Potentising and application of a *Combretum woodii* leaf extract with high antibacterial and antioxidant activity

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Submitted in fulfilment of the requirements of the degree of Magister Scientiae

in the

Phytomedicine Programme

Department of Paraclinical Sciences

Faculty of Veterinary Science

University of Pretoria

Pretoria

2004

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Preface

This represents a record of the work carried out in the Phytomedicine Programme, Department of Paraclinical Sciences, Faculty of Veterinary Science, University of Pretoria, under the supervision of Prof J.N Eloff and Prof C.J Botha.

The results in these studies have not been submitted in any form to any other University and represent work done by Vincent Kudakwashe Zishiri, except where the work of others is acknowledged.

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Vincent Kudakwashe Zishiri

Acknowledgements

Firstly I would like to thank God almighty for the abundant blessings he has bestowed upon me.

I would like to express my most sincere gratitude to my supervisors, Prof J.N Eloff and Prof C.J Botha, for their expert guidance throughout the course of this work.

To my father Onesimo Joseph, and mother Chiedza Barbara; I have seen as far as I have because I stood on the shoulders of giants. I appreciate your guidance, support and always cherish your unconditional love.

My brothers and sisters, colleagues, friends at home and at school who have supported me morally and otherwise.

My thanks to Havana Chikoto and Martin Mmethi for their assistance and guidance in the animal studies work.

Lastly, I dedicate this work to my lovely wife Elizabeth and daughter Idanai Venus, from whom I have asked so much and given so little during the course of this programme.

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Abbreviations used

$ABTS^+$	2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid)
AFA	Antibiotic Feed Additive
ATCC	American type culture collection
BEA	Benzene/Ethanol/Ammonium hydroxide (90/10/1 v/v/v)
C_{18} column	18-Carbon reverse phase column
CB5	Combretastatin B5
CEF	Chloroform/Ethylacetate/Formic acid (5/4/1 v/v/v)
COX-1	Cyclooxygenase enzyme 1
COX-2	Cyclooxygenase enzyme 2
dH_2O	Distilled water
DMSO	Dimethylsulphoxide
DNA	Deoxyribose nucleic acid
DPPH	2, 2,diphenyl-1-picrylhydrazyl
ELISA	Enzyme linked immunosorbent assay
EMW	Ethylacetate/Methanol/Water (40/5.4/4 v/v/v)
FCR	Feed Conversion Ratio
HKI	Hans Knoll Institute
INT	Iodonitro-tetrazolium salts
LC_{50}	Lethal concentration for 50% of the cells
LDL	Low density lipids
LNBG	Lowveld National Botanical Garden
MDA	Malondialdehyde
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
ROS	Reactive oxygen species
rpm	revolutions per minute
SEE	Serial exhaustive extraction
TEAC	Trolox equivalent antioxidant capacity
TF2	Target fraction 2
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

Given the drawbacks associated with the use of antibiotics as feed additives and the imminent banning of its use in the European Union, the aim of this project was to develop an extract that could be used as an alternative feed additive in poultry production. The desired extract preferably had to be rich in antibacterial activity to control proliferation of undesired microorganisms, and antioxidant activity to boost the immune system of the poultry.

A number of trial extraction procedures were employed on dried leaf material samples to identify the best extraction method. In preliminary extraction studies, direct extraction was performed on leaf samples from the Lowveld National Botanical Gardens (LNBG) and from University of Pretoria Botanical Garden (UP). The principle aim of preliminary studies was to identify the solvents that extracted high antibacterial and antioxidant activity while also extracting large quantities of material. The secondary objective was to test for differences in activities between samples collected from LNBG and UP. Five extractants of varying polarities; acetone, ethanol, ethylacetate, dichloromethane and hexane were used.

Antibacterial activity of all extracts was quantified by a serial dilution microplate technique while bioautography was used in qualitative analysis of the antibacterial active compounds. ATCC strains of *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis* were used as test organisms. Qualitative antioxidant activity was determined by using a DPPH assay on TLC plates.

Results from preliminary extraction studies showed larger quantities of material were present in extracts from the LNBG sample than in the UP sample. Two major antioxidant compounds (R_f values of 0.85 and 0.35 in EMW solvent system) were seen on DPPH sprayed TLC plates, while bioautography showed the presence of a number antibacterial active compounds in the acetone, ethanol and ethylacetate extracts with R_f values ranging between 0.85 and 0.56 on TLC plates developed in the EMW solvent system. MIC values of the extracts tallied with the results from bioautography. The acetone, ethanol and ethylacetate extracts had the highest antibacterial activity while the hexane extracts had the lowest activity with average MIC value of 0.55 mg/ml for both the LNBG and UP

samples. MIC values as low as 0.04 mg/ml were measured in the acetone and ethylacetate extracts of the LNBG sample against *S. aureus* and *E. faecalis*. Based on results from preliminary extraction studies, hexane was identified as a possible pretreatment solvent for application in enrichment procedures, acetone and ethanol were chosen as the main extractants and only the LNBG sample was used for future work.

Enrichment procedures were employed along two pathways; the first pathway involved the use of hexane "wash" as a pretreatment procedure prior to extraction with acetone or ethanol. The second pathway involved the use of various mixtures of acetone in water and ethanol in water as extractants. The rationale of using these various ratios was an attempt to identify solvent mixtures that would selectively extract the bioactive components or otherwise selectively remove inactive material.

A serial dilution microplate method was used to determine Minimum Inhibitory Concentrations (MICs) and the Trolox Equivalent Antioxidant Capacity (TEAC) assay was used to quantify antioxidant activity of all extracts. The optimal extract was the one developed by pretreatment with a single direct extraction with hexane prior to extraction with acetone. It had a TEAC value of 2.3, an increase in TEAC value of 283% compared to that of the crude acetone extract. The average MIC of the crude acetone extract against ATCC stains of *S. aureus, Ps. aeruginosa*, *E. coli* and *E. faecalis* had dropped from 0.15 mg/ml to 0.08 mg/ml in the optimal extract (an improvement in antibacterial activity of 87.5%).

Since the optimal extract is intended for commercial application in poultry production, its antibacterial activity was tested against *Campylobacter jejuni, Clostridium perfringens, Salmonella enteritidis, E. coli* and multi drug resistant *E. coli* isolated from chickens. Its *in vitro* toxicity was ascertained using the brine shrimp assay and the MTT cytotoxicity assay on monkey kidney cells. The optimal extract was effective against *Campylobacter jejuni* and *Clostridium perfringens* with MIC values ranging from 40 µg/ml to 80 µg/ml. It was also active against multi-resistant strains of *E. coli* and *Salmonella enteritidis* (MIC values of 125 µg/ml for both strains).

LC_{50} results from the brine shrimp assay and the MTT cytotoxicity assay on monkey kidney cells gave values of 863 µg/ml and 226 µg/ml respectively indicating low toxicity.

These results meant that though in some cases the MICs of the optimal extract were higher than befitting of typical antibiotics, due to its relatively low toxicity, large quantities of the extract may possibly be feed to achieve the desired activity without causing any toxicity in the poultry.

The major antioxidant compound was isolated by silica gel column chromatography. The isolated compound was identified by nuclear magnetic resonance and mass spectroscopy as combretastatin B5 (2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxybibenzyl), previously isolated from the seeds of *C. kraussii* and also from *C. woodii* leaves. Famakin (2002) showed this compound to be the major antibacterial compound in *C. woodii* leaves. Combretastatin B5 (CB5) demonstrated *in vitro* cytotoxicity in the MTT assay on monkey kidney cells with an LC_{50} value of 10 µg/ml. *In vitro* cytotoxicity of CB5 could be due to its antimitotic activity. The TEAC value of 7.9 found in this study means that combretastatin B5 has about 8 times the antioxidant capacity of vitamin E. This is the first report of the antioxidant activity of any of the combretastatins

Tolerance of broiler chickens to the optimal extract was assessed at clinically inferred doses of 2 mg/kg, 5mg/kg and 10 mg/kg. After 21 days of infeed-dosing with the optimal extract, none of the chickens died or showed any behavioral signs of toxicity. There were no statistically significant differences in weight gain between broilers fed the optimal extract and the positive and negative control. There was also no positive correlation between weight gain and amount of the optimal extract incorporated in feed.

Although the optimal extract did not result in significant growth promotion relative to the positive and negative control, 2 mg/kg dose regimens showed the best Feed Conversion Ratio (FCR), with a 6.2% improvement compared to the negative control. The positive control was the only other feed regimen to provide a positive FCR with an improvement of 1.73% compared to the negative control. Because purchase of feed could represent up to 80% of costs of broiler production, this is an important finding. If these results can be confirmed, the product may therefore have commercial value. Repetition of the experiment with lower doses of the optimal extract on poultry challenged with bacterial infections is required to confirm the commercial applicability of this product.

Chapter 1 Literature review

1.1 Introduction

The poultry industry is a major supplier of animal protein in the world. In South Africa, at least 10 million broilers and at least 16 million eggs are produced per week (Petersime newsletter, 2002). The recent intensive rearing for food production has led to a substantial increase in the use of veterinary medicines for therapeutic and prophylactic reasons in parallel with their use as growth promoters (Anadon and Martinez-Larranaga, 1999). Infectious diseases currently requiring extensive use of therapeutic or prophylactic drugs are respiratory and enteric diseases of poultry, pigs and calves, and mastitis in dairy cattle. Prophylactic treatment is particularly common during periods of stress on the animal, e.g. through dietary changes, loss of maternal interaction at weaning, after transport and co-mingling.

Under high-density housing conditions, infectious diseases are a major problem (Rogol *et al.*, 1985). Since 1946, it was found that low concentrations (i.e. sub-therapeutic levels) of antibiotic feed additives (AFAs) led to enhanced growth and productivity (Anderson *et al.*, 1999). Feed additives have been defined as "substances which improve both the feeding stuffs in which they are incorporated and livestock productivity". It is a prerequisite that they do not adversely affect either human or animal health or the environment (McEvoy, 2002).

Feed additives include antibiotic growth promoters, coccidiostats, binding agents and enzymes. The rationale behind including antibiotics as feed additives (when some of these were also used as veterinary medicines, e.g. tylosin), is that the dosage rates for feed additives are significantly less than for therapeutic use. Furthermore, only those compounds that are not used in human medicine have been authorised for use as growth promoters (McEvoy, 2002).

In their use as growth promoters, antibiotics are amongst others, thought to control the numbers of undesirable bacteria in the intestines, allowing a better absorption of nutrients. They also appear to prevent irritation of the intestinal lining and may enhance uptake of

nutrients from the intestines by thinning the mucosal layer (Anderson *et al.*, 1999). Literature suggests that under experimental conditions, improvements of up to 15% in weight gain or feed efficiency may be realised when antibiotics are used as feed additives (Doyle, 2001).

However, there has been increasing public concern over the possible links between veterinary drug residues in edible tissue and milk, the perception of widespread use of antimicrobial feed additives in animal feeds and the problems emanating from the transfer of antibiotic resistance and resistant genes to humans as a result of veterinary and zootechnical use in food-producing animals (Swann, 1968; Lamming, 1992; Klare *et al.*, 1995; Aarestrup *et al.*, 1986). The World Health Organisation (WHO) has also recently recommended a phasing out of the use of in-feed antibiotics used as growth promoters where such drugs are used in human therapeutics or are known to select for cross-resistance to antimicrobials used in human medicine (Anon, 1997). Consequently, in the European Union (EU), marketing authorisations for a number of such compounds, previously licensed as zootechnical feed additives, have been suspended resulting in the EU-wide prohibition on the use of avoparcin, ardacin (Anon, 1997b), spiramycin, tylosin, virginiamycin, zinc bacitracin, carbadox and olaquindox (Anon, 1998). There are now only four growth promoting antibiotics remaining that are permitted for use in animal feedingstuffs, these are avilamycin, flavophospholipol (flavomycin), salinomycin and monensin. However, it is proposed that even these be phased out by 2006 (McEvoy, 2002).

As a result of this imminent banning, there is a need to find alternatives to the use of antibiotics in animal production. Many approaches are being followed worldwide in an attempt to circumvent the development of resistant strains emanating from the continued use of antibiotics as feed additives without increasing the cost of production. New feed additives may have antibacterial activity to control proliferation of undesired microorganisms, and or antioxidant activity to boost the immune system (Surai, 2002).

1.2 The problem of antibiotic use

One of the major problems inherent in uncontrolled use of antibiotics is the development of resistant strains of bacteria. Dosages of antibiotics at levels not sufficiently high to kill all

bacteria, such as used in AFAs, promote the selection of resistant strains and this resistance can be transferred between different bacteria and between human and animal hosts (Aarestrup, 1999). There is a concern that the use of antibiotic feed additives could result in an increase in the environmental pool of resistance genes especially in the case where the same classes of antibiotics are being used in humans (Anadon and Martinez-Larranaga, 1999). The continued use of antibiotics will select for resistance genes in non-pathogenic bacteria that may later transfer the acquired resistance to different pathogenic bacterial species (Aarestrup, 1999). Thus the danger that antibiotic resistance developed in animal bacteria can be transferred to human pathogens as well as to those of zoonotic importance.

Food-producing animals such as cattle, pigs, turkeys or chickens contain microorganisms in their intestines. For example, *Salmonella* spp., *Campylobacter* spp., and *Escherichia coli* are common bacteria found in the intestines of various food-producing animals without causing any disease although all three bacteria cause food borne illness in humans. These organisms may develop resistance when continuously exposed to low levels of antibiotics administered to the animal (Rogol *et al.*, 1985). The resistant organisms can contaminate food products at slaughter and then infect humans who consume the food.

Residues are low levels of the drug and its metabolites that remain in the animal carcass or other food products of animal origin after drug administration has ceased. Residues are a problem if they persist at unacceptable levels at slaughter, as they then become available to the consumer of the food products (Dewdney *et al.*, 1991). Antibiotic residues in meat result in primary sensitization particularly in the case of ß- lactam based antibiotics, which will lead to hypersensitivity and allergic reactions when the individual is treated with this class of drugs (Dewdney *et al.*, 1991).

As a result of these concerns there is a need to find alternatives to the use of antibiotics in animal production.

1.3 **Possible solutions**.

Many strategies have been followed worldwide to circumvent the development of resistant strains as a result of continued use of antibiotics, these include developing new antibiotics, feeding animals with benign microorganisms (probiotics), enzymes such as phytase that

acid, phospholipids, amino acids, carnithine and carbohydrates. None of these products has really solved the problem, however, it may be necessary to combine two or more alternative feed ingredients or to combine a feed supplement with hygienic measures to attain the best effects (Doyle, 2001). Plant extracts have also been used as feed additives to try and counter the problems that arise form the continued used of antibiotics. Waihenya *et al.*, (2002) reported on the potential use of extracts from *Aloe* species as feed additives in chickens to help boost their immune system and fight against fowl typhoid.

1.3.1 Hygienic measures.

In recent years much emphasis has been placed on disease prevention through improved management and improved environmental conditions for livestock production as infection prevention strategies (World Health Organisation, 1998). These measures can work in Europe where agriculture is heavily subsidised but probably not in other countries where cost would inhibit sales of product where such price competition exists.

1.3.2 Probiotics.

Probiotics are bio-preparations containing living cells or metabolites of stabilised microorganisms that optimise the colonisation and composition of gut flora in both animals and humans and have an additive effect on digestive processes and immunity of the host (Fuller, 1992). They influence digestion by improving the microbial population that is beneficial to the host (Bomba *et al.*, 2002). Some bacteria produce proteins referred to as bacteriocins whose purpose is to eliminate competing bacteria.

1.3.3 Immunomodulators

These compounds can be antibodies and cytokines and they enhance the immune systems resistance to diseases (Doyle, 2001). Antibodies act against subclinical infections or competitive intestinal bacteria, while cytokines regulate immune response by mediating effects ranging from activation and differentiation of immune cells to enhancing the immune function and production of other cytokines.

1.3.4 Organic acids

A study to determine the growth promoting effects of organic acids in weaned piglets found the incorporation of organic acids in feed to generally improve growth, but the magnitude

of effect depends on the amount used and other components of the diet. Fumaric and formic acid have been used as food preservatives and can be used as feed additives (Doyle, 2001).

1.3.5 Plant extracts

Plant extracts have been known since antiquity to possess notable biological activity including antioxidant, antibacterial and antifungal properties. There is a growing interest in the use of natural products in human food and animal feed as consumer resistance to synthetic additives increases (van Wyk *et al.*, 1997). Concerns over the safety of use of synthetic additives in the food chain have led to their restricted use in several countries.

Antimicrobials of plant origin are of interest to the pharmaceutical industry for the control of microbial pathogens. Although the introduction of antibiotics dramatically improved the treatment of bacterial infections, the emergence of antibiotic resistant strains of bacteria has led to the continuing search for useful natural antimicrobials. 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 25% 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 (van Wyk *et al.*, 1997).

In this search for a solution to problems associated with the use of antibiotics as feed additives in livestock production, plant extracts developed from the leaves of *Combretum woodii* were used. Previous studies on the plant done by Famakin (2002) showed *C. woodii* leaf extracts to contain high antibacterial activity with minimum inhibitory concentration (MIC) values of 40 µg/ml against American Type Culture Collection (ATCC) strains of *Staphylococcus aureus* and *Enterococcus faecalis* and 80 µg/ml against *E. coli*. Because the active compounds appeared to be present in high concentrations, this prompted further inquiries into the plant hence this research.

1.4 Why choose plant extracts.

(1) Synergism occurs when the effect of two or more compounds applied together to a biological system is greater than the sum of the effects when identical amounts of each

constituent are used. Since plant extracts contain more than one biologically active compound, synergism may explain why the activity of an extract could be greater than that of pure compounds.

(2) Each plant is a unique chemical factory capable of synthesizing large numbers of highly complex and new chemical substances (Farnsworth, 1984). The abundance of plant species therefore provides us with a very broad spectrum of natural and novel chemical substances for our scientific inquiries.

(3) The biologically active substances derived from plants have served as templates for synthesis of pharmaceuticals. In the United States of America, about 25% of prescription drugs contain active principles that were derived from higher plants and there is increasing popularity in the use of herbal medicines (Farnsworth and Morris, 1976). It has also been estimated by the World Health Organisation that about 80% of the population of the developing countries rely exclusively on plants to meet their health care needs (Farnsworth *et al.*, 1985).

(4) Less time and resources are used in the development of an extract compared to isolation or synthesis of pure compounds; it is therefore more economical to develop biologically active extracts. Research has also shown fewer cases of undesirable side effects when dealing with extracts compared to pure compounds.

(5) The main source of the antimicrobial activity of plants is their secondary metabolites (Farnsworth, 1984). There are diverse and wide varieties of classes of these compounds therefore plant extracts may contain more than one antimicrobial compound and resistance is unlikely to develop. Some constituents may prevent the deterioration of others e.g. antioxidant compounds may preserve compounds susceptible to oxidation hence the stability of biologically active compounds in an extract may be enhanced.

1.5 Biological activity of plants

Higher plants produce hundreds to thousands of diverse chemical compounds with different biological activities (Hamburger and Hostettmann, 1991). It is believed that these compounds have an important ecological role, working as pollinator attractants and as chemical defences against insects and herbivores. They also exhibit biological activity against diverse classes of microorganisms e.g. McGaw *et al.*, (2001) reported on the anti-inflammatory, anthelmintic and antischistosomal activity of 20 *Combretum* species found in

against diverse classes of microorganisms e.g. McGaw *et al.*, (2001) reported on the anti-inflammatory, anthelmintic and antischistosomal activity of 20 *Combretum* species found in South Africa, while Gonzalez-Coloma *et al.*, (1994) detected antifeedant and insecticidal effects in plants from the Canary Islands and Japanese Lauraceae.

Plant-based antimicrobials have enormous therapeutic potential and are active against both plant and human pathogenic microorganisms (Cowan, 1999). They are supposedly effective in treatment of infectious diseases while simultaneously mitigating many of the side effects that are often associated with synthetic antimicrobials (Iwu, 1993).

A number of drugs of plant origin have been used in human medicine; typical examples include drugs like quinine. Quinine occurs in the bark of the *Cinchona officinalis* tree, apart from its usefulness in the treatment of malaria, it can also be used to relieve nocturnal leg cramps (Iwu, 1993). The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha* and related species, which has been used as an amoebicidal drug as well as for the treatment of abscesses resulting from *Escherichia histolytica* infections.

Similarly, higher plants have also played important roles in cancer therapies. Recent examples include the antileukaemic alkaloids, vinblastine and vincristine, which were both obtained from *Catharanthus roseus* and antineoplastic combretastatins from *Combretum caffrum* (Pettit *et al.*, 1987).

1.6 Sources of antimicrobial activity in plants

Secondary metabolites are the main sources of the antimicrobial activity of plants, among them, are compounds like tannins, flavonoids, terpenoids, alkaloids, and stilbenes. These compounds vary in structures and functions, but are generally derived by plants for protection against harmful pathogens in their surroundings and herbivory (Farnsworth, 1984).

1.6.1 Tannins

Tannins are water-soluble polyphenols, which differ from other natural phenolic compounds in their ability to precipitate proteins such as gelatine from solution (Bruneton,

acid in gallo-tannins or other phenolic acids derived from oxidation of gallic acid in ellagitannins. One of their molecular actions is to complex with polymers such as proteins and polysaccharides through non-specific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bonding (Haslam, 1996).
Their mode of antimicrobial action may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport protein and complex with cell wall (Cowan, 1999)

1.6.2 Flavones, flavonoids and flavonols

Flavones are phenolic structures containing one carbonyl group. The addition of a 3-hydroxyl group yields a flavonol. Flavonoids are also hydroxylated phenolic substances but occur as a C6–C3 unit linked to an aromatic ring (Goodwin and Mercer, 1972). Almost all are water-soluble; they are responsible for the colour of flowers, and fruits and sometimes leaves. Their activity is probably due to their ability to complex with extra cellular and soluble proteins and with bacterial cell wall. 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).

1.6.3 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 cyclisized.
Terpenes or terpenoids are active against bacteria and fungi (Taylor *et al.*, 1996).
The mechanism of action of terpenes is not fully understood, but it is speculated to involve membrane disruption by the lipophilic compounds.
A variety of triterpenoids have been isolated from *Combretum spp.* (Rogers and Verotta, 1996).

1.6.4 Alkaloids.

Heterocyclic nitrogen compounds are called alkaloids. Indoquinoline alkaloids, the active principle in *Cryptolepsis sanguinnolenta* has been shown to inhibit Gram-negative bacteria and yeast (Silva *et al.*, 1996). Alkaloids are active against bacteria specifically enteric

and yeast (Silva *et al.*, 1996). Alkaloids are active against bacteria specifically enteric pathogens, most notably *E. coli* (but also *Staphylococcus*, *Pseudomonas*, *Salmonella*, *Shigella*, *Streptococcus* and *Vibrio* spp.) and against *Candida* spp (Sawer *et al.*, 1995). Indoquinolizidine alkaloids and glycoalkaloids and saponins, the essential constituents of *Naclea latifolia* have antibacterial activity against Gram-negative and Gram-positive bacteria and antifungal activity (Iwu, 1993).

1.6.5 Combretastatins

Combretastatins are a group of closely related bibenzyls, stilbenes, dihydrostilbenes and phenanthrenes that have been isolated from the Combretaceae (Petit *et al.*, 1995). This group of compounds has been classified based on structure, into four classes and these are, stilbenes (A-class), dihydrostilbenes (B-class), phenanthrenequinones (C-class) and macrocyclic lactones (D-class). The first compound to be isolated in this group was isolated from *Combretum caffrum* and called combrestatin. Combrestatin caused mitotic arrest in cells in culture and interacted with tubulin, the major protein component of microtubules. It showed *in vitro* activity against the murine P388 leukaemia cell line (Pettit *et al.*, 1982).

Combretastatins are potent angiogenesis inhibitors, because of their antitumour activity these compounds have been used in the development of drugs for the treatment of cancers. The most documented drugs from this class being Combretastatin A-1 and combretastatin A-4 prodrugs (Shnyder *et al.*, 2003).

Combretastatin A-4, isolated from *C. caffrum*, is one of the most potent anti-mitotic agents that bind to tubulin. This compound has been shown to exhibit strong cytotoxicity against a variety of human cancer cells, including multi-drug resistant cell lines. The high potency of combretastatins A-1 and A-4 as angiogenesis inhibitors is due to their ability to bind to tubulin at the colchicine site. These compounds upon binding to tubulin prevent tumors from metastasizing by inhibiting their ability to grow new blood vessels. Anti-tumor vascular targeting agents are an entirely new drug class in cancer therapy. Instead of attacking malignant cells directly, these agents are aimed at attacking a tumor's blood supply, thereby depriving the tumor of the vital oxygen and nutrients necessary for its growth and survival (Bui *et al.*, 2002).

A stilbene 2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxybibenzyl (combretastatin B5) was isolated from the leaves of this plant by Famakin (2002). It showed significant antibacterial activity with MIC values against ATCC strains of *S. aureus* of 16 µg /ml and *Pseudomonas aeruginosa* (125 µg /ml), *E. faecalis* (125 µg /ml) and slight growth inhibitory activity against *E. coli*.

1.7 Antioxidant compounds

Antioxidant compounds protect the body by boosting the immune system. Their physiological role is to scavenge for free radicals (Surai, 2002). Free radicals are highly unstable and reactive species that are capable of damaging molecules such as DNA, proteins, and carbohydrates. The body is under constant attack from free radicals formed as a consequence of the body's normal metabolic activities (Surai, 2002).

Reactive oxygen species (ROS) are generated in specific organelles under normal physiological conditions (Haraguchi *et al.*, 1998). These compounds can be defined as oxygen containing free radicals such as hydrogen peroxide, hydroxyl radical and super oxide anion. Oxidative stress caused by an imbalance of ROS and antioxidants in the body damages chromosomes, inhibits key enzymes and initiates the peroxidation of lipid membranes. Oxidative stress can also be as a result of UV-radiation, some drugs whose hepatic metabolism result in the formation of hydrogen peroxide and other free radical, as well as due to air pollution (Hippeli and Elstner, 1991).

1.7.1 Phytochemical antioxidants

The therapeutic properties of certain plant extracts used in traditional medicine have been linked to their antioxidant abilities. Several natural compounds from plants exhibit antioxidant and or radioprotective properties (Tseng *et al.*, 1997). Antioxidant active compounds from plants exert their effect by enhancing the levels of antioxidant enzymes like superoxide dismutase and glutathione peroxidase or by lowering the levels of lipid peroxides in blood (Tseng *et al.*, 1997).

High antioxidant activity has been observed in pine bark (*Pinus maritima*) (Parker *et al.*, 1999), green tea leaves (*Camella sinesis*) and the leaves of *Gingko biloba* (Alan and

Miller, 2000), and grape seed (*Vitis vinifera*) extracts (Bagchi *et al.*, 1998). Flavones, proanthocyanidins, coumarins, phenylpropanoids, tannins and terpenoids, amongst others have been identified as the compounds typically responsible for the antioxidant activity of plant extracts.

1.7.2 Antioxidant compounds as feed additives

Lipid oxidation is the major cause of quality deterioration in meat and meat products. In muscles, lipid oxidation is primarily initiated in the unsaturated fatty acids of the phospholipids that are an integral part of mitochondrial and microsomal membranes (Rice and Kennedy, 1998). Protection of muscle-based food against lipid oxidation is thus dependent on the incorporation of antioxidants into the membranes.

Vitamin E has been reported as an excellent biological chain-breaking antioxidant that protects cells and tissue from lipoperoxidative damage induced by free radicals (McDowell *et al.*, 1996). The efficiency of this protection is related to the quantity of vitamin E incorporated relative to the level of unsaturated fatty acids present in membranes. Besides the presence of unsaturated fatty acids and the availability of α-tocopherol (vitamin E), the susceptibility to lipid oxidation may also depend on the presence of certain enzymes and other pro-oxidants, such as transition metals like iron and copper (Strain, 1984). Vitamin E is included into animal feed to improve performance, to strengthen immunological status, and to improve the quality of animal-originated food (McDowell *et al.*, 1996). Poultry cannot synthesize vitamin E; therefore, vitamin E requirements must be met from dietary supplements. Sahin *et al.*, (2002) reported that broilers supplemented with dietary vitamin E had a significant reduction in malondialdehyde (MDA) values, an indicator of lipid peroxidation, in serum and tissue. Vitamin E supplementation was also reported to alleviate the negative effects of heat stress on egg production and quality (Sahin *et al.*, 2002).

Vitamin C also plays an important role in animal health as an antioxidant supplement by inactivating free radicals produced through normal cellular activity and diverse stressors (Halver, 1995). The mode of action of vitamin C as an immunostimulant is not clear, although its antioxidant role and in consequence cell protection could be a mechanism to preserve blood cells, improving the general immunological system of food animals.

1.8 The Combretaceae family

1.8.1 Introduction.

Combretaceae is a pantropic family of trees, shrubs, climbers and mangroves (Carr, 1988). Many species of Combretaceae are widely distributed in southern Africa. The leaves and bark of these plants are used in traditional medicines for treating a variety of conditions including pneumonia, colds, chest coughs, fever, syphilis and mumps (Hutchings *et al.*, 1996).

1.8.2 Taxonomy

The Combretaceae family belongs to the order Myrtales consisting of 18 genera. The two largest genera in Africa include *Combretum* with about 370 species and *Terminalia* with about 200 species (Lawrence, 1951). The other genera are *Calopyxes* and *Buchenavia* comprising of 22 species each, *Quesqualis* 16, *Angioeissis* 14, *Conocarpus* 12, and *Pteleopsis* 10 species (Rogers and Verotta, 1996).

