CHAPTER 1

1 Introduction

1.1 Resistance to antibiotics-A world-wide problem

Resistance to antimicrobial agents is recognized at present as a major global public health problem (Iwu et al., 1999). 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.

The introduction of penicillin 50 years ago was followed by an extraordinary period of discovery, exuberant use, and predictable obsolescence (Calvin, 1993). Resistant bacterial strains have emerged and have spread throughout the world because of the remarkable genetic plasticity of the microorganisms, heavy selective pressures of use, and the mobility of the world population. The widespread use and misuse of antibiotics led to emergence of antibiotic-resistant bacteria. Bacteria have become resistant to antimicrobial agents because of chromosomal changes or exchange of genetic materials via plasmids and transposons among other adaptations (Table 1).

Streptococcus pneumonia, Streptococcus pyogenes and *Staphylococci*, the organisms that cause respiratory and cutaneous infections, and member of the Enterobacteriaceae and Pseudomonas families, the organisms that cause diarrhoea, urinary infection, and sepsis, are now resistant to virtually all of the older antibiotics (Harold, 1992). Despite the availability of a wide range of antibiotics (e.g. penicillins, cephalosporins, tetracycline, amino-glycosides, monobactams, carbapenems, macrolides, streptogranins and dihydrofolate reductase inhibitors), people die in hospitals because of resistant bacterial infections.

1.2 Possible solution

Due to emergence of drug resistant bacteria, the search for new antibacterial compounds with improved activity is necessary. Many indigenous plants are used in treating bacterial related diseases (Carr, 1988). Only a small fraction of these indigenous plants has been investigated. Scientific examination of plants used traditionally in bacteria infection seems to be a logical step of exploiting the antimicrobial compounds, which may be present in plants. Plant based antimicrobials represent a vast untapped source of medicines. Plant-based antimicrobials have 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 antimicrobials (Iwu et al., 1999)

Table 1 Major Mechanisms of Resistance to Antimicrobial agents (Jacoby and Archer, 1991)

Type of Resistance and Antimicrobial class	Specific Resistance Mechanism
Quinolones	Altered DNA gyrase
Rifampicin	Altered RNA polymerase
Sulfonamides	New drug-insensitive dihydropteroate synthase
Tetracycline	Ribosomal protection
Trimethoprim	New drug-insensitive dihydrofolate reductase
Vancomycin	Altered cell wall stem peptide
Detoxifying enzyme	
Aminoglycosides (amikacin, gentamicin, Kanamycin, netilmicin, tobramycin)	Acetyltransferase, nuceotidyl-transferase, phosphotransferase
β-Lactam antibiotics (Carbapenems, Cephalosporins, monobactams, Penicillins)	β-Lactamase
Chloramphenicol	Acetyltransferase
Decreased Uptake	
Diminished permeability	
β-lactam antibiotics, chloramphenicol, quinolones, tetracycline, trimethoprim	Alterations in outer membrane proteins
Active efflux	
Erythromycin	New membrane transport system
Tetracycline	New membrane transport system

1.3 Medicinal Plants-As source of Antibacterial drugs

1.3.1 Plants as sources of medicines

The use of medicinal plants 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.

Other than for purposes of scientific inquiry, plants historically have served as models in drug development for three reasons:

First, 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).

Second, the biologically active substances derived from plants have served as templates for synthesis of pharmaceuticals. Such compounds may have poor pharmacological and toxicological profiles.

Third, 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 below (Table 2).

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 long history 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 the antileukaemic alkaloids, vinblastine and vincristine, which were both obtained from *Catharantus roseus* (Nelson, 1982) and combretastatins from *Combretum caffrum* (Pettit et al., 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, 1989). Other antineoplastic agents include taxol and several derivatives of camptothecin from *Taxus brevifolia* and *Camptotheca acuminata* respectively (Monroe and Mansukkh, 1996).

Table 2 Some plant-derived preparations for medicinal use

Active compound	***Origin***	**Application**
Ephedrine	*Ephedra sinica*	Bronchodilator
Egortamine	*Ergot spp*	Migraine remedy
Hyoscyamine	*Hyoscyamus niger*	Anticholinergic
Ipatropium	*Hyoscyamus niger*	Bronchodilator
Morphine	*Papaver somniferum*	Analgesic
Penicillin	*Penicillin spp*	Antibiotic
Physostigmine	*Physostigma venenosum*	Cholinesterase inhibitor
Pilocarpine	*Pilocarpus jaborandi*	Glaucoma remedy
Quinidine	*Cinchona pubescens*	Anti arrhythmic
Quinine	*Cinchona pubescens*	Antimalarial
Reserpine	*Rauwolfia serpentina*	Antihypertensive
Salicin	*Salix spp*	Anti-inflammatory
Scopolamine	*Datura stramonium*	Antispasmodic
Sennoside A+ B	*Cassia angustifolia*	Laxative
Theophylline	*Camellia sinensis*	Bronchodilator
Vinblastine	*Catharantus roseus*	Antineoplastic

1.3.2 Plant 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 must 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; Bohlman et al., 1987).

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 antimicrobial compounds, which accumulate at sites of infection (Kuc, 1985; Carr and Klessig, 1989; Bailey and Mansfield, 1992; 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.

