CHAPTER 3

3. SELECTION OF PLANTS

3.1 FAMILY COMBRETACEAE

3.1.1 Taxonomy

The Combretaceae family belongs to the order Myrtales, which is sub-divided into two sub-families of which only the Combretoideae is of interest. Of the two tribes comprising this sub-family, only the Combreteae, which is further divided into three subtribes, is of relevance in Africa. The five genera from the three sub-tribes are *Combretum, Terminalia, Pteleopsis, Quisqualis* and *Lumnitzera* [Arnold and De Wet, 1993]. Reports in the literature indicate that traditional healers have confined themselves almost exclusively to the use of species form the genus *Combretum* and to a lesser extent *Terminalia* in the treatment of a wide range of illnesses [Rogers and Verotta, 1996].

3.1.2 Use in Traditional Medicine

The first published scientific study carried out was on the West-African drug "Kinkeliba", isolated from *C. micranthum* leaves and used in the treatment of biliary fever, colic and vomiting [Paris, 1942]. This plant also has cholagog and diuretic properties and shows antimicrobial properties against both Gram positive and negative organisms [Rogers and Verotta, 1996].

Some medicinal uses of the Combretaceae are listed in Table 3.1.

Table 3.1: Traditional medicinal uses of the Combretaceae [Adapted from Rogers and Verotta, 1996]

COMBRETUM SPECIES	***TRADITIONAL USE***
C. apiculatum	Snake and scorpion bite, bloody diarrhoea, leprosy, abdominal disorders, conjunctivitis and weak body
C. erythrophyllum	Fattening tonic for dogs, to reduce the size of the vaginal orifice
C. fragrans	Chest coughs, syphilis, aphrodisiac
C. glutinosum	Hepatic disease, antihypertensive, diuretic, bronchial disease
C. hereroense	Bilharzia, headache, infertility in women
C. imberbe	Coughs, colds, diarrhoea
C. microphyllum	Lunacy, lucky charm
C. molle	Hookworm, stomach ache, snakebite, leprosy, fever, dysentery, chest complaints, anthelmintic, headache, diarrhoea, convulsions, dressing for wounds, stop bleeding after childbirth and to fatten babies
C. platypetalum	Swelling caused by mumps, pneumonia, abdominal pains, diarrhoea, antiemetic, dysmenorrhoea, infertility in women, earache, epistaxis, haemoptysis
C. zeyheri	Toothache, cough, scorpion bite, bloody diarrhoea, arrest menstrual flow, eye lotion, embrocation, abdominal disorders

From data provided by Cunningham [1990] it was calculated that 20.2 tons of Combretaceae (Table 3.2) was used annually in the herbal trade in Zululand [Eloff, 1998]. This is a rough indication of its considerable popularity in traditional medicinal usage.

Table 3.2 : Total amount of medicinal plant material in tons yearly used in Kwazulu-Natal [Adapted from Eloff, 1998]

FAMILY	TOTAL	FAMILY	TOTAL
Liliaceae	131.7	Combretaceae	20.2
Myrsinaceae	84.4	Rutaceae	20.2
Anacardiaceae	56.4	Sapotaceae	20.2
Sapindaceae	49.2	Rubiaceae	17.2
Compositae	47.9	Halorrhagidaceae	17.0
Amarillidaceae	47.7	Dioscoreaceae	16.3
Umbelliferae	39.9	Canellaceae	15.8
Passifloraceae	36.9	Crassulaceae	14.2
Meliaceae	31.4	Convovulceae	12.5
Melianthaceae	29.8	Vitaceae	12.2
Lauraceae	23.1	Cycadales	11.7
Leguminosae	21.2	Ranunculaceae	11.4

3.1.3 Metabolites isolated from Combretaceae

Recently a number of clinical trials initiated with Deerghayu, a *Terminalia arjuna* based polyherbal formulation, have shown encouraging results in heart patients. Arjuna exhibits good anti-oxidant properties, reduces cardiac size and downregulates beta-receptors, therefore proving beneficial in coronary artery disease. It has also shown good antiviral and antimicrobial properties. Researchers contribute this activity to various active constituents including tannins such as gallic acid, ellagic acid and oligomeric proanthocyanidins, triterpenoid saponins, flavonoids (luteolin, arjunone, arjunolone), phytosterols and certain minerals [Narendran, 2001].

