best antimicrobial activity for further investigation.
- Isolate and characterize the compounds active against *Aspergillus fumigatus* from extracts of selected plant species.
- Determine the in *vitro* antimicrobial, antioxidant and anti-inflammatory activities and cytotoxicity of the extracts and isolated compound(s) of selected plant species.
- Evaluate the efficacy of the isolated compound(s) or crude extracts *in vivo* in a poultry model.

The degree to which these objectives were attained is discussed below.

9.1. Evaluate the antimicrobial activity of selected South African plant species

The antifungal and antibacterial activities of extracts of leaves of seven selected trees were determined. Extracts of *Loxostylis alata* had more active compounds on bioautograms against all the bacteria (*Staphylococcus aureus, Escherichia coli, Enterococcus faecalis* and *Pseudomonas aeruginosa*) and fungi (*Candida albicans, Cryptococcus neoformans, Microsporum canis, Sporothrix schenckii* and *Aspergillus fumigatus*) used as test organisms and hence were selected as the most promising species to investigate in more detail. It appeared that the same compounds (based on their identical R_f values) inhibited several bacteria and fungi. This non-specificity of activity could be due to a broad antimicrobial action of the plant compounds or the compounds could be general metabolic poisons inhibiting many types of living cells. Acetone is an intermediate polar solvent which extracts both polar and non-polar constituents of plant. The acetone extract of *Loxostylis alata* therefore contained more antibacterial and antifungal compounds than all other extracts. Acetone extracts of *Curtisia dentata* also contained more active compounds against the same bacteria and fungi tested in this work than other extracts used (Shai, 2007). In contrast, the

dichloromethane and hexane extracts of six *Terminalia* species had more antifungal compounds than the acetone extracts (Masoko *et al.* 2005).

All the extracts of the plant species used in this study had antimicrobial activity with MICs generally less than 0.1 mg/ml against at least one pathogen..The lowest MIC of 0.04 mg/ml was of the acetone extract of *Loxostylis alata* against *Sporothrix schenckii*.

9.2. Isolate and characterise the compounds active against *Aspergillus fumigatus* from extracts of selected plant species

9.2.1. Selection of the most promising tree species for further work

From the seven plant species that were screened for antimicrobial activity, *Loxostylis alata* was selected for further work because the powdered leaf of *Loxostylis alata* gave the highest yield of extract (21.8%) and had the highest average total activity (TA) of 1343 and 1414 ml/g against bacteria and fungi, respectively. Total activity is calculated by dividing the quantity extracted from one gram of plant material with the MIC value in mg/ml. This value indicates the volume to which the active constituent present in one gram of the plant material can be diluted and still inhibit the growth of the test organism. Furthermore, leaf extracts of *L. alata* had more compounds active against *Aspergillus fumigatus* and other tested microbial organisms on bioautograms. The acetone extract of the leaves of *Loxostylis alata* had an MIC of 0.07 mg/ml against *Aspergillus fumigatus*. These factors made the plant a potential source of active compounds or extracts for the treatment of *Aspergillus fumigatus* infection. To promote the sustainable use of plant and protect the environment only the leaves were used in this study.

9.2.2. Isolation and characterisation of compounds

The acetone extract (70 g) was subjected to solvent-solvent fractionation using carbon tetrachloride, hexane, chloroform, aqueous methanol, butanol and water. The carbon tetrachloride (CCl_4) fraction had the best antimicrobial activity (MIC and TA value of 0.08 mg/ml and 32012 ml/g, respectively) and therefore, chosen for further isolation of active compounds.

Using bioassay-guided fractionation two active compounds against *A. fumigatus* were isolated from the leaves of *Loxostylis alata.* ^{13}C and ^{1}H NMR spectroscopic and mass spectrometric data led to identification of the compounds as the lupane triterpenoid lupeol and β-sitosterol. Although these compounds are common secondary metabolites present in many plant species (Imam et al., 2007; Lopes et al., 1999) it is reported for the first time from *L. alata* leaves.

9.3. Determine the *in vitro* antimicrobial, antioxidant and anti-inflammatory and cytotoxicity activities of the extracts and isolated compound(s) of selected plant species

9.3.1. Antimicrobial activity

The antifungal activitiy of the fraction of *L. alata* were determined prior to the bioassay guided fractionation. The carbon tetrachloride fraction was the most active with an MIC of 0.08 mg/ml against *Aspergillus fumigatus*. Similarly, the carbon tetrachloride fraction of *L. alata* had a more lines of inhibition against all the tested microorganisms on bioautograms. The hexane and aqueous methanol fractions had few line of inhibition, while the butanol and water fractions did not have any.

