CHAPTER 1

INTRODUCTION

1.1 Introduction

Natural products and their derivatives represent more than 50% of the drugs in clinical use in the world (Cowan, 1999, Sofowora, 1984). One of the paramount reasons for pursuing natural products chemistry resides in the actual or potential pharmacological activity to be found in alkaloids, terpenoids, coumarins, flavonoids, lignans and the like. Since the advent of antibiotics in the 1950's, the use of plant derivatives as a source of antimicrobials has been virtually non-existent (Cowan, 1999). Antimicrobial plant extracts have been recognised as a future source of new antimicrobials in the event of the current downturn in the pace at which these are being derived from micro-organisms. The public is also becoming more aware of problems with overprescription and misuse of traditional antibiotics (Cowan, 1999).

Resistance to anti-microbial agents is recognized at present as a major global public health problem. Infective diseases account for approximately one-half of all deaths in countries in tropical regions. In industrialized nations, despite the progress made in the understanding of microorganisms and their control, incidents of epidemics due to drug resistant microorganisms and the emergence of hitherto unknown disease-causing microbes, pose enormous public health concerns (Iwu *et al*; 1999). The number of resistant strains of microbial pathogens is also growing since penicillin resistance and multiresistant pneumococci caused a major problem in South African hospitals in 1977 (Berkowitz, 1995). Berkowitz, (1995) referred to the emergence of drug resistant bacteria as a medical catastrophe. Leggiadro (1995) stated that effective regimens might not be available to treat some enterococci isolates and that it is critically important to develop new anti-microbial compounds for these and other organisms before we enter the post-antibiotic era.

The cost of drugs is a sizable proportion of total health expenditure in most developing countries. In some of these countries, drug related expenses account for up to 30-50% of the total cost of health care (Sofowora, 1984). This situation is becoming increasingly unbearable to many nations including South Africa. The World Health Organization (WHO), have observed that up to 80% of the rural populace in the developing countries depend on herbal or alternative medicine and requested member countries to explore safe indigenous medicines for their national health care (Sofowora, 1984).

Plants have served as a source of new pharmaceutical products and inexpensive starting materials for the synthesis of some known drugs. Components with medicinal properties from plants play an important role in conventional Western medicine. In 1984, at least 25% of the Western medicine issued in the US and Canada were derived from or modelled after plant natural products and 119 secondary metabolites were used globally as drugs (Farnsworth, 1994). It has been estimated that 14-28% of higher plant species are used medically. Only 15% of all angiosperms have been investigated chemically and 74% of pharmaceutically active plant derived components were discovered after following up on ethnomedical use of the plant (Farnsworth, 1991).

The traditions of collecting, processing and applying plant and plant-based medications have been handed down from generation to generation. In many African countries, traditional medicines, with medicinal plants as their most important components, are sold in marketplaces or prescribed by traditional healers (without accurate dose value) in their homes (Herdberg and Staugard, 1989). Because of this strong dependence on plants as medicines, it is important to study their safety and efficacy (Farnsworth, 1994). The value of ethno-medicine and traditional pharmacology is nowadays gaining increasing recognition in modern medicine because the search for new potential medicinal plants is frequently based on an ethno-medicinal basis. In the ethno-pharmacological approach, local knowledge about the potential uses of the plants is very useful as compared to the random approach where indigenous knowledge is not taken into consideration.

Compounds inhibiting microorganisms, such as benzoin and emetine have been isolated from plants (Cox, 1994). It is possible that anti-microbial compounds from plants may inhibit bacteria by a different mechanism than the presently used antibiotics and may have clinical value in the treatment of resistant microbial strains. For this reason, it is therefore important to investigate plants as alternative sources of anti-microbial compounds.

Preliminary work done on the southern African members of the section Hypocrateropsis (Eloff, 1999a) indicated that most of the members of this section had substantial antibacterial activity against Gram-positive and Gram-negative bacteria. Bioautography studies indicated that members of the section Hypocrateropsis have different antibacterial compounds from members of other sections. Substantial antibacterial activity of some species further motivated this study.

1.2 Hypothesis

Combretum species from the section Hypocrateropsis contain antibacterial compounds that can be isolated and tested for in *vivo* activity.

1.3 Aim of research

To isolate the main antibacterial compounds present in *Combretum* section Hypocrateropsis in a bioassay guided process, and to characterize these compounds chemically and biologically.

1.3.1 Objectives

- Select the most active plant species
- Select and evaluate the best fractionation procedure for isolation
- Isolate antibacterial compounds
- Determine the chemical structure of isolated compounds
- Determine the biological activity of isolated compounds
- Determine the possible of synergistic effects of isolated compounds
- Evaluate how well phytochemistry agrees with taxonomy based on morphology.

CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

Plants have served as a source of new pharmaceutical products and inexpensive starting materials for the synthesis of many known drugs. Natural products and their derivatives represent more than 50% of the drugs in clinical use in the world (Cowan, 1999, Sofowora, 1984) (Table 2-2). Although the first chemical substance to be isolated from plants was benzoic acid in 1560, the search for useful drugs of known structures did not begin until 1804 when morphine was separated from *Papaver somniferum L. (Pium).* Since then many drugs from higher plants have been discovered, but less than 100 with defined structures are in common use. Less than half of these (Table 2-1) are accepted as useful drugs in industrialized countries (Farnsworth, 1984). Considering the great number of chemicals that have been derived from plants as medicine, scientific evaluation of plants used traditionally for the treatment of bacterial infection seems to be a logical step of exploiting the anti-microbial compounds, which may be present in plants. Plant-based anti-microbials represent a vast untapped source of medicines With enormous therapeutic potential (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 anti-microbials (Iwu *et al*., 1999)

Table 2-1: Plant-derived drugs widely employed in Western medicine (Adapted from Farnsworth, 1984)

Acetyldigoxin	Ephedrine*	Pseudoephedrine*	Xanthotoxin
Aescin	Hyoscyamine	Quinidine	
Ajmalicine	Khellin	Quinine	
Allantoin*	Lanatoside	Reserpine	
Atropine	Leurocristine	Rescinnamine	
Bromelain	Lobeline	Scillarens A & B	
Caffeine	Morphine	Scopolamine	
Codeine	Narcotine	Sennosides A & B	
Colchicine	Ouabain	Sparteine	
Danthron*	Papain	Strychnine	
Deserpidine	Papaverine*	Tetrahydrocannabinol	
Digitoxin	Physostigmine	Theobromine*	
Digoxin	Picrotoxin	Theophylline*	
L-Dopa*	Pilocarpine	Tubocurarine	
Emetine	Protoveratrines A & B	Vincaleukoblastine	

* Produced industrially by synthesis

2.2 Antibiotic resistance

Resistance to anti-microbial agents is recognized at present as a major global public health problem. Infective diseases account for approximately one-half of all deaths in tropical countries. In industrialized nations, despite the progress made in the understanding of microorganisms and their control, incidents of epidemics due to drug resistant microorganisms and the emergence of hitherto unknown disease-causing microbes, pose enormous public health concerns (Iwu *et al*., 1999).

Almost since the beginning of the antibiotic era, bacterial resistance has been seen as the major obstacle to successful treatment (Iwu *et al.,* 1999). Microbial resistance to antibiotics in the clinic emerged soon after their first use in the treatment of infectious disease, and continue to pose a significant challenge for the health care sector. Resistance has now firmly emerged as a problem in the wider community. At the end of the 1960s the Surgeon General of the United States stated that: "we could close the book on infectious diseases." At the time he uttered these words the emergence of resistance did not seem to affect therapeutic options. Although *S. aureus* had become resistant to benzylpenicillin and showing resistance to thethincillin, it remained sensitive to gentamicin and infections could therefore still be treated. At the start of the next century, things looked very different. Already at least three bacterial species, capable of causing life-threatening illness (*Enterococcus faecalis, Mycobacterium tuberculosis and Pseudomonas aeruginosa*), had become resistant to every one of the 100 antibiotics, available except for vancomycin (Iwu, 1999). Vancomycin is the antibiotic of last resort for treatment of resistant infections and within the past year scientists have found strains of *Streptococcus pneumoniae* and *S. aureus* to be resistant to this antibiotic. This is attested by the spread, with associated deaths, of infection by methicillin-resistant *Staphylococcus aureus* and the increased prevalence of drug-resistant *S. pneumoniae* in patients suffering from pneumonia. Anti-microbial resistance is driven by inescapable evolutionary pressures and is therefore predictable and inevitable. The emergence in the past year of vancomycin-resistant *S. aureus* an event that has been anticipated for the past decade with great dread, punctuates this assertion. Hardly any group of antibiotics has been introduced to which some bacterium has not developed resistance (Iwu et *al*., 1999).

Recent reports have shown a marked increase in antibiotic resistance of food-poisoning bacteria due to non-rational and excessive use of antibiotics as therapeutic agents or as growth promoters in livestock. Another factor of resistance potentially lies in the use of antibiotic resistant genes as selection markers in genetically modified organisms (GMOs) (http://www.biosafety. ihe.de 1999). The main safety issue of

concern is the release of these resistant genes to sensitive organisms when these GMOs are introduced into the environment.

Due to emergence of drug resistant bacteria, the search for new antibacterial compounds with improved activity is necessary (Harold and Heath 1992a). Many indigenous plants are used in treating bacterial related diseases. Only a small fraction of these indigenous plants has been investigated (Carr and Rogers 1987).

Understanding of the mechanism of action of resistance development remains the foundation of new cycles of antibiotic discovery. Such events demonstrate that antibiotic management and new discovery must continue in the face of these pressures.

2.3 Natural products in drug discovery

Medicinal plants use is widespread (Farnsworth, 1991). The production of medicines and the pharmacological treatment of diseases began with the use of herbs (Tyler, 1997). Life saving and essential drugs from medicinal plants such as morphine, digoxin, aspirin, emetine, and ephedrine were introduced into modern therapeutics several centuries ago. However, plants have been used as drugs for over millenia by human beings. Plants historically have served as models in drug development for some major reasons: The first being that each plant is a unique chemical factory capable of synthesizing large numbers of highly complex and unusual chemical substances. In the United States of America alone, about 25% of popularity in the use of plant-derived preparations (Farnsworth and Morris, 1976). It has also been estimated by the World Health Organization (WHO) that about 80% of the population of the developing countries rely exclusively on plants to meet their health care needs (Farnsworth et al., 1985).

The second reason involves biologically active substances derived from plants have served as templates for synthesis of pharmaceuticals. Such compounds may have poor pharmacological and toxicological profiles. While the reason concerns the fact that highly active secondary plant constituents have been instrumental as pharmacological tools to evaluate physiological processes (Farnsworth, 1984). There are numerous illustrations of plant-derived drugs.

Despite the expense involved in the development of a drug today, at least US$230 million and a time span between 10 - 20 years (Farnworth, 1984), nature remains the most reliable and most important source of novel drug molecules. Nature provides 80% of all pharmacological and therapeutic lead compounds and the NCI estimates that over 60% of the compounds currently in pre-clinical and clinical development in its

laboratories are of natural origin. Thus higher plants remain an important and reliable source of potentially useful chemical compounds not only for direct use drugs, but also as unique prototypes for synthetic analogues and as tools that can be used for a better understanding of biological processes (Farnsworth, 1984).