More substantial chemical work has, however, been done on the genus *Combretum*. In 1973, Letcher and co-workers isolated a number of substituted phenanthrenes, dihydrophenanthrenes and bibenzyls from various *Combretum* spp. [Letcher and Nhamo, 1973].

A group from the University of Durban-Westville isolated and characterised a number of novel compounds, [Pegel and Rogers 1985, Rogers and Thevan 1986, Osborne and Pegel 1985] namely triterpenes and saponins like mollic acid, jessic acid and their derivatives. The sodium salts of mollic acid glycoside isolated from *C. molle* were found to be toxic to *Biomphalaria glabrata* snails at a concentration of 12 ppm [Rogers, 1989].

More recently a series of stilbenes and dihydrostilbenes (combretastatins) with potent cytotoxic activity and acidic triterpenoids and their glycosides with molluscicidal, antifungal and anti-inflammatory activity have been isolated from *Combretum* species. Combretastatin is the first of a series of unique stilbenes highly active against the murine P-388 lymphocytic leukaemia cell line and was originally isolated from *C. caffrum* [Rogers and Verotta, 1996]. These bioactive compounds were later also found in *C. kraussi* and *C. erythrophyllum* species and clinical trials began in November 1998 [Schwikkard, 2000].

Novel pentacyclic triterpenes were isolated from *Combretum nigricans* and identified as combregenin and its glycoside, combre-glucoside [Jossang, 1996]. This species is used in folk medicine for the treatment of gastrointestinal diseases and also as a fish poison. Two triterpenes, arjunolic acid and mollic acid, and two flavonoids, 3-*O*-methylquercentin and 3-*O*-α-L-rhamnopyranosylquercetin, have been isolated and identified from *Combretum leprosum* [Facundo, 1993] and two phenanthrenes (4,7-dihydroxy-2,3,6-trimethoxyphenanthrene, 2,7-dihydroxy-3,4,6-trimethoxydihydrophenanthrene) and a dihydrostilbene (4,4'-dihydroxy-3,5-dimethoxydihydrostilbene) have been isolated from the heartwood of *C. apiculatum* [Grayer and Harborne, 1994]. These compounds showed total inhibition of *Penicillium expansum* when 20 μg was spotted on a TLC plate.

Although many compounds have been isolated and chemically identified, few plant species have been subjected to bioassays in order to determine pharmacological activity. Some of those tested however, have shown remarkable activity. *Combretum micranthum* for example, traditionally used in the treatment of malaria, has shown strong antimalarial activity against both a chloroquine-sensitive and -resistant strain of *Plasmodium falciparum* [Benoit, 1996]. Leaves of *C. paniculatum* have shown promising results in the inhibition of HIV-1 and HIV-2 replication [Asresk, 2001].

Alexander *et al*. [1992] investigated the antimicrobial activity of 12 *Combretum* spp. and found several active components in some species. They did not follow up the antimicrobial components in *C. erythrophyllum*, but found up to five different inhibitors in extracts of other *Combretum* spp. using different test organisms. Preliminary bioassays of extracts were found to be active against *S. aureus*. Breytenbach and Malan [1989] isolated three antimicrobial components from *C. zeyheri* and proposed structures for two of them. All had activity against *S. aureus*.

A number of Combretaceae species tested for antifungal activity showed promising results. Of the seven species tested, *C. nigricans* was strongly active on dermatophytes [Baba-Moussa, 1998].

