Characterization of antifungal compounds isolated from *Combretum* and *Terminalia* species (Combretaceae)

By

Peter Masoko

B.Sc (Med. Sci) (UNIN), M.Sc (Microbiology) (UNIN)

Submitted in fulfillment of the requirements for the degree

PHILOSOPHIAE DOCTOR (PhD)

In the

Faculty of Veterinary Science
Department of Paraclinical Science
Phytomedicine Programme

At the

UNIVERSITY OF PRETORIA

SUPERVISOR:	Prof J.N. Eloff
CO-SUPERVISOR:	Dr J.A. Picard

Pretoria
August 2006

DECLARATION

I, **PETER MASOKO**, hereby do declare that this thesis submitted for the award of the degree of **PHILOSOPHIAE DOCTOR (PhD**) of University of Pretoria is my independent work and it has previously not been submitted for a degree or any other examination at this of any other university.

_____

Peter Masoko

_____ day of _____ 2006

DEDICATION

This work is dedicated first of all, to my parents who were my first teachers, my younger brothers Kegomoditswe, Kabelo, Mojalefa and sister Refilwe. Secondly my grandmother Mosepele Shongwane and my late uncle Mosalagae.

ACKNOWLEDGEMENTS

This study has been a long journey that would not have been successfully made if it were for the support of various persons.

First and foremost, I would like to acknowledge the support, guidance and encouragement of my supervisor, **Prof Jacobus N. Eloff**. Without your positive comments and persistent encouragement this work would have simply floundered on and on. I truly want to thank him for listening to my frustrated rantings and bringing me back to a relative level of clarity and calm. For allowing me to disrupt his precious moments at anytime during his busy work.

This also goes to, **Dr Jackie A. Picard**, my co-supervisor, for the constructive criticism, advices, and patience during the course of this research. Her encouragement and keen interest led to satisfactory realization of this study.

I would like to extend a sincere word of thanks to **Dr Lyndy McGaw** who did the proof-reading. Thank you for your willingness and the special effort in terms of timing.

My sincere thanks also goes to **Dr Ladislaus K. Mdee**, for helping with structure elucidation and chemical characterization.

Special thanks to **Dr Joshua Dabrowski** (Pathologist), for helping with histopathology studies.

My profound gratitude goes to **Mr Patrick N. Selahle** (Technologist), for helping with rats handling and technical assistance.

Ms Denise Marais, for continuous and rapid responses to my administrative questions and queries. Always with a smile.

Ms Lita Pauw, for making sure that all necessary equipments and materials are ordered. “Baie Dankie”.

I am especially grateful to staff members of Bacteriology Section, Department of Tropical Diseases, University of Pretoria Onderstepoort Biomedical Research Centre (UPBRC) and Division of Pathology, Department of Paraclinical Sciences.

Thank you to **Mr Mathebula** (University of Limpopo, Medunsa Campus) for NMR and **Mr Ian Voster** (University of Johannesburg) for mass spectrometry.

I also appreciate the love and care my friends provided throughout this period. So everyone of you, especially to you **Calvin**, **Molatelo**, **Lerato** and **Moloisi**.

To all persons who helped me in one way or another during this work and whom I have not mentioned by nonetheless, I sincerely extend my thanks.

This study has been made possible by the financial support of **National Research Foundation** (**NRF**), **Department of Agriculture** (**SA**) and **University of Pretoria**.

Last, but far from least, I am thankful to all my family members, particularly to my parents **Johannes Motheo** (father), **Johanna Mmakepi** (mother), who have been there for me throughout my student life and for their never-ending motivation, encouragement, unreserved support and love in realizing my dreams come true.

Lastly, I would like to appreciate and to give praise to **God Almighty** for grace, wisdom and comfort throughout the time of study. Glory be to God for indeed thus far has He brought me.

"The light shines in the darkness and the darkness has never put it out" John 1.5

CONFERENCES AND PROCEEDINGS

Paper Presentation

Masoko P., Picard J. and Eloff J.N., (2004). Screening of twenty-four South African *Combretum* species (Combretaceae) for antifungal activities*. Indigenous plant use forum (IPUF) (Clanwilliam).*

Masoko P., Picard J. and Eloff J.N., (2004). Screening of twenty-four South African *Combretum* species (Combretaceae) for antifungal activities. Faculty Day, *(Faculty of Veterinary Science, University of Pretoria)*

Masoko P. and Eloff J.N., (2005). The diversity of antifungal compounds of six South African *Terminalia* species ((Combretaceae) determined by bioautography. *Indigenous plant use forum (IPUF) (Grahamatown).*

Masoko P. and Eloff J.N., (2005). The diversity of antifungal compounds of six South African *Terminalia* species ((Combretaceae) determined by bioautography. *Faculty Day, (Faculty of Veterinary Science, University of Pretoria).*

Masoko P., Picard J. and Eloff J.N. (2006). *In vivo* antifungal activity of *Combretum* and *Terminalia* extracts in rats. *Indigenous plant use forum* (*IPUF) (Gaborone, Botswana*).

Poster Presentation

Masoko P., Picard J. and Eloff J.N., (2003). Screening of antifungal activity from medicinal plants (Combretaceae). *Indigenous plant use forum (IPUF) (Rustenburg).*

Masoko P., Picard J. and Eloff J.N., (2003). Screening of antifungal activity from medicinal plants (Combretaceae). *Faculty Day, (Faculty of Veterinary Science, University of Pretoria).*

Masoko P., Picard J. and Eloff J.N., (2005). Extracts of 30 South African Combretum and Terminalia species have antifungal activities with MIC's as low as 20 µg/ml. *53rd Annual meeting of Society Medicinal Plant Research (GA) (Florence, Italy).*

MANUSCRIPTS PUBLISHED AND SUBMITTED

Masoko P., Picard J. and Eloff J.N., (2005). Screening of antifungal activity of six South African *Terminalia* species (Combretaceae). Journal of Ethnopharmacology, **99**. 301- 308.

Masoko P. and Eloff J.N., (2005). The diversity of antifungal compounds of six South African *Terminalia* species ((Combretaceae) determined by bioautography. African Journal of Biotechnology, **4(12),** 1425-1431.

Masoko P. and Eloff J.N., (2006). Bioautography indicates the multiplicity of antifungal compounds from twenty-four South African *Combretum* species (Combretaceae). African Journal of Biotechnology, **5 (18),** 1625 - 1647.

Masoko P., Picard J. and Eloff J.N., (2006). Antifungal activity of twenty-four South Africa *Combretum* species (Combretaceae) (*In Press: South African Journal of Botany*).

Masoko P., and Eloff J.N., (2006). Antioxidant activity of six *Terminalia* and twenty-four *Combretum* species found in South Africa (*In Press, Afr. J. Trad. CAM*).

Masoko P. Picard J. and Eloff J.N., (2006). Evaluation of the wound healing activity of selected *Combretum* and *Terminalia* species (Combretaceae) (*In Press, Onderstepoort Journal of Veterinary Research*).

Masoko P., Mdee L.K. and Eloff J.N., (2006). Biological activity of two related triterpenes isolated from *Combretum nelsonii* (Combretaceae) leaves (*Prepared for J. of Ethnopharmacology*).

Eloff J.N. and **Masoko P**., (2006). Resistance of fungal pathogens to solvents used in bioassays. (*Prepared for South African Journal of Botany*).

Most of the chapters in this thesis have been written in the form of a manuscript for publication and will be submitted.

LIST OF ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ATCC	American type culture collection
BEA	Benzene/Ethanol/Ammonium hydroxide (90/10/1 v/v/v)
C_{18} column	18-Carbon reverse phase siliga gel column
CEF	Chloroform/Ethylacetate/Formic acid (5/4/1 v/v/v)
CsA	Cycosporin A
DEPT	Distortionless enhancement by polarization transfer
DAC	Dicationic aromatic compounds
DCM	Dichloromethane
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribose nucleic acid
DPPH	2, 2,diphenyl-1-picrylhydrazyl
EF3	Elongation factor
ELISA	Enzyme linked immunosorbent assay
EMW	Ethylacetate/Methanol/Water (40/5.4/4 v/v/v)
GGT	Geranylgeranytransferase
GS	Glucan synthase
HMBC	Heteronuclear multiple bond correlation
HMQC	Heteronuclear multiple quantum coherence
HPLC	High performance liquid chromatography
INT	Iodonitro-tetrazolium salts
LC_{50}	Lethal concentration for 50% of the cells
LPO	Lactoperoxidase
LNBG	Lowveld National Botanical Garden
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye
NaCl	Sodium chloride
NADH	Nicotinamide Adenine Dinucleotide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NCCLS	National Committee for Clinical Laboratory Standards
NMR (^{13}C and ^{1}H)	Nuclear magnetic resonance (carbon 13 and proton)
PBS	Phosphate buffer saline
R_f	Retardation factor

rpm	revolutions per minute
SEE	Serial exhaustive extraction
TLC	Thin layer chromatography
UP	University of Pretoria
UV	Ultra violet radiation
v/v	volume per volume
VLC	Vacuum liquid chromatography
WHO	World Health Organisation

SUMMARY

Several investigations into the antimicrobial activity of members of the Combretaceae have been undertaken in recent years. Although the antibacterial properties of various species of *Combretum, Terminalia* and *Pteleopsis* have been investigated in depth, this is not the case for their antifungal properties. Due to the increasing importance of fungal infections the aim is to address this by focusing on antifungal activities of Combretaceae species. This was done by focusing on the following objectives:

1. Developing minimum inhibitory concentration (MIC) and bioautography procedures for fungi to be used in the laboratory in order to screen *Combretum* and *Terminalia* species for antifungal activity.
2. Selecting three or four species for further investigation based on antifungal activity and availability.
3. Isolating the antifungal compounds from one or more of the selected species.
4. Determining the chemical structure and *in vitro* biological activity of the antifungal compound.
5. Developing and applying a protocol and determining *in vivo* antifungal activity of *Combretum* and *Terminalia* extracts and isolated compounds in rats infected with different fungal pathogens.

Leaves of 24 *Combretum* and 6 *Terminalia* species were collected in the Lowveld National Botanical Gardens (LNBG) in Nelspruit. After the dried plants were milled to a fine powder, they were extracted with hexane, dichloromethane, acetone and methanol. Chemical constituents of the 120 extracts were analyzed by thin layer chromatography (TLC). The TLC plates were developed with one of the three eluent systems developed in our laboratory that separate components of Combretaceae extracts well 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) and Benzene/ethanol/ammonia hydroxide (90:10:1) [BEA] (non-polar/basic). To detect the separated compounds, vanillin-sulphuric acid-methanol was sprayed on the chromatograms and heated at 110 ^{o}C to optimal colour development. Methanol was the best extractant, extracting a greater quantity of plant material than any of the other solvents. There was similarity in the chemical composition of the non-polar compounds of extracts using extractants of varying polarity

Qualitative analysis of antioxidant activity, the 2, 2,diphenyl-1-picrylhydrazyl (DPPH) assay on TLC plates was used as a screen test for the radical scavenging ability of the compounds present in the different 120 extracts. TLC-DPPH screening method indicated the presence of

antioxidant compounds in some of the extracts tested, with *C. woodii* and *C. hereroense* showing the most prominent antioxidant activity. Methanol and acetone extracted the most antioxidant compounds based on DPPH TLC. I*n vitro* studies coupled with the phytochemical analysis confirm that the extracts had antioxidant activity.

The solvent tolerance of the microorganisms was tested using the following solvents; DMSO, acetone, methanol and ethanol. In order to determine the maximum concentration at which different solvents would allow the test microorganisms to reach normal growth, different concentrations from 10 to 100% were used. Uninhibited growth was evaluated as no toxic effects of the solvent. Methanol and ethanol were found to be toxic. The growths of the fungi were not affected by DMSO and acetone concentrations up to 60%.

A serial microdilution assay was used to determine the minimum inhibitory concentration (MIC) values for plant extracts using tetrazolium violet reduction as an indicator of growth. This method had previously been used only for antibacterial activities. To apply it to measuring antifungal activities, a slight modification was made to suit fungal growth conditions. The following fungal pathogens were used: yeasts (*Candida albicans* and *Cryptococcus neoformans*), thermally dimorphic fungi (*Sporothrix schenckii*) and moulds (*Aspergillus fumigatus* and *Microsporum canis*). To determine MIC values, growth was checked after 24 and 48 hours to determine the end point. The MIC values of most of the extracts were in the order of 0.08 mg/ml and some had values as low as 0.02 – 0.04 mg/ml after 24 hours incubation.

TLC plates were loaded with 100 μg (5 μl of 20 mg/ml) of each of the extracts. The prepared plates were developed in the three different mobile systems used: CEF, BEA and EMW. The chromatograms were dried for a week at room temperature under a stream of air to remove the remaining solvent. The TLC plates developed were inoculated with a fine spray of the concentrated suspension containing approximately 10^9 organisms per ml of actively growing fungi e.g. conidia for *A. fumigatus* and yeast cells (blastocysts) for the other fungi in a Biosafety Class II cabinet (Labotec, SA) cupboard. The plates were sprayed until they were just wet, and after drying were sprayed with a 2 mg/ml solution of INT. White areas 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 fungi.

During this study we experienced a number of difficulties. Firstly I found that preparing cultures some days before spraying them makes it difficult to get good results, possibly due to quick mycelial overgrowth and blockage of the spray gun with mycelia. The new method

was developed. This procedure led to reduced overgrowth of the mycelia. In the study of biologically active compounds from extracts, it was indicated that the extracts had antifungal compounds.

Fractionation and bioassay-guided isolation of the antifungal compounds was undertaken on the crude extracts of *C. nelsonii,* based on very low MIC's of the crude extracts on all tested pathogens, it had several compound which are active against all pathogens, lastly it is one of the *Combretum* species which have never being worked on. Antifungal compound was successfully isolated from the leaves of *C. nelsonii*. The structure was elucidated.

After structure elucidation bioassays of isolated active compounds was done to confirm that the compound isolated is the one expected, and how active the compound is, on its own. The compound was very active against all tested pathogens.

Cytotoxicity of the acetone extracts of *C. imberbe*, *C. nelsonii*, *C. albopunctactum* and *T. sericea* were evaluated using Brine shrimp (*Artemia salina*) assay and tetrazolium-based colorimetric assay (MTT assay) on Vero monkey kidney cells. These four extracts were chosen because of the good *in vitro* antifungal activity of crude extracts and there was intention of using them in *in vivo* studies in animal models. The results on brine shrimps indicated that the four leaf extracts have LC_{50} values above 20 µg/ml, the recommended cut-off point for detecting cytotoxic activity. Using MTT assay it was found that the four extracts did not suppress mitochondrial respiration in monkey kidney cells. Only *C. imberbe* was closer to the cut-off value (200 µg/ml), which was used by other authors. In searching for cytotoxic activity to the criteria of the American National Cancer Institute, the LC_{50} limit to consider a crude extract promising for further purification is lower than 30 µg/ml.

In vivo antifungal activity was investigated on the wound irritancy and efficacy of the four most promising, *Combretum nelsonii*, *Combretum imberbe*, *Combretum albopunctactum* and *Terminalia sericea* extracts applied topically to skin wounds in fungal infected skin wound of rat model. Wound irritancy and wound healing were evaluated by macroscopical, physical and histological methods. Aspects evaluated include wound healing, erythema, exudate formation and possible toxic effects of the extracts. Twenty rats were used in two pilot studies (Exploratory studies and Infection with different pathogens). During the pilot studies rats were not irritated by treatment of infection. The wound healed within three weeks. Only one rat was terminated due to weight loss and it was found that nasal discharge was due to external factors, which were not related to the experiment.

The clinical treatment of skin infected with pathogens continues to be a major problem especially in immuno-compromised patients. Therapeutic agents selected for the treatment of infected wounds had ideally shown antifungal activity on *in vitro* studies. I investigated whether these agents would improve phases of wound healing without producing deleterious side effects. All the parameters showed that the crude extracts and amphotericin B were effective in decreasing formation of the exudate, increasing crust formation and that they have antifungal activities used in *in vivo* studies. Acetone extract of leaves of *C. nelsonii*, *C. albopunctactum*, *C. imberbe* and *T. sericea* possessed remarkable growth inhibitory activities against fungal pathogens. Acetone extracts of leaves and isolated compound demonstrated wound healing properties comparable with that of antibiotic powder (amphotericin B).

The results of this study in general indicate that the *Terminalia* and *Combretum* species possess substantial antifungal properties. This explains the use of these plants in folk medicine for the treatment of various diseases related to fungal infections.

TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 5

Minimum Inhibitory Concentration (Papers)

Chapter 6

Bioautography (Papers)

Chapter 7

Chapter 8

Chapter 9

Chapter 11

LIST OF TABLES

Chapter 7

Chapter 9

Chapter 11

CHAPTER 1

Literature review

1. INTRODUCTION

In the 1990s, drug resistance had become an important problem in a variety of serious infectious diseases of humans including human immunodeficiency virus (HIV) infection, tuberculosis, and other bacterial infections. At the same time, there have been dramatic increases in the incidence of fungal infections, which are probably the results of alterations in immune status associated with the acquired immunodeficiency syndrome (AIDS) epidemic, cancer chemotherapy and organ and bone marrow transplantation. The rise in the incidence of fungal infections has exacerbated the need for the next generation of antifungal agents, since many of the currently available drugs have undesirable side effects, are ineffective against new or re-emerging fungi, or lead to the rapid development of resistance. Antifungal drug resistance is quickly becoming a major problem in certain populations, especially those infected with HIV, in whom drug resistance of the agent causing oropharyngeal candidiasis is a major problem (Graybill, 1988).

Resistance to antimicrobial agents has important implications for morbidity, mortality and health care costs all over the world. Substantial attention has been focused on developing a more detailed understanding of the mechanism of antimicrobial options, new antimicrobial options for the treatment of infections caused by resistance organisms and methods to prevent the emergence and spread of resistance in the first place. The study of resistance to antifungal agents has lagged behind that of antibacterial resistance for several reasons. Prior to the late 1980's with the rise of AIDS, fungal infections were rare (Wey et al., 1988).

These developments and the associated increase in fungal infections intensified the search for new, safer, and more efficacious agents to combat serious fungal infections. One of the options in tackling this problem is by ethnopharmacological approach.

Ethnopharmacology is the cross-cultural study of how people derive medicines from plants, animals, fungi, or other naturally occurring resources. Up to now, the field has focused mostly on developing drugs based on the medicinal use of plants by indigenous people. The "discovery" that indigenous knowledge about medicinal plants may hold clues for curing "western" diseases has become one of the most widely used arguments for conserving cultural and biological diversity (Farnsworth, 1988). Due to the potential for profit, some drug companies have teamed up with botanists, anthropologists, biochemists, conservation

organizations, and governments of less-developed countries to protect biologically diverse areas and search for new drugs.

Medicinal plant research is urgently needed. The AIDS virus, the crisis of bacterial resistance to antibiotics, and other recent developments have increased the value of indigenous medicinal plant knowledge, which may hold clues for solving these deadly problems. Indigenous medicinal plant knowledge is also critical because synthetic chemical processes have proved inadequate for dealing with the rapid evolution of pathogens. Unfortunately, many opponents of medicinal plant research that involves indigenous people have chosen to ignore the fact that "western" medicine relies on plants and traditional knowledge for clues to cure our worst diseases.

In addition, plant species are disappearing, and many indigenous people have stopped transmitting traditional medicinal knowledge to their children. In many places, the current generation represents our last chance to find ways that indigenous people can benefit from their knowledge instead of simply liquidating their biological resources to join a global economy in which they are at a serious disadvantage, including not being able to afford "western" medicines. New and innovative programs of benefits sharing between indigenous people and biomedical scientists are intended to achieve this goal. (Casagrande, 2000).

Medicinal plant research includes much more than the discovery of new drugs. Recently, the field has been expanding to also include such diverse subjects as negotiation of power based on medicinal plant knowledge (Garro, 1986) and the co-evolution of humans and plants (Alcorn, 1981). The field also provides opportunities to study how human interaction with biological diversity is influenced by human psychology, cognition, and evolution.

1.1. Medicinal plants

According to the World Health Organization (WHO), a medicinal plant is defined as any plant which contains substances that can be used for therapeutic purposes or which contain precursors of chemopharmaceutical semisynthesis (World Health Organization, 1979).

Traditionally used medicinal plants produce a variety of compounds of known therapeutic properties (Chopra *et al*., 1992, Harborne and Baxter, 1995, Ahmad and Beg, 2000). The substances that can either inhibit the growth of pathogens or kill them and have no or low toxicity to host cells are considered candidates for developing new antimicrobial drugs. In recent years, antimicrobial properties of medicinal plants are increasingly reported from different parts of the world (Nimri *et al*., 1999, Saxena and Sharma, 1999). Higher plants are still regarded as potential sources of new medicinal compounds. Throughout the world,

plants are used traditionally to treat many ailments, particularly infectious diseases, such as diarrhoea, fever and colds, as well as for the purposes of birth control and dental hygiene (Mitscher *et al*., 1987). In addition, many psychoactive substances used in traditional medicine are of plant origin (Deans and Svodoba, 1990).

More than 80% of the population in developing countries depend on plants for their medical needs (Farnsworth, 1988, Balick *et al*., 1994). Medicinal and poisonous plants have always played an important role in African society. Traditions of collecting, processing and applying plants and plant-based medications have been handed down from generation to generation (von Maydell, 1996). In South Africa, and also in many other African countries, traditional medicines, with medicinal plants as their most important components, are sold in marketplaces or prescribed by traditional healers in their homes (Fyhrquist, 2002). Because of this strong dependence on plants as medicines, it is important to study their safety and efficacy (Farnsworth, 1994).

The value of ethnomedicine and traditional pharmacology is gaining increasing recognition in modern medicine because the search for new, potential medicinal plants is more successful if the plants are chosen on an ethnomedical rather than a random basis. It has been estimated that 74% of pharmacologically active plants-derived components were discovered after the ethnomedical uses of the plants were investigated (Farnsworth and Soejarto, 1991).

1.1.1. Approaches for selecting medicinal plants

Four different approaches of selecting plants for pharmacological screening, are known, and are as follows: (1) 'random approach' which involves the collection of all plants found in that area; (2) 'phytochemical targeting' which entails the collection of all members of a plant family known to be rich in bioactive compounds; the (3) 'ethno-directed' sampling approach, based on traditional medicinal use(s) of the plant; (4) 'chemotaxonomic approach' and a method based on 'specific plant parts' such as seeds (Cotton 1996, Khafagi and Dewedar, 2000).

1.1.2. Importance of medicinal plants

Plants were once a primary source of all the medicine in the world and they still continue to provide mankind with new remedies. Natural products and their derivatives represent more than 50% of all drugs in clinical use in the world (van Wyk *et al*., 1997). Well-known examples of plants derived medicine include quinine, morphine, codeine, aspirin, atropine,

reserpine and cocaine. Recently, important new drugs such as taxol and vincristine have been developed. Taxol is a highly effective drug against breast cancer and was recently also approved for the treatment of ovarian cancer. It is a diterpenoid originally extracted from the bark of the pacific yew (*Taxus brevifolius*). Quinine is an alkaloid from the bark of the quinine tree (*Cinchona pubescens*), and is an effective remedy for malaria. Atropine and various tropane alkaloids are extracted from deadly nightshade and other plants for example *Datura stramonium*. Extracted alkaloids are used in eyedrops and in skin patches to treat motion sickness, and are injected to treat Parkinsonism (van Wyk *et al*., 1997). South Africa's contribution to world medicine includes Cape aloes (*Aloe ferox*), buchu (*Agathosma betulina*) and devil's claw (*Harpagophytum procumbens*) (van Wyk *et al*., 1997) and many more.

1.1.3. Traditional herbal medicine

In Africa, the use of plants to treat various ailments in humans and animals has been extensively documented by scientists. Herbalists use stems, leaves, roots and shoots of plants to prepare extracts, decoctions, concoctions, mixtures, potions, creams, infusions and pastes, which are then used to cure all sorts of afflictions. The variety of plants used in a community reflects the duration of a people's presence in a certain location, their medicinal knowledge, the diversity of plants present and the availability of plants with a possible medicinal use. Unfortunately, discovering the potential of a herb is not easy and can often only be done by careful and time-consuming experimentation. By this process, many people have discovered herbs to be effective against diseases. People in different places have independently discovered some of these remedies.

Many herbal remedies cure disease not understood by 'Western' medicine, i.e. diseases of the spirit, curses and spells. Although many cures are often available against the most common and easily diagnosed illnesses within a community, not all are effective. Some however do contain effective ingredients, which may be applied in Western medicine.

Primarily, healers use herbal medicine to cure diseases of the body and the spirit of their patients. This group of herbal remedy users can be split into subgroups, namely the traditional healer, who is usually a male whose family tradition it is to be the healer or doctor. He can cure diseases both of the body and spirit, using different remedies for children and women. Another subgroup, which usually consists of the wives of the healers, is concerned with the problems of women within the community. Wives of healers can advise about pregnancy and childbirth as well as herbal remedies to fight menstrual pains and the spiritual well-being of the unborn child or the young baby. A last subgroup is the normal person within

the community who has a basic knowledge of the herbs in its vicinity to cure such minor illnesses as colds, fevers, muscle aches, headaches, sore throats and joint pains. He may also be knowledgeable about plants that can be used to cure diseases of cattle or pets.

1.1.4. Ethnobotanical research

Ethnobotanical research is done primarily for three reasons, including an ethnological, developing and pharmaceutical motivation (Portillo et al., 2001).

1.1.4.1. Ethnological

In ethnological research, the investigator records how the plants are used, their use and beliefs. The anthropologist does not test the effectiveness of the plants, nor does he/she devise ways in which the plants can be put in better use (Kårehed, 1997).

1.1.4.2. Developing

The reason for ethnobotanical research is to document the knowledge of the healers in the community to save it for future generations. Many traditional healers are old and have no successors. People tend to think that Western medicine is better, and young people move to the cities where they have easy access to this medicine. Traditional knowledge should be written in a local language. It is most of the times impossible to document all the knowledge of the traditional healer. This makes it necessary to make through observations of the community in order to be able to make a good selection of plants that may be of use for future generations. A common way of selecting plants for documentation is to interview several traditional healers and to search for consensus (Schlage, 2000). This is done from the perspective that it would be more likely that a certain cure actually works, if it is used by more than one traditional healer (Mahunnah, 1996).

Within this motivation for research, one can also include the study of the role of traditional medicine in relation to modern medicine. Many people in developing countries have limited access to health clinics or hospitals, but ready availability of traditional healers. These healers play an important role in these societies as an institution to consult before attending a hospital or clinic, thereby reducing the number of patients going to the hospitals, as well as allowing medical facilities to be shared among a greater number of people.

1.1.4.3. Pharmaceutical

Scientists could chemically screen all possible plants to find new pharmaceuticals to be used in Western medicine. However, the knowledge of the chemical functioning of the human body is by far not extensive enough yet and a lot of possibilities are missed that way.

Therefore ethnobotanical research is a good way to start. In this kind of research the consensus of healers is also used very often (Schlage, 2000, Leaman, 1995). This might be a good way to find a number of plants that probably contain interesting chemicals, but there is a risk of missing the less commonly-known cures used by the traditional healers. In this research, plant taxonomy also plays an important role. If a plant contains bioactive chemicals it is definitely worthwhile looking at its close relatives. Using this kind of research a lot of important pharmaceuticals are found. Some examples are quinine, aspirin, and several HIV-blockers (Portillo *et al*., 2001).

The Combretaceae plant family has been used for medicinal purposes all over South Africa. In the present study, attention will be focused on this plant family.

1.2. Combretaceae

The plants in this family are used for many medicinal purposes by traditional healers. They include treating abdominal disorders, backache, bilharzia, chest coughs, colds, conjunctivitis, diarrhoea, dysmenorrhoea, earache, fattening babies, fever, headache, hookworm, infertility in women, leprosy, pneumonia, scorpion and snake bites, swelling caused by mumps, syphilis, toothache, gastric ulcers, venereal diseases, heart diseases, cleansing the urinary system, dysentery, gallstones, sore throats, nosebleeds and general weakness (Hutchings *et al*., 1996, van Wyk *et al*., 1997, McGaw *et al*., 2001).

The Combretaceae family belongs to the order Myrtales consisting of 18 genera, the largest of which are *Combretum*, with about 370 species, and *Terminalia*, with about 200 species (Lawrence, 1951). The other genera are smaller; e.g. *Calopyxes* and *Buchenavia* comprise 22 species each, *Quesqualis* 16, *Angioeissis* 14, *Conocarpus* 12 and *Pteleopsis* 10 species (Rogers and Verotta, 1996). The genus *Combretum* has two subgenera, which are subgenus *Combretum* and subgenus *Cacoucia* with several sections in each subgenus (Carr, 1988). (**Table 1.1**).

Species from the genus *Combretum,* and to a lesser extent *Terminalia,* are most widely used for medicinal purposes. These genera are widespread all over Africa including southern Africa and Asia, where some are often the dominant species (Carr, 1988). They are easily characterized by the wing-shaped appendages of the fruits, and are either trees, shrubs or climbers (Rogers and Verotta, 1996). The leaves and the bark of *Combretum* species are predominantly used. Fruits do not feature in medicine owing to their reported toxicity to humans.

Members of the family are often tanniferous and produce ellagic and gallic acids and frequently also proanthocyanins (Cronquist, 1981). They are sometimes cyanogenic and often accumulate triterpenoids, especially as saponins, but are without iridoid compounds. Mucilaginous secretory cells or canals are often present in the parenchymatous tissues and sometimes even in the wood. Solarity or clustered crystals of calcium oxalate frequently occur in some cells of the parenchymatous tissues, those in leaves often taking the form of stellate idioblasts.

Their leaves are simple, petiolate or sessile, opposite, alternate, verticillate, whorled, without stipules or very small, with margins entire (in one instance sometimes crenulate), with indumentum comprising hairs, stalked glands, and scales. The inflorescences are axillary, terminal, spicate (sometimes panicullate or subcapitulate). The flowers are sessile, or pedicellate, bisexual or sometimes unisexual, usually actinomorphic, and male on the same inflorescence.

Table 1.1. The Combretaceae family (Carr, 1988)

THE COMBRETACEAE FAMILY		
***Combretum* L**	**Section *Spathulipetala* Engl. & Diels**	**Section *Oxystachya* Excell**
SUBGENUS *Combretum*	*C. zeyheri* Sond.	*C. oxystachyum* Welw. Ex Laws.
Section *Hypocrateropsis* Engl. & Diels	**Section *Ciliatipetala* Engl. & Diels**	**Section *Poivrea* (Comm. Ex DC)**
C. celastroides Welw. Ex Laws.	*C. albopunctatum* Suesseng.	*C. bracteosum* (Hochst.)
C. imberbe Wawra	*C. apiculatum* Sond.	*C. mossambicense* (Klotzsch)
C. padoides Eng. & Diels.	*C. edwardsii* Exell. (provisional)	**Section *Megalantherum* Excell**
Section *Combretastrum* Eichl	*C. moggii* Excell. (provisional)	*C. wattii* Excell.
C. umbricola Engl	*C. molle* R. Br.	
Section *Angustimarginata* Engl. & Diels	C. *petrophilum* Retief	***Terminalia* L.**
C. caffrum (Eckl. & Zeyh.) Kuntze	*C. psidioides* Welw.	
C. erythrophyllum (Burch.) Sond.	**Section *Fusca* Engl. & Diels**	**Section *Abbreviatae* Excell**
C. kraussii Hochst. (incorporating *C. nelsonii* Duemmer)	*C. coriifolium* Engl. & Diels.	*T. prunioides* Excell.
C. vendae Van Wyk	**Section *Breviramea* Engl. & Diels**	*T. randii* Bak.f.
C. woodii Duemmer	*C. hereroense* Schinz.	*T. stuhlmannii* Engl.
Section *Macrostigmatea*	**Section *Elaegnoida***	**Section *Psidioides***

Engl. & Diels	**engl. &Diels**	**Excell**
C. engleri Schinz.	*C. elaeagnoides* Klotzsch.	*T. brachystemma* Welw. Ex Hierr
C. kirkii Laws		*T. sericea* Burch. Ex DC
Combretum sp. nov.(provisional)	**SUBGENUS *Cacoucia* (AUBL.)**	*T. trichopoda* Diels.
Section *Metallicum* Excell & Stace	**Section *Lasiopetala* Engl. & Diels**	**Section *Platycarpae* Engl. & Diels**
C. collinum Fresen	*C. obovatum* F.Hoffm.	*T. gazensis* Bak.f.
Section *Glabripetala* Engl. & Diels	**Section *Conniventia* Engl. Diels**	*T. phanerophlebia* Engl. & Diels
C.	*C. microphyllum* Klotzsch	*T. mollis* Laws
	C. paniculatum Vent.	*T. sambesiaca* Engl. & Diels
	C. platypetalum Welw. Ex Laws	*T. stenostachya* Engl. & Diels

The perianth arises from near the summit of a tubular epigynous zone; calyx of usually four or five distinct to slightly connate sepals; corolla commonly of four or five distinct petals, occasionally absent. The androecium of 4-10 stamens is adnate to the epigynous zone, commonly in two cycles, often strongly exserted. The gynoecium is a single compound pistil of 2-5 carpels; style and stigma 1; ovary inferior, with 1 locule containing 2(-6) apical ovules pendulous on long funiculi. The nectary is usually a disk (often hairy) above the ovary. The fruit is 1-seeded, often a flattened, ribbed, or winged drupe. The receptacles are usually in two parts, the lower containing the ovary, the upper terminating in four or five sepals. The style is centrally situated on a disc (Carr, 1988).

1.2.1. Ethnopharmacology of Combretaceae

There is a large variation in the chemical composition and antibacterial activity among different genera and species in the Combretaceae. Seven species of Combretaceae used in traditional medicine in West Africa have been investigated for their antifungal activity against the pathogenic fungi. Phytochemical screening revealed that these plants are particularly rich in tannins and saponins, which might be responsible for their antifungal activity (Baba-Moussa *et al*., 1999).

1.2.2. Antimicrobial activity of the Combretaceae

Species of Combretaceae contain compounds with potential antimicrobial properties (Eloff, 1999). In the last two decades a series of stilbenes and dihydrostilbenes (the combretastatins) with potent cytototoxic activity and acidic triterpenoids and their glycosides with molluscicidal, antifungal, antimicrobial activity have been isolated from species of

Combretum (Rogers and Verotta, 1996; Eloff *et al*., 2005a). There is a large variation in the chemical composition and antimicrobial activity among different genera and species in the Combretaceae.

Leaf extracts of *Combretum padoides, Combretum celestroides, Combretum hereroense, Combretum obovatum*, *C. zeyheri, C. erythrophyllum, Combretum paniculatum, Combretum edwarsii, C. apiculatum* and *C. imberbe* have been shown to have some activity against *S. aureus, Bacillus subtilis, E. coli, Serratia marcescens*, *Mycobacterium phlei* and *Saccharomyces cerevisiae* (Alexander, 1992).

Eloff (1999) investigated the antibacterial activity 27 southern African members of Combretaceae including *C. woodii*, using minimum inhibitory concentrations (MICs) and total quantities extracted. All the plants tested exhibited antibacterial activity against *S. aureus, E. coli, E. faecalis* and *P. aeruginosa,* while Rogers and Verotta (1996) reported the leaves of *C. molle* and *C. imberbe* to possess anti-inflammatory and molluscicidal activity against *Biomphalaria glabrata*.

1.2.3. Phytochemistry of the Combretaceae

Members of the family are often tanniferous and produce ellagic and gallic acids and frequently proanthocyanins. They are sometimes cyanogenic and often accumulate triterpenoids, especially as saponins (Hutchings *et al*., 1996).

Chemical studies of the *Combretum* genus have yielded acidic triterpenoids and their glycosides, phenanthrenes, amino acids and stilbenes (Pellizzoni *et al*., 1993). A series of closely related bibenzyls, stilbenes and phenanthrenes have been isolated from *C. caffrum* (Petit *et al*., 1995). Some of these stilbenes have been found to be anti-mitotic agents that inhibit both tubulin polymerisation and binding of colchicine to tubulin. Flavonoids have been isolated from *C. micranthum* leaves (Rogers and Verotta, 1996).

The fruits of *Terminalia cheluba* have yielded complex esters of gallic acid e.g. corilagin (Haslam, 1996). The aerial parts and fruits of *C. zeyheri* have been found to contain ursolic acid, and a compound named as CZ 34 and L-3 (3-aminomethylphenyl) alanine (Breytenbach and Malan, 1998). With the exception of the simple indole alkaloids that Harman and Eleagnine isolated from the roots of *Galago senegalensis*, there have been no other reports on the presence of alkaloids contained by Combretaceae (Rogers and Verotta, 1996).

Anti-inflammatory and molluscicidal compounds such as mollic acid –D – glycoside and imberbic acid have been isolated from *C. molle* and *C. imberbe* respectively (Pegel and Rogers, 1985). The saponin, jessic acid linked to α-L-arabinose has been isolated from *Combretum eleagnoides* leaves (Osborne and Pegel, 1984).

1.3. Some of the work done on Combretaceae family in Phytomedicine Programme

Our laboratory has developed methods on screening and activities of Combretaceae. Some of the work was as follows:

(i) *Selection of plants to investigate*

An analysis was made of approaches to be followed towards selecting plants for research and gene banking. Plants used as phytomedicines in Africa and were also analysed and the Combretaceae made up a major group. (Eloff, 1998a)

(ii) *Selection of best extraction procedure*

Several extractants were tested and evaluated on many different parameters. Acetone was found to be the best extractant. (Eloff, 1998b)

(iii) *Selection of best purification procedures*

The solvent solvent fractionation procedure used by the USA National Cancer Institute was tested and refined and several TLC separation procedures were also developed. (Eloff, 1998c)

(iv) *Developing a novel way of determining antibacterial activity*

It could be shown that the traditional agar diffusion assays for determining activity of plant extracts did not work. A new serial dilution microplate assay using INT was developed. (Eloff, 1998 d)

(v) *Antibacterial activity of Combretum erythrophyllum*

Using the techniques developed above we could show that *Combretum erythrophyllum* contains at least 14 antibacterial compounds. [Martini and Eloff 1998]. Extracts had MIC values as low as 50 μg/ml.

(vi) *Antibacterial activity and stability of 27 members of Combretaceae*

Acetone leaf extracts of 27 species of *Combretum, Terminalia, Pteleopsis* and *Quisqualis* all had antibacterial activity ranging from 0.1 –6 mg/ml. Storing extracts for 6 weeks at room temperature did not affect MIC values (Eloff, 1999).

(vii) *Stability of antibacterial activity in C. erythrophyllum*

Leaves of *C. erythrophyllum* stored in herbaria for up to 92 years did not lose any antibacterial activity (Eloff, 1999).

(viii) *A proposal for expressing antibacterial activity*

MIC values do not give any indication of the activity present in a plant. A proposal was made that "total activity" should be determined by dividing the quantity extracted from 1 g of plant material in mg by the MIC in mg/ml. The resultant value in ml /g gives the highest dilution to which a plant extract can be diluted and still inhibited the growth of the test organism (Eloff 2000).

(ix) *Other biological activities of Combretum species*

The anti-inflammatory anthelminthic and antischistosomal activity of 20 *Combretum* species was determined. There was very little antischistosomal activity, low to medium anthelminthic activity and medium to strong anti-inflammatory activity in extracts of the different species (McGaw *et al.* 2001)

(x) *Antibacterial activity of Marula bark and leaves*

Both leaf and bark extracts had antibacterial activity and there were two main bioactive compounds i.e. a very polar and a very non-polar compound (Eloff, 2001).

(xi) *The stability and relationship between antibacterial and anti-inflammatory activity of southern African Combretum species*

Both antibacterial and anti-inflammatory activity was stable and there was a reasonable correlation between antibacterial and anti-inflammatory activity indicating that similar compounds may be responsible for the biological activities (Eloff *et al*., 2001).

(xii) *Extraction of antibacterial compounds from Combretum microphyllum*

Several extractants were tested to determine if any extractant selectively extracted antibacterial compounds. The three most promising extractants were di-isopropyl ether,

ethanol, ethyl ether, acetone and ethyl acetate. The activity towards Gram negative and Gram-positive bacteria was similar (Kotze and Eloff, 2002)

(xiii) *Isolation of antibacterial compounds from C. erythrophyllum*

Martini *et al*., (2004a) isolated and characterized seven antibacterial compounds. Four were flavanols: kaemferol, rhamnocitrin, rhamnazin, quercitin 5,3 -dimethylether] and three flavones apigenin, genkwanin and 5-hydroxy-7,4'-dimethoxyflavone.

All test compounds had good activity against *Vibrio cholerae* and *Enterococcus faecalis*, with MIC values in the range of 25-50 μg/ml. Rhamnocitrin and quercetin-5,3-dimethylether showed additional good activity (25 μg/ml) against *Micrococcus luteus* and *Shigella sonnei*. Toxicity testing showed little or no toxicity towards human lymphocytes with the exception of 5-hydroxy-7,4-dimethoxyflavone (Martini *et al*., 2004b). This compound is potentially toxic to human cells and exhibited the poorest antioxidant activity. Both rhamnocitrin and rhamnazin exhibited strong antioxidant activity with potential anti-inflammatory activity. Although these flavonoids are known, this was the first report of biological activity with some of these compounds.

(xiv) *Variation in the chemical composition*

Variation in the chemical composition, antibacterial and anti-oxidant activity of fresh and dried Acacia leaf extracts (Katerere and Eloff, 2004).

(xv) *Isolation of antibacterial compounds from C. woodii*

The stilbene 2, 3, 4-trihydroxyl, 3, 5, 4-trimethoxybibenzyl (combretastatin B5) from the leaves of *C. woodii* was isolated. It showed significant activity against *S. aureus* with an MIC of 16 μg/ml MIC of 16 μg/ml [*Ps. aeruginosa (125* μg/ml), *E. faecalis* (125 μg/ml) and slight activity against *E. coli*.] (Eloff *et al*., 2005a,b). This is the first report of the antimicrobial activity of combretastatin B5.

(xvi) *Isolation of antibacterial compounds from C. apiculatum*

For his M.Sc study Serage (2003) isolated and elucidated the structures of two flavanones alpinetin, pinocembrin, and one chalcone flavokawain-from the leaves of *C. apiculatum subsp apiculatum*. All the compounds had substantial activity against the bacterial pathogens tested.

(xvii) *Isolation of antibacterial compound from Terminalia sericea*

In his PhD study Kruger (2003) investigated eleven extractants and seven *Terminalia spp* to find the best extractant and species to use for isolating antibacterial compounds. He isolated terminoic acid from *Terminalia sericea* and showed that it could be used as a topical agent.

(xviii) *Use of Urginea sanguinense in ethnoveterinary medicine*

Pretreatment of bulbs of *Urginea sanguinense* used in ethnoveterinary medicine influences chemical composition and biological activity (Naidoo *et al*., 2004).

(xix) *Use of Gunnera perpensa extracts in endometriosis*

McGaw *et al*., (2005) checked whether the use of *Gunnera perpensa* extracts in endometriosis were related to antibacterial activity.

(xx) *Use of Peltephorum africanum extracts in veterinary medicine*

The rationale for using *Peltephorum africanum* (fabaceae) extracts in veterinary medicine was investigated (Bizimenyera *et al*., 2005).

(xxi) *Toxic effects of the extracts of Allium sativum bulbs on adults of Hyalomma marginatum rufipes and Rhipicephalus pulchellus.*

In vitro investigation of the toxic effects of the extracts of *Allium sativum* bulbs on adults of *Hyalomma marginatum rufipes* and *Rhipicephalus pulchellus* (Nchu *et al*., 2005).

(xxii) *Screening of sixteen poisonous plants*

Sixteen poisonous plants were screened for antibacterial, anthelmintic and cytotoxic activity *in vitro* (MacGaw and Eloff, 2005).

(xxiii) *Antibacterial and antioxidant activity of Sutherlandia frutescens*

Antibacterial and antioxidant activity of *Sutherlandia frutescens* (Fabaceae) were investigated (Katerere and Eloff, 2005a).

(xxiii) *Identification of anti-babesial activity*

Anti-babesial activity of four ethnoveterinary plants were identified *in vitro* (Naidoo *et al*., 2005).

(xxiv) *Management of diabetes in African traditional medicine*

Management of diabetes in African traditional medicine in Soumyanath (Katerere and Eloff, 2005b).

1.4. Existing antifungal drugs

The information in this section was compiled from the following publications: (Wills *et al*., 2000; White *et al*., 1998; Ghannoum and Rice, 1999; Tkacz and Didomenico, 2001, Didomenico, 1999).

There has been extensive research on the development of antifungal drugs, but only six of these of these antifungal agents were licensed for use in 1995. These include only polyene amphotericin B, three azoles, miconazole, ketoconazole, fluconazole and itraconazole and one pyrimidine synthesis inhibitor flucytosine (5-FC) (Espinel-Ingroff and Pfaller, 1995).

Polyenes act by binding to ergostel present in the fungal cell membrane, causing osmotic instability and loss of membrane integrity. The azoles on the other hand inhibit fungal cytochrome P450-dependent enzymes, with resulting impairment of ergosterol synthesis and depletion in the fungal cell membrane (Espinel-Ingroff and Pfaller, 1995). Fluconazole is a water-soluble bifluorinated triazole, with low binding affinity for plasma protein. It distributes extensively throughout the body, and readily diffuses into saliva. This drug is highly successful in the treatment of AIDS patients who had relapsed after amphotericin B and flucytosine (5-FC) treatment (Drouhet and Dupont, 1989).

However, it has been found that treatment with these drugs, especially for extended periods, can lead to problems with toxicity to the patients (amphotericin B) or with the development of resistant pathogenic organisms during the course of therapy (5-fluorocystine) (Boonchird and Flegel, 1982). Since the incidence of these opportunistic infections is on the increase, attempts are made to develop new chemotherapeutic agents or a combination of agents to treat the causative fungus.

Due to the sterol-binding action of amphotericin B in the fungal cell membrane, renal damage is found to occur in more than 80% of patients and can be permanent in patients receiving larger doses of the drug (Clark and Hufford, 1993). Flucytosine in combination with amphotericin B is designed to reduce the dosage of amphotericin and to eliminate the development of resistance to flucytosine. However, it has been noted that flucystoine toxicity may increase when it is used in combination with amphotericin B (Clark and Hufford, 1993).

The above-mentioned problems therefore illustrate the need for antifungal compounds with low or no toxicity, and natural products are an important potential source of the compounds.

1.4.1. Novel antifungal medicine

Fungi, like their hosts are eukaryotic organisms, making it more difficult to select intracellular fungal targets whose inhibition would not also be deleterious to the host cell. Of the four classes of antifungal compounds currently in use, three affect ergosterol, namely polyenes, azoles, and allylamines. Fluoropyrimidine 5-fluorocytosine (5-FC) achieves its specificity through a converting enzyme not present in mammalian cells. **Table 1.2** shows general overview of presently used antifungal agents.

Table 1.2. An overview of antifungal agents (Didomenico, 1999)

Compound/Class	**Mode of action**	**Comments**
Amphotericin B/polyene	Selective binding to ergosterol, major sterol of fungal membranes	Fungicidal Broad spectrum Intravenous Little resistance observed Significant nephrotoxicity
Abelcet/polyene Ambisome Amphotec	Selective binding to ergosterol, major sterol of fungal membranes	Liposomal formulation of AMB Similar efficacy as AMB Reduced toxicity observed
Nyotran/nystatin	Selective binding to ergosterol major sterol of fungal membranes	Liposomal formulation of nystatin Lowered toxicity compared to nystatin
5- Fluorocytosine (5-FC)/nucleoside analog	Selective conversion to toxic intermediate	Most often given in combination with AMB for: Cryptococcal meningitis Poor activity against filamentous fungi Significant resistance observed
Miconazole/azoles Ketoconazole	Selective inhibition of fungal cytochrome P450-dependent lanosterol-14-α-demethylase	Static activity against yeast, dimorphic fungi, dermatophytes General fungistatic activity
Fluconazole/triazoles Itraconazaole Voriconazole Posaconazole UR-9825 SYN-2869 BMS-207147	Selective inhibition of fungal cytochrome P450-dependent lanosterol-14-α-demethylase	Broad spectrum including *Aspergillus* spp. FLU-resistant *C. albicans* strains and non-albicans strains increasing Efficacious in immune compromised models
LY303366?candins Caspofungin FK-463	Fungal β-1,3-glucan synthase inhibitors	Partly fungicidal Broad spectrum except for *Cryptococcus, Fusarium, Sporothrix, Trichosporon* Efficacious in immune compromised models
BMS181184/pradimicins	Calcium-dependent binding to mannoproteins in cell wall	Broad spectrum except for *Fusarium* Oral Hepatotoxicity led to

		discontinution
Nikkomycin/nikkomycins	Chitin synthase inhibitors	Liposomal formulation of nikkomycin Limited spectrum for fungi Effective against cells with high chitin content
Terbinafine/allylamines	Squalene epoxidase inhibitors	Fungicidal Active against dermatophytes Topical and oral formulations
Basifungin/aureobasidins	Inositol-P ceramide synthase inhibitor	Fungicidal Broad spectrum
Sordarin/Sordarins	Selectively binds to fungal EF2/ribosomal stalk proteins	Fungicidal Broad spectrum

AMB, amphotericin B

1.4.1.1. Inhibitors of fungal cell membranes

Polyenes

The only polyene approved for systemic use is Amphotericin B (AMB). Its primary advantages include its fungicidal activity against most clinically relevant pathogens, and the low occurrence of resistance. The primary disadvantage of AMB is its nephrotoxicity. Ambisome, Abelcet and Amphocil/Amphotech all exert relatively similar efficacies with fewer side effect than AMB (Walsh *et al*., 1998). Composition of the lipid bilayer containing the polyenes appears to contribute to slight differences in efficacy as a result of both redistribution of the antifungal drug to tissues and the selective release of active AMB from the complex (Boswell *et al*., 1998).

Azoles

There is a wide variety of azoles that have *in vitro* efficacy, but only a few have had significant clinical utility. Azoles inhibit cytochrome P450-dependent lanosterol 14-alpha-demethylase, causing accumulation of methylated sterols, depletion of ergosterol, and inhibition of cell growth (Koltin and Hitchcock, 1997). Sensitivity of other P450-dependent enzymes accounts for their primary mode of toxicity. Although azoles demostrate a broad spectrum of activity with less toxicity than AMB, they are not generally fungicidal but rather fungistastic.

Aureobasidins

Basifungin is a cyclic depsipeptide with good *in vitro* and *in vivo* activity against a number of pathogenic fungi including most *Candida* species, *Cryptococcus neoformans*, *Histoplasma capsulatum* and *Blastomyces dermatidis*, with poor activity against *Aspergillus* spp. and dermatophytes (Takesako *et al*., 1993). This compound inhibits phosphatidyl-

inositol:ceramide phospho-inositol transferase (IPC synthase), which is encoded by an essential gene (Nagiec *et al*., 1997). Other natural products, kafrefungin and rustmicin also inhibit IPC synthase (Mandala *et al*., 1997).

1.4.1.2. Inhibitors of fungal cell wall

The fungal cell wall is an ideal target for the search for novel, fungicidal compounds. Several of the enzymes involved in the biosynthesis of the cell wall are unique to fungi, including chitin and glucan synthases (Georgopapadakou, 1997)

Echinocandins and pneumocandins

β-1,3-Glucan synthase is the target of both the echinocandins and pneumocandins (Radding *et al*., 1998). Indianapolis is a derivative of cilofungin, an early echinocandin B analog that has a limited spectrum. LY303366 compound is both orally and parenterally active and more potent. It has *in vitro* and *in vivo* activity against numerous clinical isolates of *C. albicans*, *B. dermatididis, H. capsulatum, A. fumigatus* and the cystic form of *Pneumocystis carinii* (Espinel-Ingroff, 1998). Caspofungin has partly fungicidal activity *in vitro* against some *Candida* spp. and some dimorphic fungi.

Nikkomycins

Members of this class of compound have been known for many years. They appear to act competitively as substrate analogs of UDP-N-glucosamine in preventing the synthesis of chitin. Although chitin synthesis is an essential function, multiple isozymes present in fungi add a level of complexity. The potency of an inhibitor may depend on the isoform's relative effectiveness in building a cell wall as well as its affinity to a given enzyme. Nikkomycin has a relatively narrow spectrum as a solo agent but has been shown to have either additive or synergistic effects in combination with azoles against a number of human pathogens (Li and Rinaldi, 1999).

Pradimicins

The pradimicin family of antifungals exerts its selectivity by calcium-dependent binding of cell surface mannoproteins leading to cell membrane leakages and loss of viability (Watanable *et al*., 1996). These compounds exhibit broad *in vitro* and *in vivo* activity (Oki *et al*., 1992). In a direct comparison with AMB, the compound is 40- to 50-fold less active, but also 130-fold less toxic (Oki *et al*., 1992). Azole and 5FC-resistant strains remain susceptible. The pradimicins have demonstrated antiviral activities *in vitro*, via a critical interaction with mannose-containing polysaccharides on the viral coat surfaces.

Geranylgeranyltransferase inhibitors

Cell wall integrity requires a functional geranylgeranyltransferase (GGT). A human ortholog has been identified, there is only about 20 % homology between the fungal and mammalian GGT therefore it may be possible to obtain specificity in action. There are number of selective active-site inhibitors targeted specifically against GGT in the micromolar to nanomolar range, and some appear fungicidal.

1.4.1.3. Inhibitors of protein synthesis

Sordarins

The search for suitable, unique targets within the fungal ribosome is challenging, (with the exception of elongation factor (EF3)), due to the structural and sequence similarity between fungal and mammalian ribosomal RNAs, subunits and soluble factors. The EF3 120 kDa soluble factor was originally discovered in *S. cerevisiae* and has subsequently been identified in other fungal pathogens (Uritani *et al*., 1999). Sordarins are highly specific inhibitors of fungal translation. Several derivatives are active against *C. albicans* (Aviles *et al*., 1998). The ability of the sordarins to selectively inhibit fungal translation underscores the possibility that other essential proteins, as well as EF2, may be important targets in antifungals.

1.4.1.4. N-myristoyltransferase inhibitors

The transfer of myristate, a 14-carbon fatty acid, from CoA to the terminal glycine of certain proteins has been shown to be essential in *C. albicans, C. neoformans* and other fungi (Weinberg *et al*., 1995). A number of inhibitors targeted towards N-myristoyltransferase (NMT) are known.

1.5. New potential targets for antifungal development

Information in this section is compiled from several reviews (Wills *et al*., 2000; White *et al*., 1998; Ghannoum and Rice, 1999; Tkacz and Didomenico, 2001, Didomenico, 1999).

There is an attempt to find sensitive fungicidal targets with potential for selectivity over mammalian cells. In this section I will attempt to examine in-depth several of these focused strategies on antifungal development (**Figure 1.1.**).

1.5.1. The fungal cell wall

The fungal cell wall acts as the interface between the fungus and its environment. It has several roles, which include providing the fungus with its shape and supporting it against osmotic forces. It acts as a filter, controlling the secretion and uptake of molecules into the cell. Some enzymes are also responsible for enzymatic conversion of nutrients into metabolisable forms, prior to their entry into the protoplast (Pebery, 1990). This structure is not only important to viability of the fungal cell, it is also unique to fungi and not present in mammalian cells. These features make it an ideal antifungal target.

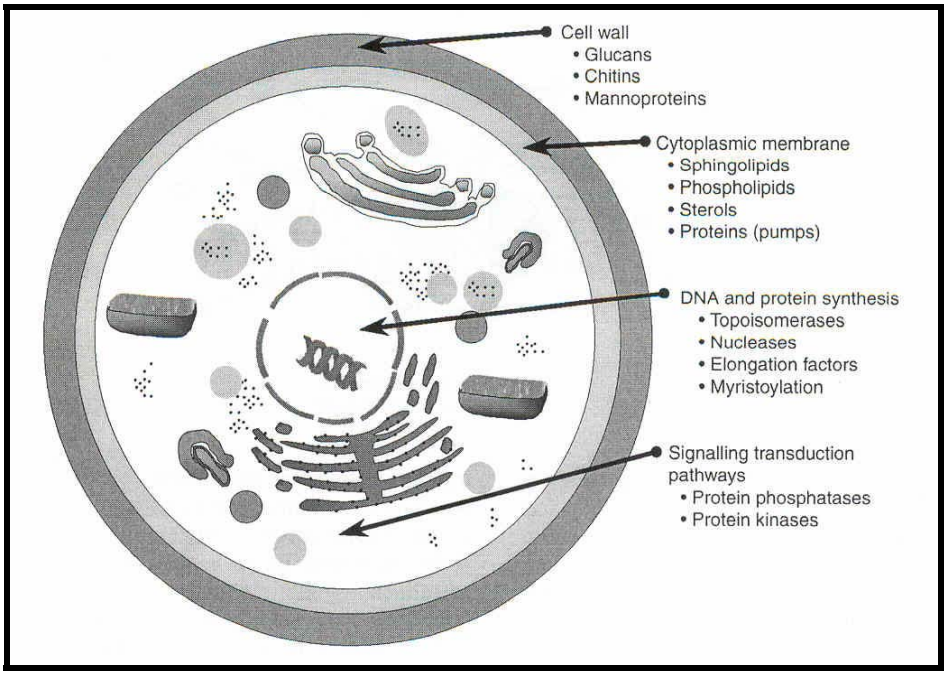


Figure 1.1. Schematic view of emerging targets for antifungal drug development (Wills *et al*., 2000).

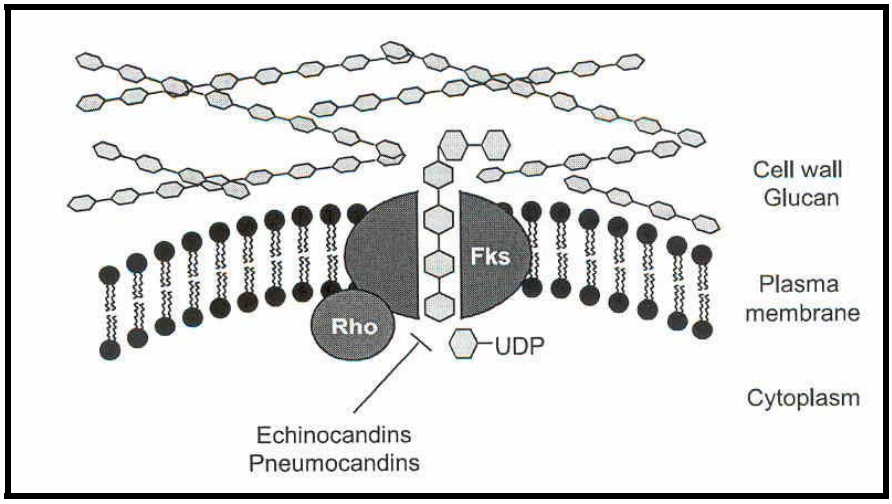
1.5.1.1. (1,3)-β-D-Glucan synthase

The β-Glucans are an abundant class of polysaccharides that are involved in structural, functional and certain morphological roles at the fungal cell surface (Fleet and Phaff, 1981). The membrane bound-enzyme (1,3)-β-D glucan synthase (GS) catalyses the synthesis of (1,3)-β-glucan, an essential glucose polymer found in fungi. It forms a fibril composed of three helically entwined linear polysaccharides, which provide rigidity and integrity to the cell structure. Since the (1,3)-β-glucan structure is not found in mammalian cells, the GS enzyme has become a target for research into antifungal agent development (Inoue *et al*., 1995). The current proposed model for GS is shown in **Figure 1.2**.

1.5.1.2. Chitin synthase

Chitin is a major structural component of the cell walls of many fungi. It is a (1-4)-β-linked homopolymer of *N*-acetyl-D-glucosamine, and is produced by chitin synthase from the nucleotide UDP-GlcNAc and follows the reaction (Cabib, 1987):

$2n$ UDP-GlcNAc → (GlcNAc-β-(1-4)-GlcNAc)$_n$ + $2n$ UDP



Fks: Glucan sunthase complex; Rho: GTP-binding regulatory subunit; UDP: Uridine diphosphate

Figure 1.2. Working model of glucan synthase (Wills *et al*., 2000)

In *S. cerevisiae*, the cell wall is relatively poor in chitin, but the primary septum, that separates the mother and daughter cells, and bud scars are mostly composed of chitin (Cabib *et al*., 1997). It is also found in the cell wall and plays a role in cell wall integrity. Chitin synthesis is cell cycle regulated, and the amount and distribution of chitin in the cell wall changes as the cell proceeds from vegetative growth to diploid formation and then sporulation. Since chitin is not present in mammalian cells, it has the potential to be a highly selective target for therapeutic use.

1.5.1.3. Mannoproteins

Mannose constitutes a major portion of the cell wall of many fungi, as well as the glycoproteins that form the protective capsule in *C. neoformans.* The biosynthetic pathway of this polysaccharide may be important to its survival in the host. Mannoproteins are formed by *O*-linkages joining mannose and small oligosaccharides to the hydroxyl groups of the amino acids serine or threonine. A second type of linkage connects high molecular weight and highly branched mannoproteins to the protein moiety via an N-acetylglucosamine and asparagines (Ballou, 1990). Once mannose has been synthesised, dolichol phosphate mannose synthase transfers mannose from GDP-mannose to dolichol phosphate, forming Dol-P-mannose, a key intermediate in protein glycosylation (Herscovics and Orlean, 1993).

The glycosylation of proteins occurs in the rough endoplasmic reticulum, after which they are transported to the cell wall. All these steps might become antifungal drug targets.

1.5.2. The fungal cytoplasmic membrane

The fungal plasma membrane is similar to its mammalian counterpart. It contains phospholipids, sphingolipids, sterols and proteins. The key factors for the plasma membrane to function are its fluidity, its rigidity and its transport mechanisms, determined by lipid composition, sterol composition and protein composition, respectively.

1.5.2.1. Sphingolipids

Sphingolipids are essential components of all eukaryotic plasma membranes and modulation of them exerts a deep impact on cell viability (Hannun and Luberto, 2000). Although the presence and role of sphingolipids are common to these two organisms, their biosynthetic pathways differ. These differences may represent a new suitable target for the development of antifungal agents. Sphingolipid synthesis and metabolism appear to be conserved among non-pathogenic and pathogenic fungi (Zhong *et al*., 2000).

1.5.2.2. Phospholipids

The fungal phospholipid pathway is structurally similar to the mammalian counterpart (Daum *et al*., 1998). The only difference is the synthesis of phosphatidylserine, which is synthesised from CDP-diacylglycerol in fungi, but from phosphotidylethanolamine and serine in mammalian cells (Klig *et al*., 1988). Presently there is no specific target or compound reported that inhibits fungal phospholipid biosynthesis.

1.5.2.3. Ergosterol synthesis

The ergosterol biosynthesis pathway and its target sites for antifungal agents are known. Azole antifungal agents prevent the synthesis of ergosterol by inhibition of the cytochrome P450-dependent enzyme, lanosterol demethylase (also referred to as 14α-sterol demethylase or $P450_{DM}$) (Ghannoum and Rice, 1999). This enzyme is also found in mammalian cells where it plays an important role in cholesterol synthesis (Koltin and Hitchcock, 1997). However, azoles possess a much greater affinity for the fungal enzyme than their mammalian counterparts, and as such are currently the most widely used antifungal agents.

1.5.2.4. Plasma membrane ATPase

The plasma membrane ATPase (P-ATPase) is encoded by the PMA1 gene and controls both efflux and influx of cations (H^+, Ca^+, Na^+, and K^+) across the plasma membrane. The fungal Pmal enzyme differs considerably from the mammalian and plant enzymes, especially in transmembrane segments 1, 2, 3, and 4 (Monk *et al*., 1995). Site-directed mutagenesis of these regions frequently results in lethal mutations in *S. cerevisiae*. These observations suggest that the P-ATPase pumps can be considered potential targets for the development of new antifungal agents.

1.5.2.5. Antifungal peptide

Antifungal peptide molecules appear to act mainly on plasma membrane synthesis. A different class of peptides, lipopepetides, affect mainly cell wall synthesis (Balkovec, 1994). These peptides may help both dissect important targets in the plasma membrane and themselves become antifungal agents.

1.5.3. DNA and protein synthesis

1.5.3.1. Topoisomerases

Topoisomerases control the topological state of DNA by introducing transient DNA breaks (single-strand DNA for Type I and double-strand DNA for Type II) that allow for the manipulation of DNA strands (Wang, 1971). Topoisomerases stabilise the nicked DNA strands by forming a covalent phosphate-tyrosine linkage with either the 3'- or 5'- end of the DNA. Topoisomerase-specific inhibitors stabilise this covalent protein-DNA linkage, effectively slowing the religation of catalysis and ultimately leading to DNA damage and cell death (Lima and Mondragon, 1994). Studies on *C. albicans* and *C. neoformans* have revealed that topoisomerase I (*TOP1*) is essential for viability (Del-Poeta *et al*., 1999; Jiang *et al*., 1997), so *TOP1* appears critical for viability. Fungal TOP1 enzymes contain an amino acid insertion, located in the linker domain region, not found in the mammalian enzyme.

1.5.3.2. Nucleases

The dicationic aromatic compounds (DACs) are pentamidine derivatives that have been shown to posess excellent *in vitro* and *in vivo* activity against pathogenic microorganisms (Tidwell *et al*., 1993). These compounds have *in vitro* antifungal activity against *C. neoformans* and *C. albicans*. Several of these agents exhibited excellent *in vitro* fungicidal activity against a *C. albicans* mutant strain containing a fluconazole-resistant mechanism (Del-Poeta *et al*., 1998). Since these compounds have been administered safely to animals,

they have the potential of being developed into potent antifungal agents for general use in humans.

1.5.3.3. Protein synthesis

Several well-characterised compounds are known to inhibit the RNA polmerases and elongation factors required for transcription and protein synthesis. The evaluation of the degree to which these compounds are selective to fungi will determine whether this class of compounds has the potential of becoming novel antifungal agents. Elongation factor 3 (EF-3) is a unique and essential requirement of the fungal translation machinery. Non-fungal organisms do not have and do not require a soluble form of the EF-3 for translation (Kovalchuke and Chakraburtty, 1994), therefore, it is an ideal antifungal target (Kovalchuke *et al*., 1998). No inhibitors of EF-3 have been identified (Wills *et al*., 2000).

1.5.3. Signal transduction pathways

The signal transduction cascades in fungi have become very attractive since their components are now emerging as targets for new natural antifungals. Cardenas *et al* (1998) studied the mechanism of action of five natural products, cycosporin A (CsA), FK506, rapamycin, wortmannin and genldanamycin on signalling and found that they targeted calcineurin-mediated signal transduction.

1.5.4.1. Calcineurin

Calcineurin is a serine/threonine-specific Ca^{2+}-calmodulin-activated protein phosphatase that is conserved from yeast to man (Hemenway and Heitman, 1999). Calcineurin is the target of CsA and FK506 in T-cells, *C. albicans*, *C. neoformans* and *A. fumigatus* (Odom *et al*., 1997). A number of non-immunosuppressive FK506 and CsA analogues have been described, including L-685, 818 (18-OH, 21-ethyl-FK506), which retain antifungal activity *in vitro* via inhibition of calcineurin (Odom *et al*., 1997). If these non-immunosuppressive CsA analogues have antifungal activity they will need to be tested in animal models for antifungal efficacy.

1.5.5. Virulence factors

1.5.5.1. Melanin

Melanin is produced by the enzyme laccase and has been thought to be major virulence factor in the pathogenic fungus *C. neoformans* (Liu *et al*., 1999). Melanin production has also been discovered in other pathogenic fungi, including the dematiaceous fungi, which produce compounds classified as phaeohyphomycoses (Fothergill, 1996). The focus on *C.*

neoformans and its melanin production has two potential benefits, firstly it facilitates understanding of the function of melanin in yeast cells within the host, and secondly with further understanding of biochemistry and molecular biology of melanin, it could become a unique target for antifungal drugs against *C. neoformans* and other dematiaceous fungi.

1.5.5.2. Mannitol

Other than mannose, another possible metabolic target associated with virulence in *C. neoformans* is the mannitol pathway. Chaturvedi *et al*. (1996) isolated one mutant with decreased mannitol production and found it to be more susceptible to polymorphonuclear leukocyte killing. Further studies are needed to understand and validate the role of the mannitol pathway in fungal virulence.

1.5.5.3. Phospholipases

Phospholipases are a group of enzymes that hydrolyse specific ester linkages in glycerophospholipids. Invasion of the host cells by microbes involves penetration and damage of the outer cell envelope. This happens by enzymatic or physical means, and phospholipases are involved in the cell disruption process that occurs during infection. The enzyme could promote the pathogen entering into the host cell (Ibrahim *et al*., 1995). Extracellular phospholipases have been found to be implicated with pathogenecity in fungi including *C. albicans, C. glabrata, Penicillum notatum, A. fumigatus and C. neoformans*. The potential of these enzymes as targets for drug design is still under development.

1.6. Major groups of antimicrobial compounds from plants

The information in this section is summarised from Cowan (1999).

1.6.1. Phenolics and Polyphenols

Simple phenols and phenolic acids.

Some of the simplest bioactive phytochemicals consist of a single substituted phenolic ring. Cinnamic and caffeic acids are common representatives of a wide group of phenylpropane-derived compounds that are in the highest oxidation state (**Figure 1.3**). The common herbs tarragon and thyme both contain caffeic acid, which is effective against viruses (Wild, 1994), bacteria (Brantner *et al*., 1996), and fungi (Duke, 1985). Catechol and pyrogallol are both hydroxylated phenols, shown to be toxic to microorganisms. Catechol has two 2-OH groups, and pyrogallol has three. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms, with evidence that increased

hydroxylation results in increased toxicity (Geissman, 1963). The mechanisms thought to be responsible for phenolic toxicity to microorganisms include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins (Mason and Wasserman, 1987). Phenolic compounds possessing a C3 side chain at a lower level of oxidation and containing no oxygen are classified as essential oils and often cited as antimicrobial as well. Eugenol is a well-characterized representative found in clove oil (**Figure 1.4**). Eugenol is considered bacteriostatic against both fungi (Duke, 1985) and bacteria (Thomson, 1978).

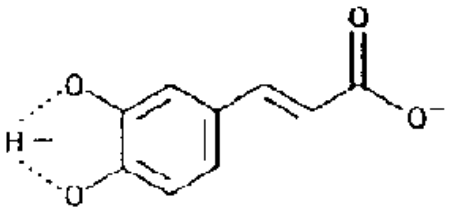


Figure 1.3. Caffeic acid

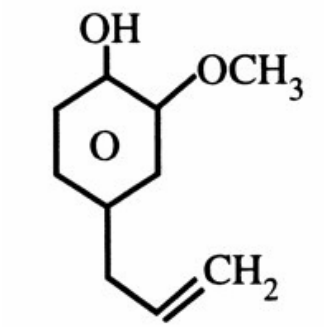


Figure 1.4. Eugenol

1.6.2. Quinones.

Quinones are aromatic rings with two ketone substitutions (**Figure 1.5**). They are ubiquitous in nature and are characteristically highly reactive. These compounds,

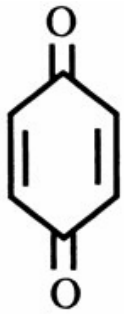


Figure 1.5. Quinone

being coloured, are responsible for the browning reaction in cut or injured fruits and vegetables and are an intermediate in the melanin synthesis pathway in human skin (Schmidt, 1988). The switch between diphenol (or hydroquinone) and diketone (or quinone) occurs easily through oxidation and reduction reactions. The individual redox potential of the particular quinone-hydroquinone pair is very important in many biological systems; witness the role of ubiquinone (coenzyme Q) in mammalian electron transport systems. Vitamin K is a complex naphthoquinone. Its antihaemorrhagic activity may be related to its ease of oxidation in body tissues (Harris, 1963). Hydroxylated amino acids may be made into quinones in the presence of suitable enzymes, such as a polyphenoloxidase (Vamos-Vigyazo, 1981).