The genus *Combretum* has two subgenera these being subgenus *Combretum* and subgenus *Cacoucia. Combretum woodii* the plant under investigation is a member of the subgenus *Combretum,* and the section *Angustimarginata.* Other members of this section are *C. caffrum*, *C. erythrophyllum*, *C. kraussii* and *C. vendae* (Carr, 1988).

1.8.3 Ethnobotanical use of the Combretaceae

Hutchings *et al.*, (1996) listed many ethnobotanical uses of different *Combretum* spp. for example, *Combretum apiculatum* has been used for abdominal disorders and conjunctivitis; *Combretum erythrophyllum* and *Combretum hereroense* for abdominal pain and venereal disease; *C. kraussii* for wounds; *C. molle* for wounds, stomach complaints and diarrhoea; *Combretum zeyheri* for diarrhoea. Oliver–Bever (1983) indicated that *C. micranthum* is used for sores and abscesses and that *Combretum racemosum* is used against internal parasites.

Many other members of the Combretaceae are used for a variety of medicinal purposes by traditional healers. This includes treating dysmenorrhoea, earache, fattening babies, fever, headache, hookworm, infertility in women, leprosy, pneumonia, scorpion bite, snake bite,

swelling caused by mumps, syphilis, toothache, gastric ulcer, heart diseases, to cleanse the urinary system, dysentery, gallstones, sore throats, nosebleeds and general weakness (Hutchings *et al.*, 1996; van Wyk *et al.*, 1997).

There is no report in literature of the ethnobotanical uses of *C. woodii*; however, the presence of antimicrobial components in *Combretum* spp. explains the basis of some of their ethnobotanical uses.

1.8.4 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 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).

1.8.5 Antimicrobial activity of the Combretaceae

Species of Combretaceae contain compounds with potential antimicrobial properties (Eloff, 1999). 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). In the last two decades a series of stilbenes and dihydrostilbenes (the combretastatins) with potent cytotoxic activity and acidic triterpenoids and their glycosides with molluscicidal, antifungal and antimicrobial activity have been isolated from species of *Combretum* (Rogers, 1996). 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 of 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*.

1.9 Work on the Combretaceae done in the Phytomedicine Programme

Antibacterial activity of Combretum erythrophyllum

At least 14 antibacterial compounds were present in *Combretum erythrophyllum* extracts with minimum inhibitory concentrations (MICs) as low as 50 µg/ml to *Staphylococcus aureus* (Martini and Eloff, 1998).

Antibacterial activity and stability of 27 members of Combretaceae

Acetone extracts prepared from the leaves 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)

Stability of antibacterial activity in C. erythrophyllum

Leaves of *C. erythrophyllum* stored in herbaria for up to 92 years had the same antibacterial activity as fresh leaves collected from the area (Eloff, 1999).

Other biological activities of Combretum species

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

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).

Extraction of antibacterial compounds from Combretum microphyllum

Several extractants were tested to determine if any extractant selectively extracted antibacterial compounds. The most promising extractants were di-isopropyl ether, ethanol, ethyl ether, acetone and ethyl acetate. The activities towards Gram-negative and Gram-positive bacteria were similar (Kotze and Eloff, 2002).

Isolation of antibacterial compounds from C. erythrophyllum

Martini (2002) isolated and characterized seven antibacterial compounds. Four were flavonols: 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 sonei*. *In vitro* toxicity testing showed little or no toxicity towards human lymphocytes with the exception of 5-hydroxy-7, 4'-dimethoxyflavone. This compound is potentially toxic to human cells and exhibited the poorest antioxidant activity.

Isolation of antibacterial compounds from C. apiculatum

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

1.10 Combretum woodii

Combretum woodii is a deciduous tree or shrub with a height of 8–12 metres. It grows on steep rocky slopes, canyon margins, ravines, sand forest, dry forest, closed forest, woodland, rocky hillsides, mountain grassland, and low to medium altitudes (up to 1200 m). Its common names in South Africa are bastard forest bush willow, or iWapu (Zulu).

There is no report in the literature of the ethnobotanical uses of *C. woodii*, however, McGaw *et al.*, (2001) reported the water, acetone, and ethylacetate extracts of *C. woodii* leaves to possess some anti-inflammatory activity while Eloff (1999) reported its antibacterial activity.

Famakin (2002) demonstrated various extracts from the leaves of *C. woodii* to possess high antibacterial activity. A stilbene, 2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxybibenzyl (combretastatin B-5) was isolated from the leaves of *C. woodii* as the main antibacterial compound.

The activities of *Combretum* species are mainly due to stilbenoids, triterpenoids and flavonoids (Rogers, 1996). Triterpenoids and saponins are well known for their antimicrobial and anti-inflammatory activity (Bruneton, 1995). The anti-inflammatory and antimicrobial activity of *C. woodii* could be ascribed to these compounds.

Figure 1:1 *Combretum woodii* from which leaves were collected in Lowveld National Botanical Garden

1.11 Aims and objectives

- An extract with high antibacterial and antioxidant activity will be developed from the leaves of *C. woodii* by using simple bioassay guided extraction and enrichment procedures.
- The major biologically active compound(s) will be isolated and characterized.
- The *in vitro* toxicity as well as tolerance levels in chickens of the extract will be determined and its potential use as a feed additive in poultry production to replace the use of growth promoting antibiotics will be assessed.

Chapter 2 General Materials and Methods

2.1 Plant material

Plant leaf material was collected from trees at the Lowveld National Botanical Garden in Nelspruit (LNBG) as well as the Manie van der Schyff botanical garden at the University of Pretoria main campus (UP). Herbarium voucher specimens are kept at both institutes. The leaves were air dried under shade at room temperature and afterwards finely ground into a powder using a Jankel and Kunkel model A10 mill. The powder was stored in a sealed container at room temperature.

2.2 Extraction procedures

The choices of solvents used in an extraction are based on polarity and toxicity especially in the case of bioassay guided work (Houghton and Raman, 1998) and the Phytomedicine laboratory experience in this field (Eloff, 1998, Kotze and Eloff, 2000). In this research different extractants and extraction procedures were employed based on the desired outcome.

2.2.1 Preliminary extraction studies

In preliminary extractions, a direct extraction method was employed on one g of finely ground leaf samples from LNBG and UP in 10 ml each of five solvents of varying polarity. Technical grade solvents (Merck) of hexane, dichloromethane, ethylacetate, acetone, and ethanol were used with vigorous shaking for 10 minutes.

2.2.2 Extract enrichment procedures

These procedures involved serial extractions aimed at removing or selectively excluding inactive compounds from the final extract as enrichment procedures. Only leaf material from LNBG sample was used in these procedures and the final extracts were dissolved in acetone or in ethanol. Two extraction pathways were adopted. The first pathway involved enrichment of the extract by pretreating the leaf samples with a series of extractions with hexane (defatting) prior to extraction with acetone or ethanol. The second pathway involved the use of a variety of acetone in water and ethanol in water mixtures as solvents of extraction.

2.2.2.1 Hexane "wash"

One g samples of material were serially extracted with 10 ml each of hexane and then acetone or ethanol. The residue was dried before the next extraction. Extraction with hexane was done until there was no colour change between the extracts.

2.2.2.2 Acetone in water and ethanol in water mixtures

One g samples of *C. woodii* leaf powder from LNBG were extracted in 10 ml each of acetone, ethanol, water, and 20%, 40%, 60%, 80% acetone or ethanol in water mixtures.

2.2.3 Optimal extraction method

Three pretreatment procedures that had been identified in the enrichment procedures were employed before subsequent extraction with acetone and ethanol. One g samples of leaf material from the LNBG sample were serially extracted with 10 ml each of solvent as outlined in Table 2.1 to ascertain the best extraction method.

Table 2:1: Table showing pretreatment procedures employed in best extraction method

Pretreatment	Extract
Hexane "wash"	Acetone or ethanol extract
20% acetone or ethanol in water "wash"	80% acetone or ethanol in water extract
Hexane "wash" first followed by 20% acetone or ethanol in water "wash"	Acetone or ethanol extract

In all the extraction procedures, the extracts were developed by shaking the leaf samples in solvent for 5 minutes, centrifuging at 3600 rpm for 5 minutes and filtering to remove particles that did not settle on centrifugation. The solvents in the supernatant were removed under a cold air stream at 7 °C and the extracts reconstituted in acetone or where stated, in ethanol, to a concentration of 10 mg/ml.

2.3 Phytochemical analysis

The chemical constituents of the extracts were analysed using aluminium backed thin layer chromatography (TLC (Merck, Kieselgel 60 F_{254})) plates. The plates were prepared in triplicates and 10 µl of the different extract solution concentrated to 10 mg/ml applied and allowed to develop in three solvent systems within closed tanks. The following solvent systems were used to separate 100 µg (10 µl of 10 mg/ml) of the different extracts:

1. Benzene: Ethanol: Ammonium hydroxide (36:4:0.4) (BEA) [non-polar/basic].
2. Chloroform: Ethylacetate: Formic acid (20:16:4) (CEF) [medium polarity/acidic].
3. Ethylacetate: Methanol: Water (40:5.4:4) (EMW) [polar/neutral].

These solvent systems were used because they are optimized to separate components of extracts of Combretaceae members (Eloff, 1998a).

The TLC plates were air-dried and the separated components were visualised using the following spray reagents:
(1) Vanillin–sulphuric acid (0.1 g vanillin, 28 ml methanol, 1 ml sulphuric acid) for detection of higher alcohols, phenols, steroids, and essential oils (Stahl, 1969).
The plates were heated at 105 °C until the development of colour is complete.
(2) 2, 2,diphenyl-1-picrylhydrazyl (DPPH) – spray (0.2% DPPH in methanol) for detection of antioxidant compounds. The plates are left to dry in the fumehood.

2.4 Bioautography method

The bioautography procedure described by Begue and Kline (1972) was used. Dried TLC (Merck, Kieselgel 60 F_{254}) plates were sprayed with a concentrated suspension of actively growing cells of American Type Culture Collection (ATCC) strains of *Staphylococcus aureus* (ATCC 29213), *Enterococcus faecalis* (ATCC21212), *Pseudomonas aeruginosa* (ATCC 25922) and *Escherichia coli* (ATCC 27853) obtained from the Microbiology Laboratory, Faculty of Veterinary Science, University of Pretoria. The plates were sprayed with bacteria until they were wet and opaque before incubation overnight at 38 °C in a clean chamber at 100% relative humidity. Following incubation, the plates were sprayed with 2 mg/ml solution of p–iodonitrotetrazolium violet (INT). Clear zones against a red background on TLC plates indicated inhibition of growth after incubation for about an hour (Begue and Kline, 1972).

2.4.1 Preparation of TLC plates for bioautography

TLC plates were prepared in duplicates (3x2) for each microorganism. One hundred µg of extract was loaded on the plates and developed in the different mobile systems mentioned in 2.3. The TLC plates were dried over night under a stream of air to remove the remaining solvents that might kill the bacteria before spraying the plates.

2.4.2 Preparation of bacteria

A 10 ml dense culture of fresh bacteria was centrifuged at 3600 rpm for 10 minutes to concentrate the bacteria. The supernatant was discarded and the pellet resuspended in 2 to 4 ml of fresh Mueller Hilton broth. This increased the concentration of bacteria in the cultures and also provided a fresh supply of the growth media.

2.5 Quantification of Antibacterial activity

The minimum inhibitory concentrations (MICs) were determined using the microplate serial dilution method (Eloff, 1998b). For the preliminary tests, specific ATCC strains of two Gram-positive (*S. aureus* (ATCC 29213) and *E. faecalis* (ATCC21212)) and two Gram-negative (*P. aeruginosa* (ATCC 25922) and *Escherichia coli* (ATCC 27853)) bacteria were used. The selection of test organism strains is based on the recommendation of the National Committee for Clinical Laboratory Standards (NCCLS) that these bacterial strains should be used as clinical laboratory standards. These species of bacteria are also the major cause of nosocomial infections in hospitals (Sacho *et. al.*, 1993).

The optimal extract was tested for activity against *Campylobacter jejuni,* anaerobic *Clostridium perfringens, Escherichia coli* and multi-drug resistant strains of *Escherichia coli* as well as *Salmonella enteritidis* isolated from poultry. In these tests gentamicin was used as the positive control and the dissolution solvent (acetone) as the negative controls. INT solution (0.2 mg/ml) was used as the indicator dye. The work was done in the laminar flow cabinet to limit contamination of the culture.

The experimental design for MIC determination was as follows:
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).
Tests group 2: Positive control, it contained the pathogen plus gentamicin.
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.

2.5.1 Dilution of extracts

Two-fold serial dilutions were dispensed into 96-well microplates as follows, distilled water (100 µl) was placed in each well using a Socorex multichannel micropipette and 100 µl of a 10 mg/ml extract concentration was placed in each of the first well of the relevant series of dilution, and thereby diluting the extracts in these wells by 50%, 100 µl was removed from it and placed into the next well. The concentration in this well would then be 25% of the original; the next would be 12,5% and so on. The process was repeated all the way to the bottom of the plate. One hundred µl from the last row was discarded to ensure that all the wells contain 100 µl of the extract. Each column therefore had a series of two-fold dilutions of the extract concentration.

2.5.2 Addition of bacteria

Cultures of *S. aureus, E. faecalis, P. aeruginosa*, and *E. coli* grown overnight at 37 °C and stored in the refrigerator for up to 10 days before testing were used (Eloff, 1998b). One hundred µl of broth containing the relevant bacteria was placed in each of the wells and mixed by squirting the bacteria into wells. The microplates were incubated overnight at 37 °C. After incubation, 40 µl of 0.2 mg/ml INT was added to each well. The microplates were visually examined for colour change after 60 minutes of incubation.
The lowest concentration where growth is inhibited was noted and recorded, and the MIC values of the extract were calculated from the original concentration of the extracts.

2.6 Antioxidant activity

Qualitative and quantitative analysis of the antioxidant activity was done using the DPPH assay and TEAC assay respectively.

2.6.1 Qualitative 2, 2,diphenyl-1-picrylhydrazyl (DPPH) assay on TLC

One hundred µg of extracts were loaded on TLC (Merck, Kieselgel 60 F_{254}) plates and developed in the three solvent systems mentioned in 2.3. The plates were dried and sprayed with 0.2% DPPH in methanol 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.

2.6.2 Quantitative Trolox Equivalent Antioxidant Capacity (TEAC) assay

Quantitative analysis of antioxidant activity was performed on the tentative best extracts and the isolated compound (CB5) using the TEAC assay. The method involves prior generation of the radical monocation 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$). The blue/green chromophore $ABTS^{\cdot+}$ is produced through the reaction between ABTS and potassium sulphate (Re *et al.*, 1999). The addition of antioxidants to the free radical reduces it to a colourless $ABTS^{\cdot+}$, a reaction that depends on the concentration of the antioxidant and the duration of the reaction. The extent of decolourisation as a percentage inhibition of $ABTS^{\cdot+}$ with time is calculated using a spectrophotometer and compared to the reactivity of Trolox against $ABTS^{\cdot+}$ under the same conditions (Re *et al.*, 1999).

2.6.2.1 Preparation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\cdot+}$)

Mixing 192 mg of $ABTS^{\cdot+}$ with 50 ml of water made a 7 mM stock solution of $ABTS^{\cdot+}$. The ABTS free radical was produced by reacting ABTS stock solution with 33 mg (2.45 mM) of potassium sulphate (final concentration). The solution was prepared 12-16 hours before use and stored at 4 °C, until required.

2.6.2.2 Experimental procedure

Different concentrations of the test extracts and Trolox were prepared by serially diluting one mg/ml of each sample.

The prepared $ABTS^{\cdot+}$ solution was diluted with ethanol to an absorbency of 0.7 ± 0.02 at 734 nm (ethanol used as blank) after which one ml was added to 10 µL of the one mg/ml solution of Trolox. The absorbance reading was taken after 6 minutes of reaction time. This was repeated for the remaining concentrations of Trolox and all the other extracts. All determinations were carried out in triplicate.

2.7 *In vitro* toxicity studies on the optimal extract

In vitro toxicity profiling of the optimal extract was determined by using the brine shrimp assay and the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide dye (MTT) cytotoxicity assay.

2.7.1 Brine shrimp assay

The brine shrimp assay was performed as described by Desmarchelier *et al.*, (1996). Brine shrimp (*Artemia salina*) eggs were hatched in the dark in a beaker filled with salty water (3.8 g NaCl in 100 ml distilled water). After 48 hours, the phototrophic nauplii were collected by 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 helped 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 ten shrimp were transferred to each well of a 96–well microplates containing the samples. The concentrations at which the optimal 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 (salty water) were determined under the microscope.

Podophyllotoxin was used as positive control and acetone as the negative control
The toxicity of the optimal extract to brine shrimps was determined in triplicate and the average percentage of live shrimps calculated.

2.7.2 MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

Logarithmically growing monkey kidney cells (Vero) were seeded into 96-well microplates (200 µl/well at a density of 0.5–10 x 10^3 cells/ml) and exposed to various concentrations (0.001-1 mg/ml) of the optimal extract that had been reconstituted in ethanol for 120 hours. The cytotoxicity of the extract at the different concentrations was analyzed in triplicate. Minimal Essential Medium (Highveld Biological) supplemented with 0.1% gentamicin and 5% fetal calf serum was used as the growth medium and ethanol was used as the negative control and Berberine chloride (Sigma) was used as positive control. After

incubation with the test-compound for 120 hours, MTT-solution (5 mg/ml in PBS) was added (30 µl/well). Plates were further incubated for 4 hours at 37°C. After incubation the plates were centrifuged at 2000 rpm for 10 minutes. The growth medium was carefully removed from each well and 150 µl PBS solution was added into each well followed by centrifugation at 2000 rpm again. The formazan crystals formed were dissolved by adding 50 µl/well of DMSO to each well. Absorbance was measured using an ELISA reader (Titertele Multiscan MCC/340) at 540 nm and a reference wavelength of 690 nm.

2.8 Isolation of compounds from *C. woodii* leaves

2.8.1 Serial exhaustive extraction

Serial exhaustive extraction (SEE) was taken as the first fractionation step in the isolation of the major antioxidant compound from the leaves of *C. woodii*. In preliminary serial exhaustive extraction studies 10 g of leaves were extracted serially with 100 ml each of various solvents. Three trial runs were performed to determine the best series of solvents to use. The series were set up and run as follows:

1: Hexane, dichloromethane, acetone and methanol.

2: Hexane, ethylacetate, acetone and methanol.

3: Hexane, acetone and methanol.

The best extraction series was chosen based on quantities extracted and results from DPPH assay on TLC.

Subsequently one kg of material was subjected to serial exhaustive extraction using hexane, acetone and methanol (series 3). Extraction with each solvent was carried out until there was no noticeable colour change in the extract.

2.8.2 Vacuum liquid column chromatography

Column chromatography was used to further simplify the acetone fraction from serial exhaustive extraction. A large column (60 cm x 5 cm) was packed with silica gel 60 (63-200 µm). The acetone fraction from SEE was dried in a rotary evaporator to determine mass of the fraction to be used for column chromatography.

About 20 g of the acetone fraction was dissolved in a small volume of hexane and applied to a column packed with 200 g silica gel 60. The sample was introduced at the top of the

The mobile phase was added in portions to fill the top of the column. The components of the acetone fraction were eluted through the column under a vacuum (VLC).

Table 2:2: Solvent mixtures used in column chromatography

Elution system
Hexane
10% Chloroform in hexane
20% Chloroform in Hexane
40% Chloroform in Hexane
60% Chloroform in hexane
80% Chloroform in Hexane
100% Chloroform
10% Methanol in chloroform
20% Methanol in Chloroform
40% Methanol in Chloroform
60% Methanol in Chloroform

Elutes from each solvent mixture were collected into different flasks to render 11 fractions. The composition of each fraction was analysed using TLC.

2.8.3 Small column separation

Fractions containing the main antioxidant compound (fraction 10 and 11) were combined to make target fraction 2 (TF2). Four g sample of TF2 was subjected to further fractionation on a small column (60 cm x 2 cm) packed with 40 g of silica gel 60 and eluted with hexane/ethylacetate (1:2) mixture under gravity. The resultant eluents were collected in 250 small test tubes and placed in the fume cabinet under a stream of air to concentrate the fractions for further analysis on TLC. The antioxidant compound was isolated by crystallization from eluents in test tubes with pronounced antioxidant activity.

2.8.4 Structure elucidation of the active fraction

^{13}C and ^{1}H Nuclear Magnectic Resonance (NMR) and Mass Spectra (MS) techniques were used to elucidate the structure of the isolated compound. Spectroscopic studies were

2.8.4 Structure elucidation of the active fraction

^{13}C and ^{1}H Nuclear Magnectic Resonance (NMR) and Mass Spectra (MS) techniques were used to elucidate the structure of the isolated compound. Spectroscopic studies were done at the Hans Knöll Institute (HKI) in Jena, Germany. ^{13}C and ^{1}H NMR was done on a 300 MHz Varian NMR machine (Oxford instruments) and MS was done using a triple quadrupole mass spectrometer (Quattro (VG Biotech, England); EIMS, 70 eV direct inlet)

2.8.5 *In vitro* cytotoxicity and antioxidant activity of CB5

The antioxidant activity and cytotoxicity of the isolate was quantified using the TEAC assay and the MTT assay respectively. The methods used are outlined in sections 2.6 and 2.7.2.

2.9 *In vivo* toxicity studies on broiler chickens

2.9.1 Study design

A controlled, randomised, 5-treatment parallel group design was used. Three treatment groups were different dose regimens of the optimal extract plus the positive and negative control.

2.9.2 Trial animals

Healthy broiler chickens (185) sourced at one day old from a commercial hatchery (Eagle's pride) were used. Of this number, 10 birds were kept to compensate for up to 2 mortalities per treatment group, thus ensuring that at least 35 birds were evaluated per treatment group. The birds were housed at the Research Facilities of the Poultry Reference Centre, Faculty of Veterinary Sciences, University of Pretoria, where they were raised under conditions similar to those of commercial chickens. Feed and water were supplied *ad libitum*. The starter feed was a standard ration fed to broilers without AFAs. The grower feed was the standard ration plus the extract. Ten birds from the group that received the highest dose were subjected to pathological examination.

2.9.3 Calculation of dose

MIC of optimal extract on chicken pathogens was in the order of 0.1 mg/ml for many of the poultry pathogens [Table 6.1]. A 2 Kg bird has a gut volume of 100 ml and eats 3 Kg of feed over whole period. Therefore three Kg of feed should contain sufficient extract to yield 0.1 mg/ml.

2.9.4 Treatment

To bracket the clinically inferred dose (3.3 mg/Kg), chickens were provided with 2, 4 and 10 mg/Kg doses. The broiler chickens were placed in the following treatment groups:

a) Optimal extract at 2 mg/kg dose
b) Optimal extract at 4 mg/kg dose
c) Optimal extract at 10 mg/kg dose
d) Untreated controls using feed without any AFAs
e) Positive controls – treated with bacitracin

The optimal extract was included in the grower feed starting from age 21 days up until age 42 days. The positive control was included from age 21 days and withdrawn at age 35 days.

Chapter 3 Preliminary extraction studies

3.1 Introduction

The main aim of preliminary extraction studies was to identify the solvents that extracted the largest quantities of material while also extracting high antibacterial and antioxidant activity in their extracts. 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). 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 and Eagles, 1996).

Many scientists extract dried plant materials using solvents that are liquid at room temperature, however, other techniques such as steam distillation and the use of supercritical fluids or pressurised gas may be employed (Houghton and Raman, 1998).

Extraction can be direct, which involves bringing the plant material in contact with the solvent for a period of time; or serial which involves the use of many solvents of varying polarities successively on the same material. Soxhlet extraction can be very useful for the exhaustive extraction of material with a particular solvent, but this cannot be used for thermolabile compounds. The problem may be overcome by extracting under reduced pressure.

The Combretaceae is particularly rich in stilbenes, alkaloids and flavonoid compounds (Rogers, 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.

Many solvents can be used to achieve the desired result, but the extract was intended for use in poultry, therefore it was necessary to select solvents that are non-toxic or otherwise would be easy to remove by evaporation. Preliminary extraction studies should be

regarded as a selection process and further studies were conducted using the solvents that presented the desired properties.

The secondary objective was to compare the biological activities of extracts from plant samples collected from two locations. Since it was necessary for us to have positive identification and a record of the plants we were using, we worked with plants growing in botanical gardens. Leaf material samples were collected from the Manie van der Schyff Botanical Garden at the University of Pretoria main campus (UP) and from the Nelspruit Lowveld National Botanical Garden (LNBG).

The antibacterial and antioxidant activities of the extracts from these two samples were determined using the MIC method and the DPPH assay on TLC respectively.

3.2 Extraction method

A direct extraction method was performed with 5 solvents of varying polarities; acetone, ethanol, ethylacetate, dichloromethane and hexane were used. These solvents were chosen based on their safety in application or their ease of evaporation.

One gram of samples were mixed with 10 ml each of the extractant in a shaker for 5 minutes and centrifuged at 3600 rpm for five minutes to separate the marc from the extract. The extracts were dried under a cold air fan, weighed and reconstituted in acetone to a concentration of 10 mg/ml

3.3 Results and discussion

Table 3:1: Amount in milligrams extracted in a single direct extraction of one gram of sample in 10 ml solvents, for the Nelspruit Lowveld National Botanical Garden (LNBG) and University of Pretoria main campus garden (UP) samples.

	Acetone	Ethanol	Ethylacetate	Dichloromethane	Hexane	Average
LNBG sample	82	88	87.5	81	38	75.3
UP sample	68.5	71	76.5	77.5	35.5	65.8

There was not much difference in quantities extracted between acetone, ethanol, ethylacetate and dichloromethane extracts of either sample. However, larger quantities of material were present in the extracts from the LNBG sample (75.3 mg/g) compared to the UP sample (65.8 mg/g).

Hexane extracted the lowest quantities (approximately half the quantity extracted by the other four solvents) while ethanol extracted the largest quantities (8.8%), followed by ethylacetate (8.75%), acetone (8.2%) and dichloromethane (8.1%) for the LNBG sample. Conversely for the UP sample the largest quantities were in the dichloromethane extract (7.75%), followed by ethylacetate (7.65%), ethanol (7.1%), acetone (6.85%) and hexane (3.55%). In general, acetone, ethanol, ethylacetate and dichloromethane all extracted comparable quantities for their respective samples.

3.4 Phytochemical analysis

3.4.1 Introduction

Thin layer chromatography (TLC) was used to investigate the composition of the various extracts. 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 value = distance moved by analyte / distance moved by solvent front

For semi-quantitative analysis, the size and colour intensity of zones on TLC plates are related to the amount of substance present. The amount of substance initially applied to the plate must be an accurately measured volume of a solution of known concentration. The zone produced after development must exhibit a measurable function proportional to the amount of substance present. This relationship can be measured by visual inspection, examining the size and intensity of the sample zone in relation to those of standards applied in the same volume.

The detection methods used in TLC are determined largely by the nature of substances present in the mixture. Although some components of an extract may be coloured and thus easy to visualize if the stationary phase is white, the vast majority will have little or no colour and other methods have to be used to make them visible. The two most common methods are examination under UV-light and the use of spray reagents to produce fluorescent or, more commonly, coloured derivatives. This latter method usually requires subsequent heating of the plate (Houghton and Raman, 1998).

3.4.2 Method

Ten µl of each extract (100 µg) was loaded onto TLC (Merck, Kieselgel 60 F_{254}) plates and developed in three solvent systems as indicated in 2.3 above. The air-dried plates were then sprayed with vanillin-sulphuric acid reagent and heated in an oven at 105 °C for two minutes to enable visual detection of extract composition.

3.4.3 Results and discussion

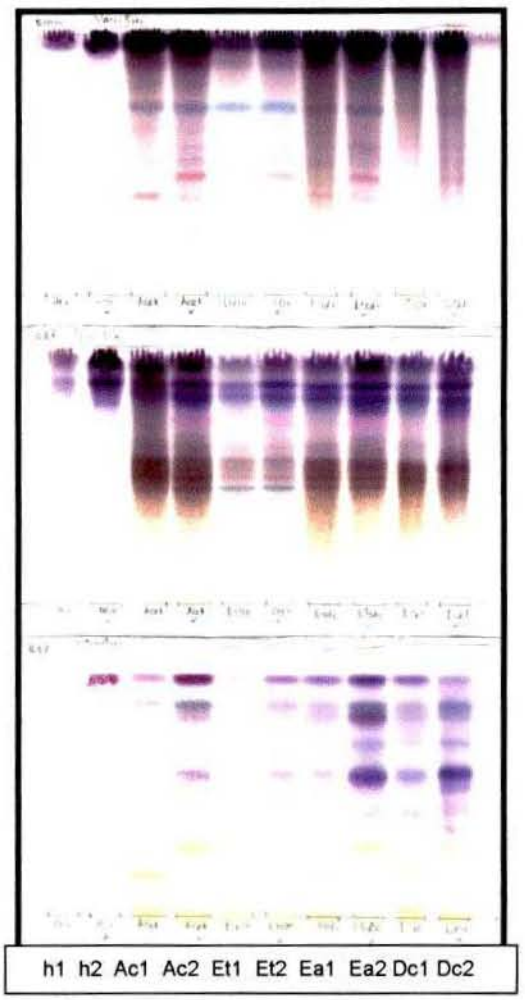


Figure 3:1:TLC profiles of the LNBG sample (1) and UP sample (2) developed side by side in EMW (top), CEF (centre), and BEA (bottom) solvent systems and sprayed with vanillin–sulphuric acid. **Lanes from left to right**: Hexane extracts (h), acetone extracts (Ac), ethanol extracts (Et), ethylacetate extracts (Ea) and dichloromethane extracts (Dc).

