# PHARMACOLOGICAL INVESTIGATION OF SOME TREES USED IN SOUTH AFRICAN TRADITIONAL MEDICINE

by

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# DECLARATION

The experimental work described in this thesis was conducted in the Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal Pietermaritzburg, from June 2003 to August 2005 under the supervision of Professor J. van Staden and Doctor E.E. Elgorashi.

These studies are the result of my own investigations, except where the work of others is acknowledged and have not been submitted in any other form to another University.

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I declare the above statement to be true.

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# ABSTRACT

South Africa is home to a wide diversity of cultural groups, all of which utilize the flora for a variety of purposes. This is true with regard to traditional medicine systems which are similar to those of the rest of Africa south of the Sahara, with diviners (*sangomas*) and herbalists (*inyangas*) as the key health providers. In addition, the Country is rich in plant diversity with some 30 000 species of flowering plants - almost one tenth of the worlds recorded higher plants. This incorporates a large diversity of plants including trees, shrubs, herbs, bulbs and corms.

The adverse effects of traditional medicinal plants and natural products are not well documented in the literature. Recently, many plants used as food or in traditional medicine have been shown to be potentially mutagenic using *in vitro* assays. Thus, the scientific evaluation of traditional medicine and medicinal plants is very important to validate claims made on safety and efficiency of such usages.

After a survey of the available ethnobotanical literature, ten trees used in South African traditional medicine were selected. These species were: *Acacia niolotica* subspecies *kraussiana, Acacia sieberiana, Albizia adianthifolia, Combretum kraussii*, *Faidherbia albida, Ficus sur*, *Prunus africana, Salix mucronata*, *Terminalia sericea* and *Trichilia dregeana*. Plant parts including leaf, root and bark were collected from each of the selected trees (exceptions were *Albizia adianthifolia*, *Faidherbia albida*, *Terminalia sericea* and *Prunus africana*) and extracted using ethyl acetate, ethanol and water individually to ensure the extraction of compounds over a wide range of polarities. The extracts (in total, 78) were screened for antibacterial, anti-inflammatory (COX-1 and COX-2) and anti-acetylcholinesterase activities and investigated for their potential mutagenic effects using the Ames test.

Antibacterial activity was detected using the disc-diffusion and micro-dilution assays. The extracts were tested against Gram-positive bacteria: *Bacillus subtilis, Staphylococcus aureus, Micrococcus luteus* and Gram-negative bacteria: *Escherichia coli* and *Klebsiella pneumoniae*. Of the 78 different plant extracts

tested (final amount of plant material was 1 mg per disc), 84% showed activity against Gram-positive bacteria. From this percentage, 20% also showed activity against Gram-negative bacteria. The best inhibition was observed with ethyl acetate and ethanol root extracts of *Terminalia sericea* against both Gram-positive and Gram-negative bacteria.

In the micro-dilution assay, 55% of the plant extracts showed minimum inhibitory concentration (MIC) values ≤ 1.56 mg/ml against Gram-positive and/or Gram-negative bacteria. The ethyl acetate bark extract of *Acacia sieberiana* and the root and bark ethyl acetate extracts of *Acacia nilotica* inhibited bacterial growth of both Gram-positive and Gram-negative bacteria at concentrations ≤ 0.8 mg/ml. The aqueous leaf extracts of *Acacia sieberiana* had a low MIC value (0.3 mg/ml) against Gram-negative *Kleibsiella pneumoniae* and the ethyl acetate extracts of the root inhibited growth of *Escherichia coli* with an MIC value of 0.1 mg/ml. However, these two extracts showed no activity in the disc-diffusion assay. The MIC values of the neomycin (control) were 0.8 µg/ml and 3.1 µg/ml against *Kleibsiella pneumoniae* and *Escherichia coli* respectively.

In the anti-inflammatory test, 70% of the plant extracts from different plant parts (leaf, root, bark) of the tree investigated showed strong inhibition in both the COX-1 and COX-2 bioassays. The COX-2 inhibitory effects of aqueous extracts were generally lower when compared to the organic solvent extracts. However, water extracts of *Acacia nilotica* was an exception (≥ 90%).

In the acetylcholinesterase inhibitory test, 21% of the plant extracts were active at concentrations ≤ 1 mg/ml using the micro-plate assay. The lowest $IC_{50}$ value was 0.04 mg/ml obtained with an ethanol bark extract of *Combretum kraussii*. The $IC_{50}$ value of the galanthamine (positive control) was 2 µM.

None of the investigated plants showed any potential mutagenic effects with S*almonella typhymurium* strain TA 98 using the Ames test.

Using bioassay-guided fractionation, anolignan B was isolated from the ethyl acetate root extract of *Terminalia sericea*. Antibacterial activity of anolignan B was determined using the microdilution assay. The compound possessed activity

against both Gram-positive and Gram-negative bacteria. The lowest MIC value (3.8 µg/ml) was observed with *Staphylococcus aureus*. MIC value of the neomycin was 1.5 µg/ml.

Anti-inflammatory activity of anolignan B was detected using the COX-1 and COX-2 bioasays. The compound showed strong inhibitory activity against COX-1 and weaker activity against COX-2. The $IC_{50}$ values were 1.5 mM and 7.5 mM with COX-1 and COX-2 respectively. The $IC_{50}$ values of indomethacin were 0.003 mM and 0.186 mM against COX-1 and COX-2 respectively.

There were no potential mutagenic effects showen by anolignan B against *Salmonella typhimurium* strain TA 98 in the Ames test.

Isolation of anolignan B from *Terminalia* species and the antibacterial and anti-inflammatory activities observed in this work have not been reported previously and could therefore be recorded as novel biological activities for this compound. These results also support the idea that the use of ethnobotanical data can provide a valuable short cut by indicating plants with specific uses which might likely be sources of biologically active chemicals.

# CONFERENCE PROCEEDINGS AND PUBLICATIONS FROM THIS THESIS

## A) ORAL PAPERS:

I.M.S. Eldeen, E.E.Elgorashi, J.van Staden. 2003. Screening of some useful trees in South African traditional medicine for anti-bacterial, anti-inflammatory activities and potential mutagenic effects. 5th Annual Meeting of the Research Centre for Plant Growth and Development. School of Biological and Conservation Sciences, Pietermaritzburg.

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## LIST OF ABBREVIATIONS

AChE. ..... Acetylcholinesterase
ATCC ..... American Type Culture Collection
BSA... ..... Bovine serum albumin
CNS... ..... Central Nervous System
COSY ..... Correlation Spectroscopy
COX .. ..... Cyclooxygenase
DTNB ..... Dithiobis-(2-nitrobenzoic acid)
Hmqc. ..... Hetero-nuclear Multiple Quantum Coherence
HRMS ..... High Resolution Mass Spectrometry
Hsqc.. ..... Hetero-nuclear Single Quantum Coherence
ICRAF ..... International Centre for Research in Agroforestry
INT .... ..... *p*-Iodonitrotetrazolium violet
MH..... ..... Mueller-Hinton
MIC.... ..... Minimal Inhibitory Concentration
MS..... ..... Mass Spectrometry
NMR.. ..... Nuclear Magnetic Resonance
NSAIDs ..... Non-steroidal anti-inflammatory drugs
OA..... ..... Osteoarthritis
RA ..... ..... Rheumatoid arthritis
$R_F$ ..... ..... Ratio of fractionation
TLC ... ..... Thin Layer Chromatography
UNEP ..... United Nation Environmental Programme
UV ..... ..... Ultraviolet
VLC ... ..... Vacuum Liquid Chromatography
WHO . ..... World Health Organization
4-NQO..... 4-Nitroquinoline1-oxide

# CHAPTER 1
# LITERATURE REVIEW

## 1.1. TREES AS SUSTAINABLE SOURCES OF NATURAL PRODUCTS

Higher plants accumulate extractable organic substances in quantities sufficient to be economically useful as chemical feedstock such as industrial oils, resins, tannins, saponins, natural rubber, gums, waxes and dyes. These compounds serve as raw materials for various scientific, technological, and commercial applications (BALANDRIN *et al.*, 1985). Plant chemicals are often classified as either primary or secondary compounds; proteins and nucleic acids are generally excluded from this classification (LEA and LEEGOOD, 1999). Primary metabolic compounds are substances widely distributed in nature, occurring in one form or another in virtually all organisms. In higher plants such compounds are often concentrated in seeds and vegetative storage organs and are needed for physiological development because of their role in basic cell metabolism (BALANDRIN *et al.*, 1985; LEA and LEEGOOD, 1999). These compounds are frequently used as industrial raw materials, foods, or food additives such as vegetable oils, fatty acids (used for making soaps and detergents) and carbohydrates such as sucrose, starch, pectin, and cellulose (BALANDRIN *et al.*, 1985).

Secondary metabolic compounds are biosynthetically derived from primary metabolites but are more limited in distribution in the Plant Kingdom. Such compounds have no apparent function in a plant's primary metabolism but often have an important ecological role. They act as pollinator attractants, represent chemical adaptations to environmental stresses, or serve as chemical defenses against microorganisms, insects and other predators (RICE, 1984). These compounds are frequently accumulated by plants in smaller quantities when compared to primary metabolites. In addition, secondary metabolites, in contrast to

primary metabolites, tend to be synthesized in specialized cell types and at distinct developmental stages of the plant, making their extraction and purification difficult (RICE, 1984). As a result, secondary metabolites that are used commercially as biologically active compounds (pharmaceuticals, flavors, fragrances, and pesticides) are generally higher value-lower volume products than primary metabolites (BALANDRIN *et al.*, 1985).

## 1.2. TROPICAL FORESTS AS PHARMACEUTICAL FACTORIES

Plants growing in tropical forest habitats have to develop and survive under intense competition for resources and nutrients. They have also had to develop an extraordinary array of defenses, most of them chemical, to protect themselves from viral diseases, fungal pathogens, insects and mammalian predators (BALANDRIN *et al.*, 1985). The biodiversity of tropical forest plant species, coupled with the chemical diversity found within each plant leads one to the conclusion that tropical plants are perhaps the most valuable sources of new bioactive chemical entities. It has been estimated that between 14% and 28% of the world's higher plants are used medicinally. Only 15% of all angiosperms have been investigated chemically, and about 74% of all pharmacologically active plant-derived components have been discovered following ethnomedicinal leads (ARNOLD *et al.,* 2002). To date the Plant Kingdom has received too little attention as a resource of potentially useful bioactive compounds (BALANDRIN *et al.*, 1985). Tropical forests could therefore, be considered as one of the nature's main potential storehouses of raw materials for both traditional and modern medicine (MYERS, 1992; Di STASI *et al.*, 2002).

Generally plants offer a host of analgesics, antibiotics, heart drugs, enzymes, hormones, diuretics, anti-parasitic compounds, ulcer treatment products, dentifrices, laxatives, dysentery treatments, and anti-coagulants, among many others (BALANDRIN *et al.*, 1985). The total number of plant-derived products in modern pharmacopoeias amount to several thousand, including such well-known

trade products as emetine, scopolamine and pilocarpine. Among the most important materials with which pharmacologists manufacture drugs are alkaloids (complex bio-compounds produced by many categories of plants). Of all biomes, tropical forests contain the highest proportion of alkaloid-bearing plants, and the alkaloid yield of these plants is higher than elsewhere (LEVIN, 1976). Moreover, the capacity of plants to produce alkaloids is under strict genetic control, which means that provided we can safeguard sufficient genetic variability in the wild, genetic engineers may be able to devise improved varieties of alkaloid-producing plants in order to better serve the needs of modern medicine (MYERS, 1992).

Tropical forests provide an abundance of antibiotic and other compounds which have many medicinal applications, including cocaine, reserpine, quinine, ipecac, ephedrine, caffeine, and nicotine (MYERS, 1992). However, it is frequently forgotten that natural products often serve as chemical models for the design and complete synthesis of new drug entities. For instance, meperidine (Demerol), pentazocine (Talwin), and propoxyphene (Darvon) are all synthetic analgesic drugs for which opiates such as morphine and codeine were the models. Aspirin is an example of a simple derivative of the naturally occurring salicylic acid originally derived from willows (*Salix* sp.) (MYERS, 1992). Some other plant species from tropical forests have been identified as containing anti-fertility compounds. Extracts of these plant species offer special promise for safer and more effective contraceptive pills, suitable for both males and females (MYERS, 1992). A leading candidate is the greenheart tree that flourishes in the forests of Guyana, where women of several tribes use the nuts from these trees as a reliable contraceptive. Among other promising plants are forest components from Haiti, Bangladesh, Papua New Guinea, Fiji, Cameroon, Madagascar, Colombia, and Brazil (COUNTE, 1996).

## 1.3. PROSPECTS FOR DISCOVERING NEW BIOACTIVE COMPOUNDS FROM PLANTS

The serious and systematic study of the chemistry and biological properties of natural products has been a major factor in the development of modern synthetic organic chemistry (KINGHORN and BALANDRIN, 1993). In spite of the advances in extraction technology, separation science (chromatographic techniques), and analytical and spectroscopic instrumentation, still little is known about the secondary metabolism of most of the higher plant species. This is especially true in the case of tropical floras. Although the tropics contain most of the world's plant species, more than half of these are still unknown and most have never been surveyed for chemical constituents (BALANDRIN *et al.*, 1985; COUNTE, 1996).

Prior to the 1950s, drugs were often developed from natural resources. However, they were discovered in a haphazard and time-intensive manner. From the 1950s to the 1980s, pharmaceutical companies found that synthetic chemicals and genetic engineering were more profitable than screening plants. Therefore, natural resource research slowed. Recently, however, the drug discovery process has reverted to more traditional methods.

The usefulness of natural resources in drugs is derived in part from the fact that tropical plants often have strong chemical defenses to repel predators (MYERS, 1992). Although the simpler of these compounds have been synthesized in the laboratory, pharmaceutical companies are realizing that it would be impossible to replicate all of nature's more complex compounds (KINGHORN and BALANDRIN, 1993). By using new bioassays and technology to test natural products, the identification of certain natural resources as prospective drugs has become easier and more accurate. Thus, the drug discovery process has moved back into the rain forest and in doing so, increased the number of reasons to exploit the environment.

## 1.4. ETHNOBOTANY

The word ethnobotany has proved to be a difficult term to define. It was stated firstly by JOHN HARSHBERGER in 1895 as "the use of plants by aboriginal peoples" (SCHULTES and REIS, 1995). Recently, ethnobotany has been defined as "folk botany", or the description of the various methods by which local people utilize plants. Even though two or more groups of people share the same environment, each group will have its own traditional methods of utilizing a particular plant. This is true in Africa where the large Continent has a variety of ethnic groups north to south, and an equally great variety of vegetation from bare deserts to tropical rain forests (PRANCE *et al.*, 1987). As a result of considerable attention that focused not only on how plants are used, but also on how they are perceived and managed, ethnobotany is considered to encompass all studies which concern the mutual relationships between plants and traditional people (COTTON, 1996).

One of the most important uses of plants is as producers of unique and diverse chemical compounds (SCHULTES and REIS, 1995). The investigation of the use of plant-derived chemicals has been an important part of ethnobotany in seeking to document indigenous knowledge about biological effects of plants and to develop new chemical products ranging from sugar substitutes to pharmaceuticals. However, one of the challenges facing ethnobotanists is how to integrate the chemical, pharmacological, and ecological studies of plant-derived remedies into ethnomedical studies that focus on the social and symbolic functions of folk medicine (PRANCE *et al.*, 1987).

Eventually, ethnobotany was accepted as an interdisciplinary science. One of the important aspects for its future is the maintenance of closer working relationships between different disciplines such as botany, chemistry and pharmacology (PRANCE *et al*, 1987).

### 1.4.1. SOCIO- ETHNOBOTANY

Socio-ethnobotany is one of the newer developments in ethnobotany dealing with the question of how indigenous people can be compensated for sharing their ethnobotanical knowledge with the industrialized world (SCHULTES and REIS, 1995). Given their extensive range of knowledge of medicinal plants, indigenous people have traditionally been the ultimate resource for retrieving this information for purposes of application to modern medicine (BUTLER, 2005).

Large numbers of people, especially among the black population in Africa, consult traditional healers. However, the knowledge of medicinal plants use is largely undocumented and could be lost since it is passed on by word of mouth from one generation to the next. When an old traditional doctor dies, it means that a complete "library" is lost (NEUWINGER, 1996). Therefore, ethnobotanists will contribute through documentation of such knowledge to the development and evaluation of different chemical products ranging from sugar to pharmaceutical substitutes (SCHULTES and REIS, 1995).

### 1.4.2. ETHNOPHARMACOLOGY

The relationship between man and plants has been very close throughout the development of all civilizations. Many higher plants produce economically important organic compounds such as oils, resins, tannins, rubber, gums, waxes, dyes, flavors and fragrances, pesticides and pharmaceuticals (HOSTETTMANN and LEA, 1987). Although most of the pharmacological classes of drugs include natural product prototypes, higher plants as one of the major sources of such products have been much less surveyed for chemical or biologically–active constituents (FARNSWORTH, 1984). Consequently higher plants remain as unique "templates" that can serve as starting points for synthetic analogues and as interesting tools which could be applied to achieve a better understanding of

biological processes (HOSTETTMANN and LEA, 1987). Therefore, screening of plant extracts followed by bioassay-guided fractionation for isolation of pure constituents becomes a successful strategy for investigation of medicinal properties of higher plants (HOSTETTMANN and LEA, 1987).

## 1.5. TRADITIONAL MEDICINE

Traditional medicine is a term loosely used to describe ancient and culture-bound health practices that existed before the application of science to health matters in official and scientific medicine (FARNSWORTH, 1988). It is essentially plant-based. Plants have long provided mankind with a source of medicinal agents, with natural products once serving as the source of all drugs. Depending on plants as the source of medicine is prevalent in developing countries where traditional medicine plays a major role in health care (SHALE *et al*, 1999). Over the last few years physiochemical and pharmacological investigations have been carried out on a number of frequently used plants to establish a scientific rational for their usage in traditional medicine (CUNNINGHAM, 1988).

A large proportion of the population of developing countries use traditional medicine as their primary health care resource due to the high cost of western pharmaceuticals and health care (TAYLOR *et al.*, 2001). Traditional medicine is also more acceptable from cultural and spiritual perspectives (GRACE *et al.*, 2002). The rational use of traditional medicine often relies on mysticism and intangible forces such as witchcraft with some aspects based on spiritual and moral principles. This is true especially in African, Chinese and Japanese traditional medicine (GEDDES and GROSSET, 1997). However, the Chinese and Japanese traditional medicine systems are further advanced in many respects than African traditional medicine.

Traditional Chinese medicine has a long history dating back several thousand years. Its origin is associated with the legendary testing of many herbs

for their medicinal properties by the folk hero, Shen Nong (CHEN, 1995; HUANG *et al.*, 2004). With the development of theory and clinical practice, China has accumulated a rich body of empirical knowledge about the use of medicinal plants for the treatment of various diseases. Many active components have been discovered from Chinese medical products for anticancer, anti-bacterial, anti-fungal, anti-viral and promoting the immunological function activity such as camptothecin, taxol, vinblastine, vincristine, tripterygium (HUANG *et al.*, 2004). Moreover, the clinical medicinal experience of more than 2000 years provides an integrated system for diagnosis and treatment. It opens short cuts for discovering new drugs from natural products (HUANG *et al.*, 2004).

The widely used Japanese herbal medicine system known as Kampo has attracted world wide attention. Kampo is a general term for the unique system of traditional medicine developed in Japan based on Chinese origins (IKEGAMI *et al.*, 2004). Although Kampo has much to offer to modern medicine, researchers need to be aware of the fact that Kampo has its own distinctive methodology and that it is not appropriate simply to take Kampo formulae and indiscriminately introduce them into modern medical practice (IKEGAMI *et al.*, 2004). The most important aspect contributing to the widespread use of Kampo is its efficiency in treatment of certain disorders and disease syndromes. It is today still serving many of the health needs of the Japanese people and is used in parallel with modern western medical treatment (IKEGAMI *et al.*, 2004).

In Africa traditional medicine belongs to what is classified as personal systems. In these approaches supernatural causes ascribed to angry deities, ghosts, ancestors and witches predominate, in contrast with the naturalistic systems where illness is explained in impersonal, systemic terms (IWU, 1994). In the African system of medicine, healing is associated with the utilization of human energy, the environment and the cosmic balance of natural forces as tools. Therefore, plants play a participatory role in healing, while a healer's power is determined not only by the number of efficacious herbs used but also the

magnitude of his/her understanding of the natural laws and his/her ability to utilize them for the benefit of the patients. Treatment is therefore not limited, to the sterile use of different leaves, roots, fruits, barks, grasses but can include various objects such as minerals, insects, bones, feathers, shells, eggs, powders and smoke from different burned objects for the cure and/or prevention of diseases. For example, if a sick person is given a leaf infusion to drink, he or she should believe in the organic properties of the plant and in the magical or spiritual forces imbibed by nature in all living things, ancestors, spirits and gods (IWU, 1994).

The African Continent is still a promising source of leads for the development of new therapeutic agents due to a long history of human civilization and centuries-old record of the use of plants as medicine. Many scientific groups are exploring the African flora for new compounds with pharmacological activities. Such efforts have lead to the isolation of several biological active molecules that are at various stages of development as pharmaceuticals (PRANCE, 1994). Recently, some major Medical Aid Schemes have included traditional healing in their cover. This increases the need for scientific evaluation of the methods and products used by traditional healers (FARNSWORTH, 1988; WHO, 2004).

### 1.5.1. TRADITIONAL MEDICINE IN SOUTH AFRICA

South Africa is home to a wide diversity of cultural groups, all of which utilize the flora for a variety of purposes (ARNOLD *et al.,* 2002). This is true with regard to traditional medicine systems which are similar to those of the rest of Africa south of the Sahara, with diviners (*sangomas*) and herbalists (*inyangas*) as the key health providers (JÄGER and VAN STADEN., 2000). In addition, the country is rich in plant diversity with some 30 000 species of flowering plants - almost one tenth of the worlds recorded higher plants. Of these 80% are endemic (FENNELL *et al.*, 2004). A large part of the population uses some form of traditional medicine. This incorporates a large diversity of plants including trees,

shrubs, herbs, bulbs and corms. Surveys have estimated that 20 000 tonnes of plant material are traded annually in the South African province of KwaZulu-Natal alone. These plants are consumed by more than 28 million people who consult one of the estimated 200 000 traditional healers, especially in rural areas (ARNOLD *et al.,* 2002; VERSCHAEVE *et al.*, 2004). The exponential growth of the South African population in the later half of the Twentieth Century has led to an almost exponential increase in the demand for medicinal plants (FENNELL *et al.*, 2004).

## 1.6. INFECTIOUS DISEASES

Infectious diseases are the leading cause of death worldwide (BANDOW *et al.*, 2003). This situation is very common in tropical countries where insect-borne diseases are common and in underdeveloped areas where standards of hygiene and nutrition are low. Although their incidence has been markedly decreased by public health measures and their ill effects reduced by specific methods of treatment, infections are still major causes of time lost in all economical fields. The morbidity due to any particular parasite varies enormously in different parts of the world owing to geographical and environmental factors which influence the host: parasite relationship. The main groups of organisms involved are metazoa, protozoa, fungi, bacteria and viruses. Infections due to metazoa and protozoa are encountered mainly in tropical areas. Thus, malaria, amoebic dysentery, sleeping sickness, kalazar and helminthes infections are common diseases in tropical Africa, Central and South America and large parts of Asia. The range of bacterial diseases is very large due to the wide range of bacterial species causing diseases. *Streptococcus* and *Staphylococcus* infections are widespread and produce similar diseases in every part of the inhabited world. Some bacterial infections may be acute such as diphtheria and tetanus; others are chronic such as tuberculosis, syphilis and leprosy (BANDOW *et al.*, 2003).

### 1.6.1. CHEMOTHERAPY OF INFECTIOUS DISEASES

The concept of chemotherapy is almost as old as the science of bacteriology. Since the later years of the Nineteenth Century attempts were made to treat infections such as tuberculosis by the injection of other organisms or their products. Antibiotics are substances that inhibit or suppress the growth and activity of microorganisms. They are divided into two main categories: the first includes antibiotics, mainly with a general use. Their therapeutic values are well established. Examples in this category include penicillin, erythromycin, streptomycin, the tetracycline and chloramphenicol. The second category consists of those antibiotics in restricted use either because the indications for them are more limited or they are deliberately kept in reserve. There antibiotics can be classified as antibiotics mainly effective against Gram-positive bacteria, antibiotics mainly effective against Gram-negative bacteria, antibiotics effective against both Gram-positive and Gram-negative bacteria and antibiotics effective against fungal infections (GLAZER and NIKAIDO, 1994).

### 1.6.1. MULTI-DRUG RESISTANCES

The clinical efficacy of many existing antibiotics is now being threatened by the emergence of multi-drug resistant pathogens. Multiple-drug resistance has become a real issue in pharmacotherapeutics as there are an increasing number of diseases which are exhibiting various levels of drug resistance, including bacterial infections. Plants used in traditional medicine have the potential to provide pharmacologically active natural products with compounds that act on novel molecular targets that circumvent the established resistance mechanisms (CLEMENTS *et al.*, 2002; ELGORASHI and VAN STADEN, 2004). Higher plants represent a bountiful source of new prototypic bioactive agents (KINGHORN and BALANDRIN, 1993). The ecological rationale is that plants produce compounds which confer an antimicrobial defense against microbes in their own environment

and makes further exploitation of this source for antibiotic leads worthwhile (GIBBONS, 2003).

## 1.7. INFLAMMATION DISORDER

The use of medicinal substances to relieve pain, fever and inflammation dates back to ancient Egypt where a decoction of dried leaves of myrtle was applied to the back and abdomen of patients (BALANDRIN *et al.,* 1993).

Inflammation is a disorder involving localized increases in the number of leukocytes and a variety of complex mediator molecules. The inhibitions of numerous rate-limiting processes could be important in the successful treatment of an inflammatory disorder (MANTRI and WITIAK, 1994). Prostaglandins are ubiquitous substances that initiate and modulate cell and tissue responses involved in physiological process such as platelet aggregation and inflammation. Their biosynthesis has also been implicated in the pathophysiology of cardiovascular diseases, cancer and inflammatory diseases (SMITH and DeWITT, 1995). Synthesis of prostaglandins is a key factor in the inflammation process. The primary enzyme responsible for prostaglandin synthesis is cyclooxygenase which occurs in three isoforms, COX-1, COX-2 and the recently discovered "COX-3" (DAVIES *et al.*, 2004). COX-1 is responsible for the production of prostanoids that maintain mucosal blood flow, promote mucose secretion, inhibit neutrophil adherence and maintain renal blood flow (WALLACE and CHIN, 1997). COX-2 is effectively absent in healthy tissues and is induced in migratory and other cells by pro-inflammatory agents such as cytokines, mitogens and endotoxins under pathological conditions such as inflammation (MITCHELL *et al.*, 1994). Furthermore, experimental evidence has shown that COX-2 promotes survival of colonic adenomas and colonic cancer. In addition, its expression is associated with the deposition of beta- amyloid protein in Alzheimer's disease (LIPSKY, 1999). COX-3 is a newly discovered COX variant enzyme. Some researchers believe that the term COX-3 is confusing as it has not yet been confirmed as an independent

third COX gene (DAVIES *et al.*, 2004). Furthermore, differences between COX-1 and COX-2 are both genetic and pharmacological while a sequence of the proposed COX-3 was attributed to a clone of the COX-1 gene with alternative splicing, resulting in the retention of nitron-1 in the mature RNA (DAVIES *et al.*, 2004). The proposed COX-3 was found to be enzymatically active in synthesizing prostaglandins from arachidonic acid and possesses approximately 20% of the activity of COX-1 when expressed recombinantely in insect cells (SIMMONS, 2003).