Literally thousands of phytochemicals with inhibiting effects on microorganisms have shown *in-vitro* activity. One may argue that these compounds have not been tested *in vivo* and therefore activity cannot be claimed, but one must take into consideration that many, if not all, of these plants have been used for centuries by various cultures in the treatment of diseases. Another argument could possibly be that at very high concentrations, any compound is likely to inhibit the growth of microorganisms. Firstly, if this is the case, the high concentrations required would no doubt have serious side effects on the patient unfortunate enough to contract an illness. Secondly, these compounds are compared with those of standard antibiotics already available in the market. This means that the concentrations used must compare favourably to those that have already passed the test. A summary of useful anti-microbial phytochemicals is given in **Table 2-2** (Cowan, 1999).

Asiaticode, an anti-microbial compound isolated from *Centella asiatica* (used traditionally in skin diseases and leprosy), has been studied in normal as well as delayed-type wound healing. The results indicated significant wound healing in both models. Another compound, cryptolepine, isolated from *Crytolepis sanguinolenta* and active against *Campylobacter* species, has been used traditionally in Guinea Bissau in the treatment of hepatitis and in Ghana for the treatment of urinary and upper respiratory tract infections and malaria.

Table 2-2: Plants containing chemotherapeutic activity

Common name	Scientific name	Compounds	Class	Activity	RT[d]
Alfalfa	*Medicago sativa*	?		Gram-positive	2.3
All specie	*Pimenta dioica*	Eugenol	Essential oil	General	2.5
Aloe	*Aloe barbandensis, Aloe vera*	Latex	Complex mixture	Corynebacterium	2. 7
Apple	*Malus sylvestris*	?		Salmonella	
Ashawagandha	*Withania somnifera*	Phloretin, Withafarin A	Flavonoid derivatives	General Bacteria, fungi,*S. aureus*	3
Aveloz	*Euphorbia tirucalli*	?	Flavonoid derivatives	General	1
Bael tree	*Aegle mamelos*	Essential oils	Terpenoids	Fungi	
Balsam pear	*Mormordica charantia*	?	Terpenoids	General	1
Barberry	*Berberis vulgaris*	Beriberine	Alkaloids	Bacteria, Protozoa	2
Basil	*Ocimum basilicum*	Essential oils	Terpenoids	Salmonella, bacteria	2.5
Bay	*Laurus nobilis*	Essential oils	Terpenoids	Bacteria, fungi	0.7

Common name	Scientific name	Compounds	Class	Activity	RT[d]
Betel pepper	*Piper betel*	Catechols, eugenol	Essential oils	General	1
Black pepper	*Piper nigrum*	Alkaloid	Alkaloids	Fungi, lactosbacillus, E. coli	1
Blueberry	*Vaccinium spp.*	Fructose	Monosaccharide	E. coli	
Brazillian pepper	*Schenus terebinthithifolius*	Terebinthone	Terpenoids	General	
Buchu	*Barosma setulina*	Essential oils	Terpenoids	General	2
Burdock	*Articum lappa*	?	Polyacetylene, tannin	Bacteria, fungi, virus	1
Buttercup	*Ranunculus bulbosus*	Protoanemonin	Lactone	General	2
Caraway	*Carum carvi*	?	Coumarins polyphenols	Bacteria, fungi, virus	1
Cascara sagrada	*Rhamnus purshiana*	Tannins	Coumarins polyphenols	Bacteria, fungi, virus	
Cashew	*Anacardium pulsatilla*	Salicylic acids	Anthraquinone, polyphenols	P.acnes, bacteria, fungi	
Castor bean	*Ricinus communis*	?		General	0*
Chamomile	*Matricaria chamomilla*	Anthemic acid	Phenolics acid	*M. tuberculosis*, *S. typhimurius*	
Chapparal	*Larrea tridentate*	?	Coumarins	*S. aureus*, virus	2
Chili pepper	*Capsicum annuum*	Capsaicin	Terpenoids	Bacteria	2
Clove	*Syzigium aromaticum*	Eugenol	Terpenoids	General	1.7
Coca	*Erythroxylum coca*	Cocaine	Alkaloid	Gram-negative & positive cocci	1.7
Cockle	*Agrostemma githago*	?		General	1
Coltsfoot	*Tussilago farfarva*	?		General	2
Coriander, cilantro	*Coriandrum sativum*	?		Bacteria, fungi	
Cranberry	*Vaccinium spp*	Fructose	Monosaccharides	Bacteria	
Dandelion	*Taraxacum officinale*	?	?	C. Albicans	
Dill	*Anethum graveolens*	Essential oil	Terpenoid		
Echinacea	*Echinaceae angustifolia*	?		General	
Eucalyptus	*Eucalyptus globules*	Tannin	Polyphenol	Bacterial, virus	1.5
Fava bean	*Vicia faba*	Fabatin	Thionin	Bacteria	
Gamboge	*Garcinia hanbyryi*		Resin	General	0.5
Garlic	*Allium sativum*	Allicin, ajoene	Sulfoxide, sulphated		
Glory lily	*Gloriosa superba*	Colchicine	Alkaloid	General	0*
Goldenseal	*Hydrastis candensis*	Berberine, hydrastin	Alkaloid	Bacteria, Giadia duodenale	2
Gotu cola	*Centella asiatica*	Asiatocoside	Terpenoid	*M. leprae*	1.7
Grapefruit tea	*Citrus paradisa*		Terpenoid	Fungi	
Green tea	*Camellia sinensis*	Catechin	Flavonoids	General, shigella,	
Ginseng	*Panax notoginseng*		Saponins	Schenchi	2.7
Harmel, rue	*Peganum harmala*	?		Bacteria, fungi	1
Hemp	*Cannabis sativa*	Beta-resercyclic acid	Organic acid	Bacteria, virus	
Henna	*Lawsiana intermis*	Garlic acid	Phenolics	*S. aureus*	1.5
Hops	*Humulus lupulus*	Lupulone	Phenolic acid	General	3
Hyssop	*Hyssopus officinalis*		Terpenoid	Viruses	
(Japanese) herb	*Rabdosia trichocarpa*	Trichorabdal A	Terpene	*Helicobacter pylori*	
Lantana	*Lantana camara*	?		General	

Common name	Scientific name	Compounds	Class	Activity	RT[d]
Lavender-cotton	*Santolina*	?		Gram-positive bacte	
Legume	*Millettia thonningii*	Alpinumisoflav0ne	Flavones		
Lemon balm	*Melissa officinalis*	Tannins	Polyphenols	Viruses	
Lemon verbena	*Aloysia*	Essential oil	Terpenoid		1.5
Licorice	*Glycyrrhiza glabra*	Glabrol	Phenolic alcohol	*M tuberculosis*	
Officinalis	*Thevetia peruviana*	?		*S. aureus*	2
Mace, nutmeg	*Myristica fragrans*	?		General	1.5
Marigold	*Calendula*	?		Bacteria	2.5
Mesquite	*Prosopis juliflora*	?		General	1.5
Mountain tobacco	*Arnica montana*	Helamins	Lactones	General	2
Oak	*Quercus rubra*	Tannins	Polyphenol		
Olive oil	*Olea europaea*	Hexanal	Aldehyde	General	
Onion	*Allium cepa*	Allicin	Sulfoxide	Bacteria, *Candida*	
Oregon grape	*Mahonia aquifolia*	Berberine	Alkaloid		2
Pao d´arco	*Tabebuia*	Sesquiterpenes	Terpenoids	Fungi	1
Papaya	*Carica papaya*	Latex	Mix of terpenoids , alkaloid		
Pasque-flower	*Anemone pulsatila*	Anemonins	Lactone	Bacteria	0.5
Perpermint	*Mentha pipeerita*	Menthol	Terpenoids	Bacteria	
Periwinkle	*Vica minor*	Reserpine	Alkalods	General	1.5
Poppy	*Papaver somniferum*	Opium	Alkaloids, others	General	0.5
Potato	*Solanum tuberosum*	?		Bacteria, fungi	2
Quinine	*Chincona sp.*	Quinine	Alkaloids		2
Rosemary	*Rosemarinus officinalis*	Oil	Terpenoid	General	
Savory	*Satureja montana*	Carvacrol	Terpenoid	General	2
Thyme	*Thymus vulgaris*	Caffeic acids, thymol	Terpenoids, phenolic acid	Viruses, bacteria, fungi	
Tree bard	*Podocarpus nagi*	Totarol	Flavonoids	Gram-positive bacteria	
Yellow dock	*Rumex crispus*	?		*S.aureus, E. Coli, Samonella*	

[d] Relative toxicity: 0=no toxic activity. < 1= toxic, > 3= very toxic, **RT=** Relative Toxicity[d] . Adapted from (Cowan, 1999)

2.4 Plants as a potential source of antibiotics

The use of medicinal plants is widespread (Farnsworth, 1994). The production of medicines and the pharmacological treatment of diseases began with the use of herbs (Tyler, 1997). Life saving and essential drugs from medicinal plants such as morphine, digoxin, aspirin, emetine, and ephedrine were introduced into modern therapeutics several centuries ago. However, plants have been used as drugs for over millenia by human beings.

Other than for purposes of scientific inquiry, plants historically have served as models in drug development for three reasons: (a) Each plant is a unique chemical factory capable of synthesizing large numbers of highly complex and unusual chemical substances. In the United States of America alone, about 25% of prescription drugs contain active principles that are still extracted from higher plants and there is increasing popularity in the use of plant-derived preparations (Farnsworth and Morris, 1976). It has also been estimated by the World Health Organization (WHO) that about 80% of the population of the developing countries rely exclusively on plants to meet their health care needs (Farnsworth *et al*., 1985). (b) The biologically active substances derived from plants have served as templates for synthesis of pharmaceuticals. Such compounds may have poor pharmacological and toxicological profiles. (c) Many highly active secondary plant constituents have been instrumental as pharmacological tools to evaluate physiological processes (Farnsworth, 1984).

There are numerous illustrations of plant-derived drugs. Some selected examples are presented in **Table 2-3:** The isoquinoline alkaloid emetine obtained from the underground part of *Cephaelis ipecacuanha* and related species has been used for many years as an amoebicidal drug as well as for the treatment of abscesses resulting from *Escherichia histolytica* infections. Another important drug of plant origin with a longhistory of use is quinine. This alkaloid occurs in the bark of the cinchona tree. Apart from its usefulness in the treatment of malaria, it can be used to relieve nocturnal leg cramps (Iwu *et al*., 1999).

Similarly, higher plants have also played important roles in cancer therapies. Recent examples include, combretastatins from *Combretum caffrum* (Pettit and Shigh, 1987). In the last two decades a series of stilbenes and dihydrostilbenes (the combretastatins) with potent cytototoxic activity, and acidic triterpenoids and their glycosides with molluscicidal, antifungal, antimicrobial activity, have been isolated from species of *Combretum* (Rogers, 1989b). Other antineoplastic agents include taxol and several derivatives of camptothecin from *Taxus brevifolia* and *Camptotheca acuminate,* respectively.