Lupeol had reasonable activity against all the tested pathogens with MICs ranging from 29-120 µg/ml. Similarly, lupeol had good activity against *C. albicans, S. aureus, E. coli* and *Enterococcus faecalis* in a similar study (Shai 2007). The isolated compounds from *L. alata* leaves are widely distributed in most plants and hence are not novel in character. The activity of the isolated compounds were lower against *A. fumigatus* than that of the crude extract of *L. alata*. Unless these compounds are destroyed during chemical elution by chromatographic means or bioautography, it appears that synergism plays an important role in the activity of the plant against *A. fumigatus*. The other compounds involved in synergism appear not to be active on their own.

9.3.2. Antioxidant activity

Antioxidants may boost the immune response and by so doing assist the body to combat microbial infections by reversing several conditions associated with immune deficiencies. This prompted us to investigate the antioxidant activity of the plant using both ABTS and DPPH radicals. The extract of *L. alata* had a trolox equivalent antioxidant capacity (TEAC) value of 1.94 and an EC_{50} value of 3.58 ± 0.23 µg/ml, which compares favourably with that of Trolox and L-ascorbic acid (positive controls) of 1 and 1.59 ± 0.80 µg/ml, respectively. The aqueous methanol, butanol and water fractions had antioxidant activities with EC_{50} values of 1.82 ± 0.03, 1.05 ± 0.06 and 0.62 ± 0.03 µg/ml respectively in the DPPH assay. The reference compound (L-ascorbic acid) had an EC_{50} of 0.94 ± 0.11 µg/ml. The lower the EC_{50} value of a substance, the more effective its antioxidant activity. Similarly, the TEAC values of aqueous methanol, butanol, water, chloroform, carbon tetrachloride and hexane fractions were 1.55, 2.21, 2.97, 0.45, 0.12 and 0.56, respectively. A TEAC value greater than 1 indicates better antioxidant activity than that of the reference compound (trolox).

As determined later increased white cell counts (neutrophils and lymphocytes) were recorded in broiler chicks treated with the extract of *L. alata*. Antioxidants in the extract of *L. alata* may have been responsible

for the antifungal action of the extract against *Aspergillus fumigatus* by boosting the host immune response via stimulation of white cell production.

9.3.3. Anti-inflammatory assay

Antioxidants limit the progression of inflammatory diseases by scavenging free radicals produced during inflammation (Knight, 2000). The anti-inflammatory action of *L. alata* was therefore also investigated. Moreover, *L. alata* is used in traditional medicine to treat inflammation and boost the host immune response (Pell, 2004). The action of phospholipase A_2 on cell wall leads to the production of free arachidonic acid (AA). The AA is converted to thromboxane A_2 by cyclooxygenase 1. Thromboxane A_2 is a powerful inducer of platelet aggregation (Vane and Botting, 1987). Inhibition of cyclooxygenase 1 in platelets will limit or stop the production of thromboxane A_2 and prostaglandins (inflammatory mediators). These effects will then prevent platelet aggregation. Platelets therefore serve as a model for *in vitro* anti-inflammatory testing of the plant extracts. Moreover, platelets have an important role in acute inflammation by releasing arachidonic acid (AA) metabolites and PAF (Holmsen et al., 1977; Vincent et al., 1977).

Platelet aggregation inhibition is achieved if the inhibitory agents are able to cross the cell membrane barrier of platelets. If extracts prevented platelet aggregation in this study it means that compounds have the capacity to move across the cell membrane barrier. Lipid soluble drugs are usually non-ionised in solution and have the capacity to diffuse across cellular membranes (Wilkinson, 2006). The intermediate polariry of compounds in some extracts may explain the activiy or inactivity of some of the extracts on platelet aggregation. The extract of *Loxostylis alata* had activity against *in vitro* adrenaline-induced platelet aggregation with EC_{50} of 0.35 ± 0.03 µg/ml which compares favourably with that of aspirin a standard antiplatelet agent. That action could also be beneficial in preventing thromboembolic disorders.

Pathogenic fungi (dermatophytic, subcutaneous, and systemic) have the ability to produce eicosanoids (prostaglandins and leukotrienes) both from host derived arachidonic acid. Host-derived eicosanoids can enhance fungal colonization and the development of inflammation during fungal infections (Noverr et al., 2002).

