

# Chapter 1

## Introduction

### 1.1 Introduction

With the increasing incidence of diseases caused by viruses and other pathogenic microorganisms, as well as the development of drug resistance, there is an urgent need to search for alternatives from plants and other sources to combat these pathogens. The development of a new antiviral drug is difficult taking into account poor selective toxicity and fast development of resistant viral variants with the existing drugs. Frequencies of viral resistance to antiviral drugs are increasing and the difficulty of viral latency remains unsolved (Kott *et al.*, 1999). The ease of national and international travel means that resistant organisms can be transported easily, making it a global problem. Despite all efforts by health bodies the threat of viral and other infectious diseases persists, making the search for more effective and efficient drugs ever more pressing.

In the past decade, considerable attention has been given to screening of plant extracts for possible anti-HIV activity. Such endeavours have been undertaken with the aim of isolating bioactive compounds as an alternative source to chemical synthesis. Screening of plant extracts for antiretroviral activity has given interesting results in that most plant-derived anti-HIV active compounds inhibit the replication of the virus by interfering with one or more of the ten steps of the HIV replicative cycle (Vlietinck *et al.*, 1998; Mathee *et al.*, 1999). Anti-HIV active compounds of plant origin do not fall into a certain class of compounds but rather possess diverse chemical structures (Vlietinck *et al.*, 1995). The therapeutic efficacy of a handful of these compounds, such as the benzylisoquinoline alkaloid, papaverine (Bassetti *et al.*, 1989) and the saponin, glycyrrhizin (Hattori *et al.*, 1989), has been studied in AIDS patients. It is evident therefore that plants can be useful sources or leads for the discovery of novel anti-HIV compounds.

In a study by Rajbhandari *et al.* (2001), 23 medicinal plant species were tested against influenza virus and herpes simplex virus. Antiviral activity was found in 15 of the tested extracts and for the influenza virus, seven plant extracts had antiviral activity with an inhibitory concentration 50 (IC<sub>50</sub>) ranging between 10 and 87 µg/ml. For the herpes simplex virus, 12 plant extracts were active with the highest antiviral IC<sub>50</sub> = 10 µg/ml. Many plants have therefore been shown to have antiviral and other antimicrobial activities.

## 1.2 Antimicrobial resistance

### 1.2.1 Antiviral resistance

During the last few decades, significant advances have been made in the development and use of antiviral agents for the successful treatment of a number of viral infections. An expanding array of antiviral drugs is currently available for the management of infections caused by herpes simplex virus types 1 and 2 (HSV-1 and HSV-2), cytomegalovirus (CMV), varicella-zoster virus (VSV), influenza A virus, respiratory syncytial virus (RSV), human immunodeficiency virus type 1 (HIV-1), papillomaviruses, and hepatitis B and C viruses. The increased number and use of antiviral agents, however, has led to the emergence of drug-resistant viruses, particularly in immunocompromised patients e.g. patients with AIDS, hematological malignancy or those who have undergone organ transplantation (Duguid *et al.*, 1978).

Clinical situations that favour the development of resistance include long-term suppressive therapy, recurrent intermittent therapy, and the use of less than optimum doses of an antiviral agent. Generally, the emergence and isolation of drug resistant viruses is associated more with the therapeutic use of antiviral agents and does not seem to be caused by prophylactic treatment. As more patients fail to respond to appropriate therapy and additional antiviral agents are produced, it will also become important for diagnostic virology laboratories to provide rapid and practical antiviral susceptibility testing to assist physicians in defining drug resistance and choosing appropriate alternative therapies (Duguid *et al.*, 1978).

### 1.2.2 Antibiotic resistance

The widespread use of antibiotics, both for human consumption and animal production, has fostered the development of resistance in a variety of pathogenic bacteria (Dessen *et al.*, 2001). The emergence of bacterial strains that exhibit resistance to a variety of antibiotics, i.e. strains that are multi-drug resistant, is becoming the major cause of treatment failure of infections worldwide. The treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) generally requires vancomycin as a last resort, while enterococcal strains that no longer respond to vancomycin have been identified (Novak *et al.*, 1999). Most antibiotics used in humans originated from natural templates produced by particular species of bacteria or fungi as a mechanism of competition to ensure their own survival (e.g. to gain a larger share of environmental food supplies by killing competitors). As the ability to produce lethal chemicals was

developed by microorganisms, so was the counter-measure in this war for survival - namely antibiotic resistance. For example, in natural environments such as the soil, bacteria can develop resistance through mutation, or can exchange genetic information (including resistance genes) with great facility and relatively low species specificity, thus permitting the transmission of molecular determinants of resistance to other microbes with great ease. Mechanisms of resistance fall into three main categories: the inactivation of the antibiotic by modification of its active chemical moiety; the specific modification of the macromolecular target (i.e. by mutagenesis of key residues); and the prevention of antibiotics from reaching their targets through decreased uptake or active antibiotic efflux (Walsh, 2000).

### 1.2.3 Factors contributing to development of resistance to antimicrobial drugs

#### 1.2.3.1 Failure to use narrow-spectrum antibacterial drugs

The widespread and often inappropriate use of broad-spectrum antibiotics in the outpatient setting is recognized as an important contributing factor to the spread of resistance (Hancock, 2005). Optimal and judicious selection of antibiotics for the therapy of infectious diseases requires clinical judgment and detailed knowledge of pharmacological and microbiological factors (Hancock, 2005). When the infecting microorganism has been identified, it seems appropriate to institute definitive antibiotic therapy with a narrow spectrum, low toxicity agent as an anti-resistance measure (Hancock, 2005).

Several investigations indicate that some infections, such as community acquired pneumonia (CAP) and urinary-tract infections, can usually be successfully treated with narrow-spectrum antibiotics, especially if the infections are not life threatening (Gleason *et al.*, 1997; Cunha, 1997; Ailani *et al.*, 1999).

#### 1.2.3.2 Colonization pressure in hospitals

The risk of acquisition of a particular bacterial infection as a function of the proportion of people colonized has been called “colonization pressure”, and has been described as a major variable affecting the spread of methicillin-resistant *Staphylococcus aureus* (MRSA) (Merrer *et al.*, 2000) and vancomycin-resistant *Enterococcus faecalis* (VREF) (Bontem *et al.*, 1998). The widespread adoption of antibiotic control measures and promotion of strict adherence to infection-control procedures are necessary to prevent the colonization pressure observed in hospitals, especially intensive care units (ICU) (Weinstein, 2001). Quantitative analysis of VREF transmission in an ICU indicates that staffing levels have a critical role in transmission, and that a productive alliance between patients and staff is a very

effective means of decreasing transmission, such that the level of adherence to hand hygiene is an inverse function of the endemic level of VREF colonization (Austin *et al.*, 1999).

Alcohol-based hand rubs seem to be promising as hand-disinfectant agents, but maintaining compliance may require continuous educational reinforcement, monitoring, and feedback to health-care workers (Weinstein and Hayden, 1998). An alternative approach to the colonization pressure problem is to encourage the use of disposable examination gloves during contacts with patients and their environment (Weinstein *et al.*, 2001; Badri *et al.*, 1998).

### 1.2.3.3 Length of hospital and ICU stays

Prolonged length of hospital stays appears to predispose people to infection with antibiotic resistant bacteria (Bontem *et al.*, 1998; Trouillet *et al.*, 1998). This predisposition may result, in part, from the greater likelihood over time of becoming colonized with such bacteria or the generally poorer underlying immune status of the most seriously ill patients. In addition, the use of invasive devices, such as endotracheal tubes, intravascular catheters, and urinary catheters, seems to encourage such infections (Richards *et al.*, 1999; Kollef *et al.*, 1997a). The rising presence of antibiotic-resistant infections among people in long-term treatment facilities can also be an important source for the entry of resistant bacteria into the ICU (Wiener *et al.*, 1999). Furthermore, outbreaks of antibiotic-resistant bacterial infections resulting from inadequate infection-control practices, failure to recognize the presence of antibiotic resistance, or use of contaminated equipment are also key factors promoting the spread of resistance (Rahal *et al.*, 1998; Alfieri *et al.*, 1999; Reboli *et al.*, 1996). A reduction in the duration of mechanical ventilation could decrease the incidence of ventilator-associated pneumonia and consequently reduce the length of hospital or ICU stays (Kollef *et al.*, 1997b).

### 1.2.3.4 Antibiotic misuse in agriculture

One of the most fundamental measures that could be taken to minimize antibiotic resistance is to eliminate supplementation of animal feeds with antibiotics, including tetracyclines, macrolides, and quinoline derivatives (Cunha, 2003). Resistant strains arising from this source can enter the human population through infection of farm workers, contamination of the ground water, or consumption of colonized animal and poultry products (Hancock, 2005).

Farm practices involving the use of antibiotics as feed additives and prophylactics should be carefully reviewed to eliminate the use of those agents that give rise to cross-resistance to antibiotics used in human medicine (Hancock, 2005). Certain countries have banned some feed additive use and the WHO has recommended the discontinuation of the use of antibiotics as growth promoters because of the evidence of health risks in human beings (Levy, 1998). Surveillance to give early warnings of emerging problems would allow more time to evaluate prevention and control. Better education of practitioners, both in the community and in hospitals, and the phasing out of the use of antibiotics in animal husbandry and agriculture would be important steps towards limiting resistance (Hancock, 2005).

#### 1.2.3.5 The FAAIR initiative

The aim of the Facts about Antibiotics in Animals and their Impact on Resistance (FAAIR) initiative, developed by the Alliance for the Prudent Use of Antibiotics (APUA), is to introduce scientific evidence to the policy debate on antimicrobial use in agriculture and the risk it poses to human, animal, and ecological health (Levy, 2000). APUA convened an expert scientific advisory panel from a variety of fields in research and medicine. The committee concluded that the elimination of non-therapeutic use of antimicrobials in food animals and in agriculture would lower the burden of antibiotic resistance in the environment, with consequent benefits to human and animal health (Barza, 2002). All uses of antimicrobials in animals, agriculture and human beings contribute to the global pool of antimicrobial-resistance genes in the environment.