Table 2-3: Some plant-derived preparations for medicinal use

Active compound	Origin	Application
Ephedrine		Bronchodilator
Egortamine	*Ergot spp*	Migraine remedy
Hyoscyamine		Anticholinergic
Ipratropium		Bronchodilator
Morphine	*Papaver somniferum*	Analgesic
Physostigmine	*Physostigma venenosum*	Cholinesterase inhibitor

Active compound	Origin	Application
Pilocarpine	*Pilocarpus jaborandi*	Glaucoma remedy
Quinidine	*Cinchona pubescens*	Anti arrhythmic
Quinine	*Cinchona pubescens*	Antimalarial
Reserpine	*Rauwolfia serpentine*	Antihypertensive
Salicin	Salix spp	Anti-inflammatory
Scopolamine	*Datura stramonium*	Antispasmodic
Sennoside A+ B	*Cassia angustifolia*	Laxative
Theophylline	*Camellia sinensis*	Bronchodilator
Vinblastine	*Catharantus roseus*	Antineoplastic

2.5 Plants and antibacterial production

An antibiotic has been defined as a chemical compound derived from or produced by living organisms, which is capable, in small concentrations of inhibiting the growth of micro-organisms (Evans, 1989). This definition limited antibiotics to substances produced by microorganisms but the definition could now be extended to include similar substances present in higher plants. Plants have many ways of generating antibacterial compounds to protect them against pathogens (Kuc, 1990). External plant surfaces are often protected by biopolymers e.g. waxes, and fatty acid esters such as cutin and suberin. In addition, external tissues can be rich in phenolic compounds, alkaloids, diterpenoids, steroid glycoalkaloids and other compounds, which inhibit the development of fungi and bacteria (Kuc, 1985). Cell walls of at least some monocotyledons also contain antimicrobial proteins, referred to as thionins (Carr and Klessig 1989).

Plant cells containing sequestered glycosides release them when ruptured by injury or infection. These glycosides may have antimicrobial activity against the invading pathogens or may be hydrolyzed by glycosidases to yield more active aglycones. In the case of phenolic compounds, these may be oxidized to highly reactive, antimicrobial quinones and free radicals (Kuc, 1985; Dean and Kuc, 1987). Thus, damage to a few cells may rapidly create an extremely hostile environment for a developing pathogen. This rapid, but restricted disruption of a few cells after infection can also result in the biosynthesis and accumulation of phytoalexins, which are low molecular weight anti-microbial compounds, which accumulate at sites of infection (Kuc, 1985; Carr and Klessig, 1989; Dean and Kuc, 1987). Some phytoalexins are synthesized by the malonate pathway others by the mevalonate, or shikimate pathways, whereas still others require participation of two or all three of the pathways. Phytoalexins are degraded by some pathogens and by the plant; thus they are transient constituents and their accumulation is a reflection of both synthesis and degradation rates.

Biopolymers are also often associated with the phytoalexin accumulation at the site of injury or infection. These biopolymers include: lignin, a polymer of oxidized phenolic compounds; callose, a polymer of β-1, 3-linked glucopyranose; hydroxyproline-rich glycoproteins, and suberin. They provide both mechanical and chemical restriction of development of pathogens (Kuc 1985; Carr and Klessig, 1989; Rao and Kuc, 1990).
The macromolecule produced after infection or certain forms of physiological stress includes enzymes, which can hydrolyse the walls of some pathogens including chitinases, β-1,3-glucanases and proteases (Carr and Klessig, 1989). Unlike the phyoalexins and structural biopolymers, the amounts of these enzymes increase systemically in infected plants even in response to localized infection. These enzymes are part of a group of stress or infection-related proteins commonly referred to as pathogenesis-related (PR) proteins. The function of many of these proteins is unknown. Some may be defense compounds while others may regulate the response to infection (Carr and Klessig, 1989; Boller, 1987; Rao and Kuc, 1990).

Another group of systemically produced biopolymer defense compounds comprises the peroxidases and phenoloxidases (Hammerschmidt *et al*., 1982; Rao and Kuc, 1990). Both can oxidize phenols to generate protective barriers to infection, including lignin. Phenolic oxidation products can also cross-link to carbohydrates and proteins in the cell walls of plants and fungi to restrict further microbial development (Stermer and Hammerschmidt, 1987). Peroxidases also generate hydrogen peroxide, which is strongly antimicrobial. Associated with peroxidative reactions after infection is the transient localized accumulation of hydroxyl radicals and super oxide anion, both of which are highly reactive and toxic to cells.

Plants therefore have several mechanisms to counter anti-microbial attack. Some of the anti-microbial compounds in plants may be exploited for use against bacterial diseases in man. Plants have developed an arsenal of weapons to survive attacks by microbial invasions. These include both physical barriers as well as chemical ones, i.e. the presence or accumulation of anti-microbial metabolites. These are either produced in the plant (prohibitins) or induced after infection, the so-called phytoalexins. Since phytoalexins can also be induced by abiotic factors such as UV irradiation, they have been defined as ‘antibiotics formed in plants via a metabolic sequence induced either biotically or in response to chemical or environmental factors (Grayer *et al.,* 1994).

When an infection or damage to a plant takes place, a number of processes are activated and some of the compounds produced become activated immediately whereas phytoalexins take two three days to be produced. Sometimes it is difficult to determine whether the compounds are phytoalexins or prohibitins and moreover, the same compound may be a preformed anti-microbial in one species and a phytoalexin in

another (Grayer *et al.,* 1994). Since the advent of antibiotics in 1950s, the use of plant derivatives as anti-microbials has been virtually non-existent but that pace is rapidly on the increase as we begin to realize the need for new and effective treatments. The worldwide spending on finding new anti-infective agents is expected to increase 60% as from 1993 and plant source are especially being investigated (Grayer *et al.,* 1994).

2.6 The Family Combretaceae

2.6.1 Taxonomy

The family Combretaceae belongs to the order Myrtales and consists of 600 species of trees and shrubs in 20 genera, which include *Anogeisssus, Bucida*, *Combretum, Quisqualis, Terminalia* and *Thiloa,* and are found throughout the tropics and sub-tropics. The largest genus is *Combretum*, with about 370 species, while *Terminalia* the second largest, and has about 200 species. They occur in most parts of Africa and are often the dominant vegetation (Rogers, 1996). The other genera are much smaller, including *Calopyxes* and *Buchenavia* which have 22 species each and *Quisqualis*, *Anogeissi*s, *Conocarypis*, and *Pteleopsis* each with 16, 14, 12 and 10 species, respectively (Rogers and Verotta, 1996). The sub generic classification for southern Africa *Combretaceae* according to Carr (1988) is indicated in **(Fig. 2-1).**

In general, the genus of *Combretum* has 4-5-winged, ridged, angled, sessile or stipitate fruit while *Terminalia* has 2-winged fruit. Hybridization is a common occurrence in both genera. This results in the formation of numerous subspecies that look, on visual inspection, quite different. For example C*. albopunctatum* and *C. apiculatum* are similar in many respects, and so are *C. psisiodes* and *C. molle*, and *T. mollis* as well as *T. stenostachya (*Carr, 1988).

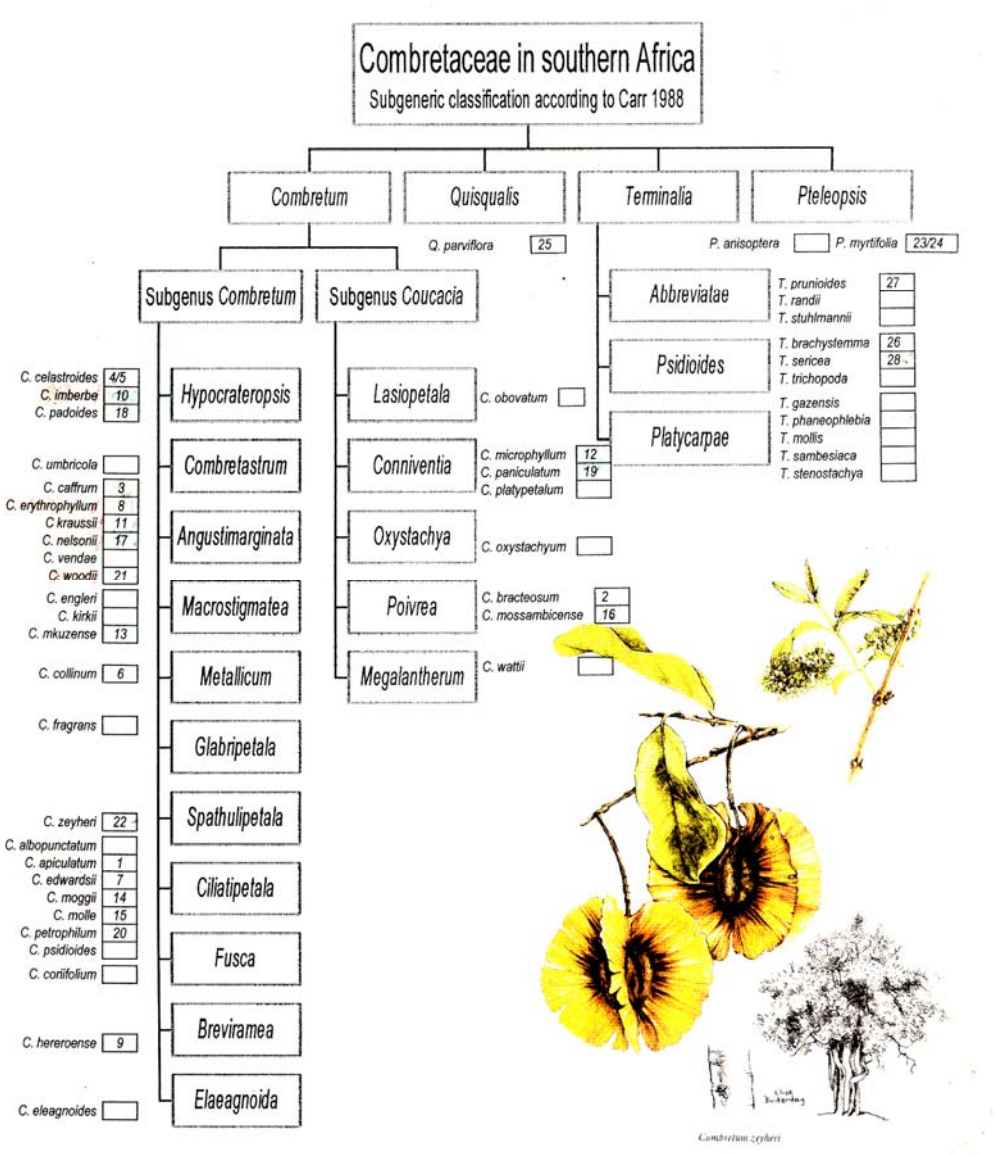


Figure 2-1: The sub generic classification for South Africa Combretaceae according to Carr (1988)

