CHAPTER 8

8. DISCUSSION AND CONCLUSION

In the course of this study a number of secondary metabolites were extracted from *Combretum erythrophyllum.* These compounds are not novel structures but are reported for the first time in this species. Although apigenin has previously been isolated in *C. apiculatum* [Katerere, unpublished 2001] none of the other compounds appear to have been isolated from Combretaceae. All the compounds isolated were flavonoids i.e. three flavones and four flavonols and since Rogers [1998] managed to isolate cycloartanes from *C. erythrophyllum,* one needs consider why this biased isolation took place. One reason might lie in the solvents used in initial extraction of crude material as well as in the solvent/solvent extraction. Also many samples, which might have contained other compounds were not analysed due to insufficient quantities or were not clean enough for spectroscopic analysis and subsequently discarded. Each of the procedures used in the extraction and characterisation of these compounds is discussed below.

8.1 EXTRACTION AND ISOLATION PROCEDURE

In order to test the activity of extracts, the extract must be dissolved in a solvent that will not inhibit the growth of the test organism. This may cause complications if the active fractions are lipophilic compounds that are insoluble or only sparingly soluble in water. According to Malone [1983], a major problem arises from the injection of imperfectly soluble extracts or solvent solubilized materials into what should be a purely aqueous isotonic pharmacological system. Acetone and DMSO have been used to dissolve lipophilic compounds because these solvents are miscible with water. DMSO is apparently not as toxic to bacteria as acetone but the high boiling point [189°C] makes it very difficult to recover the active compound from a DMSO solution. Dried extracts are frequently not soluble enough in DMSO.

Acetone was investigated as solvent for extraction of crude material as it is miscible with water, is volatile and has low toxicity to bioassays used [Eloff, 1998]. Another advantage of acetone is the ease of removal from fractions collected within a relatively short period.

Snyder and Kirkland [1979] grouped solvents in 8 groups depending on the dipole as well as their ability to act as proton-donors or –acceptors. Acetone forms part of group VIa. Other solvents in this same group that are miscible with water are dioxane and to a lesser degree ethyl acetate and may be useful as extractants for dried plant material.

8.1.1 Analysis of extracts

To investigate the number of components in the extracts several TLC systems were developed. In order to obtain well separated components it is essential to select the right mobile phase and it was decided to use primarily three systems: 2A:3MDC for separation of polar components; BEA for non-polar and CEF for both polar and non-polar components. EMW was also attempted in the separation of the highly polar components. Each of the four systems was employed at different stages of extraction.

8.1.2 Solvent/solvent extraction

This procedure involves the separation of compounds with different polarities and since the desired compounds will be soluble in either one or another solvent, this technique is meant to “clean” the solution by removing contaminants.
Although hexane extracted the largest percentage of the dry weight, it contained the fewest inhibitory compounds giving the false impression that isolation of those compounds would be a simple procedure. Hexane is usually employed as a defatting solvent to remove unwanted waxes and oils from the crude material, which may interfere with further analysis, making further separation easier by removing a large portion of “unwanted” material.

It is possible that since acetone was used as initial extraction of crude material and not hexane, as is usually done; many compounds may not have been extracted resulting in primarily flavonoid isolation.

8.1.3 Column Chromatography

Column chromatography is the most commonly used preparative technique to isolate chemical compounds. Initially it was thought essential to develop a good eluting system in order to separate components optimally; therefore much care was taken to develop a good eluting system with the hexane fraction. A combination of acetone:DCM (1:1) and hexane/DCM (1:1) was initially chosen as the former separated the polar and the latter the non-polar components and both components are volatile and easily removed from the fractions. After combination of fractions a second column using BEA as mobile phase was used to separate fraction C. Since benzene is a toxic substance, care was taken to work only in the fume-hood, which made the extraction procedure and drying of samples tedious.

The chloroform fraction contained many antimicrobial compounds as seen with the bioautography and was subsequently chosen for separation via column chromatography. Although much care was taken with the hexane fraction to develop a good mobile system it was decided to remove non-polar components with a solvent such as hexane and then gradually introduce another solvent e.g. dichloromethane in small quantities to 100%. This achieved a good separation. It is sometimes necessary to include a third solvent to elute non-mobile components and therefore methanol was gradually added towards the end of separation with both the hexane and chloroform fractions. Care was used that the concentration of methanol did not increase above 50% in some cases as it has a tendency to dissolve silica gel, which subsequently interferes with structural elucidation. Another problem encountered with methanol was the difficulty in drying of the extracts.

Active fractions CE36, CE46 and CE51 were eluted with 10% hexane in DCM; Seph51 and CE144 with 5% methanol in DCM and IIIa90 and IIIa150 with 10% methanol in DCM.

The different stationary phases used in this study were silica gel, Sephadex and Toyopearl, size-exclusion gels. The reason for these three phases was primarily experimental in order to determine the best effect on separation. The best separations were achieved with silica and Sephadex. Toyopearl was easy to use since it came in a suspension and easy to clean but the separation was poor.