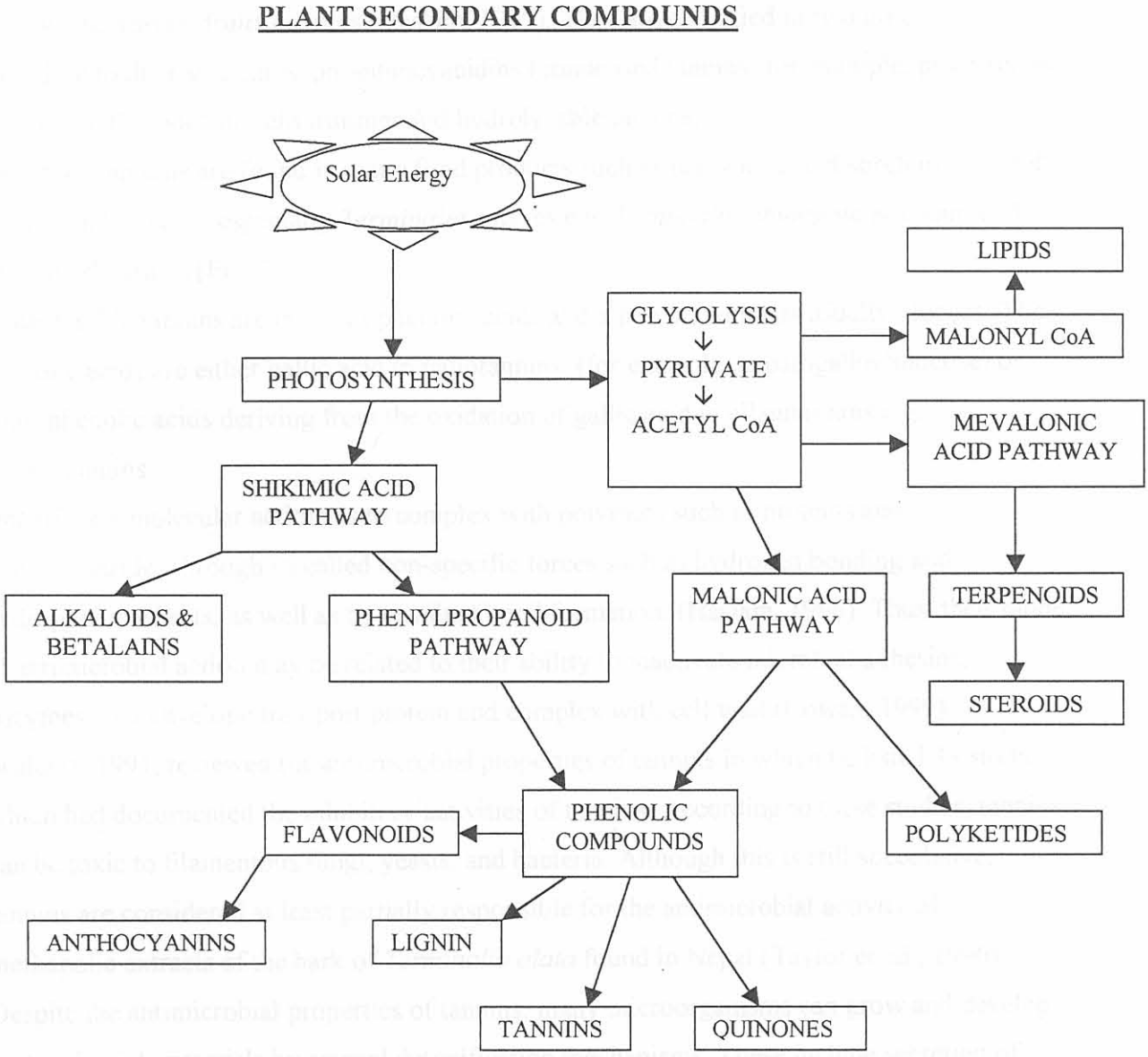
Biopolymers are also often associated with the phytoalexin accumulation at the site of injury or infection. These biopolymers include: lignin, a polymer of oxidized phenolic compounds; callose, a polymer of β-1, 3-linked glucopyranose; hydroxyproline-rich glycoproteins, and suberin. They provide both 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; Boller, 1987; Rao and Kuc, 1990). 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 antimicrobial attack. Some of the antimcrobial compounds in plants may be exploited for use against bacterial diseases in man.

Fig. 1 Synthesis of plant secondary compounds.



1.4 Antimicrobial activity of some plant constituents- An overview

Some plant secondary metabolites which are synthesized by photosynthesis via various pathways have antibacterial activities [Fig. 1].

1.4.1 Tannins

Tannins are water-soluble polyphenols, which differ from other natural phenolic compounds in their ability to precipitate proteins such as gelatin from solution (Bruneton, 1995). They differ in that respect from simpler phenols such as catechol, pyrogallol, gallic acid, catechin and other flavanols. Tannins are commonly found in a large array of higher plant species of both herbaceous and woody types. They accumulate in large amounts (often more than 10 % of the dry weight) in particular organs or tissues, which can be almost any plant: bark, wood, leaves, fruits or roots (Haslam, 1989). They are classified in two groups according to their structures, proanthocyanidins (condensed tannins) for example, procyanidin trimmer, and prodelphinidin trimmer and hydrolysable tannins.

Proanthocyanidins are found in many food products such as tea, cocoa, and sorghum of carob pods. Combretaceae especially *Terminalia* species e.g. *Terminalia oblongata* is a source of condensed tannins [Fig. 2].

