

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 olaquinox (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 β -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 from the continued use 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 cyclized.

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 *Cryptolepis sanguinolenta* 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 (*Camellia 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 edwardsii*, *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, quercetin 5,3'-dimethylether and three flavones apigenin, genkwanin and 5-hydroxy-7, 4'-dimethoxyflavone.

All test compounds had good activity against *Vibrio cholerae* and *E. faecalis*, with MIC values in the range of 25-50 µg/ml. Rhamnocitrin and quercetin-5, 3'-dimethylether showed

additional good activity (25 µg/ml) against *Micrococcus luteus* and *Shigella sonnei*. *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.

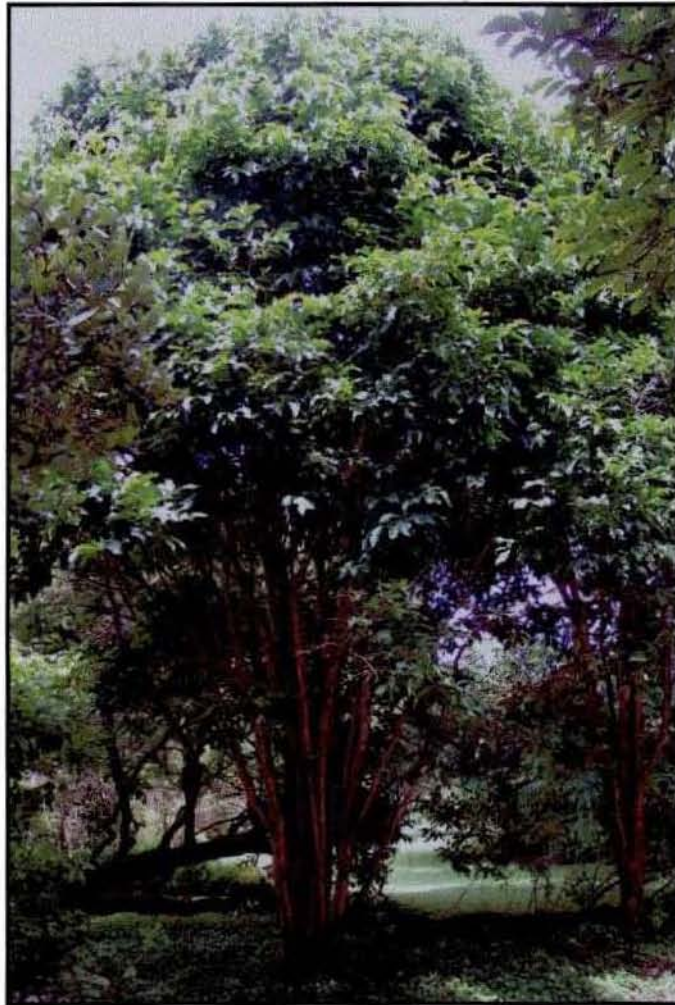


Figure 1:1 *Combretum woodii* from which leaves were collected in Lowveld National Botanical Garden

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 LN BG 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 LN BG 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 F₂₅₄)) 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 F₂₅₄) 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.

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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 F₂₅₄) 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⁺). The blue/green chromophore ABTS⁺ 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⁺, 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⁺ with time is calculated using a spectrophotometer and compared to the reactivity of Trolox against ABTS⁺ under the same conditions (Re *et al.*, 1999).

2.6.2.1 Preparation of 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS⁺)

Mixing 192 mg of ABTS⁺ with 50 ml of water made a 7 mM stock solution of ABTS⁺. 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⁺ 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 μ L 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 10³ 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 μ l/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 μ l PBS solution was added into each well followed by centrifugation at 2000 rpm again. The formazan crystals formed were dissolved by adding 50 μ l/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 μ m). 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

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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.