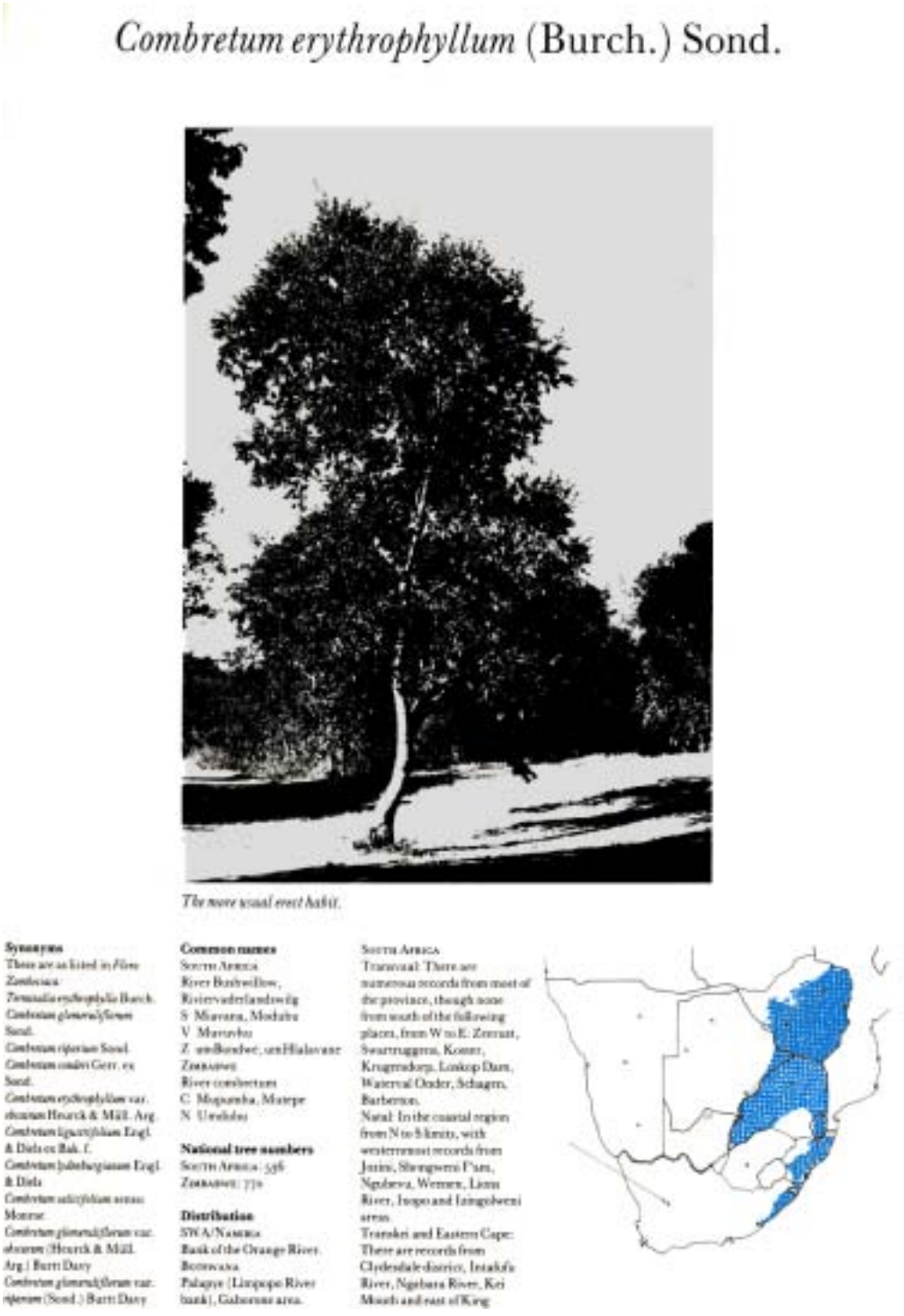
3.2 PRESENT STUDY

3.2.1 Background on *Combretum erythrophyllum* (Burch.) Sond.

Combretum erythrophyllum (see figures 3.1 and 3.2) was nominated as the tree of the year in 1995. It is commonly known as the river bushwillow and is adapted to a great variety of climatic conditions. It occurs in almost the entire eastern part of South Africa and as can be deduced from its name, the river bushwillow prefers riverbanks as its natural habitat leaning over the water in true willow fashion. In the west it occurs mainly along the Orange River. The growth form is mostly multi-stemmed and bushy with branches growing out horizontally, sometimes lying on the ground and with a large number of upright branches sprouting up from these. The tree flowers in winter to late spring, just after the young leaves have appeared [http://www.sa-embassy]. In autumn the leaves sometimes turn red, which explains the scientific name. The gum is found to be slightly antibacterial and can be applied to sores in a powdered form [pamphlet, National Botanical Gardens, Pretoria].

In Zulu medicine, unspecified parts are used in pregnancy to facilitate labour, leaves are used for coughs and abdominal pain and the bark for infertility and during pregnancy. Small doses of root have been administered to dogs as a fattening tonic. In Zimbabwe the roots are used as a cure and prophylactic against venereal disease and inserted vaginally as an aphrodisiac and to reduce the size of the vaginal orifice. This practice is not recommended as many women have died after vaginal insertion of powdered roots, with symptoms including abdominal pain, severe vomiting and confusion. Roots have a purgative effect and the fruit can produce persistent hiccoughs. The poisonous principle in the fruit has not yet been identified. The gum is used for tanning and has a slight positive test for antibiotic activity. Extracts from leaves have significant antimicrobial activity against *Branhamella catarrhalis, Mycobacterium phlei* and *Serratia marecescens* [as quoted by Hutchings et al, 1996].

