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1D	1- dimensional
2D	2-dimensional
AIDS	Acquired Immune Deficiency Syndrome
APUA	Alliance for the Prudent Use of Antibiotics
ATCC	American Tissue Culture Center
BEA	Benzene, ethyl acetate, acetone
BuOH	Butanol
Bs	*Bacillus subtilis* ATTC 6633
Ca	*Candida albicans* BMSY 212
CAP	Community Acquired Pneumonia
CC	Cytotoxic concentration
CEF	Chloroform, ethyl acetate, formic acid
CFIDS	Chronic Fatigue Immune Deficiency Syndrome
CMV	Cytomegalovirus
CNS	Central Nervous System
COSY	Correlation Spectroscopy
CPE	Cytopathic effect
CRFK	Crandell Feline Kidney cells
DCM	Dichloromethane
DEPT	Distortionless Enhancement by Polarization Transfer
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPH	1,1-diphenyl-2-picryl hydrazyl
DS	Double-stranded
EBV	Epstein-Barr Virus
Ec	*Escherichia coli* SG 458
EC_{50}	Effective Concentration 50
EDTA	Ethylene Diamine Tetraacetic Acid
EMW	Ethyl acetate, methanol, water
FAAIR	Facts about Antibiotics in Animals and their Impact on Resistance

FAWE	Formic acid, acetic acid, water and ethyl acetate
GMK	Green Monkey Kidney cells (Vero)
HIV	Human immunodeficiency virus
HKI	Hans-Knöll Institute
HMBC	Heteronuclear Multiple Bond Correlation
HMQC	Heteronuclear Multiple Quantum Coherence
HPLC	High Performance Liquid Chromatography
HSV	Herpes Simplex Virus
IC_{50}	Inhibitory Concentration 50
ICTV	International Committee on Taxonomy of Viruses
ICU	Intensive Care Unit
INT	p-Iodonitrotetrazolium chloride
MDCK	Madin-Darby Canine Kidney cells
ME	Myalgic Encephalomyelopathy
MEM	Minimum Essential Medium
MeOH	Methanol
MIC	Minimum Inhibitory Concentration
MRSA	Methicillin-resistant *Staphylococcus aureus*
MS	Mass Spectrometry
MTT	3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide
Mv	*Mycobacterium vaccae* IMET 10670
MW	Molecular Weight
NMR	Nuclear Magnetic Resonance
NOESY	Nuclear Overhauser Enhancement Spectroscopy
Pa	*Pseudomonas aeruginosa* K 799/61
PBS	Phosphate Buffer Solution
PEP	Pyruvate
Pn	*Penicillium notatum* JP 36
RNA	Ribonucleic acid
RSV	Respiratory Syncytial Virus
Sa	*Staphylococcus aureus* SG 511
SAR	Structure Activity Relationship
SARS	Severe Acute Respiratory Syndrome

Ss	*Sporobolomyces salmonicolor* SBUG 549
TA	Total activity
$TCID_{50}$	Tissue Culture Infectious Dose 50
TLC	Thin Layer Chromatography
THF	Tetrahydrofuran
UV	Ultraviolet
VREF	Vancomycin-resistant *Enterococcus faecalis*

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_{50}) ranging between 10 and 87 µg/ml. For the herpes simplex virus, 12 plant extracts were active with the highest antiviral IC_{50} = 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). 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). 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 38ºC). 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/mmwrhtm/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/).

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).

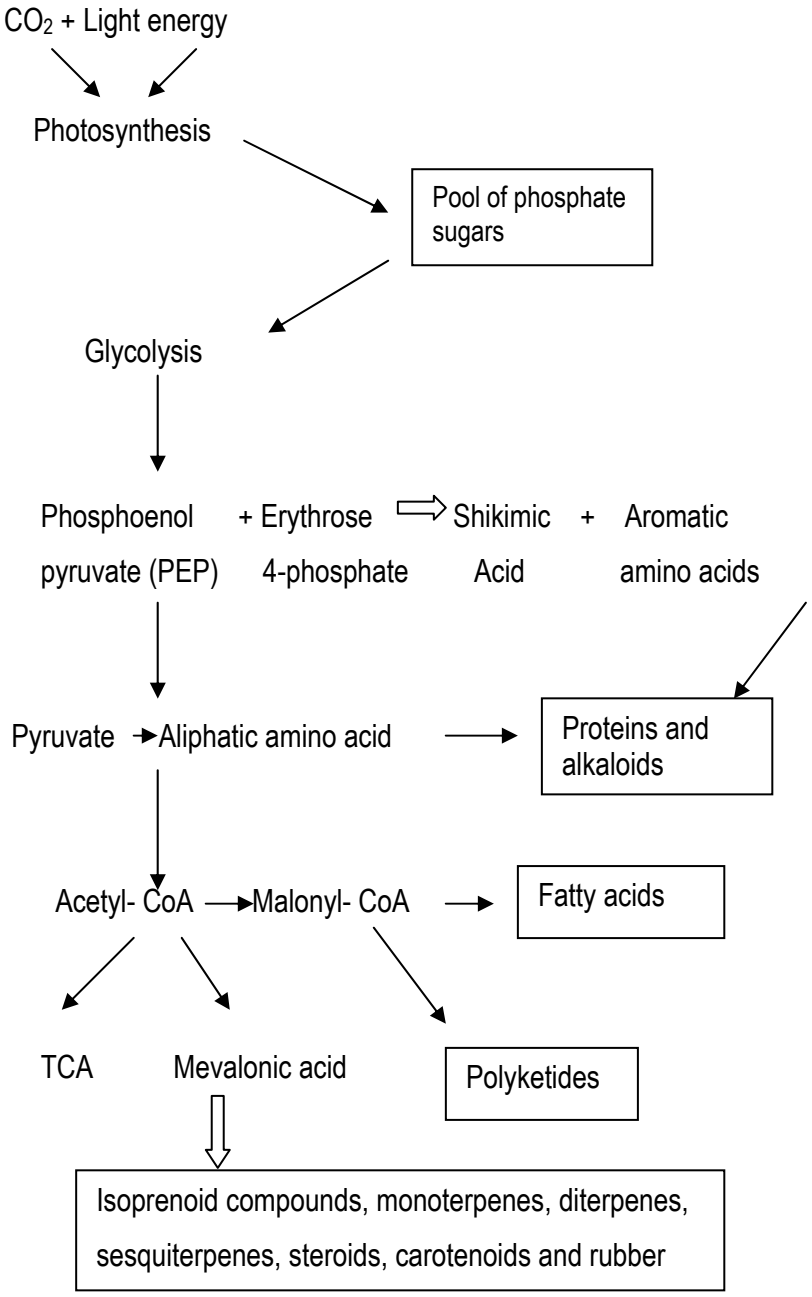
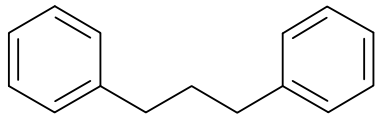


Fig 2.1. Relationship between intermediate metabolism and secondary compounds in plants

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).

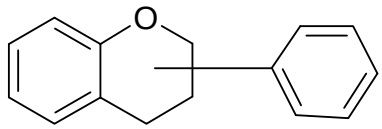
2.2.1 Flavonoids

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.

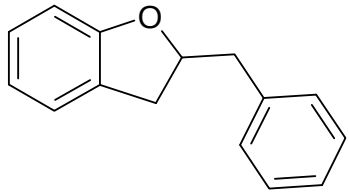


The skeleton above can be represented as the C_6-C_3-C_6 system.

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_{15} 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_2) 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:

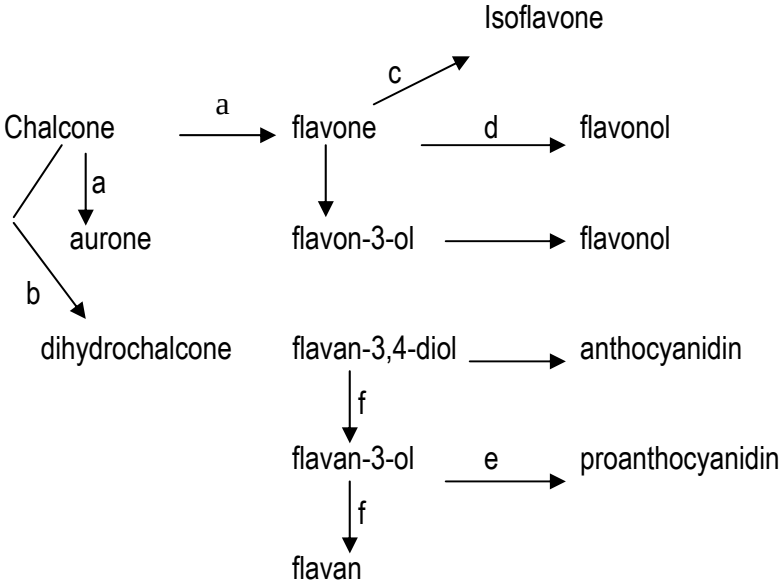


Fig 2.2. Biosynthetic relationship of flavonoids

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_{10}, C_{20}, C_{30} and C_{40} respectively) as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). When they contain additional elements, usually oxygen, they are termed terpenoids. They differ from fatty acids in that they contain extensive branching and are cyclized. Examples of common terpenoids are menthol and camphor (monoterpenes), farnesol and artemisinin (sesquiterpenoids). Artemisinin and its derivative, α-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).

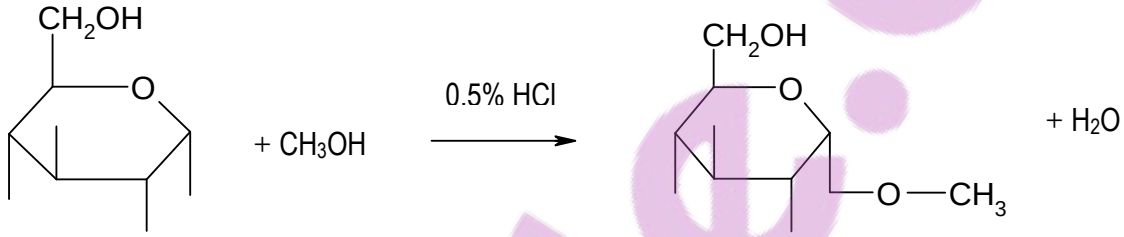
Terpenoids are active against bacteria (Taylor *et al.,* 1996), fungi (Suresh *et al.,* 1997) and viruses (Xu *et al.,* 1996). Their mechanism of action is not fully understood. Capsaicin, a constituent of chili peppers, is bactericidal to *Helicobacter pylori,* although possibly detrimental to the human 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, combre-glucoside, 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.

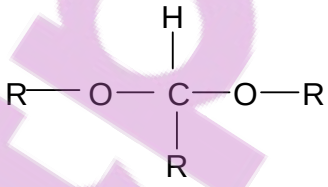
2.2.3 Glycosides

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.



Glucoside Methyl-glucoside

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:

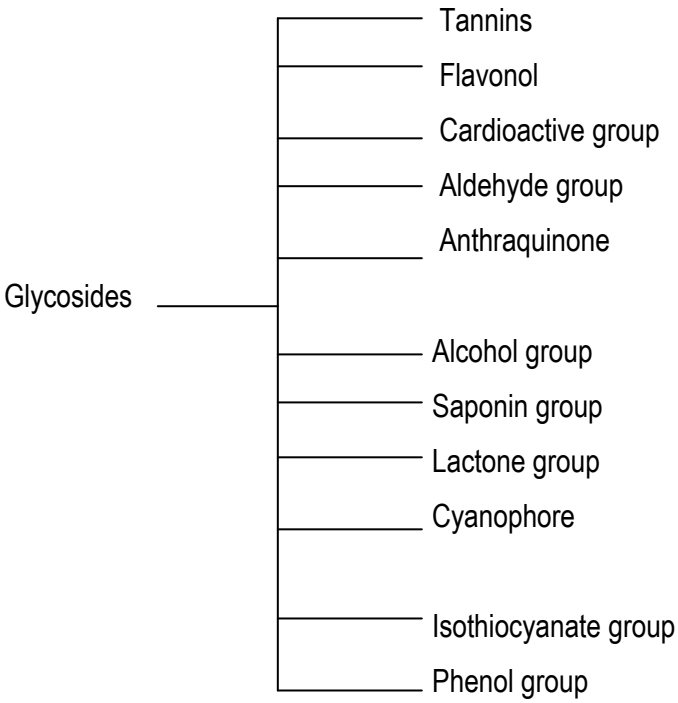


Fig 2.3. Classification of glycosides (www.friedli.com)

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.

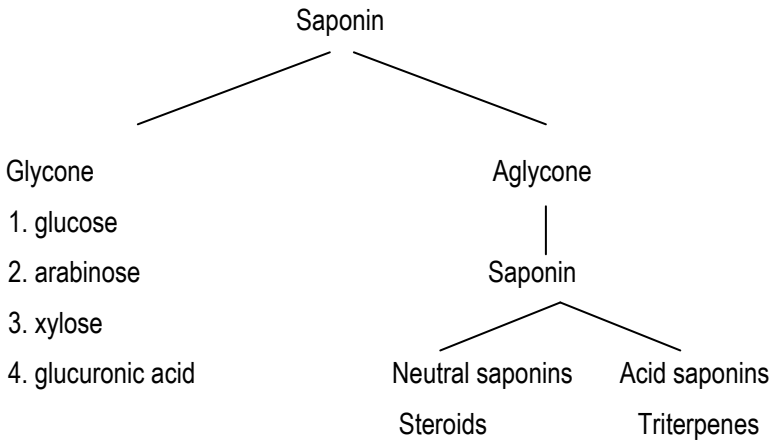


Fig 2.4. Types of saponins (www.friedli.com)

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.

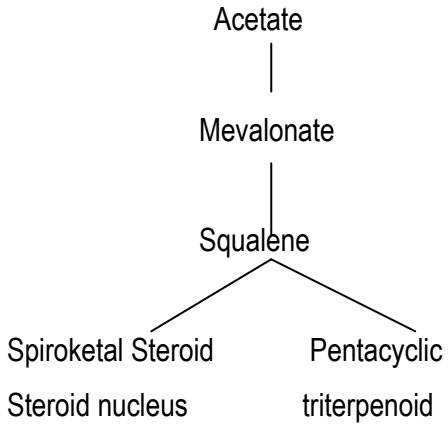
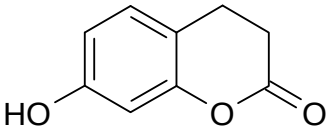


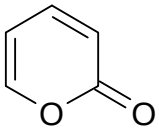
Fig 2.5. Pathway to triterpenes (www.friedli.com)

2.2.4 Coumarins

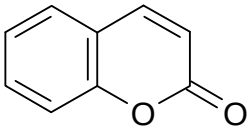
Coumarins are phenolic substances made of fused benzene and α – pyrone rings (O'Kennedy and Thornes, 1997). They are responsible for the characteristic odour of hay. Their fame has come mainly from their antithrombotic (Thastrup *et al.,* 1985), anti-inflammatory (Piller, 1975) and vasodilatory activity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

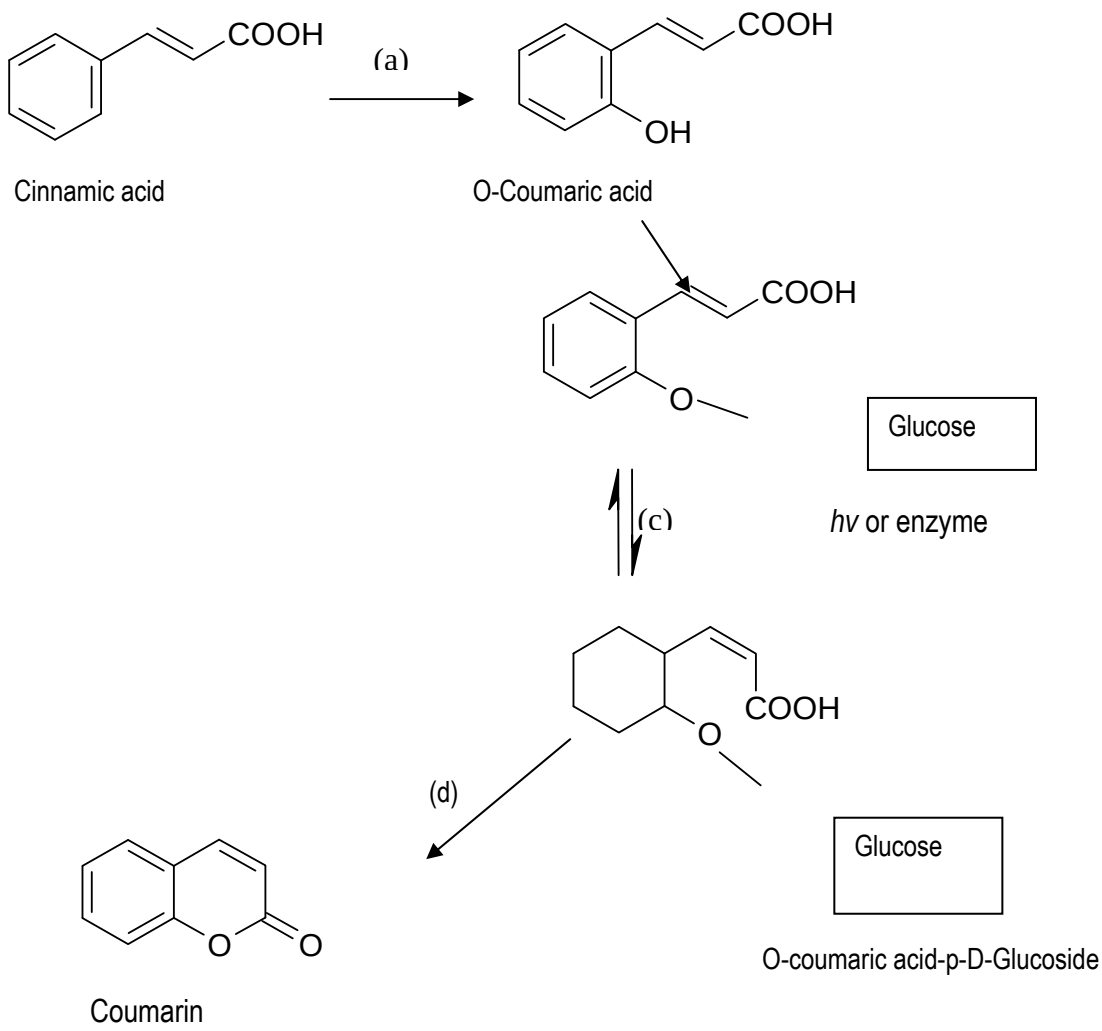
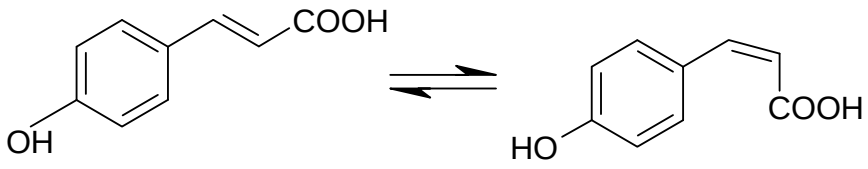


Fig 2.6. Pathway to formation of coumarins (www.friedli.com)

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.



Trans form Cis form

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	Rescinnamide
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	Vincaleukoblastine
L- Dopa	Protoveratrines 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 19th 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	*Carum carvi*		Coumarins	Bacteria, fungi,viruses	2.3
Cascara sagrada	*Rhammus purshiana*	Tannins	Polyphenols Anthraquinone	Viruses, bacteria,fungi	1.0
Eucalyptus	*Eucalyptus globules*	Tannin	Polyphenols Terpenoids	Bacteria, viruses	1.5
Hemp	*Cannabis sativa*	β-Resercyclic acid	Organic acid	Viruses, bacteria	1.0
Thyme	*Thymus vulgaris*	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).

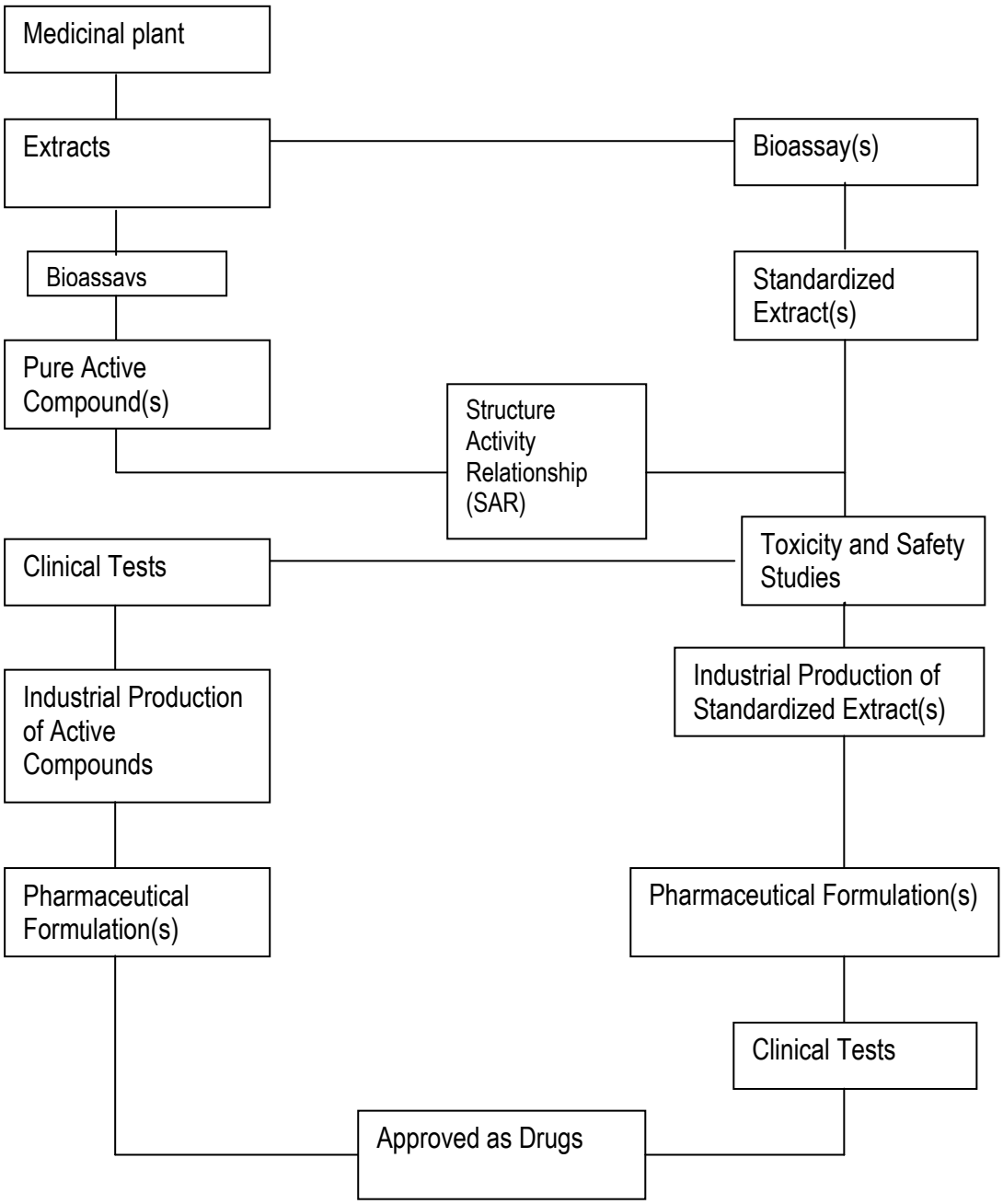


Fig 2.7. Flow chart for the study of plants used in traditional medicine (Vlietinck and Vanden Berghe, 1991)

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 *Combretum*	Section *Spathulipetala* Engl. & Diels	Section *Oxystachya* Exell
	C. zeyheri Sond	*C. oxystachyum* Welw. Ex Laws
Section *Hypocrateropsis* Engl. & Diels		
C. celastroides Welw. Ex Laws	Section *Ciliatipetala* Engl. & Diels	Section *Poivrea* (Comm. Ex DC.) G. Don
C. imberbe Wawra	*C. albopunctatum* Suesseng.	*C. bracteosum* (Hochst.) Brandis
C. padoides Engl. & Diels	*C. apiculatum* Sond.	*C. mossambicense* (Klotzsch) Engl.
	C. edwardsii Exell (provisional)	
Section *Combretastrum* Eichl.	*C. moggii* Exell (provisional)	Section *Megalantherum* Exell
C. umbricola Engl.	*C. molle* R. Br.	*C. wattii* Exell
	C. petrophilum Retief	
Section *Angustimarginata* Engl. & Diels	*C. psidiodes* Welw.	Section *Lasiopetala* Engl. & Diels
C. caffrum (Eckl. & Zeyh.) Kuntze		*C. obovatum* F. Hoffm.
C. erythrophyllum (Burch.) Sond	Section *Fusca* Engl. & Diels	
C. kraussii Hochst. (incorporating *C. nelsonii* Duemmer)	*C. coriifolium* Engl. & Diels	Section *Conniventia* Engl. & Diels
C. vendae Van Wyk		*C. microphyllum* Klotzsch
C. woodii Duemmer	Section *Breviramea* Engl. & Diels	*C. paniculatum* Vent.
	C. hereroense Schinz	*C. platypetalum* Welw. Ex Laws
Section *Macrostigmatea* Engl. & Diels		Section *Metallicum* Exell & Stace
C. engleri Schinz	Section *Elaegnoida* Engl. & Diels	*C. collinum* Fresen.
C. kirkii Laws.	*C. elaeagnoides* Klotzsch	
C. mkuzense (provisional)		Section *Glabripetala* Engl. & Diels
	SUBGENUS *Cacoucia* (AUBL.) EXELL & STACE	*C. fragrans* 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 sonei* 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 1α, 23β-dihydroxy-12-oleanen-29-oic-acid-3β-O-2, 4-diacetylrhamnopyranoside from *Combretum imberbe* and 1α, 3β-dihydroxy-12-oleanen-29-oic-acid--23β-O-α-4-acetylrhamnopyranoside 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 ($C_{15}H_{12}O_4$), flavokawain ($C_{17}H_{16}O_4$), and alpinetin ($C_{16}H_{14}O_4$). 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.

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_{50}) 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.

Chapter 3

Materials and Methods

3.1 Plant collection

Plant material (leaf, stem and bark) of *Combretum paniculatum* was collected from the garden of Prof J.N. Eloff (Murrayfield, Pretoria) and identified by Prof Eloff. Collection was done in April 2004 when leaves were still green. A voucher specimen is deposited in the medicinal plant herbarium of the Pharmacology and Toxicology Section of the Department of Paraclinical Sciences at the University of Pretoria.

3.2 Preparation and extraction of plant material

The leaves were carefully examined and old, insect-damaged or fungus-infected leaves, twigs and flowers removed. Healthy leaves were spread out and dried in the laboratory at room temperature for about ten days. The plant material was ground to a fine powder using a Jankel and Kunkel model A 10 mill.

Small scale extraction was done using several solvents, from non-polar to polar. Extraction was done in small quantities (3 g to 30 ml solvent) in order to determine the solvent that extracted the most active components. Separate aliquots of plant material were used for each solvent. The following solvents were used: hexane, carbon tetrachloride, ethanol, acetone, dichloromethane, tetrahydrofuran, methanol and water. Extracts were reconstituted to 10 mg/ml for biological assays.

3.3 Analysis by thin layer chromatography (TLC)

In TLC, 100 µg of the plant extract (10 µl of 10 mg/ml) was separated on Merck TLC F_{254} analytical plates using as eluents solvent systems of different polarities, namely BEA (benzene/ethanol/ammonium hydroxide (90:10:1), CEF (chloroform/ethyl acetate/formic acid (5:4:1) and EMW (ethyl acetate/methanol/water (40:5.4:4). Separated components were visualized under visible and UV light at wavelengths of 254 nm and 365 nm (Camac Universal UV lamp TL-600). Plates were afterwards sprayed with *p*-anisaldehyde or vanillin sulphuric acid spray reagents and heated for about

five minutes at 100°C for development of colour (Wagner and Bladt, 1996). The two spray reagents were respectively prepared thus: 1 ml of *p*-anisaldehyde mixed in 18 ml of ethanol and 1 ml of concentrated sulphuric acid. For vanillin, 0.1 g of vanillin was dissolved in 28 ml methanol and 1 ml of sulphuric acid was added (Wagner and Bladt, 1996).

3.4 Bioassay-guided isolation

A bioassay-guided isolation method was used with the objective of isolating the antibacterially active compounds. Leaves of the plant were extracted and fractionated with the activity of the fractions identified by bioautography. The extracts were spotted on thin layer chromatography (TLC) plates and developed in the solvent system that separated the compounds most effectively. This was done initially using BEA, CEF and EMW. Duplicate chromatograms were dried overnight and sprayed with a concentrated suspension in Müller-Hinton (MH) broth of actively growing cells of Gram-positive (*Staphylococcus aureus* and *Enterococcus faecalis)* or Gram-negative (*Escherichia coli* and *Pseudomonas aeruginosa)* bacteria. The plates were incubated at 37°C overnight in a chamber at 100% relative humidity. Plates were sprayed with a 2 mg/ml solution of *p*-iodonitrotetrazolium (INT) salt. Clear zones on the chromatogram indicated inhibition of growth after incubating for one hour (Begue and Kline, 1972). The INT is metabolized to a coloured formazan product by actively growing cells.