Hydrolysable tannins are esters of phenolic acids and a polyol, which is usually glucose. The phenolic acids are either gallic acid in gallotannins, (for example, hepatogalloylglucose) or other phenolic acids deriving from the oxidation of gallic acid in ellagitannins e.g. pedunculagins.

One of their molecular actions is to complex with polymers such as proteins and polysaccharides through so called non-specific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation. (Haslam, 1996). Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport protein and complex with cell wall (Cowan, 1999).

Scalbert, 1991, reviewed the antimicrobial properties of tannins in which he listed 33 studies which had documented the inhibitory activities of tannins. According to these studies, tannins can be toxic to filamentous fungi, yeasts, and bacteria. Although this is still speculative, tannins are considered at least partially responsible for the antimicrobial activity of methanolic extracts of the bark of *Terminalia alata* found in Nepal (Taylor et. al., 1996).

Despite the antimicrobial properties of tannins, many microorganisms can grow and develop on tannin-rich materials by several detoxification mechanisms. These include secretion of tannin-binding polymers, tannin-resistant enzymes, oxidation of tannins, siderophores, and

tannin biodegradation (Scalbert, 1991). Further, tannins are non-specific in their activity and present absorption problems.

2, 3- (S)-Hexahydroxydiphenoyl-4, 6-(S, S)-gallagylglucose (punicalagin) from *Terminalia oblongata*

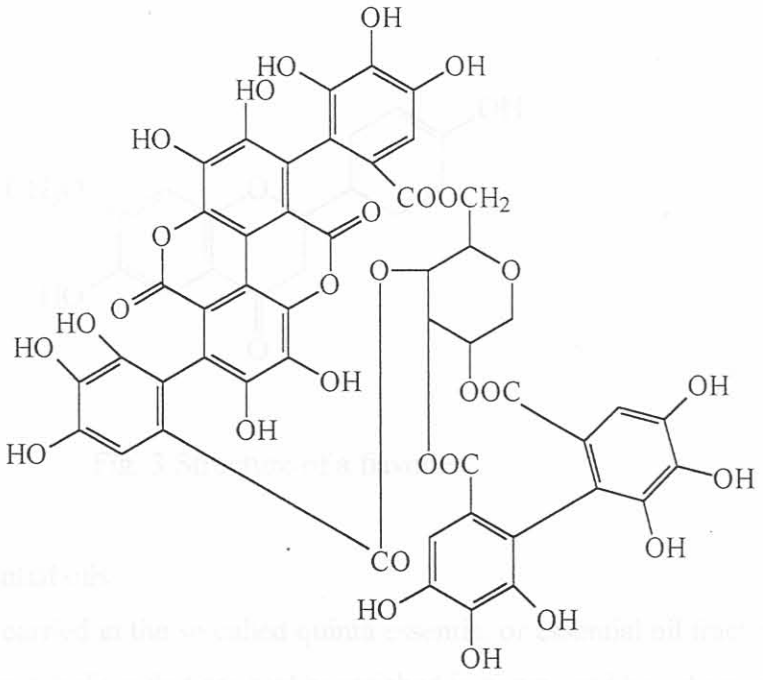


Fig. 2 Structure of a condensed tannin

1.4.2 Flavones, flavonoids and flavonols

Flavones are phenolic structures containing one carbonyl group [Fig. 3]. The addition of a 3-hydroxyl group yields a flavonol. Flavonoids are also hydroxylated phenolic substances but occur as a C_6-C_3 unit linked to an aromatic ring. They are found in fruits and vegetables essential for processing vitamin C and needed to maintain capillary walls. They may aid in protecting against infections (Mosby Medical Encyclopedia, 1997). Almost all are water-soluble; they are responsible for the colour of flowers, and fruits and sometimes leaves. Biflavonoids are dimers of flavonoids. The majority of natural biflavonoids are dimers of flavones and flavonones.

Since they are known to be synthesized by plants in response to microbial infections, they have been found in vitro to be effective antimicrobial substances against a wide range of microorganisms (Cowan, 1999). Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls.

Flavonoids isolated from the leaves of *C. micranthum* have been shown to have antimicrobial activity against both Gram positive and Gram negative microorganisms (Rogers and Verrotta, 1996).

Flavone from *Terminalia arjuna*

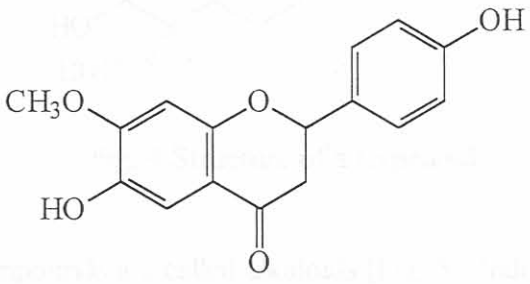


Fig. 3 Structure of a flavone.

1.4.3 Terpenoids and essential oils

The fragrance of plants is carried in the so called quinta essentia, or essential oil fraction. These oils are secondary metabolites that are highly enriched in compound based on an isoprene structure. They are called terpenes, their general chemical structure is $C_{10}H_{16}$, and they occur as diterpenes, triterpenes and tetraterpenes (C_{20}, C_{30}, and C_{40}), as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When the compounds contain additional elements, usually oxygen, they are termed terpenoids.