The principal pharmacological effect of non-steroidal anti-inflammatory drugs (NSAIDs) is due to their ability to inhibit prostaglandin synthesis by binding reversibly and irreversibly to the enzyme. Their major toxicities are due to their ability to block synthesis of the housekeeping prostaglandin as a result of inhibition of COX-1 (PORTANOVA *et al.*, 1996). The selective anti-inflammatory drugs such as celecoxib and valdecoxib and rofecoxib (removed from the market recently), are highly selective for COX-2 (375-fold and 1000-fold respectively) in comparison with the traditional NSAIDs (LANE, 1997; SIMON, 1998). Scientific evidence has shown that the proposed COX-3 could be inhibited selectively by using analgesic/antipyretic drugs such as acetaminophen, phenacetin and antipyrine. It is potently inhibited by some of the non-steroidal anti-inflammatory drugs. Thus, inhibition of the proposed COX-3 could represent a primary central mechanism by which these drugs decrease pain and possibly fever (CHANDRASEKHARAN et al., 2002). Celecoxib has been approved for osteoarthritis (OA) and rheumatoid arthritis (RA). Rofecoxib was removed from the market early in 2004 as it was confirmed that rofecoxib increased the risk of serious cardiovascular events including heart attacks and strokes, blood clots, ulceration and kidney damage. It is important that the results of clinical studies with one drug in a given class does not necessarily apply to other drugs in the same class (FOOD and DRUG ADMINSTRATION, 2004).

## 1.8. THE CENTRAL NERVOUS SYSTEM AND COGNITIVE FUNCTION

The human brain is made up of billions of nerve cells called neurons that share information with one another through neurotransmitters. Neurotransmitters are chemicals that transmit information across the junction (synapse) which separates one neuron from another neuron or muscle. Neurotransmitters are stored in the neuron's bulbous end (axon). When an electrical impulse traveling along the nerve reaches the axon, the neurotransmitter is released and travels across the synapse, either prompting or inhibiting continued electrical impulses along the nerve (FOYE *et al.*, 1995; BOEVE *et al.*, 1999). There are more than 300 known neurotransmitters. These include chemicals such as acetylcholine, norepinephrine, adenosine triphosphate, endorphins and gases (LEWIS and ELVIN-LEWIS, 2003). Neurotransmitters transmit information within the brain and from the brain to all parts of the body. It is estimated that a typical neuron has up to 15,000 synapses (ALZHEIMER'S DISEASE, 2002).

Neurotransmitters carrying messages bind to specific receptor sites on the receiving end of dendrites of adjacent neurons. Receptors are proteins (molecules that determine the physical and chemical traits of cells and organisms) that recognize and bind to chemical messengers from other cells. When the receptors are activated, they open channels into the receiving nerve cell's interior or start other processes that determine what the receiving nerve cell will do. Some neurotransmitters inhibit nerve cell function making a neuron less likely to act. Other neurotransmitters stimulate nerve cells by priming the neuron to become active or send a message. In this way, signals travel back and forth across the brain in a fraction of a second (ALZHEIMER'S DISEASE, 2002). Millions of signals flash through the brain all the time (Figure 1.1).

Acetylcholine is an organic molecule liberated at nerve endings as a neurotransmitter. It is produced by the synthetic enzyme choline acetyltransferase that uses acetyl co-enzyme A and choline as substrates for the formation of

acetylcholine in specific cells known as cholinergic neurons. Dietary choline and phosphatidylcholine serve as the sources of free choline for acetylcholine synthesis. Upon release, acetylcholine is metabolized into choline and acetate by acetylcholinesterase and other non-specific esterases. Acetylcholine release can be excitatory or inhibitory depending on the type of tissue and the nature of the receptor with which it interacts. The principle role of acetylcholinesterase (AChE) is termination of impulse transmission at cholinergic synapses by rapid hydrolysis of acetylcholine (BAR-ON *et al.*, 2002). Due to the essential biological role of acetylcholinesterase in the mediation of vertebrate and invertebrate nervous transmission, it has been medicinally targeted in treatments for Alzheimer's disease, myasthenia gravis, glaucoma, pesticides, and snake venom toxins (PERSONENI *et al.*, 2001; SUSSMAN *et al.*, 2003).

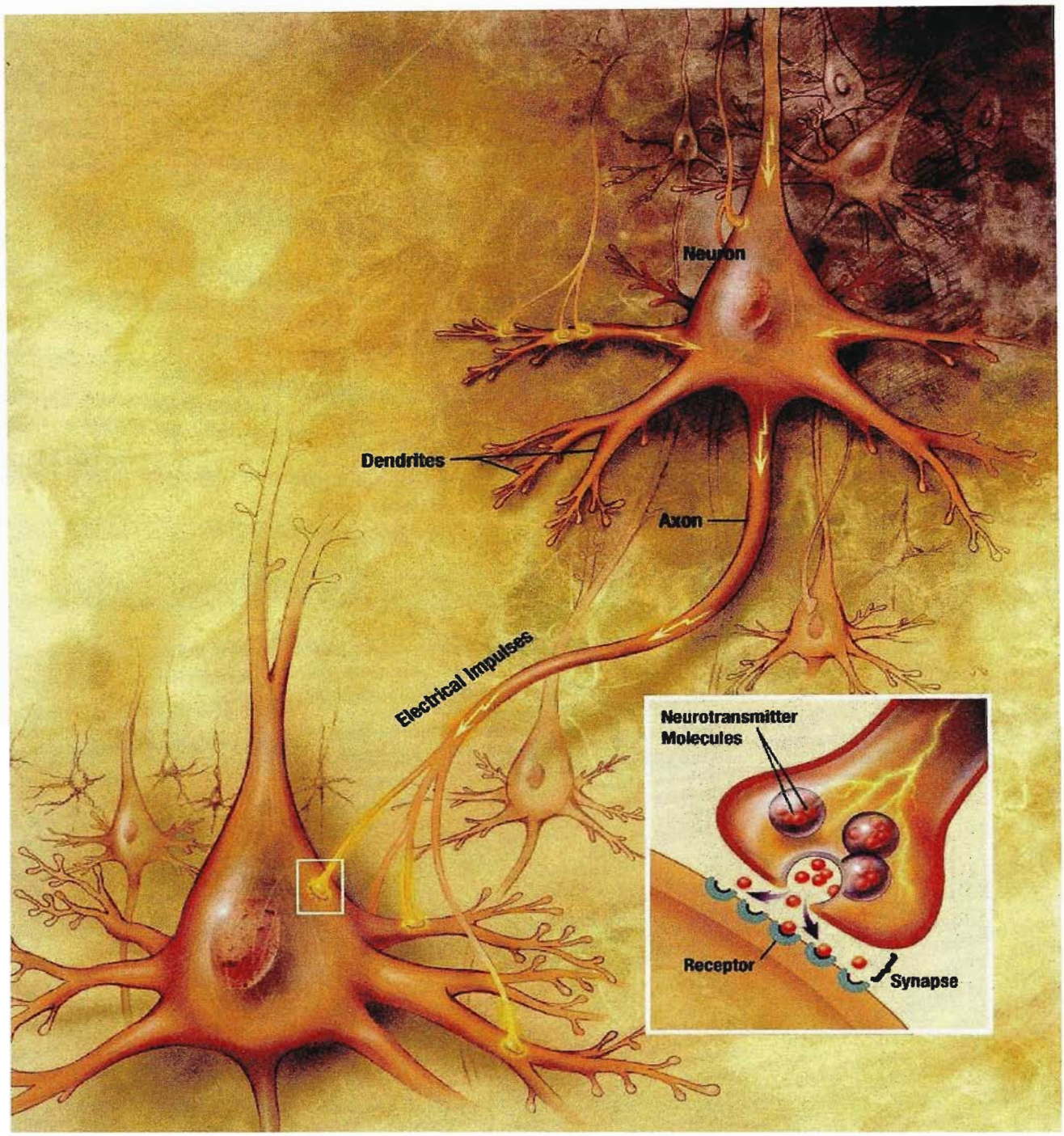


**Figure 1.1** Schematic presentation of neuron and neurotransmitter action

Source: ALZHEIMER'S DISEASE (2002)

### 1.8.1. ACETYLCHOLINESTERASE AND ALZHEIMER'S DISEASE

Alzheimer's disease was first discovered by Alois Alzheimer in 1906. It is an irreversible, progressive brain disorder that occurs gradually and results in memory loss, unusual behaviors, personality changes and a decline in thinking abilities (HOWES and HOUGHTON, 2003; SUSSMAN *et al.*, 2003). These losses are related to the death of brain cells and the breakdown of the connections between them. The course of this disease varies from person to person, as does the rate of decline. Alzheimer's disease advances by stages, from early, mild forgetfulness to severe dementia which is the loss of mental function. In most people with Alzheimer's disease, symptoms first appear after the age of sixty (ALZHEIMER'S DISEASE, 2002).

The pathological features that have been identified in the central nervous system in Alzheimer's disease are senile plaques and neurofibrillary tangles, inflammatory processes, neurotransmitter disturbances and insufficient cholinergic functions. This cholinergic insufficiency has been correlated with the severity of Alzheimer's disease (FOYE *et al.*, 1995; GREENBLATT *et al.*, 1999; PERSONENI *et al.*, 2001; HOWES and HOUGHTON, 2003). This serves as the rationale for use of cholinergic agonists and cholinesterase inhibitors for the symptomatic treatment of Alzheimer's disease in its early stages (ENZ *et al.*, 1993; GREENBLATT *et al.*, 1999). The survival of nerve cells in the brain depends on the healthy functioning of several processes all working in harmony. These processes involve nerve cell activities related to intercellular communication, cellular metabolism and cell and tissue repair. Some of these nerve cells are involved in thinking, learning, remembering and planning (FOYE *et al.*, 1995; HOWES and HOUGHTON, 2003).

### 1.8.2. ANTI-CHOLINESTERASE DRUGS

Approaches to enhance cholinergic function have included stimulation of cholinergic receptors or prolonging the availability of acetylcholine released into the neuronal synaptic cleft by inhibiting the acetylcholinesterase enzyme. Traditionally, plants have been used to enhance cognitive function and to alleviate other symptoms associated with Alzheimer's disease (HOWES and HOUGHTON, 2003). Acetylcholinesterase inhibitor drugs are effective in delaying neurocognitive decline in people with mild to moderate severity of Alzheimer's disease. However, their effect is only to alleviate symptoms and they do not achieve any permanent improvement (LEWIS and ELVIN-LEWIS, 2003). The use of anti-inflammatory agents has also been suggested to delay the progression of Alzheimer's disease. Several studies have shown that patient treated with non-steroidal anti-inflammatory drugs may have a reduced risk of developing Alzheimer's disease. The synthetic drug tacrine was the first acetylcholinesterase inhibitor to be licensed but its routine use has been restricted due to its hepatotoxicity. The use of tacrine has been eclipsed by newer drugs such as rivastigmine, adonepezil and recently galanthamine (GREENBLATT *et al.*, 1999; FAJEMISIN, 2002; HOWES and HOUGHTON, 2003).

Galanthamine is an alkaloid found in the bulbs and flowers of the common snowdrop (*Galanthus nivalis*) and several other members of the Amaryllidaceae. The active ingredient was discovered accidentally during the early 1950's. The plant extracts were used initially to treat nerve pain and poliomyelitis. Based on the knowledge of the action of galanthamine on both peripheral and the central nervous system, many countries in Eastern Europe had acknowledged its use in the treatment of myasthenia gravis and muscular dystrophy, residual poliomyelitis paralysis symptoms, trigeminal neuralgia and other forms of neuritides (HEINRICH and LEE TEOH, 2004). As a natural compound, galanthamine has been tested for use in anesthesiology and for treatment of many human ailments, from facial nerve paralysis to schizophrenia (GREENBLATT *et al.*, 1999). The double action of

galanthamine as both an anti-cholinesterase and a nicotinic activator has rendered it a promising candidate drug for the treatment of Alzheimer's disease (GREENBLATT *et al.*, 1999). Currently, galanthamine has been approved for the treatment of Alzheimer's disease in the United States, many European countries and some Asian countries (HEINRICH and LEE TEOH, 2004).

## 1.9. TOXICITY OF NATURAL PRODUCTS

Although plant extracts have been used in the treatment of diseases, utilizing knowledge that had been accumulated over centuries, recent scientific research has shown the presence of potentially toxic and mutagenic substances in some useful medicinal plants (VERSCHAEVE *et al.*, 2004). It has been demonstrated that several potential side effects such as allergic reactions, cramps, diarrhoea, fever, gastrointestinal disturbances, headaches, hematuria and vomiting may be experienced when administering some types of traditional medicine (IKEGAMI *et al.*, 2004).

### 1.9.1. MUTAGENICITY AS AN ASPECT OF TOXICITY

The genetic material of a cell beside DNA molecules and chromosomes includes all structures that play a role in correct transmission of the genetic information of the cell to its daughter cells (ROCHE FACETS, 2005). Substances that have a toxic effect on the genetic material of cells and can thus alter an organism's genome, are said to be genotoxic (ROCHE FACETS, 2005). Alterations in the structure and function of DNA are believed to play a crucial role in the production of cancer by chemicals (CLIVE *et al.*, 1983).

Carcinogenesis is a multistage process that may take years to evolve and a number of different factors influence the progression from a normally functioning cell to an invasive neoplastic tumor (INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY, 1985). A carcinogen is defined as an agent that significantly increases the frequency of malignant neoplasm in a population. Carcinogens may

be physical, chemical, or biological agents (CLIVE *et al.*, 1983). The complex mechanisms by which chemicals induce malignant are not fully understood. However, evidence suggests the occurrence of four major stages following an adequate exposure of a mammal (including man) to a chemical carcinogen: (a) transport of the chemical from the site of entry into the body and, in many cases, metabolic modification of the chemical (principally in the liver) to a more reactive form; (b) interaction of the molecule or its reactive metabolite with the molecular target in the cell; (c) expression of the DNA damage as a potentially carcinogenic lesion; and (d) progression, influenced by modifying factor(s) and proliferation to form a malignant tumor (INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY, 1985).

All chemicals that produce DNA damage leading to mutations or cancer are described as genotoxic (ROCHE FACETS, 2005). Such substances increase the error rate in the reduplication of the genome and induce mutations by damaging the organism's DNA (ROCHE FACETS, 2005). Mutations in the germ cells are passed on to the organism's offspring and can cause congenital or hereditary defects. Mutations in somatic cells can result in cell death, an increased risk of diseases and even cancer (WITT and BISHOP, 1996). As it has been known for many years that some chemicals can cause cancer in man, attention has been given recently to the possibility that chemicals may also produce mutations in human germ cells thus influencing the frequency of genetic or heritable diseases (INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY, 1985).

In addition, there are many compounds that occur naturally which are known to be mutagenic and/or carcinogenic (UNITED NATION ENVIRONMENTAL PROGRAMME, 2000). Although there is no definitive evidence that exposure to such chemicals is responsible for any of the known human genetic disorders, experimental evidence from other mammals have shown that some of these chemicals can produce both chromosomal and gene mutations of the type that are

associated with human genetic diseases (GAO *et al.*, 2005).There is little direct evidence to suggest that man is any less susceptible than other mammals to the effects of exposure to mutagenic chemicals (ROCHE FACETS, 2005).

As thousands of chemicals, including pharmaceutical products, domestic and food chemicals, pesticides and petroleum products are present in the environment and new chemicals are being introduced each year, it is very important to investigate potential mutagenic effects of chemicals to which people are exposed (INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY, 1996).

## 1.10. SUMMARY

Higher plants are remarkable in their ability to produce a vast array of diverse metabolites varying in chemical complexity and biological activity. These products have historically served as templates for the development of many important classes of drugs. Efforts to develop new, clinically effective pharmaceutical agents have traditionally relied on many approaches including strategies to discover new pharmaceutical agents that do not share the same toxicities, cross resistance or mechanism of action as existing agents (KINGHORN and BALANDRIN, 1993).

Nearly one-fifth of the world's population suffers from a number of diseases which still lack curative agents. Rural populations are more disposed to traditional ways of treatment because of its easy availability and cheaper cost (McCHESNEY, 1993; SHALE *et al.*, 1999). Traditional healers in Africa use large numbers of plants to treat different kinds of diseases such as bacterial diseases, inflammation and cognitive disorders. Although cognitive disorders and Alzheimer's disease are not very common in Africa compare with the industrialized communities, an ethnopharmacological approach may be useful in providing leads to identify promising compounds that can be developed as new drugs for the treatment of such diseases.

The adverse effects of traditional medicinal plants and natural products are not well documented in the literature. Recently, many plants used as food or in traditional medicine have been shown to be potentially mutagenic using *in vitro* assays (VERSCHAEVE *et al.*, 2004). This raises concern about the potential mutagenic hazards resulting from the long-term use of such plants (INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY, 1996).

The use of ethnobotanical data can provide a valuable short cut in identification of potential new promising compounds by indicating plants with specific traditional uses which might likely be sources of biologically active constituents. There are numerous chemically uninvestigated plants with reported ethnomedicinal uses. These native uses could serve as pre-existing clinical tests (GENTRY, 1993).

## 1.11. AIMS AND OBJECTIVES

During a review of traditional medicinal literature the importance of trees and their secondary metabolites was clearly evident. Insufficient attention has been given to trees as sustainable sources of promising bioactive compounds that may serve as chemical models for the design and synthesis of new drug entities. In this study, attention was given to trees which were selected on the basis of reported biological activities in the literature. The tree species studied together with their ethnobotanical uses in South African traditional medicine are outlined in Chapter 2.

The aims of this study were to:

1. Screen the plant extracts for;
    - •Antibacterial activity;
    - •Anti-inflammatory activity; and
    - •Anti-acetylcholinesterase activity;
2. Investigate potential mutagenic effects of the extracts; and where possible
3. Isolate and identify some of the active compounds.

# CHAPTER 2
# MORPHOPLOGICAL DESCRIPTIONS AND TRADITIONAL USES OF TREE SPECIES STUDIED

## 2.1. SELECTION OF THE TREE SPECIES STUDIED

The selection of the tree species in this study was based on their uses in African traditional medicine with special reference to South Africa (Table 2.1). This information was gleaned from the available literature. The major sources were: The Medicinal and Poisonous Plants of Southern and Eastern Africa (WATT and BREYER-BRANDWIJK, 1962); Zulu Medicinal Plants: An inventory (HUTCHINGS *et al.*, 1996); Medicinal Plants of South Africa (VAN WYK *et al.*, 1997); Poisonous Plants of South Africa (VAN WYK *et al.*, 2002); Photographic Guide to Trees of Southern Africa (VAN WYK *et al.,* 2000); and Making the Most of Indigenous Trees (VENTER and VENTER, 1996).

## 2.2. BOTANICAL NAMES AND MORPHOLOGICAL DESCRIPTIONS

### 2.2.1. *Acacia nilotica* (L.) Willd. ex Del. subsp. *kraussiana* (BENTH.) Brenan.

Voucher specimen: Eldeen 8 (Figure 2.1).

Common names: Black thorn tree; Readheart tree; Scented thorn tree.

Zulu names: *ubobe; ubombo; umnqawe, umqawe.*

Habitat: Widespread species, usually found on heavy soils throughout the bushveld in South Africa (VAN WYK *et al.*, 2002).

**Table 2.1** Botanical name, family, voucher specimen and traditional uses of tree species screened for biological activity

| Family and botanical name | Synonyms | Traditional uses and References |
|---|---|---|
| Mimosaceae<br>*Acacia nilotica* (L.) Willd. ex Del. subsp.*kraussiana* (BENTH.) Brenan | *Acacia arabica* (Lam.) Willd. var. *kraussiana* Benth.<br>*A. benthamii* Rochebr.<br>*A. nilotica* subsp. *subalata* Brenan. | Bark decoctions are taken by the Zulu for dry coughs in South Africa (WATT and BREYER-BRANDWIJK, 1962). Roots are used in various parts of Africa for respiratory ailments including tuberculosis (SAWHNEY *et al.*, 1978; KHAN *et al.*, 1980). Bark decoctions are used for eye complaints in Tanzania, Senegal, Liberia and Guinea (SAWHNEY *et al.*, 1978). |
| *Acacia sieberiana* Dc. var.*woodii* (Burtt Davy) Keay & Brenan | *A. amboensis* Schinz.<br>*A. lasiopetala* Burtt Davy.<br>*A. sieberiana* DC. var. *vermoesenii* (De Wild.) & Brenan Keay | In South Africa, bark infusions are administered for pain in the back (HUTCHINGS *et al.*, 1996). Root infusions are used as anti-septics in Zimbabwe (GELFAND *et al.*, 1985; HUTCHINGS *et al.*, 1996). Leaves are used in Nigeria for inflammation (HUTCHINGS *et al.*, 1996). |
| *Faidherbia albida* (Del.) A. Chev. | *Acacia albida* Del.<br>*A. mossambicensis* Bolle.<br>*A. gyrocarpa* Hochst.<br>*A.saccharata* Benth. | In South Africa, bark is traditionally used to prevent stomach disorders (MABOGO, 1990). Bark decoctions are taken for diarrhoea in Tanzania and Namibia (HUTCHINGS *et al.*, 1996). In West Africa, bark and leaves are used for colds, ophthalmia and diarrhoea. (WATT and BREYER-BRANDWIJK, 1962). |

**Table 2.1** (*continued*)

| Family and botanical name | Synonyms | Traditional uses and References |
|---|---|---|
| Fabaceae<br>*Albizia adianthifolia* (Schumach.) W. F. Wight. | *A. fastigiata* (E. Mey.) *B.* Oliv.<br>*Inga fastigiata* (E. Mey.) Oliv.<br>*Mimosa adianthifolia* Schumach.<br>*Zygia fastigiata* E. Mey. | In South Africa, roots are pounded in a little cold water and used to make drops for inflammation of eyes (HUTCHINGS *et al.*, 1996). Powdered bark is taken as snuff for headaches, stomachache and as purgatives (PUJOL, 1990). In Liberia, unspecified parts are used for internal parasites while leaves are used for dysentery in Madagascar and Kenya (JENKINS, 1987). In Sudan a root decoction is used for fever (ELDEEN, 2001). |
| Rosaceae<br>*Prunus africana* (Hook.f.) Kalkm. | *Pygeum africanum* Hook.f. | South African healers use the bark as an effective drug for chest pain, diarrhoea and fever (PUJOL, 1990). Extracts of powdered bark is traditionally drunk as tea for genito-urinary complaints, allergies, inflammation, kidney diseases, malaria, fever and stomachache among many other uses in folkloric African medicine (INTERNATIONAL CENTRE FOR RESEARCH IN AGROFORESTRY, 2004). |
| Combretaceae<br>*Terminalia sericea* Burchel ex Dc. | *Terminalia phanerophlebia* Engl. Driels. | Root decoctions are mainly used as a traditional Tswana remedy for stomachache and diarrhoea. The bark is also used for treating diabetes and wounds (HUTCHINGS *et al.*, 1996). In Tanzania, roots are used for stomachache and bilharzia. In Zambia, the Mankoya use the root for swollen painful eyes (NEUWINGER, 1996). |

**Table 2.1** (*continued*)

| Family and botanical name | Synonyms | Traditional uses and References |
|---|---|---|
| *Combretum kraussii* Hochst. | *C. nelsonii* DÜmmer. | In South Africa fresh or dry leaves are used as dressings for wounds. The inert bark infusions are administered orally for various stomachache complaints and fever in various parts of Africa (NEUWINGER, 1996). |
| Moraceae *Ficus sur* Forssk. | *F. capensis* Thunb. *F. mallotocarpa* Warb. | Root and bark decoctions are adminstered for pulmonary tuberculosis (WATT and BREYER-BRANDWIJK, 1962). Roots are used for colic, bark is used for influenza in Tanzania, while the root is used for skin diseases in Sudan (HUTCHINGS *et al.*, 1996). |
| Meliaceae *Trichilia dregeana* Sond. | | An infusion of the bark is taken for hot pain in the back, stomachache and fever (WATT and BREYER-BRANDWIJK, 1962). In tropical Africa, the root is used as a remedy for fever and as a purgative (WATT and BREYER-BRANDWIJK, 1962). |
| Salicaceae *Salix mucronata* Thunb. | | In South Africa the plant parts are traditionally used to treat rheumatism and fever (ROOD, 1994). Europeans in South Africa use a decoction of the leaves for muscular rheumatism and rheumatic fever (WATT and BREYER-BRANDWIJK, 1962). |

Usually a single stemmed tree 5-6 m high, but often branching from low down to form a compact rounded to flattened crown. Bark is rough and deeply fissured on older branches and stems. Leaves are twice–compound, hairy, up to 50 x 25 mm, with 4-11 pairs of pinnate and 7-36 pairs of leaflets. Spines paired from a common base; slightly curved backwards. Flowers are deep yellow and in round heads. Fruits are non-splitting pods, scented constricted between the seeds (VAN WYK *et al.*, 2002).

**Figure 2.1** *Acacia nilotica* (L.) Willd. ex Del. subsp.*kraussiana* (BENTH.) Brenan.

### 2.2.2. *Acacia sieberiana* Dc. var.*woodii* (Burtt Davy) Keay& Brenan.

Voucher specimen: Eldeen 1 (Figure 2.2).

Common names: Natal camel thorn; Paper bark acacia.

Zulu names: *umkhamba; umkhambati, umkhaya.*

Habitat: Medium altitude bushveld areas, and grassland, often in deep soil, along rivers (CARR, 1976).

One of the biggest umbrella–shaped acacias of Africa, reaching up to 25 m with a diameter of 60 cm. The bark is strongly fissured, yellow to cream-colored on young trees and twigs, scaly on old trees. Thorns are auxiliary and in pairs, straight, white, up to 12 cm long. Leaves are bipinnate, rachis 6-12 cm long, 10-25 pairs of pinnae with 15-50 pairs of leaflets on each. Flowers are cream-colored or light-yellow globose heads (diameter 1.5 cm) on peduncles, groups of 3-6 at the axils. Pods are thick, woody glabrous, brown or reddish brown at maturity, shining like varnish, straight or slightly curved (CARR, 1976).

**Figure 2.2** *Acacia sieberiana* Dc. var.*woodii* (Burtt Davy) Keay & Brenan.

### 2.2.3 *Albizia adianthifolia* (Schumach.) W. F. Wight.

Voucher specimen: Eldeen 6 (Figure 2.3).