The crude acetone extract of *L. alata* and the isolated compounds were screened against cyclooxygenase enzymes -1 and -2 (COX-1 and -2).). Both COX -1 and -2 regulate the biosynthesis of prostaglandins from arachidonic acid. COX-1 is a constitutive form and has a clear physiological function while COX-2 is induced by inflammatory mediators. It is the inhibition of prostaglandin synthesis by COX-1 and -2 that is responsible for the anti-inflammatory actions of NSAIDs. The crude extract of *L. alata* and lupeol inhibited COX-1 in a dose dependent manner (IC_{50} = 92.5 ± 1.6 and 134.0 ± 5.1 µM, respectively). Neither the crude extract nor the 2 isolated compounds had an inhibitory effect on COX-2. In addition, COX-1 selective inhibitors have beneficial anti-thrombotic effect. Inhibition of COX-1 by both the crude extract and lupeol

may exert beneficial anti-thrombotic effect and protect from heart diseases. Furthermore, as evidence the crude extract of *Loxostylis alata* inhibited equine platelet aggregation.

9.3.4. Cytotoxic activity

The acetone extracts of the selected plants had comparably low toxicity against Vero Monkey kidney cells and equine red blood cells *in vitro* except for the extract of *C. harveyi* which had a higher haemagluttination assay titre value indicating toxicity of the plant extract. The viability of the cells was between 70-90% when the extract of *L. alata* was used at concentrations of 0.01-0.001 mg/ml. Over 90% of Vero cells treated with berberine (reference compound) at the concentration of 0.01 mg/ml were not viable. Similarly, the extract of *L. alata* had a haemagglutination titre (HA) of 0.8 at a concentration as high as 1.25 mg/ml. These values indicate low toxicity of the extract of *L. alata*. The isolated compounds (lupeol and β-sitosterol) were relatively non-toxic with an LC_{50} of 76.66 ± 4.13 and 136.60 ± 7.20 µg/ml, respectively compared to the reference compound berberine (LC_{50} of 6.36 ± 0.81 µg/ml). β-sitosterol which occurs as sterol in many plants is potentially useful in improving human health as an anti-inflammatoriy, antipyretic, immunomodulator, or antineoplastic agent.The two compounds isolated from *L. alata* had no mutagenic effect on *Salmonella*/ microsome tester strains TA98 and TA 100.

9.4. Evaluate the efficacy of the crude extract *in vivo* in a poultry model

The crude acetone extract of *Loxostylis alata* was investigated for its safety and *in vivo* antifungal activity against experimental aspergillosis in broiler chicks. At a dose of 300 mg/kg, the extract had some toxic effect causing depression, decrease feed intake, diarrhoea, weight loss and death in broiler chicks. The method employed for toxicity testing in this study allows for the determination of defined exposure ranges where lethality is expected since death or appearance of toxic signs of a proportion of the animals is the major endpoint.

Antifungal infection was assessed by comparing the degree and severity of clinical signs, lesion scores and fungal re-isolation observed from treated chicks with those observed from infected chicks not treated with the extract. The extract at a dose of 100 and 200 mg/kg significantly reduced ($p \leq 0.05$) the lesions due to aspergillosis and the quantity of *Aspergillus* organisms isolated from infected chicks in a dose dependent fashion. Moreover, the extract of *L. alata* at a dose of 200 mg/kg enhanced the survival of broiler chicks by 75% since 6 out of the 8 infected chicks survived compared to only 2 out of 8 (25%) in the infected untreated control group. The survivability of chicks treated with ketaconazole (positive control drug) at a

dose of 60 mg/kg was 87.5%. The *L. alata* extract appears to have substantial value in treating and curing aspergillosis, a major health problem facing the poultry industry.

9.5. Conclusions

The crude acetone extract at a dose of 200 mg/kg was as effective as ketoconazole (positive control). The pharmacological action of *Loxostylis alata* could possibly be attributed to the combined action of both antioxidant and antifungal compounds present in the crude extract. 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 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.