In addition to providing a source of stable free radicals, quinones are known to complex irreversibly with nucleophilic amino acids in proteins (Stern *et al*., 1996), often leading to inactivation of the protein and loss of function. Probable targets in the microbial cell are surface-exposed adhesins, cell wall polypeptides, and membrane-bound enzymes.

1.6.3. Flavones, flavonoids, and flavonols.

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones) (**Figure 1.6**). 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. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described above for quinones. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al*., 1996).

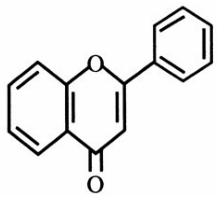


Figure 1.6. Flavone

Catechins are the most reduced form of the C3 unit in flavonoid compounds, and these flavonoids have been extensively researched due to their occurrence in oolong green teas. Flavonoid compounds exhibit inhibitory effects against multiple viruses. Numerous studies have documented the effectiveness of flavonoids such as swertifrancheside, glycyrrhizin (from licorice), and chrysin against HIV (Pengsuparp *et al*., 1995). More than one study has

found that flavone derivatives are inhibitory to respiratory syncytial virus (RSV) (Kaul et al., 1985). Kaul et al. (1985) provide a summary of the activities and modes of action of quercetin, naringin, hesperetin, and catechin in *in vitro* cell culture monolayers. While naringin was not inhibitory to herpes simplex virus type 1 (HSV-1), poliovirus type 1, parainfluenza virus type 3, or RSV, the other three flavonoids were effective in various ways.

1.6.4. Tannins

Tannin is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin and other proteins 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 (**Figure 1.7**). Tannins may be formed by condensations of flavan derivatives which have been transported to woody tissues of plants. Alternatively, tannins may be formed by polymerization of quinone units (Geissman, 1963). This group of compounds has received a great deal of attention in recent years, since it was suggested that the consumption of tannin-containing beverages, especially green teas and red wines, can cure or prevent a variety of ills (Serafini *et al*., 1994).

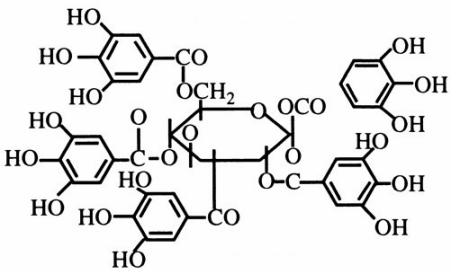


Figure 1.7. Tannins

1.6.5. Coumarins

Coumarins (**Figure 1.8**) are phenolic substances made of fused benzene and α-pyrone rings (O'Kennedy and Thorne, 1997). They are responsible for the characteristic odour of hay. As of 1996, at least 1,300 had been identified (Hoult and Paya, 1996). Their fame has come mainly from their antithrombotic, anti-inflammatory, and vasodilatory activities (Namba, 1988). Warfarin is a particularly well-known coumarin which is used both as an oral anticoagulant and, interestingly, as a rodenticide (Keating and O'Kennedy, 1997). It may also

have antiviral effects (Berkada, 1978). Coumarins are known to be highly toxic in rodents and mammals, therefore are treated with caution by the medical community.

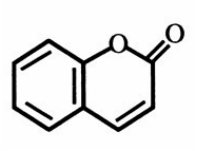


Figure 1.8. Coumarins

Coumarin was found *in vitro* to inhibit *Candida albican*s. 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. More specifically, coumarin has been used to prevent recurrences of cold sores caused by HSV-1 in humans (Berkada, 1978) but was found ineffective against leprosy. Hydroxycinnamic acids, related to coumarins, seem to be inhibitory to Gram-positive bacteria (Fernandez *et al*., 1996). Also, 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).

1.6.6. Terpenoids and Essential Oils

The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compounds based on an isoprene structure (**Figure 1.9**). They are called terpenes, their general chemical structure is C_{10} H_{16} , and they occur as monoterpenes, diterpenes, triterpenes, and tetraterpenes (C_{20} ,C_{30} , and 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 acetate units, and as such they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized.

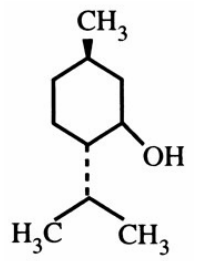


Figure 1.9. Terpenoids

Examples of common terpenoids are menthol and camphor (monoterpenes) and farnesol and artemisin (sesquiterpenoids). Artemisin and its derivative a-arteether, also known by the

name qinghaosu, find current use as antimalarials (Vishwakarma, 1990). Terpenenes or terpenoids are active against bacteria (Amaral *et al*., 1998, and Barre *et al*., 1997), fungi (Ayafor *et al*., 1994), viruses (Fujioka and Kashiwada, 1994), and protozoa (Ghoshal *et al*., 1996). In 1977, it was reported that 60% of essential oil derivatives examined to date were inhibitory to fungi while 30% inhibited bacteria (Chaurasia and Vyas, 1977). The triterpenoid betulinic acid is just one of several terpenoids which have been shown to inhibit HIV. The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. Accordingly, Mendoza *et al*. (1997) found that increasing the hydrophilicity of kaurene diterpenoids by addition of a methyl group drastically reduced their antimicrobial activity.

1.6.7. Alkaloids

Heterocyclic nitrogen compounds are called alkaloids (**Figure 1.10**). The first medically useful example of an alkaloid was morphine, isolated in 1805 from the opium poppy *Papaver somniferum* (Fessenden and Fessenden, 1982); the name morphine comes from the Greek Morpheus, god of dreams. Codeine and heroin are both derivatives of morphine. Diterpenoid alkaloids, commonly isolated from the plants of the Ranunculaceae, or buttercup family, are commonly found to have

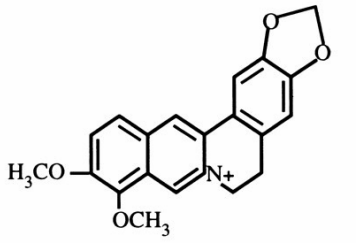


Figure 1.10. Berberine

antimicrobial properties (Omulokoli *et al*., 1997). Solamargine, a glycoalkaloid from the berries of *Solanum khasianu*m, and other alkaloids may be useful against HIV infection (McMahon *et al*., 1995) as well as intestinal infections associated with AIDS (McMahon *et al*., 1995). While alkaloids have been found to have microbicidal effects (including against *Giardia* and *Entamoeba* species), the major antidiarrheal effect is probably due to their effects on transit time in the small intestine.

Berberine (**Figure 1.10**) is an important representative of the alkaloid group. It is potentially effective against trypanosomes and plasmodia (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).

1.6.8. Lectins and Polypeptides

Peptides which are inhibitory to microorganisms were first reported in 1942 by Balls and colleagues. 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. Recent interest has been focused mostly on studying anti-HIV peptides and lectins, but the inhibition of bacteria and fungi by these macromolecules, such as that from the herbaceous *Amaranthu*s, has long been known (De Bolle, 1996). Thionins are peptides commonly found in barley and wheat and consist of 47 amino acid residues. They are toxic to yeasts and Gram-negative and Gram-positive bacteria (Fernande de Caleya *et al*., 1972). Thionins AX1 and AX2 from sugar beet are active against fungi but not bacteria (Kragh *et al*., 1995). Fabatin, a newly identified 47-residue peptide from fava beans, appears to be structurally related to g-thionins from grains and inhibits *E. coli*, *P. aeruginos*a, and *Enterococcus hirae* but not *Candida* or *Saccharomyces* (Zhang and Lewis, 1997). The larger lectin molecules, which include mannose-specific lectins from several plants, MAP30 from bitter melon, GAP31 from *Gelonium multiflorum*, and jacalin (Lee-Huang et al., 1995), are inhibitory to viral proliferation (HIV, cytomegalovirus), probably by inhibiting viral interaction with critical host cell components.

1.7. FUNGI

Fungi are eukaryotic microorganisms, which are heterotrophic and essentially aerobic with limited anaerobic capabilities. Fungi synthesize lysine by the L-aadipic acid biosynthetic pathway. They possess chitinous cell walls, plasma membranes containing ergosterol, 80SrRNA and microtubules composed of tubulin. Fungi grow as yeasts, moulds or a combination of both (i.e. dimorphism). They lack chlorophyll and are classified into a separate kingdom.

1.7.1. Structure

Fungi can grow as yeasts and/or as moulds or both. The latter is known as dimorphism. Yeasts are single-celled forms that reproduce by budding, whereas moulds form multicellular hyphae. Many human and animal fungal pathogens exhibit thermal dimorphism in that they exist as yeast cells at 37 °C and as moulds at 25°C. Dimorphism is regulated by factors such as temperature, CO_2 concentration, pH, and the levels of cysteine or other sulfhydryl-containing compounds, depending upon the dimorphic fungus.

1.7.1.1. Yeast

Yeasts are unicellular fungi. The precise classification is a field that uses the characteristics of the cell, ascospore and colony. Physiological characteristics are also used to identify species. One of the more well-known characteristics is the ability to ferment sugars for the production of ethanol. Budding yeasts are true fungi of the phylum *Ascomycetes*, class *Hemiascomycetes*. The true yeasts belong to one main order *Saccharomycetales*.

Yeasts are characterized by a wide dispersion of natural habitats, and are common on plant leaves and flowers, in soil and salt water. Yeasts are also found on the skin surfaces and in the intestinal tracts of warm-blooded animals, where they may live symbiotically or as parasites. In humans, *Candida albicans* causes vaginal infections, diaper rash and thrush of the mouth and throat.

Yeasts multiply as single cells that divide by budding (e.g. *Saccharomyces*) or direct division (fission, e.g. *Schizosaccharomyces*), or they may grow as simple irregular filaments (mycelium). In sexual reproduction most yeasts form asci, which contain up to eight haploid ascospores. These ascospores may fuse with adjoining nuclei and multiply through vegetative division or, as with certain yeasts, fuse with other ascospores.

1.7.1.2. Moulds

Moulds are microscopic, plant-like organisms, composed of long filaments called hyphae. Mould hyphae grow over the surface and inside nearly all substances of plant or animal origin. Included in this group are the familiar mushrooms and toadstools. When mould hyphae are numerous enough to be seen by the naked eye they form a cottony mass called a mycelium.

Moulds reproduce sexually by spores and asexually by conidia. Spores are in certain aspects like seeds; they germinate to produce a new mould colony when they land in a suitable place. Unlike seeds, they are very simple in structure and never contain an embryo. Spores are produced in a variety of ways and occur in a bewildering array of shapes and sizes. In spite of this diversity, spores are quite constant in shape, size, colour and form for any given mould, and are thus very useful for mould identification.

1.7.1.3. Dimorphic fungi

The dimorphic fungi have two forms, which are: (1) Yeast - (parasitic or pathogenic form). This is the form usually seen in tissue, in exudates, or if cultured in an incubator at 37 ^{o}C. (2) Mycelium - (saprophytic form). The form observed in nature or when cultured at 25 ^{o}C. Conversion to the yeast form appears to be essential for pathogenicity in dimorphic fungi. Fungi are identified by several morphological or biochemical characteristics, including the appearance of their fruiting bodies. The asexual spores may be large (macroconidia, chlamydospores) or small (microconidia, blastospores, arthroconidia).

Fungal infections appear as systemic mycoses with the exception of *S. schenckii* and usually begin by inhaling spores from the mould form. After germination in the tissues, the fungus grows in a non-mycelial form. For example, *Coccidioides immitis* (cause of coccidiodomycosis) produces hyphae and arthrospores when it grows in arid soil but grows as endosporulating spherules (a spherule filled with yeast-like spores) in the lung. *Histoplasma capsulatum*, the cause of histoplasmosis on the other hand, produces hyphae and tuberculate macroconidia in soil contaminated with bird or bat droppings but grows as an encapsulated yeast in the lungs. *Blastomyces dermatitidis* the cause of blastomycosis produces hyphae and conidiospores in soil contaminated with bird droppings but grows as a thick-walled yeast in the body.

1.7.2. Classification

Classification of fungi are mainly based on reproductive structures. Asexual structures are referred to as anamorphs; sexual structures are known as teleomorphs; and the whole fungus is known as the holomorph. Two independent, coexisting classification systems, one based on anamorphs and the other on teleomorphs are used to classify fungi. Fungal infections are usually classified according to the type and degree of tissue involvement and the host response to the pathogen. Fungi can also be classified as exogenous or endogenous depending on the route of infection. Endogenous fungi can cause infections if the host immune system is depressed. Such endogenous infections may originate from normal flora or via reactivation of a previous infection. Classification may be based on the interaction of the organism and the host immune response. Primary pathogens can cause disease even if the host immune system is intact while opportunistic pathogens generally cause disease only in immunocompromised persons.

1.7.2.1 Clinical classification of the mycoses

Fungal diseases may be discussed in a variety of ways. They can be divided into the clinical taxonomy: superficial mycoses, subcutaneous mycoses, systemic mycoses and opportunistic mycoses.

The superficial mycoses (or cutaneous mycoses) are fungal diseases that are confined to the outer layers of the skin, nail, or hair (keratinized layers), rarely invading the deeper tissue or viscera. The fungi involved are called dermatophytes. The subcutaneous mycoses are confined to the subcutaneous tissue and only rarely spread systemically. They usually form deep, ulcerated skin lesions or fungating masses, most commonly involving the lower extremities. The causative organisms are soil saprophytes, which are introduced through trauma to the feet or legs. The systemic mycoses may involve deep viscera and become widely disseminated. Each fungus type has its own predilection for various organs, which will be described as individual diseases are discussed. The opportunistic mycoses are infections due to fungi with low inherent virulence. The etiologic agents are organisms, which are common in all environments.

1.7.3. Multiplication

Fungi may reproduce sexually or asexually. Spores may be either sexual or asexual in origin. Sexual spores include ascospores, basidiospores, oospores and zygospores, which are used to determine phylogenetic relationships. Sexual reproduction occurs by the fusion of two haploid nuclei (karyogamy), followed by meiotic division of the diploid nucleus. Asexual spores are produced in sac-like cells called sporangia and are called sporangiospores. Asexual reproduction results from division of nuclei by mitosis.

1.7.4. Pathogenesis

Fungi have developed many mechanisms to colonize human hosts. The ability to grow at 37°C is one of the most important. Production of keratinase allows dermatophytes to digest keratin in skin, hair and nails. Dimorphism allows many fungi that exist in nature as moulds to change to a yeast form in the host and thus become pathogenic. In contrast, *Candida albicans* exists in the yeast form as normal flora but becomes invasive in the filamentous form. In addition, the antiphagocytic properties of the *Cryptococcus neoformans* capsule and the adherence abilities of *C. albicans* allow pathogenic potential for these fungi.

Fungi may spread locally, such as dermatophytes on the skin or eumycotic mycetomas in subcutaneous tissue. *Sporothix schenckii*, another subcutaneous pathogen, spreads via local

lymphatics. The fungi-producing systemic mycoses mainly cause pulmonary infections. These fungi are phagocytosed by alveolar macrophages but are not destroyed. Instead the fungi are spread hematogenously to distant sites in the body. An exception is *Cryptococcus neoformans*, which disseminates without being phagocytosed. The pathogenesis of some fungi may be at least partly due to the host's reaction to the organism such as the allergic reactions elicited by some fungi.

1.7.5. Host Defenses

While some fungi have more pathogenic potential than others, the immunologic status of the host is of paramount importance in determining whether an organism will cause disease and will help determine the severity of the infection. Both humoral and cell mediated immunity (CMI) are important in control of fungal infections, but CMI appears to be more important since patients with defects in CMI usually suffer more severe fungal infections than do persons with depressed humoral immunity. Nonspecific barriers to fungal infection must be crossed, however, before specific immune responses to fungi are elicited. These primary barriers to fungal infection include intact skin, naturally occurring long-chain unsaturated fatty acids, competition with normal bacterial flora and epithelial turnover rate. In addition the mucous membranes are covered with fluids containing antifungal substances. Furthermore, many epithelial cells of the mucous membranes contain cilia that actively remove microorganisms.

1.7.6. Epidemiology

Whereas some fungi such as *Sporothrix schenckii* are found worldwide, it is most commonly encountered in persons engaged in professions or hobbies where the organism might gain entry into subcutaneous tissues via trauma (e.g. gardeners). Other fungi would be most commonly seen in persons living in or visiting specific geographic regions (e.g. *Coccidioides immitis* in the desert southwestern United States). More specific examples of the role of the environment in fungal infections include the increased rate of candidal vaginitis in women taking systemic antibacterial drugs and increased prevalence of mycotic mycetomas in barefoot persons living in tropical countries. While immunocompromising conditions result in increases in opportunistic fungal infections, the specific underlying disease partially determines the prevalence of such infections. For example, the rhinocerebral syndrome (a deeply invading, life threatening form of zygomycosis, also known as mucormycosis) might be seen in persons suffering from diabetic ketoacidosis while histoplasmosis would be more common in AIDS patients.

1.7.7. Diagnosis

1. Skin scrapings suspected to contain dermatophytes or pus from a lesion can be mounted in 20% KOH on a slide and examined directly under the microscope.

2. Skin testing (dermal hypersensitivity) used to be popular as a diagnostic tool, but this use is now discouraged because the skin test may interfere with serological studies, by causing false positive results. It may still be used to evaluate the patient's immunity, as well as a population exposure index in epidemiological studies.

3. Serology may be helpful when it is applied to a specific fungal disease; there are no screening antigens for 'fungi' in general. Because fungi are poor antigens, the efficacy of serology varies with different fungal infections. The most common serological tests for fungi are based on latex agglutination, double immunodiffusion, complement fixation and enzyme-linked immunoassays (ELISA). While latex agglutination may favor the detection of IgM antibodies, double immunodiffusion and complement fixation tests usually detect IgG antibodies. Some ELISA tests are being developed to detect both IgG and IgM antibodies. There are some tests, which can detect specific fungal antigens, but they are just coming into general use.

4. Fungi can be identified in tissue or exudate smears by using fluorescing stain such as cocalcifluor white or specifically with direct immunoflorescent staining methods

5. Biopsy and histopathology. A biopsy may be very useful for the identification and as a source of the tissue-invading fungi. Either the Gomori methenamine silver (GMS) stain is used to reveal the organisms, which stain black against a green background or Periodic Acid Schiff (PAS) fungi stain a dark pink against blue background.

6. Culture. A definitive diagnosis requires a culture and identification. Pathogenic fungi are usually grown on Sabouraud dextrose agar (Difco). It has a slightly acidic pH (~5.6); cyclohexamide, penicillin, streptomycin or other inhibitory antibiotics are often added to prevent bacterial contamination and saprophytic fungal overgrowth. Two cultures are inoculated and incubated separately at 25 ^{o}C and 37 ^{o}C to reveal dimorphism. The cultures are examined macroscopically and microscopically. They are not considered negative for growth until after 4 weeks of incubation.

1.7.8. Treatment

Mammalian cells do not contain the enzymes that will degrade the cell wall polysaccharides of fungi. Therefore, these pathogens are difficult to eradicate by the animal host defense mechanisms. Because mammals and fungi are both eukaryotic, the cellular milieu is

biochemically similar in both. The cell membranes of all eukaryotic cells contain sterols; ergosterol in the fungal cell membrane and cholesterol in the mammalian cell membrane. Although one of the first antimycotic agents (oral iodides) were used in 1903, the further development of such agents has been left far behind the development of anti-bacterial agents. The selective toxicity necessary to inhibit the invading organism with minimal damage to the host has been difficult to establish within eukaryotic cells. The primary antifungal agents are:

Amphotericin B.

A polyene antimycotic. It is usually the drug of choice for most systemic fungal infections. It has a greater affinity for ergosterol in the cell membranes of fungi than for the cholesterol in the host's cells. Once bound to ergosterol, it causes disruption of the cell membrane and death of the fungal cell. Amphotericin B is usually administered intravenously (patient usually needs to be hospitalized), often for 2-3 months or as a slow release lipid-bond compound subcutaneously. As it is often toxic it is nowadays used together with other antifungals. The drug is rather toxic; thrombo-phlebitis, nephrotoxicity, fever, chills and anemia frequently occur during administration.

Azoles

The azoles (imidazoles and triazoles), including ketoconazole, fluconazole, and itraconozole, are being used for muco-cutaneous candidiasis, dermatophytosis, and for some systemic fungal infections. Fluconazole is presently essential for the treatment of AIDS patients with cryptococcosis. The general mechanism of action of the azoles is the inhibition of ergosterol synthesis. Oral administration and reduced toxicity are distinct advantages.

Griseofulvin

Griseofulvin is a very slow-acting drug, which is used for severe skin and nail infections. Its effect depends on its accumulation in the stratum corneum where it is incorporated into the tissue and forms a barrier, which stops further fungal penetration and growth. It is administered orally. The exact mechanism of action is unknown.

5-fluorocytosine

5-fluorocytosine (Flucytosine or 5-FC) inhibits RNA synthesis and has found its main application in cryptococcosis. It is administered once daily.

1.8. Fungal pathogens used in this study

1.8.1. *Candida albicans*

Candida is a yeast and the most common cause of opportunistic mycoses worldwide. It is also a frequent colonizer of human skin and mucous membranes. *Candida* is a member of normal flora of skin, mouth, vagina, and stool. As well as being a pathogen and a colonizer, it is found in the environment, particularly on leaves, flowers, water, and soil.
It is a dimorphic fungus, most of the time it exists as oval, single yeast cells (10 – 12 μm in diameter), which reproduce by budding. Most yeasts do not produce mycelia but *Candida* has a trick up its sleeve. Normal room temperatures favour the yeast form of the organism, but under physiological conditions (body temperature, pH, and the presence of serum) it may develop into a hyphal form. Pseudohyphae**,** composed of chains of cells, are also common. Chlamydospores may be formed on the pseudomycelium.

Although *Candida* most frequently infects the skin and mucosal surfaces, it can cause systemic infections manifesting as pneumonia, septicaemia or endocarditis in severely immunocompromised patients. There does not appear to be a significant difference in the pathogenic potential of different *Candida* strains, therefore establishment of infection appears to be determined by host factors and not the organism itself. However, the ability to assume various forms may be related to the pathogenicity of the organism. Fortunately, several drugs are available to treat serious systemic infections, e.g. itraconazole and fluconazole.

1.8.1.a. Pathogenicity and Clinical Significance

Infections caused by *Candida* spp. are in general referred to as candidiasis. The clinical spectrum of candidiasis is extremely diverse. Almost any organ or system in the body can be affected. Candidiasis may be superficial and local or deep-seated and disseminated (Beilsa *et al*., 1987). Disseminated infections arise from hematogenous spread from the primarily infected locus. *C. albicans* is the most pathogenic and most commonly encountered species among all (Bodey, 1996). Its ability to adhere to host tissues, produce secretory aspartyl proteases and phospholipase enzymes, and transform from yeast to hyphal phase are the major determinants of its pathogenicity. Several host factors predispose to candidiasis (Bodey *et al*.,1992).

Candidiasis is mostly an endogenous infection, arising from overgrowth of the fungus inhabiting in the normal flora. However, it may occasionally be acquired from exogenous sources (such as catheters or prosthetic devices) (Band and Maki, 1979) or by person-to-person transmission (such as oral candidiasis in neonates of mothers with vaginal

candidiasis or endophthalmitis following corneal transplantation from an infected donor) (Behrens-Baumann, 1991).

1.8.2. *Aspergillus fumigatus*

Aspergillus is a filamentous, cosmopolitan and ubiquitous fungus found in nature. It is commonly isolated from soil, plant debris, and indoor air environment. *Aspergillus* colonies are downy to powdery in texture. The surface colour may vary depending on the species.

A. fumigatus is a thermotolerant fungus and grows well at temperatures over 40°C. This property is unique to *Aspergillus fumigatus* among the *Aspergillus* species.

1.8.2.a. Pathogenicity and Clinical Significance

Aspergillus spp. are well-known to play a role in three different clinical settings in man: (i) opportunistic infections; (ii) allergic states; and (iii) toxicoses. Immunosuppression is the major factor predisposing to development of opportunistic infections (Ho and Yuen, 2000). These infections may present in a wide spectrum, varying from local involvement to dissemination and as a whole called aspergillosis. Among all filamentous fungi, *Aspergillus* is in general the most commonly isolated one in invasive infections. It is the second most commonly recovered fungus in opportunistic mycoses following *Candida*.

Almost any organ or system in the human body may be involved. Onychomycosis, sinusitis, cerebral aspergillosis, meningitis, endocarditis, myocarditis, pulmonary aspergillosis, osteomyelitis, otomycosis, endophthalmitis, cutaneous aspergillosis, hepatosplenic aspergillosis, as well as *Aspergillus* fungaemia, and disseminated aspergillosis may develop (Denning, 1998 and Arikans *et al*., 1998). Nosocomial occurrence of aspergillosis due to catheters and other devices is also likely (Lucas *et al*., 1999). Construction in hospital environments constitutes a major risk for development of aspergillosis particularly in neutropaenic patients (Loo et al., 1996).

Aspergillus spp. may also be local colonizers in previously developed lung cavities due to tuberculosis, sarcoidosis, bronchiectasis, pneumoconiosis, ankylosing spondylitis or neoplasms, presenting as a distinct clinical entity, called aspergilloma (Hohler *et al*., 1995). Aspergilloma may also occur in kidneys (Halpern et al., 1992).
Some *Aspergillus* antigens are fungal allergens and may initiate allergic bronchopulmonary aspergillosis particularly in atopic host (Germand and Tuchais, 1995). Some *Aspergillus* spp. produces various mycotoxins. These mycotoxins, by chronic ingestion, have proven to

possess carcinogenic potential particularly in animals. Among these mycotoxins, aflatoxin is well-known and may induce hepatocellular carcinoma. It is mostly produced by *Aspergillus flavus* and contaminates foodstuff, such as peanuts (Mori *et al*., 1998).

In birds, respiratory infections may develop due to *Aspergillus*. It may induce mycotic abortion in the cattle and the sheep (St-Germain and Summerbell, 1996). Ingestion of high amounts of aflatoxin may induce lethal effects in poultry animals fed with grain contaminated with the toxin. Since *Aspergillus* spp. are found in nature, they are also common laboratory contaminants.

1.8.3. *Sporothrix schenckii*

Sporothrix schenckii is a thermally dimorphic fungus, which is distributed worldwide and isolated from soil, living and decomposing plants, woods, and peat moss. *S. schenckii* is an occasional cause of human infections. Despite the existence of the fungus worldwide, infections due to *S. schenckii* are more common in certain geographical areas. Peru is an area of hyperendemicity for *S. schenckii* infections (Pappas *et al*., 2000).

At 25°C, colonies grow moderately rapidly. They are moist, leathery to velvety, and have a finely wrinkled surface. From the front and the reverse, the colour is white initially and becomes cream to dark brown in time ("dirty candle-wax" color). At 37°C, colonies grow moderately rapidly. They are yeast-like and creamy. The color is cream to beige. The conversion of the mould form to the yeast form is required for definitive identification of *S. schenckii* (Larone, 1995; and Sutton 1998). *Ophiostoma stenoceras* is the teleomorph of *Sporothrix* sp.

1.8.3.a. Pathogenicity and Clinical Significance

S. schenckii is the causative agent of sporotrichosis ("rose handler's disease") (Rex and Okhuysen, 2000). Sporotrichosis is a subcutaneous infection with a common chronic and a rare progressive course. The infection starts following entry of the infecting fungus through the skin via a minor wound and may affect an otherwise healthy individual. Following entry, the infection may spread via the lymphatic route. Nodular lymphangitis may develop (Kostman and DiNubile, 1993). Interestingly, an epidemic of sporotrichosis after sleeping in a rust-stained camping tent has been reported and the tent was identified as the source of infection (Campos et al., 1994). Patients infected with *S. schenckii* may be misdiagnosed as pyoderma gangrenosum due to the large ulcerations observed during the course of sporotrichosis (Byrd et al., 2001).

1.8.4. *Cryptococcus neoformans*

Cryptococcus neoformans is an encapsulated yeast that can cause disease in apparently immunocompetent, as well as immunocompromised, hosts. Most susceptible to infection are patients with T-cell deficiencies (Kwong-chung, 1992). *C. neoformans* var. *neoformans* causes most cryptococcal infections in humans. *C. neoformans* var. *neoformans* is found worldwide; its main habitats are debris around pigeon roosts and soil contaminated with decaying pigeon or chicken droppings. Not part of the normal microbial flora of humans, *C. neoformans* is only transiently isolated from persons with no pathologic features (Mitchell and Perfect, 1995). *C. neoformans* var *gitii* is found in the subtropics in decaying bark and affects both immunocompetent and immunocompromised persons.

Colonies of *C. neoformans* are fast growing, soft, glistening to dull, smooth, usually mucoid, and cream to slightly pink or yellowish brown in colour. The growth rate is somewhat slower than *Candida* and usually takes 48 to 72 h. It grows well at 25°C as well as 37°C. Ability to grow at 37°C is one of the features that differentiates *C. neoformans* from other *Cryptococcus* spp. However, temperature-sensitive mutants that fail to grow at 37°C *in vitro* may also be observed. At 39-40°C, the growth of *Cryptococcus neoformans* starts to slow down (Larone, 1995).

1.8.4.a. Pathogenicity and Clinical Significance

C. neoformans is the causative agent of cryptococcosis. Given the neurotropic nature of the fungus, the most common clinical form of cryptococcosis is meningoencephalitis. The course of the infection is usually subacute or chronic. Cryptococcosis may also involve the skin, lungs, prostate gland, urinary tract, eyes, myocardium, bones, and joints (Durden *et al*., 1994).

The most commonly encountered predisposing factor for development of cryptococcosis is AIDS (Abadi *et al*., 1999). Less commonly, organ transplant recipients or cancer patients receiving chemotherapeutics or long-term corticosteroid treatment may develop cryptococcosis (Urbini et al., 2000).

1.8.6. *Microsporum canis*

Microsporum canis grows rapidly and the diameter of the colony reaches 3 to 9 cm following incubation at 25°C for 7 days on Sabouraud dextrose agar. The texture is woolly to cottony

and flat to sparsely grooved. The color is white to yellowish from the front and deep yellow to yellow-orange from the reverse.

1.8.6.a. Pathogenicity and Clinical Significance

M. canis is a zoophilic dermatophyte of world-wide distribution which is a frequent cause of ringworm in humans, especially children. Invades hair, skin and rarely nails. Cats and dogs are the main sources of infection. Invaded hairs show an ectothrix infection and usually fluoresce a bright greenish-yellow under Wood's ultra-violet light.

1.9. Aim and Objectives

Several investigations into the antimicrobial activity of members of the Combretaceae have been undertaken in recent years. Although the antibacterial properties of various species of *Combretum, Terminalia* and *Pteleopsis* (Basséne *et al*., 1995, Silva *et al*., 1996, Baba-Moussa *et al*., 1998) have been investigated in depth, this is not the case for their antifungal properties (Bhatt and Saxena, 1979, Baba-Moussa et al., 1998). Due to the increasing importance of fungal infections the aim is to fill this gap to a degree by focusing on antifungal activities of Combretaceae species.

Objectives

1. Developing minimum inhibitory concentration (MIC) and bioautographic procedures for fungi to be used in the laboratory in order to screen *Combretum* and *Terminalia* species for antifungal activity.
2. Selecting three or four species for further investigation based on antifungal activity and availability.
3. Isolating the antifungal compounds from one or more of the selected species.
4. Determining the chemical structure and *in vitro* biological activity of the antifungal compound.
5. Developing and applying a protocol and determining *in vivo* antifungal activity of *Combretum* and *Terminalia* extracts and isolated compounds in rats

1.9.1. Hypothesis

The genera *Combretum* and *Terminalia* contain antifungal compounds that can be isolated by bioassay guided fractionation. The chemical structures can be determined and these compounds will have antifungal activity that may be useful in human or animal medicine.

CHAPTER 2

Extraction and TLC profiles

2.1. Introduction

Many solvents can be used to extract antifungal compounds. Since the extracts are intended for use in microbial bioassay and *in vivo* study on rats, it was necessary to select solvents that are non-toxic or otherwise would be easy to remove by evaporation before subsequent assays. The choice of solvent also depends on the ability of solvents to extract the largest quantities of material while also extracting high antifungal and antioxidant activity. An important factor governing the choice of solvents used in an extraction is the type of phytochemical groups that are to be extracted (Houghton and Raman, 1998). In this case it is not known if antibacterial triterpenoids (Angeh, 2005), flavonoids (Martini *et al*., 2004b) or bibenzyls (Eloff *et al*., 2005b) isolated from other Combretaceae may be responsible for antifungal activity.

Several researchers have used different solvents while extracting compounds from plants, for example 80% ethanol in water solution (Vlietinck *et al.*, 1995), ethanol-water (50:50,v/v), methanol (Taylor *et al.*, 1996), petroleum ether, chloroform, ethanol, methanol and water (Salie *et al,* 1996). Cowan (1999) indicated that water, ethanol, methanol, chloroform, methylene dichloride and acetone have been used to isolate antimicrobial compounds from plants. Eloff (1998a) evaluated several solvents and concluded that acetone was the best extractant for antibacterial compounds from *C. erythrophyllum* and *Anthocleista grandiflora*.

The Combretaceae is particularly rich in triterpenoids, flavonoid and stilbenes (Rogers and Verotta, 1996). These compounds are intermediate polar compounds and as such would be extracted by intermediate polar solvents like diethyl ether, ethylacetate, acetone, ethanol and methanol. However, most antibacterial compounds isolated from the Combretaceae are non-polar (Kotze and Eloff, 2002), while most antioxidant compounds are polar (Re *et al*., 1999) hence the need to extract with a wide range of solvent polarities if antifungal and antioxidant compounds are to be extracted.

2.1.1. Extraction

The extraction step is the least developed part of most analytical procedure, and today Soxhlet extraction (developed by F. Soxhlet in 1879) is still used in many routine laboratories. In the last decade there has been an increasing demand for new extraction techniques, amenable to automation, with shortened extraction times and reduced organic solvent consumption — preventing pollution in analytical laboratories and reducing sample

preparation costs (Wan and Wong, 1996). Driven by these purposes advances in sample preparation have resulted in a number of techniques such as microwave-assisted extraction (MAE), supercritical fluid extraction (SFE) and pressurised liquid extraction ((PLE, Dionex trade name ASE, for accelerated solvent extraction) (Björklund *et al*., 2000).

The most commonly studied parameters that have an effect on the extraction process are solvent composition, solvent volume, extraction temperature, extraction time and matrix characteristics such as water content.

2.1.2. Choice of solvents

A correct choice of solvent is fundamental for obtaining an optimal extraction process. When selecting a solvent consideration should be given to the interaction of the solvent with the matrix and the analyte solubility in the solvent. Preferably the solvent should have a high selectivity towards the analyte of interest and exclude unwanted matrix components (Eskilsson and Björklund, 2000). Another important aspect is the compatibility of the extraction solvent with the analytical method used for the final analysis step. Optimal extraction solvents cannot be deduced directly from those used in conventional procedures. We need to know the type of compounds we are targeting in terms of polarity, if not, we must use solvents of different polarities to have a wide range of compounds.

Traditional doctors in southern Africa and all over the world use water extract to cure different diseases. Some are using boiling water, but that can lead to denaturing of some of the compounds, which are heat sensitive. This naturally sets some limitations to the type and amount of compound to be extracted relative to their polarity (Eloff, 1998a).

2.1.3. Solvent volume

The important factor is the ratio of extractant to the sample to be extracted. The solvent volume must be sufficient to ensure that the entire sample is immersed, especially when having a matrix that will swell during the extraction process. Generally in conventional extraction techniques a higher volume of solvent will increase the recovery, because the extraction depends on the partition between the phases. A larger extractant phase leads to a lower consequently better partition from the sample. It is also better to extract repeatedly with a smaller volume than once with a larger volume (Eloff, 1998b). For efficient extractant a rates of 5 – 10 ml of extractant per gram of sample repeated three times extracts practically all soluble compounds from Combretum species (Kotze and Eloff, 2002). Hydrocarbons

have been extracted from sediment samples in the range of 1–15 g with solvent volumes between 10 and 30 ml (Vázquez Blanco *et al*., 2000).

2.1.4. Temperature

Extraction temperature is one of the parameters, which is not surprising since an increase in temperature shortens the establishment of the time needed for partition equilibrium, for all extraction techniques. When extraction is conducted at high temperature, the temperature may reach well above the boiling point of the solvent. This can only occur at high pressures in closed containers. These elevated temperatures result in improved extraction efficiencies, since desorption of analytes from active sites in the matrix will increase (Chee, 1997). Additionally, solvents have higher capacity to solubilize analytes at higher temperatures, while surface tension and solvent viscosity decrease with temperature, which will improve sample wetting and matrix penetration, respectively. Room temperature was found to be optimum temperature to extract different compounds, usually there is little effect when heat is used, only when compound of interest are volatile, there is a need to use less heat (Lopez-Avila *et al*., 1994).

2.1.5. Extraction time and shaking

There is an inverse relationship between the time required for efficient extraction and size of the sample particles (Kotze and Eloff, 2002). Extraction times differs depending on the amount of the sample used. Often 10 min are sufficient for extracting 1 to 3 g samples, which is exemplified by the extraction of organic pollutants (Lopez-Avila *et al*., 1994), but even 3 min have been demonstrated to give full recovery for pesticides from soils and sediments (Onuska and Terry, 1993). In the extraction of sulfonylurea herbicides from soils it was demonstrated that increasing the extraction time from 5 to 30 min did not adversely affect the recovery (Font *et al*., 1998). This was also found by Stout *et al*. (1998) when extracting the fungicide dimethomorph from soil. No difference in recovery was found using 3 or 45 min extraction time. When extracting amino acids from food, no improvement in the extraction efficiency was observed applying longer irradiation times (Kovács et *al*., 1998). Additionally there was no evidence of breakdown or alteration of the amino acids caused by longer extraction times. With thermolabile compounds, long extraction times may result in degradation. The optimum time for most plants extracts is 10 minutes for 1 –2 grams sample (Kotze and Eloff, 2002).

2.1.6. Analysis of compounds in extracts

Chromatography and other related techniques are used to analyze plants extracts. The diversity of compounds and their content vary with not only the species but also with the growing conditions, the season when plants are harvested, the process methods and storage duration.

Although there is a wide choice of other chromatographic methods for plant extracts analysis (GC, HPLC), thin layer chromatography remains a valid and simple analytical procedure for semi-qualitative detection and quantitative determination of plant extracts and their metabolites in the environmental samples (Sherma, 2000).

Thin layer chromatography (TLC) is widely used to analyze compounds recovered from natural materials. TLC does not require expensive instrumentation, nor do samples generally need extensive purification prior to analysis and several extracts can be run on at a time. Compounds can be separated with good resolution, and methods are readily adaptable for applications ranging from high throughput to preparative-scale work. Both normal and reversed-phase adsorbents have been used with a variety of mobile-phase solvent systems.

Separated substances are visualized by UV absorption, chromogenic reaction with spray reagents, or bioautography, in which suspensions of indicator organisms in agar or broth are overlaid on chromatograms to detect bioactive spots (Homans and Fuchs, 1970). Compound identity is confirmed by appearance, distance traveled relative to the solvent front (R_f value), and co-chromatography with standards in at least two different solvent systems. Quantities can be estimated from spot size and intensity, or size of the inhibition zone for bioautography, at various dilutions relative to known amounts of standards run on the same plate (Fried and Sherma, 1982).

2.2. Materials and Methods

2.2.1. Plant collection

Leaves of *Combretum* and *Terminalia* species were collected in the Lowveld National Botanical Gardens (LNBG) in Nelspruit, South Africa in Summer of 2003. Summer is a good period to collect leaves, because new leaves are growing in number during this season. Voucher specimens and origins of the trees are kept in the Garden Herbarium. *Combretum* species collected are listed in **Table 2.1** and *Terminalia* species in **Table 2.2** below. More

information on the origin and references of these plants are presented elsewhere (Eloff, 1999).

Table 2.1. *Combretum* species collected for antifungal and antioxidant screening.

***Combretum* L**	
Section	**Species**
***Hypocrateropsis* Engl. & Diels**	*C. celastroides* Welw. Ex Laws (i) *C. celastroides* ssp. *celastroides* (ii) *C. celastroides* ssp. *orientale*
	C. imberbe Wawra
	C. padoides Eng. & Diels
***Angustimarginata* Engl. & Diels**	*C. caffrum* (Eckl. & Zeyh) Kuntze
	C. erythrophyllum (Burch.) Sond.
	C. kraussii Hochst
	C. woodii Duemmer
	C. nelsonii Duemmer
***Metallicum* Excell & Stace**	*C. collinum* Fresen (i) *C. collinum* ssp. *suluense* (ii) *C. collinum* ssp. *taborense*
***Spathulipetala* Engl. & Diels**	*C. zeyheri* Sond.
***Ciliatipetala* Engl. & Diels**	*C. albopunctactum* Suesseng.
	C. apiculatum Sond. (i) *C. apiculatum* ssp. *apiculatum*
	C. edwardsii Exell (provisional)
	C. moggii Excell (provisional)
	C. molle R. Br.
	C. *petrophilum* Retief
***Breviramea* Engl. & Diels**	*C. hereroense* Schinz
***Conniventia* Engl. Diels**	*C. microphyllum* Klotzsch
	C. paniculatum Vent.
***Poivrea* (Comm. Ex DC)**	*C. bracteosum* (Hochst)
	C. mossambicense (Klotzsch)
	C. acutifolium

Table 2.2. *Terminalia* species collected for antifungal and antioxidant screening.

***Terminalia* L.**	
Section	Species
Abbreviate Exell	*T. prunioides* M.A.Lawson
Psidiodes Exell	*T. brachystemma* Welw. ex Hiern
	T. sericea Burch ex DC
Platycarpae Eng. Diels emend Exell	*T. gazensis* Bak.f.

	T. mollis Laws
	T. sambesiaca Engl.&Diels

2.2.2. Plant storage

Leaves were separated from stems, and dried at room temperature. Most scientists have tended to use dried material because they are fewer problems associated with large-scale extraction of dried plants rather than fresh plant material (Eloff, 1998a). 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.

Some of the reasons dried leaves were chosen to work with were, the time delay between collecting plant material and processing it makes it difficult to work with fresh material because differences in water content may affect solubility or subsequent separation by liquid-liquid extraction. The secondary metabolic plant components should be relatively stable especially if it is to be used as an antimicrobial agent, and many if not most plants are used in the dried form by traditional healers (Eloff, 1998a).

2.2.3. Extractants

When choosing the extractants the following parameters were considered, polarity (polar, intermediate or non-polar); the ease of subsequent handling of the extracts; the toxicity of the solvent in the bioassay process, the potential health hazard of the extractants (Eloff, 1998b). The following extractants, methanol (polar); acetone (intermediate polarity); dichloromethane (intermediate polarity) and hexane (Non-polar) were chosen on the basis of the above parameters.

2.2.4. Extraction procedure

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 3-5 minutes in a Labotec model 20.2 shaking machine at high speed. After centrifuging at 1643 x g for 10 minutes the supernatant was decanted into pre-weighed labeled containers. The process was repeated 3 times to exhaustively extract the plant material and the extracts were combined. Extraction was done at room temperature,

because temperature was found not to have effect on antibacterial activity of Combretaceae species (Eloff, 1999). The solvent was removed under a stream of air in a fume cupboard at room temperature, to quantify the extraction.

2.2.5. Phytochemical analysis

Chemical constituents of the extracts were analyzed by thin layer chromatography (TLC) using aluminium-backed 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 that separate components of Combretaceae extracts well 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 (90:10:1): [BEA] (non-polar/basic) (Kotze and Eloff, 2000).

The dried extracts of the solvents were reconstituted to a concentration of 10 mg/ml in acetone. Acetone was the solvent of choice owing to its wide extraction capacity and low toxicity towards the test organisms in the bioassay procedures (Eloff, 1998b).

Approximately 100 µg aliquots (10 µl of a 10 mg/ml solution) of each of the extracts were loaded in 1 cm bands on three 10 x 10 cm TLC plates (Merck, silica gel 60 F_{254}) and each of these was developed with EMW, CEF or BEA. The extracts were applied approximately 1 cm from the bottom of the plates with a micropipette and allowed to develop for 8 to 9 cm in a tank containing eluent. The atmosphere in the tank was saturated by placing filter paper wetted with the eluent against the walls of the tanks, which were then sealed with lids.

Once developed, the separated compounds were observed under Camac Universal TL-600 UV light at 360 nm and 254 nm and the fluorescing (360 nm) or quenching (254 nm) compounds marked. To detect the separated compounds, vanillin-sulphuric acid (0.1 g vanillin (Sigma): 28 ml methanol: 1 ml sulphuric acid) was sprayed on the chromatograms and heated at 110 ^{o}C to optimal colour development.

2.3. Results

2.3.1. Extraction of raw material

The mass that each solvent extracted from 1 g leaf material of *Terminalia* species was

determined, calculated as % extracted and recorded in **Table 2.3** and of *Combretum* species in **Table 2.4**.

Table 2.3. The percentage mass (%) of *Terminalia* species extracted with four extractants from dried powdered leaves.

***Terminalia* species**	**Percentage mass residue extracted (%)**				
	Acetone	**Hexane**	**DCM**	**Methanol**	**Average**
T. prunioides	7.3	1.1	1.8	27.3	**9.4**
T. brachystemma	7.5	2.5	2.8	26.8	**9.9**
T. sericea	5.5	1.6	2.2	31.4	**10.2**
T. gazensis	2.4	0.3	0.6	3.3	**1.7**
T. mollis	4.7	0.6	0.9	47.3	**13.4**
T. sambesiaca	11.2	1	0.9	22.5	**8.9**
Average	**6.43**	**1.18**	**1.53**	**26.43**	**8.9**

Methanol (26.43 %) extracted the most material in terms of mass from all six *Terminalia* species, followed by acetone with average mass of 6.43 %; hexane and DCM extracted the lowest mass, which were 1.18 and 1.53 % respectively. The highest average mass extracted was from *T. mollis* (13.4 %) and *T. sericea* (10.2 %), and the lowest yield was from *T. gazensis* with 1.7 %. The total percentage mass extracted was 8.9 %. The 47.3 % extracted by methanol from *T. mollis* leaves is an extraordinary high value.

Table 2.4. The percentage mass (%) of *Combretum* species extracted with four extractants from dried powdered leaves.

***Combretum* species**	**Percentage mass residue extracted (%)**				
	Acetone	**Hexane**	**DCM**	**Methanol**	**Average**
C. celastroides ssp. *celastroides*	3.8	1.2	1.7	12.9	**4.9**
C. celastroides ssp. *orientale*	4.2	1.5	2.4	18.2	**6.6**
C. imberbe	5.1	0.9	2.9	9.6	**4.6**
C. padoides	3.5	1.5	2.5	24.5	**8.0**
C. caffrum	18.8	2.1	3.7	17.1	**10.4**
C. erythrophyllum	8	2.7	4.6	8.9	**6.1**
C. kraussii	6.5	0.9	1.7	24.9	**8.5**
C. woodii	5.9	1.2	4.1	20.4	**7.9**
C. collinum ssp. *suluense*	2.2	1.8	1.4	7.1	**3.1**
C. collinum ssp. *taborense*	4.7	1.8	2.1	18	**6.7**
C. zeyheri	3.4	0.9	1.9	18.8	**6.3**
C. albopunctactum	3	1.1	2.1	9.6	**4.0**
C. apiculatum ssp. *apiculatum*	8.9	1.7	2.3	35.2	**12.0**
C. edwardsii	2.9	0.8	1.1	23	**7.0**
C. moggi	7.2	1.5	2	24.2	**8.7**
C. molle	20.9	1.4	2.4	24	**12.2**
C. petrophilum	12.7	2.3	3	40.6	**14.7**

C. hereroense	8	0.7	1.2	32.9	**10.7**
C. microphyllum	1.7	1	1	29.9	**8.4**
C. paniculatum	1.5	0.6	1.5	17.7	**5.3**
C. bracteosum	2.2	1	1.9	8.9	**3.5**
C. mossambicense	5.8	2.8	3.8	11.8	**6.1**
C. acutifolium	4.1	1.4	2.3	18.9	**6.7**
C. nelsonii	5.8	2.3	4.3	18.9	**7.8**
Average	**6.28**	**1.46**	**2.41**	**19.83**	**7.5**

Methanol (19.83 %) extracted the most material in terms of mass from twenty-four *Combretum* species as in *Terminalia* species. This was followed by acetone with an average extracted mass of 6.28 %, almost similar to that of *Terminalia* species. Hexane and DCM extracted the lowest mass, which were 1.46 % and 2.41 % respectively. The highest average mass extracted was from *C. petrophilum* (14.7 mg), *C. molle* (12.2 %) and *C. apiculatum* ssp. *apiculatum* (12.0 %), and the lowest yields were from *C. collinum* ssp. *suluense* and *C. albopunctactum* with 3.1 and 4.0 % extracted respectively. The total percentage mass extracted was 7.5 %, lower than that of the *Terminalia* species with a difference of 1.4 %. In this case methanol extracted an extraordinary high concentration of 40.6 % from *C. petrophilum* leaves.

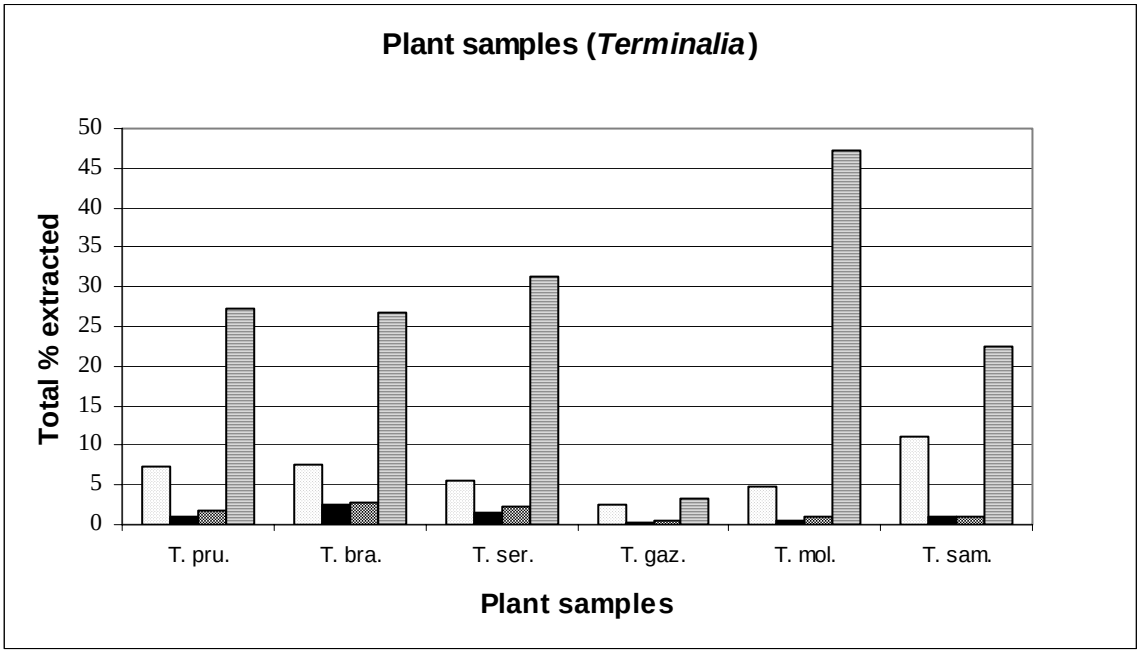


Fig 2.1. Percentage of powdered Terminalia leaf samples extracted by acetone ⬚ , hexane ■ , dichloromethane ▩ ,and methanol ▤ from the six *Terminalia* species: T. pru. = *T. prunioides*, T. bra. = *T. brachystemma*, T. ser. = *T. sericea*, T. gaz. = *T. gazensis*, T. mol = *T. mollis* and T. sam = *T. sambesiaca.*

The total percentages extracted of the *Combretum* species using different solvents (acetone, hexane, DCM and methanol) are shown in **Figure 2.2.** Methanol was the best extractant,

extracting a greater quantity of plant material than any of the other solvents. Total percentages extracted with methanol of *C. apiculatum* ssp. *apiculatum*, *C. petrophilum*, *C. hereroense* and *C. microphyllum* were between 25 and 41%. From **Figure 2.2** it appears that hexane and dichloromethane are more selective extractants for *Combretum* species, because for all the species, the total percentage extracted was below 5%. The total percentage extracted with acetone was better in 10 of the *Combretum* species tested, ranging from 5 to 21%.

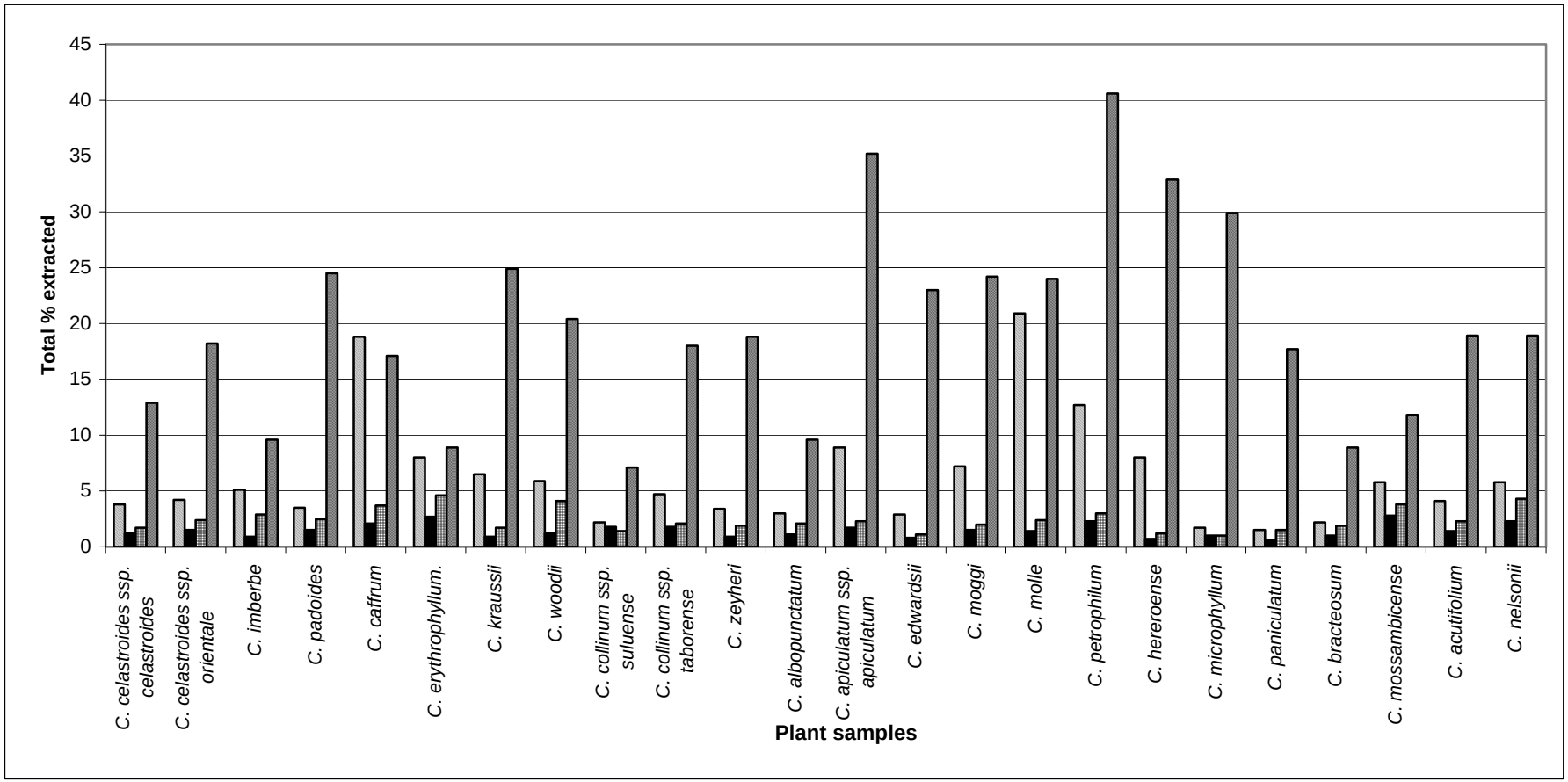


Figure 2.2. Percentage of powdered *Combretum* species leaf extracted by acetone , hexane , dichloromethane , and methanol .

2.3.2. Phytochemical analysis

In all the *Terminalia* and *Combretum* extracts vanillin spray reagent was chosen for visualization of compounds (**Figure 2.3a** to **Figure 2.3g)**.

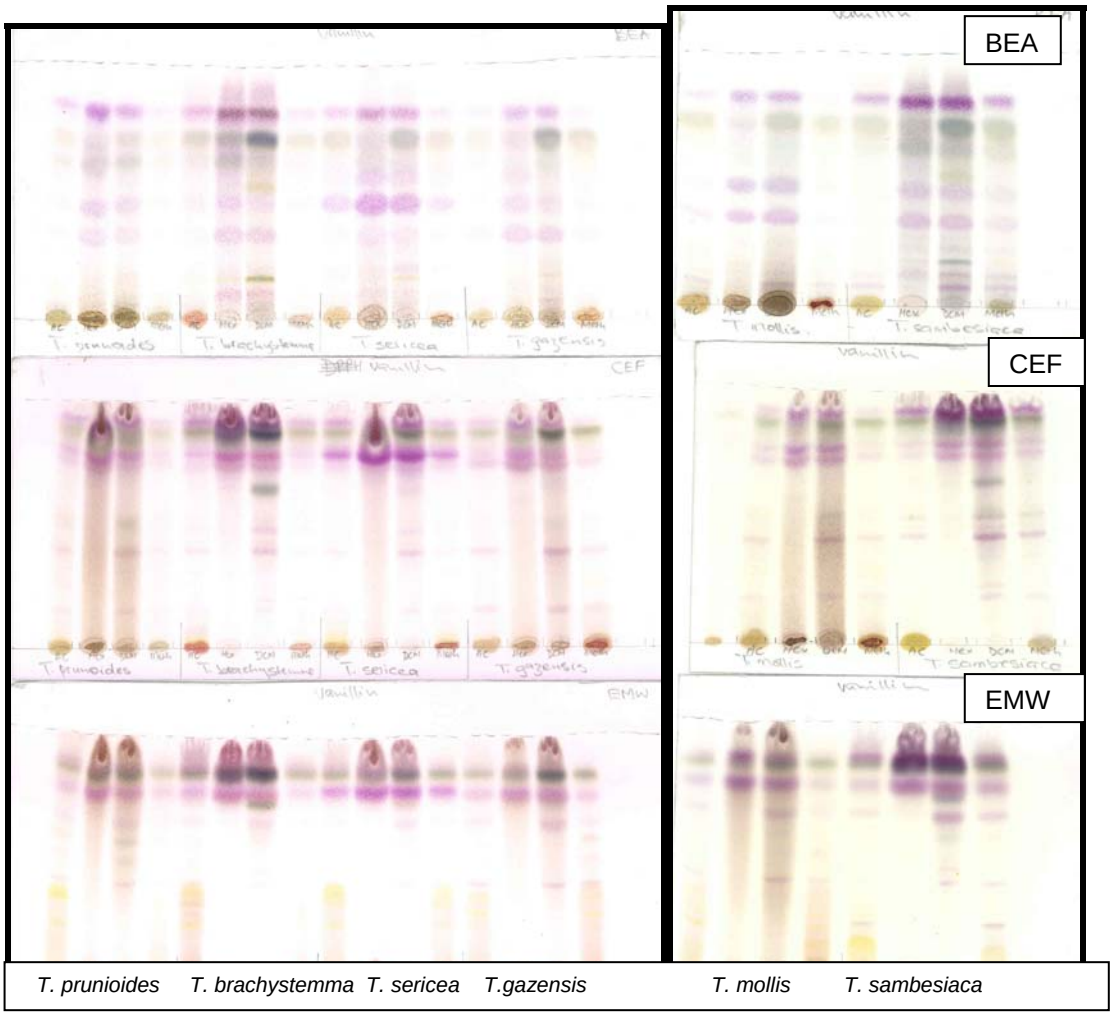
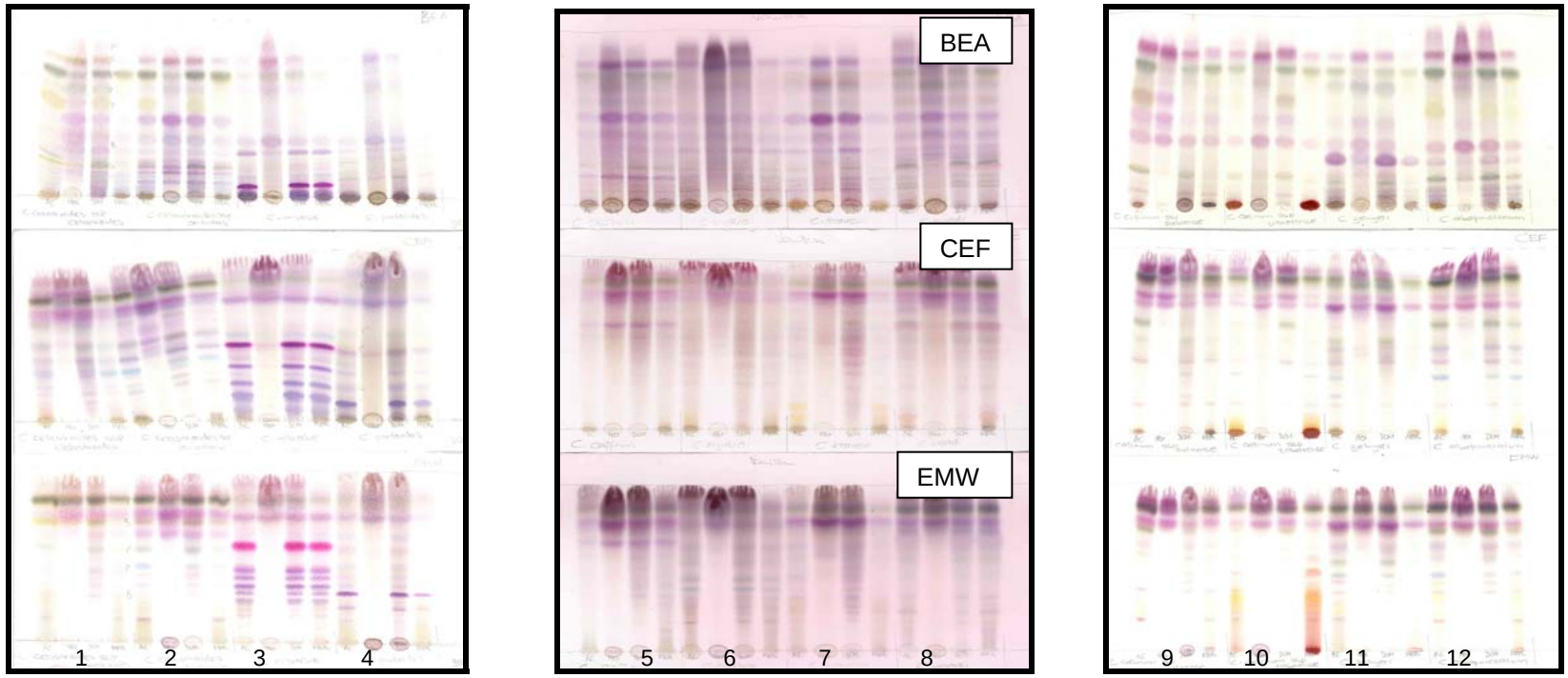


Figure 2.3a. Chromatograms of *Terminalia* species developed in BEA (top), CEF (centre), and EMW (bottom) solvent systems and sprayed with vanillin–sulphuric acid to show compounds extracted with acetone (Ac), hexane (Hex), dichloromethane (D) and methanol (Met), in lanes from left to right for each group.

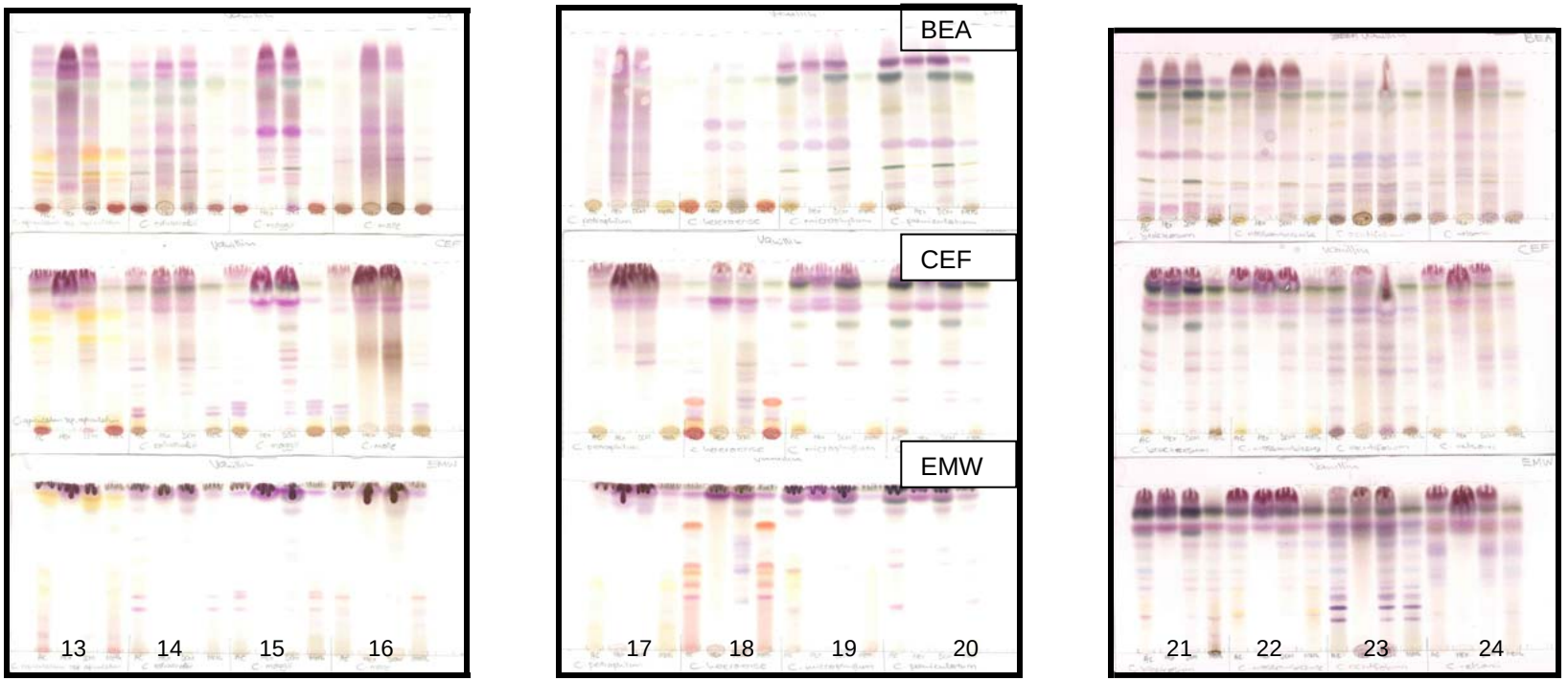


1 *C. celastroide* ssp *celastroides*
2 C. celastroides ssp*. orientale*
3 *C. imberbe*
4 C. padoides

5 *C. caffrum*
6 *C. erythrophyllum*
7 *C. kraussii*
8 *C. woodii*

9 *C. collinum* ssp. *suluense*
10 *C. collinum* ssp. *taborense*
11 *C. zeyheri*
12 *C. albopunctatum*

Figure 2.3b. Chromatograms of *Combretum* species developed in BEA (top), CEF (centre), and EMW (bottom) solvent systems and sprayed with vanillin–sulphuric acid to show compounds extracted with acetone (Ac), hexane (Hex), dichloromethane (D) and methanol (Met), in lanes from left to right for each group.



13 *C. apiculatum* ssp. *apiculatum*
14 *C. edwardsii*
15 *C. moggii*
16 *C. molle*

17 *C. petrophilum*
18 *C. hereroense*
19 *C. microphyllum*
20 *C. paniculatum*

21 *C. bracteosum*
22 *C. mossambicense*
23 *C. acutifolium*
24 *C. nelsonii*

Figure 2.3c. Chromatograms of *Combretum* species developed in BEA (top), CEF (centre), and EMW (bottom) solvent systems and sprayed with vanillin–sulphuric acid to show compounds extracted with acetone (Ac), hexane (Hex), dichloromethane (D) and methanol (Met), in lanes from left to right for each group

2.4. Discussion

Six *Terminalia* species and twenty-four *Combretum* species were selected for antifungal activity and antioxidant screening based on their use in traditional medicinal treatments for both domestic animals and humans in southern Africa, as well as their availability.

The majority of traditional healers use water to isolate active compounds from these plants, because water is not harmful to domestic animals and humans and is generally the only extractant available. Water extracts were not used as test substances in our study because in all our previous work water extracts had no antimicrobial activity and it is tedious to remove water from extracts. This naturally sets some limitations to the type and amount of compound to be extracted relative to their polarity (Eloff, 1998). Using only water leads to difficulties in extracting non-polar active compounds. Success in isolating compounds from plant material is largely dependent on the type of the solvent used in the extraction procedure (Lin *et al*., 1999).

The total percentages of the *Terminalia* species extracted using different solvents (acetone, hexane, DCM and methanol) are shown in **Figure 2.1.** Methanol was the best extractant, extracting a greater quantity of plant material than any of the other solvents. There was a major difference in the methanol extractability of *T. gazensis* leaves compared with all the other species. This difference is not related to the sectional division of the species (Carr, 1988).

After evaporation of extracting solvents, the hexane, dichloromethane and methanol extracts were redissolved in acetone because this solvent was found not to be harmful towards bacteria (Eloff, 1998b). I found that acetone was also not harmful towards fungi at concentrations used in the plant extracts (**Chapter 4**).

Of the four solvents used, methanol extracted more chemical compounds from leaves of the *Terminalia* and *Combretum* species, but the extract probably contained highly polar compounds that may not that interesting for clinical application. Baba-Moussa *et al* (1999)has found that methanol extracts of Combretaceae family contains tannins. Because tannins have low bioavailability, the potential value of tannins as a systemic antifungal compound is low. Some scientists have concluded

that there is therefore not much scope for investigating the Combretaceae for antimicrobial compounds.