Problems encountered with column chromatography were not so much in the procedure involved but in the resulting fractionation. Some compounds appeared to be more complex after passing them through a column than they had been prior to separation. Columns II and III were based on selected fractions obtained from column I, and column IIIa on the first four fractions of column III. The results should have depicted a separation of the compounds as presented on TLC but in some cases more compounds appeared despite the use of the same mobile phases for elution. This increase in compounds could be due to an increase in the concentration of the solution applied to the TLC plate although Rf values of compounds from column IIIa differed dramatically from the parent column. It might also be possible that decomposition of compounds had taken place and artefacts had formed which contributed to a more complex chromatogram.

8.1.4 Preparative TLC

Preparative TLC was carried out on sample 2C of the hexane fraction because of its relative low complexity as seen with TLC. Although its MIC value lay in the order of 0.18 mg/ml, the mass was calculated at 120 mg and was selected for PTLC. Seven fractions were scraped off and eluted first with acetone and then 1% acetic acid in methanol to ensure full compound recovery. MICs were calculated using *S. aureus* as test organism and lay in the order of 0.09 – 1.31 mg/ml.

Fraction VII was not tested since the extract smelled very strongly of acetic acid and the component could not be recovered. Although all extracts were fairly active, the complexity was still too high for structural elucidation and since the masses ranged between 6 and 21 mg, it was impractical to purify them further. No further experiments with these extracts were carried out.

PTLC was also attempted to purify some extracts obtained from the chloroform fraction but most of these separations did not prove fruitful, as the resulting fractions were never clean enough for further analysis. The only separation, which resulted in NMR and MS analysis was that of fraction C3, which was relatively clean before application to the PTLC plate, however still no structure could be elucidated for this sample.

8.1.5 GC/MS

This type of analysis requires that the sample to be tested be converted to gas under the influence of a high temperature (215°C). Fraction 8 of the hexane fraction had begun to precipitate in solution and was considered a good candidate for GC/MS. Since no prior idea of structure was known, it was decided, under recommendation, to use a steroid analysis. Both the supernatant and precipitate were tested and compared with similar results. Although one steroid structure was seen with the precipitate, it had disappeared upon silinization with TMSI. Silinization converts polar compounds to non-polar compounds so they may be picked up and analysed by the apparatus. Disappearing of compounds could be due to either unbinding of the compound or misanalysis of original structure. Many of the compounds appeared to be long chain fatty acids, which explains why hexane is used as a defatting solvent. Several fatty acids were identified, including octacosane, tetradecanoic acid, stearic acid and eicosanoic acid. Since these compounds were not pure, bioactivity could not be tested and more focus was placed on the chloroform fraction. Waxes and fatty acids tend to precipitate under reduced temperatures and some exhibit high antimicrobial activity possibly indicating that the major source of antimicrobial compounds in *C.*

erythrophyllum might lie in the waxy, outer layers of the leaf material and warranting further investigation into this area.

8.1.6 HPLC

HPLC was used to determine the purity of the Toyopearl isolated compounds. In preparation for application onto the reverse phase column, samples were spotted on RP-TLC plates and eluted with varying ratios of methanol:water. Seeing that the best separation was obtained with 10% water/methanol, this was the choice eluting solvent for the HPLC analysis. All the samples were dissolved in acetone and since mobile phases interfere with compound analysis it is essential to determine where these peaks will appear in relation to the compound peaks. Various flow rates and sample dilutions were tested in an attempt to obtain the optimal separation but with little success. Identification of the compound peaks was not possible since the solvent peaks absorbed at similar wavelengths and therefore impossible to differentiate between them. Another problem was the small quantity of sample, which retarded further experimentation and this type of analysis was subsequently abandoned.

8.2 IDENTIFICATION

Identification of compounds usually involves a combination of various techniques including nuclear magnetic resonance (NMR), mass spectrometry (MS), ultraviolet (UV) and infrared (IR) spectrometry. Other ways of confirming the identification of the compound include calculation of Rf values in different solvent systems and determination of the melting point. In this study NMR was used as a tool for identifying the structure and confirmed with ^{1}H-^{1}H-COSY-LR and MS. Rf values were calculated in some cases using CEF, BEA and 2A:3MDC as the mobile phases. UV and IR were not required as structural elucidation was complete with the techniques used.

8.2.1 Nuclear magnetic resonance (NMR)

The most general technique is ^{1}H NMR spectroscopy but with advances and greater availability of ^{13}C NMR, the past few years has seen an increase in ^{13}C NMR. This technique provides information on the carbon skeleton of the molecule and is regarded as complementary to ^{1}H NMR since it relates more closely to the structural environment of the flavonoid nucleus [Wollenweber, 1988].

^{13}C NMR performed in this study was a DEPT experiment, which failed to detect all the carbon resonances and therefore values were not obtained for all compounds. This didn't affect the structural elucidation since ^{1}H NMR was more than effective in determining the compound structures.

^{1}H-^{1}H-COSY-LR is Long Range COrrelation SpectrometrY designed to give a two dimensional pattern of the protons in relation to one another. This technique was of value in confirming the positions of methoxyl groups on the A- and C-ring.