The use of antibiotics by physicians in hospitals and elsewhere requires an acute awareness of the increasing problems with resistant organisms. This awareness is especially important given the limited availability of fundamentally new antibiotics. Thus, unnecessary use of antibiotics has public health implications. Such use may serve to select for resistant organisms that may be carried to other, more vulnerable patients, and produce serious, difficult-to-treat infections. Antibiotic control programmes can be an effective means to prevent inappropriate use of antibiotics in hospitals. Newer antibiotics should be included in such programmes to delay the emergence of bacterial resistant strains by limiting unnecessary use of such drugs (Hancock, 2005).

### 1.3 Viruses and viral diseases

Viral diseases were largely untreated 40 years ago. Now, effective and safe therapies are available. This has led to significant improvements in the quality of life for large numbers of patients. New viral

diseases are, however, continuing to emerge and established viruses have been shown to develop resistance to available therapies making this a fertile area for continued drug discovery. In this study, greater attention will be paid to literature concerning the antiviral activity of plants compared to antibacterial activity, as antiviral explorations of plant compounds are comparatively few.

We tend to think of viruses as the smallest of the microorganisms responsible for infectious diseases. Yet, strictly speaking, they are not microorganisms, for they are not cells. Unlike bacteria, they contain no organelles and have no metabolism, consisting basically of a nucleic acid genome enclosed in a protective coat of protein. They are metabolically inert until they enter the host cell upon which they are absolutely dependent for their replication. It is true that most viruses do carry a nucleic acid polymerase which can, under certain experimental situations, transcribe messenger RNA from the viral genome in a test-tube, and that certain additional steps involved in viral replication have been successfully accomplished *in vitro*. However, under natural circumstances, viruses are capable of multiplying only inside living cells. They are thus obligate intracellular parasites, which utilize the organelles and metabolic pathways of the host cell for their own reproduction.

### 1.3.1 Structure of viruses

The genome of a virus consists of either DNA or RNA. 'The latter is unique in biology, no other living creature employs RNA as its repository of genetic information' (Duguid *et al.*, 1978). Furthermore, viral nucleic acid (NA), whether DNA or RNA, may be single- or double-stranded. It may be linear or circular. It may consist of a single "polycistronic" molecule, analogous to a chromosome comprised of a string of genes; alternatively the genome may be "segmented", occurring as a number of distinct molecules, each being a separate gene. Moreover, single-stranded viral nucleic acid may be of positive or negative "polarity". If positive it represents meaningful information and the viral RNA acts as messenger RNA; if negative, i.e. complementary RNA, messenger RNA must first be transcribed by a transcriptase carried in the virion (Duguid *et al.*, 1978). The nature of the viral nucleic acid dictates the strategy of viral replication. It also forms the basis of viral classification (Duguid *et al.*, 1978).

Systematic attempts to develop taxonomic criteria for classifying viruses began only in the 1960s and since that time have been under the control of the International Committee on Taxonomy of Viruses (ICTV). This has been achieved on the basis of a number of parameters which may be grouped under two main headings: (1) the nature of the genome and (2) the structure of the virion.

Viruses are God's creatures and no matter how much we try to eradicate them, they will always be there. There are viruses of all vertebrates, not only mammals (Fenner *et al.*, 1974). There are viruses of invertebrates (Gibbs, 1973), protozoa (Diamond and Mattern, 1976), and algae (Sherman and Brown, 1978). Indeed, there is reason to believe that the number of distinct species of viruses on earth far exceeds the number of species of all living things (Diamond and Mattern, 1976). Every species that has been studied, e.g. man, monkey, mouse, *Escherichia coli*, has yielded dozens of different viruses. By no means have all of them produced disease, though many do under certain circumstances. There are many other viruses with significant impacts in humans and animals and some of them will be discussed.

### 1.3.2 HIV and AIDS

As an example of the potentially devastating effect of viruses, HIV is an extremely important virus. In June 1981, scientists in the United States reported the first clinical evidence of a disease that would later become known as acquired immunodeficiency syndrome, or AIDS. Twenty-five years later, the AIDS epidemic has spread to every corner of the world, but the continuous struggle to control the epidemic has also yielded a growing list of breakthroughs (UNAIDS, 2006; [www.unaids.org](http://www.unaids.org)). An estimated 38.6 million people world-wide were living with HIV at the end of 2005. An estimated 4.1 million became newly infected with HIV and an estimated 2.8 million lost their lives to AIDS. Overall, the HIV incidence rate (the proportion of people who have become infected with HIV) is believed to have peaked in the late 1990s and to have stabilized subsequently, notwithstanding an increasing incidence in several countries (UNAIDS, 2006; [www.unaids.org](http://www.unaids.org)). Favourable trends in incidence in several countries are related to changes in behaviour and prevention programmes. Changes in incidence along with rising AIDS mortality have caused global HIV prevalence (the proportion of people living with HIV) to level off.

The numbers of people living with HIV have continued to rise due to population growth and, more recently, the life-prolonging effects of antiretroviral therapy. In sub-Saharan Africa, the region with the largest burden of the AIDS epidemic, data also indicate that the HIV incidence rate has peaked in most countries. However, the epidemics in this region are highly diverse and especially severe in southern Africa, where the epidemics are still expanding. Among the notable new trends are the recent declines in national HIV prevalence in two sub-Saharan African countries (Kenya and Zimbabwe), urban areas of Burkina Faso, and similarly in Haiti, in the Caribbean, alongside indications of significant behavioural change, including increased condom use, fewer partners and delayed sexual debut. In the rest of sub-

Saharan Africa, the majority of epidemics appear to be leveling off, but are still at exceptionally high levels in most of southern Africa (UNAIDS, 2006).

Africa remains the global epicenter of the AIDS pandemic. South Africa's AIDS epidemic, one of the worst in the world, shows no evidence of a decline. Based on its extensive antenatal clinic surveillance system, as well as national surveys with HIV testing and mortality data from its civil registration system, an estimated 5.5 million people were living with HIV in 2005. An estimated 18.8% of adults (age 15-49) were living with HIV in 2005 (UNAIDS, 2006). Almost one in three pregnant women attending public antenatal clinics were living with HIV in 2004 and trends over time show a gradual increase in HIV prevalence. There are no clear signs of declining HIV prevalence elsewhere in southern Africa, including Botswana, Namibia and Swaziland, where exceptionally high infection levels continue.

### 1.3.3 Other viral infections

Severe acute respiratory syndrome (SARS) is a viral respiratory illness caused by a coronavirus, called SARS associated coronavirus (SARS – CoV). SARS was first reported in Asia in February 2003. In a few months, the illness spread to more than two dozen countries in North America, South America, Europe, and Asia before the SARS outbreak of 2003 was contained. SARS begins with a high fever (temperature greater than 38°C). Other symptoms may include headache, an overall feeling of discomfort, and body aches. Some people may have mild respiratory symptoms at the outset. About 10 to 20 percent of patients have diarrhea. After 2 to 7 days, SARS patients may develop a dry cough. Most patients develop pneumonia ([www.cdc.gov/mmwr/preview/mmwrhtml/mm5249a2.htm](http://www.cdc.gov/mmwr/preview/mmwrhtml/mm5249a2.htm)).

Avian influenza or “bird flu” is an infectious disease of animals (usually birds, and less commonly pigs) caused by type A strains of the influenza virus. Transmission to humans is rare, but there is recent cause for concern. In mid 2003, the largest and most severe avian flu outbreak in history began in South East Asia, caused by a sub-type of the virus called H5N1 and resulting in widespread transmission to poultry and some documented transmission to humans. Transmission of H5N1 to humans is of particular concern because it mutates rapidly and may therefore change into a form that is highly infectious for humans and more easily spread. Unlike normal seasonal influenza, H5N1 can cause severe disease in humans ([www.who.int/csr/disease/avianinfluenza/country/en/](http://www.who.int/csr/disease/avianinfluenza/country/en/)).

Acute respiratory infections caused by viruses are the major cause of morbidity and mortality in children all over the world. Respiratory syncytial virus (RSV) is a common cause of pneumonia and bronchitis in



infants, in young children, and even adults (Hruska *et al.*, 1982; Treanor and Falsey, 1999). It can also be devastating to immunocompromised populations (Wyde, 1998). In addition, re-infections are a common event, suggesting that naturally acquired immunity does not provide long lasting protection (Dubovi *et al.*, 1981).

Plant extracts contain many antimicrobial compounds (Cowan, 1999). Some of these compounds may be useful in treating viral infections. In the next chapter, a literature review on the therapeutic use of plant compounds will be presented, followed by the aims and objectives of this study.

## Chapter 2

### Literature review on the therapeutic use of plant compounds

#### 2.1 Natural product drug discovery

For decades, natural products have been a wellspring of drugs and drug leads. When you have no idea where to begin in a drug discovery programme, nature is a good starting point. According to a survey by Newman and co-workers (1997) of the National Cancer Institute, 61% of the 877 small molecule new chemical entities introduced as drugs worldwide during 1981–2002 can be traced to or were inspired by natural products (Newman *et al.*, 2003). These include natural products (6%), natural product derivatives (27%), synthetic compounds with natural-product-derived pharmacophores (5%), and synthetic compounds designed on the basis of knowledge gained from a natural product (i.e. a natural product mimic, 23%).

Two shortcomings of natural products are the difficulty in chemical derivatization and the small quantities available from nature. Natural products will not solve all the problems because in many cases, natural products may have negative side effects or insufficient biological activity. Synthetic chemists must investigate modification of lead compounds. It makes a lot of sense to be guided by natural products, and derivatives with validated biological relevance.

In a field that has been ravaged by herbivores, some plants, although they are without protective structures, are untouched. Perhaps they are distasteful or toxic and are therefore protected against natural enemies. Very well preserved plants may be chemically interesting. Extra-organismal interactions make up a chemical web that keeps the environment working the way it does. With the techniques now available, chemical ecology, which is the study of the chemical interactions between organisms, is poised to look at nature in a new way. To understand biotic interactions at a molecular level is both a great opportunity and a major challenge for chemists.