Terpenoids are synthesized from acetate units, and as such, they share their origins with fatty acids. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are methanol and camphor (monoterpenes) and farnesol and artemisin (sesquiterpenoids).

Terpenes or terpenoids are active against bacteria and fungi (Taylor et. al., 1996). The mechanism of action of terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds. A rich variety of triterpenoid acids has been isolated from *C. molle* and *C. imberbe* (Rogers and Verotta, 1996) [Fig. 4].

Pentacyclic terpenoid from *C. molle*

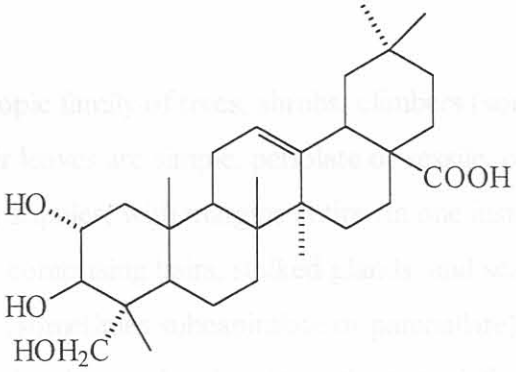


Fig. 4 Structure of a terpenoid

1.4.4 Alkaloids

Heterocyclic nitrogen compounds are called alkaloids [Fig. 5]. Indo quinoline alkaloids, the active principals in *Cryptolepsis sanguinnolenta* has been shown to inhibit Gram negative bacteria and yeast (Silva, 1996). Additional studies have shown this plant to have bactericidal activity. Recent *in vitro* studies have shown activity against bacteria specifically, enteric pathogens, most notably *E. coli* (but also *Staphylococcus*, *Pseudomonas*, *Salmonella*, *Shigella*, *Streptococcus* and *Vibrio* spp) and some activity against *Candida* spp (Sawer, 1995). Indo-quinolizidine alkaloids and glycoalkaloids and saponins, the essential constituents of *Naclea latifolia* have antibacterial activity against Gram positive and Gram negative bacteria and antifungal activity (Iwu, 1993). They are most effective against *Cornebacterium diptheriae, Streptobacillis sp., Streptococcus* sp., *Neisseria* sp., *Pseudomonas aeruginosa, Salmonella* sp. (Deeni 1991).

Berberine

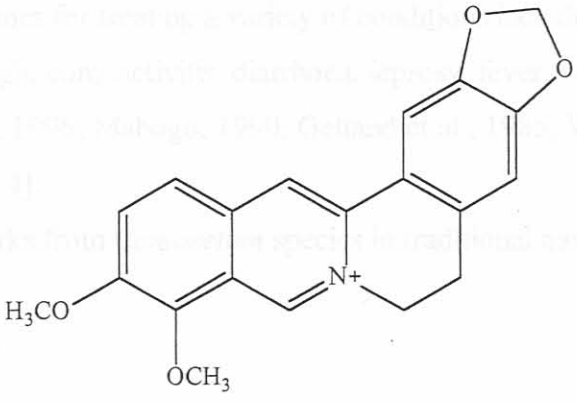


Fig. 5 Structure of an alkaloid.

1.5 Combretaceae

1.5.1 Introduction

The Combretaceae is a pantropic family of trees, shrubs, climbers (sometimes spinose) and mangrove (Carr, 1988). Their leaves are simple, petiolate or sessile, opposite, alternate, verticillate, whorled, without stipules, with margins entire (in one instance sometimes crenulate), with indumentum comprising hairs, stalked glands, and scales. The inflorescences are axillary, terminal, spicate (sometimes subcapitulate or panicullate). The flowers are sessile, or pedicellate, bisexual or bisexual and male on the same inflorescence. The receptacles are usually in two parts, the lower containing the ovary, the upper terminating in four or five sepals. The number of petals may be four or five or absent. The stamen may be eight or ten. The style are centrally situated on disc (Carr, 1988).

1.5.2 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 smaller; e.g. *Calopyxes* and *Buchenavia* comprise 22 species each, *Quesqualis* 16, *Angioeissis* 14, *Conocarpus* 12 and *Pteleopsis* 10 species (Rogers and Verotta, 1996). The genus *Combretum* has two subgenera, these being subgenus *Combretum* and sub genus *Cacoucia*. *C. woodii*, a member of subgenus *Combretum* was investigated in this study [Table 3].

1.5.3 Use of Combretaceae in Traditional medicine

Many species of Combretaceae are widely distributed in Southern Africa. These plants are used in traditional medicines for treating a variety of conditions including pneumonia, syphilis, colds, chest cough, conjunctivitis, diarrhoea, leprosy, fever, wound dressings, and mumps (Hutchings et al., 1996; Mabogo, 1990; Gelfand et al., 1985; Watt and Breyer-Brandwijk, 1962) [Table 4].

The use of leaves and barks from *Combretum* species in traditional medicine is widespread.

Table 3 The subgeneric classification the genus *Combretum* in South Africa according to Carr 1988.