All the compounds sent for NMR had precipitated out of solution after column chromatography had been performed and the test-tubes had been left to dry. In total 54 samples were sent for analysis and seven compounds were identified, all of them flavonoids (three flavones and four flavonols). Most of the compounds were replicas or mixtures and some were not clean enough for structural elucidation.

8.2.2 Mass Spectrometry (MS)

High Resolution Electron Impact Mass Spectrometry (HREIMS) serves as a valuable aid in determining the structures of flavones and flavonols, especially when only very small quantities are available. Most aglycones are sufficiently volatile at probe temperatures of 100-300°C to allow for successful MS without derivatization [Wollenweber, 1980]. Kingston [1973] suggested that this type of mass spectrometry provides more structural information than chemical ionization and must therefore remain the method of choice for these compounds.

HREIMS was used to confirm the structures identified with NMR and led to the characterisation of seven flavonoids.

8.3 FOCUS ON FLAVONOIDS

Flavonoids were isolated by Albert Szent-Gyorgi, MD of Hungary and researched during the 1940s and '50s [Berkoff, 1998]. They are 15-carbon compounds consisting of two aromatic rings A and B and an oxygen containing heterocyclic ring C. Depending on the oxidation level of ring C, they are classified into several groups, i.e. chalcones, flavanones, flavones, isoflavones and flavonols, and are collectively known as the “yellow pigments” and the coloured “anthocyanin pigments” [Ibrahim, 2000]. The widely occurring flavonols, i.e. kaempferol, quercetin and myricetin, do not contribute to the yellow colour since they are more or less colourless at the pH values normally found in cells and are often abundant in white, ivory or cream coloured petals. Flavonols are therefore isolated from a range of yellow-petalled plants in order to assess their contribution, if any, to flower colour. A significant proportion of yellow-petalled plants that have been examined contain both carotenoid and flavonoid, pigments produced by quite unrelated biosynthetic pathways. It is also clear that for a flavonol to contribute to yellow flower colour it must possess some structural feature absent from the commonly-occurring flavonols [Harborne, 1965].

Flavonoid compounds may undergo further enzymatic hydroxylation, methylation, glycosylation, sulfonation, acylation and/or prenylation reactions, thus resulting in the immense diversity of flavonoid structures which amount to 12 subcategories [Berkoff, 1998] and more than 5000 identified compounds that are found in nature [Ibrahim, 2000].

Flavonoids are present mainly as glycosides, which are water-soluble and located in cell sap but Wollenweber [1980] suggests that an increasing number of free aglycones are being found in various plant species.

8.3.1 Biosynthesis

Enzymatic and genetic studies, coupled with the use of efficient methods for isolation and identification of flavonoids, have allowed the elucidation of the pathways involved in the biosynthesis of the different groups of flavonoids [Ibrahim, 2000].

All flavonoids derive their carbon skeletons from two basic compounds, malonyl-CoA (synthesised from the glycolysis intermediate acetyl-CoA and carbon dioxide) and the CoA ester of a hydrocinnamic acid [Figure and Table 8.1]. Both of these precursors are derived from carbohydrates [Heller and Forkmann, 1988]. The aromatic ring B and its adjacent 3-carbon side-chain are derived from L-phenylalanine via the shikimate pathway, whereas ring A is formed by the head-to-tail condensation of three acetate units via the polyketide pathway that is proposed for the biosynthesis of phloroglucinol and resorcinol derivatives [Ibrahim, 2000] leading to the formation of a C15 chalcone intermediate [Heller and Forkmann, 1988].

Flavonoids, aurones and other diphenylpropanoids are derived from the C-15 chalcone intermediate and the first flavonoid, naringenin, is formed by stereospecific action of chalcone isomerase on this compound. Oxidative rearrangement of this flavonone yields an isoflavone, genistein, which is catalysed by isoflavone synthase. Introduction of a double bond between C-2 and C-3 of the flavonone leads to the abundant class of flavones of which the first and most simple one is apigenin. Dihydroflavonols (dihydrokaempferol) are formed by direct hydroxylation of flavanones in the 3-position, which is catalysed by flavanone 3-hydroxylase.

Dihydroflavonols are biosynthetic intermediates in the formation of flavonols, catechins, proanthocyanidins and anthocyanidins. The large class of flavonols (e.g. kaempferol) is formed by introduction of a double bond between C-2 and C-3 of the dihydroflavonols [Heller and Forkmann, 1988].