There was similarity in the chemical composition of the non-polar compounds of extracts using extractants of varying polarity. This may indicate the presence of saponin-like compounds in the leaves. Saponins are a vast group of glycosides, widely distributed in higher plants. Their surface-active properties are what distinguish these compounds from other glycosides. They dissolve in water to form colloidal solutions that foam upon shaking. Saponins have also been sought after in the pharmaceutical industry because some form the starting point for the semi-synthesis of steroidal drugs. They are believed to form the main constituents of many plant drugs and folk medicines, and are considered responsible for numerous pharmacological properties (Estrada *et al*., 2000).

In all the extracts a number of compounds were observed. Because of the number of samples (120 extracts) I decided not to count all the compounds visualized, but present only the chromatograms. Thin layer chromatography (TLC) was used to fingerprint the plant extracts. This allowed for a comparison of the R_f values and thus aided in the identification of biologically active bands on the chromatograms, used for bioautography. The R_f value can however provide corroborative evidence to identity of a compound. If the two or more compounds have the same R_f values in several solvent systems they are most likely, although not necessarily, the same compounds.

The three eluent systems (**Section 2.2.5**) differed in separating the different polarity compounds. The EMW mobile system separates polar and neutral compounds well, the BEA mobile system separates non-polar compounds best and the CEF mobile system separates intermediate polarity and acidic compounds best. Before spraying the TLC plates with vanillin spray reagent, the plates were observed under UV and visible light, which identified fluorescent-quenching compounds in herbal extracts. UV light usually identifies fluorescing compounds with many double bonds and the visible light only detects coloured compounds, usually with conjugated bonds. Compounds containing aromatic rings adsorb UV light at 254 nm and therefore quench the florescence of the pigment included in the silica gel.

TLC can be used for qualitative as well as semi-quantitative analysis of crude extracts for identification of constituents (Houghton and Raman, 1998). Qualitative

analysis is done by comparing the retardation factor (R_f value) on the TLC against a reference value of a standard.

$$R_f \text{ value} = \frac{\text{distance moved by analyte}}{\text{distance moved by solvent front}}$$

After spraying the TLC plates with vanillin-sulphuric acid, many different compounds could be observed. It is difficult to identify certain compounds on these plates, but one can compare the R_f values of the compounds seen on the plates with the R_f values of compound isolated from *Combretum* or *Terminalia* species, to check resemblance between specific compounds in an extract. Different factors influence the chemical composition of the material and subsequently also influence the results of this study. Such factors may include the season when the plant has been harvested, together with effects of variation in growth conditions. As our study is based on the leaves only, and other parts of the plants were not considered, the results may underestimate the activity of the plant species involved.

CHAPTER 3

Antioxidants

3.1. Introduction

Combretum extracts are used for antimicrobial applications. Very low *in vitro* antimicrobial activities were frequently found in water extracts. Water extracts more polar compound and most antioxidants are polar. Plant extracts containing antioxidant compounds may protect patient indirectly by stimulating the immune system. Therefore, I decided to investigate the presence of antioxidant compounds in *Combretum* and *Terminalia* species.

Oxidation in living organisms is essential for the acquirement of energy in catabolism. However, free radicals produced as a result of this process can result in cell death and tissue damage. Free radicals apparently play a role in aging and in diseases such as atherosclerosis, diabetes, cancer and cirrhosis (Halliwell and Gutteridge, 1999).

Free radicals are continuously produced by our body's use of oxygen such as in respiration and some cell-mediated immune functions. Free radicals are also generated through environmental pollutants, cigarette smoke, automobile exhaust, radiation, air-pollution, pesticides, etc. (Li and Trush, 1994). Normally there is a balance between the quantity of free radicals generated in the body and the antioxidant defense systems that scavenge/quench these free radicals preventing them from causing deleterious effects in the body (Nose, 2000). The antioxidant defense systems in the body can only protect the body when the quantity of the free radicals is within the normal physiological level. But when this balance is shifted towards more free radicals, increasing their burden in the body either due to environmental condition or infections, it leads to oxidative stress, which may result in tissue injury and subsequent diseases (Finkel and Holbrook, 2000).

Plants (fruits, vegetables, medicinal herbs, etc.) contain a wide variety of free radical scavenging molecules, such as phenolic compounds (e.g. phenolic acids, flavonoids, quinones, coumarins, lignans, stilbenes, tannins), nitrogen compounds (alkaloids, amines, betalains), vitamins, terpenoids (including carotenoids), and some other endogenous metabolites, which are rich in antioxidant activity (Zheng and Wang, 2001 and Cai *et al*., 2003). Epidemiological studies have shown that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic, antitumor, antimutagenic, anticarcinogenic, antibacterial, or antiviral activities to a greater or lesser extent (Owen *et al*., 2000 and Sala *et al*., 2002).

Some species of the Combretaceae family, have been found to have antioxidant activities. *Terminalia chebula* extracts have different levels of antioxidant activity for anti-lactoperoxidase (LPO), anti-superoxide radical formation and free radical scavenging activities (Cheng *et al*., 2003).

Terminalia arjuna is a large tree distributed throughout India and its bark is used as cardioprotective agent in hypertension and ischaemic heart diseases. The bark powder is reported to exert hypocholesterolaemic and antioxidant effects in humans (Gupta *et al*., 2001). Extracts of both *Terminalia sericea* and *Gunnera perpensa* showed possible scavenging activity in a concentration dependant manner. Water extracts demonstrated higher activity than the methanol extracts (Mabogo, 1990). Several galloyl quinic acid derivates have been isolated from the galls of *Guiera senegalensis* (Bouchet *et al*., 1996) and have shown antioxidant activity (Bouchet *et al*., 1998).

Masoko *et al*., (2005), have reported that six *Terminalia* species tested possess antioxidant activity. But less work has been done on *Combretum* species. Although many synthetic chemicals, such as phenolic compounds are strong radical scavengers, they usually have side effects (Imaida *et al*., 1983). Antioxidant substances obtained from natural sources may be of great interest in the near future.

3.1.1. Antioxidant screening

The most commonly used methods for measuring antioxidant activity are those that involves the generation of a free radical species, which are then neutralized by antioxidant compounds (Arnao *et al*., 2001). Free radicals are the main focus in research related to antioxidants and oxidative stress. They are reactive species (oxidants), generated internally and externally, that can have adverse effects on physiological function. A free radical is defined as an atom or molecule having at least one unpaired electron. Free radicals generally abstract electrons from other molecules, thereby inducing a chain reaction of electron abstraction and radical formation.

In qualitative analysis of antioxidant activity, the 2, 2,diphenyl-1-picrylhydrazyl (DPPH) assay on TLC plates was used as a screen test for the radical scavenging ability of the compounds present in the different extracts. DPPH is a purple coloured compound that does not dimerize and can hence be prepared in crystalline form. It is a stable free radical and following interaction with antioxidants, they either transfer electrons or hydrogen atoms to it thus neutralizing its free radical character (Naik *et al*., 2003).

The DPPH method measures electron-donating activity of other compounds in the mixture and hence provides an evaluation of antioxidant activity due to free radical scavenging. Any molecule that can donate an electron or hydrogen to it will reacts with DPPH, thus bleaching its colour, through reduction from a purple compound to a light yellow compound by electrons from oxidant compounds. Reaction of DPPH with hydroxyl groups involves a homolytic substitution of one of the phenyl rings of DPPH yielding 2-(4-hydroxyphenyl)-2-phenyl-1-picryl hydrazine as a major product whilst 2-(4 nitrophenyl)-2phenyl-1-picrylhydrazine is also formed via a series of secondary processes which is shown from **figure 3.1**. The concentration of DPPH at the end of a reaction will depend on the concentration and structure of the compound being scavenged (Naik *et al*., 2003).

The main objective was to evaluate the antioxidant activity of various extracts from *Combretum* and *Terminalia* species, and to choose one with the promising antioxidant to do further studies.

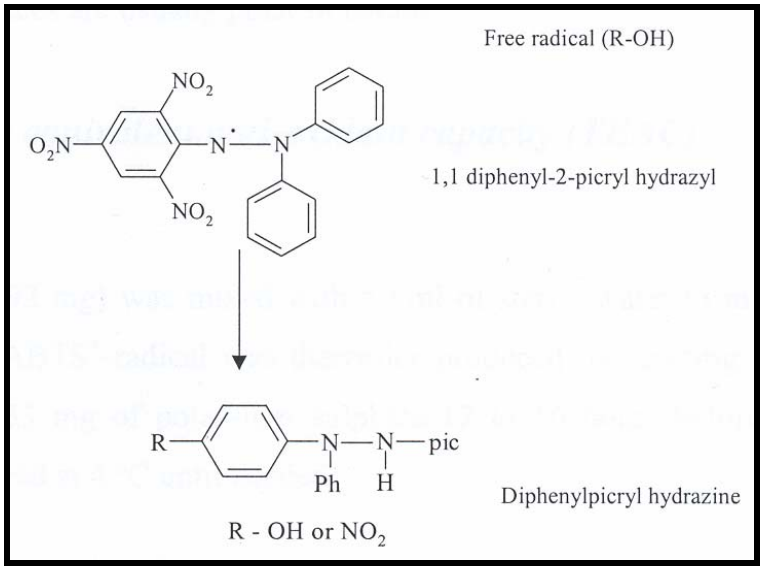


Figure 3.1. Reaction of DPPH with hydroxyl groups of free radical (R-OH) to produce 2-(4-hydroxyphenyl)-2-phenyl-1-picryl hydrazine and R-NO2, 2-(4 nitrophenyl)-2phenyl-1-picrylhydrazine

3.2. Materials and Methods

3.2.1. TLC-DPPH antioxidant screening

This method is generally used for the screening of potential antioxidant activity in crude plant extracts. It involves the chromatographic separation of the crude plant extract, after which

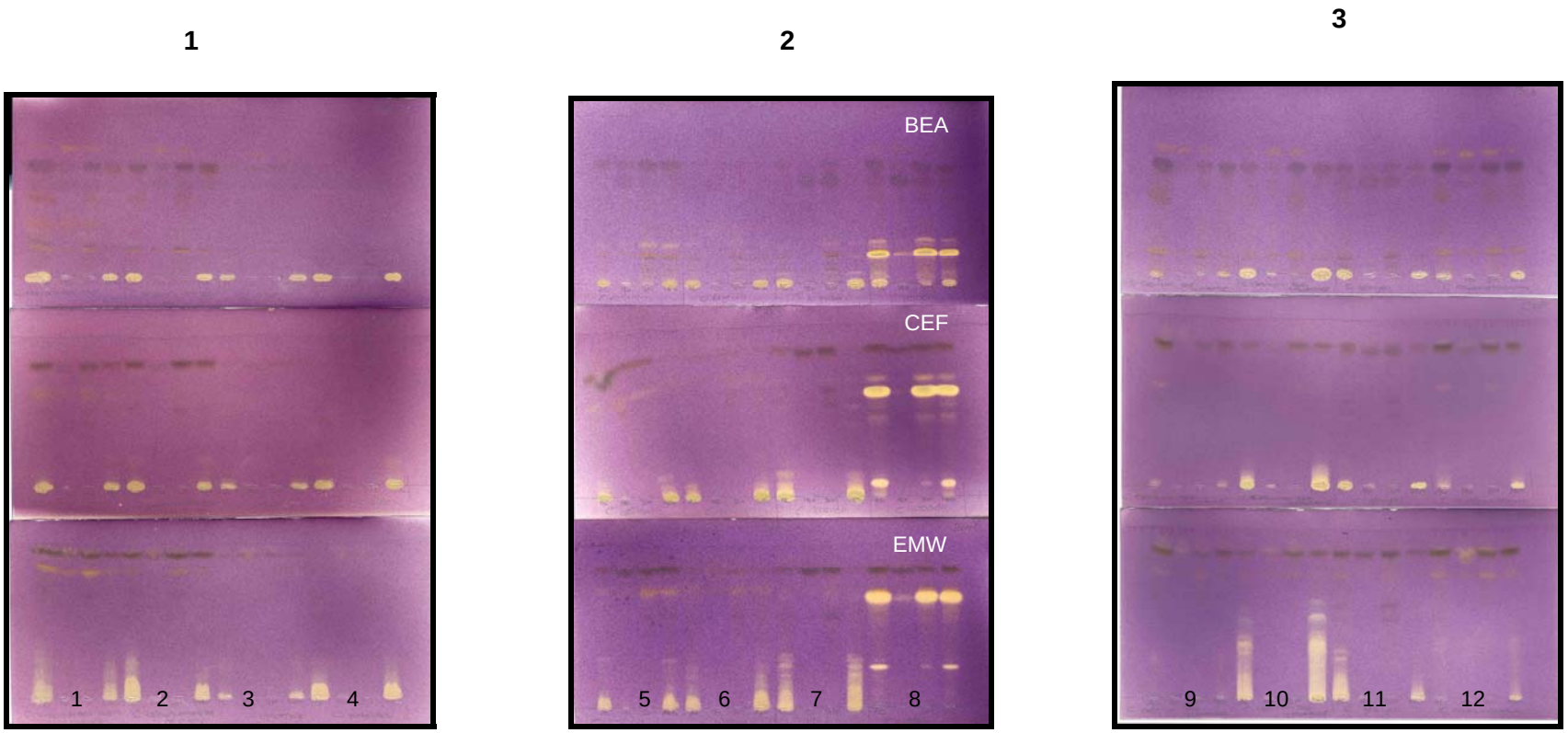
the developed chromatogram is sprayed with a coloured radical solution and the presence of antioxidant compounds is indicated by the disappearance of the radical's colour. Ten microlitres of each extract was loaded as a 1 cm band on the origin of the TLC (Merck, silica gel 60 F_{254}) plates. Plates were developed using BEA, CEF and EMW (**Section 2.2.5**). Plates were viewed under UV (254 and 360 nm) light to locate the UV active compounds. To detect antioxidant activity, chromatograms were sprayed with 0.2 % 1.1 diphenyl-2-picryl-hydrazyl (Sigma®)(DPPH) in methanol, as an indicator (Deby and Margotteaux, 1970) until just wet, and dried in the fumehood. The presence of antioxidant compounds was detected by yellow spots against a purple background on TLC plates sprayed with 0.2% DPPH in methanol.

3.3. Results and Discussion

TLC-DPPH screening method indicated the presence of antioxidant compounds in some of the extracts tested, with *C. woodii* and *C. hereroense* showing the most prominent antioxidant activity (**Figures 3.2a** to **3.2c**). Visualization of the compound with antioxidant activity enabled the localization and the subsequent identification of the potential active compounds.

Results of chromatograms sprayed with 0.2 % DPPH are presented in **Figures 3.2a** to **3.2c**. The acetone and methanol extracts had antioxidant activity after spraying chromatogram. Hexane and dichloromethane extracts apparently did not have any antioxidant activity in *Terminalia* species but hexane and dichloromethane extracts of *Combretum* showed activity, although most of them were very polar. Most of antioxidant compounds were observed in EMW.

C. woodii (**Figure 3.2a(2)**) had very clear antioxidant active compounds from acetone, DCM and methanol extracts. The most prominent compounds were at R_f values 0.20 (BEA), 0.65 (CEF) and 0.73 (EMW). *C. kraussii* also had antioxidant activity especially in EMW. In **Figure 3.2a(3)** only *C. collinum* ssp. *taborense* had antioxidant compounds in EMW from acetone and methanol extracts. The acetone extract of *C. zeyheri* had active compounds with less activity. **Figure 3.2b(4)** had less active compounds calorimetrically determined , but *C. apiculatum* ssp. *apiculatum* showed a number of them and *C. molle* and *C. moggii* thus have some activity.



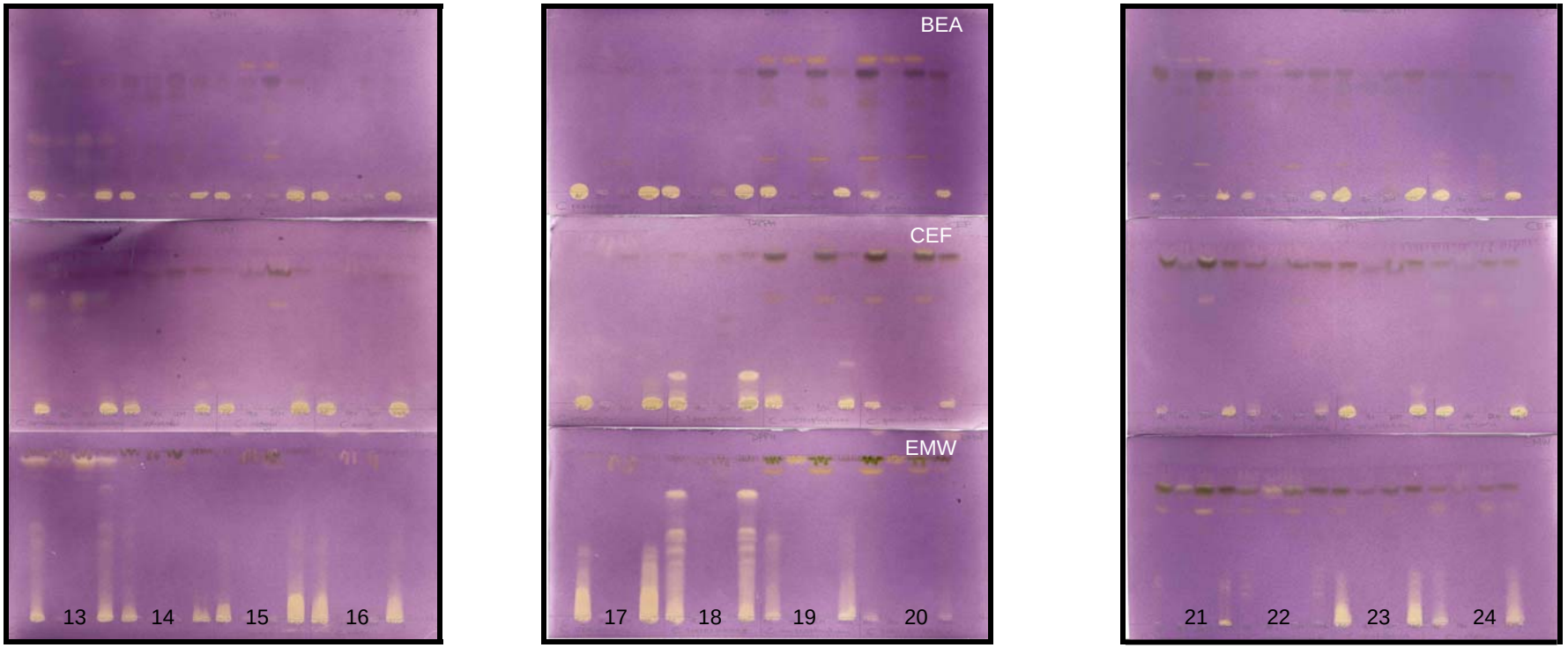
1 *C. celastroide* ssp *celastroides*
2 C. celastroides ssp. *orientale*
3 *C. imberbe*
4 C. padoides

5 *C. caffrum*
6 *C. erythrophyllum*
7 *C. kraussii*
8 *C. woodii*

9 *C. collinum* ssp. *suluense*
10 *C. collinum* ssp. *taborense*
11 *C. zeyheri*
12 *C. albopunctatum*

Figure 3.2a. Chromatograms of *Combretum* species developed in BEA (top), CEF (centre), and EMW (bottom) solvent systems and sprayed with 0.2% DPPH in methanol, clear zones indicate antioxidant activity of compounds extracted with acetone (Ac), hexane (Hex), dichloromethane (D) and methanol (Met), in lanes from left to right for each group.

4 **5** **6**

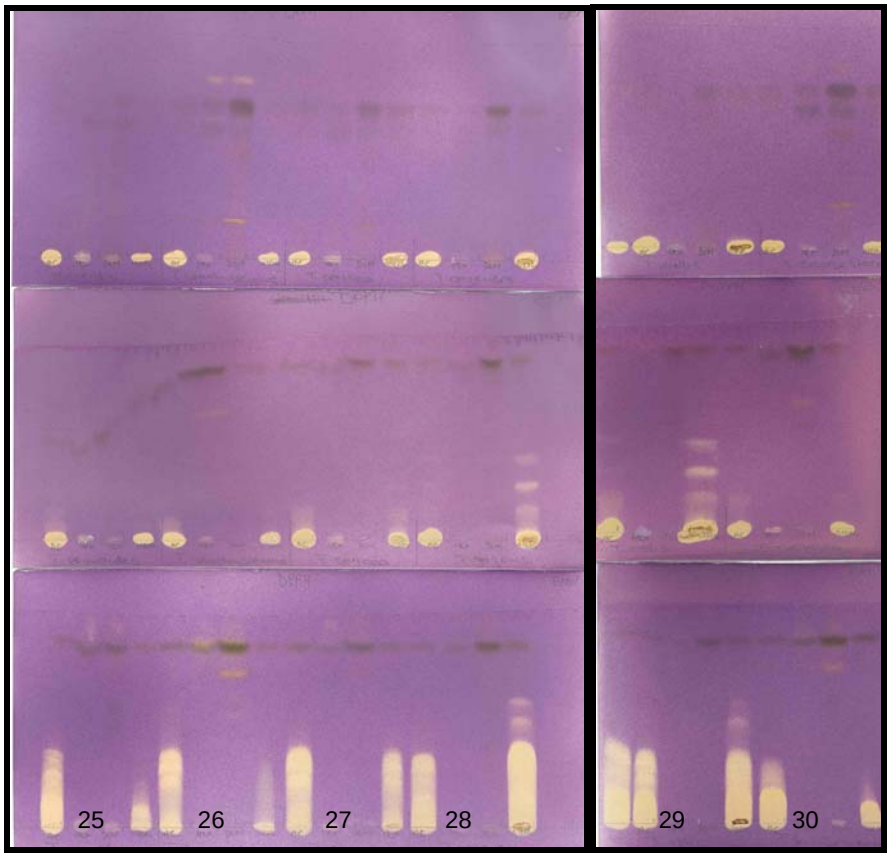


13 *C. apiculatum* ssp. *apiculatum*
14 *C. edwardsii*
15 *C. moggii*
16 *C. molle*

17 *C. petrophilum*
18 *C. hereroense*
19 *C. microphyllum*
20 *C. paniculatum*

21 *C. bracteosum*
22 *C. mossambicense*
23 *C. acutifolium*
24 *C. nelsonii*

Figure 3.2b. Chromatograms of *Combretum* species developed in BEA (top), CEF (centre), and EMW (bottom) solvent systems and sprayed with 0.2% DPPH in methanol, clear zones indicate antioxidant activity of compounds extracted with acetone (Ac), hexane (Hex), dichloromethane (D) and methanol (Met), in lanes from left to right for each group.



25 *T. prunioides*
26 *T. brachystemma*
27 *T. sericea*
28 *T. gazensis*
29 *T. mollis*
30 *T. sambesiaca*

Figure 3.2c. Chromatograms of *Terminalia* species developed in BEA (top), CEF (centre), and EMW (bottom) solvent systems and sprayed with 0.2% DPPH in methanol, clear zones indicate antioxidant activity of compounds extracted with acetone (Ac), hexane (Hex), dichloromethane (D) and methanol (Met), in lanes from left to right for each group.

C. hereroense (**Figure 3.2b(5)**) also had good number of antioxidant compounds to isolate active compounds from, and these compounds are clearly shown in CEF and EMW systems. *C. petrophilum* and *C. microphyllum* have antioxidant compounds. In **Figure 3.2b(6)** none of the tested *Combretum* species had prominent activity, but *C. acutifolium* had less activity in EMW system.

All *Terminalia* species (**Figures 3.2c**) had activity in the acetone and methanol extracts. *T. gazensis* and *T. mollis* methanol extracts had a number of antioxidant compounds in CEF and EMW. The degree of activity of all the samples tested was determined qualitatively from observation of the yellow colour intensity, which indicate antioxidant activity (**Table 3.1**). Only *C. woodii* and *T. mollis* showed activity in other extracts, other

than acetone and methanol, that is in DCM and hexane, respectively

BEA and CEF solvent systems had fewer active compounds, and active compounds were only observed with EMW as eluent for *Combretum* species (**Table 3.2**) and *Terminalia* species (**Table 3.3**). *C. hereroense* had the highest number of active compounds (16), followed by *C. collinum* ssp. *taborense* (10). Acetone extracts of all tested *Combretum* species had 53 active band, methanol extracts had 55, and DCM extracts had only 3 from *C. woodii* (**Table 3.2**). There are differences in species in same section. In *Metallicum* section *C. collinum* ssp *suluense* did not have antioxidant activity but *C. collinum* ssp *taborense* had 10 active bands, in *Connivetia* section *C. microphyllum* had 6 active bands and *C. paniculatum* had nothing, and in *Poivrea* section *C. acutifolium* had 4 and *C. bracteosum* and *C. mossambicense* had nothing. It appears that the presence of antioxidant compounds does not correlate well with taxonomy based on morphological characters.

Six tested *Terminalia* species had the same number of active compounds in the acetone extracts (24) (**Table 3.3**) extracts, and in methanol (23). *T. mollis* hexane leaf extracts had 4 antioxidant compounds, and it was the only species with activity in the hexane extract. Again species in same sections have different number of active compounds. In *Psidiodes* section, *T. brachystemma* had 6 active compounds and *T. sericea* had 8. In *Platycarpae* section *T. sambesiaca* had 6 active compounds and *T. gazensis* and *T. mollis* had 11 and 14 respectively.

Table 3.1. Qualitative DPPH assay on TLC of the 30 plants studied

Plant species	Extractants			
	Acetone	Hexane	DCM	Methanol
***Combretum* species**				
C. celastroides ssp. *celastroides*	++	-	-	++
C. celastroides ssp. *orientale*	++	-	-	++
C. imberbe	++	-	-	++
C. padoides	++	-	-	++
C. caffrum	++	-	-	++
C. erythrophyllum	++	-	-	++
C. kraussii	++	-	-	++
C. woodii	+++	-	+++	+++
C. collinum ssp. *suluense*	-	-	-	-
C. collinum ssp. *taborense*	+++	-	-	+++
C. zeyheri	++	-	-	+
C. albopunctactum	-	-	-	+
C. apiculatum ssp. *apiculatum*	++	-	-	++

C. edwardsii	++	-	-	+
C. moggi	+	-	-	++
C. molle	++	-	-	++
C. petrophilum	++	-	-	++
C. hereroense	+++	-	-	+++
C. microphyllum	+	-	-	+
C. paniculatum	-	-	-	-
C. bracteosum	-	-	-	-
C. mossambicense	-	-	-	-
C. acutifolium	++	-	-	++
C. nelsonii	++	-	-	++
***Terminalia* species**				
T. prunioides	+++	-	-	++
T. brachystemma	+++	-	-	+
T. sericea	+++	-	-	+++
T. gazensis	+++	-	-	+++
T. mollis	+++	+++	-	+++
T. sambesiaca	+++	-	-	+

The degree of activity, determined qualitatively from observation of the yellow colour intensity: weak (+), moderate (++), strong (+++) and no activity (-)

Table 3.2. Number of antioxidant bands present in all *Combretum* species tested on EMW solvent systems and extractants.

***Combretum* species**	**Extractants**				**Total**	**Section**
	Acetone	**Hexane**	**DCM**	**Methanol**		
C. celastroides ssp. *celastroides*	3			3	**6**	**H**
C. celastroides ssp. *orientale*	3			1	**4**	**H**
C. imberbe	1			1	**2**	**H**
C. padoides	2			2	**4**	**H**
C. caffrum	1			1	**2**	**A**
C. erythrophyllum	1			2	**3**	**A**
C. kraussii	3			3	**6**	**A**
C. woodii	3		3	3	**9**	**A**
C. nelsonii	3			3	**6**	**A**
C. collinum ssp. *suluense*	0			0	**0**	**M**
C. collinum ssp. *taborense*	4			6	**10**	**M**
C. zeyheri	3			1	**4**	**S**
C. albopunctactum	0			1	**1**	**C**
C. apiculatum ssp. *apiculatum*	3			6	**9**	**C**
C. edwardsii	3			1	**4**	**C**
C. moggi	2			2	**4**	**C**
C. molle	2			2	**4**	**C**
C. petrophilum	3			4	**7**	**C**
C. hereroense	8			8	**16**	**B**

C. microphyllum	3			3	**6**	**Co**
C. paniculatum	0			0	**0**	**Co**
C. bracteosum	0			0	**0**	**P**
C. mossambicense	0			0	**0**	**P**
C. acutifolium	2			2	**4**	**P**
TOTAL	**53**		**3**	**55**	**108**	

H, *Hypocrateropsis*; A, *Angustimarginata*; M, *Metallicum*; C, *Ciliatipetala*; B, *Breviramea*, Co, *Connivetia*; P, *Poivrea*

Table 3.3. Number of antioxidant bands present in all *Terminalia* species tested on EMW solvent systems and extractants.

***Terminalia* species**	**Extractants**				**Total**	**Section**
	Acetone	**Hexane**	**DCM**	**Methanol**		
T. prunioides	4			2	**6**	**A**
T. brachystemma	4			2	**6**	**Ps**
T. sericea	4			4	**8**	**Ps**
T. gazensis	4			7	**11**	**Pl**
T. mollis	4	4		6	**14**	**Pl**
T. sambesiaca	4			2	**6**	**Pl**
TOTAL	**24**	**4**		**23**	**51**	

A, *Abbreviatae*; Ps, *Psidiodes*; Pl, *Platycarpae*

3.4. Conclusion

The leaves of Combretaceae family are known for their pharmacological activity and in this chapter it has been shown that many extracts also contain several anti-oxidant compounds. Plants with the best antioxidant effects were *C. woodii*, *C. collinum* ssp. *Taborense, C. hereroense, T. gazensis* and *T. mollis* Methanol and acetone extracted the most antioxidant compounds based on DPPH TLC. I*n vitro* studies coupled with the phytochemical analysis confirm that the extracts possessed potential antioxidant activity. Qualitative DPPH assay on TLC method established was successfully used in this study to systematically assess the total antioxidant capacity of the *Combretum* and *Terminalia* species extracts on a large scale, being simple, fast, reliable, inexpensive, and also very adaptable to both hydrophilic and lipophilic antioxidants systems.

Chapter 4
Solvent toxicity

4.1. Introduction

If plants are selected based on ethnomedicinal use, the extraction procedure used in folk medicine must be kept in mind. However, a search for biological activity several solvents of different polarity can be used to isolate all the possible active compounds present. Since the chemical composition of the plant is unknown, the nature of the solvent used affects the composition of the crude extract. Solvents frequently used include methanol, ethanol, acetone, water, ethyl acetate and dichloromethane or combinations thereof. Non-polar solvents yield more lipophilic components, while alcoholic solvents give a larger spectrum of a polar material (Stecher, 2003). Ethanol and water are the most widely used solvents based on hygiene and availability. However acetone is usually used in preference as solvent for extraction because it extracts polar and non-polar components from the plant material, is miscible with water, very volatile, has low toxicity in antimicrobial bioassays and is easily removed from the plant material at low temperature (Eloff, 1998a).

To quantify antimicrobial activities, extracts have to be dried. Frequently it is difficult to resolubilize extracts even in the solvent originally used. Although acetone is an excellent extractant for a wide range of polarity compounds, in our experience especially relatively polar or non-polar extracts are completely soluble in acetone. In serial dilution assays the solvent has to be miscible with water. Water frequently does not dissolve the intermediate polarity or non-polar components of a dried extract. A detergent such as Tween 80 could be added, but a detergent could be toxic to microorganisms. An alternative is to use solvents such as methanol, ethanol or dimethyl sulfoxide (DMSO). To avoid solvents affecting the toxicity of an extract, they should first be tested for any effects against the target fungi.

4.2. Method

4.2.1. Solvents used

Four solvents with different polarities were used, i.e. dimethyl sulfoxide, acetone, methanol and ethanol.

4.2.2. Bioassays

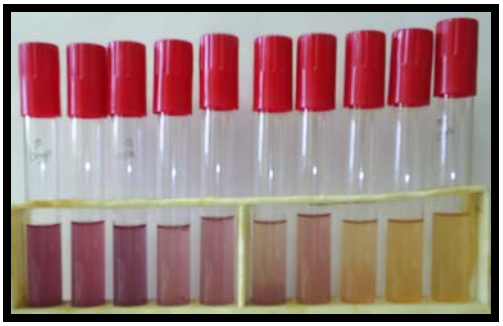
Different concentrations of DMSO, acetone, methanol and ethanol were prepared in sterilized test tubes from 10% to 100%. Dilutions of solvents were made with sterile distilled water. Fungal test organisms (**Section 5**) were prepared in Sabourand dextrose broth. One milliliter of each culture was transferred into test tubes and mixed well. Four hundred microlitres of 2 mg/ml of *p*-iodonitrotetrazolium violet (Sigma®) (INT) dissolved in water was added to each of the test tubes. Test tubes were incubated for three to five days at 35 ^{o}C at 100% relative humidity to ensure adequate colour development.

4.3. Results

Toxicity of different solvents on tested fungi was investigated using macrodilution method and *p*-iodonitrotetrazolium violet (INT) as indicator. With some fungi the differences in response to acetone were easier to notice than with other (**Figure 4.1**). Where fungal growth was inhibited, the solution in the tube remained clear or had a distinct decrease in colour after incubation with INT.

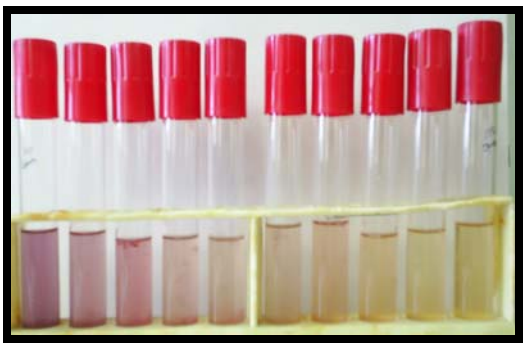
Aspergillus fumigatus

Candida albicans



Cryptococcus neoformans

Microsporum canis



Sporothrix schenckii

Figure 4.1. Test tubes of 10% to 100% acetone from left to right for each group mixed with different fungi and 2 mg/ml of *p*-iodonitrotetrazolium violet (INT) as an indicator. Purple colours indicate fungal growth and clear tubes indicate no growth.

Macrodilution assay was chosen, because it was easy to use different percentages i.e. starting from 100 to 10%. With serial microplate assay we used (Eloff, 1998) it is difficult to have values above 25%. The results of all the solvents are presented in **Table 4.1**.

Only the visual results with acetone were presented in **Figure 4.1**. The results of all the solvents are presented in **Table 4.1**.

Table 4.1. Toxicity of different solvents on tested fungi

Concentrations (%)	DMSO					Acetone					Ethanol					Methanol				
	C. albicans	*C. neoformans*	*M. canis*	*S. schenckii*	*A. fumigatus*	*C. albicans*	*C. neoformans*	*M. canis*	*S. schenckii*	*A. fumigatus*	*C. albicans*	*C. neoformans*	*M. canis*	*S. schenckii*	*A. fumigatus*	*C. albicans*	*C. neoformans*	*M. canis*	*S. schenckii*	*A. fumigatus*
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
30	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
40	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	-	-	+
50	+	+	+	-	+	+	+	+	+	+	-	+	-	-	-	+	+	-	-	+
60	+	+	+	-	-	+	+	+	+	+	-	-	-	-	-	-	+	-	-	-
70	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-
80	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
90	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+ growth, - no growth

The MIC values are then calculated using the known density of 100% of the solvent. For example, if the density of acetone is 0.8 gm/ml then at 0.4 ml/ml it equals 0.4 X 0.8 g/ml = 0.32 g/ml = 320 mg/ml. From this it follows that the concentration of 100% acetone is 800 mg/ml. The MIC values were calculated from **Table 4.1** and results are presented in **Table 4.2**.

Different solvents were toxic to different fungi at different concentrations and **Table 3.2** had MIC values at which solvents kill different fungi. DMSO was toxic to *S. schenckii* in 40% (0.40 ml/ml) and *A. fumigatus* in 50% (0.50 ml/ml). *C. albicans* and *M. canis* can still survive in 60% (0.60 ml/ml), but *C. neoformans* can survive in 70% acetone. Among the tested solvents acetone was found not to be toxic to fungi tested, as they can all survive in concentrations of 60% to 70%. Methanol was relatively toxic to *M. canis* and *S. schenckii*, both at 20% and ethanol was toxic to *M. canis* at 20%.

Table 4.2. MIC values and equivalent concentrations of different solvents against tested fungi

Microorganisms	MIC values (mg/ml) and % final concentration				
	DMSO	**Acetone**	**Ethanol**	**Methanol**	**Average**
C. albicans	660 (60%)	474 (60%)	324 (40%)	395 (50%)	**465**
C. neoformans	770 (70%)	553 (70%)	405 (50%)	474 (60%)	**553**
M. canis	660 (60%)	474 (60%)	162 (20%)	158 (20%)	**365**
S. schenckii	440 (40%)	553 (70%)	324 (40%)	158 (20%)	**370**
A. fumigatus	550 (50%)	474 (60%)	324 (40%)	395 (50%)	**438**
Average	**616**	**512**	**304**	**320**	

On average DMSO is by far the least toxic of all the solvents tested followed by acetone. *C. neoformans, C albicans* and *A. fumigatus* survived under higher concentrations of methanol (**Figure 3.2**). The average MIC of DMSO on all tested fungi was 616 mg/ml, followed by acetone with 512 mg/ml, methanol with 304 mg/ml and ethanol with 320 mg/ml (**Figure 3.3**).

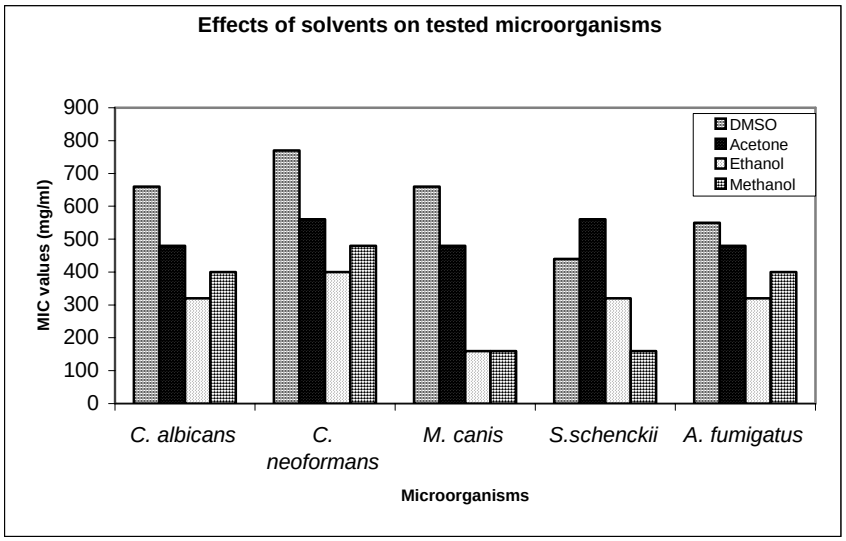


Figure 4.2. Effects of solvents on tested fungi

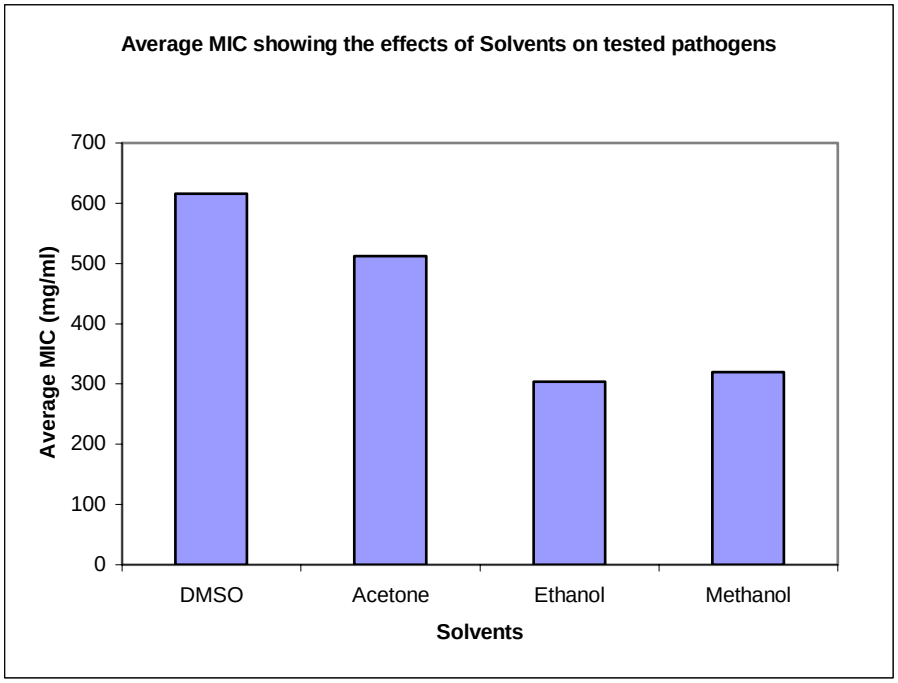


Figure 4.3. Average MIC showing the effects of solvents on tested fungi

C. neoformans and *C. albicans* were resistant to solvents, with an average MIC of 553 and 465 mg/ml respectively, followed by *A. fumigatus* (436 mg/ml). *M. canis* and *S. schenckii* were very sensitive with an average MIC of 365 and 370 mg/ml respectively (**Figure 4.4**).

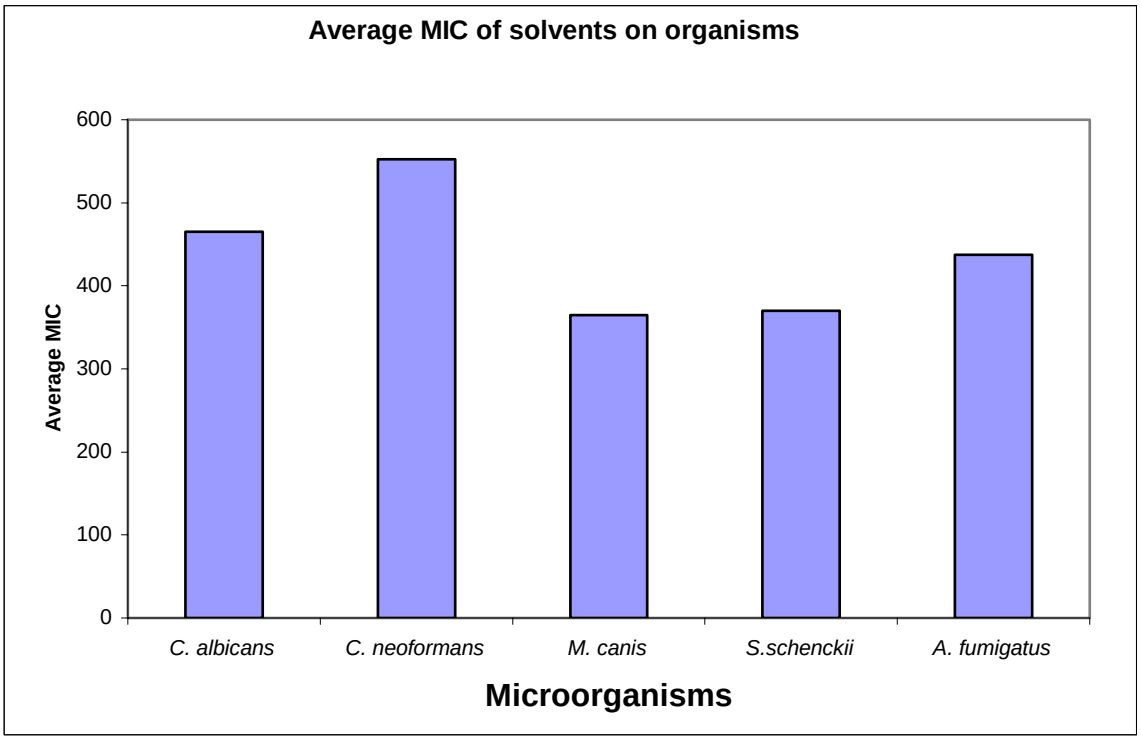


Figure 4.4. Average MIC of solvents on tested fungi

4.4. Discussion

Screening, isolation, and identification of novel compounds depends on the solubility and charge properties of the extractant. Microbial strains able to tolerate and survive in the presence of toxic organic solvent concentrations were underdeveloped until the last 5 years (Ojala *et al*., 2000). This was because of the difficulties of maintaining cell viability on highly toxic organic solvent environment and as a result of the anthropomorphic view of microbial life (e.g. aqueous media, 37 °C, pH 7.0). Also, organic solvent-tolerant microorganism, and viable cells for enzyme production in extreme environments, such as organic solvents, have received little attention, but is now growing up as a new area of extremophiles.

The solvent tolerance of the microorganisms was tested using the following solvents; DMSO, acetone, methanol and ethanol. In order to determine the maximum concentration at which different solvents would allow the test microorganisms to reach normal growth, different concentrations from 10 to 100% were used. Uninhibited growth was evaluated as no toxic effects of the solvent. Methanol and ethanol were found to be toxic to tested fungi with average MIC's of 304 and 320 mg/ml respectively as expected, based on previous studies on bacteria by Eloff (1998b). DMSO and acetone appear to be good solvents to use for bioassays, but acetone

was used in bioassays because of reasons stated earlier. The major difficulty with DMSO is the boiling point (189 ^{o}C), which is very high, fortunately it is relatively volatile and can be removed under high vacuum. If further work has to be done on extracts and it is fatty soluble in acetone that is the solvent of choice, if not DMSO may be useful.

Surprisingly, *C. neoformans, C. albicans* and *A. fumigatus* managed to survive higher concentrations of methanol, which was found to be toxic in previous studies (Heipieper *et al*., 1991). However, it was toxic to *M. canis* and *S. schenckii*. The two yeasts, *C. albicans* and *C. neoformans* were very resistant with average MIC's of 465 and 553 mg/ml respectively.

Solvent toxicity was explained considering the lipid-rich cellular membrane as the main organic solvent target by the Hansch parameter or log *P*, which is defined as the logarithm of the partition coefficient of solvent in octanol–water phase system. Organic solvents with log *P* between approximately 1 and 5 are considered extremely toxic for microorganisms. Nevertheless, the limits of solvent toxicity to the cells apparently are not strict and depend not only on strains and species assayed, but also of experimental conditions (e.g. medium, pH, temperature, ionic strength, inoculums) (Ojala *et al*., 2000).

Carlson *et al* (1991) demonstrated clearly that increasing concentrations of 6 alcohols inhibit fungal pathogens (as carried out by *Saccharomyces cerevisiae*); a correlation with increased partition coefficients into a hydrophobic milieu was also evident. This would tend to suggest that the action of these alcohols is primarily located at a hydrophobic site, possibly at the membrane.

It is well known that modest concentrations of ethanol and other alcohols lead to reduced fermentation and growth rates of organisms, which produce them, and that high concentrations are cytotoxic. While much research has been carried out (Lovitt *et al*, 1988), the methods by which these organic solvents affect the cell are poorly documented; in many cases they are simply cited as being multi- target or non-specific in their action. It is however generally agreed that the cell membrane is one of, if not "the", primary target for organic solvents, as we have seen with the differences in yeasts and moulds in our experiments.

DMSO was the least toxic of the solvents used with an average MIC of 616 mg/ml (56%) followed by acetone 512 mg/ml (64%), methanol 320 mg/ml (40%) and ethanol 304 mg/ml (38%). The danger of using ethanol or methanol is evident from the inhibition by 20% ethanol or methanol of *M. canis* and *S. schenckii*. In general the two moulds appeared to be most

resistant. Acetone was the only extractant that could be used with safety at a 50% concentration.

4.5. Conclusion

There was a variable susceptibility of the fungi to the solvents with C. neoformans was not resistant and S. schenckii was most susceptible. In spite of this it was found that DMSO and secondarily acetone can be used in fungal bioassays at higher concentrations than ethanol and methanol. Thus I recommend that where possible the use of ethanol and methanol be avoided in these tests.

CHAPTER 5

Antifungal assays (Minimum Inhibitory Concentration)

5.1. Introduction

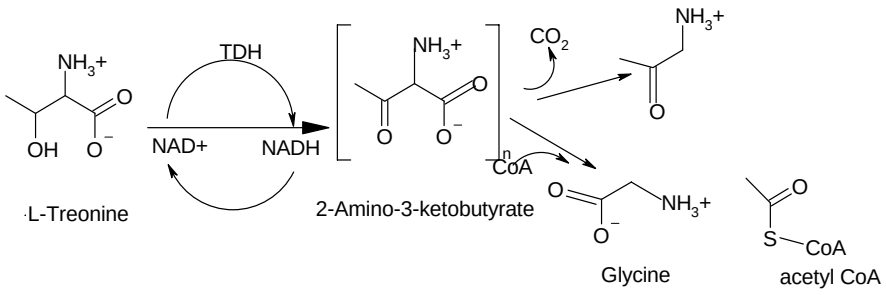
In its most general terms, susceptibility testing refers to the idea of mixing a fungal culture with an antifungal agent and seeing what happens. In general, we are interested in determining the lowest concentration of an antifungal agent that appears to inhibit growth (minimum inhibitory concentration, MIC) the fungus. If this level is low enough, then the drug may work against an infection. The approach to testing is different for yeasts and moulds.

Dilution methods are used to determine the MIC of antimicrobial agents and are the reference methods for antimicrobial susceptibility testing against which other methods, such as disk diffusion are calibrated. In dilution tests, microorganisms are tested for their ability to produce visible growth on a series of agar plates or in test tubes or microplate wells of broth containing dilutions of the microbial agent.

In determining the MIC values growth indicators are used and not turbidity because plant extracts are frequently turbid or causes precipitates when mixed with microbial growth media. In this project INT was used.

5.1.1. p-iodonitrotetrazolium violet (INT) Reaction

The reaction is based on the transfer of electrons from NADH, a product of the threonine dehydrogenase [TDH] catalyzed reaction, to the tetrazolium dye [p-iodonitrotetrazolium violet]. Threonine dehydrogenase [TDH] from bacteria/fungi catalyses the NAD-dependent oxidation of threonine to form 2- amino-3-ketobutyrate and NADH. During the active growth of bacteria/fungi, an electron is transferred from NADH [which is colourless in the visible range] to p-iodonitrotetrazolium violet resulting in a formazan dye, which is purple in colour. Therefore, the clear zone(s) on the chromatogram indicate areas of inhibition [zones where



no active growth of bacteria has taken place].

Reaction pathway for the assay of TDH

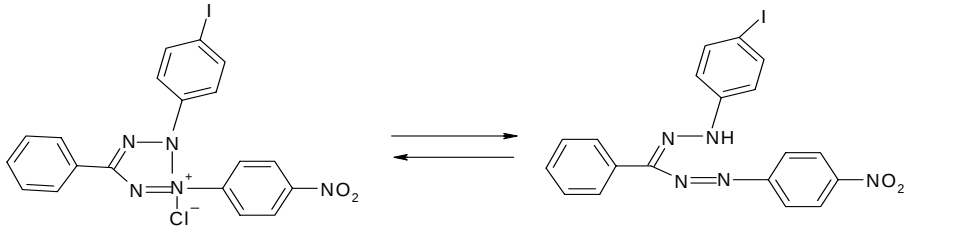


Figure 5.1. INT, coupling reagent for the colorimetric assay

5.2. Materials and Method

5.2.1. Fungal test organisms

Five fungi were obtained from the Bacteriology Laboratory, Department of Veterinary Tropical Diseases, Faculty of Veterinary Science and used as test organisms. These fungi represent the different morphological forms of fungi, namely yeasts (*Candida albicans* and *Cryptococcus neoformans*), thermally dimorphic fungi (*Sporothrix schenckii*) and moulds (*Aspergillus fumigatus* and *Microsporum canis*). They are the most common and important disease-causing fungi of animals. *Candida albicans* was isolated from a Gouldian finch, *C. neoformans* from a cheetah, and *Aspergillus fumigatus* from a chicken, all of which suffered from a systemic mycosis. *Microsporum canis* was isolated from a cat with dermatophytosis and *S. schenckii* from a horse with cutaneous lymphangitis. Not one of the animals had been treated prior to sampling. All fungal strains are maintained on Sabouraud dextrose agar (Oxoid, Basingstoke, UK).

5.2.2. Minimum inhibitory concentration

5.2.2.1. Microdilution assay

A serial microdilution assay (Eloff, 1998c) was used to determine the minimum inhibitory concentration (MIC) values for plant extracts using tetrazolium violet reduction as an indicator of growth. This method had previously been used only for antibacterial activities (Eloff, 1998c; McGaw et al., 2001). To apply it to measuring antifungal activities, a slight modification was made to suit fungal growth conditions. Residues of the different extracts were dissolved in acetone to a concentration of 10 mg/ml. The plant extracts (100 µl) were serially diluted 50% with water in 96 well microtitre plates (Eloff, 1998c). Fungal cultures were transferred into fresh Sabouraud dextrose broth, and 100 µl of this was added to each well. Amphotericin B was used as the reference antibiotic and positive control, and appropriate solvent blanks were included as negative control. As an indicator of growth, 40 µl

of 0.2 mg/ml of *p*-iodonitrotetrazolium violet (Sigma®) (INT) dissolved in water was added to each of the microplate wells. The covered microplates were incubated for two to three days at 35 ^{o}C and 100% relative humidity. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth after 24 and 48 hours.

5.2.2.2. The experimental design

Tests group 1: Consisted of the pathogen plus different concentrations of the extracts. This group was used to determine activity in the extract (MIC value). For each plant the following extracts were used, hexane, DCM, acetone and methanol.

Tests group 2: Positive control, it contained the pathogen plus Amphotericin B.
This group was used to ensure that the pathogen was not a resistant strain and also to compare relative activities with the extracts.

Tests group 3: A pure culture containing only the pathogen. This was necessary to distinguish poor growth from inhibition and to ensure that the laboratory conditions under which the pathogens had been placed did not affect its growth.

Tests group 4: A negative control containing the pathogen together with the dissolution solvents. This ensured that the extraction solvents had no inhibitory effects on the pathogens.

.

Results in this chapter are represented by two papers, which are: Masoko *et al*., (2005) and Masoko *et al*., (2006).

CHAPTER 6

Bioautography

6.1. Introduction

Bioautography is probably the most important detection method for new or unidentified antimicrobial compounds and has found widespread application in the search for new antimicrobials. It is based on the biological (antibacterial, antifungal, etc.) effects of the substances under study. This assay had the advantage of being quick, easy to perform, relatively cheap, requiring no sophisticated infrastructure, only requiring micrograms of test compound and results are easy to interpret. In this study TLC separation of the crude plant extracts in combination with bioautography was used as a bioassay-guided isolation method in order to screen for and identify compounds with fungal activity within the tested samples.

The diversity of antifungal compounds of six *Terminalia* species determined by bioautography was presented by published article (Masoko and Eloff, 2005). The results of the *Combretum* species were submitted for publication and the paper is in press: Bioautography indicates the multiplicity of antifungal compounds from twenty-four South African *Combretum* species (Combretaceae) (Masoko and Eloff, 2006).

CHAPTER 7

Extraction and isolation of antifungal compounds

7.1. Introduction

Plants are a good source of compounds that can be used for medicinal and other purposes because they protected themselves against fungal attack by synthesizing chemical compounds. However among the thousands of compounds present only a small number of these can be used. Thus it is time consuming and often difficult to use classical methods of extraction and complete separation of individual compounds and then test these isolated compounds for biological activities. The more practical route is to screen plant extracts for a specific biological activity, select the promising compounds and purify them further. In this bioassay guided fractionation and column chromatography were mainly used. From results obtained in previous chapters *C. nelsonii* was selected for isolation of antifungal compounds. *C. nelsonii* was also chosen because it contained more active compounds had high activity against all tested pathogens and it was active at low concentrations.

7.2. Materials and methods

7.2.1. Extraction procedure

A number of factors were taken into consideration in selecting solvents that were to be used in the serial exhaustive extraction (**Section 2.1**). The choice of solvent also depended on what was planned with the extract. The effect of solvent on subsequent bioassay was an important factor.

The *Combretum nelsonii* leaves were carefully examined and old, insect damaged, fungus-infected leaves were removed. Healthy leaves were dried at room temperature. Once the leaves were dry, they were ground to a fine powder of c. 1.0 mm diameter. Material was stored in a closed container at room temperature.

The defatting process by hexane is important in the isolation process since non-polar compounds will be extracted fast in this process. Hence, serial exhaustive extraction was used on *C. nelsonii* leaf powder with hexane as a starting solvent followed by dichloromethane (DCM), acetone and methanol as extractants. The polarity of solvents gradually increased and ranged from a non-polar solvent (hexane) to a more polar solvent (methanol). This was to ensure that a wide polarity range of compounds could be extracted in the process.

Dried powdered leaves (502 g) of *C. nelsonii* were exhaustively extracted in a serial manner with solvents of increasing polarity. Finely ground plant material (502 g) was initially extracted in a Labotec model 20.2 shaking machine at high speed with 5 litres of hexane. The solvent was allowed to extract for 1 hour while shaking. Rotarvapour was used to concentrate the extracts. The solvent was recovered and reused for the next extraction. before being decanted. The same quantity of solvent was added to the marc and shaken once again for an hour. The process was repeated three times. The marc was allowed to dry and the process of extraction was repeated three times with dichloromethane, then acetone, and finally methanol.

The extracts were vacuum filtered through Whatman (no. 2) filter paper using a Buchner funnel, and most of the solvent was removed by vacuum distillation in a Buchi rotary evaporator at 60^0C. Once concentrated to a small volume, the extracts were placed in pre-weighed beakers and allowed to dry completely in front of a cool stream of air. The mass extracted with each solvent was calculated. To determine chemical profile by TLC, 20 mg of each extract was weighed into a pill vial and made up to a concentration of 10 mg/ml by re-dissolving in acetone.

7.2.2. Analysis by TLC

The chemical profile of extracts was determined by TLC using aluminum backed thin layer chromatography plates (Merck, silica gel 60 F_{254}). The following three solvent systems were used to develop the plates: EMW, CEF and BEA (**Section 2.1.5**).

7.2.3. Bioautography

Bioautography was done according to Begue and Kline (1972) with modifications as explained in **Section 6.1.2** and fungal test organisms (**Chapter 5**) were used.

7.2.4. Microdilution assay

The serial dilution microplate dilution (Eloff, 1998) method was used to determine the Minimum Inhibitory Concentration (MIC) values of the extracts against each test fungal species with modifications as explained in **Chapter 5**.

7.2.5. Total activity

Total activity in ml/mgindicates the degree to which the active extracts, fractions or compounds in one gram of plant material can be diluted and still inhibit the growth of the test organisms (Eloff, 2000). This makes it possible to quantify the efficiency of fractionation and determine loss or gain of activity (Eloff, 2004). It was calculated as explained in **Chapter 5**.

7.2.6. Isolation

Since the acetone and DCM fractions of *C. nelsonii* had a high number of antifungal compounds they were subjected to column chromatography starting with the acetone fraction.

7.2.6.1. Open column chromatography

Column chromatography was used to further simplify the acetone fraction from serial exhaustive extraction. The acetone fraction from *C. nelsonii* was dried in a rotary evaporator to determine the mass of the fraction to be used for column chromatography.

The dry method for packing of chromatographic columns was used; silica gel 60 was poured slowly into a column (15.5 cm x 10 cm), on top of a small amount of cotton wool. The dry sample of acetone fraction (12.38 g) of *C. nelsonii* was then placed neatly on top of the silica in the column. Filter paper cut to the internal diameter of the column and cotton-wool were neatly placed on top of the sample to prevent disturbance at the surface during solvent introduction. Fifteen elution systems were added slowly in the order as in **Table 7.1**. With the addition of solvent (1.2 L) into the column, the vacuum was switched on. The solvent was allowed to run through the column; until the 1.2 L had been collected in the beakers through a separating funnel. The beakers were allowed to evaporate overnight under a cool stream of air and TLC analysis was then carried out.

Table 7.1. Solvent mixtures used in column chromatography

Elution system	
Hexane:	100 %
Hexane: Ethyl acetate	90 %
	80 %
	70 %
	50 %
	30 %
	10 %
Ethyl acetate	100%
Ethyl acetate: Methanol	90 %
	80 %
	70 %
	60 %
	50 %
	40 %
Methanol	100%

The composition of each fraction was analysed using TLC.

7.2.6.1. Analysis and grouping of fractions

After vacuum liquid chromatography, beakers were placed under a stream of air to facilitate concentration of the fractions for TLC analysis and bioassays. After about 50% of the volume of the eluent had been evaporated, the volume was measured and 5 ml was collected from each beaker into a pre-weighed pill vial and allowed under a stream of air to dry rapidly. The mass of each fraction was calculated and the concentration (10 mg/ml) determined. Fractions were analysed by TLC (**Chapter 2**).

7.2.6.2. Combination of fractions

From TLC results, fractions were combined, based on the similarity of their chemical profile. Combined fractions were placed under an air current to facilitate drying and crystallization. Once dry, the fractions were weighed to calculate the total mass fractionated and the crystallized fractions were washed with a combination of solvents to obtain pure compounds. Active fractions were combined and subjected to further column chromatography.

In order to select the best mobile phase for eluting the 80% ethyl acetate fraction and 90% ethyl acetate fractions, 5 µl of a 10 mg/ml (i.e. 50 µg) solution was placed in a narrow band c. 1 cm wide on TLC plates and developed with various combination of solvents. The solvent that exhibited the most favourable separation of compounds was chosen.

7.3. Results of Vacuum Liquid Chromatography

7.3.1. Extraction

Finely ground, dried *C. nelsonii* leaves (502 g) were serially extracted with hexane, DCM, acetone and methanol as indicated above. The following masses in **Table 7.2.** were obtained.

Table 7.2. The mass (g) of *C. nelsonii* leaf powder serially extracted with four extractants from 502 g.

Extractants		**Mass residue extracted (g)**		
		Mass	**Total**	**Total activity**
Hexane	I	4.29	6.93	99
	II	1.88		
	III	0.76		
DCM	I	9.32	16.29	407.25
	II	3.66		
	III	3.31		
Acetone	I	10.59	12.38	213
	II	1.31		
	III	0.48		
Methanol	I	28.35	40.58	676.3
	II	8.11		
	III	4.12		
TOTAL			**76.18**	**1395.55**

The total mass extracted was 76.18 g from 502 g of *C. nelsonii*. Methanol (40.58 g) extracted the highest mass from *C. nelsonii*, followed by DCM (16.29 g); acetone (12.38 g) and hexane (6.93 g) extracted the lowest mass. Success in isolating compounds from plant material is largely dependent on the type of the solvent used in the extraction procedure (Lin *et al*., 1999). The total mass extracted using different solvents (acetone, hexane, DCM and methanol) are shown in **Figure 7.1.** Methanol was the best extractant, extracting a greater quantity of plant material than any of the other solvents. The important factor is actually not quantity, but the biological activity.

7.3.2. Phytochemical analysis

The separated compounds on TLC chromatograms were made visible by spraying with vanillin-sulphuric acid and heating at 105 ^{o}C (**Figure 7.2**). The BEA separation system had

number of compounds followed by CEF, and EMW had the least number of compounds, which means more polar compounds were separated. The DCM extract contained more compounds in CEF and EMW as compared to other extracts. Greatest separation was noticed in CEF.

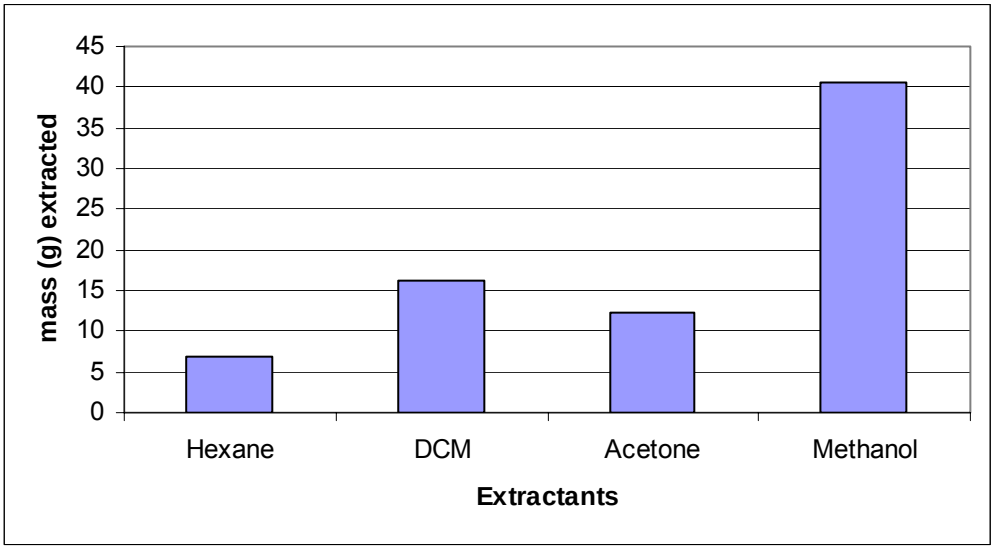


Figure 7.1. Mass serially extracted by hexane, DCM, acetone, and methanol from *C. nelsonii*

7.3.3. Quantitative antifungal activity

All of the extracts had substantial antifungal activity against different pathogens tested (**Table 7.3**). *C. neoformans* and *M. canis* were the most sensitive microorganisms with an average MIC value of 0.02 mg/ml, followed by *C. albicans* (0.04 mg/ml). The least sensitive were *A. fumigatus* and *S. schenckii* with average MIC's of 0.09 and 0.10 mg/ml respectively (**Table 7.3**).

Table 7.3. Minimum Inhibitory Concentration (MIC) of *C. nelsonii* extracts after 24 H.

Microorganisms	MIC values (mg/ml)												
	Hexane			DCM			Acetone			Methanol			Average
	I	II	III	I	II	III	I	II	III	I	II	III	
C. albicans	0.02	0.04	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.08	0.08	0.08	**0.04**
C. neoformans	0.04	0.02	0.04	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	**0.02**
A. fumigatus	0.16	0.16	0.16	0.04	0.04	0.04	0.08	0.08	0.16	0.04	0.08	0.08	**0.09**
M. canis	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	**0.02**
S. schenckii	0.08	0.08	0.08	0.08	0.08	0.08	0.08	0.16	0.16	0.02	0.16	0.16	**0.10**
Average	**0.06**	**0.06**	**0.07**	**0.04**	**0.04**	**0.04**	**0.04**	**0.06**	**0.08**	**0.04**	**0.07**	**0.07**	
Total Average	**0.07**			**0.04**			**0.06**			**0.06**			

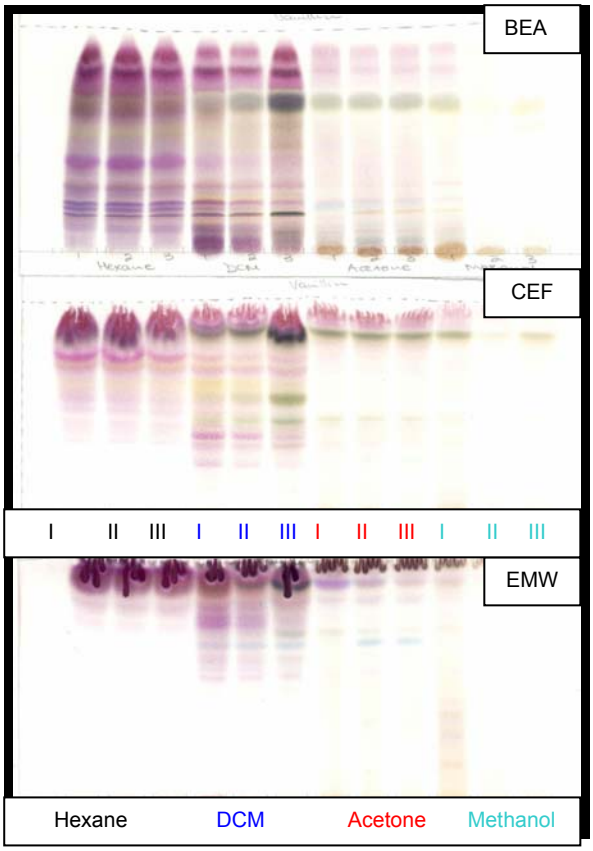


Figure 7.2. Chromatograms of *C. nelsonii* extracts developed in BEA (top), CEF (centre), and EMW (bottom) solvent systems and sprayed with vanillin–sulphuric acid to show compounds extracted with acetone, DCM, hexane and methanol.

DCM extracts had the highest average antifungal activity with the average MIC of 0.04 mg/ml, followed by acetone (0.06 mg/ml) and methanol (0.06 mg/ml), then hexane (0.07 mg/ml).

Total activity was also calculated. The reason for this is explained in **Chapter 5.** Not only the MIC but also the quantity in fraction is important. Extracts with higher values are most promising to work with. All extracts had substantial total activity against *C neoformans* and *M. canis*, with average of 307 and 317 ml/g respectively, followed by *C. albicans* with 185 ml/g (**Table 7.4**). *S. schenckii* and A*. fumigatus* were less sensitive with average total activity of 161 and 122 ml/g respectively. Methanol extracts had the highest average total activity (461 ml/g) and hexane the lowest with 65 ml/g. The first methanol extract had the highest average total activity of 1063 ml/g and the lowest was the third acetone extract with 16 ml/g.

Table 7.4. Total activity in ml/g of *C. nelsonii* extracts after 24 hours incubation at 37 °C.

Microorganisms	Total activity (ml/g)												Average
	Hexane			DCM			Acetone			Methanol			
	I	II	III	I	II	III	I	II	III	I	II	III	
C. albicans	215	47	19	466	183	166	530	66	24	354	101	52	**185**
C. neoformans	107	94	19	466	183	166	530	66	24	1418	406	206	**307**
A. fumigatus	27	12	5	233	92	83	132	16	3	709	101	52	**122**
M. canis	215	94	38	466	183	166	530	66	24	1418	406	206	**317**
S. schenckii	54	24	10	117	46	41	132	8	3	1418	51	26	**161**
Average	**123**	**54**	**18**	**350**	**137**	**124**	**371**	**44**	**16**	**1063**	**213**	**108**	
Total Average	**65**			**204**			**143**			**461**			

Methanol had the highest total activity and that looks promising but the difficult part is to remove methanol from the extracts and chemistry of polar compounds are difficult to work with.

7.3.4. Quantitative analysis of antifungal compounds

The extracts were analysed by bioautography for quantitative analysis of antifungal compounds on the chromatograms. Chromatograms were sprayed with *C. albicans* (**Figure 7.3a**), *C. neoformans* (**Figure 7.3b**), *S. schenckii* (**Figure 7.4a**), *A. fumigatus* (**Figure 7.4b**) and *M. canis* (**Figure 7.4c**).