The chromatographic profiles of the LNBG and UP samples were generally similar in terms of numbers of compounds visible on the chromatograms, the size of bands and their colour intensities when the plates were sprayed with vanillin–sulphuric acid reagent [Figure 3.1], suggesting similar composition and occurrence of the compounds in approximately the same quantities in the two samples

3.5 Antioxidant screening

3.5.1 Introduction

Various assays have been used to test for antioxidant activity but the mostly widely used methods are those that involve generation of free radical species that are then neutralised by antioxidant compounds (Arnao *et al.*, 2001). 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 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 react with DPPH, thus quenching its absorption, DPPH is therefore reduced 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. 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).

3.5.2 Method

One hundred µg of each extract was loaded on TLC (Merck, Kieselgel 60 F_{254}) plates and developed in three solvent systems described in 2.3. The dried plates were sprayed with 0.2% DPPH in methanol and the presence of antioxidant compounds was detected by yellow bands against a purple background.

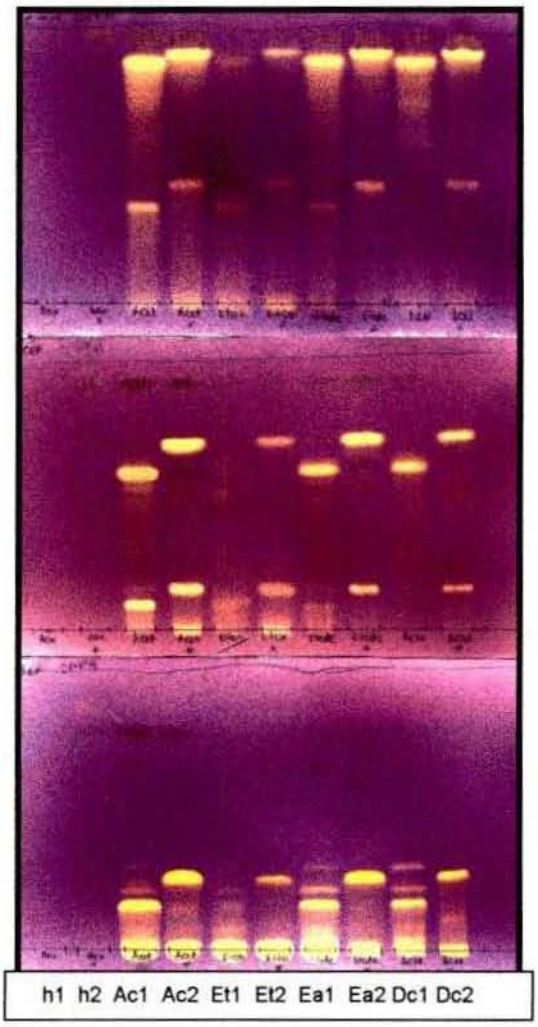


Figure 3:2: TLC profiles of the LNBG sample (1) and UP sample (2) developed side by side in EMW (top), CEF (centre), and BEA (bottom) solvent systems and sprayed with 0.2% DPPH in methanol. **Lanes from left to right**: Hexane extracts (h), acetone extracts (Ac), ethanol extracts (Et), ethylacetate extracts (Ea) and dichloromethane extracts (Dc).

TLC plates sprayed with 0.2% DPPH in methanol revealed the presence of two major antioxidant compounds that appeared immediately after spraying [Figure 3.2]. Though the antioxidant compounds had different R_f values for the different samples, the colour intensities and reaction speed with which they formed yellow bands of inhibition on TLC plates were similar suggesting similar radical scavenging characteristics. Hexane extracts did not possess antioxidant compounds.

Table 3:2: R_f values of the 2 major antioxidant compounds present in the acetone, ethanol, ethylacetate and dichloromethane extracts of *C. woodii* leaves when developed in EMW, CEF and BEA, for the UP and the LNBG samples.

	EMW		**CEF**		**BEA**	
	1st	2nd	1st	2nd	1st	2nd
U. P sample	0.90	0.40	0.69	0.15	0.25	0
LNBG sample	0.85	0.35	0.56	0.08	0.15	0
Difference	0.05	0.05	0.13	0.07	0.1	0

The antioxidant active compounds in the UP sample had higher R_f values compared to the LNBG sample by a consistent magnitude of 0.05 when developed in EMW solvent system [Table 3.2]. This means that the two samples have different antioxidant compounds as evidenced by their different R_f values, but the difference may be because the compounds in one sample occurred as derivatives e.g. glycoside or hydroxylated derivatives, in the other. The large difference in R_f values in either sample when developed in CEF meant that CEF is the best separation system for the antioxidant compounds in *C. woodii* leaves and also suggests that these compounds are of intermediate polarity. BEA (a non-polar) system was the least ideal system for separation of the antioxidant compounds of *C. woodii* extracts with only one of the two major compounds moving up on TLC.

3.6 **Bioautography**

Bioautography of the extracts from the two samples was performed to identify the antibacterial compounds present in them. Clear zones against a red background on the chromatogram sprayed with 2 mg/ml INT indicated growth inhibition.

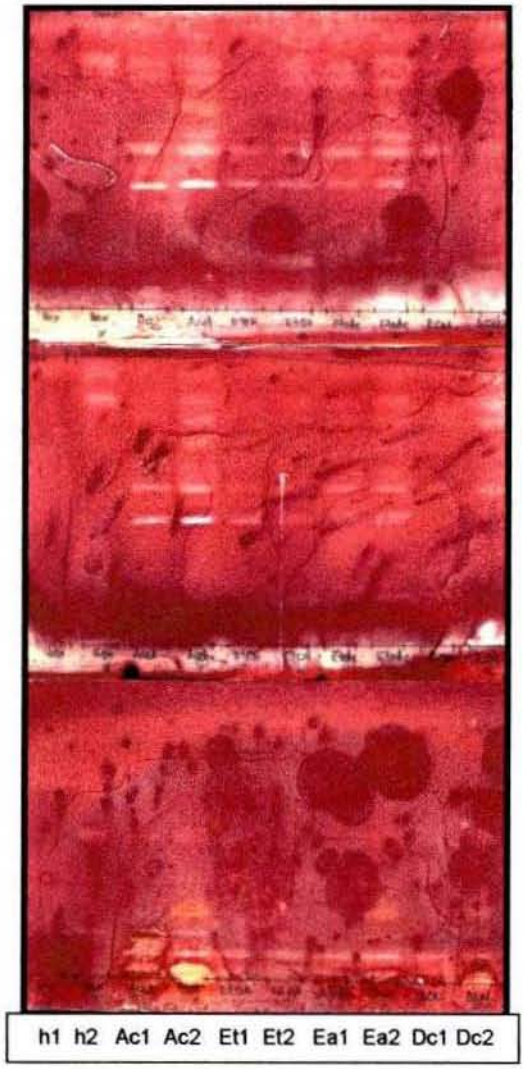


Figure 3:3 Bioautography profiles of the LNBG sample (1) and UP sample (2) developed side by side in EMW (top), CEF (centre), and BEA (bottom) solvent systems and sprayed with *S. aureus*. **Lanes from left to right**: Hexane extracts (h), acetone extracts (Ac), ethanol extracts (Et), ethylacetate extracts (Ea) and dichloromethane extracts (Dc).

Bioautography worked well with the two Gram-positive bacteria, *E. faecalis* and *S. aureus* [Figure 3.3] but not as well with the Gram-negative bacteria *E. coli* and *P. aeruginosa*. Antibacterial compounds were present in the acetone, ethanol and ethylacetate extracts (about 6 bands of inhibition on TLC developed in EMW solvent system), while the dichloromethane extracts showed fewer zones of inhibition and the hexane extracts had the lowest number of antibacterial compounds (one band of inhibition on TLC developed in EMW solvent system) [Figure 3.3].

The best separation of antibacterial compounds was obtained with EMW and CEF solvent systems suggesting the antibacterial compounds to be intermediate polar to polar compounds. It was necessary to completely remove formic acid from TLC plated developed in CEF solvent systems because it is quite toxic to bacteria, therefore plates developed in this solvent system were allowed to dry for two days before spraying with bacteria.

Bioautography technique has a drawback in that coloured compounds may mask the growth inhibition of the bacteria. Some of the more polar components have a green, yellow or brown colour and it is possible that some of these compounds may also inhibit the growth of one or more of the test organisms but their colour may mask the presence of clear zones of growth inhibition. This situation occurred with *Combretum erythrophyllum* extracts (Martini and Eloff, 1998).

3.7 Quantification of antibacterial activity of C. woodii leaf extracts

3.7.1 Introduction

Agar diffusion assays cannot be used to determine the antibacterial activities of plant extracts. The technique works well with a single compound (Hewitt and Vincent, 1989), but when examining extracts containing unknown compounds there are problems leading to false negative and false positive results (Eloff, 1998b).

An alternative technique is serial dilution of the extracts in a number of test tubes followed by addition of test organisms to determine the minimum inhibitory concentration (MIC) for the test organism using turbidity as an indicator of growth. This technique requires large quantities of extracts and is therefore not useful in bioassay-guided isolation of antimicrobial compounds (Eloff, 1998b).

Eloff (1998b) developed a microdilution technique that involves the use of 96-well microplates and tetrazonium salts to indicate bacterial growth. The method worked well with *S. aureus, E. faecalis, P. aeruginosa*, and *E. coli* and is useful in bioassay work because it requires small quantities of materials. This method was used in the quantification of the antibacterial activity of various *C. woodii* extracts.

3.7.2 Method

The minimum inhibitory concentration (MIC) values were determined using the microplate dilution method (Eloff, 1998b). ATCC strains of *S. aureus, E. faecalis, P. aeruginosa* and *E. coli* were used as test organisms and the method was performed as outlined in 2.5.

3.7.3 Results and discussion

Table 3:3: Amount in mg extracted from one gram samples and MIC values in mg/ml for *C. woodii* leaf extracts of hexane (H), dichloromethane, (Dc), ethylacetate (Ea), acetone (Ac) and ethanol (Et) for the LNBG (N) and UP (M) samples against ATCC strains of the four test bacteria.

	H(N)	H(M)	Ea(N)	Ea(M)	Dc(N)	Dc(M)	Ac(N)	Ac(M)	Et(N)	Et(M)
Quantity	38	35.5	87.5	76.5	81	77.5	82	68.5	88	71
					MIC in mg/ml					
E. coli	0.31	0.31	0.08	0.16	0.08	0.16	0.16	0.16	0.04	0.08
S. aureus	0.63	0.63	0.08	0.08	0.16	0.16	0.04	0.16	0.16	0.16
E. faecalis	0.63	0.63	0.04	0.08	0.08	0.08	0.04	0.08	0.08	0.16
P. aeruginosa	0.63	0.63	0.31	0.31	0.63	0.63	0.31	0.31	0.31	0.31
Average MIC	0.55	0.55	0.13	0.16	0.24	0.26	0.14	0.18	0.15	0.18

MIC values for the extracts tallied with the results from bioautography with the acetone, ethanol and ethylacetate extracts showing the highest bacterial growth inhibitory properties while the hexane extract showed the least antibacterial activity [Table 3.3]. MICs as low as 0.04 mg/ml were realized in the acetone and ethylacetate extracts of the LNBG sample against *S. aureus* and *E. faecalis*.

3.8 Reciprocal of MIC value

One way of expressing antibacterial activity on a positive scale is to express it as the reciprocal of MIC. The units of this measure are ml/mg and the value indicates the volume to which one mg of extract can be diluted in ml and still inhibit bacterial growth.

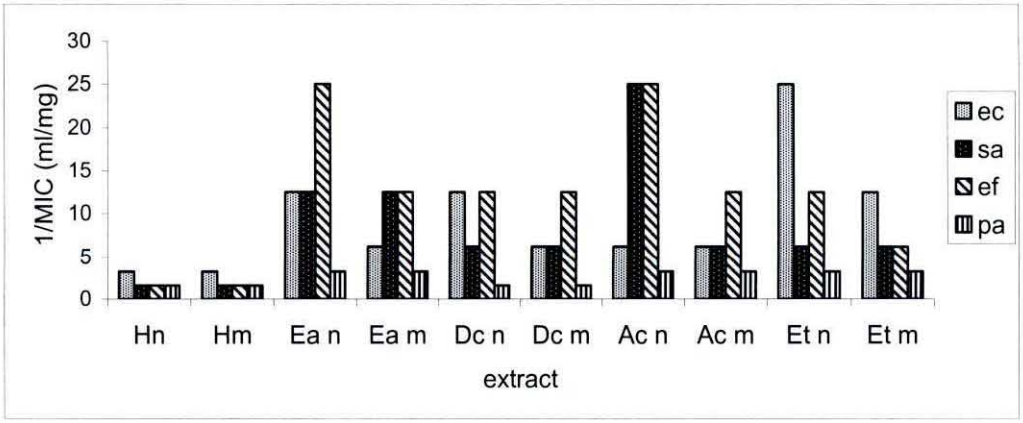


Figure 3:4: Reciprocal of the MIC values of the extracts against *E. coli* (ec), *S. aureus* (sa), *E. faecalis* (ef), and *P. aeruginosa* (pa) for hexane (H), dichloromethane, (Dc), ethylacetate (Ea), acetone (Ac) and ethanol (Et) extracts from the LNBG at Nelspruit (n) and UP main campus (m) samples.

The reciprocal of the MIC values showed the acetone extract from LNBG sample (Ac n) to possess the most potent antibacterial compounds against the four tested bacteria with the highest activity against *S. aureus* and *E. faecalis,* while hexane extracts showed the least potency. Except in the hexane extracts where antibacterial activity of the two samples was similar, greater activity was realized in the LNBG sample compared to the UP sample for all the other extracts [Figure 3.4].

3.9 Total activity

The reciprocal of the MIC value is related to the potency of antibacterial compounds present, but it does not take account of the quantity of material in the extract. Total activity is an arbitrary measure of the quantity of antibacterial compounds present in the extract and is calculated by dividing the mass in mg of the extract by the MIC in mg/ml, thus, the unit of this arbitrary measure is ml. Total activity therefore indicates the volume to which the bioactive compounds present in the extract can be diluted and still inhibit growth of bacteria (Eloff, 1999).

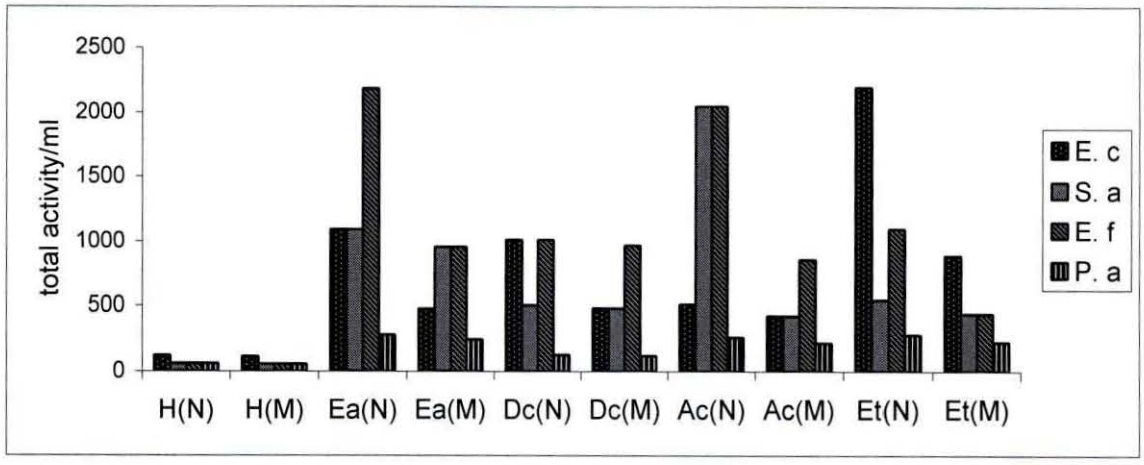


Figure 3:5 Total activity of the four test pathogens *E. coli* (E.c), *S. aureus* (S.a), *E. faecalis* (E.f), and *P. aeruginosa* (P.a) for extracts of hexane (H), dichloromethane, (Dc), ethylacetate (Ea), acetone (Ac) and ethanol (Et) extracts from the LNBG at Nelspruit (n) and UP main campus (m) samples.

Acetone, ethanol and ethylacetate extracts from the LNBG sample showed the best total activity values with the ethanol extract possessing the highest average total activity with a value of 2440 ml. In all the extracts the total activity for the LNBG sample was greater then the total activity of the UP sample and in some cases (for the acetone and ethanol extracts) was more than two fold higher.

The highest average total activity was found in intermediate polar solvents ethylacetate, ethanol and acetone, dichloromethane had lower total activity values compared to the other intermediate polarity solvents while the hexane extracts had the lowest total activity values [Figure 3.5]. This suggests that the antibacterial compounds present in *C. woodii* leaves are intermediate polar compounds and agrees with the observations made in bioautography [Figure 3.3].

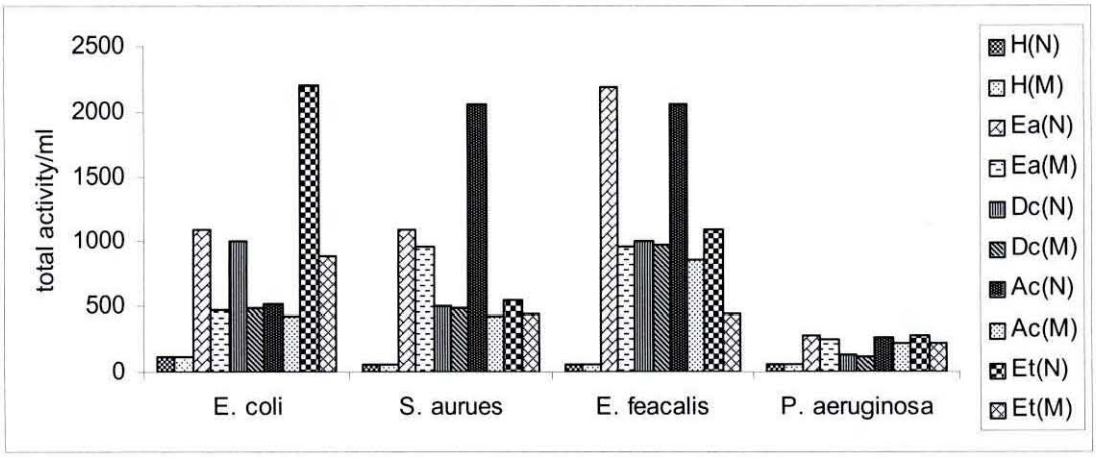


Figure 3:6 Total activity of *C. woodii* extracts of hexane (H), dichloromethane, (Dc), ethylacetate (EA), acetone (Ac) and ethanol (Et) for the LNBG (N) and UP (M) garden samples against four test pathogens.

3.10 General discussion and conclusion

Drying is the most common method of preservation and is achieved by leaving the material in warm dry air, however, the time delay between collecting and processing may affect extractability. Increases in exposure to light, oxygen, temperature and moisture can accelerate deterioration and also affect extractability (Houghton and Raman, 1998).

The method employed for extraction has the following advantages; it is quick, easy to use and compounds that are heat sensitive are extracted successfully from the leaf material. The time frame in which the procedure is performed limits the possible degradation of compounds due to prolonged exposure to solvent. The great limitation of this method is that a number of compounds may not be extracted due to the short time frame during which the samples are in contact with the solvents. However, for its use in these studies, the advantages of direct extraction far outweighed its limitations.

Preliminary extraction studies showed that for all five test solvents, larger quantities of material were present in extracts from the LNBG sample as compared to samples from UP [Table 3.1].

TLC plates developed in CEF solvent system showed the largest numbers of compounds and the best resolution compared to EMW and BEA [Figure 3.1]. This result suggests that the leaves of *C. woodii* have more intermediate polar compounds and tallies with the conclusion in *C. microphyllum* (Kotze and Eloff, 2002).

The fact that polar solvents like ethanol, ethylacetate, dichloromethane and acetone also extracted similar concentrations of non-polar compounds, as did hexane [Figure 3.1], suggested the presence of saponin compounds with polar and non-polar ends, which solubilize in either polar or non-polar solvents (Bruneton, 1995).

MIC and total activity values showed extracts from the LNBG sample to possess more potent antibacterial properties when compared to extracts from the UP sample [Table 3.2]. For these reasons, the LNBG sample was used for further work.

Data from TLC analysis of LNBG sample extracts revealed the presence of two major antioxidant compounds (one with R_f values of 0.85 and the other with an R_f value of 0.35 in EMW separation system) [Figure 3.2], while bioautography showed the presence of a number of antibacterial active compounds in the acetone, ethanol and ethylacetate extracts with R_f values ranging from 0.85 to 0.46 for the chromatogram developed in the EMW system [Figure 3.3]. These results meant that the antibacterial and antioxidant activity of *C. woodii* leaves occurred in compounds whose R_f values lie between 0.85 and 0.35 on TLC plates developed in EMW solvent system.

Hexane extracted 4% of the original material of *C. woodii* leaves [Table 3.1]. TLC plates sprayed with 0.2% DPPH in methanol showed that hexane extracts did not contain any antioxidant active compounds [Figure 3.2], while the bioautograms of the hexane extracts showed one zone of inhibition [Figure 3.3]. All the compounds present in the hexane extract were located near the front of the chromatogram of a TLC plate developed in EMW and sprayed with vanillin spray reagent and had R_f values greater than 0.84 [Figure 3.1]. Thus, hexane could be used to remove ~ 4% of material that is largely inactive from the leaves of *C. woodii* before subsequent extraction with selected solvents. This "washing" procedure would effectively enrich the extract by removing inactive components.

Gram-negative bacteria are generally more resistant to antibiotics and antimicrobials because their cell wall is thicker compared to Gram-positive bacteria.

The two Gram-positive organisms, *S. aureus* and *E. faecalis* as expected showed high sensitivity to the different extracts. The Gram-negative *E. coli* showed sensitivity that compared to that of the two Gram-positive bacteria while *P. aeruginosa* was less sensitive to the test extracts [Figure 3.6]. Because *E. coli* is an important poultry pathogen these results are promising. The quantity of antibacterial compounds present in one g of *C. woodii* leaves diluted to two litres would theoretically still inhibit the growth of *E. coli*, *S. aureus* and *E. faecalis*. The high total activity value for some of the extracts indicates their potential use as replacements for antibiotic feed additives.

Acetone and ethanol are preferred solvents in bioassay application for reasons of safety, cost and availability. Since both solvents extracted large quantities and retained high activity in their extracts, further work was carried out using acetone and ethanol as the main extractants.

Chapter 4 Enrichment procedures

4.1 Introduction

The primary objective of this project was to develop an extract containing high antibacterial and antioxidant activity. In enrichment procedures studies, the aim was to identify solvents that could be used individually, in mixtures or serially as pretreatment or final extractants in a simple extraction procedure.

Many enrichment procedures have been published during the last two decades. The first procedures were extremely time-consuming and error-prone comprising up to 35 partitioning steps or column chromatography steps and not validated (Lobstein-Guth *et al.*, 1983). The first validated method for sample clean-up procedures was published in 1991 (van Beek *et al.*, 1991). An aqueous leaf extract of *Gingko biloba* was enriched over a combination of a polyamide and an 18-carbon (C_{18}) column. The desired phenols remained on the polyamide column while remaining impurities were removed on the C_{18} column. Other researchers have made use of solvent-solvent partitioning steps by means of separatory funnels (Lang and Wai, 1999). The major disadvantage of this procedure is the limited solubility of some compounds in the extraction solvents used. Another technique for extract enrichment involves the use of silica gel in column chromatography.

Bioassay-guided fractionation on column separation simplifies extracts based on polarity of their components. Although the entire procedure may be lengthy, the sample clean up is robust and has been applied successfully by other researchers (van Beek and Taylor, 1996). The effective fractionation of relevant compounds also depends on the sample preparation prior to column chromatographic separation. Alternative extraction procedures to the conventional e.g. the use of supercritical fluids, may also be helpful. However, because of the commercial nature of this project, other factors like cost of setting up and running the separation, the time it will take and quantities that can be yielded have to be considered.

In this project we investigated low cost procedures that would yield an extract with high antioxidant and antibacterial activity.

4.2 Pathway 1

The first pathway was based on results from preliminary extraction studies. Leaf extracts of *C. woodii* contained significant amounts of nonpolar compounds that were largely inactive.

Hexane could be used to defat (remove ~ 4% of non-polar material that is largely inactive from the leaves of *C. woodii*) before extraction with acetone or ethanol for reasons cited in the discussion in Chapter 3. This defatting procedure would effectively enrich the extract by means of removing the inactive components.

4.3 Pathway 2

The second pathway involved the use of various mixtures of acetone in water and ethanol in water solvents to evaluate their application as pretreatment or final extractants. To avoid too many polar impurities, in almost all approaches water is an important constituent of the solvent initially used for the extraction of compounds (Fuzzati *et al.*, 2003). Normally an organic solvent like methanol or acetone is added to improve the rate of extraction because, while most polar compounds are water-soluble, some compounds are poorly soluble in 100% water at room temperature.

4.4 Hexane "wash" (defatting)

A single direct extraction with 10 ml of hexane was performed on one g sample of leaf material from LNBG as a pretreatment procedure before subsequent extraction with ethanol or acetone on the plant residue. The extract was dried at room temperature under a fan and reconstituted in acetone to a concentration of 10 mg/ml.

4.4.1 Results

The resultant hexane-pretreated extracts of acetone and ethanol were analysed together with their respective crude extracts and the hexane extracts to determine how much was removed in the pretreatment and what changes were realised in the pretreated extracts. The crude acetone and ethanol extracts were therefore reference extracts.

4.4.2 Phytochemical analysis

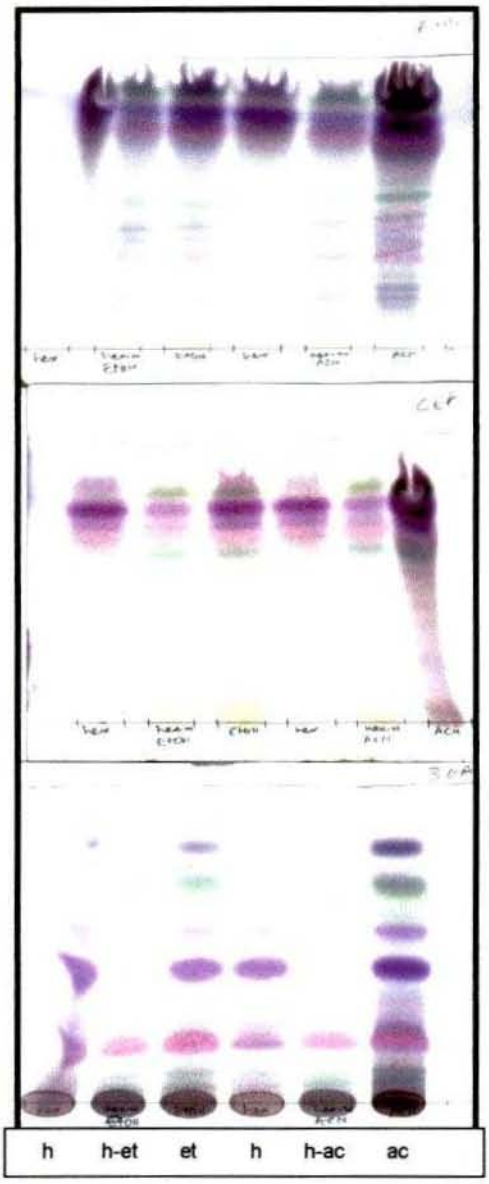


Figure 4:1 TLC profiles of the hexane "wash" extracts developed in EMW (top), CEF (centre), and BEA (bottom) solvent systems and sprayed with vanillin–sulphuric acid reagent. **Lanes from left to right**: Hexane "wash" extracts (h), hexane-pretreated ethanol extract (h-et) and its acetone equivalent (h-ac), crude ethanol extract (et) and crude acetone extract.

Hexane "washing" did not completely remove the inactive components lying at the top of the chromatogram developed in EMW, however, their concentration was reduced as evidenced by the reduction in colour intensity of these compounds in hexane pretreated extracts.