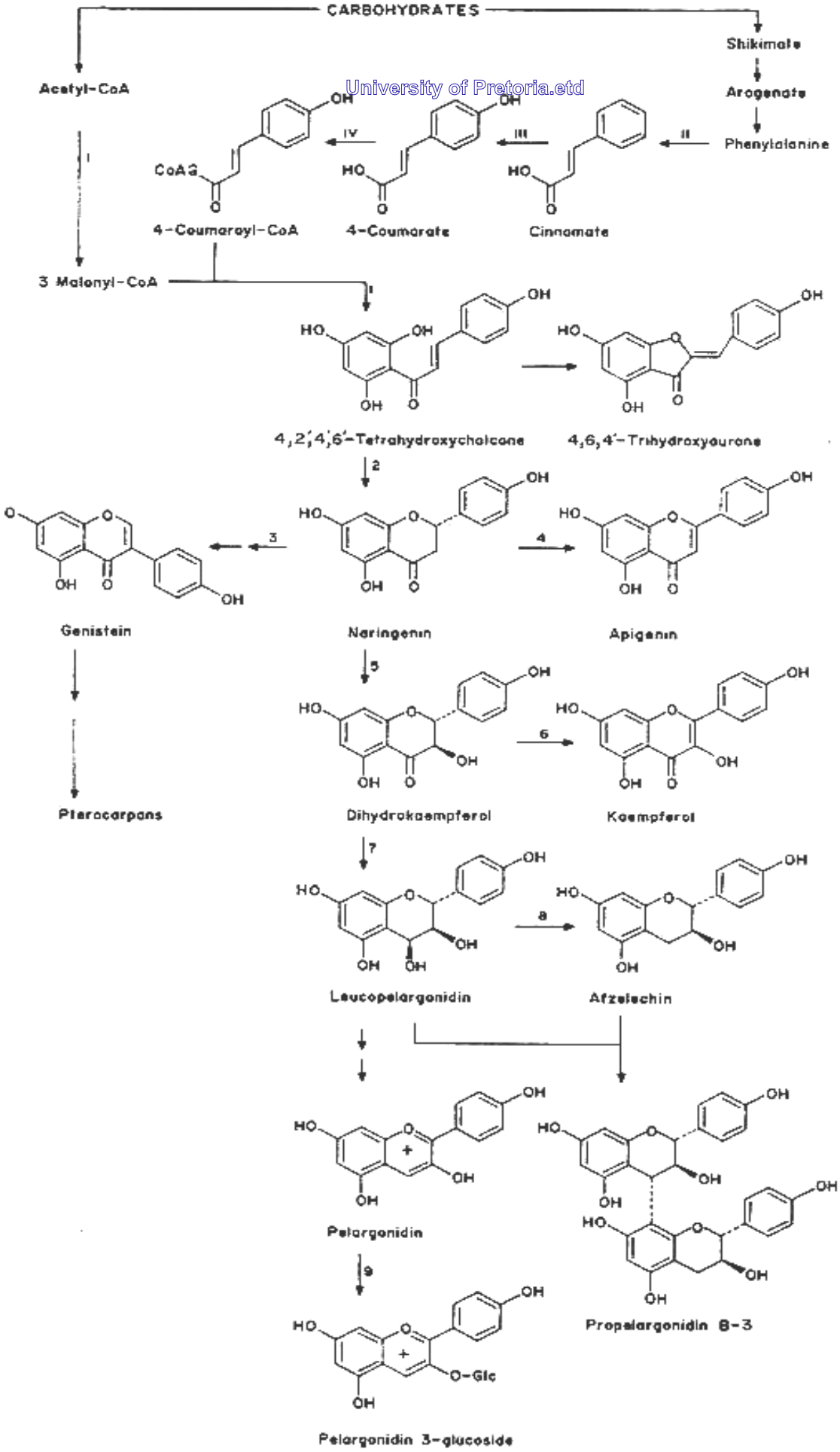

Figure 8.1: Biosynthesis pathway of flavonoids [From Heller & Forkman, 1988]

Table 8.1: List of enzymes leading to various flavonoid classes [adapted from Heller and Forkmann, 1988]

Enzymes
Non-flavonoid precursors
I Acetyl-CoA carboxylase
II Phenylalanine ammonia-lyase
III Cinnamate 4-hydroxylase
IV 4-Coumarate: CoA ligase
Flavonoid classes
1 Chalcone synthase
2 Chalcone isomerase
3 2-Hydroxyisoflavanone synthase
4 Flavone synthase
5 (2S)-Flavanone 3-hydroxylase
6 Flavonol synthase
7 Dihydroflavonol 4-reductase
8 Flavan-3,4-cis-diol 4-reductase
9 Anthocyanidin/flavonol 3-O-glucosyltransferase

8.3.1.1 Pathways to precursors of flavonoid formation:

Acetyl –CoA carboxylase catalyses the reaction in the presence of ATP and Mg^{2+} and has been extensively studied in relation to fatty acid biosynthesis [Lowenstein as quoted by Heller and Forkmann, 1988].

A great number of phenolic compounds in plants are derived from the hydrocinnamic pathway and in many plants specific classes can be stimulated by exogenous factors. UV irradiation for example induces flavonoid glycoside synthesis in parsley cell cultures whereas furanocoumarin phytoalexins are formed on treatment of parsley cells with fungal cell wall preparations (so-called elicitors). In soybean and many other legumes, the same elicitors give rise to the formation of isoflavonoid phytoalexins and *Arachis* of stilbenoids.

The second enzyme of the hydrocinnamic acid metabolism, cinnimate 4-hydroxylase, is located at the endoplasmic membrane and consists essentially of a cytochrome P-450 and NADPH-cytochrome P-450 reductase. Cytochrome P-450 inhibitors such as ketokonazole inhibit this enzyme.