Chemical relevance is revealed by chemical profiling. Crude extracts are analyzed by high-performance liquid chromatography with mass spectrometric, light scattering, and ultraviolet detection. Mass spectrometry gives the molecular weight and structural information. Light scattering estimates the amount of material represented by each peak, because a minimum amount is needed to build good libraries that can be used for years. UV absorption gives additional insight on the compound's structure.

Data are fed to internally developed software that compares all the peaks in an extract with all the peaks that the software has seen. At the end, the software ranks the extracts on the basis of the number of new peaks. The high-ranking extracts will very probably contain new compounds and will be taken further to purification and structure elucidation. The technique is useful in determining whether a collection of biological materials is chemically interesting. Typically, only 10 to 20% of the initially acquired biological samples qualify for further processing by this profiling step.

## 2.2 Secondary metabolites

Fig 2.1 shows the interaction between primary and secondary plant metabolism. Secondary metabolites are molecules that are not necessary for the growth and reproduction of a plant, but may serve some role in herbivore deterrence due to astringency, or they may act as phytoalexins, killing bacteria that the plant recognizes as a threat. Secondary compounds are often involved in key interactions between plants and their abiotic and biotic environments (Facchini *et al.*, 2000).

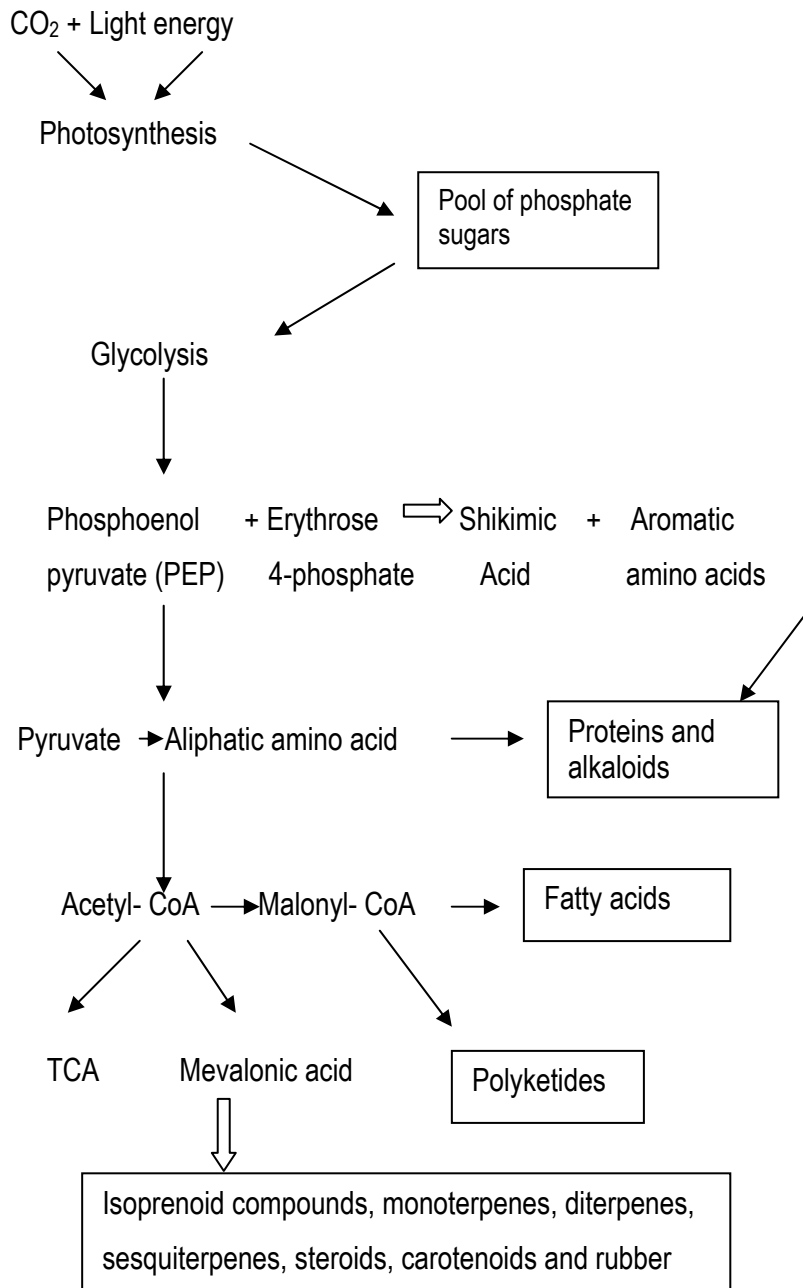
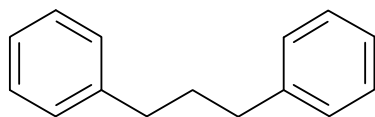


Fig 2.1. Relationship between intermediate metabolism and secondary compounds in plants

Throughout history secondary metabolites of plants have been utilized by humanity. There are approximately four major classes of secondary compounds that are significant to humans. These classes are the flavonoids, alkaloids, phenylpropanoids and terpenoids (Edwards and Gatehouse, 1999).

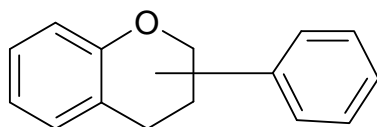
## 2.2.1 Flavonoids

The flavonoids are a large group of natural products widespread in higher plants, and are also found in some lower plants including algae. The flavonoids are phenolic compounds possessing 15 carbon atoms and comprise two benzene rings joined by a linear three carbon chain.

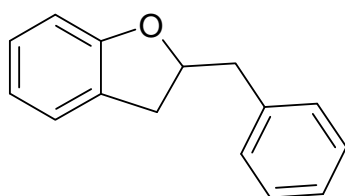


The skeleton above can be represented as the C<sub>6</sub>-C<sub>3</sub>-C<sub>6</sub> system.

Flavonoids constitute one of the most characteristic classes of compounds in higher plants. Many flavonoids are easily recognized as flower pigments in most angiosperm families (flowering plants). However, their occurrence is not restricted to flowers but includes all parts of the plant. The chemical structure of flavonoids is based on a C<sub>15</sub> skeleton with a chromane ring being a second aromatic ring B in position 2, 3 or 4.



In a few cases, the 6-membered heterocyclic ring C occurs in an isomeric open form or is replaced by a five-membered ring e.g. 2-benzyl-coumarone.



Aurones (2-benzyl-coumarone).

The oxygen bridge involving the central carbon atom (C<sub>2</sub>) of the 3C-chain occurs in a rather limited number of cases, where the resulting heterocyclic ring is of the furan type. Various subgroups of flavonoids are classified according to the substitution patterns of ring C. Both the oxidation state of the heterocyclic ring and the position of ring B are important in the classification.

Examples of the 6 major subgroups are:

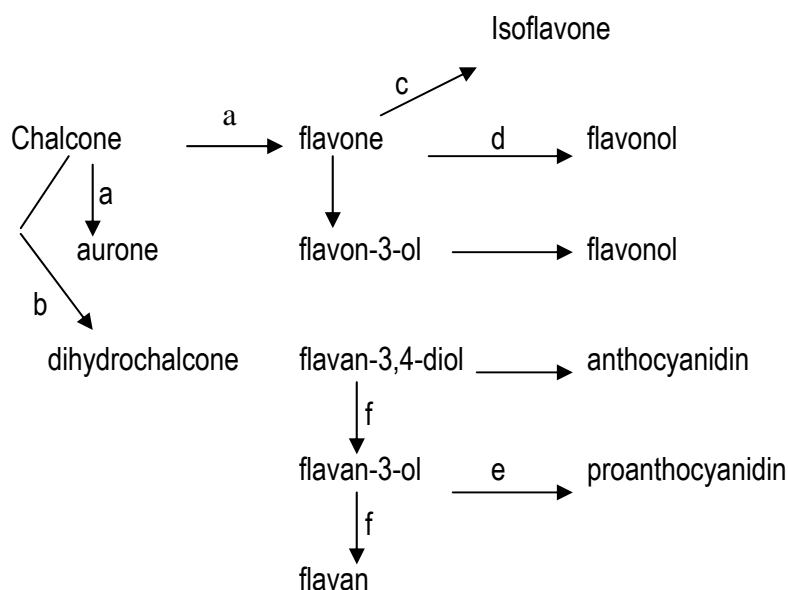


Fig 2.2. Biosynthetic relationship of flavonoids

a = cyclisation, b = bioreduction, c = aryl migration, d = dehydrogenation, e = hydroxylation, f = dehydroxylation.

Flavonoids are low molecular weight substances found in all vascular plants. In the broad sense they are virtually universal plant pigments. The anthocyanidins are responsible for flower colour in the majority of angiosperms, but colourless flavonoids are also widespread and abundant. They are phenylbenzopyrones with an assortment of basic structures usually found conjugated to sugars although the forms have been identified in nature. Flavonoids occur in several structurally and biosynthetically related classes and are important constituents of the human diet, being derived largely from fruits, vegetables, nuts, seeds, stems and flowers (Harborne, 1977).

While several members of the flavonoid family are known to possess antiviral and anti-inflammatory properties, vasculo-protector and anti-thrombotic action, spasmolytic activity, estrogenic actions, antioxidant and liver protecting effects (Middleton and Kandaswami, 1994), very little was known before 1989 on the effects of this class of compounds on the central nervous system (CNS). Some flavonoids, like quercetin and gossypin, have recently been shown to possess sedative and analgesic effects (Picq *et al.*, 1991). Another flavonoid, and biflavonoid derivatives, isolated from *Ginkgo biloba* have been shown to increase blood flow (Danser *et al.*, 1933), and reduce neuronal oxidative metabolism (Quisumbing, 1951).

## 2.2.2 Triterpenoids

Triterpenoids have been shown to have antibacterial activity and a number of triterpenes have been isolated from plants (Rogers, 1998; Angeh, 2005). Rogers (1998) isolated seven novel triterpenoids from *Combretum erythrophyllum* and Angeh (2005) isolated two new triterpenoids. Terpenes can occur as monoterpenes, diterpenes, triterpenes, and tetraterpenes (C<sub>10</sub>, C<sub>20</sub>, C<sub>30</sub> and C<sub>40</sub> respectively) as well as hemiterpenes (C<sub>5</sub>) and sesquiterpenes (C<sub>15</sub>). 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 (monoterpenes), farnesol and artemisinin (sesquiterpenoids). Artemisinin and its derivative,  $\alpha$ -arteether, find current use as antimalarials.