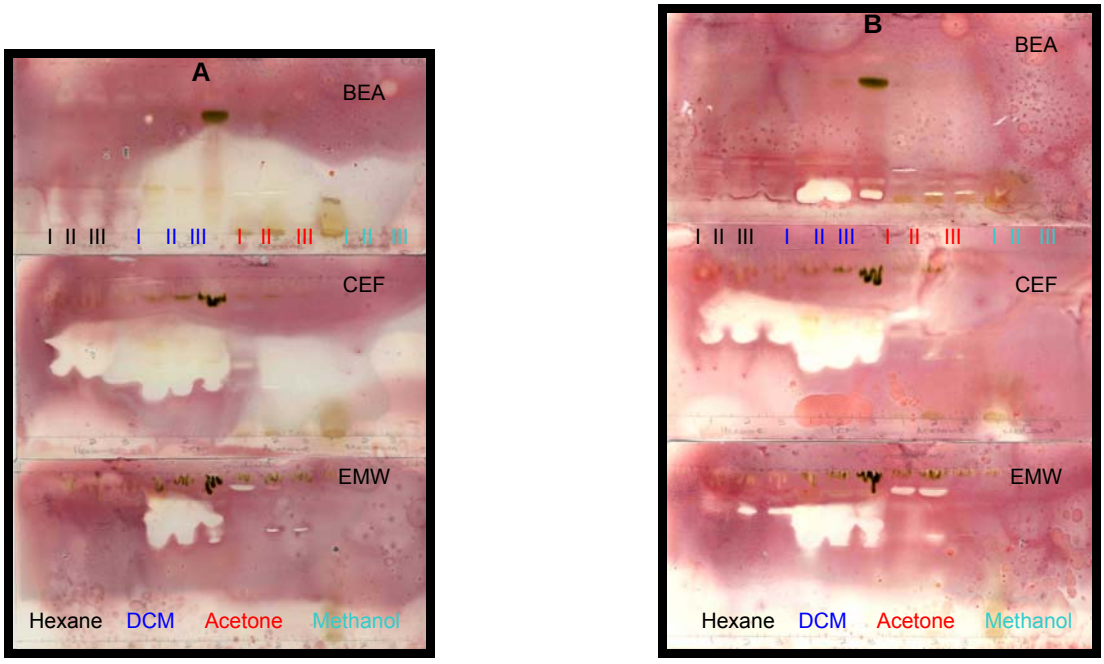


Figure 7.3. Bioautography of *C. nelsonii* extracts separated by BEA (top), CEF (Centre) and EMW (Bottom) and sprayed with *C. albicans* (A) and *C. neoformans* (B). White areas indicate active compounds that inhibited the growth. (I, first extraction; II, second extraction; III, third extraction).

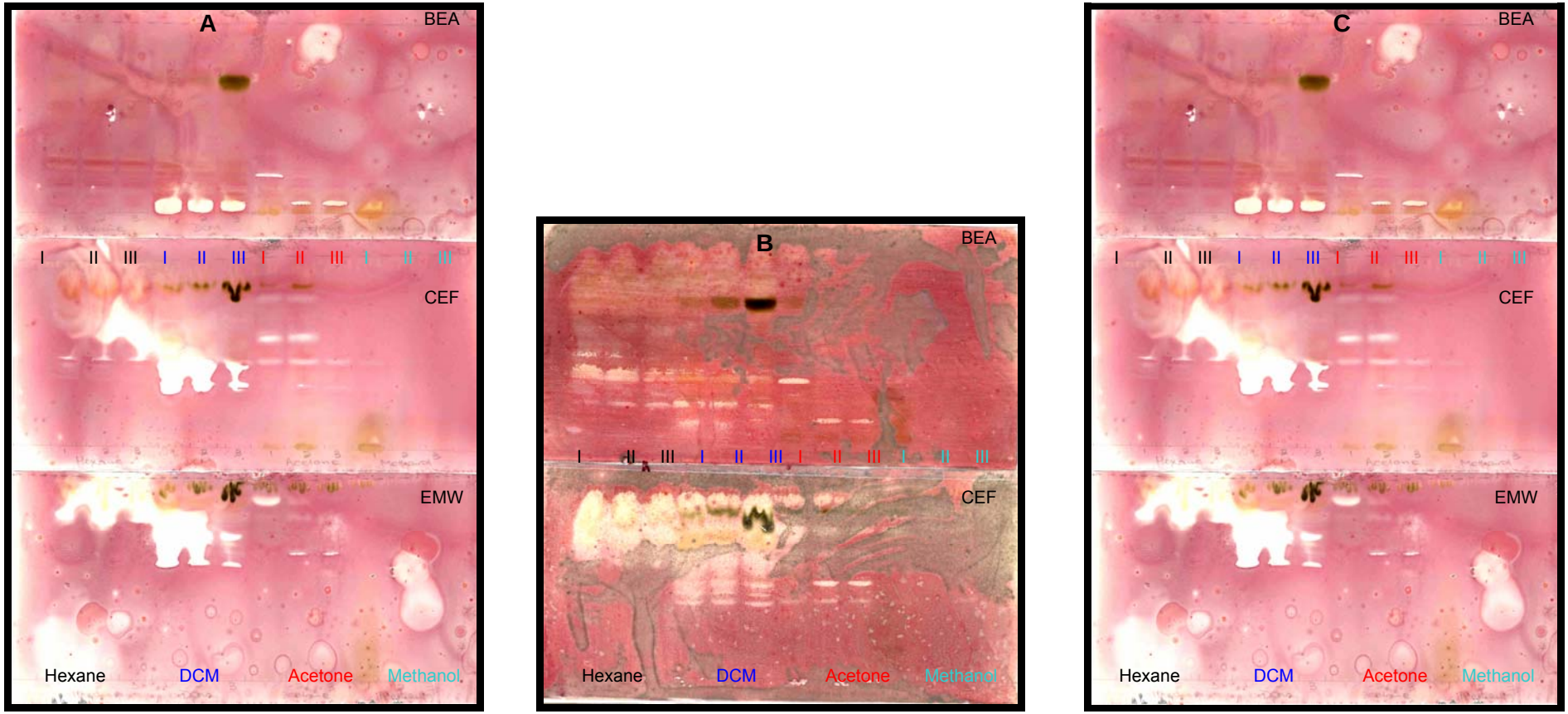


Figure 7.4. Bioautography of *C. nelsonii* extracts separated by BEA (top), CEF (Centre) and EMW (Bottom) and sprayed with *S. schenckii* (A), *A. fumigatus* (B) and *M. canis* (C). White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth. (I, first extraction; II, second extraction; III, third extraction).

DCM extracts of *C. nelsonii* contain very active compounds against all tested microorganisms, followed by acetone extracts. The CEF separation system separated active compounds against all organisms, and it will be used in the next experiments. BEA separated active compounds only in DCM extracts. Hexane extracts had more activity against *C. albicans* and *C. neoformans* in CEF only, and for *S. schenckii* and *M. canis* in both CEF and EMW. Methanol extracts didn't have any activity and were discarded. Acetone and DCM extracts were used for column chromatography because they had more active compounds. It was decided not to use the hexane extracts as they were oily and had lower activity than the acetone and DCM. When leaves were extracted with methanol alone, there was activity, but when methanol was used in serial exhaustive extraction the was no activity. It might be due synergistic effects of compounds in the cruse extract.

7.3.5. Fractionation of VLC fractions

Acetone extracts (12.38 g) of *C. nelsonii* were combined and subjected to the column (23 cm X 3 cm). Isolation was done as indicated in **Section 7.2**; several eluents were used (**Table 7.1**). The masses of all fractions were recorded (**Table 7.5**). Hexane: Ethyl acetate (80:20) and Ethyl acetate: Methanol (90:10) fractions had the highest masses of 1.119 and 1.524 g respectively. The lowest mass was in the Ethyl acetate: methanol (40:60), 0.130 g fraction. DCM fractions will be discussed in the later stages.

Table 7.5. The mass (g) of *C. nelsonii* acetone and DCM extracts fractions recovered by VLC with different eluents.

Eluent	Percentages (%)	Mass (g)	
		Acetone	DCM
Hexane:	100%	0.347	0.243
Hexane: Ethyl acetate	90%	0.868	3.065
	80%	1.119	1.624
	70%	0.842	0.715
	50%	0.607	2.252
	30%	0.690	1.808
	10%	0.303	0.589
Ethyl acetate	100%	0.204	0.253
Ethyl acetate: Methanol	90%	1.524	1.250
	80%	0.964	1.024
	70%	0.568	0.930
	60%	0.334	0.687
	50%	0.189	0.437
	40%	0.130	0.128
Methanol	100%	0.190	0.360
Total		**8.879**	**15.365**

Out of the 12.38 g of *C. nelsonii* acetone extract used, I managed to collect 8.88 g using different eluent systems. All the plates were separated with CEF because more active compounds were found in CEF separation (**Figure 7.3** to **7.4**). Phytochemical analysis of the isolates was done (**Figure 7.5**). Bioautography was done on all isolates to locate active compounds (**Figure 7.6** to **7.9**).

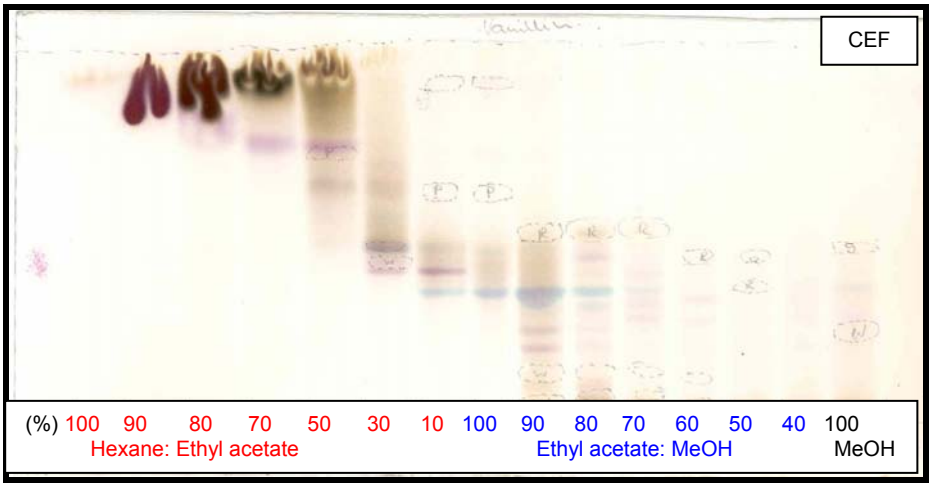


Figure 7.5. Chromatograms of *C. nelsonii* acetone extracts developed in CEF solvent systems and sprayed with vanillin–sulphuric acid to show compounds isolated with different eluent systems.

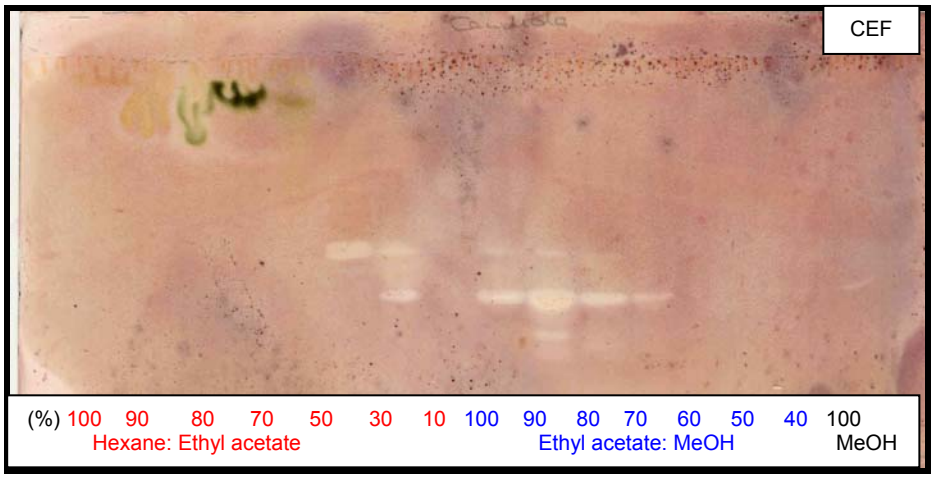


Figure 7.6. Bioautography of *C. nelsonii* acetone extracts separated by CEF and sprayed with *C. albicans.* White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *C. albicans.*

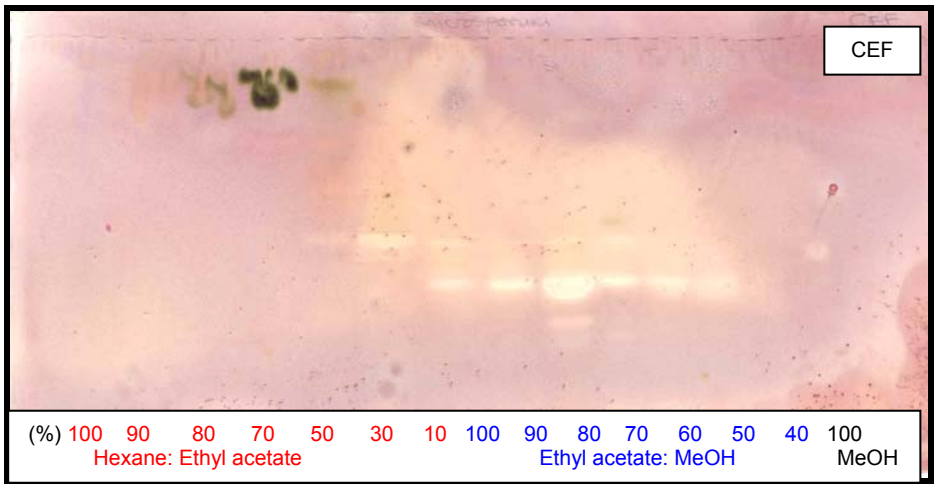


Figure 7.7. Bioautography of *C. nelsonii* acetone extracts separated by CEF and sprayed with *M. canis.* White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *M. canis.*

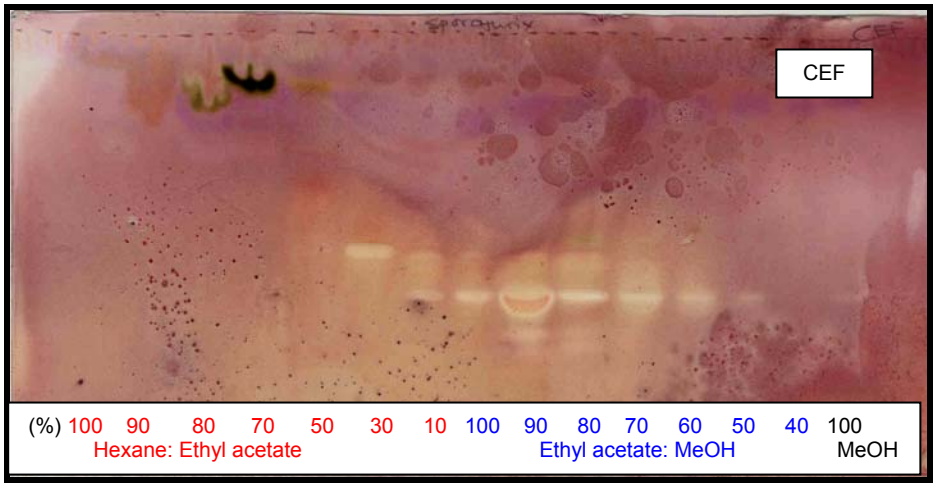


Figure 7.8. Bioautography of *C. nelsonii* acetone extracts separated by CEF and sprayed with *S. schenckii.* White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *S. schenckii.*

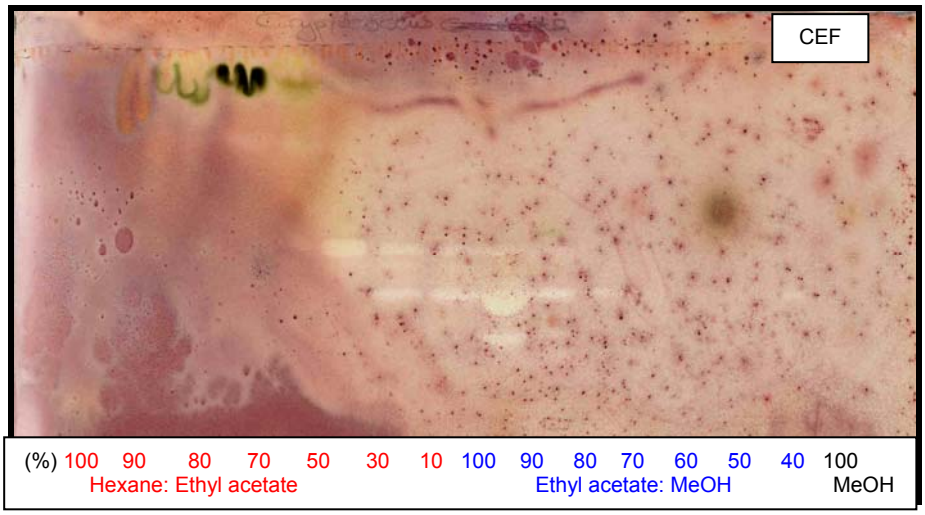


Figure 7.9. Bioautography of *C. nelsonii* acetone extracts separated by CEF and sprayed with *C. neoformans.* White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *C. neoformans.*

Antifungal compounds against the four fungi tested were present in 50 to 10% hexane in ethyl acetate and 100 to 70% ethyl acetate in methanol fractions (**Figures 7.6** to **7.9**). *A. fumigatus* results were not clear although there was inhibition (results not presented). Only the active compounds in **Figure 7.6** to **7.9** were blue after treating with the vanillin spray reagent (**Figure 7.5**). The most active compound was located in 90% ethyl acetate in methanol fraction. In the following experiments the blue compound was consequently followed for isolation.

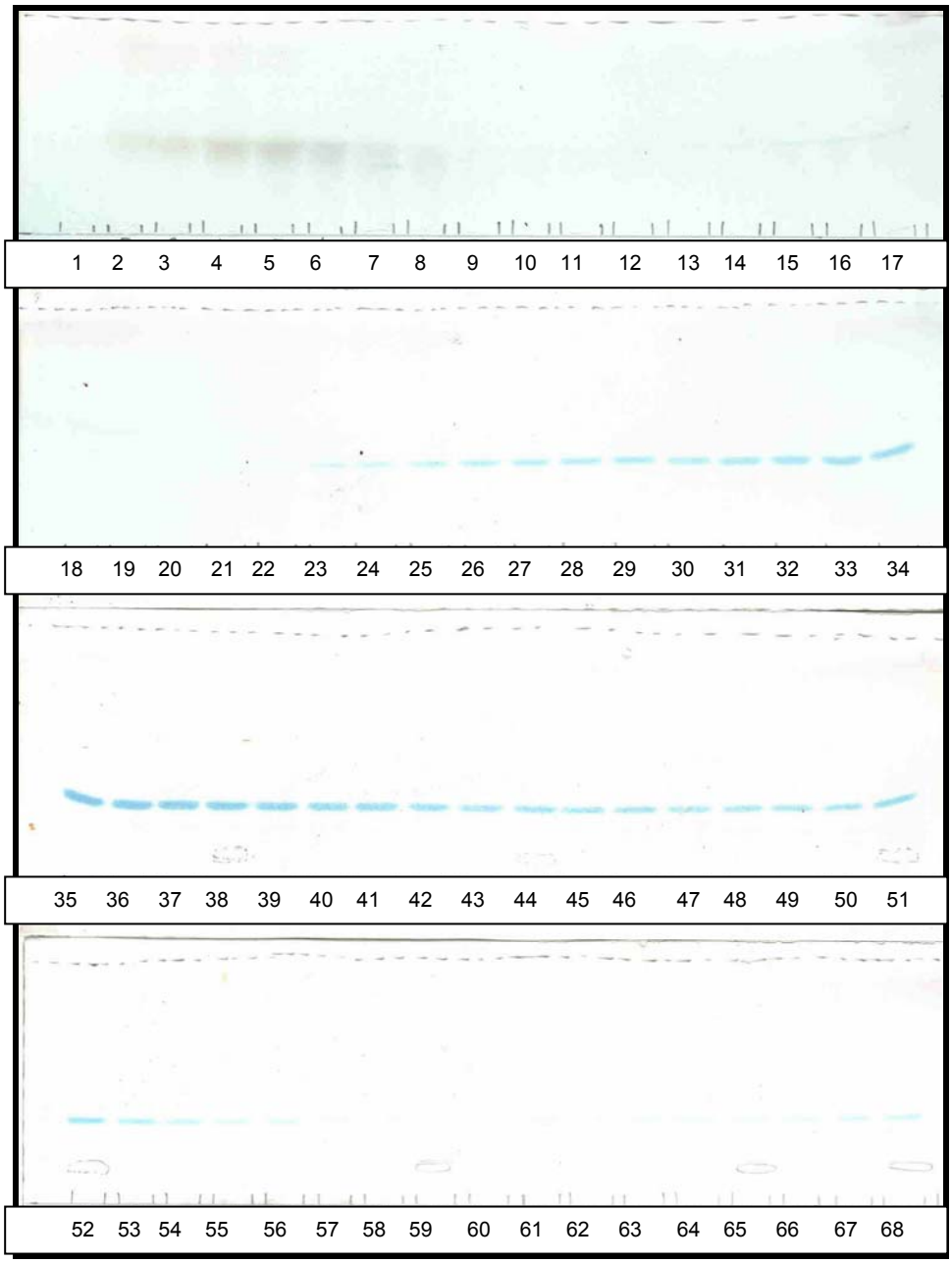


Figure 7.10. Chromatograms of *C. nelsonii* acetone extracts isolated with 90% ethyl acetate and developed in CEF solvent systems and sprayed with vanillin–sulphuric acid to show compounds isolated with different eluent systems.

The fractions from 90 and 80% ethyl acetate in methanol were combined. The masses were 1.524 and 0.964 g respectively, resulting in a total of 2.488 g. After evaluating several eluent systems by TLC, 90% ethyl acetate in hexane was the best, in separating compounds.

Column was prepared as in **Section 7.2.6.1.** Different fractions were collected as indicated in **figure 7.10**. Fractions were combined **Section 7.2.6.2**. Fractions were combined as indicated in the flow chart of the overview of isolation of active compounds (**figure 7.11**).

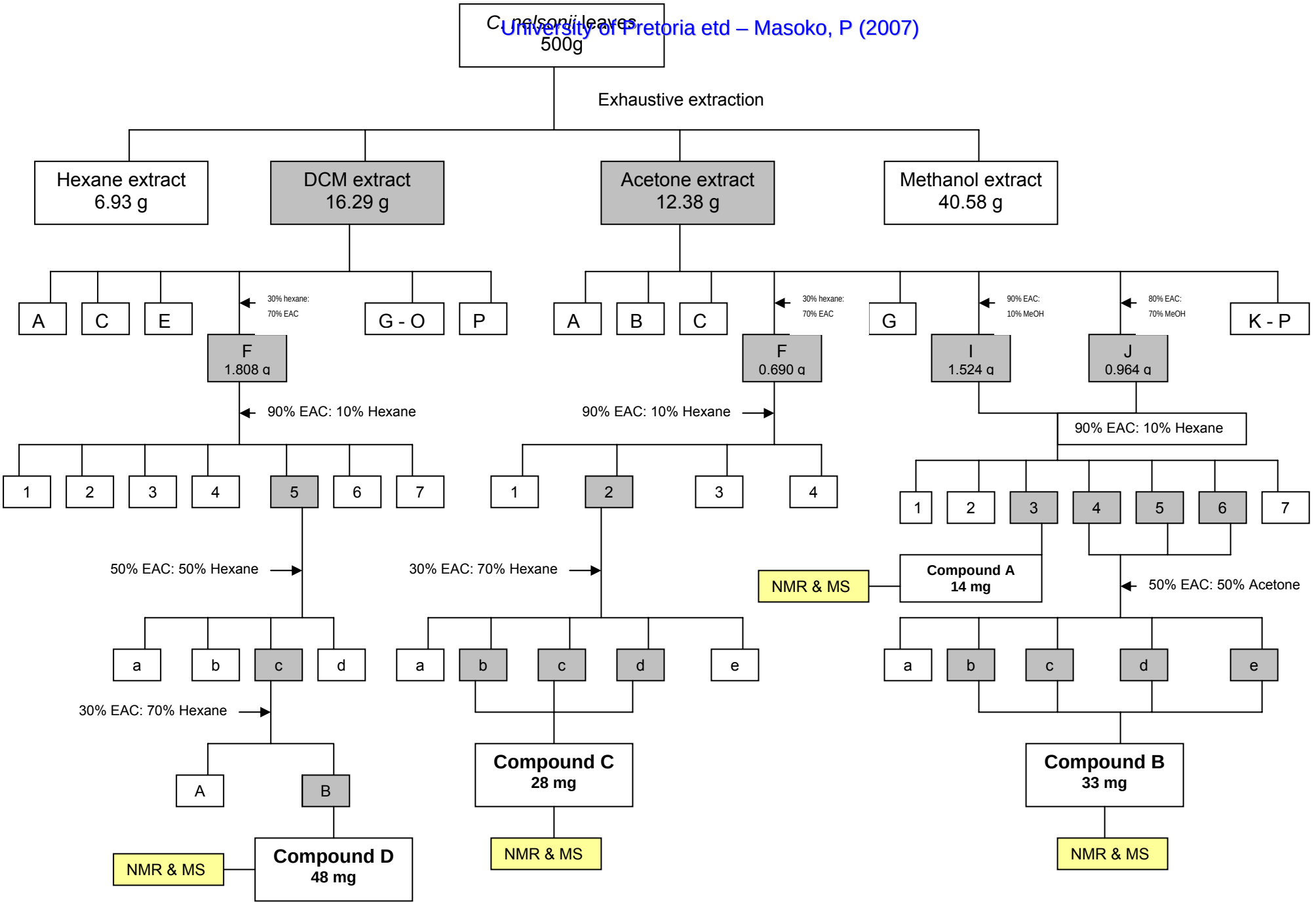


Figure 7.11. Overview of isolation process of four active compounds

7.3.6. DCM Fractions

DCM extracts (21.45 g) from the serial extraction were combined and separated on a Si gel column (12 X 10 cm) by eluting (**Section 7.2.6.1**) with different solvents (**Table 7.1**). The different masses were recorded in **Table 7.5**.

From the 21.45 g of *C. nelsonii* DCM extract used, I managed to collect 15.237 g using different eluent systems. All the plates were separated with CEF because more active compounds were found in CEF separation (**Figure 7.2** to **7.6**). The fractions were analyzed by TLC (**Figure 7.12**). Bioautography was done on all isolates to locate active compounds (**Figure 7.13** to **7.17**).

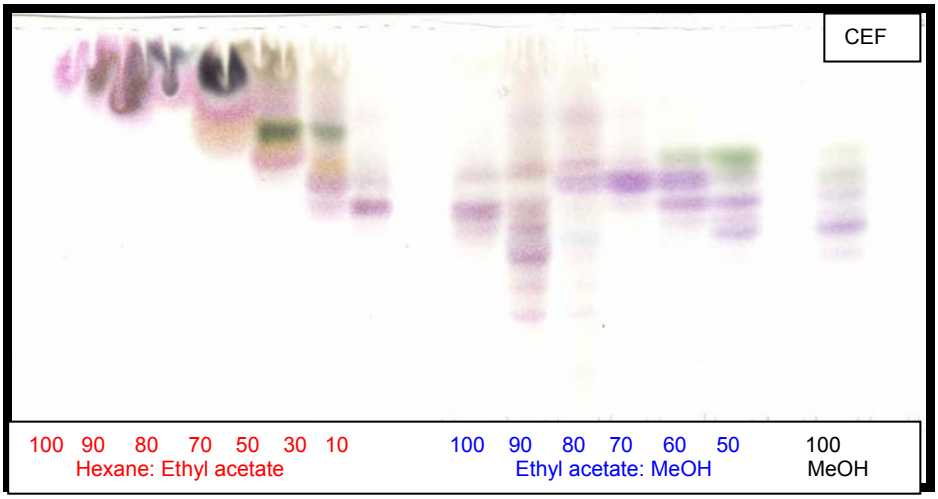


Figure 7.12. Chromatograms of *C. nelsonii* DCM extracts developed in CEF solvent systems and sprayed with vanillin–sulphuric acid to show compounds separated with different eluent systems.

Figure 7.13. Bioautography of *C. nelsonii* DCM extracts separated by CEF and sprayed with *A. fumigatus.* White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *A. fumigatus.*

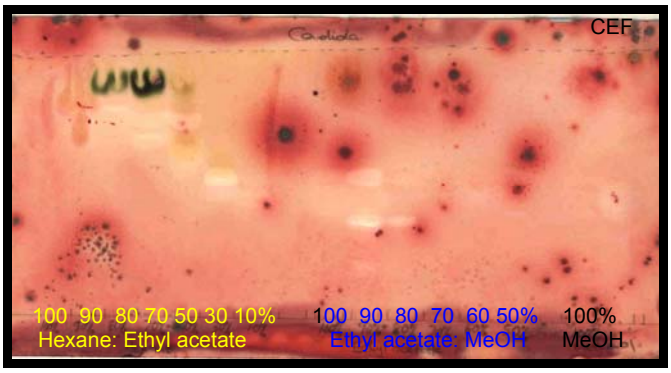


Figure 7.14. Bioautography of *C. nelsonii* DCM extracts separated by CEF and sprayed with *C. albicans.* White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *C. albicans.*

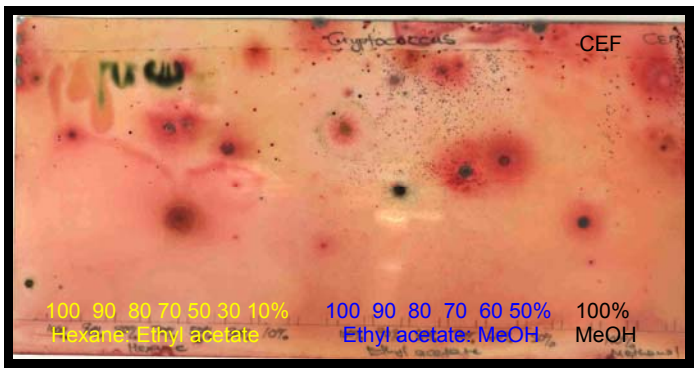


Figure 7.15. Bioautography of *C. nelsonii* DCM extracts separated by CEF and sprayed with *C. neoformans.* White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *C. neoformans.*

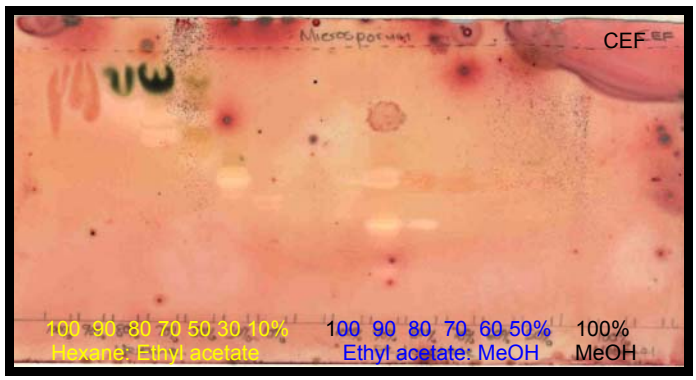


Figure 7.16. Bioautography of *C. nelsonii* DCM extracts separated by CEF and sprayed with *M. canis.* White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *M. canis*

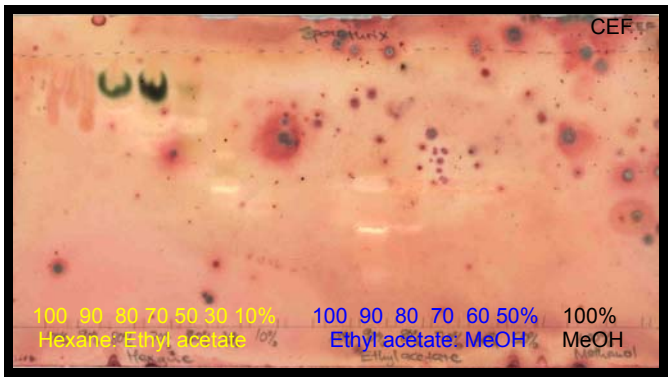


Figure 7.17. Bioautography of *C. nelsonii* DCM extracts separated by CEF and sprayed with *S. schenckii.* White areas indicate where reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *S. schenckii.*

Active compounds were found in fractions eluted with 80, 70, 50 and 30% hexane in ethyl acetate, and in 90 and 80% ethyl acetate in methanol in *C. albicans, C. neoformans, M. canis* and *S. schenckii*, but in *A. fumigatus* they were found in fractions eluted by 90, 80, 70% ethyl acetate in methanol. Ethyl acetate (90 and 80%) in methanol fractions were combined and subjected to a column and eluted with 90% ethyl acetate in hexane (**Figure 7.18**). The following fractions were combined based on similarity of chromatograms. An overview of isolation is presented in **figure 7.11**. the blue compounds after isolation were again the most active against the fungi tested.

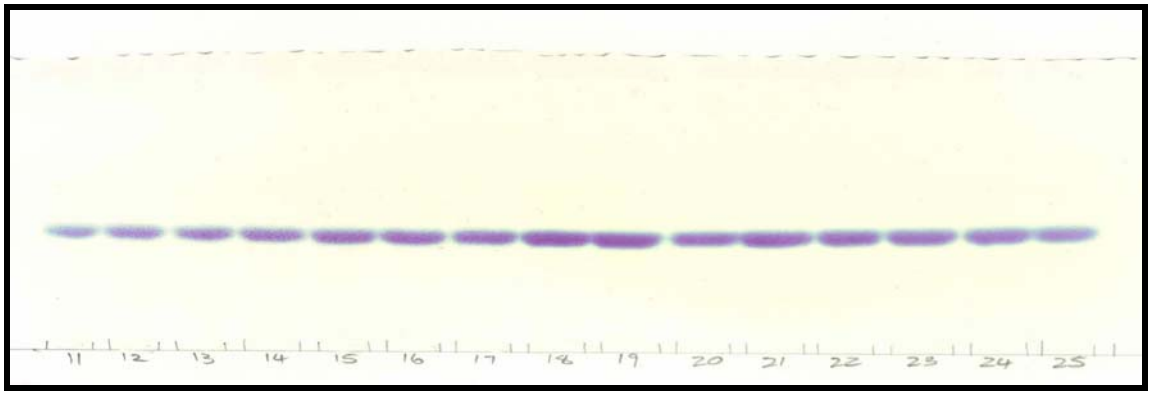


Figure 7.18. Chromatograms of combined fractions of *C. nelsonii* DCM extracts isolated with 80% ethyl acetate in methanol and developed in CEF solvent systems and sprayed with vanillin–sulphuric acid to indicate composition of fractions.

7.4. Discussion and Conclusion

Bioassay-guided fractionation on silica gel 60 (63-200 um) in column chromatography resulted in the successful isolation of the major antifungal compounds present in the leaves of *C. nelsonii*.

A number of active compounds have been isolated from different *Combretum* species. Most of the work so far has been done on antibacterial compounds. The stilbene 2, 3, 4-trihydroxyl, 3, 5, 4-trimethoxybibenzyl (combretastatin B5) was isolated from the leaves of *C. woodii*. It had significant activity against *S. aureus* with an MIC of 16 μg/ml [*Ps. aeruginosa (125* μg/ml), *E. faecalis* (125 μg/ml) and slight activity against *E. coli*.] (Eloff *et al*., 2005a,b). A variety of triterpenoids have been isolated from *Combretum* spp. (Rogers and Verotta, 1996). Terpenes or terpenoids are active against bacteria and fungi (Taylor *et al.*, 1996). Flavonoids isolated from the leaves of *Combretum micranthum* have been shown to have antimicrobial activity against both Gram-positive and Gram-negative microorganisms (Rogers and Verrotta, 1996).

Anti-inflammatory and molluscicidal compounds such as mollic acid –D – glycoside and imberbic acid have been isolated from *C. molle* and *C. imberbe* respectively (Pegel and Rogers, 1985). The saponin, jessic acid linked to α-L-arabinose has been isolated from *Combretum eleagnoides* leaves (Osborne and Pegel, 1984). Chemical studies of the *Combretum* genus have yielded acidic triterpenoids and their glycosides, phenanthrenes, amino acids and stilbenes (Pellizzoni *et al*., 1993). A series of closely related bibenzyls, stilbenes and phenanthrenes have been isolated from *C. caffrum* (Petit *et al*., 1995).

Martini *et al*., (2004a) isolated and characterized seven antibacterial compounds. Four were flavanols: kaemferol, rhamnocitrin, rhamnazin, quercitin 5,3 -dimethylether] and three flavones apigenin, genkwanin and 5-hydroxy-7,4'-dimethoxyflavone.
All test compounds had good activity against *Vibrio cholerae* and *E. faecalis*, with MIC values in the range of 25-50 μg/ml. Rhamnocitrin and quercetin-5,3-dimethylether showed additional good activity (25 μg/ml) against *Micrococcus luteus* and *Shigella sonnei*. Toxicity testing showed little or no toxicity towards human lymphocytes with the exception of 5-hydroxy-7,4-dimethoxyflavone (Martini *et al*., 2004b). This compound is potentially toxic to human cells and exhibited the poorest antioxidant activity. Both rhamnocitrin and rhamnazin exhibited strong antioxidant activity with potential anti-inflammatory activity. Although these flavonoids are known, this was the first report of biological activity with some of these compounds.

Serage (2003) isolated and elucidated the structures of two flavanones alpinetin, pinocembrin, and one chalcone flavokawain-from the leaves of *C. apiculatum subsp apiculatum*. All the compounds had substantial activity against the bacterial pathogens tested.

Angeh (2005) isolated 8 compounds with antibacterial activity from *Combretum* section, Hypocrateropsis. Two new pentacyclic triterpenoids (1α, 24β-dihydroxyl-12-oleanen-29-oic acid-24β-O-α-L-2, 4-diacetylramnopyranoside and 1α, 23β-dihydroxyl-12-oleanen-29-oic acid-23β-O- α-L-4-acetylramnopyranoside) and six known triterpenoids (1α, 3β-dihydroxyoleanen-12-29-oic, 3-hydroxyl-12-olean-30-oic, 3, 30-dihydroxyl-12-oleanen-22-one, 1,3, 24-trihydroxyl-12-olean-29-oic acid, (1α, 22β-dihydroxyl-12-oleanen-30-oic acid) and (24-ethylcholesta-7, 22,25-trien-3-ol-O-β-D-glucopyranoside). All eight compounds had moderate (MIC of 60 µg/ml) to strong (10 µg/ml) antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Mycobacterium vaccae*.

In present study I managed to isolate compounds with antifungal activity from *Combretum nelsonii*. This provides a scientific base for the use of this plant in folk medicine and could be the basis of the novel antifungal.

The structure elucidation of the isolated compound will be dealt with in the next chapter.

Chapter 8

Structure elucidation

8.1. Introduction

8.1.1. Nuclear Magnetic Resonance (NMR)

NMR is a valuable structure elucidation tool for organic and biological molecules. Besides qualitative information, NMR can provide valuable quantitative information about a sample. A normal liquid state 1D ^{1}H NMR spectrum is commonly recognized as a reliable method for quantification. Other nuclei have also been utilized with one-dimensional experiments, both in liquids and solid state (Martin *et al*., 1980 and Harris 1985).

The major limitation of NMR spectroscopy is the rather low detection sensitivity, rendering the experiments time-consuming compared to other methods used for molecular structure determination or verification such as X-ray crystallography or mass spectroscopy. This is because the sensitivity of the NMR signal depends on the small difference in the populations of the Zeeman energy levels. The separations between the nuclear spin states are small, corresponding to energies in the radiofrequency range. The population difference is given by the Boltzman distribution. For ^{1}H nuclei at room temperature and magnetic field of 10 T the difference in the population is in the order of 1 in 10^{5} which means that most of the nuclei do not contribute to the NMR signal. This is in contrast to optical spectroscopic methods such as, for instance, infrared (IR) spectroscopy where basically a single photon can be detected.

For a high-resolution NMR investigation using a conventional probe operating at ambient temperature the required amount of substance is often milligrams. In many applications the available amount of sample is limited, or the inherent solubility of the substance of interest may be low, or a dilute solution is required because the sample may tend to aggregate at higher concentrations. In such cases, the cryogenic probe technology moves the lower limit of the feasible sample concentration to the microgram and micromolar range. For biological macromolecules, the change in the sample requirement from the millimolar to the micromolar sample concentration range greatly increases the number of compounds that can be studied by NMR.

The strength of NMR spectroscopy is given by its multifarious applications, which range from statistical analysis of mixtures to the determination of three-dimensional structures for molecules of biological interest. The information content of NMR at the atomic level is both comprehensive and diverse. Thus, to improve the sensitivity has always been an important

development goal from the point of methodology and engineering. In an NMR experiment, the signal-to-noise ratio is usually augmented through computer averaging of accumulated transients. In the signal averaging, however, the signal-to-noise ratio is proportional to the square root of the number of transients. Consequently, a 3–4-fold sensitivity increase as provided by cryogenic probes, entails a 9–16-fold reduction in measurement time (Kovacs *et al*., in Press).

8.1.2. Mass spectrometry (MS)

Mass spectrometry is the most sensitive and selective method for molecular analysis and can yield information on the molecular weight as well as the structure of the molecule. Combining chromatography with mass spectrometry provides the advantage of both chromatography as a separation method and mass spectrometry as an identification method. In mass spectrometry, there is a range of methods to ionize compounds and then separate the ions (Gong *et al*., 2001a). Common methods of ionization used in conjunction with gas chromatography are electron impact (EI) and electron capture ionization (ECI). EI is primarily configured to select positive ions, whereas ECI is usually configured for negative ions (ECNI). EI is particularly useful for routine analysis and provides reproducible mass spectra with structural information, which allows library searching. GC–MS was the first successful online combination of chromatography with mass spectrometry, and is widely used in the analysis of essential oil in herbal medicines (Guetens *et al*., 2002).

With the GC–MS, people could produce not only a chromatographic fingerprint of the essential oil of the herbal medicine but also the information related to its most qualitative and relative quantitative composition (Li *et al*., 2001). Used in the analysis of the herbal medicines, there are at least two significant advantages for GC–MS, that is: (1) with the capillary column, GC–MS has in general very good separation ability, which can produce a chemical fingerprint of high quality; (2) with the coupled mass spectroscopy and the corresponding mass spectral database, the qualitative and relatively quantitative composition information of the herb investigated could be provided by GC–MS, which will be extremely useful for the further research for elucidating the relationship between chemical constituents in herbal medicine and its pharmacology in further research (Gong *et al*., 2001b).

To fully elucidate a molecular structure, distortionless enhancement by polarization transfer (DEPT), heteronuclear multiple bond correlation (HMBC), heteronuclear multiple quantum coherence (HMQC) and correlation spectroscopy (COSY) are also done.

8.1.3. Distortionless enhancement by polarization transfer (DEPT)

DEPT is a technique that gives information about the number of protons bonded to each carbon.

8.1.4. Heteronuclear multiple bond correlation (HMBC)

HMBC gives information about weak proton-carbon J-coupling. A weak proton-carbon J-coupling indicates that the proton is two, three, or four bonds away from the carbon. This experiment gives information about which protons are near to (but not directly bonded to) different carbons. HMBC can give an enormous amount of information about molecular structure, since the long-range proton-carbon correlations can include quaternary carbons, in addition to protonated carbons.

8.1.5. Heteronuclear multiple quantum coherence (HMQC)

HMQC gives information about strong proton-carbon J-couplings. A strong proton carbon J-coupling indicates that the proton is directly bonded to the carbon. HMQC is selective for direct C-H coupling

8.1.6. Correlation Spectroscopy (COSY)

Correlation Spectroscopy (COSY) gives information about pairs of protons that are J-coupled. This usually indicates that the protons are on adjacent carbons, e.g. 3-bonds away (though protons further apart may in some cases be J-coupled).

8.2. Materials and Methods

8.2.1. Nuclear Magnetic Resonance (NMR)

After column chromatography, precipitation of some fractions began to take place. These precipitates were collected, cleaned using various solvent systems starting with non-polar solvents e.g. hexane and then introducing ethanol, methanol, ethyl acetate, chloroform and acetone. The samples were passed through a Pasteur pipette plugged with cotton wool to facilitate the removal of impurities. The clean samples were weighed and dissolved in maximum 2 ml deuterated solvents used for NMR (Merck). In these studies, acetone was used as the solvent of choice, although other solvents were also attempted, because of its ability to dissolve a wide range of compounds. The samples were then pipetted into tubes

(Milmad, economy) with the aid of a Pasteur pipette and send to Mr Mathebula of the Chemistry department, University of Limpopo, MEDUNSA campus. ^{1}H NMR was run at either 300 or 400 MHz and ^{13}C at 75 MHz using the solvent signal as the reference. Structures were elucidated by Dr Mdee (Phytomedicine Programme).

8.2.2. Mass spectrometry (MS)

High Resolution Electron Impact Mass Spectroscopy (HREIMS) was performed on samples sent for analysis using a MASPEC II system (II32/A002) at University of Johannesburg (UJ). Mr Vorster also performed DEPT, HMBC and HMQC at UJ. Dr Mdee of University of Pretoria, Phytomedicine Programme performed the analysis.

8.2. Results

From **Chapter 7**, compound D and compound B were combined and labeled **compound I** (81 mg). Compound C was then labeled **compound II**. Due to the small amount isolated of compound A, it was not further studied. NMR analysis of compound I are shown in **figures 8.1** to **8. 7**.

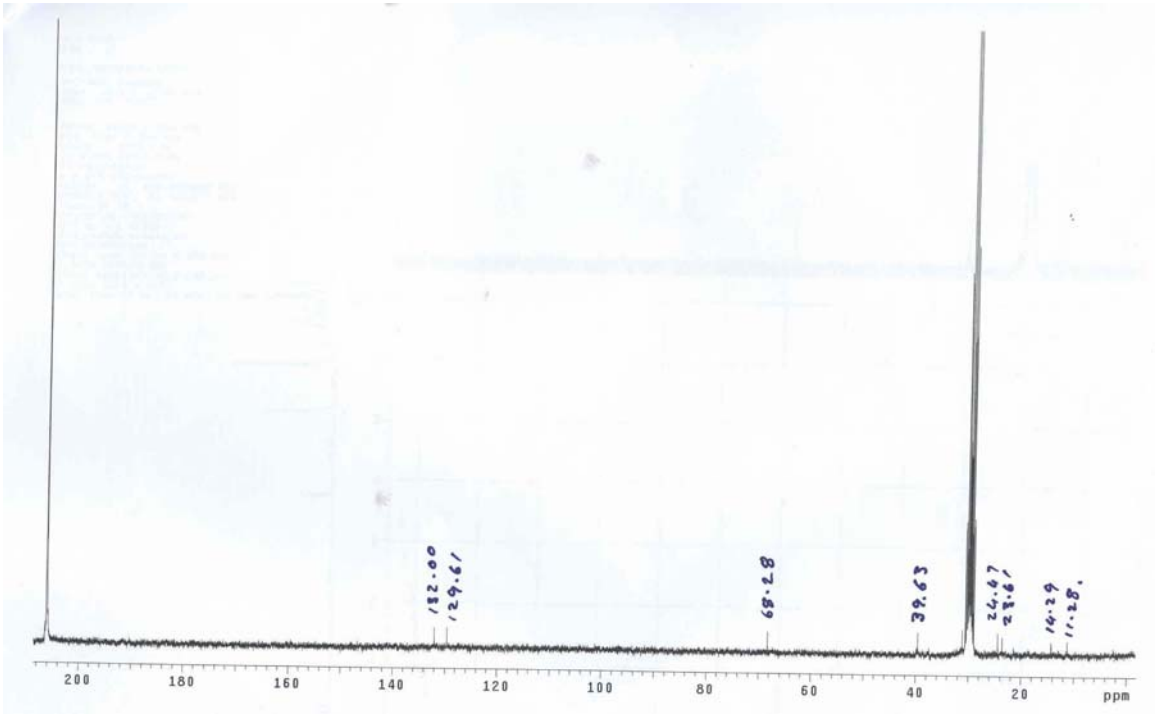


Figure 8.1. ^{13} C NMR spectrum of **Compound I**

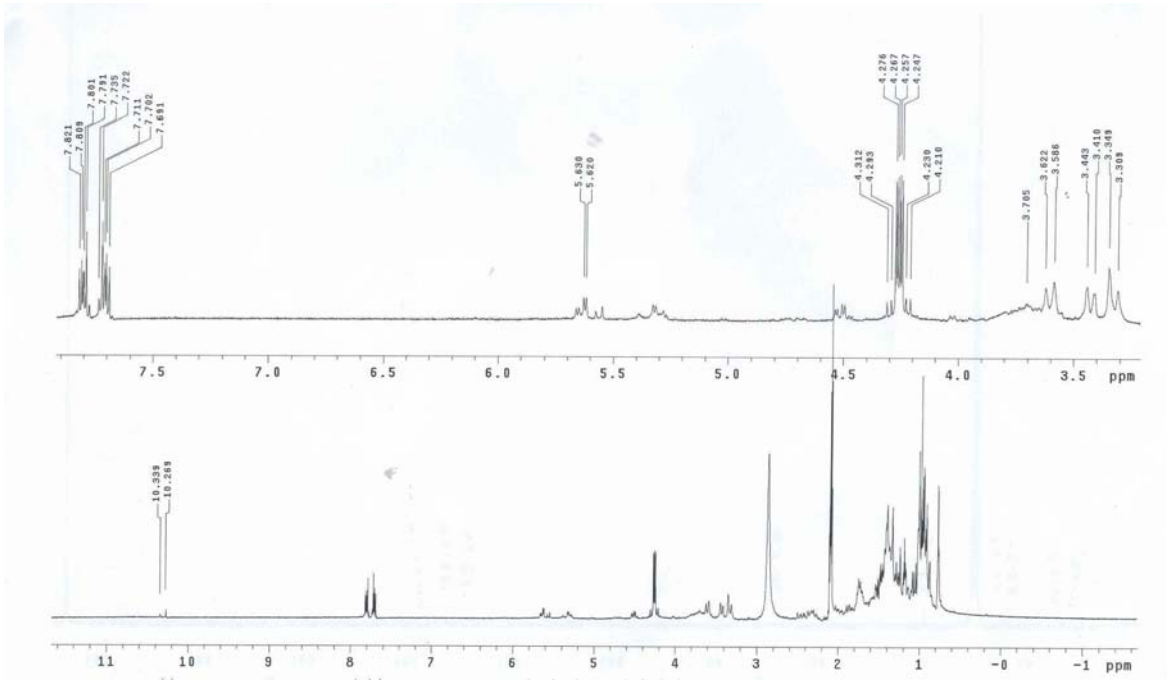


Figure 8.2. ^{1}H NMR spectrum of **Compound I**

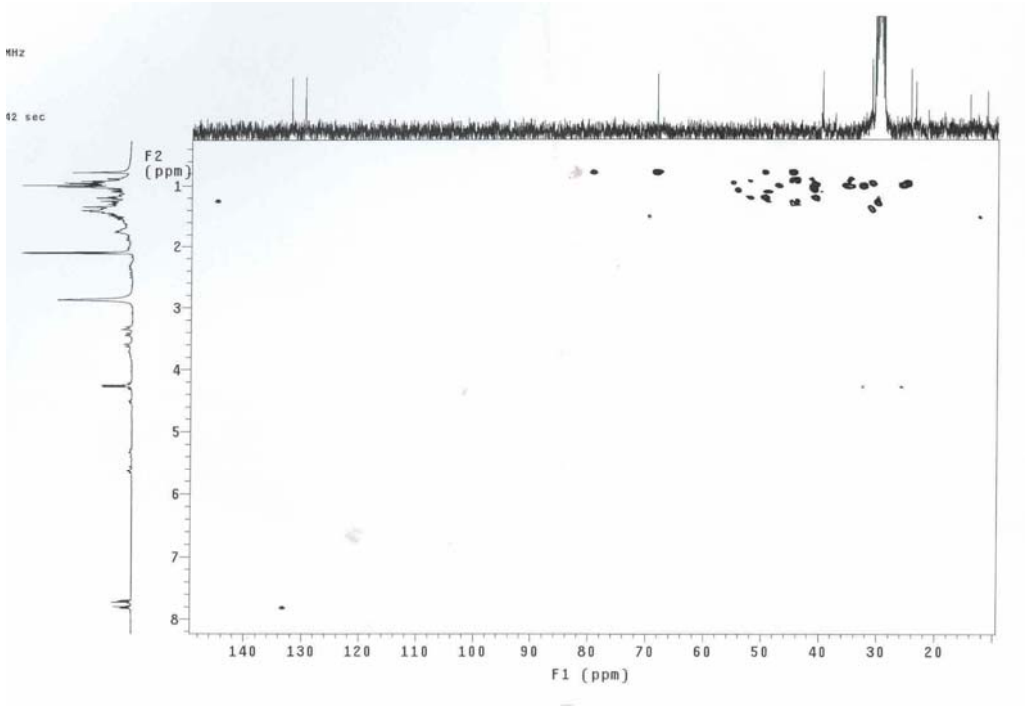


Figure 8.3. HMBC NMR spectrum of **Compound I**

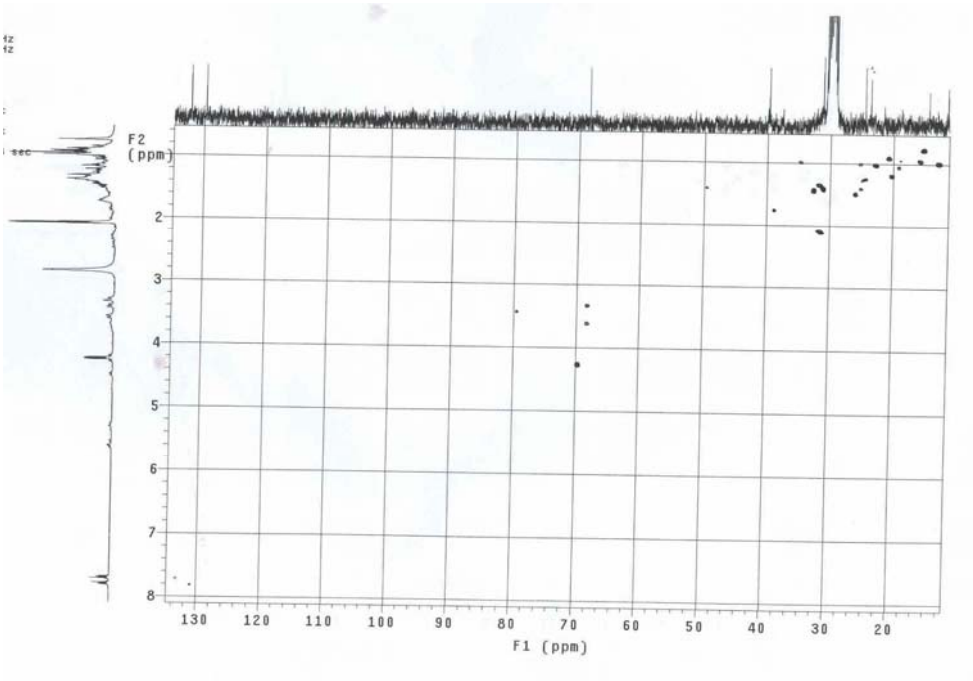


Figure 8.4. HSQC NMR spectrum of **Compound I**

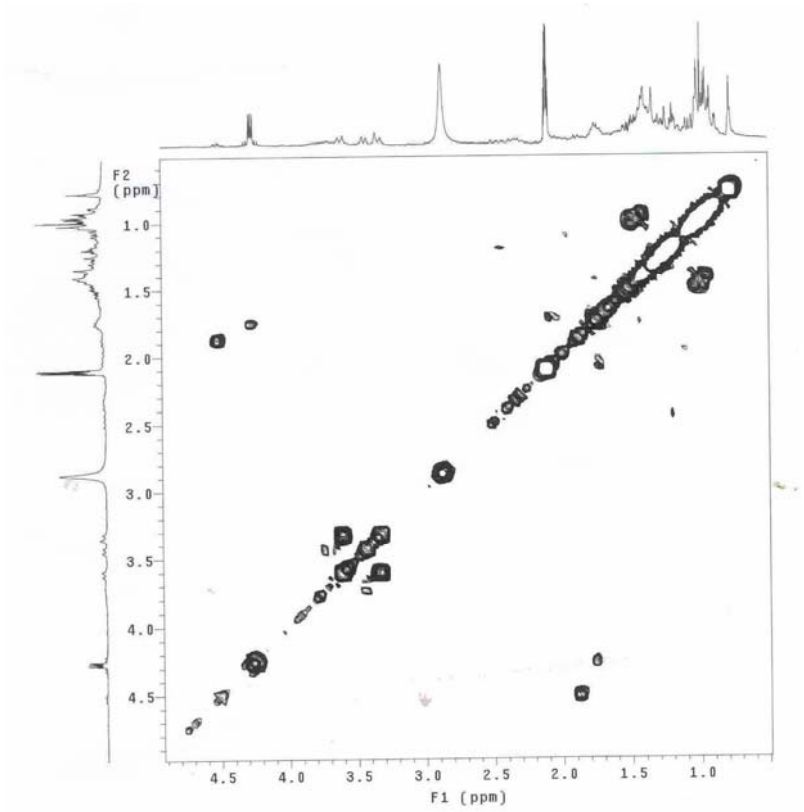


Figure 8.5. gCOSY NMR spectrum of **Compound I**

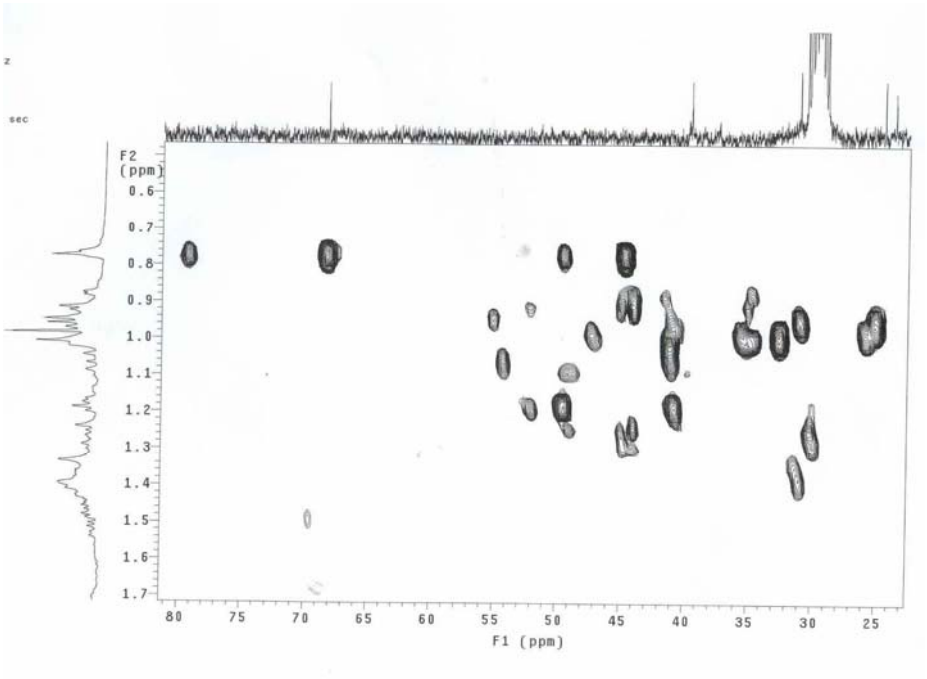


Figure 8.6. gHMBC NMR spectrum of **Compound I**

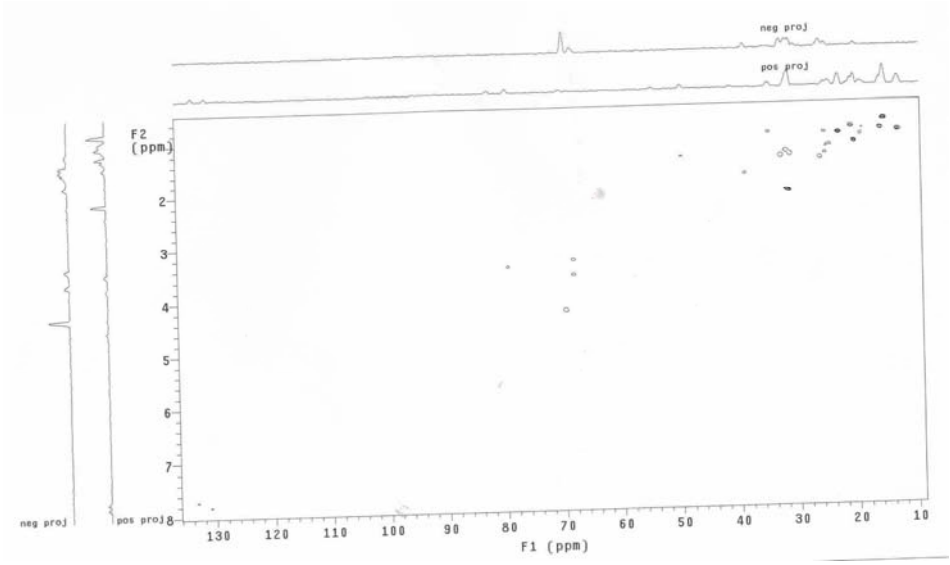


Figure 8.7. gHSQC NMR spectrum of **Compound I**

The bioassay-guided fractionation of the acetone extract by column chromatography lead to **compound II** with *m/z* 503 $(M-H)^-C_{30}H_{48}O_6$ and *m/z* 649 $(M-H)^-C_{36}H_{58}O_{10}$. The presence of one $C_6H(\delta_C 68.7, \delta_H 5.11)$ in compound II and the absence of $C_6H_2(\delta_C 18, \delta_H 1.41)$ in the ^1H, ^{13}C and DEPT spectra led to the identification of Compound II as terminolic acid.

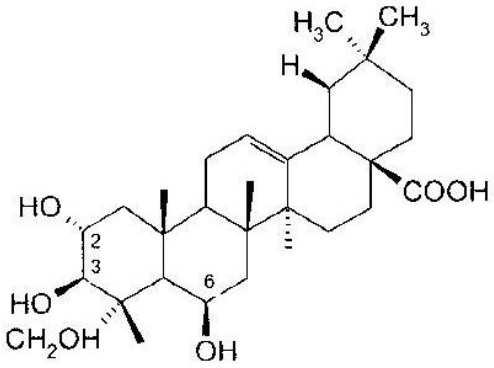
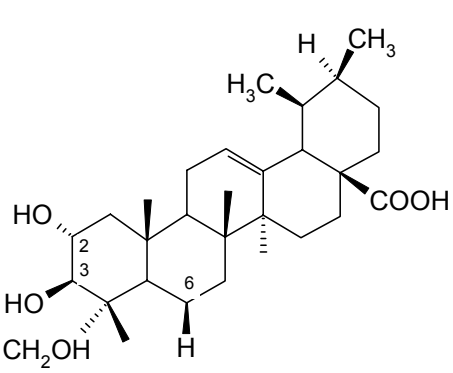
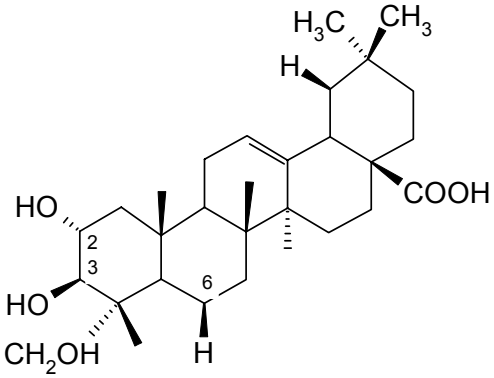


Figure 8.8. Terminolic acid

Compound I was a mixture of two inseparable compounds



Asiatic acid



Arjunolic acid

Figure 8.9. Compound **1,** a mixture of two inseparable compounds, which were asiatic acid (**1b**) and arjunolic acid (**1a**)

8.3. Discussion and Conclusion

It was the first time **compound II** (Terminolic acid) was isolated from *C. nelsonii*. It was previously isolated from *Terminalia macroptera* (Conrad *et al*., 1998) and they have studied its biological activities (*Bacillus subtilis* (5 μg/ml), *Pseudomonas fluorescens* (2.5 μg/ml) and *Cladosporium cucumerinum* (20 μg/ml)). It was also isolated from *Syzygium guineense* (Djoukeng et al., 2005) and they have found that it is active against *Escherichia coli* (6 μg/ml) and *Bacillus subtilis* (3 μg/ml). Due to lack of sufficient material, the antifungal activities of this compound were not determined and it was not further studied because it was not compound of interest. Compound 1 will be dealt with as a published paper in **chapter 10**. The title is: "**Biological activity of two related triterpenes isolated from *Combretum nelsonii* (Combretaceae) leaves".**

Chapter 9

In vitro cytotoxicity tests of the developed extracts

9.1. Introduction

Cytotoxicity testing using cell cultures is a rapid, standardized, sensitive, and inexpensive means to determine whether a material contains significant quantities of biologically harmful extractables. The high sensitivity of the tests is due to the isolation of the test cells in cultures and the absence of the protective mechanisms that assist cells within the body. A mammalian cell culture medium is the preferred extractant because it is a physiological solution capable of extracting a wide range of chemical structures, not just those soluble in water.

Toxicity is usually divided into two types, acute or chronic, based on the number of exposures to a poison and the time it takes for toxic symptoms to develop. Acute toxicity is due to short-term exposure usually a high dose, whereas chronic exposure is due to repeated or long-term exposure usually involving low doses.

Acute toxicity tests are short-term tests designed to measure the effects of a product on species during a short portion of their life span. The tests, which typically run for between 48 and 96 hours, usually measure the effects of products on the survival of a species. The results of these tests are often reported as an ‘EC50’, which is the effective concentration of a test sample that causes a specific effect to 50% of the cells. Chronic tests are used for low-level exposure for long periods, and are designed to measure effects on development, growth and reproductive success or failure.

Standard acute toxicity tests with aquatic macro-invertebrates have long played a major role in aquatic hazard and risk assessments, especially at a "screening" level of evaluation. A number of alternative tests have been proposed for rapid screening and are freshwater rotifer (*Branchionus calyciflorus*), brine shrimp (*Artemia salina*), lettuce (*Luctuca saliva*), mysid shrimp (*Mysidopsis hahia*), fathead minnow (*Pimephales promelas*) (McLaughlin, 1991). These tests are useful in situations where their rapidity and relative low cost make it practical to screen large numbers of samples for preliminary indications of toxicity. In this project the brine shrimp assay was used.

There are number of *in vitro* toxicity tests where different cell lines are used. Each test use different indicators. Some of this tests are Neutral red uptake cytotoxicity assay, one disadvantage of the Neutral Red assay is the possibility that deceptively low cell viability or cell number readings will result in those cases where a chemical has a relatively selective effect upon the lysosomes/endosomes of the cell. Hyaluronan gel cell toxicity test, a cell toxicity assay was devised to test for the effect of leachable chemicals and break-down products of the HA construct on CF-31 adhesion and proliferation (Borenfreund and Puerner, 1985). In this project the MTT, [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay was used based.

9.1.1. The brine shrimp assay

The brine shrimp lethality assay is considered a useful tool for preliminary assessment of toxicity. Brine shrimp is popularly known as sea monkeys and are crustaceans that live in saline environments. Their eggs (actually cysts), which can be inexpensively purchased from pet stores, hatch quickly and the larvae, termed a nauplius (plural, nauplii) are sensitive to small doses of biologically active chemicals. One indicator of the toxicity of a substance is LD_{50}, which refers to the amount (*i.e.,* lethal dose or concentration) of a substance that kills 50% of the test organisms. Activities are considered significant if the LD_{50} is less than 30 µg/ml (=0.03 mg/ml) (Geran *et al*., 1972). In this bioassay, the mortality of brine shrimp that are incubated in the test solution is recorded. Although the brine shrimp assay provides no information on the mechanism of action, it is a very useful preliminary tool in assessing the toxicity of extracts.

Brine shrimp assay has number of advantages like, experimental simplicity, sensitivity, reproducibility, ease of handing, lack of continuous culturing, short exposure time and lower costs. The main disadvantage is that one cannot extrapolate the results to toxicity to mammals (McGaw and Eloff, 2005).

9.1.2. The MTT cytotoxicity assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, (Mosmann, 1983), is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave the tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals which are largely impermeable to cell membranes, thus resulting in their accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals that are solubilized. The number of surviving cells is directly proportional to the level of

the formazan product created. The colour can then be quantified using a spectrophotometer (Mosmann. 1983).

The MTT cytotoxicity assay is considered a major advance in toxicity testing and it is the most widely used *in vitro* cytotoxicity assay. It is rapid, sensitive, versatile, quantitative and highly reproducible. It is also adaptable to a large-scale screening relevant for most cells. MTT reduction correlates to indices of cellular protein and viable cell number (Timmins, 2002).

However there are cell lines that do not metabolise MTT well or do not show an acceptable colorimetric profile for control cells. Production of the MTT product is dependent on the MTT concentration in the culture medium. The kinetics and degree of saturation are dependent on cell type. The assay is less effective in the absence of cell proliferation. The presence of glutathione-*S*-transferase (a normal enzyme that protects cells) can reduce the MTT independent of toxicity. These cells give high background and potentially false positives. MTT cannot distinguish between cytostatic and cytocidal effect. Individual cell numbers are not quantitated and results are expressed as a percentage of control absorbance. The test is less effective if cells have been cultured in the same media that has supported growth for a few days, which leads to underestimation of control and untreated samples. Certain types of drugs (i.e. interferon) can induce formazan production (MTT) and/or mitochondrial activity. Increased production of formazan will potentially give false positives with these drugs (Timmins, 2002).

9.2. Materials and Methods

9.2.1. Extracts tested

Acetone extracts of *C. imberbe*, *C. nelsonii*, *C. albopuntactum* and *T. sericea* were tested based on previous chapters results.

9.2.2. The brine shrimp assay

Brine shrimp (*Artemia salina*) eggs were hatched in a beaker filled with 3.8% aqueous sodium chloride in the dark. After 48 hours, the phototrophic nauplii were collected using a Pasteur pipette. Newly hatched nauplii were concentrated just above the unhatched eggs on the bottom. Since the nauplii are positively phototropic (attracted to light), shining a light in the middle of the container and shading the container at the bottom helps direct them to an area where they can be easily harvested. The nauplii were counted macroscopically in the stem of the pipette against

a lighted background. Approximately 10 – 15 shrimps were transferred to each well of 96 – well microplates containing the samples. The concentrations in which each extract was tested ranged from 0.1 – 2 mg/ml. The plates were kept in the dark. Survivors were counted after 24 hours of incubation and the percentage of deaths at each concentration and controls (salt water alone) were determined under the microscope. Podophyllotoxin (Sigma) was used as a positive control. The toxicity of the extracts to brine shrimps was determined in triplicate and the average percentage of live shrimps calculated.

9.2.3. The MTT cytotoxicity assay

Viable cell growth after incubation with test compound is determined using the tetrazolium-based colorimetric assay (MTT assay) (Mosmann, 1983). Vero kidney cells (monkey) of a subconfluent culture were harvested and centrifuged at 200 x *g* for 5 min, and resuspended in growth medium to 2.4 x 10^3 cells/ml. The growth medium used was Minimal Essential Medium (MEM, Highveld Biologicals) supplemented with 0.1% gentamicin and 5% foetal calf serum (Highveld Biologicals). A total of 200 µl of the cell suspension was pipetted into each well of columns 2 to 11 of a sterile 96-well microtitre plate. Growth medium (200 µl) was added to wells of columns 1 and 12 to minimize the "edge effect" and maintain humidity. The plates were incubated for 24 h at 37ºC in a 5% CO_2 incubator, until the cells were in the exponential growth phase. The Minimal Essential Medium was aspirated from the cells using a fine tube attached to a hypodermic needle, and replaced with 200 µl of test compound at differing concentrations (0.001 to 1 mg/ml) serial dilution prepared in growth medium. The cells were disturbed as little as possible during the aspiration of medium and addition of test compound. Each dilution was tested in quadruplicate. Untreated cells and positive control (berberine chloride, Sigma) were included. The microtitre plates were incubated at 37ºC in a 5% CO_2 incubator for 5 days.