3.5 Solvent/Solvent fractionation

This procedure simplifies extracts by fractionating the components based on polarity. The solvent/solvent group procedure used by the US National Cancer Institute as described by Suffness and Douros (1979) was applied.

3.6 Chromatography

Column chromatography is the most widely used technique to isolate the components of complex mixtures. Various stationary phases were used to separate compounds either according to polarity or size of the compounds (normal and reverse silica gel, Sephadex and Amberlite XAD-16).

3.6.1 Amberlite XAD-16

Amberlite XAD-16 is a polymeric adsorbent supplied as insoluble white beads. It is a non-ionic, hydrophobic, cross-linked polymer which derives its adsorptive properties from its macroreticular structure (containing both a continuous polymer phase and a continuous pore phase), high surface area, and the aromatic nature of its surface.

3.6.2 Chromatotron

The chromatotron is a preparative, centrifugally accelerated, radial, thin-layer chromatograph designed by the authors of Compendium of Organic Synthetic Methods. It replaces preparative TLC plates, small columns and HPLC. The sample to be separated is applied as a solution using a dropper or syringe near the center of a spinning disk coated with a thin layer of sorbent. Elution by a solvent forms circular bands of the separated components which are spun off from the edge of the rotor together with the solvent. A novel collection system brings the eluate to a single output tube. The capacity that can be introduced to the plate should not be above 1 g.

Special advantages of the chromatotron include:

- No spotting of samples or scraping of bands.
- Separations are completed rapidly, typically within 20 minutes.
- A UV transparent lid allows direct observation of UV absorbing or coloured compounds during the separation.
- The pump recycles the output to the input for increased resolving power.
- Layer thickness of 1, 2 or 4 mm of sorbent on the inner plate gives high capacity. The sorbent layer is easily regenerated *in situ* for re-use.
- Solvents are used sparingly. Gradient elution is easy. A nitrogen atmosphere prevents oxidation of samples.
- Compact (easily moved from lab to lab), few controls, no high pressures.
- Low price: half a dozen chromatotrons cost less than a single preparative HPLC (high performance liquid chromatograph).

UV transparent compounds may be detected in the eluted fractions by conventional TLC.

3.7 High pressure liquid chromatography (HPLC)

Chemical separations can be accomplished using high pressure (or performance) liquid chromatography (HPLC) utilizing the fact that certain compounds have different migration rates given a particular column and mobile phase. The chromatographer can separate compounds using HPLC, and

the extent or degree of separation is mostly determined by the choice of stationary phase and mobile phase.

In this research HPLC was also used in the isolation process. There is the option of using different types of columns and solvent systems for the separation of compounds as well as a variety of detectors to interface with the HPLC in order to achieve optimal analysis of the compound.

Detection of compounds by HPLC is a crucial part of any HPLC assay. In order to detect any compound by HPLC, a detector must first be selected. Once the detector is selected and is set to optimal detection settings, a separate assay must be developed. The parameters of this assay should be such that a clean peak of the known sample is observed on the chromatograph. The identifying peak should ideally have a reasonable retention time and should be well separated from extraneous peaks at the detection levels which the assay will be performed. To alter the retention time of a compound, several parameters can be manipulated. One is the choice of mobile phase, and another is the choice of column.

Selection of the separation system by HPLC is accomplished by researching the literature and by trial and error. A sample of a known compound must be utilized in order to assure identification of the unknown compound. Identification of compounds can be confirmed by combining two or more detection methods.

3.8 Analysis and concentration of fractions

When column chromatography was completed, the fractions collected in test-tubes were placed under a stream of air to facilitate concentration of the fractions for TLC analysis and bioassay. After concentration and reconstitution to 10 mg/ml, the contents of the test tubes were spotted on TLC plates, developed in a suitable solvent system, viewed under UV, sprayed wit a chromogenic spray reagent and heated for about 5 minutes. Those fractions with similar TLC profiles were combined. Plates were also developed for bioautography except that they were not sprayed with a spray reagent. Minimum inhibitory concentrations (MIC) of the fractions were also determined.

3.9 Antiviral activity

The crude extracts of *C. paniculatum* were tested for antiviral efficacy at the University of Pretoria. The active extracts were taken to the Hans-Knöll Institute (HKI) in Jena, Germany, where compounds were isolated from the plant material. These compounds were then tested for antiviral activity at the HKI.

3.9.1 University of Pretoria method

3.9.1.1 Cell culture

Crandell feline kidney cells (CRFK) were obtained from the Department of Veterinary Tropical Diseases, University of Pretoria. Cultures were grown in Eagle's Minimum Essential Medium (MEM) containing 10% foetal calf serum (FCS) and 0.05 mg/ml gentamicin. Confluent cell cultures were maintained at 37°C.

3.9.1.2 Virus

The virus used in this assay was feline herpesvirus type 1 (FHV-1), an enveloped virus. Enveloped viruses are highly sensitive to environmental influences. The virus used in the assay was cultured in 75 cm^2 flasks of confluent CRFK cells. Flasks were inoculated with virus stock and then incubated until approximately 90% of the monolayer showed cytopathic effect (CPE). The monolayer was then trypsinised and the resulting cell suspension centrifuged at 1000 X g for 10 minutes and the supernatant stored at -70°C.

3.9.1.3 Determination of the antiviral efficacy of the extract

The extract (see section 3.2) was diluted in sterile de-ionized water to a final dilution of 1:50. Virus stock (0.5 ml) was then mixed with 0.5 ml of extract (contact time 20 minutes). A serial 10-fold dilution was performed by taking 0.5 ml from the mixture of virus and extract and placing in tubes containing 4.5 ml MEM. The above-mentioned 10-fold dilutions were performed in 8 different tubes to give a dilution range of 1:10 to 1:1280. Two hundred µl of each dilution was placed in wells of a 96-well flat bottom microtitre plate. Eighty µl of CRFK cells (480,000 cells/ml) were added to each well in 5 replicates. Each extract test included a virus control and a toxicity test. The plates were incubated for 5 days at 37°C in a 5% CO_2 atmosphere. The CPE was observed by the use of an inverted light microscope. One hundred

percent cell damage was scored with a 4 while 75% cell damage was scored a 3 and so on. A zero indicated that the cells were unaffected. The tissue culture infectious dose 50 ($TCID_{50}$) was calculated using the Karber method (Karber, 1931).

3.9.1.4 Determination of the cytotoxicity of the extracts (MTT assay)

Viable cell growth after incubation with the test compound was determined using the tetrazolium based colorimetric assay described by Mosmann (1983). Cells (CRFK) of a sub-confluent culture were harvested and centrifuged at 200 x g for 5 min, and resuspended in growth medium to 4.8 x 10^5 cells/ml. The growth medium used was MEM supplemented with 0.1% gentamicin and 5 % foetal calf serum. A total of 200 µl of the cell suspension was pipetted into each well of a 96-well microtitre plate. The plates were incubated for 24 h at 37°C in a 5% CO_2 incubator, until the cells were in the exponential phase of growth. The MEM was aspirated from the cells using a fine tube attached to a hypodermic needle, and replaced with 200 µl of test compound at differing concentrations (serial dilution prepared in growth medium). The cells were disturbed as little as possible during the aspiration of medium and addition of test compound. Each dilution was tested in 5 replicates. The microtitre plates were incubated at 37°C in a 5% CO_2 incubator for 5 days. Untreated cells and positive control (berberine chloride, Sigma) were included.

After incubation, 30 µl MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide, Sigma, stock solution of 5 mg/ml in PBS) was added to each well and the plates incubated for a further 4 h at 37°C. After incubation with MTT the plates were centrifuged for 10 min at 1500 rpm. The medium in each well was carefully removed, without disturbing the MTT crystals in the wells, before adding 150 µl fresh PBS to each well. The microtitre plates were again centrifuged for 10 min at 1500 rpm and the PBS removed from the wells. The MTT formazan crystals were dissolved by adding 50 µl DMSO to each well. The plates were shaken gently until the MTT solution was dissolved. The amount of MTT reduction was measured immediately by detecting absorbance in a microplate reader (Titertek Multiscan MCC/340) at a test wavelength of 540 nm and a reference wavelength of 690 nm. Wells containing medium and MTT but no cells, were used to blank the plate reader. The lethal concentration 50 (LC_{50}) value was calculated as the concentration of test compound resulting in a 50% reduction of absorbance compared to untreated cells.

3.9.2 Hans-Knöll Institute (HKI) method

3.9.2.1 Cytotoxicity test to determine the maximum tolerated dose (CC_{10}) of the test compound in HeLa, MDCK and Vero cell monolayers

MDCK (Madin-Darby Canine Kidney) and Vero cells were seeded in microtitre plates and incubated for 48 hours at 37°C in 95% humidity and in the presence of 5% CO_2 to form a monolayer. The medium was then discarded and the compounds added at various concentrations (100 µl per well; 2-fold dilutions). To determine the control value (6 untreated cell controls) 100 µl medium was used in each control well respectively. Cells were stained with crystal violet/methanol 72 hours after the compounds had been added and incubation had taken place. Following elution of the stain, the optical density of the individual wells was measured with a Dynatech 570/630 plate photometer and compared with the mean values of the controls. The CC_{10} values (µg/ml) were estimated with the aid of the foregoing data provided that more than 90% of the control cells were viable.

3.9.2.2 Determination of the antiviral efficacy of the test compounds by means of inhibition of the cytopathic effect (CPE)

The test system consisted of:

1. HeLa cells infected with coxsackievirus strain B3 Nancy
2. MDCK cells infected with influenzavirus type A strain Hong Kong
3. Vero cells infected with herpes simplex virus type 1 strain K1

The replication of the viruses used in the test results in pronounced CPE leading to complete destruction of the cells. The virus-induced CPE can be inhibited by adding antiviral substances in volumes of 100 µl per well and diluted by means of 2-fold dilutions. In the test, treated and untreated cell layers were infected with a dose of virus that after 24 hours (B3 and Hong Kong) and 48 hours (K1) resulted in complete CPE in the untreated virus controls. At this stage cells that were still adhering were fixed and stained with crystal violet/formalin solution. The inhibition of the virus-induced CPE was quantified photometrically using a Dynatech plate reader following elution of the stain. The antiviral efficacy was determined by comparing the optical densities of treated and untreated virus-infected cells with the average optical densities of the controls that was set as 100%. As internal test control, a known virustatic compound was included simultaneously in each microtitre plate.

3.10 Antibacterial activity

3.10.1 Microdilution assay for MIC determination

The MIC for each plant extract against a range of bacteria was determined by serial two-fold dilution of extracts beyond the level where no inhibition of growth of test organism was observed (Eloff, 1998b). The test organisms were the Gram-positive *Enterococcus faecalis* ATCC 29212 and *Staphylococcus aureus* ATCC 29213, and the Gram-negative *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853. The assay was performed in microtitre plates by adding 100 µl of sterile water to all wells. In row A, 100 µl of extract was added with a micropipette. From row A, 100 µl was transferred to row B after using a micropipette to take up and release the liquid three times to ensure adequate mixing. The process was repeated until all the rows down the column were completed and 100 µl from row H was discarded. Two wells were used as a sterility control containing only water, whilst the growth control contained both water and test organism. Gentamicin was used as a positive control antibiotic.

After adding 100 µl of the bacterial suspension to each row (except for the sterility control), the microplate was sealed and incubated at 37^0C at 100% relative humidity overnight. The following morning 40 µl of a 0.2 mg/ml solution of INT (iodonitrotetrazolium chloride) was added to each row and the plate was returned to the incubator for at least half an hour to ensure adequate colour development. INT is a dehydrogenase activity detecting reagent, which is converted into an intensely coloured red-purple formazan by metabolically active micro-organisms.

Inhibition of growth was indicated by a clear solution or a definite decrease in colour reaction. This value was taken as the MIC of the extract. Extracts used for MIC determination were either dissolved in acetone, water or DMSO.

3.10.2 Total activity

Total activity is a measure of the amount of material extracted from a plant in relation to the MIC of the extract, fraction or isolated compound. Reasons for screening studies abound: to find new lead biologically active compounds, and to confirm the ethnomedicinal use of plants to develop phytomedicines for use as herbal medicine. In many screening studies, activities are reported non-quantitatively. Even if extract data are expressed in quantitative terms such as MIC, it is usually not

possible to compare different plants or different fractions with the results presented. To compare activity of plants or fractions, however, the quantity extracted from the plant or present in the fraction should be included in the equation (Eloff, 2004). In mathematical terms it can be expressed as:

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

The units are expressed in ml/g and indicate the degree to which the active extracts, fractions or compounds present in one gram can be diluted and still inhibit the growth of the test organisms (Eloff, 2004).

3.10.3 Bioautography

Extracts were reconstituted to a concentration of 10 mg/ml in acetone and 10 µl (100 µg) of each extract were applied as narrow lines on TLC plates. Extracts of the leaves and root bark were used for bioautography. The organic solvent was evaporated by a stream of air and plates were eluted by a solvent system that would provide efficient separation. The solvent was once more evaporated by a stream of air overnight. In the case of TLC plates run in CEF, plates were dried much longer to ensure adequate removal of formic acid. The plates were then sprayed with a suspension of actively growing bacterial cells and incubated overnight at 37^0C in a chamber at 100% relative humidity. The next morning, the plates were sprayed with a 2 mg/ml solution of INT. Inhibition of growth was indicated by clear zones on the chromatogram (Begue and Kline, 1972). This method was chosen for its simplicity, low cost, accuracy and fast results.

3.11 Antifungal activity of extracts

A serial microdilution assay with INT added as growth indicator (Masoko *et al.,* 2005) was used to determine the minimum inhibitory concentration (MIC) values for plant extracts against fungi. This method had previously been used successfully for antibacterial activities (Eloff, 1998b and McGaw *et al*., 2001). By applying the tetrazolium assay for measuring antifungal activities, a slight modification was made to suit fungal growth conditions. Extracts were resuspended to 10 mg/ml in acetone. The plant extracts (100 µl) were serially diluted in water in 96-well microtitre plates (Eloff, 1998b). Using a sterile swab, fungal cultures were transferred from Sabouraud agar plates into fresh Sabouraud dextrose broth, and 100 µl of this suspension was added to each well. Amphotericin B was used as the reference antibiotic and positive control, and appropriate solvent blanks were included. As an indicator

of growth, 40 µl of 0.2 mg/ml *p*-iodonitrotetrazolium violet (INT) dissolved in water was added to each of the microplate wells. The microplates were incubated at 37°C for 2-3 days. The MIC was recorded as the lowest concentration of the extract that inhibited antifungal growth after 24 and 48 h.

3.12 Agar diffusion method for antibacterial and antifungal activity

Nine ml of Müller-Hinton agar for bacteria and Sabouraud Dextrose Agar for fungi were poured into Petri dishes (9 cm in diameter) and inoculated with the respective test organisms to form a lawn. Wells (4 mm) were punched out of the solid agar using pipette tips, and 1 ml of 50 µg/ml test compounds and control antibiotics were placed into each well. The Petri dishes were incubated at 37°C for 20 h and the average diameter of the inhibition zone surrounding the wells was measured.

3.13 Antioxidant activity

Metabolism, like other aspects of life, involves trade-offs. Oxidant by-products of normal metabolism cause extensive damage to DNA, proteins, and lipids. It is argued that this damage (the same as that produced by radiation) is a major contributor to ageing and to degenerative diseases of ageing such as cancer, cardiovascular disease, immune-system decline, brain dysfunction, and cataracts. Antioxidant defenses against this damage include ascorbate, tocopherol, and carotenoids. Dietary fruits and vegetables are the principal source of ascorbate and carotenoids and are one source of tocopherol. Low dietary intake of fruits and vegetables doubles the risk of most types of cancer as compared to high intake and also markedly increases the risk of heart disease and cataracts. Since only 9% of Americans eat the recommended five servings of fruits and vegetables per day, the opportunity for improving health by improving diet is great (Ames *et al.*,1993).

Plant extracts may protect against infection by stimulating or protecting the immune system of the user. Immune system stimulation is associated with antioxidant activity. The most widely used methods for measuring antioxidant activity are those that involve the generation of a radical species and measurement of a range of end points as a fixed time. Two types of approaches may be followed, namely inhibition assays where the extent of the scavenging by hydrogen or electron donation of a preformed free radical is the marker of antioxidant activity as well as assays involving the presence of antioxidant systems during the generation of the radical. This study made use of the DPPH free radical assay.

DPPH (1, 1 diphenyl-2-picryl hydrazyl) is a stable purple-coloured free radical which does not dimerize and can hence be prepared in crystalline form. The DPPH method measures hydrogen atom or electron donating activity and hence provides an evaluation of antioxidant activity due to free radical scavenging. Reaction of the DPPH with the antioxidant results in a decolourisation of the free radical, which can be followed spectrophotometrically. DPPH may also be used as a TLC spray reagent in a quick screening procedure which detects the presence of antioxidants when the initial purple background on the TLC plate turns yellow in the presence of an antioxidant (Brand-Williams *et al*., 1995). In this way, the qualitative antioxidant activity can be determined easily by spraying a chromatogram with 0.2% DPPH. The yellow zones on the purple background represent compounds with antioxidant activity.

3.14 Anti-inflammatory activity

The NAD(P)-linked enzyme, 3α-hydroxysteroid dehydrogenase, has been purified to homogeneity from rat liver cytosol (Penning, 1985). This enzyme is known to reduce a variety of 3-ketosteroids, e.g. 5α-dihydrotestosterone (5α-androstan-17β-o-one), 5β-dihydrocortisone (5β-pregnan-17α, 21-diol-3, 11, 20-trione), to the corresponding 3α-hydroxysteroids, and therefore plays an important role in cortisone metabolism (Penning, 1985). A surprising property of the purified enzyme is that it is potentially inhibited by the major classes of non-steroidal and steroidal anti-inflammatory drugs in rank order of their therapeutic potency (Penning, 1985). A high correlation exists between the logarithms of the concentration of drug required to produce 50% inhibition of the purified 3α-hydroxysteroid dehydrogenase (log IC_{50} value) with the dose required to produce an anti-inflammatory response in man. These observations led to the suggestion that the extent of inhibition of 3α-hydroxysteroid dehydrogenase could be used to predict anti-inflammatory drug potency (Penning, 1985).

3.14.1 Enzyme assay

The reduction of 5β-dihydrocortisone was monitored by measuring the changes in the absorbance of the pyridine nucleotide at 340 nm. Each assay (1.0 ml) contained the following: 0.840 ml of H_2O, 0.100 ml of 1 M potassium phosphate buffer (pH 6.0), 20 µl of 9 M NADPH, 10 µl of 5 mM 5β-dihydrocortisone, and 30 µl of acetonitrile. The reactions were initiated by the addition of enzyme (30-50 µg of cytosolic protein or 0.6 µg of purified enzyme), and optical density change was observed over a period of 5 minutes. Control incubation experiments by addition of cytosol in which either the 5β-

dihydrocortisone or NADPH was absent, indicated that the presence of both substances was required before the cytosol would promote a change in absorbance at 340 nm.

The percentage inhibition of isolated compounds was detected at three concentrations (30 µg/ml, 3 µg/ml and 0.3 µg/ml). Increasing amounts of the isolated compound were added to the standard assay and the concentration of the compound required to reduce the rate of 5β-dihydroxycortisone by 50% inhibitory concentration (IC_{50}) was computed from the resulting dose-response curves.

Chapter 4

Selection of the best extractant for the plant material

4.1 Introduction

In phytochemical analysis, the botanical identity of the plant under study must be authenticated by an acknowledged authority and a voucher specimen stored so that results can be reproduced on the correct species by other scientists. Also, if taxonomy changes, the identity of the species investigated can be verified.

There are different methods available for extracting compounds from plant material. One method for obtaining organic constituents from dried plant tissue is to use a Soxhlet apparatus with a sequential range of solvents, starting in turn with ether, petroleum and chloroform (to extract lipids and terpenoids) and then using alcohol and ethyl acetate (for more polar compounds). This method is useful when working on the gram scale. The extract obtained is clarified by filtration and concentrated in a rotary evaporator. This procedure leads to exhaustive extraction but labile compounds will be destroyed by the high temperature used.

A direct cold extraction procedure of finely ground material was developed for use in the Phytomedicine Programme (Eloff, 1998a). This chapter will focus on how to select the best extractant, quantity and number of compounds separated, and whether there is a difference between compounds extracted from *C. paniculatum* and *C. microphyllum.* The aim of this chapter is to determine the best extractant to use in the isolation of the bioactive compounds and also to compare data for the closely related *C. paniculatum* and *C. microphyllum.* Germishuizen and Meyer (2003) consider *C. paniculatum* and *C. microphyllum* the same species while Carr (1988) considers them to be different species.

4.2 Extraction

Extraction was carried out on the powdered leaf material of *C. paniculatum*. This was performed on a Labotec Model 20.2 shaking apparatus with a 1:10 dry weight plant sample (g) to solvent (ml) ratio. This extraction was carried out in parallel using eight solvents ranging from non-polar to polar (hexane, carbon tetrachloride, dichloromethane, tetrahydrofuran, ethanol, acetone, methanol and water). One

gram of plant material (separate aliquots for each solvent) was placed in a polyethylene centrifuge tube and 10 ml of the extracting solvent placed into the polyethylene tube. The sample and solvent were shaken for 10 minutes on the shaker and centrifuged for 10 minutes at 1500 x g before filtering using Whatman No. 1 filter paper. The solvent was evaporated and the residue resuspended to 10 mg/ml. The plates were developed in three solvent systems, namely BEA, EMW and CEF, and the fingerprint profile observed. The composition of the solvent systems is described in section 3.3.

4.3 Results

Water extracted the largest quantity (246 mg) with a 24.6% yield followed by methanol (194 mg and 19.4% yield). The lowest quantity extracted was for dichloromethane (DCM) (15 mg) and 1.5% of material was extracted (Table 4.1). Water and methanol extracted the highest quantity of material from *C. paniculatum*, and this implies that there are many polar compounds in the sample since water and methanol are the most polar solvents among the solvents used. The values for *C. microphyllum* that were obtained from the work of Kotze and Eloff (2002) are also included in Table 4.1 for comparative purposes. In general, the results for the two plants were dissimilar.

Table 4.1. Quantity of material extracted (mg/g) from *C. paniculatum* and *C. microphyllum* (Kotze and Eloff, 2002)

Plant species	Hexane	CCl_4	DCM	THF	EtOH	Acetone	MeOH	Water
C. paniculatum	23	27	15	47	43	29	194	246
C. microphyllum	36	48	106	64	36	32	174	48

Figure 4.1 depicts TLC separations of the leaf extracts developed in EMW and sprayed with vanillin sulphuric acid spray reagent.

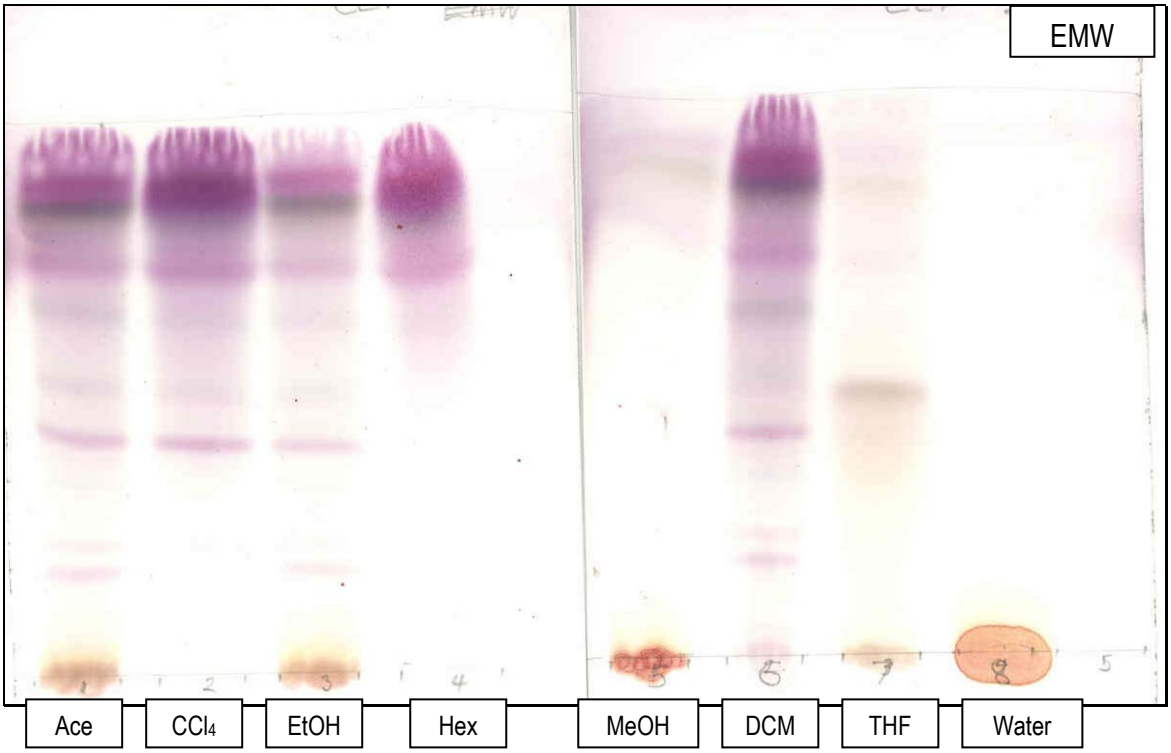


Fig 4.1. TLC plate showing separation of components present in 8 different solvent extracts of *C. paniculatum* leaf material using EMW as eluent and vanillin spray reagent

With the spray reagent used, about 11 compounds were detected by TLC in the EMW solvent system. No components visualized by the spray reagent separated in the methanol and water extract using this eluent. The retention factors of the components of the leaf extract are shown in Table 4.2.

Table 4.2. Retention factors (R_f) of compounds present in leaf extracts developed in EMW and sprayed with vanillin/sulphuric acid spray

Solvents	Retention factor (R_f)						
Hexane					0.77	0.88	
CCl_4		0.46	0.56		0.77	0.89	
DCM	0.25	0.46	0.65		0.74	0.88	
THF			0.52				
EtOH		0.46			0.77	0.87	0.91
Acetone	0.24	0.46	0.56	0.68	0.77	0.85	0.89
MeOH	0						
H_2O	0						

Acetone extracted the highest number of compounds (7), followed by dichloromethane (5). Water and methanol extracts did not move from the origin so the retention values are zero in the EMW solvent system. The TLC chemical profile of the acetone extract indicated that a wide range of compounds was extracted by acetone. The retention factors had a wider range for acetone and the major compounds isolated by the other solvents are also found in the acetone extracts. Extracts of water and methanol did not separate even with the polar EMW solvent system, meaning that the compounds isolated were highly polar and stayed at the bottom of the plate.

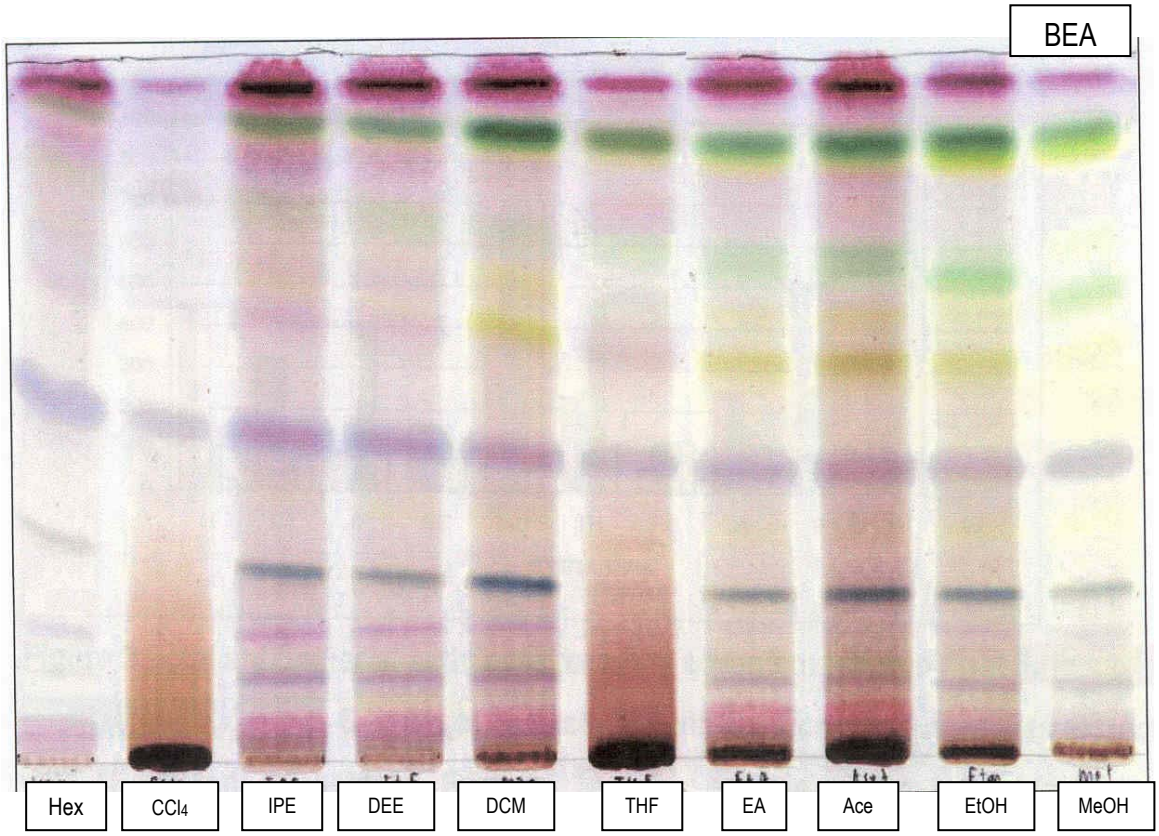


Fig 4.2. Separation of components present in 100 µg of extracts of *C. microphyllum* leaves, developed in BEA and sprayed with vanillin/sulphuric acid spray reagent (from Kotze and Eloff, 2002)

The solvents used from left to right are: hexane, carbon tetrachloride, isopropyl ether, diethyl ether, dichloromethane, tetrahydrofuran, ethyl acetate, acetone, ethanol and methanol.