Combretum Loefl	
Subgenus *Combretum*	**Subgenus *Cacoucia***
Section *Hypocrateropsis*	**Section *Lasiopetala***
C. celastroides	*C. obovatum*
C. imberbe	**Section Conniventia**
C. padoides	*C. microphyllum*
Section *Combretastrum*	*C. paniculatum*
C. umbricola	*C. platypetalum*
Section *Angustimarginata*	Section *Oxystachya*
C. caffrum	*C. oxystachytum*
C. erythrophyllum	**Section *Megalantherum***
C. kraussili	*C. wattii*
C. vendae	**Section *Poivrea***
C. woodii	*C. bracteosum*
Section *Macrostigmatea*	*C. mossambicense*
C. engleri	
C. kirkii	
C. mkuzense	
Section *Metallicum*	
C. collinum	
Section *Glabripetala*	
C. fragrans	
Section *Spathulipetala*	
C. zeyheri	
Section *Ciliatipetala*	
C. albopunctatum	
C. apiculatum	
C. edwardsii	
C. maggii	
C. molle	
C. petrophilum	
C. psidioxides	
Section *Fusca*	
C. coriifolium	
Section *Breviramea*	
C. hereroense	
Section *Elaeagnoida*	
C. elaeagnoides	

Table 4 Traditional uses of some *Combretum* species

Combretum	Medicinal uses
C. apiculatum	Snakebite, Scorpion bite, Bloody diarrhoea, leprosy, Abdominal disorders, Conjunctivitis.
C. erythropyllum	Fattening tonic for dogs. To reduce the size of vaginal orifice, Infertility, Venereal disease, To facilitate labour.
C. fragrans	Chest coughs, Syphilis, Aphrodisiacs.
C. hereroense	Bilhariziasis, Heart disease, Heartburns, Body pains, Stomach complaints.
C. kraussii	Appetite stimulant, Cleaning of urinary system.
C. microphyllum	Lunacy, Lucky charm.
C. molle	Snake bite, Stomach ache, Abortion, Wound dressing, Fattening of infants, Abdominal pains, Diarrhoea, To stop bleeding after birth, Convulsions, Backache, Headache.
C. zeyheri	Gallstones, Bloody diarrhoea, To arrest menstrual flow, Embrocation, Scorpion bite.

1.5.4 Ethnopharmacology of Combretaceae

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

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

The ethnopharmacological use of *Combretum zeyheri* against diarrhoea and eye infections has been ascribed to its antibacterial activity towards Gram- positive microbes (Breytenbach and Malan, 1989).

Combretum 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 (1999) also found that all the leaf extracts from 27 Southern African members of the Combretaceae including *Combretum woodii* exhibited antibacterial activity against *Staphylococcus aureus, Escherichia coli, Enterococcus feacalis and Pseudomonas aeruginosa.*

The leaves of *Combretum molle* and *Combretum imberbe* have been shown to have molluscicidal activity against *Biomphalaria glabrata* snails (Rogers and Verotta, 1996).

1.5.5 Phytochemistry of 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 (*C. molle, C. padoides, C. eleagnoides*), phenanthrenes (*C. hereroense, C. molle, C. apiculatum, C. caffrum*), amino acids (*C. zeyheri*), and stilbenes (*C. caffrum*)(Pellizzoni et al., 1992).

A series of closely related bibenzyls, stilbenes and phenanthrenes have been isolated from *C. caffrum*, a member closely related to *C. woodii*. Some of these stilbenes have been found to be potent antimitotic agents, which inhibited both tubulin polymerization and binding of colchine to tubulin. These stilbenes are called combretastatins. Combretastatin A-4 has been shown the most potent cancer cell growth inhibitor of the series (Petit et al., 1995). Combretastatins A-4, A-5, A-6, were also found to inhibit growth of *Neisseria gonorrhea* (Petit et al., 1995).

The anti-inflammatory and molluscicidal compounds mollic acid β-D-glucoside and imberbic acid have been isolated from *C. molle* and *C. imberbe* respectively (Pegel and Roger 1976, Roger, 1988). The saponin, jessic acid linked to α-L-arabinose has been found in *C. eleagnoides* leaves (Osborne and Pegel, 1984). Panzini et al., 1993 also identified jessic acid 3-0-β-D-xylopyranoside from the fruit of *C. molle*.

The presence of poly-cis prenols of chain length 20 - 60 isoprene units or longer in leaves of plants belonging to Combretaceae family has been shown to be the common feature of this group in most cases the polyprenols occurred in the form of fatty acid esters. The polyprenols in *Combretum molle* are in form of free alcohols (Kulcitsky et al., 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 et al., 1985, Nwauluka et al., 1975). Flavonoids have been isolated from *C. micranthum* leaves (Rogers and Verotta, 1996). The fruits of *Terminalia cheluba* have yielded complex esters of gallic acid, corilagin (Haslam, 1989).

With the exception of the simple indole alkaloids harman and eleagnine isolated from the roots of *G. senegalensis*, there have been no other reports of alkaloids from the Combretaceae. (Rogers and Verotta, 1996).

1.5.6 Selection of *C. woodii*

C. woodii belongs to the subgenus *Combretum*, the same section of very important medicinal plants such as *C. caffrum*, *C. erythrophyllum*. The activities of *Combretum* species are ascribed mainly to stilbenoids (combretastatins), triterpenoids and flavonoids (Roger, 1996). Triterpenoids and saponins are well known for their antimicrobial and anti-inflammatory activity (Bruneton, 1995). The water, acetone and ethyl acetate extracts of *C. woodii* leaves have been shown to have anti-inflammatory activity (McGaw et. al., 2001). However, there has not been any report in the literature on the antibacterial compounds that are present in *C. woodii*. It contains antibacterial compound chemically different to those in *C. erythrophyllum*, according to our preliminary results (Eloff 1998, unpublished data). It was selected for this study because of these reasons.