Triterpenoids are non-steroidal secondary metabolites. The physiological function of these compounds is generally believed to be a chemical defense against pathogens and herbivores. Throughout the plant and animal kingdom, terpenoids are known to have a wide range of functions. They can act as defensive substances in plants (allomones) and animals, they can be used by plants to deter herbivores or to inform conspecifics or attract natural enemies of herbivores (synomones). Plant hormones are often derivatives of terpenoids, such as cytokinins, gibberellins and abscisic acid. It is therefore expected that triterpenoids should act against certain pathogens causing human and animal diseases (Mahato and Sen, 1997). Although medicinal use of this class of compounds is 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 *et al.*, 1992).

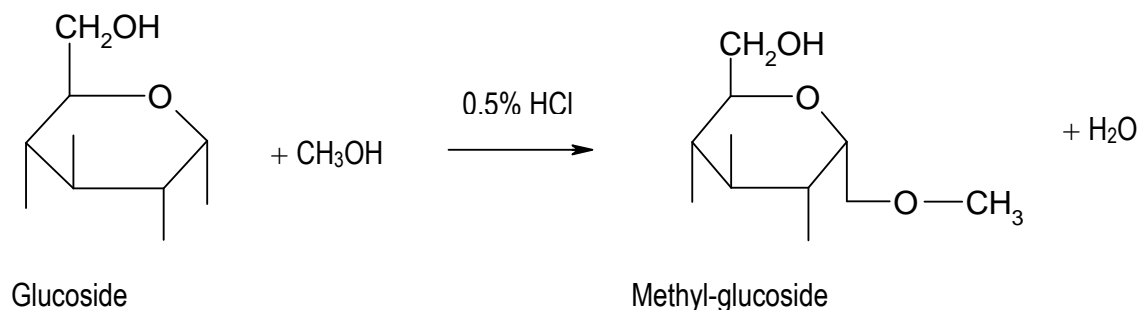
Terpenoids are active against bacteria (Taylor *et al.*, 1996), fungi (Suresh *et al.*, 1997) and viruses (Xu *et al.*, 1996). Their mechanism of action is not fully understood. Capsaicin, a constituent of chili peppers, is bactericidal to *Helicobacter pylori*, although possibly detrimental to the human gastric mucosa (Jones *et al.*, 1997). Another terpenoid called petalostemumol, isolated from the prairie clover (*Dalea* sp.) showed excellent activity against *Bacillus subtilis* and *Staphylococcus aureus* as well as *Candida albicans* (Cowan, 1999).

Terpenoids isolated from *Combretum* species include jessic acid and methyl jessate from *Combretum elaeagnoides*; imberbic acid from *Combretum imberbe* (Mahato *et al.*, 1992); combregenin, combreglucoside, arjungenin and arjunglucoside from *Combretum nigricans* (Jossang *et al.*, 1996) and arjunolic

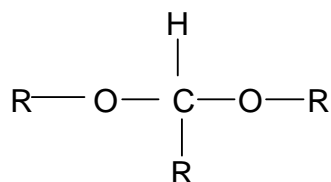
and mollic acid from *Combretum leprosum* (Facundo *et al.*, 1993), and many more which have been cited above.

### 2.2.3 Glycosides

Glycosides are compounds containing a carbohydrate and a non-carbohydrate residue in the same molecule. The carbohydrate residue is attached by an acetal linkage at carbon atom 1 to a non-carbohydrate residue or aglycone. The sugar component is called the glycone. If the carbohydrate portion is glucose, the resulting compound is a glucoside. An example is the methyl glucoside formed when a solution of glucose in boiling methyl alcohol is treated with 0.5% HCl as a catalyst.



The aglycone may be methyl alcohol, glycerol, a sterol, a phenol, etc. An acetal has two ether functions at a single carbon atom.



#### 2.2.3.1 Classification of glycosides

When the chemical nature of the aglycone group is used as the basis of systematization, the classification is as follows:



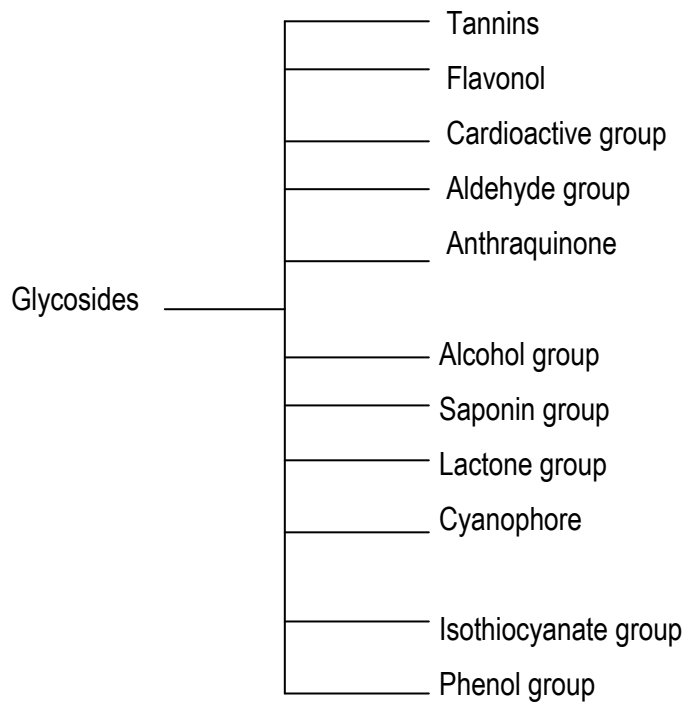


Fig 2.3. Classification of glycosides ([www.friedli.com](http://www.friedli.com))

Saponin glycosides are divided into two types based on the chemical structure of their aglycones (sapogenins). Saponins on hydrolysis yield an aglycone known as 'sapogenin'.

Saponin = Sugar (glycone) + Sapogenin (aglycone).

The so-called neutral saponins are derivatives of steroids with spiroketal side chains. The acid saponins possess triterpenoid structures.

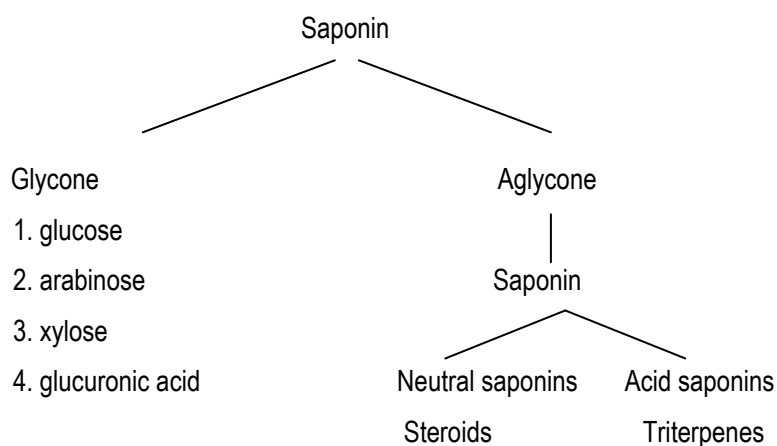


Fig 2.4. Types of saponins ([www.friedli.com](http://www.friedli.com))

The main pathway leading to both types of sapogenins is similar and involves the head-to-tail coupling of acetate units. However, a branch occurs after the formation of the triterpenoid hydrocarbon, squalene, that leads to steroids in one direction and to cyclic triterpenes in the other.

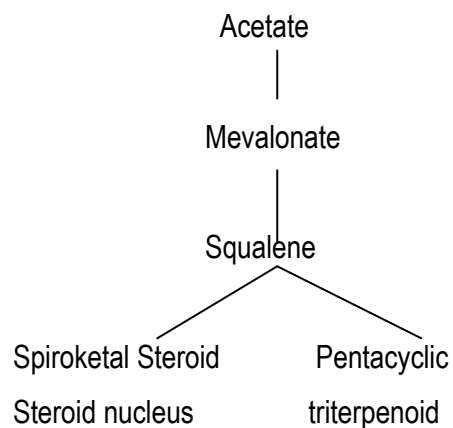
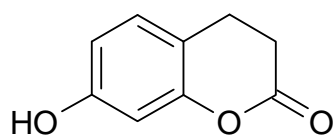


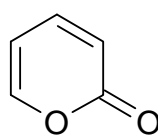
Fig 2.5. Pathway to triterpenes ([www.friedli.com](http://www.friedli.com))

#### 2.2.4 Coumarins

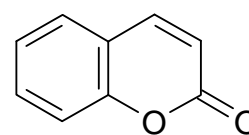
Coumarins are phenolic substances made of fused benzene and  $\alpha$  – pyrone rings (O’Kennedy and Thornes, 1997). They are responsible for the characteristic odour of hay. Their fame has come mainly from their antithrombotic (Thastrup *et al.*, 1985), anti-inflammatory (Piller, 1975) and vasodilatory activity (Namba *et al.*, 1988).



2H-1-benzopyran-2-one



2- pyrone



Benzo-2-pyrone

The benzo-2-pyrone nucleus of the simple coumarins derives from the phenylacrylic skeleton of cinnamic acids.