Evidence suggests that 4-coumarate is the main or even exclusive physiological precursor for flavonoids in many plants [Heller and Forkmann, 1988].

8.3.1.2 Individual steps to flavonoid classes

Chalcone synthase is regarded as the key enzyme of flavonoid biosynthesis with the intermediate product being the chalcone naringenin. Chalcone synthases from several plants are more or less strongly inhibited by the flavanone formed in the reaction. A possible role for this effect may be in protecting the cell from phytotoxic concentrations of this product.

With a few exceptions, the various flavonoid classes are derived from a flavanone intermediate. Flavanones are formed from chalcones by isomerization and only flavanones with an (*S*)-configuration act as substrates for the enzymes of the flavonoid pathway. Spontaneous isomerization of some chalcone to flavanone *in vivo* may lead to formation of moderate amounts of anthocyanins leading to an orange colour in the flowers.

In certain plant species desaturation of flavanone to flavone is catalysed by a microsomal enzyme requiring NADPH as cofactor. Both the parsley and the flower enzyme catalyse the reaction from (2*S*)-naringenin to apigenin and (2*S*)-eriodictyol to luteolin. The mechanism of the double-bond formation is still unclear. It is suggested that a 2-hydroxyflavanone is formed in the first step and that water is eliminated via a dehydratase.

Flavanone 3-hydroxylase requires 2-oxoglutarate, Fe^{2+} and ascorbate as cofactors and is rather unstable under normal conditions. Flavonol synthesis most probably proceeds via a 2-hydroxy intermediate with subsequent dehydration similar to the scheme proposed for the conversion of flavanones to flavones. Such intermediates have been shown spontaneously to eliminate water, giving rise to the respective flavonols. Dihydrokaempferol and dihydroquercetin are substrates for the *in vitro* formation of kaempferol and quercetin respectively [adapted from Heller and Forkmann, 1988].

8.3.2 Biological roles

The most powerful antioxidants are to be found among the flavonoids and 18 flavonoids are now acknowledged as having 20 times the potency of vitamin C and 50 times the potency of vitamin E. Flavonoids are essential for processing vitamin C and are needed to maintain capillary walls as well as protect against infection. Rutin is an example of a flavonoid that helps increases capillary strength and ensures fast healing. A deficiency can result in a tendency to bruise easily [Berkoff, 1998].

Research supports the antioxidant and antibacterial properties of flavonoids and their ability to reduce the risk for developing heart disease, possibly due to inhibition of oxidation of low-density lioprotein (LDL). They also inhibit inflammation by decreasing the release of inflammatory mediators and by stabilizing cell membranes [Berkoff, 1998].

Many possess antifungal properties, especially the nonpolar polymethylated flavones and the prenylated isoflavones as well as various chalcones and flavanones. Some exhibit antiallergic and anti-inflammatory activity; and 3-methylquercetin is recognised as a potent, selective inhibitor of viral RNA synthesis.

The pterocarpan phytoalexins, which have antimicrobial activities and characteristic of members of Leguminosae are included in soybean, alfalfa and chickpea [Ibrahim, 2000]. Gallates (in green tea) have also shown powerful inhibition in the growth of staphylococcus [Berkoff, 1998].

They play an important role in plant growth and development, inhibit the activity of certain enzymes, affect cellular protein phosphorylation and act as regulators of hormonal transport, as determinants of pollen germination and functionality, as stress metabolites in response to wounding and nutrient deficiencies, as potent scavengers of free radicals and in the protection against UV radiation [Ibrahim, 2000].

Genistein and daidzein are examples of two powerful antioxidants and cancer inhibitors, proven to lower the risks for breast and prostrate cancer [Berkoff, 1998].

Flavonoids can also act as signalling molecules that regulate the induction and expression of Rhizobium nodulation genes that are involved in nitrogen fixation by leguminous plants. Several flavonoid compounds have been implicated in plant-insect interactions, in which they act as feeding stimulants, feeding deterrents and oviposition stimulants with the potential role of improving host-plant resistance to herbivory [Ibrahim, 2000].

There are currently no dosage or toxicity levels set for flavonoids and an average American diet contains about 1000 mg per day [Berkoff, 1998].

8.3.2.1 Impact on mammalian biology

Where flavonoid structure-activity relationships have in most cases been detected, they have been implicated for immunity, inflammation and carcinogenicity. *In vitro* they inhibit the activity of a number of mammalian enzymes including histidine decarboxylase, alleviating histamine-induced gastric acid secretions; hyaluronidase, responsible for the breakage of glucosaminidic bonds involved in tumour cell invasiveness and hypersensitivity phenomena; the catecholamine-metabolising enzyme COMT resulting in adrenaline-sparing effect; lens aldose reductase, implicated in the pathogenesis of cataracts in diabetic animals; protein kinase C (serine/threonine phosphorylation) involved in a wide range of cellular activities including tumour promotion, mitogenesis, inflammation and T lymphocyte functions; and lipoxygenase which metabolises arachidonic acid released from membrane phospholipids to vasoactive leukotrienes that are involved in signal transduction and cell division processes related to immune systems and activated by hormones, neurotransmitters and growth.