C. woodii Duemmer is a deciduous tree or shrub with a height of 8 - 12 m [Fig. 6].

It is found in South Africa: Transvaal, Kruger national park, Natal, Ndumu, Mkuze game reserve,) and Swaziland: Lebombo, Nhlemen [Fig. 7].

It grows in steep rocky slopes, canyon margins, ravines, sand forest, dry forest, closed forest, riverine woodland, rocky hillsides, mountain grassland, and at low to medium altitudes (up to 1200 m).

Common names in South Africa are Bastard Forest Bush willow, Basterbosvaderlandswilg (Afrikaans), iWaphu (Zulu), Mbondvo sehlatsi (Siswati).

The bark is mostly smooth light grey (almost white) and a slightly darker, biscuit-tinged grey. In places, however, the outer covering lifts in large flakes, exposing a biscuit to ginger sub-surface, which is also smooth.

Leaves are often alternate on extending shoots but usually opposite to sub- opposite when born on new lateral twigs. Lamina shape varies somewhat. It may be broadly elliptic with base bluntly tapered and apex rounded or broadly elliptic to obovate with a rounded taper to the base and the apex bluntly acuminate with slight attenuation or elliptic, tapering to the base and acuminate and attenuate at the apex.

The size is also variable, from 65 x 25 mm with petiole 2.5 mm to 150 mm x 75 mm and an 8 mm petiole. Calculated average dimensions are 79 x 38 x 6 mm. The leaf keel is arched and the lamina is vee-ed about the midrib. There can be considerable marginal undulations. Lamina texture is papyraceous. The upper surface of the leaf is medium green with the underside appreciably paler. Foliage may develop yellow and red coloration in the autumn and (during flowering) leaves near inflorescences are sometimes markedly blanched. Venation on the upper surface is flush to slightly rise and a yellowish green. Main and lateral veins can be readily discerned but secondary venation is sometimes indistinct.

The inflorescences are borne singly in axis along (and particularly near the terminal of short, new, leafy, lateral shoots) from August to first week in December. Spikes may be glabrous except for small light- coloured scales, dense to rather obscure, with in some cases scattering hairs, or puberulous. Petals are spathulate, glabrous, whitish and are about 1 mm long. Filamens are exserted up to 4.5 mm. Anthers, yellowish brown, are, 0.8 mm long. The style projects up to 3 mm and has an apical swelling. The disc has whitish hairs, sometimes long and projecting to the same extent as the petals.

The fruit is 4-winged (occasionally 5- or 6-winged) with outline broadly elliptic to sub-circular. Some fruit has dense, minute, yellowish to light brown scales evenly over the body and wings, but scales are usually closely spaced on the body and are more scattered on the wings. With the unripe fruit, there is a tingling mainly on the body but spread to the wings. When ripe, wings are biscuit- coloured and the body a light brown color. The seed is cigar shaped up to 12 mm long and 3.5 mm across at its greatest diameter. There are four longitudinal grooves, the testa is appreciably wrinkled and the colour is cinnamon.

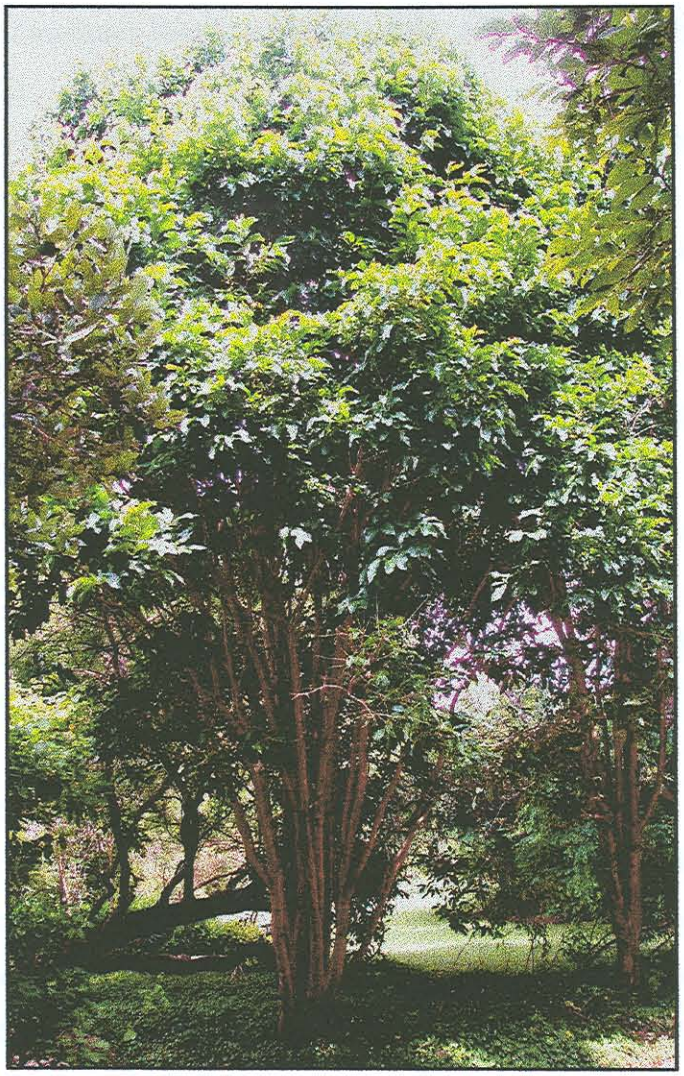


Fig. 6 *Combretum woodii* from which leaves were collected in Lowveld National Botanical Garden