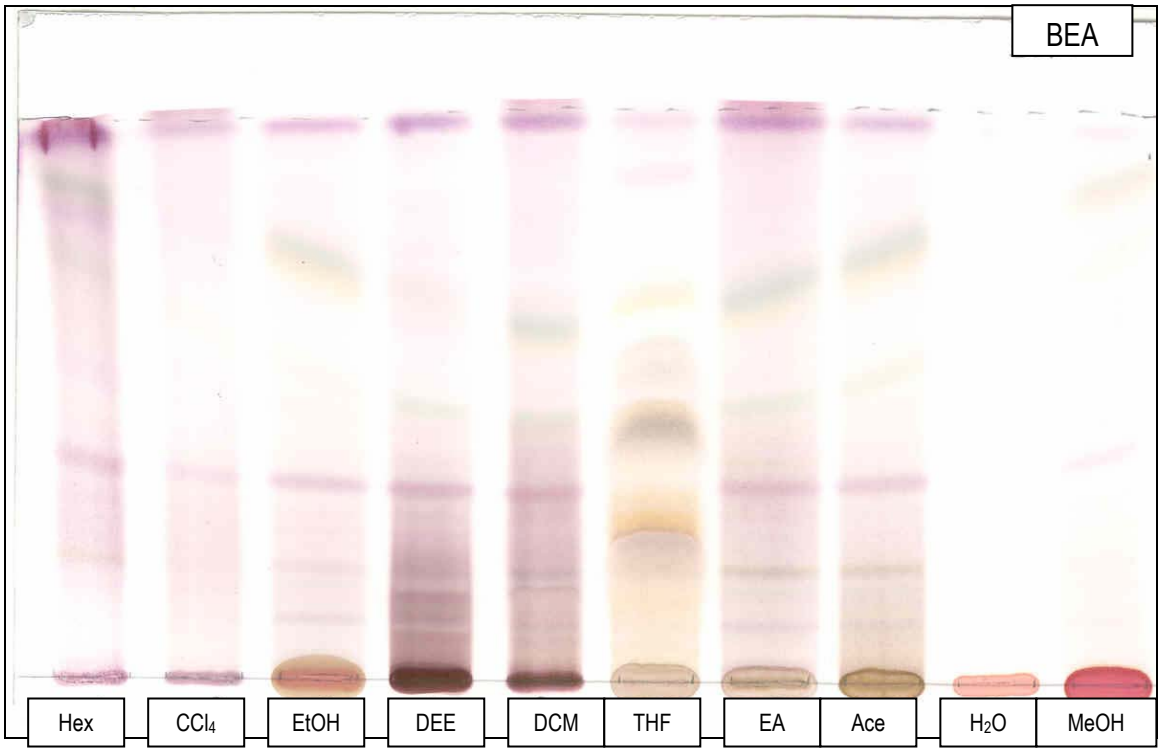


Fig 4.3. Separation of components present in 100 µg of extracts of *C. paniculatum* leaves developed in BEA and sprayed with vanillin/sulphuric acid spray reagent

Lanes from left to right represent compounds extracted by hexane, carbon tetrachloride, ethanol, diethyl ether, dichloromethane, tetrahydrofuran, ethyl acetate, acetone, water and methanol. No components separated in the water and methanol extract.

4.4 Discussion

The amount of material extracted per gram of powdered *C. paniculatum* leaves ranged from 15 mg to 246 mg (Table 4.1). These values are different from those obtained by Kotze and Eloff (2002) working on the closely related *C. microphyllum* (26 to 174 mg). The amounts of material extracted by water and DCM for the two species were notably different but the amounts for the other solvents were closer. This may be related to the season of collection of plant material, when sugars (polar and soluble in water) may have been converted to non-polar metabolites (soluble in DCM).

For *C. paniculatum,* water, methanol and THF extracted the most components while for *C. microphyllum*, methanol, DCM and THF extracted the most compounds. *C. microphyllum* extracts were separated into many more compounds using TLC analysis than *C. paniculatum* extracts. The

differences shown in the fingerprints and the amounts of material extracted from the two plants supports the view of Carr (1988) that the plants represent different species. The differences in amounts of material extracted from *C. microphyllum* and *C. paniculatum* and in TLC analysis of fingerprint profiles can be seen in Table 4.1, Fig 4.2 and Fig 4.3 respectively.

TLC was used to determine the chemical composition of the extracts. The solvent systems used were BEA, CEF and EMW. The acetone extract had as many as seven compounds on the TLC fingerprint profile compared to the other solvents used for extraction. Dichloromethane, carbon tetrachloride, hexane and tetrahydrofuran extracts were separated into 5, 4, 2, and 1 compounds respectively by TLC in the EMW solvent system. Water and methanol extracts did not separate into compounds visible with the spray reagent.

As far as the selection of the extractant of choice is concerned, Eloff (1998a) found that acetone was the best extractant, especially for the Combretaceae family. This conclusion was confirmed in work done on several *Combretum* and *Terminalia* species investigated in the Phytomedicine Programme. Based on the presence of compounds visualized with the vanillin spray reagent, acetone extracted the largest number of different compounds from *C. paniculatum* leaves in this study. The ease of evaporation and the fact that it is not toxic to bacteria at the concentrations used in the bioassays in this study makes acetone a good solvent to work with. Asres *et al.* (2001) also used acetone as extractant for the leaves of *C. paniculatum*, and the extract exhibited antiviral activity against HIV-2 with an EC_{50} of 3.0 µg/ml and a selectivity index of 32. Acetone was therefore selected as the initial extractant in further work.

Chapter 5

Determination of antimicrobial, cytotoxic and antioxidant activities of extracts

5.1 Introduction

Effective therapies for HIV infection are being sought far and wide, in the natural world as well as in laboratories. *In vivo* anti-HIV studies of glycyrrhizin, from glycyrrhiza plants (the source of licorice), extended the life of retrovirus-infected mice from 14 to 17 weeks (Watanbe *et al*., 1996). A crude extract of the cactus *Opuntia streptacantha* had a marked antiviral effect *in vitro*, and toxicity studies performed in mice, horses, and humans found the extract to be safe (Ahmad *et al*., 1996).

Human mycoses, especially in immunocompromised patients, are not always successfully treated due to the ineffectiveness or toxicity of the available antifungal drugs (Barrett-Bee and Ryder, 1992). Similarly some bacterial infections, especially those produced by *Staphylococcus aureus*, may be difficult to manage. Therapy with several types of antibiotics (Hiramatsu *et al*., 1997) is frequently accompanied by side effects and microbial resistance, such as is the case with methicillin-resistant *Staphylococcus aureus* (MRSA). New and potent antimicrobial agents are still needed and should be actively sought (Chambers, 1997; Selitrennikoff, 1992). Belachew (1993) showed that the direct aqueous extract of the leaf of *C. paniculatum* showed bacterial inhibition against *S. aureus* which was better than that shown by the antibiotic neomycin.

Cytotoxicity measures the toxicity of extracts to cells. It is measured as functions of fundamental biochemical pathways leading to cell death (Mosmann, 1983). Only one in ten drug candidates make it through the development process. One-third of these failures are due to unacceptable toxicity levels (Ricerca, 1998). A cytotoxicity assay is a rapid and cost-effective tool to sort out the likely failures before a compound is entered into the costly development process and to help choose the optimal candidate (Wallin and Arscott, 1998). Cytotoxicity testing is a rapid, standardized, sensitive and inexpensive means to determine whether a material contains significant quantities of biologically harmful extractables. The high sensitivity of the tests is due to the isolation of the test cells in cultures and the absence of the protective mechanisms that assist cells within the body (Ricerca, 1998).

5.2 Methods

The leaf, stem bark and root bark extracts were tested for various biological activities, and initial concentrations for each extract were 100 mg/ml.

5.2.1 Antiviral activity

The procedure for this test described in section 3.9.1 was followed to determine antiviral activity of the extracts. The virucidal activity of the diluted extract was calculated by comparing the infectivity titre obtained in the absence of the extract (log_{10} $TCID_{50}$ of the controls) while the infectivity titre was determined after the interaction of virus and extract. The difference between the two values was equal to the reduction of the infectivity titre. The Karber method was used for calculating the $TCID_{50}$ (Karber, 1931).

5.2.2 Antibacterial activity

The procedure for this test is described in section 3.10.1.

5.2.3 Bioautography

The method used for this test is described in section 3.10.3.

5.2.4 Antioxidant activity

The TLC DPPH spray method described in section 3.13 was used to determine the presence of antioxidant compounds in the extract.

5.2.5 Cytotoxicity (MTT assay)

After scoring the percentage cell damage in the antiviral test, with the use of a light microscope, the MTT method was used as an additional test to determine cytotoxicity. This procedure is described in section 3.9.1.4.

5.3 Results

5.3.1 Antiviral and cytotoxic activities of extracts

Fig 5.1 shows the antiviral and cytotoxic activities of the acetone extract of the leaves. The virus used was feline herpesvirus (type 1) and the host cells were Crandell feline kidney cells (CRFK). The cells were destroyed by virus (plate C) up to the fifth column of the microtitre plate (i.e. up to a dilution of 10^{-5}). The extract alone (plate B) damaged cells in the first column only (at a dilution of 10^{-1}). For the extract antiviral test (A), the cells were damaged in the first column and partly in the second column. It was concluded that the extract diminished cell damage by the virus by three 10-fold dilutions (a thousand-fold). The deep purple colour indicates wells where the cells are viable and the lighter coloured wells are those in which the cells were partly destroyed.

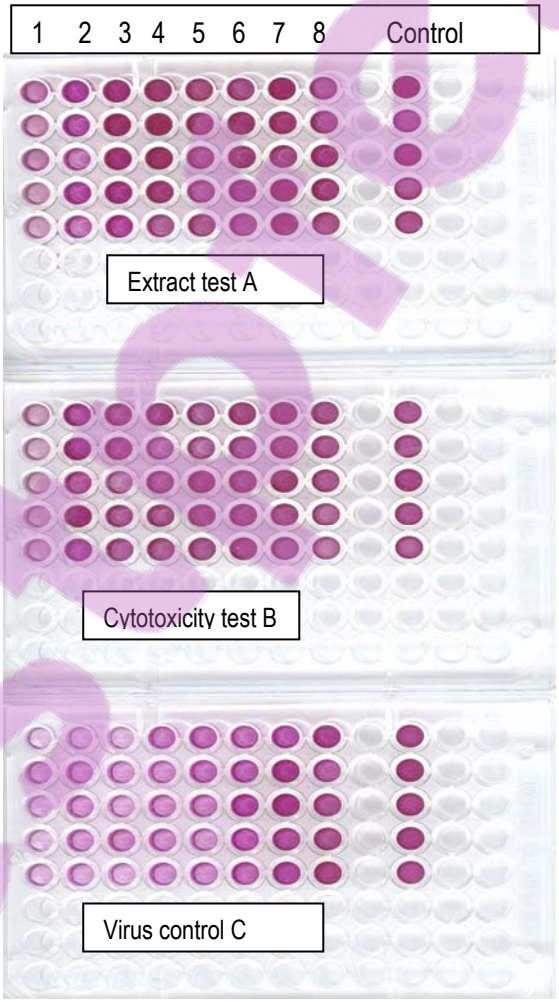


Fig 5.1. Microplate representation of acetone leaf extract test (A), cytotoxicity (B) and virus control (C)

The results of the various extracts tested are shown in Table 5.1.

Table 5.1. Antiviral activity of *C. paniculatum* extracts against FHV-1

Part of plant	Extract test (b)	Virus titre (a) ($TCID_{50}$)	Antiviral activity (c = a - b)
Leaf (acetone)	$10^{-1.5}$	$10^{-4.5}$	10^{3}
Leaf (water)	$10^{-2.5}$	$10^{-5.5}$	10^{3}
Leaf (80% methanolic)	$10^{-1.5}$	$10^{-4.5}$	10^{3}
Stem bark (water)	$10^{-2.5}$	$10^{-6.1}$	$10^{3.6}$
Root bark (water)	$10^{-2.5}$	$10^{-5.9}$	$10^{3.4}$

The results are reported as tissue culture infectious dose 50% ($TCID_{50}$) in log_{10} values. The criterion of efficacy for virucides stipulated by both the DVV (1984) and AFNOR (1989) is a 4.0 log_{10} reduction in virus titre. However, Sattar and Springthorpe (1991) suggested that a 3.0 log_{10} reduction, if achieved under realistic conditions, is an adequate measurement of product efficacy.

5.3.2 Antibacterial activity

Leaves of the plant were extracted in 8 solvents and their MICs determined against four bacterial organisms. The lowest average MIC value was obtained for DCM and THF (0.275 mg/ml) extracts (Table 5.2). The highest MIC values were those of the water and hexane extracts. This is similar to the situation in practically all members of the Combretaceae examined to date. *E. faecalis* was the most sensitive bacterium while the least sensitive species to the extracts was *S. aureus.* The MIC values for the different extractants ranged from 0.156 to 2.5 mg/ml. There was only one value of 2.5 mg/ml and this was for the water extract against *S. aureus.* All the extracts had a substantial antibacterial activity towards the four bacteria examined with average values of 0.47, 0.59, 0.57 and 0.29 mg/ml for *E*. coli, *S. aureus, P. aeruginosa* and *E. faecalis.* Plant extracts are frequently much more active against Gram-positive bacteria (Vlietinck *et al*., 1995), but this was not true for *C. paniculatum* extracts.

The highest total activity according to Kotze and Eloff (2002) while investigating *C. microphyllum* was for the methanol extract followed by the DCM extract, while for *C. paniculatum,* the highest total activity was for the water extract followed by the methanol extract. The total activity values for *C. paniculatum* leaf extracts are reported in Table 5.2.

Table 5.2. MIC (mg/ml) and total activity (TA, ml/g) values of *C. paniculatum* leaf extracts prepared using eight extractants

	Acetone		CCl_4*		EtOH		C_6H_{12}		MeOH		DCM		THF		water	
	MIC	TA														
Qty (mg) extracted per gram	29		27		43		23		194		15		47		246	
E. coli	0.315	92	0.63	43	0.315	136	1.25	18	0.315	615	0.315	47	0.315	149	0.315	780
S. aureus	0.63	46	0.63	43	0.63	68	1.25	18	0.63	310	0.315	47	0.315	149	0.315	780
P. aeruginosa	0.156	185	0.315	85	0.156	275	0.63	36	0.315	615	0.315	47	0.156	301	2.5	98
E. faecalis	0.156	185	0.156	175	0.315	136	0.315	73	0.63	310	0.156	96	0.315	149	0.315	780
Average	0.313	127	0.432	86.5	0.354	153.7	0.861	36.3	0.47	462.5	0.275	59.3	0.275	187	0.861	609.5

* CCl_4 = carbon tetrachloride, EtOH = ethanol, C_6H_{12} = hexane, MeOH = methanol, DCM = dichloromethane, THF = tetrahydrofuran

5.3.3 Bioautography of extracts

Figure 5.2 shows a bioautographic plate of extracts prepared using 10 solvents, developed in CEF.

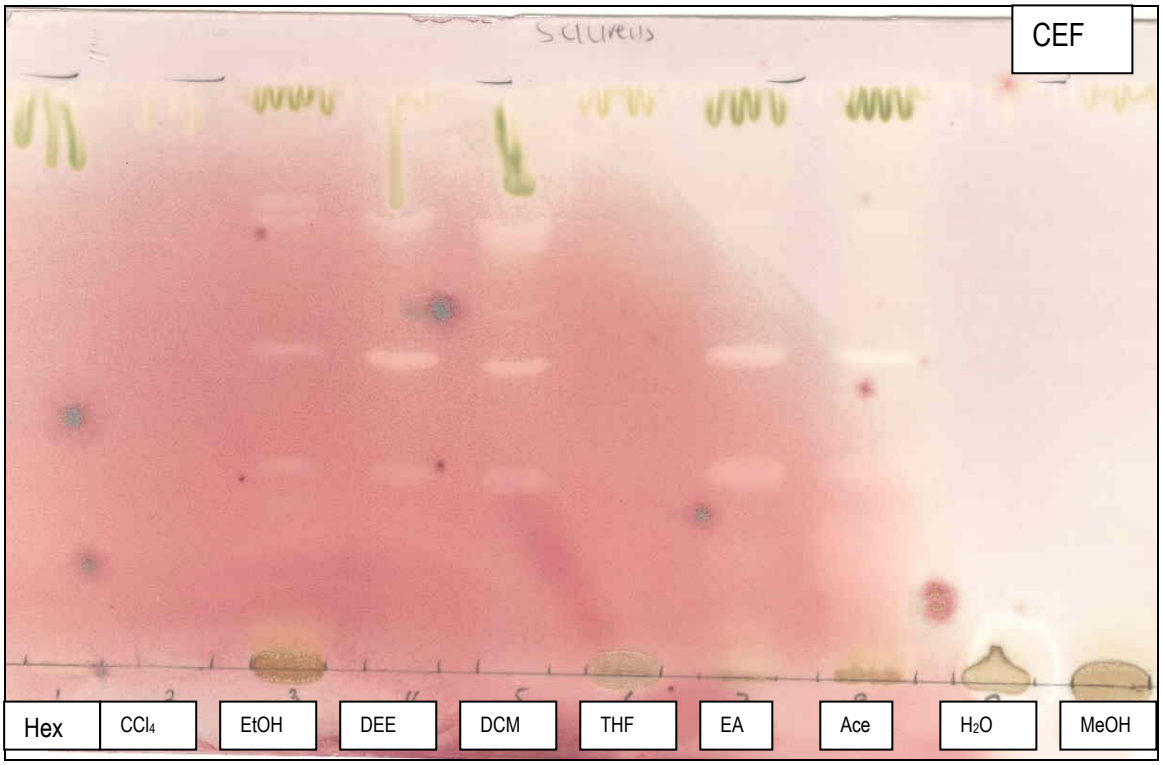


Fig 5.2. Bioautography of ten different leaf extracts of *C. paniculatum* against *S. aureus* in CEF

The solvents from left to right are: hexane, carbon tetrachloride, ethanol, diethyl ether, dichloromethane, tetrahydrofuran, ethyl acetate, acetone, water and methanol. Inhibition zones are the clear zones on the purple background.

5.3.4 Antioxidant activity of extracts

Results of the antioxidant activity of extracts of leaves prepared using five different solvents are shown in Fig 5.3, and those of the root bark extracts in Fig 5.4. The clear zones in the purple background are areas where components exhibit antioxidant activity after spraying with DPPH. This is a rapid, qualitative method of determining antioxidant activity of extracts.

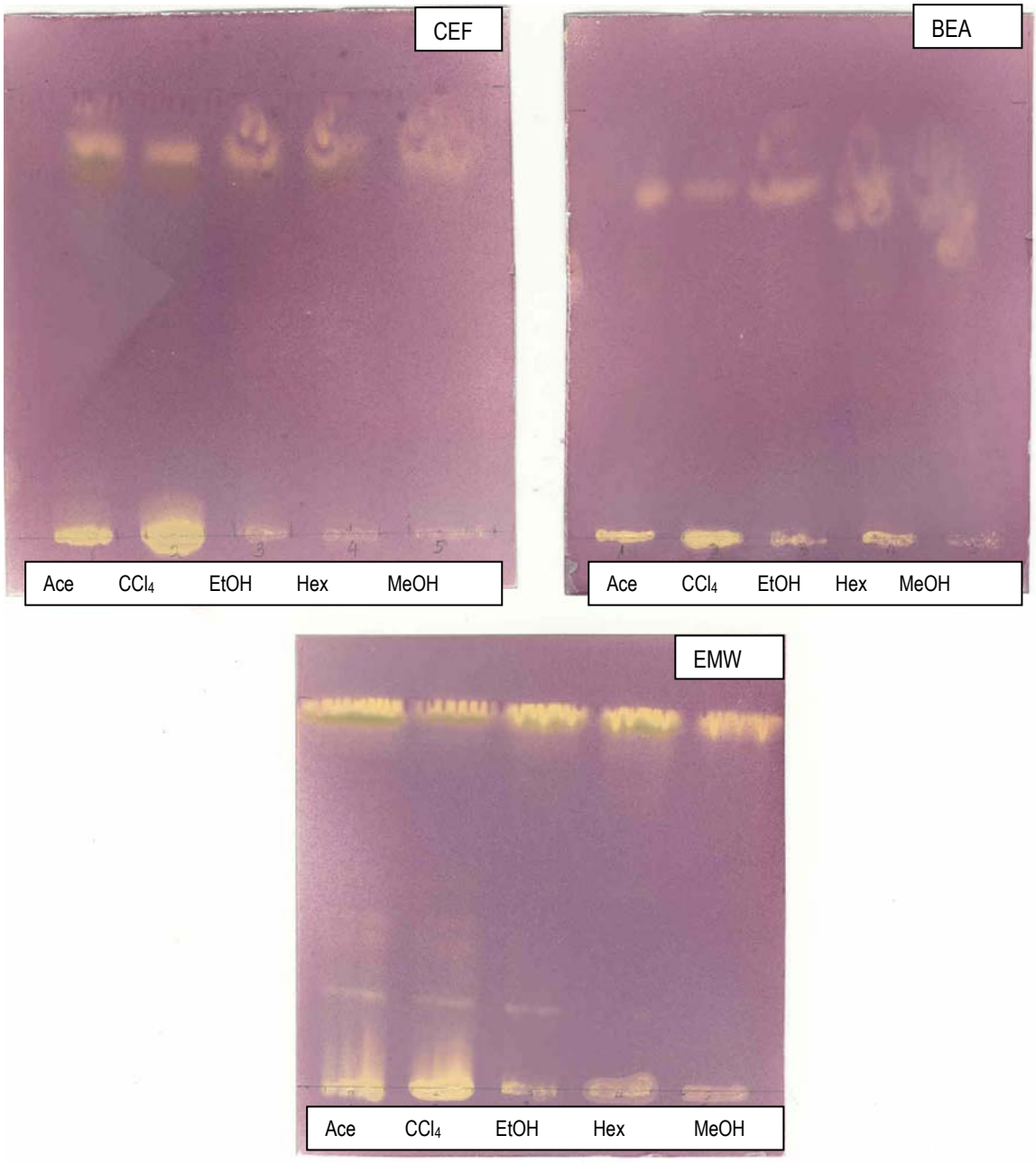


Fig 5.3. DPPH spray of leaf extracts in different solvents

The chromatograms were developed in three solvent system (CEF, BEA and EMW) and sprayed with DPPH.

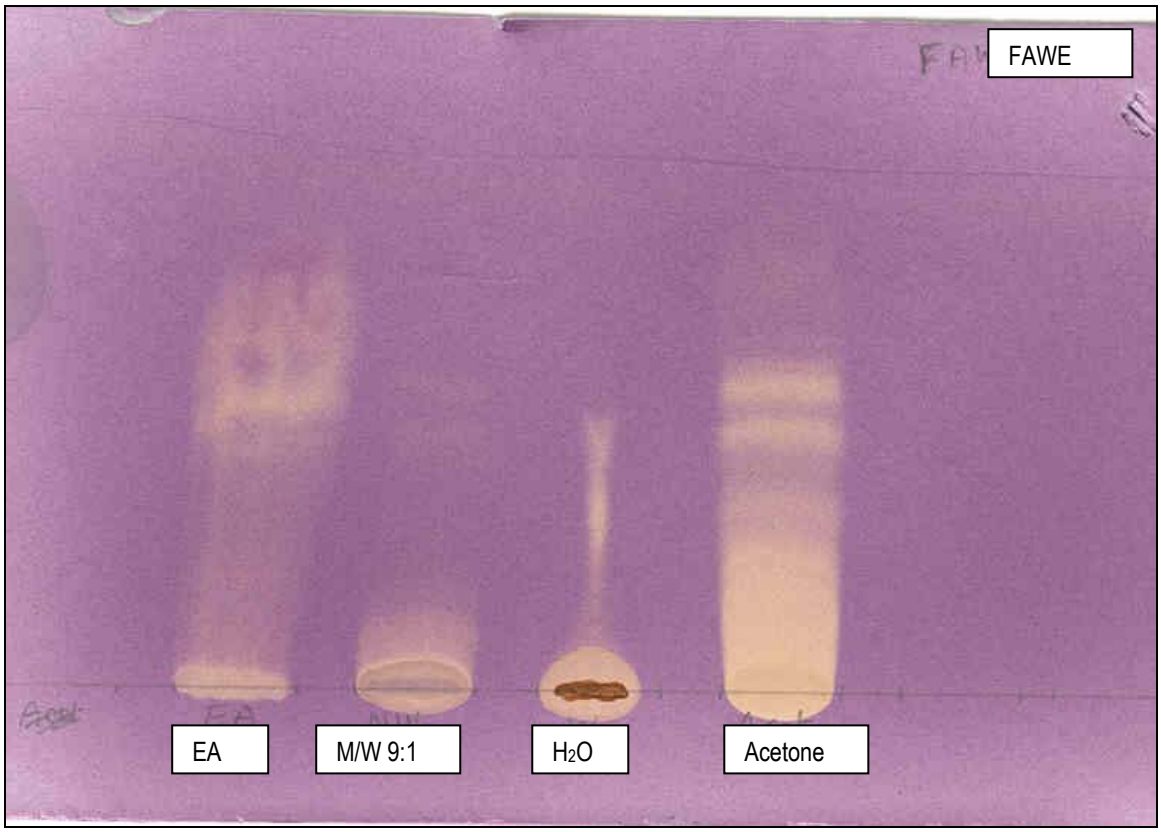


Fig 5.4. DPPH spray of the root bark extracts of *Combretum paniculatum* prepared using four solvents

5.4 Discussion

The antiviral activity of the leaf, stem bark and root bark was determined microscopically by examination for cytopathic effect (CPE) and also with the aid of the colorimetric MTT assay. The antiviral activity was the difference between the virus titre in the absence and in the presence of the extract (δ log $_{10}$ $TCID_{50}$/ml). CPE was determined by microscopic examination of the cells and the results used to calculate the $TCID_{50}$ values.

The antiviral activity of the stem bark was $10^{3.6}$ which represented a reduction in viral titre of 3.6 log_{10}. This was followed by the root bark with a $TCID_{50}$ of $10^{3.4}$ representing a 3.4 log_{10} reduction. The criterion of efficacy for virucides stipulated by both the DVV (1984) and AFNOR (1989) is a 4.0 log_{10} reduction in virus titre. The effective concentration 50 (EC_{50}) for the acetone extract of the leaf was 2.8 µg/ml against feline herpesvirus (FHV-1). The EC_{50} was determined by calculating the concentration where 50% of the cells were destroyed. This was done by visual observation with a light microscope.

It is very interesting that water and acetone extracts had a similar antiviral activity. This is contrary to our experience with antibacterial and antifungal activity of Combretaceae extracts, where aqueous extracts were always inactive. The antiviral activity found in the extracts prepared using polar extractants indicates that tannins or polysaccharides may be responsible for the antiviral activity.

Asres *et al.* (2001) reported that the acetone extract of the leaf of *C. paniculatum* inhibited the replication of HIV-2 with an EC_{50} value of 3.0 µg /ml and selectivity index of 32. This confirms that the acetone extract of the leaves of *C. paniculatum* inhibits the replication of both HIV-2 (Asres *et al*., 2001) and FHV-1 (shown in the present study). Due to the good activity for the extracts, it is expected that the pure compounds, once inactive compounds have been removed, will be more active.