Chapter 10

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CHAPTER 11: APPENDIX

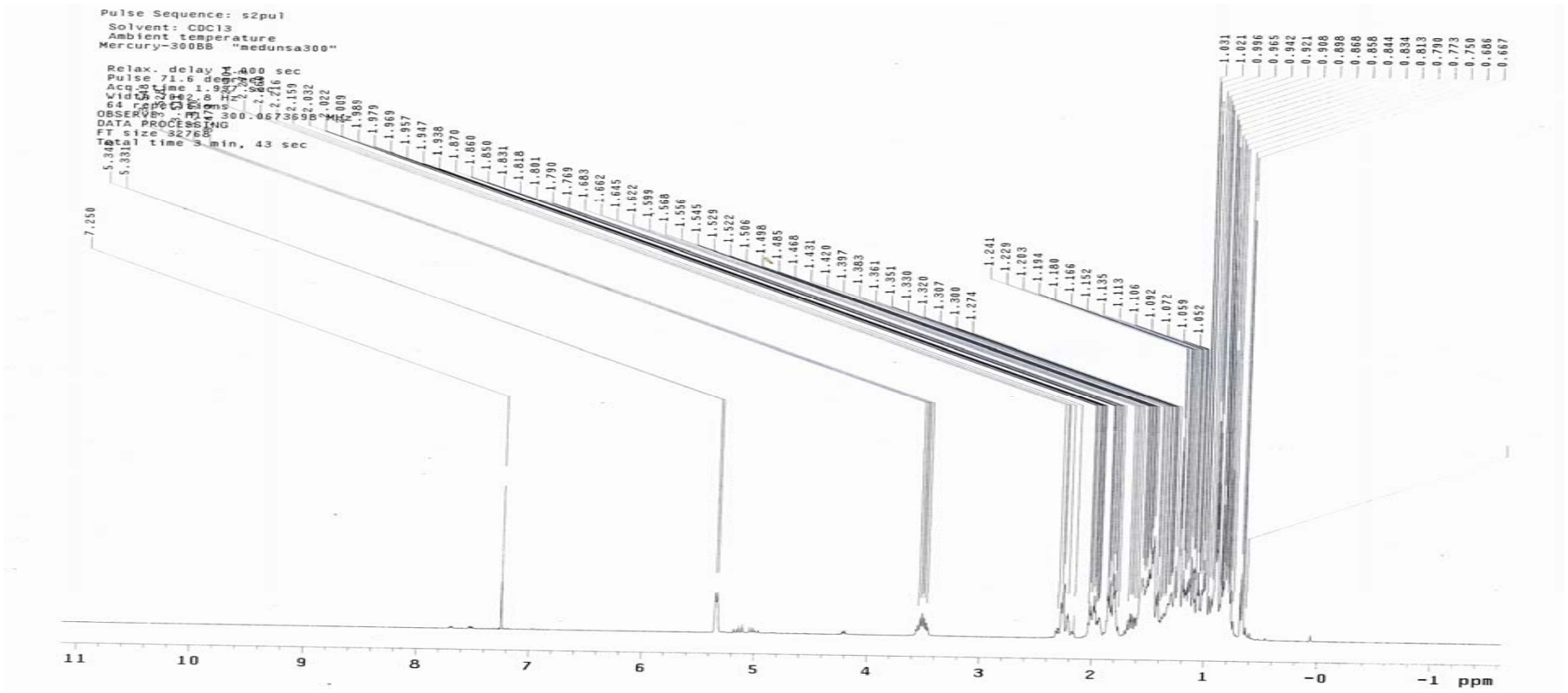

Figure A.1. The ^{13}C NMR spectrum of lupeol isolated from the leaves of *Loxostylis alata*