2.6.2 Taxonomy of the section Hypocrateropsis

The section Hypocrateropsis is made up of 4 species as described below:

2.6.2.1 Combretum imberbe Engl. & Diels (mutsviri) (Leadwood)

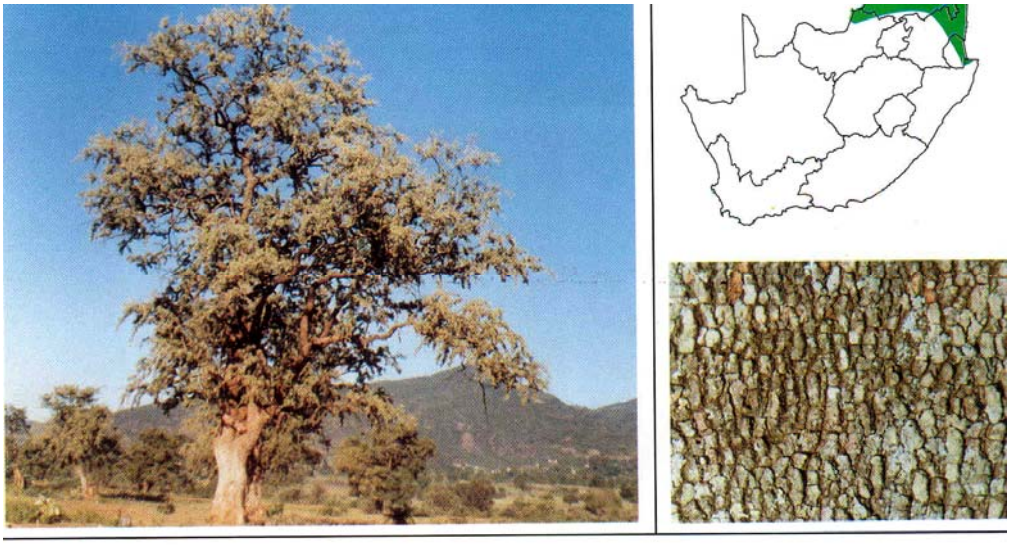


Figure 2-2: *C. imberbe* **Leadwood/hardekool (Steyn, 1994)**

Combretum imberbe **(Fig. 2-2)** is sometimes a shrub, but most frequently a small to large tree 7-15 m in height, with a grayish appearance. It occurs at medium to low altitudes, in mixed woodland and bushveld, often along rivers or dry watercourses, particularly on alluvial soils. **Bark**: Sometimes pale grey, smoothish and cracked in rectangular flakes, but mostly black and rough with characteristic deep longitudinal furrows and irregular transverse cracks, producing a mesh rather like crocodile skin. **Leaves:** occurs on short, opposite, often spine-tipped twigs; small, obovate to oblong, 2, 5-8x1-3 cm, usually 4 x 2 cm, grey-green, hairless, with silvery, microscopic scales densely covering both surfaces, thinly textured to rather leathery, 4-7 pairs of lateral veins visible on both surfaces; apex broadly tapering to round, often with a fine hair-like tip; base broadly to narrowing tapering; and petiole 4-10 mm long. **Flowers:** Cream to yellow, sweetly scented, in rather slender spikes 4-8 cm long, in the axils of the leaves or sometimes forming a terminal head, or panicle (Nov.-Mar.). **Fruits:** 4-winged, seldom exceeding 1,5 x 1,5 cm, somewhat round or often D-shaped in outline, apical peg distinct, densely covered with silvery scales, characteristically pale yellow drying to pale straw-coloured, giving the tree a distinct appearance (Feb. onward).

The heartwood is dark and extremely hard, heavy and durable. It is difficult to work and rapidly blunts and breaks tools, so it is not suitable for furniture; however, it bunts well and has been used for ornaments. It makes good fencing standards, railway sleepers and mine props. The wood is so hard that blades for hoes

were made from it before metal became available. The wood burns slowly; the ash has high lime content and has been used as toothpaste and is also suitable for whitewash (Carr, 1988)

2.6.2.2 Combretum padoides Engl. & Diel

Combretum padoides **(Fig. 2-3)** is a shrub, often thicket-forming, using its long trailing branches to scramble into adjacent trees, or a small tree 3-5 m in height; occurring at low altitudes in hot dry areas, in riverine fringes, on rocky hills and along escarpments in mixed woodland and bushedveld. **Bark**: light brownish grey, smoothish, flaking. Leaves: elliptic to narrowly so, 3-10 x 3-4, 5 cm, thinly textured, dull green, 6-8 pairs of lateral veins, yellowish and conspicuous with hairs along the veins below, net veining indistinct, with scales on the undersurface giving the appearance of skin that have been stippled or dotted with a white ballpoint pen; apex tapering to a point; base tapering to round; margin entire, wavy; petiole slender, up to 10 mm long**. Flowers**: creaming yellow, disc in the center hairy, in simple or branched, axillary or terminal, rather loose spikes up to 10 cm long, often in profusion (Dec.-Feb). Fruits: 4-winged, 1-1,5 cm long, pale yellow green drying to light brown, on very slender stalks about 3 mm long, often in profusion (Mar. -Jun).

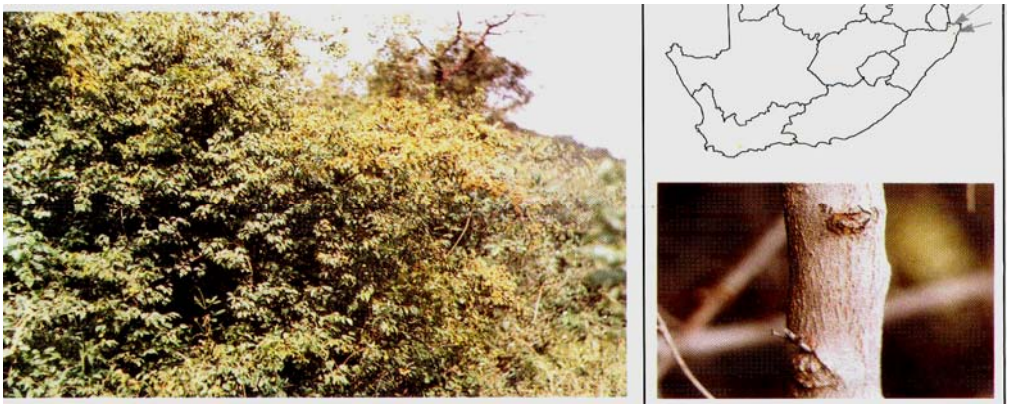


Figure 2-3: *C. padoides* **(Steyn, 1997)**

2.6.2.3 Combretum celastroides Welw Ex M.A. Lawson

A dense, straggling shrub about 4 m in height, or a tree up to 7 m; occurring in dry wood land, on rocky hillsides, frequently on Kalahari sand. **Back**: creamy brown or grey with patches of lichen; rather smooth. **Leaves:** elliptic to broadly obovate, 2,5-14 x 1-8 cm, but usually about 4-5 cm long, thinly leathery, green, becoming beautiful deep red to plum-coloured in autumn, upper surface hairless with small dots visible against the light, undersurface with or without hairs (depending on the subspecies) but with scales that give

it the appearance of skin stippled or dotted with white boil point pen, 4-6 pairs with hair-tuft domatia-veining indistinct; apex bluntly acuminate; base tapering to rounded; margin entire; petiole up to 8 mm long. **Flowers:** greenish to yellow, disc in the center hairless, glossy; in rather sparse axillary spikes, usually about 5-8 cm long but sometimes reaching 12 cm (Dec.-Mar.). **Fruit:** 4-winged, 1-2 cm long, the wings becoming bright red while the body of the fruit remains greening yellow, drying to golden brown with satiny sheen, with a distinct apical peg.

Ssp. *celastroides*: Occurring in Kalahari wood land and jesse-bush in Zimbabwe and extending into Botswana and the more arids area in Namibia; leaves usually 5-10 cm long with a finely velvety undersurface; flowers large, the disc measuring up to 4 mm diameter.

Ssp. *orientale* Excell **(Fig. 2-4)**: Usually occurring on sand or silt at 50-500 m in coastal sand forest, lowveld savanna in the northern Limpopo and adjacent areas in Zimbabwe and Mozambique, and also in northern Kwazulu natal; Leaves about 5 x 2 cm, almost hairless except for hair-tuft domatia on the under surface; Flowers smaller, the disc measuring 2-2,5 mm in diameter. This ssp. is rather similar to *C. padoides*, but the later has a different habit, often with long trailing branches scrambling into adjacent trees, the fruits are smaller with apical peg very short to absent, and the disc is hairy.

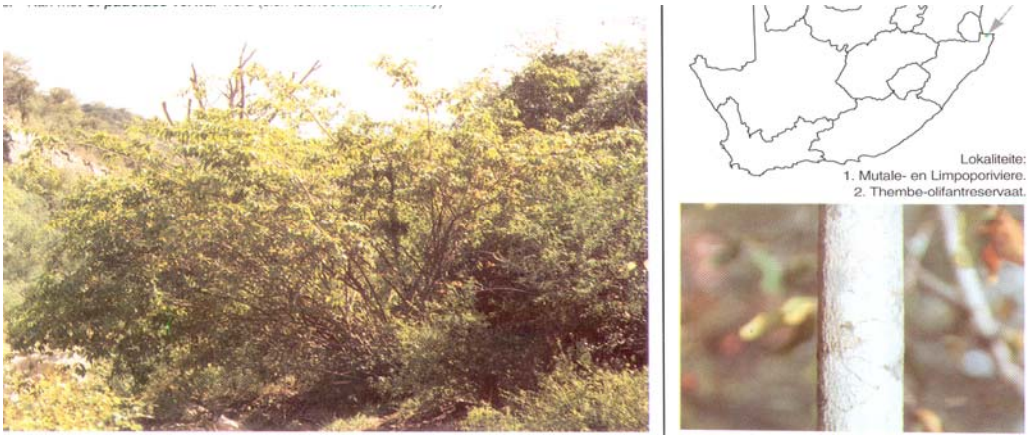


Figure 2-4: *C. celastroides* **ssp.** *orientale* **(Steyn, 1994)**

2.7 Ethnopharmacology of Combreteceae

Species of Combretaceae contain compounds with potential antimicrobial properties (Eloff, 1999a).

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

Combretum species occurring in southern Africa are used for many medicinal purposes. These include treating abdominal disorders (eg abdominal pains, diarrhea) backache, bilharziasis, chest coughs, colds, conjunctivitis, dysmenorrhoea, earache, fattening babies, fever, headache, hookworm, infertility in women, leprosy, pneumonia, scorpion and snake bite, swelling caused by mumps, syphilis, toothache and general weakness (Hutchings *et al*., 1996).