The antibacterial MIC values obtained for *C. paniculatum* were lower than those obtained for other members of the Combretaceae family. The average MIC values ranged between 0.28 and 0.86 mg/ml. Eloff (1999) found that members of the Combretaceae inhibited bacterial growth and MIC values ranged from 0.1 to 6 mg/ml with an average of 2.01 mg/ml. Gram-positive strains were slightly more sensitive with an average MIC value of 1.8 mg/ml while activity of Gram-negative strains averaged 2.22 mg/ml.

In a publication investigating 63 medicinal plant species from Ethiopia using the agar plate well-diffusion method using a sample concentration of 1000 µg/ml, Belachew (1993) showed that the direct aqueous extract of the leaves of *C. paniculatum* had activity against *S. aureus* superior to that obtained with the antibiotics neomycin and nystatin. Bioautography of the crude leaf extracts showed compounds with bacterial growth inhibition for acetone and ethyl acetate extracts in this study.

The leaf extracts were spotted on TLC, developed and sprayed with 0.2% DPPH to reveal antioxidant activity. Almost all the fractions had antioxidant compounds, but acetone and carbon tetrachloride had bigger zones, indicating a higher antioxidant activity compared to the other solvents (Fig 5.3). The acetone and ethyl acetate root bark extracts had many more compounds with antioxidant activity than the other extracts (Fig 5.4). Based on R_f values, the antioxidant compounds were different from the antibacterial compounds.

5.5 Conclusion

The antiviral activity of extracts of *C. paniculatum* against FHV-1 was meaningful. For an extract that is a crude product, a 3.6 $\log_{10}$ reduction is a promising result. The acetone extract of the leaves inhibited

viral growth by 3 $\log_{10}$. The EC_{50} value obtained (2.8 µg/ml) for FHV-1 was close to that obtained by Asres *et al.* (2001) of 3.0 µg/ml against HIV-2. The acetone and water extracts had antiviral activity, hence it was decided to use these two solvents for bulk extraction.

The MIC values of the leaf extract against *S. aureus, E. faecalis, P. aeruginosa,* and *E. coli* ranged between 0.156 to 2.5 mg/ml. This was close to the values (0.8 - 1.6 mg/ml) found by Eloff (1999) of *C. paniculatum* growing in the Lowveld Botanical Garden. MICs of the root bark extracts ranged between 0.31 and 1.25 mg/ml. These values were higher than those obtained for some other members of the Combretaceae family (Eloff, 1999). The MIC values obtained by Kotze and Eloff (2002) while investigating *C. microphyllum* were in the range of 0.29 to 1.25 mg/ml which was in the range obtained for *C. paniculatum.*

Chapter 6

Preliminary isolation study

6.1 Introduction

After selecting the best extractant for the extraction process, the next step was to carry out a preliminary separation procedure to simplify the complex crude extract and then to verify the antibacterial activity of the fractions before large-scale isolation. In this chapter, investigation of the activity of the root bark and leaf extracts is described, as both these extracts may be a source of bioactive compounds. Bioautography remains a useful technique to reveal compounds with antibacterial activity, and purification of the extracts and possible subsequent concentration of active compounds may reveal more plant constituents that inhibit bacterial growth.

A plant extract contains many compounds that may be seen in visible light or under ultraviolet conditions. Chromogenic spray reagents are also useful to show different types of compounds and to gain an indication of which types of compounds are present in the plant extract.

6.2 Methods

6.2.1 Extraction and preliminary column chromatography

Ten grams of dried, ground leaf material were weighed and placed in a polyethylene tube containing 100 ml acetone. The tube and contents were shaken for ten minutes. The extract was filtered using Whatman No.1 filter paper. This process was repeated three times on the marc and in each case the supernatant was filtered and combined. The extract (1.6 g) was dried and mixed with 4 g silica gel as stationary phase. The silica gel column (2.2 cm by 30 cm) was eluted with 100 ml of each of the following solvents: 100% hexane, 75:25 (hexane:acetone), 50:50 (hexane:acetone), 25:75 (hexane:acetone), 100% acetone and 90:10 (acetone:methanol). The fractions were analyzed using TLC and similar fractions were combined.

6.2.2 Solvent/solvent fractionation of root bark

This procedure simplifies extracts by fractionating the components based on polarity. The solvent/solvent group procedure used by the US National Cancer Institute as described by Suffness and Douros (1979) was applied. After extracting the root bark with acetone, the extract was dried in a Buchi RE-120 rotary evaporator under reduced pressure. This extract was dissolved in a 1:1 mixture of chloroform and water and the two phases were separated in a separatory funnel. Fig 6.1 below is a flow chart of the process used for solvent/solvent fractionation of the root bark.

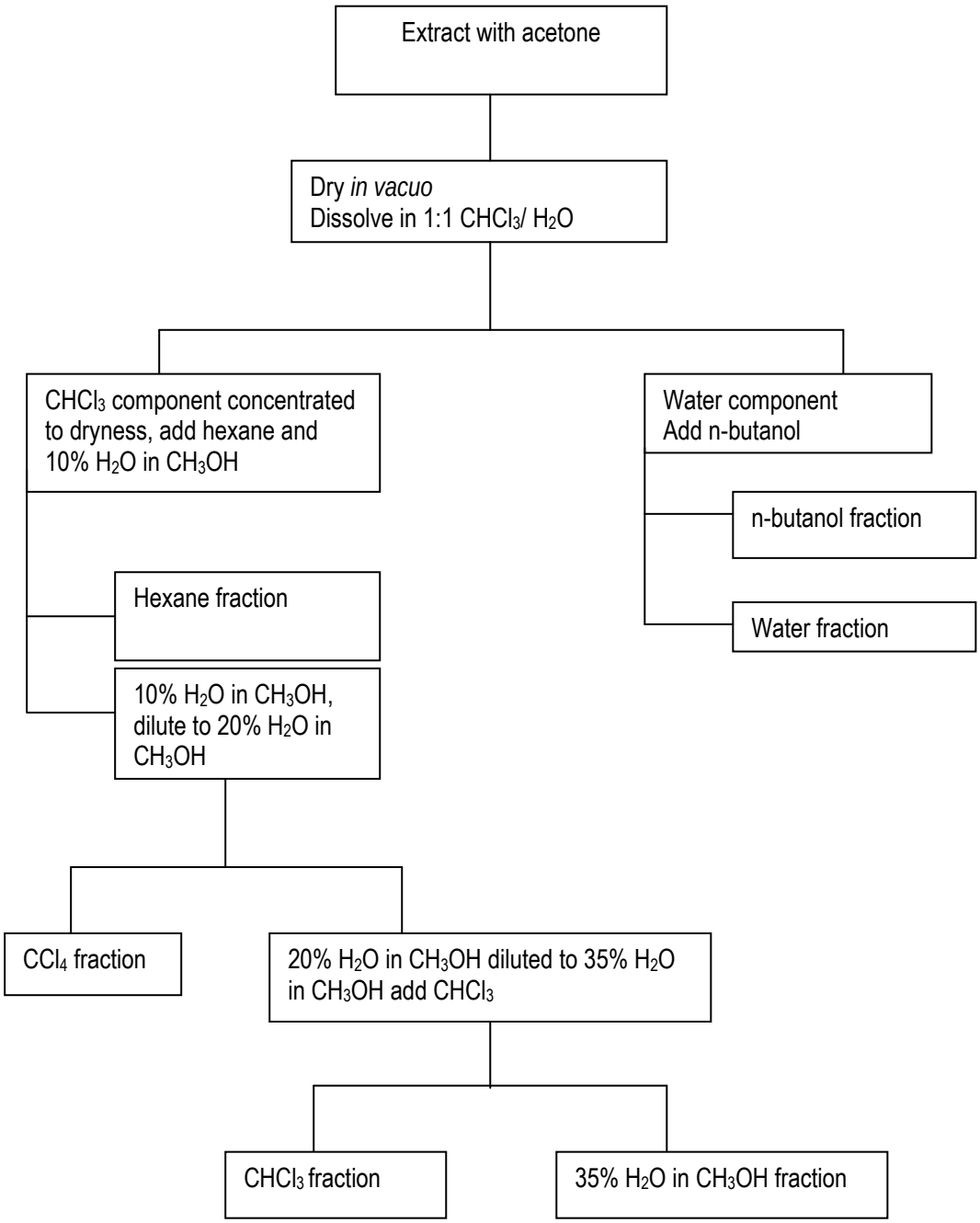


Fig 6.1. Flow chart of solvent/solvent fractionation of root bark

The water fraction was mixed with an equal volume of n-butanol in a separatory funnel to yield the water and butanol fractions. The chloroform fraction was dried in a vacuum rotary evaporator and extracted with an equal volume of hexane and 10% water:methanol mixture. This yielded the hexane fraction, and the 10% water:methanol mixture was diluted to 20% water:methanol by addition of water. This was then mixed with carbon tetrachloride in a separatory funnel giving the carbon tetrachloride fraction. The 20% water:methanol fraction was further diluted to 35% water:methanol and mixed with chloroform to yield the chloroform and water:methanol fractions. In all cases, equal volumes of solvents were used and separation repeated with small quantities of solvent to facilitate separation.

6.2.3 Bioactivity testing

All the resulting fractions from the separation of the leaf and root bark extracts were tested for antibacterial and antifungal activity (sections 3.10.1 and 3.11) as well as bioautography (section 3.10.3).

6.3 Results and Discussion

The column chromatography of the acetone extract of the leaves resulted in a reasonable separation of plant components. Fig 6.2 is a chromatogram of combined similar fractions of the leaf acetone extract separated by column chromatography. The solvent system used to develop the plate was CEF, and vanillin-sulphuric acid was used as the detection agent.

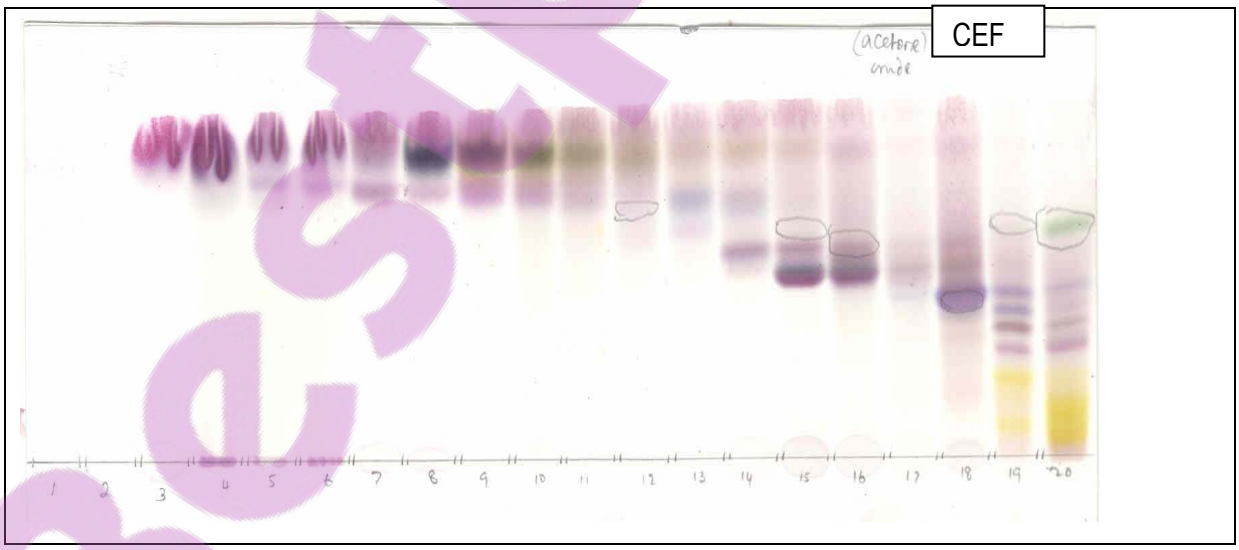


Fig 6.2. Chromatogram of combined fractions after spraying with vanillin/sulphuric acid (leaf acetone extract)

Bioautography was done with the combined fractions and compounds with bacterial growth inhibition were observed as clear zones against the purple background. Figures 6.3, 6.4, and 6.5 show bioautography using the test organisms *S. aureus, P. aeruginosa* and *E. coli* respectively.

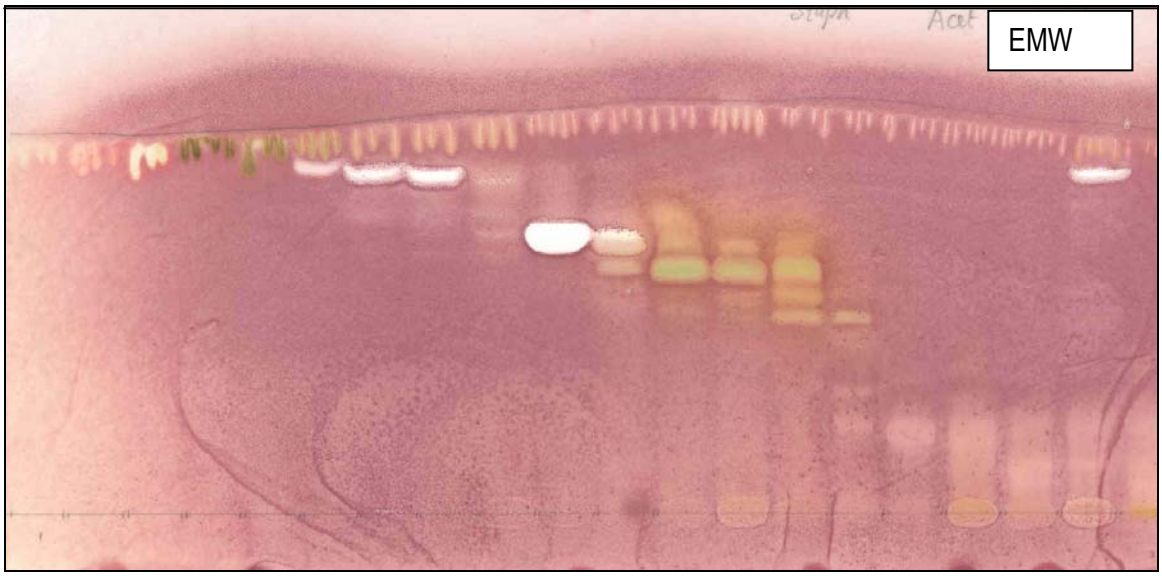


Fig 6.3. Bioautography of acetone fractions against *S. aureus* after column chromatography

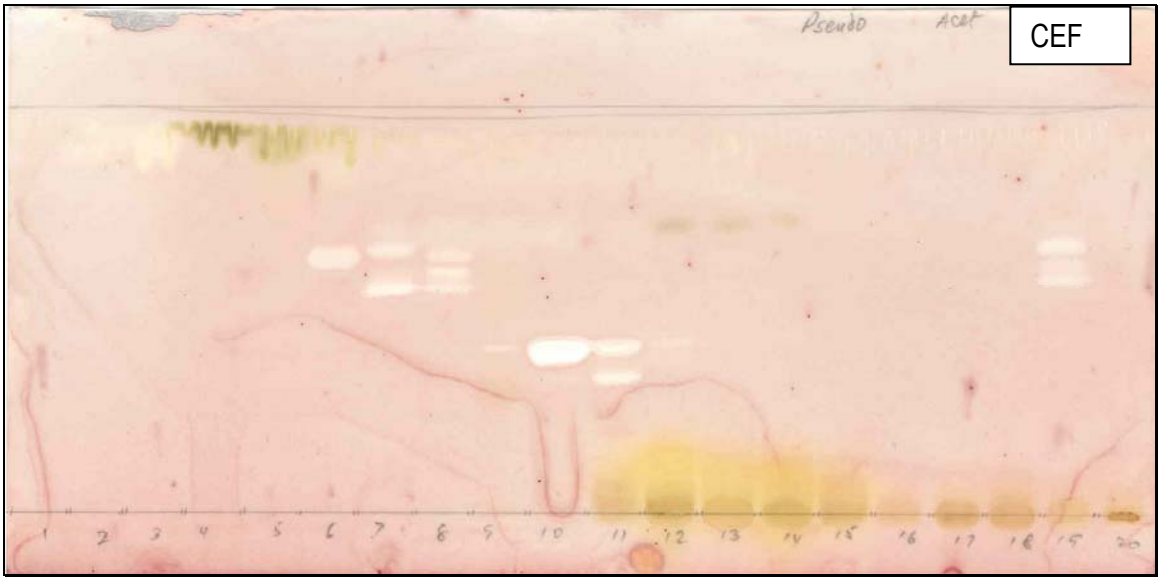


Fig 6.4. Bioautography of acetone fractions against *P. aeruginosa* after column chromatography

The acetone fraction contained compounds that were active against both Gram-positive and Gram-negative bacteria. The bioautograms confirm that purification of the acetone extract revealed many more compounds with antibacterial activity than the crude extract.

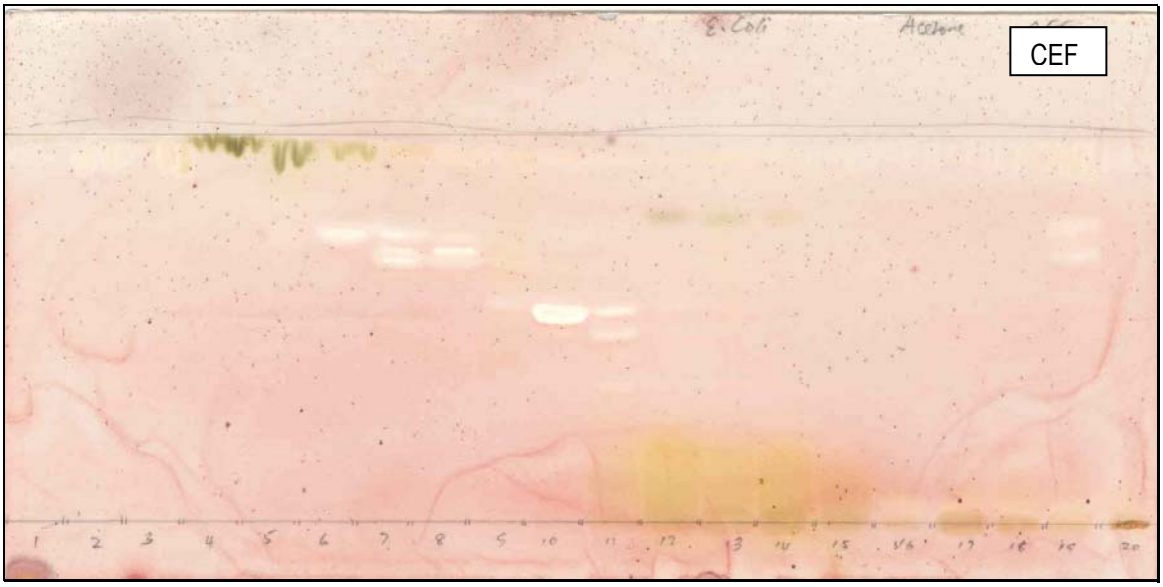


Fig 6.5. Bioautogram of combined fractions of acetone extract fractions against *E. coli* after column chromatography

From the above bioautograms, it will be observed that the compounds that inhibited *P. aeruginosa* and *E. coli* have similar R_f values. The same compounds probably cause bacterial inhibition against the two pathogens.

Fig 6.6 is a chromatogram of the root bark fractions after solvent/solvent fractionation. The chloroform and carbon tetrachloride fractions separated into many more compounds compared to the other extracts. The R_f values of components from the solvent/solvent fractionation of the root bark extract are given in Table 6.1.

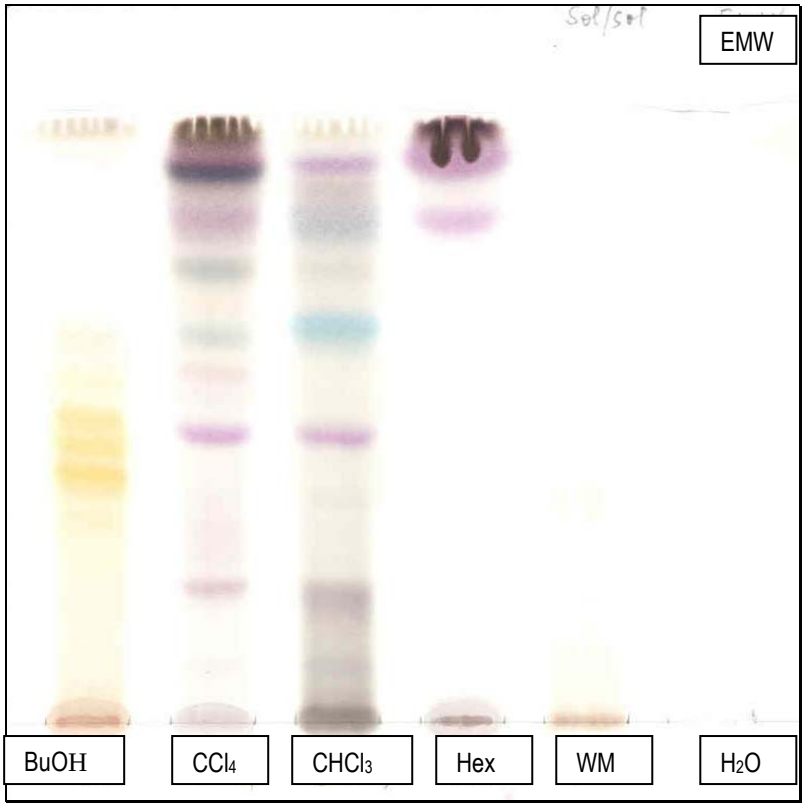


Fig 6.6. Fractions from solvent/solvent fractionation of the root bark after spraying with vanillin/sulphuric acid spray reagent

Table 6.1 R_f values of components from solvent/solvent fractionation of the root bark

Solvents	R_f values							
BuOH	0.47	0.52	0.55					
CCl_4	0.31	0.53		0.68	0.77	0.84	0.90	0.94
$CHCl_3$	0.30	0.52		0.69		0.83	0.92	
Hexane						0.84		

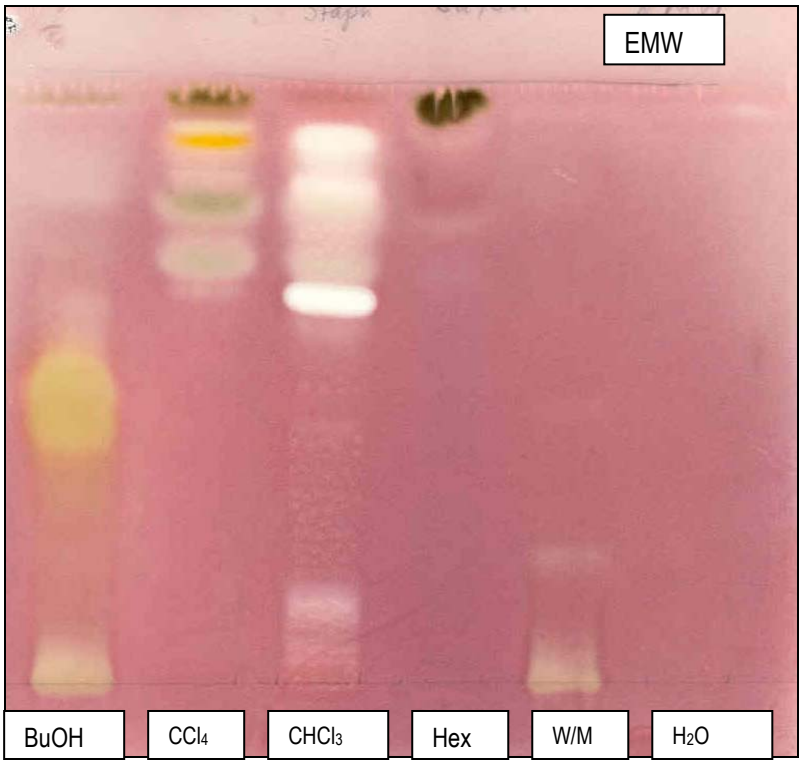


Fig 6.7. Bioautography of solvent/solvent fractions of root bark against *S. aureus*

The root bark was partitioned using six solvents and bioautography was carried out against *S. aureus*. As observed in Fig 6.7, the chloroform fraction had more compounds with antibacterial activity compared to the other fractions. The carbon tetrachloride fraction had a few areas of bacterial inhibition and the rest of the fractions did not show any inhibition of bacterial growth. The R_f values for the chloroform fraction were as follows: 0.14, 0.64, 0.81, and 0.9. For the carbon tetrachloride fraction, the values were as follows: 0.71 and 0.93. There was little activity in the butanol and water:methanol fractions.

The root bark of the plant was extracted using three solvents and the MIC and total activity values of these extracts are presented in Table 6.2. The lowest MIC value was obtained against *E. faecalis* and the highest against *E.coli.* The organisms were equally sensitive to the water and acetone extracts. The same compounds responsible for antibacterial activity are probably present in both the acetone and water extracts.

Table 6.2. MIC (mg/ml) and TA (ml/g) values of root bark extracts against four bacterial strains

Organisms	Solvents					
	Acetone		Ethyl acetate		Water	
	MIC	TA	MIC	TA	MIC	TA
S. aureus	0.63	35.4	1.25	17.8	0.31	72
P. aeruginosa	0.31	72	1.25	17.8	0.63	35.4
E. faecalis	0.31	72	0.63	35.4	0.31	72
E. coli	1.25	17.8	1.25	17.8	0.63	35.4
Average	0.62	49.3	1.09	22.05	0.63	53.7

The total activity of the water extract was generally the highest because water extracted the highest quantity of compounds from the plant material although the MIC was the highest.

The root bark fractions from solvent/solvent partitioning were tested for antifungal activity (Table 6.4 and Fig 6.8). The values obtained were not good compared to values obtained for other *Combretum* and *Terminalia* species (Masoko *et al.,* 2006). In several cases MIC values as low as 0.02 mg/ml were obtained against several fungal pathogens with these extracts, and the leaf extract was more active compared to the root bark as reported by Masoko *et al,* (2006).

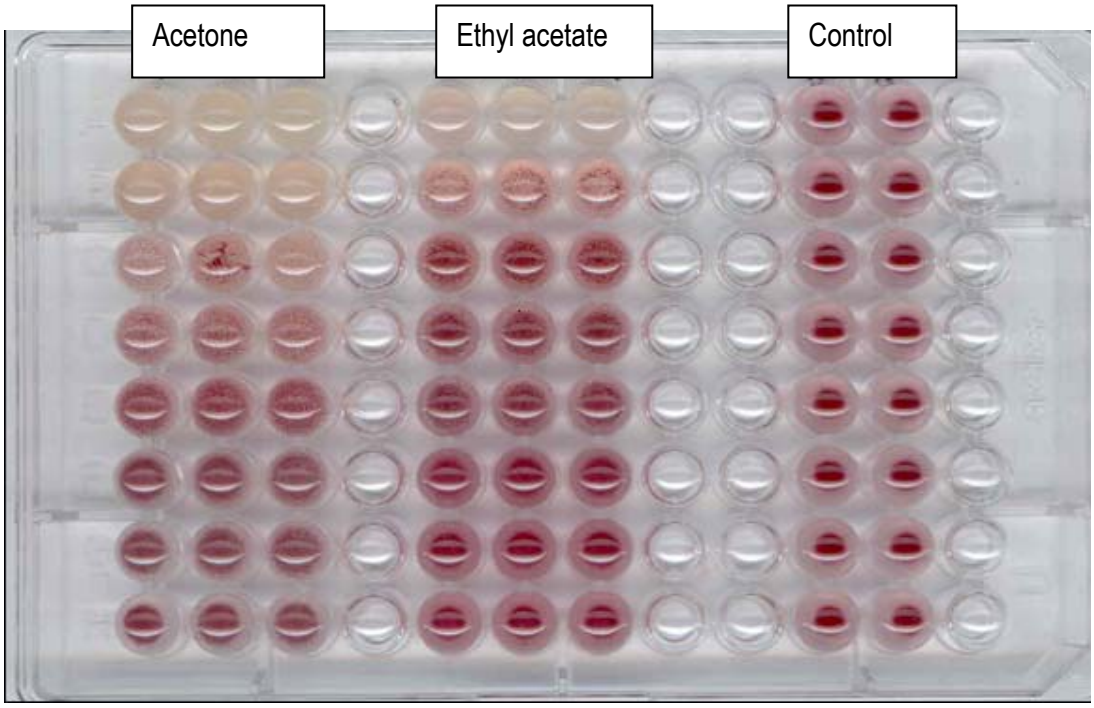


Fig 6.8. Representative microplate of root bark extracts against *Candida albicans*

Table 6.3. MIC (mg/ml) of root bark fractions obtained from solvent/solvent fractionation against *Candida albicans*, *Microsporum canis, Aspergillus fumigatus, Cryptococcus neoformans* and *Sporothrix schenckii.*

Organisms	CCl_4	H_2O (RB)[1]	BuOH	Hexane	W/MeOH	H_2O (Leaf)	$CHCl_3$
C. albicans	0.63	2.5	0.63	0.63	1.25	0.63	0.63
M. canis	0.63	0.63	0.31	0.31	0.16	0.63	0.31
A. fumigatus	2.5	2.5	0.63	2.5	1.25	2.5	2.5
C. neoformans	0.16	2.5	0.63	0.16	0.16	0.16	0.31
S. schenckii	0.16	1.25	0.16	0.63	0.16	0.63	0.16
Average	0.816	1.876	0.472	0.846	0.596	1.198	0.782

[1]RB = root bark

The butanol fraction from the solvent/solvent partitioning had the lowest average MIC value of 0.472 mg/ml while the highest was that of the water extract at 1.198 mg/ml. The lower the MIC value, the more active the extract. The extracts had good activity against *Cryptococcus neoformans* and *Sporothrix schenckii.* The lowest average MIC value was that against *Aspergillus fumigatus* with an average MIC value of 1.87 mg/ml. The MIC of the root bark for antifungal activity ranged from 0.16 to 2.5 mg/ml. The crude acetone leaf extracts of *C. paniculatum* were more active according to Masoko *et al.* (2006), with values as low as 0.02 mg/ml. This may indicate that there were synergistic effects in the crude extracts compared to the fractions generated by solvent/solvent fractionation.