4.4.3 DPPH assay

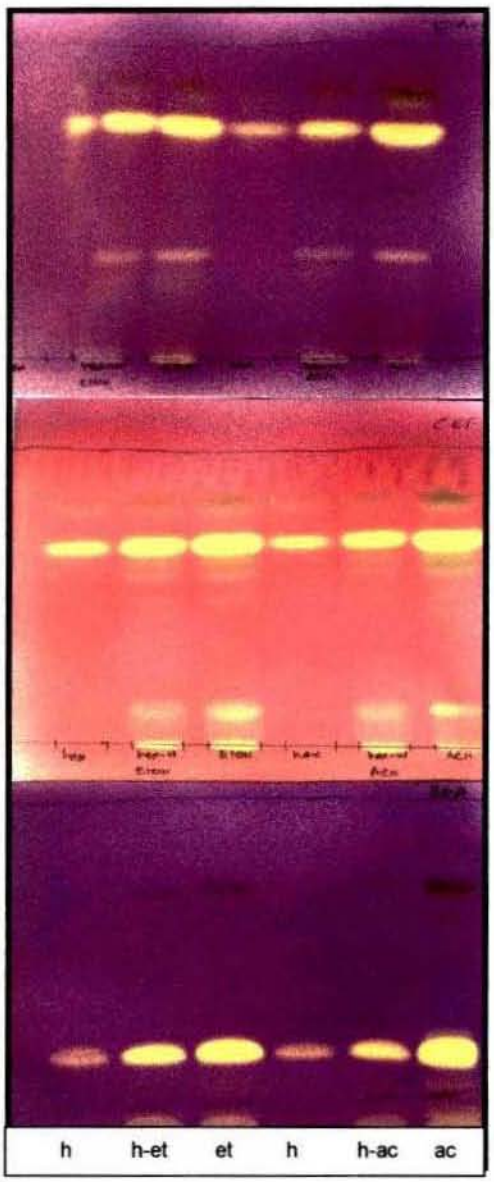


Figure 4:2 TLC profiles of the hexane wash extracts developed in EMW (top), CEF (centre), and BEA (bottom) solvent systems and sprayed with 0.2% DPPH in methanol. **Lanes from left to right**: Hexane "wash" extracts (h), hexane-pretreated ethanol extract (h-et) and its acetone equivalent (h-ac), crude ethanol extract (et) and crude acetone extract.

Contrary to earlier observations, the hexane extracts contained one of the two major antioxidant compounds. However, the compound was extracted in low concentrations as evidenced by the small size and lower intensity of the zone of inhibition. The deviation

from earlier observations could have been caused by a more rigorous extraction procedure in the latter.

4.4.4 MIC and total activity determination

Table 4:1: Amount extracted in mg from one gram, MIC values in mg/ml and total activity values (ml) of hexane "wash" (H), acetone (ac) and ethanol (et), and hexane-pretreated acetone (h-ac) and ethanol (h-et) extracts.

	H	Ac	h-ac	Et	h-et
Quantities	33	82	87	86	92
MIC values in mg/ml					
E. coli	0.63	0.16	0.08	0.08	0.08
S. aureus	0.63	0.16	0.08	0.16	0.08
E. faecalis	0.31	0.04	0.04	0.08	0.04
P. aeruginosa	0.63	0.31	0.16	0.31	0.31
Average MIC	0.55	0.17	0.08	0.16	0.13
Total activity values in ml					
E. coli	52	513	1088	1075	1150
S. aureus	52	513	1088	538	1150
E. faecalis	106	2050	2175	1075	2300
P. aeruginosa	52	265	544	277	297
Average	66	835	1495	741	1224

4.4.5 Discussion

Pretreatment with hexane resulted in an increase in the quantities of materials that were present in the subsequent extracts of acetone and ethanol compared to their crude extracts.

The MIC values of hexane-pretreated acetone and ethanol extracts were enhanced especially against *S. aureus* where MICs were improved by 100% (0.16 mg/ml to 0.08 mg/ml) [Table 4.1], while the changes in sensitivity of *E. coli* and *P. aeruginosa* to the hexane-pretreated extracts were marginal where they were realized. The magnitude of change in average MIC was larger for the acetone extracts (0.17 mg/ml to 0.08 mg/ml) while change in average MIC in the ethanol extracts was less significant (from 0.16 mg/ml to 0.13 mg/ml) [Table 4.1], average MIC values of the acetone extracts were improved to a larger extent compared to ethanol extracts.

The hexane-pretreated extracts showed a significant increase in total activity values compared to their crude extracts. Total activity is doubled against *S. aureus* for hexane-pretreated extracts (513 ml to 1088 ml in acetone extract and from 538 ml to 1150 ml ethanol extracts)[Table 4.1]. In all instances, the total activity of the pretreated extracts was higher when compared to their crude extracts, while there were slight differences in average total activity values between the pretreated acetone and ethanol extracts.

The slight difference in total activity values of hexane-pretreated acetone and ethanol extracts even as relatively large differences in average MIC values were realized can be explained since total activity takes into account both the MIC value and quantity extracted. The poorer MICs of the ethanol extracts were compensated by their larger extraction quantities relative to acetone.

TLC plates sprayed with 0.2% DPPH in methanol showed one of the two major antioxidant compounds to be present in the hexane "wash" extract [Figure 4.2], a result that deviates from earlier observations [Figure 3.2], this result could have arisen owing to increased exposure of solvent to leaf sample or a more vigorous shaking procedure compared to previous extractions

The overall observation from hexane "wash" studies was that pretreatment with hexane prior to extraction with acetone or ethanol not only increases the quantities present in the extracts, but also improves significantly on the antibacterial activity of these extracts.

4.5 Ethanol in water and acetone in water extracts

4.5.1 Introduction

Most biological assays are carried out in water therefore problems arise if the active compounds are only lipid soluble. An alternative is to use a water miscible general solvent like acetone or ethanol. Eloff (1998a) reported that acetone gave the best results in bioassays, due to its low toxicity to test organisms when compared to other solvents such as methanol, chloroform and dichloromethane. Acetone and to a lesser extent ethanol dissolve many hydrophilic and lipophilic components, are miscible with water, volatile and have low toxicity to bioassays.

Ethanol or acetone in water mixtures are safer solvents in extraction procedures and could result in products that may safely be incorporated in feed. They are also useful as extraction solvents because they are inexpensive and readily available. In preliminary extraction studies, ethanol and acetone as individual extractants had high antibacterial activity and high extraction yields. In work on extraction of proanthocyanidins from grape seed, Pekic *et al.*, (1998) concluded that the presence of water in solvent mixtures increased permeability of seed tissue enabling better extraction. Results on other Combretaceae indicate that 100% water does not extract antibacterial compounds (Kotze and Eloff, 2002), however, water extracts antioxidant compounds.

The rationale of using various ratios of solvent in water was to identify mixtures that would selectively extract the bioactive components or otherwise mixtures that would selectively extract inactive material, because different ratios of solvent in water have different polarities and strengths, they can be used to extract different components. Once identified, the suitable solvent mixtures would be used as final extractants or as pretreatment extractants depending on their applicability.

4.5.2 Methods

Various mixtures of acetone in water and ethanol in water were developed and tested as described in 2.2.2, phytochemical analysis was performed as outlined in 2.3 (vanillin spray) and 2.6.1 (DPPH assay).

4.5.3 Results

4.5.3.1 Phytochemical analysis of acetone in water extracts

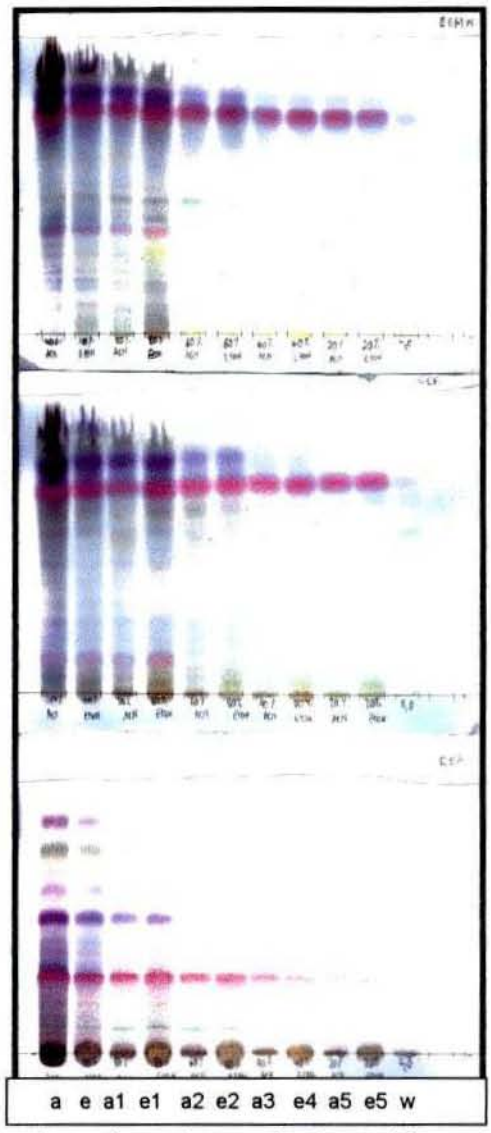


Figure 4:3 TLC profiles for acetone in water and ethanol in water extracts developed side by side in EMW (top), CEF (centre) and BEA (bottom) and sprayed with vanillin–sulphuric acid. **Lanes from left to right**: 100% acetone (a), 100% ethanol (e), 80% acetone in water (a1), 80% ethanol equivalent (e1) 60% acetone in water (a2) 60% ethanol (e2), 40% acetone in water (a3) 40% ethanol equivalent (e3), 20% acetone in water (a4) 20% ethanol equivalent (e4) and water (W) extracts.

As the water content of the solvent mixtures increased, the number of vanillin active compounds that were extracted decreased. All the solvents except water extracted a compound that gave a deep red in colour with vanillin spray and had an R_f value of 0.85 in EMW solvent system.

4.5.3.2 DPPH assay

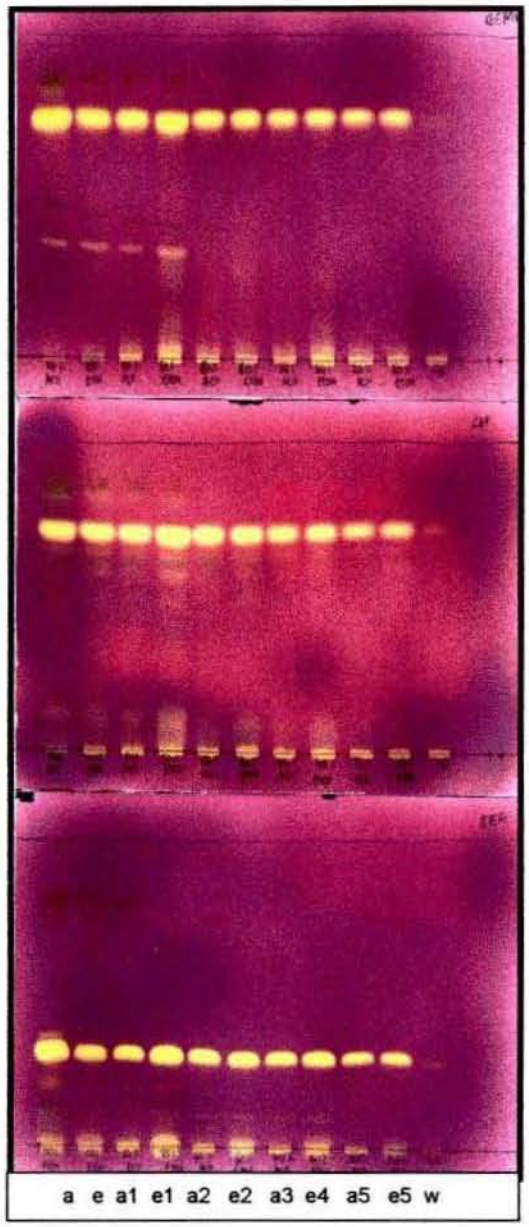


Figure 4:4 TLC profiles for acetone in water and ethanol in water extracts developed side by side in EMW (top), CEF (centre) and BEA (bottom) and sprayed with 0.2% DPPH in methanol. **Lanes from left to right**: 100% acetone (a), 100% ethanol (e), 80% acetone in water (a1), 80% ethanol equivalent (e1) 60% acetone in water (a2) 60% ethanol (e2), 40% acetone in water (a3) 40% ethanol equivalent (e3), 20% acetone in water (a4) 20% ethanol equivalent (e4) and water (W) extracts.

Only 100% acetone, 100% ethanol, 80% acetone and 80% ethanol in water mixtures retained the two major antioxidant compounds. The compound that gave a deep red colour

with vanillin spray reagent and had an R_f value of 0.85 in EMW solvent system was one of the major antioxidant compounds.

4.6 MIC determination of acetone in water and ethanol in water extracts.

The antibacterial activities of acetone, ethanol and acetone or ethanol in water and water extracts were quantified using the 96-well microtitre plate technique (Eloff, 1998b), as described in 2.5. Ten mg/ml solutions of the extracts were tested against *S. aureus*, *E. coli*, *E. faecalis* and *P. aeruginosa*.

4.6.1 MIC and total activity values of acetone in water extracts

Table 4:2: Amount in mg extracted from one g samples and MIC values in mg/ml of 100% acetone (Ace), 80% acetone in water (80% Ace), 60% acetone in water (60% Ace), 40% acetone in water (40% Ace), 20% acetone in water (20% Ace) and water extracts of *C. woodii* leaves.

	Ace	80% Ace	60% Ace	40% Ace	20% Ace	Water
Quantity	84	167	169	164	128	68
			MIC values (mg/ml)			
E. coli	0.16	0.16	0.16	0.31	0.63	1.25
S. aureus	0.16	0.16	0.31	0.31	0.63	2.5
E. faecalis	0.08	0.08	0.16	0.31	0.63	1.25
P. aeruginosa	0.63	0.31	0.63	0.63	0.63	2.5
Average MIC	0.26	0.18	0.32	0.39	0.63	1.88

Eighty percent acetone extracts had higher antibacterial potency compared to the acetone extracts with average MIC of 0.18 mg/ml compared to 0.26 mg/ml respectively. As the water content of the extracts increased from 60% acetone in water to water, the antibacterial activity of the extracts decreased and in all cases the average MICs were poorer than that of 100% acetone extract. The lowest antibacterial activity was present in the water extract.

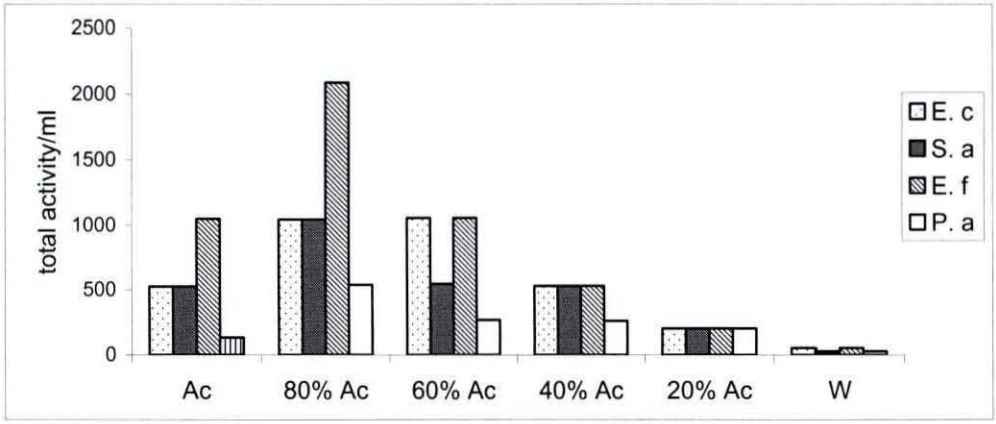


Figure 4:5 Total activity of *C. woodii* extracts of 100% acetone (Ac), 80% acetone in water (80% Ac), 60% acetone in water (60% Ac), 40% acetone in water (40% Ac), 20% acetone in water (20% Ac) and water (W) against *S. aureus* (S.a), *E. faecalis* (E.f), *E. coli* (E.c), and *P. aeruginosa* (P.a).

The highest total activity values were in 80% acetone in water extracts followed by 60% acetone in water and 100% acetone. Though 100% acetone extracts had the best MIC values, 80% and 60% acetone in water solvents extracted larger quantities hence the better total activity values.

4.6.2 **MIC and total activity values of ethanol in water extracts.**

Table 4:3: Amount in mg extracted from one gram and MIC values in mg/ml of 100% ethanol, 80% ethanol in water (80% Ethanol), 60% ethanol in water (60% Ethanol), 40% Ethanol in water (40% Ethanol), 20% Ethanol in water (20% Ethanol) and water extracts of *C. woodii* leaves.

	Ethanol	**80% Ethanol**	**60% Ethanol**	**40% Ethanol**	**20% Ethanol**	**Water**
Quantity	92	184	198	168	149	72
			MIC values in mg/ml			
E. coli	0.16	0.16	0.31	0.31	0.31	1.25
S. aureus	0.16	0.16	0.16	0.31	1.25	1.25
E. faecalis	0.08	0.04	0.16	0.31	0.63	1.25
P. aeruginosa	0.63	0.31	0.31	0.63	0.63	1.25
Average MIC	0.26	0.17	0.24	0.39	0.71	1.25

The average MIC values of the 80% ethanol in water and 60% ethanol in water extracts were better compared to the ethanol extracts. Eighty% and 60% ethanol in water extracts showed good activity against *P. aeruginosa* (MIC values of 0.31 mg/ml for both extracts).

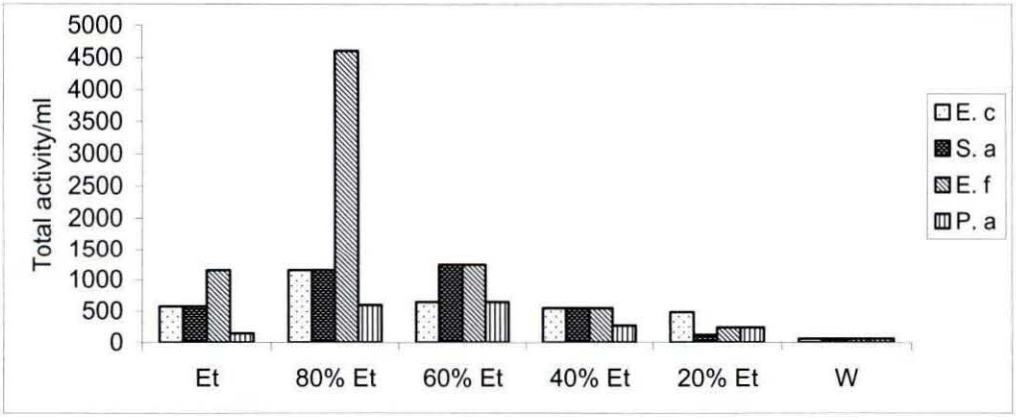


Figure 4:6 Total activity of *C. woodii* extracts of 100% ethanol (Et), 80% ethanol in water (80% Et), 60% ethanol in water (60% Et), 40% Ethanol in water (40% Et), 20% Ethanol in water (20% Et) and water (W) against *S. aureus* (S.a), *E. faecalis* (E.f), *E. coli* (E.c) and *P. aeruginosa* (P.a).

The highest total activity values were in 80% ethanol in water extracts followed by 60% ethanol in water and 100% acetone. As observed with acetone in water extracts, the antibacterial activity of the ethanol in water extracts decreased as the water content of the extractant increased after 80% ethanol in water.

4.7 Summary discussion and conclusions

Hexane is often used as a defatting solvent, removing oils and fatty acids from test samples. The subsequent increase in quantities extracted and improvement in activity values realized in hexane-pretreated extracts could be because; hexane had removed the fatty acid composition of the leaves resulting in rupturing of cellular membranes consequently exposing more compounds in the leaves of *C. woodii* to better extraction with acetone and ethanol.

Acetone and ethanol in water extracts had fewer vanillin-active compounds on TLC plates as the water content of the mixture increased [Figure 4.1] and the DPPH assay showed that only the acetone, ethanol and 80% acetone or ethanol in water extracts contained the two major antioxidant compounds while the other mixtures extracted only one (with R_f 0.85 in EMW solvent system) [Figure 4.2]. The water extract had a few vanillin active compounds and showed no antioxidant active compounds in the DPPH assay [Figures 4.1 and 4.2]. The absence of antioxidant compounds in the water extract was surprising as antioxidant compounds are usually considered to be water-soluble. Possibly the antioxidant compounds though water-soluble, may be present within cellular organelles whose membranes did not rupture in water.

Different ratios of acetone or ethanol in water extractants resulted in larger quantities of material being extracted compared to the individual extraction abilities of acetone, ethanol or water [Tables 4.2 and 4.3]. The quantity extracted gradually increased, reaching a maximum when 60% acetone or 60% ethanol in water solvents were used. This increase could be because the presence of water in the extraction solvents brought water-soluble compounds in contact with acetone or ethanol enabling better extraction.

Eighty percent acetone or ethanol in water extracts possessed the most potent antibacterial activity of the mixtures and had similar or better MIC values to their respective crude extracts [Tables 4.2 and 4.3]. Because they extracted larger quantities while retaining similar MIC values, 80% mixtures had higher total activity values. This result means that 80% acetone and 80% ethanol in water solutions are better solvents for extraction of the antibacterial compounds in the leaves of *C. woodii* compared to their

individual solvents. It is interesting that 70 % ethanol has been used frequently in the extraction of amino acids from plants (JN Eloff, personal communication)

Antibacterial activity of the extracts decreased as the water content of the mixtures increased with the water extracts having the lowest antibacterial activity against the test organisms. This result tallies with the observations in *Combretum microphyllum* (Kotze and Eloff, 2002). Twenty percent acetone or ethanol in water solvent mixtures extracted a large quantity of material (~ 15%), but their extracts had very little antibacterial and antioxidant activity. This meant that these solvent mixtures could be employed as pretreatment solvents in extract enrichment.

The low antibacterial activity of water extracts *in vitro* does not support its common use as the principal extraction solvent by traditional healers. The healing properties of their remedies could possibly be linked to antioxidant activity because antioxidants are usually water-soluble. Another possibility is that the extraction procedures employed during preparation of these remedies allows for more rigorous extractions e.g. boiling. Extraction procedures also often involve soaking the plant material in water over days, this might allow for fermentation thus changing the extractant from water to an alcoholic mixture that will better extract the antibacterial compounds.

Although water extracted c.7% of the material from the leaves of *C. woodii*, the extracts had no antioxidant activity in the DPPH assay and very little antibacterial activity. It was not considered for application as a pretreatment extractant because it would be difficult to remove traces of water remaining in the extract prior to extraction with acetone or ethanol, and also because water extracts did not retain many vanillin active compounds as seen on TLC plates sprayed with vanillin reagent. The 7% that was extracted by water could have been some salts and sugars.

In summary, these extraction studies provided a clean-up step using 20% acetone in water and 20% ethanol in water while 80% acetone or ethanol in water mixtures were identified as possible final extractants.

Chapter 5 Optimal extraction method

5.1 Introduction

Tentative sample clean-up (pretreatment) extractants using 20% acetone or ethanol in water mixtures and the possible use of 80% acetone or ethanol in water mixtures as final extractants were identified, while pretreatment with hexane improved on the extractability of compounds present in leaf samples and led to higher antibacterial activity in final extracts. Eighty percent acetone in water and 80% ethanol in water were better extractants than their individual solvents. All this data was utilized to design tentative optimal extraction methods.

Once the pretreatment measures to be employed in tentative optimal extraction methods were identified, the next important questions to be answered that effectively became the objectives of this chapter were:

(1): How many times should pretreatment procedures be performed on leaf sample to ensure best extraction results?

(2): How much activity would be lost in pretreatment procedures?

(3): How much was gained in the final extract?

The pretreatment procedures were designed and performed as outlined in the Table 5.1.

Table 5:1: Table showing the tentative best extraction methods

Extract	Pretreatment	Expected effects	Possible draw backs
Acetone (1a) or Ethanol (1e)	Direct extraction with hexane (hexane – "wash")	• Removes 4% of inactive material that had high R_f values in EMW system. • Increase extractability by breaking cell membranes	• Pretreatment will not remove inactive compounds with low R_f values in EMW system. • Hexane is not miscible with the final extractants; traces of hexane in sample after pretreatment may affect subsequent extractions.
80% acetone (2a) or 80% ethanol in water (2e)	Direct extraction with 20% acetone/ethanol in water (20% - "wash")	• Selectively extract polar compounds with 80% mixture leaving the non-polar inactive material that had high R_f values in EMW system. • 20% mixture will remove the inactive compounds with low R_f values in EMW system. • Presence of water in extractants increases extractability of water soluble compounds.	• Antioxidant activity will decrease because 20% solvent mixtures extracted some antioxidant compounds • If quantities lost are cumulative (4% + 15%), a lot of material will be lost in pretreatment. This increases chances of losing substantial activity
Acetone (3a) or Ethanol (3e)	Serial extraction with hexane first then 20% acetone/ethanol in water	• Hexane "wash" removes inactive material that had high R_f values in EMW system. • 20% mixture will remove inactives with low R_f values in EMW system. • Increased extractability due to effect of hexane and the water mixtures.	• Hexane is not miscible with the final extractant • Loss of antioxidant activity in 20% "wash".
Crude acetone (4a) / Crude ethanol (4e)	Not applicable	• Crude used as control for comparison.	• Inactive compounds not removed

5.2 Pre-treatment procedures

To determine the optimal number of times "washing" should be carried out before final extraction with acetone or ethanol, pretreatment extractions was repeatedly performed on one g samples in 10 ml of extractant until no visible colour change in the extract was observed.

Table 5:2: Amount in mg extracted from one gram of leaf material during 3 serial extraction procedures on leaf sample using hexane, 20% acetone (20% Acn), and 20% ethanol (20% EtOH).

	Hexane	20% Acn	20% EtOH
1st	36	168	189
2nd	10	36	41
3rd	6	16	13
Total	52	220	243

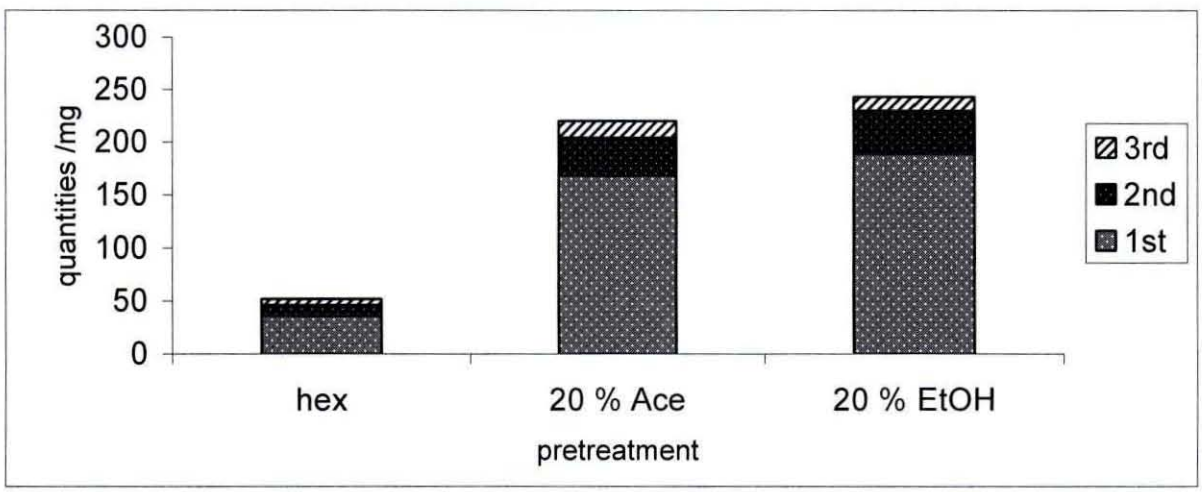


Figure 5:1 Quantities present in the three serial extractions employed in each pretreatment procedure.

Low quantities were extracted by the second and especially the third extractions.

5.2.1 Phytochemical analysis

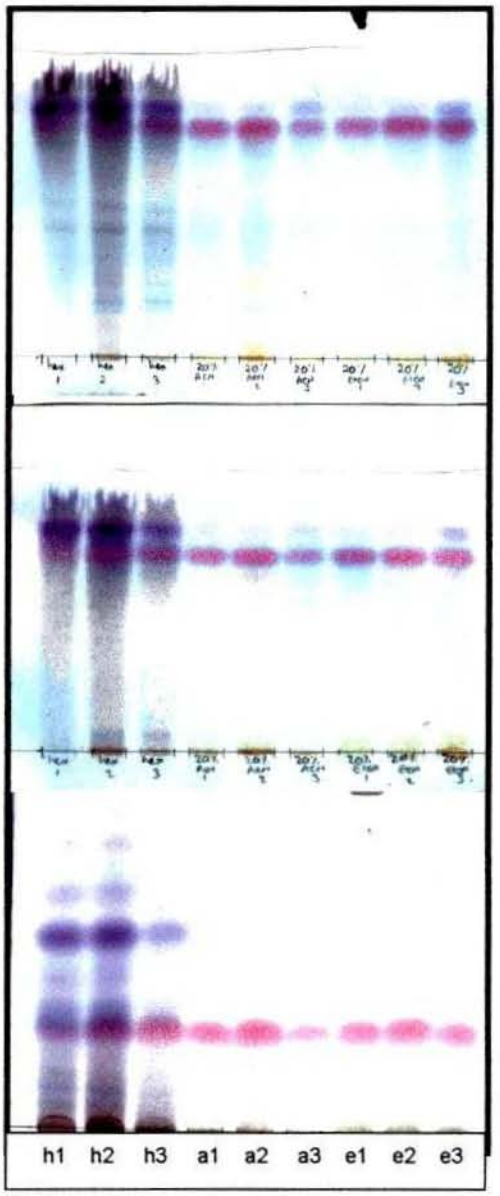


Figure 5:2 TLC profiles of 100 µg of pretreatment extracts developed in EMW (top), CEF (centre) and BEA (bottom) and sprayed with vanillin-sulphuric acid. **Lanes from left to right**: 1st, 2nd and 3rd hexane extracts (h1, h2 and h3 respectively); 1st, 2nd and 3rd 20% acetone in water extracts (a1, a2, a3 respectively) and 1st, 2nd and 3rd 20% ethanol in water extracts (e1, e2, e3 respectively).