The ethnopharmacological use of *C. zeyheri* against diarrhoea and eye infections has been ascribed to its antibacterial activity towards Gram- positive microbes (Breytenbach and Malan, 1989).
C. erythrophyllum has been shown to possess many antibacterial compounds and some of these had activities higher than chloramphenicol and ampicillin (Martini and Eloff, 1998). Eloff (1999a) also found that all the leaf extracts from 27 Southern African members of the Combretaceae including *C. woodii* exhibited antibacterial activity against *S. aureus, E. coli, E. feacalis and P. aeruginosa*. The leaves of *C. molle* and *C. imberbe* have been shown to have molluscicidal activity against *Biomphalaria glabrata* snails (Rogers and Verotta, 1996).

2.8 Phytochemistry/chemistry and biological activity of Combretaceae

Phytochemistry, or plant chemistry, has developed in recent years as a distinct discipline, somewhere in between natural product organic chemistry and plant biochemistry and is closely related to both. It is concerned with the enormous variety of organic substances that are elaborated and accumulated by plants and deals with the chemical structures of these substances, their biosynthesis, turnover and metabolism, distribution and their biological functions. The range and number of discrete molecular structures produced by plants is huge and such is the present rate of advancement of our knowledge of them that a major problem in phytochemical research is the collection of existing data on each particular class of compound. Because the number of known substances is so large, this section deals with a summary of the structural variation existing within each class of compound, outlining those compounds that are commonly occurring and illustrating the chemical variation within representative formulae. Classification is based on the presence of certain functional groups. Phytochemical work on *Combretum* and *Terminalia* start since the

early 1970s and extends well into the present. This section also outlines the phytochemical constituents that have been isolated in detail and considers the sources. The biological activities of some of these constituents are indicated as well.

2.8.1 Tannins

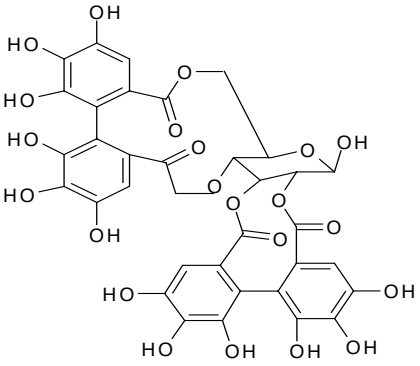
Plant polyphenols (vegetable tannins) are secondary metabolites widely distributed in the plant kingdom. They are based upon two broad structural themes: Hydrolysable tannins (HT) and Proanthocyanidins (PA) (often called condensed tannins)

2.8.1.1 Classes of tannins

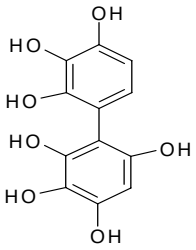
2.8.1.1.1 Hydrolysable tannins (HTs)

Hydrolysable tannins are molecules with a polyol (generally D-glucose) as a central core. The hydroxyl groups of these carbohydrates are partially or totally esterified with phenolic groups like gallic acid (gallotannins) or ellagic acid (ellagitannins). Hydrolysable tannins are usually present in low amounts in plants. Some authors define two additional classes of hydrolysable tannins: taragallotannins (gallic acid and quinic acid as the core) and caffetannins (caffeic acid and quinic acid**)**

Gallotannins: The phenolic groups that esterify with the core are sometimes constituted by dimers or higher oligomers of gallic acid (each single monomer is called galloyl). Each HT molecule is usually composed of a core of D-glucose and 6 to 9 galloyl groups. In nature, there is an abundance of mono and di-galloyl esters of glucose (MW about 900) that are not considered to be tannins. At least 3 hydroxyl groups of the glucose must be esterified to exhibit a sufficiently strong binding capacity to be classified as tannin. The most famous source of gallotannins is tannic acid obtained from the twig galls of *Rhus semialata*. It has a penta galloyl-D-glucose core and five more units of galloyl linked to one of the galloyl of the core (Harbone, 1994).

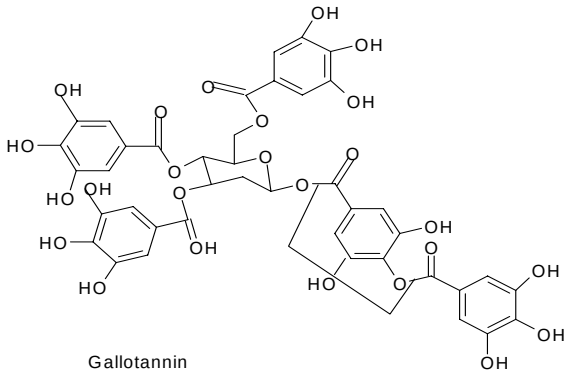


Ellagitannin

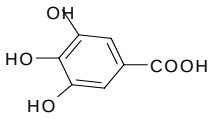


Hexahydroxydiphenic acid

Ellagitannins: The phenolic groups consist of hexahydroxydiphenic acid, which spontaneously dehydrates to the lactone form, ellagic acid. Molecular weight range: 2000-5000.



Gallotannin



Gallic acid

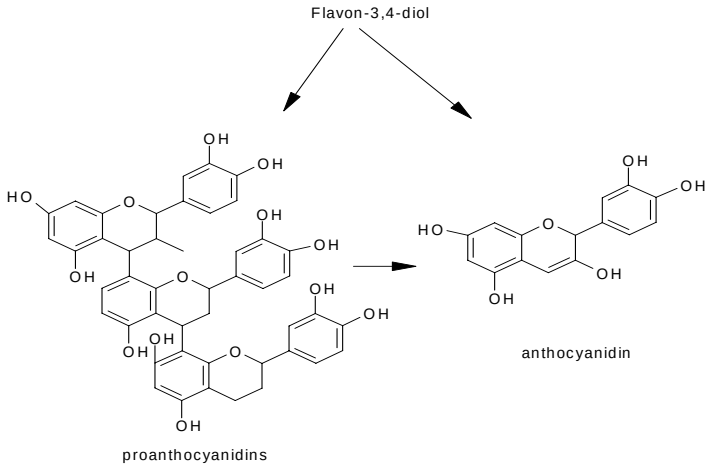
Hydrolysed by mild acids or mild bases to yield carbohydrate and phenolic acids. Under the same conditions, proanthocyanidins (condensed tannins) do not hydrolyze. Hydrolysable tannins are also hydrolyzed by hot water or enzymes (i.e. tannase).

2.8.1.1.2 Proanthocyanidins (condensed tannins)

Proanthocyanidins are more widely distributed than HTs. They are oligomers or polymers of flavonoid units (i.e. flavan-3-ol) linked by carbon-carbon bonds not susceptible to cleavage by hydrolysis. Proanthocyanidins are more often called condensed tannins due to their condensed chemical structure.

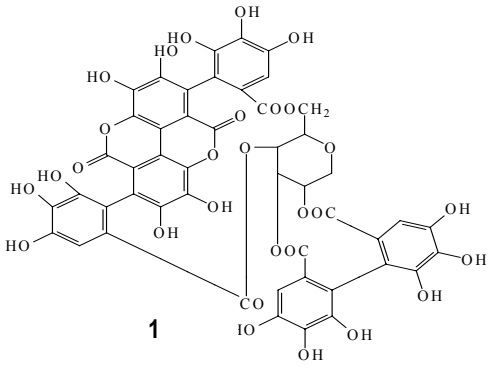
However, HTs also undergo condensation reaction. The term, condensed tannins, is therefore potentially confusing. The term, proanthocyanidins, is derived from the acid catalyzed oxidation reaction that produces red anthocyanidins upon heating PAs in acidic alcohol solutions. The most common anthocyanidins produced are cyanidin (flavan-3-ol, from procyanidin) and delphinidin (from prodelphinidin). Proanthocyanidins may contain from 2 to 50 or greater flavonoid units; PA polymers have complex structures because the flavonoid units can differ for some substituent and because of the variable sites for interflavan bonds. Anthocyanidin pigments are responsible for the wide array of pink, scarlet, red, mauve, violet, and blue colors in flowers, leaves, fruits, fruit juices, and wines. Proanthocyanidin is the preferred name for condensed tannins (or flavolans), a series of flavan-3-ol oligomers that are usually based on a C-C link from the 8-position of one flavan unit to the 4-position of a second unit. As with the monomeric leucoanthocyanidins, they produce colored anthocyanidins on heating with mineral acid, but they have the additional property of binding to protein. The best-known proanthocyanidins are procyanidins, based on catechin and/or epicatechin units, and oligomers up to the hexamer have now been found in plants.

Two common procyanidin dimers are described as epicatechin- (4, 8)-catechin and *ent*- epicatechin- (4, 8)-epicatechin respectively. A considerable number of doubly linked proanthocyanidins are known, where there is a second linkage through C-2 to O-7. The naming of such compounds can be accommodated in the same general way, e.g. one such compound is epicatechin- (2, 7, 4, 8)-epicatechin. Many Oligomeric proanthocyanidins with molecular sizes greater than the hexamer have been isolated from plants but their stereochemistries have yet to be determined (Harbone, 1994).



2.8.2 Tannins chemistry of Combretaceae

Gallic acid and its derivatives are commonly occurring constituents of the Combretaceae. A number of elaborate tannins have been isolated mainly from *Terminalia* e.g. the diphenoyl-gallagylglucose (**1**) isolated from *T. oblongata* and an ellagitannin from the leaf of *T. calamansanai* as well as derivatives from *T. catappa* and *T. chebula* ((Pettit *et al*., 1996).

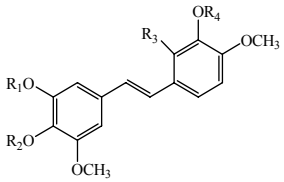


1

These compounds as refered to by Keterere, 2001, have been shown to have anti-tumour activity in numerous studies. However the pharmacological activity induced by tannins (with the recent exception of ellagitannins) is, in general, non-specific and thus of little clinical potential (Pettit *et al.*, 1996).

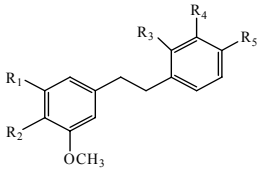
2.8.3 Stilbenoids

These compounds, which are biogenetically related, have generated immense interest because of their biological potency and structural simplicity. They were first isolated from *Combretum* species by Letcher and Nhamo (1973). Later in the 1980, Pettit and colleagues isolated stilbenes from a methylene chloride–methanol extract of the leaves, fruit and stemwood of *C. caffrum* (Cape bush willow tree) (Pettit *et al.,* 1982). They called these compounds combretastatins (2)-(25) which have since been isolated from *C. kraussii, C. molle, C. psidiodes* and *C. apiculatum* (Malan *et a.l*, 1993). (See **4** and **Table 2).** Combrestatins have been designated as A, B, C and D according to their chemical structures. Combretastatins A and B are almost identical but differ in that the former has an ethylene bridge joining the two benzyl groups and is chemically identified as a stilbene. The latter, meanwhile, has an ethane-type bond and is based on the dihydrostilbenes (also called bibenzyls).