The solvent/solvent fractionation of the root bark produced fractions containing 4 compounds with antibacterial activity (with R_f = 0.14, 0.64, 0.81 and 0.9) for the chloroform fraction, two for the carbon tetrachloride fraction (R_f = 0.71 and 0.91) and one for the water:methanol fraction (R_f = 0.21). The butanol fraction exhibited antibacterial activity at the origin (R_f = 0).

6.4 Conclusion

Initial extraction of the leaves using different solvents proved that acetone extracted more compounds with antibacterial activity. The acetone extract of the leaves was purified using a silica gel column and bioautography of the combined fractions revealed many more compounds with antibacterial activity.

The MIC of the leaf extracts was in the range 0.28 to 0.86 mg/ml while those of the root bark ranged between 0.42 and 1.04 mg/ml. It can be deduced from this that the leaf extract was more active compared to the root bark extract.

The root bark was subjected to solvent/solvent partitioning and the fractions evaluated for antibacterial and antifungal activity. Four antibacterial compounds were found in the chloroform fraction while the carbon tetrachloride fraction had two compounds that exhibited antibacterial activity. It appears from the retention factors that similar bioactive compounds occur in the root bark and leaves.

Antifungal activity of the root bark was disappointing, and the acetone extract of the leaf was more inhibitory to fungal growth. It is easier and more sustainable to use leaves to prepare bulk extracts for isolation of compounds, so the next phase was the large-scale extraction and isolation of bioactive compounds from leaves of *C. paniculatum*.

Chapter 7

Isolation of compounds from *C. paniculatum* leaves

7.1 Introduction

Plants have an almost limitless ability to synthesize aromatic substances, many of which are phenols or their oxygen-substituted derivatives (Geissman, 1963). Most are secondary metabolites, of which at least 12 000 have been isolated, a number estimated to be less than 10% of the total (Schultz, 1988). In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects, and herbivores (Cowan, 1999).

The separation of plant constituents is generally performed using one or a combination of chromatographic techniques including column chromatography, thin layer chromatography and high-pressure liquid chromatography. The choice of technique depends largely on the solubility properties and volatilities of the compounds to be separated. The complexity of plant extracts can be simplified using different separation techniques based on size or polarity. For preparative work, column chromatography coupled with an automatic fraction collector is frequently used. This procedure can yield purified components in larger amounts.

7.2 Methods

7.2.1 Extraction and isolation

The air-dried leaf material (1100 g) was pulverized and extracted using 70% acetone in water. The extract was concentrated in a rotary evaporator and placed in a column containing XAD-16 as stationary phase. The column was sequentially eluted with 2 500 ml each of acetone, methanol and water. The fractions were concentrated in a rotary evaporator and analysed using TLC. The acetone fraction contained the green pigment. Sixty grams of the methanol extract was mixed with same amount of silica gel and placed in a column (3.0 by 45 cm) containing XAD-16 as stationary phase.

The dried fraction eluted by 100% methanol was placed onto a silica gel column after concentration and eluted with varying ratios of dichloromethane in methanol beginning with 100% dichloromethane. The

column was eluted with 2 000 ml of each of the solvents. Ten fractions were collected and fraction 2 was concentrated and run in a column packed with silica gel and eluted with hexane:ethyl acetate (9:1). This yielded compound 1. The column was further eluted with a 20:1 dichloromethane:methanol (2 000 ml) and 12 fractions were collected. Fraction 15 was dried and separated in another silica gel column and eluted in a 30:1 dichloromethane:methanol mixture (1 000 ml) and compound 2 was obtained.

Further elution of the column with 10:1 dichloromethane:methanol (2 000 ml) gave 12 fractions from which fraction 29 was dried and fractionated in a Sephadex column using 4:1 dichloromethane:methanol (2 000 ml) together with a few drops of acetic acid. Thirty-three fractions were collected and fraction 13 was dried and run in a silica column using 15:1 dichloromethane:methanol (1 000 ml) as eluent. This resulted in the isolation of compound 5. In each case, the fractions were analyzed by TLC and fractions showing a similar profile of compounds were combined.

The objective was to isolate antibacterial compounds, but when pure compounds were found in some fractions, they were also analyzed for possible antiviral activity. A flow chart of the process that led to the isolation of compounds 1, 2, 5, and 6 is presented in Fig 7.1.

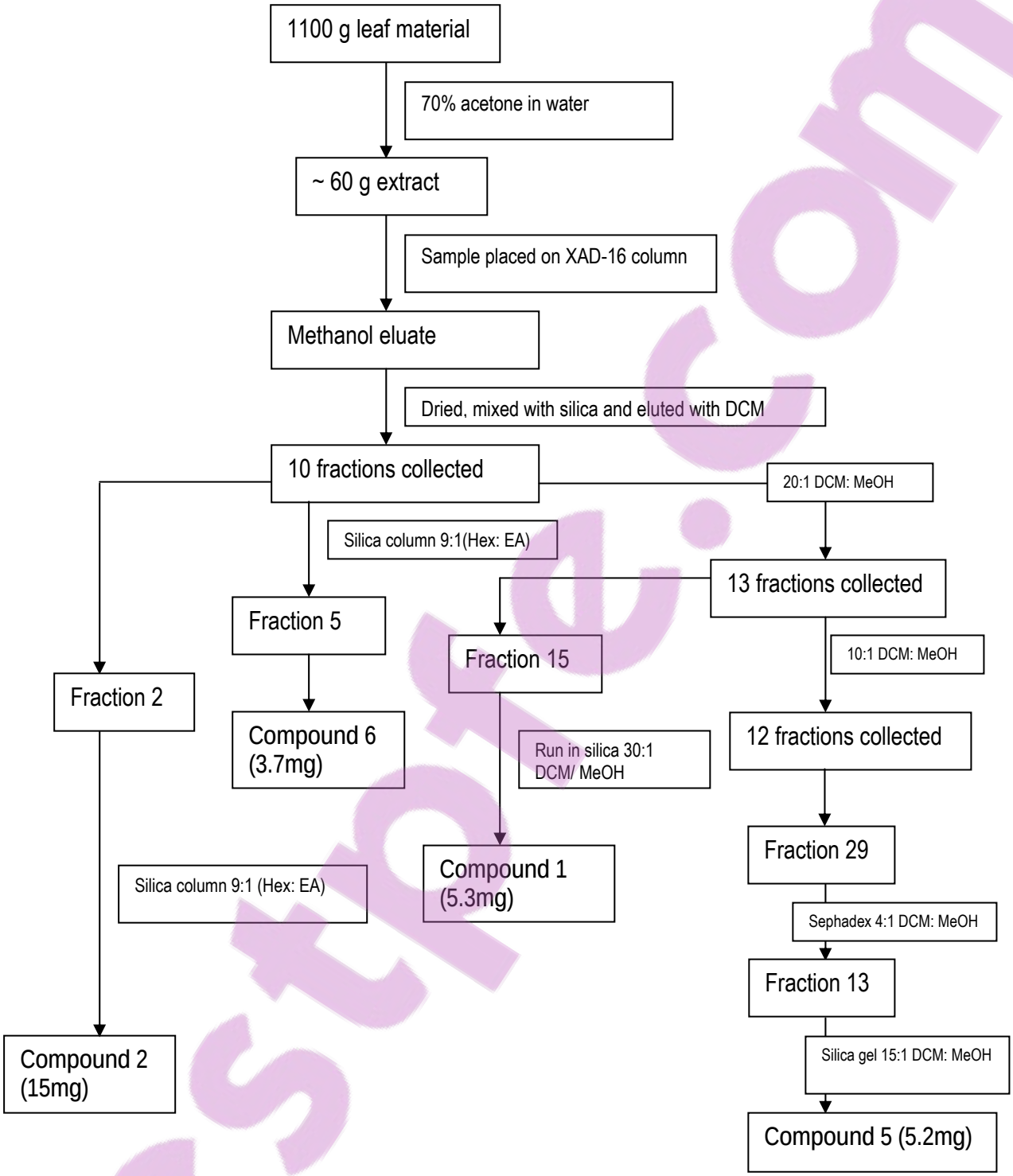


Fig 7.1. Flow chart for isolation of compounds 1, 2, 5 and 6

A portion of the methanol extract (50 g) was partitioned in ethyl acetate (60 ml) and water (60 ml). The ethyl acetate fraction was evaporated under reduced pressure and a mixture of 20 g sample and 20 g silica gel was placed in a silica gel column. The column was eluted with 100% dichloromethane (2 000

ml) and 11 fractions were collected. Elution was continued with a 20:1 DCM:MeOH (2 000 ml) mixture and 13 fractions were collected. Fraction 16 was dried and fractionated in a Sephadex column using methanol (1 000 ml) as eluent, and fractions were collected and separated using TLC. Fractions 9 and 10 were dried, combined and separated in a silica gel column eluting with a 40:1 DCM:MeOH (1 000 ml) mixture. This resulted in the isolation of compound 4. Elution was continued with a 10:1 DCM:MeOH (1 000 ml) mixture. After TLC analysis, fractions 25, 26 and 27 were combined, dried and fractionated by preparative HPLC with a mixture of acetonitrile and water (HPLC grade, 400 ml of each) as eluents. Purification was carried out in a Merck-Hitachi apparatus (Germany). An ODS column (250 X 4.0 mm I.D., 5 µm) was employed at a temperature of 30ºC, flow rate of 1.0 ml/min and wavelength of 254 nm. The fractions were dissolved in methanol and injected into the HPLC column. This resulted in the isolation of compounds 3, 7, 8 and 9. A flow chart of the isolation process resulting in the isolation of compounds 3, 4, 7, 8 and 9 is presented in Fig 7.2.

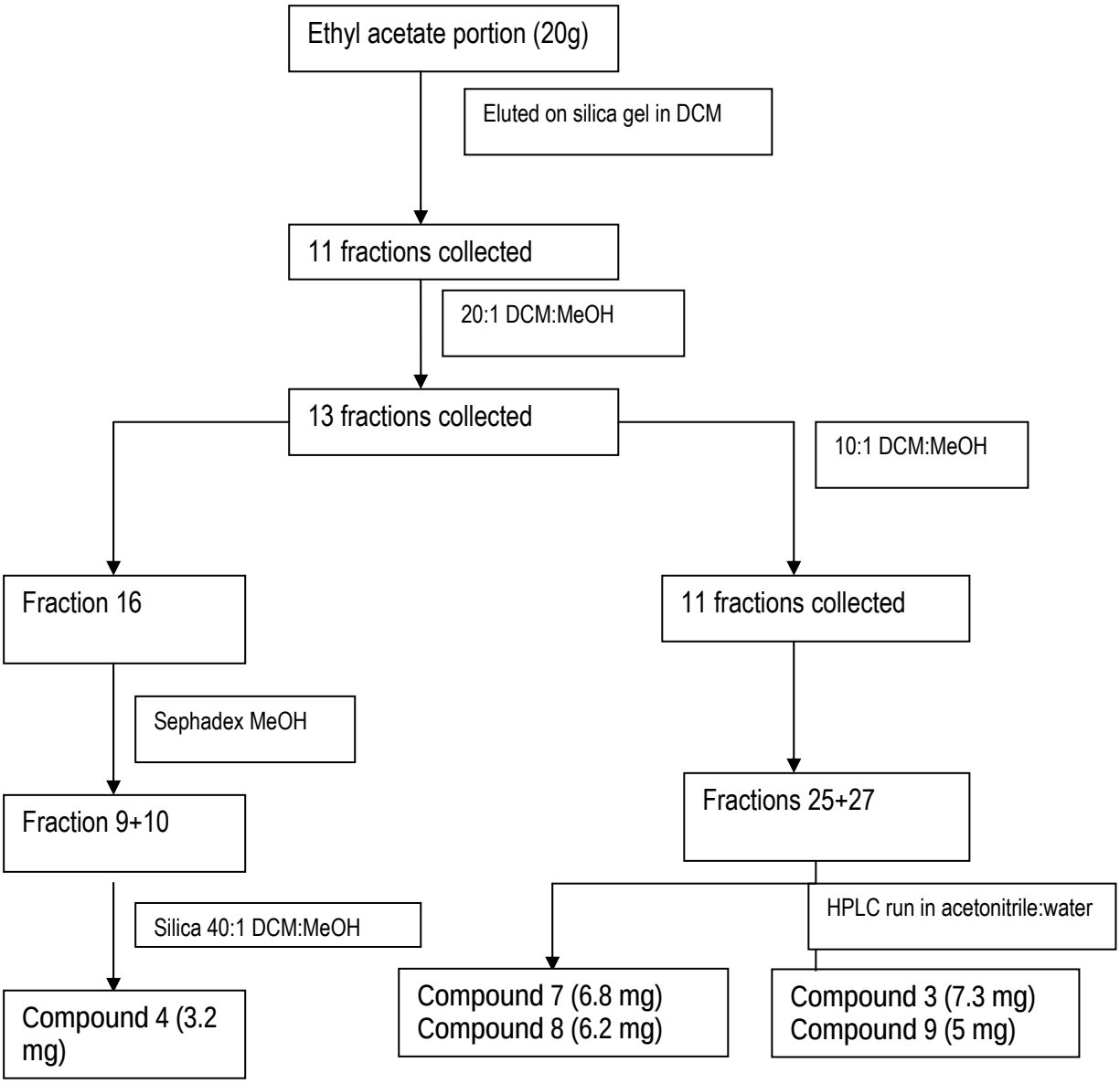


Fig 7.2. Flow chart of isolation of compounds 3, 4, 7, 8 and 9

7.3 Results and Discussion

Initial extraction using different solvents proved that acetone extracted the most components with antibacterial activity. Bioautography supported the conclusion and therefore acetone was used to extract the bulk sample for the isolation process. Water was mixed with acetone (30:70) because in the extraction process water extracted the largest quantity. A combination of acetone and water was therefore used to maximize the number of antibacterial compounds and amount of material extracted from the plant.

The compounds were isolated via initial antibacterial bioassay-guided extraction. Compounds that crystallized after column chromatography were also collected. The isolation process was continued without determining whether other fractions collected thereafter were active or not. This was because the facilities for bioassay-guided isolation were not available at that stage of the project. In bioassay-guided fractionation, fractions are subjected to bioautography or other bioassay in order to target compounds that exhibited antibacterial activity. At the HKI, where this portion of the research was performed, biological assays are carried out in another facility by other staff and sometimes one had to wait for a week to get results. This would have slowed down the isolation process and it was therefore decided to aim to isolate as many compounds as possible.

Nine compounds were isolated using column chromatography and HPLC. R_f values for some of the compounds are shown in Table 7.1. Compounds 3, 7 and 8 did not migrate from the bottom of the TLC plate.

Table 7.1. R_f values of the isolated compounds

Compounds	R_f values	Solvent system used for elution
1	0.26	BEA
2	0.43	Hexane:chloroform (8:2)
3	0.46	EMW
4	0.62	CEF
5	0.75	CEF
6	0.65	Hexane:DCM (8:2)
7	0	EMW
8	0	EMW
9	0	EMW

Compounds 7, 8 and 9 were polar and were isolated by the use of HPLC apparatus with water and acetonitrile as eluent. The R_f values of the isolated compounds were not compared to those occurring in the crude extract because they were developed in different solvent systems.

7.4 Conclusion

Using column chromatography and HPLC, nine compounds were isolated from the acetone:water extract of *C. paniculatum* leaves. All the compounds reacted with *p*-anisaldehyde-sulphuric acid spray reagent. Many other compounds were present but the quantity of fractions was too small to enable characterization of these compounds.

Chapter 8

Determination of chemical structures, biological activity and cytotoxicity of isolated compounds

8.1 Introduction

The elucidation of chemical structures involves using a combination of different techniques including nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry (MS), ultraviolet (UV) and infrared (IR) spectrometry. Two key points for solving complex problems are that no single spectrum will entirely solve a problem and that all information must be used simultaneously. It is preferable to get key structural facts from each spectrum and then assemble the pieces.

The best starting point is to obtain a molecular formula from the high resolution mass spectrum, or from the molecular ion mass and the number of signals seen in the proton and carbon NMR spectra. Second, determine the number of double bonds and/or rings present by calculating the degree of unsaturation. Third, determine functional groups and other molecular fragments present from the ^{1}H and ^{13}C NMR spectra. Fourth, assemble the pieces in all reasonable combinations. Fifth, eliminate wrong structures and verify the correct structure by reanalyzing the NMR spectra against the proposed structures.

8.2 Structure elucidation

For structural elucidation purposes, the compounds isolated were subjected to instrumental analysis. The NMR (^{1}H and ^{13}C) was determined at the HKI using DRX-500, DNMR, DRX-500 (Bruker, Germany), and TMS as internal standard. Isolated compounds were dried in a freeze-drier, weighed and dissolved in a suitable solvent e.g. deuterated solvent for NMR. Methanol, chloroform and dimethyl sulfoxide were used depending on the solubility of the compounds. The solutions were pipetted into NMR tubes and analyzed for ^{1}H, ^{13}C, distortionless polarization transfer (DEPT), correlated spectroscopy (COSY), heteronuclear multiple quantum coherence (HMQC) and heteronuclear multiple bond connectivity (HMBC). Mass spectrometry (MS) analysis was performed on the samples using a quadruple Mass Spectrometer.

8.3 Biological activity and cytotoxicity

The isolated compounds were tested for antiviral, antibacterial and antifungal activities as described in sections 3.9.2, 3.10.1 and 3.12 respectively. Cytotoxicity was also determined using the cell line MTT assay (section 3.9.1.4).

8.4 Results and Discussion

8.4.1 Identification of isolated compounds

Compound 1

Compound 1 was isolated as a white amorphous powder. The molecular formula was $C_{27}H_{46}O$, based on ESI-MS m/z: 387.5 $[M+H]^+$ together with ^1H and ^{13}C NMR data, corresponding to 5° unsaturation in the molecule. The ^{13}C together with DEPT spectrum exhibited 27 carbon signals: δ 140.8 (s), 121.7 (d), 71.8 (d), 56.8 (d), 56.1 (d), 50.2 (d), 45.9 (d), 42.3 (t), 39.8 (t), 37.2 (t), 36.5 (s), 36.1 (d), 33.9 (t), 31.9 (d), 31.7 (t), 29.2 (d), 28.2 (t), 26.2 (t), 24.3 (t), 23.1 (t), 21.1 (t), 19.8 (q), 19.4 (q), 19.0 (q), 18.7 (q), 11.9 (q), 11.8 (q). The ^1H NMR spectrum exhibited the signal of olefine protons δ 5.34, m, ^1H one oxide methine group δ 3.50, m, ^1H, six methyl groups δ 1.02, 0.95, 0.92, 0.90, 0.85, 0.66. Compound 1 showed characteristic steroid signals in δ 0.66 and δ 3.50. After comparing spectral data with literature, the structure was deduced as cholest-5-en-3-ol (Fig 8.1) (Aldrich Library, 1992).

Cholest-5-en-3-ol is a characteristic sterol of higher animals. It occurs either free or as esters, of fish liver oils, egg yolk, bile, bran, and gallstones. It is a constituent of the scent material of cotton-top tamarin monkeys (*Saguinus oedipus*), and of *Macoma balthica*. It is also used as a pharmaceutical aid (emulsifying agent). Exposure to very high doses has teratogenic effects (Dictionary of Natural Products, 2006). The compound is also found in virtually all plant oils, for example rapeseed oil (*Brassica napa*), soybean oil (*Glycine max*) and wheatgerm oil (*Triticum* spp.).

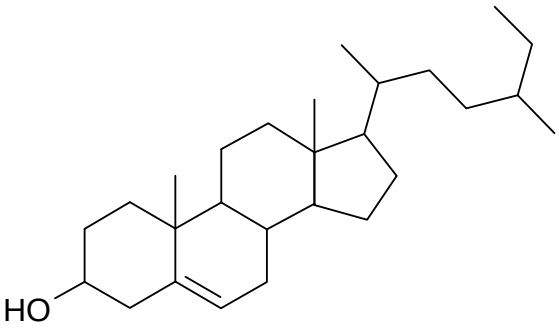


Fig 8.1. Structure of cholest-5-en-3-ol

Compound 2

Compound 2 was obtained as a colourless oil, with a molecular formula of $C_{20}H_{20}O$. From ESI-MS m/z: 319.24 $[M+ Na]^+$, 615.5 $[2M+ Na]^+$ together with ^1H and ^{13}C NMR spectral data, corresponding to 1° of unsaturation in the molecule. The ^{13}C NMR together with DEPT spectrum exhibited 20 carbon signals: δ 140.1 (s), 123.1 (d), 59.3 (t), 39.8 (t), 39.3 (t), 37.4 (t), 37.2 (t), 37.0 (t), 36.6 (t), 32.9 (d), 32.6 (d), 27.9 (q), 25.1 (t), 24.7 (t), 24.2 (t), 22.6 (q), 22.5 (q), 19.7 (q), 19.6 (q), 16.1 (q). The ^1H NMR spectra exhibited the signal of an olefine with protons δ 5.40, td, 1H, and oxide methylyne group δ 4.10, d, J=6.9, 2H, methylene group δ 1.95, d, J= 7.7, 2H, 5 methyl group δ 1,65, 0.86, 0.84, 0.83, 0.80. According to the data above and the degree of unsaturation in the molecule, it should be a long chain compound containing a double bond. After comparing the spectra with literature, the structure was deduced as 2-phyten-1-ol (Fig 8.2) (3, 7, 11, 15- tetramethyl-2-hexadecen-1-ol) (Goodman *et al.,* 1973; Skilleter and Kekwick, 1970).

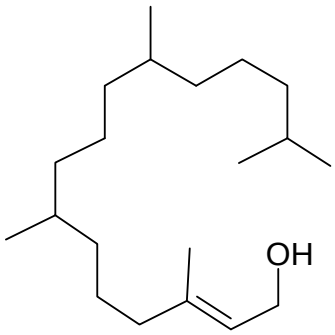


Fig 8.2. Structure of 2-phyten-1-ol

The compound is a constituent of nettles, *Leucas volkensii,* alga, *Perilla* spp, *Solidago virga-aurea, Tetragonia tetragonoides* (New Zealand spinach), *Garcilaria andersoniana, Megaceros flagellaris* and

other plants. It is an anticancer agent (colon and gastric cancer) and used in the preparation of vitamin E and K. The lethal dose 50 (LD_{50}) (rat, oral) is > 5000 mg/kg (Dictionary of Natural Products, 2006).

Fig 8.3 shows the TLC analysis of 2-phyten-1-ol. The R_f of 2-phyten-1-ol is 0.43 in hexane:chloroform (4:1).

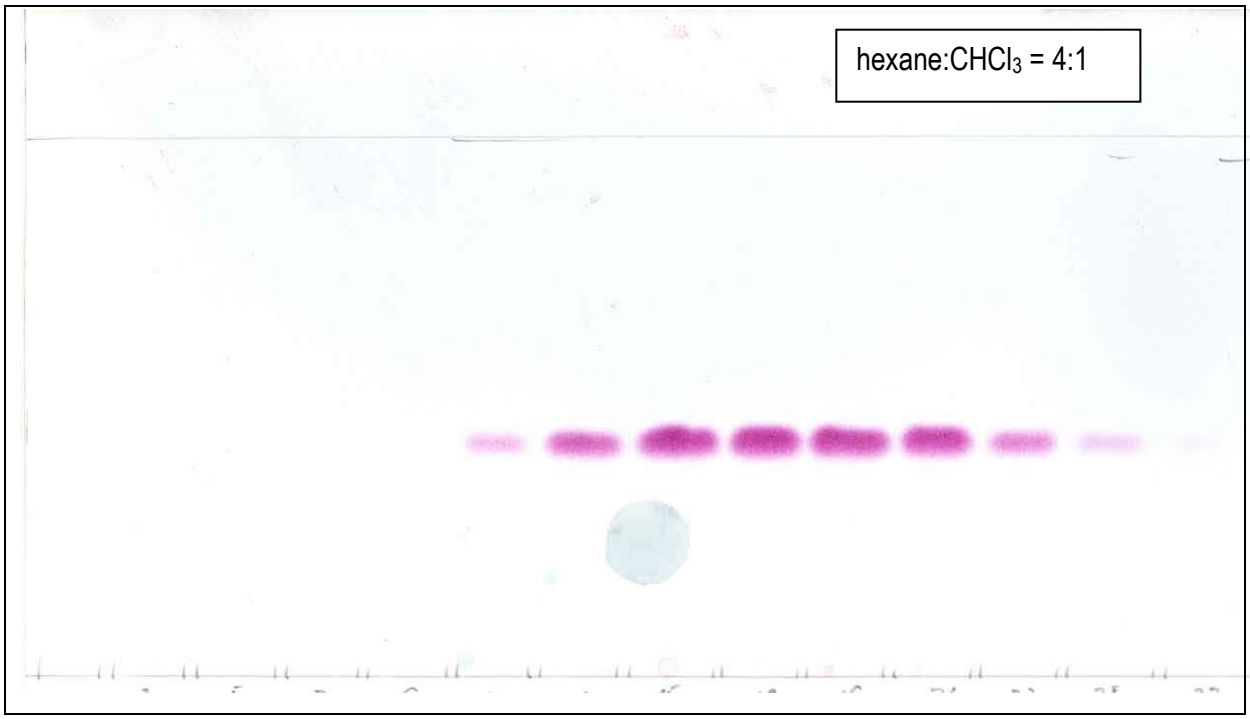


Fig 8.3. TLC of 2-phyten-1-ol

Compound 3

Compound 3 was obtained as a yellow amorphous powder, with a molecular formula of $C_{21}H_{20}O_{12}$, from ESI-MS m/z 464.9 $[M+H]^+$, 950.8 $[2M+Na]^+$ together with ^1H and ^{13}C spectra data, corresponding to 7° of unsaturation in the molecule. The ^{13}C NMR and DEPT spectrum exhibited 21 carbon signals: δ 179.4 (s), 165.9 (s), 162.9 (s), 159.0 (s), 158.4 (s), 149.8 (s), 135.6 (s), 123.2 (d), 122.9 (s), 117.6 (d), 115.9 (d), 105.6 (s), 104.4 (d), 99.8 (d), 94.7 (d), 78.3 (d), 78.1 (d), 75.7 (d), 71.2 (d), 62.5 (t). The ^1H NMR spectra aromatic proton signals δ 7.55, 6.88, 6.36, 6.17, pyranglucose signals in δ 5.23, 3.31-3.75. Since compound 3 has the characteristic signal δ 179.4, 162.9 as position 2 and 4 of isoflavonoids, the structure was assigned as an isoflavonoid. From 2D-NMR studies, the structure was determined as 3-glucopyranosyloxy-3', 4, '5, 7- tetrahydroxyflavone (quercetin-3-glucopyranoside, Fig 8.4).

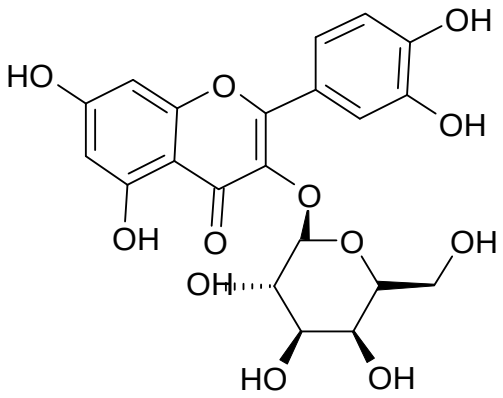


Fig 8.4. Structure of quercetin-3-glucopyranoside

Quercetin-3-glucopyranoside occurs widely in plants. It is used as a diuretic, antioxidant, and has antifungal activity. This compound has been isolated from *Gaultheria miqueliana* and many other plant species. Quercetin-3-glucopyranoside is present in red wine (Dictionary of Natural Products, 2006).