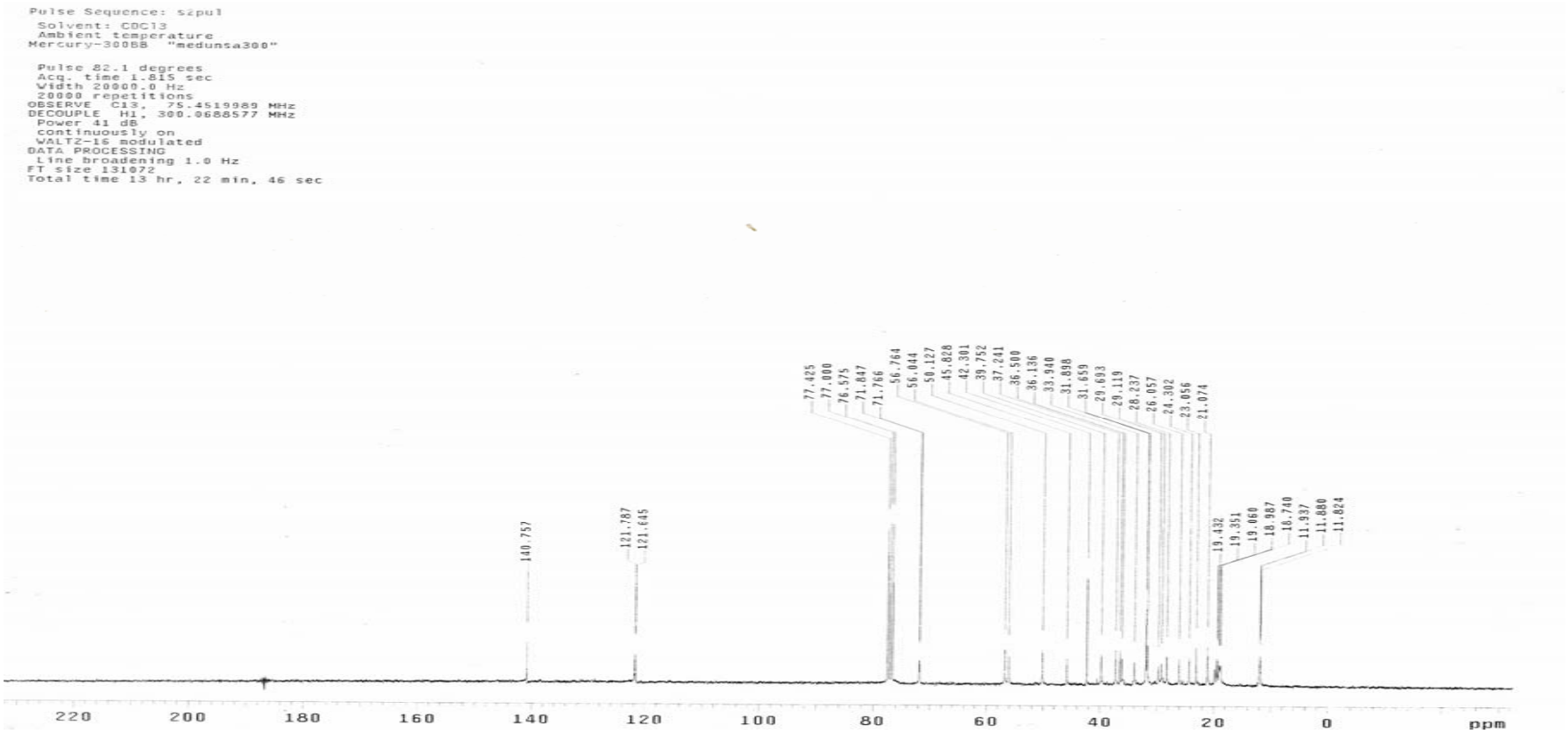

Figure A.2. The ^{1}H NMR spectrum of lupeol isolated from the leaves of *Loxostylis alata*