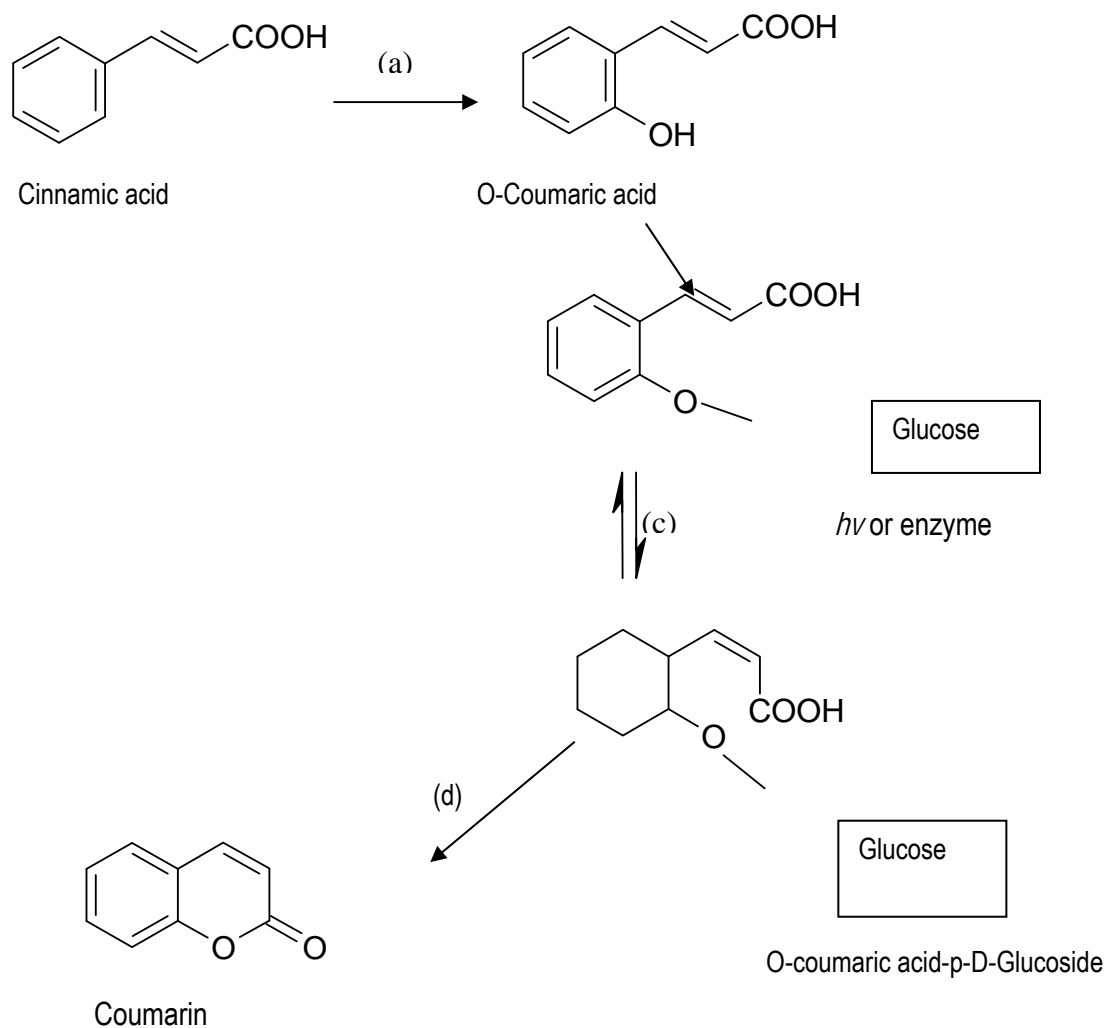
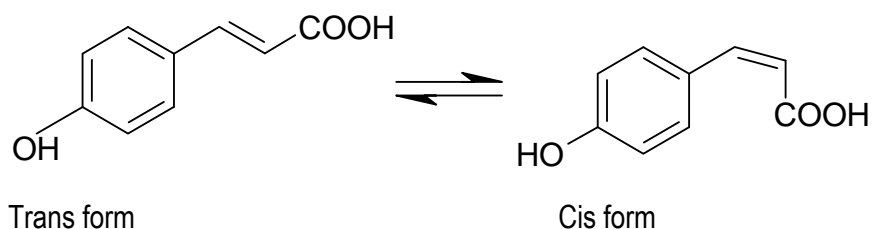


Fig 2.6. Pathway to formation of coumarins ([www.friedli.com](http://www.friedli.com))

The coumarin structure is derived from coumaric acid via ortho-hydroxylation (a), trans-cis isomerization of the side chain double bond (b) and (c), and lactonisation (d). The trans form is stable and could not cyclise, therefore there should be isomerization of some sort and the enzyme isomerase is implicated.



Umbelliferone, esculetin and scopoletin are the most widespread coumarins in nature.

## 2.2.5 Plant-derived drugs employed in Western medicine

Plants are a rich source of drugs either as direct remedies or as templates for the production of drugs. The first chemical substance to be isolated from plants was benzoic acid in 1560, but the search for useful drugs of known structure did not begin until 1804 when morphine was separated from *Papaver somniferum* L (Opium). Since then many drugs from higher plants have been discovered (Farnsworth, 1984). Less than 10 of these well-established drugs are produced commercially by synthesis although laboratory synthesis has been described for most of them (Farnsworth, 1984).

Table 2.1. Plant-derived drugs widely employed in Western medicine (adapted from Farnsworth, 1984)

Acetyldigoxin	Hyoscyanine	Quinine
Aescin	Khellin	Rescinamide
Ajmalicine	Lanatoside C	Reserpine
Allantoin	Leurocristine	Scillarenes
Atropine	A-Lobeline	Scopolamine
Bromelain	Morphine	Sennosides A & B
Caffeine	Narcotine	Sparteine
Codeine	Ouabain	Strychnine
Colchicine	Papain	Tetrahydrocannabinol
Danthron	Papaverine	Theobromine
Deserpidine	Physostigmine	Theophylline
Digitoxin	Picrotoxin	Tubocurarine
Digoxin	Pilocarpine	Vincalokoblastine
L- Dopa	Protoveratrine A and B	Xanthotoxin
Emetine	Pseudoephedrin	
Ephedrine	Quinidone	

## 2.3 A brief history of pharmacology

### 2.3.1 Historical development

Synthetic organic chemistry was born in 1828, when Friedrich Wohler synthesized urea from inorganic substances and thus demolished the vital force theory. In the early nineteenth century, it was thought that organic compounds were produced from their elements by laws different from those governing the formation of inorganic compounds. This then led to the belief that organic compounds were produced under the influence of a 'vital force' and that they could not be produced artificially. The birth of pharmacology is not as clear-cut. In the early 19<sup>th</sup> century, physiologists performed many pharmacological studies. Francois Magendie studied the action of nux vomica (a strychnine-containing plant drug) on dogs, and showed that the spinal cord was the site of its convulsant action. In 1842, Claude Bernard discovered that the arrow poison curare acts at the neuromuscular junction to interrupt the stimulation of muscle by nerve impulses (Sneader, 1985).

Pharmacology is one of the cornerstones of the drug discovery process. The medicinal chemist may create the candidate compound, but the pharmacologist is the one who tests it for physiological activity. A promising compound is investigated by many other scientists - toxicologists, microbiologists, clinicians - but only after the pharmacologist has documented a potential therapeutic effect.

The main tasks of pharmacologists in the search for and development of new medicines are

- Screening for desired activity,
- Determining mode of action, and
- Quantifying drug activity when chemical methods are not available (Anonymous, 2005a).

### 2.3.2 The herbal approach to viral infection

The herbal approach involves stimulating the immune system to produce more immune cells and immune chemicals, and the use of antiviral herbs to disrupt the replication cycle of the virus. To support the immune system, herbalists have traditionally employed herbs that contain chemicals known as high molecular weight heterogeneous polysaccharides. Certain types of these chemicals enhance the body's general immunity, for instance by increasing the total number of lymphocytes and helper T-cells or the

activity of natural killer cells or macrophages or by increasing the number of immune stimulating messenger molecules known as cytokines (e.g. interferon and interleukins) (Davies, 2003).

Immune-enhancing polysaccharides have been identified in herbs such as Siberian ginseng, astragalus, liquorice, bladderwrack and saw palmetto (Davies, 2003). Traditionally, immune enhancing herbs like these are combined with those that increase the action of the eliminative channels. Detoxification and elimination is fundamental to the enhancement of immune function. Herbs to support the eliminative channels include marshmallow and rehmannia for the kidneys, mullein and lobelia for the lungs, burdock and fenugreek for the skin and barberry and gentian for digestion. The addition of herbs containing insulin, for instance burdock and elecampane, helps to balance blood sugar levels which are essential because fluctuations, either too high or too low, can considerably compromise immunity (Davies, 2003).

The role of these immune-enhancing herbs as part of a broad systemic treatment cannot be over-estimated. Ultimately the body's own defenses are strengthened and recovery is therefore faster and more complete. However, specific antiviral plants can also be of significant use in dealing with the speeding up of the destruction of the virus by the immune system, which is ultimately the best way of overcoming a viral infection.

Antiviral compounds from herbs interrupt the virus replication cycle at various stages. For example, the chemical known as prunellin (a sulphated polysaccharide) from Self-Heal (*Prunella vulgaris*) blocks the receptor used by the HIV virus so the virus cannot attach to host cell surface receptors. A different chemical from nettle roots inhibits the same virus but by preventing the genetic information from the virus fusing with the host cell's genome (Davies, 2003).

Some compounds work by interfering with the enzymes needed to make copies of virus components. Pokeweed antiviral protein works in this way, as does baicalin (from plantain) and skullcap (Davies, 2003). Acute infection is however not the only way a virus interacts with its host. Over the many generations of co-evolution, viruses and hosts have adapted to each other. Even the common cold was a deadly plague 5,000 years ago. Some viruses, instead of precipitating an acute episode, lie dormant within the host's body. No viral proteins or particles are produced and these latent viruses remain in a state of suspended animation until the immune system weakens through stress, bacterial infection, accumulated toxins, or nutritional deficiency, when the virus makes the most of the lowered line of defense to establish itself. The herpesviruses are the archetypal examples of persistent latent viral infection. Cold sores come courtesy of this very process of persistent latent viral infection. Post viral

syndromes like chronic fatigue immune deficiency syndrome (CFIDS), a disease previously known as just chronic fatigue syndrome (CFS) or myalgic encephalomyelopathy (ME) also appear to be persistent viral infections. Epstein-Barr virus (EBV - the virus that causes glandular fever), herpesvirus number 6 (HSV6) and a retrovirus (the same type of virus as HIV and human leukaemia viruses) have all been put forward as the possible cause.