Flavones are phenolic structures containing one carbonyl group (as opposed to the two carbonyls in quinones). The addition of a 3-hydroxyl group yields a flavonol (Fessenden and Fessenden, 1982). Flavonoids are also hydroxylated phenolic substances but occur as a C_6 - C_3 unit linked to an aromatic ring. They are known to be synthesized by plants in response to microbial infection (Dixon *et al.*, 1983); it should not be surprising that they have been found *in vitro* to be effective antimicrobial substances against a wide array of microorganisms (Cowan, 1999). Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls, as described for quinones. More lipophilic flavonoids may also disrupt microbial membranes (Tsuchiya *et al*., 1994). Catechol and pyrogallol, both hydroxylated phenols, have been shown to be toxic to microorganisms (Cowan, 1999). Catechol has two –OH groups, and pyrogallol has three. The site(s) and number of –OH groups on the phenol group are thought to be related to their relative toxicity to microorganisms with evidence that increased hydroxylation results in increased toxicity (Geissman, 1963). In addition, some authors have found out that more highly oxidized phenols are more inhibitory (Scalbert, 1991; Urs and Dunleavy, 1975).

Flavonoid compounds exhibit inhibitory effects against multiple viruses. Numerous studies have documented the effectiveness of flavonoids such as swertifranchoside (Pensuparp *et al*., 1994), glycyrrhizin from licorice (Watanbe *et al.*, 1996), and chrysin (Critchfield *et al*., 1996) against HIV. More than one study has found that flavone derivatives are inhibitory to respiratory syncytial virus (RSV) (Barnard *et al*., 1993; Kaul *et al*., 1985). Kaul *et al.* (1985) provided a summary of the activities and

modes of action of quercetin, naringin, herperetin, and catechin in *in vitro* cell culture monolayers. While naringin was not inhibitory to herpes simplex virus type 1 (HSV-1), polio virus type 1, parainfluenza virus type 3, or RSV, the other 3 flavonoids were effective in various ways (Cowan, 1999). An isoflavone found in a West African legume, alpinumisoflavone, prevents schistosomal infection when applied topically (Perrett *et al*., 1995).

Flavonoids lacking hydroxyl groups on their β-rings are more active against microorganisms than those with the –OH groups (Chabot *et al*., 1992). This finding supports the idea that the microbial target is the membrane. Lipophilic compounds would be more disruptive of this structure. However, several authors have also found the opposite effect, i.e. the more hydroxylation, the greater the antimicrobial activity (Sato *et al*., 1996).

Compound 4

Compound 4 was obtained as a brown amorphous powder, with molecular formula $C_9H_8O_3$. From ESI-MS m/z: 162.9 $[M-H]^+$ together with ^1H and ^{13}C NMR spectra data. The ^{13}C NMR and DEPT spectrum exhibited 9 carbon signals: δ 171.2 (s), 161.0 (s), 146.4 (s), 131.0 (d, CHx2), 127.3 (s), 116.9 (d, CHx2), 115.9 (d). The ^1H NMR spectra showed aromatic protons δ 7.60 (d, J= 15.0, 1H), 7.44 (d, J= 9.0, 2H) 6.81 (d, J= 9.0, 2H), 6.29 (d, J= 15.0, 1H). Proton NMR data showed a Z- form double bond and a benzo- group. Carbon-13 NMR showed the structure also contains a carboxyl acid group. From the analysis of molecular formula, NMR data and literature, the structure was deduced as 3-(4-hydroxyphenyl)-2- propenoic acid, or p-coumaric acid (Fig 8.5) (Aldrich Library, 1992).

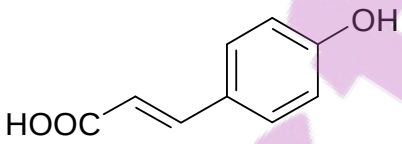


Fig 8.5. Structure of p-coumaric acid

p-Coumaric acid is widespread in plants, e.g. peel of black cherry (*Prunus serotina)*, lentil seeds, red clover (*Trifolium pretense*) and *Daviesia latifolia.* It is also found in *Larix sibirica* bark and *Miscanthus floridulu.* It shows cytostatic activity, and is an immunoactive agent, and inhibitor of stilbene oxidase (Dictionary of Natural Products, 2006).

Coumarins are phenolic substances made of fused benzene and α-pyrone rings (O' Kennedy *et al.*, 1997). Coumarins are responsible for the characteristic odour of hay. As of 1996, at least 1300 had been identified (Hoult and Paya, 1996). Their fame has come mainly from their antithrombic (Thastrup *et al*., 1985), anti-inflammatory (Piller, 1975), and vasodilatory (Namba *et al*., 1988) activities. Warfarin is a particularly well-known coumarin which is used both as an oral anticoagulant and as a rodenticide (Keating and O'Kennedy, 1997). It may also have antiviral effects (Berkada, 1978). Coumarins are known to be highly toxic to rodents (US Department of Health and Human Services, 1992) and therefore are treated with caution by the medical community. It appears that toxic coumarin derivatives may be safely excreted in the urine in humans (Weinmann, 1997).

Several other coumarins have antimicrobial properties. Thornes, while working at the Boston Hospital in 1954, sought an agent to treat vaginal candidiasis in pregnant patients and coumarin was found *in vitro* to inhibit *Candida albicans* (Thornes, 1997). During subsequent *in vivo* tests on rabbits, the coumarin-spiked water supply was inadvertently given to all the animals in the research facility and was discovered to be a potent contraceptive agent when breeding programmes started to fail (Thornes, 1997). Its estrogenic effects were later described (Soine, 1964). As a group, coumarins have been found to stimulate macrophages (Casley-Smith, 1997), which could have an indirect negative effect on infections. More specifically, coumarin has been used to prevent recurrences of cold sores caused by HSV-1 in humans (Berkada, 1978) but was found ineffective against leprosy (Thornes, 1997). Hydrocinnamic acids, related to coumarins, seem to be inhibitory to Gram-positive bacteria (Fernandez *et al*., 1996). Also, phytoalexins, which are hydroxylated derivatives of coumarins, are produced in carrots in response to fungal infection and can be presumed to have antifungal activity (Hoult and Paya, 1996).

General antimicrobial activity has been documented in woodruff (*Galium odorantum)* extracts (Thomson, 1978). All in all, data about specific antibiotic properties of coumarins are scarce, although many reports give reason to believe that some utility may reside in these phytochemicals (Bose, 1958; Hamburger and Hostettmann, 1991; Scheel, 1972).

Compound 5

Compound 5 was obtained as a pale yellow amorphous powder, with molecular formula $C_{17}H_{12}O_8$, from ESI-MS m/z: 345.1 $[M-H]^+$. Three methoxyl groups δ 4.38 (s, 3H), 4.21 (s, 3H), 4.01 (s, 3H), and two aromatic protons δ 7.66 (s, 1H), 7.74 (s, 1H) were found in proton NMR spectra. Comparing the

spectrum with literature, the structure was determined to be 2, 3, 8-tri-O-methylellagic acid (Fig 8.6) (Skilleter and Kekwick, 1970).

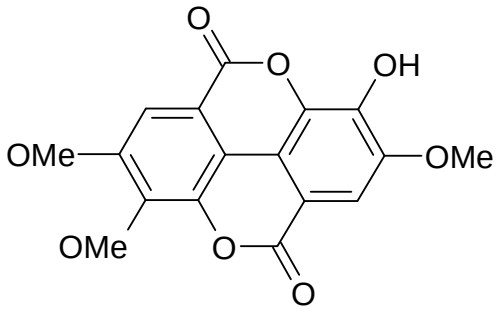


Fig 8.6. Structure of 2, 3, 8-tri-O-methylellagic acid

This compound is a constituent of the haemolymph of *Nasutitermes exitiosus,* and *Eugenia maire* (Dictionary of Natural Products, 2006).

Tannin is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution, a property known as astringency (Cowan, 1999). Their molecular weights range from 500 to 3000 (Haslam, 1996), and they are found in almost every plant part: bark, wood, leaves, fruits, and roots (Scalbert, 1991). They are divided into two groups, hydrolysable and condensed tannins. Hydrolysable tannins are based on gallic acid, usually as multiple esters with D-glucose; while the more numerous condensed tannins (often called proanthocyanidins) are derived from flavonoid monomers. Tannins may be formed by polymerization of quinone units (Geissman, 1963). This group of compounds has received a great deal of attention in recent years, since it was suggested that the consumption of tannin-containing beverages, especially green teas and red wines, can cure or prevent a variety of ills (Serafini *et al*., 1994).

Many human physiological activities, such as stimulation of phagocytic cells, host-mediated tumour activity, and a wide range of anti-infective actions, have been assigned to tannins (Haslam, 1996). One of their molecular actions is to complex with proteins through so-called nonspecific forces such as hydrogen bonding and hydrophobic effects, as well as by covalent bond formation (Haslam, 1996; Stern *et al*., 1996). Thus, their mode of antimicrobial action may be related to their ability to inactivate microbial adhesions, enzymes, cell envelope transport proteins, etc. Tannins also complex with polysaccharides (Ya *et al*., 1988). The antimicrobial significance of this particular activity has not been explored. There is also evidence for direct inactivation of microorganisms: low tannin concentrations

modify the morphology of germ tubes of *Crinipellis perniciosa* (Brownlee *et al*., 1990). Tannins in plants inhibit insect growth (Schultz, 1988) and disrupt digestive events in ruminal animals (Butler, 1988).

Scalbert (1991) reviewed the antimicrobial properties of the tannins, listing 33 documenting the inhibitory activities of tannins. According to these studies, tannins can be toxic to filamentous fungi, yeasts, and bacteria. Condensed tannins have been determined to bind cell walls of ruminal bacteria, preventing growth and protease activity (Jones *et al*., 1994). Although this is still speculative, tannins are considered to be at least partially responsible for the antibiotic activity of methanolic extracts of the bark of *Terminalia alata* found in Nepal (Taylor *et al*., 1996). This activity was enhanced by UV light activation (320 to 400 nm at 5 W/m^2 for 2h). At least two studies have shown tannins to be inhibitory to viral reverse transcriptase (Kaul *et al*., 1985; Nonaka *et al*., 1990).

Tannins may also directly affect the metabolism of microorganisms, as suggested by modification of the morphology of the germ tube of *Crinipellis perniciosa* at low tannin concentrations (0.063 g/l) (Akpata and Akinrimisi, 1977). A drastic change in the morphology and growth pattern of bacteria was observed when they were grown in the presence of sub-inhibitory concentrations (0.6 g/l) of tannic acid or carob pod extract (Casley- Smith, Casley-Smith, 1997). *Pseudomonas fluorescens, Escherichia coli* or *Cellvibrio fulcus* formed chains of filaments whereas most cells were single when grown in the absence of tannins; the morphology of other bacterial species, although also subject to tannin inhibition, was not affected (Scalbert, 1991).

Some moulds develop easily on the surface of tannin-rich woods such as quebracho (Mcunier and Vancy, 1903) or European oak (Scalbert, 1991). Moulds such as *Aspergillus niger,* or *Penicillium glaucum* grow on the surface of the liquid of tannery pits (Mcunier and Vancy, 1903).

Compound 6

Compound 6 was obtained as a white powder, and its molecular formula was established as $C_{28}H_{51}O$. It is also known as (3 beta)-stigmast-5-en-3-ol; 22:23-dihydrostigmasterol; 24beta-ethyl-delta-5-cholesten-3beta-ol. Beta-sitosterol (Fig 8.7) is extremely insoluble in aqueous media and poorly soluble in lipid media. It is found in nature in ester and glycoside forms, both of which forms are more soluble than beta-sitosterol itself.

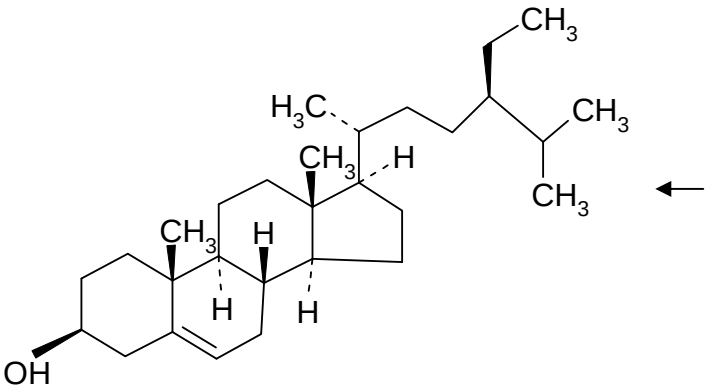


Fig 8.7. Structure of beta-sitosterol

Compound 6 is the most abundant phytosterol in the diet. It is also widely distributed in the plant kingdom and found in such botanicals as *Serenoa repens* (saw palmetto), *Curcurbita pepo (*pumpkin seed) and *Pygeum africanum* (Anonymous, 2005b).Chemically, beta-sitosterol is a very close relative of cholesterol. It differs from cholesterol by the presence of an ethyl group at the 24th carbon position of the side chain. Beta-sitosterol has possible activity in promoting prostate health. It also has cholesterol-lowering activity (Anonymous, 2005b).

Compound 7

Compound 7 was obtained as a yellow amorphous powder with molecular formula $C_{15}H_{14}O_7$. The ^{13}C-NMR and DEPT spectrum exhibited 15 carbons, δ 28.5, 69.2, 83.3, 95.9, 96.7, 101.2, 107.6, 132.0, 134.4, 147.3 (2 carbons), 157.2, 158.0, 158.2 (2 carbons). The proton chemical shifts are: 3.97 (m, H3), 4.55 (d, J= 7.2 Hz, H2), 5.88 (d, J= 2.2 Hz, H8), 5.94 (d, J= 2.2 Hz, H6), 6.4 (s, H2", H6'').

This compound, gallocatechin (Fig 8.8), was isolated by reverse phase chromatography on preparative HPLC eluting with water and acetonitrile. Hussein *et al*. (1999) reported this compound to be active against HIV-1 with an IC_{50} greater than 100 µg/ml. Mahmood *et al.* (1993) reported a 97% HIV-1 inhibition at 1 µg/ml. The ELISA antiviral assay method was used for the test. It is widespread in plants, occurring in broad beans, green tea, redcurrants and gooseberries. Gallocatechin possesses antiscorbutic, and antioxidant properties (Dictionary of Natural Products, 2006).

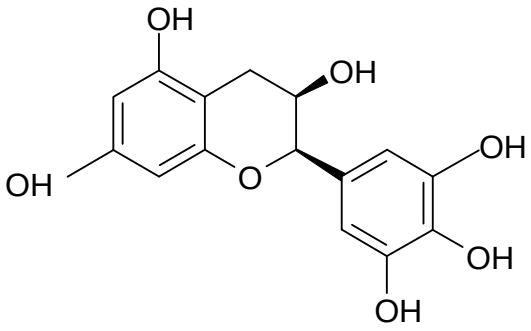


Fig 8.8. Structure of gallocatechin

Compound 8

Compound 8 was obtained as yellow needles and its molecular formula was determined to be $C_{15}H_{10}O_5$ and molecular weight 270. The ^{13}C- NMR and DEPT spectrum led to the conclusion that this compound had 15 carbons with the following chemical shifts: C-2 (163.8), C-3 (102.8), C-4 (181.8), C-5 (161.9), C-6 (98.8), C-7 (164.1), C-8 (94.0), C-9 (157.3), C-10 (103.7), C-1'(121.3), C-2' (128.4), C-3' (116.0), C-4' (161.5), C-5' (116.0), C-6' (128.4). ^1H- NMR 6.2 (d, H 6), 6.5 (d, H8), 6.8 (s, H3), 6.9 (d, H3', H6'), 7.9 (d, H2', H6'), 10.4 (s, O−H, 4'), 10.9 (s, O−H, 7), 13 (O−H, 5). This compound, apigenin (Fig 8.9) is found free or as glycosides in the stems, roots, leaves, seeds or fruits of a very wide range of plant species and has been found also in some fossil leaf tissues. It is used as an ethanol solution for photometric determination of aluminum, rare earth elements, beryllium, zirconium and cadmium. It shows antineoplastic activity *in vitro*, antispasmolytic agent and anti-inflammatory activity. It is also an enzyme inhibitor and superoxide scavenger (Dictionary of Natural Products, 2006).

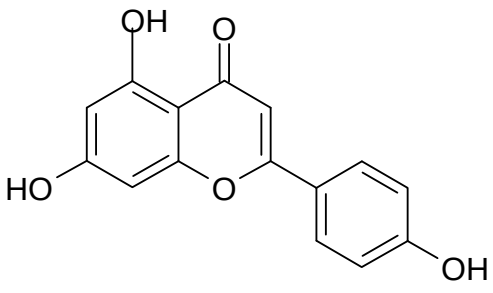


Fig 8.9. Structure of apigenin

Compound 9

Compound 9 (cosmosiin, Fig 8.10) was isolated as a yellow powder with formula $C_{21}H_{20}O_{10}$ and molecular weight 432. The ^{13}C, DEPT and ^{1}H NMR led to the conclusion that this compound had 21 carbons with the following chemical shifts: C-4 (181.7), C-2 (164.3), C-7 (162.8), C-5 (161.5), C-4' (161.0), C-9 (156.8), C-2', 6'(128.3), C-1' (120.8), C-3', 5' (116.0), C-10 (105.4), C-3 (102.9), C-!" (100.2), C-6 (99.5), C-8 (94.9), C-5" (77.1), C-3" (76.5), C-2'' (73.1), C-4'' (69.8), C-6'' (60.8). The ^{1}H NMR spectra had the following shifts: 6.2 (1H, d), 6.5 (1H, d), 6.8 (1H, s), 6.9 (2H, d), 7.9 (2H, d), 10.4 (1H, s) O-H (4'), 10.9 (1H, s) O-H (7), 13 O-H (5).

The compound is a constituent of flowers of *Cosmos bipinnatus, Zinnia elegans* and other plant species. It is an anti-HIV agent (Dictionary of Natural Products, 2006). Apigenin-7-O-glucoside has been shown to exhibit a moderate antiamoebic activity with IC50 value of 22.3 ± 3.2 µg/ml (Cimanga *et al.,* 2006).

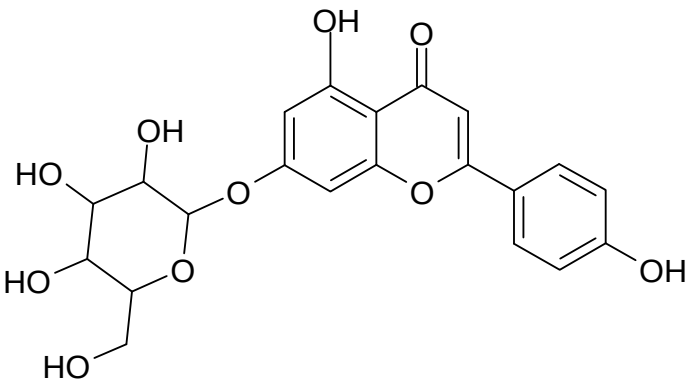


Fig 8.10. Structure of cosmosiin

Representative NMR spectra of the isolated compounds are attached in Appendix 1.

8.4.2 Biological activity of isolated compounds

8.4.2.1 Antiviral activity

The antiviral activity of extracts of *C. paniculatum* was determined using feline herpesvirus type 1 (an enveloped virus). The best result for the extracts was a 3.6 $\log_{10}$ reduction for the stem bark. It is very

interesting that water and acetone extracts had a similar antiviral activity. The acetone and water extract of the leaves all had a 3 log_{10} reduction of virus titre. The water extract of the root bark had a 3.4 log_{10} reduction value.

All the isolated compounds were tested for antiviral activity against Coxsackie virus B3 (CVB3), influenza virus A, and Herpes simplex virus type 1 (HSV1) and there was no activity.
It is surprising that no antiviral activity was found for compound 7 (gallocatechin) because Hussein *et al.* (1999) found this compound to be active against HIV-1 with an IC_{50} greater than 100 µg/ml.

It was a disappointment that there was no good activity with any of the isolated compounds despite the promising results obtained for the water and acetone extracts of the leaves against the sensitive feline herpesvirus. The isolated compounds did not show as significant a reduction in virus titre as that obtained for the water and acetone extracts of the leaves. This could be due to synergistic effects of various constituents of the plant material. The acetone extract of the leaves has previously been shown to inhibit HIV-2 replication with an EC_{50} of 3.0 µg/ml and selectivity index of 32 (Asres *et al.,* 2001). The acetone extract of the leaves inhibited feline herpesvirus with an EC_{50} of 2.8 µg/ml.

8.4.2.2 Antibacterial and antifungal activities

MIC values for some of the isolated compounds are presented in Table 8.1.

Table 8.1. MIC values (µg/ml) of some isolated compounds

Compound	*S. aureus*	*E. coli*	*E. faecalis*	*P. aeruginosa*
Methylellagic acid	>250	62.5	125	125
Isoquercitin	62.5	31.25	62.5	62.5
Gallocatechin	62.5	62.5	31.25	125
p-coumaric acid	>250	>250	>250	>250
Gentamicin	0.8	0.4	0.4	1.6

The MIC values of the compounds tested against the four species of bacteria ranged between 31.25 µg/ml to >250 µg/ml.

The agar diffusion method (described in section 3.12) was used in a separate group of experiments for the determination of antibacterial and antifungal activities against the microorganisms listed in Table 8.2.

A standard quantity of 50 µg of isolated compounds was placed in each agar well and the inhibition zones in the bacterial or fungal lawn measured after overnight incubation.

Table 8.2. Organisms tested for activity against isolated compounds

Organisms	Code
Bacteria	
Bacillus subtilis ATCC 6633 (IMET 10880)	Bs
Staphylococcus aureus (IMET 10760) SG 511	Sa
Escherichia coli SG 458	Ec
Pseudomonas aeruginosa K799/61	Pa
Mycobacterium vaccae IMET 10670	Mv
Fungi	
Sporobolomyces salmonicolor SBUG 549	Ss
Candida albicans BMSY 212	Ca
Penicillium notatum JP 36	Pn

Table 8.3. Results of antimicrobial activity on isolated compounds (diameter of inhibition zone in mm)

Compounds	Bs	Sa	Ec	Pa	Mv	Ss	Ca	Pn
Cholest-5-en-3-ol	10	0	15p	0	18p	23	0	13p
2-phyten-1-ol	10	0	14p	0	33p	22	0	13p
Gallocatechin	0	0	16p	0	16p	24	0	13p
Apigenin	0	0	14p	0	18p	27	0	13p

Diameter of inhibition zone

0-15 mm = No activity

16-20 mm = Moderate activity

21-25 mm = Good activity

>25 mm = Strong activity

p = Few colonies present in the inhibition zone (moderate activity)

Cholest-5-en-3-ol had good activity against *Sporobolomyces salmonicolor* (23 mm) and moderate activity against *Mycobacterium* vaccae (18p). The compound 2-phyten-1-ol had a strong activity against *Mycobacterium vaccae* (33p) and good activity against *Sporobolomyces salmonicolor* (22 mm). p-Coumaric acid has been found to be active against *Escherichia coli* and *Staphylococcus aureus* at pH

5.0, 6.0 and 7.0 and *Bacillus cereus* at pH 6.0, 6.5 and 7.0. p-Coumaric acid was generally the most effective inhibitor tested causing more than 99.9% inhibition of *E. coli* at 1000 µg/ml (pH 5.0, 48 hr), *S. aureus* at 500 µg/ml (pH 5.0, 48 hr), *B. cereus* at 500 µg/ml (pH 5.0, 48 hr), and B. cereus at 500 µg/ml (pH 7.0, 9 hr) (Herald and Davidson, 1983). Inhibition increased as pH decreased with *E. coli* and *S. aureus* but not in the case of *B. cereus. Bacillus cereus* appeared to be the most susceptible strain with 1000 µg/ml of the compounds tested causing > 99% inhibition at all three pH's (Herald and Davidson, 1983). It has been shown that extracts of some *Combretum* species (*C. glutinosum, C. hispidium, C. molle* and *C. nigricans*) have antifungal effects against dermatophytes as well as *Candida albicans* (Baba-Moussa *et al*., 1999). Baba-Moussa *et al.* (1999) proposed that tannins and saponins might be responsible for this activity and this might explain the good activity of cholest-5-en-3-ol and 2-phyten-1-ol.

Compound 7 (gallocatechin) had a strong activity against *Sporobolomyces salmonicolor* (24 mm) and a moderate activity against *Mycobacterium vaccae* (16p) and *E. coli* (16p).
Compound 8 (apigenin) had a strong activity against *Sporobolomyces salmonicolor* (27 mm) and moderate activity against *Mycobacterium vaccae* (18p). Yoichi *et al.* (2000) suggested that apigenin and the related flavonoids are potentially useful for the development of therapeutic treatments of MRSA infections. They also found that apigenin tested against the following organisms: *S. aureus, Bacillus cereus, E. faecalis, Acinetobacter calcoacetics, Citrobacter freundii, Enterobacter cloacae, E. coli, Proteus mirabilis, P. vulgaris* and *Salmonella typhimurium* resulted in MICs greater than 250 µg/ml. For both MRSA and methicillin-sensitive *Staphylococcus aureus* (MSSA) strains, the MIC for apigenin ranged between 3.9 to 15.6 µg/ml (Yoichi *et al.,* 2000).

Compound 9 (apigenin-7-O-glucoside) had a strong activity against *Sporobolomyces salmonicolor* (27 mm) and a moderate activity against *Mycobacterium vaccae* (18 p).

8.4.2.3 Cytotoxic activity of isolated compounds

The cytotoxic effects of the compounds were tested *in vitro* against HeLa, MDCK (Madin-Darby Canine Kidney) and GMK (Green Monkey Kidney) cell lines. The results are presented in Table 8.4.

Table 8.4. Cytotoxicity of isolated compounds

Compounds	HeLa	MDCK	GMK
Cholest-5-en-3-ol	25	>50	>50
2-phyten-1-ol	12.5	>50	>50
Isoquercitin	12.5	>50	>50
P- coumaric acid	25	>50	>50
Methylellagic acid	12.5	>50	>50
Beta-sitosterol	25	>50	>50
Gallocatechin	12.5	>50	>50
Apigenin	25	>50	12.5

Toxicity Categories:

CC_{10} > 100 µg/ml = Slight cytotoxicity

CC_{10} 10- 100 µg/ml = Moderate cytotoxicity

CC_{10} 1-10 µg/ml = Strong cytotoxicity

CC_{10} < 1 µg/ml = Extreme cytotoxicity

The compounds were more toxic to HeLa cell lines than MDCK and GMK cells. The GMK cell lines were the least affected with most CC_{10} values greater than 50 µg/ml. With the isolated compounds, the cytotoxicity was in the moderate toxicity range (CC_{10} = 12 µg/ml to > 50 µg/ml) compared to some fractions. The compound 2-phyten-1-ol is known to have anticancer activity (colon and gastric cancer) (Dictionary of Natural Products, 2006). It is also used in the preparation of Vitamins E and K (Dictionary of Natural Products, 2006). This is probably the reason this compound has strong antiproliferative activity.

There are very few reports on the antiproliferative and cytotoxic effects of the constituents of the Combretaceae. This study indicates that some members of the Combretaceae have antiproliferative and cytotoxic components, justifying further research.

8.5 Conclusion

There was very little antiviral activity observed for the two isolated compounds tested against feline herpesvirus. The extracts were more active compared to these two compounds, implying synergism. The hypothesis that antibacterial compounds isolated from *C. paniculatum* will have antiviral activity

could not be substantiated because not one of the isolated antibacterial compounds had good antiviral activity. It is much easier to use antibacterial activity in bioassay-guided fractionation. Since the hypothesis has been proven wrong, in future it will better to carry out antiviral activity at each stage of the isolation, though it is painstakingly slow. The aqueous extracts may contain antiviral compounds and are worth investigating further.

In the Phytomedicine Programme, we have frequently found that the biological activity of known compounds isolated earlier is not known. Consequently several biological activities of isolated compounds were determined where sufficient material was available.

Nine compounds were isolated and their structures determined by the aid of instrumental analysis. Our hypothesis that one can isolate potential antiviral compounds using antibacterial activity since it is easier to test for antibacterial activity was not substantiated because none of the isolated compounds had good antiviral activity. However, the compounds had some antibacterial and antifungal activity. A broad spectrum of antibacterial activity against Gram-positive and Gram-negative pathogens was shown.

The compounds were tested for antiviral activity against three viruses, namely Coxsackie virus (CVB3), Influenza virus A and herpes simplex virus type 1 (HSV1) but there was no activity. As the compounds isolated from the leaves of *Combretum paniculatum* in this study did not have antiviral activity, this implies that there could be a synergistic effect, or that the compound(s) with antiviral activity were not isolated. It is recommended that further research be carried out with a much larger quantity of plant material so that the antiviral compounds that were potentially not isolated owing to presence in small quantities may be isolated using antiviral assays to guide the fractionation process.