The colour of vanillin-active compounds were more intense in the second extractions (h2, a2 and e2) for all pretreatment procedures

5.2.2 DPPH assay

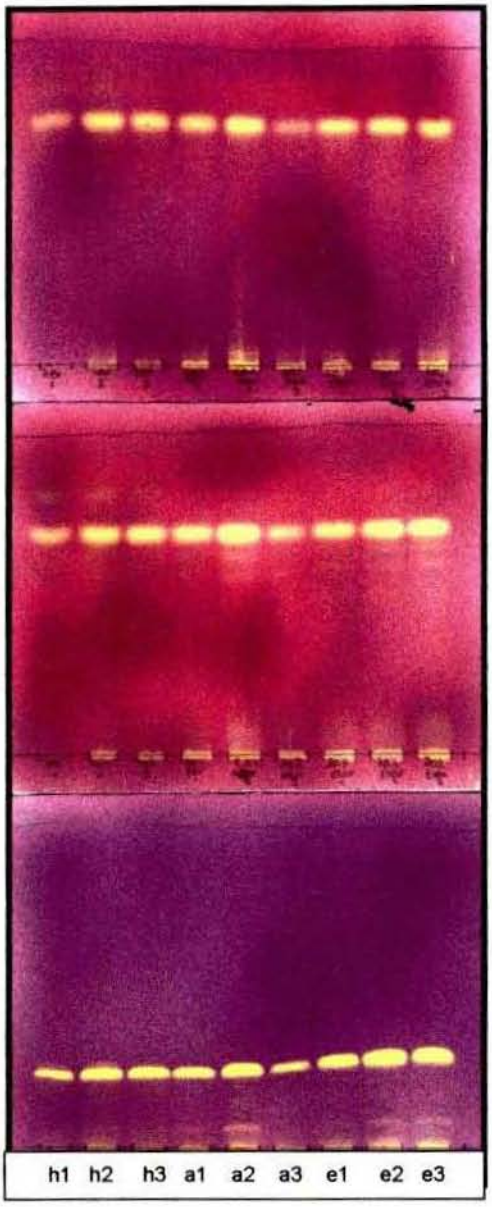


Figure 5:3TLC profiles of 100 µg of pretreatment extracts developed in EMW (top), CEF (centre) and BEA (bottom) and sprayed with 0.2% DPPH in methanol. **Lanes from left to right**: 1st, 2nd and 3rd hexane extracts (h1, h2 and h3 respectively); 1st, 2nd and 3rd 20% acetone in water extracts (a1, a2, a3 respectively) and 1st, 2nd and 3rd 20% ethanol in water extracts (e1, e2, e3 respectively).

All pretreatment extracts contained one of the two major antioxidant compounds, and in all cases, the first extractions (h1, a1, and e1) contained little quantities of this compound relative to the other extracts as evidenced by the smaller size of the inhibition zone.

5.2.3 MIC and total activity lost in pretreatment procedures

To quantify antibacterial activity that would be lost in the serial pretreatment extractions, the MICs of 10 mg/ml extracts after 1st, 2nd, and 3rd extractions in each pretreatment procedure were determined individually using *S. aureus*, *E. coli*, *E. faecalis* and *P. aeruginosa* as test organisms. The MIC method was performed as outlined in 2.5.

Table 5:3: Amount in mg extracted from one g sample in three serial extractions of each pretreatment procedure, MIC values in mg/ml and total activity values (ml) of Hexane "wash", 20% acetone "wash" and 20% ethanol "wash" on *C. woodii* leaves.

	Hexane "wash"			20% Acetone "wash"			20% Ethanol "wash"		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
Quantity	36	46	52	168	204	220	189	230	243
	MIC values (mg/ml)								
E. coli	0.63	0.31	0.31	0.63	0.63	0.63	0.63	0.31	0.31
S. aureus	0.63	0.31	0.31	0.63	0.63	0.63	1.25	1.25	1.25
E. faecalis	0.63	0.31	0.31	0.63	0.31	0.31	0.63	0.31	0.31
P. aeruginosa	0.63	0.63	0.31	0.63	0.63	0.63	0.63	0.63	0.63
Average	0.63	0.39	0.31	0.63	0.55	0.55	0.79	0.63	0.63

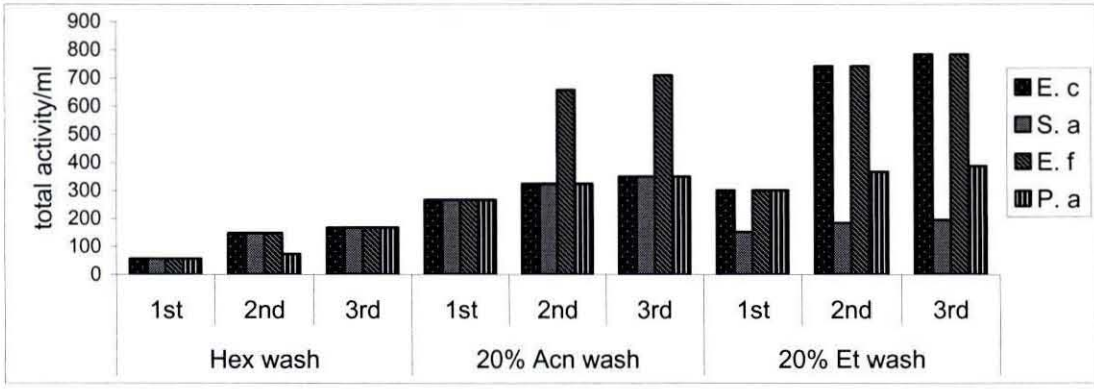


Figure 5:4: Total activity in 1st, 2nd and 3rd serial extracts from the pre-treatment procedures against *S. aureus* (S.a), *E. faecalis* (E.f), *E. coli* (E.c), and *P. aeruginosa* (P.a)

Better MICs were observed in the second and third extractions [Table 5.3] meaning more antibacterial compounds were removed during the second and third extractions.

Since less quantities of material were extracted in the second and third extractions [Table 5.2], and more antibacterial [Table 5.3] and antioxidant [Figure 5.3] activity was being lost in them, it was decided that pretreatment procedures should be performed only once.

Once the optimal number of pretreatment extractions and the antibacterial activity that would be lost in pretreatment was known, the extraction procedures outlined in table 5.1 were adopted as tentative best extraction methods, applying only a single extraction for each pretreatment and allowing the sample to dry before extraction with the final extractant.

Using 10 mg/ml concentrations, phytochemical analysis on the tentative optimal extracts was performed and their antibacterial and antioxidant activity was quantified using MIC method and the TEAC assay as outlined in 2.5 and 2.6 respectively.

5.3 Phytochemical analysis of tentative optimal extracts

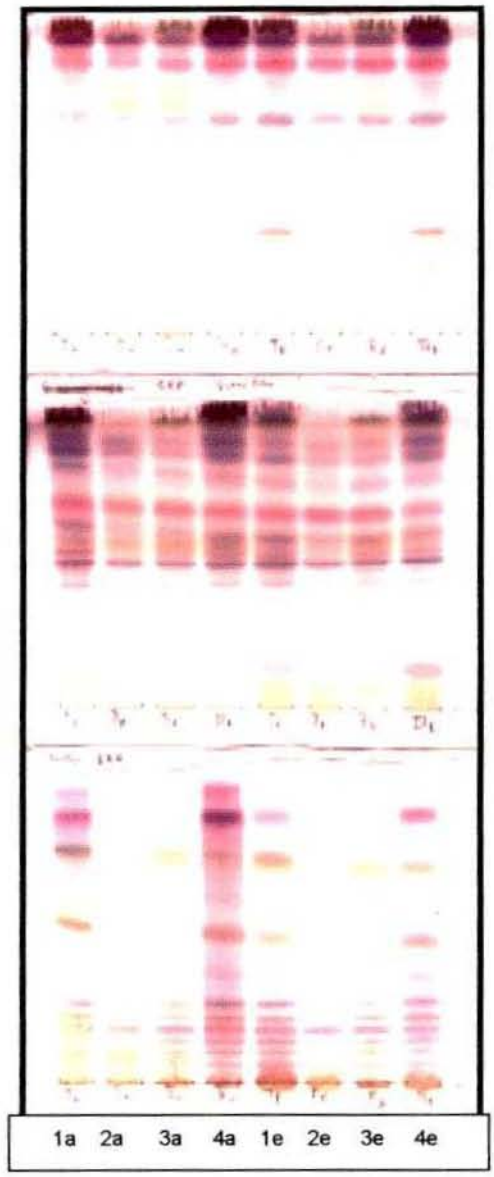


Figure 5:5 TLC profiles of tentative optimal extracts developed in EMW (top), CEF (centre), and BEA (bottom) and sprayed with vanillin-sulphuric acid. **Lanes from left to right:** hexane-pretreated acetone extract (1a), hexane-pretreated ethanol extract (1e), 80% acetone extract pretreated with 20% acetone in water (2a), 80% ethanol extract pretreated with 20% ethanol in water (2e), acetone extract pretreated with hexane first then 20% acetone (3a), ethanol extract pretreated with hexane first then 20% ethanol in water (3e), acetone extract (4a) and ethanol extract (4e).

5.3.1 DPPH assay

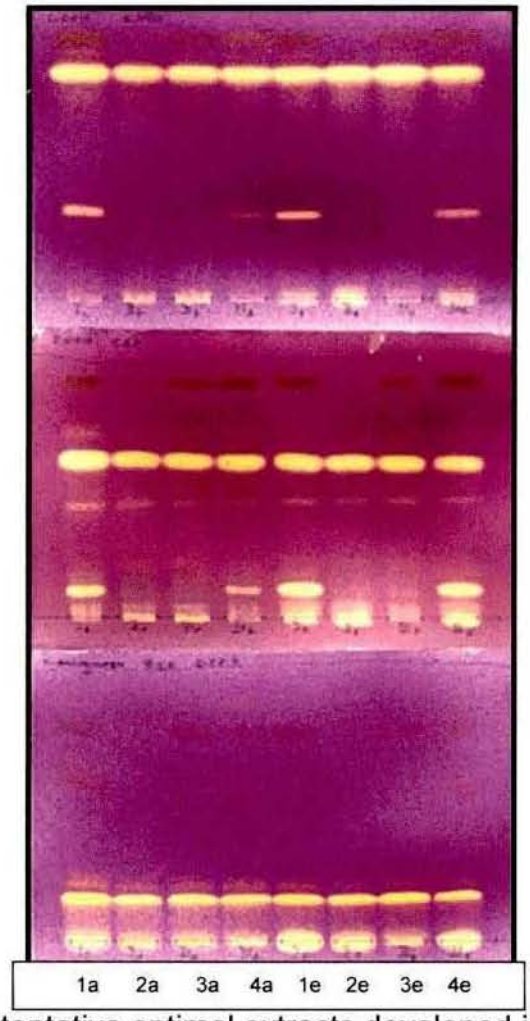


Figure 5:6 TLC profiles of tentative optimal extracts developed in EMW (top), CEF (centre), and BEA (bottom) and sprayed with 0.2% DPPH in methanol. **Lanes form left to right**: hexane-pretreated acetone extract (1a), hexane-pretreated ethanol extract (1e), 80% acetone extract pretreated with 20% acetone in water (2a), 80% ethanol extract pretreated with 20% ethanol in water (2e), acetone extract pretreated with hexane first then 20% acetone (3a), ethanol extract pretreated with hexane first then 20% ethanol in water (3e), acetone extract (4a) and ethanol extract (4e).

Only extracts labelled 1a and 1e contained the same number of antioxidant active compounds as the crude acetone (4a) and crude ethanol (4e) extracts, while the other extracts contained fewer zones of inhibition. This meant that antioxidant activity was being lost in extraction procedures that resulted in extracts labelled 2 and 3 for both acetone (a) and ethanol (e).

5.3.2 Bioautography

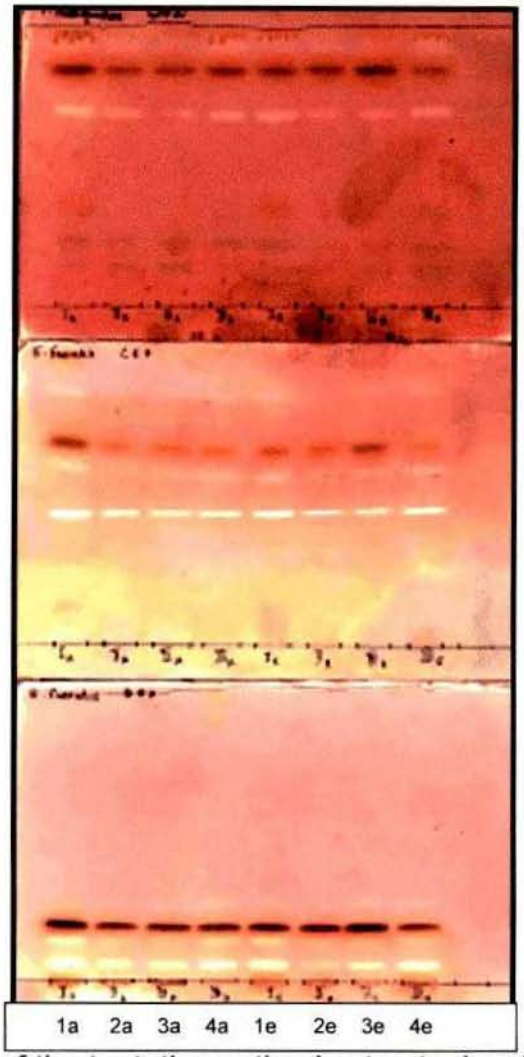


Figure 5:7: Bioautography of the tentative optimal extracts developed in EMW (top), CEF (centre), and BEA (bottom) solvent systems and sprayed with *S. aureus*. **Lanes from left to right:** hexane-pretreated acetone extract (1a), hexane-pretreated ethanol extract (1e), 80% acetone extract pretreated with 20% acetone in water (2a), 80% ethanol extract pretreated with 20% ethanol in water (2e), acetone extract pretreated with hexane first then 20% acetone (3a), ethanol extract pretreated with hexane first then 20% ethanol in water (3e), acetone extract (4a) and ethanol extract (4e).

Extracts 1a and 1e had the same number of antibacterial compounds (3 zones of inhibition) as did acetone (4a) and ethanol (4e) extracts on plates developed in CEF solvent system while extracts 2 and 3 had fewer (2) zones of antibacterial compounds by bioautography.

5.4 MIC and total activity values of the tentative best extraction method

Qualitative analysis on bioautography had shown extracts 2 and 3 to possess lower numbers of antibacterial compounds compared to the crude acetone and ethanol extracts while hexane pretreated extracts had the same numbers of antibacterial compounds [Figure 5.7], however, bioautography results were not sufficient for one to identify the extract that contained the most antibacterial potency. The MIC method was employed to quantitatively discern the antibacterial activity of the different extracts. Ten mg/ml concentrations of tentative best extracts were tested against *S.aureus*, *E. coli*, *E. faecalis* and *P. aeruginosa* as described in 2.5.

5.4.1 Results

Table 5:4: Amount in mg extracted from one gram of leaf sample, the MIC values in mg/ml and total activity values in ml of the hexane-pretreated acetone extract (1a), hexane-pretreated ethanol extract (1e), 80% acetone extract pretreated with 20% acetone in water (2a), 80% ethanol extract pretreated with 20% ethanol in water (2e), acetone extract pretreated with hexane first then 20% acetone (3a), ethanol extract pretreated with hexane first then 20% ethanol in water (3e), acetone extract (4a) and ethanol extract (4e).

	1a	2a	3a	4a	1e	2e	3e	4e
Quantity	84	119	128	82	87	139	110	92
MIC values in mg/ml								
E. coli	0.08	0.16	0.16	0.16	0.08	0.16	0.31	0.16
S. aureus	0.04	0.16	0.16	0.08	0.08	0.16	0.31	0.16
E. faecalis	0.04	0.08	0.16	0.04	0.04	0.16	0.16	0.08
P. aeruginosa	0.16	0.16	0.31	0.31	0.16	0.16	0.63	0.31
Average MIC	0.08	0.14	0.20	0.15	0.09	0.16	0.35	0.18
Total activity values in ml								
	1a	**2a**	**3a**	**4a**	**1e**	**2e**	**3e**	**4e**
E. coli	1050	744	800	513	1088	869	355	575
S. aureus	2100	744	800	1025	1088	869	355	575
E. faecalis	2100	1488	800	2050	2175	869	688	1150
P. aeruginosa	525	744	413	265	544	869	175	297
Average	1444	930	703	963	1223	869	393	694

Table 5:5: Changes in average MIC values of the tentative optimal extracts compared to their crude extracts.

	Activity lost in pre-treatment (ave MIC)	Activity of untreated extract (ave MIC)	Activity of developed extract (ave MIC)	% Improvements in ave MIC.
1a	0.63	0.15	0.08	87.5
2a	0.63	0.15	0.14	7.14
3a	0.63	0.15	0.20	-25
1e	0.63	0.18	0.09	100
2e	0.79	0.18	0.16	12.5
3e	0.71	0.18	0.35	-48.6

5.4.2 Discussion and conclusions

Improvement in average MIC values were realized in hexane-pretreatment acetone and ethanol extracts (1a and 1e, respectively) and 80% acetone or ethanol in water extracts developed by pretreatment with 20% "wash" (2a and 2e, respectively) while extracts labelled 3 had lower antibacterial activity compared to their crude extracts [Table 5.5].

The most significant improvement in antibacterial activity was realized in extracts labelled 1 (87.5% and 100% for the hexane-pretreated acetone (1a) and ethanol (1e) extracts respectively), though the most antibacterial activity was lost in their pretreatment procedure (average MIC value of 0.63 mg/ml) relative to the other extraction procedures (0.71 and 0.79 mg/ml). The improvement in average MIC for extracts labelled 2 were 7.14% and 12.5% for the acetone and ethanol extracts respectively [Table 5.5]. Antibacterial activity was lost in pretreatment procedures employed in development of extracts labelled 3 as illustrated by the poor average MIC values compared to the crude extracts.

Based on these results, extraction procedures labelled 1 were identified as possibly the best procedures. Since the extraction procedures of extracts labelled 2 resulted in improvements in average MICs and the principle aim was to develop an extract with high antibacterial and antioxidant activity, this extraction procedure would be considered if the antioxidant activity of extracts prepared by procedure 2 far outweighs that of extracts prepared by procedure 1.

5.5 Quantification of the antioxidant activity of tentative best extraction method

5.5.1 Introduction

Generally the DPPH assays on TLC of the developed extracts were similar with at least two major antioxidant compounds visible in all extracts. Differences in antioxidant activity, if any, would therefore be discerned by methods that quantify antioxidant activity.

The most widely used methods for measuring antioxidant activity are those that involve the generation of a radical species and measurement of a range of end points at a fixed time or over a range (Re *et al.*, 1999). Two types of approaches may be followed, namely the inhibition assays where the extent of the scavenging by hydrogen or electron donation of a preformed free radical is the marker of antioxidant activity as well as assays involving the presence of antioxidant system during the generation of the radical (Re *et al.,* 1999). One difficulty in assessing antioxidant activity is to select which method to be used. Different methods seem to give different results and methods have their advantages and disadvantages.

This study made use of the Trolox equivalent antioxidant capacity (TEAC) assay. This assay is frequently used to rank antioxidants and for the construction of structure activity relationships. It has the major advantage that it is applicable to both aqueous and lipophilic systems (Re *et al.,* 1999). TEAC is a decolourization assay that measures antioxidant activity in relation to Trolox, a water-soluble vitamin E analogue (Re *et al.*, 1999).

5.5.2 Method

The TEAC assay was performed as outlined in 2.6.2.

5.5.3 TEAC results

A Trolox standard line was prepared by plotting percentage inhibition of the $ABTS^+$ radical against concentration of Trolox. The curve had a gradient of 169.98 and a percentage fit of 98.5%.

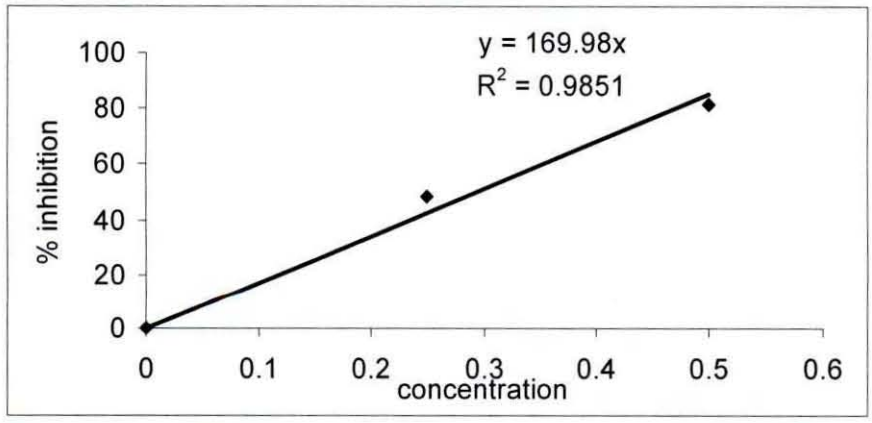


Figure 5:8 Standard curve of% inhibition of $ABTS^+$ against concentration of Trolox after 6 minutes of reaction time.

Data from the tentative optimal extracts was analyzed in a similar manner and the gradient obtained for the different extracts was divided by the gradient of Trolox resulting in a Trolox equivalent value.

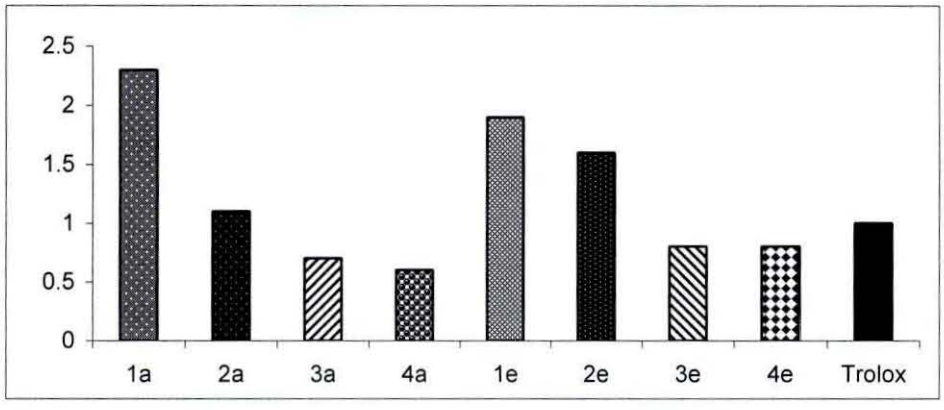


Figure 5:9 Comparison of TEAC values of the hexane-pretreated acetone extract (1a), hexane-pretreated ethanol extract (1e), 80% acetone extract pretreated with 20% acetone in water (2a), 80% ethanol extract pretreated with 20% ethanol in water (2e), acetone extract pretreated with hexane first then 20% acetone (3a), ethanol extract pretreated with hexane first then 20% ethanol in water (3e), acetone extract (4a) and ethanol extract (4e).

Table 5:6: Magnitude of change in TEAC values of the tentative optimal extracts compared to their respective crude extracts.

Extract	TEAC value	Un-pretreated (crude) extracts	% Increase In TEAC
1a	2.3	0.6	283.3
2a	1.1	0.6	83.3
3a	0.7	0.6	16.7
1e	1.9	0.8	137.5
2e	1.6	0.8	100
3e	0.8	0.8	0

Except for extract labelled 3e, the tentative optimal extracts had higher antioxidant activity compared to their respective crude extracts. Extract labelled 1a had the highest antioxidant activity with a TEAC value of 2.3, and a percentage increase in TEAC value of 283% compared to the crude acetone extract, while extract labelled 1e was second best with a TEAC value of 1.9 and percentage improvement of 137.5% from the crude ethanol extract.

5.5.4 Discussion and conclusions

The TEAC assay is widely applied to assess the total amount of radicals that can be scavenged by an antioxidant, i.e. the antioxidant capacity, however TEAC values reported in the literature are variable. It appears that the TEAC value largely depends on the assay conditions. The most important reason for this variation is that the reaction of an antioxidant with $ABTS^{+}$ usually does not reach completion within the time span applied (van den Berg *et al.*, 1999). Furthermore, the TEAC assay measures the antioxidant capacity of the parent compound plus that of the reaction products. These reaction products may have a considerable contribution to the TEAC value (Arts *et al.*, 2004).

The major limitation of this procedure is that the concentration range of the antioxidant that can be used is relatively small. A too high antioxidant concentration would need too much $ABTS^{+}$, giving a too high absorbance for a reliable fit of the curve. A too low concentration of the antioxidant would result in a decrease in $ABTS^{+}$ absorbance too low to be measured accurately (van den Berg *et al.*, 1999).

Despite its drawbacks, the TEAC assay is a useful tool for tracking down unknown antioxidants in complex mixtures. For this application, the TEAC assay has been used with success (van Overveld *et al.*, 2000).

Though the majority of enriched extracts showed an increase in antioxidant activity as evidenced by their improved TEAC values compared to their respective crude extracts, only extracts labelled 1a, 2a, 1e and 2e had higher TEAC values than Trolox. Extract 1a had a TEAC value 2.3 times greater than that of the Vitamin E analogue. The lower TEAC values in the extracts developed by pretreatment procedures involving the use of 20% acetone or ethanol in water mixtures could be explained by the fact that DPPH assay on TLC showed 20% acetone or ethanol in water mixtures to retain one of the two major antioxidant compounds in large quantities as evidenced by the large zone of inhibition. This meant that one of the two major antioxidant compounds was lost in these pretreatment procedures hence the lower TEAC values.

Based on MIC values and the results obtained from the TEAC assay, the hexane-pretreated acetone extract (extract labelled 1a) was selected as the optimal extract.

Chapter 6 *In vitro* antibacterial and toxicity tests on the optimal extract

6.1 Introduction

The optimal extract is intended for commercial application as a feed additive in the broiler industry thus, the next important steps were to test its *in vitro* activity against common poultry pathogens and to ascertain the toxicity profile of the enriched extract.

Some enteric bacteria in poultry are zoonotic and may cause diseases in humans e.g. *Campylobacter spp.* cause abdominal pain and diarrhoea in man and *Salmonella spp.* cause typhoid (Aarestrup, 1999). A newsletter article from the meat industry insights dated October 23 1997 stated that *Salmonella enteritidis, Campylobacter jejuni* and *Clostridium perfringens* have been found in 79% of fresh and frozen chickens bought in butcheries and supermarkets in the United States of America. The continued supplementation of antibiotics in feed may lead to development of resistant strains of these bacteria that can be transmitted to humans via the food chain (Aarestrup, 1999). Perpetual use of existing drugs also results in the development of multi drug-resistant strains of bacteria.

The best way to circumvent the proliferation of resistance would be to develop novel antimicrobial agents, which inhibit these new bacterial strains (Coleman, 2004). The effectiveness of the optimal extract in this regard was assessed against two strains each of *Campylobacter jejuni, Clostridium perfringens, Salmonella enteritidis, E. coli* and multi drug-resistant *E. coli* isolated from chickens.

To establish whether the optimal extract had the potential to be toxic in poultry, basic toxicity screening test were performed using the brine shrimp assay and the MTT assay on cell culture. These assays were chosen because they are relatively cheap and easy to conduct.

6.2 Antibacterial activity of the best extract against poultry enteric bacteria

6.2.1 Introduction

Campylobacter jejuni is one of the most common bacterial causes of diarrhoeal illness in humans. It grows best at the body temperature of a bird, and seems to be well adapted to birds, which carry it without becoming ill. The bacterium is fragile; it cannot tolerate drying and can be killed by oxygen. It grows only if there is less than the atmospheric concentration of oxygen present. Many chicken flocks are infected with the organism but show no signs of illness. *Campylobacter* can be easily spread from bird to bird through a common water source or through contact with infected faeces. When an infected bird is slaughtered, *Campylobacter* can be transferred from the intestines to the meat (Frediani-Wolf and Stephan, 2003).

Clostridium perfringens is an anaerobic, spore-forming rod. It is widely distributed in the environment and frequently occurs in the intestines of humans and many domestic and feral animals. Spores of the organism persist in soil, sediments, and areas subject to human or animal faecal pollution (Johansson *et al.*, 2004).

Escherichia coli is a Gram-negative bacterium. There are hundreds of strains of this bacterium. Although most of them are harmless and live in the intestines of healthy humans and animals, some strains produce toxins and can cause severe illness. Infection often leads to bloody diarrhoea, and occasionally to kidney failure. Most illness has been associated with eating undercooked, contaminated meat. Infection can also occur after drinking raw milk and after swimming in or drinking sewage-contaminated water (Schroeder *et al.*, 2004).

Most types of *Salmonella* live in the intestinal tracts of animals and birds and are transmitted to humans by contaminated foods of animal origin. *Salmonella enteritidis* infects the ovaries of healthy appearing hens and contaminates the eggs before the shells are formed, it can therefore be inside normal-appearing eggs, and if the eggs are eaten raw or undercooked, the bacterium can cause illness (Wierup, 2000).

6.2.2 Method

The MIC values were determined as described in section 2.5. Antibacterial activity of the optimal extract was tested against two strains each of *Campylobacter jejuni, Clostridium perfringens, Salmonella enteritidis, E. coli* and multi-drug resistant *E. coli* isolated from chickens obtained from the Microbiology Laboratory, Faculty of Veterinary Science, University of Pretoria. *E. coli* ATCC 28922 was used as a control organism and was tested against gentamicin. The organisms were all grown under aerobic conditions, therefore gentamycin, which requires oxygen for activity, was a valid choice as positive control.

6.2.3 MIC results and discussion

Table 6:1: MIC values in mg/ml, total activity in ml and the mean and standard deviation (SD) of the MIC values.