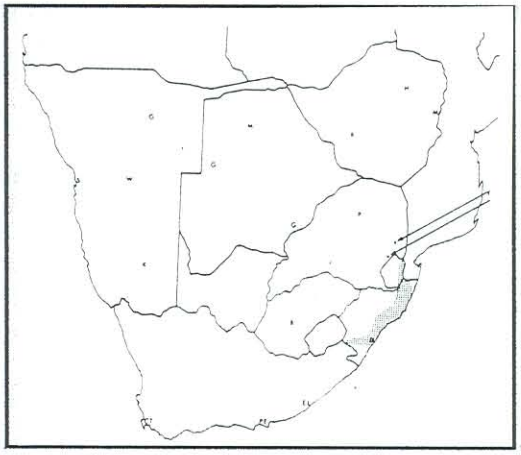


Fig. 7 Distribution of *C. woodii* in Southern Africa

1.6 An overview of methods that have been employed to isolate compounds from plants.

1.6.1 Plant materials.

Fresh or dried plant material can be used as a source of extract for secondary plant metabolites. It is preferable to use dried material because:

- Plant material not rapidly dried may be attacked by fungi, which could alter the chemical composition of the components drastically.
- The time delay between collecting plant material and processing it makes it difficult to work with fresh material because differences in water content may affect solubility or subsequent separation by solvent-solvent extraction.
- There are fewer problems associated with large-scale extraction of dried plant material than with fresh material.
- Dried plant materials are mostly used by traditional healers, so it may be necessary to follow their method of plant harvesting and preparation.

1.6.2 Extraction techniques

An important factor governing the general and specific method used in an extraction is the type of phytochemical groups that are to be extracted [Table 5]. The main groups of compound to be considered are fixed oils, fats and waxes, volatile or essential oils, carotenoids, alkaloids, glycosides, aglycones, phenolic compounds, polysaccharides and proteins.

Many workers have used different extractants while screening for antimicrobial activity of medicinal plants. These include, for instance, 80% ethanol solution (Vlietinck et. al., 1995), ethanol-water (50:50,v/v)(Baba-Mousa et al., 1999), methanol (Taylor et al., 1995), petroleum ether, chloroform, ethanol, methanol and water (Salie et. al., 1996). Many scientists employ Soxhlet extraction of dried plant material using solvents with increasing polarity. This may be suitable for compounds that can withstand the temperature of the boiling solvent, but cannot be used for thermolabile compounds. The problem can be overcome by extracting under reduced pressure. Acetone is an ideal solvent for use in extractions. It dissolves many hydrophilic and lipophilic components. It is miscible with water, volatile and has low toxicity (Eloff, 1997).

Different phytochemical groups are extracted by different extractants according to Houghton and Raman (1998) [Table 5]

Table 5 Type of phytochemicals extracted by different solvents (Houghton and Raman, 1998)

Polarity	Solvent	Phytochemicals extracted
Low	Hexane	Waxes, Fats, Volatile oils
Low	Chloroform	Alkaloids, Aglycones, Volatile oils
Medium	Diethyl ether	Alkaloids, Aglycones
Medium	Ethyl acetate	Alkaloids, Aglycones, Glycosides
Medium	Acetone	Alkaloids, Aglycones, Glycosides
Medium	Ethanol	Glycosides
Medium	Methanol	Sugars, Amino acids, Glycosides
High	Water	Sugars, Amino acids, Glycosides
High	Aqueous acid	Sugars, Amino acids, Bases
High	Aqueous alkali	Sugars, Amino acids, Acids.

1.6.3 Isolation and analysis of constituents

The isolation and analysis of phytochemical constituents or fractions with antibacterial activity are carried out by means of thin layer chromatography (TLC), column chromatography, nuclear magnetic resonance spectroscopy (NMR), and mass spectroscopy. TLC is used for qualitative analysis of crude extract for identification of isolated constituents present in an extract by comparison with reference substances and /or data in the literature. For a given substance, a useful parameter to measure is its retardation factor or the Rvalue on TLC. This is the ratio of the distance from the baseline (point of application) to the center of the zone divided by the distance from the baseline to the solvent front.

Ultraviolet detection of UV active compounds on TLC plates at UV light (254 nm and 366 nm) can be carried out [Table 6].

Table 6 Examples of colours detected under UV for some phytochemical preparation according to Wagner and Bladt (1996).

Type of compound	UV-254nm	UV-365nm
Alkaloids	Pronounced quenching of some alkaloids of indoles, quinolines, isoquinolines, and purines. Weak quenching e.g. atropine alkaloids	Blue, blue-green or violet fluorescence. Yellow fluorescence e.g. Colchicine, berberine.
Flavonoids	All flavonoids cause fluorescence quenching. Caffeic acid, its derivatives and isoflavones show quenching	Dark yellow, green or blue fluorescence depending on the structure type Caffeic acid, its derivatives and isoflavones fluoresce blue
Triterpenes and Essential oils	Compound containing at least two conjugated double bonds quench fluorescence and appear as dark zones against light green fluorescent background of the TLC plate	No characteristic fluorescence of terpenoids and propylphenols is noticed
Saponins	No saponins are detectable	No detection

Column chromatography separates constituents of a mixture according to the polarity as well as several other factors. Adsorption chromatography using silica gel as a stationary phase is used for analysis of components.