After incubation, 30 µl MTT (Sigma, stock solution of 5 mg/ml in PBS) was added to each well and the plates were incubated for a further 4 h at 37ºC. After incubation with MTT the plates were centrifuged for 10 min at 1500 rpm. The medium in each well was carefully removed, without disturbing the MTT crystals in the wells, followed by adding 150 µl fresh phosphate buffer saline (PBS) to each well. The microtitre plates were again centrifuged for 10 min at 1500 rpm and the PBS removed from the wells. After washing with PBS, the MTT formazan crystals were dissolved by adding 50 µl DMSO to each well. The plates were shaken gently until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (Titertek Multiscan MCC/340) at a test wavelength of 540 nm

and a reference wavelength of 690 nm. The wells in column 1, containing medium and MTT but no cells, were used to blank the plate reader. The LC_{50} values were calculated as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells.

9.2.4. Statistics

The line regression tool was used from Microsoft Excel.

9.3. Results

9.3.1. The brine shrimp assay

Brine shrimp assay results were analysed by plotting percentage mortality of brine shrimps against the different concentrations of the *C. nelsonii, C. imberbe, C. albopuntactum* and *T. sericea* extracts tested. The curves plotted for each extract had a percentage fit of 98, 73, 96 and 83% respectively. The equations of the curves are clearly indicated on the following figures (**Figure 9.1 – 9.4**). These four extracts were chosen because of their high antifungal activity based on MIC and bioautography assays.

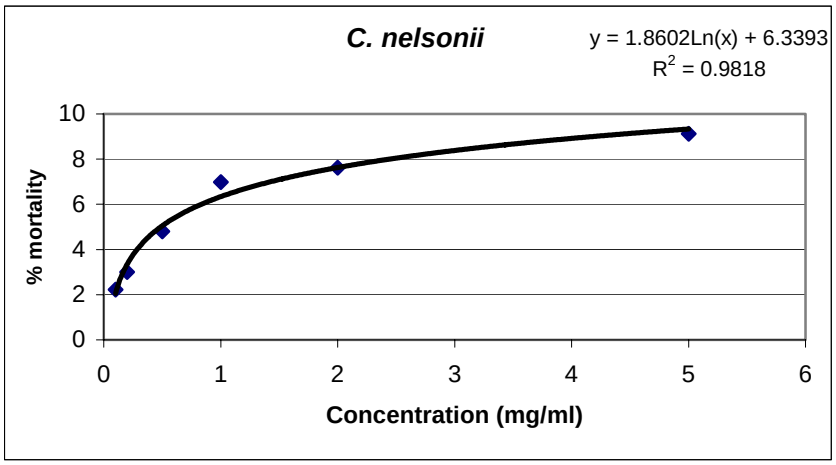


Figure 9.1. Brine shrimp assay mortality after exposure to *C. nelsonii* extract

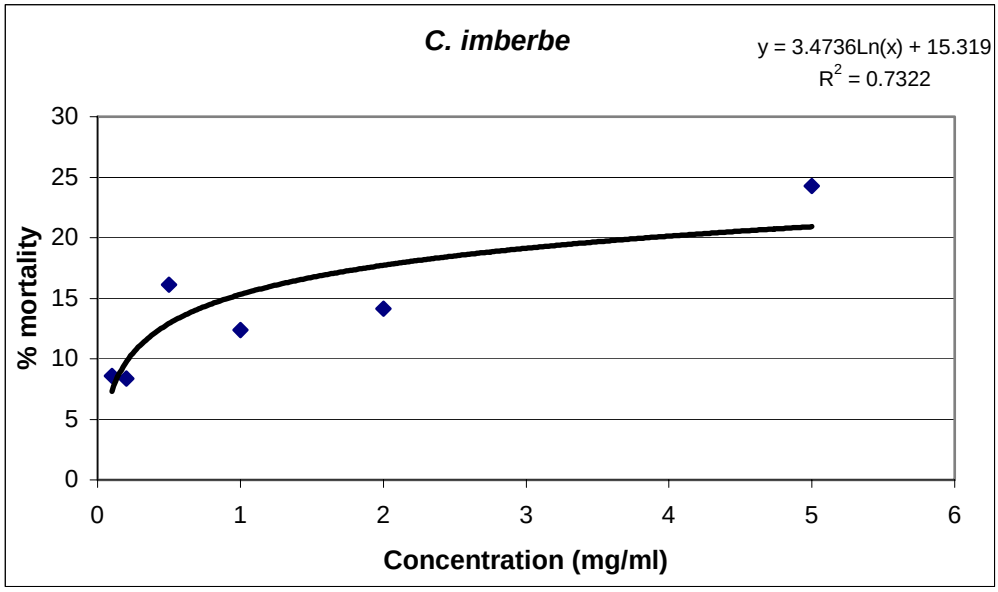


Figure 9.2. Brine shrimp assay mortality after exposure to *C. imberbe* extract

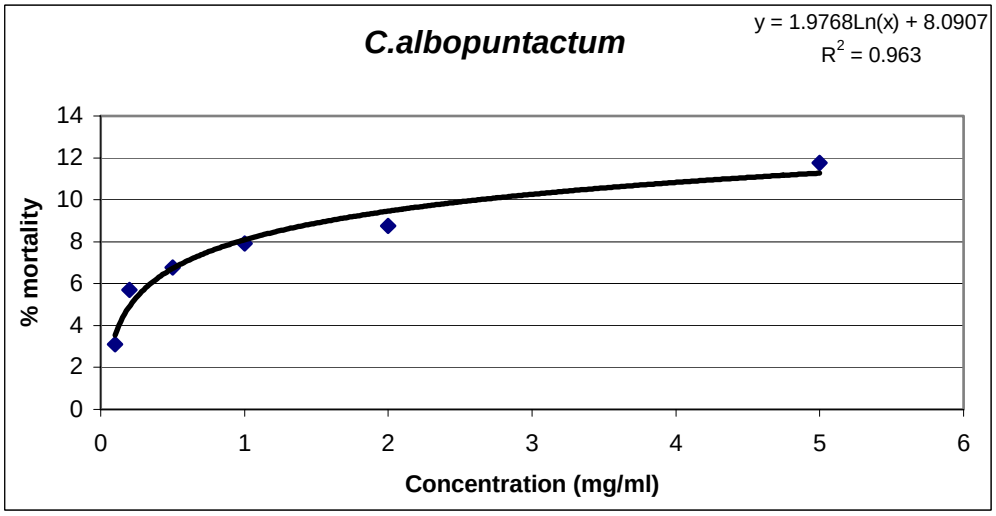


Figure 9.3. Brine shrimp assay mortality after exposure to *C. albopunctactum* extract

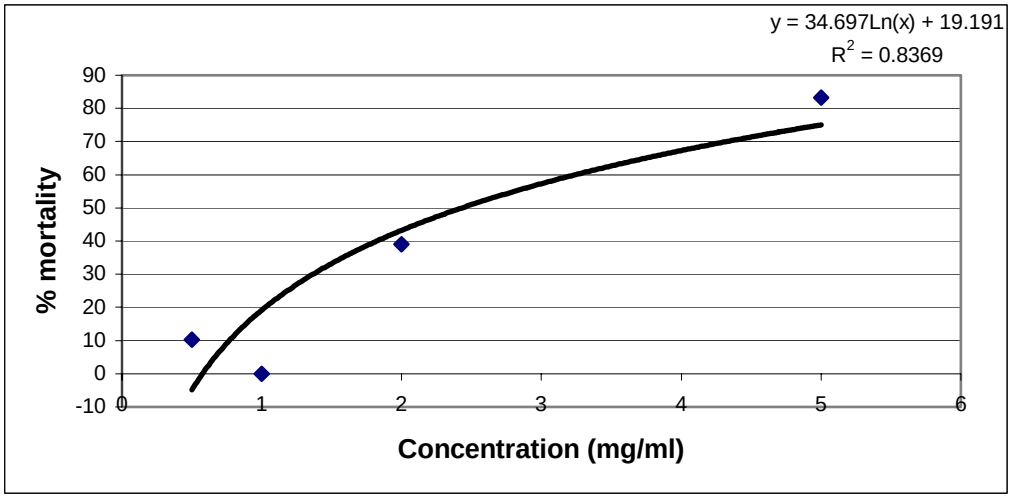


Figure 9.4. Brine shrimp assay mortality after exposure to *T. sericea* extract

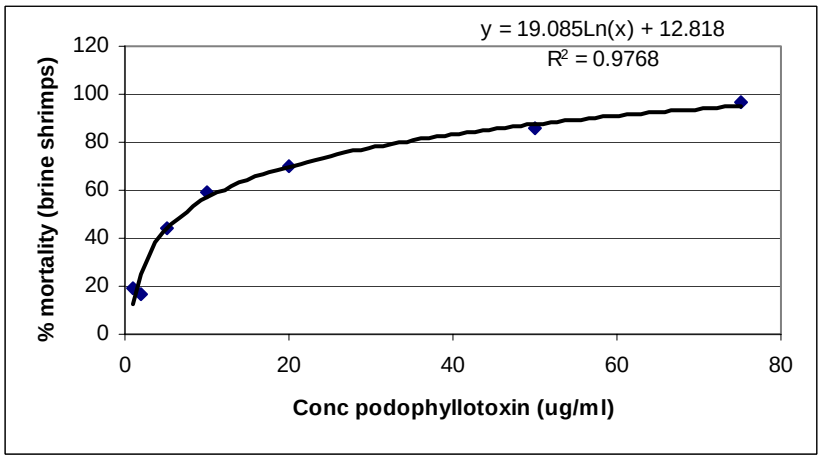


Figure 9.5. Brine shrimp assay curve of Podophyllotoxin (Positive control)

The LC_{50} value was calculated by substituting 50% for y into the curve equations. *In vitro* studies using the brine shrimp assay showed that *C. nelsonii, C. imberbe, C. albopuntactum* and *T. sericea* extracts to be relatively non toxic with LC_{50} values of 3.16, 2.30, 3.05 and 2.43 mg/ml respectively compared to 7 µg /ml for the podophyllotoxin standard.

9.3.2. The MTT cytotoxicity assay

The curve for the berberine chloride standard had a 91.64% fit and gave an equation of y = -0.4386x + 1.0009. The LC_{50} was calculated by substituting for y half the value of absorbance at 540 nm for the control (0.721).

The LC_{50} value for berberine chloride was therefore 4.347 µg/ml (published results give an LC_{50} value of 10 µg/ml) (**Figure 9.6**).

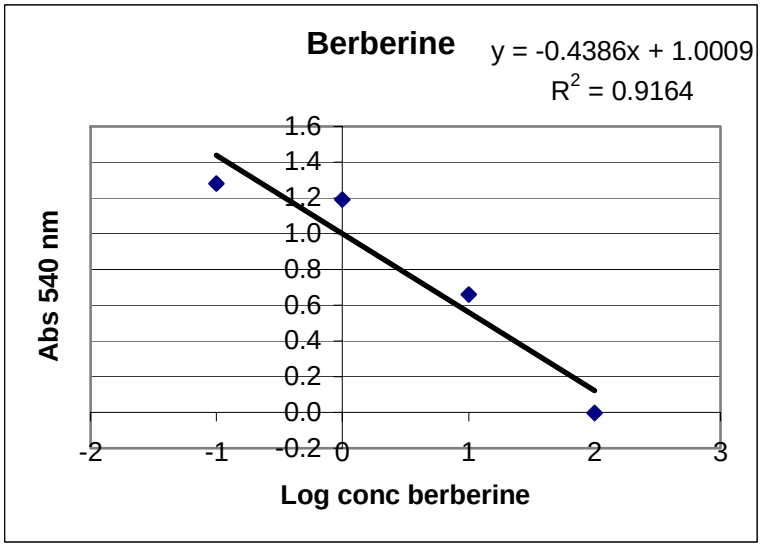


Figure 9.6. MTT cytotoxicity assay curve for Berberine chloride.

Cytotoxicity of the tested extracts was analysed at 540 nm for 1 mg/ml, 0.1, 0.01 and 0.001 mg/ml concentrations.

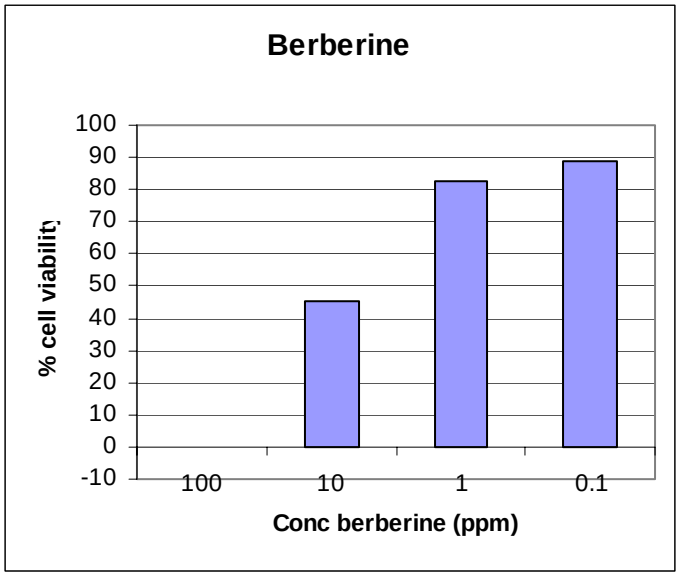


Figure 9.7. Percentage cell viability of berberine different concentration

Table 9.1. Results showing absorbance values at 540 nm for the various optimal extract concentrations.

Extracts	Conc.(mg/ml)	Log conc.	Ave abs 540	SD
C. imberbe	1	0.000	0.019	0.008
	0.1	-1.000	1.182	0.039
	0.01	-2.000	1.214	0.076
	0.001	-3.000	1.296	0.106
C. nelsonii	1	0.000	0.025	0.008
	0.1	-1.000	0.756	0.367
	0.01	-2.000	1.191	0.132
	0.001	-3.000	1.214	0.116
T. sericea	1	0.000	0.123	0.081
	0.1	-1.000	0.841	0.053
	0.01	-2.000	1.165	0.111
	0.001	-3.000	1.168	0.103
C. albopunctactum	1	0.000	0.003	0.005
	0.1	-1.000	1.086	0.085
	0.01	-2.000	1.146	0.055
	0.001	-3.000	1.225	0.063

The results were analysed by plotting the logarithm of different concentrations of the extract versus absorbance values at 540nm. The *C. imberbe*, *C. nelsonii*, *T. sericea* and *C. albopunctactum* curves had a percentage fit of 67.29, 86.39, 82.38 and 69.37 % respectively and the equation of the curves were y = -0.3861(x) + 0.3485; y = -0.4001(x) + 0.1961; y = -0.346(x) + 0.3054 and y = -0.3724(x) + 3063 respectively.

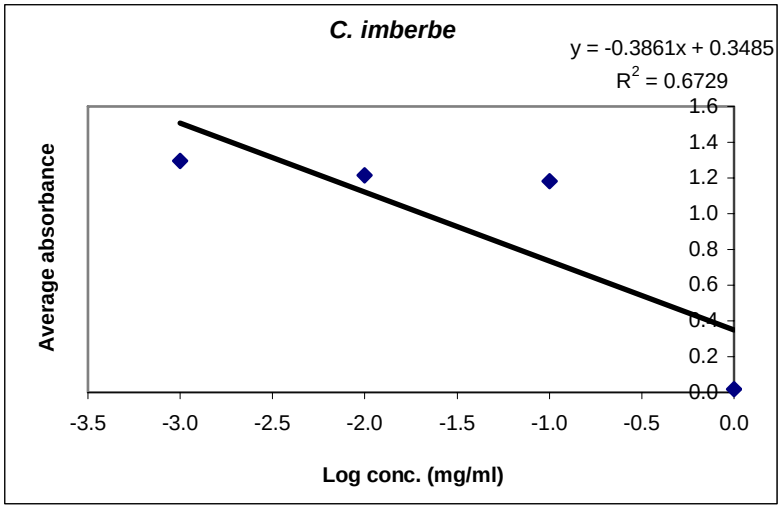


Figure 9.8. MTT cytotoxicity activity of *C. imberbe* extract against Vero cells.

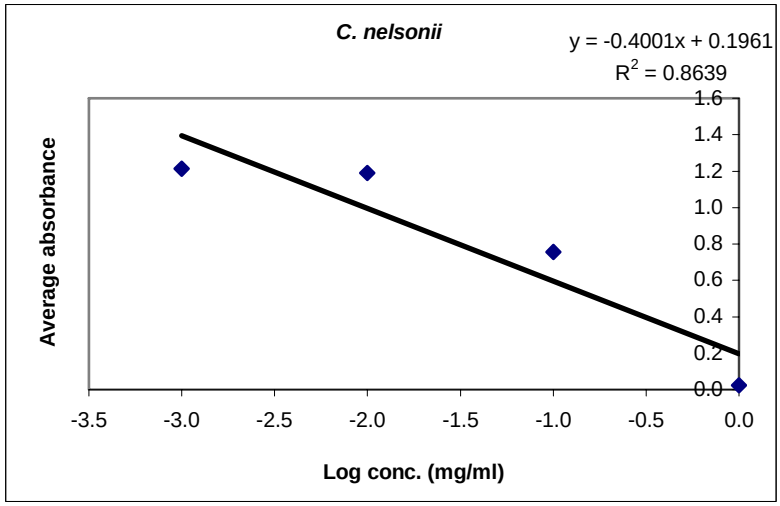


Figure 9.9. MTT cytotoxicity activity of *C. nelsonii* extract against Vero cells.

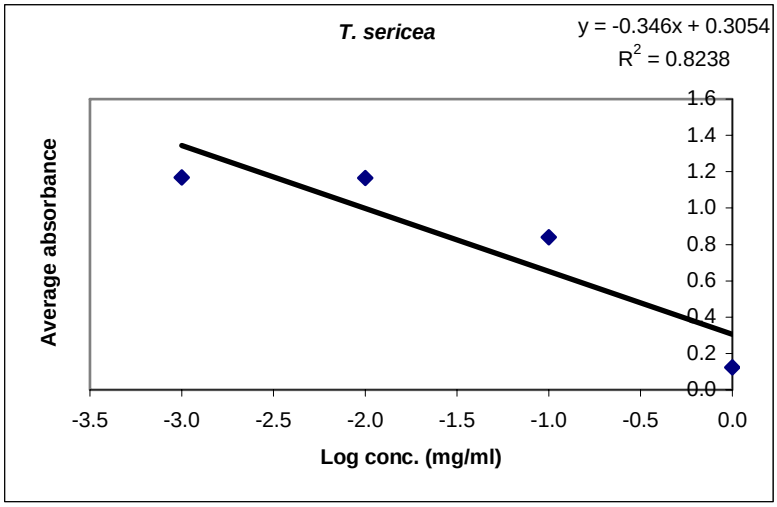


Figure 9.10. MTT cytotoxicity activity of *T. sericea* extract against Vero cells.

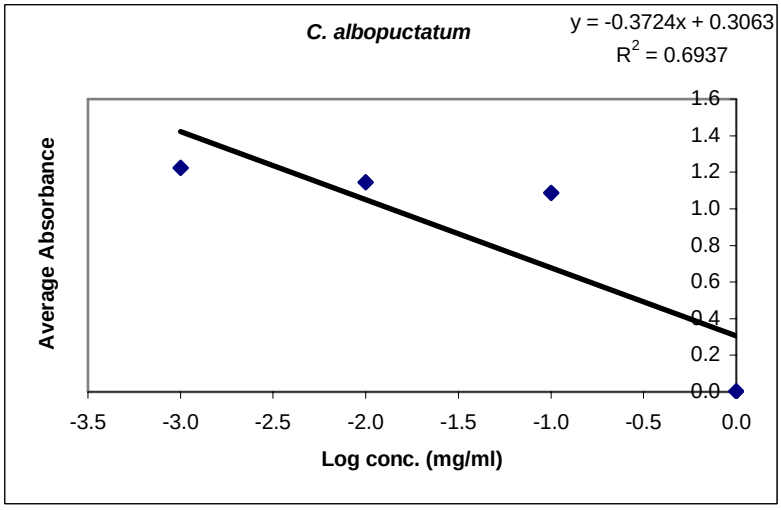


Figure 9.11. MTT cytotoxicity activity of *C. albopunctactum* extract against Vero cells.

LC_{50} untreated was calculated by substituting for y by half the value of absorbance at 540 nm for the control (0.645). The LC_{50} was a relatively non-toxic value of 168.6, 75.7, 102.9 and 121.7 µg/ml for *C. imberbe*, *C. nelsonii*, *T. sericea* and *C. albopunctactum* respectively compared to 4.347 µg/ml of the berberine chloride standard.

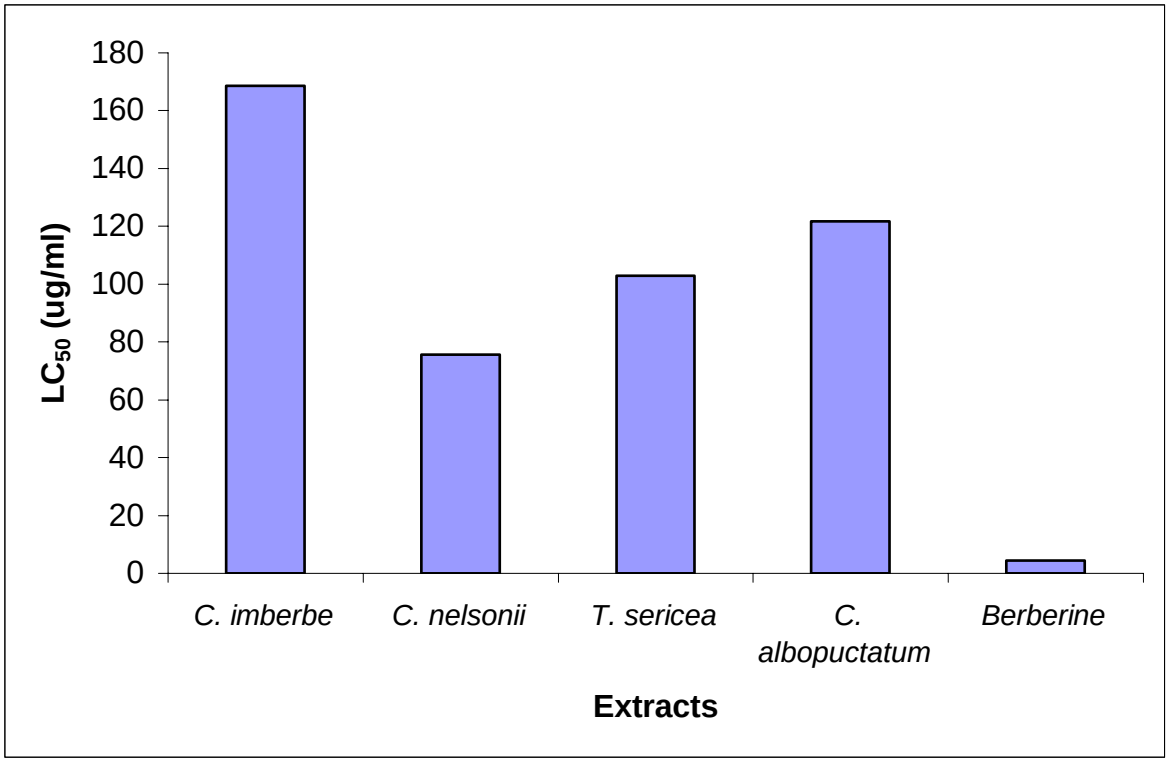


Figure 9.12. LC_{50} of the tested extracts

The relative safety margin indicates the number of times the effective concentration is lower than the LC_{50} concentration of the optimal extract and is calculated using the LC_{50} and MIC values.

Table 9.2. Relative safety margin (using LC_{50} value from the brine shrimp assay and the MTT cytotoxicity assay) of the optimal extract.

Microorganisms	**MIC (mg/ml)**				**LC_{50}/MIC**							
					Brine Shrimp assay				**MTT assay**			
	C. imberbe	*C. nelsonii*	*T. sericea*	*C. albopuntactum*	*C. imberbe*	*C. nelsonii*	*T. sericea*	*C. albopuntactum*	*C. imberbe*	*C. nelsonii*	*T. sericea*	*C. albopuntactum*
C. albicans	2.5	0.04	0.64	0.64	1.26	57.50	4.77	3.80	0.09	1.89	0.16	0.19
C. neoformans	0.16	0.04	0.08	0.08	19.75	57.50	38.13	38.38	1.05	1.89	1.29	1.52
A. fumigatus	2.5	0.16	0.16	0.08	1.26	14.38	19.06	30.38	0.07	0.47	0.64	1.52
S. schenckii	2.5	0.02	0.02	0.08	1.26	115.0	152.5	30.38	0.07	3.79	5.15	1.52
M. canis	0.04	0.02	0.02	0.02	79.00	115.0	152.5	121.5	4.22	3.79	5.15	6.09
Average	**1.54**	**0.06**	**0.18**	**0.18**	**20.5**	**71.9**	**73.4**	**44.9**	**1.1**	**2.37**	**2.48**	**2.17**

9.4. Discussion

The *in vitro* cytotoxicity of three *Combretum* and one *Terminalia* species extract were investigated. These four extracts were chosen because of their good *in vitro* antifungal activity and we considered of using them in *in vivo* studies in animal models. The toxicity of the extracts to *Artemia salina* nauplii and monkey Vero cells were evaluated, because herbal medicines are perceived as safe, yet there is little knowledge on the potential toxicity of these indigenous plants. Responses varied for the different extracts and between the two assays, but brine shrimps responded less sensitively than the monkey Vero cells. Only acetone extracts were used, because it was found not to be toxic to fungi. In this study it was used as control and it was found not to have effect on *A. salina* nauplii and Vero cells at the concentrations used.

The results on brine shrimps indicated that the four leaf extracts have LC_{50} values above 20-30 µg/ml, the recommended cut-off point for detecting cytotoxic activity (Geran *et al*., 1972). Podophyllotoxin toxin standard had LC_{50} of 7 µg /ml, which is well within the cut-off value.

A crucial point in discussing the relevance of LC_{50} values obtained in the brine shrimp assay is the question of whether the mortality data can be tied to a more specific activity. A general correlation of brine shrimp toxicity with special types of bioactivity seems invalid. However, in various cases it has been shown to be possible. Fang *et al*. (1991) investigated the usefulness of the brine shrimp assay as an antitumour pre-screen for plant extracts and was able to determine a positive correlation between brine shrimp lethality and cytotoxicity towards 9KB cells (cell line derived from the human carcinoma of the nasopharynx used as an assay for antineoplastic agents), while Solis *et al*., (1993) found the brine shrimp assay was not predictive for compounds requiring metabolic activation, since the brine shrimp lack the necessary cytochrome P-450 enzyme. No published work on whether brine shrimp can be used to detect specific activity of antimicrobial agents was found.

The MTT assay was done using one cell line (monkey Vero cells). It is known that different cell lines might exhibit different sensitives towards a cytotoxic compound or extracts. The use of more than one cell line is therefore considered necessary in the detection of cytotoxic compounds or extracts. Kamuhabwa *et al* (2000), used three human cells (HeLa, HT29 and A431) of different histological origin in their study and slight differences were observed.

However, in certain cell types the situation seems to be more complex than that. In a study with a cell line derived from rat brain tumours it was reported that the mitochondria are not the exclusive site of MTT reduction (Liu *et al*., 1997). It was observed that various sub-cellular fractions could reduce MTT when supplied with NADH or NADPH and the intracellular MTT formazan granules did not accumulate in mitochondria, endoplasmic reticulum, or Golgi apparatus, but partially co-localize with endosomes or lysosomes. Furthermore, based on inhibition experiments it was concluded that the investigated cellular MTT-reductase is an N-ethylmaleimide sensitive flavin oxidase. Although these studies made the exclusive role of mitochondria in MTT reduction questionable, they did not question the validity and usefulness of the MTT assay because even if the MTT assay measures endocytosis, it would be based on a fundamental feature of living cells (Liu *et al*., 1997).

The LC_{50} value for berberine chloride was 4.347 µg/ml, which is toxic. The normal LC_{50} of berberine is 0.141-0.148 µg/ml (Vennestrom and Klayman, 1988). *C. imberbe* had the highest LC_{50} of 168.6 µg/ml and *C. nelsonii* had the lowest which was 75.7 µg/ml. *C. albopunctactum*, *T. sericea* and *C. nelsonii* were 1.38, 1.64, and 2.23 less active than *C. imberbe*. The choice of cell number initially plated into the 96 well plate was determined, such that the control cells undergo

8-9 divisions during the incubation period before reaching 80 –90 % confluency. The number of cells in the well was ruled out as one of the causes of high values based on the above reason. Again number of cells were sufficient to enable detection of cell death and growth inhibition effects. If larger cell numbers and shorter assay times are used the cultures rapidly become confluent and cells destined to die as a result of the toxic effect of the test extract may still be metabolically active at the point where cell number is estimated. These can result in overestimation of survival and an underestimation of the toxic potential of the extracts.

The MTT assay is a well-established method used to assess mitochondrial competence (Freshney, 2000). Using this assay we found that the four extracts did not suppress mitochondrial respiration in monkey kidney cells. Only *C. imberbe* was closer to the cut-off value (200 µg/ml), which was used by other authors. However according to the criteria of the American National Cancer Institute, the LC_{50} limit to consider a crude extract promising for further purification to isolate biological active (toxic) compounds is lower than 30 µg/ml (Suffness and Pezzuto, 1990).

MTT assay formation of the formazan product correlated well with the number of surviving cells, although not always in a strictly linear fashion. The assessment of results was carefully interpreted. If surviving fraction is calculated directly from the ratio of absorbances, an estimation and not an absolute value, of the cell numbers will occur. Calibration curves of each extract were constructed, but at some point some of the values were far from the curves, maybe that is one of the reason the values were high. The MTT assay has several drawbacks: it is not readily adaptable for use with static cell populations or those of low mitochondrial activity. Certain compounds may selectively affect the mitochondria of the cells resulting in a greatly overestimated/ underestimated level of toxicity. Different cell lines are likely to give different absorbance levels when at similar degrees of confluence. Lastly MTT is mutagenic and, therefore must be handled with care.

The relative safety margin was calculated because these extracts were to be used in *in vivo* in rats. The relative safety margin indicates the number of times the effective concentration is lower than the LC_{50} concentration of the optimal extract and is calculated using the LC_{50} and MIC values. The extracts were relatively non-toxic, which means that the relative safety margin (LC_{50}/MIC) of the optimal extract was large (Table 9.2). This allows for large quantities of the optimal extract to be incorporated in treatment without causing toxic reactions. This will be discussed in detail in the following chapters.

The relative safety margins (RSM) in the MTT assay were high compared to those in the brine shrimp assay. RSM of *C. imberbe*, *T. sericea* and *C. albopunctactum* in *C. albicans* were 0.09, 0.16 and 0.19 respectively. *C. nelsonii* and *T. sericea* had high RSM values against *S. schenckii* and *M. canis*, therefore high amount of the material must be used in treatment. The *C. imberbe* acetone extract had the lowest RSM values, which means a lower amount can be used in the treatment of the test pathogen. The results of the brine shrimp assay correspond with those of the MTT assay with very low RSM values. But this was expected because the same MIC values were used.

Are these “toxic” concentrations relevant in traditional use? When most of these plants are used in traditional medicine, an infusion of about 50 g (estimated) leaves are soaked in 1 L of water for 24 h and taken orally, three times a day which is way below the detected toxicity. Even in the absence of information on the pharmacokinetics of the extracts, it is evident that the concentrations at which we observed either inhibition of mitochondrial respiration or loss of the cell membrane integrity are never relevant in traditional use. It is, however, important to know them as more concentrated forms may well be formulated as medicines even herbal medicines

9.5. Conclusion

We found that extracts of four plants used in traditional medicine are not toxic at therapeutic levels. Responses varied for the different extracts and bioassays but the brine shrimp assay generally responded less sensitively to the impact of the cyanotoxins than the monkey Vero cells. Therefore, further investigations are now needed to establish the exact mechanism of action and identify the bio-ingredients of each extract in order to explain the therapeutic efficacy, and this will be covered in the next chapters. Further extensive biological evaluations will also be carried out.

Chapter 10

Biological activity of isolated compound

10.1. Introduction

After structure elucidation of isolated active compounds, it is important to confirm that the compound isolated is the one targeted in the crude extract and not a minor compound. It is also important to determine the biological activity. Frequently known compounds have biological activities that have not previously been determined. It is also important to determine the toxicity of the isolated compounds.

Biological activities will be presented in the paper format, title: **Biological activity of two related triterpenes isolated from Combretum nelsonii (Combretaceae) leaves**. (Prepared for Journal of Ethnopharmacology)

CHAPTER 11

In vivo antifungal activity of *Combretum* and *Terminalia* extracts and isolated compounds in rats

11.1. Introduction

Wound healing consists of an orderly progression of events that establishes the integrity of the damaged tissue. The process of wound healing is essential to prevent the invasion of damaged tissue by pathogens and to partially or completely reform the damaged tissue (Sumitra *et al*., 2005). The process of wound healing is promoted by several plant products (Suguma *et al*., 1999), containing active principles like triterpenes, alkaloids, flavonoids (Sharma *et al*., 1990) and biomolecules (Chithra *et al*., 1995). These agents usually influence one or more phases of healing processes. Wound healing properties of two tropical plants *Centella asiatica* (Suguma *et al*., 1996) and *Terminalia chebula* (Suguma *et al*., 2002) have been demonstrated on dermal wound healing in rats.

In earlier studies we found *Combretum* and *Terminalia* extracts had remarkable antifungal activity against *Candida albicans, Cryptococcus neoformans, Microsporum canis, Sporothrix schenckii* and *Aspergillus fumigatus* having minimum inhibitory concentration (MIC) as low as 0.02 and 0.04 mg/ml (Masoko *et al*., 2005 and 2006). The next stage is to evaluate the *in vivo* topical antifungal activity of some of these plant extracts against fungal infections in an animal model. Cytotoxicity using cell lines and the brine shrimp mortality assay were determined (**Chapter 9**) and extracts were not toxic based on these assays. We assume that the extracts applied topically will not have systemic activity, but this has to be confirmed.

A member of the Phytomedicine Programme (Kruger, 2004) had developed a method to test crude extracts and an isolated compound on rats infected with *Staphylococcus aureus*. The animal experiment was carried out at the Onderstepoort Veterinary Institute (OVI) of the Agricultural Research Centre (ARC) in 2002. This work led to a patent and licensed product. Several improvements based on his method were used in this fungal infection study.

11.1.1. Aim

Previous experiments on extracts *of Combretum nelsonii, Combretum imberbe, Combretum albopunctactum* and *Terminalia sericea* (Masoko *et al*., 2005 and Masoko *et al*., 2006), indicated

excellent *in vitro* activity against *C. albicans, C. neoformans, M. canis, S. schenckii* and *A. fumigatus.* The next stage in the potential use of leaf extracts or isolated compounds from these species was to determine *in vivo* activity of *Combretum* and *Terminalia* extracts. A non-infected and fungal infected skin wound model in rats had to be developed to test irritation and effectivity. The study was divided into two pilot studies and main study, each with its aims as follows:

Pilot study I (Local irritancy and wound healing study)

Aim: To establish whether an aqueous cream used as vehicle has any irritant effect.
To determine irritant/ tolerance effect of 10% and 20% crude extracts in cream based on rats.
To determine the degree of wound healing within three weeks.

Pilot study II (Infection with different pathogens)

Aim: To determine the effects of plant extracts in aqueous creams on irritation and the infection and wound healing.
To determine the rate and extent of infection from different pathogens.
To investigate possibility of systemic infection.

Main study (Confirmation study)

Aim: To determine if suppressing the immune system of the rats would make them more susceptible to fungal infection.
To determine healing activity of the extracts and isolated compound under these condition.
To determine antifungal activity of extracts on infected wounds.

11.1.2. Objective

To investigate the effects of *Combretum nelsonii*, *Combretum imberbe*, *Combretum albopunctactum* and *Terminalia sericea* extracts applied topically on skin wounds in control and rat skin wounds infected with fungal pathogens. Wound irritancy and wound healing will be evaluated by physical and histological methods. Aspects evaluated will include wound healing, erythema, exudate formation, crust formation, possible toxic effects of the extracts and histopathology.

11.2. Materials and methods

The research was approved by the Research and Animal Use and Care Committee of the University of Pretoria (VI 010/05 approval number).

11.2.1. Selection of rats

Healthy male Wistar rats weighing 150-200 g were used. The test was conducted using a single gender as a way of reducing variability and to minimize the numbers required (OECD, 2000). At the commencement of the study, each rat was 8 – 12 weeks old and the weight variation of animals used did not exceed ± 20% of the mean weight of all previously dosed animals (National Institute of Environmental Health Sciences, 2001).

11.2.2. Housing and feeding conditions

Rats were kept at the University of Pretoria Biomedical Research Centre at Onderstepoort and housed in separate cages at a temperature of 22 ^{o}C (± 2 ^{o}C) and relative humidity (50% - 60%) in a light/dark cycle of 12 hours. The rats were fed conventional rodent diets with an unlimited supply of drinking water (National Institute of Environmental Health Sciences, 2001). Environmental enrichment e.g. bedding (wood wool), were provided to keep rats busy. Previous work suggests that the provision of enrichment items, which give laboratory rats the opportunity to perform exploratory and gnawing activities, is an effective way to improve their welfare and to distract them from tampering with dressings (Zhu *et al*., 2006).

11.2.3. Preparation of animals

Cages of the rats were labeled with numbers to facilitate identification. Rats were kept in their cages for at least 5 days prior to treatment to allow for acclimatization to the laboratory conditions (Spielmann *et al*., 1999). They were also handled daily in this period. The rats used in pilot studies were not immunosuppressed but the 24 used for the experiment were immunosuppressed 4 days before challenge by subcutaneous injection of 500 µg of estradiol valerate. Estradiol pretreatment is known to inhibit innate and acquired immune defenses (Carlsten *et al*., 1991).

11.2.4. Wound creation

The hair on the back area was removed by cutting it with electrical clippers. The area was disinfected using 0.5% chlorhexidine in 70% alcohol and allowed to dry. Rats were anaesthesized with isoflurane (0.01- 0.05 µg/kg). Six evenly spaced circular wounds were made on each rat using 6 mm diameter punch biopsies (Simosen *et al*., 2002). The whole process was carried out in a biosafety class II cabinet to limit infection and prevent infection of people.

11.2.5. Induced fungal infections

The fungi (*Candida albicans, Cryptococcus neoformans, Microsporum canis* and *Sporothrix schenckii*) were grown for 5-7 days on Sabouraud agar slant at 30°C. Thereafter the fungal material was scraped aseptically from slants, and pooled in 30 ml of sterile water and briefly homogenized. Volumes of 100 µl of the fungal suspension was introduced onto the test area. The area was covered with an occlusive wrapping (Transpore^{R}) and left to incubate for 48 hours. After 48 hours the test products were introduced and the resultant inhibition of growth or healing quantified on the basis of erythema, exudate and physical size of the lesion on a Monday, Wednesday and Friday for 3 weeks. Infection by fungi was clinically detected by the presence or absence of the swelling, erythema, pain and ulceration of the inoculation sites. Rise in body temperature, not eating for 24 hours and weight loss were also notes as clinical signs.

11.2.6. Preparation of extracts

C. nelsonii, C. imberbe, C. albopunctactum and *T. sericea* powdered leaves were extracted with acetone. Extracts were dried at room temperature under a stream of cold air and ground with mortar and pestle and then mixed with aqueous cream consisting of distilled water, white petroleum jelly, mineral oil, emulsifying wax and phenoxyethanol to a concentration of 10% (1 g/10 g cream) and 20% (2 g/ 10 g) and kept at 4 ^{o}C until use.

11.3. Pilot studies

11.3.1. Exploratory studies

Procedure: In Pilot study 1 the treatments were applied as shown in **Table 11.1** on sites of the rats.

Table 11.1. Treatment in topical to study skin tolerance.

Sites on the rat	Treatment
A	No treatment
B	Cream only
C	10% *C. nelsonii* crude extract
D	20% *C. nelsonii* crude extract

These sites were randomly allocated when the treatment was repeated. Treatment shown above was repeated using *C. imberbe*, *C. albopunctactum* and *T. sericea* extracts. Two rats were used for each plant extract *i.e*. eight rats were used. Rats were weighed and new creams and creams with crude extracts were applied, every Monday, Wednesday and Friday. The lesions were also measured. All rats were observed daily for any indication of interference with the wound dressing. Severe irritation and enlargement of the wound lead to the termination of that specific treatment. If there are no signs of irritation the experiment was terminated after three weeks when wounds had completely healed. Treatment with best effect was used in subsequent experiments if no adverse effects are found. The lesions were evaluated, temperature and weight measured.

11.3.2. Infection with different pathogens

Procedure: In Pilot study 2 different infections were treated as follows on **Table 11.2**. The study was double blinded

Table 11.2. Treatment of different rats in efficacy experiment.

Site on the rat	Cream	*C. albicans*	Extracts	Amphotericin B
A	-	-	-	-
B	√	√	-	-
C	√	√	-	√
D	√	√	I	-
E	√	√	N	-
F	√	√	P	-
G	√	√	T	-

T= *T. sericea* crude extract, N= *C. nelsonii* crude extract, I= *C. imberbe* crude extract, P= *C. albopunctactum* crude extract, concentration with best effect of crude dried acetone extract in cream, √ = Added, - = Not added.

Wounds were created in the same fashion as pilot study I. Twenty percent of the extracts was chosen from first pilot. Once the wounds were created on each rat, 6 of the 7 wounds were infected with 0.1 ml of the fungal suspension. Three rats were allocated to each fungus, namely *C. albicans, C. neoformans, M. canis* and *S. schenckii*. Treatments as laid down in **Table 11.2** were initiated after 48 hours. The rats were inspected three times a week for any signs of systemic infection by determining mass, food intake, and temperature by means of a microchip.

11.3.2.1. Parameters of infection/recovery

The erythema and exudate were evaluated three times a week using the score provided in **table 11.3.**

Table 11.3. Evaluation of erythema and exudate

Score	Erythema	Exudate
0	No red colour at all	No exudate
1	Light red just visible	Exudate just visible
2	Clearly red	Easily visible
3	Dark red, not whole area	Substantial quantity
4	Dark red wide spread	Large quantity

11.3.3. Confirmation study (Treatment experiment)

Once all the necessary precautions were taken, the experiment was carried out with treatments shown in **Table 11.4**. Pilot study 2 was repeated with the following amendments:

Rats were immunocompromised.

Isolated compound was added as part of treatment.

Rats were infected by pads soaked in cultures

Table 11.4. Treatment of different rats in efficacy experiment.

Site on the rat	Cream	*C. albicans*	Extracts	Isolated compound	Amphotericin B	No. of rats
A	√	√	-	-	-	01 – 06
B	√	√	-	-	√	01 – 06
C	√	√	I	-	-	01 – 06
D	√	√	N	-	-	01 – 06

E	√	√	P	-	-	01 – 06
F	√	√	T	-	-	01 – 06
G	√	√	-	√	-	01 – 06
Site on the rat	**Cream**	***C. neoformans***	**Extracts**	**Isolated compound**	**Amphotericin B**	**No. of rats**
A	√	√	-	-	-	07 – 12
B	√	√	-	-	√	07 – 12
C	√	√	I	-	-	07 – 12
D	√	√	N	-	-	07 – 12
E	√	√	P	-	-	07 -12
F	√	√	T	-	-	07 – 12
G	√	√	-	√	-	07 – 12
Site on the rat	**Cream**	***M. canis***	**Extracts**	**Isolated compound**	**Amphotericin B**	**No. of rats**
A	√	√	-	-	-	13 – 18
B	√	√	-	-	√	13 - 18
C	√	√	I	-	-	13 – 18
D	√	√	N	-	-	13 – 18
E	√	√	P	-	-	13 – 18
F	√	√	T	-	-	13 – 18
G	√	√	-	√	-	13 – 18
Site on the rat	**Cream**	***S. schenckii***	**Extracts**	**Isolated compound**	**Amphotericin B**	**No. of rats**
A	√	√	-	-	-	19-24
B	√	√	-	-	√	19-24
C	√	√	I	-	-	19-24
D	√	√	N	-	-	19-24
E	√	√	P	-	-	19-24
F	√	√	T	-	-	19-24
G	√	√	-	√	-	19-24

T= *T. sericea* crude extract, N= *C. nelsonii* crude extract, I= *C. imberbe* crude extract, P= *C. albopunctactum* crude extract in all cases 20 % of crude dried acetone extract in cream, √ = Added, - = Not added.

11.3.3.1. Treatment of different sites on individual rats

Aqueous cream consisting of distilled water, white petroleum jelly, mineral oil, emulsifying wax and phenoxyethanol was used to prepare different mixtures.

Site A: Treated with aqueous cream only (negative control),
Site B: Treated with antibiotics e.g. amphotericin B mixed with aqueous cream (positive control),
Site C: Treated with *C. imberbe* extracts mixed with aqueous cream
Site D: Treated with *C. nelsonii* extracts mixed with aqueous cream
Site E: Treated with *C. albopunctactum* extracts mixed with aqueous cream
Site F: Treated with *T. sericea* extracts mixed with aqueous cream
Site G: Treated with isolated compound mixed with aqueous cream

Wounds were medicated with a local application (enough to cover the wound) of each cream. This study was randomised, and blinded by mixing cream with different compounds/extracts, as well as changing the sites on the rats. The person applying the treatments did not know which treatment was being used so that bias in evaluation was removed.

11.3.3.2. Administration of doses

The test material was formulated into topical creams by blending in a mortar and pestle. A 1% concentration in aqueous cream of the isolated compounds were used while a 20% of the crude extract (Kruger and Eloff, 2004), also in emulsifying cream was used. The positive control was 0.1% amphotericin B. The wound was cleaned every 48 hours with clean cotton-wool and the creams were applied to the wounds for 3 weeks, or until the positive control (wound treated with amphotericin B) has 100 % healed or until the untreated controls have healed, whatever was the latest.

11.3.3.3. Observations

Each animal served as its own control with five test sites for the crude and isolated compound, one as a positive control with Amphotericin B and one site as a negative control. The presence of factors such as erythema, exudate, swelling, ulceration, crust formation, healing and infection were checked. The measuring of the size of the lesion relative to that of the negative and positive controls or the complete healing of the lesion served as the means of measurement of the antimicrobial activity. Every Monday, Wednesday and Friday at the same time, each rat was taken out of the cage, given anaesthetic, the dressing removed and the different parameters were measured. Thereafter the test samples and control treatment were applied and new dressings were applied to each test rat and it was placed in its cage.

11.3.3.4. Daily observations on weekdays

Observations were systematically recorded with individual records kept for each rat. Rats were observed individually at least once during the first 30 minutes after dosing and periodically during the first 24 hours (with special attention given during the first four hours). The rats were observed daily for up to 3 weeks. The time at which any abnormalities were observed as well as when they disappear were noted. Observations included changes in skin and fur, diarrhoea, lethargy, unusual sleepiness, weight loss and coma. After the completion of the experiment rats were euthanased by CO_2 inhalation and a necropsy was done. The liver, heart, lungs, intestine, lymph nodes and kidney of the rats were checked by the pathologist for gross abnormalities.

11.4. Evaluation of lesions

The mass of each rat, body temperature and lesion characteristics were recorded three times a week. The study was blinded (person doing the evaluation did not know the treatment).

11.4.1. Lesion size measured

The lesion sizes of each rat were measured with the same callipers three times a week using the horizontal and vertical diameters.

11.4.2. Recording of data

The data for each rat was recorded on a single form for the three weeks period. The recording sheets and results are shown on Tables 11.5 and 11.6 (appendix)

11.5. Pathological and histopathological studies

Histopathological studies were done with the help of a pathologist (**Dr Joshua Dabwroski**) at the end of the experiment. Wound tissue specimens from treated and non-treated rats were collected in 10% buffered formalin and after processing 6 µm thick sections were cut and stained with haematoxylin and eosin (McManus and Mowry, 1965). Sections were qualitatively assessed under the light microscope and graded in respect of congestion, oedema, infiltration of polymornuclear leukocytes (PMNLs) and monocytes, necrosis, fibroblast proliferation, collagen formation, angiogenesis and epithelisation (Shukla *et al*., 1999). Necropsies were performed and the presence of fungi were determined using the PAS stain.

Schematic presentation of the methods is presented from slide 1 to slide 31 (Figure 11.1 to 11.3)

11.6. RESULTS

11.6.1. Pilot 1 (Local irritancy and wound healing study)

Results for first Pilot study are presented in the paper: **Evaluation of the wound healing activity of selected *Combretum* and *Terminalia* species (Combretaceae)** (*Submitted to Onderstepoort Journal of Veterinary Research)*

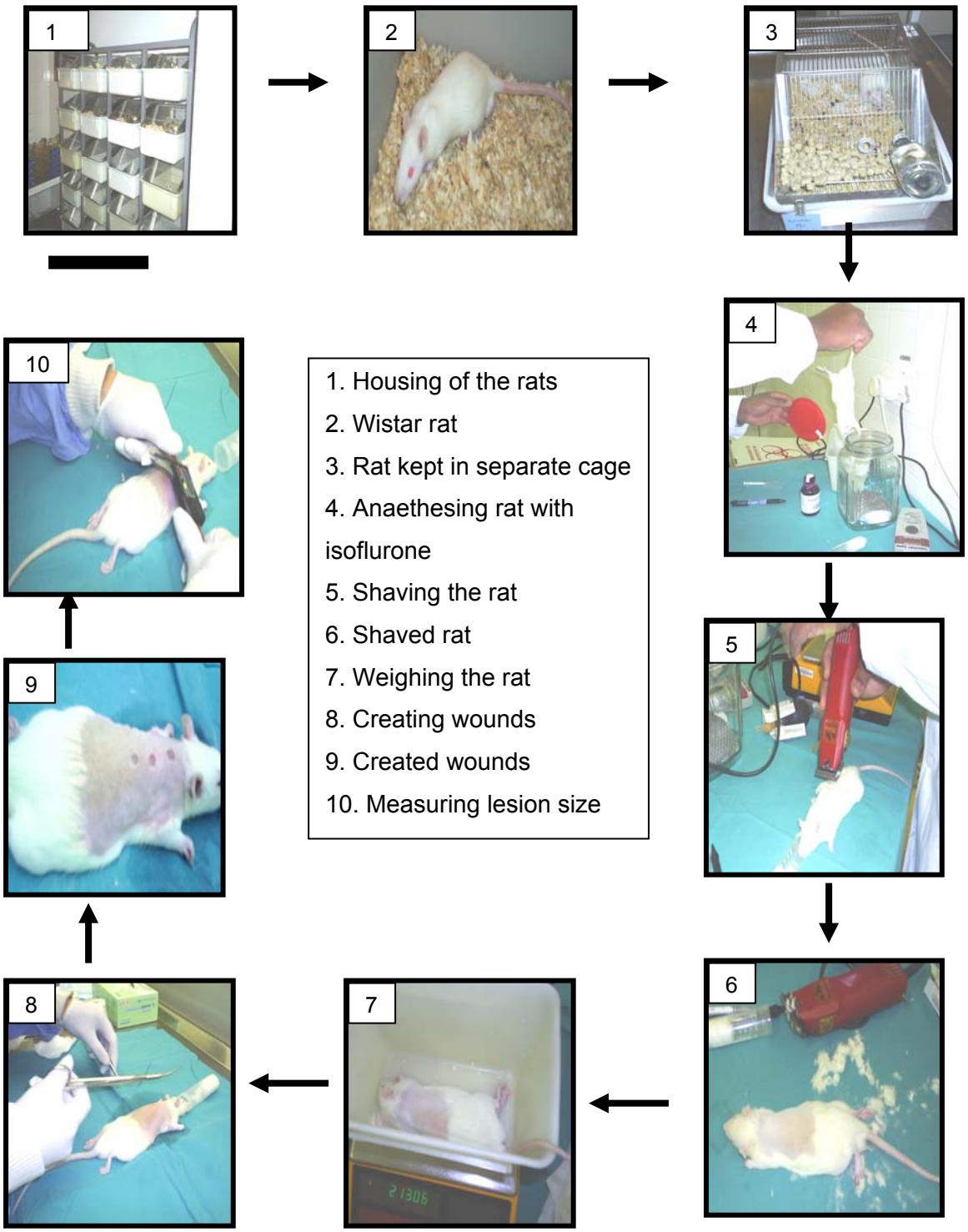


Figure 11.1. Wounds creation

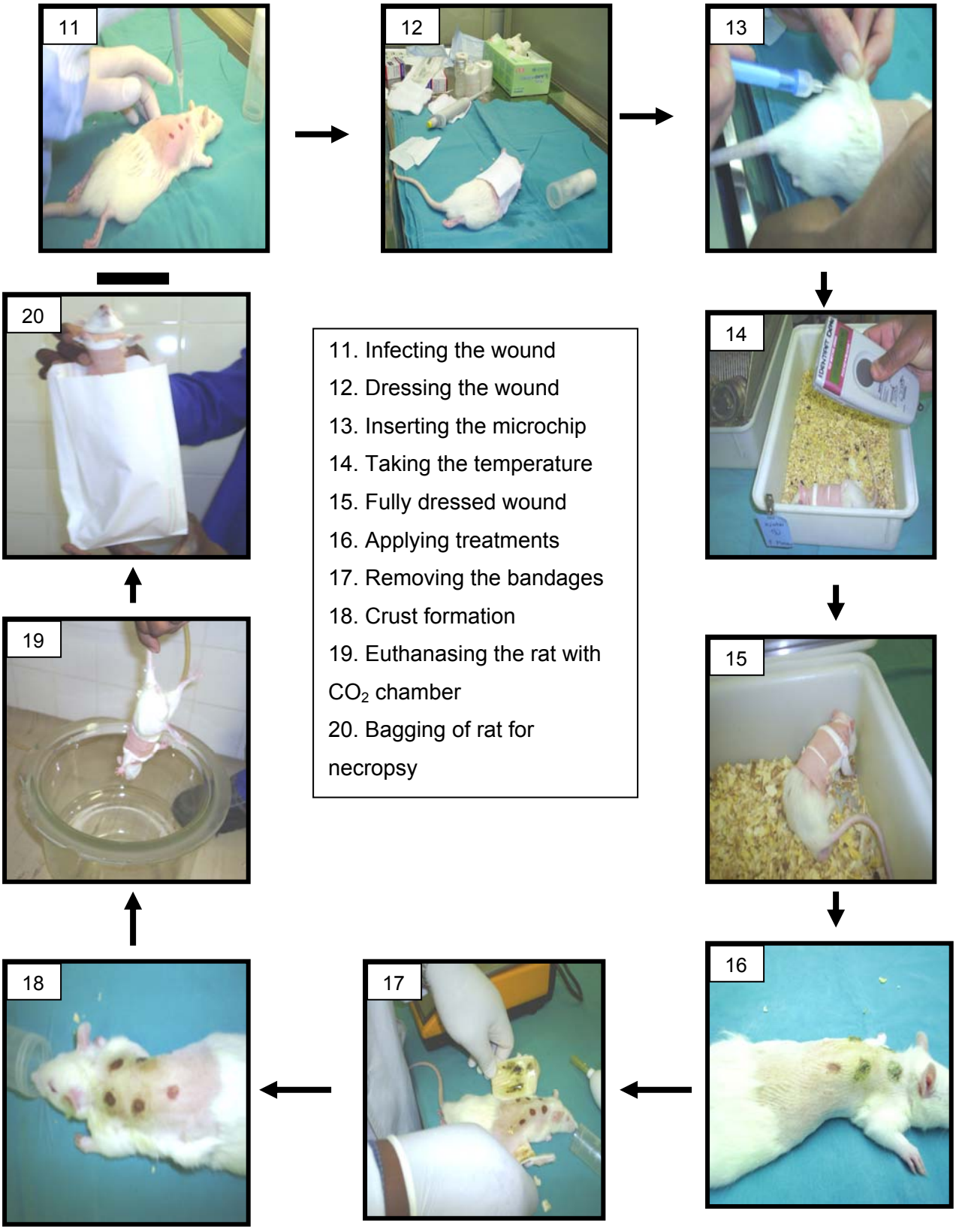


Figure 11.2. Wound treating and dressing.

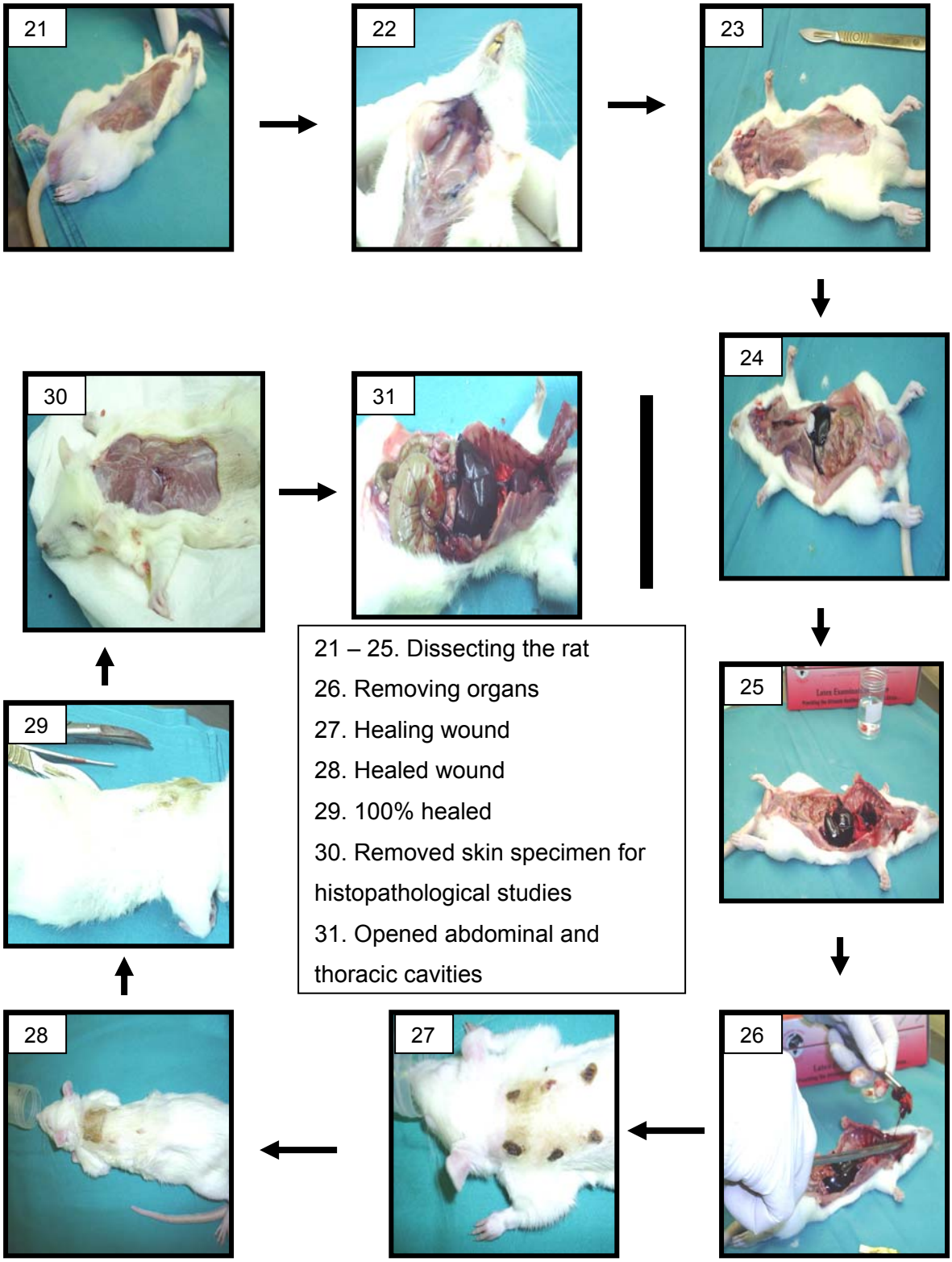


Figure 11.3. Wound healing and necropsy.

11.6.2 Pilot study 2 (Infection with different fungal pathogens)

The aim of pilot study II was to determine the effects of plant extracts in aqueous creams on the infection and wound healing and to determine the rate and extent of infection from different pathogens. Results were recorded in **Table 11.2** (**Appendix**). Rats 1 to 3 were infected with *C. albicans*, 4 to 6 with *C. neoformans*, 7 to 9 with *M. canis* and 10 to 12 with *S. schenckii*.

Although there was an initial weight loss in all the rats, all except Rat 11 gradually recovered the weight lost (**Figure 11.10a-b**). As Rat 11 was suffering from weight loss and pneumonia it was terminated and a necropsy was done on it.

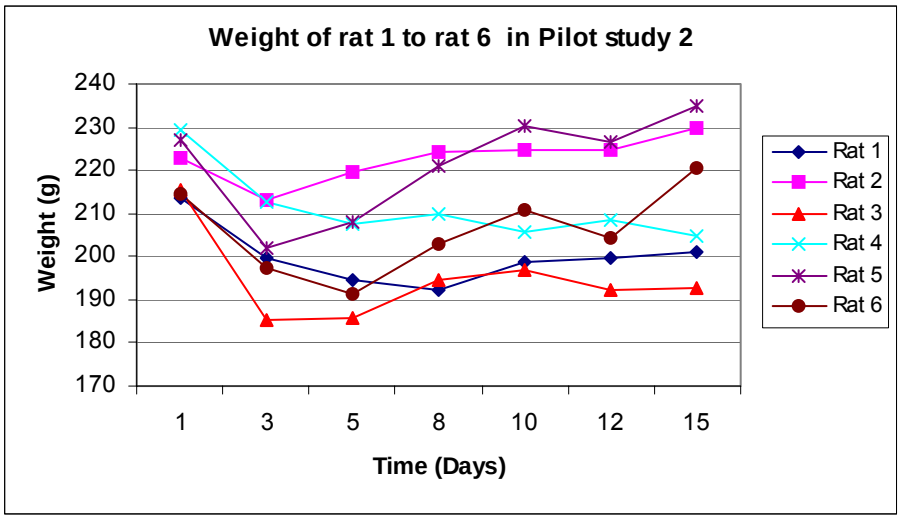


Figure 11.10a. Weights of rats 1 to 6 in pilot study 2

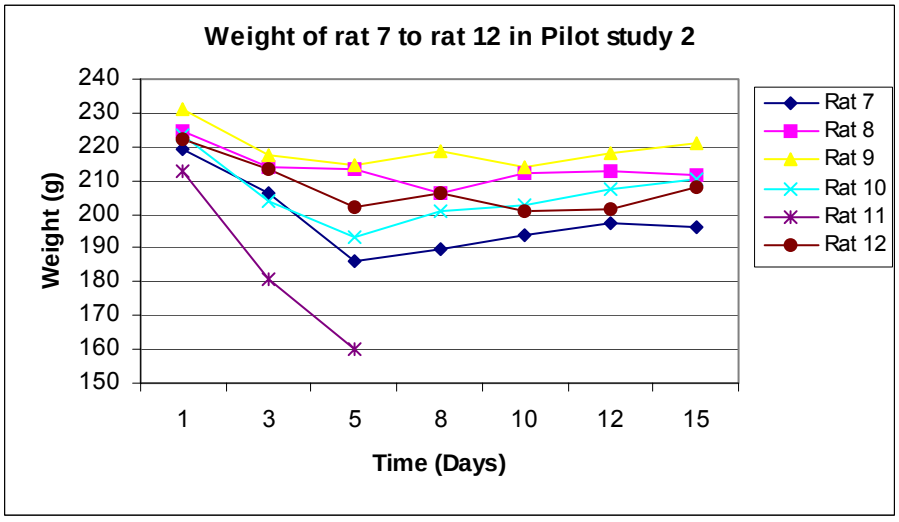


Figure 11.10b. Weights of rats 7 to 12 in pilot study 2

Temperatures of all 12 rats were within the normal parameters, even of rat 11, which was terminated. In all instances the initial temperature was low. That was because of temperatures were measured immediately after inserting the microchips and prolonged anaesthesia (**Figure 11.11a-b**).

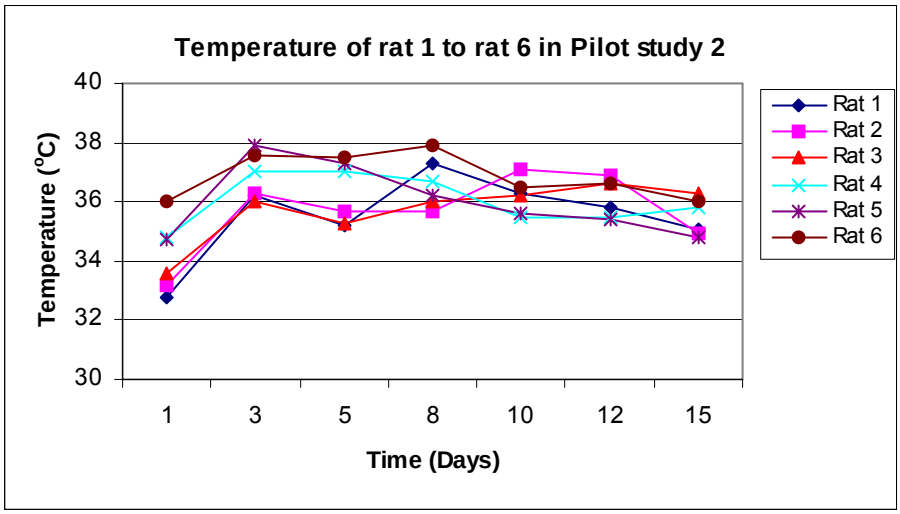


Figure 11.11a. Temperatures of rats 1 to 6 in pilot study 2

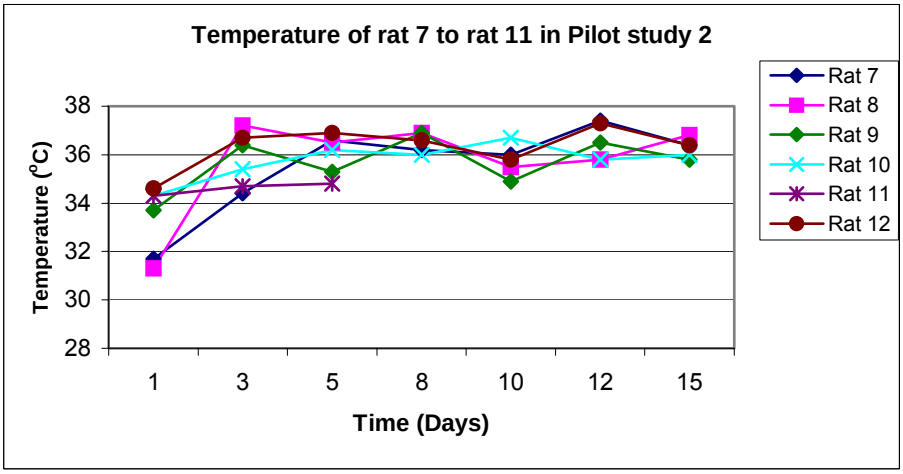


Figure 11.11b. Temperatures of rats 7 to 12 in pilot study 2

Lesion sizes were measured. The rats were group according to the fungal pathogens they were infected with, *C. albicans* (**Figure 11.12a**), *C. neoformans* (**Figure 11.12b**), *M. canis* (**Figure 11.12c)** and *S. schenckii* (**Figure 11.12d**). The lesion sizes were calculated the same way as in pilot study I. Amphotericin B was used as the positive control and the negative control was the untreated lesion but infected. In rats infected with *C. albicans* amphotericin B had the best activity

and *C. albopunctactum* had worst activity, but after Day 8 it was within the same activity as other treatments.

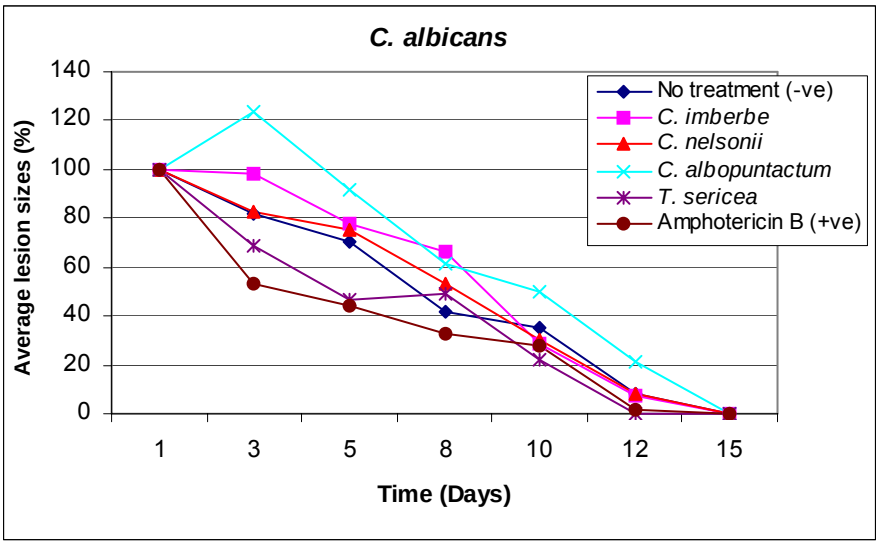


Figure 11.12a. The average lesion size of lesions infected with *C. albicans* and treated with four extracts and Amphotericin B (positive control).

Lesion sizes in rats infected with *C. neoformans* followed the same format of healing, although *T. sericea* had better activity and C. imberbe had the least activity. After Day 10 wounds without treatment were the ones with higher lesion sizes (**Figure 11.12b**).

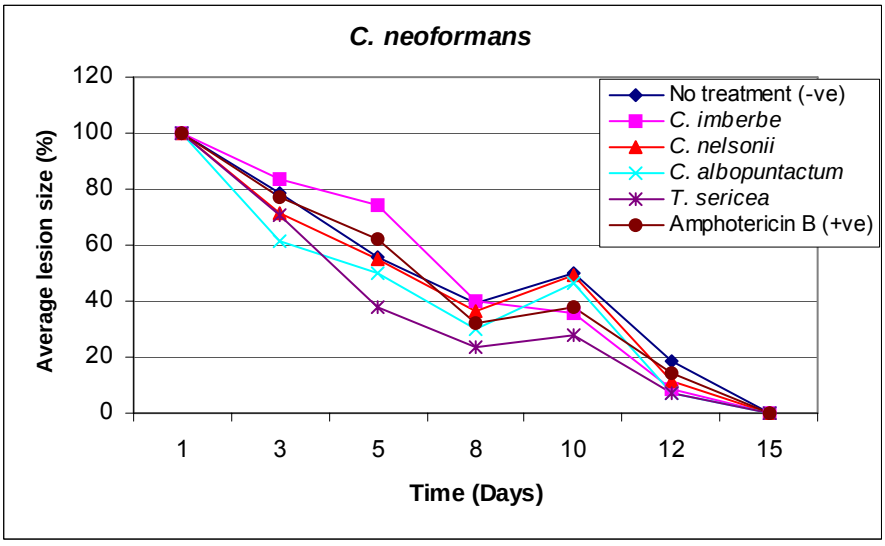


Figure 11.12b. The average lesion size of lesions infected with *C. neoformans* and treated with four extracts and Amphotericin B (positive control).