	MIC values (mg/ml)			Mean	S.D	Total
	1	2	3			activity (ml)
Campylobacter jejuni 26/7	0.08	0.04	0.08	0.07	0.02	1260
Campylobacter jejuni B603/96	0.16	0.16	0.31	0.21	0.07	400
Clostridium perfringens B805/96	0.04	0.04	0.08	0.05	0.02	1575
Clostridium perfringens Bs2	0.08	0.08	0.08	0.08	0.00	1050
E. coli ATCC 28922	0.08	0.08	0.08	0.08	0.00	1050
E. coli P1016/04	0.16	0.16	0.16	0.16	0.00	525
E.coli P1008/04	0.31	0.16	0.16	0.21	0.07	400
E. coli P1493 (multi resistant)	0.63	0.63	0.63	0.63	0.00	133
E. coli P1497 (multi resistant))	0.63	0.63	1.25	0.84	0.29	100
Salmonella enteritidis 215	1.25	1.25	1.25	1.25	0.00	67
Salmonella enteritidis 57/2001	1.25	1.25	1.25	1.25	0.00	67

The MIC can give an indication as to the concentration of antimicrobial needed at the site of infection to inhibit the growth of the pathogen. MIC values of the optimal extract against poultry pathogens were understandably higher than values expressed for single antibiotics as outlined by the National Committee for Clinical Laboratory Standards (NCCLS, 1994). Values for enrofloxacin for example, varied between 0.03 µg/ml and 4 µg/ml, oxytetracycline from 0.5 µg/ml to 64 µg/ml and for gentamicin between 0.25 and 32 µg/ml against the same ATCC strains of *E. coli, S. aureus* we used and other common pathogens.

Campylobacter jejuni and *Clostridium perfringens* were relatively sensitive with MICs varying from 40 µg/ml to 80 µg/ml. Though less sensitive than *Campylobacter jejuni* and *Clostridium perfringens*; *E. coli* and *Salmonella enteritidis* also showed some sensitivity with MIC values of 1250 µg/ml for both strains against to the optimal extract.

6.3 *In vitro* toxicity tests of the optimal extract

In vitro assays are important and useful tools in toxicity assessment of various compounds and extracts not only because they significantly reduce evaluation time, but also because they are usually less expensive, more quantitative and more reproducible than *in vivo* studies. They are also useful and necessary for screening purposes to define dose and time-dependent cytotoxicity, considered primarily as the potential of a compound to induce cell death, in different cell types (Eisenbrand *et al.*, 2002). On the other hand, the possibility of obtaining a false positive result is of particular concern when using bioassays. False positives may also be due to compounds that are apparently non-toxic to higher animals, but toxic to lower biological organisms, tissue or cells (Pangrahi, 1993). For the reversed argument false negatives may also be obtained. This study made use of the brine shrimp assay and the MTT assay on monkey kidney cells to assess *in vitro* cytotoxicity of the optimal extract.

6.3.1 The brine shrimp assay

The brine shrimp assay is an indicator of cytotoxicity and may be a predictor of effects on cancer cells. Fang *et al.*, (1991) found a positive correlation between brine shrimp toxicity and 9KB (human nasopharyngeal carcinoma) cytotoxicity. Although the brine shrimp assay is rather inadequate regarding the elucidation of the mechanism of action, it is a useful tool in assessing the toxicity of extracts.

6.3.1.1 Method

The brine shrimp assay was performed as outlined in 2.7.1.

6.3.1.2 Results

Brine shrimp assay results were analysed by plotting percentage mortality of brine shrimps against the different concentrations of the extract. The curve had a percentage fit of 93.39% and the equation of the curve was, $y = 27.452 \text{ Ln } (x) + 54.045$.

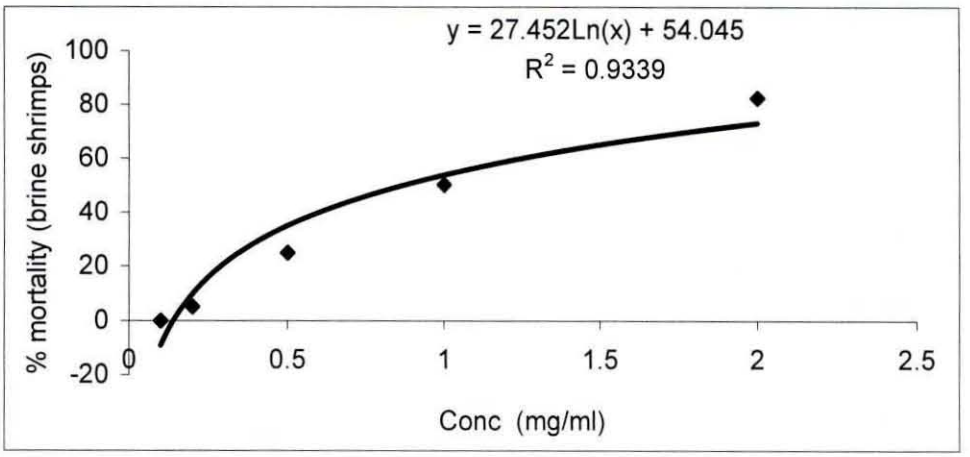


Figure 6:1 Brine shrimp assay curve.

The LC_{50} value was calculated by substituting 50% for y into the equation of the curve. The optimal extract was relatively non-toxic with an LC_{50} value of 863 µg/ml compared to 7 µg /ml for the Podophyllotoxin standard.

6.3.2 The MTT cytotoxicity assay

The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, first described by 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 its accumulation within healthy cells. Solubilisation of the cells by the addition of a detergent results in the liberation of the crystals that are also 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 simple colorimetric assay. The results were read on a multiwell scanning spectrophotometer (Mosmann, 1983).

6.3.2.1 Method

The MTT assay was performed as outlined in section 2.7.

6.3.2.2 Results

The curve for Berberine chloride standard had a 91.66% fit and gave an equation of y = -0.3234x + 0.6284. The LC_{50} was calculated by substituting for y half the value of absorbance at 540 nm for the control (0.459)

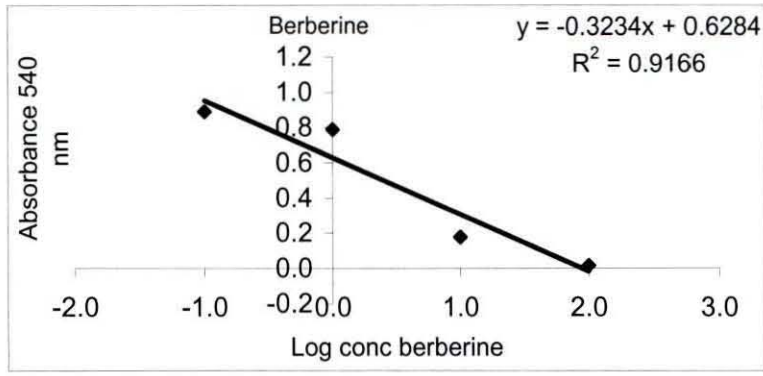


Figure 6:2 MTT cytotoxicity assay curve for Berberine chloride.

The LC $_{50}$ value for Berberine chloride was therefore 3.3 µg/ml (published results give an LC_{50} value of 10 µg/ml).

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

Table 6:2: Results showing absorbance values at 540 nm for the various optimal extract concentrations

Conc.	Log conc.	Ave abs 540nm	SD
1	0.000	0.290	0.029
0.1	-1.000	0.485	0.040
0.01	-2.000	0.946	0.042
0.001	-3.000	0.926	0.052

The results were analysed by plotting the logarithm of different concentrations of the extract versus absorbance values at 540nm. The curve had a percentage fit of 87.67% and the equation of the curve was, y = -2368 (x) + 0.3062.

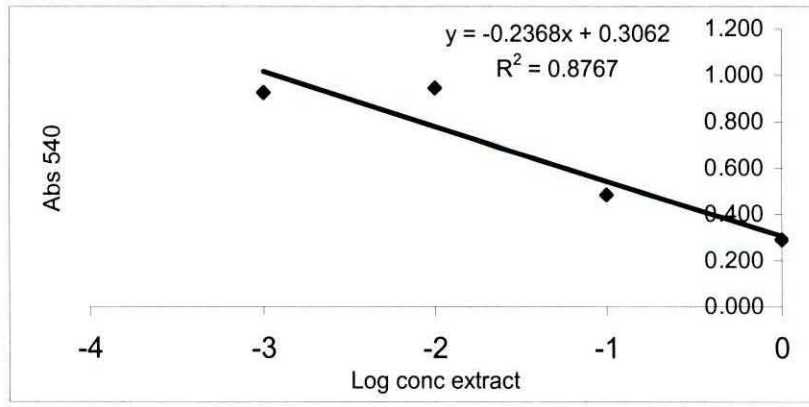


Figure 6:3 MTT cytotoxicity assay curve for the best extract.

LC_{50} was calculated by substituting for y by half the value of absorbance at 540 nm for the control (0.459). The LC_{50} was a relatively non-toxic value of 226 µg/ml compared to 3.3 µg/ml of the Berberine chloride standard.

6.4 Discussion and conclusions

The optimal extract had cytotoxic effects in the brine shrimp assay with an LC_{50} of 863 µg /ml. The MTT cytotoxicity assay on monkey kidney cells gave an LC_{50} value of 226 µg /ml. The LC_{50} values from these two assays are close and comparable.

According to the prevailing view of the MTT assay, respiratory chain activity is the sole MTT reducing activity and thus the marker for viable cells. 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).

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 was 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 to be predictive of KB-cell cytotoxicity except for compounds requiring metabolic activation, since the brine shrimp lack the necessary cytochrome P-450 enzyme.

Several cytotoxic stilbenes have been isolated from *C. caffrum* a plant closely related to *C. woodii* and these compounds have different degrees of *in vitro* cytotoxicity against various tumor cell lines and are considered potential lead compounds for the development of new chemotherapeutic agents. A *cis* – stilbene, combretastatin B5 has also been isolated from *C. woodii* by Famakin (2002) but its cytotoxicity was not evaluated, however, the wide application of combretastatins in cancer therapy is owing to their antineoplastic activity. The toxic effect of the optimal extract on monkey kidney cells and brine shrimp may be due to the antineoplastic or antimitotic activity of combretastatin B5.

Relative safety margin indicates of 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 6:3 Relative safety margin (using LC_{50} value from the brine shrimp assay) of the optimal extract.

	Average MIC (mg/ml)	LC_{50} / MIC
Campylobacter jejuni	0.14	6.2
Clostridium perfringens	0.07	12.3
E. coli (ATCC 28922)	0.08	10.8
E. coli	0.19	4.5
Multi-resistant *E. coli*	0.79	1.1
Salmonella enteritidis	1.25	0.7

Although the MIC values of the optimal extract are above the MIC values of currently used antibiotics, the optimal extract was relatively non-toxic, the latter result means that the relative safety margin (LC_{50}/MIC) of the optimal extract was large, this allows for large quantities of the optimal extract to be incorporated in feed without causing toxic reactions in the broilers The effectiveness of the extract as a feed additive would further be aided by its high antioxidant activity.

Plant extracts contain thousands of compounds; an activity 10-1000 times lower on mass basis than currently used antibiotics is still exciting. Because a pure compound with inactive components removed may have excellent biological activities. In the next section an attempt was made to isolate the main antioxidant compound that seems to occur at a very high concentration.

Chapter 7 Isolation of compounds

Because the optimal extract is intended for commercial application in the broiler industry, its activity should be ascribed to analytically determined active principles. The primary aim of isolation work was to isolate, determine structure and quantify activity of the major antioxidant compound that gave a deep red colour with vanillin-sulphuric acid reagent and had an R_f value of 0.84 in EMW solvent system.

Famakin (2002) isolated a stilbene, 2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxybibenzyl from the leaves of *C. woodii* that had high antibacterial activity. The R_f value of this compound on TLC developed in EMW system was 0.82 and very close to the R_f value of the target antioxidant compound (0.85 in EMW solvent system). In order to isolate the major antioxidant compound, serial exhaustive extraction was used as the first fractionation step, further fractionation and isolation was done using column chromatography.

7.1 Serial exhaustive extraction

Serial exhaustive extraction (SEE) is a mild technique that aims at fractionating a crude extract without introducing chemical changes. It was chosen as the first fractionation step in the isolation of the major antioxidant compound in the leaves of *C. woodii*. Because different solvents extract different phytochemical groups, serial extractions using solvents of varying polarities simplify fractions and enhanced the isolation of compounds from the complex crude extracts.

Three extraction series were adopted in preliminary serial exhaustive extraction; each series had solvents in a range of polarities varying from non-polar, intermediate polarity to polar solvents. In all cases, one plant sample was extracted successively with different extractants. The serial extractions were performed as follows:
Series 1: hexane, dichloromethane, acetone and methanol as extractants:
Series 2: hexane, ethylacetate, acetone and methanol.
Series 3: hexane, acetone and methanol

Ten g of leaf powder were extracted three times with each solvent by vigorous shaking in 100 ml of the various solvents for 30 minutes. The best serial extraction series was

determined based on the quantities extracted and the antioxidant activity present after each series had been completed.

Table 7:1: Amount in milligrams extracted from 10 g samples in the three serial exhaustive extraction series.

	Series 1	Series 2	Series 3
Hexane	258	285	293
Ethylacetate		628	
Dichloromethane	512		
Acetone	763	471	1243
Methanol	946	643	686
Total extracted	2479	2027	2222

Although the total quantity of material extracted in series 1 was higher than in the other two series, the acetone fraction in series 3 extracted the largest amount of the individual extractants in the various series.

7.2 TLC analysis

One hundred µg of each fraction was loaded on TLC (Merck, Kieselgel 60 F_{254}) plates and developed in the three solvent systems described in section 2.3. The plates were sprayed with vanillin-sulphuric acid reagent and 0.2% DPPH in methanol for visual detection of antioxidant compounds in the different fractions.

series of solvents described in 2.8.2 were used as eluents. This gave rise to 11 fractions from column elution when each of the solvent systems was used.

The different fractions resulting from VLC were not well resolved on TLC plates developed in EMW, CEF or BEA solvent systems and also to identify the solvent systems to be used in the next column separation on fraction TF2, it was necessary to develop a TLC system which would give good resolution of components in the fractions from VLC.

7.3.1 Development of a TLC separation system for column chromatography

Various TLC analyses were done on TF2 fraction in an attempt to determine an effective solvent system for column chromatography. One hundred µg each of the dried fractions was applied to the TLC plates for this purpose. Various ratios of different solvent mixtures were used. The following solvent systems were evaluated: chloroform/hexane; chloroform/ethylacetate; ethylacetate/hexane and chloroform/methanol combinations in different ratios, these solvents were chosen because of their varying polarities and selectivities (Snyder and Kirkland, 1979).

The ethylacetate and hexane combination in the ratio of 2:1 gave the best resolution and was chosen as the best solvent system for the next column separation.

test tubes. About 250 test tubes were collected and placed in a fume cupboard under a stream of air to concentrate the fractions. TLC analysis of column fractions was carried out with the intention to combine fractions with the similar compounds based on colour and R_f values.

7.3.3 TLC analysis of column fractions

Thin layer chromatography was carried out on the collected fractions to determine their complexities. After about 50% of the volume of the eluents evaporated, TLC analysis of every fourth test tube was carried out in EMW solvent system. Depending on the extent to which evaporation of the eluents in the different fractions had transpired 5–20 μl was applied on TLC.

Based on TLC analysis, the tubes were grouped into three as follows:

Group A: Test tubes 1–80
Group B: Test tubes 81–160
Group C: Test tubes 161–250.

Table 7:2 Amount in mg of grouped fractions obtained from column separations of TF2

	Test tube	Amount in mg
Group A	1-80	9
Group B	81-160	19
Group C	161-250	42

Mass spectroscopy confirmed the bibenzylic nature of the isolated compound. It gave a molecular ion at m/z 320 corresponding to $C_{17}H_{20}O_6$ and a major fragment at m/z 167 ($C_9H_{11}O_3$). The fragments are tropylium derivatives of the phenolic ring that is typical of bibenzyls (Letcher and Nhamo, 1972). This suggested that one ring contained two methoxyl groups and one hydroxyl group and the other one methoxyl group and two hydroxyl groups.

Table 7:3: ^{1}H-NMR (300MHz) and ^{13}C-NMR (75MHz) spectra data for isolated compound. Data obtained in $CDCl_3$

	Chemical shift (δ, ppm)		
Position	^{1}H	^{13}C	^{13}C (DEPT)
1	-	133.5	
2	6.39	56.2	CH
3	-	146.9	
4	-	132.3	
5	-	105.3	CH
6	6.39	56.3	CH
1'	-	121.6	
2'	-	142.1	
3'	-	132.8	
4'	6.55	145.3	
5'	-	102.4	CH
6'	6.35	120.2	CH
1a	3.8	36.5	CH3
1'a	3.8	32	CH3
3 OMe	2.82	76.6	
5 OMe	2.82	76.9	
4' OMe	2.82	77.4	

The exact positions of the hydroxyl and methoxyl groups around the two aromatic rings were ascertained from the chemical shift and the splitting patterns of signals of the aromatic protons. The isolated active compound was divided into aromatic rings arbitrarily labelled as 'A' and 'B' [Figure 8.11]. Mass spectroscopy of the isolated compound gave fragment of m/z 167 ($C_9H_{11}O_3$) representing aromatic ring A. It has two methoxyl and one-hydroxyl functions.

For ring A, ^{1}H-NMR showed a singlet at 6.394 ppm, which correspond to two protons. These are *meta*-coupled and magnetically equivalent and also imply that ring A is probably symmetrical. Therefore, the protons were placed at position 2 and 6. Positions 3, 5, 6 would have to be oxygenated as seen from the mass spectroscopic fragment m/z 167 ($C_9H_{11}O_3$).

The other fragment of m/z 153 ($C_8H_9O_3$) from mass spectroscopy represents the aromatic ring B. The proton at 6.55 ppm is *ortho*- coupled to that at 6.35 ppm. This is implied from the coupling constant J=8.4. The protons were placed at 5' and 6' leaving the other positions to be taken up by two hydroxyl and a methoxyl group.

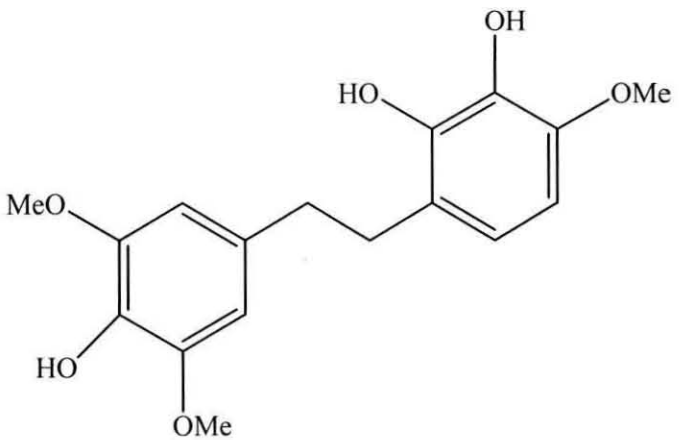


Figure 7:7 Structure of isolated active compound.

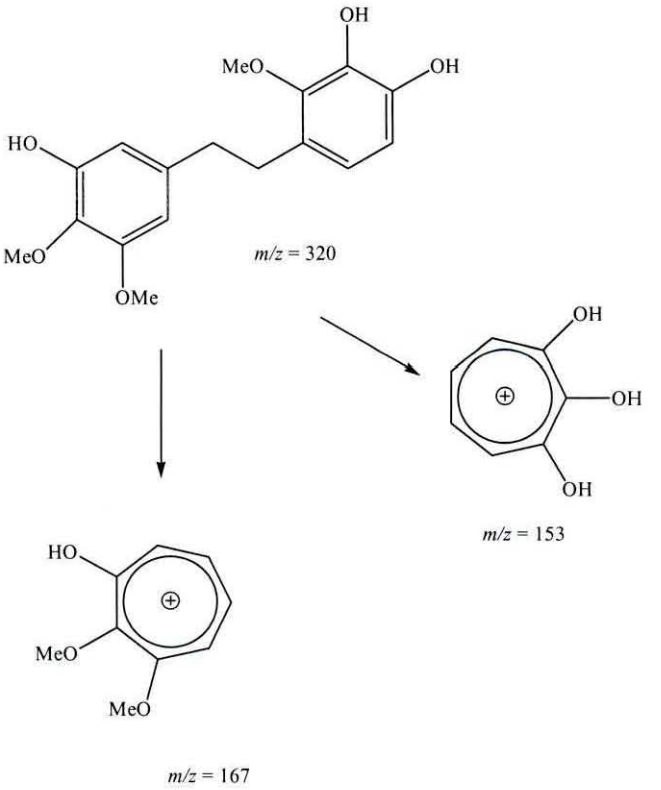


Figure 7:8 The isolated active compound and its fragmentation into two tropylium ions

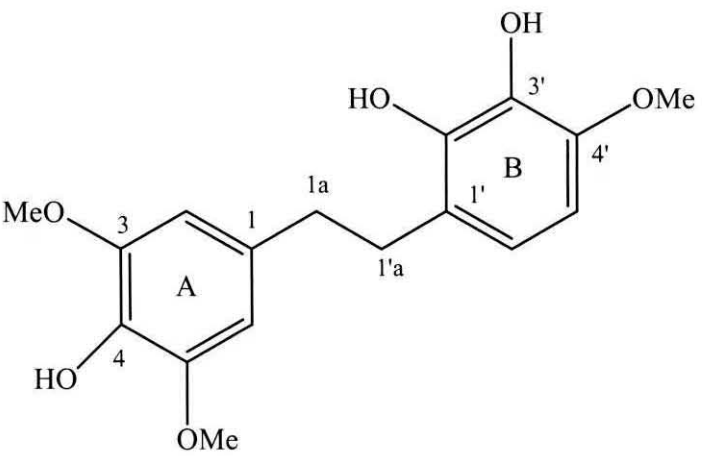


Figure 7:9 Isolated active compound with its two aromatic rings labelled as 'A' and 'B'

The isolated compound was proposed to be a stilbene, 2', 3', 4-trihydroxyl, 3, 5, 4'-trimethoxybibenzyl and identified as combretastatin B5 (CB5). This is the same as the major antibacterial compound isolated by Famakin (2002). This compound and its 2'-O-

glucoside have also been previously isolated from seeds of *Combretum kraussii*. Apart from C. *krausii*, this compound has not yet been found in any other plant. There are no previous reports on the antioxidant activity of this compound.

7.6 Antioxidant activity and cytotoxicity of combretastatin B5

The cytotoxicity and antioxidant activity of CB5 was quantified using the MTT assay on monkey kidney cell cultures and TEAC assay respectively. Stilbenes have been found in many families of higher plants such as Combretaceae, Liliaceae, Moraceae and Cyperaceae. They play important roles in plants especially in heartwood protection and in dormancy and growth inhibition. Certain stilbenoids, besides being toxic to insects and other organisms, have mammalian antifeedant and nematicidal properties (Croteau *et al.*, 2000, Gorham *et al.*, 1995, Schroder, 1999).

Stilbenes in general are known antioxidants whose occurrence in plants have been reported (Packer *et al.*, 1999 and Su *et al.*, 2002) mainly in grapes and wines (Burns *et al.*, 2002). The most documented antioxidant stilbene is resveratrol. In addition, stilbenes possess cyclooxygenase-I and-II (COX-1 and COX-2) inhibitory effects (Su *et al.*, 2002), as well as affecting lipid peroxidation (Stivala *et al.*, 2001), low density lipid (LDL) oxidation and vasodilation capacities (Burns *et al.*, 2002).

Combretastatins are stilbenes, dihydrostilbenes and phenanthrenes that have been isolated from the Combretaceae family (Petit *et al*, 1995). The most documented activity of combretastatins is their ability to cause mitotic arrest in cells in culture and to interact with tubulin, the major protein component of microtubules hence their wide use as anticancer drugs (Pettit *et al.*, 1987; Schwikkard *et al.*, 2000; Shnyder *et al.*, 2003), but there is no report in literature on the antioxidant activity of combretastatins.

7.6.1 TEAC assay of CB5

The TEAC assay was performed to quantify the antioxidant activity of combretastatin B5 as out lined in 2.6.2. A Trolox standard line was prepared by plotting percentage inhibition of the $ABTS^{+}$ radical against concentration of Trolox. The standard curve had a gradient of 155.89 and a percentage fit of 99.9%. A curve was also plotted for the isolated compound. The CB5 curve had a gradient of 1229.4 and percentage fit of 98.4%.

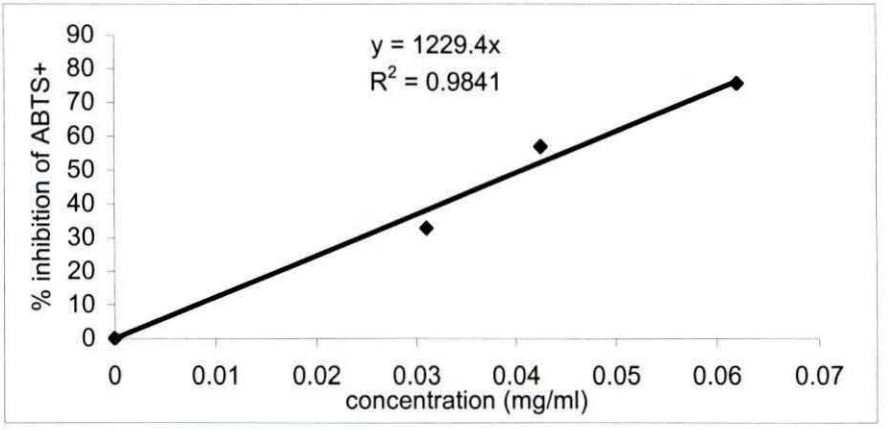


Figure 7:10: CB5 TEAC curve

The TEAC value of combretastatin B5 was calculated by dividing the gradient of its curve by the gradient of the Trolox curve to give a value of 7.886.

This result means that combretastatin B5 is 7.9 times a better antioxidant than the water-soluble vitamin E analogue (Trolox). This is the first report of the antioxidant activity of combretastatin B5.

7.6.2 MTT assay of CB5

In vitro cytotoxicity of CB5 was analysed using monkey kidney cells of the Vero type as outlined in 2.7.2 at 0.1, 0.01, 0.001 and 0.0001 mg/ml concentrations.
The Berberine chloride standard was also analysed at the same concentrations and gave an LC_{50} value of 3.002 µg/ml.

Table 7:4 Absorbance values at 540 nm for CB5 in the MTT assay

Log conc	Ave abs 540 nm	SD
-1	0.035	0.01
-2	0.537	0.08
-3	0.888	0.03
-4	0.906	0.05

In vitro cytotoxicity results of CB5 were analysed by plotting the logarithm of the concentrations against their absorbance values at 540 nm. The resultant curve had a percentage fit of 87.95% and the equation of the curve was, y = -0.2965(x) – 0.1497.

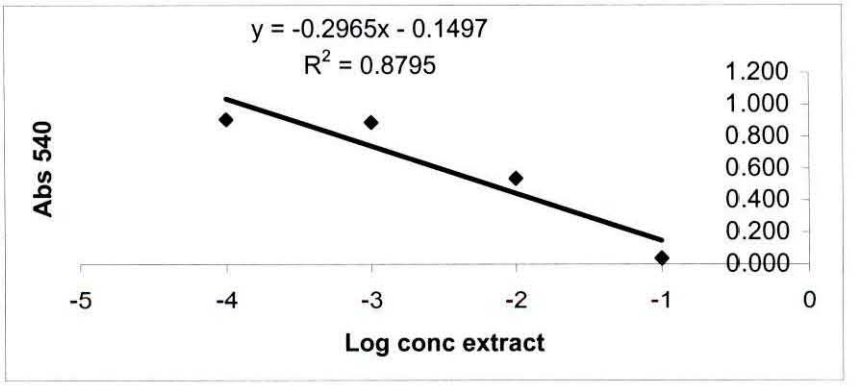


Figure 7:11 MTT assay curve for CB5

LC_{50} was calculated by substituting for y by half the value of absorbance at 540 nm for Berberine control (0.436). LC_{50} of CB5 was therefore 10.58 µg/ml.

7.7 Discussion

Bioassay-guided fractionation on silica gel 60 (63-200 um) in column chromatography resulted in the successful isolation of the major antioxidant compound present in the leaves of *C. woodii*. Combretastatin B5 had a TEAC value which was 7.89 times better than the water-soluble vitamin E analogue. The high TEAC value of CB5 is not surprising because most antioxidant compounds are polyphenolic compounds. The presence of many hydroxyl groups in the structure of CB5 accounts for its high antioxidant activity.

Combretastatins are aromatic compounds with antineoplastic and cell growth inhibitory properties. They cause mitotic arrest in cells in culture by interacting with tubulin, the major protein component of microtubules, at the colchicine binding-site (Pettit *et al.*, 1982). The MTT assay on monkey kidney cells gave an LC_{50} value of 10.58 µg/ml for CB5, a value that is similar to the reported LC_{50} value of the Berberine standard (10 µg/ml). The cytotoxic effect of Berberine is largely due to its antimitotic effect (Lin *et al.*, 1999, Fukuda *et al.*, 1999). It affects cell growth by interfering with spindle formation. The demonstrated cytotoxicity of CB5 could also be largely due to the same effect.