Figure 3.1: *Combretum erythrophyllum* (Adapted from Carr]



Combretum erythrophyllum (Burch.) Sond.

The more usual erect habit.

Synonyms
These are as listed in *Flora Zambesiaca*
Terminalia erythrophylla Burch.
Combretum glomeruliflorum Sond.
Combretum riparium Sond.
Combretum randii Gerr. ex Sand.
Combretum erythrophyllum var. *obovatum* Heurck & Müll. Arg.
Combretum ligustrifolium Engl. & Diels ex Bak. f.
Combretum paludosum Engl. & Diels
Combretum salicifolium sensu Monroe
Combretum glomeruliflorum var. *obovatum* (Heurck & Müll. Arg.) Burtt Davy
Combretum glomeruliflorum var. *riparium* (Sond.) Burtt Davy

Common names
South Africa
River Bushwillow,
Rivierwaterlandswilg
S Muvaru, Modubu
V Murovhu
Z umBondwe, umHlalovane
Zimbabwe
River combretum
C Mupamba, Mutepe
N Umholoti

National tree numbers
South Africa: 536
Zimbabwe: 770

Distribution
SWA/Namibia
Bank of the Orange River.
Botswana
Palapye (Limpopo River bank), Gaborone area.
South Africa
Transvaal: There are numerous records from most of the province, though none from south of the following places, from W to E: Zeerust, Swartruggens, Koster, Krugersdorp, Loskop Dam, Waterval Onder, Schagen, Barberton.
Natal: In the coastal region from N to S limits, with westernmost records from Jozini, Shongweni Farm, Ngubevu, Wemmer, Lions River, Inspo and Izingolweni areas.
Transkei and Eastern Cape: There are records from Clydesdale district, Intaka's River, Ngabara River, Kei Mouth and east of King

Figure 3.2: Common names and distribution of *C. erythrophyllum* [Adapted from Carr]

Seven novel triterpenoid acids and lactones were isolated in the search for compounds responsible for the toxicity of this plant [Rogers, 1998]. The representative compound was given the name erythrophyllic acid [Lawton and Rogers, 1991]. Attempts to saturate one of the triterpenoids by hydrogenation, using Adams catalyst, resulted in two unusual hydrogenation products, 3-oxo-9,19-cycloart-11-en-21-oic acid and 23-hydroxycycloart-11-en-21-oic acid. The water-soluble sodium salt fractions were found to be active against *Citrobacter, Enterobacter* and *Salmonella* species [Mocktar, 1997].

3.2.2 Background to Triterpenoids

Since triterpenoids have shown to exhibit antibacterial activity [Mocktar, 1997] and Rogers [1998], as mentioned above, isolated seven novel triterpenoids from *Combretum erythrophyllum*, it is necessary to include a more detailed background to these secondary metabolites and their functional role in plants. This is also a continuation of a previous study, which isolated five triterpenoids [Martini, 1998].

Terpenoids are also called terpenes and can occur as monoterpenes, diterpenes, triterpenes and tetraterpenes (C_{10}, C_{20}, C_{30} and C_{40} respectively) as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When they contain additional elements, usually oxygen, they are termed terpenoids. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are menthol and camphor, as well as artemisin, a compound currently used in the treatment of cerebral malaria [Cowan, 1999].

Triterpenoids are non-steroidal secondary metabolites. The physiological function of these compounds is generally believed to be a chemical defence against pathogens and herbivores. It is therefore expected that they should act against certain pathogens causing human and animal diseases [Mahato, 1997]. Although medicinal uses of this class of compounds are rather limited, possibly due to their hydrophobic nature, recent work in this regard indicates their great potential as drugs. Moreover, despite the remarkable diversity already known to exist, new variants continue to emerge [Mahato, 1992].