Common names: Flat crown; Platkroon

Zulu names: *igowane, umbhelebhele, umgadankawu; umgadenkawu, usolo.*

Habitat: Woodland, usually associated with coastal and montane forest. It occurs along the eastern parts of South Africa and is also widely distributed in tropical Africa (VAN WYK *et al.*, 2002).

The flat-crown is a large tree up to 20 m in height with a characteristic flattened and spreading crown. The smooth or rough bark is grey to yellowish–brown. Leaves are alternate, bipinnately compound; leaflets are usually rectangular in shape. Pods are oblong and thin with prominent veins (VAN WYK *et al.*, 2000).

**Figure 2.3** *Albizia adianthifolia* (Schumach.) W. F. Wight.

### 2.2.4. *Combretum kraussii* Hochst.

Voucher specimen: Eldeen 9 (Figure 2.4).

Common names: Bosvaderslandswilgerb room; Forest bush willow.

Zulu names: *umdubu* (*-wehlathi*).

Habitat: Forest, often as conopy constituents and in associated woodland (VAN WYK *et al.*, 2000).

Medium-sized to large deciduous or semi-deciduous tree, multi-stemmed or with a single trunk that branches low down; crown is rounded; foliage dark green; with reddish autumn colors; bark is smooth grey; leaves are opposite simple, elliptic to obovate and hairless. Flowers are in auxiliary spikes and produce 4-winged fruits. (VAN WYK *et al.*, 2000).

**Figure 2.4** *Combretum kraussii* Hochst.

### 2.2.5. *Faidherbia albida* (Del.) A. Chev.

Voucher specimen: Eldeen 2 (Figure 2.5).

Common names: Ana tree; Anaboom.

Zulu names: *umhlalankwazi; umkhaya-womfula.*

Habitat: Bushveld, usually on alluvial floodplains, river banks and along pans, swamps or dry watercourses with a high water table (CARR,1976).

Large tree, attaining heights of 15-25 m occasionally. It remains leafless for most of the wet season, only coming into leaf in the early dry season. Leaves are alternate, bipinnately compound. Spines stipule, straight, and in axillary's pairs. Diameter of the tree may reach more than 1 m. Branches are characteristically light grey to whitish. Bark is deeply fissured with age (CARR, 1976).

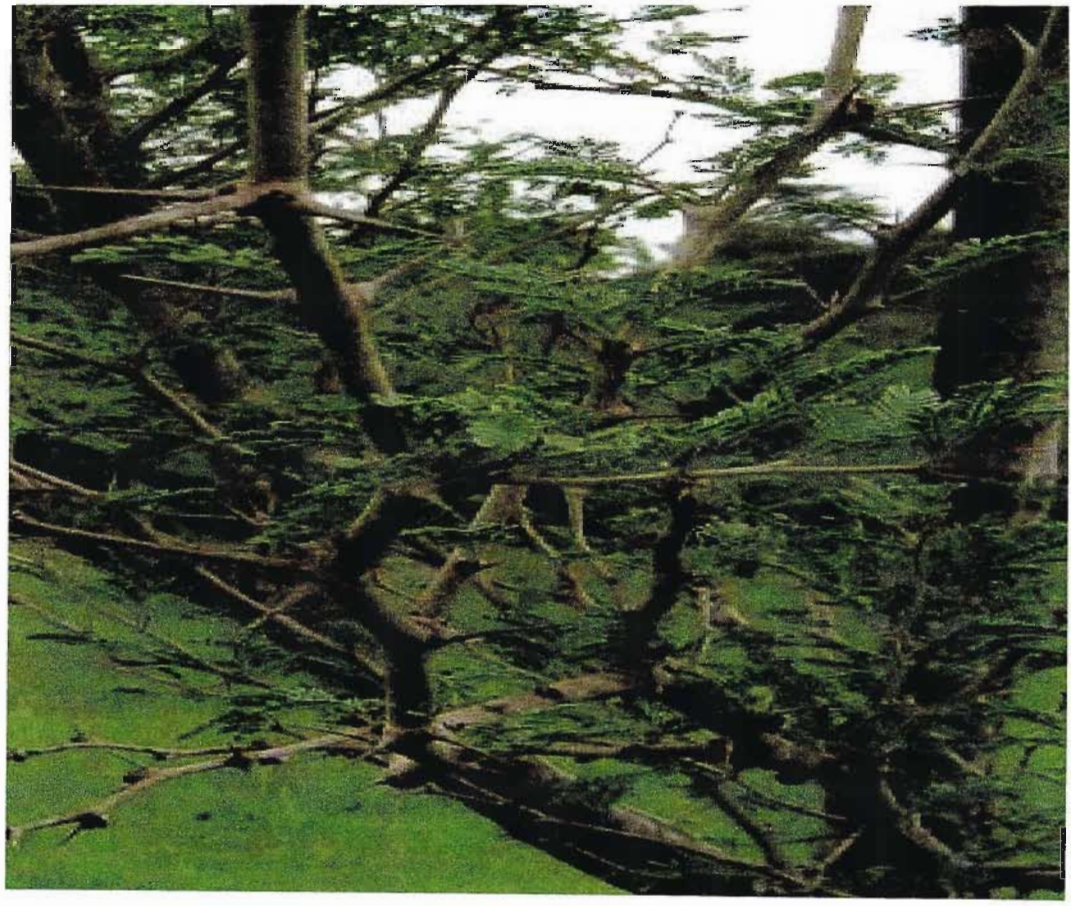

**Figure 2.5** *Faidherbia albida* (Del.) A. Chev.

### 2.2.6. *Ficus sur* Forssk.

Voucher specimen: Eldeen 4 (Figure 2.6).
Common names: Bosvy, Bush/ cape fig; Grootvy; Komaan; Suurvy.
Zulu names: *ingobozweni; intombi-kayibhinci-umkhiwane.*
Habitat: Forest and bushveld, usually along streams and in moist ravines (VAN WYK *et al.*, 2000).

An evergreen tree up to 35 m tall with a dense rounded crown with milky latex in all parts. Bark smooth and light grey. Leaves are copper-colored when young. They are simple, margins wavy, irregularly toothed and furrowed on upper surface. Stipules are thinly texured, grow as long as 8-10 mm and envelope the growth tips. Fruits found in large clusters on long, branched stalks on the old wood (Figure 2.7) or some times on the roots (VENTER and VENTER, 1996).

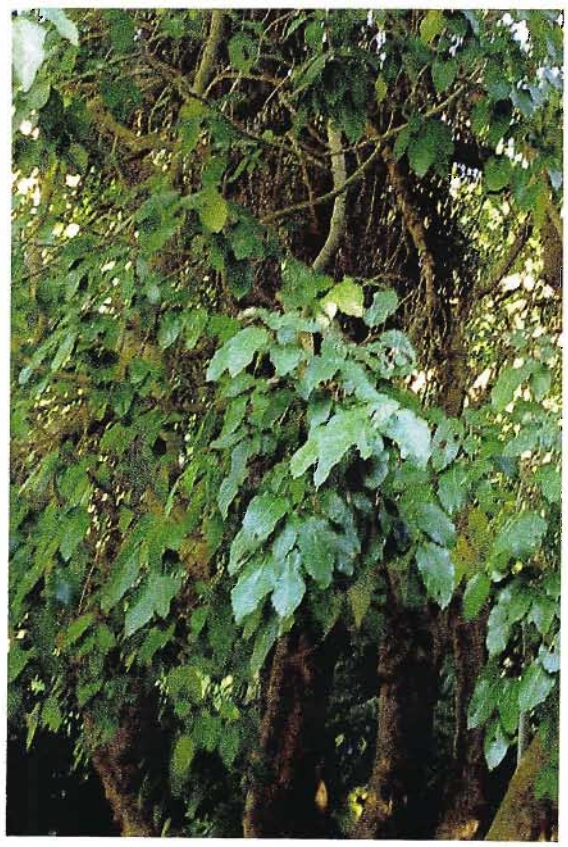

**Figure 2.6** *Ficus sur* Forssk.

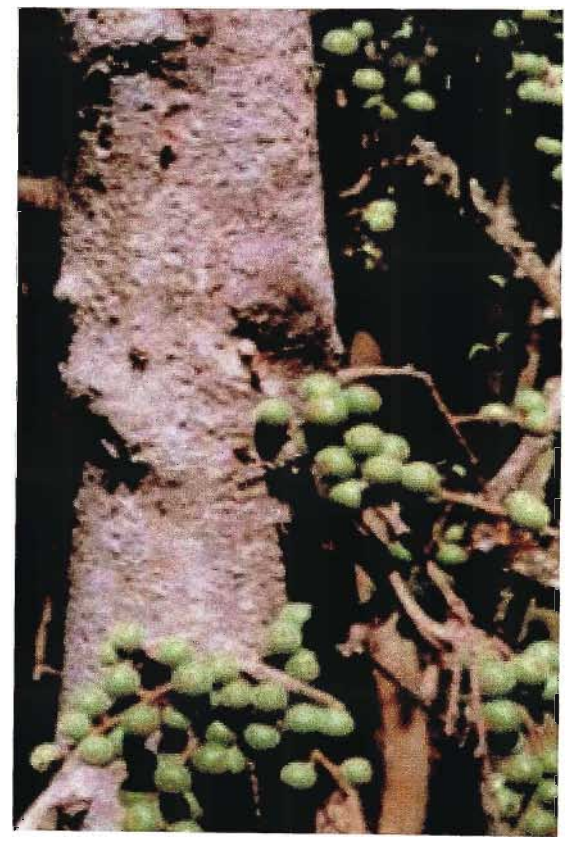

**Figure 2.7** Fruiting branches of *Ficus sur.*

### 2.2.7. *Prunus africana* (Hook.f.) Kalkm.

Voucher specimen: Eldeen 10 (Figure 2.8).

Common names: Bitter almond; Betteramandelboom; Nuweamandelhout.

Zulu names: *inyazangoma-elimnyama; umdumezulu; umdumizula.*

Habitat: The tree is found mainly in afromontane forests along the mistbelt regions of South Africa and occurs further north into Tropical Africa (VON BREITENBACH, 1986).

Tall forest tree which may reach a height of 30 m often with buttress roots. Bark is coarse, with a dark brown and black colour. Flowers white small in elongated clusters followed by reddish-brown berries (VAN WYK *et al.*, 2000).

**Figure 2.8** *Prunus africana* (Hook.f.) Kalkm.

### 2.2.8 *Salix mucronata* Thunb.

Voucher specimen: Eldeen 7 (Figure 2.9).

Common names: Safsaf wilger

Zulu names: umNyezane

Habitat: Along streams and rivers. It grows over large parts of South Africa specially in the eastern and northern east coast of the Northern Province (VAN WYK *et al.*, 2000).

Tree or shrub of up to 12 m in height. Bark is dark brown, rough and fissured. Leaves are alternate, simple and lanceolate. Flowers are small in short male and female spikes, yellowish-green. Fruits are small brown capsules (VENTER and VENTER, 1996).

**Figure 2.9** *Salix mucronata* Thunb.

### 2.2.9 *Terminalia sericea* Burchel ex Dc.

Voucher specimen: Eldeen 3 (Figure 2.10).

Common names: Lebombo cluster-leaf/terminalia, Silver cluster-leaf.

Zulu names: *amangwe(-amhlophe, -amnyama)*.

Habitat: Bushveld, on deep sandy soils, often in dense stands (VAN WYK *et al.*, 2000).

Small to medium–sized tree of about 5-8 m in height. Bark is grey to pale brown and coarsely fissured. Leaves are characteristically silver-haired and crowded near the branch tips. Flowers are cream-coloured with unpleasant smell (VAN WYK *et al.*, 2000).

**Figure 2.10** *Terminalia sericea* Burchel ex Dc.

### 2.2.10. *Trichilia dregeana* Sond.

Voucher specimen: Eldeen 5 (Figure 2.11).

Common names: Bosrooiessenhout, Natal forest mahogany

Zulu names: *ixolo; umathunzini; umkhuhla, umkhuhlu.*

Habitat: Coastal and montane forest. The species is widely distributed in the north eastern parts of South Africa along the coastal parts of KwaZulu-Natal (VAN WYK *et al.*, 2000).

Evergreen tree about 10 m in height with a dense rounded crown and smooth bark. Leaves are alternate, imparipinnate. Tips sharply pointed, essentially hairless below; side veins 7-12 pairs widely spaced (VAN WYK *et al.*, 2000).

**Figure 2.11** *Trichilia dregeana* Sond.

## 2.3. COLLECTION OF PLANT MATERIAL

Plant material was collected in August 2003 from the National Botanical Gardens Pietermaritzburg and Botanical Garden of the University of KwaZulu-Natal Pietermaritzburg. Voucher specimens were deposited in the Herbarium of the University of KwaZulu-Natal. The plant material was dried in an oven at 50°C for 7 days and finely ground to powders using an A 10 Analysis Mill (Janke and Kunkel, I KA®- Labortechnik). The powdered material was stored in brown paper bags at room temperature until extraction.

## 2.4. EXTRACTION

The powdered plant material (10 g) was extracted individually using ethyl acetate, ethanol and water (10 ml/g) respectively by sonication for 1 h and then filtered through Whatman No.1 filter paper. The filtrates were air-dried under a fan at room temperature.

# CHAPTER 3
# PHARMACOLOGICAL SCREENING OF PLANT EXTRACTS OBTAINED FROM TREES USED IN SOUTH AFRICAN TRADITIONAL MEDICINE

## 3.1. INTRODUCTION

Screening implies a large number of assays, often, though not always, tuned so as to indicate simply the presence or absence of a biological response (O'NEILL and LEWIS, 1993). The target may be a particular cell type, an enzyme believed to be a key regulator of a specific biosynthetic pathway, a receptor–ligand interaction or a molecule involved in gene transcription (HOSTETTMANN and LEA, 1987).

Screening of plant extracts as a source of biologically–active compounds has become increasingly important for both traditional and systematic medicine. Plant extracts contain hundreds of discrete compounds with pharmacological potential (GIBBONS, 2003). Therefore the search to find new bioactive chemicals has renewed interest in plant species world wide and has stimulated a long-overdue renaissance of activities in the areas of plant natural product chemistry, pharmacognosy, and ethnomedical sciences (O'NEILL and LEWIS, 1993).

The chances of succeeding in identifying biological active compounds are increased by testing large numbers of samples across a wide range of target screenings. An alternative is to use a selection strategy that relies on ethnobotanical information to identify particular species reported to induce significant pharmacological effects (KINGHORN and BALANDRIN, 1993). In screening programmes, any plant part or combination of parts are acceptable. However, many plants concentrate certain secondary metabolites in specific organs. Therefore, in terms of anticipated chemical diversity, it is quite legitimate to screen samples from different parts of the same plant (KINGHORN and BALANDRIN, 1993).

The tree species selected in this study are reported to have different classes of chemical compounds such as alkaloids, triterpenoids, acetylhistamine and flavonoids. Some of these types or other related compounds are reported to have many medicinal applications including anti-infectious, anti-inflammatory and neurocognitive drugs (Chapter one). Pharmacological screening of these trees followed by bioactivity-guided fractionation might lead to the isolation and identification of promising bioactive compounds.

Traditional healers in South Africa and Africa generally use parts or different combinations of plant parts from the selected plants-as well as many other plants- for treating different diseases such as respiratory ailments, stomach disorders, colds, kidney diseases, allergies, fever, malaria, wounds and inflammations (Chapter two). These ailments can be classified as infections and/or inflammation disorders. As it is not possible to cover all diseases in this study, the extracts from selected trees were screened for antibacterial, anti-inflammatory and anti-cholinesterase activities and then tested for potential mutagenic effects. These assays have been established and well developed in the laboratory of the Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal.

## 3. 2. MATERIALS AND METHODS

### 3.2.1. ANTIBACTERIAL ACTIVITY

Antibacterial assays can be classified into three categories: diffusion, dilution and bioautographic assays. Disc-diffusion is an assay based essentially on the placing of filter paper discs impregnated with the antibiotic or plant extract on the surface of agar immediately after inoculation (RIOS *et al.*, 1988). This technique was originally standardized by BAUTER *et al.* (1966) and subsequently modified in the report of the World Health Organization (1977). For minimum inhibitory concentrations (MIC) of active plant extracts, a very sensitive 96-well micro-titer plate

method described by ELOFF (1998) can be used (TAYLOR *et al.*, 2001). Bioautography combines thin layer chromatography (TLC) with bioassay *in situ*, therefore allowing the localization of active compounds (Chapter four).

### i) DISC-DIFFUSION ASSAY FOR DETERMINATION OF ANTIBACTERIAL ACTIVITY OF PLANT EXTRACTS OBTAINED FROM TREES USED IN SOUTH AFRICAN TRADITIONAL MEDICINE

The disc-diffusion assay (RASOANAIVO and RATSIMAMANGA-URVERG, 1993) was used to determine antibacterial activity of the plant extracts using three Gram-positive bacteria: *Bacillus subtilis* (ATCC No. 6051), *Staphylococcus aureus* (ATCC No.12600), *Micrococcus luteus* (ATCC No. 4698) and two Gram-negative bacteria: *Escherichia coli* (ATCC No. 11775) and *Kleibsiella pneumoniae* (ATCC No. 13883).

The test organisms were grown in Mueller-Hinton (MH) broth (2.1 g/100 ml distilled water) overnight in a water bath at 37 °C. The overnight broth was subcultured and incubated again for 6 h in a water bath at 37 °C. Base plates were prepared by pouring 10 ml of autoclaved Mueller-Hinton (MH) agar (Biolab) into sterile Petri dishes (9 cm) and allowing them to settle. Molten autoclaved MH agar that had been kept at 48 °C was inoculated with a broth culture ($10^6$-$10^8$ /ml) of the test organism and then poured over the base plate. Ten µl of plant extract (100 mg/ml) were applied to each filter paper disc (6 mm diameter, Whatman No. 3) to give a final amount of 1 mg plant extract per disc. The discs were air dried and placed on top of the agar layer. Four replicates of each extract were tested (4 discs per plate) with a neomycin disc (0.2 mg/ml) as a reference. The plates were then incubated for 18 h at room temperature. Antibacterial activity is expressed as a ratio of the inhibition zone produced by the plant extract to the inhibition zone produced by the neomycin reference.

### ii) MICRO-DILUTION ASSAY FOR DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC) OF PLANT EXTRACTS OBTAINED FROM TREES USED IN SOUTH AFRICAN TRADITIONAL MEDICINE

The serial dilution technique described by ELOFF (1998) using 96-well micro plates to determine the MIC of extracts was used. Two ml cultures of four bacterial strains: two Gram-positive bacteria: *Bacillus subtilis and Staphylococcus aureus* and two Gram-negative bacteria: *Escherichia coli* and *Kleibsiella pneumoniae* were prepared and placed in a water bath overnight at 37 °C. The overnight cultures were diluted with sterile MH broth (1 ml bacteria /50 ml MH broth). The extracts were resuspended to a concentration of 50 mg/ml with sterile distilled water for the aqueous extracts and with ethanol for the organic extracts. For each of the four bacteria, 100 µl of redissolved extract were two-fold serially diluted with 100 µl sterile distilled water in a sterile 96-well microtitre plate (Greiner Labortechnik). A similar two-fold serial dilution of neomycin (Sigma) (0.1 mg/ml) was used as a positive control against each bacterium. One hundred µl of each bacterial culture were added to each well. The plates were covered and incubated overnight at 37 °C. To indicate bacterial growth, 50 µl of 0.2 mg/ml *p*-iodonitrotetrazolium violet (INT) was added to each well and the plates were incubated at 37 °C for 30 min. Bacterial growth in the wells was indicated by a red colour, whereas a clear well indicated inhibition by the tested substances. The MIC was taken as the lowest concentration of plant extract to elicit an inhibitory effect on the growth of the test bacterium.

## 3.2.2. ANTI-INFLAMMATORY ACTIVITY OF PLANT EXTRACTS OBTAINED FROM TREES USED IN SOUTH AFRICAN TRADITIONAL MEDICINE

As indicated in Chapter one, prostaglandin synthesis-inhibition can be evaluated using the cyclooxygenase (COX) assays. This is an example of a mechanism-based assay that uses subcellular structure to detect inhibitors of inflammation (HAMBURGER and HOSTETTMANN, 1991). This bioassay tests for the inhibition of the COX enzyme by crude plant extracts or pure subtances. The assay

coupled to bioassay guided fractionation of plant extracts has led to the characterization of several new inhibitors of the COX enzymes (NOREEN *et al.*, 1998).

Inhibition of prostaglandin biosynthesis by the plant extracts was investigated using both the COX-1 and COX-2 assays. The basic protocol is the same for both assays, allowing a comparison of the inhibitory effects of the extracts on the two enzymes.

### i) **CYCLOOXYGENASE-1 (COX-1) BIOASSAY**

The COX-1 bioassay was performed according to the modified method of WHITE and GLASSMAN (1974) as modified by JÄGER *et al.* (1996). The COX-1 enzyme (isolated from ram seminal vesicles) (Sigma-Aldrich) was activiated with co-factor solution and pre-incubated on ice for 5 min. Co-factor soultion was prepared for COX-1 by adding 3 mg L-adrenalin, 3 mg reduced glutathione and 100 µl of hematin to 10 ml 0.1 M Tris buffer, pH 8.0. Residues of plants extracts were resuspended to a concentration of 10 mg/ml using water and ethanol for aqueous and non aqueous extracts respectively. Sixty µl of the enzyme/co-factor solution were added to 20 µl of plant extract (20 µl of aqueous extract solution, 2.5 µl of ethanolic extract solutions + 17.5 µl water) and pre-incubated for 5 min at room temperature. Twenty µl of [$^{14}$C] arachidonic acid (Sigma-Aldrich) were added to the tested samples and incubated at 37 °C for 10 min. The final concenration of each of the tested plant material was 250 µg/ml. After incubation, the reaction was terminated by adding 10 µl 2N HCl. Four µl of a 0.2 mg/ml carrier solution of unlabelled prostaglandins ($PGE_2$:$PGF_{2\alpha}$ 1:1; v/v) were added. Prostaglandins were separated from the unmetabolised arachidonic acid by silica gel column chromatography. The samples were loaded onto individual pasture pipette packed with silica gel (particle size 0.063-0.200 mm, Merck). Four ml of hexane:dioxane:acetic acid (350:150:1 v/v) mixture were added, one ml at a time, to the column to elute the arachidonic acid. The prostaglandins were then eluted by the addition of 3 ml ethyl acetate:methanol (425:75 v/v) mixture and received in

scintillation vials individually. Four ml scintillation solution were added to each vial containing the prostaglandins and radioactivity was measured using a Beckman L S 6000LL scitillation counter.

### ii) CYCLOOXYGENASE-2 (COX-2) BIOASSAY

The COX-2 assay described by NOREEN *et al.* (1998) with slight modifications (ZSCHOCKE and VAN STADEN, 2000) was followed. Human recombinant COX-2 containing a six histidine sequence near the N-terminus isolated from a Baculovirus over expression system in S*f* 21 cells was used (Sigma-Aldrich). The same protocol as for COX-1 was followed except that in the preparation of co-factor solution. Co-factor solution for COX–2 was prepared by adding 6 mg L-adrenalin, 3 mg reduced glutathione and 100 µl of hematin to10 ml 0.1 M Tris buffer, pH 8.0. (hematin is prepared to give a final concentration of 1 µM in the assay).

In each test assay, four controls were run. Two were background in which the enzyme was inactivated with HCl before the addition of [$^{14}$C]arachidonic acid and two were solvent blanks (untreated sample). Indomethacin, a Non-Steroidal Anti-Inflammatory Drug was included in each test assay as a standard (5 µM for the COX-1 assay and 200 µM for the COX-2 assay). Both assays were performed in duplicate with double determinations for each extract per assay. Results were expressed as mean ± standard deviation.

### iii) CALCULATION OF INHIBITION

The percentage inhibition by the tested solutions was obtained by analysing the amount of radioactivity in these solutions relative to that present in the solvent blank for both COX-1 and COX-2 as follows:

$$\text{Inhibition (\%)} = \left[1 - \left[\frac{\text{radioactivity}_{\text{sample}} - \text{radioactivity}_{\text{background}}}{\text{radioactivity}_{\text{blank}} - \text{radioactivity}_{\text{background}}}\right]\right] \times 100$$

### 3.2.3. ACETYLCHOLINESTERASE ENZYME (AChE) INHIBITORY ACTIVITY OF PLANT EXTRACTS OBTAINED FROM TREES USED IN SOUTH AFRICAN TRADITIONAL MEDICINE

Inhibition of acetylcholinesterase activity by plant extracts was investigated using the thin layer chromatography (TLC) and microplate assays. These assays are based on Ellman's method (ELLMAN *et al.*, 1961) with modifications. Both are principally based on a photometric method for determining acetylcholinesterase activity of tissue extracts, homogenates and cell suspensions. The enzyme activity is measured by observing the increase of a yellow colour produced from thiocholine when it reacts with the dithiobisnitrobenzoate ion. It is based on coupling of the following reactions:

Acetylthiocholine $\xrightarrow{\text{AChE enzyme}}$ thiocholine + acetate

Thiocholine + dithiobisnitrobenzoate $\longrightarrow$ yellow colour

The latter reaction is rapid and the assay is extremely sensitive and can be used with either small amounts of tissue or low concentrations of enzyme (ELLMAN *et al.*, 1961; RHEE *et al.*, 2001; RHEE *et al.*, 2003).

#### i) PREPARATION OF REAGENTS

Acetylthiocholine iodide (ATCl), acetylcholinesterase (AChE) obtained from electric eels (type VI-S lypophilized powder), 5, 5-dithiobis [2-nitrobenzoic acid] (DTNB), and galanthamine were obtained from Sigma-Aldrich. Methanol was purchased from Saarchem, Merck Laboratory Supplies (PTY) LTD (South Africa). The following buffers were used; Bufer A: 50 mM Tris-HCl, pH 8; Buffer B: 50 mM Tris-HCl, pH 8 containing 0.1% bovine serum albumin (BSA); Buffer C: 50 mM Tris-HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2.6H_2O$.

## ii) DETERMINATION OF ANTI-CHOLINESTERASE ENZYME ACTIVITY OF THE PLANT EXTRACTS USING THIN LAYER CHROMATOGRAPHY (TLC) PLATES

The TLC plate assay for determination of AChE activity was based on the method described by ELLMAN *et al.* (1961). Ten µl aliquots of plant extracts (resuspended to a concentration of 10 mg/ml using ethanol) and 5 µl 0.2 mM galanthamine hydrobromide were spotted on three TLC plates (20X20 cm silica gel 60 $F_{254}$, 1 mm) and developed in chloroform:methanol (8:2 v/v). One of the TLC plates was used as a reference, the second was used for the assay and the third was used for false positive test. Enzyme inhibitory activities of the extracts was detected by spraying the substrate (1 mM ATCI in buffer A), dye (1 mM DTNB in buffer A) and enzyme (3 U/ml AChE in buffer A) onto the TLC plate (assay). After 2-5 min a yellow background appeared with white spots for AChE inhibiting compounds. These were observed and recorded within 5 min. A false-positive reaction was determined by mixing the enzyme with the substrate before spraying onto the second TLC plate. Plant extracts with a false positive activity were obseved as a white spot on the yellow background of the TLC plate (false positive).