A major pitfall of the MTT assay seems to be restricted to polyphenols with antioxidant properties. At a lower, pre-apoptotic concentration range, these compounds, in spite of their growth inhibition properties, can induce in certain cell types an increase of the MTT-reducing activity that is not related to the number of living cells (Bernhard *et al.*, 2003). The result of this is the presence of an increased MTT-reducing activity in a slower growing cell fraction, compared to the faster growing untreated control cells.

Unfortunately insufficient material of CB5 was available to test its activity on bacteria isolated from poultry to determine to what degree the antibiotic activity of the optimal extract could be ascribed to CB5. Famakin (2002) found that the MIC value of CB5 against *S. aureus* was 16 μg/ml, *E. faecalis* was 125 μg/ml, *P. aeruginosa* was 125 μg/ml and *E. coli* had an MIC value of 250 μg/ml. CB5s antibacterial activities against *S. aureus*, *E. faecalis* and *P. aeruginosa* were higher than chloramphenicol and ampicillin antibiotics.

There was insufficient CB5 available to carry out *in vivo* experiments on poultry; however, it may be possible to synthesize CB5 for future *in vivo* experiments. Based on results from *in vitro* studies, the ability of the optimal extract to replace AFAs remains a possibility and this was examined in the next section.

Chapter 8 Tolerance and productivity studies in chickens

8.1 Introduction

Plants have been widely used in ethnomedicine around the world. A multitude of plant compounds is readily available and already being used by farmers for medication. Commercial application of these plants could be in the near future a common place. Some of the antimicrobial remedies currently used in livestock production have been reported and are listed in Table 8.1.

Table 8:1 Herbal remedies of potential use as antimicrobial agents in animal production (Cowan, 1999; Tedesco, 2001),

Common name	Scientific name	Compound	Class	Activity
Luceme	*Medicago sativa*			Gram + bacteria
Aloe	*Aloe vera*	Latex	Complex mixture	Corynebacterium, *Salmonella*, *Streptococcus*, *Staphylococcus*
Eucalyptus	*Eucaliptus globules*	Tannin	Polyphenol terpenoid	Bacteria
Fava bean	*Vicia faba*	Fabatin	Thionin	Bacteria
Garlic	*Allium sativum*	Allicin, ajoene	Sulfoxides, sulfate terpenoids	Bacteria
Green tea	*Camelia sinensis*	Chatechin	Flavonoid	Shigella, *Vibrio*, *S. mutans*, Viruses, Bacteria, Fungi
Oak	*Quercus rubra*	Tannins Quercitin	Polyphenols flavonoid	General
Onion	*Allium cepa*	Allicine	Sulfoxide	Bacteria
Rosemary	*Rosmarinus officinalis*	Essential oil	Terpenoid	General

The optimal extract demonstrated low *in vitro* cytotoxicity in the brine shrimp and MTT assays with LC_{50} values of 863 µg /ml and 226 µg /ml respectively. However, these findings alone are not enough for one to draw a conclusion on the potential toxicity of the extract hence the need to do *in vivo* tests that in effect would also assess the possible

application of the optimal extract to replace AFAs (growth promoting additives) without eliciting toxic effects.

Although plant extracts from the *Combretum* spp. have been effectively and safely used in human medicine both therapeutically and prophylatically (Hutchings *et al.*, 1996) and in ethnoveterinary medicine e.g. the use of galls from *Guiera senegalensis*, a member of the Combretaceae, to treat fowl pox virus (FPV) infections in chickens (Lamien *et al.*, 2004), there was a need to establish whether the target animals, the broiler chicken, could tolerate the optimal extract. Tolerance levels by broiler chickens to the extract were evaluated using the repeated dose toxicity procedure. The dosing regimens employed were inferred from *in vitro* studies since there are no recommended doses for *C. woodii* extracts in literature.

8.2 Method

In vivo toxicity studies were carried out as outlined in 2.9. All five treatment groups had 36 broilers except the 5 mg/kg treatment group that had 35 birds.

8.3 Results and discussion

8.3.1 Growth promoting effect

Due to the large variation in masses of the birds, there were no statistically significant differences in growth. This study detected no positive correlation in weight gain with amount of the optimal extract added in the finisher feed, thus the effect of the additives was not influenced by their concentrations in the diet. Compared to broilers diets without any feed additives (negative control) and diets incorporated with the antibiotic growth promoter bacitracin (positive control), the growth promoting effect of the optimal extract tended to be inferior throughout the dosing period [Figure 8.1].

However, the 2 mg/kg dose regimen of the optimal extract and the bacitracin positive control improved the Feed Conversion Ratio (FCR) of broiler chickens compared to the negative control. The improvement in FCR was larger with the former (6.2%) compared to the latter (1.7%). Five mg/kg and 10 mg/kg dose regimes of the optimal extract resulted in both, a reduced weight gain and FCR compared to the positive and negative control [Table 8.4]. It may be that the lowest dose of the optimal extract administered was already too

high. Throughout the 21 days dosing period until the last seven days, broilers tended to have greater improvements in weight when fed diets without any additives than diets with additives [Figure 8.1]. However, at the end of the 21 days dosing period, bacitracin tended to improve growth and the feed to weight gain ratio by 2.08% and 1.73%, respectively.

8.3.2 Health of the broilers

During the dosing period, none of the dose regimens of the optimal extract resulted in death or any visible toxic reactions in the broiler chickens. There was also no statistical significant decrease in the weight gain. Further analyses of the possible toxic reactions elicited by the optimal extract are investigated by post-mortem.

Table 8:2 Average weight of birds, standard deviation and variability over the 21 days dosing period

	Treatment group	Average mass of birds (g)	Standard deviation	Variability coefficient
From day 21	2 mg/kg	654	0.030	4.6
	5 mg/kg	624	0.032	5.1
	10 mg/kg	654	0.042	6.5
	Positive control	640	0.029	4.6
	Negative control	623	0.040	6.4
Day 28	2 mg/kg	1008	0.119	11.8
	5 mg/kg	964	0.080	8.3
	10 mg/kg	991	0.103	10.4
	Positive control	1027	0.082	8.0
	Negative control	973	0.076	7.8
Day 35	2 mg/kg	1390	0.069	5.0
	5 mg/kg	1358	0.057	4.2
	10 mg/kg	1387	0.080	5.7
	Positive control	1496	0.061	4.1
	Negative control	1420	0.077	5.4
Day 42	2 mg/kg	1822	0.184	10.1
	5 mg/kg	1726	0.149	8.7
	10 mg/kg	1783	0.111	6.2
	Positive control	1840	0.073	4.0
	Negative control	1848	0.109	5.9

Table 8:3 Average feed intake, weight gain and Feed Conversion Ratio (FCR) of birds in the different treatment groups over 21 days

	2 mg/kg	5 mg/kg	10 mg/kg	Neg control	Pos control
Feed intake over 21 days	66.258	64.919	71.028	72.681	72.798
Intake / bird over 21 days	1.8405	1.85483	1.973	2.01892	2.02217
Weight gain over 21 days	1.1682	1.10189	1.14013	1.20028	1.22486
FCR	1.5755	1.68332	1.73051	1.68204	1.65094

Table 8:4 Growth performance of broiler chickens in response to the optimal extract fed for 3 weeks, positive and negative controls. Values in brackets denote standard deviations.

Diet	Ave weight gain (g/bird)	Feed intake (g/bird)	% Improvement in weight gain	Food conversion (g feed/g gain)	% Improvement in FCR
Negative control	1200(0.184)	2019	-	1.68	-
2 mg/kg	1168 (0.184)	1840	-2.67	1.576	6.190476
5 mg/kg	1102 (0.149)	1855	-8.17	1.683	-0.17857
10 mg/kg	1140 (0.111)	1973	-5.00	1.731	-3.03571
Bacitracin (control)	1225(0.109	2022	2.08	1.651	1.72619

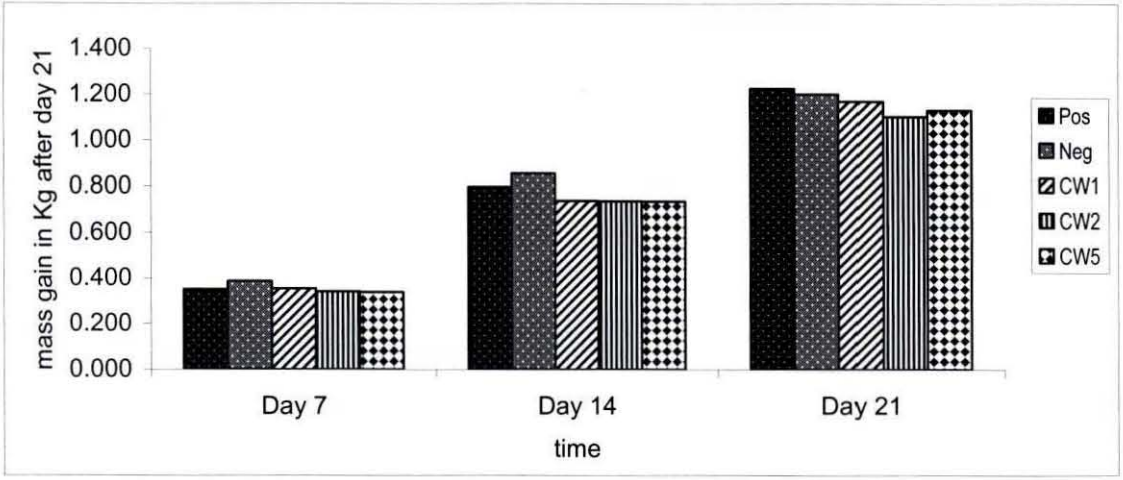


Figure 8:1 Weight gain after different periods of birds dosed with bacitracin (Pos), no feed additive (Neg), 2 mg/kg optimal extract (CW1), 5 mg/kg (CW2) and 10 mg/kg (CW5) optimal extract in feed.

8.3.3 Implications

The sustainability of broiler farming is dependent on both environmental implications and economic viability although these two factors are in direct conflict in many situations. Based on the assumption that the antimicrobial component of the optimal extract is novel and may readily control emergence of resistant strains of poultry pathogens, use of the optimal extract may be beneficial in the broiler industry if it becomes more effective as a growth promoter, no matter the benefits derived from its use in terms of controlling emergence of resistant pathogens. Based on the results presented in this work, there is room for eventual application of extracts from *C. woodii* in the broiler industry if methods that can improve extract potency are designed.

Because even the positive control had no statistically significant increase in productivity, the results probably mean that the hygienic situation under which the experiment was carried out did not lead to infection of the chickens. This experiment should be repeated on chickens challenged by prior infection in future experiments, possibly starting with a lower dose based on the feed conversion results [Table 8.4].

Chapter 9 General conclusion

Extracts of *C. woodii* leaves have *in vitro* antibacterial and antioxidant activity. Before investigating it in animals, attempts were made to increase the activities. After investigating a series of pretreatment and treatments, an extract with higher antibacterial and antioxidant activity (optimal extract) was developed by employing a single pretreatment extraction with hexane on *C. woodii* leaf material prior to extraction with acetone. This extract led to an improvement in antibacterial activity by 87.5% and antioxidant activity improved by 283.3% compared to the crude acetone extract. The optimal extract had a TEAC value of 2.3 in the TEAC assay; this result means it had 2.3 times the antioxidant capacity of vitamin E. Its average MIC against enteric poultry pathogens (*Campylobacter jejuni, Salmonella enteritidis, Clostridium perfringens* and *E.coli*) was in the order of about 0.1 mg/ml.

When the *in vitro* toxicity was determined, the extract was relatively nontoxic in both the brine shrimp assay (LC_{50} of 863 µg/ml) and MTT assay on monkey kidney cells (LC_{50} of 226 µg/ml). The improved extract therefore had a good activity and low levels of toxicity in *in vitro* studies.

Because the optimal extract was investigated for commercial application in the broiler industry, it was important to know the identity of the main antioxidant and antibacterial compound. Silica gel column chromatography was used to isolate the major antioxidant compound in the leaves of *C. woodii.* A stilbene, combretastatin B 5 was isolated as the major antioxidant compound. Its antioxidant activity was 7.9 times the activity of the water-soluble vitamin E analogue (Trolox). There are no previous reports of its antioxidant activity. This same compound has also been isolated from *C. woodii* by Famakin (2002) as the main antibacterial compound and from seeds of *C. kraussii* and has been found to have antimitotic properties (Pellizzoni *et. al*, 1992). The LC_{50} value of 10 µg/ml in the MTT assay could be ascribed to its antimitotic activity. Stilbenes are phytoalexins, these are antimicrobial compounds that accumulate in response to a pathogen (Kuc, 1990), and therefore the possible role of the bibenzyl in *C. woodii* is to protect the plant against any invading microorganism.

Preliminary studies and results from other researcher, (Eloff, 1999, Famakin, 2002, McGaw *et al.*, 2001) demonstrated *C. woodii* leaves to contain several antimicrobial and antioxidant compounds. Future work could be focused on identification and determination of the other active principles in the optimal extract for possible use as biomarkers in quality control. The study of the metabolism of these compounds and investigating the correlation between their activity and concentration may be useful. The successful isolation of one of the major biologically active compounds (combretastatin B5) from *C. woodii* leaves already provides a strong base for these inquiries.

In vivo toxicity studies of the optimal extract on broiler chickens demonstrated tolerance of the extract by the birds with no mortalities recorded during the dosing period and no bird showing behavioural signs of toxicity. There were no statistically significant differences between the growth promoting properties of the optimal extract and the positive and negative control. It will be worthwhile to determine the efficacy of the optimal extract as a growth promoter by repeating the experiment on chickens challenged with prior infection. This work should use lower doses of the optimal extract because 2 mg/kg dose regimen resulted in an increased Feed Conversion Ratio by 6.2 % compared to the negative control, a value that is about four percent superior to the value obtained in bacitracin-incorporated feed. If this observation can be confirmed under different conditions, this product may become a financially viable proposition.

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. woodii*.

References

Aarestrup, F. M., Ahrens, P., Madsen, M., Pallesen, L.V., Poulsen, R.L and Westh H., (1986). *Antimicrob. Agents Chemother.* 40, pp. 1938–1940.

Aarestrup, F. M., (1999). Association between the consumption of antimicrobial agents in animal husbandry and the occurrence of resistant bacteria among food animals. *J. Antimicrobial agents.12, 279-285.*

Alan, L and Miller, N. D., (2000). Antioxidant Flavonoids: Structure, Function and Clinical Usage. http://www.thorne.com/altmedrev/fulltext/flavonoids1-2.html

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.*

Anderson, D. B., McCracken, V. J., Aminov, R. I., Simpson, J. M., (1999). Gut microbiology and growth promoting antibiotics in swine. *Pig news info, 20 (4), pp 115-122.*

Anadón, A. and. Martínez-Larrañaga, M. R., (1999). Residues of antimicrobial drugs and feed additives in animal products: regulatory aspects • *Livestock Production Science, Volume 59, Issues 2-3, Pages 183-198*

Anon., (1997). Report of a WHO Meeting, WHO/EMC/ZOO/97.4, Berlin, Germany, 13–17.

Anon, (1997b). *Off. J. Eur. Communities, L351, pp. 55–59.*

Anon, (1998). *Off. J. Eur. Communities, L351, pp. 4–8.*

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

Arts, M. J. T. J., Haenen, G. R. M. M., Voss, H., Bast, A., (2004). Antioxidant capacity of reaction products limits the applicability of the TEAC assay. *J. Food and chemical toxicology, Vol 42,pp 45-49*

Baba-Moussa, F., Akpagana, K and. Bouchet, P., (1999). Antifungal activities of seven West African Combretaceae used in traditional medicine. *Journal of Ethnopharmacology, Volume 66, Issue 3, Pages 335-338*

Bagchi, D., Garg, A., Krohn, R. L., Bagchi, M., Bagchi, D. J., Balmoori, J and Stohs, S. J. (1998) Protective Effects of Grape Seed Proanthocyanidins and Selected Antioxidants against TPA-Induced Hepatic and Brain Lipid Peroxidation and DNA Fragmentation, and Peritoneal Macrophage Activation in Mice. *General Pharmacology, 30, 771-776*

Begue, W. J and. Kline, R. M., (1972). The use of tetrazolium salts in bioauthographic procedures. *Journal of Chromatography A, Volume 64, Issue 1, Pages 182-184*

Bernhard, D., Schwaiger, W., Crazzolara, R., Tinhofer, I., Kofler, R and Csordas, A., (2003). Enhanced MTT-reducing activity under growth inhibition by resveratrol in CEM-C7H2 lymphocytic leukemia cells. *Cancer letters, vol 195, Iss 2, pp 193-199.*

Bomba, A., Nemcova, R., Mudronova, D., Guba, B., (2002). The possibilities of potentiating the efficacy of probiotics, *Trends in food science and technology, 13, 121-126.*

Breytenbach, J. C., Malan, S. F., (1998). Phamacochemical properties of *Combretum zeyheri. S. Afri.J.Sci. 85, 372-374.*

Bruneton, J., (1995), Pharmacology, Phytochemistry, Medicinal Plants. Intercept limited, Andover, UK.

Bui, V. P., Hudlicky, T., Hansen, T. V., Stenstrom, Y., (2002). Direct biooxidation of arenes to corresponding catechols with *E. coli* JM109 (pDTG602). Application to synthesis of combretastatins A-1 and B-1. *CANCER RESEARCH, vol 61, pp 4312.*

Burns, J., Yokota, T., Ashihara, H., Lean, M. E. J and Crozier, A., (2002). Plant foods and herbal sources of resveratrol. *J. Agric. Food Chem.* 50, pp. 3337–3340.

Carr, J. D., (1988). Combretaceae in Southern Africa. *The Tree Society of Southern Africa, Johannesburg.*

Coleman, K., (2004). Recent advances in the treatment of Gram-positive infections, a review, *Drug Discovery Today: Therapeutic Strategies.*

Cowan, M. M., (1999). Plant Products as Antimicrobial Agents. *Clinical Microbiology Reviews, Vol. 12, 4, 564-582.*

Croteau, R., Kutchan T. M and Lewis, N. G., (2000). Natural Products (secondary metabolites). In: B.B. Buchanan, W. Gruissem and R.L. Jones, Editors, *Biochemistry and Molecular Biology of Plants*, American Society of Plant Physiologists, Rockville, MD, pp. 1250–1318.

Desmarchelier, C., Mongelli, E., Coussio, J. and. Ciccia, G., (1996). Studies on the cytotoxicity, antimicrobial and DNA-binding activities of plants used by the Ese'ejas. *Journal of Ethnopharmacology, Volume 50, Issue 2, Pages 91-96*

Dewdney, J. M., Maes, L., Raynaud, J. P., Blanc, F., Scheid, J. P., Jackson, T., Lens, S and. Verschueren, C., (1991). Risk assessment of antibiotic residues of ß-lactams and macrolides in food products with regard to their immuno-allergic potential. *Food and Chemical Toxicology, Volume 29, Issue 7, Pages 477-483*

Doyle, M. E., (2001). Alternatives to antibiotic use for growth promotion in animal husbandry. *FRI Briefings. Food Research Institute, University of Wisconsin.*

Eisenbrand, G., Pool-Zobel, B., Baker, V., Balls, M., Blaauboer, B.J., Boobis, A., Carere, A., Kevekordes, S., Lhuguenot, J. C., Pieters, R. and Kleiner, J., (2002). Methods on *in vitro* toxicology. *Food hem. Toxicol.* 40, pp. 193–236.

Eloff, J. N., (1998a). A sensitive and quick method to determine the minimum inhibitory concentration of plant extracts for bacteria. *Planta Medica. 60, 1-8.*

Eloff, J. N., (1998b). Which extract should be used for the screening and isolation of antimicrobial component from plants? *J. Ethnophamacology. 60, pp 1-8.*

Eloff, J. N., (1999). The antibacterial activity of 27 Southern African members of the Combretaceae. *SD. Afri. J. Sci. 95, 148-152.*

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, Volume 47, Issue 47, Pages 9751-9758*

Farnsworth, N. R., (1984). The role of medicinal plants in drug development. In Natural products and Drug Development, *eds. P. Krogsgaard-Larsen, S.B Christensen, H.Kofod, pp. 8-98. Balliere, Tindall and Cox, London.*

Farnsworth, N. R., Morris, R. W., (1976). Higher plants: the sleeping giants of drug development. *J. Pharm. 148, 46-52.*

Farnsworth, N. R., Akerele, O., Bingel, A. S, (1985). Medicinal plants in therapy. Bull. World

Frediani-Wolf, V and Stephan, R., (2003). Resistance patterns of Campylobacter spp starins isolated from poultry carcasses in a big Swiss poultry slaughter house. *International Journal of Food Microbiology, Volume 89, Issues 2-3, Pages 233-240*

Fuller, R., (1992). The effects of probiotics on the gut microbiology of farm animals. *In B, J. B Wood (Ed), The lactic acid bacteria. Pg 171-192.London Elsevier Applied Science.*

Fukuda, K.; Hibiya, Y.; Mutoh, M. (1999). *J. Ethnopharmacol*, vol *66*, pp227-233.

Fuzzati, N., Pace, R and Villa, F., (2003). A simple HPLC-UV method for the assay of ginkgolic acids in *Ginkgo biloba* extracts. *Fitoterapia, Volume 74, Issue 3, Pages 247-256*

Goodwin, T. W. and Mercer, E. I. (1972) Introduction to Plant Biochemistry, *Permagon Press, Oxford, New York*

González-Coloma, A., Escoubas, P., Mizutani, J and Lajide, L., (1994)
Insect growth inhibitors from *Machilus japonica*. *Phytochemistry, Volume 35, Issue 3, Pages 607-610*

Gorham, J., Tori, M and. Asakawa, Y., (1995). The biochemistry of the stilbenoids. In: J.B. Harborne, Editor, *Biochemistry of Natural Products Series*, Chapman and Hall, London

Halver, J. E., (1995). Vitamin requirement study techniques. *J. Appl. Ichthyol.* 11, pp. 215–224

Hamburger, M and Hostettmann, K., (1991). Bioactivity in plants: the link between phytochemistry and medicine *Phytochemistry, Volume 30, Issue 12, Pages 3864-3874*

Haraguchi, H., Ishikawa, H., Mizutani, K., Tamura, Y and Kinoshita, T., (1998)
Antioxidative and superoxide scavenging activities of retrochalcones in *Glycyrrhiza inflate*. *Bioorganic & Medicinal Chemistry, Volume 6, Issue 3, Pages 339-347*

Haslam, E., (1996). Natural polyphenols, (vegetable tannins) as drugs: possible
modes of action. J. *Nat. Prod. 59, 205-215.University of Natal Press Pietermaritsburg, South Africa.*

Hewitt, W., Vincent, S., (1989). Theory and application of Microbiological assay. *Academic Press, London*.

Hippeli, S and Elstner, E. F. (1991) Oxygen Radicals and air pollution. Oxidative Stress: Oxidants and antioxidants *(ed. by H. Sies), pp. 3-56. Academic Press, London, New York.*

Houghton, P. J., Raman, A., 1998. Laboratory Handbook for the Fractionation of Natural Extracts. Chapman and Hall, London.

Hutchings, A., Scott, A. H., Lewis, G., Cunningham, A. B., (1996). Zulu medicinal plants

Iwu, M. W., (1993). Handbook of African medicinal plants. *CRC Press, Boca Raton, Florida.*

Johansson, A, Greko, C, Engstrom, B.E, Karlsson, M., (2004). Antimicrobial susceptibility of Swedish , Norwegian and Danish isolates of Clostridium perfringens from poultry. *Veterinary Microbiology*, Apr 2004, Vol 99, Iss 3-4, pp 251-57

Klare, I., Heier, H., Claus, H., Reissbrodt, R and Witte, W., (1995). *FEMS Microbiol. Lett. 125, pp. 165–171*

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.

Lamien, C. E., Meda, A., Mans, J., Romito, M., Nacoulma, O. G and Viljoen, G. J., (2004). Inhibition of fowlpox virus by an aqueous acetone extract from galls of *Guiera senegalensis* J. F. Gmel (Combretaceae). *Journal of Ethnopharmacology, Volume 96, Issues 1-2,* pp 249-253

Lamming, E., (1992). The Report of the Expert Group on Animal Feedingstuffs, *HM Stationery Office, London.*

Lang, Q and Wai, C. M., (1999). *Anal. Chem.* 71, p. 2929

Lawrence, G, H, M., (1951). The Taxonomy of Vascular plants. *Macmillan, New York.*

Letcher, R. M. and. Nhamo, L. R. M., (1972). A revised structure for the tetra-substituted phenanthrene from *T. communis. Tetrahedron Letters, Volume 13, Issue 48, Pages 4869-4870*

Lin, J. G., Chung, J. G.; Wu, L. T., (1999). *Am. J. Chin. Med.*, *27*,pp265-275.

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, pp. 581–593

Lobstein-Guth, A., Briançon-Scheid and Anton, R., (1983). *J. Chromatogr.* 267, p. 431.

Majumder, P. L., Pal, S and Majumder, S., (1999). Dimeric phenanthrenes from the orchid *Bulbophyllum reptans. Phytochemistry, Volume 50, Issue 5, Pages 891-897*

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, Volume 62, Issue 3, Pages 255-263*

McDowell, L. R., Williams, S. N., Hidiroglou, N., Njeru, C. A., Hill, G. M., Ochoa, L and Wilkinson, N. S., (1996). Vitamin E supplementation for the ruminant. *Animal Feed Science and Technology, Volume 60, Issues 3-4, Pages 273-296*

McEvoy, J. D. G., (2002). Contamination of animal feedingstuffs as a cause of residues in food: a review of regulatory aspects, incidence and control. *Analytica Chimica Acta 473, 3 – 26.*

McGaw. J., Rabe, T., Sparg, S. G., Jager, A. K., Eloff, J. N., Van Staden, J., (2001). An investigation on the biological activity of *Combretum* species. *J. Ethnophamacology. 25, pp 45-50.*

Mosmann, T., (1983). Rapid colourimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, Volume 65, Issues 1-2, Pages 55-63*

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, Volume 63, Issue 1, Pages 97-104*

National Committee for Clinical Laboratory Standards. Performance standards for antimicrobial disk and dilution susceptibility tests for bacteria isolated from animals; proposed standard. (1994) NCCLS Document M31-P, vol 14, No 20.

Oliver-Bever, B., (1983). Medicinal plants in tropical West Africa III. Anti-infection therapy with higher plants. *Journal of Ethnopharmacology, Volume 9, Issue 1, Pages 1-83*

Osborne, R and Pegel, K. H., (1984). Jessic acid and related acid triterpenoids from *Combretum elaeagnoides. Phytochemistry, Volume 23, Issue 3, Pages 635-637*

Packer, L., Rimbach, G., and Virgili, F., (1999). Antioxidant activity and biologic properties of a procyanidin-rich extract from pine (pinus maritima) bark, pycnogenol. *Free Radical Biology and Medicine*, 27, 704-724.

Pangrahi, S., 1993. Bioassay of mycotoxins using terrestrial and aquatic, animal and plant species. *Food Chem. Toxic.* 31, *pp. 767–790.*

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.*

Pekic, B., Kova, V., Alonso, E and Revilla, E., (1998). Study of the extraction of proanthocyanidins from grape seeds. *Food Chemistry, Volume 61, Issues 1-2, Pages 201-206*

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.*

Petersime news letter #37, (2002). www.petersime.com/news

Pettit, G. R., Singh, S. B., Smith, C. R., (1982). Recent advances in the chemistry of plant antineoplastic constituents. Chemistry of plant antineoplastic constituents, pp105-116

Petit, G. R., Singh, S. B., Gragg, G. M., 1987. Antineoplastic agents, 122. Constituents of *Combretum caffrum.* J. Nat. Prod. 50, 386 - 391.

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. 1995. 38, pp 1666-1672.*

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M., Rice-Evans, C., (1999). Antioxidant activity applying an improved ABTS radical cation decolourization assay. *Free Radical Biology and Medicine*, 26, 1231-1237.

Rogers, C. B., Verotta L., (1996). In Biological and Pharmacological Properties of African Medicinal Plants, eds. F. Hostettmann, M. Chinyanganya, M. Maillard, J. L. Wolfender, pp. 121-141. University of Zimbabwe Publications, Harare.

Rogers, C. B., (1996). Chemistry and biological properties of the African Combretaceace. *Proceedings of the first international IOCD symposium, 25-28 February, Victoria Falls Zimbabwe.*

Rogol, M., Sechter, I., Greenberg, Z., Mizrachi, R., Shtark, Y and Alfi S., (1985). Contamination of chicken meat and environment with various serogroups of

Campylobacter jejuni/coli. International Journal of Food Microbiology, Volume 1, Issue 5, Pages 271-276

Rice, D., and Kennedy, S., (1988). Vitamin E: function and effects of deficiency. *British Veterinary Journal* 144, pp. 482–496

Sacho, H., Schoub, D. B., (1993). Current perspectives on Nosocomial Infections, [Sponsored by Glaxo Wellcome] 100pp Natal Witness Printing and Publishing, Pietermaritzburg

Sahin, K., Sahin, N and. Onderci, M., (2002). Vitamin E supplementation can alleviate negative effects of heat stress on egg production, egg quality, digestibility of nutrients and egg yolk mineral concentrations of Japanese quails. *Research in Veterinary Science, Volume 73, Issue 3, Pages 307-312*

Salie, F., Eagles, P. F. K., (1996). Preliminary antimicrobial screening of four South African Astraceae species. *J. Ethnopharmacol. 52, pp 27-33.*

Sawer, I., Berry, M., Brown, M., Ford, J., (1995). The effect of Cryptolepine on the morphology and survival of *E.coli, C.albicans* and *S.cerevisiae*. *J. Appl. Bacteriol, 79, 314-321*

Schroder, J., (1999). The chalcone/stilbene synthase-type family of condensing enzymes. In: D.H.R. Barton, K. Nakanishi and O. Meth-Con, Editors, *Comprehensive Natural Products Chemistry, Polypeptides and other Secondary Metabolites including Fatty Acids and their Derivatives*, Elsevier, Amsterdam, pp. 749–772

Schroeder, C.M, White, D.G, Meng, J.,(2004). Retail meat and poultry as a reservoir of antimicrobial resistant E. coli. *Food Microbiology*, vol 21, Iss3, pp 249-55

Schwikkard S, Zhou B, Glass T.E, Sharp J.L, Mattern M.R, Johnson R.K, Kingston D.G.I., (2000). Bioactive compounds from *Combretum erythrophyllum. J. Nat. Prod*, vol 63, pp 457-460.