Chapter 9

General Conclusions

9.1 Introduction

The development of new antiviral and antibacterial drugs is challenging, taking into account the poor selective toxicity and fast selection of resistant variants with existing drugs. Virus infection is a common problem worldwide. Herpes simplex virus is found in over 60 million people in the US, most of whom are of child-bearing age. It is therefore necessary to find alternative active compounds. Pure compounds of plant origin have been shown to exhibit antiviral and antibacterial activities.

According to ethnobotanical literature, the genus *Combretum* is used widely for a variety of conditions in African traditional medicine. Members of this genus have the following biological activities: antifungal, anti-inflammatory, antibacterial, diuretic and molluscidal (Hutchings *et al*., 1996).

Asres *et al*. (2001) reported the antiviral activity of the leaf extract of *Combretum paniculatum* against HIV-2. The acetone extract of the leaf showed a high degree of antiviral activity against HIV-2 with an EC_{50} of 3 µg/ml and selectivity index of 32. Following from this, the aim of the present work was to isolate compounds, characterize them and evaluate them for antiviral and other biological activities.

The objectives were to:
Select the best extractant for the plant material in terms of quantity and antimicrobial activity
To determine antiviral, antibacterial and cytotoxic activities of extracts
To isolate and chemically characterize compounds
To determine the antiviral and other biological activities of isolated compounds.

9.2 Selection of the best extractant for the plant material

Acetone was selected as the best extractant based on the number of compounds extracted from the plant after TLC analysis and the number of bioactive compounds on bioautography. The number of compounds in the extracts was ranked in the following order by TLC analysis: acetone (7), DCM (5), carbon tetrachloride (4), ethanol (4), hexane (2) and THF (1), where the number of compounds

visualized on TLC plates are indicated in brackets. Water and methanol extracts did not separate with the TLC solvent systems used.

Antiviral activity of the acetone and water extracts of the leaves against feline herpesvirus produced a promising result with a 3 $\log_{10}$ reduction of virus titre. Bioautography of the acetone extract was used to determine the effectiveness of acetone as a solvent for extraction of antibacterial compounds. Many more compounds had antibacterial activity in the acetone extract after separating the extracts on a column, compared to the crude acetone extract. Eloff (1999) discovered that leaves of 27 southern African members of the Combretaceae had antibacterial activity when extracted with acetone and tested against *E. coli, S. aureus, P. aeruginosa* and *E. faecalis.* Acetone was therefore selected as extractant for isolating antibacterial compounds. Water extracted a good quantity of plant material so a mixture of acetone and water (70:30) was chosen for the bulk extraction process.

The amount of material extracted from the leaves of *C. paniculatum* per gram using different solvents ranged from 15 mg to 246 mg. These values are different to those obtained by Kotze and Eloff (2002) while working on *C. microphyllum* (26 to 174 mg), a plant closely related to *C. paniculatum*. Some authorities consider *C. paniculatum* to be synonymous with *C. microphyllum* (Germishuizen and Meyer, 2003) but others (Palgrave, 2002; Carr, 1988) recognize both species. The quantities of material extracted by water and DCM for the two species were significantly different. For *C. paniculatum,* water extracted the most (246 mg) followed by methanol (194 mg) while for *C. microphyllum*, methanol extracted 174 mg followed by DCM (106 mg). The differences shown in the TLC fingerprints and the amount of material extracted from the two plants supports recognition of the two species.

9.3 Determination of the antimicrobial, cytotoxic and antioxidant activities of extracts

Antiviral activity was determined for the leaf, stem bark and root bark extracts of *C. paniculatum.* The results were determined by noting cytopathic effect (CPE) and also by using a colorimetric MTT assay. The cells were examined for CPE and virus titre was calculated using the Karber formula. The antiviral activity of the stem bark was 3.6 $\log_{10,}$ that is, 3.6 $\log_{10}$ reduction (close to 4 000 fold) of virus titre, while the root bark displayed a 3.4 $\log_{10}$ reduction. The acetone extract of the leaves displayed a 1000 fold reduction. The EC_{50} of the acetone extract of the leaves was 2.8 µg/ml against feline herpesvirus type 1. Asres *et al*. (2001) reported that the acetone extract of *C. paniculatum* leaves collected in East Africa

inhibited HIV-2 replication with an EC_{50} of 3.0 µg/ml. This confirms that the acetone extract of the leaves has antiviral activity.

The MIC values against the selected bacteria obtained for the leaf extract were lower than those obtained for other members of the Combretaceae family (Eloff, 1999). The average MIC values ranged between 0.28 to 0.86 mg/ml. Eloff (1999) found that 27 members of the Combretaceae inhibited bacterial growth with MIC values between 0.1 to 6 mg/ml and an average of 2.01 mg/ml. Gram-positive bacterial strains were slightly more sensitive with an average MIC of 1.8 mg/ml while the Gram-negative strains had a higher average MIC value of 2.22 mg/ml. Bioautography of the extracts showed that there was good bacterial growth inhibition by compounds in acetone and ethyl acetate extracts.

Antioxidant activity was determined qualitatively by spraying developed TLC plates with 0.2% DPPH. Almost all the fractions possessed antioxidant compounds. Acetone and ethyl acetate extracts had more antioxidant compounds than the other extracts. Cytotoxicity was observed only at the highest concentration of extracts tested (0.28 mg/ml).

9.4 Preliminary isolation study

A preliminary fractionation was carried out to simplify the complex crude extracts and verify their antibacterial activity. The acetone extract of the leaves was separated in a silica column and the collected fractions pooled after TLC analysis. The pooled fractions were used to determine antibacterial activity. Nine of the 20 fractions had compounds with antibacterial activity against *S. aureus, E. coli* and *P. aeruginosa*.

The antibacterial activity of the root bark extract was also investigated as this may be a source of bioactive compounds. The antibacterial activity was determined after solvent/solvent partitioning of the acetone extract of the root bark. The chloroform, carbon tetrachloride and butanol fractions were separated into many compounds by TLC. Bioautography of the fractions from solvent/solvent fractionation separated three compounds with antibacterial activity against *S. aureus* in the chloroform fraction. The chloroform fraction was the most active.

The MIC of the root bark solvent/solvent fractions was determined against four bacterial strains and the average MIC values ranged from 0.42 to 1.04 mg/ml. The fractions were tested against 5 fungal strains and the average MIC values ranged from 0.47 to 1.19 mg/ml. According to Masoko *et al.* (2006), a

crude acetone leaf extract of *C. paniculatum* against five fungal strains was more active than the root bark fractions, with MIC values in the range 0.02 to 2.5 mg/ml. Because exactly the same procedures were used for the fractions and the crude acetone extract, the results may indicate synergistic effects in crude extracts.

From bioautography analysis, it appeared that similar bioactive compounds occurred in the root bark and leaves. Due to the small quantity of root bark available, and because using roots of the plant can kill the plant, only leaves were used for large scale extraction and isolation.

9.5 Isolation of antibacterial compounds

The separation of plant constituents was carried out using a combination of chromatographic techniques, namely column chromatography, thin layer chromatography and HPLC. The bulk leaf extraction was performed with acetone and water (70:30) because acetone extracted the most antibacterial compounds and water extracted the most material from the plant. During the isolation process, any compound that crystallized was chemically and biologically characterized.

At the HKI, biological assays are carried out in another facility, slowing the isolation process considerably and the period for research at the Institute was short. Nine compounds were isolated, not strictly by bioassay-guided fractionation, and subjected to instrumental analysis for structure elucidation. The quantities of the isolated compounds ranged from 5 to 10 mg. For future work, a larger initial amount of plant material is recommended to enable the isolation of more compounds present in small quantities.

9.6 Determination of chemical structures, biological assays and cytotoxicity of compounds

Experiments using 1D NMR, 2D NMR, ^{1}H, ^{13}C, HMBC, HMQC and COSY were used for structure elucidation. Elucidation of chemical structures involved a combination of different techniques including NMR and mass spectroscopy (MS). The structures of nine compounds were determined with the aid of instrumental analysis and literature. The compounds were: cholest-5-en-3-ol, 2-phyten-1-ol, isoquercitrin, p-coumaric acid, 2, 3, 8- tri-O-methylellagic acid, beta-sitosterol, gallocatechin, apigenin and apigenin-7-glucoside.

Biological activities were determined for each isolated compound where sufficient material was available. All the compounds were tested for antiviral activity against CVB3, influenzavirus A and herpes simplex virus type 1 and there was hardly any activity. The hypothesis that antibacterial compounds isolated from *C. paniculatum* may have antiviral activity was not substantiated because not one of the isolated antibacterial compounds had any antiviral activity. It is much easier to use antibacterial activity in bioassay-guided isolation. Since the hypothesis has been proven wrong, in future it will be better to carry out antiviral activity testing at each stage of the isolation. It may also be worthwhile to concentrate on aqueous extracts based on results reported in Table 5.1.

Antibacterial and antifungal activities were determined for the compounds, and 2-phyten-1-ol showed the best activity of all the compounds. It had a strong activity against *Mycobacterium vaccae* and good activity against *Sporobolomyces salmonicolor*. 2-Phyten-1-ol is a skin irritant and an anticancer agent. Apigenin had a strong activity against *S. salmonicolor* and moderate activity against *M. vaccae*. Gallocatechin had a good activity against *S. salmonicolor* and moderate activity against *E. coli* and *M. vaccae*. Cholest-5-en-3-ol had a good activity against *S. salmonicolor* and a moderate activity against *M. vaccae*.

Regarding cytotoxicity, the compounds were generally more toxic to the HeLa cell lines than the other cells, with CC_{10} values ranging from 12.5 to 25 µg/ml. The latter values fall in the moderate toxicity category. The compounds generally were less toxic to the GMK and MDCK cell lines with toxicity values all greater than 50 µg/ml which was of moderate toxicity. Some of the compounds have known toxicity. Cholest- 5-en-3-ol at high doses has teratogenic effects. Compound 2 (2-phyten-1-ol) is a skin irritant and has a low LD_{50} (rat, oral) greater than 5000 mg/kg (Dictionary of Natural Products, 2006). Isoquercitrin is a diuretic, antifungal and antioxidant agent. The LD_{50} for p- coumaric acid has been reported to be 657 mg/kg (muscle, ipr) (Dictionary of Natural Products, 2006).

The results obtained confirm the ethnobotanical use of many *Combretum* species for antibacterial infections. In future work, many compounds that are present in low concentrations could be isolated from *C. paniculatum* by starting with a large quantity of plant material. Synergistic effects on biological activity, particularly antiviral activity, of isolated compounds could be investigated. It is also possible that the antiviral compounds were not isolated and further work should be carried out making use of antiviral assay-guided isolation.

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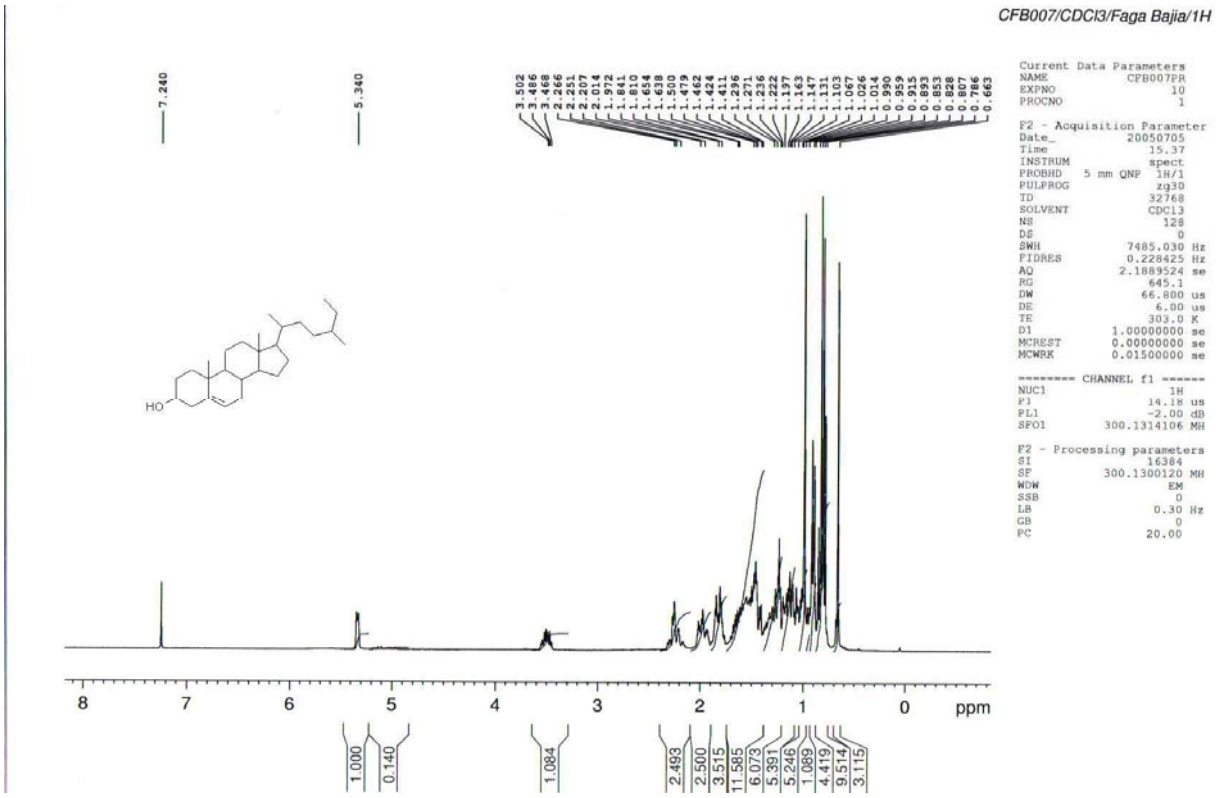
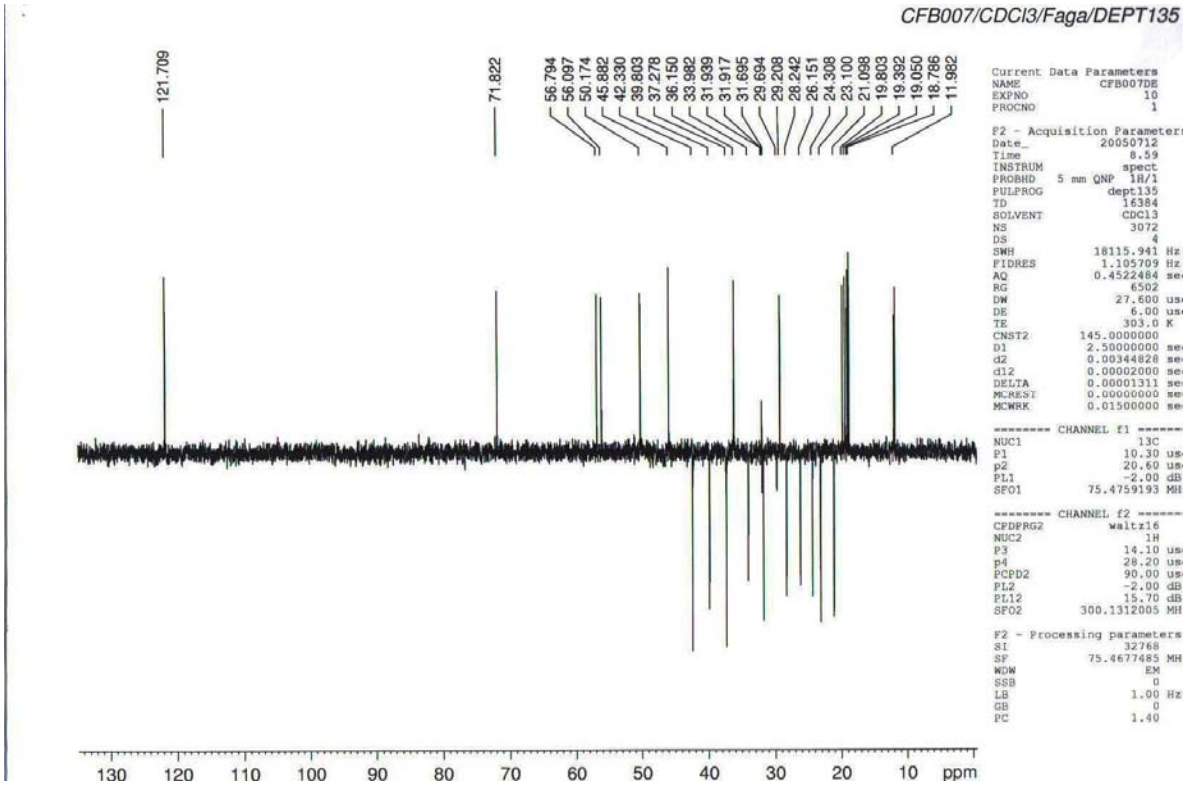
Appendix 1



Compound 1 (cholest-5-en-3-ol): ^{1}H NMR



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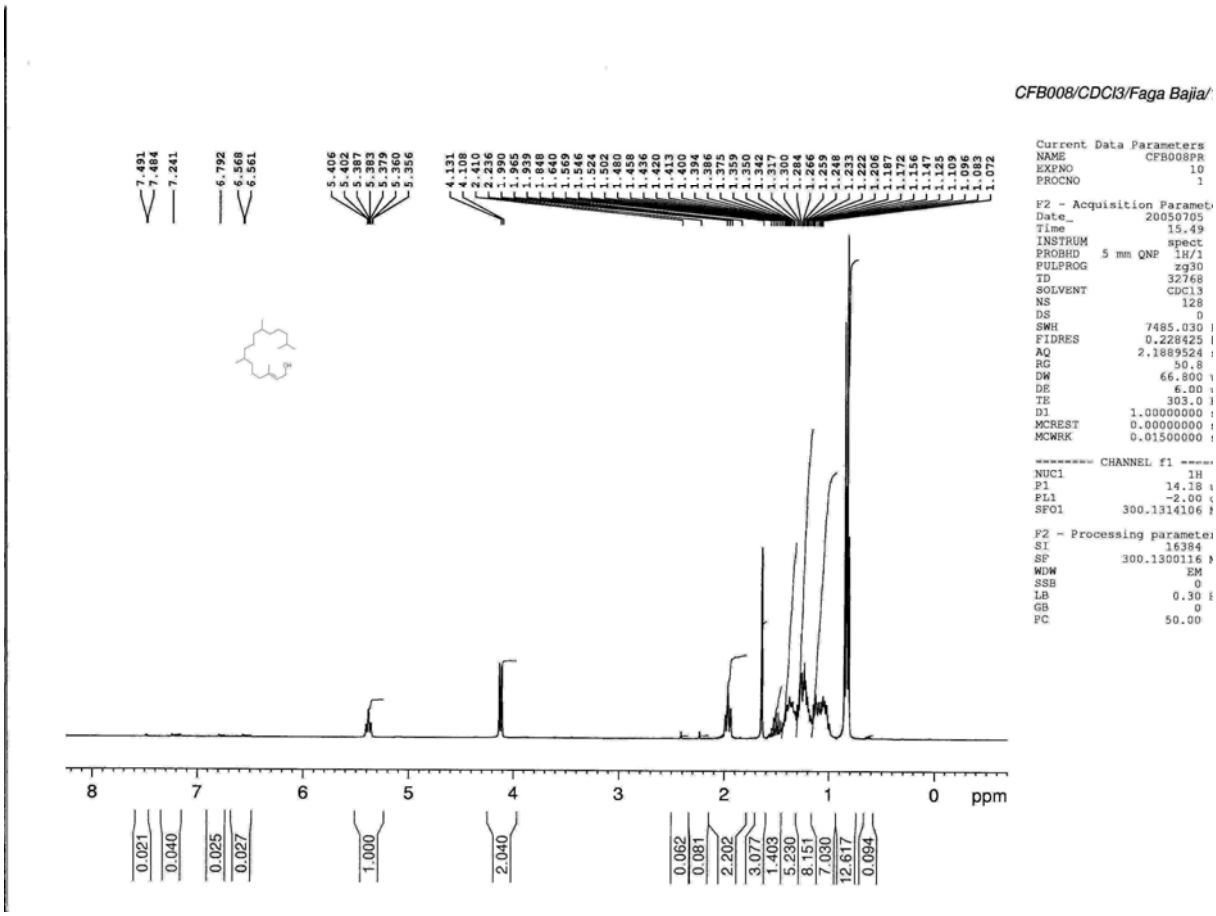
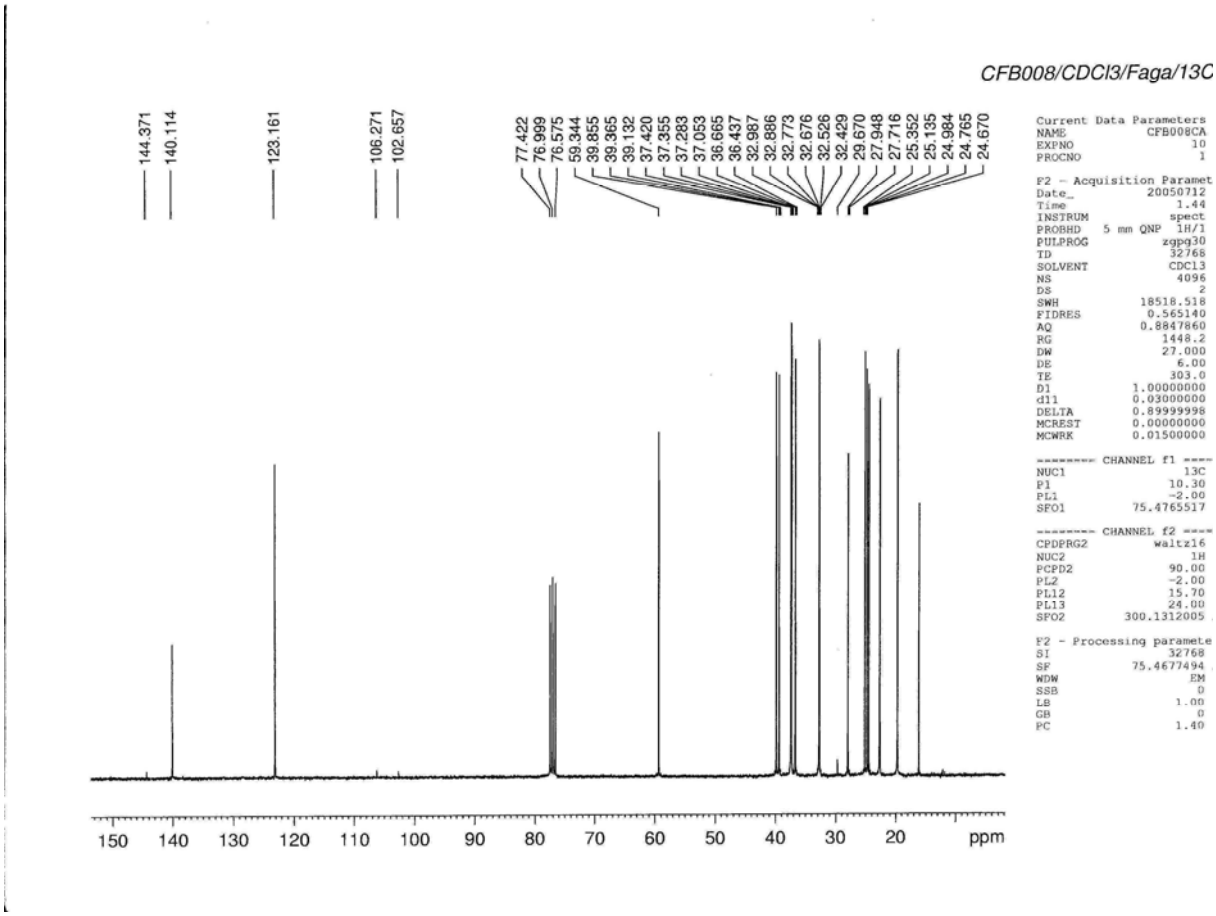
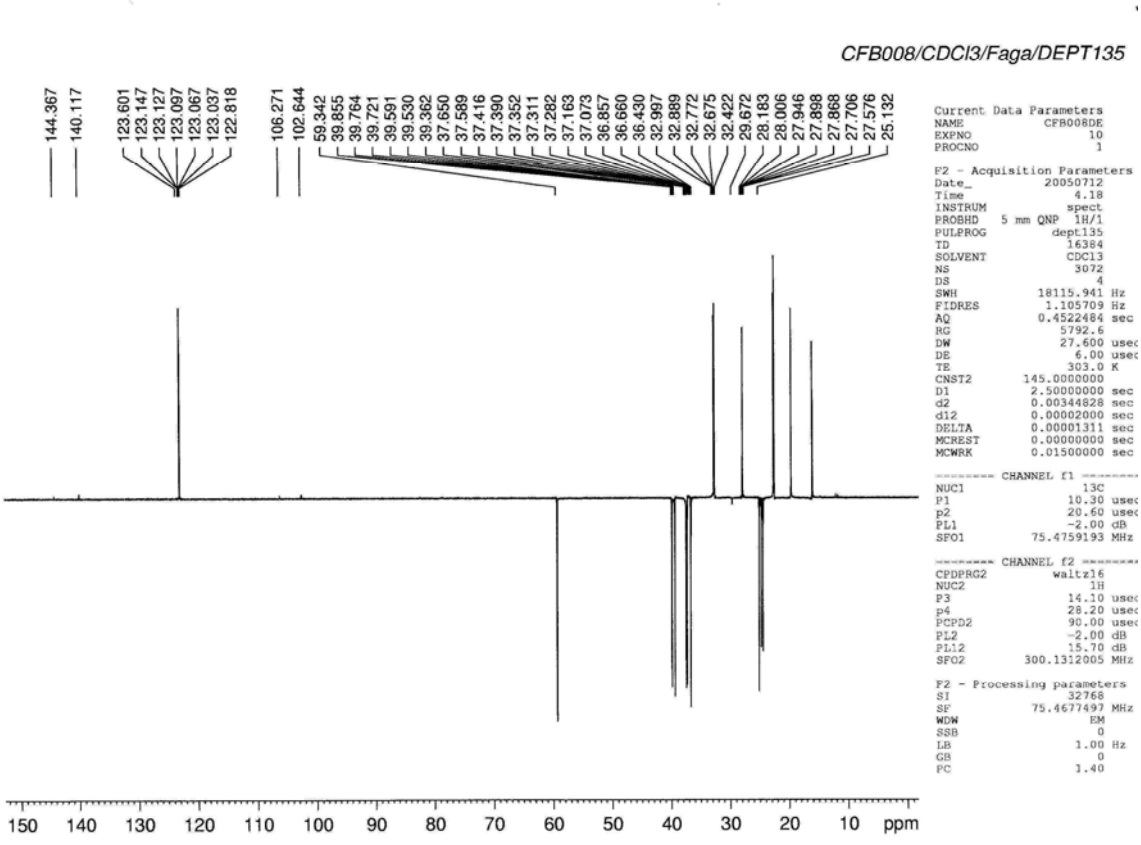