## iii) DETERMINATION OF THE MINIMUM INHIBITORY CONCENTRATION (MIC) OF ANTI-CHOLINESTERASE ENZYME ACTIVITY OF THE PLANT EXTRACTS

The microplate assay utilised an Opsys MR 96-well microplate reader. To each well, 25 µl of 15 mM ATCI in water, 125 µl of 3 mM DTNB in buffer C, 50 µl of buffer B and 25 µl of a serially diluted (two fold) plant extract (initial concentration of 1 mg/ml) were added. The absorbance was measured five times at 405 nm at 45 s intervals (background). After this, 25 µl of 0.2 U/ml of enzyme was added and the absorbance measured eight times at 45 s intervals at 405 nm (sample reading). Serially diluted galanthamine (25 µl) was used as a positive control (initial concentration of 0.02 mM) and 10% methanol in buffer A (25 µl) was used as a blank (negative control).

The rate of reaction was calculated. Any increase in absorbance due to the spontaneous hydrolysis of the substrate was corrected by subtracting the ratio of reaction before adding the enzyme (background) from the rate after adding the enzyme (sample reading). Percentage inhibition was calculated by comparing the reaction rates of the sample to the blank (10% methanol in buffer A) as follows:

$$\text{Inhibition}(\%) = \left(1 - \left(\frac{R_S}{R_B}\right)\right) X\ 100$$

$R_S$: Sample reading - background

$R_B$: Blank reading - background

The $IC_{50}$ values (concentrations of plant extracts or pure compounds resulting in a 50% reduction in activity of acetyl-cholinesterase enzyme) were calculated by regression analysis of the results for three different concentrations (1 mg/ml, 0.5 mg/ml and 0.25 mg/ml) of each sample using GraFit soft ware version 5 for Microsoft ® Windows.

### 3.2.4. MUTAGENIC EFFECTS OF THE INVESTIGATED PLANT EXTRACTS

### AMES TEST

The potential mutagenic effects of the plants was investigated using the Ames test. The Ames assay was performed with *Salmonella typhimurium* strain TA98 using the plate incorporation procedure described by MARON and AMES (1983). One hundred µl of bacterial stock were incubated in 20 ml of Oxoid Nutrient for 16 h at 37 °C on an orbital shaker. The overnight culture (0.1 ml) was added to 2 ml top agar (containing traces of biotin and histidine) together with 0.1 ml test solution (plant extract, solvent control or positive control) and 0.5 ml phosphate buffer (pH 8) (for exposure without metabolic activation).

Minimum glucose plate agar was prepared from a Difco-Bacto agar (a purified agar prepared especially for use in microbiological culture media by a process in which extraneous matter, pigmented portions and salts are reduced to a minimum) as well as Vogel-Bonner medium (50XVB) and 40% glucose. Fifteen g of the Difco-Bcto agar was added to 930 ml of distilled water and autoclaved for 20 min. Then 20 ml of sterile 50X VB salts and 50 ml of sterile 40% glucose were added. Thirty ml of this mixture was poured into each petri plate and allowed to cool. The top agar mixture containing the bacteria was poured over the surface of the minimal glucose agar plate and incubated for 48 h at 37 °C.

After incubation, the number of revertant colonies (mutants) were counted. All cultures were made in triplicate (except the solvent control where five replicates were made) for each assay. The assays were repeated twice. Absence of toxicity was examined by observing the background bacterial growth which should be normally present. The positive control used was 4-nitroquinoline 1-oxide (4-NQO) at a concentration of 2 µg/ml.

## 3.3. RESULTS

### 3.3.1. ANTIBACTERIAL ACTIVITY

#### i) DISC-DIFFUSION ASSAY

The antibacterial activities of 78 crude ethanol, ethyl acetate and aqueous extracts obtained using the disc-diffusion assay are shown in Table 3.1. Antibacterial activity is expressed as the ratio of the inhibition diameter around the disc with plant extract to the inhibition zone around the disc with the neomycin (0.2 mg/ml). Of the plant extracts tested (final amount of plant material was 1 mg per disc), 84% showed activity against Gram-positive bacteria. From this percentage, 20% showed also activity against Gram-negative bacteria. The best inhibition was observed with ethyl acetate and ethanol root extracts of *Terminalia sericea* (Figure 3.1). Some of the

tested extracts showed activity against Gram-positive bacteria and bacteriostatic effects against Gram-negative bacteria. The root extracts and the aqueous and ethanolic bark extracts of *Albizia adianthifolia,* leaf extracts of *Acacia sieberiana* and aqueous root and bark extracts of *Trichilia dregeana* showed no activity.

**Table 3.1** Antibacterial activity of plant extracts (100 mg/ml) obtained from trees used in South African traditional medicine as determined using the disc-diffusion assay. Results given are ratio of inhibition zone of plant extract to that of neomycin (0.2 mg/ml). Extracts showed ratio of inhibition zone ≥ 0.4 are considered highly active

| Plant species | Plant part analyzed | Ethyl acetate extract | | | | | Ethanol extract | | | | | Water extract | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Bacteria tested | | | | | Bacteria tested | | | | | Bacteria tested | | | | |
| | | B.s | M.l | S.a | E.c | K.p | B.s | M.l | S.a | E.c | K.p | B.s | M.l | S.a | E.c | K.p |
| *Acacia nilotica* | Leaf | **0.5** | **0.4** | **0.5** | static | static | **0.6** | **0.5** | **0.7** | static | static | 0.3 | 0.3 | 0.3 | static | static |
| subsp.*kraussiana* | Bark | **0.6** | **0.6** | **0.5** | static | static | **0.6** | **1.0** | **0.5** | static | static | 0.2 | **0.4** | **0.5** | static | **0.5** |
| | Root | **0.4** | **1.4** | **0.9** | static | static | **0.5** | **1.0** | **0.4** | static | static | 0.1 | **0.5** | **0.5** | static | 0.1 |
| *Acacia sieberiana* | Leaf | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| var.*woodii* | Bark | 0.1 | 0.1 | 0.3 | 0.3 | 0.1 | **0.4** | 0.2 | 0.3 | 0.0 | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Root | 0.1 | **0.9** | **1.5** | **0.6** | 0.0 | 0.1 | **0.5** | **1.0** | **0.6** | **0.5** | 0.1 | 0.0 | 0.2 | 0.3 | 0.1 |
| *Albizia adianthifolia* | Bark | 0.1 | 0.2 | **1.7** | 0.2 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Root | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

**Table 3.1** (*Continued*)

| Plant species | Plant part analyzed | Ethyl acetate extract | | | | | Ethanol extract | | | | | Water extract | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Bacteria tested | | | | | Bacteria tested | | | | | Bacteria tested | | | | |
| | | B.s | M.l | S.a | E.c | K.p | B.s | M.l | S.a | E.c | K.p | B.s | M.l | S.a | E.c | K.p |
| *Combretum kraussii* | Leaf | **0.4** | 0.1 | 0.2 | static | static | 0.3 | **0.4** | **0.5** | static | static | 0.1 | **0.5** | **1.6** | static | 0.2 |
| | Bark | **0.6** | 0.2 | **0.6** | static | static | 0.3 | **0.4** | **1.3** | static | static | 0.3 | **0.7** | **0.8** | static | 0.2 |
| | Root | **1.0** | **0.7** | **1.6** | static | **0.4** | 0.3 | **1.0** | **1.2** | static | 0.2 | 0.1 | 0.3 | **4.0** | 0.2 | **1.0** |
| *Faidherbia albida* | Leaf | 0.1 | 0.0 | 0.2 | 0.2 | 0.2 | 0.2 | 0.0 | 0.3 | **0.7** | 0.3 | 0.3 | 0.0 | **0.5** | **0.7** | 0.3 |
| | Bark | 0.1 | 0.0 | **0.4** | 0.1 | 0.1 | 0.1 | 0.3 | **0.8** | **1.0** | 0.3 | 0.3 | 0.0 | 0.3 | **0.3** | 0.3 |
| *Ficus sur* | Leaf | 0.3 | **0.4** | 0.3 | 0.2 | 0.1 | 0.3 | **0.4** | **0.4** | **0.4** | 0.2 | 0.1 | **0.6** | **1.0** | **0.7** | 0.0 |
| | Bark | 0.0 | 0.3 | 0.0 | **0.5** | 0.0 | 0.2 | 0.3 | **0.6** | **1.3** | 0.1 | 0.1 | 0.2 | **1.1** | **0.8** | 0.1 |
| | Root | **0.7** | 0.2 | 0.2 | 0.2 | **0.4** | 0.1 | 0.1 | 0.2 | **0.5** | **0.4** | 0.2 | 0.3 | **1.0** | **0.6** | 0.3 |
| *Prunus africana* | Leaf | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 | static | 0.3 | **0.4** | 0.0 | 0.0 | static | 0.3 | 0.2 | 0.0 | 0.0 |
| | Bark | static | **0.5** | **0.5** | 0.0 | 0.0 | static | **0.5** | **1.0** | 0.0 | 0.0 | static | 0.3 | 0.3 | static | static |

**Table 3.1** (*Continued*)

| Plant species | Plant part analyzed | Ethyl acetate extract | | | | | Ethanol extract | | | | | Water extract | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Bacteria tested | | | | | Bacteria tested | | | | | Bacteria tested | | | | |
| | | B.s | M.l | S.a | E.c | K.p | B.s | M.l | S.a | E.c | K.p | B.s | M.l | S.a | E.c | K.p |
| *Salix mucronata* | Leaf | 0.1 | 0.2 | 0.2 | 0.0 | 0.1 | **0.8** | 0.3 | 0.3 | 0.1 | 0.1 | 0.1 | 0.2 | 0.1 | 0.1 | 0.1 |
| | Bark | 0.2 | **0.5** | 0.1 | **0.5** | 0.3 | 0.1 | **0.5** | **1.0** | **1.3** | **0.5** | **0.4** | **0.6** | **1.4** | **1.8** | 0.0 |
| | Root | 0.1 | **0.5** | 0.2 | 0.3 | **0.5** | 0.1 | **0.7** | **1.0** | **1.2** | **0.5** | 0.1 | **0.6** | **0.7** | **0.8** | 0.2 |
| *Terminalia sericea* | Bark | 0.1 | 0.2 | **0.6** | **1.0** | **0.5** | 0.1 | **1.1** | **1.0** | **1.0** | 0.3 | **0.4** | **0.6** | **0.4** | **0.8** | **1.0** |
| | Root | 0.1 | **1.3** | **4.0** | **2.0** | **1.0** | **0.6** | **1.1** | **2.3** | **1.1** | **2.7** | 0.1 | **1.4** | **0.6** | **1.0** | 0.0 |
| *Trichilia dregeana* | Leaf | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | 0.1 | **0.5** | 0.2 | 0.3 | 0.0 | 0.1 | 0.3 | 0.2 | 0.3 | 0.3 |
| | Bark | 0.2 | 0.2 | 0.1 | **0.5** | 0.2 | 0.1 | 0.2 | **0.8** | **1.3** | 0.3 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |
| | Root | 0.0 | 0.2 | 0.0 | 0.0 | 0.0 | 0.1 | 0.0 | 0.1 | 0.3 | 0.1 | 0.0 | 0.0 | 0.0 | 0.0 | 0.0 |

B.s = *Bacillus subtilis,* E.c = *Escherichia coli,* K.p = *Klebsiella pneumoniae,* S.a = *Staphylococcus aureus,* M.l = *Micrococcus luteus.* Static= Bacteriostatic effect.

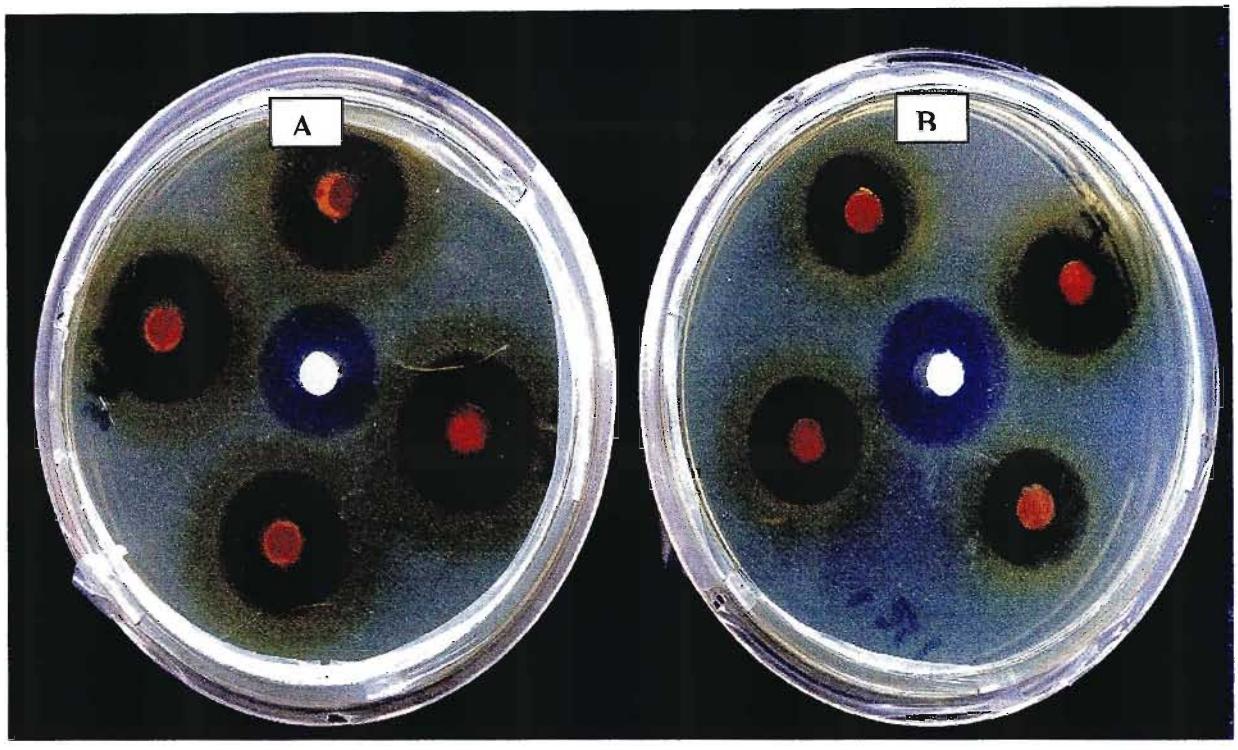


**Figure 3.1** Inhibition of bacterial growth by an ethyl acetate root extract of *Terminalia sericea* (1mg per disc) in the disc-diffusion assay against:

A: *Staphylococcus aureus* (Gram-positive).

B: *Escherichia coli* (Gram-negative).

## ii) MICRO-DILUTION ASSAY

The MIC values of the active extracts obtained by using the micro-dilution assay are shown in Table 3.2. Fifty five percent of the tested plant extracts showed minimum inhibitory concentration (MIC) values ≤ 1.56 mg/ml against Gram-positive and/or Gram-negative bacteria. The ethyl acetate bark extract of *Acacia sieberiana* and the root and bark ethyl acetate extracts of *Acacia nilotica* inhibited bacterial growth of both Gram-positive and Gram-negative bacteria at concentrations ≤ 0.8 mg/ml. The aqueous leaf extracts of *Salix mucronata* and the aqueous bark extracts of *Terminalia sericea* also inhibited growth of both Gram-positive and Gram-negative bacteria at concentrations ≤ 0.7 mg/ml. The aqueous leaf extracts of *Acacia sieberiana* had a low MIC value against Gram-negative *Kleibsiella pneumoniae* (0.3 mg/ml) and the ethyl acetate extracts of the root inhibited growth of *Escherichia coli* with an MIC value of 0.1 mg/ml. However, the two extracts showed no activity in the disc-diffusion assay.

**Table 3.2** The minimal inhibitory concentration (MIC) of antibacterial activity of plant extracts obtained from trees used in South African traditional medicine as determined by the micro-dilution assay. Values given are in mg/ml. Extracts showed MIC values ≤ 1 mg/ml are considered highly active.

| Plant species | Plant part analyzed | Ethyl acetate extract | | | | Ethanol extract | | | | Water extract | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Bacteria tested | | | | Bacteria tested | | | | Bacteria tested | | | |
| | | B.s | S.a | E.c | K.p | B.s | S.a | E.c | K.p | B.s | S.a | E.c | K.p |
| *Acacia nilotica* | Leaf | **0.8** | 1.5 | 3.1 | 3.1 | **0.8** | 1.5 | 3.1 | 3.1 | 5.0 | 6.3 | >12.5 | >12.5 |
| subsp.*kraussiana* | Bark | **0.1** | **0.1** | **0.8** | **0.8** | **0.9** | **0.1** | **0.8** | **0.8** | **0.8** | **0.8** | 6.1 | 6.1 |
| | Root | **0.2** | **0.3** | **0.8** | **0.8** | **0.8** | **0.8** | 1.5 | 1.5 | 2.5 | **0.6** | 6.5 | 6.5 |
| *Acacia sieberiana* | Leaf | >12.5 | >12.5 | >12.5 | >12.5 | **0.3** | 3.1 | **0.8** | 1.5 | **0.3** | **0.8** | 1.5 | **0.3** |
| var.*woodii* | Bark | **0.8** | **0.8** | **0.8** | **0.8** | **0.5** | **0.8** | **1.0** | **0.8** | >12.5 | >12.5 | >12.5 | >12.5 |
| | Root | **0.2** | **0.1** | **0.1** | >12.5 | **1.0** | 3.1 | 3.1 | 3.1 | 3.1 | 1.5 | 1.5 | 1.5 |
| *Albizia adianthifolia* | Bark | 6.2 | 6.2 | 12.5 | na | 3.1 | 3.1 | 6.2 | 6.2 | >12.5 | >12.5 | >12.5 | >12.5 |
| | Root | na | na | na | na | na | na | na | na | na | na | na | na |

**Table 3.2** (*continued*)

| Plant species | Plant part analyzed | Ethyl acetate extract | | | | Ethanol extract | | | | Water extract | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Bacteria tested | | | | Bacteria tested | | | | Bacteria tested | | | |
| | | B.s | S.a | E.c | K.p | B.s | S.a | E.c | K.p | B.s | S.a | E.c | K.p |
| *Combretum kraussii* | Leaf | **0.7** | 1.5 | 6.2 | 6.2 | **0.5** | 1.5 | 6.2 | 6.2 | 3.1 | 3.1 | 6.2 | 6.2 |
| | Bark | **0.6** | 3.1 | 6.2 | 0.9 | 1.3 | 1.3 | 1.5 | 1.5 | 1.5 | 1.5 | 3.1 | 3.1 |
| | Root | **0.2** | **0.8** | 1.5 | 3.1 | **0.7** | **0.8** | 1.5 | **0.5** | 3.1 | 3.1 | 3.1 | 3.1 |
| *Faidherbia albida* | Leaf | **0.8** | 6.2 | 6.2 | 6.2 | **0.7** | 1.5 | 1.5 | 3.1 | 4.0 | 1.5 | 3.1 | 6.2 |
| | Bark | 3.1 | 3.1 | 6.2 | 12.5 | **0.5** | **0.3** | 4.6 | 6.25 | **0.7** | 6.2 | 6.2 | 12.5 |
| *Ficus sur* | Leaf | 6.2 | 6.2 | 6.2 | 12.5 | **0.7** | **0.7** | 3.1 | 6.2 | 6.2 | 6.2 | >12.5 | >12.5 |
| | Bark | >12.5 | >12.5 | >12.5 | >12.5 | **0.3** | **0.1** | 2.1 | 3.1 | 1.5 | **0.5** | 6.2 | 6.2 |
| | Root | **0.3** | **0.1** | 1.5 | 1.5 | **0.5** | 1.5 | 3.1 | 6.2 | 3.1 | **0.7** | 4.6 | 1.2 |
| *Prunus africana* | Leaf | 6.2 | 6.2 | 3.1 | 6.1 | 1.5 | 2.9 | 3.1 | 6.2 | 3.1 | 5.0 | 6.2 | 6.2 |
| | Bark | 3.1 | 3.1 | 6.2 | 6.2 | 3.1 | 1.5 | 3.1 | 2.9 | 2.9 | 2.9 | 3.1 | 6.2 |

**Table 3.2** (*Continued*)

| Plant species | Plant part analyzed | Ethyl acetate extract | | | | Ethanol extract | | | | Water extract | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Bacteria tested | | | | Bacteria tested | | | | Bacteria tested | | | |
| | | B.s | S.a | E.c | K.p | B.s | S.a | E.c | K.p | B.s | S.a | E.c | K.p |
| *Salix mucronata* | Leaf | 4.7 | 6.2 | 4.7 | 6.2 | 3.1 | 1.5 | 3.1 | 3.1 | **0.2** | **0.5** | **0.5** | **0.3** |
| | Bark | 2.3 | 3.1 | 3.1 | 6.2 | **0.5** | 3.1 | 3.1 | 6.2 | 1.5 | 6.2 | >12.5 | >12.5 |
| | Root | >12.5 | >12.5 | >12.5 | >12.5 | 3.1 | 6.2 | 6.2 | >12.5 | >12.5 | >12.5 | >12.5 | >12.5 |
| *Terminalia sericea* | Bark | 1.5 | 1.5 | 1.5 | 1.5 | **0.7** | 1.5 | 3.1 | 3.1 | **0.7** | **0.7** | **0.7** | **0.7** |
| | Root | **0.3** | 1.5 | 1.5 | **0.7** | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | 1.5 | na |
| *Trichilia dregeana* | Leaf | 3.1 | 6.2 | 6.2 | 6.2 | 3.1 | 1.5 | 3.1 | 3.1 | 3.1 | 1.5 | 3.1 | 3.1 |
| | Bark | 3.1 | 3.1 | 3.1 | 6.2 | 2.3 | **1.0** | 6.2 | 3.1 | >12.5 | >12.5 | 6.2 | >12.5 |
| | Root | 6.2 | 6.2 | 6.2 | 6.2 | 6.2 | 6.2 | 6.2 | 6.2 | >12.5 | >12.5 | >12.5 | >12.5 |
| Neomycin (µg/ml) | | B.s | | | S.a | | | E.c | | | K.p | | |
| | | 0.1 | | | 0.3 | | | 3.1 | | | 0.8 | | |

Bacteria: B.s = *Bacillus subtilis;* E.c = *Escherichia coli;* K.p = *Klebsiella pneumoniae;* S.a= *Staphylococcus aureus;*

M.l= *Micrococcus luteus.* na= not active.

### 3.3.2. CYCLOOXYGENASE ENZYME INHIBITORY ACTIVITY

Results obtained from the screening assays for inhibition of COX-1 and COX-2 enzymes are shown in Table 3.3. Plant extracts were classified active using criteria based on the fact that activity of extracts obtained by aqeous solvents are generally lower than organic extracts (JÄGER *et al.*,1996). Accordingly minimum inhibition of 50% and 60% is required for water extracts and organic solvent extracts respectively to be considered significantly active. It is noteworthy that 70% of the plant extracts from different plant parts (leaf, root, bark) of the tree investigated showed strong inhibition in both the COX-1 and COX-2 bioassays. The COX-2 inhibitory effects of aqueous extracts were generally lower when compared to the organic solvent extracts. However, water extracts of *Acacia nilotica* was an exception (> 90%). Ethanolic and aqueous root extracts of *Albizia adianthifolia*, all bark and root extracts of *Terminalia sericea*, and all the root extracts and the ethanolic and aqueous bark extracts of *Combretum kraussii* only showed activity against COX-1. Ethyl acetate leaf extracts of *Trichilia dregeana* showed strong activity against COX-2 (81%) but had slightly weaker activity against COX-1.