Lesion sizes in rats infected with *M. canis* (**Figure 11.12c**), wounds treated with *C. nelsonii* increased in sizes at Day 3 and at Day 10 they were at the same healing range as others. After Day 12 they were the best as expected based on *in vitro* studies. In rats infected with *S. schenckii* (**Figure 11.12d**) wound treated with *T. sericea* took longer time to reduce size.

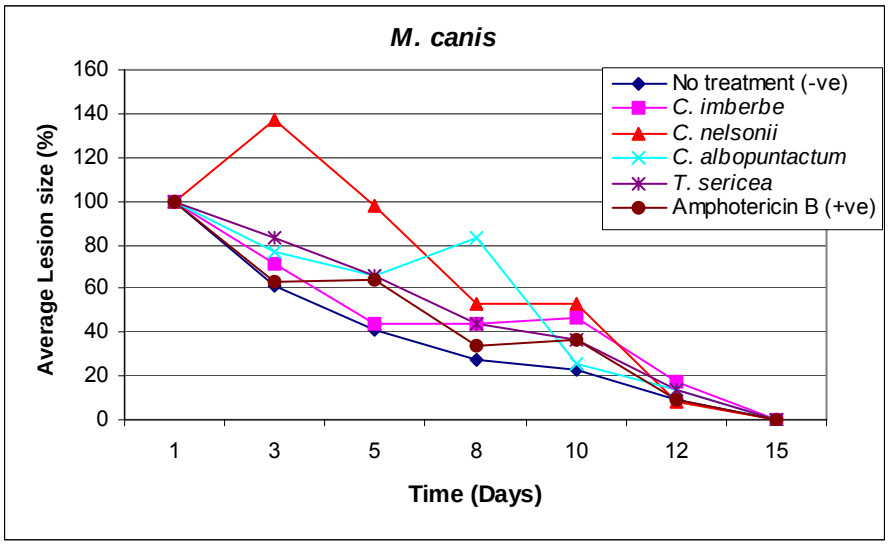


Figure 11.12c. The average lesion size of lesions infected with *M. canis* and treated with four extracts and Amphotericin B (positive control).

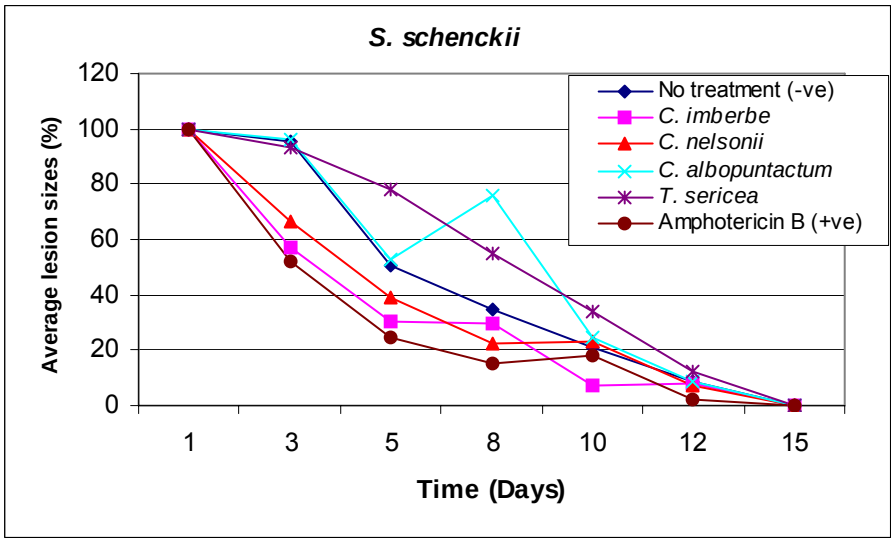


Figure 11.12d. The average lesion size of lesions infected with *S. schenckii* and treated with four extracts and Amphotericin B (positive control).

Again the resulting healing was quantified on the basis of erythema (**Figure 11.13a**), exudate (**Figure 11.14a**) and crust formation (**Figure 11.15a**). An arbitrary value was allocated, as it was difficult to measure the degree of erythema as well as the quantification of the exudate and crust formation formed. Subsequently a scale of 1 to 5 was used, 1 being the lowest and 5 being the highest formed. Averages of all 12 rats were used. Error bars were also drawn to show the confidence level of data or the deviation along a curve, erythema (**Figure 11.13b**), exudate (**Figure 11.14b**) and crust formation (**Figure 11.15b**).

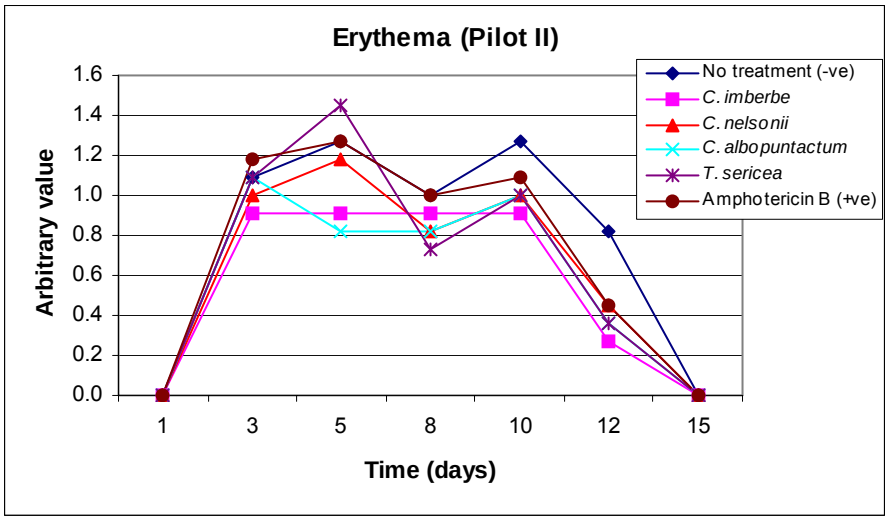


Figure 11.13a. The influence of the crude extracts and Amphotericin B (positive control) on the wound erythema of rat in pilot study 2

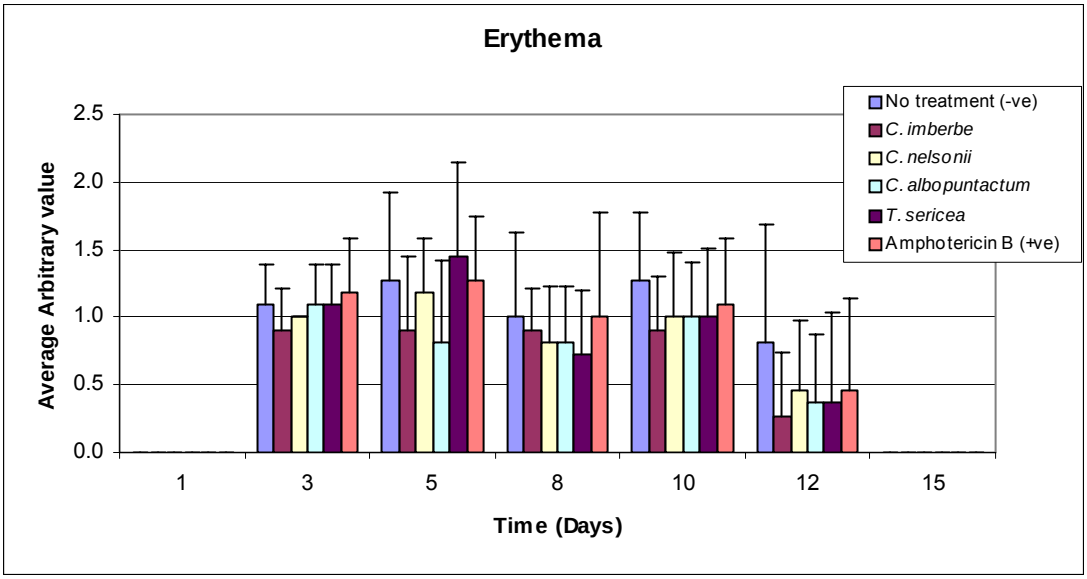


Figure 11.13b. Average arbitrary values of erythema of rats in pilot study 2 with error bars

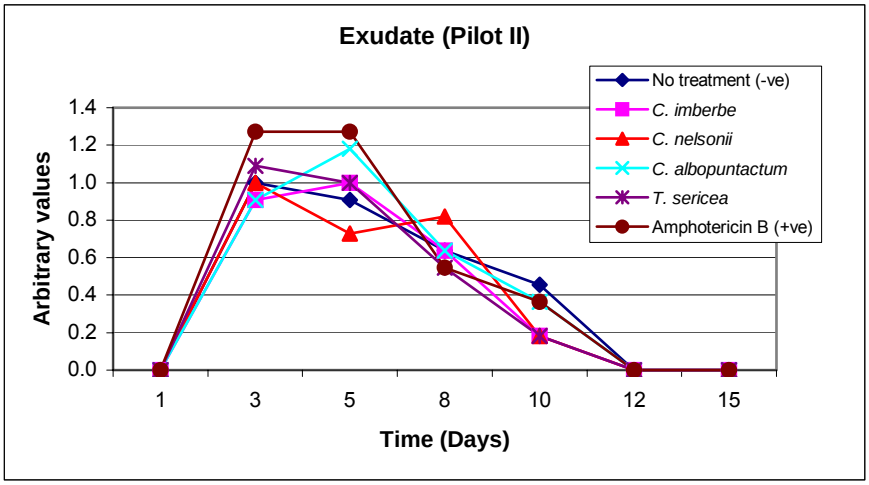


Figure 11.14a. The influence of the crude extracts and Amphotericin B (positive control) on the exudate formed of rats in pilot study 2

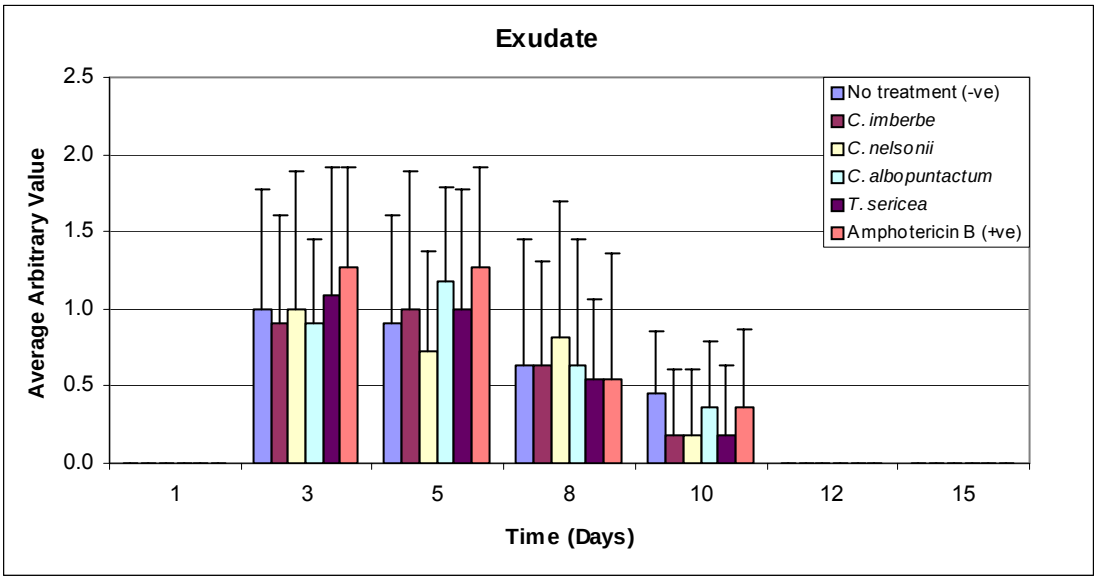


Figure 11.14b. Average arbitrary values of exudate of rats in pilot study 2 with error bars

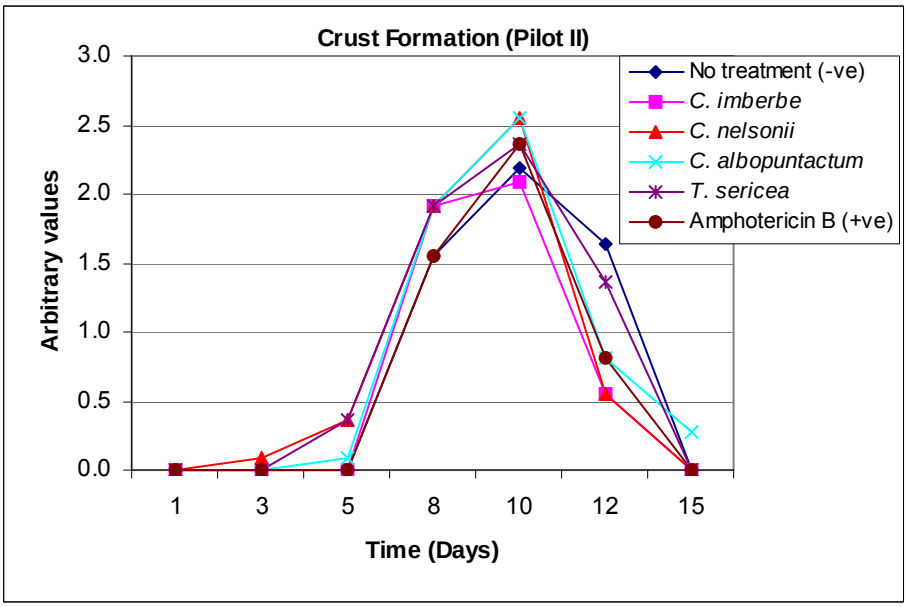


Figure 11.15a. The influence of the crude extracts and Amphotericin B (positive control) on the crust formed of rats in pilot study 2

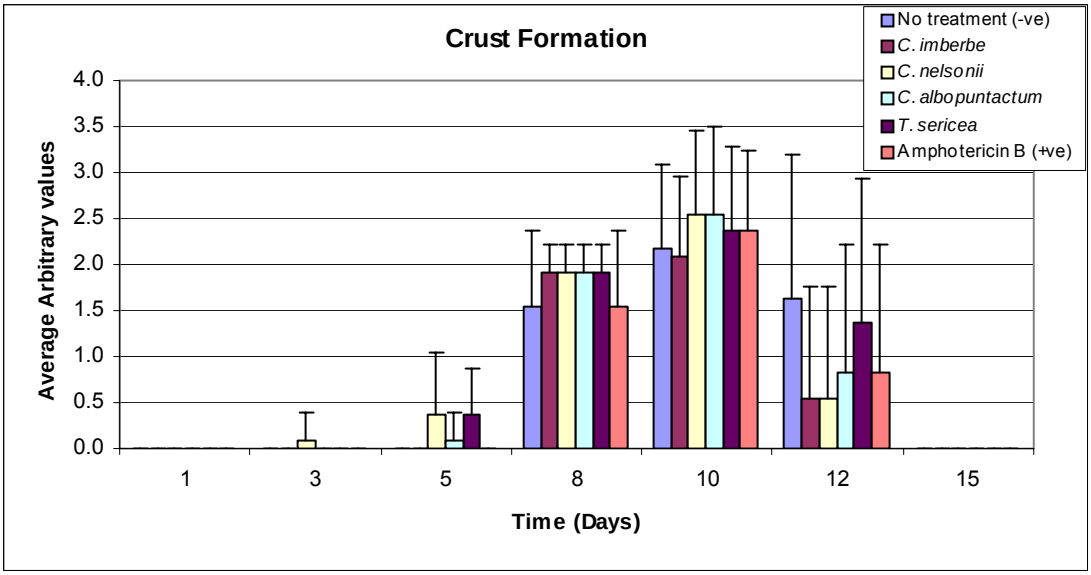
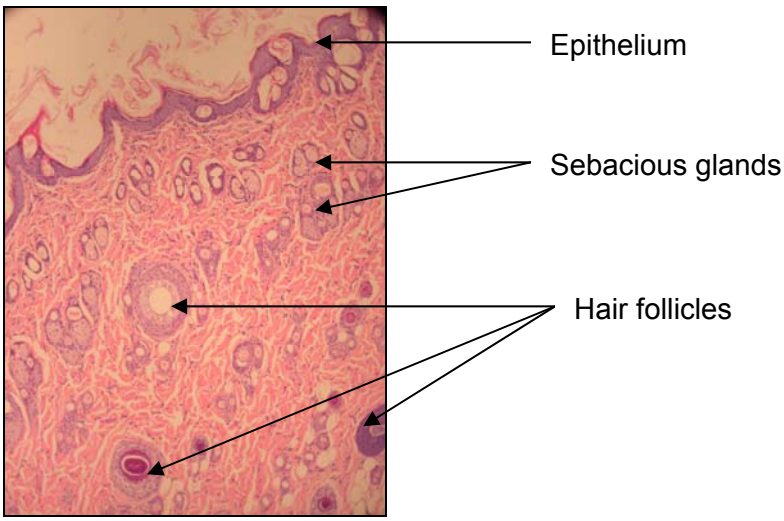


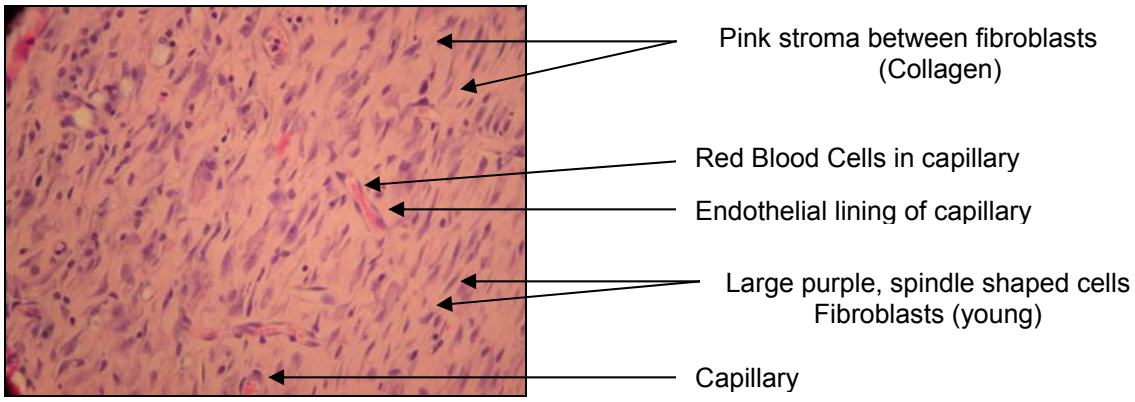
Figure 11.15b. Average arbitrary values of crust formation of rats in pilot study 2 with error bars

All 12 rats were euthanased after 3 weeks. The following observations were made, all lesions were properly 100% healed, there was no sign of inflammation underneath the skin and plant extracts in aqueous creams had wound healing properties on the infected wounds

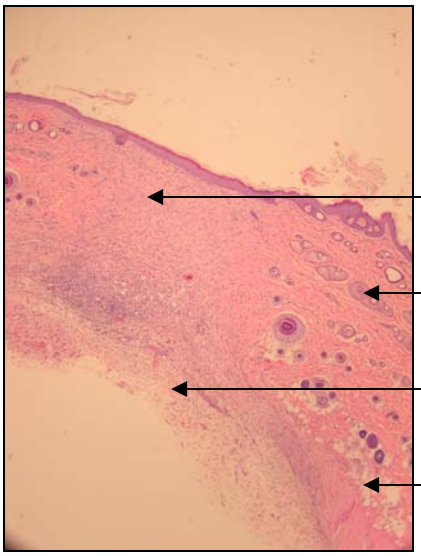
Figure 11.16 showed normal skin and the other histological observations which were fully presented in **table 11.6a** to **11.6d**. All lesions showed wound healing properties and few had fungal hyphae which indicate the treatments used had antifungal activities.



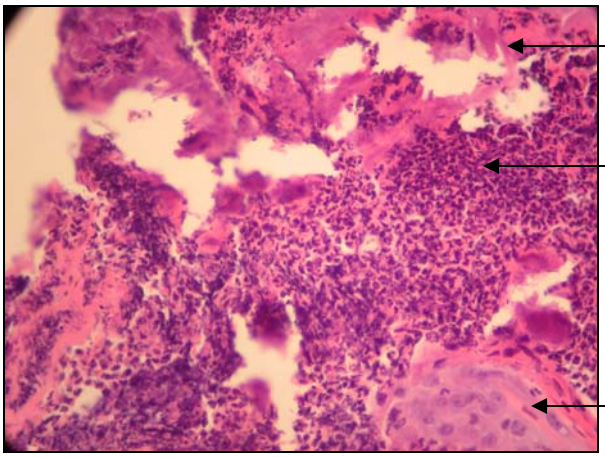
(A) Normal rat skin



(B) Fibrosis/Fibroplasia and Angiogenesis



(C) Fibrosis



(D) Degeneration of cells

Table 11.5a. Quantitative histopathological findings of wounds of rats infected with *C. albicans* after topical application of different creams (A= *T. sericea* crude extract, B= *C. nelsonii* crude extract, C= *C. imberbe* crude extract, D= *C. albopunctactum* crude extract, E= Negative control and F= Positive control (Amphotericin B))

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epitheliasation	Presence of fungal spores and hyphae**
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
1	A	1(++)	1(+)	-	-	2(++)	2(+)	2(+)	2(++)	2(+)	2	-
	B	2(++)	1(+)	-	2(±)	2(++)	2(+)	2(+)	2(++)	2(+)	2	-
2	A	2(++)	-	-	-	2(++)	-	1(++)	2(+)	2(++)	2	++
	B	3(++)	1(+)	+	2(+)	2(++)	2(+)	2(++)	2(++)	3(+++)	2	++
	C	1(++)	1(+)	-	-	2(++)	1(±)	2(+)	2(++)	1(++)	2	+
	D	1(++)	1(+)	-	-	1(+++)	1(±)	2(+)	1(++)	2(++)	2	+
	E	1(+)	1(+)	-	-	1(+)	-	±	1(++)	1(++)	2	+
	F	**Unable to evaluate accurately**										±
3	A	2(++)	-	-	-	2(++)	±	2(+)	2(+)	2(++)	2	-
	B	2(+)	2(+)	-	-	2(+)	-	+	2(+)	2(++)	2	-

*Neut's= Neutrophils; Mac's= Macrophages

These parameter were marked as follows:

- Severity: -, absent; ±, scant; +, mild; ++, moderate; +++, severe/marked
- Distribution: (1), dermal; (2), dermal and subdermal (i.e. subcutaneous skeletal muscle); (3), locally extensive (dermal and subdermal).
- Epithelialisation: 0, absent; 1, partial; 2, complete

** Fungal spores and hyphae are present only on the epidermas except where indicated otherwise

Table 11.5b. Quantitative histopathological findings of wounds of rats infected with *C. neoformans* after topical application of different creams (A= Negative control, B= *C. nelsonii* crude extract, C= Positive control (Amphotericin B), D= *C. albopunctactum* crude extract, E= *T. sericea* crude extract and F= *C. imberbe* crude extract)

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epitheliasation	Presence of fungal spores and hyphae**
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
4	A	2(++)	2(+)	-	-	2(++)	1(+)	1(+)	2(+++)	2(++)	2	+
	B	3(++)	2(+)	-	1(±)	2(++)	-	2(±)	2(+++)	2(++)	2	+
	C	2(++)	1(+)	-	1(±)	2(+)	2(+)	2(+)	2(++)	3(++)	2	+
	D	1(+)	1(+)	-	1(+)	1(++)	1(+)	1(±)	1(++)	1(+)	2	+
	E	2(++)	2(+)	-	-	2(++)	2(+)	2(++)	2(++)	2(++)	2	+
	F	3(+++)	-	-	2(+)	2(+)	2(+)	1(+)	2(+)	3(++)	2	+
5	A	2(++)	2(±)	-	1(±)	2(+++)	2(+)	2(++)	2(++)	2(++)	2	-
	B	3(++)	1(+)	+	-	3(++)	3(+)	3(+)	3(++)	2(+)	2	-

*Neut's= Neutrophils; Mac's= Macrophages

These parameter were marked as follows:

- Severity: -, absent; ±, scant; +, mild; ++, moderate; +++, severe/marked
- Distribution: (1), dermal; (2), dermal and subdermal (i.e. subcutaneous skeletal muscle); (3), locally extensive (dermal and subdermal).
- Epithelialisation: 0, absent; 1, partial; 2, complete

** Fungal spores and hyphae are present only on the epidermas except where indicated otherwise

Table 11.5c. Quantitative histopathological findings of wounds of rats infected with *M. canis* after topical application of different creams (A= *T. sericea* crude extract, B= *C. albopunctactum* crude extract, C= *C. imberbe* crude extract, D= *C. nelsonii* crude extract, E= Positive control (Amphotericin B) and F= Negative control)

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epitheliasation	Presence of fungal spores and hyphae**
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
7	A	2(+)	-	+	2(±)	2(++)	2(+)	-	2(++)	2(+)	2	-
	B	3(++)	2(+)	-	-	2(+++)	2(++)	2(++)		2(++)	2	-
9	A	3(++)	2(+)	-	-	3(++)	-	-	3(++)	3(++)	2	+
	B	3(+++)	-	-	+++	3(++)	3(++)	3(++)	3(++)	3(++)	2	+
	C	2(++)	2(+)	+	-	2(++)	1(±)	2(++)	2(++)	2(++)	2	+
	D	2(++)	2(±)	±	2(±)	2(++)	-	2(+)	2(++)	2(++)	2	+
	E	1(++)	1(++)	+	1(±)	1(++)	1(+)	1(+)	1(+++)	1(++)	2	+
	F	1(++)	1(+)	±	-	2(++)	-	-	1(++)	1(++)	2	+

*Neut's= Neutrophils; Mac's= Macrophages

These parameter were marked as follows:

- Severity: -, absent; ±, scant; +, mild; ++, moderate; +++, severe/marked
- Distribution: (1), dermal; (2), dermal and subdermal (i.e. subcutaneous skeletal muscle); (3), locally extensive (dermal and subdermal).
- Epithelialisation: 0, absent; 1, partial; 2, complete

** Fungal spores and hyphae are present only on the epidermas except where indicated otherwise

Table 11.5d. Quantitative histopathological findings of wounds of rats infected with *S. schenckii* after topical application of different creams (A= *T. sericea* crude extract, B= *C. albopunctactum* crude extract, C= *C. imberbe* crude extract, D= *C. nelsonii* crude extract, E= Positive control (Amphotericin B) and F= Negative control)

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epitheliasation	Presence of fungal spores and hyphae**
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
10	A	3(++)	1(+)	+	-	2(++)	2(±)	2(+)	3(++)	3(++)	2	+
	B	3(++)	-	-	-	2(++)	2(±)	2(+)	2(+)	3(++)	2	+
	C	1(+)	-	-	1(±)	1(+)	1(±)	1(±)	1(+)	1(+)	2	±
	D	1(+)	1(+)	-	-	1(+)	1(++)	1(+)	1(++)	1(+)	2	±
	E	2(+)	2(+)	-	-	2(++)	-	2(+)	2(++)	2(++)	2	±
	F	2(+)	1	-	-	2(+)	-	2(+)	2(+)	2(++)	2	-
11	A	3(+++)	1(++)	-	1(+++)	3(+++)	3(+++)	3(++)	3(++)	2(++)	2	++
	B	3(+++)	1(++)	-	1(+++)	3(+++)	3(++)	3(++)	3(++)	3(++)	2	+
	C	2(++)	1(++)	-	1(+++)	2(++)	-	2(+)	2(++)	2(++)	2	+
	D	1(++)	1(++)	+	1(+++)	1(+++)	1(±)	1(+)	1(++)	1(+)	2	++
	E	3(++)	1(++)	-	1(+++)	2(+++)	1(±)	2(+)	2(++)	2(++)	2	++
	F	2(++)	1(++)	+	1(+++)	2(++)	-	1(±)	2(++)	2(++)	2	++

*Neut's= Neutrophils; Mac's= Macrophages

These parameter were marked as follows:

- Severity: -, absent; ±, scant; +, mild; ++, moderate; +++, severe/marked
- Distribution: (1), dermal; (2), dermal and subdermal (i.e. subcutaneous skeletal muscle); (3), locally extensive (dermal and subdermal).
- Epithelialisation: 0, absent; 1, partial; 2, complete

** Fungal spores and hyphae are present only on the epidermas except where indicated otherwise

There was no evidence of systemic infection and there was no differences in treatments This formed the basis of starting with the main study, where the isolated compound was added as part of the treatment.

11.6.3. Confirmation study with immunocompromised rats (Main experiment)

The data of all parameters measured were recorded in **Table 11.7** (*C. albicans*), *C. neoformans* (**Table 11.8**), *M. canis* (**Table 11.9**) and *S. schenckii* (**Table 11.10**). All the tables are placed in the **Appendix** because they were big and they had too much data.

11.6.3.1. Weight

All the rats infected with different fungal pathogens i.e. *C. albicans* (**Figure 11.16**), *C. neoformans* (**Figure 11.17**), *M. canis* (**Figure 11.18**) and *S. schenckii* (**Figure 11.19**) gradually lost weight, which was not the case in pilot study (2) and this was attributed to immunosuppressive reaction and the additional treatment (isolated compound). On Day 15 all the rats gained weight except Rat 3. We decide to leave the rats for 6 additional days without handling them and an increase in weight was observed. Rat 22 lost more weight after Day 5 but gradually recovered the weight lost after Day 8

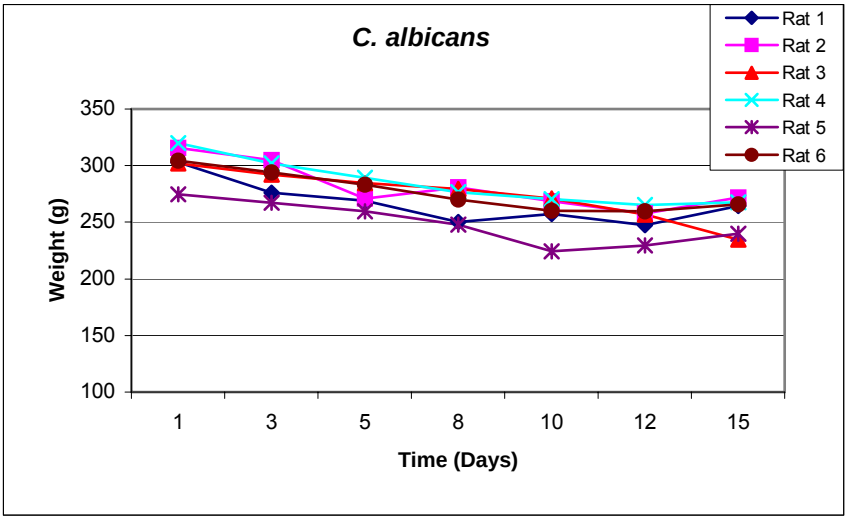


Figure 11.16. Weights of rats (1 to 6) infected with *C. albicans*.

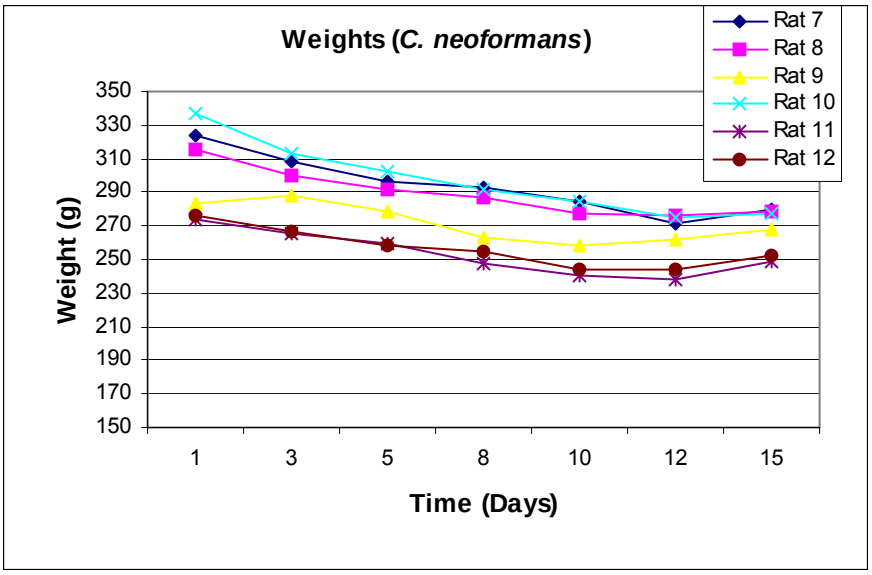


Figure 11.17. Weights of rats (7 to 12) infected with *C. neoformans.*

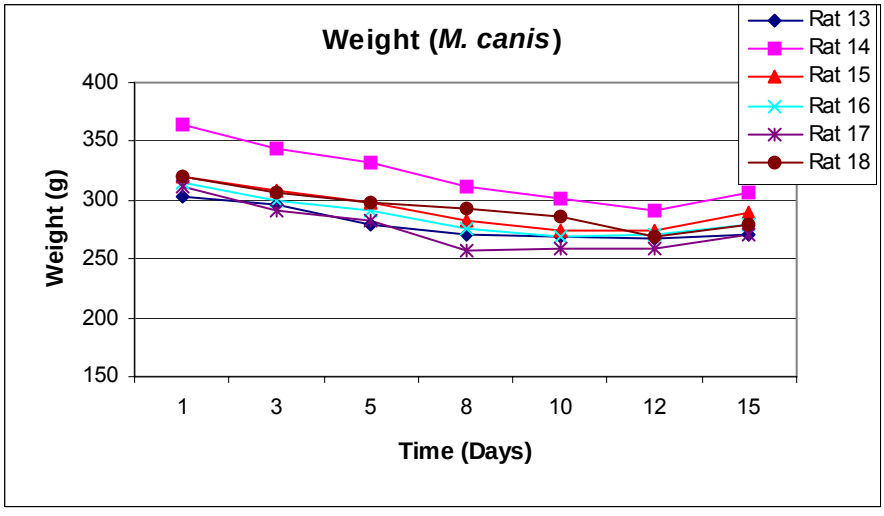


Figure 11.18. Weights of rats (13 to 18) infected with *M. canis.*

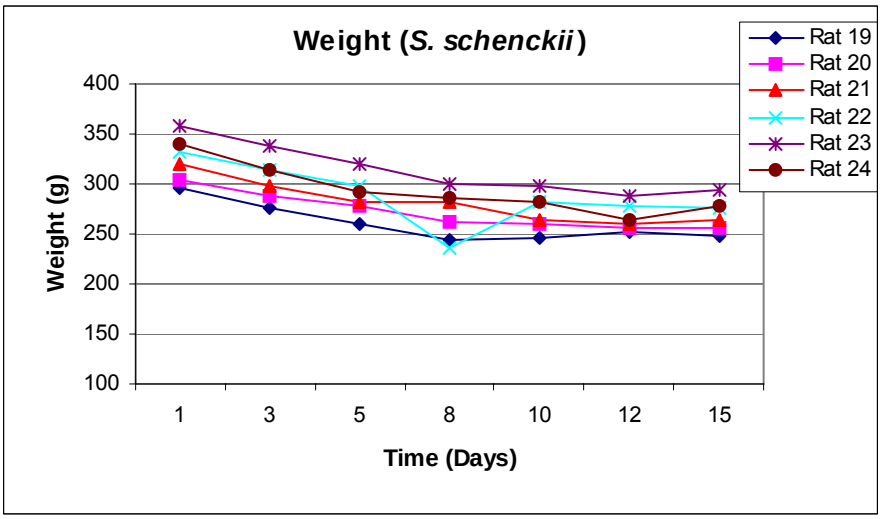


Figure 11.19. Weights of rats (19 to 24) infected with *S. schenckii*.

11.6.3.2. Temperature

The temperatures of rats infected with *C. albicans* (**Figure 11.20**), *C. neoformans* (**Figure 11.21**), *M. canis* (**Figure 11.22**) and *S. schenckii* (**Figure 11.23**) were within the expected range (35-37 ^{o}C) at the end of the experiment (Day 15). Temperature above normal were recorded for rat 24 on Day 3, rat 12 on Day 10 and rats 3 and 16 on Day 12.

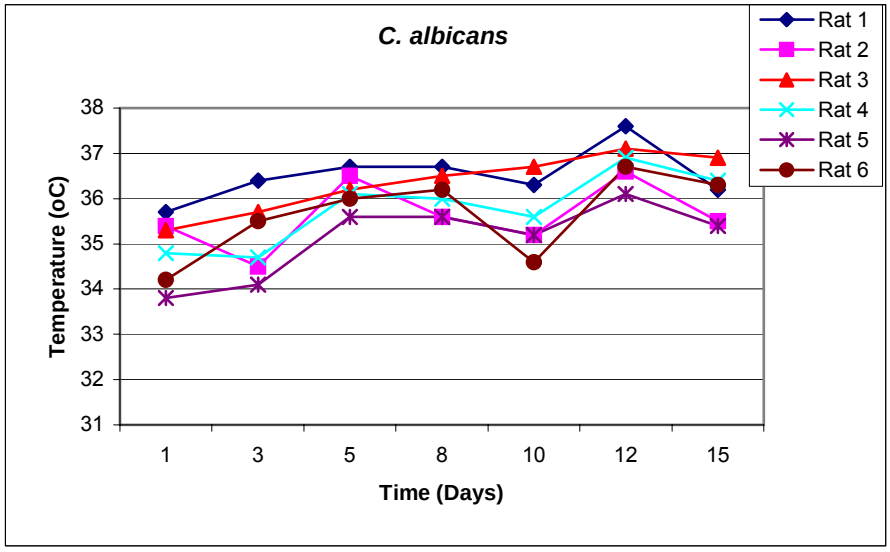


Figure 11.20. Temperatures of rats (1 to 6) infected with *C. albicans*.

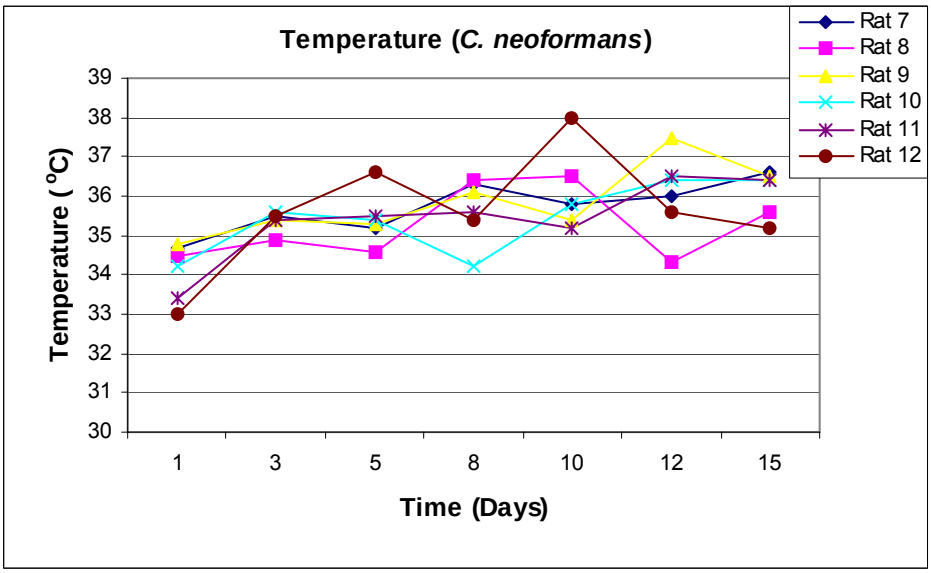


Figure 11.21. Temperatures of rats (7 to 12) infected with *C. neoformans*.

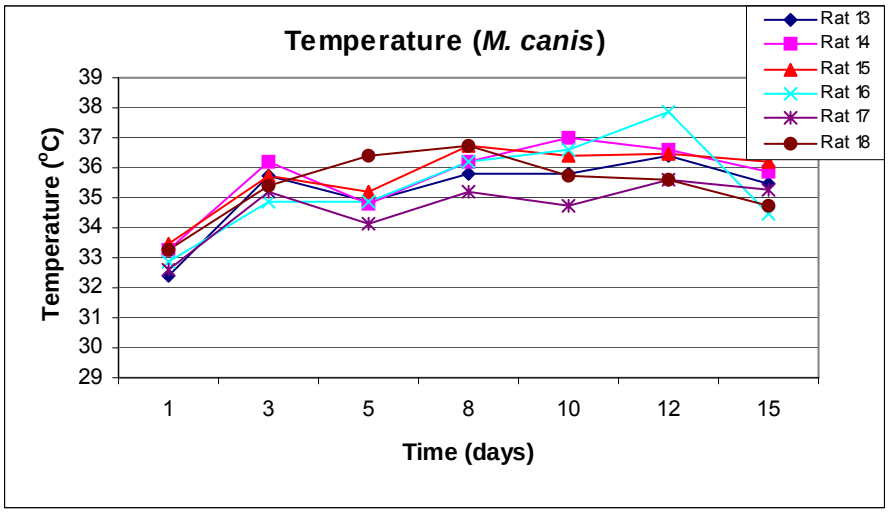


Figure 11.22. Temperatures of rats (13 to 18) infected with *M. canis.*

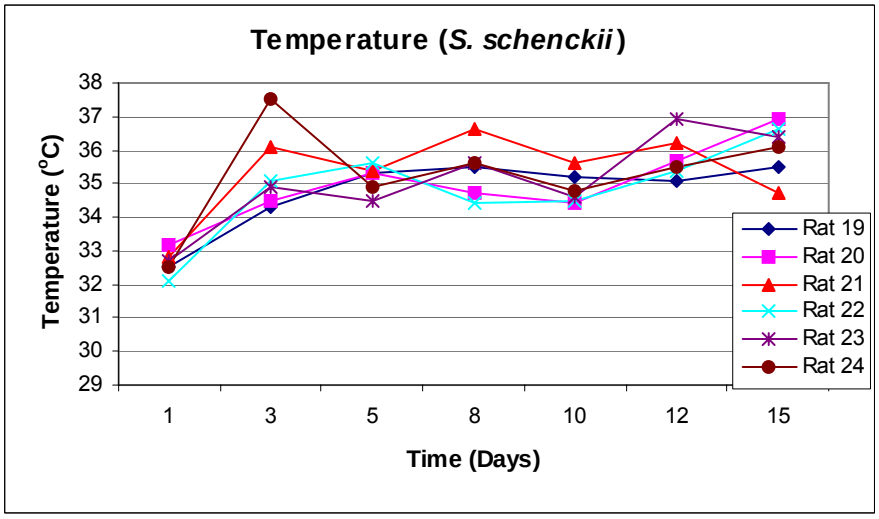


Figure 11.23. Temperatures of rats (19 to 24) infected with *S. schenckii*.

11.6.3.3. Lesion size

A circular full-thickness lesions were created on the back of rats. Lesion sizes were measured, *C. albicans* (**Figure 11.24**), *C. neoformans* (**Figure 11.25**), *M. canis* (**Figure 11.26**) and *S. schenckii* (**Figure 11.27**). The lesion sizes were calculated the same way as in pilot study I. The open lesion was healed by the process of wound contraction. The epithelial closure in all rats occurred by 17 days. The transient formation of granulation tissue was vigorous on day 12 after wounding. There was no significant difference in the contraction of lesion area treated with different extracts. In all the experiments, lesions treated with isolated compound healed faster than the extracts and amphotericin B. However, contraction was fastest in the untreated wounds.

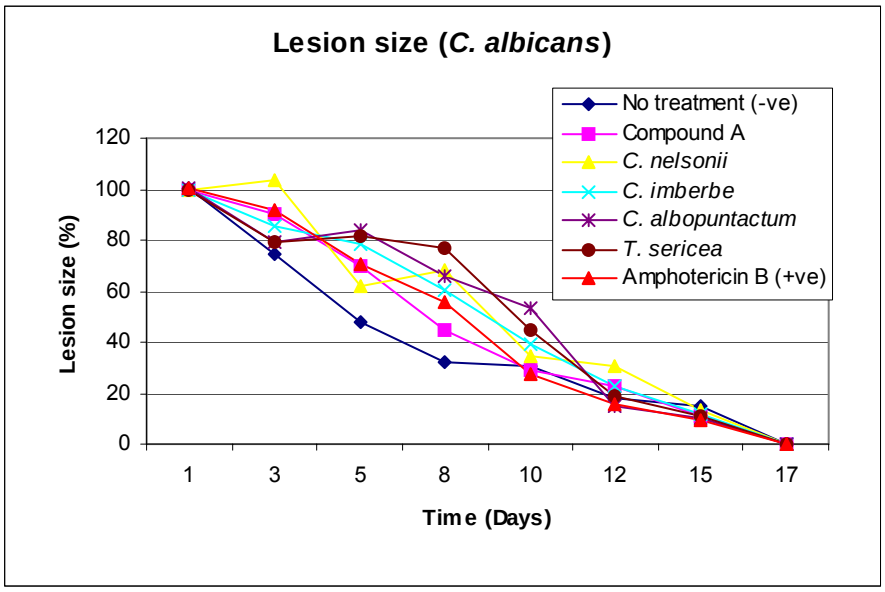


Figure 11.24. The average lesion size of lesions infected with *C. albicans* and treated with four extracts, isolated compound and Amphotericin B (positive control).

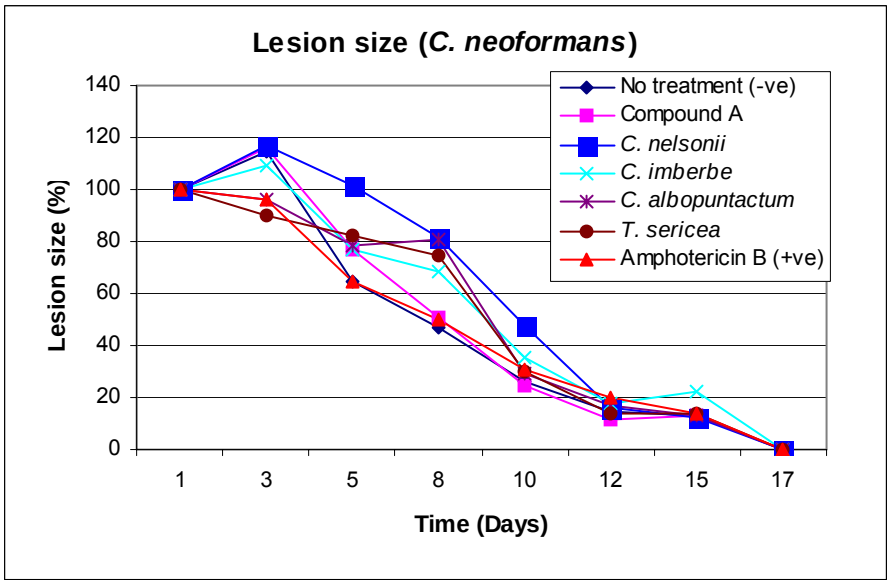


Figure 11.25. The average lesion size of lesions infected with *C. neoformans* and treated with four extracts, isolated compound and Amphotericin B (positive control).

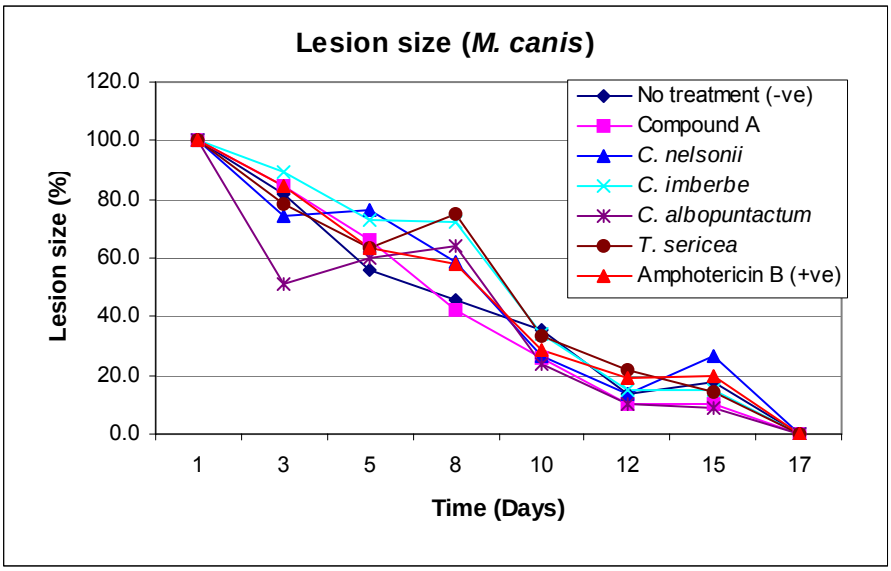


Figure 11.26. The average lesion size of lesions infected with *M. canis* and treated with four extracts, isolated compound and Amphotericin B (positive control).

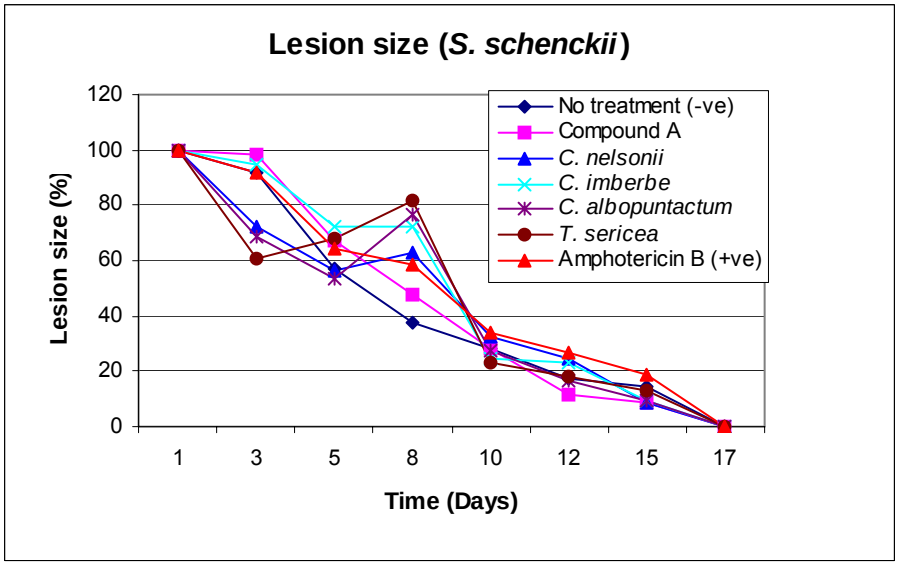


Figure 11.27. The average lesion size of lesions infected with *S. schenckii* and treated with four extracts, isolated compound and Amphotericin B (positive control).

11.6.3.4. Erythema

One fundamental property of the skin is its ability to respond to treatment. In rats populations these responses are clearly adaptive where the first response, erythema (redness) is a sign that the immune system is active and the healing process has begun. The resulting healing was quantified on the basis of erythema, *C. albicans* (**Figure 11.28a**), *C. neoformans* (**Figure 11.29a**),

M. canis (**Figure 11.30a**) and *S. schenckii* (**Figure 11.31a**). As described in Section 11.6.2. a scoring system was used to determine the degree of erythema. Subsequently a scale of 1 to 5 was used, 1 being the lowest and 5 being the highest formed. Averages of all 6 rats were used in all groups infected with different pathogens. Error bars were also drawn to show the confidence level of data or the deviation along a curve, *C. albicans* (**Figure 11.28b**), *C. neoformans* (**Figure 11.29b**), *M. canis* (**Figure 11.30b**) and *S. schenckii* (**Figure 11.31b**). The variability in the results of erythema at each lesion in rats infected with different fungal pathogens differs between the treatments used, lesion without treatment took longer time to heal in all cases. Although the differences were not statistically significant, the plant extracts tended to decrease erythema in practically all cases.

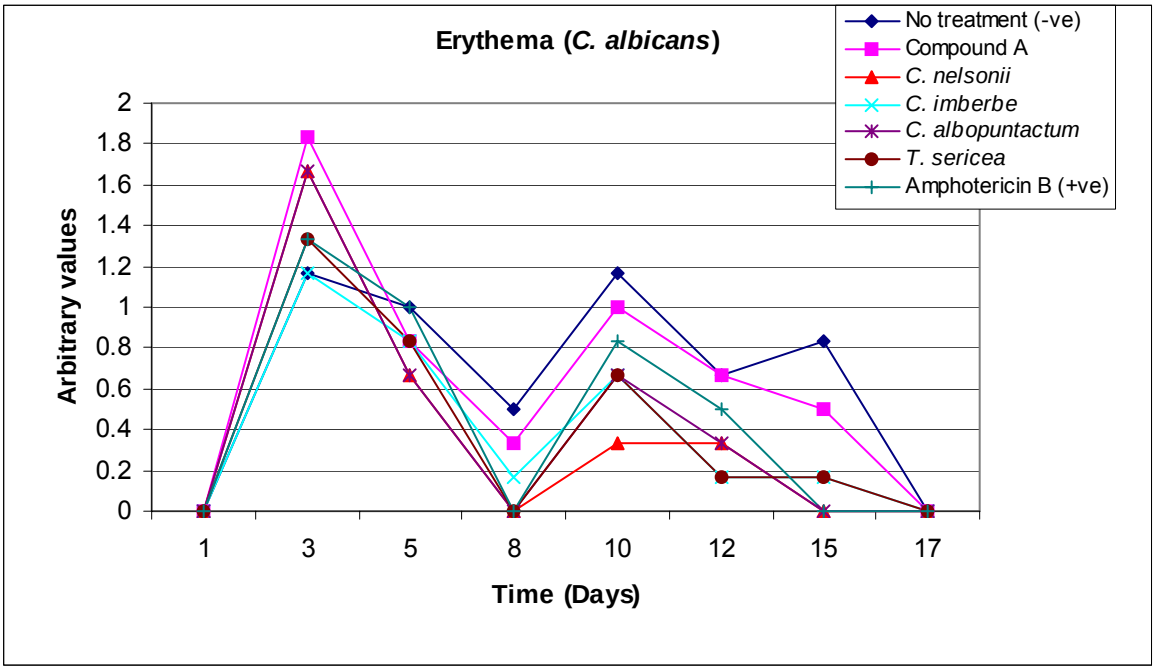


Figure 11.28a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the wound erythema of rat infected with *C. albicans*.

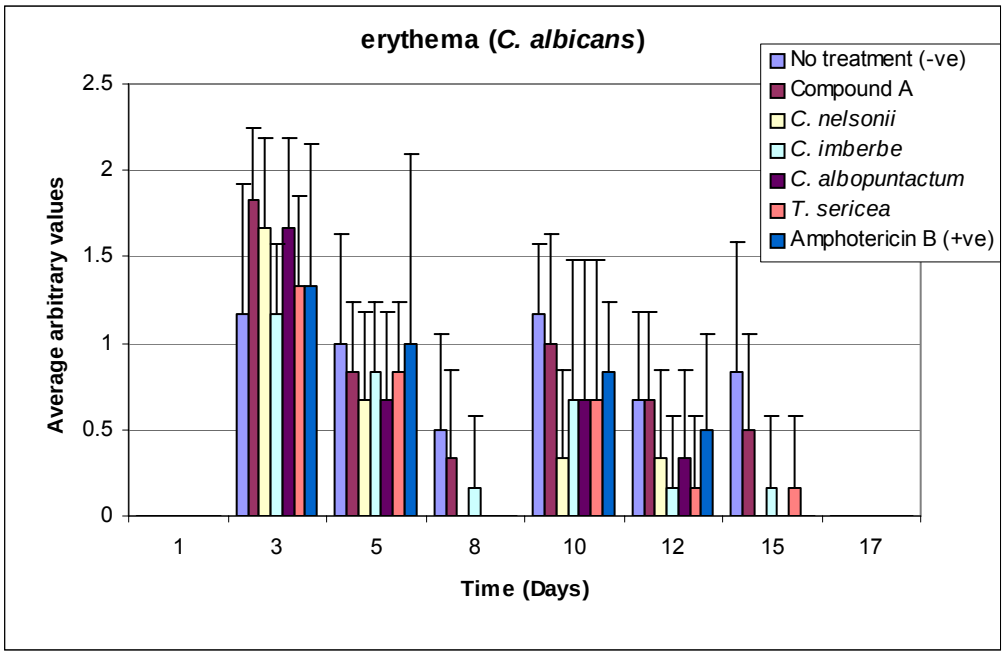


Figure 11.28b. Average arbitrary values of erythema of rats infected with *C. albicans* with error bars

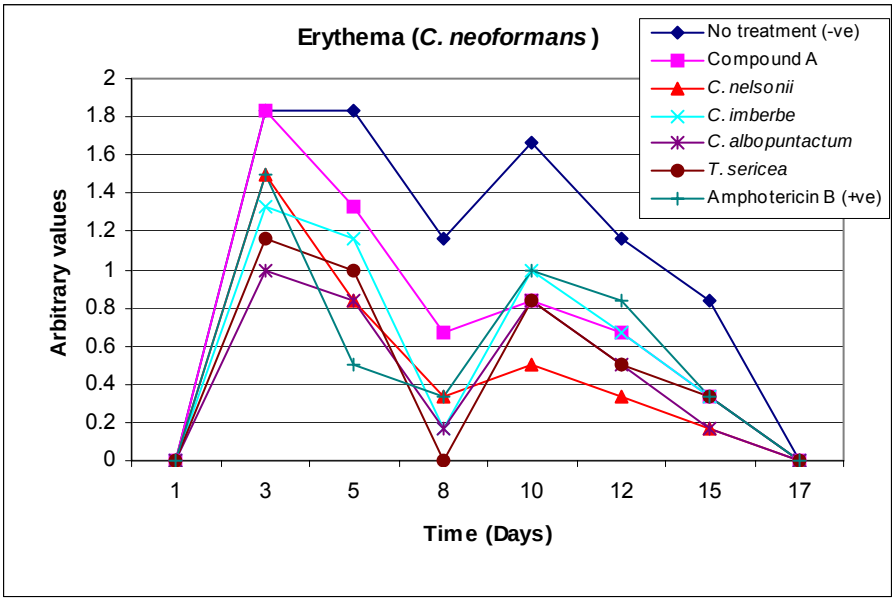


Figure 11.29a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the wound erythema of rat infected with *C. neoformans*.

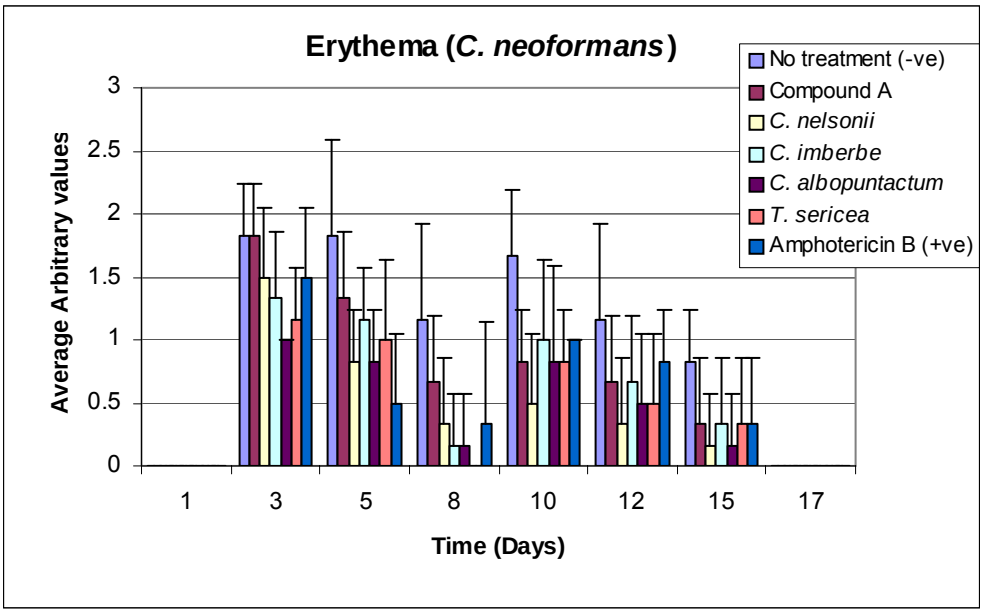


Figure 11.29b. Average arbitrary values of erythema of rats infected with *C. neoformans* with error bars

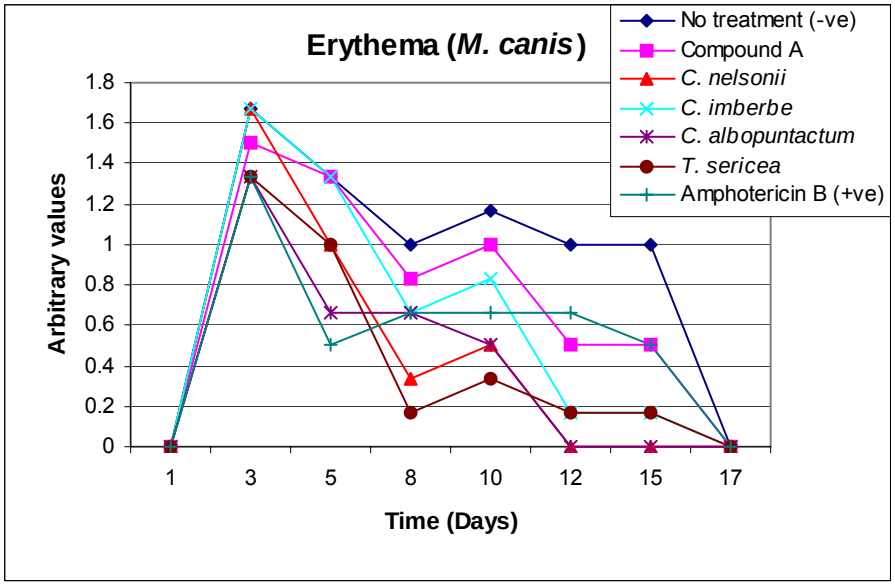


Figure 11.30a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the wound erythema of rat infected with *M. canis*.

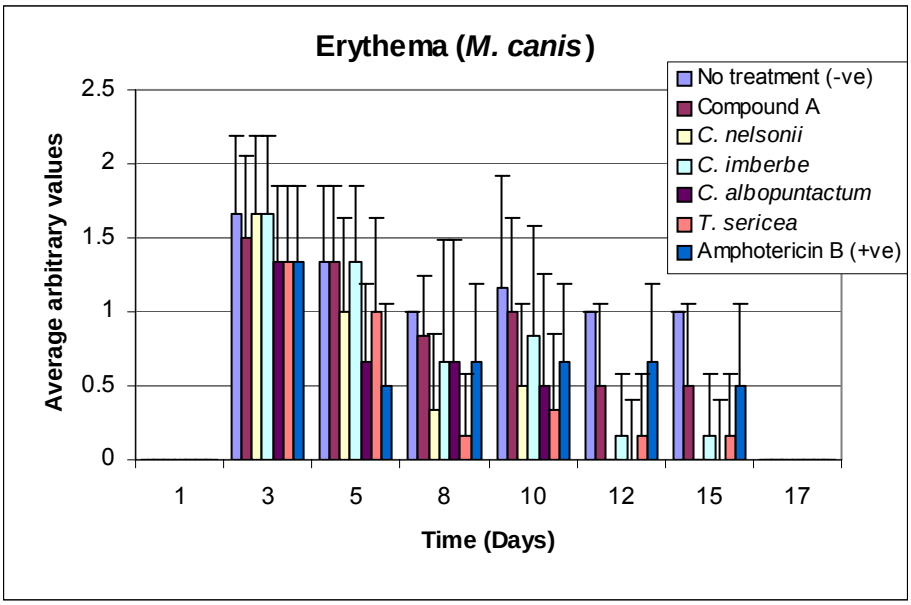


Figure 11.30b. Average arbitrary values of erythema of rats infected with *M. canis* with error bars

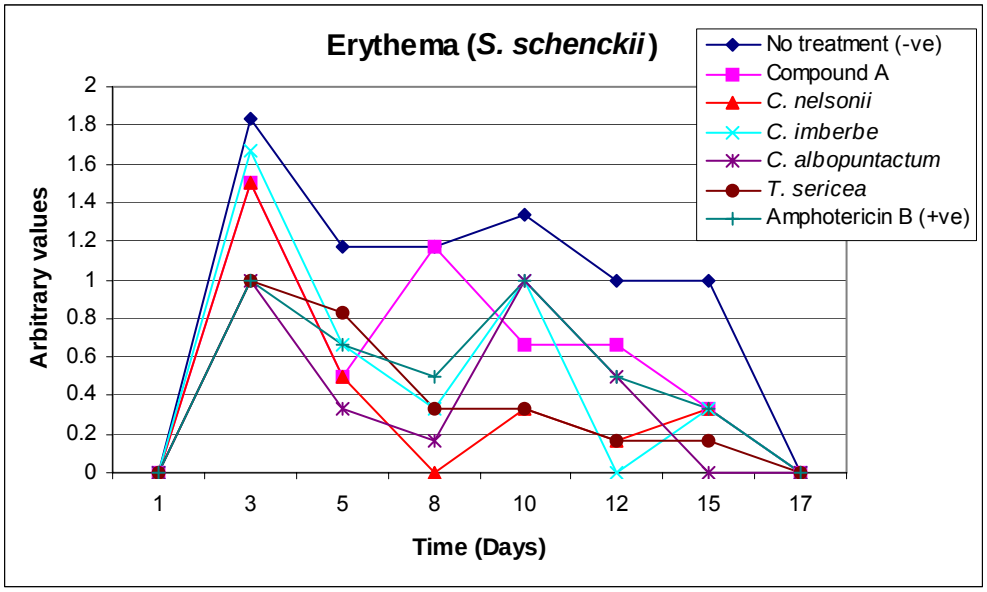


Figure 11.31a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the wound erythema of rat infected with *S. schenckii*.

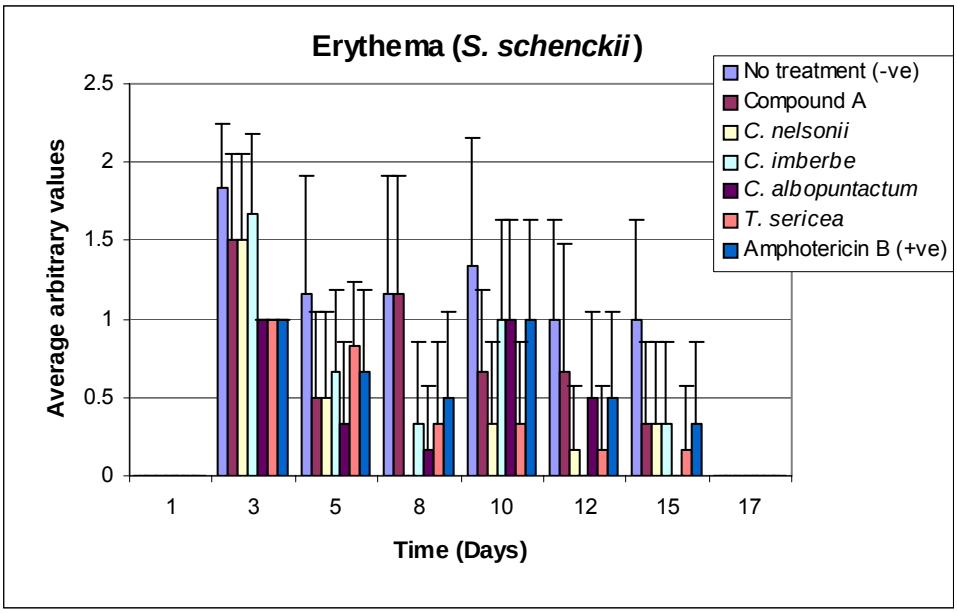


Figure 11.31b. Average arbitrary values of erythema of rats infected with *S. schenckii* with error bars

11.6.3.5. Exudate

Exudate formation was one of the parameters used to quantify the healing process, *C. albicans* (**Figure 11.32a**), *C. neoformans* (**Figure 11.33a**), *M. canis* (**Figure 11.34a**) and *S. schenckii* (**Figure 11.35a**). The same scale used in erythema was used. Error bars were also drawn to show the confidence level of data or the deviation along curves, *C. albicans* (**Figure 11.32b**), *C. neoformans* (**Figure 11.33b**), *M. canis* (**Figure 11.34b**) and *S. schenckii* (**Figure 11.35b**). Exudate formation was observed until Day 12 in rats infected with *C. albicans*, Day 8 in rats infected with *C. neoformans*, except the lesions, which were not treated. In lesions infected with *M. canis* and *S. schenckii*, exudate formation was observed until Day 10. There was less exudate formation in lesions not treated.

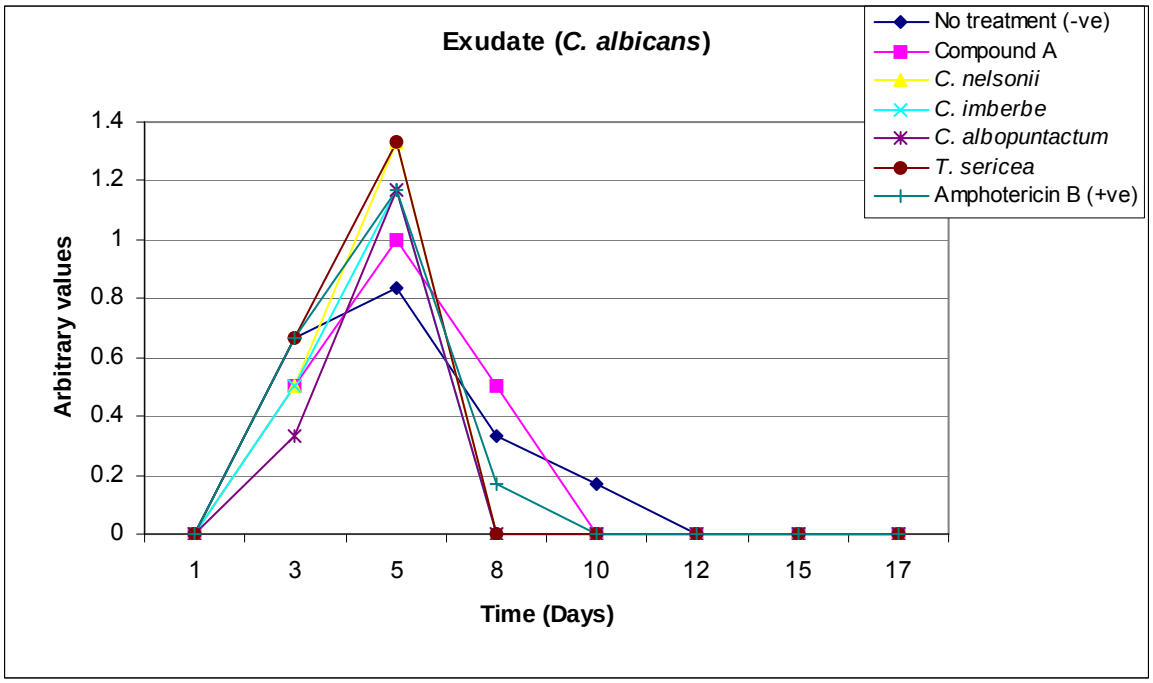


Figure 11.32a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the exudate formed of rats infected with *C. albicans*.