The worst plague of all time was not AIDS or the Bubonic plague. In 1918 influenza wiped out about 40 million people in just a matter of months. No pandemic before or since has killed more in such a short time. In that year you could have been arrested for sneezing in public (Davies, 2003). Influenza, a disease once thought to be the result of the influence of celestial transits, has been the most dreadful viral disease in human history. It is an RNA virus and so has a mutation rate up to a million times faster than DNA viruses. Only one other virus, HIV, mutates faster. Such changes mean that the needed remedies to stop the virus are continually changing, like shooting at a moving target. This process, known as antigenic drift, lies behind the yearly influenza epidemics.

Influenza virus doesn't only infect humans, but also pigs, horses, dogs and many avian species. Ducks for example often have several different strains of influenza thriving in the digestive tract with no apparent effect but such close proximity of different strains very occasionally results in a genetic reshuffle producing a recombinant strain of increased virulence. The 1918 epidemic was a recombinant strain created in a pig cell. The epidemic was referred to as swine flu. There are two proteins on the viral envelope of influenzaviruses which determine the shape of the key which needs to fit the host cell receptor to cause infection. They are known as H (for hemagglutinin) and N (for neuraminidase). There are at least 14 different subtypes of H antigen and 9 of N antigen. Each subtype of H or N is totally different - the antibodies for one are no good for the other. The combination of the two antigens determines the pathogenicity of the influenzavirus (Davies, 2003).

In the modern era of AIDS, and under the threat of biological warfare, of Ebola, of emerging viruses and persistent latent viral infection, antiviral compounds will be needed for many years to come. The briefest glance over the way the world's most deadly virus, influenza, changes might warn us to be on the lookout for new antiviral herbal and plant compounds. Alongside viral evolution the plant kingdom has been exposed to similar selection processes producing antiviral and immune stimulant properties and compounds. Many of the chemicals used by the plant to protect from viral infection also have antiviral activity in higher species. Using these antiviral herbs in combination with good nutrition and a healthy lifestyle can contribute to combat viral attacks and maintain our legacy as survivors (Davies, 2003).

### 2.3.3 Some plants with antiviral and antibacterial activities

Many plants have been tested for antiviral and antibacterial activities and Table 2.2 represents a selection of these plants.

Table 2.2. Some plants with antiviral and antibacterial activities (adapted from Cowan 1999)

Common name	Scientific name	Compound	Class	Activity	Toxicity
Caraway	<i>Carum carvi</i>		Coumarins	Bacteria, fungi, viruses	2.3
Cascara sagrada	<i>Rhammus purshiana</i>	Tannins	Polyphenols	Viruses, bacteria, fungi	1.0
Eucalyptus	<i>Eucalyptus globules</i>	Tannin	Anthraquinone Polyphenols	Bacteria, viruses	1.5
Hemp	<i>Cannabis sativa</i>	$\beta$ -Resercyclic acid	Terpenoids Organic acid	Viruses, bacteria	1.0
Thyme	<i>Thymus vulgaris</i>	Caffeic acid Thymol Tannins	Terpenoid Phenolic alcohol Flavones	Viruses, bacteria, fungi	2.5

## 2.4 Study of medicinal plants

In a drug discovery process, pure active compounds obtained by bioassay-guided isolation from extracts of medicinal plants, are subjected to structure-activity relationship studies (SAR) (Fig 2.7). Toxicity and safety studies as well as clinical tests are carried out, active compounds have to be prepared on an industrial scale, and an appropriate pharmaceutical formulation has to be developed before the compound can be approved as a drug. In a traditional medicine system, however, pharmacological evaluation of extracts from medicinal plants may lead to the establishment of standardized extracts. In this case, the industrial production of these standardized extracts can start immediately after toxicity and safety studies. After formulation of the standardized extracts clinical tests can be carried out, which may lead to approval as drugs (Vlietinck and Pieters, 2005).



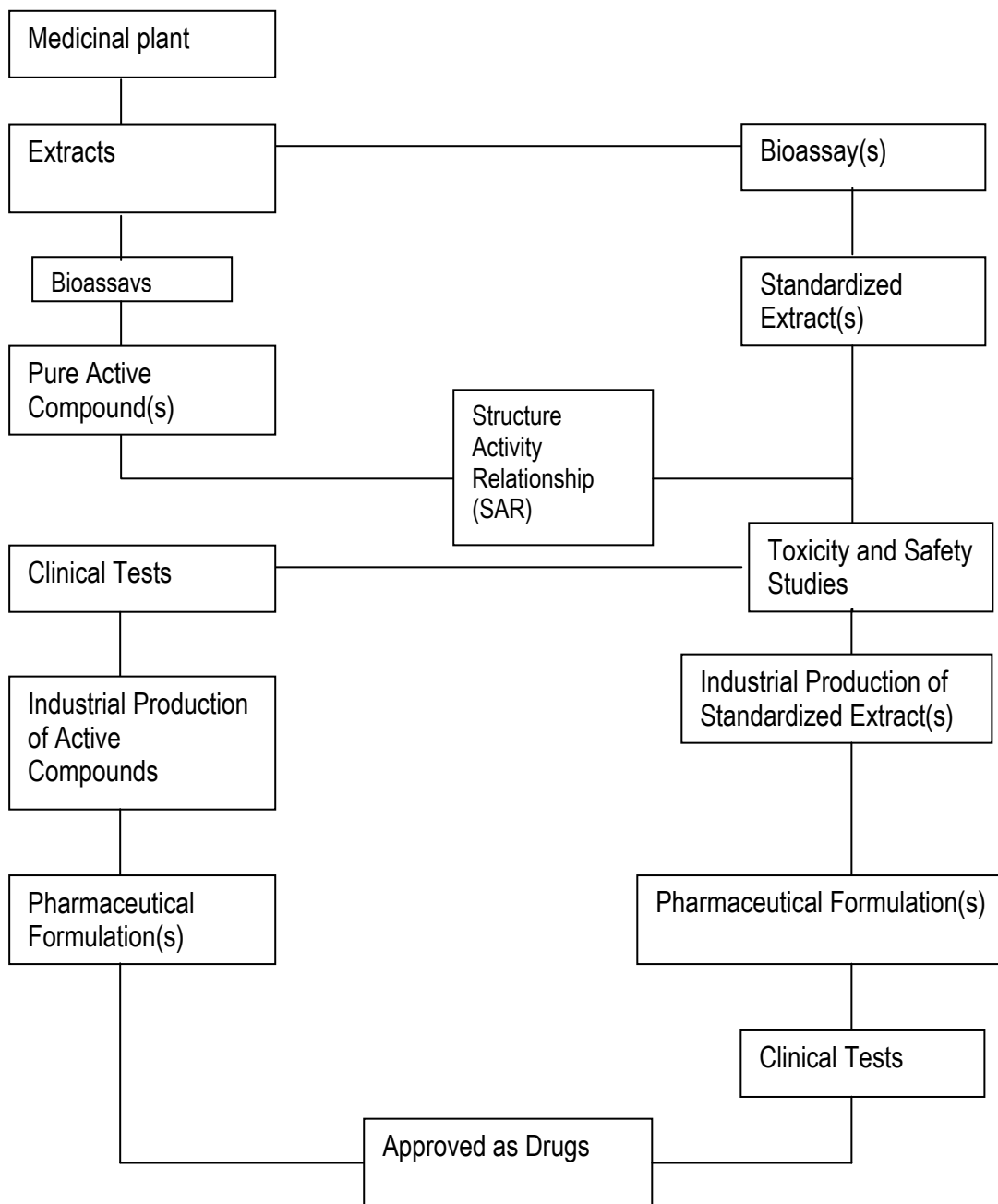


Fig 2.7. Flow chart for the study of plants used in traditional medicine (Vlietinck and Vanden Berghe, 1991)

## 2.5 Work done on the Combretaceae family

The decision to work on plants from this family was based on:

- The wide ethnomedicinal use of Combretaceae species in Africa and Asia,
- The quantity of material of Combretaceae used in the natural medicine trade in southern Africa,
- Pharmacological activity in related taxa, availability of plant material and co-operators, and

- Asres *et al.* (2001) found good anti-HIV activity with the acetone extract of the leaves of *C. paniculatum*.

Analysis of available data (Cunningham, 1990) indicates that the Combretaceae is one of the main medicinal plant families used in KwaZulu-Natal with an average of 20.2 tonnes consumed per year. Researchers of the Phytomedicine Programme at the University of Pretoria have been investigating this family, especially since Noristan found indications of antibacterial activity with *Combretum erythrophyllum* extracts (Eloff, 1999).

### 2.5.1 The Combretaceae family

The two most important genera of the Combretaceae are *Combretum* and *Terminalia*. The genus *Combretum* has two subgenera, these being subgenus *Combretum* and subgenus *Cacoucia*. With subgenus *Combretum*, trichomes present are scales with or without hairs but with subgenus *Cacoucia* the trichomes are stalked glands, accompanied or not by hairs. Scales when examined under 10 X magnification can be seen to have differing configurations. Some appear to be recessed, others flush, many apparently crateriform, some doomed and still others protruding. However, a microscopic examination shows that apart from size and shape variations there is a series of different arrangements of the cells which make up the scales. The kind of arrangements - type of scale - may have taxonomic significance (Carr, 1988).

Species in the subgenus *Combretum* have been grouped into sections (Table 2.3), those in each section often having certain similar plant morphological characteristics. Stace (1969 and 1980) has shown that those species in a particular section all have similar or nearly similar scale types and has thus established that the plant characters and scale types are often correlated. Species in the subgenus *Cacoucia* have also been allocated to different sections, and microscopic examination of the stalked glands has shown that the type and size of gland varies according to the section, and this in turn has resulted in some revision of sections and the establishment of a new section. It will thus be apparent that characters of the trichomes provide an additional aid to identification; even fragmentary specimens of *Combretum*, when examined microscopically, can usually be assigned at least to sectional level (Carr, 1988). While the sections and the structural details of scales and stalked glands are of no real importance for the normal process of identification, the appearance of scales in particular, as discernable with the aid of magnification, should be taken into account as this can in some cases be diagnostic.

Table 2.3. Sectional division in southern African members of Combretaceae (adapted from Carr, 1988).