Shnyder, S. D., Cooper, P. A., Pettit, G. R., Lippert, J. W., Bibby, M. C., (2003). Combretastatin A-1 phosphate potentiates the antitumour activity of cisplatin in a murine adenocarcinoma model. *ANTICANCER RESEARCH, vol 23 (2B), pp 1619-1623.*

Silva, O., Duarte, A., Cabrita, J., Pimementel, M., Diniz, A., Gomes, E., (1996). Antimicrobial activity of Guinea-Bissau traditional remedies. *J. Ethnopharmacology. 50, pp 55-59.*

Snyder, L. R., Kirkland, J. J., (1979). Introduction to modern liquid chromatography. John Wiley, New York.

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, pp. 250–252

Stahl, E., (1969). Thin Layer Chromatography, *2nd edition, Springer-Verlag, Berlin, Heidenberg, New York*

Stivala, L. A., Savio, M., Carafoli, F., Perucca, P., Bianchi, L., Maga, G., Forti, L., Pagnoni, U. M., Albini, A., Prosperi, E and Vannini, V., (2001). Specific structural determinants are responsible for the antioxidant activity and the cell effects of resveratrol. *J. Biol. Chem.* 276 25, pp. 22586–22594

Strain, J. J., (1984). Newer aspects of micronutrients in chronic disease: copper. *Proceedings of the Nutritional Society* 53, pp. 583–598.

Su, B. N., Cuendet, M., Hawthorne, M. E., Kardono, L. B. S., Riswan, S., Fong, H. H. S., Metha, R. G., Pezzuto, J. M and Kinghorn, A. D., (2002). Constituents of the bark and twigs of Artocarpus with cyclooxygenase inhibitory activity. *J. Nat. Prod* 65, pp. 163–169.

Surai, P. F., (2002). Natural antioxidants in avian nutrition and reproduction, *Nottingham University Press, pp 5-9*

Swann, M. M., (1968). Joint Committee on the Use of Antibiotics in Animal Husbandry and Veterinary Medicine, *HM Stationery, Office London*

Taylor, R. S., Manadhar, N. P., Towers, G. H. N., (1996). Screening of selected medicinal plants of Nepal of antimicrobial activities. *J. Ethnopharmacol. 46, pp 153-159.*

Tedesco, D. (2001). *Zoo. Nutr. Anim.* 27, p. 111.

Tseng, T. H., Kao, E. S., Chu, C. Y., Chou, F. P., Lin Wu, H. W and. Wang, C. J., (1997) Protective effects of dried flower extracts of *Hibiscus sabdariffa* L. against oxidative stress in rat primary hepatocytes. *Food and Chemical Toxicology, Volume 35, Issue 12, Pages 1159-1164*

Van Beek, T. A., Scheeren, H. A., Rantio, T., Melger, W. C and. Lelyveld, G. P., (1991). *J. Chromatogr.* 543, p. 375.

Van Beek, T. A. and. Taylor, L.T., (1996). *Phytochem. Anal.* 7, p. 185

Van den Berg, R., Haenen, R. M. M., van den Berg, H and Bast, A., (1999) Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. *Food Chemistry, Volume 66, Issue 4, Pages 511-517*

Van Overveld, F. W. P. C., Haenen, R. M. M., Rhemrev, J., Vermeiden, J. P and Bast, A., (2000). Tyrosine as important contributor to the antioxidant capacity of seminal plasma. *Chemico-Biological Interactions, Volume 127, Issue 2, Pages 151-161*

Van Wyk, B. E., van Oudtshoorn, B. and Gericke, N., 1997. Medicinal plants of South Africa, Briza Publications, Pretoria, South Africa.

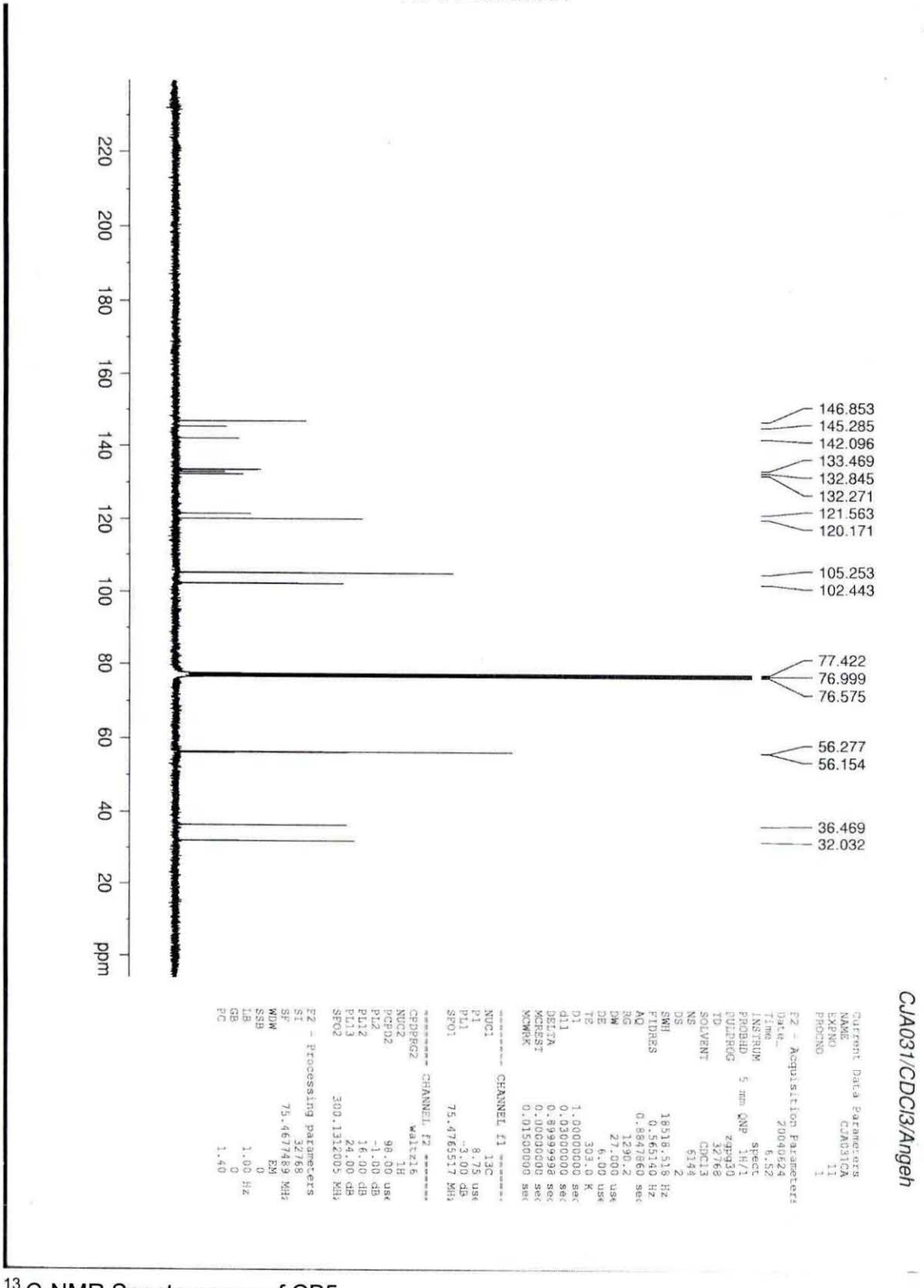
Vlietinck, A. J., Van Hoof, L., Tott, J., Lasure, A., Van den Berghe, D., Rwangabo, P. C., Mvukiyumwami, J., (1995). Screening of hundred Rwandese medicinal plants for antimicrobial and antiviral properties. *J. Ethnopharmacol. 46, pp 31-47.*

Waihenya, R. K., Mtambo, M. M. A and. Nkwengulila, G., (2002). Evaluation of the efficacy of the crude extract of *Aloe secundiflora* in chickens experimentally infected with Newcastle disease virus *Journal of Ethnopharmacology, Volume 79, Issue 3, Pages 299-304*

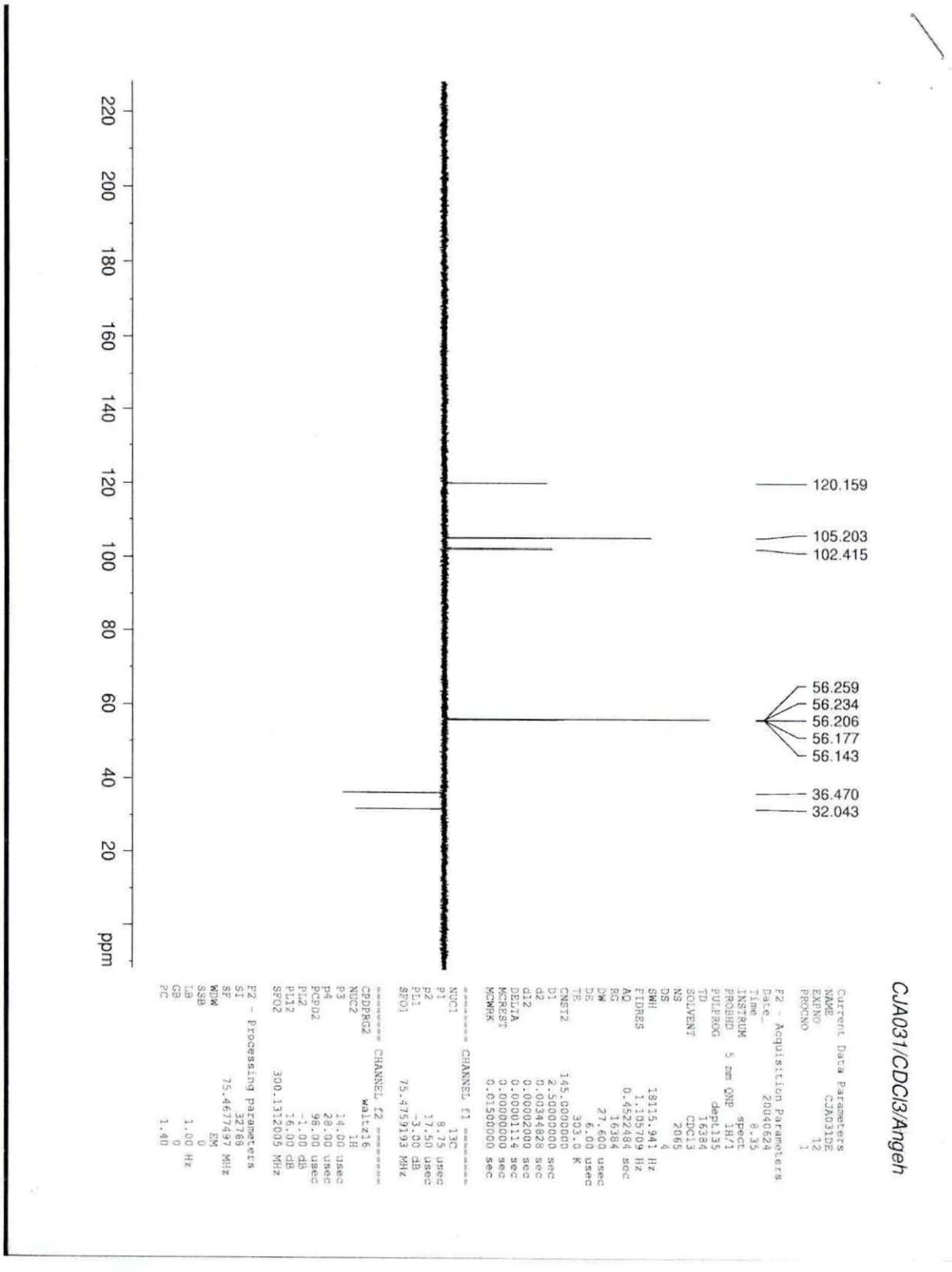
Wierup, M., (2000). The control of microbial diseases in animals: alternatives to the use of antibiotics, *International Journal of Antimicrobial Agents, Volume 14, Issue 4, Pages 315-319*

World Health Organization. 63, pp 965-981.

APPENDIX A



^{13}C-NMR Spectroscopy of CB5



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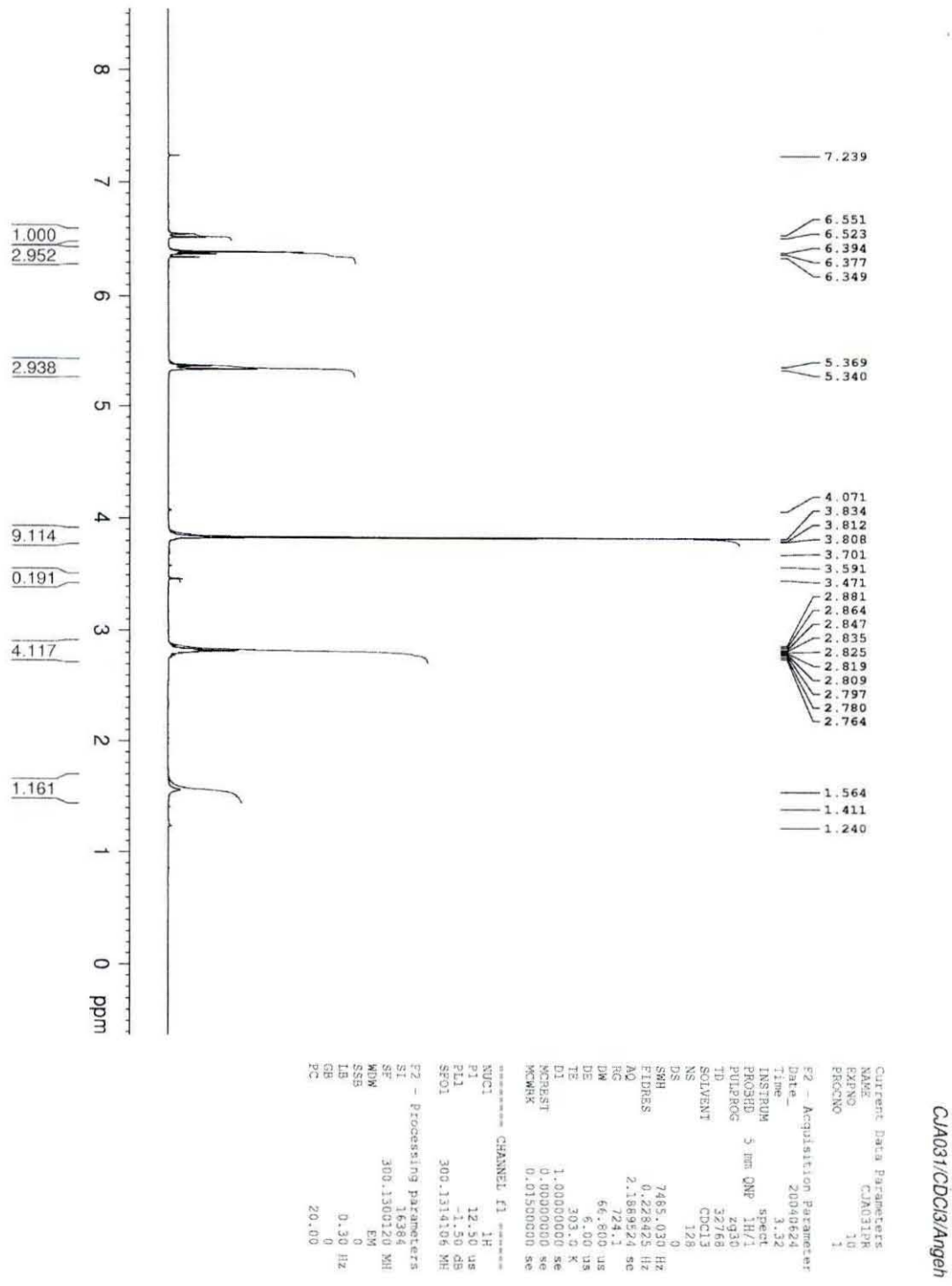
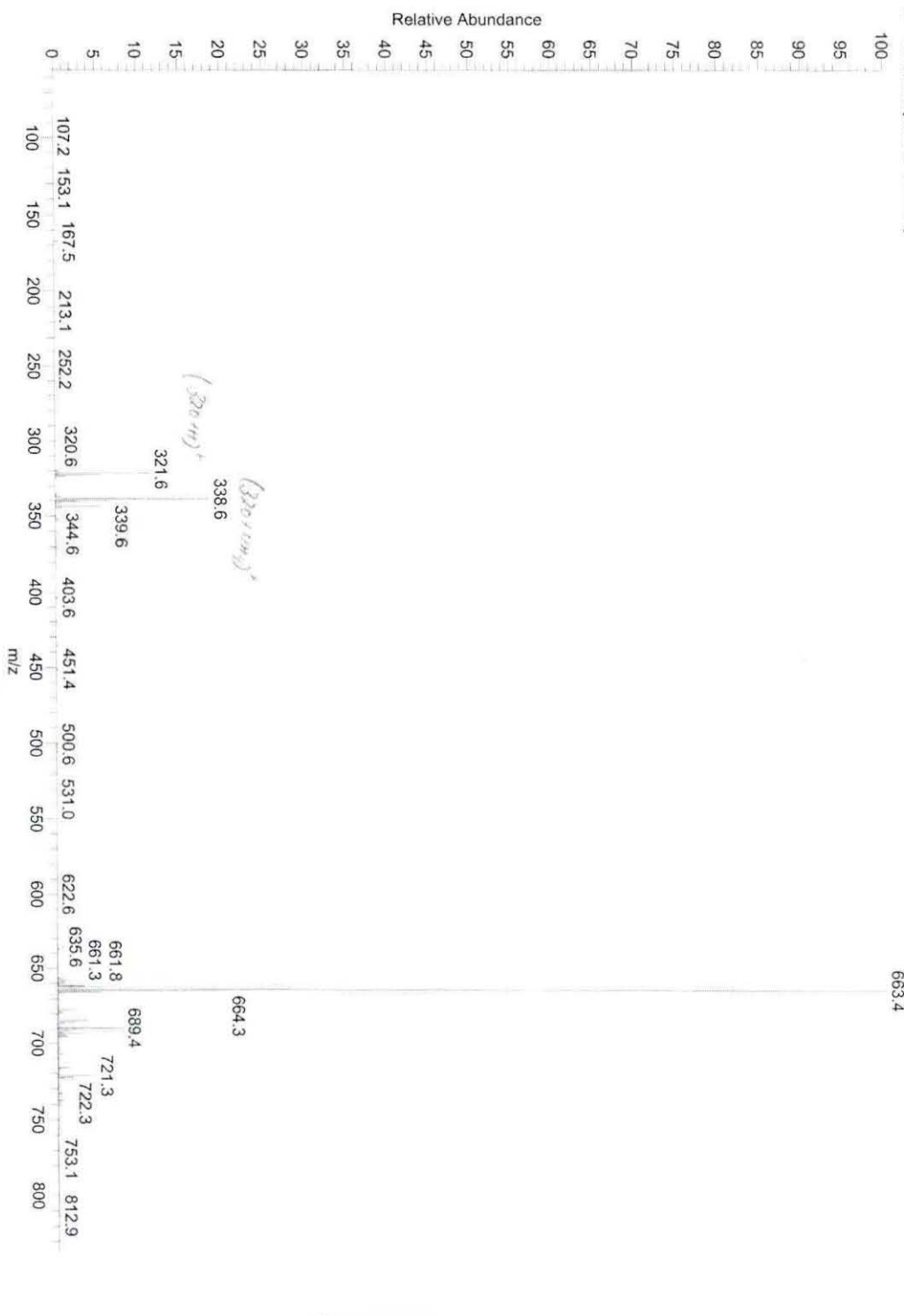


^{1}H-NMR Spectroscopy of CB5



Mass spectrometry of CB5

APPENDIX B

Weight of birds in 2 mg/kg treatment group (A), 5 mg/kg treatment group (B), 10 mg/kg treatment group (C), negative control (D) and positive control (E) at day 21 (left) and day 28 (right).

	A	B	C	D	E	A	B	C	D	E
1	0.65	0.396	0.527	0.75	0.74	1.031	0.603	0.99	1.079	1.096
2	0.593	0.599	0.68	0.643	0.549	1.154	0.839	1.25	1.005	0.88
3	0.684	0.724	0.653	0.636	0.63	1.093	1.036	1.03	1.007	0.915
4	0.608	0.435	0.622	0.656	0.518	1.028	0.853	0.96	1.077	0.872
5	0.552	0.537	0.729	0.706	0.577	1.039	0.952	0.927	0.904	0.997
6	0.681	0.514	0.628	0.602	0.7	1.131	1.157	1.19	0.82	0.606
7	0.699	0.594	0.701	0.585	0.689	0.999	0.975	1.001	1.053	0.895
8	0.661	0.502	0.675	0.675	0.656	0.647	1.053	0.878	1.001	0.79
9	0.672	0.682	0.56	0.573	0.655	1.048	0.991	0.889	1.002	0.859
10	0.699	0.517	0.69	0.619	0.539	0.969	1.071	0.971	1.039	0.81
11	0.724	0.672	0.661	0.58	0.654	1.036	0.908	1.16	1.057	1.09
12	0.6	0.618	0.69	0.557	0.36	1.079	1.094	1.004	1.058	1.017
13	0.618	0.587	0.454	0.677	0.482	0.902	1.154	1.047	1.107	0.98
14	0.609	0.639	0.545	0.65	0.687	0.986	0.907	0.8	0.999	0.952
15	0.667	0.624	0.669	0.675	0.604	1.079	0.605	0.83	1.072	1.123
16	0.663	0.725	0.652	0.66	0.619	0.954	1.092	0.961	0.999	1.179
17	0.671	0.689	0.675	0.699	0.68	1.008	0.796	1.03	0.932	0.879
18	0.708	0.729	0.763	0.603	0.569	1.052	1.004	0.95	0.952	0.962
19	0.785	0.691	0.641	0.545	0.72	1.089	1.092	0.99	0.978	1.079
20	0.657	0.703	0.801	0.725	0.674	1.158	0.872	0.927	0.905	0.999
21	0.748	0.527	0.691	0.676	0.63	0.978	0.977	0.97	1.03	0.923
22	0.584	0.685	0.701	0.684	0.782	1.034	1.053	1.012	0.984	0.99
23	0.693	0.696	0.699	0.621	0.548	0.953	0.894	1	1.043	0.905
24	0.642	0.642	0.765	0.66	0.576	1.019	0.873	0.855	1.172	1.074
25	0.684	0.658	0.784	0.576	0.695	0.915	0.882	1.06	1.077	0.888
26	0.68	0.637	0.648	0.585	0.607	0.934	1.079	1.08	1.179	0.919
27	0.722	0.747	0.63	0.622	0.62	1.199	1.1	1.049	0.88	1.092
28	0.681	0.734	0.688	0.645	0.54	1.009	1.075	0.706	0.984	0.972
29	0.659	0.565	0.649	0.62	0.593	0.868	1.002	1.067	1.043	0.973
30	0.602	0.675	0.624	0.69	0.709	0.889	0.959	0.969	1.135	0.987
31	0.68	0.619	0.655	0.641	0.618	0.903	0.939	1.16	1.128	1.026
32	0.686	0.623	0.609	0.739	0.598	1.134	0.969	1.015	1.106	0.973
33	0.565	0.572	0.525	0.693	0.721	1.12	0.903	0.93	1.002	1.135
34	0.65	0.607	0.553	0.609	0.667	1.076	1.028	0.958	1.026	0.993
35	0.42	0.668	0.676	0.49	0.597	0.755	0.965	1.086	1.052	1.084
36			0.613	0.679	0.64				1.067	1.124

Weight of birds in 2 mg/kg treatment group (A), 5 mg/kg treatment group (B), 10 mg/kg treatment group (C), negative control (D) and positive control (E) at day 35 (left) and day 42 (right).

	A	B	C	D	E	A	B	C	D	E
1	1.482	1.326	1.515	1.619	1.345	2.438	2.001	2.13	2.007	2.17
2	1.347	1.533	1.515	1.383	1.429	2.101	1.96	2.017	1.998	2.087
3	1.498	1.423	1.438	1.491	1.557	2.087	1.959	1.99	1.992	2.071
4	1.371	1.31	1.09	1.516	1.306	2.041	1.95	1.986	1.967	2.056
5	1.509	1.419	1.399	1.458	1.384	2.039	1.939	1.968	1.948	1.999
6	1.436	1.432	1.42	1.599	1.251	2.018	1.89	1.948	1.938	1.998
7	1.439	1.541	1.446	1.453	1.515	2.006	1.876	1.943	1.928	1.989
8	1.231	1.325	1.325	1.439	1.431	2.002	1.866	1.907	1.927	1.982
9	1.61	1.451	1.378	1.586	1.609	1.997	1.846	1.902	1.922	1.974
10	1.554	1.578	1.599	1.43	1.381	1.978	1.844	1.9	1.902	1.973
11	1.474	1.503	1.286	1.436	1.553	1.929	1.84	1.885	1.895	1.969
12	1.292	1.326	1.36	1.237	1.328	1.908	1.839	1.876	1.886	1.947
13	1.106	1.412	1.156	1.566	1.531	1.907	1.811	1.858	1.885	1.902
14	1.408	1.305	1.47	1.427	1.312	1.901	1.801	1.832	1.885	1.889
15	1.263	1.277	1.429	1.46	1.277	1.895	1.797	1.831	1.875	1.855
16	1.376	1.235	1.365	1.475	0.958	1.876	1.769	1.812	1.868	1.839
17	1.385	1.496	1.501	1.506	1.362	1.858	1.754	1.81	1.864	1.836
18	1.424	1.264	1.35	1.442	1.362	1.839	1.748	1.805	1.852	1.829
19	1.543	1.491	1.235	1.542	1.442	1.825	1.706	1.798	1.843	1.815
20	1.403	1.285	1.428	1.526	1.455	1.825	1.705	1.769	1.826	1.809
21	1.358	0.993	1.519	1.302	1.635	1.795	1.704	1.763	1.817	1.806
22	1.472	1.271	1.268	1.54	1.504	1.794	1.698	1.752	1.817	1.805
23	1.457	1.296	1.399	1.535	1.654	1.79	1.68	1.733	1.809	1.801
24	1.378	1.489	1.248	1.447	1.487	1.775	1.64	1.725	1.808	1.792
25	1.413	1.331	1.454	1.603	1.309	1.765	1.629	1.723	1.807	1.79
26	1.608	0.906	1.442	1.542	1.348	1.748	1.625	1.693	1.802	1.786
27	1.586	1.215	1.356	1.512	1.383	1.699	1.619	1.69	1.791	1.785
28	1.358	1.416	1.332	1.396	1.385	1.615	1.605	1.671	1.786	1.752
29	1.628	1.568	1.529	1.52	1.583	1.598	1.598	1.667	1.782	1.752
30	1.506	1.177	1.172	1.442	1.209	1.591	1.588	1.639	1.755	1.752
31	0.941	1.298	1.379	1.521	1.549	1.585	1.584	1.614	1.749	1.726
32	1.352	1.403	1.256	1.525	1.364	1.575	1.58	1.599	1.733	1.709
33	1.326	1.462	1.37	1.685	1.507	1.501	1.526	1.545	1.709	1.676
34	1.286	1.243	1.271	1.601	1.351	1.28	1.213	1.543	1.671	1.64
35	0.825	1.533	1.725	1.733	1.644	1.203	1.208	1.453	1.65	1.609
36			1.495	1.347	1.415			1.399	1.562	1.368

Feed weighed in (in) and weighed out (out) in Kg for 2 mg/kg treatment group (Group A), 5 mg/kg treatment group (Group B), 10 mg/kg treatment group (Group C), negative control (Group D) and positive control (Group E) for the 21 day dosing period.

	Group A		Group B		Group C		Group D		Group E	
	out	in	out	in	out	in	out	in	out	in
Day 21										
16/11/2004										
17/11/2004										
18/11/2004		10		10		10		10		10
19/11/2004										
20/11/2004										
21/11/2004		3.89		3.844		3.832		3.561		2.875
22/11/2004	1.65	8.35	2.103	7.897	0.902	10	0.244	9.756	0.62	9.38
Day 28										
23/11/2004										
24/11/2004		5		5		5		5		5
25/11/2004		5.5		5.5		5.5		5.5		5.5
26/11/2004		5.575		5.069		4.34		5.57		4.945
27/11/2004		7		8		7		7		7
28/11/2004										
29/11/2004	4.883	5.5	4.964	5.5	3.294	5.5	2.276	5.5	2.514	5.5
Day 35										
30/11/2004		6.16		7		7		7		7
01/12/2004										
02/12/2004		9		9		9		9		9
03/12/2004		5.5		5.5		5.5		5.5		5.5
04/12/2004										
05/12/2004		5.5		5.5		5.5		5.5		5.5
06/12/2004	4.184		5.824		2.948		3.686		1.268	