**Table 3.3** Inhibition (%) of prostaglandin synthesis by plant extracts (250 µg/ml) obtained from trees used in South African traditional medicine as determined by the cyclooxygenase (COX-1 and COX-2) assays. Results given are mean ± S.D. Inhibitions of prostaglandins by indomethacin (5 µM for COX-1 and 200 µM for COX-2) were 80±1.9% for COX-1 and 69±2.4% for COX-2.

| Plant species | Plant part analyzed | Inhibition (%) COX-1 | | | Inhibition (%) COX-2 | | |
|---|---|---|---|---|---|---|---|
| | | Ethyl acetate extract | Ethanol extract | Water extract | Ethyl acetate extract | Ethanol extract | Water extract |
| *Acacia nilotica* subsp.*kraussiana* | Leaf | 93±3.1 | 96±1.9 | 96±3.1 | 89±2.9 | 89±2.9 | 90±2.7 |
| | Bark | 94±3.0 | 96±1.7 | 97±5.5 | 88±3.1 | 96±4.6 | 96±1.3 |
| | Root | 87±2.8 | 91±2.6 | 95±5.2 | 89±4.6 | 94±3.8 | 97±5.5 |
| *Acacia sieberiana* var.*woodii* | Leaf | 80±0.8 | 85±3.9 | 61±2.4 | 77±1.1 | 88±1.5 | 50±3.3 |
| | Bark | 81±5.1 | 88±3.6 | 76±1.7 | 70±4.4 | 76±4.9 | 52±1.3 |
| | Root | 67±4.0 | 58±3.0 | 40±2.5 | 13±2.3 | 48±3.8 | 48±4.9 |

**Table 3.3** (*continued*)

| Plant species | Plant part analyzed | Inhibition (%) COX-1 | | | Inhibition (%) COX-2 | | |
|---|---|---|---|---|---|---|---|
| | | Ethyl acetate extract | Ethanol extract | Water extract | Ethyl acetate extract | Ethanol extract | Water extract |
| *Albizia adianthifolia* | Bark | 87±7.4 | 75±3.3 | 65±4.2 | 87±4.4 | 81±1.4 | 58±4.0 |
| | Root | 88±5.9 | 90±3.7 | 61±3.1 | 84±3.9 | 22±3.4 | 36±2.9 |
| *Combretum kraussii* . | Leaf | 90±1.9 | 93±3.1 | 71±1.6 | 96±2.7 | 90±5.1 | 52±3.6 |
| | Bark | 97±2.1 | 85±2.9 | 69±4.6 | 90±1.5 | 34±3.8 | 41±2.9 |
| | Root | 90±1.2 | 73±4.1 | 60±3.1 | 20±4.0 | 29±2.1 | 39±2.4 |
| *Faidherbia albida* | Leaf | 80±5.3 | 88±4.4 | 91±1.6 | 81±2.0 | 82±0.9 | 53±1.6 |
| | Bark | 71±2.2 | 82±5.1 | 80±2.6 | 74±2.2 | 76±2.1 | 57±1.0 |

**Table 3.3** (*continued*)

| Plant species | Plant part analyzed | Inhibition (%) COX-1 | | | Inhibition (%) COX-2 | | |
|---|---|---|---|---|---|---|---|
| | | Ethyl acetate extract | Ethanol extract | Water extract | Ethyl acetate extract | Ethanol extract | Water extract |
| *Ficus sur* | Leaf | 66±1.9 | 15±1.9 | 22±0.9 | 60±3.3 | 24±4.4 | 29±1.5 |
| | Bark | 79±3.1 | 82±5.5 | 89±4.4 | 22±5.2 | 16±2.9 | 19±4.3 |
| | Root | 84±2.9 | 80±3.8 | 73±3.1 | 21±1.7 | 13±7.1 | 43±2.2 |
| *Prunus africana* | Leaf | 88±3.4 | 90±2.8 | 55±5.2 | 84±2.5 | 84±4.1 | 51±1.0 |
| | Bark | 90±4.1 | 96±1.1 | 60± 3.1 | 79±1.9 | 88±1.2 | 58±2.4 |
| *Salix mucronata* | Leaf | 82±2.4 | 80±3.2 | 71±1.1 | 74±6.1 | 78±2.6 | 67±5.7 |
| | Bark | 78±2.1 | 93±4.3 | 83±4.7 | 82±1.1 | 70±3.1 | 56±2.3 |
| | Root | 94±1.7 | 87±2.9 | 61±7.8 | 74±1.8 | 77±6.3 | 58±1.1 |

**Table 3.3** (*continued*)

| Plant species | Plant part analyzed | Inhibition (%) COX-1 | | | Inhibition (%) COX-2 | | |
|---|---|---|---|---|---|---|---|
| | | Ethyl acetate extract | Ethanol extract | Water extract | Ethyl acetate extract | Ethanol extract | Water extract |
| *Terminalia sericea* | Bark | 90± 3.9 | 72±1.8 | 59±4.0 | 41±3.5 | 20±2.7 | 23±2.3 |
| | Root | 85±3.2 | 78±2.4 | 55±2.1 | 37±1.6 | 32±2.6 | 28±6.1 |
| *Trichilia dregeana* | Leaf | 57±1.1 | 46±3.5 | 25±3.3 | 81±2.1 | 22±6.2 | 17±6.3 |
| | Bark | 78±0.9 | 46±3.0 | 27±2.6 | 19±1.9 | 20±4.1 | 33±5.1 |
| | Root | 37±2.4 | 27±1.9 | 22±4.1 | 11±3.7 | 49±3.1 | 13±3.1 |

### 3.3.3. ACETYLCHOLINESTERASE ENZYME INHIBITORY ACTIVITY

Results of acetylcholinesterase enzyme inhibitory activity of the tested plant extracts are shown in Table 3.4. Plant extracts showed activities only in the preliminary TLC assay were not considered active. Of the 78 crude extracts tested, 21% showed activity in both TLC and the microplate assay. At the concentration of 1 mg/ml, ethyl acetate leaf extract of *Combretum kraussii* showed the highest activity (96%) amongst the tested extracts and an $IC_{50}$ value of 0.2 mg/ml in the the microplate assay. Moderate activities (<60%) were observed with all leaf extracts of *Acacia nilotica*, the ethanolic bark extract of *Albizia adianthifolia* and the ethyl acetate bark extract of *Trichilia dregeana*. The lowest $IC_{50}$ value (0.04 mg/ml) was obtained with the ethanolic bark extract of *Combretum kraussii*. The percentage inhibition of galanthamine (positive control) at concentration of 20 µM was 93% ($IC_{50}$ value = 2.0 µM).

### 3.3.4. MUTAGENIC EFFECTS

#### AMES TEST

Results obtained using the Ames test for different plant parts tested against the *Salmonella typhimurium* strain TA 98 are shown in Table 3.5. The results are based on the number of induced revertant colonies detected. Plant extracts are considered active if the number of induced revertant colonies is double the revertant colonies of the blank (negative control). According to this criterion, none of the investigated plants showed any potential mutagenic effects.

**Table 3.4** Inhibition of acetylcholinesterase enzyme activity by plant extracts (1 mg/ml) obtained from trees used in South African traditional medicine as determined by the micro-plate assay. Only the extracts of the six species that showed biological activity are listed. Results given are percentage inhibition and $IC_{50}$ values. The values are expressed as mean ± S.D.

| Plant species | Plant part analyzed | Inhibition (%) and $IC_{50}$ values (mg/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Plant extracts tested at concentration of 1 mg/ml | | | | | |
| | | Ethyl acetate | $IC_{50}$ | Ethanol | $IC_{50}$ | Galanthamine (20 µM) | $IC_{50}$ (µM) |
| *Acacia nilotica* subsp.*kraussiana* | Leaf | 53±3.7 | 0.7±0.1 | 56±6.3 | 0.5±0.1 | 93±3.2 | 2.0±0.1 |
| | Bark | 41±2.1 | 1.3±0.12 | na | - | | |
| *Acacia sieberiana* var.*woodii* | Root | 60±4.3 | 0.4±0.06 | 62± 4.1 | 0.4±0.03 | | |
| *Albizia adianthifolia* | Bark | 61±5.1 | 0.4±0.17 | 53±2.2 | 0.7± 0.09 | | |
| | Root | 45± 2.1 | 1.2±0.08 | 51±3.4 | 0.9±0.1 | | |

**Table 3.4** (*continued*)

| Plant species | Plant part analyzed | Inhibition (%) and IC $_{50}$ values (mg/ml) | | | | | |
|---|---|---|---|---|---|---|---|
| | | Plant extracts tested at concentration of 1 mg/ml | | | | | |
| | | Ethyl acetate | $IC_{50}$ | Ethanol | $IC_{50}$ | Galanthamine (20 µM) | $IC_{50}$ (µM) |
| *Combretum kraussii* | Leaf | 96±4.6 | 0.2±0.1 | 88±3.1 | 0.2± 0.11 | | |
| | Bark | 82±6.1 | 0.14±0.09 | 83±4.5 | 0.04±0.07 | | |
| | Root | 81±4.1 | 0.2±0.07 | 82±5.2 | 0.13±0.03 | | |
| *Salix mucronata* | Bark | 82±3.9 | 0.3±0.09 | na | - | 93±3.2 | 2.0±0.1 |
| *Trichilia dregeana* | Bark | 55±4.4 | 0.8±0.08 | na | - | | |

na= not active.

**Table 3.5** Mutagenic effects of plant extracts obtained from trees used in South African traditional medicine as determined by the Ames test using *Salmonella typhimurium* strain TA 98. Number of induced revertant colonies were recorded

| Plant species | Plant part analyzed | Plant extract concentration (mg/ml) tested | | | | | | | | | Positive Control | Negative Control (Blank) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ethyl acetate | | | Ethanol | | | Water | | | | |
| | | 0.05 | 0.5 | 5 | 0.05 | 0.5 | 5 | 0.05 | 0.5 | 5 | | |
| *Acacia nilotica* | Leaf | 20±3 | 25±4 | 19±3 | 30±4 | 28±3 | 34±4 | 30±4 | 19±4 | 29±2 | 191±8 | 31±2 |
| subsp.*kraussiana* | Bark | 22±5 | 20±2 | 26±3 | 31±2 | 24±2 | 31±3 | 24±3 | 22±2 | 22±5 | 189±6 | 29±1.2 |
| | Root | 18±2 | 23±3 | 27±2 | 24±5 | 29±3 | 30±4 | 27±3 | 26±3 | 27±4 | 181±9 | 34±3 |
| *Acacia sieberiana* | Leaf | 32±3 | 29±5 | 37±3 | 26±4 | 39±4 | 36±5 | 30±4 | 37±3 | 33±2 | 203±6 | 35±1 |
| var.*woodii* | Bark | 30±2 | 34±2 | 38±6 | 38±3 | 28±3 | 36±3 | 34±4 | 31±1 | 38±5 | 210±8 | 38±3.1 |
| | Root | 33±4 | 27±4 | 31±2 | 27±5 | 27±4 | 31±4 | 39±1 | 36±4 | 32±1 | 198±4 | 36±2 |
| *Albizia adianthifolia* | Bark | 28±4 | 34±3 | 29±1.3 | 24±1.9 | 21±6 | 32±4.1 | 38±1.1 | 29±2 | 29±3 | 204±10 | 34±3 |
| | Root | 33±7 | 31±2 | 25±4 | 31±3 | 28±3 | 29±7 | 31±4 | 31±3 | 31±1 | 201±5 | 33±4 |

**Table 3.5** (*continued*)

| Plant species | Plant part analyzed | Plant extract concentration (mg/ml) tested | | | | | | | | | Positive Control | Negative Control (Blank) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ethyl acetate | | | Ethanol | | | Water | | | | |
| | | 0.05 | 0.5 | 5 | 0.05 | 0.5 | 5 | 0.05 | 0.5 | 5 | | |
| *Combretum kraussii* | Leaf | 39±9 | 34±5 | 36±6 | 29±7 | 34±8 | 37±6 | 26±4 | 31±5 | 28±3 | 182±12 | 35±7 |
| | Bark | 25±11 | 36±8 | 26±8 | 29±10 | 32±9 | 32±10 | 31±5 | 36±2 | 30±1 | 201±9 | 32±4 |
| | Root | 33±7 | 40±4 | 37±5 | 35±9 | 39±8 | 38±7 | 34±6 | 30±7 | 34±6 | 196±17 | 28±2 |
| *Faidherbia albida* | Leaf | 23±4 | 13±1 | 23±4 | 22±3 | 27±5 | 29±4 | 24±2 | 26±3 | 24±2 | 163±7 | 27±4 |
| | Bark | 19±2 | 18±5 | 22±6 | 24±1 | 27±3 | 27±2 | 22±4 | 22±4 | 29±1 | 162±6 | 35±3 |
| *Ficus sur* | Leaf | 27±3 | 30±2 | 26±4 | 28±3 | 22±4 | 24±3 | 30±1 | 38±2 | 36±1 | 192±4 | 31±2 |
| | Bark | 29±4 | 27±4 | 31±3 | 29±2 | 27±6 | 29±4 | 27±4 | 31±2 | 37±2 | 184±6 | 29±1 |
| | Root | 34±1 | 34±1 | 36±1 | 23±3 | 26±2 | 29±3 | 26±1 | 36±3 | 34±3 | 179±4 | 33±2 |

**Table 3.5** (*continued*)

| Plant species | Plant part analyzed | Plant extract concentration (mg/ml) tested | | | | | | | | | Positive Control | Negative Control (Blank) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Ethyl acetate | | | Ethanol | | | Water | | | | |
| | | 0.05 | 0.5 | 5 | 0.05 | 0.5 | 5 | 0.05 | 0.5 | 5 | | |
| *Prunus africana* | Leaf | 29±2 | 31±2 | 33±1 | 29±3 | 34±2 | 28±4 | 29±6 | 33±2 | 33±1 | 167±8 | 31±2 |
| | Bark | 34±4 | 35±1 | 31±4 | 31±1 | 36±2 | 26±1 | 34±1 | 36±2 | 38±2 | 179±3 | 27±1 |
| *Salix mucronata* | Leaf | 19±3 | 16±1 | 23±4 | 20±3 | 27±4 | 29±4 | 24±2 | 26±3 | 24±3 | 163±2 | 27±4 |
| | Bark | 22±4 | 17±5 | 21±6 | 24±1 | 26±3 | 27±2 | 22±4 | 27±2 | 26±2 | 159±6 | 29±1 |
| | Root | 18±2 | 25±3 | 25±1 | 26±5 | 20±1 | 29±3 | 28±4 | 22±4 | 29±1 | 167±9 | 35±3 |
| *Terminalia sericea* | Bark | 29± 9 | 31±7 | 26±3 | 34±2 | 31±2 | 27±3 | 35±9 | 34±2 | 26±6 | 177±11 | 29±3 |
| | Root | 22±6 | 29±5 | 24±8 | 29±4 | 35±1 | 34±7 | 28±4 | 30±3 | 27±5 | 189±8 | 31±2 |
| *Trichilia dregeana* | Leaf | 31±.9 | 39±4 | 40±2 | 29±5 | 35±3 | 22±1 | 34±4 | 23±4 | 30±5 | 187±9 | 34±3 |
| | Bark | 35±5 | 34±3 | 38±3 | 36±4 | 33±2 | 24±7 | 37±2 | 32±3 | 37±7 | 170±11 | 35±5 |
| | Root | 33±.8 | 37±1 | 31±2 | 38±3 | 39±4 | 29±6 | 21±5 | 33±3 | 30±2 | 194±7 | 39±3 |

## 3.4. DISCUSSION AND CONCLUSION

The plants investigated in this study contain different types of chemical compounds. Alkaloids, tannins, lactones, triterpenoids and hydroxystilbene glycoside, are found in *Combretum* and *Terminalia* species (HUTCHINGS *et al.*, 1996; McGAW *et al.*, 2000). Albilocin, flavonoids, acetylhistamine and imidazoleatic acid occur in *Albizia adianthifolia* (MAAT and BEYERMAN,1983) and limonoids are present in *Trichilia* species (MULHOLLAND and TAYLOR, 1980). Tannins, ethyl galate and flavonoids the octasanol, ß-amyrine and α-betulin are found in *Acacia* species (AYOUB, 1984; ABDELNABI *et al.*, 1992; NEUWINGER, 1996) and ferulic esters, terpenoids, phytosterols, and amygdalin occur in *Prunus africana* (HUTCHINGS *et al.*, 1996). Of these compounds, ß-glycyrrhertic acid and other derivatives, albilocin, flavonoids, limonoids, α-betulin, ß-sitosterol triterpenoids, hydroxystilbene glycoside phytosterols, amygdalin and stilbenoids are known to have anti-inflammatory and/or antibacterial activity (UBERTI *et al.*, 1990; BRUNETON, 1995; HUTCHINGS *et al.*, 1996). These compounds may be responsible for the observed activities in the crude extracts investigated for antibacterial and anti-inflammatory activities in the present study.

Tannins are reported to be responsible for the majority of false positive activities obtained in assays. They affect highly purified enzyme-based targets due to their strong ability to bind with proteins, this action leads to inhibition of prostaglandin synthesis through blocking the cyclooxygenase enzymes (O'NEILL and LEWIS, 1993). This may explain the high COX-1 and COX-2 activities observed with extracts from *Acacia* species.

The obtained results showed different levels of activities against both Gram-positive and Gram-negative bacteria. Gram-negative bacteria which are responsible for a large number of infectious diseases, have a unique outer membrane that contains lipopolysaccharides which render them impermeable to certain antibacterial compounds (CLEMENTS *et al.*, 2002). Some of the plant extracts (*Terminalia* and *Acacia* species) showed antibacterial activity against both Gram-positive and Gram-negative bacteria at

low MIC values (Table 3.2). This gives an indication of presence of promising antibacterial compounds to be isolated. These findings confirm previous reports about antibacterial properties of *Acacia* species (KHAN *et al.*, 1980) and *Terminalia* species (BRUNETON, 1995).

Despite the inhibition of COX-2 showed by some of the plant extracts, most of the investigated plant parts showed strong inhibition of COX-1 (with the exception of *Trichilia dregeana*). It has been reported that COX-1 inhibition reduces the production of prostaglandins in the stomach which causes gastric ulceration and other serious side effects in the body (WALLACE and CHIN, 1997; HENRY *et al.*, 2002). This implies that if these plants are used in traditional medicine, they could potentially cause negative side effects. Many cases of adverse crude drug interactions such as allergic reactions, cramps, diarrhoea, fever, gastrointestinal disturbances, headaches, hematuria and vomiting may be experienced when administering some kind of traditional medicine as a result of insufficient patient awareness or improper use of the traditional medicine (WHO, 2004). Although, the investigated plants were reported to have anti–inflammatory and antibacterial effects (BRUNETON, 1995; NEUWINGER, 1996; HUTCHINGS *et al.*,1996), some did not show strong activity in the current study. However, negative results do not mean an absence of bioactive constituents nor that the plant is inactive. Active compound(s) may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed (TAYLOR *et al.*, 2001). Lack of activity can thus only be proven by using larger doses (FARNSWORTH, 1993). Alternatively, if the active principle is present in high enough quantities, there could be other constituents exerting antagonistic effects or negating the positive effects of the bioactive agents (JÄGER *et al.*, 1996). In the case of no antibacterial activity, extracts may be active against other bacterial species that were not tested (SHALE *et al.*, 1999). Some extracts did not show antibacterial activity with the disc-diffusion assay while they inhibited bacterial growth in the micro-dilution assay (aqueous leaf extracts of *Acacia sieberiana*). This could be attributed to the effects of agar on the diffusion of the active compounds.

Organic extracts generally (with some exceptions) showed stronger activity in the antibacterial and anti-inflammatory bioassays when compared to aqueous extracts. As water is the major solvent used in the traditional administration of the plant, it suggests that water is not the most effective solvent. However, considering the high dosage normaly administered by the traditional healers, water can still be considered as an effective extracting solvent for traditional remedies (SHALE *et al.*, 1999).

Extracts from roots and bark of the tree species investigated in this study generally showed stronger activity in the antibacterial bioassays (with some exceptions) when compared to leaves. In the anti-inflammatory and acetylcholinesterase bioassays the extracts showed slight differences between bark, root and leaves in their biological activities against cyclooxygenase 1&2 and acetylcholinesterase enzymes (exceptions were *Ficus sur* (COX-1), *Trichilia dregeana* (COX-2), *Acacia sieberiana* and *Salix mucronata* (acetylcholinesterase)). These findings in general support the use of bark in traditional medicine. It has been reported that bark products constitute nearly one third of plant material used in South African traditional medicine (GRACE *et al.*, 2002). Bark also may have been favoured historically as it is readily accessible compared to roots and availability unaffected by seasons as the situation with leaves, fruits and flowers (CUNNINGHAM, 1988). However, leaves are the most commonly used plant part in other regions of Africa (GRACE *et al.*, 2002)

Alkaloids are known to exhibit a number of pharmacological and/or biological properties. For example, galanthamine is an alkaloid recently introduced for clinical use as an anti-cholinesterase drug for the treatment of Alzheimer's disease. The plant extracts that showed acetylcholinesterase inhibitory activity are known to contain different types of alkaloids. *Combretum* species have saponaretine and quaternary amino bases comprising two major alkaloids, combretines A and B and stereoisomers of betonicine (BRUNETON,1995). *Albizia adianthifolia* yielded acetylhistamine and imidazoleatic acid and other minor imidazole compounds (HUTCHINGS *et al.*, 1996). The presence of alkaloids might explain the inhibition of acetylcholinesterase enzyme activity observed by these plant extracts in the present study. Some of the plant extracts

that showed activity against acetylcholinesterase were also found active against cyclooxygenase enzyme in this study. These findings support the suggestion that anti-inflammatory agents could also be used to delay neurodegenarative disorders such as Alzheimer's disease (LÓPEZ *et al.*, 2002; HOWES and HOUGHTON, 2003).

Although none of the investigated plants showed any potential mutagenic effects, this does not necessarily mean absence of adverse effects that justifies their use as a safe traditional medicine. Recent investigations have revealed that many plants used as food or in traditonal medicine have mutagenic effects when tested in *in vitro* assays (ELGORASHI *et al.*, 2003). Mutation mechanisms are also extremely complex and cannot be detected easily. Therefore accurate evaluation of mutagenic activity of medicinal plants using developed and sophisticated tools is urgently needed (DEBNATH et *al.*, 1991).

In general the results obtained are in line with the traditional uses of the plants as crude anti-bacterial and anti-inflammatory drugs. However, traditional healers seldom use a single plant in their extracts. In many cases the therapeutic benefits are attributed to the consumption of plant mixtures in which different plant parts are prepared and/or consumed in combination or in sequence (ETIKIN, 1986; TAYLOR *et al.*, 2001). Modern and traditional health care often exist side by side but seldom cooperate. There is a lack of standardization in traditional medicine with respect to raw materials, methods of production and in quality control of the finished product (ANAND and NITYANAND, 1984). The rational use of traditional medicine often relies on mysticism and intangible forces such as witchcraft, with some aspects based on spiritual and moral principles (Chapter one). While these may be valid psychologically, they cannot be rationalized scientifically (ADDAE-MENSAH, 1992). Thus, pharmacological screening of medicinal plants is very important to provide a scientific basis for the continued use of the plants and to provide society with potential sources of new, effective and safe drugs.

The ethyl acetate root extract of *Terminalia sericea* was selected for further investigation to isolate active compound(s) possibly responsible for the antibacterial and anti-inflammatory activities observed by the plant extracts in this study.

# CHAPTER 4

# ISOLATION AND IDENTIFICATION OF AN ANTIBACTERIAL COMPOUND FROM THE ETHYL ACETATE ROOT EXTRACT OF *TERMINALIA SERICEA*

## 4.1. INTRODUCTION

Plants have been selected and used empirically as natural drugs for centuries (Chapter one). A drug is a chemically identified substance derived from plants or animals, or produced by synthesis (COUZINIER and MAMATAS, 1986). Most drugs were originally discovered from folk knowledge that was disseminated by indigenous cultures where herbal medicines are usually employed as crude aqueous or ethanol extracts (HOSTETTMANN *et al.*, 1995; HOUGHTON and RAMAN, 1998). However, in many cases the activity of crude extracts could be increased by an equivalent dose of the isolated active constituents (Chapter three). Currently considerable attention is focused on higher plants as promising sources of new leads of active compounds that can be used in the pharmaceutical industry. This is largely attributed to the availability of advanced and sophisticated analytical tools that can be used for isolation, purification and identification of desirable bioactive compounds (WU *et al.*, 2004).

## 4.2. ANALYTICAL METHODS USED FOR ISOLATION, PURIFICATION AND IDENTIFICATION OF BIOACTIVE COMPOUNDS

### 4.2.1. CHROMATOGRAPHIC TECHNIQUES

Chromatography can be defined as a method of analysis in which flow of a solvent or gas promotes the separation of substances by differential migration from a narrow initial zone in a porous sorptive medium (HEFTMANN, 1961). It has become a central technology in many fields of applied science such as the

synthesis of drugs and pharmaceutics, purification of natural products, food and clinical chemistry (NEVEROVA and VAN EYK, 2005).

Technically, chromatography involves a sample (or sample extract) being dissolved in a mobile phase (which may be a gas, liquid or supercritical fluid) which is then passed through an immobile stationary phase (CHROMATOGRAPHY, 2005). In all chromatographic separations, components of the mixture interact with the solvent and the sorbent (HEFTMANN, 1961). These interactions determine the degree of sorption of particular substances as well as the effectiveness of the separation. Separation of complex mixtures therefore depends upon their differential penetration into the porous sorbent (CHROMATOGRAPHY, 2005).

Substances detected during chromatography can be characterized by their migration relative to that of the wash liquid. This ratio is called the "$R_F$ value" (HEFTMANN, 1961). Each substance has a different $R_F$ value for each sorbent and solvent combination. Thus to use the $R_F$ value as a factor for characterization, all conditions such as solvent, sorbent, porosity, concentration of the solute and the temperature should be recorded (HEFTMANN, 1961). As many similar chemical substances such as amino acids and carbohydrates are separable by various modifications to the chromatographic system, there is no standard chromatographic system which can be used as a perfect method (HEFTMANN, 1961). However, chromatographic methods are still widely used as analytical tools due to their high separation abilities prior to detection of individual components (HEFTMANN, 1961; CAO *et al.*, 2004).

### i) THIN LAYER CHROMATOGRAPHY (TLC)

Thin layer chromatography (TLC) continues to be widely used for the standardization of natural plant product purification. It is suitable for the analysis of complex and dirty samples with poor detection characteristics (CAO *et al.*, 2004). The stationary phase in the TLC technique is fixed to aluminum, glass or

plastic sheets. The sample, or a mixture, to be analyzed is loaded near the bottom of the plate. As the mobile phase (the solvent) moves up the plate, components of the mixture are distributed between the adsorbent on the plate and the solvent. Therefore, components can be separated into individual compounds on the plate (CHROMATOGRAPHY, 2005).

### ii) COLUMN CHROMATOGRAPHY

Column chromatography is one of the oldest and most useful methods for the separation and purification of different components in a mixture. The theory of column chromatography is analogous to that of thin-layer chromatography. As the solvent flows through the column, the mixture distributes between the adsorbent and non-stationary phase, separating the components within the solute (HEFTMANN, 1961; CHROMATOGRAPHY, 2005).

## 4.2.2. NUCLEAR MAGNETIC RESONANCE (NMR)

Nuclear magnetic resonance (NMR) spectroscopy is well known as the most powerful spectroscopic technique for obtaining the detailed structural information of molecules and is used to discern the structural differences of compounds of the same molecular mass (WU *et al.*, 2004). The physical foundation of NMR spectroscopy lies in the magnetic properties of atomic nuclei. The interaction of the nuclear magnetic moment of a nucleus with an external magnetic field leads according to the rules of quantum mechanics, to a nuclear energy level diagram as the magnetic energy of the nucleus is restricted to certain discrete values called *eigenvalues*. Associated with the *eigenvalues* are the *eigenstates* (GÜNTHER, 1980). Through a high-frequency transmitter, transitions between *eigenstates* within the energy level diagram can be stimulated. The absorption of energy can be detected, amplified and recorded as spectral line, the so- called resonance signals (GÜNTHER, 1980). In this way a spectrum can be generated for a compound containing atoms whose nuclei have

non-zero magnetic moments such as the proton $^{1}H$. However, the carbon nucleus,($^{12}C$), which is so important in organic chemistry, has no magnetic moment and therefore NMR studies with carbon are limited to the isotope $^{13}C$ (GÜNTHER, 1980).

### 4.2.3. MASS SPECTROMETRY (MS)

Mass spectrometry is a complex discipline that has evolved through many stages, in terms of both the chemical problems that can be tackled and the instrumentation employed (RUSSELL and EDMONDSON, 1997). It is an analytical spectroscopic tool primarily concerned with the separation of molecular and atomic species according to their mass (RUSSELL and EDMONDSON, 1997). Mass spectrometry can achieve molecular weight and fragmentation and even molecular formula using high resolution mass spectrometry (HRMS) through which identification or confirmation of the molecular formulas of new compounds can be achieved. It can also be used in the analysis of many types of samples from elemental to large proteins and polymers. Mass spectrometry therefore can provide accurate mass measurements, identify an unknown compound through determination of the molecular mass, identify functional groups present in the molecule on the basis of specific fragments or ions and help to elucidate the structure of molecules (RUSSELL and EDMONDSON, 1997; GLISH and VACHET, 2003).

### 4.2.4. BIOAUTOGRAPHIC GUIDED-FRACTIONATION FOR ISOLATION OF BIOACTIVE COMPOUND FROM PLANT CRUDE EXTRACT

In the isolation of biologically active compounds from natural sources, it is evident that rapid and efficient detection of such compounds is of critical importance during the discovery processes (RIOS *et al.*, 1988; HOSTETTMANN *et al.*, 1995). Bioautography is one of the most important detection methods for new or unidentified compounds based on the biological effects of the substances under

study (RIOS *et al.*, 1988). The bioautography procedure involves an agar-diffusion technique in which antibacterial compounds are transferred from the chromatographic plate to an inoculated agar plate.