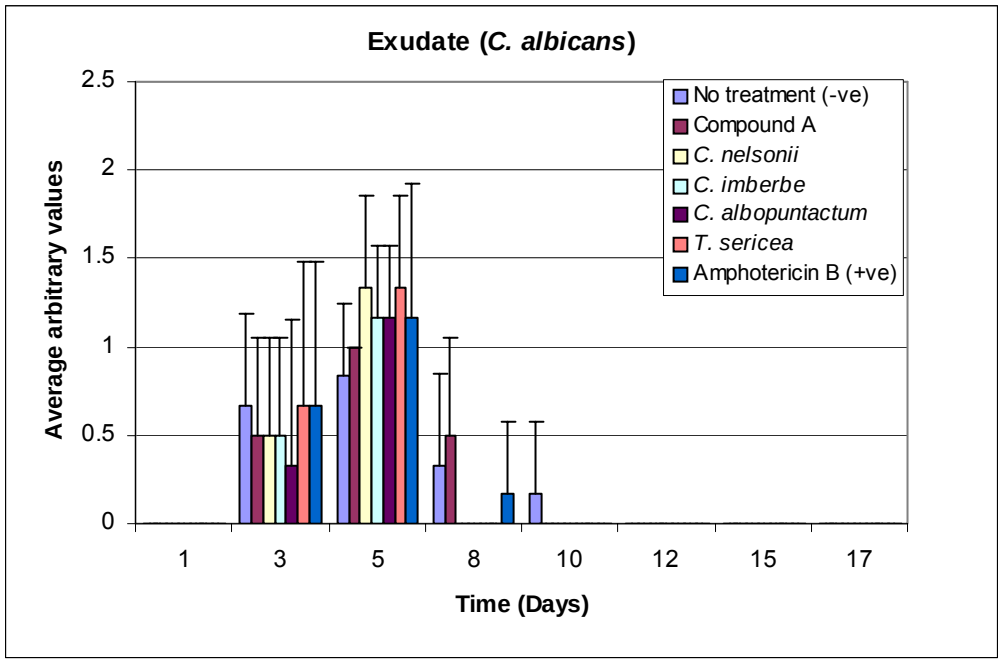


Figure 11.32b. Average arbitrary values of exudate of rats infected with *C. albicans* with error bars

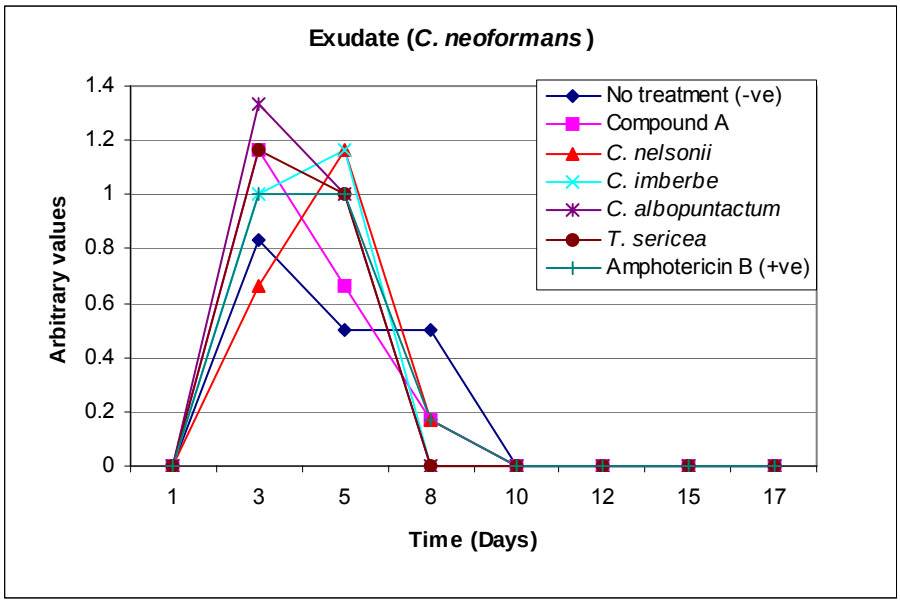


Figure 11.33a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the exudate formed of rats infected with *C. neoformans*.

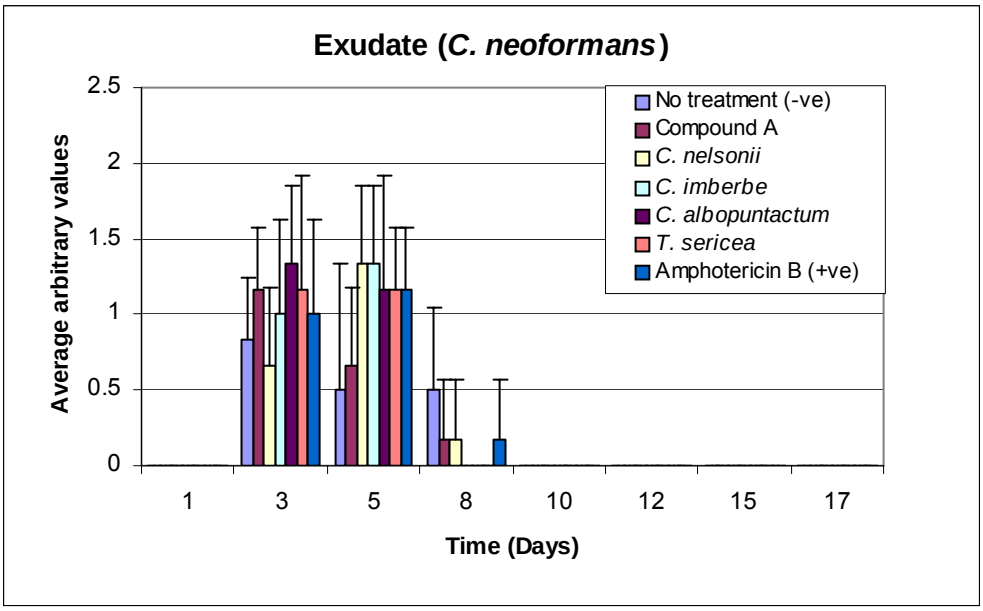


Figure 11.33b. Average arbitrary values of exudate of rats infected with *C. neoformans* with error bars

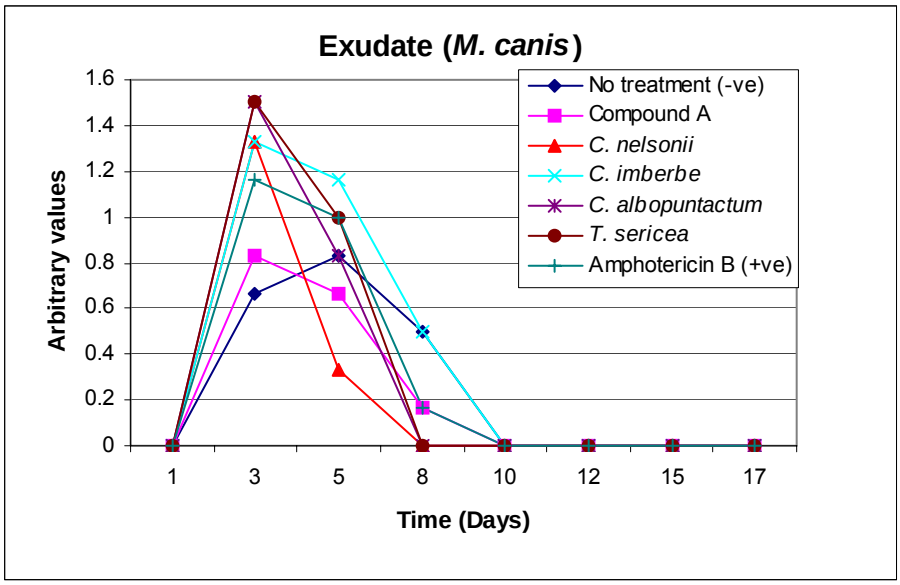


Figure 11.34a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the exudate formed of rats infected with *M. canis*.

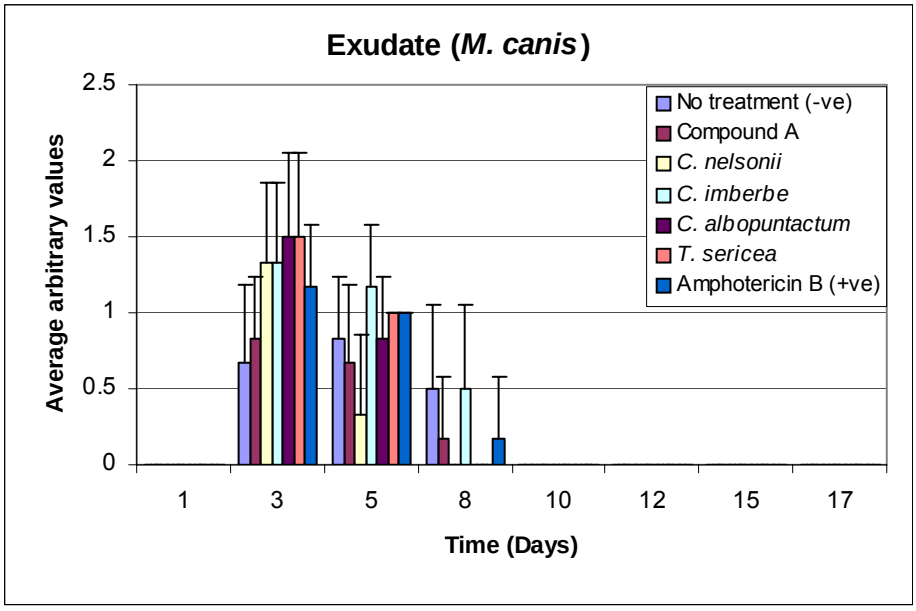


Figure 11.34b. Average arbitrary values of exudate of rats infected with *M. canis* with error bars

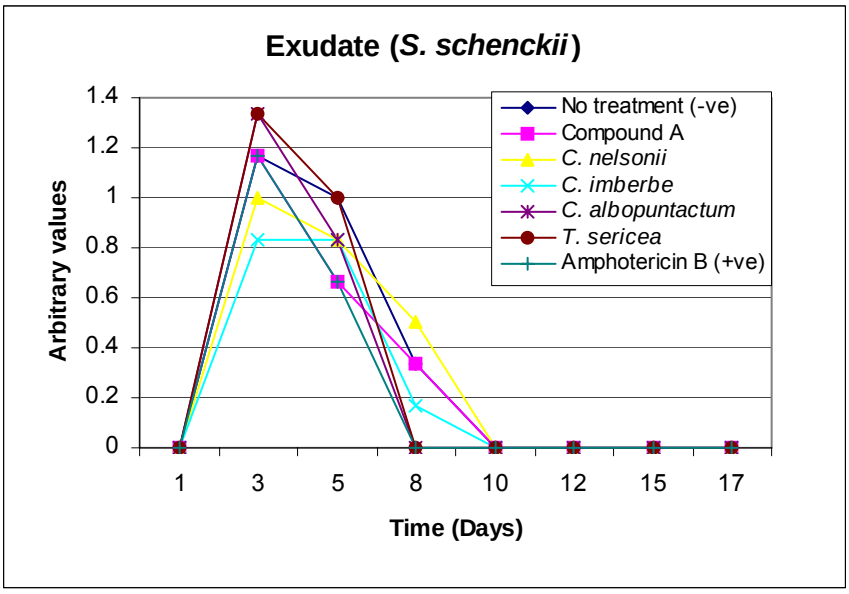


Figure 11.35a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the exudate formed of rats infected with *S. schenckii*.

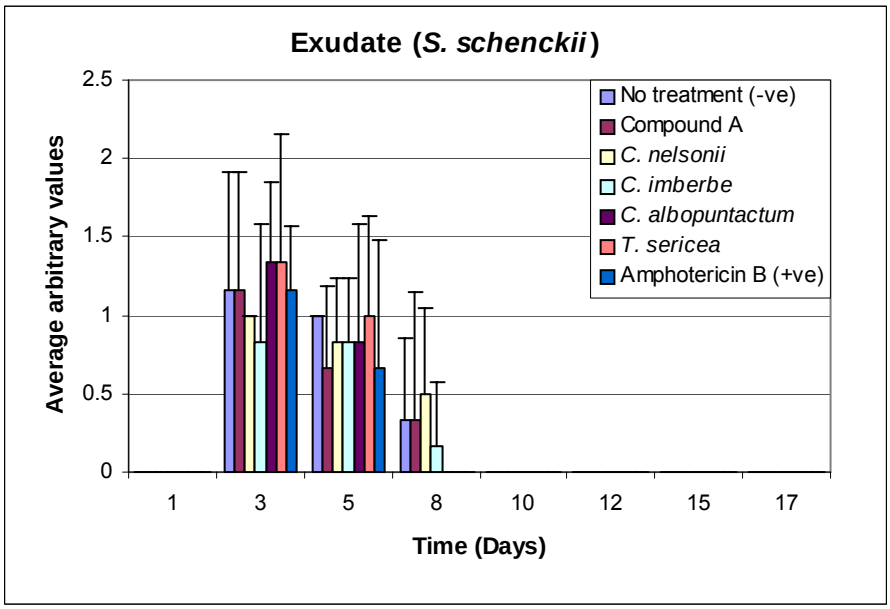


Figure 11.35b. Average arbitrary values of exudate of rats infected with *S. schenckii* with error bars

11.6.3.6. Crust Formation

Wound healing process was also quantified by crust formation, *C. albicans* (**Figure 11.36a**), *C. neoformans* (**Figure 11.37a**), *M. canis* (**Figure 11.38a**) and *S. schenckii* (**Figure 11.39a**). The

same scale used in erythema was used. Error bars were also drawn to show the confidence level of data or the deviation along curves, *C. albicans* (**Figure 11.36b**), *C. neoformans* (**Figure 11.37b**), *M. canis* (**Figure 11.38b**) and *S. schenckii* (**Figure 11.39b**). The treated group presented a rigid, dark and thick crust. It is probably due to proteins and wound exudates interconnected with the extract constituents favouring the local homeostasis and protecting the new tissue by forming an external cover that furnished mechanic protection. The crust formation in all infected rats follow the same patter. i.e. crust start forming after Day 3 until Day 15. There was no marked difference in crust formation of all the treatments.

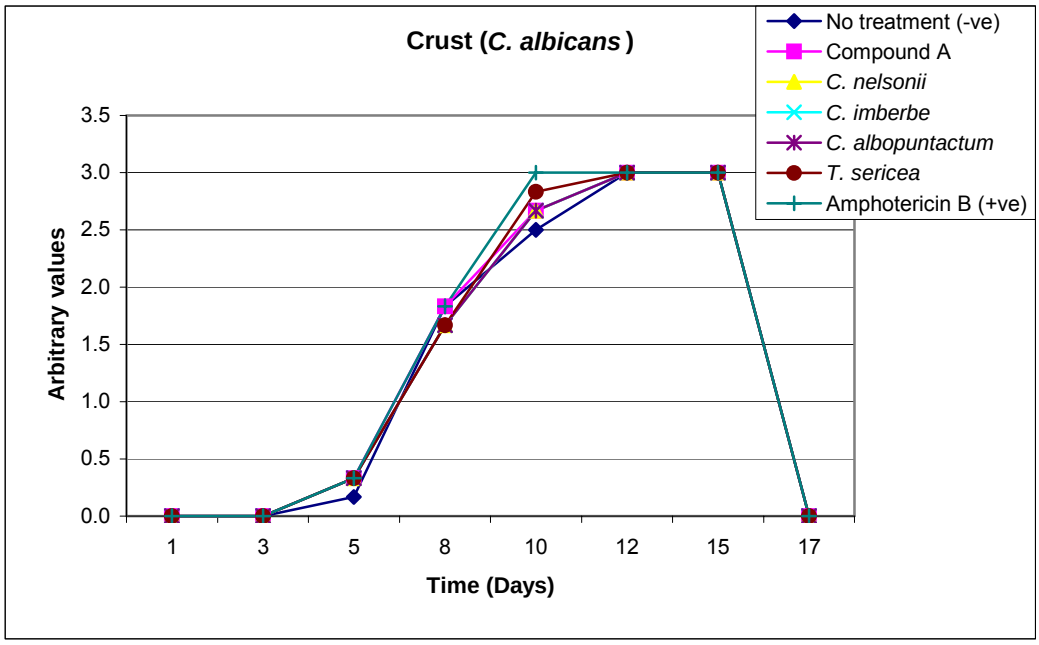


Figure 11.36a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the crust formed of rats infected with *C. albicans*.

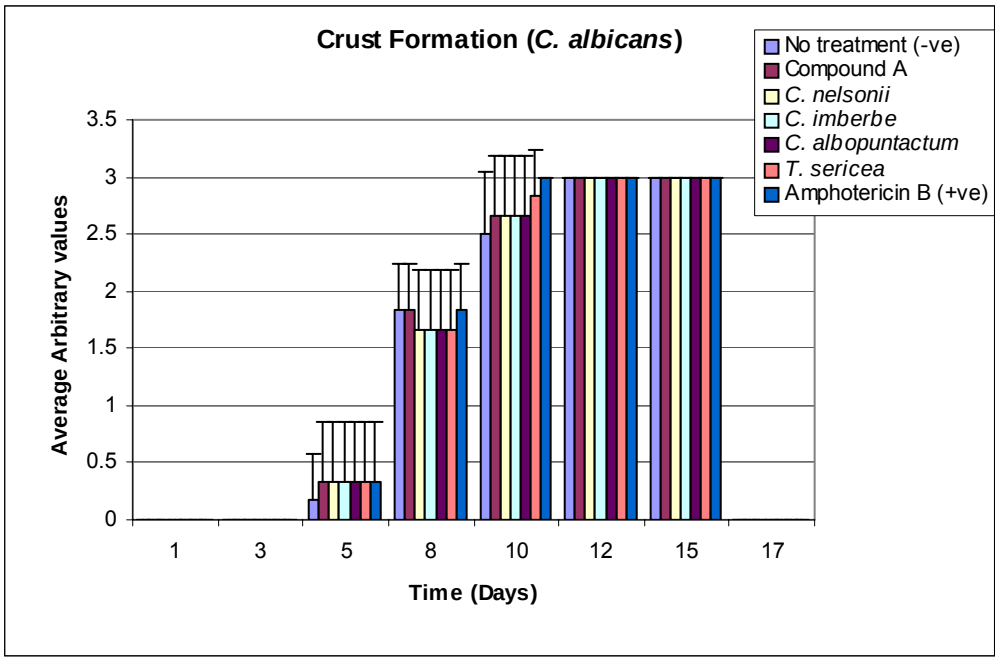


Figure 11.36b. Average arbitrary values of crust formation of rats infected with *C. albicans* with error bars

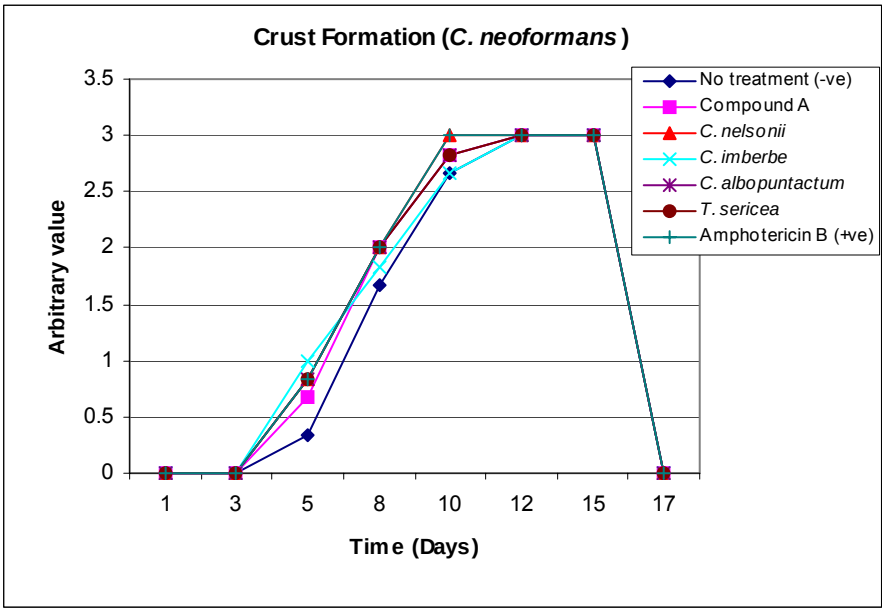


Figure 11.37a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the crust formed of rats infected with *C. neoformans*.

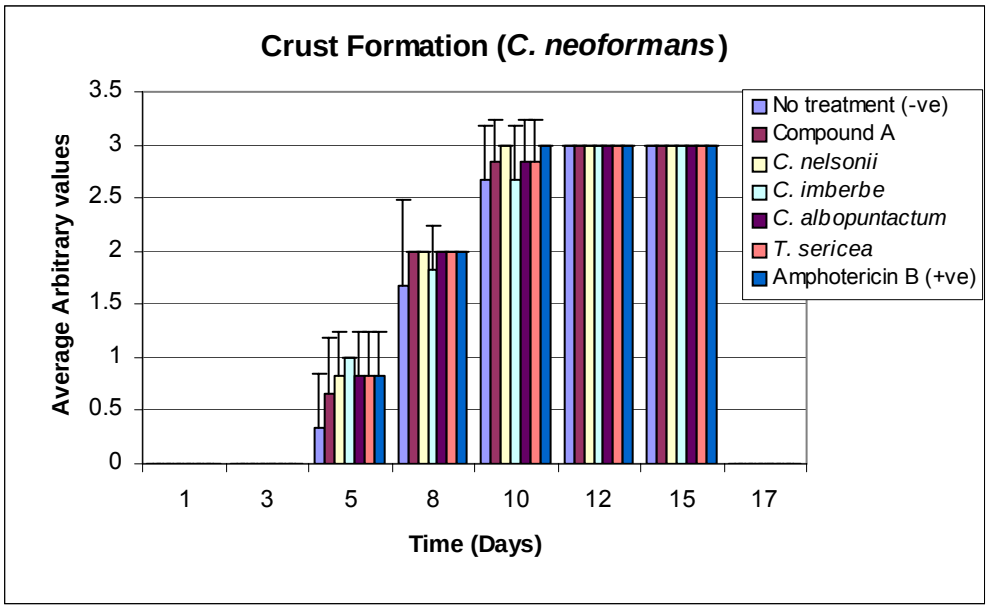


Figure 11.37b. Average arbitrary values of crust formation of rats infected with *C. neoformans* with error bars

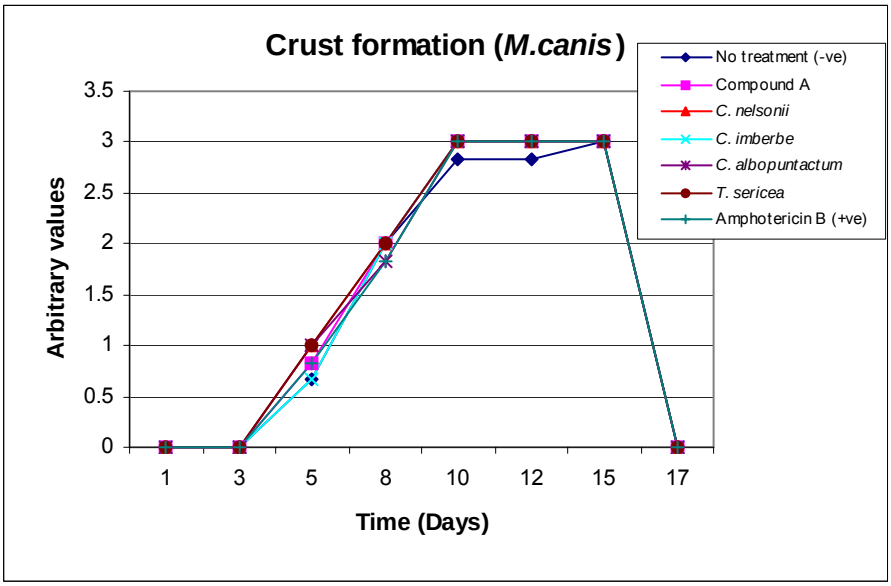


Figure 11.38a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the crust formed of rats infected with *M. canis*.

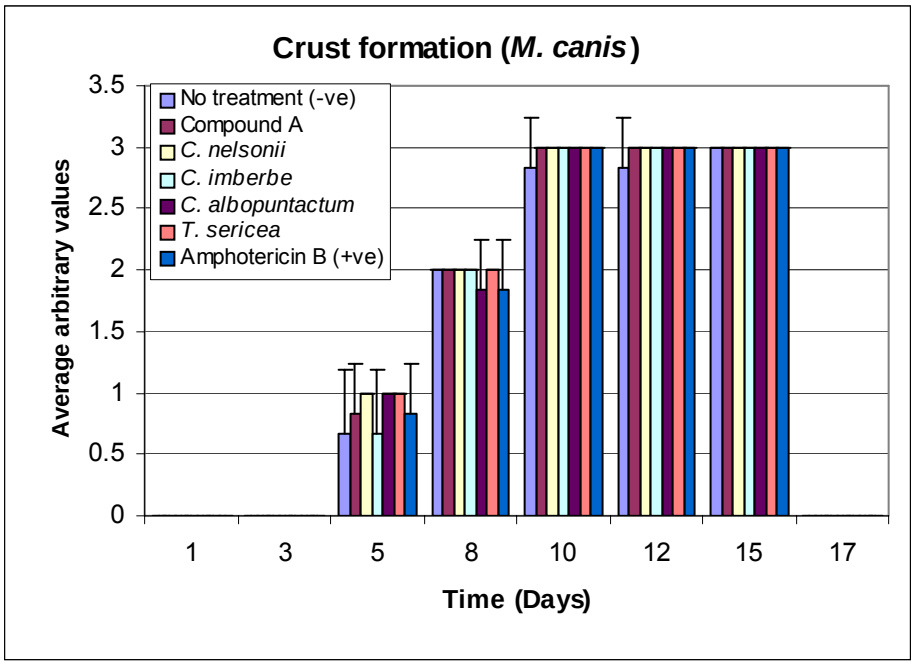


Figure 11.38b. Average arbitrary values of crust formation of rats infected with *M. canis* with error bars

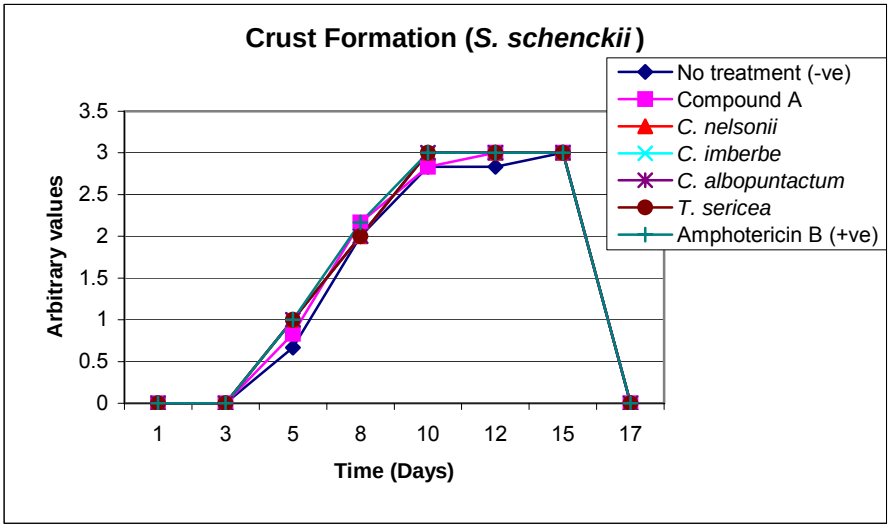


Figure 11.39a. The influence of the crude extracts, isolated compound and Amphotericin B (positive control) on the crust formed of rats infected with *S. schenckii*.

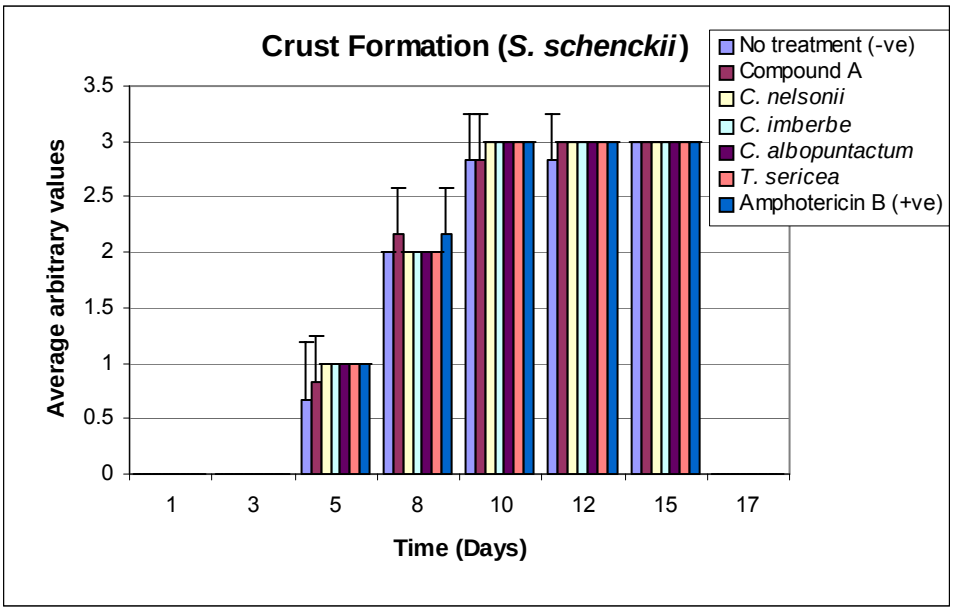


Figure 11.39b. Average arbitrary values of crust formation of rats infected with *S. schenckii* with error bars

11.6.4. Comparisons of lesions sizes

Comparison of lesion sizes was done to check the effect of treatments on fungal pathogens. Isolated compound (**Figure 11.40**) was very active against *M. canis* and *S. schenckii*. Amphotericin B (**Figure 11.41**) had almost similar activity against tested pathogens. All extracts were very active against *M. canis* and *S. schenckii* and least active against *C. albicans* and *C. neoformans.* (**Figure 11.42 – 11.45**).

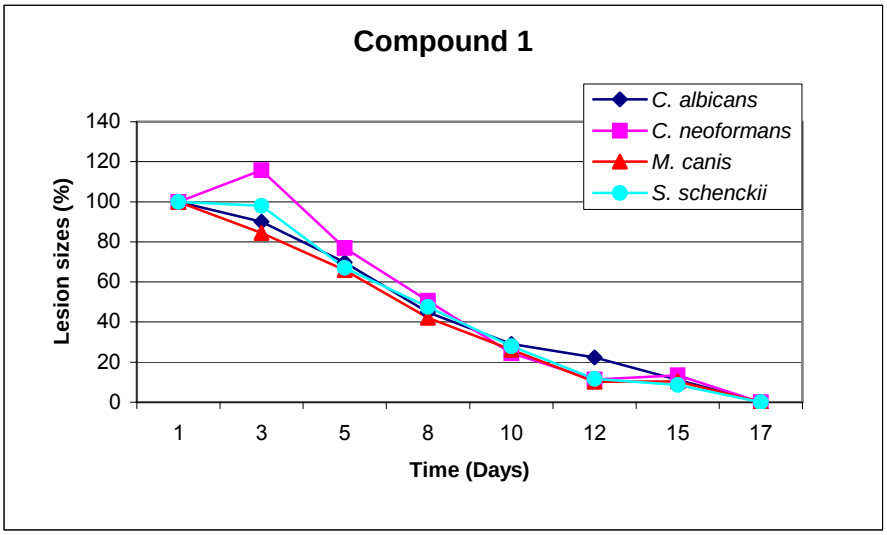


Figure 11.40. Effect of compound 1 on fungal pathogens.

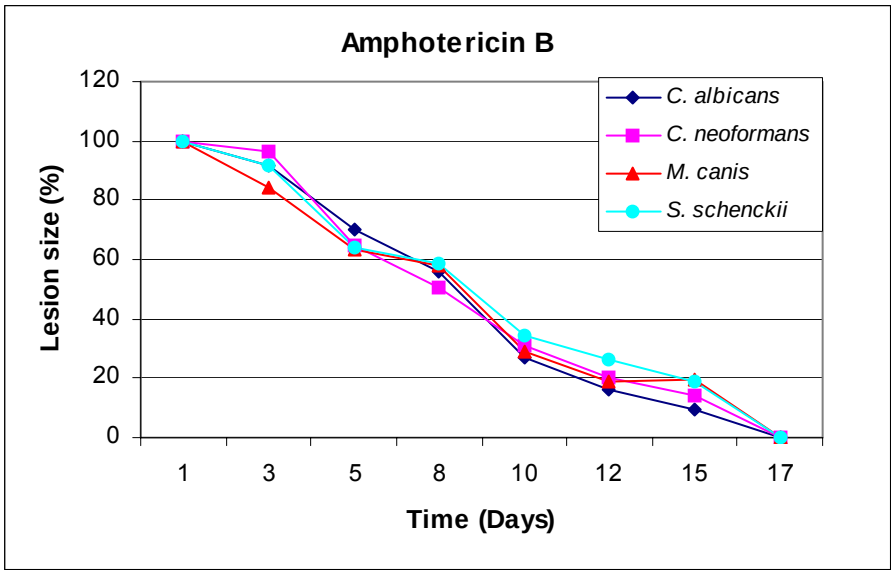


Figure 11.41. Effect of amphotericin B on fungal pathogens.

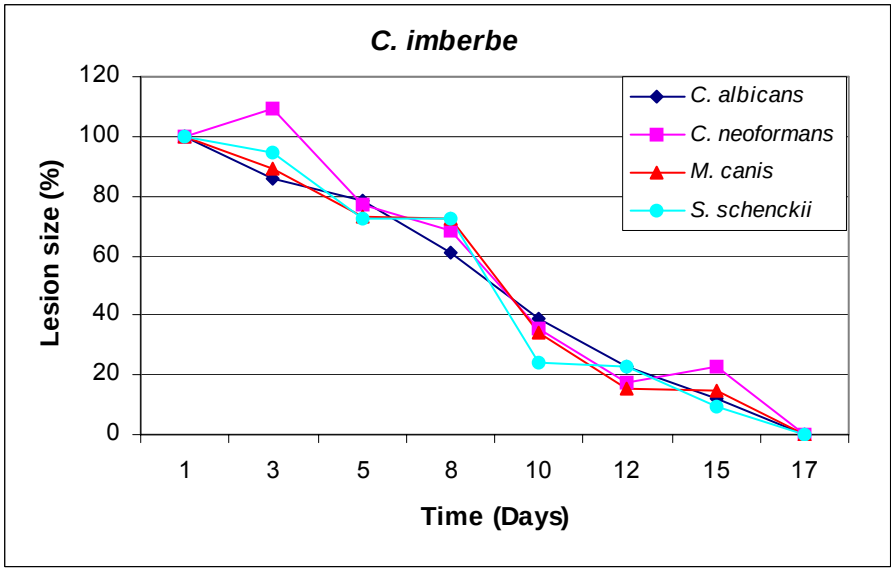


Figure 11.42. Effect of *C. imberbe* acetone extract on fungal pathogens.

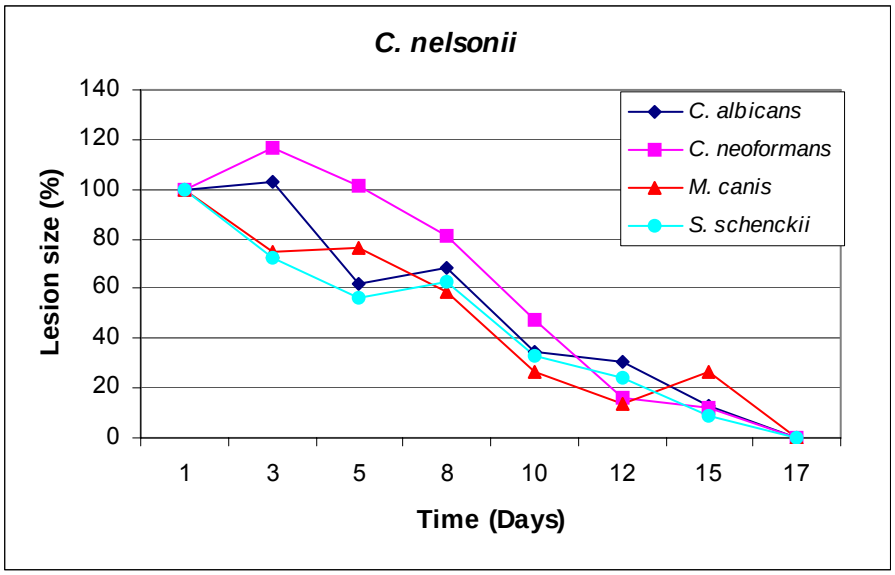


Figure 11.43. Effect of *C. nelsonii* acetone extract on fungal pathogens.

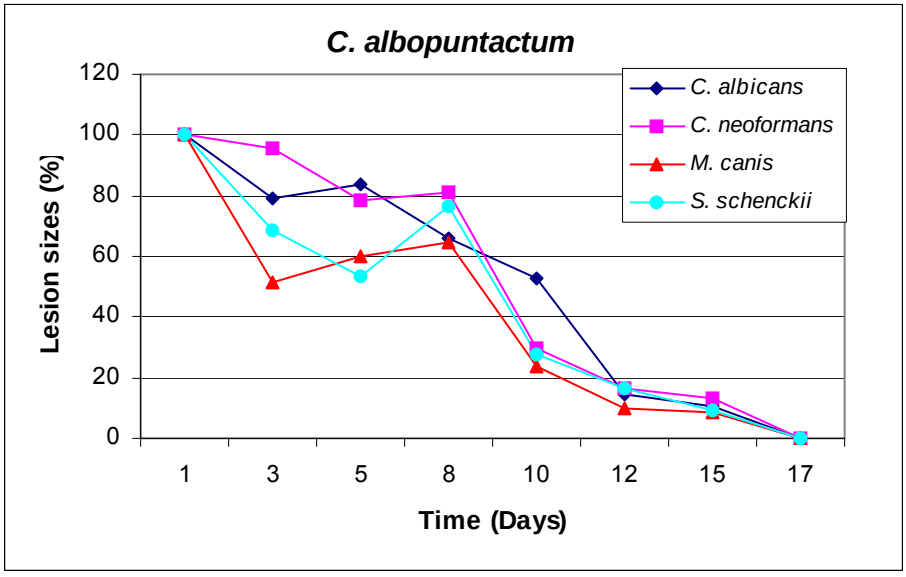


Figure 11.44. Effect of *C. albopunctactum* acetone extract on fungal pathogens.

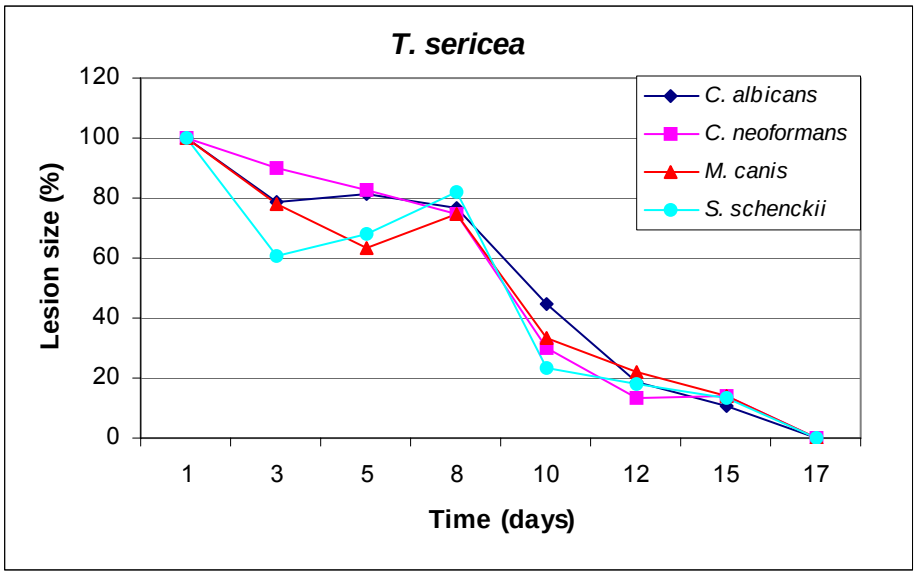


Figure 11.45. Effect of *T. sericea* acetone extract on fungal pathogens.

11.6.5. Histopathological findings

Quantitative histopathological findings were determined on 4 rats, each rat representing the group i.*e.* first six rat were infected with *C. albicans,* rat 7 to rat 12 with *C. neoformans,* rat 13 to 18 with *M. canis* and rat 19 to rat 24 with *S. schenckii* (**Table 11.6a – d**). The clumps of fungi were observed in all treatment and controls in all infected rats with different fungal pathogens. Epithelialisation was observed in the dermus except for the rats infected with *C. neoformans,* whereby it was observed on the epidermus on the wound treated with *C. nelsonii* and the untreated wound. Clumps of degenerating neutrophils, necrotic changes in the upper dermis with loss of epidermis were also observed up to day 17. Scant fungi were noted in all the wounds indicating that infection had occurred, but had generally cleared. Exceptions were treatments with isolated compound and *T. sericea* on the *S. schenckii* infected wounds where there were high numbers of fungi.

11.7. Discussion

These experiments were designed to afford a simple *in vivo* method for comparing the relative effectiveness of various plant extracts against fungal pathogen wound infectious. The duration of therapy and the dosage employed determined the end point of the experiment, and gave an index of their relative effectiveness. The preliminary survey of therapy reveals that a considerable

number of plant extracts are effective locally in the prevention of fungal infection. The choice depended upon consideration of toxicity that was determined in previous chapters.

Table 11.6a. Quantitative histopathological findings of wounds of rats infected with *C. albicans* after topical application of different creams (A= Negative control (Untreated), B= Compound 1 (Mixture of asiatic acid and ajurnolic acid), C=*C. nelsonii* crude extract, D= *C. imberbe* crude extract, E= *C. albopunctactum* crude extract and F= *T. sericea* crude extract and G = Positive control (Amphotericin B))

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epithelialisation	Presence of fungal spores and hyphae
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
1	A	2(++)	0	0	1&2(+++)	3(+)	3(±)	3(±)	3(++)	3(++)	2	1(±)
	B	2(++)	0	0	2(±)	3(++)	3(±)	3(±)	3(+++)	3(++)	2	1(±)
	C	2(+)	2(+)	0	1(+++);2(±)	2(+)	2(±)	2(±)	2(+++)	2(+)	2	1(±)
	D	2(++)	0	0	2(±)	2(++)	2(±)	2(±)	2(+++)	2(++)	2	1(±)
	E	2(++)	0	0	2(±)	2(+++)	2(±)	0	2(+++)	2(+)	2	1(±)
	F	2(+)	2(±)	0	2(±)	2(+)	2(++)	0	2(++)	2(+)	2	1(±)
	G	2(+)	0	0	0	2(++)	0	0	2(+++)	2(+)	2	1(±)

*Neut's= Neutrophils; Mac's= Macrophages

These parameter were marked as follows:

- Severity: -, absent; ±, scant; +, mild; ++, moderate; +++, severe/marked
- Distribution: (1), dermal; (2), dermal and subdermal (i.e. subcutaneous skeletal muscle); (3), locally extensive (dermal and subdermal).
- Epithelialisation: 0, absent; 1, partial; 2, complete

Table 11.6b. Quantitative histopathological findings of wounds of rats infected with *C. neoformans* after topical application of different creams (A= Negative control (Untreated), B= Compound 1 (Mixture of asiatic acid and ajurnolic acid), C=*C. nelsonii* crude extract, D= *C. imberbe* crude extract, E= *C. albopunctactum* crude extract and F= *T. sericea* crude extract and G = Positive control (Amphotericin B))

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epithelialisation	Presence of fungal spores and hyphae
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
1	A	3(++)	2(+)	0	1&2(++)	3(++)	3(±)	0	3(++)	3(++)	1	1(±)
	B	2(++)	0	0	0	2(+)	2(±)	0	2(+)	2(++)	2	1(±)
	C	2(+++)	1(++)	0	1(++);2(+)	2(+++)	2(+)	2(+)	2(+++)	2(++)	1	1(±)
	D	2(++)	2(±)	0	0	2(+++)	2(+)	2(±)	2(++)	2(+)	2	1(±)
	E	3(++)	2(±)	0	2(+)	3(++)	3(+)	3(+)	3(++)	3(++)	2	1(±)
	F	2(++)	2(±)	+	0	2(+)	3(±)	0	3(++)	2(++)	2	1(±)
	G	2(++)	2(±)	±	2(±)	2(++) & 3(+)	2(+)	0	2(+)	2(+)	2	1(±)

*Neut's= Neutrophils; Mac's= Macrophages

These parameter were marked as follows:

- Severity: -, absent; ±, scant; +, mild; ++, moderate; +++, severe/marked
- Distribution: (1), dermal; (2), dermal and subdermal (i.e. subcutaneous skeletal muscle); (3), locally extensive (dermal and subdermal).
- Epithelialisation: 0, absent; 1, partial; 2, complete

Table 11.6c. Quantitative histopathological findings of wounds of rats infected with *M. canis* after topical application of different creams (A= Negative control (Untreated), B= Compound 1 (Mixture of asiatic acid and ajurnolic acid), C=*C. nelsonii* crude extract, D= *C. imberbe* crude extract, E= *C. albopunctactum* crude extract and F= *T. sericea* crude extract and G = Positive control (Amphotericin B))

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epithelialisation	Presence of fungal spores and hyphae
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
1	A	3(++)	3(±)	0	0	3(++)	3(±)	3(±)	3(++)	3(++)	2	1(±)
	B	3(++)	0	0	3(±)	3(+)	3(±)	0	3(++)	3(++)	2	1(±)
	C	2(++)	2(±)	±	2(±)	2(+++)	2(±)	0	2(++)	2(++)	2	1(±)
	D	2(++)	2(±)	0	2(±)	2(++)	2(+)	3(+)	2(++)	2(++)	2	1(±)
	E	2(++)	2(++)	+	2(+)	2(+++)	2(±)	0	2(+++)	2(++)	2	1(±)
	F	2(++)	0	0	0	2(++)	2(+)	0	2(+)	2(++)	2	1(±)
	G	3(+++)	0	0	2(±)	3(++)	2(±)	0	3(+++)	2(++)	2	1(±)

*Neut's= Neutrophils; Mac's= Macrophages

These parameter were marked as follows:

- Severity: -, absent; ±, scant; +, mild; ++, moderate; +++, severe/marked
- Distribution: (1), dermal; (2), dermal and subdermal (i.e. subcutaneous skeletal muscle); (3), locally extensive (dermal and subdermal).
- Epithelialisation: 0, absent; 1, partial; 2, complete

Table 11.6d. Quantitative histopathological findings of wounds of rats infected with *S. schenckii* after topical application of different creams (A= Negative control (Untreated), B= Compound 1 (Mixture of asiatic acid and ajurnolic acid), C=*C. nelsonii* crude extract, D= *C. imberbe* crude extract, E= *C. albopunctactum* crude extract and F= *T. sericea* crude extract and G = Positive control (Amphotericin B))

Rat Nr.	Treatment	Fibrosis	Necrosis	Hypertrophy of subcutaneous muscle fibers	Infiltration of					Angiogenesis	Epithelialisation	Presence of fungal spores and hyphae
					Neut's*	Lymphocytes/ Plasma cells	Eosinophils	Mast cell	Mac's*			
1	A	4(++)	4(±)	0	1(+++)	4(+++)	4(+)	4(+)	4(++)	3(++)	1	1(±)
	B	4(++)	1(++)	0	1(+++)	4(+++)	4(+)	4(±)	4(++)	3(++)	0	1(±)
	C	2(+++)	0	0	2(+)	2(+++)	2(+)	0	2(++)	2(+++)	1	1(±)
	D	2(+++)	2(±)	0	0	2(+++)	2(+)	0	2(++)	2(+++)	2	2(±)
	E	2(+++)	2(+)	0	2(+)	2(+++)	2(+)	0	2(+++)	2(+++)	2	1(±)
	F	2(++)	2(+)	0	0	2(+++)	2(+)	0	2(+)	2(++)	2	2(+++)
	G	4(+++)	4(++)	0	2(++)	4(+++)	4(±)	0	3(+++)	4(++)	1	4(+++)

*Neut's= Neutrophils; Mac's= Macrophages

These parameter were marked as follows:

- Severity: -, absent; ±, scant; +, mild; ++, moderate; +++, severe/marked
- Distribution: (1), dermal; (2), dermal and subdermal (i.e. subcutaneous skeletal muscle); (3), locally extensive (dermal and subdermal).
- Epithelialisation: 0, absent; 1, partial; 2, complete

During the study, I realised that the treatments used had wound healing properties as well. Inngjerdingen *et al*. (2004) reported that some Combretaceae species had wound healing activities when the plant powder was applied directly on the wound. The treatments were usually repeated every day until the wounds were healed. In this study I treated the rats with the selected leaf extracted every second day. Wound healing is a multifactorial process where microbial infections and the formation of free radicals may contribute to retard or inhibit its resolution. Free radicals can oxidise the endogenous inhibitors or proteases, this reduces their ability to inhibit elastase and the proteases responsible for the deterioration of the extra-cellular matrix (Kudi *et al*., 1999). The possibility of the wound healing due to free radicals was eliminated in previous chapters, where the antioxidant activity of the selected plants was studied. The selected plant extracts and compound **1** did not have antioxidant activity based on the DPPH assay.

The main focus of the chapter was to investigate the antifungal activities of the four selected acetone extracts (*C. nelsonii*, *C. imberbe*, *C. albopunctactum* and *T. sericea*) and compound **1** on wounds infected with *C. albicans*, *C. neoformans*, *M. canis* and *S. schenckii*. The clinical treatment of skin infected with fungi has become a major problem especially in immuno-compromised patients. Therapeutic agents selected for the treatment of infected wounds had ideally shown antifungal activity on *in vitro* studies. I also checked whether these agents would improve phases of wound healing without producing deleterious side effects.

This study describes some unique features with respect to the therapeutic effect of leaf extracts of selected plants on dermal wound of rats infected with fungal pathogens. Plants products are potential agents for wound healing and the treatment of fungal infections (Masoko *et al*., 2005 and Masoko *et al*., 2006) and largely preferred because of their widespread availability, low toxicity and their effectiveness as crude preparations. We have reported that *Combretum* and *Terminalia* species have antifungal activity (Masoko *et al*., 2005 and 2006 and Masoko and Eloff, 2005 and 2006). These findings prompted us to further investigate *in vivo* activity of the four most active extracts. The study was divided into 2 pilot studies and the main experiment.

Pilot study I (Local irritancy and wound healing study)

In this pilot study the created wound were not infected. I wanted to establish whether an aqueous cream used as vehicle had no irritant effect on the rat, to determine irritant/ tolerance effect of 10 % and 20 % crude extracts in cream based on rats and to determine degree of wound healing within three weeks. Rats did not show any sign of irritancy to any of the treatments. They

maintained their normal temperature and often an initial weight loss gained, indicating that they tolerated the procedures well and that systemic infection did not occur. There was no swelling and ulcerations as well. The wounds were 100% healed after three weeks.

I found that the extracts of *C.imberbe* and *C. nelsonii* were superior in wound healing abilities. The wound treated with *C. imberbe* extracts healed faster than the control. Wound without treatment lesion size increased after day 3 but it started decreasing after day 5, at day 10 it was within the same range as others. The wound without treatment and cream only lesion size increased after Day 10. *C. nelsonii* extract healed the wound faster but the controls lesion sizes increased after Day 8. In the wound treated with *T. sericea*, the cream only healed the wound faster, before Day 8 the extract lesion size decreased faster than the cream only but after Day 8 wound treated with cream only had smaller wound size. From these results the use of 20% extracts was recommended as they ought to have a better antifungal effect based on MIC values and did not have a negative effect on wound healing as was the case of *C. albopunctactum* where the 10% extract was found to have a slightly negative effect on wound healing. Crude extracts were better in reducing the erythema of the wounds. The average erythema of wounds treated with *C. imberbe* was lower than the other extracts. After Day 10 the wound without treatment had high average erythema formation. The crude extracts and had lower average erythema than the untreated wounds. Exudate formation was more formed from extracts used. All the treatments decreased the exudate formation after 12 days. Crust formation took longer in the untreated wounds.

Pilot study II (Infection with different pathogens)

The aim of this part of the study was to determine the effect of plant extracts in aqueous cream on fungal infection of wounds and subsequent healing. Since three of the four fungi tested are known to cause deep or systemic infection, it was also necessary to determine whether this occurred. From the four acetone extracts used, amphotericin B was added as the positive control and the negative control was the infected wound but not treated. The rats were left for 48 hours after infection before topical treatment was applied. This was done to encourage infection. All 12 rats lost weight (**Figure 11.10**) within 48 hours of infection and started picking up weight after the first treatment, with exception of Rat 11 which continued to lose weight. Therefore Rat 11, infected with *S. schenckii* was euthanased. All rats infected with *S. schenckii* had a nasal discharge. Histopathological results of the lymph nodes of Rat 10 showed moderate lympoid hyperplasia with infiltration of moderate numbers of lymphocytes, plasma cells, mild number of mast cells and

eosinophils. Lymph node was large (4x2 mm). (MD: Moderate lymph node hyperplasia with lymphodenitis, mild, subacute).

After Rat 11 was terminated, necropsy was performed and the following observations were made: stomach and intestine were empty which means the rat was not eating and that led to weight loss; lymph nodes were enlarged, and lungs showed a diffuse pneumonia, other organs (lliver, thymus, heart, spleen, kidney and pancreas) were normal. Specimens were sent to Pathology Section for histopathological examination and some to Bacteriology Section. After bacteriological examination it was found that the nasal discharge was not due to fungi but bacterial infection. It was then concluded that the rat had pneumonia. Histopathological results of the lungs showed moderate congestion, moderate to severe interstitial pneumonia with infiltration of polymorphonuclear leukocytes and moderate alveolar oedema. No significant lesions in other organs examined other than moderate generalised congestion were observed.

Temperature of the remaining 11 rats was within the expected range of 34 to 37 ^{o}C (**Figure 11.11**).

While all rats survived for the duration of the experiment, technical difficulties reduced the sample size to 11. Wound sites were evaluated for erythema, exudate and crust formation during the period of the study. There were no signs of erythema surrounding any wound sites throughout the experiment. All of the dressings applied during the experiment did not adhere to the wound bed. Since the experimental dressings remained securely fastened, all wounds were therefore included in the calculations.

Crude extracts and amphotericin B reduced the erythema (**Figure 11.13**) of the wounds. The average erythema of wounds treated with *C. imberbe* was lower than the other extracts including amphotericin B. The average erythema of amphotericin B increased on Day 5 and again on Day 10 but went down after Day 10. The crude extracts and amphotericin B had lower average erythema than the untreated, therefore they can be regarded as being effective in reducing erythema caused by fungal infection.

More exudate was formed (**Figure 11.14**) from treatment with amphotericin B followed by *C. albopunctactum*. All the treatment decreased the exudate formation after 12 days. This results correlate with the one of erythema. Untreated wounds were the last to form crust (**Figure 11.15**), which was expected. Wounds treated with *C. nelsonii* were the first ones to form crust.

There was difference in lesion size of wounds infected with different fungal pathogens. In wounds infected with *C. albicans* (**Figure 11.12a**) and in *S. schenckii* (**Figure 11.12d**), amphotericin B decreased lesion size quicker compared to crude extracts. Wounds treated with *C. albopunctactum* had the least effect in reducing the lesion sizet. In *C. neoformans* (**Figure 11.12b**) *T. sericea* was the best in reducing lesion size, and in *M. canis* (**Figure 11.12c**), *C. nelsonii* was the best.

All these parameters showed that the crude extracts and amphotericin B were effective in decreasing formation of the exudate, increasing crust formation and that they have antifungal activities even when use in *in vivo* studies.

A specimen sample of skin tissues of each group of rats were taken out from the healed wounds of the animals in the above excision wound model for histopathological examination. All lesions showed wound healing activity with few or no fungal hyphae. Lesions of Rat 11 which was euthanased before the end of the experiments had high presence of fungal hyphae because it didn't finish treatment.

In pilot study 2 all the wounds healed almost at the same time even the untreated, therefore it was concluded that it was due to the strong immune system of the rats and wound contraction being the primary means of wound closure.

Confirmation study (Main study)

There was no evidence of systemic infection caused by the irritant effects in the second pilot study, therefore I continued with the main study where 24 rats were used. The rats were divided in four groups based on fungal pathogens. All the treatments used in the second pilot study were used but the compound 1 a mixture of asiatic acid and arjunolic acid was added. In the main study rats were immunocompromised by subcutaneous injection of 500 µg of estradiol valerate. Estradiol pretreatment is known to inhibit innate and acquired immune defenses (Carlsten *et al*., 1991).

Acetone extracts of leaves of *C. nelsonii*, *C*. *albopunctactum*, *C. imberbe* and *T. sericea* possess remarkable growth inhibitory activities against fungal pathogens (**Chapters 5** and **6**). Acetone extracts of leaves and mixture of asiatic acid and arjunolic acid demonstrated wound healing properties comparable with that of antibiotic powder (amphotericin B). Even the untreated wounds

healed but not at the same rate as the treated wounds. It is important to note that throughout the period of wound treatment, the extracts did not cause irritation or pain to the animals as the rats neither showed any signs of restlessness nor scratching/biting of wound site when the extracts were applied.

All the rats lost weight in this study until Day 12 and started increasing weight from Day 15. It was assumed that it was due to immunocompromising the rats. After 3 weeks of the experiment, bandages were removed and all rats were kept for another week. Thereafter it was found that all have gained weight except Rat 6 where the mass remained constant. The temperatures were also within the normal ranges. All other parameters measured were the same as explained in second pilot study.

Mixture of asiatic acid and arjunolic acid (**Figure 11.40**) and *C. nelsonii* (**Figure 11.42**) were very active against *M. canis* and *S. schenckii*. Wounds infected with *C. neoformans* took longer time to reduce size. Amphotericin B (**Figure 11.41**) had almost same activity against all tested pathogens. *C. imberbe* (**Figure 11.42**) and *T. sericea* (**Figure 11.45**) had similar impact on the pathogens. *C. albopunctactum* (**Figure 11.44**) was very active against *M. canis*.

After 3 weeks all 24 rats were euthanized with CO_2 and necropsies performed. From rats infected with *C. albicans* (Rat 1 to 6) organs were taken from Rat 1 for histopathological studies. The lungs of Rat 3 lungs had block specks and the intestines were blue. The lungs were taken to Bacteriology lab for culturing. Organs of Rat 7 together with lungs of Rat 10 were taken for histopathological studies. Organs in group 2 (rats infected with *C. neoformans*) (Rats 7 to 12) were normal. Again organs of Rat 13 in group 3 (rats infected with *S. schenckii*) (Rats 13 to 18) and liver of Rat 16 were taken to Pathology section, together with organs of Rat 19 and the left lymph node of Rat 23 in group 4 (rats infected with *M. canis*) (Rats 19 –24).

Unfortunately some of the results of histopathological studies are not reported here due a delay in evaluation of the samples by the pathologists at Onderstepoort. Only results of four rats were discussed and some of the results will be included in a publication to be written. The following comparisons were made from histopathology results: generally more fibrosis with crude extracts, but an exception is Amphotericin B treatment of *S. schenckii*. The other parameters of healing i.e. angiogenesis and epithelisation were present or complete with the exception of S. schenckii infections where the extracts performed better. Possibly a synergistic effect. No noticeable differences in wound necrosis. Neutrophils were evident and in deeper levels in the untreated.

Plasma cells, lymphocytes and macrophages were the most predominant cell types. These are the most predominant cells in fungal infections and are also the more common ones in chronic infections. Macrophages are also the most active cell in wound healing acting as potent wound debriders.

A close examination of tissue sections revealed that there was marked infiltration of lymphocytes, eosinophils, neutrophils, mast cells and macrophages and enhanced proliferation of fibroblasts as a result of treatments. Increased cellular infiltration observed from hematoxylin and eosin (H&E) staining in treated rats may be a result of chemotactic effect enhanced by the extract, which might have attracted inflammatory cells toward the wound site. Increased cellular proliferation may be a result of the mitogenic activity of the plant extract, which might have significantly contributed to healing process. Early dermal and epidermal regeneration in treated rats also confirmed that the extract had a positive effect toward cellular proliferation, granular tissue formation and epithelialisation.

Histopathological studies of the wounds bring prominent aspects, that both the antibiotic and the plant extracts individually are capable of healing the wound. What needs to be further worked out is the relative quantities of plant extracts that are necessary for optimal effect, the maximum period for which the extract can be kept stable. Whether such preparations should be and could be sterilized is also an aspect, which may be pertinent. And finally the most important question that arises from the study is “which constituent of extracts evokes wound healing effect”. Bioactivity may also be associated with some other components such as prostaglandin precursors or some other molecule; an identification and isolation of such molecule may also be desirable. Until such a possibility is brought to reality, plant extracts in their natural form may be our only choice. The isolated mixture of asiatic acid and arjunolic acid from *C. nelsonii* which showed high activity in *in vitro* studies did not have the same effect in *in vivo* studies. Rat infected with *M. canis* and treated with the mixture, formation of the fibroblast and infiltration of the cells occurred in deeper tissues. Same with rat infected with *S. schenckii*, wound treated with mixture, formation of fibroblast occurred in extensive deeper tissues and there was delay of epithelisation.

In some instances there were more traces of fungal hyphae in wound treated with amphotericin B compared to extracts i.e. rat infected with *S. schenckii*. Maybe this antibiotic was not the right choice, as the control and the presumption is that any other antibiotic could have behaved in a manner similar to amphotericin B but certainly that needs to be experimentally confirmed. Amphotericin B was selected as it is the most potent broad spectrum antifungal that is available.

However, it is not usually used as a topical treatment, the inidazoles are better for that. These aspects if at all considered important will have to be studied separately.

Some organ samples were also studied. In most instances the lungs showed diffuse, sub-acute mono-morphonuclear (mainly lympho-plasmacytic, with lesser numbers of macrophages) interstitial pneumonia with moderate to severe, diffuse haemorrhage. Spleen showed mild red pulp hyperplasia with many haemosiderin laden macrophages. Small intestine showed mild to moderately increased numbers of eosinophils within the lamina proprium of the small intestine wall. Some fungal spores were seen within the lumen, but no signs of any reaction were visible. Prescopular lymph nodes of Rat 23 infected with *S. schenckii* showed moderate cortical hyperplasia. A handful (4 or 5) of fungal spores was seen in two foci just below the capsule, two of them within a macrophage phagosome. This is a common mode of spread of this fungus and as was exhibited rodents are particularly susceptible to *S. schenckii* infections.

The pulmonary lesions are commonly seen in experimental rodents, and are possibly as a results of various environmental factors/stressors. The moderate amounts of haemosiderin within splenic macrophages may possibly be a result of the pulmonary haemorrhage or the wounds created for the experiment. The few fungal spores within the lymph nodes of rat 23 are most likely a result of lymphatic drainage from the experimental wounds as opposed to direct infection, as there were no signs of inflammation in the tissues surrounding the lymph nodes. The occasional fungal spores seen on all of the epidermal surface of the skin samples can be regarded as incidental. These spores were always in association with normal skin, hardly ever would they be seen over the lesion itself.

Lung tissues of Rats 3 and 10 were cultured on blood agar and MacConkey agar. After Gram staining, wet preparation and haemolytic tests, the conclusion was reached to the effect that the lungs were infected with a Streptococcus sp an opportunistic pathogen.

Healing is a physiological process and does not normally require much help but still wounds cause discomfort and are prone to infection and other complications. Therefore, use of agents expediting healing is indicated. Further, some diseases like diabetes, immunocompromised conditions, ischaemia and conditions like malnourishment, ageing, local infection, local tissue damage due to burn or gun shot wounds lead to delay in healing. Such conditions often require the use of agents, which can facilitate the healing process (Mensah *et al*., 2001).

The rat model descripted in these studies was used for the first time to test for fungal pathogens in our group. I have observed that rats must be immunocompromised to ensure a localized fungal infection. I have demonstrated that all the extracts used have antifungal activity. Although amphotericin B gave better results the isolated mixture of asiatic acid and arjunolic acid gave promising results and thus can be considered for future treatment due to the toxicity of amphotericin B. Exudate formation, erythema and lesion size are good parameters to consider for wound healing. Ulcerations did not occur indicating that wound healing progressed normally. Generally the technique works and it can be used as the model for future studies. The main objective was to test plant extracts activity on infected animals. I didn't experienced any systemic infection except in one rat and the infection was not from the fungal pathogens used.

11.8. Conclusion

The results of this study have confirmed the antifungal potentials of crude extracts and wound healing properties of selected plants and mixture of asiatic acid and arjunolic acid on rat model. The extracts of these plants may possibly further be developed into phytomedicines for the management of septic wounds, because they did not show any signs of irritancy to rats. The model used was successful as there were no systemic infections in all the rats and the wounds healed within three weeks.

Rat models infected have often been used for the determination of the wound healing properties of various dressings and topical formulations, it is generally acknowledged that these models may not reflect accurately the biological processes occurring in humans during wound healing, likely due to significant inter-species skin differences in morphology and function (Dorsett-Martin, 2004). Still there is a potential to consider using it for animal and human infections.

In conclusion, treatment from the leaves of selected plants exhibited significant pro-healing activity in the infected wound when topically applied on rats by affecting various stages of healing process.

CHAPTER 12

General discussion and Conclusion

Six *Terminalia* species and twenty-four *Combretum* species were evaluated for antifungal activity and antioxidant screening based on the use of the two genera in traditional medicinal treatments for both domestic animals and humans in southern Africa, as well as their availability. The total percentages of the *Terminalia* species extracted using different solvents (acetone, hexane, DCM and methanol) were determined. Methanol extracted a greater quantity of plant material. There was a major difference in the methanol extractability of *T. gazensis* leaves compared with all the other species. This difference is not related to the sectional division of the species (Carr, 1988).

The leaves of Combretaceae family are known for their pharmacological activity and in this study, I have shown that many extracts also contain several anti-oxidant compounds. Methanol and acetone extracted the largest number of different antioxidant compounds based on DPPH TLC. *In vitro* studies coupled with the phytochemical analysis confirm that the extracts possessed potential antioxidant activity.

The solvent tolerance of the microorganisms was tested using the following solvents; DMSO, acetone, methanol and ethanol. In order to determine the maximum concentration at which different solvents would allow the test microorganisms to reach normal growth, different concentrations from 10 to 100% were used. Based on MIC DMSO was the least toxic of the solvents used with an average MIC of 616 mg/ml (56%) followed by acetone 512 mg/ml (64%), methanol 320 mg/ml (40%) and ethanol 304 mg/ml (38%). The danger of using ethanol or methanol is evident from the inhibition by 20% ethanol or methanol of *M. canis* and *S. schenckii*. In general the two moulds (*M. canis* and *S. schenckii*) appeared to be most resistant. Acetone was the only extractant that could be used with a safety margin at a 50% concentration.

The serial dilution microplate method used for detecting antibacterial activity worked well with fungi after slight modifications. The antifungal activity of some of the extracts were at concentrations that could be therapeutically useful already, leading to the distinct possibility that some of the extracts may be applied clinically for dermatophyte infections e.g. *M. canis.* If one extrapolates from *in vitro* to *in vivo* activity especially in topical applications, it means that an acetone leaf extract from 1 g of *T. sericea* leaves diluted to 2.7 L would still inhibit the growth of *M. canis*. Extracts from other species had values as high as 6.05 L/g. The results of this study indicate that the *Combretum* species assayed possess substantial antifungal

properties. If there are no synergistic effects and the antifungal compounds comprise 0.1 % of the total mass, the antifungal compound should have an MIC of 0.02 to 0.2 µg/ml. The results of this study support several of the traditional medicinal uses of *Combretum* species all over Africa. I found that adding of INT from the beginning of the MIC experiment allowed the early detection of growth of the fungi tested, which overcomes the difficulties of previous methodologies. The acetone extracts with the highest activity were from *C. imberbe*, *C. nelsonii*, *C. albopunctactum* and *T. sericea*, and were considered to be good candidates for the *in vivo* tests.

T. sericea holds promise for isolating antifungal compounds, because hexane and DCM extracts of *T. sericea* have compounds inhibiting growth of all pathogens, especially the compound at R_f value of 0.46. It is followed by *T. brachystemma*, which also had compounds (R_f = 0.46), which inhibited growth of all tested pathogens.

Extracts of *Combretum* species in the Section *Hypocrateropsis* had high number of total active antifungal compounds, ranging from 56 to 62. These values were high because, in some instances, the same compound was active against different fungi and it was observed in more than one of the three TLC solvent systems used and was present in more than one extract. For comparative purposes we counted all visible compounds. That explains high numbers reported. *C. celastroides* ssp. *celastroides* and *C. clelastroides* ssp. *orientale* possess 62 active compounds each. Section *Angustimarginata* follows section *Hypocrateropsis* with active compounds ranging from 37 to 43, more active compound being in *C. nelsonii* (43). *Metallicum* section, which is made up of *C. collinum* ssp. *suluense* and *C. collinum* ssp. *taborense* did not contain active compounds against all tested microorganisms. *C. zeyheri* in *Spathulipetala* section contained 26 active compounds. In *Ciliatipetala* section, *C. albopunctactum* contained more active compounds than other species in the same section, with 43 compounds. It is followed by *C. apiculatum* ssp. *apiculatum* and *C. petrophilum* with 20 and 14 compounds respectively. In *Breviramea* section we had only one species, *C. hereroense,* with 16 active compounds. *Connivetaia* section species, C*. microphyllum* and *C. paniculatum* had a similar number of active compounds, 7 and 8 respectively. *C. mossambicense* and C. *acutifolium* in *Poivrea* section, had the most active compounds, which were 25 for each species, and *C. bracteosum* had 14. That was the biggest difference in the section as compared to others where species in the section had almost the same number of compounds.

C. neoformans was the most sensitive organism against all *Combretum* species, with 367 compounds active against it. This was followed by *C. albicans* and S*. schenckii* with 339 and

314 compounds active against them respectively. *M. canis* had 298 compounds active against it, followed by *A. fumigatus* with only 192 compounds being active against it.

The results indicate that bioautography is probably the most important detection method for new or unidentified antifungal compounds, because it is based on the biological effects of the substances under study. In the study of biologically active compounds from natural sources, it is evident that rapid and sufficient detection of such compounds is a critically important aspect of the discovery process. Bioautography is a method that makes it possible to localise antifungal activity on the chromatogram. Bioautography results confirmed the low MIC values obtained in the previous chapters (Masoko *et al*., 2005).

Bioassay-guided fractionation on silica gel 60 (63-200 µm) in column chromatography resulted in the successful isolation of the highly active compound I (later shown to be a 3:4 mixture of asiatic acid and arjunolic acid) from the leaves of *C. nelsonii*. This very active "Compound **1**" was used in the *in vivo* assay.