(2)- (6)

Primary structure of Combretastatins A



(7)-(25)

Primary structure of Combretastatins B

The work by Letcher and Nhamo (1973) as refered to by Keterere, 2001 was mainly concerned with the chemical analysis (including organic synthesis) of constituents of *C. apiculatum, C. psidiodes, C. molle.* Letcher and Nhamo (1973) also identified several unknown substituted phenanthrenes and 9, 10-dihydrophenanthrenes (26)-(44) (See **Table 2-6**) in addition to isolating a few stilbenes. Some of these compounds have since been also isolated from related species (Malan and Swinny, 1993). Combretastatin B5 has been isolated as the main antibacterial compound present in high concentration in leaves of *Combretum woodii* (Eloff *et al.,* 2005).

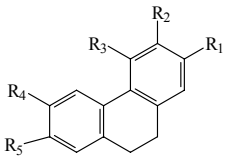
Table 2-4: Derivatives of stilbenes (Combrestatins) isolated from Combretaceae

	R_1	R_2	R_3	R_4		Plant source
2	CH_3	CH_3	OH	H	C-A1	*C. caffrum C. kraussii*
3	$-CH_2-$	$-CH_2-$	OH	H	C-A2	*C. caffrum*
4	H	CH_3	H	OH	C-A3	*C. caffrum*
5	H	CH_3	H	CH_3	C-A6	*C. caffrum*
6	CH_3	CH_3	OH	OGlc		*C. kraussii*

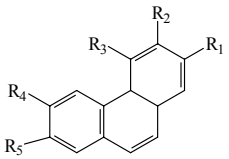
Table 2-5: Derivatives of dihydrostilbenes (Combrestatins) isolated from Combretaceae

	R_1	R_2	R_3	R_4	R_5		Plant
7	OCH_3	OCH3	OH	OH	CH_3	C-B1	*C. caffrum C. kraussii*
8	$O-CH_2-O$	$O-CH_2-O$	OH	OH	CH_3	C-B2	C. caffrum
9	OCH_3	OCH_3	H	OH	H	C-B3	*C. caffrum*
10	OCH_3	H	H	OH	H	C-B4	*C. caffrum*
11	OCH_3	H	H	OH	CH_3		*C. caffrum*
12	OCH_3	H	H	H	H		*C. caffrum*
13	OCH_3	OCH_3	H	H	H		*C. psidiodes, C. caffrum*

	R_1	R_2	R_3	R_4	R_5		Plant
14	OH	H	-	H	H		*C. apiculatum*
15	OH	H	H	OH	CH_3		C. apiculatum
16	OH	OCH_3	H	OCH_3	H		*C .apiculatum*
17	OH	OH	H	OCH_3	CH_3		*C. apiculatum*
18	OH	OCH_3	O-Glc	OH	H		*C. apiculatum*
19	OH	OH	OH	H	CH_3		*C. apiculatum*
20	OH	OCH_3	-Glc	H	H		*C. apiculatum C. molle*
21	OCH_3	OH	H	H	H		*C. apiculatum,C.psidiodes*
22	OCH_3	OCH_3	CH_3	OH	O-Glc		*C. kraussi*
23	OCH_3	OH	CH_3	OH	OH	C-B5	*C. kraussi*
24	OCH_3	OCH_3	CH_3	OH	O-Glc		*C. kraussi*
25	OCH_3	OH	CH_3	OH	O-Glc		*C. kraussi*



26-44



26-44

Table 2-6: Derivatives of phenanthrenes isolated from Combretaceae

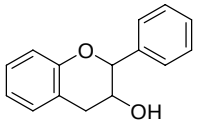
	R_1	R_2	R_3	R_4	R_5	Plant
26	OCH_3	OCH_3	OH	OH	OH	*C. apiculatum*
27	OH	OCH_3	OCH_3	OH	OCH_3	*C. apiculatum*
28	OCH_3	OCH_3	OH	OCH_3	OH	*C. apiculatum, C. molle*
29	OCH_3	OCH_3	OCH_3	OCH_3	OH	*C. caffrum*
30	OH	OH	OCH_3	OCH_3	OH	*C. apiculatum*
31	OCH_3	OH	OCH_3	OH	OH	*C. apiculatum*
32	OCH_3	OH	OCH_3	OCH_3	OH	*C. apiculatum*
33	OCH_3	OH	OCH_3	OCH_3	OCH_3	*C. apiculatum*
34	OCH_3	H	OH	OCH_3	OH	*C. apiculatum*
35	OCH_3	OCH_3	OH	OCH_3	OH	*C. apiculatum, C. molle*
36	OH	OCH_3	OCH_3	OH	OCH_3	*C. apiculatum C. molle*
37	OCH_3	OCH_3	OH	OH	OH	*C. apiculatum,C. molle*
38	OH	OCH_3	OCH_3	OH	OCH_3	*C. apiculatum, C. molle*
39	OCH_3	OCH_3	OH	OH	OH	*C. apiculatum, C. molle*
40	OCH_3	OCH_3	OCH_3	OH	OH	*C. apiculatum,C. caffrum,*

	R_1	R_2	R_3	R_4	R_5	Plant
41	OH	OCH_3	OCH_3	OCH_3	OCH_3	*C. caffrum*
42	OH	OCH_3	OCH_3	OCH_3	OH	*C. caffrum,C. apiculatum, C. psidiodes*
43	OCH_3	OCH_3	OCH_3	OCH_3	OH	*C.apiculatum, C. psidiodes, C. caffrum*
44	OCH_3	OH	OCH_3	OCH_3	OCH_3	*C. apiculatum*

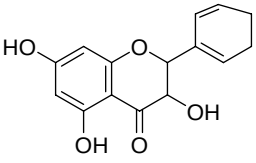
2.8.4 Flavonoids

Flavonoids are a group of polyphenolic compounds, which are widely distributed through out the plant kingdom. To date about 3000 varieties of flovonoidsare known. Many have low toxicity in mammals and some of them are widely used in medicine for maintenance of capillary integrity. Flavonoids exhibit several biological effects such as antiinflammatory, antihepatotoxic and anti-ulcer actions. They also inhibit enzymes such as aldose reductase and xanthineoxidase. They are potent antioxidants and have free radical scavenging abilities. Many have antiallergic, antiviral actions and some of them provide protection against cardiovascular mortality. They have been shown to inhibit the growth of variouscancer cell lines *in vitro*, and reduce tumour developmentin experimental animals (Narayana, *et al*., 2000)

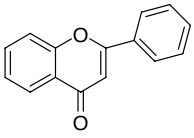
Structure and classification of flavonoids: Flavonoids occur as aglycones, glycosides and methylated derivatives. The flavonoid aglycone consists of a benzene ring (A) condensed with a sixmemberedring (C), which in the 2-position carries a phenyl ring (B) as a substituent. Six-memberring condensed with the benzene ring is either aa-pyrone (flavonols and flavonones) or its dihydroderivative (flavanols and flavanones). The position of the benzenoid substituent divides theflavonoid class into flavonoids (2-position) and isoflavonoids (3-position). Flavonols differ from flavonones by hydroxyl group the 3-position and aC2-C3 double bonds. Flavonoids are often hydroxylatedin position 3,5,7,2', 3', 4', 5'. Methyl ethers and acetyl esters of the alcohol group are known to occur in nature. When glycosides are formed, the glycosidic linkage is normally located in positions 3 or 7 and the carbohydrate can be L-rhamnose, D-glucose, glucor-hamnose, galactose or arabinose. The most common flavonoids are listed in **Table 2-7.**



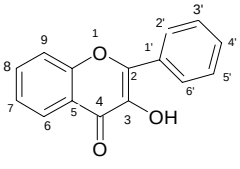
Flavan-3-ol



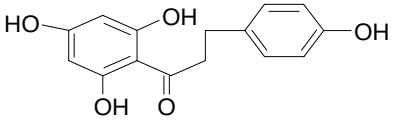
Favanolols



Flavone



Flavonol



Chalchone

Table 2-7; Nomenclature of the subclasses of favanoids (Narayana *et al.,* 2000),

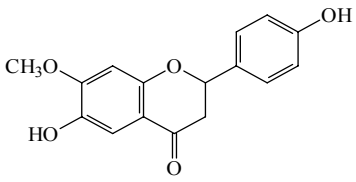
	3	5	7	2'	3'	4'	5'
Flavonols:							
Kaempferol	OH	OH	OH	H	H	OH	H
Morin	OH	OH	OH	OH	H	OH	H
Rutin	O-R¹	OH	OH	H	OH	OH	H
Myricetin	OH	OH	OH	H	OH	OH	OH
Quercetin	OH	OH	OH	H	OH	OH	H
Quercetrin	O-Rh	OH	OH	H	OH	OH	H
Myricitrin	O-Rh	OH	OH	H	OH	OH	OH
Spirenoside	OH	OH	OH	H	OH	O-Glu	H
Galangin	OH	OH	OH	H	H	H	H
Robinin	O-R¹	OH	OH	H	H	OH	H
Kaempferide	OH	OH	OH	H	H	O-Me	H
Fisetin	OH	H	OH	H	OH	OH	H
Rhamnetin	OH	OH	O-Me	H	OH	OH	H
Flavonones:							
Hesperitin	H	OH	OH	H	OH	O-Me	H
Naringin	H	OH	O-R	H	H	OH	H
Naringenin	H	OH	OH	H	H	OH	H
Eriodictyol	H	OH	OH	H	OH	OH	H
Hesperidin	H	OH	O-Me	H	OH	O-Me	H
Pinocembrin	H	OH	OH	H	H	H	H
Likvirtin	H	H	OH	H	H	O-Glu H	H
Flavones:							
Rpoifolin	H	OH	O-R	H	H	OH	H
Apigenin	H	OH	OH	H	H	OH	H
Tangeretin	H	O-Me	O-Me	H	H	O-Me	H
Flavone	H	H	H	H	H	H	H
Baicalein	H	OH	OH	H	H	H	H
Luteolin	H	OH	OH	H	OH	OH	H
Chrysin	H	OH	OH	H	H	H	H
Techtochrysin	H	OH	O-Me	H	H	H	H
Diosmetin	H	OH	OH	H	OH	O-Me	H
Diosmin	H	OH	O-R¹	H	OH	O-Me	H
Flavanolols:							
Silibinin	OH	OH	OH	H	H	O-L-O -	H
Silymarin	OH	OH	OH	H	H	O-L-O -	H
Taxifolin	OH	OH	OH	H	OH	OH	H
Pinobanksin	OH	OH	OH	H	H	H	H
Flavan-3-o1s:							
Catechin	OH	OH	OH	H	OH	OH	H
Isoflavones:							
Genistein	-	OH	OH	H	H	OH	H
Daidzin	-	H	O-Glu	H	H	OH	H

-O-Me = Methoxy -O-Glu = Glucosyl -O-R' = Alkoxy -O-L-O = Selane

2.8.4.1 Flavonoids chemistry of Combretaceae

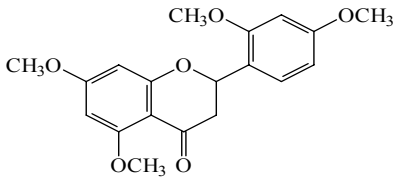
The flavonoids, luteolin, has been isolated from *Terminalia arjuna* and shown to be active against a murine P388 lymphocytic leukemia and human cancer cell line as well as inhibiting the growth of *Neisseria gonorrhoeae* (Pettit *et al*, 1996). Luteolin has been shown to be both an anti-tumour promotor and mutagen.