Certain flavonoids exert antitoxic and hepatoprotective effects on X-irradiation-induced capillary permeability of rat intestine with the promise of use of related compounds as antagonists in the treatment of liver diseases and those associated with vascular permeability and capillary fragility.

Some of the endocrine effects include those of quercetin and hesperitin in reducing the sensitivity of membrane calcium ATPase to thyroid-hormonal stimulation of human RBCs and that of phyto-oestrogenic activity of the clover isoflavione formononetin, which competitively antagonises oestradiol binding to cytoplasmic oestrogen receptors thus causing infertility syndrome in sheep.

Most flavonoids with a free 3-hydroxyl group such as kaempferol and quercetin are mutagenic in different strains of *Salmonella typhimurium, E. coli* and *Saccharomyces cerevisiae*, but do not appear mutagenic in mammals. Quercetin has been reported to inhibit many biochemical events associated with tumour promotion such as alteration of protein kinase C and lipoxygenase activities. In general they inhibit carcinogenesis by acting as “blocking agents” via (1) inhibiting metabolic P450-mediated activation of the carcinogen to its reactive intermediates, (2) inducing the enzymes involved in detoxification of the carcinogen, and/or (3) binding to reactive forms of the carcinogen. They are known to inhibit several biochemical events associated with the transformation of non-malignant fibroblasts to sarcoma cells [Ibrahim, 2000].

8.3.4 Pharmacokinetics (adapted from Williamson, 2000]

Flavonoids and cinnamates are secondary metabolites synthesised by plants for defensive purposes i.e. they act as defence against UV light, protect against pathogen attack and are involved in repair of injury as well as play important structural roles in the plant cell wall. Most plants store phenolics attached to a hydrophilic moeity such as a sugar. This renders them more soluble and easily handled by the plant but less biologically active. Certain phenolics are particularly bioactive and have pronounced effects on mammalian cells including antioxidant activity, modulation of gene expression, enzyme inhibition and receptor binding. These effects have led to the belief that a diet rich in fruit and vegetables contribute to health. There still remain large gaps in our knowledge of uptake and metabolism of dietary phenolics and particularly on the biological effects of the phenolic metabolites.

8.3.3.1 ABSORPTION

Many phenolic aglycones are hydrophobic and passively pass through biological membranes. Linkage to a sugar or organic acid increases the water solubility and limits passive diffusion, therefore if phenolic aglycones are absorbed in the small intestine some form of transport mechanism must exist. Flavonols and flavones are absorbed from ingested food and appear rapidly in the plasma (<0.7h). This means that absorption occurs primarily in the small intestine although additional absorption from the colon may still occur. Some are absorbed more rapidly than others indicating that an attached sugar will affect the rate of absorption as the sugar can vary in type and position. It may be possible for the flavonol glycosides to be selectively absorbed in the gut. There is an interaction of some flavonol glycosides with the sodium-dependant glucose transporter (SGLT1) of the rat small intestine. They might however interact by inhibiting SGTL1 but not themselves be transported across the membrane. There is also considerable interspecies variation in SGLT1

properties and therefore conflicting date suggests that further evidence is required to determine the role of sugar transporters in flavonoid glycoside uptake.

8.3.3.2 METABOLISM

The presence of quercetin glucosides have been reported in plasma but positive proof of identification has not yet been provided. The first stage of metabolism is likely to be deglycosylation of the flavonoid glycoside, which is essential if further metabolism is to occur. This could take place either outside the cell, in the gut lumen or inside the enterocyte after transport. The cytosolic β-glucosidase present in cell-free extracts of human small intestine and liver can hydrolyse carious phenolic glucosides.

Once absorbed, phenolics will be metabolised by phase-I or phase-II enzymes. Cytochrome P450 mono-oxygenase dependant activities that may be involved include hydroxylation and demethylation. Two or more hydroxyl groups in the B-ring prevented further hydroxylation. Demethylation is observed when a methyl group is present in the 4’ position but not in the 3’ position. Several investigations of quercetin metabolism in rate have found 3’- and 4’-methylquercetin in the bile and/or the urine. The 3’-position (relative to 4’) for methylation appears to be the preferential site for catechol-O-methyltransferase activity with a ratio of >2:1. There is no evidence for methylation at any other hydroxyl groups of the flavonoids apart from the catechol grouping on the B-ring.

The liver plays a major role in the metabolism, however certain flavonoid glucosides are deglycosylated by human intestinal mucosa at a rate potentially higher than that of the liver. Conjugation with glucuronide or sulphate is the most likely metabolic routes. Conjugation increases solubility and increases molecular mass, important for excretion in the bile. Sulphation is the major pathway for many at low concentrations, although this pathway can easily become saturated, whereas glucuronidation will be dominant at higher concentrations.

8.3.3.3 EXCRETION

The major route for excretion of flavonol conjugates is probably through the bile back into the small intestine. Flavonol glucuronides will reach the colon where gut microflora release the aglycone leading to reabsorption and enterohepatic circulation.