Bioautography can be divided into three general methods: contact, direct and immersion bioautography (RIOS *et al.*, 1988). Contact bioautographic is based on the diffusion of separated compounds through TLC plates which are placed on the surface of large nutrient agar plates inoculated with micro-organisms that are sensitive to antibiotics. Antibiotics diffuse into the agar layer and inhibit the growth of the test micro-organisms. In direct bioautography, a micro-organism suspension in liquid medium is sprayed on a developed chromatoplaque after removing the solvents (RIOS *et al.*, 1988). With the immersion bioautography method, a solidified agar is used with a dried TLC plate, while other agar medium is cooled to 48 °C and seeded with a test micro-organism and poured on surface of the TLC plate (BETINA, 1973). Contact bioautography is the method most often employed but it has certain disadvantages and requires the use of suitable microbiological equipment. The problem of the differential diffusion of compounds from the chromatogram to the agar plate is simplified by direct bioautographic detection (RIOS *et al.*, 1988). In both immersion and direct bioautography, inhibition zones are observed directly on the TLC plate (RIOS *et al.*,1988).

## 4.3. ISOLATION OF ANTIBACTERIAL COMPOUNDS FROM AN ETHYL ACETATE ROOT EXTRACT OF *TERMINALIA SERICEA*

*Terminalia sericea* is a shrub or medium-sized deciduous or semi-deciduous tree found abundantly in the tropical and warm temperate regions and is characteristic of sandy savannah areas in Africa (Chapter two, section: 2.2.9 and Figure 2.10). Dried fruits of *Terminalia sericea* are used traditionally in Africa for the treatment of tuberculosis (NEUWINGER, 1996). A decoction of the root and bark of the tree is used in South Africa in multi-component preparations to treat bacterial infections, dysentery and infected wounds (Chapter two Table 2.1).

A decoction of the root is also used for stomach troubles, diarrhoea, wounds, inflammation and sexually transmitted diseases (NEUWINGER, 1996; MOSHI and MBWAMBO, 2005; TSHIKALANGE *et al.*, 2005). Both aqueous and organic extracts from leaves, root and bark of *Terminalia sericea* were found to have antibacterial activity (MOSHI and MBWAMBO, 2005). Several pentacyclic triterpenoids have been isolated from *Terminalia* species, of which sericic acid and a sericoside are the main compounds in the root and reported to have antimicrobial activity (BRUNETON, 1995). However, relatively, few publications have reported on the bioactive compounds of the tree (BOMBARDELLI *et al.*, 1974; STEENKAMP *et al.*, 2004).

The widespread use of *Terminalia sericea* in indigenous medicine for different ailments, as well as the significant antibacterial and anti-inflammatory activities exhibited by extracts obtained from roots of *Terminalia sericea* in this study (Chapter three) justified further attempts to isolate and identify the active compound(s) from the ethyl acetate root extracts of this tree.

### 4.3.1. MATERIAL AND METHODS:

#### 4.3.1.1. PLANT MATERIAL AND BULK EXTRACTION

Roots of *Terminalia sericea* Burch. Ex. DC (Combretaceae) were collected in August 2003 from the National Botanical Garden Pietermaritzburg (Chapter two section 2.2.9 and Figure 2.10). A voucher specimen (Eldeen 3) was deposited in the herbarium of The University of KwaZulu-Natal Pietermaritzburg. The collected material was dried in an oven at 50 °C for 7 days and grounded to a powder. The dried and powdered roots (327 g) were extracted with 1.5 L ethyl acetate in a volumetric flask (5000 ml) by sonication for 1 h and then shaken in an orbital shaker overnight. This procedure was repeated thee times using the same plant material. The extract was filtered using Whatman No.1 filter paper and concentrated to dryness under reduced pressure.

### 4.3.1.2. BIOASSAY-GUIDED FRACTIONATION FOR ISOLATION OF ANTIBACTERIAL COMPOUND(S) FROM THE ETHYL ACETATE ROOT EXTRACT OF *TERMINALIA SERICEA*

#### i) VACUUM LIQUID CHROMATOGRAPHY (VLC)

Vacuum liquid chromatography (VLC) using silica gel (Merck 230-400 mesh) was employed as the first isolation step. A column 27 cm in length with an internal diameter of 6 cm was packed with 150 g silica gel. A gradient of hexane:ethyl acetate solvent system (400 ml per fraction) was used. Initially 100% hexane was used, and then further reduced to 50% hexane in 5% increments. Finally 100% (400 ml) ethyl acetate and followed by 100% (400 ml) methanol was used. The 13 fractions obtained were collected and dried under reduced pressure.

The dried fractions were redissolved at a concentration of 40 mg/ml using ethyl acetate. Aliquots of all the fractions (5 µl) were spotted on two TLC plates (20X20 cm silica gel 60 $F_{254}$, 0.25 mm) and developed with hexane: ethyl acetate (1:1 v/v). The plates were dried and then viewed under $UV_{366}$ and $UV_{254}$. Visible bands were marked. One plate was used as a reference while the second plate was used for direct bioautographic assay.

#### ii) DIRECT BIOAUTOGRAPHIC ASSAY

The direct bioautographic assay was performed according to the method of SLUSARENKO *et al.* (1998). An overnight culture of *Staphylococcus aureus* strain was grown in Mueller-Hinton (MH) broth (2.1 g/100 ml) in a water bath at 37 °C. This was then centrifuged at 3000 g for 10 min. The pellet was re-suspended in 10 ml of MH broth and sprayed onto the thin layer chromatography (TLC) plate. The plate was then placed on damp tissue in a metal tray covered with plastic to allow 100% humidity. The tray was left in the oven at 35 °C

overnight to allow the bacteria to grow. The following day, a 2 mg/ml INT (*p*-iodonitrotetrazolium) solution was sprayed onto the bacteria-covered plate which was then placed back in the oven in 100% humidity for 30 min. The INT stained the bacteria dark red so that it was clearly visible where bacterial growth had not been inhibited. The band that remained in a clear zone possessed compounds with antibacterial activity. Fractions with antibacterial activity which seemed to be caused by the same compounds (having the same $R_F$ value) were combined. The most active antibacterial fraction was used for further purification using gravity assisted column chromatography.

### iii) GRAVITY-ASSISTED COLUMN CHROMATOGRAPHY

Dried residue (279 mg) obtained from the most active antibacterial fraction as detected using the bioautographic assay was applied to gravity assisted column chromatography. A column 75 cm in length with an internal diameter of 3 cm was packed with 200 g silica gel (Merck 230-400 mesh). A gradient starting at 19:1, 9:1, 4:1, 3:2 hexane: ethyl acetate (750 ml) followed by 1:1 hexane: ethyl acetate was used. The column was finally washed with 750 ml 100% ethyl acetate. A fraction collector (Gilson FC203B) was set to collect fractions of approximately 10 ml each. Fractions obtained were placed in volumetric vials and air dried. TLC combined with the bioautography assay was again used to detect the active compounds.

### iv) PREPARATIVE THIN LAYER CHROMATOGRAPHY

Dried fractions collected during gravity-assisted column chromatography were re-dissolved at a concentration of 40 mg/ml using ethyl acetate and spotted on two TLC plates (5 µl per spot). The plates were developed to a height of 18 cm using the solvent system hexane: ethyl acetate (3:1 v/v). As before, the plates were viewed under $UV_{366}$ and $UV_{254}$ and visible bands were marked. One plate

was used as a reference and the second was subjected to the bioautographic assay. Similar fractions were combined as described earlier. Five µl of the fraction showing the highest antibacterial activity were spotted on a TLC plate and run in different solvent systems to achieve the best separation. The remained of the combined fraction (66 mg) was loaded onto two TLC plates (20X20 cm silica gel 60 $F_{254}$, 0.25 mm) and run in the desired solvent. Bands showing antibacterial activity were scraped off the plates and applied to a small column packed in two pasture pipettes with glass wool and celite and eluted with distilled methanol. Antibacterial activity of the isolates was determined using the bioautographic assay. The isolates were sent to the School of Chemistry, University of KwaZulu-Natal, Howard College Campus, Durban for identification.

### 4.3.2. RESULTS

#### 4.3.2.1. BULK EXTRACTION

The bulk extraction with ethyl acetate of the dried, powdered roots (327 g) of *Terminalia sericea* yielded 7 g residue. This residue was separated using vacuum liquid chromatography.

#### 4.3.2.2. BIOASSAY-GUIDED FRACTIONATION FOR ISOLATION OF ANTIBACTERIAL COMPOUND(S) FROM THE ETHYL ACETATE ROOT EXTRACT OF *TERMINALIA SERICEA*

The residue obtained from the bulk extraction was separated using VLC from which thirteen fractions were collected. The bioautography plate of these fractions against *Staphylococcus aureus* is shown in Figure 4.1. White areas indicate the presence of antibacterial compounds as the lack of bacterial growth cannot convert the INT salt to a red product. Three combinations were made from fractions that appeared to have antibacterial activity caused by the same compounds as follows: (**1**) combination of fractions 3 and 4 with $R_F$ value of 0.9

(20 mg), (**2**) combination of fractions 5 to 11 (279 mg). These fractions appeared to have at least three active compounds with $R_F$ values of 0.8, 0.5 and 0.3 respectively and (**3**) fraction 12 (91 mg).

Combination **2** (fraction 5-11 (279 mg) was chosen for further separation using the gravity assisted column chromatography. The bioautographic plate of the fractions obtained from the gravity assisted column chromatography is shown in Figure 4.2. Similar fractions were combined. The fraction showing the highest antibacterial activity (66 mg) was loaded on two TLC plates and developed using solvent system hexane: ethyl acetate: acetic acid (2:1:0.05 v/v). Three antibacterial substances were collected. They were labeled A (14 mg), B (3 mg) and C (2 mg). A bioautographic plate of the isolated substances tested against *Staphylococcus aureus* is shown in Figure 4.3. Due to insufficient quantity of substance B and C, only substance A could be chemically elucidated.

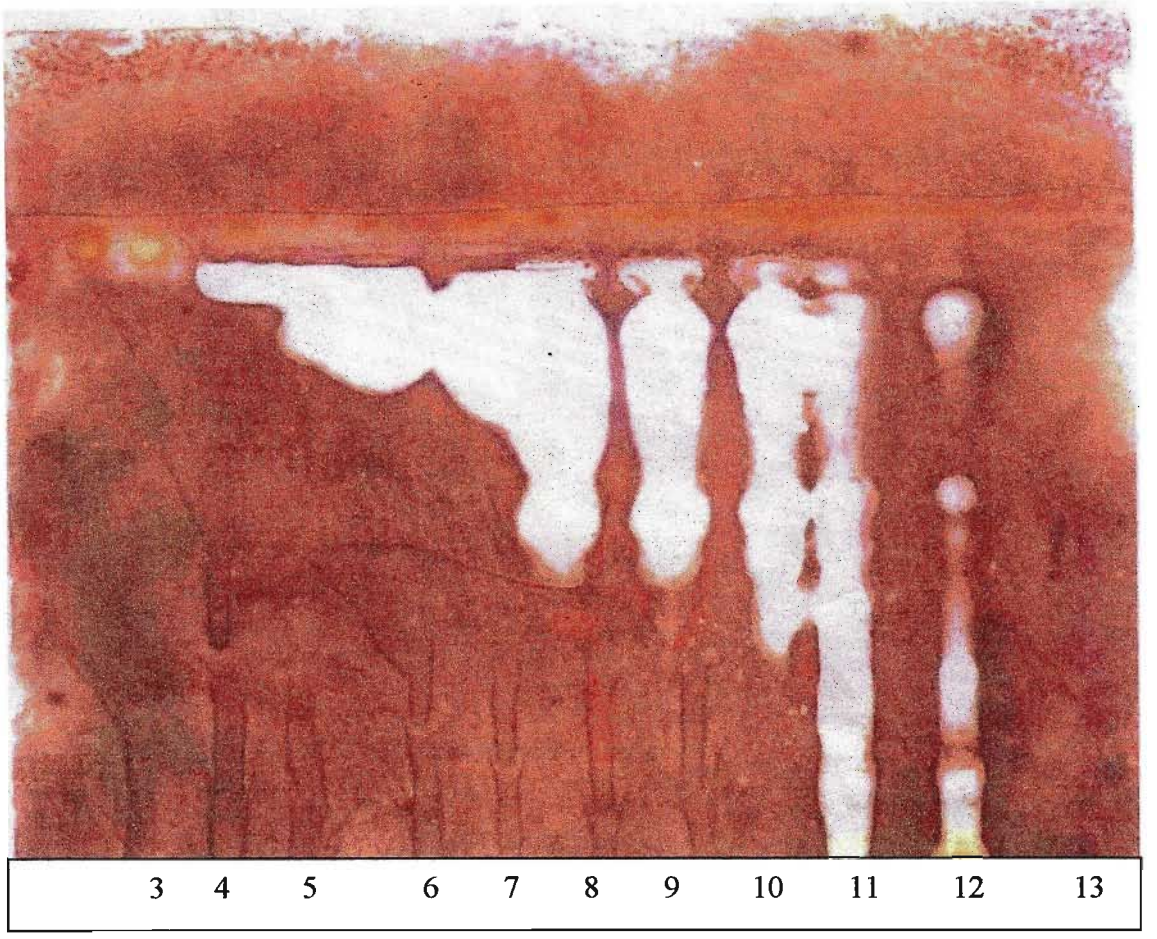


**Figure 4.1** Antibacterial activity (white spots) of the thirteen fractions collected from a VLC against *Staphylococcus aureus* as detected in the bioautographic assay. The TLC plate was developed using hexane: ethyl acetate (1:1 v/v) solvent system.

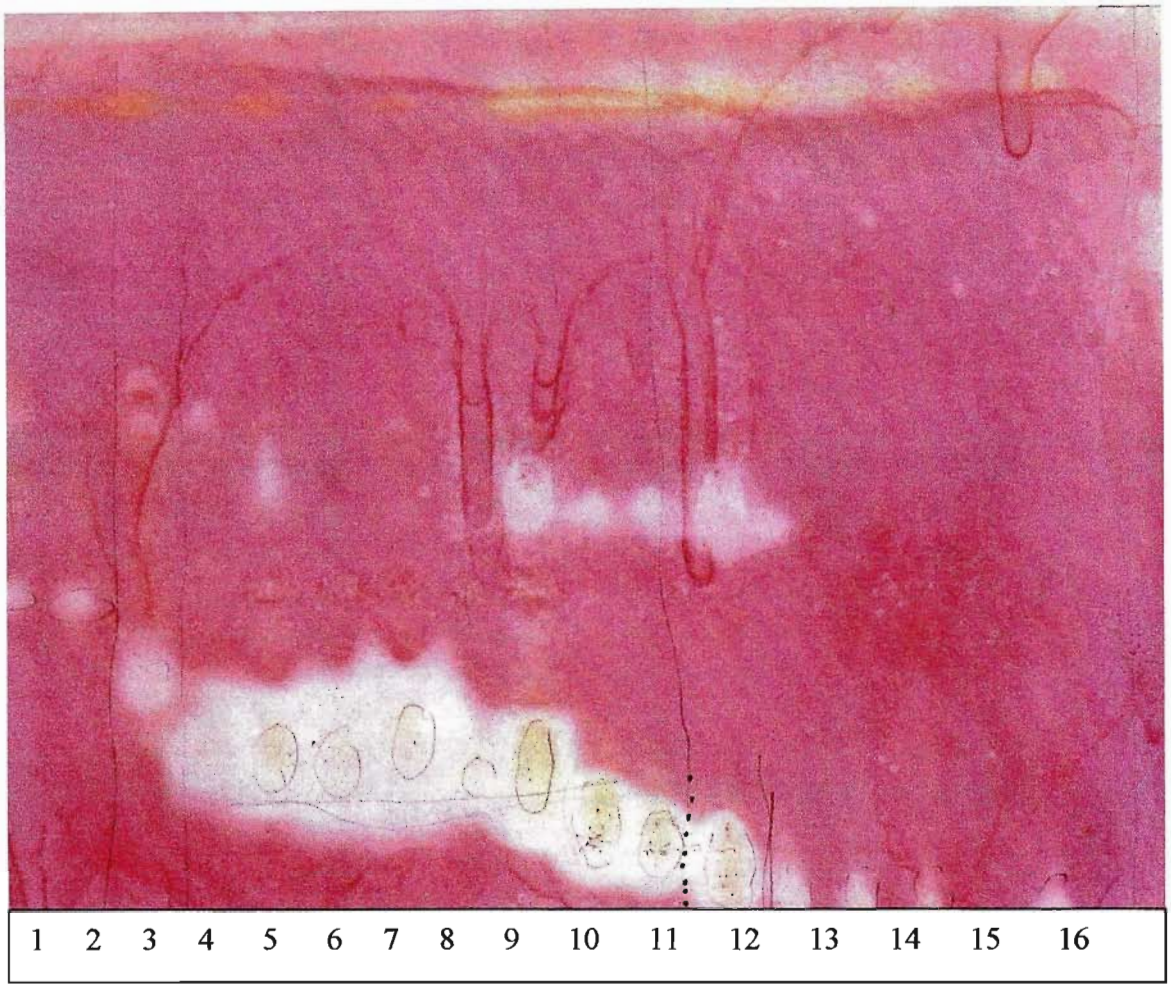


**Figure 4.2** Antibacterial activity of the fractions collected from gravity-assisted column chromatography against *Staphylococcus aureus* as detected during the bioautographic assay. The TLC plate was developed using solvent system hexane: ethyl acetate (1:1v/v).

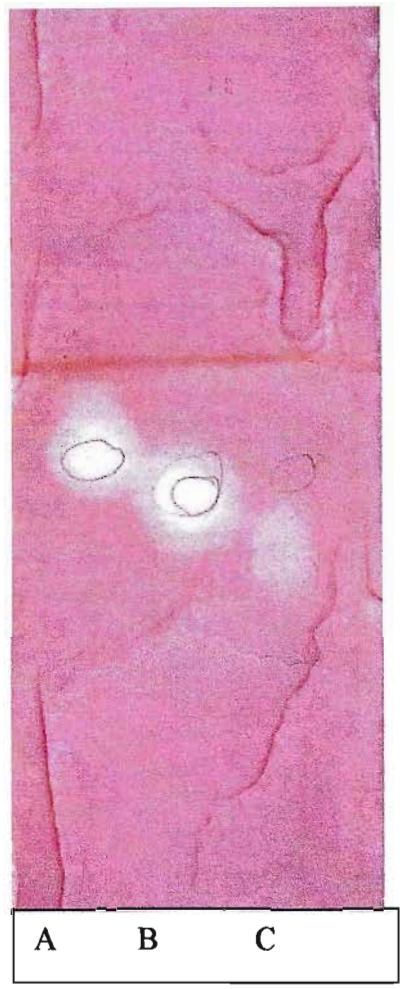


**Figure 4.3** Antibacterial activity of the three isolated substances (A, B and C) against *Staphylococcus aureus* as detected during the bioautographic assay where the TLC plate was developed in hexane: ethyl acetate (2:1 v/v).

## 4.4. IDENTIFICATION OF THE ACTIVE COMPOUND ISOLATED FROM THE ETHYL ACETATE ROOT EXTRACT OF *TERMINALIA SERICEA*

The pure compound isolated from the ethyl acetate root extract of *Terminalia sericea* was identified as anolignan B (Figure 4.4) using 2 D NMR and mass spectrometric analysis (Figures 4.5, 4.6, 4.7, 4.8 and 4.9.) and then confirmed by comparing NMR data against literature (RIMANDO *et al.*, 1994). LRMS of the isolated compound indicated a molar mass of 266 g $mol^{-1}$. The $^{13}C$ NMR spectrum showed only seven resonances, including those ascribed to a *para*-disubstituted aromatic ring (δ 156.5 (C), δ 132.0 (C), δ 130.7 (2CH), δ 115.9 (2 CH)), an alkyl methylene group (δ 40.9 ($CH_2$)) and a terminal methylene group (δ 147.9 (C), δ 115.4 ($CH_2$). The $^1H$ NMR spectrum, in conjunction with the HSQC and COSY spectra, confirmed the *para*- disubstituted aromatic ring with a pair of doublets at δ 6.92 (*J*=8.3 Hz) and δ 6.66 (*J*=8.3 Hz) each integrating to two protons. The HMBC and NOESY spectra indicated that a $CH_2$ group (δ 3.47) was joined to the aromatic ring and the protons of this methylene group showed vinylic coupling with the non-equivalent protons of a terminal methylene group ( δ 5.21, δ 4.84). A hydroxyl group was placed at the *para*-position on the benzene ring. This accounted for a molecular formula of $C_9H_9O$ or mass of 133, half of the found mass. Thus the molecule had to be symmetrical and in the absence of a proton at C-2', could only be joined to the second part at this position to give anolignan B.

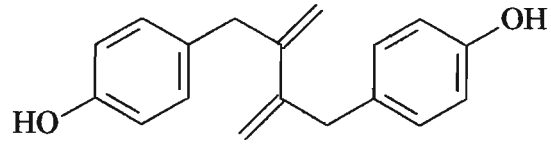


**Figure 4.4** Structure for anolignan B (2,3-bis-(4-hydroxybenzyl)-1,3-butadiene) isolated from ethyl acetate root extract of *Terminalia sericea*