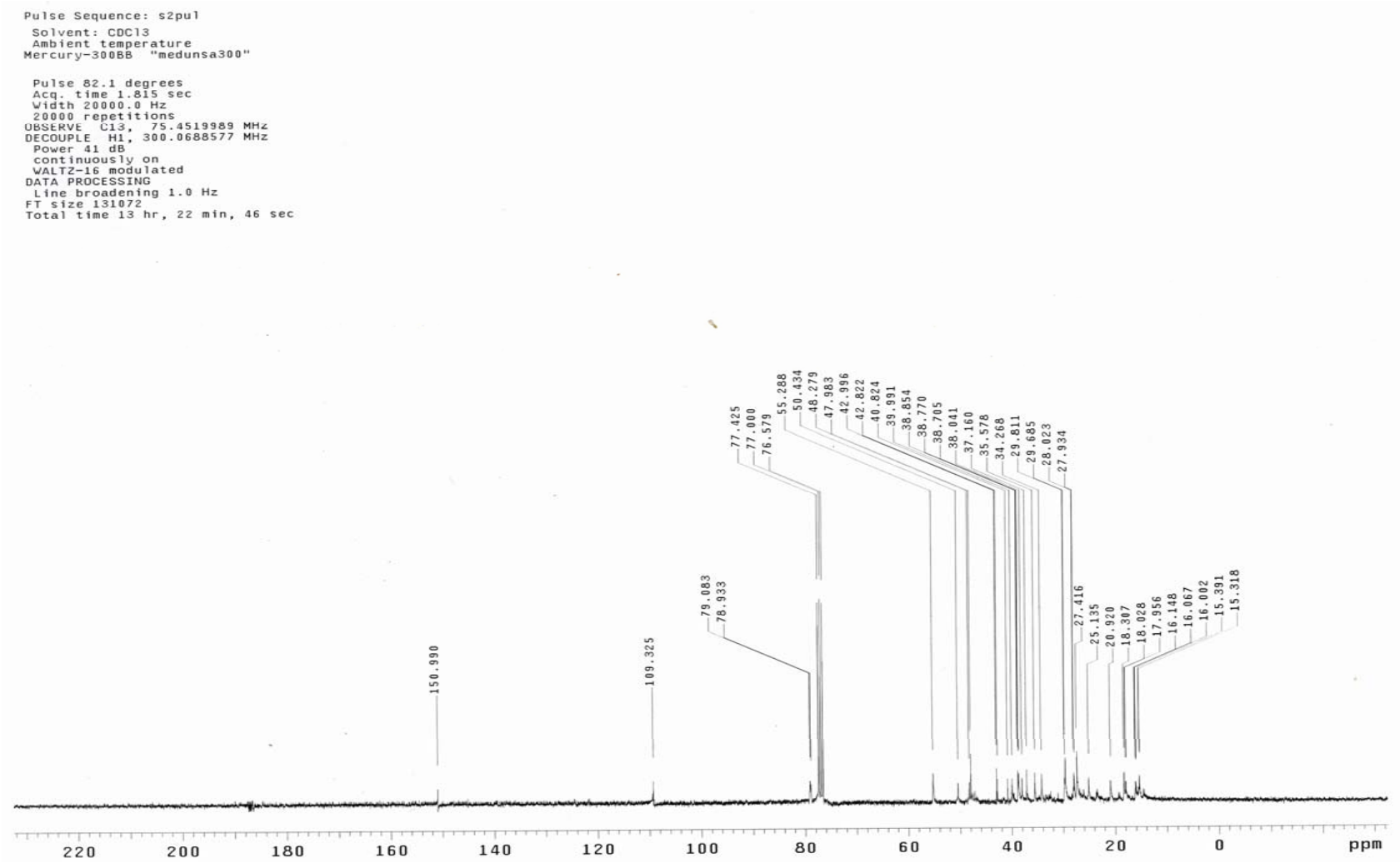

Figure A.3. The ^{13}C NMR spectrum of β-sitosterol isolated from the leaves of *Loxostylis alata*