The *in vitro* cytotoxicity of three *Combretum* and one *Terminalia* species extract were investigated. Responses varied for the different extracts and between the two assays, but brine shrimps were less sensitive than the Vero monkey kidney cells. Only acetone extracts were used, because it was found not to be toxic on fungi. In this study it was used as control and it was found not to have effect on *A. salina* nauplii and Vero cells at the concentrations used. These values were all higher than the berberine chloride control of 4.35 µg/ml. The value was higher than that of berberine in the literature of 0.141-0.148 µg/ml (Vennestrom and Klayman, 1988). *C. imberbe* extracts was the least toxic with an LC_{50} of 168.6 µg/ml and *C. nelsonii* extracts were the most toxic with an LC_{50} of 75.7 µg/ml, LC_{50} values for *C. albopunctactum* and, *T. sericea* were 102.9 and 121.7 µg/ml respectively (Masoko, 2006)

The relative safety margins (RSM) in the MTT assay were high compared to those in the brine shrimp assay. RSM of *C. imberbe*, *T. sericea* and *C. albopunctactum* in *C. albicans* were 0.09, 0.16 and 0.19 respectively. *C. nelsonii* and *T. sericea* had high RSM values against *S. schenckii* and *M. canis*, therefore high amount of the material must be used in treatment.

In the *in vivo* experiments the mixture of asiatic acid and arjunolic acid (Compound **1)** and *C. nelsonii* extracts were very active on *M. canis* and *S. schenckii.* Wounds infected with *C. neoformans* took longer time to reduce in size. Amphotericin B had almost same activity against all tested pathogens. *C. imberbe* and *T. sericea* extracts had similar impact on

pathogens. *C. albopunctactum* extracts were very active against *M. canis*. Plants used for the treatment of wounds can have different properties like anti-inflammatory, antimicrobial, healing, analgesic, haemostatic and immuno-modulating activities. The immune system is an important factor in the process of healing of wound.

Acetone extracts of leaves of *C. nelsonii*, *C. albopunctactum*, *C. imberbe* and *T. sericea* possess remarkable growth inhibitory activities against fungal pathogens. Acetone extracts of leaves and isolated compound demonstrated wound healing properties comparable with that of the antibiotic amphotericin B. Even the untreated wound healed but not at the same rate as the treated wounds. As earlier suggested, healing in this untreated group may be due to a strong immune system. It is important to note that throughout the period of wound treatment, the extracts did not cause irritation or pain to the animals as the rats neither showed any signs of restlessness nor scratching/biting of wound site when the extracts were applied.

After structure elucidation, the isolated compound were terminolic acid and "Compound **1**". Terminolic acid was not studied further as "Compound **1**" was present in a high concentration and was very active. The structure of Compound **1** was elucidated by using NMR and MS. It was found that Compound **1** is a mixture (3:4) of two isomers by intensities and number of its NMR signals. The MS gave a molecular ion peak at *m/z* 487 (M–H) relating to $C_{30}H_{48}O_5$. The isomers were labeled Compound **1a** and **1b**. ^{1}H NMR data of **1a** and **1b** were similar, with the main differences noted in the DEPT experiment, where **1a** and **1b** possessed ten and nine CH_2, six and eight CH, respectively, with seven methyl groups for each compound. The ^{13}C NMR spectra showed 30 carbon atoms for each. The chemical shift of the carbon atoms C_{12} and C_{13} at δ 122.4; 144.4 and δ 125.7; 138.8 for **1a** and **1b**, respectively, suggested the presence of two classes of triterpenes, the oleanane and ursane. The ^{1}H and ^{13}C NMR data of Compound **1** were similar to those recorded for arjulonic acid **1a** and asiatic acid **1b** (Facundo et al., 2005). Asiatic acid and arjunolic acid are well known but it is the first that the two compounds are isolated from *C. nelsonii*.

A variety of triterpenoids have been isolated from *Combretum spp.* (Rogers and Verotta, 1996). Terpenes or terpenoids are active against bacteria and fungi (Taylor *et al.*, 1996). From our group, Martini *et al*., (2004a) isolated and characterized seven antibacterial compounds. Four were flavanols: kaemferol, rhamnocitrin, rhamnazin, quercitin 5,3 -dimethylether] and three flavones apigenin, genkwanin and 5-hydroxy-7,4'-dimethoxyflavone. All test compounds had good activity against *Vibrio cholerae* and *E. faecalis*, with MIC values in the range of 25-50 μg/ml. Angeh (2005) isolated 8 compounds with antibacterial activity

from *Combretum* section, Hypocrateropsis. All eight compounds had moderate (MIC of 60 µg/ml) to strong (10 µg/ml) antibacterial activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Mycobacterium vaccae*.

The mixture of asiatic acid and arjunolic acid had excellent antifungal activity against all tested pathogens i.e. Mixture of asiatic acid and arjunolic acid had the lowest MIC value against *M. canis* and *S. schenckii* which was 0.2 µg/ml, followed by *C. neoformans* which was 0.4 µg/ml. *A. fumigatus* was the most resistant with the highest MIC value of 1.6 µg/ml. LC_{50} of compound A was 10.58 µg/ml, a value that is similar to the reported LC_{50} value of the berberine standard (10 µg/ml), this is an indication that it is as toxic as berberine. Because it is effective at such a low dose the therapeutic index of 50 may be acceptable.

This study indicates that the C*ombretum* and *Terminalia* species assayed possess substantial antifungal properties. This explains the use of these plants in folk medicine for the treatment of various diseases related to fungal infections. The aims of the project have largely been attained and it appears that there may be scope for continuing work on plant extracts of *C. nelsonii* or *C. imberbe* due to its higher therapeutic index.

CHAPTER 13

References

Abadi, J., Nachman, S., Kressel, A.B., and Pirofski, L., 1999. Cryptococcosis in children with AIDS. Clin Infect Dis. **28**, 309-313.

Ahmad, I. and Beg, A.Z., 2001. Antimicrobial and phytochemical studies of 45 Indian medicinal plants against multi-drug resistant human pathogens. J. of Ethnopharmacology, **74**, 113-123**.**

Alcorn, J. B. 1981. Huastec Noncrop Resource Management: Implications for Prehistoric Rain Forest Management. *Human Ecology* **9,** 395-417.

Alexander, D. M., Bhana, N., Bhika, K. H., and Rogers, C. B., 1992. Antimicrobial testing of selected plant extracts from *Combretum* species. S. Afr. J. Sci. **88**, 342 - 344.

Aly, R., Hay R.J., Del Palacio A, and Galimberti R., 2000. Epidemiology of tinea capitis. Med Mycol. **38**, 183-188.

Aman, S., Haroon T.S., Hussain I, Bokhari M.A., and Khurshid K., 2001. Tinea unguium in Lahore, Pakistan. Med Mycol. **39**, 177-180.

Amaral, J. A., Ekins, A., Richards, S. R., and Knowles, R. 1998. Effect of selected monoterpenes on methane oxidation, denitrification, and aerobic metabolism by bacteria in pure culture. Appl. Environ. Microbiol. **64,** 520-525.

Angeh, J. 2005. Isolation and characterization of antibacterial compounds present in members of *Combretum* section, Hypocrateropsis. PhD Thesis, University of Pretoria.

Arnao, M. B., Cano, A. and Acosta, M. 2001. The hydrophilic and lipophilic contribution to total antioxidant activity. Food Chemistry, **73**, 239-244.

Arikan, S., Uzun O., Cetinkaya Y., Kocagoz S., Akova M. and Unal, S. 1998. Primary cutaneous aspergillosis in human immunodeficiency virus- infected patients: Two cases and review. Clin Infect Dis. **27**, 641-643.

Aviles, P., Aliouat, E.M., Martinez, A., San Roman, R., Guillen, M.J., Ferrer, S., Gomez delas Heras, F. and Gargallo-Viola D. 1998. Pharmacokinetics/ Pharmacodynamic (PK/PD) study of sordarin derivatives against *Pneumocystic carinii* pneumonia in a cortcosteroid-treated rat model. American Society for Microbiology. J-56.

Ayafor, J. F., Tchuendem, M. H. K. and Nyasse, B. 1994. Novel bioactive diterpenoids from *Aframomum aulacocarpos*. J. Nat. Prod. **57,** 917-923.

Baba-Moussa, F., Akpagana, K. and Bouchet, P. 1999. Antifungal activities of seven West African Combretaceae used in traditional medicine. J. of Ethnopharmacology, **66**, 335-338.

Balick, M.J., Arvigo, R. and Romero, L., 1994. The development of an enthnobiomedical forest reserve in Belixe: its role in the preservation of biological and biological and cultural diversity. Conservation Biology, **8**, 316-317.

Balkovec, J.M. 1994. Lipopeptide antifungal agents. Exp. Opin. Invest. Drug, **3**, 65 –82.

Ballou, C.E. 1990. Isolation, characterization and properties of *Saccharomyces cerevisiae mnn* mutants with non-conditional protein glycosylation defects. Meth. Enzym. **185**, 440 – 470.

Basséne, E., Mahamat, B., Lo, M., Boye, C. and Faye B. 1995. Comparaison de l'activite antibacterienne de trios Combretaceae *Combretum micranthum, Guiera senegalensis* et *Terminalia avicennioides*. Fitoterapia, **66**, 86-87.

Berkada, B. 1978. Preliminary report on warfarin for the treatment of herpes simplex. J. Irish Coll. Phys. Surg. **22,** 56.

Bhat, S.K. and Saxena, V.K., 1979. Efficacy of successive extracts of seeds of *Anogeissus leiocarpus* against some human pathogenic fungi. Indian drugs, **16**, 263-264.

Bizimenyera, E.S., Swan, G.E., Chikoto, H. and Eloff, J.N., 2005. Rationale for using *Peltephorum africanum* (Fabaceae) extracts in veterinary medicine? Journal of the South African Veterinary Association. **76**, 54 – 58.

Björklund, E., Bøwadt ,S. and Nilsson, T. 2000. Trends Anal. Chem. **19**, 434.

Boonchird, C. and Flegel, T.W. 1982. *In vitro* activity of eugenol and vanillin against *Candida albicans* and *Crytococcus neoformans*. Canadian Journal of Microbiology, **28**, 1235-1241.

Borenfreund, E. and Puerner, J.A. 1985. Toxicity determined *in vitro* by morphological alterations and neutral red absorption. *Toxicology Letters,* **24,** 119–124.

Boswell, G.W., Buell, D. and Berersky, I. 1998. AmBiosome (Liposomal Amphotericin B): a comparative review. J Clin. Pharmacol., **36**, 589-592.

Bouchet, N., Levesque, J., Blond, A., Bodo, B. and Pousset, K. 1996. 1,3-di-*O*-Galloyiquinic acid fron *Guiera senegalensis*. Phytochemistry. **42**, 189 – 190.

Bouchet, N., Barrier, L. and Fauconneau, B. 1998. Radical scavenging activity and antioxidant properties of tannins from *Giuera senegalensis* (Combretaceae), Phytotherapy Research, **12**, 159 - 162.

Brantner, A., Males, Z., Pepeljnjak, S. and Antolic. A. 1996. Antimicrobial activity of *Paliurus spina-christi* mill. J. Ethnopharmacol. **52,**119 -122.

Breytenbach, J. C. and Malan, S. F. 1998. Phamacochemical properties of *Combretum zeyheri*. S. Afri.J.Sci. **85**, 372-374.

Byrd, D. R., el-Azhary, R.A., Gibson, L.E., and Roberts, G.D., 2001. Sporotrichosis masquerading as *pyoderma gangrenosum*: case report and review of 19 cases of sporotrichosis. J Eur Acad Dermatol Venereol. **15**, 581- 584.

Cabib, E. 1987. the synthesis and degradation of chitin. Adv. Enzymol. Relat. Areas Mol. Biol. 59, 59 –101.

Cabib, E., Dragon, T. and Drgonova, J., 1997. The yeast cell wall, a dynamic structure engaged in growth and morphogenesis. Biochem. Soc. Trans. **25**, 200 - 204.

Cai, Y.Z., Sun, M. and Corke, H. 2003. Antioxidant activity of betalains from plants of the Amaranthaceae. Journal of Agricultural and Food Chemistry **51(8),** 2288 - 2294.

Campos, P., Arenas, R., and Coronado, H., 1994. Epidemic cutaneous sporotrichosis. Int. J. Dermatol. **33**, 38 - 41.

Cardenas, M.E., Sanfridson, A., Cutler, N.S. and Heitman, J. 1998. Signal-transduction cascades as targets for therapeutic intervention by natural products. Trends Biotechnol. **16(10),** 996 -1000.

Carlson, D., Haurie, A and Leizarowitz, A. 1991. Infinite Horizon Optimal Control: Deterministic and Stochastic Systems (second ed.), Springer, Berlin.

Carr, J.D., 1988. Combretaceae in Southern Africa. The Tree Society of Southern Africa, Johannesburg.

Casagrande, D. G. 2000. Human Taste and Cognition in Tzeltal Maya Medicinal Plant Use. Journal of Ecological Anthropology. **4**, 57-69.

Casley-Smith, J. R. and Casley-Smith. J. R. 1997. Coumarin in the treatment of lymphoedema and other high-protein oedemas, p. 348. *In* R. O'Kennedy, and R. D. Thornes (ed.), Coumarins: biology, applications and mode of action. John Wiley & Sons, Inc., New York, N.Y.

Chaurasia, S. C. and Vyas, K. K. 1977. In vitro effect of some volatile oil against *Phytophthora parasitica* var. *piperina*. J. Res. Indian Med. Yoga Homeopath. **1977,** 24-26.

Chee K.K., Wong, M.K. and Lee, H.K. 1997. Environ. Monit. Assess**. 44**.

Cheng H.Y., Lin, T.C., Yu, K.H., Yang, C.M. and Lin, C.C. 2003. Antioxidant and free radical scavenging activities of *Terminalia chebula*. Biological and Pharmaceutical Bulletin **26**, 1331 - 1335.

Chopra, R.N., Nayer, S.L. and Chopra, I.C., 1992. Glossary of Indian Medicinal Plants, 3rd edn. Council of Scientific and Industrial Research, New Delhi, India.

Clark, A.M. and Hufford, C.D., 1993. Discovery and development of novel prototype antibiotics for opportunistic infections related to acquired immunodeficiency syndrome. *In*: A.D. Kinghorn and M.F. Balandrin (eds). Human medicinal agents from plants. American Chemical Society, Washington DC.

Conrad, J., Vogler, B., Klaiber, I, Roos, G, Walter, U and Kraus, W. 1998. Two triterpene esters from *Terminalia macroptera* bark. Phytochemistry, **48(4),** 647-650.

Cotton, C.M., 1996. Ethnobotany: Principles and Application. Wiley, Chichester, UK.

Cowan, M.M. 1999. Plant products as antimicrobial agents. Clinical Mirobiology Reviews. **12(4),** 564 - 582.

Cronquist, A., 1981. An integrated system of classification of flowering plants. Columbia University Press, New York, USA.

Daum, G., Lees, N.D., Bard, M. and Dickson, R., 1998. Biochemistry, cell biology and molecular biology of lipids of *Saccharomyces cerevisiae*. Yeast, **14(16),** 1471-1510.

Deans, S.G. and Svoboda, K.P., 1990. Biotechnology and bioactivity of culinary and medicinal plants. AgBiotech News and Information, **2**, 211-216.

De Bolle, M. F., Osborn, R. W. Goderis, I. J., Noe, L., Acland, D., Hart, C. A., Torrekens, S., Van Leuven, F. and Broekart, N. F. 1996. Antimicrobial properties from *Mirablis jalapa* and *Amaranthus caudalus*: expression, processing, localization and biological activity in transgenic tobacco. Plant Mol. Biol. **31,** 993 -1008.

Del-Poeta M., Bixel, A.S. and Barcheisi, F. 1998. In vitro activity of dicationic aromatic compounds and fluconazole against *Cryptococcus neoformans* and *candida* ssp. J. Antimicrob. Chemother., **44(2),** 223 - 228.

Del-Poeta, M., Toffaletti, D.L. and Rude, T.H. 1999. Topoisomerase I is essential in *Cryptococcus neoformans*: role in pathobiology and as an antifungal target. Genetics, **152,** 167-178.

Denning, D. W., 1998. Invasive aspergillosis. Clin Infect Dis. **26**, 781- 803.

Deby, C. and Margotteaux, G., 1970. Relationship between essential fatty acids and tissue antioxidant levels in mice. C R Seances Soc Biol Fil., **165**, 2675 -2681.

Djoukeng, J.D., Abou-Mansour, E., Tabacchi, R., Tapondjou, A.L., Bouda, H. and Lontsi, D. 2005. Antibacterial triterpenes from *Syzygium guineense* (Myrtaceae). Journal of Ethnopharmacology, **101,** 283-286.

Didomenico, B. 1999. Novel antifungal drugs. Current Opinion in Microbiology. **2**, 509 - 515.

Drouhet, E. and Dupont, B., 1989. Fluconazole for the treatment of fungal diseases in immunosuppressed patients. Annals, **544**, 564 -570.

Duke, J. A. 1985. Handbook of medicinal herbs. CRC Press, Inc., Boca Raton, Fla.

Durden, F. M. and Elewski, B., 1994. Cutaneous involvement with Cryptococcus neoformans in AIDS. J. Amer. Acad. Dermatol. **30**, 844 -848.

Eloff, J.N., 1998a. Conservation of Medicinal Plants: Selecting Medicinal Plants for research and gene banking. Monographs in systematic Botany from the Missouri Garden 71, 209-222 *In* : Conservation of plants Genes III: Conservation and utilisation of African plants. Robert P. Adams and Janice E. Adams, eds., Missouri Botanical Garden Press, St.Louis, USA.

Eloff, J.N., 1998b. Which extractant should be used for the screening and isolation of antimicrobial components from plants? J. of Ethnopharmacology, **60**, 1- 8.

Eloff, J.N., 1998c. The presence of antibacterial compounds in *Anthocleista grandiflora* [Loganiaceae] South African Journal of Botany, **64**, 209 -212.

Eloff, J.N., 1998d. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plants extracts for bacteria. Planta Medica, **64**, 711-713.

Eloff, J.N., 1999. The antibacterial activity of 27 Southern African members of the Combretaceae. South African Journal of Science, **95**, 148 -152.

Eloff, J.N., 2000. A proposal on expressing the antibacterial activity of plant extracts – a small first step in applying scientific knowledge to rural primary health care in South Africa. S. Afr. J. Sci. **96**, 116 - 118.

Eloff, J.N., 2001. Antibacterial activity of Marula (*Scerocarya birrea* (A. Rich.) Hochst. Subsp. Caffra (Sond.) Kokwaro) (Anacardiaceae) bark and leaves. J. of Ethnopharmacology, **76**, 305 - 308.

Eloff, J.N., Jäger ,A.K and van Staden, J., 2001. The stability and relationship between anti-inflammatory activity and antibacterial activity of southern African Combretum species. S. Afr. J. Sci. **97**, 291 - 293.

Eloff, J.N., 2004. Quantifying the bioactivity of plant extracts during screening and bioassay-guided fractionation. Phytomedicine, **11**, 370 - 371.

Eloff J.N., Famakin, J.O., and Katerere, D.R.P., 2005a. *Combretum woodii* (Combretaceae) leaf extracts have high activity against Gram-negative and Gram-positive bacteria. African J. of Biotechnology, **4**, 1161 - 1166.

Eloff, J.N., Famakin, J.O., and Katerere, D.R.P., 2005b. Isolation of an antibacterial stilbene from Combretum woodii (Combretaceae). African J. of Biotechnology, **4**, 1166 - 1171.

Eskilsson, C.S. and Bjorklund, E., 2000. Analytical-scale microwave-assisted extraction (Lecture Notes), Lund Unversity Sweden.

Estrada, A., Cammarao, P.L. and Coates-Estrada, R. 2000. Birds species richness in vegetation fences and in strips of residual rain forest vegetation at Los Tuxtlas, Mexico. Biodivers. Conserv, 9, 1399 - 1416.

Espinel-Ingroff, A. and Pfaller, M.A., 1995. Antifungal agents and susceptibility testing. *In*: P.R. Murray, E.J. Baron, M.A. Pfaller, F.C. Tenover, R.H. Yolken (eds). Manual of clinical microbiology. ASM Press, Washington D.C., 1405 -1414.

Espinel-Ingroff, A. 1998. Comparison of in vitro activities of the new triazole SCH 56592 and the echinocandins MK-0991 (L-743,872) and LY 303366 against opportunistic filamentous and dimorphic fungi and yeast. J Clin Microbiol. **36**, 2950 -2956.

Famakin, J. O. 2002. Investigation of antimicrobial compounds present in *Combretum woodii*, MSc thesis, University of Pretoria.

Fang, X., Anderson, J. E., Chang, C and McLaughlin, J. L., (1991). Three new bioactive styryllactones from *goniothalamus giganteus* (Annonaceae). Tetrahedron, **47(47),** 9751-9758

Farnsworth, N.R., 1988. Screening plants for new medicines. *In*: Wilson, E.O. (Ed), Biodiversity. National Academic Press, Washington, DC, pp 83 -97.

Farnsworth, N.R. and Soejarto, D.D., 1991. Global importance of medicinal plants. *In*: Akerele O., Heywood V. and Synge H. (Eds), Conservation of Medicinal Plants. Cambridge University Press, Cambridge.

Farnsworth, N.R., 1994. The ethnobotanical approach to drug discovery: strengths and limitations. *In*: Prance G.T. (Ed), Ethnobotany and the search for new drugs. *In*: Ciba Foundation Symposium, **185**. Chicherster, pp. 42 -59.

Fernandes de Caleya, R., Gonzalez-Pascual, B., Garcia-Olmedo, F. and Carbonero, P. 1972. Susceptibility of phytopathogenic bacteria to wheat purothionins in vitro. Appl. Microbiol. **23,** 998 -1000.

Fessenden, R. J. and J. S. Fessenden. **1982.** Organic chemistry, 2nd ed. Willard Grant Press, Boston, Mass

Finkel, T. and Holbrook, N.J. 2000. Oxidants, oxidative stress and biology of ageing. Nature, **408**, 239 - 247.

Flee, G.H. and Phaff, H.J., 1981. In Encyclopaedia of Plant Physiology: Plant Carbohydrates II. 13b. Tanner W. *et al*. (Eds.), Springer-Verlag, Germany, 416 - 440.

Font, N., Hernández, F., Hogendoorn, E.A., Baumann, R.A. and van Zoonen, P., 1998. J. Chromatogr. *A* **798**, 179.

Fothergill, A.W. 1996. Identification of dematiaceous fungi and their role in human diseases. Clin.Infest. Dis. **22(2),** S179 - S184.

Fried, B. and Sherma, M. 1982. In: Cazes, Editor. Chromatographic Science Series, **17**. Thin-layer Chromatography, Marcel Dekker, New York.

Fyhrquist, P., Mwasumbi, L., Haeggstrom, C. -A., Vuorela, H., Hiltunen, R. and Vuorela, P. 2002. Ethnobotanical and antimicrobial investigation of some species of *Terminalia* and *Combretum* (Combretaceae) growing in Tanzania. J. of Ethnopharmacology, **79**, 169 -177.

Garro, L.C. 1986. Intracultural Variation in Folk Medical Knowledge: A Comparison Between Curers and Noncurers. American Anthropologist. **88**, 351-370.

Geissman, T. A. 1963. Flavonoid compounds, tannins, lignins and related compounds, p. 265. *In* M. Florkin, and E. H. Stotz (ed.), Pyrrole pigments, isoprenoid compounds and phenolic plant constituents, vol. 9. Elsevier, New York, N.Y.

Georgopapadakou, N.H. 1997. Antifungal targeted to the cell wall. Exp Opin Invest Drugs. **6**, 147-150.

Germaud, P., and Tuchais, E., 1995. Allergic bronchopulmonary aspergillosis treated with itraconazole. Chest. **107**, 883.

Ghannoum, M.A. and Rice, L.B., 1999. Antifungal agents: mode of action, mechanisms of resistance and correlation of these mechanisms with bacterial resistance. Clin. Microbial. Rev., **12,** 501-517.

Ghoshal, S., Krishna Prasad, B. N. and Lakshmi, V. 1996. Antiamoebic activity of *Piper longum* fruits against *Entamoeba histolytica* in vitro and in vivo. J. Ethnopharmacol. **50,** 167-170.

Gong F., Liang, Y.Z. Hui, C. Chau F.T. and Chan B.T.P. 2001a. *J. Chromatogr. A* **905**, 193.

Gong F., Y.Z. Liang, Q.S. Xu and Chau F.T. 2001b. *J. Chromatogr. A* **909**, 237.

Graybill, J.R. 1998. The long and the short antifungal therapy. Infect. Dis. Clin. North Am. **2**, 805 - 825.

Guetens G., De Boeck, G. Wood, M. Maes, R.A.A. Eggermont, A.A.M. Highley, M.S. van Oosterom, A.T. de Bruijn E.A. and Tjaden U.R. 2002. *J. Chromatogr. A* **976**, 229.

Gupta, S.K., Dua, A. and Vohra, B.P. 2003. *Withania somnifera* (Ashwagandha)attenuates antioxidant defense in aged spinal cord and inhibits copper induced lipid peroxidation and protein oxidative modifications. Drug Metabolism and drug Interaction, **19**, 211- 222.

Halliwell, B and Gutteridge, J.M.C. 1999. Free radicals in biology and medicine. Free radicals in Biology and Medicine, 3rd ed. Oxford University Press, New York.

Hannun, Y.A. Luberto, C. Halpern, M., Szabo, S., Hochberg, E., Hammer, G.S., Lin, J., Gurtman, A.C., Sacks, H.S., Shapiro, R.S., and Hirschman, S.Z., 1992. Renal aspergilloma: an unusual cause of infection in a patient with the acquired immunodeficiency syndrome. Am. J. Med. **92**, 437-440.

Harborne, S.B. and Baxter, H., 1995. Phytochemical Dictionary. A handbook of bioactive compounds from plants. Taylor and Francis, London.

Harris, R. S. 1963. Vitamins K, *In* M. Florkin, and E. Stotz (ed.), Pyrrole pigments, isoprenoid compounds and phenolic plant constituents, vol. 9. Elsevier, New York, N.Y.

Harris, R.K. 1985. Quantitative aspects of high-resolution solid-state nuclear magnetic resonance spectroscopy, *Analyst* **110**, 649–655.

Haslam, E. 1996. Natural polyphenols (vegetable tannins) as drugs: possible modes of action. J. Nat. Prod. **59,** 205-215.

Heipieper, H. J., Keweloh, H. and Rehm, H. J. 1991. Influence of phenols on growth and membrane permeability of free and immobilized *Escherichia coli*. Applied Environmental Microbiology, 57,1213 -1217.

Hemenway, C.S. and Heitman, J. 1999. Calcineurin, structure, function and inhibition. Cell Biochem. Biophys., **30,** 115 -151.

Herscovics, A. and Orlean, P. 1993. Glycoprotein biosynthesis in FASEB J**. 7**, 540 - 550.

Ho, P. L., and Yuen, K.Y., 2000. Aspergillosis in bone marrow transplant recipients. Crit Rev Oncol Hematol. **34**, 55 - 69.

Hohler, T., Schnütgen, M., Mayer, W.J, and Mayer zum Büschenfeld, K.H., 1995. Pulmonary aspergilloma in a patient with AIDS. Thoras. **50**, 312-313.

Homans, A.I. and Fuchs, A. 1970. Direct bioautography on thin-layer chromatograms as a method for detecting fungitoxic substances. Journal of Chromatography A. **51**, 327 - 329.

Houghton, P.J. and Raman, A. 1998. Laboratory handbook for the fractionation of natural extracts, St Edmundsbury Press, Great Britain.

Hoult, J. R. S. and M. Paya. 1996. Pharmacological and biochemical actions of simple coumarins: natural products with therapeutic potential. Gen. Pharmacol. **27,** 713 -722.

Hutchings, A., Scott, A.H., Lewis, G. and Cunninghan, A. 1996. Zulu Medicinal Plants, An inventory, University of Natal Press, Pietermarizburg, South Africa.

Imaida, K., Fukushima, S., Shirui, T., Ohtani, M., Nakanishi, K. and Ito, N. 1983. Promoting actions of butylated hydroxy anisole and butylated hydroxytoluene on 2-stage urinary bladder carcinogenesis and inhibition of y-glutamyl transpeptidase-positive foci development in the liver of rats. Carcinogenesis, **4**, 895 - 899.

Inoue, S.B., Takewaki, N. and Takasuka, T. 1995. Characterization and gene cloning of 1,3-β-D-glucan synthase from *Saccharomyces cerevisiae*. Eur. J. Biochem, **231**, 845 - 854.

Jäger, A.K., Hutchings, A. and van Staden, J. 1996. Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. J. of Ethnopharmacology, **52**, 95 -100.

Jiang, W., Gerhold, D. and Kmiec, E.B. 1997. The topoisomerase I gene from *Candida albicans*. Microbiology, **143,** 377 - 386.

Kårehed, J. 1997. An ethnobotanical study among the Maasai of the Loita Hills, Kenya. MFS, 14

Katerere, R.P.D. and Eloff, J.N. 2004. Variation in the chemical composition, antibacterial and antioxidant activity of fresh and dried *Acacia* leaf extracts. South African J. of Botany. **70**, 303 - 305.

Katerere, R.P.D. and Eloff, J.N., 2005. Management of Diabetes in African traditional medicine in Soumyanath A (Ed.) Traditional Medicine for Modern Times. Vol 6.

Kaul, T. N., Middletown, Jr., E. and Ogra, P. L. 1985. Antiviral effect of flavonoids on human viruses. J. Med. Virol. **15,** 71-79.

Keating, G. J., and O'Kennedy, R. 1997. The chemistry and occurrence of coumarins, p. 348. *In* R. O'Kennedy, and R. D. Thornes (ed.), Coumarins: biology, applications and mode of action. John Wiley & Sons, Inc., New York, N.Y.

Khafagi, K.I. and Dewedar, M. 2000. The efficiency of random versus ethno-directed research in the evaluation of Sinai medicinal plants for bioactive compounds. J. of Ethnopharmacology, **71**, 365 -376.

Klig, L.S., Homann, M.J. and Kohlwein, S.D., 1988. *Saccharomyces cerevisiae* mutant with a partial defect in the synthesis of CDP-diacylgycerol and altered regulation of phospholipids biosynthesis. J. Bacteriology, **172,** 4407- 4414.

Koltin, Y. and Hitchcock, C.A. 1997. The search for new triazole antifungal agents. Curr. Opin. Chem. Bio., **1**, 176 -182.

Kotze, M. and Eloff, J. N., 2002. Extraction of antibacterial compounds from *Combretum microphyllum* (Combretaceae). South African Journal of Botany **68**, 62-67.

Kruger, J. 2004. Isolation and characterization of antibacterial compounds from three Terminalia spp. Ph. D thesis, University of Pretoria.

Kostman, J. R. and DiNubile, M.J. 1993. Nodular lymphangitis: a distinctive but often unrecognized syndrome. Ann. Intern. Med. **118**, 883-888.

Kovács, Á., Ganzler, K. and Simon-Sarkadi, L., 1998. *Z.* Lebensm.-Unters. Forsch. *A* **207**, 26.

Kovacs, H., Moskau, D and Spraul, M. 2005. Cryogenically cooled probes, a leap in NMR technology. Progress in Nuclear Magnetic Resonance Spectroscopy, *In Press.*

Kovalchuke, O. and Chakraburtty, K. 1994. Comparative analysis of ribosome-associated adenosinetriphosphate (ATPase) from pig liver and the ATPase of elongation factor 3 from *Saccharomyces cerevisiae*. Eur. J. Biochem., **226,**133 -140.

Kovalchuke, O., Kambampati, R., Blades, E. and Chakraburtty, K. 1998. Competition and cooperation amongst yeast elongation factors. Eur. J. Biochem., **258(3),** 986 - 993.

Kragh, K. M., Nielsen, J. E. Nielsen, K. K., Dreboldt, S. and Mikkelsen. J. D. 1995. Characterization and localization of new antifungal cysteine-rich proteins from Beta vulgaris. Mol. Plant-Microbe Interact. **8,**424 - 434.

Kreger-Van Rij, N.J.W. (ed) 1984. The Yeasts: a taxonomic study. 3rd Edition. Elsevier Science Publishers B.V., Amsterdam,The Netherlands.

Kwon-Chung, KJ. 1992. Cryptococcosis. In: Kwon-Chung KJ, Bennett JE, editors. Medical mycology. Philadelphia: Lea & Febiger, 397 - 446.

Larone, D. H. 1995. Medically Important Fungi - A Guide to Identification, 3rd ed. ASM Press, Washington, D.C.

Lawrence, G.H.W. 1951. The taxonomy of vascular plants. Macmillan, New York.

Leaman, D.J. 1995. Malaria Remedies of the Kenyah of Apo Kayan, East Kalimantan, Indonesian Borneo: A quantitative assessment of local consensus as an indicator of biological efficacy. Journal of Ethnopharmacology, **49**, 1-16.

Lee-Huang, S., Huang, P. L. Huang, P. L. Bourinbaiar, A. S. Chen, H. C. and Kung, H. F. 1995. Inhibition of the integrase of human immunodeficiency virus (HIV) type 1 by anti-HIV plant proteins MAP30 and GAP31. Proc. Natl. Acad. Sci. USA **92,** 8818-8822.

Li, Y. and Thrush, M.A. 1994. Reactive oxygen dependent DNA damage resulting from the oxidation of phenolic compounds by a copper redox cycle, Cancer Research, **54**, 1895s – 1898s.

Li, R.K. and Rinaldi, M.G. 1999. *In vitro* antifungal activity of nikkomycin Z in combination with fluconazole or itraconazole. Antimicrob Agents Chemother. **45**, 1401-1405.

Li X.N., Cui, H., Song, Y.Q. and Liang, Y.Z. 2001. *Acta Pharm. Sin.* **36,** 215.

Lima, C.D. and Mondragon, A. 1994. Mechanism of Type II DNA topoisomerase: a tale of two gates. Structure, **2,** 559-560.

Lin, J. G., Chung, J. G. and Wu, L. T., 1999. Am. J. Chin. Med., **27**, 265-275.

Liu, Y., Peterson, D. A., Kimura, H and. Schubert, D. (1997). Mechanism of cellular 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) reduction. *J. Neurochem.* **69**, 581- 593.

Liu, J., Farmer, J.D. and Lanes, W.S. 1999. Calcineurin is a common target of cyclophilin-cyclosporin A and FKBP-FK506 complexes. Cell, 66**(4),** 807-815.

Loche,r C.P., Burch, M.T., Mower, H.F., Berestecky, J., Davis, H., van Poel, B., Lasure ,A., Vanden Berghe, D.A. and Vlietinck, A.J., 1995. Anti-microbial activity and anti-complement activity of extracts obtained from selected Hawaaiian medicinal plants. J. of Ethnopharmacology, **49**, 23 -32.

Loo, V. G., Bertrand, C., Dixon, C., Vitye, D., DeSalid, A., McLean, A.P.H., Bronx, A., and Robson, H.G., 1996. Control of construction-associated nosocomial aspergillosis in an antiquated hematology unit. Infect. Control Hosp. Epidemiol. **17**, 360 -364.

Lopez-Avila, V., Young, R. and Beckert, W.F. 1994. Anal. Chem. **66,** 1097.

Lovitt, R.W., Shen, G.-J. and Zeikus, J.G. 1988. Ethanol production by thermophilic bacteria: biochemical basis for ethanol and hydrogen tolerance in *Clostridium thermohydrosulfuricum*. Journal of Bacteriology. 170, 2809 -2815.

Lucas, G. M., Tucker, P., and Merz, W.G., 1999. Primary cutaneous Aspergillus nidulans infection associated with a Hickman catheter in a patient with neutropenia. Clin Infect Dis. **29**, 1594 -1596.

Mabogo, D.E.N. 1990. The ethnobotany of the Vhavenda, M.Sc. thesis. Univeristy of Pretoria.

Mahunnah, R.L.A. 1996. Tanzania's policy on biodiversity prospecting and drug discovery programs. Journal of Ethnopharmacology, **51**, 221-228.

Mandala, S.M., Thornton, R.A., Rosenbach, M., Milligan, J., Garcia-Calvo, M., Bull, H.G. and Kurtz, M.G. Khafrefungin, a novel inhibitor of sphingolipid synthesis. J Biol Chem. 272, 32709 -32714.

Martini, N. 2002. Isolation and characterization of antibacterial compounds from *Combretum erythrophyllum,* Ph. D thesis, University of Pretoria.

Martini, N. and. Eloff, J. N. 1998. The preliminary isolation of several antibacterial compounds from *Combretum erythrophyllum* (Combretaceae). Journal of Ethnopharmacology, **62(3),** 255 -263.

Martini, N., Katerere, D.R.P. and Eloff, J.N., 2004a. Seven flavonoids with antibacterial activity isolated from *Combretum erythrophyllum*. South African J. of Botany, **70**, 310 - 312.

Martini, N., Katerere, D.R.P. and Eloff, J.N., 2004b. Biological activity of five antibacterial flavonoids isolated from *Combretum erythrophyllum* (Combretaceae). J. of Ethnopharmacology, 93, 207 -212.

Martin, M.L., Martin, G.L. and Delpuech J.J. 1980. Practical NMR Spectroscopy, Heyden, London, Chapter 9.

Masika, P.J. and Afolayan, A.J., 2002. Antimicrobial activity of some plants use for the treatment of livestock disease in the Eastern Cape, South Africa. J. of Ethnopharmacology, **84**, 129 -134.

Masika, P.J., van Averbeke, W. and Sonandi, A., 2000. Use of herbal remedies by small-scale farmers to treat livestock diseases in central Eastern Cape Province, South Africa. Journal of South African Veterinary Association, **35(2)**, 87- 91.

Masoko, P., Picard, J. and Eloff, J.N., (2005). Screening of antifungal activity of six South African *Terminalia* species (Combretaceae). Journal of Ethnopharmacology, **99**. 301- 308.

Masoko, P. and Eloff J.N., (2005). The diversity of antifungal compounds of six South African *Terminalia* species ((Combretaceae) determined by bioautography. African Journal of Biotechnology, **4(12),** 1425 -1431.

Masoko, P., Picard, J. and Eloff, J.N., (2006). Antifungal activity of twenty-four South Africa *Combretum* species (Combretaceae) (*In Press, South African Journal of Botany*).

Mason, T. L., and Wasserman, B. P. 1987. Inactivation of red beet beta-glucan synthase by native and oxidized phenolic compounds. Phytochemistry **26,** 2197 -2202.

McGaw, L.J. and Eloff, J.N., 2005. Screening of sixteen poisonous plants for antibacterial, anthelmintic and cytotoxic activity in vitro. South African J. of Botany, **71**, 302 - 306.

McGaw, L.J., Gehring, R., Katsoulis, L and Eloff, J.N., 2005. Is the use of *Gunnera perpensa* extracts in endometriosis related to antibacterial activity? Onderstepoort J. Veterinary Research, **72**, 129 - 134.

McGaw, L.J., Rabe, T., Sparg, S.G., Jäger, A.K., Eloff, J.N. and van Staden, J. 2001. An investigation of the biological activity of *Combretum* species. J. of Ethnopharmacology, **75**, 45 - 50.

McLaughlin, J.L. 1991. Crown gall tumours on potato discs and brine shrimp lethality: two simple bioassays for higher plants screening and fractionation. *In*: K. Hosttetmann, Editor, Methods in plant biochemistry **9**, Academic Press, London , 1- 32.

McMahon, J. B., Currens, M. J., Gulakowski, R. J., Buckheit, R. W. J., Lackman-Smith, C. Hallock, Y. F. and Boyd, M. R. 1995. Michellamine B, a novel plant alkaloid, inhibits human immunodeficiency virus-induced cell killing by at least two distinct mechanisms. Antimicrob. Agents Chemother. **39,** 484 - 488.

Mendoza, L., Wilkens, M. and Urzua, A. 1997. Antimicrobial study of the resinous exudates and of diterpenoids and flavonoids isolated from some Chilean *Pseudognaphalium* (Asteraceae). J. Ethnopharmacol. **58,** 85 -88.

Mitchell, T.G. and Perfect, J.R. 1995. Cryptococcosis in the era of AIDS—100 years after the discovery of *Cryptococcus neoformans*. Clin Microbiol Rev, **8**, 515 - 48.

Mitsher, L.A., Drake, S., Goliapudi, S.R. and Okwute, S.K., 1987. A modern look at folkloric use of anti-infective agents. Journal of Natural Products, **50**, 1025 -1040.

Monk, B.C., Mason, A.B., Kardos, T.B. and Perlin, D.S. 1995. Targeting the fungal plasma membrane proton pump. Acta Biochim. Pol. **42,** 481- 496.

Mori, T., Matsumura, M., Yamada, K., Irie, S., Oshimi, K., Suda, K., Oguri, T., and Ichinoe M. 1998. Systemic aspergillosis caused by an aflatoxin-producing strain of Aspergillus flavus. Med Mycol. **36**, 107-112.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods **65**, 55-63.

Nagiec, M.M., Nagieec, E.E., Baltisberger, J.A., Wells, G.B., Lester, R.L. and Dickson, R.C. 1997. Sphingolipid synthesis as a target for antifungal drug: complementation of the inositol phosphorylceramide synthase defect in a mutant strain of *Saccharomyces cerevisiae* by the *AUR1* gene. **272**, 32709 -32714.

Naidoo, V.R., Katerere, R.D.P., Swan, G.E. and Eloff, J.N., 2004. Pretreatment of bulbs of *Urginea sanguinense* used in ethnoveterinary medicine influences chemical composition and biological activity. Pharmaceutical Biology, **42**, 529 - 533.

Naidoo, V.R., Zweygarth, E., Eloff, J.N. and Swan, G.E. 2005. Identification of anti-babesial activity for four ethnoveterinary plants in vitro. Veterinary Parasitology **130**, 9 - 13.

Naik, G. H., Priyadarsini, K. I., Satav, J. G., Banavalikar, M. M., Sohoni, D. P., Biyani, M. K and Mohan, H., 2003. Comparative antioxidant activity of individual herbal components used in Ayurvedic medicine. Phytochemistry, **63**, 97-104.

Namba, T., Morita, O., Huang, S.-L., Goshima, K., Hattori, M., and Kakiuchi. N. 1988. Studies on cardio-active crude drugs. I. Effect of coumarins on cultured myocardial cells. Planta Med. **54,**277-282.

Nchu, F., Magano, S.R. and Eloff, J.N. 2005. *In vitro* investigation of the toxic effects of the extracts of *Allium sativum* on adults of *Hyalomma marginatum rufipes* and *rhipicephalus pulchellus*. J. of the Soth African Veterinary Association, **71**, 302 - 306.

Nimri, L.F., Meqdam, M.M. and Alkofahi, A., 1999. Antibacterial activity of Jordanian medicinal plants. Pharmaceutical Biology **37(3)**, 196 -201.

Nose, K. 2000. Role of reactive oxygen species in regulation of physiological functions, Biological and pharmaceutical Bulletin, **23**, 897 - 903.

Odom, A., Del-Poeta, M., Perfect, J. and Heitman, J. 1997. The immunosuppressant FK506 and its nonimmunosuppressive analog L-685,818 are toxic to *Cryptococcus neoformans* by inhibition of a common target protein. Antimicrob. Agents Chemother. **41,** 156 -161.

Ojala, T., Remes, S., Haansuu, P., Vuorela, H., Hiltunen, R., Haahtela, K. and Vuorela, P. 2000. Antimicrobial activity of some coumarin containing herbal plants growing in Finland. J. of Ethnopharmacology, **73(1 –2),** 299 - 305.

O'Kennedy, R., and Thornes, R. D. (ed.). 1997. Coumarins: biology, applications and mode of action. John Wiley & Sons, Inc., New York, N.Y.

Oki T., Kakushima, M., Hirano, M., Takahashi, A., Ohta, S., Masuyoshi, S., Hatori, M. and Kamei, H. 1992. *In vitro* and *in vivo* antifungal activities of BMS-181184. J Antibiot. 36, 1512 -1517.

Omulokoli, E., Khan, B. and Chhabra, S. C. 1997. Antiplasmodial activity of four Kenyan medicinal plants. J. Ethnopharmacol. **56,** 133 -137.

Onuska, F.I. and Terry, K.A. 1993. Chromatographia **36**, 191.

Osborne, R and Pegel, K. H., 1984. Jessic acid and related acid triterpenoids from *Combretum elaeagnoides*. Phytochemistry, **23,** 635 - 637.

Owen, R.W., Giacosa, A., Hull, W.E., Haubner, R., Speegelhalder, B. and Bartsch H. 2000. The antioxidant/anticancer potential of phenolic compounds isolated from olive oil, European Journal of Cancer, **36,** 1235 - 1247.

Pappas, P. G., Tellez, I., Deep, A.E., Nolasco, D., Holgado, W., and Bustamante, B., 2000. Sporotrichosis in Peru: Description of an area of hyperendemicity. Clin Infect Dis. **30**, 65 -70.

Peberby,J.F. 1990. Fungal cell walls. In: Biochemistry of cell walls and membranes in fungi. Kuhn P.J et al. (Eds.), Spring-Verlag, New York, USA, 5 -24.

Pegel, K. H., Rogers, C. B., 1985. The characterization of mollic acid 3d –D-Xyloside and its genuine aglycone mollic acid, two novel 1a-hydroxycycloartenoids from *Combretum molle*. J. Chem. Soc., Perkin Transaction, **1**, 1711-1715.

Pellizzoni, F., Verotta, L., Rogers, C., Pedrotti, B., Balconi, G., Erba, E., Dincalci, M., 1993. Cell growth inhibitor constituents from *Combretum kraussii*. Natural Product Letters, **14**, 273 -280.

Pengsuparp, T., Cai, L., Constant, H., Fong, H. H., Lin, L. Z., Kinghorn, A. D., Pezzuto, J. M. G., Cordell, A., Ingolfsdottir, K. and Wagner. H. 1995. Mechanistic evaluation of new plant-derived compounds that inhibit HIV-1 reverse transcriptase. J. Nat. Prod. **58,** 1024 -1031.

Petit, G. R., Singh, S. B., Niven, M. L., Hamel, E., Schmidt, J. M., Hogan, F., 1995. Antineoplastic agents. 291. Isolation and synthesis of combretastatins A-4, A-5, and A-6 (1a). J. Med Chem. **38**, 1666 -1672.

Phillipson, J. D., and O'Neill, M. J. 1987. New leads to the treatment of protozoal infections based on natural product molecules. Acta Pharm. Nord. **1,** 131-144.

Portillo, A., Vila, R., Freixa, B., Adzet,T. and Canigueral, S. 2001, Antifungal activity of Paraguayan plants used in traditional medicine. J Ethnopharmacol. **76,** 93 -98

Rios, J.L., Recio, M.C. and Villar, A., 1988. Screening methods for natural products with antimicrobial activity, a review of the literature. J. of Ethnopharmacology. **23**, 127-149.

Rabe, T. and van Staden, J., 1997. Antibaterial activity of South African plants used for medicinal purposes. J. of Ethnopharmacology, **56**, 81-87.

Radding, J.A., Heidler, S.A. and Turner, W.W. 1998. Photoaffinity analog of the semisynthetic echocandin LY303366: identification of echinocandin targets in *Candida albicans*. Antimicrob Agents Chemother. **42**, 1187-1194

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. and Rice-Evans, C., 1999. Antioxidant activity applying an improved ABTS radical cation decolourization assay. Free Radical Biology and Medicine, **26**, 1231-1237.

Rex, J. H., and Okhuysen P.C., 2000. *Sporothrix schenckii*, p. 2695-2698. *In* G. L. Mandell, J. E. Bennett, and R. Dolin (ed.), Mandell, Douglas and Bennett's Principles and Practice of Infectious Diseases, 5th ed. Churchill Livingstone, New York.

Rippon, J.W. 1988. Medical Mycology. 3rd Edition. W.B. Saunders Co., Philadelphia, USA.

Rogers, C.B. and Verotta, L., 1996. Chemistry and biological properties of the African Combretaceae. In: Hostettman K., Chinyanganga F., Maillard M. and Wolfender J.-L. (Eds), Chemistry,Biological and Pharmacological properties of African Medicinal Plants. University of Zimbabwe Publications, Harare, Zimbabwe.

Sala, A., Recio, M.D., Giner, R.M., Manez, S., Tournier, H., Schinella, G. and Rios, J.L. 2002. Anti-inflammmatory and antioxidant properties of *Helichrysum italicum*, Journal of Pharmacy and Pharmacology, **54,** 365 - 371.

Saleem, A., Ahotupa, M. and Pihlaja, K. 2001. Total phenolics concentration and antioxidant potential of extracts of medicinal plants of Pakistan, Zeischrift für Naturforschung, ***C* 53**, 973 - 978.

Salie, F. and Eagles, P.F.K., 1996. Preliminary antimicrobial screening of four South African Astraceae species. J. Ethnopharmacol. **52**, 27 - 33.

Saxena, V.K. and Sharma, R.N., 1999. Antimicrobial activity of essential oil of *Lantana aculeate*. Fitoterapia, **70**, 59 - 60.

Scalbert, A. 1991. Antimicrobial properties of tannins. Phytochemistry **30,** 3875 - 3883.

Schage, C. 2000. Medicinal plants of the Washambaa (Tanzania):Documentation and Ethnopharmacological evaluation. Plant Biology, **2,** 83-92.

Schultz, J. C. 1988. Tannin-insect interactions, p. 553. *In* R. W. Hemingway, and J. J. Karchesy (ed.), Chemistry and significance of condensed tannins. Plenum Press, New York, N.Y.

Serafini, M., A. Ghiselli, and A. Ferro-Luzzi. 1994. Red wine, tea and anti-oxidants. Lancet **344,** 626.

Silva, O., Duarte, A., Cabrita, J., Pimentel, M., Diniz, A. and Gomes, E., 1996. Antimicribial activity of Guinea-Bissau traditional remedies. J. of Ethnopharmacology, **50**, 55 -59.

Sheldon, J., Balick, M.J. and Laird, S.A., 1997. Medicinal Plants: Can utilization and conservation coexist? The New York Botanical Garden, Bronx, New York, USA.

Sherma, J., 2000. Thin layer chromatography in food and agricultural analysis. J. of Chromatography A. **880**, 129 - 147.

Slusarenko, A.J., Longland, A.C. and Whitehead, I.M., 1989. A convenient, sensitive and rapid assay for antibacterial activity of phytoalexins. Botanica Helvetica, **99**, 203-207.

Smith, A., 1895. A contribution to South African Materia Medica, third ed. Lovedale Press, Lovedale.

Solis, P.N., Wright, C.W., Anderson, M.M., Gupta, M.P. and Phillipson, J.D., 1993. A microwell cytotoxicity assay using *Artemia salina* (brine shrimp). *Planta Medica* **59**, 250-252.

Stecher G., Huck C.W., Stöggl M.W. and Bonn G.K. 2003. Phytoanalysis: a challenge in phytomics. Trac Trends in Analytical Chemistry, **22**, 1-14.

Stern, J.L., Hagerman, A.E. Steinberg, P.D. and Mason, P. K. 1996. Phlorotannin-protein interactions. J. Chem. Ecol. **22,** 1887-1899.

St-Germain, G. and Summerbell R. 1996. Identifying Filamentous Fungi - A Clinical Laboratory Handbook, 1st ed. Star Publishing Company, Belmont, California.

Stout, S.J., Babbitt, B.W., DaCunha, A.R. and. Safarpour, M.M. 1998. *J. AOAC Int.* **81**, 1054.

Stuffness, M. and Douros, J. 1979. Drugs of Plant Origin. Methods in cancer research, 73-126

Sutton, D. A., Fothergill, A.W. and Rinaldi M.G. (ed.). 1998. Guide to Clinically Significant Fungi, 1st ed. Williams & Wilkins, Baltimore.

Takesako, K., Kuroda, H., Inoue, T., Haruna, F., Yoshikawa, Y., Kato, I., Uchida, K., Hiratani, T. and Yamaguchi, H. 1993. Biological properties of aureobasidin A, a cyclic depsipeptide antifungal antibiotic. J Antibiot. **46**, 1414 -1420.

Taylor, R. S., Manadhar, N. P. and Towers, G. H. N., 1996. Screening of selected medicinal plants of Nepal of antimicrobial activities. J. Ethnopharmacol. **46**, 153 -159.

Thomson, W. A. R. (ed.). 1978. Medicines from the Earth. McGraw-Hill Book Co., Maidenhead, United Kingdom.

Tidwell, R.R., Jones, S.K. and Naiman, N.A. 1993. Activity of cationically substituted bis-benzaimidazoles against experimental *Pneumocystis carinii* pneumonia. Antimicrob. Agents Chemother. **37(8),** 1713 -1716.

Timmins, M. 2002. BD oxygen biosensor system vs existing methods of measuring toxicology, www.bdbiosciences.com.

Tkacs, J.S. and Didomenico, B. 2001. Antifungals: what's in the pipeline. Current Opinion in Microbiology. **4**, 540 - 545.

Tsuchiya, H., Sato, M., Miyazaki, T., Fujiwara, S., Tanigaki, S., Ohyama, M., Tanaka, T. and Linuma. M. 1996. Comparative study on the antibacterial activity of phytochemical flavanones against methicillin-resistant *Staphylococcus aureus*. J. Ethnopharmacol. **50,** 27-34.

Urbini B., Castellini, C., Rondelli, R., Prete, A., Pierinelli, S., and Pession, A., 2000. Cryptococcal meningitis during front-line chemotherapy for acute lymphoblastic leukemia. Haematologica. **85**, 1103 -1104.

Uritani, M., Shoumura, Y. and Yamada, M. 1999. Detection and analysis of translation elongation factor 3 genes from various yeast. Biosci Biotechnol Biochem. 63, 769 -772.

Vamos-Vigyazo, L. 1981. Polyphenol oxidase and peroxidase in fruits and vegetables. Crit. Rev. Food Sci. Nutr. **15,** 49 -127.

Van Wyk, B. –E., van Oudtshoorn, B. and Gericke N., 1997. Medicinal plants of South Africa, Briza Publications, Pretoria, South Africa.

Vázquez Blanco, E., López Mahía, P. Muniategui Lorenzo, S., Prada Rodríguez, D. and Fernández Fernández, E. 2000. Fresenius J. Anal. Chem. **366**, 283.

Vennestrom, J.L., and Klayman, D.L. 1988. Protoberberine alkaloids as antimalarials. Journal of Medicinal Chemistry Jun., **31(6),** 1084 -1087.

Vishwakarma, R. A. 1990. Stereoselective synthesis of beta-arteether from artemisinin. J. Nat. Prod. **53,** 216-217.

Vlietinck, A. J., Van Hoof, L., Tott, J., Lasure, A., Van den Berghe, D., Rwangabo, P. C. Mvukiyumwami, J. 1995. Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. J. Ethnopharmacol. **46**, 31- 47.

von Maydell, H.-J., 1996. Trees and shruds of the Sahel. Verlag Josef Margraf, Weikersheim, p. 562.

Walsh, T.J., Yeldandi, V., McEvoy, M., Gonzalez, C., Chanock, S., Freifeld, A., Seibel, N.I., Whitcomb, P.O., Jarosinski, P. and Boswel, G. 1998. Safety tolerence and pharmacokinetics of a small unilamellar liposomal formulation of smphotericin B (AmBisome) in neutropenic patients. Antimicrob Agents Chemother. **42**, 2391-2398.

Wan, H.B. and Wong, M.K. 1996. J. Chromatogr. *A* **754,** 43.

Wang, J.C. 1971. Interaction between DNA and an *Escherichia coli* protein omega. J. Mol. Biol., **55,** 523-533.

Watanabe, M., Gomi, S., Tohyama, H., Ohtsuka, K., Shibahara, S., Inouye, S., Kobayashi, H., Suzuki, S., Kondo, S., Takeuchi, T and Yamaguchi, H. 1996. Binding of benanomicin A to fungal cells in reference to its fungicidal action. J Antibiot. **49**, 366 - 373.

Weinberg, R.A., McWheter, C.A., Freeman, S.K., Wood, D.C. and Gordon J.I. 1995. Genetic studies reveal that myristolCoA:protein N-myristoltransferase is an essential enzyme in *Candida albicans*. Mol Microbiol. 6, 241-250.

Weitzman, I., and Summerbell, R.C. 1995. The dermatophytes. Clin Microbiol Rev. **8**, 240-259.

Wey, S.B., Mori, M., Pfaller, M., Woolson, R.F. and Wenzel, R.P. 1988. Hospital-acquired candidemia. The attributable mortality and excess length of stay. Arch. Intern. Med. **148**, 2642 - 2645.

White, T.C., Marr, K.A. and Bowden, R.A. 1998. Clinical, cellular and molecular factors that contribute to antifungal drug resistance. Clin. Microbiol. Rev. **11,** 383 - 398.

WHO: World Health Organisation, 1979. The selection of essential drugs. Second report of the WHO Expert Committee. WHO Technical Report Series, **641**, pp 1- 44.

Wild, R. (ed.). 1994. The complete book of natural and medicinal cures. Rodale Press, Inc., Emmaus, Pa.

Williams, V.L. 1996. The Witwatersrand muti trade. Veld and Flora**, 82**, 12-14.

Wills, E.A., Redinbo, M.R., Perfect, J.R. and Del-Poeta, M. 2000. New potential targets for antifungal development. Antifectives, Ashley Publication Ltd.

Zhang, Y., and Lewis, K. 1997. Fabatins: new antimicrobial plant peptides. FEMS Microbiol. Lett. **149,** 59 - 64.

Zheng, W. and Wang, S. 2001. Antioxidant activity and phenolic composition in selected herbs. Journal of Agricultural and Food Chemistry, **49**, 5165 - 5170.

Zong, W., Jeffries, M.W. and Geogopapadakou, N.H., 2000. Inhibition of inositol phosphorylceramide synthase by aureobasidin A in *Candida* and *Aspergillus* species. Antimicrob. Agents Chemother. **44,** 651- 653.

Chapter 13
Appendix

Table 13.1. Weights of rats in Pilot study 1.

Days	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Rat 8
M	197	196.5	211.96	187.68	189	195.45	205.02	202.73
W	194	193.5	205.07	186.18	181.96	191.34	201.24	197.81
F	165.18	194.8	200.74	193.27	187.1	194.19	196.6	195.33
M	202.38	201.21	200.74	204.07	204.21	191.96	188.45	211.96
W	200.26	191.6	192.72	199.02	205.04	200.97	188.11	217
F	209.32	191.74	200.74	202.66	198.7	202.98	199.23	215.85
M	216.51	204.73	202	209.41	218.43	211.66	213.53	227.08

Table 13.2. Temperature of rats in Pilot study 1.

Days	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6	Rat 7	Rat 8
M	35.3	36	35.9	35.6	38.8	36.5	35.5	37
W	35.3	36	36.6	35.7	38.8	36.5	35.5	37
F	37.7	36	36.2	36.2	35.7	34.8	36.2	36.3
M	34.8	36.1	35.2	35.7	36	35.4	36.1	36.3
W	35.5	36.7	36	35.8	35.3	35.6	36.2	35.5
F	35.2	36.2	35.7	35.2	36.6	37	35.8	36.2
M	35.6	35.3	36.3	35.5	35.8	36.5	35.4	36.2

Table 13.3. Lesion sizes of rats in pilot study 1.

Rats No.	Days	Untreated	Cream	10% Extract	20% Extract
1	1	19.91	70.26	17.76	6.79
	3	19.91	70.26	17.76	6.79
	5	21.63	59.47	24.50	10.87
	8	12.25	33.24	16.22	7.96
	10	5.27	13.55	3.20	7.76
	12	2.20	6.23	1.45	3.79
	15	0.00	0.00	0.00	0.00
2	1	46.92	51.70	26.43	21.29
	3	46.92	51.70	26.43	21.29
	5	43.12	29.72	25.07	31.09
	8	33.48	23.54	16.55	29.93
	10	29.37	11.59	2.90	6.22
	12	14.13	9.72	29.88	23.76
	15	0.00	0.00	0.00	0.00
3	1	23.62	40.81	32.59	29.61
	3	23.62	40.81	32.59	29.61
	5	16.60	35.82	19.11	32.31
	8	12.69	22.02	31.27	30.69
	10	1.17	5.00	21.48	19.78
	12	0.00	3.28	1.08	1.85
	15	0.00	0.00	0.00	0.00
4	1	22.21	40.40	9.26	17.96
	3	22.21	40.40	9.26	17.96
	5	19.97	23.23	2.99	17.62
	8	22.70	18.23	10.92	27.95

	10	7.56	10.97	3.78	10.01
	12	10.71	2.63	8.84	15.10
	15	0.00	0.00	0.00	0.00
5	1	21.04	25.12	13.72	21.05
	3	21.04	25.12	13.72	21.05
	5	20.07	22.34	10.74	19.13
	8	17.44	10.63	10.50	12.68
	10	11.83	7.19	3.61	8.32
	12	1.91	2.39	18.14	9.25
	15	0.00	0.00	0.00	0.00
6	1	43.85	31.45	74.05	46.30
	3	43.85	31.45	74.05	46.30
	5	33.82	17.90	69.38	32.44
	8	37.48	10.98	42.21	17.10
	10	11.97	5.79	9.49	10.26
	12	2.96	1.82	1.30	3.59
	15	0.00	0.00	0.00	0.00
7	1	38.16	33.94	19.45	17.30
	3	38.16	33.94	19.45	17.30
	5	42.66	39.26	12.88	17.96
	8	35.86	20.50	10.79	10.42
	10	16.97	11.66	0.32	6.88
	12	5.73	5.75	4.15	1.93
	15	0.00	0.00	0.00	0.00
8	1	31.37	70.89	35.19	25.78
	3	31.37	70.89	35.19	25.78
	5	36.64	51.45	18.40	22.59
	8	32.25	37.21	26.40	18.17
	10	14.15	30.67	21.33	13.56
	12	4.74	5.49	0.74	1.75
	15	0.00	0.00	0.00	0.00

Table 13.4. Average lesion sizes of rats in pilot study 1.

Days	Control	Cream	10%	20%
1	30.9	45.6	28.6	23.3
3	30.9	45.6	28.6	23.3
5	29.3	34.9	22.9	23.0
8	25.5	22.0	20.6	19.4
10	12.3	12.1	8.3	10.3
12	5.3	4.7	8.2	7.6
15	0.0	0.0	0.0	0.0

Table 13.5. Average exudate of rats in pilot study 1.

Days	Control	Cream	10%	20%
M	0.000	0.000	0.000	0.000
W	1.000	1.375	1.500	1.375
F	0.625	0.750	1.250	1.375
M	0.250	0.625	0.750	0.750
W	0.500	0.625	0.375	1.000
F	0.125	0.125	0.250	0.250
M	0.000	0.000	0.000	0.000

Table 13.6. Average exudate of rats in pilot study 1.

Days	Control	Cream	10%	20%
M	0	0	0	0
W	1	1.25	1	0.875
F	1	1.125	0.75	0.5
M	0.75	1	0.5	0.625
W	0.875	0.75	0.125	0.25
F	0.375	0	0.125	0.375
M	0	0	0	0

Table 13.7. Average crust formation of rats in pilot study 1.

Days	Control	Cream	10%	20%
M	0.00	0.00	0.00	0.00
W	0.00	0.00	0.00	0.00
F	0.50	0.88	0.63	0.63
M	3.00	2.88	3.00	3.00
W	2.63	2.63	3.00	3.00
F	1.88	1.50	1.13	2.25
M	0.00	0.00	0.38	0.38

Table 13.8. Weight of rats in pilot study 2.

Days	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6
M	213.36	222.68	215.51	229.49	227.14	214.33
W	199.54	212.92	185.46	212.66	202.12	197.29
F	194.59	219.68	185.93	207.53	208.24	191.19
M	192.3	224.37	194.76	209.96	221.17	203.04
W	198.94	224.73	197.08	205.55	230.41	210.87
F	199.44	224.6	192.19	208.35	226.52	204.17
M	200.83	229.77	192.75	204.82	235.03	220.62
Days	Rat 7	Rat 8	Rat 9	Rat 10	Rat 11	Rat 12
M	219.09	224.47	231.25	223.71	212.78	222.42
W	206.15	213.95	217.48	203.98	181.01	213.32
F	186.33	213.28	214.79	192.98	160.1	201.86
M	189.4	206.43	218.88	200.87		205.96
W	194	211.99	214.16	202.74		201.07
F	197.08	212.76	218.03	207.36		201.5
M	196.13	211.42	221.04	210.47		207.98

Table 13.9. Temperature of rats in pilot study 2.

Days	Rat 1	Rat 2	Rat 3	Rat 4	Rat 5	Rat 6
M	32.8	33.2	33.6	34.8	34.7	36
W	36.2	36.3	36	37	37.9	37.6
F	35.2	35.7	35.3	37	37.3	37.5
M	37.3	35.7	36	36.7	36.2	37.9
W	36.3	37.1	36.2	35.5	35.6	36.5
F	35.8	36.9	36.6	35.5	35.4	36.6
M	35.1	34.9	36.3	35.8	34.8	36
Days	Rat 7	Rat 8	Rat 9	Rat 10	Rat 11	Rat 12
M	31.7	31.3	33.7	34.3	34.3	34.6
W	34.4	37.2	36.4	35.4	34.7	36.7
F	36.6	36.5	35.3	36.2	34.8	36.9
M	36.2	36.9	36.9	36		36.6
W	36	35.5	34.9	36.7		35.8
F	37.4	35.8	36.5	35.8		37.3
M	36.4	36.8	35.8	36		36.4

Table 13.10. Average exudate of rats in pilot study 2.

Days	No treatment (-ve)	*C. imberbe*	*C. nelsonii*	*C. albopunctactum*	*T. sericea*	Amphotericin B (+ve)
M	0.00	0.00	0.00	0.00	0.00	0.00
W	1.00	0.91	1.00	0.91	1.09	1.27
F	0.91	1.00	0.73	1.18	1.00	1.27
M	0.64	0.64	0.82	0.64	0.55	0.55
W	0.45	0.18	0.18	0.36	0.18	0.36
F	0.00	0.00	0.00	0.00	0.00	0.00
M	0.00	0.00	0.00	0.00	0.00	0.00

Table 13.11. Average erythema of rats in pilot study 2.

	No treatment (-ve)	*C. imberbe*	*C. nelsonii*	*C. albopunctactum*	*T. sericea*	Amphotericin B (+ve)
M	0.00	0.00	0.00	0.00	0.00	0.00
W	1.09	0.91	1.00	1.09	1.09	1.18
F	1.27	0.91	1.18	0.82	1.45	1.27
M	1.00	0.91	0.82	0.82	0.73	1.00
W	1.27	0.91	1.00	1.00	1.00	1.09
F	0.82	0.27	0.45	0.36	0.36	0.45
M	0.00	0.00	0.00	0.00	0.00	0.00

Table 13.12. Average crust formation of rats in pilot study 2.

	No treatment (-ve)	*C. imberbe*	*C. nelsonii*	*C. albopunctactum*	*T. sericea*	Amphotericin B (+ve)
M	0.00	0.00	0.00	0.00	0.00	0.00
W	0.00	0.00	0.09	0.00	0.00	0.00
F	0.00	0.00	0.36	0.09	0.36	0.00
M	1.55	1.91	1.91	1.91	1.91	1.55
W	2.18	2.09	2.55	2.55	2.36	2.36
F	1.64	0.55	0.55	0.82	1.36	0.82
M	0.00	0.00	0.00	0.27	0.00	0.00

Table 13.13. Standard deviation of crust formation of rats in pilot study 2.

	No treatment (-ve)	*C. imberbe*	*C. nelsonii*	*C. albopunctactum*	*T. sericea*	Amphotericin B (+ve)
M1	0	0	0	0	0	0
W1	0	0	0.30151134	0	0	0
F1	0	0	0.67419986	0.301511345	0.50452498	0
M2	0.820199532	0.301511345	0.30151134	0.301511345	0.30151134	0.8202
W2	0.91035266	0.867349805	0.90230216	0.956985714	0.92037378	0.877612
F2	1.566698904	1.213559752	1.21355975	1.401298099	1.5666989	1.401298
M3	0	0	0	0	0	0

Table 13.14. Standard deviation of exudate of rats in pilot study 2.

	No treatment (-ve)	*C. imberbe*	*C. nelsonii*	*C. albopunctactum*	*T. sericea*	Amphotericin B (+ve)
M1	0	0	0	0	0	0
W1	0.774596669	0.70064905	0.894427191	0.53935989	0.831209415	0.646669791
F1	0.70064905	0.894427191	0.646669791	0.603022689	0.774596669	0.646669791
M2	0.809039835	0.674199862	0.873862898	0.809039835	0.522232968	0.820199532
W2	0.403672487	0.430045968	0.422086435	0.421853153	0.452346709	0.500134063
F2	0	0	0	0	0	0
M3	0	0	0	0	0	0

Table 13.15. Standard deviation of erythema of rats in pilot study 2.

	No treatment (-ve)	*C. imberbe*	*C. nelsonii*	*C. albopunctactum*	*T. sericea*	Amphotericin B (+ve)
M1	0	0	0	0	0	0
W1	0.301511345	0.301511345	0	0.301511345	0.30151134	0.404519917
F1	0.646669791	0.53935989	0.40451992	0.603022689	0.68755165	0.467099366
M2	0.632455532	0.301511345	0.40451992	0.404519917	0.46709937	0.774596669
W2	0.506615573	0.396086952	0.47604791	0.40972332	0.50179929	0.495528042
F2	0.873862898	0.467099366	0.52223297	0.504524979	0.67419986	0.687551651
M3	0	0	0	0	0	0

Table 13.16. Average lesion size of rats in pilot study 2

	No treatment (-ve)	*C. imberbe*	*C. nelsonii*	*C. albopunctactum*	*T. sericea*	Amphotericin B (+ve)
M1	28.99791818	29.22276364	31.0538273	34.4117	31.1346273	29.68434545
W1	23.04898182	23.78405455	28.8677182	30.77445455	23.9417909	18.90043636
F1	16.30921818	17.92761818	22.5626818	23.35464545	16.6266455	14.93672727
M2	9.879681818	14.31259091	13.5489091	20.17067273	12.6590818	8.610918182
W2	9.896963636	9.528672727	11.9718364	14.07238182	8.81677273	9.171309091
F2	3.679309091	3.139681818	2.92155455	5.019481818	2.29633636	2.386018182
M3	0	0	0	0	0	0

Table 13.17. Standard deviation of lesion size of rats in pilot study 2.

	No treatment (-ve)	*C. imberbe*	*C. nelsonii*	*C. albopuntactum*	*T. sericea*	Amphotericin B (+ve)
M1	7.855258962	6.576222346	7.84316015	9.837250478	8.37141455	6.953621752
W1	11.18249669	12.9477218	12.2763443	16.5237778	9.24352609	8.740044979
F1	8.920585354	11.64572105	10.1871795	15.84566595	6.33718333	7.140911291
M2	5.43769399	9.479187358	7.8051266	12.07611199	6.58194067	5.531751752
W2	8.381626493	7.368666187	7.23478569	11.34026506	4.69611613	8.627557854
F2	5.14245172	3.667864916	2.88671152	7.447729108	2.82305341	3.24383612
M3	0	0	0	0	0	0