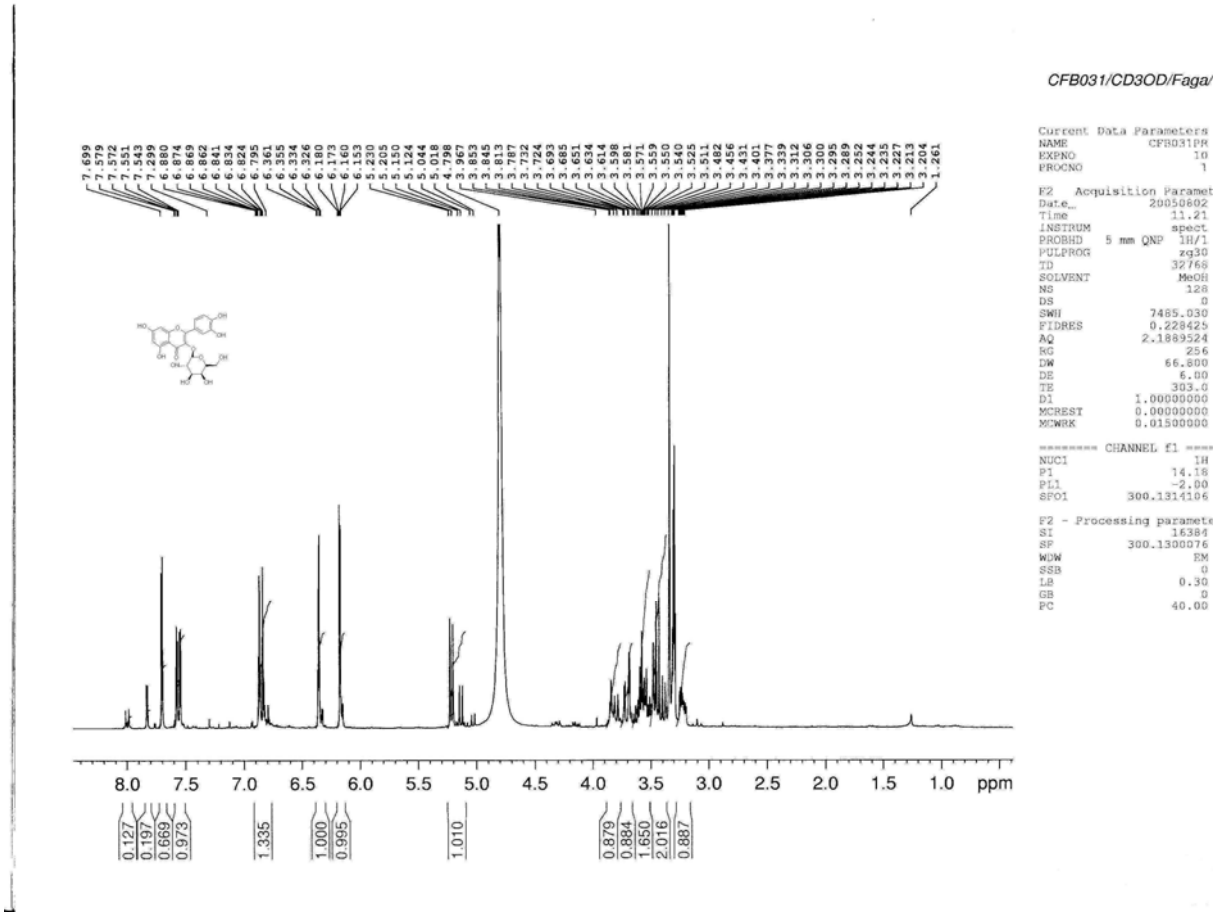
Compound 2 (2-phyten-1-ol): ^{1}H NMR



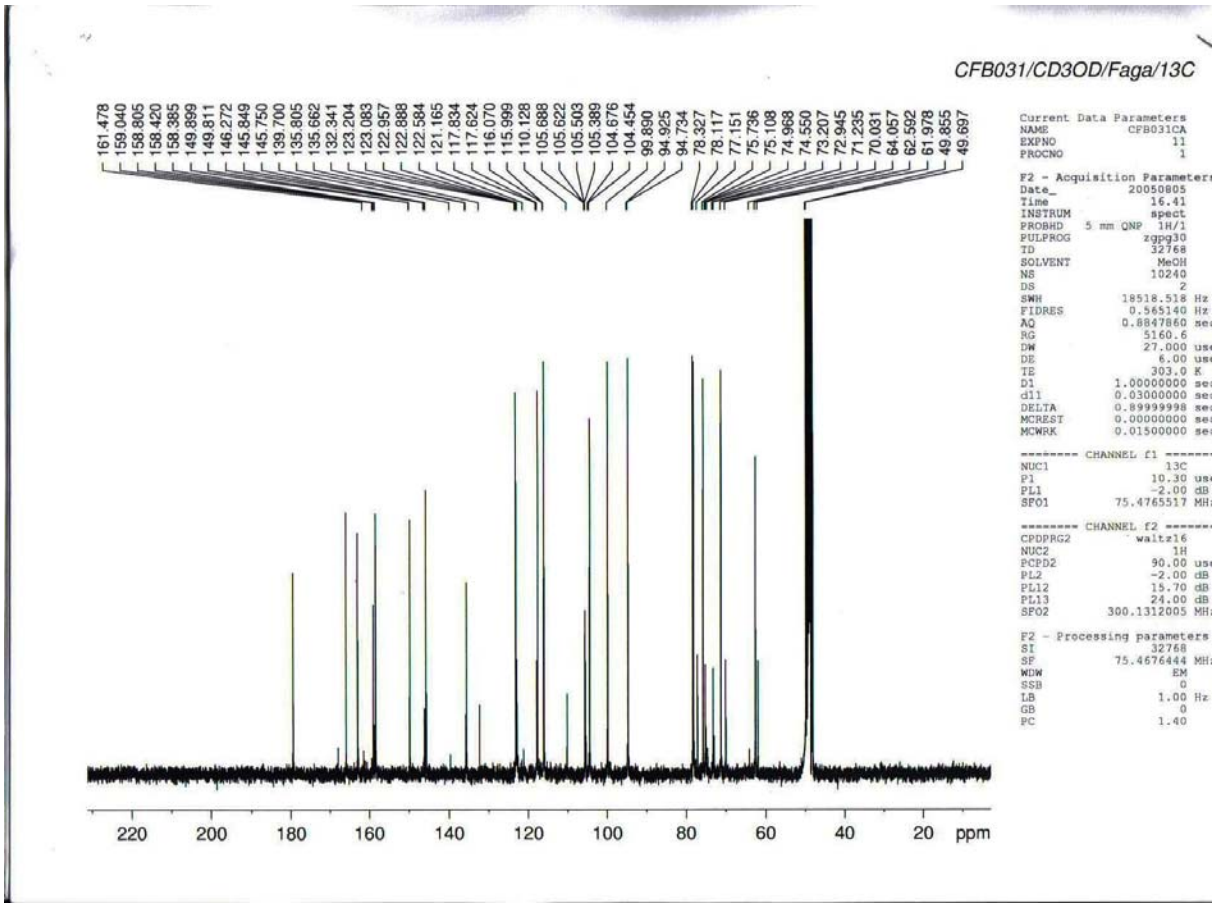
Compound 2 (2-phyten-1-ol): ^{13}C NMR



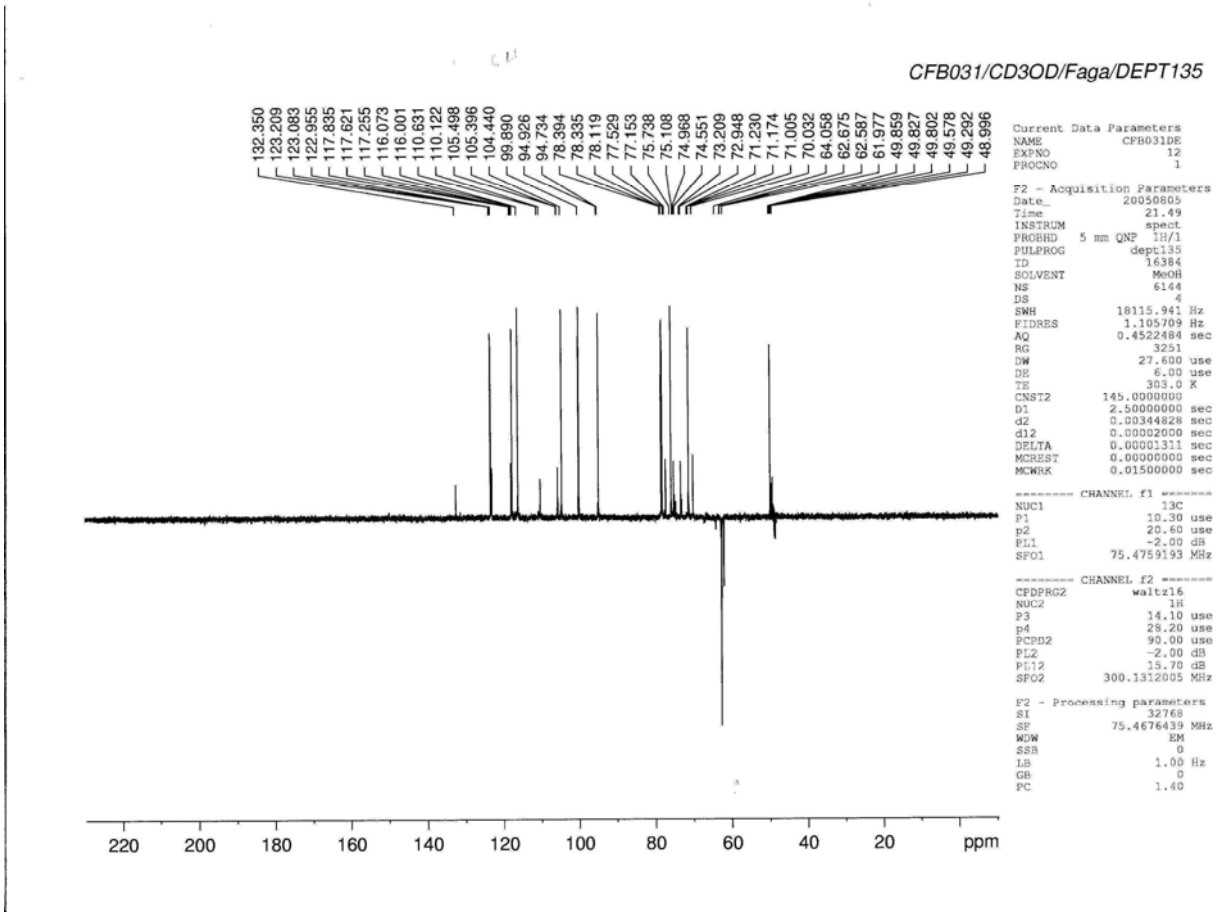
Compound 2 (2-phyten-1-ol): DEPT



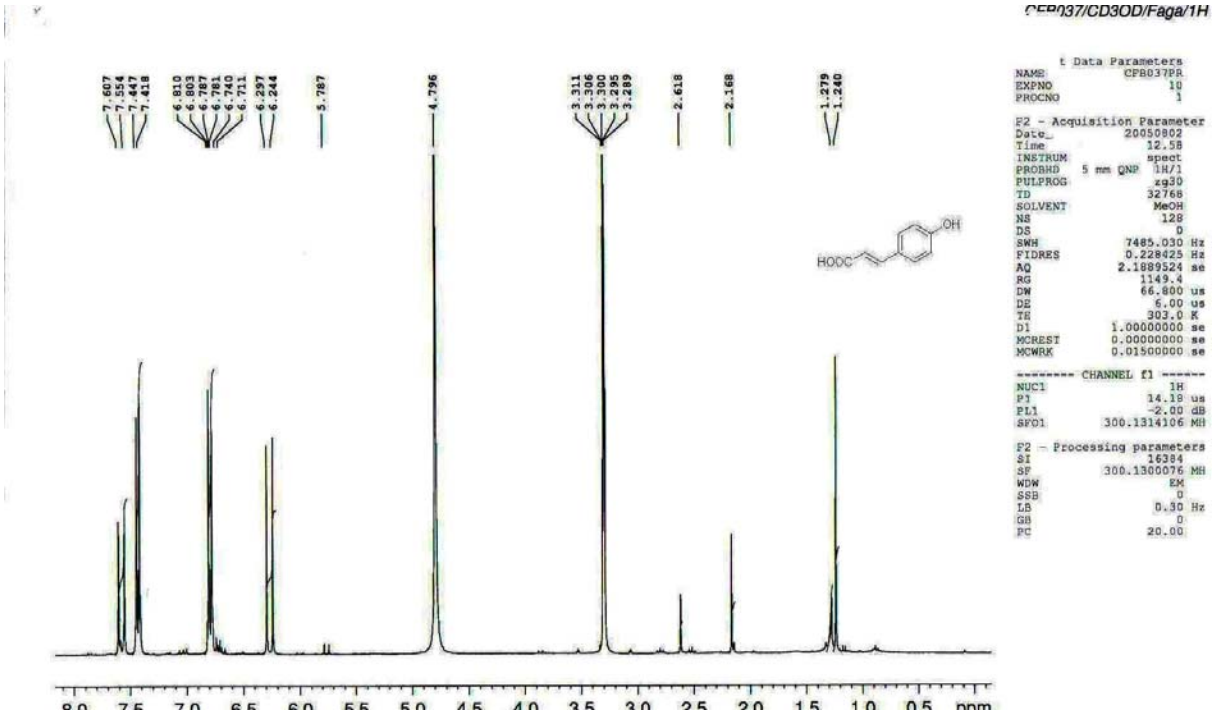
Compound 3 (quercetin-3-glucopyranoside): ^{1}H NMR



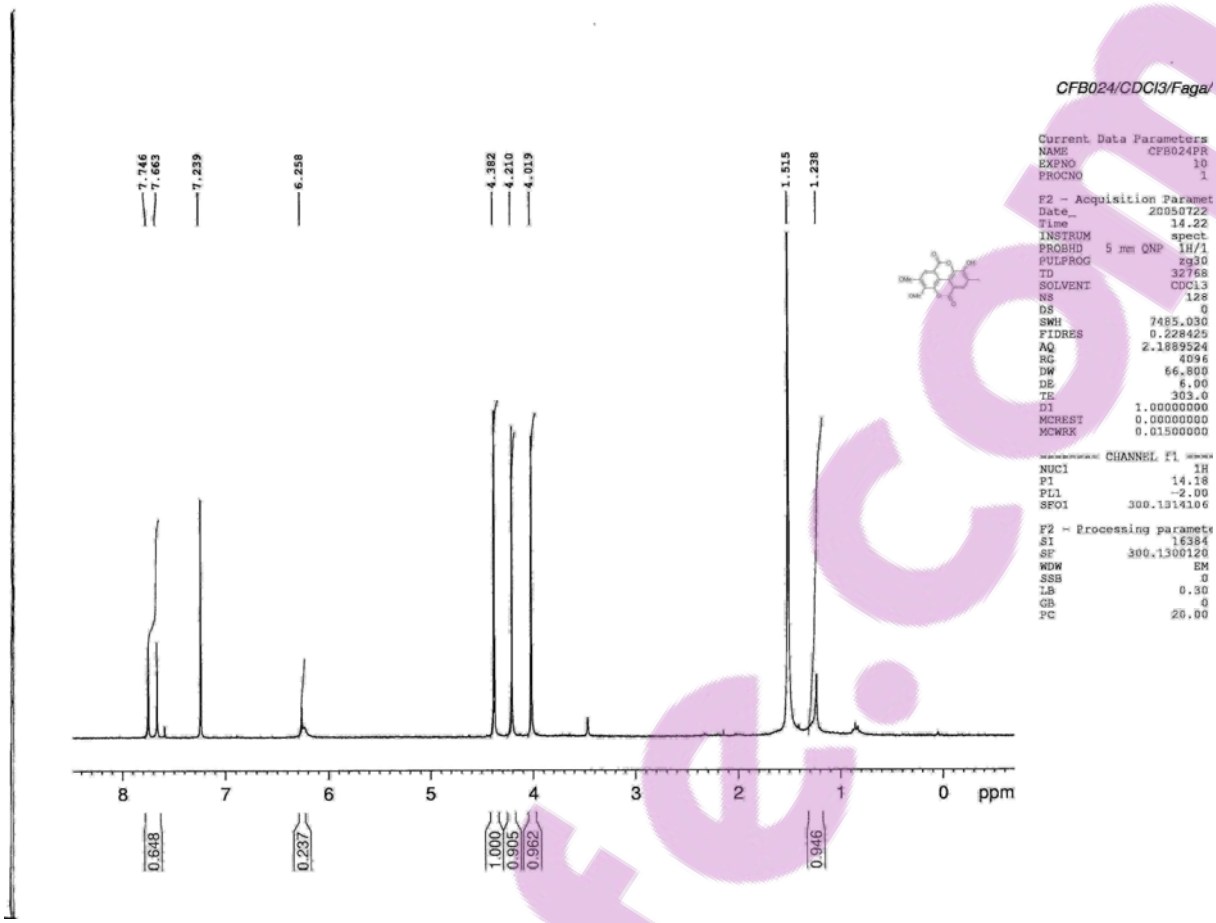
Compound 3 (quercetin-3-glucopyranoside): ^{13}C NMR



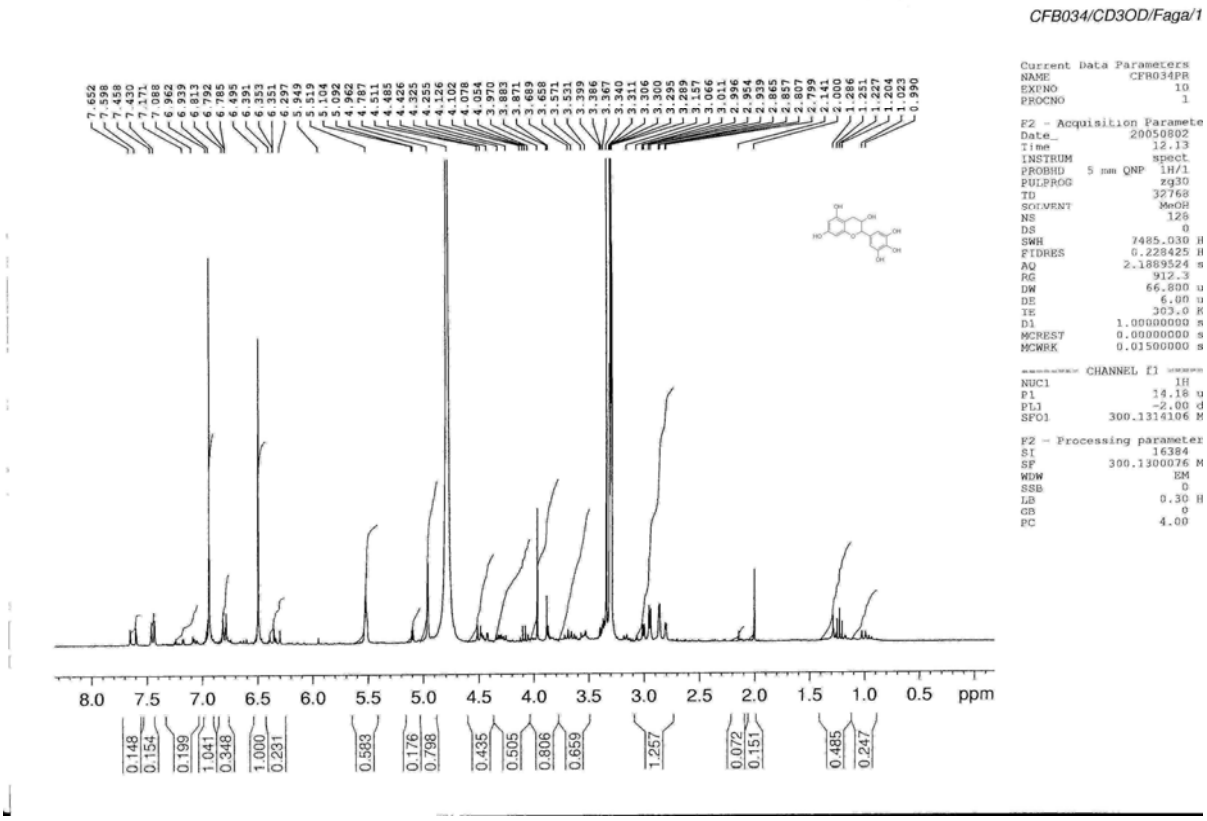
Compound 3 (quercetin-3-glucopyranoside): DEPT



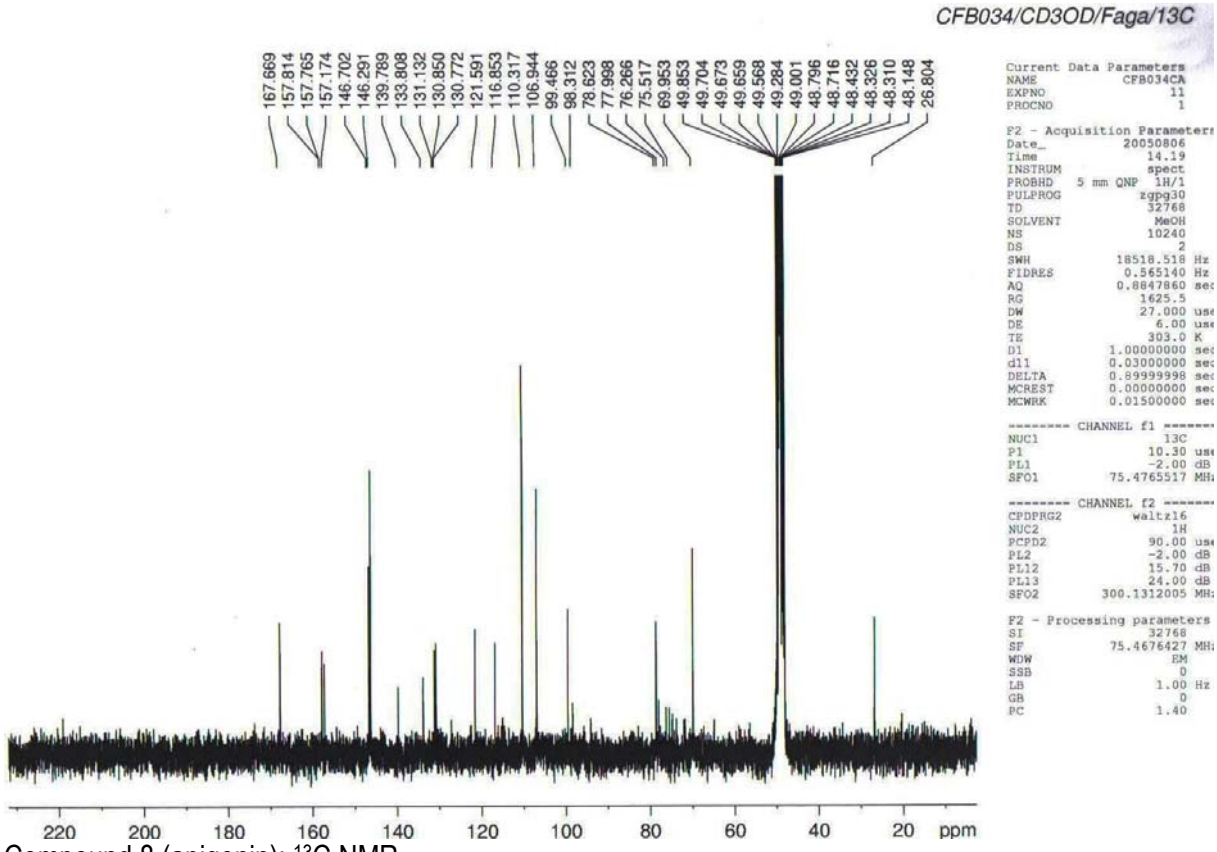
Compound 4 (p-coumaric acid): ^{1}H NMR



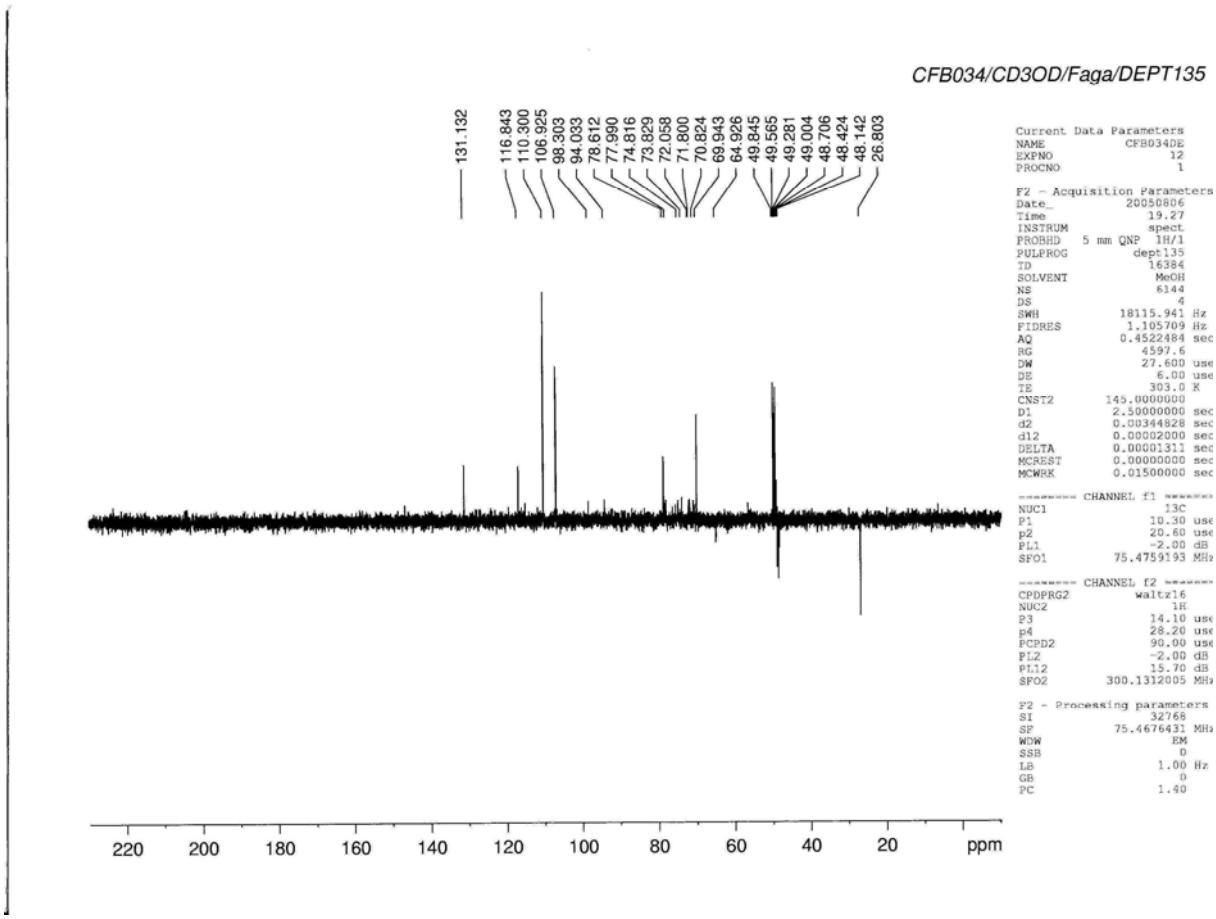
Compound 5 (2,3,8-tri-O-methylellagic acid): ^{1}H NMR



Compound 8 (apigenin): ^{1}H NMR



Compound 8 (apigenin): ^{13}C NMR



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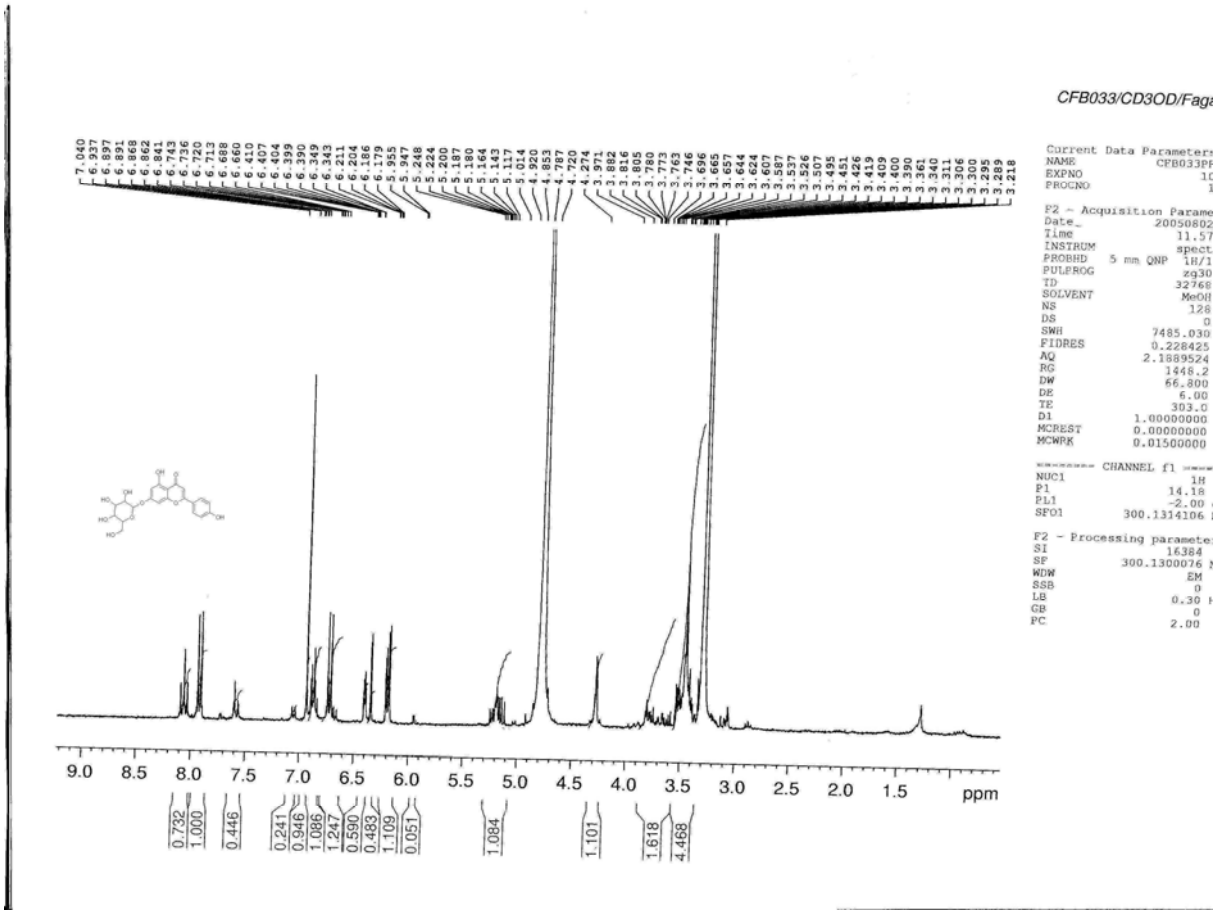

Compound 9 (cosmosiin): ^{1}H NMR