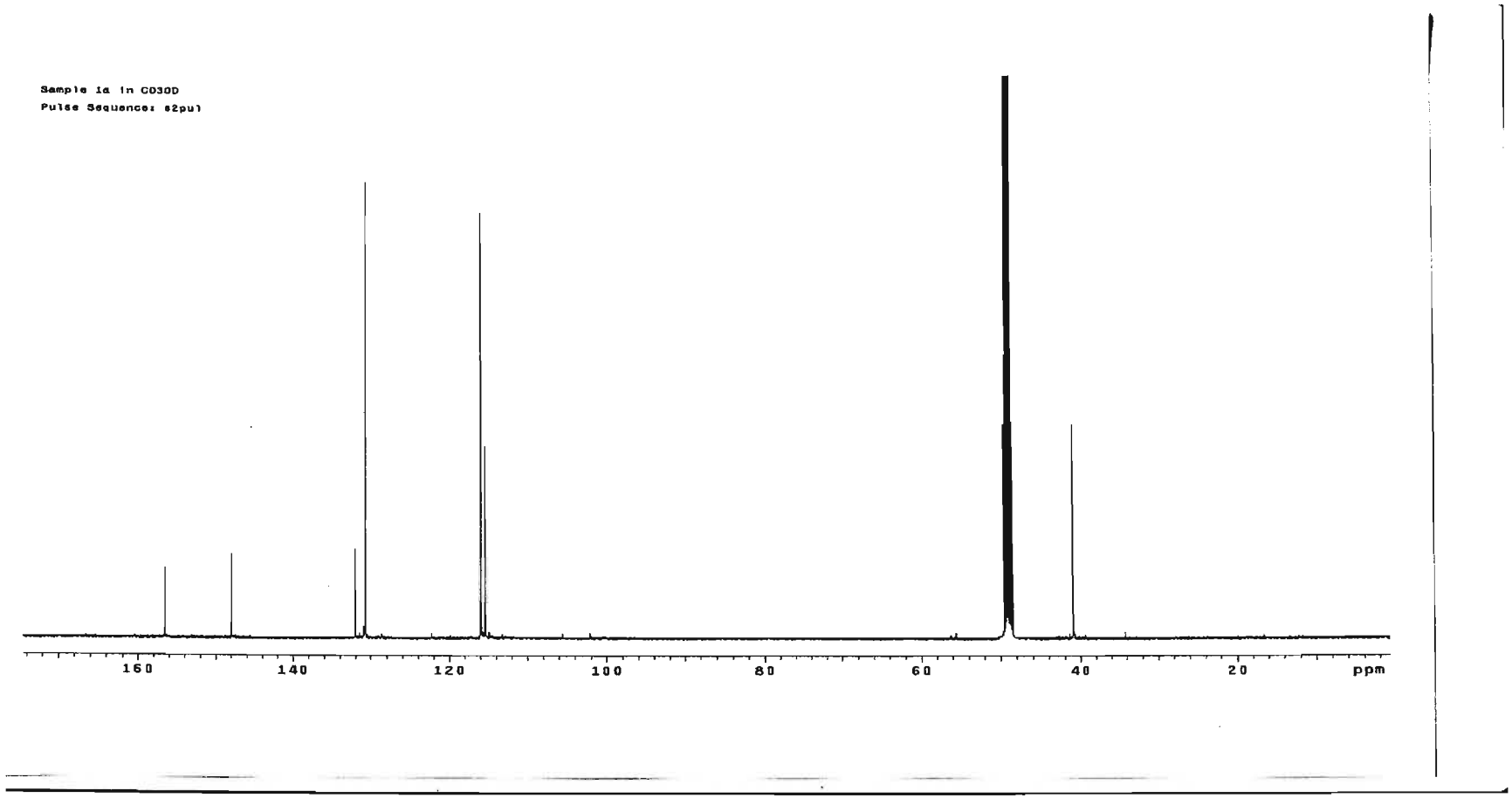


**Figure 4.5** $^{13}C$ spectrum of anolignan B (2,3-bis-(4-hydroxybenzyl)-1,3-butadiene) in $CD_3OD$

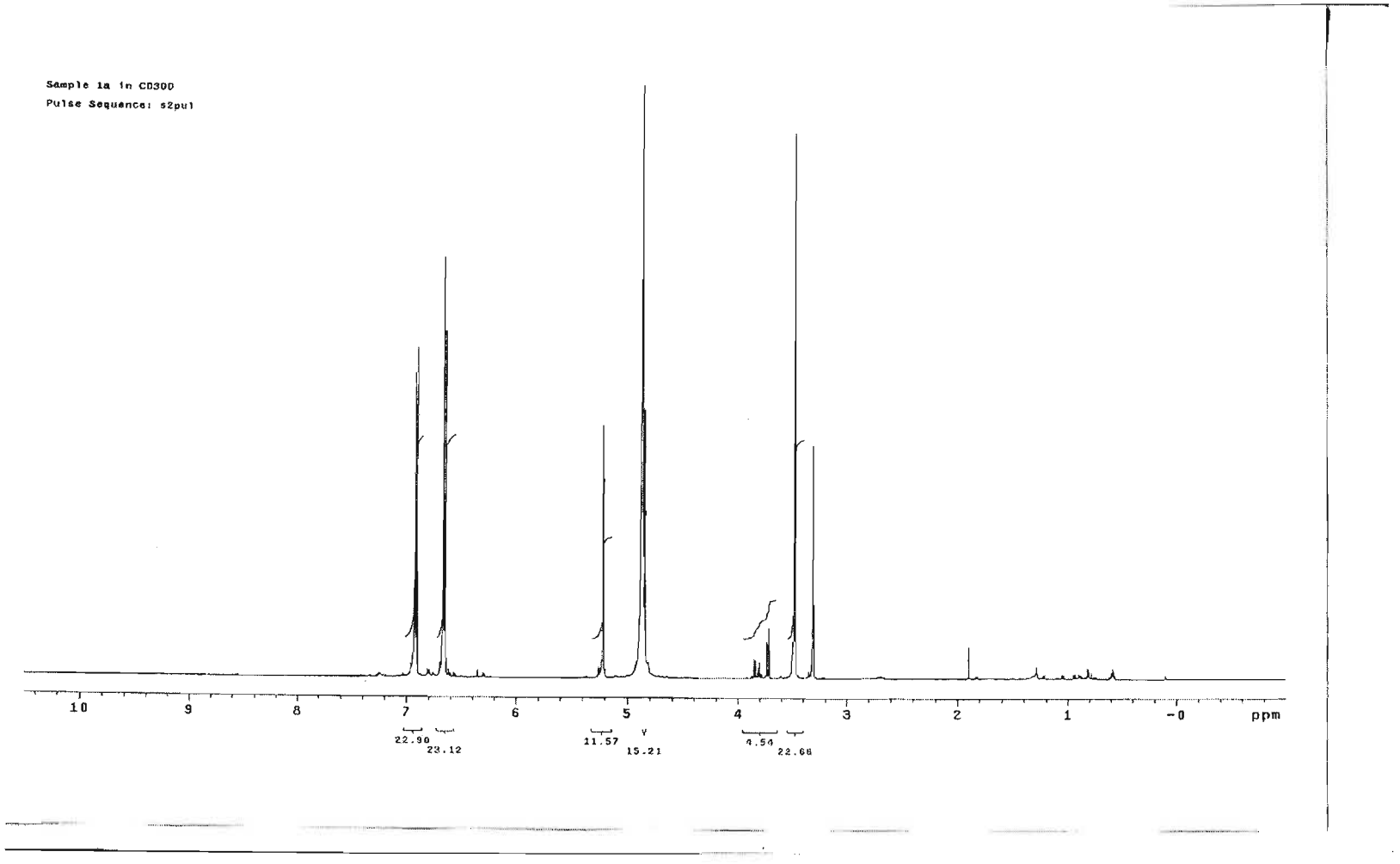


**Figure 4.6** $^{1}H$ spectrum of anolignan B (2,3-bis-(4-hydroxybenzyl)-1,3-butadiene) in $CD_3OD$

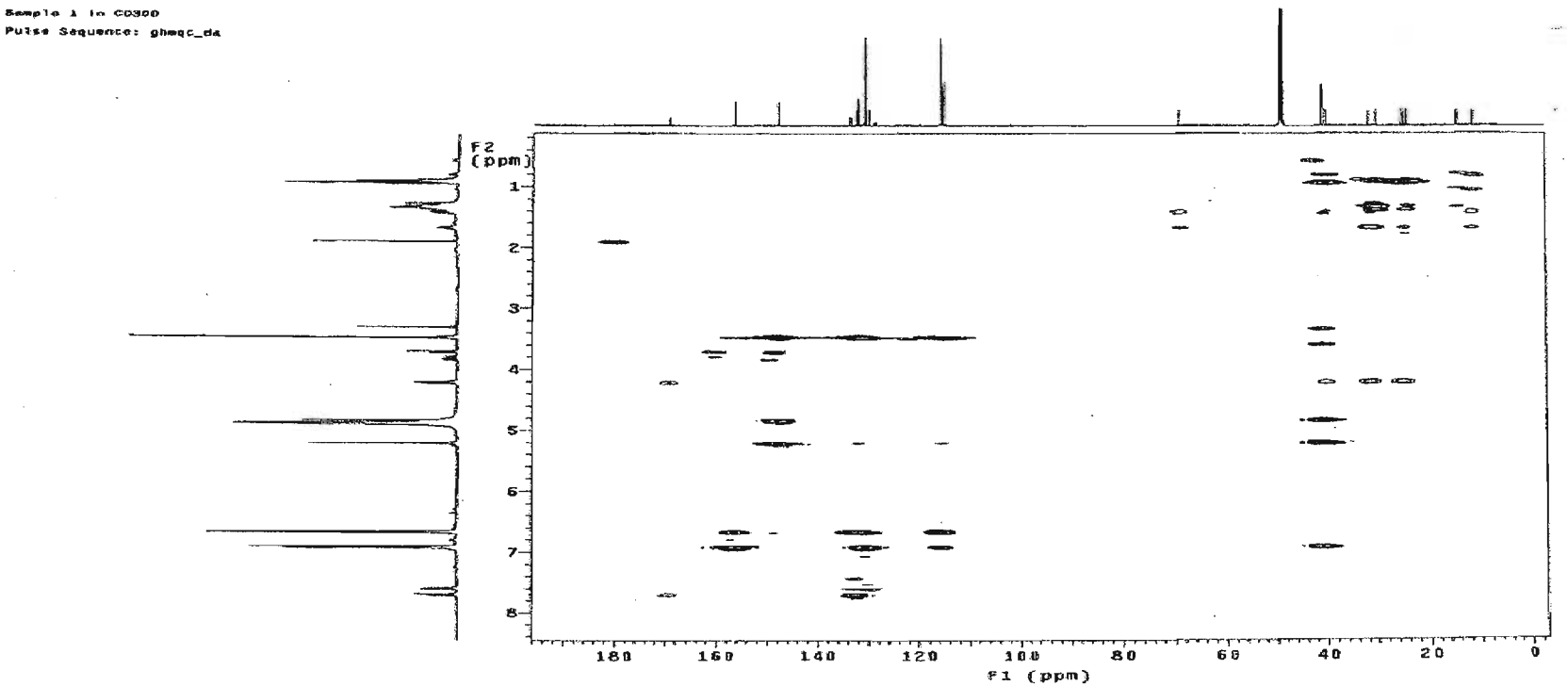


**Figure 4.7** ghmqc spectrum of inolignan B (2,3-bis-(4-hydroxybenzyl)-1,3-butadiene) in $CD_3OD$

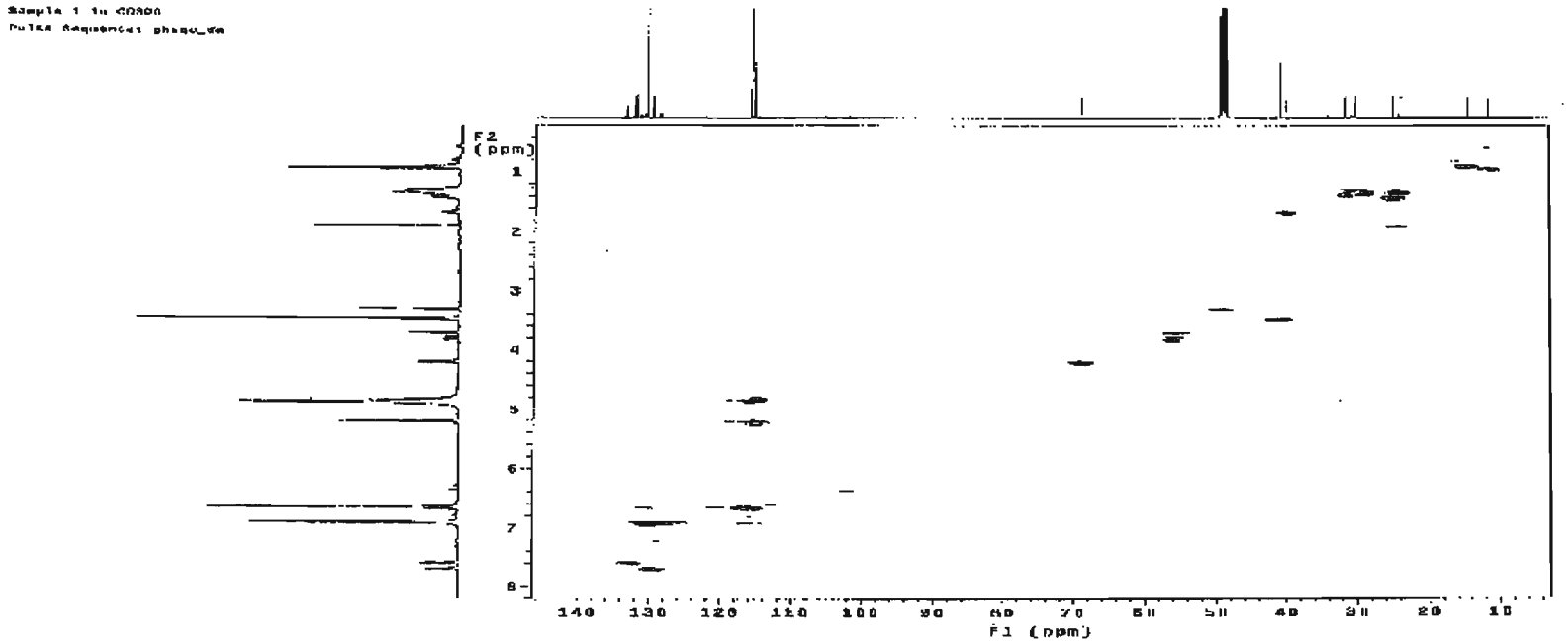


**Figure 4.8** ghsqc spectrum of anolignan B anolignan B (2,3-bis-(4-hydroxybenzyl)-1,3-butadiene) in $CD_3OD$

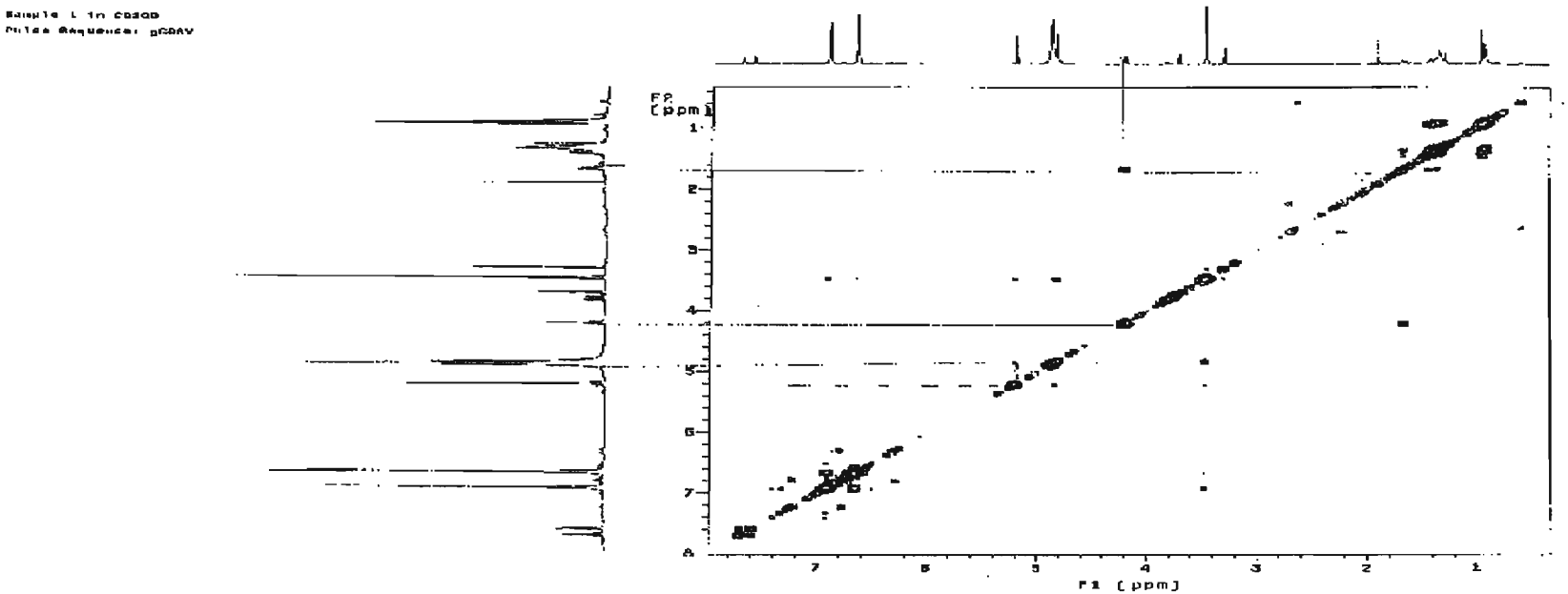


**Figure 4.9** gCOSY spectrum of anolignan B anolignan B (2,3-bis-(4-hydroxybenzyl)-1,3-butadiene in $CD_3OD$

## 4.5. DETERMINATION OF BIOLOGICAL ACTIVITY OF ANOLIGNAN B

### 4.5.1. ANTIBACTERIAL ACTIVITY

#### MICRO-DILUTION ANTIBACTERIAL ASSAY

The same serial dilution technique described by ELOFF (1998) as mentioned earlier in Chapter three (Section 3.2.1) was used to determine the minimum inhibitory concentration (MIC) for antibacterial activity of anolignan B. The pure compound was redissolved to a concentration of 1 mg/ml with ethanol then serially diluted (two fold) with 100 µl sterile distilled water before adding 100 µl of each of the tested bacteria.

### 4.5.2. ANTI-INFLAMMATORY ACTIVITY

#### CYCLOOXYGENASE ASSAYS

Anti-inflammatory activity anolignan B was determined using both the COX-1 and COX-2 assays as described earlier in Chapter three (Section 3.2.2). The percentage inhibition of anolignan B was calculated by comparing the amount of radioactivity present in the sample to that in the solvent blank. Regression analysis was used to calculate $IC_{50}$ values (concentration of an active compound resulting in a 50% reduction in activity of a micro-organism or enzyme target) using GraFit soft ware version 5 for Microsoft ® Windows. Three different concentrations of anolignan B were tested in each bioassay. In the COX-1 the concentrations tested were 3.7, 1.8 and 0.9 mM. In the COX-2 assay the concentrations of the anolignan B tested were 9.2, 4.6 and 2.3 mM.

### 4.5.3. GENOTOXICITY TEST

#### AMES TEST

The potential mutagenic effects of anolignan B was detected using the Ames test. The Ames assay was performed with *Salmonella typhimurium* strain TA98 using the plate incorporation procedure described by MARON and AMES (1983) as mentioned earlier in Chapter three (Section 3.2.4). The compound was tested at three different concentrations: 100, 50 and 25 µg/ml.

## 4.6. RESULTS

### 4.6.1. ANTIBACTERIAL ACTIVITY

The minimal inhibitory concentration values (MIC) for antibacterial activity of the tested pure compound obtained using the micro-dilution assay, are shown in Table 4.1. The compound was active against both Gram-negative and Gram-positive bacteria. MIC values obtained against Gram-positive bacteria *Bacillus subtilis* and *Staphylococcus aureus* were 3.8, and 7.7µg/ml respectively. With Gram-negative bacteria, the compound had MIC values of 31, and 15 µg/ml towards *Escherichia coli* and *Klebsiella pneumoniae* respectively.

**Table 4.1** Minimum inhibitory concentration (µg/ml) for antibacterial activity of anolignan B compared to the neomycin standard

| Bacterial strain | MIC (µg/ml) | |
|---|---|---|
| | Anolignan B | Neomycin |
| *Bacillus subtilis* | 3.8 | 0.097 |
| *Staphylococcus aureus* | 7 | 1.56 |
| *Escherichia coli* | 31 | 3.125 |
| *Klebsiella pneumoniae* | 15 | 0.78 |

### 4.6.2. ANTI-INFLAMMATORY ACTIVITY

Anti-inflammatory activity of anolignan B in the COX-1 and COX-2 assays expressed as percentage inhibition and $IC_{50}$ values are given in Table 4.2. At a concentration of 3.7 mM, the compound showed strong inhibitory activity against COX-1 (81%). The $IC_{50}$ value was 1.5 mM in the COX-1 assay. However, anolignan B showed weaker inhibitroy activity against COX-2, with an $IC_{50}$ value of 7.5 mM.

**Table 4.2** Inhibition (%) of prostaglandin synthesis by anolignan B and $IC_{50}$ values as determined by cyclooxygenase (COX-1 and COX-2) assays. Indomethacin was tested at concentrations of 0.005 mM and 0.2 mM against COX-1 and COX-2 respectively.

| Compound | COX-1 | | COX-2 | |
|---|---|---|---|---|
| | Inhibition (%) (3.7 mM) | $IC_{50}$ value (mM) | Inhibition (%) (9.2 mM) | $IC_{50}$ value (mM) |
| Anolignan B | 81 | 1.5 | 71 | 7.5 |
| Indomethacin | 78 | 0.003 | 70.2 | 0.186 |

### 4.6.3. MUTAGENIC EFFECTS

Results obtained from the mutagenicity test of anolignan B as detected by Ames test using the *Salmonella* strain TA98 are shown in Table 4.3. Based on the number of induced revertant colonies, the compound tested would be mutagenic if the number of the induced revertant colonies is double the revertant colonies of the blank (negative control). Accordingly, no mutagenic effect was detected by the compound in this test.

**Table 4.3** Mutagenic effects of anolignan B as determined by the Ames test using *Salmonella typhimurium* strain TA 98. The number of revertant colonies induced was recorded.

| Compound tested | Concentration (mg/ml) tested | | |
|---|---|---|---|
| | 1 | 0.5 | 0.25 |
| Anolignan B | 22±8 | 29±4 | 31±3 |
| Positive control (2 µg/ml) | 167±9 | | |
| Negative control (Blank) | 28±6 | | |

## 4.7. DISCUSSION AND CONCLUSION

Bioassay-guided fractionation of the ethyl acetate extract of *Terminalia sericea* roots using column chromatography followed by preparative TLC led to the isolation of anolignan B. Anolignan B was first isolated from *Anogeissus acuminate* and reported as a constituent acting with anolignan A to inhibit the enzyme HIV-1 reverse transcriptase. It was also reported to have *in vitro* cytotoxic effects against a fibro sarcoma cell line (RIMANDO *et al.*, 1994). As *Anogeissus acuminate* taxonomically belongs to the Combretaceae, isolation of anolignan B from *Terminalia sericea* supports the idea that plants belonging to the same genus or family may have similar types of secondary compounds. Therefore, taxonomy can also provide useful clues when searching for bioactive compounds (GENTRY, 1993).

In the antibacterial test, anolignan B showed stronger activity against Gram-positive than Gram-negative bacteria. Gram-negative bacteria which are responsible for a large number of infectious diseases have a unique outer membrane that contains lipopolysaccharides which render them impermeable to certain antibacterial compounds (CLEMENTS *et al.*, 2002).

As anolignan B was previously reported to be active against HIV-1 reverse transcriptase enzyme, it is interesting that it also showed inhibitory effects against COX-1 and COX-2 enzymes in the present study. Suppression of COX-1 might cause adverse side-effects including a reduction in mucosal blood flow and mucous secretion, delay in the healing of ulcers and a reduction in renal blood flow (mentioned earlier in Chapter three).

$IC_{50}$ values obtained in this study for indomethacin were comparable to previous reported values in the literature (TAYLOR and VAN STADEN 2002) where, 0.0031 and 0.188 mM were recorded for COX-1 and COX-2 respectively.

Isolation of anolignan B from *Terminalia* species and the antibacterial and anti-inflammatory activities observed in this work have not been reported previously and could therefore be recorded as novel biological activities for this compound. This is an interesting result based on the fact that novelty of chemical structure is less important than the novelty of biological activity (CLARK and HUFFORD, 1993). The previously reported biological activities of *Terminalia sericea* were mainly attributed to triterpenoids, saponins and tannins (BOMBARDELLI *et al.*, 1974; CLARK and HUFFORD, 1993; BRUNETON, 1995).

From the results presented here, anolignan B did not have comparable activities to the antibiotic drug neomycin, nor to indomethacin which is a standard anti-inflammatory drug. However as no potential mutagenic effect was detected, the observed biological activity could be considered as a good scientific base for evaluation and formulation of the traditional uses of *Terminalia sericea* as an antibacterial and anti-inflammation agent. It validates claims made on safety, efficacy and quality of such uses. Thus, the obtained results contribute positively to the active movement towards integration of traditional healers into official health care systems (NEUWINGER, 1996; MOSHI and MBWAMBO, 2005; TSHIKALANGE *et al.*, 2005).

On the other hand, our findings highlight the importance of studying the biological activity of secondary metabolites of trees in terms of non-timber forest products. As most species of trees are still much less surveyed for chemical or biological active constituents when compared to lower plants, such studies can lead to the discovery of new sources of valuable products for both traditional and industrial applications.

# CHAPTER 5
# GENERAL CONCLUSION

## 5.1. INTRODUCTION

Plants have played an integral part in the evolution of human cultures. The physical and chemical properties of plants provide a wealth of raw materials which fulfill many medicinal and material requirements. Trees in particular are remarkable in their ability to produce a vast array of diverse metabolites varying in chemical complexity and biological activity. These products have historically served as templates for the development of many important classes of drugs such as antibiotics, heart drugs, inflammation, enzymes, ulcer treatments amongst many others.

Traditional knowledge is becoming increasingly recognized as a valuable source of information on the use of and ecology of many plant species. Western understanding of traditional knowledge has changed considerably as various approaches to its study have been developed.

While much of the early research was of a predominantly utilitarian nature and discussed only the ways in which particular plants were used within traditional communities, recently there have been a number of attempts to integrate this valuable ethnobotanical knowledge with the Western scientific system through formal collaboration between traditional and non-traditional researchers.

## 5.2. BIOLOGICAL ACTIVITY OF PLANT EXTRACTS OBTAINED FROM TREES USED IN SOUTH AFRICAN TRADITIONAL MEDICINE

Traditional healers in South Africa and Africa generally used plant parts or combinations of plant parts from the studied trees and others for treating

different ailments which can be classified as infectious and/or inflammation disorders. The screening of plant extracts in this study was motivated by the necessity of a scientific evaluation of the traditional remedies as well as the potential discovery of new drugs that could be developed for use in clinical medicine.

Results obtained from the antibacterial bioassays presented in Chapter three showed different levels of activities against Gram-positive and Gram-negative bacteria by the plant extracts screened in this study. Gram-negative bacteria which are responsible for a large number of infectious diseases have a unique outer membrane that contains lipopolysaccharides which render them impermeable to certain antibacterial compounds. *Staphylococcus aureus* (Gram-positive) was found to be responsible for many incidents of food contamination resulting in illnesses such as vomiting, abdominal cramps and diarrhea. Some of the plant extracts (*Acacia nilotica* and *Terminalia sericea*) showed antibacterial activity against both Gram-positive and Gram-negative bacteria at low MIC values. This gave an indication of the presence of promising antibacterial compounds to be isolated.

Results obtained from the anti-inflammatory testing showed that 70% of the investigated plant extracts inhibited both COX-1 and COX-2 (Chapter three). This implied that if these plants are used in traditional medicine, they could potentially cause negative side effects such as blocking COX-1 activity.

Weak activity observed by some of the investigated plant extracts does not necessarily mean absence of bioactive agents or that the plants are inactive. Active compound(s) may be present in insufficient quantities in the crude extracts to show activity with the dose levels employed. Lack of activity in this case can only be proven by testing high concentrations. On the other hands, if the active principle is present in high enough quantities, there could be other constituents

exerting antagonistic effects or negating the positive effects of the bioactive agents.

No potential mutagenic effects were observed in the investigated plant extracts (Chapter three). This does not necessarily mean the absence of adverse effects that justifies their use as a safe traditional medicine. Toxicity and mutagenicity of plants and their active compounds need to be better understood due to the complexity of mutation mechanisms. Given the potential exposure of humans to natural products from plants as food and/or beverages, accurate evaluation of mutagenic activity of medicinal plants using developed and sophisticated tools are needed.

Generally the results obtained are in line with the traditional uses of the investigated plants as crude antibacterial and anti-inflammatory drugs. However, traditional healers seldom use a single plant in their extracts. In many cases the therapeutic benefits are attributed to the consumption of plant mixtures in which different plant parts are prepared and/or consumed in combination or in sequence. On the other hand, organic solvents are used widely in laboratory tests while water is a major solvent used in traditional medicine. Therefore, it is not easy to make an accurate comparison between the laboratory results and the extracts used by traditional healers.

## 5.3. ISOLATION OF AN ANTIBACTERIAL COMPOUND FROM THE ETHYLE ACETATE ROOT EXTRACT OF *TERMINALIA SERICEA*

The isolation and identification of the antibacterial compound (anolignan B) from the ethyl acetate root extract of *Terminalia sericea* was described in Chapter four. Anolignan B was first isolated from *Anogeissus acuminate* and reported as a constituent acting with anolignan A to inhibit the enzyme HIV-1 reverse transcriptase (Chapter four). As *Anogeissus acuminate* and *Terminalia sericea* both taxonomically belong to the Combretaceae, isolation of anolignan B from *Terminalia sericea* supports the idea that plants belonging to the same

genus or family may have similar types of secondary compounds. Taxonomy therefore can also provide useful clues when searching for bioactive compounds.

Bioassay-guided fractionation revealed the presence of other antibacterial compounds in the ethyl acetate root extract of *Terminalia sericea*. These deserve further study to identify the active compounds.

Isolation of anolignan B from *Terminalia* species and the antibacterial and anti-inflammatory activities observed in this work have not been reported previously and could therefore be recorded as novel biological activities for this compound. As no potential mutagenic effect was detected with anolignan B, the observed activity could be considered as a good scientific base for evaluation and formulation of the traditional uses of *Terminalia sericea* as an antibacterial and anti-inflammation agent (Chapters two, three and four). It partly validates claims made on safety, efficacy and quality of such uses.

## 5.4. CONCLUSION

Plants produce secondary metabolites as defenses against animals, parasites, bacteria, and viruses. Such chemicals had served as sources of medicinal agents for thousands of years, from these an impressive number of modern drugs have been isolated.

Results of this study support the idea that the most direct way to identify promising natural products is through ethnobotanical knowledge.

The results presented in this thesis represent an extensive investigation into some trees used in South African traditional medicine. The study provides scientific verification of the efficiency of the tested plants for their use in traditional medicine. The study also highlights the importance of secondary metabolites from trees as promising source of bioactive constituents.

One bioactive compound has been isolated and identified from the root of *Terminalia sericea.* There is much potential for further research in this field, as many of the investigated plants have showed interesting biological activities such as: *Acacia nilotica* subsp. *kraussiana* (antibacterial and anti-inflammatory activities) *Trichilia dregeana* (anti-inflammatory activity (COX-2)), and *Combretum kraussii* (anti-cholinesterase activity).

# REFERENCES


ABDELNABI, O.M., REISINGER, E.C., REINTHALER, F.F., STILL, F., FIBEL, U., KREIS, G.J. 1992. Antimicrobial activity of *Acacia nilotica* (L.) Willed ex Del. var. *nilotica* (Mimosaceae). Journal of Ethnopharmacology 37, 77-79.

ADDAE-MENSAH, I. 1992. Towards a Rational Scientific Basis for Herbal Medicine a Phytochemist's Two-Decades Contribution. Accra: Ghana University Press. ISBN 9964-3-0203-7.

ALZHEIMER'S DISEASE: Unraveling the Mystery. 2002. The Changing Brain in Alzheimer's Disease. U.S.A. Department of Health and Human Services, National Institutes of Health. NIH Publication Number: 02-3782.

ANAND, N., NITYANAND, S. 1984. Integrated approach to development of new drugs from plants and indigenous remedies. In: Krogsgaard, P., Christensen, S., Kofod, H. (Eds.), Natural Products and Drug Development. Copenhagen: Munksgaard. ISBN 87-1609563-4.