Arjunolone (1) and arjunone (2), two flavanones, have also been isolated from *T. arjuna* which is widely used in India and surrounding countries for many medical conditions arising from cardiovascular insufficiency.



(1)

Arjunolone



(2)

Arjunon

A study of the leaves of the South American plant, *C. leprosum* showed two flavonoids, 3-O-methylquercertin and 3-O-α-L-rhamnopyranosylquercetrin (quercetrin). Seven antibacterial flavonoids were subsequently isolated by bioassay-guided fractionation, i.e. apigenin; genkwanin; 5-hydroxy-7, 4-dimethoxyflavone, rhamnocitrin; kaempferol; quercetin-5, 3-dimethylether; rhamnazin from *C. erythrophyllum* (Martini *et al*., 2004)

2.8.5 Terpenoids

Terpenoids are all based on the isoprene molecule CH_2=C (CH_3)-CH=CH_2 and their carbon skeletons are built up from the union of two or more of these C_5 units. They are then classified according to whether they contain two (C_{10}), three (C_{15}), four (C_{20}), six (C_{30}) or eight (C_{40}) such units. They range from the essential oil components, volatile mono- and sesquiterpenes (C10 and C15) through to the less volatile diterpenes (C_{20}) to the involatile triterpenoids and steroids (C_{30}) and carotenoids pigments (C_{40}). Chemically, terpenoids are generally lipid soluble and are located in the cytoplasm of the plant cell. Essential oils sometimes occur in special granular cells on the leaf surface, whilst carotenoids are especially associated with the chloroplast. Terpenoids are normally extracted from plant tissue with light petroleum ether or chloroform.

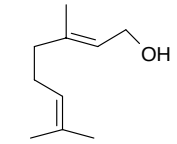
A considerable number of different functions have been ascribed to plant terpenoids. Their growth regulating properties are very well documented; two of the major classes of growth regulator are the sesquiterpenoids, abscisins and the diterpenoid-based gibberellins. The important contribution of carotenoids to plant colour is well known and it is almost certain that these C_{40} terpenoids are also involved as accessory pigments in photosynthesis. The mono- and sesquiterpenes provide plants with their distinctive smell. Also, certain nonvolatile terpenoids have been implicated as sex hormones among the fungi (Harbone, 1994).

2.8.5.1 *Classes of terpenes*

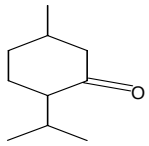
2.8.5.1.1 *Terpene essential oils*

Chemically, this group of terpenes can be divided into classes; the mono- and the sesquiterpenes. The monoterpenes can further be divided into three groups depending on whether they are acyclic (geraniol), monocyclic (limonene) or have functional groups alcohol (menthol), aldehyde or ketone.

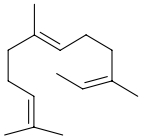
Like the monoterpenes, the sesquitepenes fall chemically into groups according to the basic carbon skeleton; the common ones are acyclic (farnesol), monocyclic (y-bisabolene) or bicyclic (beta-selinene, carotol).



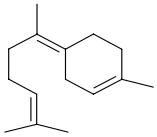
Geraniol
(Acyclic)



Menthone
(monocyclic)



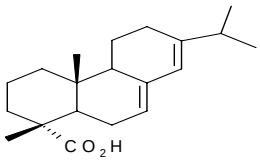
Farnesol



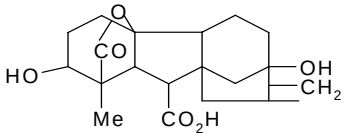
y-bisabolene

2.8.5.1.2 Diterpenoids and Gibberellins

Diterpenoids comprise a chemically heterogenous group of compounds, all with a carbon skeleton based on four isoprene units; most have a very limited distribution. Many classes of diterpenes are known, among them resin diterpenes, toxic diterpenes and gibberellins. The resin compounds are protective in nature and are exuded from wood trees or latex of plants. The toxic diterpenes occur in the foliage of the leaves of some plants and are responsible for the poisonous nature of the foliage. The gibberellins are a group of hormones, which generally stimulate growth and are known to be widespread in plants (Goodwin, 1981).



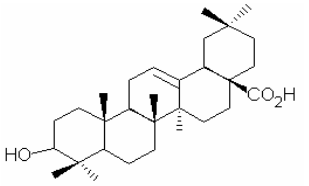
Abietic acid (resin)



Gibberellic acid

2.8.5.1.3 Triterpenoids and Steroids

Triterpenoids are compounds with a carbon skeleton based on six isoprene units. They are relatively complex cyclic structures, most being either alcohols, aldehydes or carboxylic acids. They are colourless, crystalline, often with a high melting point, optically active substances that are generally difficult to characterize because of their lack of chemical reactivity. Triterpenoids can be divided into at least four groups of compounds: true terpenes, steroids, saponins and cardiac glycosides. The latter two groups are terpenes or steroids that occur mainly as glycosides. Many triterpenes are known in plants and new ones are regularly being discovered and characterized. So far only a few are known to be widely distributed. This is true of the pentacyclic triterpenes alpha and beta-amyrin and the derived acids, ursolic and oleanic acids. These and related compounds occur especially in the waxy coating of leaves and fruits such as apple and pear have a protective function of repelling insect and microbial attack. Steroids are triterpenes, which are based on the cyclopentane perhydrophenanthrene ring system. At one time, steroids were considered to be mainly animal substances (sex hormones) but in recent years such compounds have been detected in plant tissues e.g stigmasterol is a phytosterol. Saponins are glycosides of both triterpenes and steroids and have been detected in several families of plants. They are surface-active agents with soap-like properties and can be detected by their ability to cause foaming and to haemolyse blood cells. The glycoside patterns of the saponins are often complex; many have as many as five sugar units attached and glucuronic acid is a common component (Goodwin, 1981).



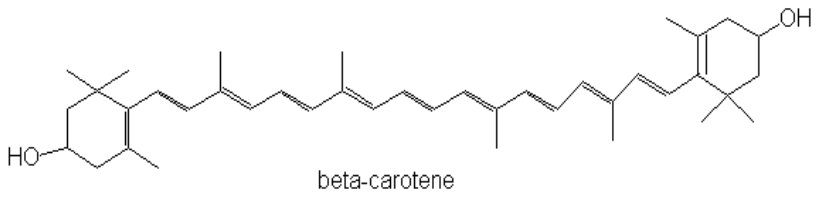
Oleanic acid

Stigmasterol

2.8.5.1.4 Tetraterpenoids (Carotenoids)

Carotenoids that are C40 tetraterpenoids are a widely distributed group of lipid-soluble pigments, found in all kinds of plants. In plants, carotenoids have two principal functions: as accessory pigments in photosynthesis and as coloring matters in flowers and fruits. In flowers, they mostly occur as yellow colours, while in fruits, they may, in addition, be orange or red. Well-known carotenoids are either simple unsaturated hydrocarbon based on lycopene or their oxygenated derivatives known as as xanthophylls. The chemical structure of lycopene consists of a long chain of eight isoprene units joined head to tail to form a completely conjugated system of alternate double bonds which is the chromophore giving it colour. Cyclization of lycopene at one

end gives y-carotene while cyclization at both ends provides the bicyclic hydrocarbon beta-carotene. Beta-carotene is the most common of all these pigments.



beta-carotene

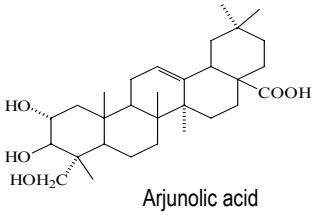
2.8.5.2 Terpenoids chemistry of Combretaceae

Triterpenoids are the most ubiquitous non-steroidal secondary metabolites in terrestrial and marine flora and fauna (Mahato and Nandy, 1991). Their medicinal use is rather limited but there remains some potential for application.

The Combretaceae as refered to by Keterere, 2001, has yielded a number of different pentacyclic triterpenoid structures, including oleanoic and ursanoic acids, friedelins, cycloartanes and dammaranes. The fruit of *C. molle*, when extracted with acetone, yielded arjunolic acid (**1**) arjungenin (**2**) and arjunglucoside (**3**), pentacylic triterpenoids previously found in *Terminalia arjuna (*Panzini, 1993).

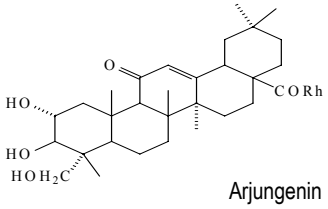
The ether extract of *C. imberbe leaves* has yielded a complex mix of triterpenoids and their glycosides (Roger, 1988). Rogers and Subramony (1988) isolated friedelin, epifriedelin and betulinic

acid from the bark of *C. imberbe* and an oleanene-based pentacyclic triterpene with its glycosides from the leaves (**4**)-(**5**).



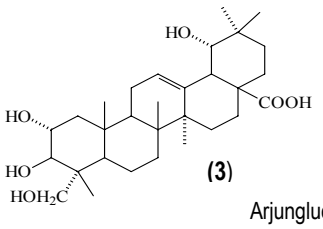
Arjunolic acid

(**1**)



Arjungenin

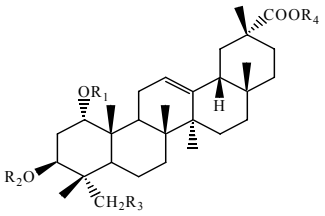
(**2**)



(**3**)

Arjunglucoside

The latter was characterized as 1α, 3β-dihydroxyolean-12-en-29-oic acid, and named imberbic acid.



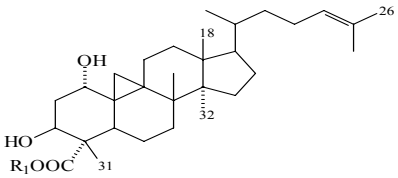
Compound	R 1	R 2	R 3	R 4
4	H	H	O-Rh	H
5	Ac	H	O-Rh	H

23-hydroxyimberbic acid 23-O-α-L-rhamnopyranoside (**4**)

23-hydroxyimberbic acid 23-O-α-L-rhamnopyranoside-1-acetate (**5**)

The ether extract of *C. edwardsii* leaves yielded the xyloside and arabinoside of mollic acid, previously isolated from *C. molle* and also reported to be present in a South American species *C. leprosum* (Rogers, 1988)

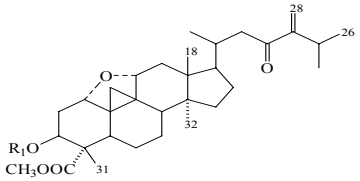
The isolation of oleanene- type pentacyclic triterpenoids containing 29–carboxy-1α-hydroxy groups from *C. molle*, *C. edwardsii, C. eleagnoides, C. imberbe*, *C. apiculatum, C. kraussi* and *C. padoides* confirms chemotaxonomically significant bifurcation in triterpenoid synthesis in the *Combretum* species (Rogers and Verotta, 1996). Both imberbic acid and mollic acid have anti-inflammatory and molluscicidal activity (Panzini *et al*, 1993). Molic acid (**6**)-(**7**) has been isolated from *C. molle*. Jessic acid (**8**)-(**9**) was previously isolated from *C. eleagnoides*. It was later found in the acetone fraction of *C. molle* fruit as the 3-O-β-D-xylopyranoside.