***Combretum* Loefl**

SUBGENUS <i>Combretum</i>	Section <i>Spathulipetala</i> Engl. & Diels <i>C. zeyheri</i> Sond	Section <i>Oxystachya</i> Exell <i>C. oxystachyum</i> Welw. Ex Laws
Section <i>Hypocrateropsis</i> Engl. & Diels <i>C. celastroides</i> Welw. Ex Laws <i>C. imberbe</i> Wawra <i>C. padoides</i> Engl. & Diels	Section <i>Ciliatipetala</i> Engl. & Diels <i>C. albopunctatum</i> Suesseng. <i>C. apiculatum</i> Sond. <i>C. edwardsii</i> Exell (provisional) <i>C. moggii</i> Exell (provisional) <i>C. molle</i> R. Br. <i>C. petrophilum</i> Retief <i>C. psidiodes</i> Welw.	Section <i>Poivrea</i> (Comm. Ex DC.) G. Don <i>C. bracteosum</i> (Hochst.) Brandis <i>C. mossambicense</i> (Klotzsch) Engl.
Section <i>Combretastrum</i> Eichl. <i>C. umbricola</i> Engl.		Section <i>Megalantherum</i> Exell <i>C. wattii</i> Exell
Section <i>Angustimarginata</i> Engl. & Diels <i>C. caffrum</i> (Eckl. & Zeyh.) Kuntze <i>C. erythrophyllum</i> (Burch.) Sond <i>C. kraussii</i> Hochst. (incorporating <i>C. nelsonii</i> Duemmer) <i>C. vendae</i> Van Wyk <i>C. woodii</i> Duemmer	Section <i>Fusca</i> Engl. & Diels <i>C. coriifolium</i> Engl. & Diels	Section <i>Lasiopetala</i> Engl. & Diels <i>C. obovatum</i> F. Hoffm.
Section <i>Macrostigmaea</i> Engl. & Diels <i>C. engleri</i> Schinz <i>C. kirkii</i> Laws. <i>C. mkuzense</i> (provisional)	Section <i>Breviramea</i> Engl. & Diels <i>C. hereroense</i> Schinz  Section <i>Elaegnoida</i> Engl. & Diels <i>C. elaeagnoides</i> Klotzsch	Section <i>Conniventia</i> Engl. & Diels <i>C. microphyllum</i> Klotzsch <i>C. paniculatum</i> Vent. <i>C. platypetalum</i> Welw. Ex Laws Section <i>Metallicum</i> Exell & Stace <i>C. collinum</i> Fresen.
	SUBGENUS <i>Cacoucia</i> (AUBL.) EXELL & STACE	Section <i>Glabripetala</i> Engl. & Diels <i>C. fragrans</i> F. Hoffm.

## 2.5.2 Taxonomy

The family Combretaceae belongs to the order Myrtales and consists of 600 species of trees and shrubs in 20 genera, which include *Anogeissus*, *Bucida*, *Combretum*, *Quisqualis*, *Terminalia*, and *Thiloa*. They are found throughout the tropics and sub-tropics. Six genera are found in southern Africa and they are as follows: *Combretum*, *Lumnitzera*, *Pteleopsis*, *Quisqualis*, *Meiostemon* and *Terminalia*.

The largest genus is *Combretum*, with about 370 species, while *Terminalia* is the second largest, and has about 200 species. They occur in most parts of Africa and are often the dominant vegetation

(Rogers and Verotta, 1996). The other genera are much smaller, including *Calopyxes* and *Buchenavia* which have 22 species each and *Quisqualis*, *Anogeissis Conocarypis* and *Pteleopsis*, each with 16, 14, 12 and 10 species, respectively (Rogers and Verotta, 1996).

In general, the genus *Combretum* has a 4-5 winged, ridged, angled, sessile or stipitate fruit while *Terminalia* has 2-winged fruit. Hybridization is a common occurrence in both genera. This results in the formation of numerous species that on visual inspection look quite different. For example, *C. albopunctatum* and *C. apiculatum* are similar in many respects, and so are *C. psidiodes* and *C. molle*, and *Terminalia mollis* as well as *T. stenostachya* (Carr, 1988). If the classification used (based mainly on morphological parameters) approximates a natural classification, there should be a good correlation between the taxonomy and occurrence of plant secondary compounds in related species, genera, or families.

Several members of the family Combretaceae have been used traditionally to treat bacterial diseases in southern Africa (Watt and Breyer-Brandwijk, 1962; Hutchings *et al.*, 1996). Different degrees of antibacterial activity in the different species may have some taxonomic predictive value (Eloff, 1999). The Phytomedicine group of the University of Pretoria has successfully used the chemotaxonomic approach to reveal significant biological activity of many members of this family (Eloff, 1999; Martini and Eloff, 1998; Eloff *et al.*, 2001; McGaw *et al.*, 2001; Kotze and Eloff, 2002; Martini *et al.*, 2004; Masoko *et al.*, 2005).

Most of the research conducted at the Phytomedicine Programme comprises studies on the antibacterial activity of plants (including the Combretaceae). This is as a consequence of determining the best extractant (Eloff, 1998a) and the development of the rapid and reproducible serial dilution method (Eloff, 1998b) used for obtaining MIC values of plant extracts against bacterial species.

### 2.5.3 Evaluation of the antibacterial activity of different species

Our research group has been working on the Combretaceae family for a long time and of late some other plant species are under investigation. A study was carried out with 27 species of *Combretum*, *Terminalia*, *Pteleopsis*, and *Quisqualis*. Leaves were collected, dried, milled, and extracted with acetone. The MIC of extracts was determined by the microplate serial dilution technique using *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa* and *Escherichia coli* as test organisms. All extracts inhibited the growth of the four test isolates with MIC values generally between

0.1 and 6 mg/ml and an average of 2.01 mg/ml. After storing extracts for six weeks at 7°C there was a slight loss of activity with MIC values increasing from 1.75 mg/ml to 2.24 mg/ml. The Gram-positive strains were slightly more sensitive (with an average MIC value of 1.8 mg/ml) than the Gram-negative strains (with an average MIC of 2.2 mg/ml). Based on the MIC values and the total content in each plant, the seven plants with the highest antibacterial activity were *C. molle*, *C. petrophyllum*, *C. moggii*, *C. erythrophyllum*, *C. padoides*, *C. paniculatum*, and *C. mossambicense* (Eloff, 1999).

#### 2.5.4 *Combretum erythrophyllum*

Work on the extracts of *C. erythrophyllum* after liquid/liquid extraction revealed that there was inhibition of four test organisms to different degrees, with *Staphylococcus aureus* the most sensitive (100%) followed by *Enterococcus faecalis* (36%), *Escherichia coli* (11%), and *Pseudomonas aeruginosa* (3%). The lowest MIC value obtained was for *Staphylococcus aureus* (0.05 mg/ml) at this stage of purification, compared with MIC values of 0.08 mg/ml and 0.16 mg/ml for ampicillin and chloramphenicol respectively (Martini and Eloff, 1998). Based on the results obtained for *C. erythrophyllum*, other members of the Combretaceae were examined.

Leaves of *C. erythrophyllum* were investigated in more detail and seven antibacterial compounds were isolated. Four of these compounds were identified as flavonols, namely 5,7,4'-trihydroxyflavonol (kaempferol), 5,4'-dihydroxy-7-methoxyflavonol (rhamnocitrin), 5,4'-dihydroxy-7,5'-dimethoxyflavonol (rhamnazin), and 7,4'-dihydroxy-5,3'-dimethoxyflavonol (quercetin-5,3'-dimethylether) and three were identified as flavones, namely 5,7,4'-trihydroxyflavone (apigenin), 5,4'-dihydroxy-7-methoxyflavone (genkwanin), and 5-hydroxy-7,4'-dimethoxyflavone. Six of these flavonoids were reported for the first time in the Combretaceae (Martini *et al.*, 2004).

The biological activities of five of these compounds were examined and all had good activity against *Vibrio cholerae* and *Enterococcus faecalis* with MIC values in the range of 25-50 µg/ml. Rhamnocitrin and quercetin-5-3'-dimethylether also inhibited *Micrococcus luteus* and *Shigella sonnei* at 25 µg/ml. With the exception of 5-hydroxy-7, 4'- dimethoxyflavone, the flavonoids were not toxic for human lymphocytes. Genkwanin, rhamnocitrin, quercetin-5, 3'-dimethylether, and rhamnazin had higher inflammatory activity than the positive control mefenamic acid. Although these flavonoids are known, this is the first report of biological activity with several of these compounds (Martini *et al.*, 2004).

#### 2.5.5 *Combretum woodii*

Dried leaves of *C. woodii* were extracted with ten different solvents to determine the best extractant for subsequent isolation and characterization of antibacterial compounds. Ethyl acetate was the best extractant with average MIC values of 0.08 mg/ml for the four pathogens followed by acetone and DCM with values of 0.14 mg/ml. The average MIC values for the positive controls were 0.13 mg/ml (ampicillin) and 0.12 mg/ml (chloramphenicol) (Eloff *et al.*, 2005). Acetone extracts of *C. woodii* leaf powder were separated by solvent/solvent partitioning into six fractions. The highest total activity was in the chloroform fraction. This fraction contained mainly one compound active against *Staphylococcus aureus*. This compound was isolated by bioassay-guided fractionation using silica gel open column chromatography and identified by NMR and MS as the stilbene 2', 3', 4'-trihydroxyl-3,5,4'-trimethoxybibenzyl (Combretastin B5) previously isolated from the seeds of *C. kraussii*. It showed significant activity against *Staphylococcus aureus* with an MIC of 16 µg/ml but lower activity towards *Pseudomonas aeruginosa* (125 µg/ml), *Enterococcus faecalis* (125 µg/ml), and slight activity against *Escherichia coli*. This is the first report of the antimicrobial activity of Combretastin B5. Its concentration in the leaves was in the order of 5-10 mg/g which makes the use of non-polar leaf extracts a viable proposition in treating some infections, particularly in resource-poor settings (Eloff *et al.*, 2005).