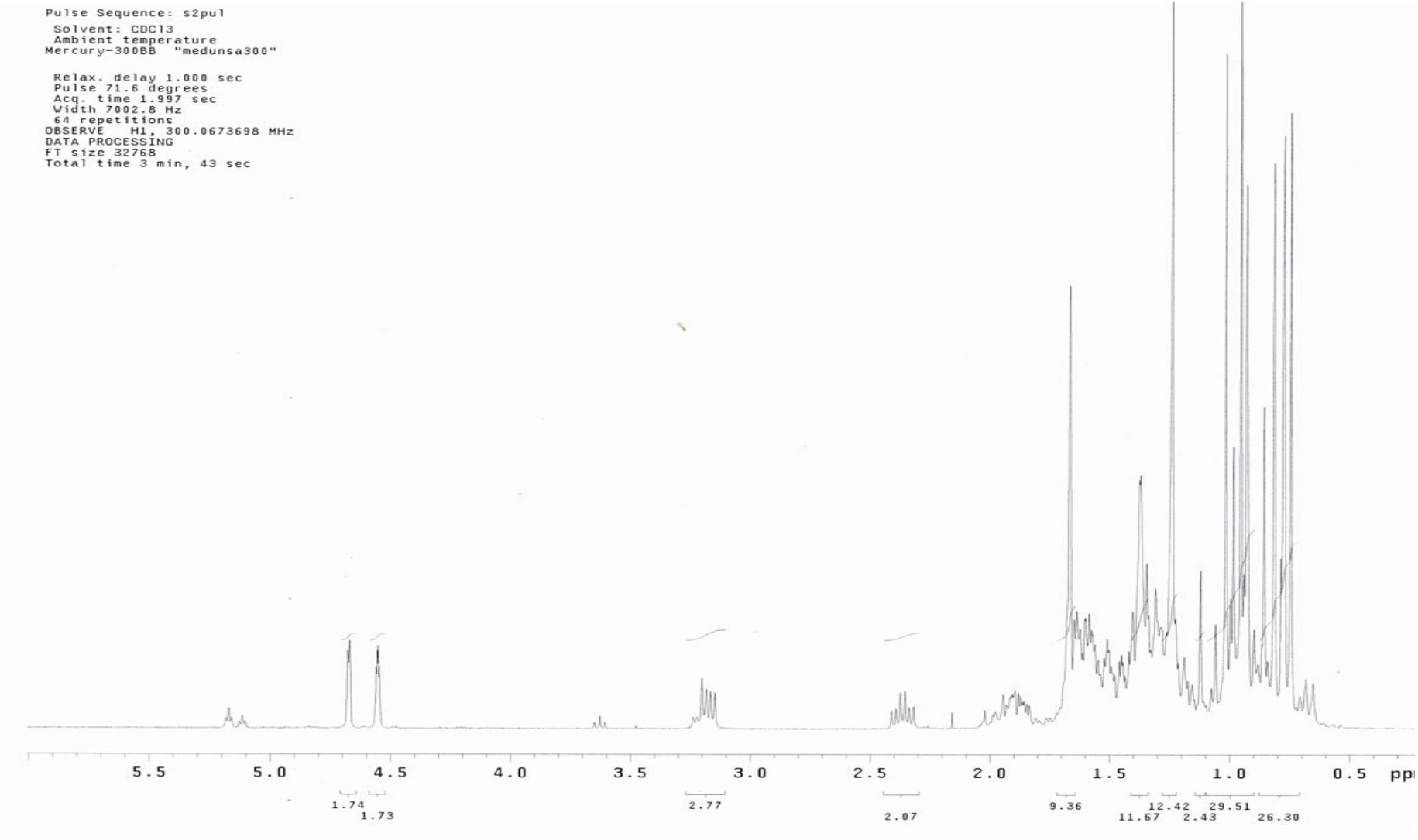

Figure A.4. The ^{1}H NMR spectrum of β-sitosterol isolated from the leaves of *Loxostylis alata*

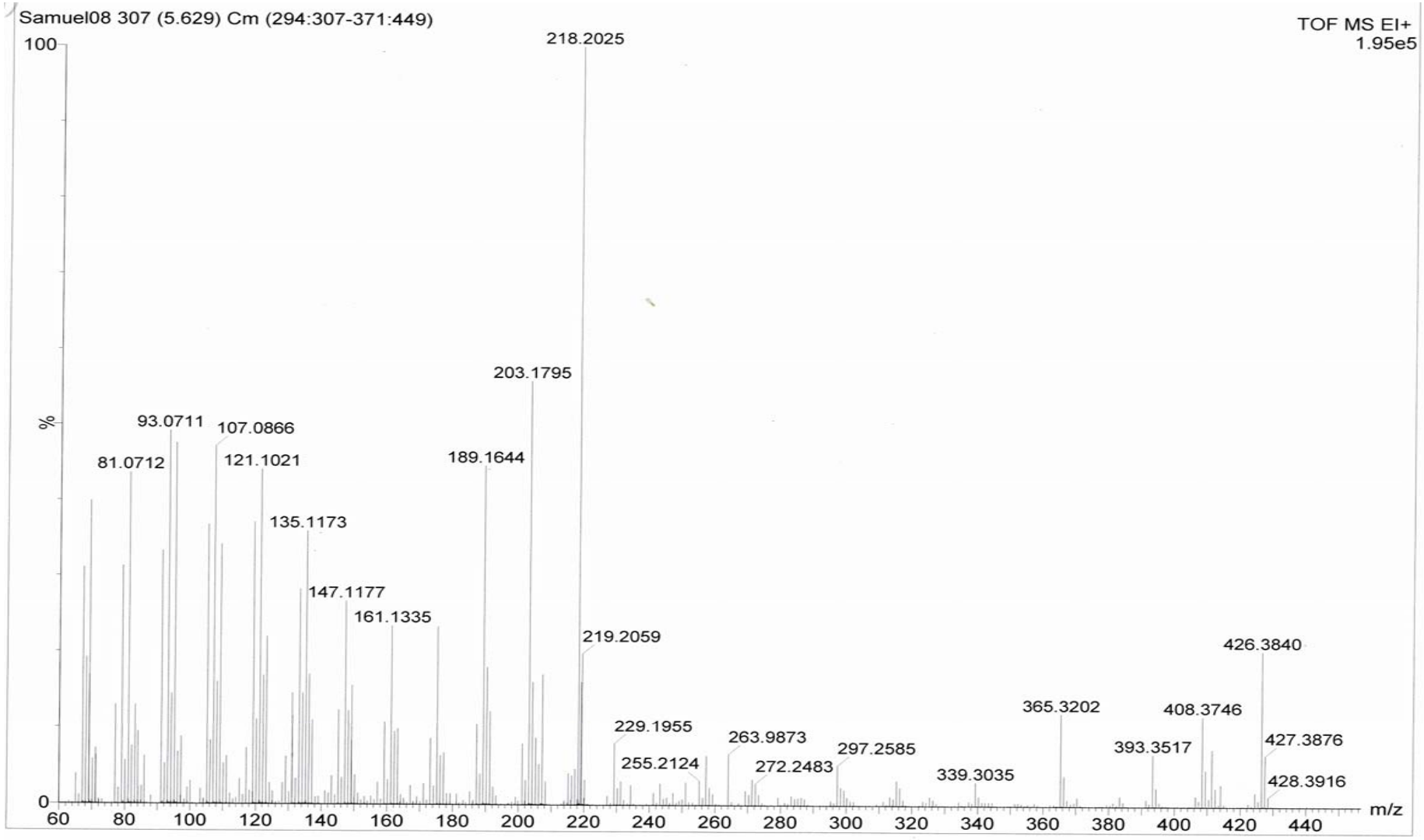

Figure A.5 The MS spectrum of lupeol isolated from the leaves of *Loxostylis alata*