Due to the wide occurrence and structural diversity of triterpenoids, evaluation of their biological activity has always attracted attention and although application of these compounds is very limited, extensive exploratory activities have been underway during recent years. A number of triterpenoids have been found to have antitumour and anticancer activity. Possibly the best-known one is ursolic acid, which was found to inhibit tumour promotion in mouse skin [Mahato, 1997]. *In vitro* and *in vivo* studies of carbenoxelone have also demonstrated intrinsic mineralocorticoid activity in rats. This compound increased the overall mean of prostaglandin E_2 concentrations in gastric juice as well as decreasing the gastric juice. Glycyrrhetinic acid was studied in hyperlipemia and caused significant changes in lipid metabolism. It has been found to have hypolipemic and antiartherosclerotic activity greater than that of the established antiartherosclerotic polysponin. Other activities include anti-inflammatory activity, analgesic and antipyretic properties. Some have been found to prevent liver injury and lower urinary tract infections by increasing the mucopolysaccharide layer in the bladder. Antitussive and expectorant activities, antiviral and antimicrobial effects have also been well documented [Mahato, 1992].

Terpenoids are found to be active against bacteria, fungi, viruses and protozoa. Their mechanism of action is not fully understood but it is speculated that these lipophilic compounds cause disruption of membranes because by increasing the hydrophilicity of kaurene diterpenoids, antimicrobial activity was drastically reduced.

Capsaicin, a constituent of chilli peppers, is bactericidal to *Helicobacter pylori*, although possibly detrimental to the gastric mucosa. Another terpenoid called petalostemumol, isolated from the prairie clover (*Dalea* sp.) shows excellent activity against *Bacillus subtilis* and *S. aureus* as well as *Candida albicans*. Some other examples are cited in Table 2.4 [Cowan, 1999].

Currently the terpenoids isolated from *Combretum* species include jessic acid and methyl jessate from *C. elaegnoides*; imberbic acid from *C. imberbe* [Mahato, 1992]; combregenin, combre-glucoside, arjungenin and arjunglucoside from *C. nigricans* [Jossang, 1996]and arjunolic and mollic acid from *C. leprosum* [Facundo, 1993]. So far the tritepenoids isolated from *C. erythrophyllum* seem to belong to almost exclusively two distinct groups, namely 30-carboxy-1α-hydroxycycloartanes and 29-carboxy-1α-hydroxyoleanes [Rogers 1998] [Figure 3.1].

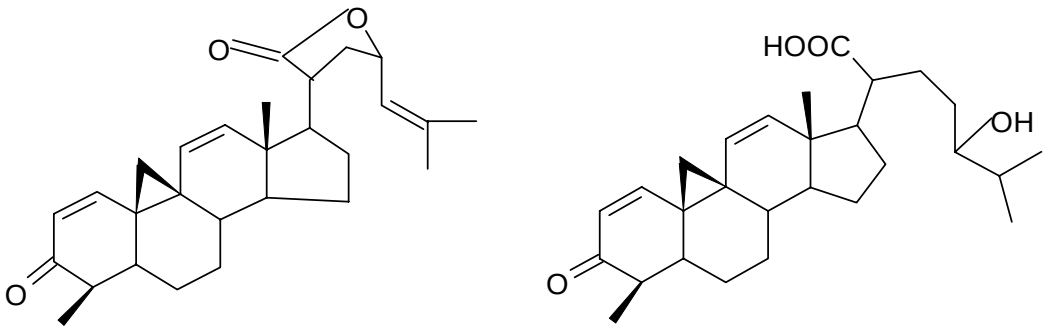


Figure 3.3: Cycloartane dienone lactones from *C. erythrophyllum*

3.3 PREVIOUS ISOLATION AND IDENTIFICATION WORK

This study is a continuation of work done previously [Martini, 1998]. Previous work involved the extraction of crude material with acetone and subsequently solvent/solvent extraction using various solvents (discussed further in chapter 4). The carbon tetrachloride and chloroform fractions were further fractionated by column chromatography (CC) and preparative TLC (PTLC). This method afforded five compounds but unfortunately due to the minute quantities extracted, structural elucidation was not possible. It was, however, established by means of NMR that these five compounds were triterpenoids and appeared to be novel structures. This was not validated however, but inspired continuation of the study.

3.4 AIM OF STUDY

The primary objective of this study is to isolate sufficient quantities of bioactive compounds, to elucidate their structures and determine biological activity.

Secondary objectives include toxicity screening of the pure compounds using an LDH method as well as determining antioxidant and anti-inflammatory activity using lymphocytes isolated from healthy volunteers. It has been suggested that the phytochemistry of a plant varies with the environment and the plant's maturity. It was decided to investigate this variability by determining antimicrobial activity of plant material sourced from various regions around Pretoria and variations in activity between young leaves versus older and mature material.