Pharmacokinetic knowledge is extremely important if flavonoids are to be used in the treatment of disease. In microbial infections they need to be adequately absorbed and distributed to the site of infection where they are required to have selective working against the invading microorganism without damaging host cells. Excretion routes may determine whether the flavonoid will for example be effective in urinary tract infections.

Of all seven flavonoids isolated in this study, all showed varying degrees of inhibition against some of the selected organisms. Bioassays performed were all *in vitro* and hence the selectivity of these compounds for microorganisms in relation to host cells is not known. Since they do not appear toxic, it is necessary to determine their pharmacokinetic profiles in test animals at a later stage.

8.3.4 Presence of flavonoids in Combretaceae

The order Myrtales including Myrtaceae, Combretaceae and Thymeleaeceae contains 17 known flavones and 1 flavonol [Wollenweber, 1988]. With regard to accumulation of free aglycones in certain families and/or genera, Combretaceae has been found to have 1 flavone and 3 flavonols [Wollenweber, 1980] of which a few examples are given on the following page.

Terminalia arjuna	(1) 5,7,2',4'-OMe flavone present in fruit
	(2) 5,7,2',4'-OMe arjunone in fruits
Calicopteris floribundus	(3) 3,4-OH-3,6,7,8-OMe flavone
	[Calicopterin (Thapsin) in leaf]
	(4) 3,6,7,8,4'-OMe in leaf
	(5) 3,6,7,8,3'-OMe in leaf

Recently apigenin was identified in *C. apiculatum* [Katerere, unpublished 2001] but no known reports on isolated flavonoid compounds have been recorded in *C. erythrophyllum* and it is assumed that this is a first report of such findings.

Certain substitutions of the aromatic rings of flavonoids are more common in certain families than in others and the frequencies of the substitutions at each of the carbon atoms is shown in Table 8.1.

Table 8.1: Frequencies of the classical O- and C-substitutions at each of the ten substituted carbon atoms of the flavone/flavonol nucleus [adapted from Wollenweber, 1988].

	Frequencies (%)									
Substitutions	C-3	C-5	C-6	C-7	C-8	C-2'	C-3'	C-4'	C-5'	C-6'
Flavones										
H	99	10	46	10	57	76	53	30	78	94
OH	0	70	10	43	8	15	22	32	6	2
OMe	0	20	41	47	34	9	25	37	16	4
C-Me	1		3		1					
Flavonols										
H	0	7	48	1	56	85	44	14	70	100
OH	38	79	10	51	11	9	31	45	16	0
OMe	62	14	34	48	28	6	25	41	14	0
C-Me			8		5					

At C-3, hydroxyl and methoxyl substitutions are of equal frequency in ferns, Betulaceae, Fabaceae and Saxifragaceae; 3-methoxyl derivatives are more abundant in Asteraceae, Cistaceae and Geraniales and dominant in Didiereaceae, Laminaceae and Solanaceae. Hydroxylation at C-5 is commonly present among flavonols. 6-hydroxyl substitution is poorly represented in most taxa but in contrast 6-O-methylation appears to be an important element of flavone diversity. 6-C-Methyl derivatives are characteristic for Myrtales and ferns. There are reports of 7-deoxyflavonols in Rutaceae and especially Myrtales. Substitution at C-8 as absent or rare except for the flavonols from Asteraceae, Lamiaceae, Rutinaceae and Solanaceae. Lamiaceae, Myrtales and Solanaceae are remarkable for the presence of 2'-O-substitution in about 20% of flavonols. They are important since 40-50% of the flavonols from these taxa do not have 4'-O-substituents. O-substitution at C-5' is rare in flavonols and 6'-O-substitution is mainly found in Laminaceae. The C-6 position of flavonols is rarely if ever O-substituted in Cistaceae, Geraniales and Solanaceae. In contrast it is most commonly substituted in Asteraceae and Didiereaceae. O-substitution is rare in flavonols, overall frequency being 20% [Wollenweber, 1988].

Of the seven flavonoids identified in this study, all three flavones have a hydrogen group at C-3 and all four flavonols are hydroxylated. At C-5, all flavonoids are hydroxylated except for the flavonol CE46 and all have a hydrogen group at C-6. Hydroxyl substitutions at C-7 are seen in one flavone and two flavonols, the others being methoxyl substitutions. Hydrogen groups are present in all the flavonoids at C-8, C-2' and C-3' and hydroxyl groups in all flavonols at C-5'. Two flavones (apigenin and genkwanin) have hydroxyl substitutions at C-5'. At C-6' all flavonoids have a hydrogen group.

8.4 BIOASSAYS

8.4.1 Antimicrobial activity

Since phenolic substances generally have significant antimicrobial activity, it is assumed that their function in tissues where they accumulate might be to provide chemical barriers to invading micro-organisms. Swain postulated that the methylated, lipophilic flavonoids are especially suitable as protection against microorganisms because of their ease in penetration of the bacterial membranes [Wollenweber, 1980].