#### 2.5.6 *Combretum microphyllum*

*C. microphyllum* is very closely related to *C. paniculatum* and some authorities consider *C. paniculatum* to be synonymous with *C. microphyllum* (Germishuizen and Meyer, 2003), but others (Palgrave, 2002; Carr, 1988) recognize both species.

In a study to investigate the antibacterial activity of *C. microphyllum*, dried ground leaves were extracted with a series of extractants of varying polarity (i.e. hexane, carbon tetrachloride, di-isopropyl ether, ethyl ether, methylene dichloride, tetrahydrofuran, ethanol, ethyl acetate, methanol and water). Thin-layer chromatography (TLC) was used to determine chemical composition, and antibacterial activity of extracts was determined by a microplate serial dilution method. The solvents extracted from 2.6 to 17.4% of the dry weight. Methanol, dichloromethane, and tetrahydrofuran extracted the most components. The minimum inhibitory concentration (MIC) for the extracts varied from 0.01 to 1.25 mg/ml with four test organisms, namely *E. coli*, *P. aeruginosa*, *E. faecalis* and *S. aureus* (Kotze and Eloff, 2002). The extracts had similar activity towards Gram-negative and Gram-positive bacteria. Di-isopropyl ether, ether, ethanol, ethyl acetate, and acetone extracted compounds with high antibacterial

activity with a lower quantity of other nonactive compounds, and appear to be useful for isolating bioactive compounds (Kotze and Eloff, 2002).

In another application of simplifying plant extracts using selective extraction, there is a rationale for using extracts to treat infectious diseases in preference to single compounds. It is likely that interactions between various compounds present in an extract result in synergistic effects which lead to heightened activity (Williamson, 2001). There is a distinct possibility that active principles with differing mechanisms of action may be present in a crude extract, thus slowing the onset of antibiotic resistance. Therefore, it may be worthwhile to seek to potentize plant extracts for anti-infectivity in preference to solely aiming for isolation of active compounds. Enhancing the activity of plant extracts by selectively removing bulky nonactive components is a relatively simple process (Kotze and Eloff, 2002). These potentized preparations may find application chiefly in the arena of primary health care for humans and animals in developing countries.

#### 2.5.7 Unpublished work on other members of Combretaceae

In his PhD study, Angeh isolated three antibacterial compounds, a new oleanene-type triterpenoid glycoside and two known triterpenoids from *Combretum padoides* (Angeh, 2005). He also isolated a new antibacterial pentacyclic triterpenoid and four antibacterial triterpenoids with known structures from the leaves of *Combretum imberbe* (Angeh, 2005). The new triterpenoids are 1 $\alpha$ , 23 $\beta$ -dihydroxy-12-oleanen-29-oic-acid-3 $\beta$ -O-2, 4-diacetylramnopyranoside from *Combretum imberbe* and 1 $\alpha$ , 3 $\beta$ -dihydroxy-12-oleanen-29-oic-acid--23 $\beta$ -O- $\alpha$ -4-acetylramnopyranoside from *Combretum padoides*.

#### 2.5.8 *Combretum apiculatum*

In a study carried out by Serage (2003) to investigate the antibacterial activity of *C. apiculatum*, dried ground leaves were extracted with a series of extractants of varying polarity. Thin-layer chromatography (TLC) was used to determine chemical composition, and antibacterial activity was determined by a serial dilution method. The solvents extracted from 0.8 to 14.4% of the dry weight. Tetrahydrofuran (THF) [72 mg], acetone [66 mg] and dichloromethane (DCM) [32 mg] extracted the most components. The MICs ranged between 0.04 to 2.5 mg/ml with four test organisms. Total activity was highest for acetone (762 ml/g) and least for water (3 ml/g).

$$\text{Total activity (ml/g)} = \frac{\text{Amount extracted from 1 gram (mg)}}{\text{MIC (mg/ml)}}$$

In a bioassay-guided isolation, three compounds were isolated and their names were as follows: pinocembrin (C<sub>15</sub>H<sub>12</sub>O<sub>4</sub>), flavokawain (C<sub>17</sub>H<sub>16</sub>O<sub>4</sub>), and alpinetin (C<sub>16</sub>H<sub>14</sub>O<sub>4</sub>). These compounds had MIC values in the range 40 to 600 µg/ml (Serage, 2003).

### 2.5.9 *Combretum paniculatum*

*C. paniculatum* was the plant selected for the present study. This species occurs in the eastern geographical division only, in the following places: Inyanga, Mutare, Chimanimani, Ngorema reserve, Chipinge, and Glencoe forest reserve.

#### *Combretum paniculatum* Vent.

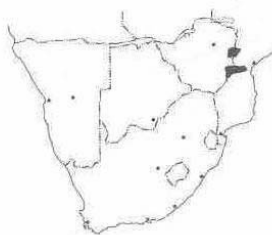


Fig 2.8. Distribution of *C. paniculatum* in southern Africa (Carr, 1988)

There are occurrences of the shrub in Zambia, Mozambique, and Malawi. This species is also distributed in wetter parts of the tropics in Africa (Carr, 1988). Common names are; baye, begie, gabai, gopo-gopo, mukopo-kopo, ndiritsamboko, okan, orutara bugu, shaga, and shikaalikanga.

Synonymous Latin binomials for this plant are:

*Combretum abbreviatum*

*Combretum pincianum*

*Combretum ramosissimum* (Napralert database)

#### 2.5.9.1 Ethnomedical information on *C. paniculatum*

The dried leaves of this plant are used to treat diarrhea, malaria and fevers. The hot water root extracts are used as an anthelmintic and in cases of retained placenta. Decoctions are used to treat pulsating



anterior fontanelle in infants, venereal disease and menorrhagia, and twigs are used as an appetizer. (Napralert database).

### 2.5.9.2 Description of *C. paniculatum*

*C. paniculatum* is a several-stemmed liana and can climb up and over adjacent vegetation to a height of 15 m or more. The foliage is dense, dark green and rather shiny and is not shed during winter. Its brilliant scarlet flowering resembles that of *C. microphyllum*. It is possible that this species, or some earlier form of it, was the progenitor of *C. microphyllum*, which at one stage was regarded as a subspecies, and *C. platypetalum*. Both of these species, having similar flowers and fruit, are certainly very closely related and *C. microphyllum*, also being a liana, might be confused with this species. The main differences are: *Combretum paniculatum* is found in a more mesic habitat, current growth stems have only a sparse indumentum, and it has stalked glands, as opposed to being puberulous to pubescent as is the case with *C. microphyllum* (Carr, 1988). The foliage is darker and shinier, with emerging leaves plum-coloured. Leaves are generally larger and are not deciduous. The upper receptacle of the flower is more cylindrical than that of *C. microphyllum* and the fruit is generally larger and is not notched at the base. The main stems are fairly smooth and a grayish biscuit colour with white to purplish brown striations, lifting slightly at their edges and comprising strips of persisting outer covering.

Flowering usually takes place some time between mid August and the end of September.

Inflorescences are produced in the axils both on the previous year's wood and on current extensions.

With leaves mostly being opposite, inflorescences are arranged likewise. They usually extend horizontally and can vary from a simple, slender spike up to 40mm long, to a panicle with up to 10 branches of about the same length, to a panicle up to 170mm long with as many as 10 pairs of spikes, simple or branched, usually opposite and horizontal, and with a spike at the terminal.

The fruit is a 4-winged samara, the wing outline broadly elliptic to broadly obovate to sub-circular, with base rounded and sometimes slightly decurrent along the stripe, the apex truncate to rounded and often shallowly and widely notched. Parasitisation is minimal with the fruit ripening fast, but one has to be available to collect it as it ripens and before it is dispersed by wind. Seed can be easily extracted from the fruit and should be soaked for a few hours before sowing (Carr, 1988).

### 2.5.9.3 Previous work on *C. paniculatum*

Eloff (1999) reported that the MIC of the acetone leaf extract was 1.56 mg/ml. McGaw *et al.* (2001) extracted and screened *C. paniculatum* leaves for anti-inflammatory, anthelmintic, antibilharzia (antischistosomal) and DNA-damaging activity. For water extracts of the leaves, anti-inflammatory activity showed 54% inhibition, there was no anthelmintic activity and the MIC for antischistosomal activity was 25 mg/ml. The acetone extract had a better anti-inflammatory inhibition of 73% and for the anthelmintic activity, 80-90% of the nematodes were alive after exposure to the extract. The ethyl acetate extract had the best anti-inflammatory activity of 76%, and no anthelmintic activity.

*Combretum paniculatum* extracts have been screened for activity against HIV-1 and HIV-2. The acetone extract showed a high degree of antiviral activity against HIV-2, with an effective concentration 50 (EC<sub>50</sub>) of 3 µg/ml, and a selectivity index of 32 (Asres *et al.*, 2001). Among the 21 plant species investigated, the acetone extract of the leaves of *C. paniculatum* had the best activity against HIV-2. The decision to work on this species was based on (a) its known antiviral activity (b) Asres *et al.* (2001) apparently did not continue with isolation work and (c) the Phytomedicine Programme has delivered many publications and has isolated many antibacterial and antifungal compounds from other plants.

## 2.6 Hypothesis

1. Because *Combretum paniculatum* has been shown to have antiviral activity (Asres *et al.*, 2001), it is possible to isolate and characterize antiviral compounds from leaf extracts.
2. Antibacterial compounds are easier to isolate using bioassay-guided fractionation since antibacterial activity is much easier to determine than antiviral activity.
3. The antibacterial compounds isolated from *Combretum paniculatum* will have antiviral activity. Some plant species and compounds from plants have been reported to have both antibacterial and antiviral activities (Table 2.2, section 2.3.3).

## 2.7 Aim of study

The aim of this study is to isolate antibacterial compounds from *C. paniculatum*, to characterize the isolated antibacterial compounds, and to test the compounds for antiviral and antifungal activity.

## 2.8 Objectives

The aim will be reached by addressing the following;

- Selection of the best extractant for the plant material
- Determination of antiviral and cytotoxic activities of extracts
- Preliminary isolation study
- Isolation of antibacterial compounds
- Determination of the chemical structures of isolated compounds
- Determination of the antiviral and other biological activities of the isolated compounds.