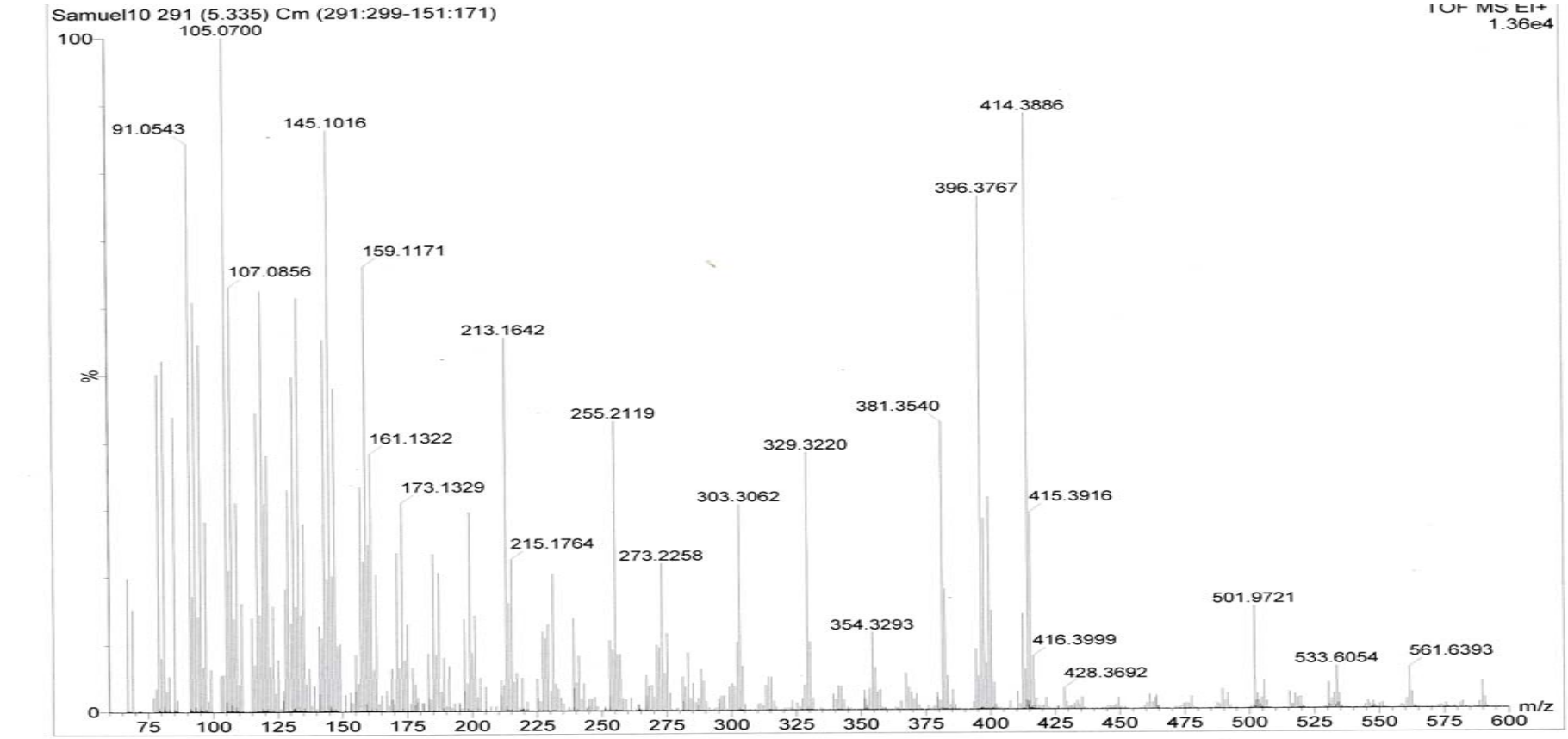

Figure A.6 The MS spectrum of β-sitosterol isolated from the leaves of *Loxostylis alata*