Flavonoids were reported by scientists in some studies to be more active against Gram-negative bacteria contrary to other literature reports indicating that Gram-positive are selectively inhibited by flavonoids and isoflavonoids derived from plants. Basile [1999] suggests that the pattern of selectivity towards Gram-positive bacteria is not restricted to compounds from plants but is a general observation amongst most antibiotics. All isolated flavonoids in this study were found to inhibit some of the selected bacteria to varying degrees and did not exhibit greater activity against either Gram-positive nor –negative organisms. Activity confirms the ethnobotanical use of this plant.

8.4.2 Cytotoxicity

Flavonoids are consumed in great quantities from the foods we eat and there are no reported toxicity levels for these compounds. Many flavonoids have however been found to be toxic to cancer cells and are currently being investigated for this use. They are believed to be implicated in the lower risk of some forms of cancer observed in Asian countries due to their capacity to control cell proliferation, act on certain regulatory enzymes as protein kinases or topoisomerases [Lopez-Lazaro, 2000a].

Genkwanin was reported to be cytotoxic to human nasopharynx carcinoma (IC_{50} 30.6 μM). Their hydroxyl groups at C-5 and C-4' and methoxy groups at C-7 and C-3' are important for inhibition of calf thymus topoisomerase I activity [Zahir, 1996]. Activity against human promyelocytic leukaemia (HL-60) cells lay in the order of 18.3 μM (ED_{50}) [Suh, 1995].

Two new rhamnazin glycosides were found to stabilize the cleavage complex human DNA topoisomerase I at concentrations in the 100-250 μM range acting as topoisomerase I poisons and a test against 3 human cancer cell lines: TK-10 (renal adenocarcinoma), MCF-7 (breast adenocarcinoma) and UACC-62 (melanoma) produced a dose-dependant inhibition of cell growth at concentration in the 10^{-6} to 10^{-4} M and was the most cytotoxic of all flavonoids and extracts tested [Lopez-Lazaro, 2000].

8.4.3 Antioxidant/anti-inflammatory

Many flavonoids exhibit remarkably high radical-scavenging activity indicating perhaps that a diet rich in these flavonoids would reduce cancer-promoting actions of these radicals and possibly other diseases and conditions caused by oxygen-related radicals [Sawa, 1999].

8.5 SUMMARY OF RESULTS

CODE	IDENTITY	MIC (µg/ml) GRAM +	MIC (µg/ml) GRAM -	ANTIOXIDANT ACTIVITY	TOXICITY
CE144	Apigenin	NT	NT	NT	NT
IIIa150	Genkwanin	25 - >100	50 - > 100	GOOD	NON-TOXIC
CE36	5-hydroxy-7,4'-dimethoxyflavone	50 - > 100	25 - > 100	POOR	POTENTIALLY TOXIC
Seph51	Kaempferol	NT	NT	NT	NT
IIIa90	Rhamnocitrin	25 - > 100	25 - > 100	STRONG	NON-TOXIC
CE51	Rhamnazin	25 - > 100	50 - > 100	STRONG	NON-TOXIC
CE46	Quercetin-5,3'-dimethylether	25 - > 100	50 - > 100	GOOD	NON-TOXIC

NT = NOT TESTED

8.6 CONCLUSION

The aim of this study was to isolate and identify the antimicrobial components from *Combretum erythrophyllum* leaf extracts and confirm the ethnobotanical use of this plant. In comparison to other *Combretum* species, *C. erythrophyllum* appears far more complex and to isolate all antimicrobial compounds is a challenging task. This is primarily because most of the compounds isolated (even those not identified) have a potential to be antimicrobial in the correct dose. Waxes isolated from the hexane fraction are strongly antimicrobial but cannot be utilised in medicine due to the non-specificity for host and bacterial cells as well as their poor pharmacokinetic profile.

In this study seven flavonoids were identified of which three were flavones, namely apigenin, genkwanin and 5-hydroxy-7,4'-dimethoxyflavone and four were the flavonols kaempferol, rhamnocitrin, rhamnazin and quercetin-5,3'-dimethylether. All these compounds are reported for the first time in *C. erythrophyllum*. Although these flavonoids did exhibit moderate antimicrobial activity, it was hardly the anticipative results. Many of these compounds are produced in response to infection (phytoalexins) and quantities produced are very small making isolation difficult. For this reason large quantities of crude material is required for proper evaluation.

As tropical forests are destroyed and tribal people acculturated, our ability to discover new pharmaceutical agents is seriously being compromised. There has been a decline in natural product research over the past few decades, combined with a reduction in global plant biodiversity [Balick, 1990]. Plants remain an untapped reservoir of potentially useful chemical compounds as drugs, as unique templates that could serve as a starting point for synthetic work, or as tools for understanding the biological processes better [Farnsworth, 1984]. Baker [1984] suggests that the question of importance is whether the traditional screens of pharmaceutical industries are adequate to ensure the detection of novel substances with novel mechanisms of action. The answer in many cases is NO. For this reason ethnobotanical studies are of increasing importance and with further work, *Combretum erythrophyllum* could contribute more towards medical science than just supplying shade on a sunny day.