Compound	R 1
6	H
7	Me

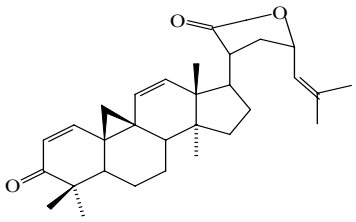
Derivatives of jessic acid, a cycloartane triterpenoid, have been found; the most chemically interesting being methyl jessate 1α, 11α-oxide (**8**) isolated from the hexane fraction of dry leaves and which shows a boat-like oxide bridge.

Combretum erythrophyllum as refered to by Kerere, 2001, has yielded some unusual cycloartane dienone lactones (10) and (11) from the leaves (Rogers, 1996).

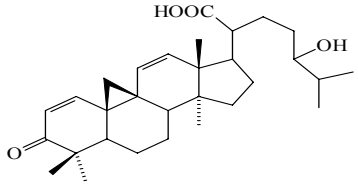


Compound	R 1
8	H
9	Ac

From this survey of phytochemical constituents it may be concluded that terpenoids not only appear to be taxonomic markers to establish biogenetic relationships, but also are the most commonly occurring compounds in the Combretaceae.



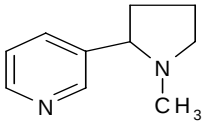
(**10**)



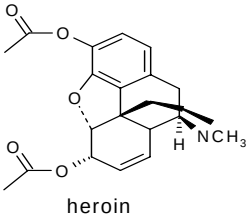
(**11**)

2.8.6 Alkaloids

Alkaloids are the largest group of secondary plant compounds (more than 12,000) and many exhibit marked physiological and pharmacological properties. Alkaloids are a heterogeneous group of compounds, which defy any adequate definition. In general, alkaloids are basic compounds containing one or more heterocyclic nitrogen atoms (e.g. nicotine).



nicotine



heroin

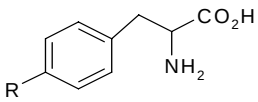
Most alkaloids are derived from amino acid precursors. Classification of alkaloids is problematic with some authors preferring a classification based on chemical structure (e.g. pyridine, tropane, pyrrolizidine alkaloids), while others base theirs on biosynthetic origin. The latter will be followed here (Harbone, 1994).

2.8.6.1 Classes of alkaloids

2.8.6.1.1 Ornithine and lysine derivatives

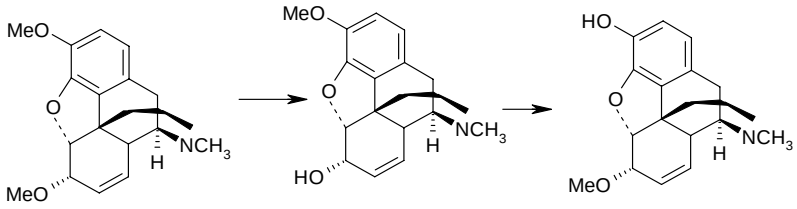
The ornithine and lysine derived alkaloids are represented here by nicotine (ornithine derived).
Cocaine and nicotine affect the central nervous system. Cocaine acts by inhibiting the re-absorption of dopamine (a neurotransmitter) and the consequent accumulation of dopamine results in a sense of well being. Nicotine acts on the cholinergic receptor either centrally, at autonomic ganglia and the neuromuscular junction causing stimulation or inhibition depending on the dose.

2.8.6.1.2 Phenylalanine and tyrosine derivatives



phenylanine R=H
tyrosine R=OH

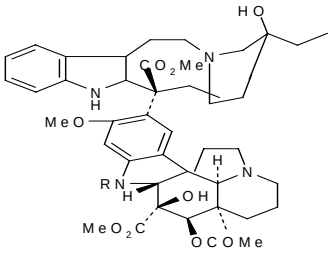
The phenylalanine and tyrosine derived alkaloids are represented by what has traditionally been known as the opiates: thebaine, codeine, and morphine from *Papaver somniferum* (opium poppy). Opium is a crude extract from ripe *Papaver* capsules and contains all three of these alkaloids with morphine in the greatest concentration. The biosynthetic route to morphine has been established beginning with the baine and following successive O-demethylation, ending with morphine. Note that this is an unusual biosynthetic sequence as successive methylation is the expected route. All these compounds have analgesic properties but differ in their potency.



thebaine codeine morphine

Heroin is a synthetic derivative of morphine and was originally considered a wonder drug.

2.8.6.1.3 Tryptophan derivatives

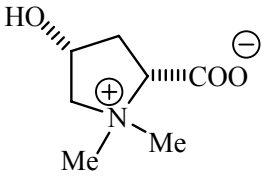


Vinca alkaloids vinblastine (R=Me) and vincristine (R=CHO)

The tryptophan derivatives include the vinca alkaloids vinblastine (R=me) and the related compound vincristine (R=CHO), both of which are potent chemotherapeutic agents. The Madagascar periwinkle (*Catharanthus roseus*) is a natural source of these compounds (Harbone, 1994).

2.8.6.2 Combretaceae alkaloids chemistry

No other alkaloids are known to have been isolated from the Combretaceae, apart from the simple betaine (1) isolated from *C. micrathum* and simple indole alkaloids from the species of a related genus, *Guiera senegalensis*This may be attributed to the inability of the separation methods to detect polar constituents.



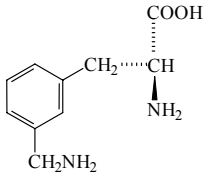
(**1**)

Hydroxyproline betaine, an alkaloid from the leaves of *C. micranthum*

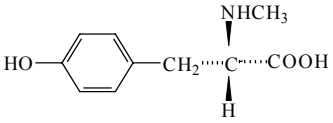
2.8.7 Other constituents

The fruit of *C. zeyheri* as refered to Kerere, 2001, has shown the presence of the amino acids, L-3-(3'–hydroxymethylphenyl) alanine (**1**), its glucoside and L-N-methyltyrosine β-D-glucopyranoside (**2**) (Panzini *et al.,* 1993).

The hexane extract of *C. apiculatum* fruit produced a compound identified as the anti-oxidant, 2, 6-di-*ter*-butyl-p-cresol (BHT), also isolated from *C. zeyheri* leaves.



(**1**)



(**2**)

This compound is toxic to brine shrimp but there are doubts about its authenticity as a plant metabolite, though it is reported to have been isolated from the family Annonaceae (Panzini *et al.,* 1993).

2.9 Methods developed and results obtained in the phytomedicine programme

2.9.1 Bioautography and MIC methods

The bioautography is a fast and quick guide toward bioassay-guided isolation and fractionation of antibacterial compounds and fractions respectively. In this approach, the activity of plant extracts on bacteria is determined on chromatography plates, in accordance with the bioautography procedure of (Begue and Kline 1972). This method is explained in **section 4.2.2** (page 51).

In an attempt to quantify the activity of the extracts, the microplate dilution method is used to determine the Minimum Inhibitory Concentration (MIC) (Eloff 1998c). This method makes use of a 2-fold serial dilution of extracts beyond the level where no inhibition of growth is observed. This method is explained in **section 4.2.3** (page 52)

2.9.2 Overview of activities in Combretaceae research in the phytomedicine group

2.9.2.1 Selection of plants

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

2.9.2.2 Selection of best extraction procedure

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

2.9.2.3 Selection of best purification procedures

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

2.9.2.4 Developing a novel way of determining antibacterial activity

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

2.9.2.5 Antibacterial activity of C. erythrophyllum

Using the techniques developed above it was shown that *C. erythrophyllum* contains at least 14 antibacterial compounds (Martini and Eloff 1998). Extracts had MIC values as low as 50 μg/ml.

2.9.2.6 Antibacterial activity and stability of 27 members of Combretaceae

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

2.9.2.7 Stability of antibacterial activity in C. erythrophyllum

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

2.9.2.8 A proposal for expressing antibacterial activity

MIC values do not give any indication of the activity present in a plant. A proposal was made that "total activity" should be determined by dividing the quantity extracted from 1 g of plant material in mg by the MIC in mg/ml. The resultant value in ml/g gives the highest dilution to which a plant extract can be diluted and still inhibited the growth of the test organism. The same proposal can be used to evaluate the efficacy of different steps in a bioassay guided isolation (Eloff, 2004).

2.9.2.9 Isolation of antibacterial compound from C. woodii

C. woodii acetone leave extracts had a better MIC values than ampicillin and chloramphenicol. The antibacterial compound combretastatin B5 was isolated and characterized. This compound had an MIC of 16 ug/ml against *S. aureus* and was present at a concentration of 5-10 mg/g in leaf extract (Eloff *et al.,* 2005)

2.9.2.2.10. Isolation of antibacterial compounds from C. erythrophyllum

For her PhD study Martini (2002) isolated and characterized seven antibacterial compounds. Four were flavanols: kaemferol, rhamnocitrin, rhamnazin, quercitin 5, 3 -dimethyl ether] and three flavones apigenin, genkwanin and 5-hydroxy-7, 4'-dimethoxyflavone (Martini *et al.,* 2004).
All test compounds had good activity against *Vibrio cholerae* and *Enterococcus faecalis*, with MIC values in the range of 25-50 μg/ml. Rhamnocitrin and quercetin-5,3-dimethylether showed additional good activity (25 μg/ml) against *Micrococcus luteus* and *Shigella sonei*. 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. Both rhamnocitrin and rhamnazin exhibited strong antioxidant activity with potential anti-inflammatory activity. Although these flavonoids are known, this was the first report of biological activity with some of these compounds (Martini *et al.*, 2004).

2.9.2.11 Other biological activities of Combretum species

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

2.9.3 Other work done on *C. imberbe* **and** *C. padoides*

Rogers and Subramony (1988) have isolated pentacyclic triterpene acids from the leave of *C. imberbe*. Related glycoside have also been isolated, all based on the olean-12-en-29-oate aglycone which has been given the trivial name imberbic acid (Roger, 1988).

Katerere *et al.,* (2002) isolated novel glycoside derivatives of hydroxyimberbic acid from the leaves of *C. imberbe*. These compounds had antibacterial activity against few strains of nosocomial organisms.

Rogers (1989b) isolated triterpenoid desmosides and 25 (27)-dehydroporiferasterol from the leaves of *C. padoides.* The biological activities of these compounds were never investigated.
Eloff (1998a) investigated the antibacterial activity of some 27 members of Combretaceae found *C. imberbe* and *C. padoides* among ther to have many antibacterial compounds and good antibacterial activity after quantification.