Nuclear magnetic resonance spectroscopy (NMR) essentially provides a means of determining the structure of an organic compound by measuring the magnetic moments of its hydrogen atoms. In most compounds, hydrogen atoms are attached to different groups (as – CH_2, -, $-CH_3$-, -CHO, $-NH_2$-, -CHOH-, etc.) and the NMR spectrum provides a record of the number of hydrogen or carbon atoms in these different situations. It can give any direct information on the nature of skeleton of the molecule; this must be obtained in the first

instance by application of other spectral techniques. NMR spectroscopy will be used for structural elucidation of isolated compounds in the fractions.

Therefore, both proton and carbon NMR are employed for structural elucidation of the pure compounds isolated.

Mass spectroscopy has a value, in that with only microgram amounts of material, it can provide an accurate molecular weight and it may yield a complex fragmentation which is usually characteristic of (and may identify) that particular isolated compound.

1.6.4 Assay of plant extracts

Most workers use agar diffusion assays to determine the antibacterial activity of plant extracts. The technique works well with defined inhibitors (Hewit and Vincent, 1989), but when examining extracts containing unknown components, there are problems leading to false positive and false negative results (Eloff, 1998). The antimicrobial effect may be inhibited or increased by extrinsic factors or contaminants. The agar type, salt concentration, incubation temperature, and molecular size of the antimicrobial component influence results obtained with agar diffusion assays (Marsh and Goode, 1994). This technique also does not distinguish between bactericidal and bacteristatic effects and minimum inhibitory concentration (MIC) cannot be determined. An alternative technique most widely used in general microbial assay is serial dilution of the extract in a number of test tubes followed by the addition of the test organism to determine to minimum inhibitory concentration for the test organism using turbidity as an indication of growth. This technique requires relatively large quantities of extracts and is therefore not useful in bioassay guided isolation of antimicrobial compounds (Eloff, 1998).

Eloff, 1998 developed a micro-dilution technique using 96-well micro plates and tetrazolium salts to indicate bacteria growth. He found that *p*-Iodonitrotetrazolium violet (0.2 mg/ml) gave better results than tetrazolium red or thiazolium blue. The method is quick, worked well with *Staphylococcus aureus*, *Enterococcus faecalis, Pseudomonas aeruginosa,* and *Escherichia coli* and with non- aqueous extracts from many different plants.

This method is useful in screening plants for antimicrobial activity and for the bioassay-guided isolation of antimicrobial compounds from plants (Eloff, 1998).

Some antibacterial agents are assayed by agar diffusion method and / or turbidimetry [Table 7].

Table 7 Pharmacopoeia methods for microbiological assay of some antibacterial agents

Antibiotic	British Pharmacopoeia (1980)	United States CFR (1980)
Amoxycillin	-	agar diffusion assay
Bactracin	agar diffusion assay	-
Capreomycin	agar diffusion assay	turbidimetry
Carbenicillin	-	agar diffusion assay
Cephalexin	-	agar diffusion assay
Chloramphenicol	agar diffusion assay	turbidimetry
Clindamycin	agar diffusion assay	-
Cloxacillin	agar diffusion assay	-
Doxycycline	agar diffusion assay	turbidimetry
Erythromycin	agar diffusion assay	agar diffusion assay
Gentamicin	agar diffusion assay	agar diffusion assay
Neomycin	agar diffusion assay, turbidimetry	agar diffusion assay
Polymyxin B	agar diffusion assay	agar diffusion assay
Rifampicin	-	agar diffusion assay
Streptomycin	agar diffusion assay, turbidimetry	agar diffusion assay, turbidimetry
Tetracycline	agar diffusion assay, turbidimetry	turbidimetry
Tobramycin	agar diffusion assay, turbidimetry	turbidimetry
Vancomycin	agar diffusion assay	agar diffusion assay

Bioautography is another method of studying antimicrobial activity. Among the numerous *in vitro* methods for studying the antimicrobial activity of plant extracts, bioautography has found widespread applications, especially for the detection of new compounds in complex plant extracts. The methods allows for the detection of spots of growth inhibition of cultures directly on the extract thin layer chromatography plate previously dispersed with a broth culture containing the microorganism.

1.7 Problem statement and Hypothesis

The growing number of resistant strains of microbial pathogens is a worldwide problem, which justifies the search for new antibiotics from indigenous plants.

Many *Combretum* species are used in traditional medicine for diseases related to bacterial infections, *Combretum woodii* contains different antibacterial compounds from other *Combretum* species examined in our laboratory (Eloff, 1998, unpublished data) hence the motivation to investigate *C. woodii* for antibacterial activity.

By applying standard bioassay guided procedures, I will be able to isolate and characterize one or more of these compounds.

1.8 Aim and Objectives of the study

To isolate and characterize antibacterial compounds in *Combretum woodii* by:

- Extracting the compounds from with several extractants.
- Testing the biological activity of the extracts against the common pathogens.
- Determining the best preliminary group separation technique.
- Fractionating the compound(s) responsible for activity using a bioassay guided process.
- Isolating the antibacterial compound(s).
- Determining its biological activity the chemical structure of the isolated compound(s).