ARNOLD, T. H., PRENTICE, C. A., HAWKER, L. C., SNYMAN, E. E., TOMALIN, M., CROUCH, N.R., POTTAS-BIRCHER, C. 2002. Medicinal and Magical Plants of Southern Africa: an annotated checklist. Pretoria National Botanical Institute. ISBN 1-919795-62-6.

AYOUB, S.M.H. 1984. Polyphenolic molluscicides from *Acacia nilotica*. Planta Medica 50, 532-540.

BALANDRIN, M. F., KINGHORN, A. D., FARNSWORTH, N. R. 1993. Plant-derived natural products in drug discovery and development. An overview. In: Kinghorn, A.D., Balandrin, M.F. (Eds.), Human Medicinal Agents from

Plants. ACS Symposium Series 534. American Chemical Society, Washington, USA. ISBN 0-8412-2705-5.

BALANDRIN, M. F., KLOCKE, J. A., WURTELE, E. S., BOLLINGER, W. H. 1985. Natural plant chemicals: Source of industrial and medicinal materials. Science 228, 1154-1160.

BANDOW, J. E., BROTZ, H., LEICHERT, L. I. O., LABISCHINSKI, H., HECKER, M. 2003. Proteomic approach to understanding antibiotic action. Antimicrobial Agents and Chemotherapy 47, 948-955.

BAR-ON, P., MILLARD, C. B., HARD, M., DVIR, H., ENZ, A., SUSSMAN, J. L., SILMAN, I. 2002. Kinetic and structural studies on the interaction of cholinesterase with the anti-Alzheimer drug rivastigmine. Biochemistry 41, 3555-3564.

BAUTER, A.W., KIRBY, W.M.M., SHERRIS, J.C., TURCK, M. 1966. Antibiotic susceptibility test by a standardized single disc method. The American Journal of Clinical Pathology 45, 493-496.

BETINA, V. 1973. Bioautography in paper and thin-layer chromatography and its scope in the antibiotic field. Journal of Chromatography 78, 41-51.

BOEVE, B. F., MARAGANORE, D. M., PARISI, J. E. 1999. Pathologic heterogeneity in clinically diagnosed corticobasal degeneration. Neurology 53, 795-800.

BOMBARDELLI, E., BONATI, A., GABETTA, B., MUSTICH, G. 1974. Triterpenoids of *Terminalia sericea*. Phytochemistry 13, 2559-2562.

BRUNETON, J. 1995. Pharmacognosy, Phytochemistry, Medicinal Plant. Intercept, Hampshire. Paris: Lavoisier. ISBN: 2-7430-0028-7.

BUTLER, T. 2005. Shamans and Robots: Bridging the past and future of ethnobotany and bioprospecting. www.mongabay.com/tina.htm. A web site aims to raise interest in and appreciation of wild lands and wildlife.

CAO, Y., CHU, Q., YE, J. 2004. Chromatographic and electrophoretic method for pharmaceutically active compounds in *Rhododendron dauricum*. Journal of Chromatography B 812, 231-240.

CARR, J.D. 1976. The South African Acacias. Johannesburg: Conservation Press. ISBN 20.00.

CHANDRASEKHARAN, N.V., DAI, H., TUREPUROOS, K.L., EVANSON, N.K., TOMSIK, J., ELTON, T.S., SIMMONS, D.L. 2002. Cox-3, a cyclooxygenase-1 variant inhibited by acetaminophen and other analgesic/antipyretic drug: Cloning, structure, and expression. PNAS 99, 13926-13931.

CHEN, K. 1995. Trends in pharmacology. Science 16, 182-187.

CHROMATOGRAPHY. 2005. Introductory Theory. Organic Chemistry Lab-Lab Techiques Contents. Colorado. www.orgchem.colorado.edu/handbksupport/chrom.html.

CLARK, A. M., HUFFORD, C.D. 1993. Discovery and development of novel prototype antibiotics for opportunistic infections related to aquired immunodeficiency syndrome. In: Kinghorn, A.D., Balandrin, M.F. (Eds.), Human Medicinal Agents from Plants. ACS Symposium Series 534. American Chemical Society, Washington, USA. ISBN 0-8412-2705-5.

CLEMENTS, J. M., COIGNARD, F., JOHNSON, I., CHANDLER, S., PALAN, S., WALLER, A., WIJKMANS, J., HUNTER, M.G. 2002. Antibacterial activities and characterization of novel inhibitors of LpxC. Antimicrobial Agents and Chemotherapy 46, 1793-1799.

CLIVE, D., MCCUEN, R., SPECTOR, J. F.S., PIPER, C., MAVOURIM, K.H. 1983. Report of the Gene-Toxicity program on L5178Y. Mutation Research 115, 225-251.

COTTON, C. M. 1996. Ethnobotany Principles and Applications. Chichester, Sussex: Wiley. ISBN 047195537x.

COUNTE, L. A. 1996. Shaman pharmacetuticals approach to drug development. In: Balick, M.J., Elisabetsky, E., Laird, A.S. (Eds.), Medicinal Resources of the Tropical Forest- Biodiversity and Its Importance to Human Health. New York: Columbia University Press. ISBN (not found).

COUZINIER, J. P., MAMATAS, S. 1986. Basic and applied research in the pharmaceutical industry into natural substances. In: Barton, D., Ollis, W. D. (Eds.), Advances in Medical Phytochemistry. Proceedings of the International Symposium on Medicinal Phytochemistry, Morocco. London: John Libbey. ISBN 0-861960920.

CUNNINGHAM, A. B. 1988. An investigation of the herbal medicine trade in NATAL/KwaZulu. Investigational Report No 29. Pietermaritzburg: University of Natal, Institute of Natural Resources. ISBN 0.00.

DAVIES, N.M., GOOD, R. L., ROUPE, K.A., YÁÑEZ, J. A. 2004. Cyclooxygenase-3: axion, dogma, normaly, enigma or splice error? - not as easy as 1,3,3. Journal of Pharmaceutical Science 7, 217-226.

DEBNATH, A.K., LÓPEZ De COMPADRE, R.L., DEBNATH, G., SHUSTERMAN, A.J., HANSCH, C. 1991. Structure-activity relationship of mutagenic aromatic and hetroaromatic nitro compounds. Correlation with molecular orbital energies and hydrophobicity. Journal of Medicinal Chemistry 34, 786-797.

Di STASI, L. C., OLIVEIRA, G. P., CARVALHAES, M. A., QUEIROZ-JUNIOR, M., TIEN, O.S., KAKINAMI, S. H., REIS, M. S. 2002. Medicinal plants popularly used in the Brazilian tropical atlantic forest. Fitoterapia 73, 69-91.

ELDEEN, I. M.S. 2001. Study on the tannin contents of *Acacia nilotica* subsp. *tomentosa, nilotica* and *adansonii* with special reference to their traditional uses. M.Sc. Thesis. University of Khartoum, Sudan.

ELGORASHI, E. E., TAYLOR, J. L. S., MAES, A., VAN STADEN, J., De KIMPE, N., VERSCHAEVE, L. 2003. Screening of medicinal plants used in South African traditional medicine for genotoxic effects. Toxicology Letters 143, 195-207.

ELGORASHI, E. E., VAN STADEN, J. 2004. Pharmacological screening of six Amaryllidaceae species. Journal of Ethnopharmacology 90, 27-32.

ELLMAN, G.L., COURTNEY, D., ANDIES, V., FEATHERSTONE, R.M. 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology 7, 88-95.

ELOFF, J. N. 1998. A sensitive and quick microplate method to determine the minimal inhibitory concentration of plant extracts for bacteria. Planta Medica 64, 711-713.

ENZ, A., AMSTUTZ, R., BODDEKE, H., GMELIN, G, MALANOWSKI, J. 1993. Brain selective inhibition of acetylcholinesterase: a novel approach to therapy for Alzheimer's disease. Progress Brain Research 98, 431-438.

ETIKIN, N.L. 1986. Multidisciplinary Perspectives in Interpretation of Plants used in Indiginous Medicine and Diet. New York: Redgrave Publishing Company. ISBN 0-91317802-0.

FAJEMISIN, B. 2002. Acetylcholinesterase inhibitors: Donepezil, Rivastigmine, Tacrine or Galantamine for non Alzeheimer's dementia. In: Foxcroft, D., Muthu, V. (Eds.), STEER: Succinct and Timely Evaluated Evidence Reviews 2 (2). Wessex Institute for Health Research and Development, University of Southampton. http://www.signpoststeer.org/

FARNSWORTH, N. R. 1984. The role of medicinal plants in drug development. In: Krogsgaard, P., Christensen, S. B., Kofod, H. (Eds.), Natural Products and Drug Development. Munksgaard, Copenhagen. ISBN 87-16-09563-4.

FARNSWORTH, N. R. 1993. Biological approaches to the screening and evaluation of natural products. In: Rasoanaivo, P., Ratsimamanga-Urverg, S. (Eds.), Biological Evaluation of Plants with Reference to the Malagasy Flora. Monograph for the IFS-NAPRECA workshop on bioassays, Antananarivo, Madagascar 72-83, 9-43.

FARNSWORTH, N. R.1988. Screening plants for new medicine. In: Wilson, E.O., Frances, M. P. (Eds.), Biodiversity. Washington, D.C: National Academy of Sciences. ISBN 0-309-03783-2.

FENNELL, C. W., LIGHT, M. E., SPARG, S. G, STAFFORD, G. I., VAN STADEN, J. 2004. Assessing African medicinal plants for efficacy and safety: Agricultural and storage practices. Journal of Ethnopharmacology 95, 113-121.

FOOD AND DRUD ADMINSTRATION. 2004. Vioxx (rofecoxib) questions and answers. Department of Health and Human service. Centre for Drug Evaluation and Research.U.S.A. www.fda.gov/cder/drug/infopage/vioxx/vioxxQA.htm.

FOYE, W. O., LEMKE, T. I., WILLIAMS, D. A. 1995. Principles of Medicinal Chemistry. 4th ed. Harcourt Brace, London. ISBN 0-683-30737-1.

GAO, X., QIAN, M., EATON, J.W., McGREGOR, W. G. 2005. Cytoxic and mutagenic effects of tobacco-borne free fatty acids. J.G. Brown Cancer Centre and Departments of Medicine and Pharmacology/ Toxicology. University of Louisville, Louisville, KY 40202. Web reference: www.umanitoba.ca/faculties/dentistry/oral-biology.

GEDDES and GROSSET . 1997. Guide to natural healing. David Dale House, New Lanark MC11 9D J, Scotland. ISBN 185534 3339.

GELFAND, M., MAVI, S., DRUMMOND, R.B., NDEMERA, B. 1985. The Traditional Medical Practitioner in Zimbabwe. Mambo Press, Gweru, Zimbabwe. ISBN 0-86922-350-x.

GENTRY, A. H. 1993. Tropical forest biodiversity and the potential for new medicinal plants. In: Kinghorn, A. D., Balandrin, M.F. (Eds.), Human Medicinal Agents from Plants. Acs Symposium Series 534. American Chemical Society, Washington. USA. ISBN 0-8412-2705-5.

GIBBONS, S. 2003. An overview of plant extracts as potential therapeutics. Expert Opinion on Therapeutic Patents 13, 489-497.

GLAZER, N.A., NIKAIDO,H.1994. Microbial biotechnology, Fundamental of Applied Microbiology. New York: Free man. ISBN 0-7167-2608-4.

GLISH, G. L., VACHET, R.W. 2003. The basic of mass spectrometry in the twenty-first century. Drug Discovery 2, 140-150.

GRACE, O. M., PRENDERGAST, H.D.V., VAN STADEN, J., JÄGER, A. K. 2002. The status of bark in South African traditional health care. South African Journal of Botany 68, 21-30.

GREENBLATT, H. M., KRYGER, G., LEWIS, T., SILMAN, I., SUSSMAN, J. L. 1999. Structure of acetylcholinesterase complexed with (-) galanthamine at 2.3

Å resolution. Federeration of European Biochemical Societies (FEBS) Letters 463, 321-326.

GÜNTHER, H. 1980. NMR Spectroscopy: An Introduction. John Wiley and Sons. Chichester. New York. Brisbane. Toronto. ISBN 0 471275808.

HAMBURGER, M., HOSTETTMANN, K. 1991. Bioactivity in plants: The link between phytochemistry and medicine. Phytochemistry 30, 3864-3874.

HEFTMANN, E. 1961. Chromatography: Adsorption Partition Ion Exchange Elektrochromatography. Reinhold Publishing Corporation. New York; Chapman Hall, LTD., London. ISBN 8.81.

HEINRICH, M., LEE TEOH, H. 2004. Galanthamine from snowdrop - the development of a modern drug against Alzheimer's disease from local Caucasian knowledge. Journal of Ethnopharmacology 92, 147-162.

HENRY, G.E., MOMIN, R.A., NAIR, M.G., DeWITT, D.L. 2002. Antioxidant and cyclooxygenase activities of fatty acids found in food. Journal of Agriculture and Food Chemistry 50, 2231-2234.

HOSTETTMANN, K., MARSTON, A., MAILLARD, M., HAMBURGER, M. 1995. Phytochemistry of Plants Used in Traditional Medicine. Proceedings of The Phytochemistry Society of Europe. Oxford Science Publications. Clarendon Press. Oxford. O.U.P. ISBN 0-19-857775-3.

HOSTETTMANN, K., LEA, P. J. 1987. Biologically Active Natural Products. Proceedings of the Phytochemical Society of Europe. 27. Oxford Science Publications. ISBN 0-19-854196-1.

HOUGHTON, P. J., RAMAN, A. 1998. Laboratory Handbook for the Fractionation of Natural Extracts. Chapman and Hall, London SE1 8HN, Uk. ISBN 0 412749106.

HOWES, M. R., HOUGHTON, P. J. 2003. Plants used in Chinese and Indian traditional medicine for improvement of memory and cognitive function. Pharmacology, Biochemistry and Behavior 75, 513-527.

HUANG, H., KONG, L., LI, X., CHEN, X., GUO, M., ZOU, H. 2004. Strategy for analysis and screening of bioactive compounds in traditional Chinese medicines. Journal of Chromatography B 812, 71-84.

HUTCHINGS, A., SCOTT, A.H., LEWIS, G., CUNNINGHAM, A.B. 1996. Zulu Medicinal Plants. An Inventory. University of KwaZulu-Natal Press, Pietermaritzburg. ISBN 0-86980-893-1.

IKEGAMI, F., FUJII,Y., SATOH, T. 2004. Toxicological considerations of Kampo medicines in clinical use. Toxicology 198, 221-228.

INTERNATIONAL CENTRE FOR RESEARCH IN AGROFORESTRY (ICRAF). 2004. *Prunus* Net Database. In: World Agroforestry Centre, Transforming lives and land escape. Web reference: http://www.worldagroforestry.org/Sites/TreeDBS/Prunus/prunus.htm

INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY (IPCS).1985. Environmental Health Criteria 51. Guide to Short-Term Tests For Detecting Mutagenic and Carcinogenic Chemicals. Published under the joint sponsorship of the United Nations Environment Programme, the International Labour Organization, and the World Health Organization Geneva.

INTERNATIONAL PROGRAMME ON CHEMICAL SAFETY (IPCS). 1996. Harmonization of Methods for the Prediction and Quantification of Human Carcinogenic/Mutagenic Hazard, and for Indicating the Probable Mechanism of Action of Carcinogens. Mutation Research 352, 153-157.

IWU, M.M. 1994. African medicinal plants in the search for new drugs based on ethnoobotanical leads. In: Prance, G. T., Marsh J. (Eds.), Ethnobotany and The Search for New Drugs. Chichester, Sussex: Wiley Ciba Foundation Symposium 185. ISBN 0-471-95024-6.

JÄGER, A. K., VAN STADEN, J. 2000. The need for cultivation of medicinal plants in southern Africa. Outlook on Agriculture 29, 283-284.

JÄGER, A.K., HUTCHINGS, A., VAN STADEN , J. 1996. Screening of Zulu medicinal plants for prostaglandin-synthesis inhibitors. Journal of Ethnopharmacology 52, 95-100.

JENKINS, M.D.1987. Madagascar: An environmental profile. Prepared by the IUCN Conservation Monitoring Centre. Gland. Switzerland. ISBN 2-88032-607-9.

KHAN, M.R., NDAALIO, G., NKUNYA, M.H.H., WEVERS, H., SAWNEY, A.N. 1980. Studies on medicinal plants. Part 1, preliminary screening of medicinal plants for antibacterial activity. Planta Medica 38, 91-97.

KINGHORN, A. D., BALANDRIN, M.F. 1993. Human Medicinal Agents from Plants. ACS Symposium Series 534. American Chemical Society, Washington, USA. ISBN 0-8412-2705-5.919795-626.

LANE, N. E. 1997. Pain management in OA: The role of COX-2 inhibitors. Journal of Rheumatology 24, 20-24.

LEA, P. J., LEEGOOD, R. C. 1999. Plant Biochemistry and Molecular Biology. 2nd ed. Chichester, Sussex: Wiley. ISBN 0-471-97683-0.

LEWIS, W.H., ELVIN-LEWIS, M.P. 2003. Medical Botany: Plant Affecting Human Health. Wiley & Sons, Inc. Hoboken, New Jersey. 2nd ed. ISBN 0-471-62882-4.

LEVIN, D.A. 1976. Alkaloid-bearing plants: An ecogeographic perspective, American Naturalist 110, 261-284.

LIPSKY, P. 1999. The clinical potential of COX-2 specific inhibitors. The American Journal of Medicine106(5B), 51S-57S.

LÓPEZ, S., BASTIDA, J., VILADOMAT, F., CODINA, C. 2002. Acetylcholinesterase inhibitory activity of some Amaryllidaceae alkaloids and *Narcissus* extracts. Life Sciences 71, 2521-2529.

MAAT, L., BEYERMAN, H.C. 1983. The imidazole alkaloids. In: Brossi, A. (Eds.), The Alkaloids, Chemistry and Pharmacology. Academic Press, San Diego. ISBN 0-12-469521-3.

MABOGO, D.E.N. 1990. The Ethnobotany of the Vhavenda. M.Sc. Thesis, University of Pretoria In : TAYLOR, J. L. S. 1999. An investigation into the Biology and Medicinal properties of *Eucomis* species. Ph.D. Thesis. University of Natal.

MANTRI, P., WITIAK, D.T. 1994. Inhibition of cyclooxygenase and 5-lipoxygenase. Current Medicinal Chemistry 1, 328-355.

MARON, D. M., AMES, B. N. 1983. Revised methods for the *Salmonella* mutagenicity test. Mutation Research 113, 173-215.

McCHESNEY, J. D. 1993. Biological and chemical diversity and the search for new pharmaceuticals and other bioactive natural products. In: Kinghorn, A. D. and Balandrin, M. F. (Eds.), Human Medicinal Agents from Plants. ACS Symposium Series 534. American Chemical Society, Washington, USA, ISBN 0-8412-2705-5.919795-626.

McGAW, L. J., JÄGER, A.K., VAN STADEN, J. 2000. Anti-bacterial, anthelmintic and anti-amoebic activity in South African medicinal plants. Journal of Ethnopharmacology 72, 247- 263.

MITCHELL, J. A., AKARASEREENONT, P., THIEMERMANN, C., FLOWER, R. J., VANE, J. R. 1994. Selectivity of nonsteroidal anti-inflammatory drugs as inhibitors of constitutive and inducible cyclooxygenase. Proceedings of the National Academy of Science of the United States of America 90, 11693-11697.

MOSHI, M.J., MBWAMBO, Z.H. 2005. Some pharmacological properties of extracts of *Terminalia sericea* roots. Journal of Ethnopharmacology 97, 43-47.

MULHOLLAND, D.A., TAYLOR, D.A.H. 1980. Limonoids from the seed of the Natal mahogany, *Trichilia dregeana*. Phytochemistry 19, 2421-2425.

MYERS, N.1992. Traditional medicines from tropical forests. The primary source: Tropical forest and our future. New York: Norton 56, 15-17.

NEUWINGER, H. 1996. African Ethnobotany Poisons and Drugs Chemistry. Chapman and Hall Gm bH, D-69469 Weinheim Bundes Republik Deutschland. ISBN 3-8261-0077-8.

NEVEROVA, I., VAN EYK, J. E. 2004. Role of chromatographic techniques in proteomic analysis. Journal of Chromatography B 815, 51-63.

NOREEN, Y., RINGBORM, T., PERERA, P., DANIELSON, H., BOHLIN, L. 1998. Development of a radiochemical cyclooxygenase–1 and–2 *in vitro* assay for identification of natural products as inhibitors of prostaglandin biosynthesis. Journal of Natural Products 61, 2-7.

O'NEILL, M.J., LEWIS, J.A. 1993. The renaissance of plant research in the pharmaceutical industry. In: Kinghorn, A.D., Balandrin, M.F. (Eds.), Human Medicinal Agents from Plants, ACS Symposium series 534. Washington: American Chemical Society. ISBN 0-8412-27055.

PERSONENI, C. D., BENTLEY, P. D., FLETCHER, R. J., KINKAID, A., KRYGER, B. P., TAYLOR, A., TAYLOR, R., TAYLOR, J., VINER, R., SILMAN, I.,

SUSSMAN, J. L., GREENBLATT, H. M., LEWIS, T. 2001. A structure-based design approach to the development of novel, reversible AChE inhibitors. Journal of Medicinal Chemistry 44, 3203-3215.

PORTANOVA, J. P., ZHANG, Y., ANDERSON, G. D. 1996. Selective neutralization of prostaglandin E2 blocks inflammation, hyperalgesia and interleukin 6 production *in vivo*. Journal of Expert Medicine 184, 883-891.

PRANCE, G.T., BALEE, W., BOOM, M. B. 1987. Quantitative ethnobotany and the case for conservation in Amazonia. Conservation Biology 1, 296-310.

PRANCE, G.T.1994. Ethnobotany and The Search for New Drugs. Wiley, Chichester Sussex: Wiley Ciba Foundation Symposium 185. ISBN 0-471-95024-6.

PUJOL, J. 1990. Nature Africa: The Herbalist Handbook: African Flora, Medicinal Plants. Durban: Jean Pujol Natural Healers Foundation. South Africa. ISBN 0-620-15148.

RASOANAIVO, P., RATSIMAMANGA-URVERG, S. 1993. Biological evaluation of plants with reference to the Malagasy flora. Napreca workshop on bioassays, Antananarivo. Madagascar 72-83, 9-43.

RHEE, I. K., VAN De MEENT, M., INGKANINAN, K., VERPOORTE, R. 2001. A screening for acetylcholinesterase inhibitors from Amaryllidaceae using silica gel thin-layer chromatography in combination with bioactivity staining. Journal of Chromatography A 915, 217-223.

RHEE, I. K., VAN RIJN, R. M., VERPOORTE, R. 2003. Qualitative determination of false-positive effects in the acetylcholinesterase assay using thin layer chromatography. Phytochemical Analysis 14, 127-131.

RICE, E. L. 1984. Allelopathy. Academic Press, New York, 2nd ed. ISBN 0-12-587055-8.

RIMANDO, A. M., PEZZUTO, J. M., FARNSWORTH, N. R. 1994. New lignans from *Anogeissus acuminata* with HIV-1 reverse transcriptase inhibitory activity. Journal of Natural Products 57, 896-904.

RIOS, J. L., RECIO, M.C., VILLAR, A. 1988. Screening methods for natural products with antimicrobial activity: A review of the literature. Journal of Ethnopharmacology 23,127-149.

ROCHE FACETS, F. 2005. Genotoxicology: does a substance alter genetic material? *Salmonellae* help in the assessment of the risk posed by a drug. http://www.roche.com/pages/facets/15/genotoxicology.htm

ROOD, B. 1994. Uit die Veldapteek. Tafelberg Uitgewers Cape Town. South Africa.

RUSSELL, D.H., EDMONDSON, R.D. 1997. High-resolution mass spectrometry and accurate mass measurements with emphasis on the characterization of peptides and proteins by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Journal of Mass Spectrometry 32, 263-276.

SAWHNEY, A. N., KHAN, M.R., NDAALIO, G., NKUNYA, M.H.H., WEVERS, H. 1978. Studies on the rationale of African traditional medicine 2. Preliminary Screening of medicinal plants for antigonococcal activity. Journal of Scientific Research 21, 189-192.

SCHULTES, R. E., REIS, S. 1995. Ethnobotany Evolution of a Discipline. Portland. ISBN 0- 931146-28-3.

SHALE, T.L., STRIK, W.A., VAN STADEN, J. 1999. Screening of medicinal plants used in Lesotho for antibacterial and anti-inflammatory activity. Journal of Ethnopharmacology 67, 347-354.

SIMMONS, D.L. 2003. Variants of cyclooxygenase-1 and their roles in medicine. Thrombosis Research 110, 265-268.

SIMON, L. S., LANZA, F. L., LIPSKY, P. E. 1998. Preliminary study of the safety and efficacy of SC-58635 a novel cyclooxygenase 2 inhibitor. Arthritis Rheumatology 41, 1591-1602.

SLUSARENKO, A.J., LONGLAND, A.C., WHITEHEAD, I. M. 1998. A convenient, sensitive and rapid assay for anti-bacterial activity of phytoalexins. Botanica Helvetica 99, 203-207.

SMITH, W. L., DeWITT, D. L. 1995. Biochemistry of prostaglandin endoperoxide H synthase–1 and synthase-2 and their differential susceptibility to NSAIDs. Seminar in Nephrology 15, 179-194.

STEENKAMP, V., MATHIVHA, E., GOUWS, M. C., VAN RENSBURG, C. E. J. 2004. Studies on antibacterial, antioxidant and fibroblast growth stimulation of wound healing remedies from South Africa. Journal of Ethnopharmacology 95, 353-357.

SUSSMAN, J. L., BEN-MORDEHAI-ZEEV, T., SILMAN, I. 2003. Acetylcholinesterase in motion: Visualizing conformational changes in crystal structures by a morphine procedure. Biopolymers 68, 395-406.

TAYLOR, J. L. S., RABE, T., McGAW, L. J., JÄGER, A. K., VAN STADEN, J. 2001. Towards the scientific validation of traditional medicinal plants. Plant Growth Regulation 34, 23-37.

TAYLOR, J. L. S., VAN STADEN, J. 2002. COX-1 and COX-2 inhibitory activity in extracts prepared from *Eucomis* species, with further reference to extract from *E. autumnalis autumnalis*. South African Journal of Botany 68, 80-85.

TSHIKALANGE, T. E., MEYER, J. J. M., HUSSEIN, A. A. 2005. Antimicrobial activity, toxicity and the isolation of a bioactive compound from plants used to

treat sexually transmitted diseases. Journal of Ethnopharmacology 96, 515-519.

UBERTI, E., MARTINELLI, E. M., PIFFERI, G., GAGLIARDI, L. 1990. HPLC analysis of *n*-docosyl ferulate in *Pygeum africana* extract and pharmaceutical formulations. Fitoterapia 61, 342-347.

UNITED NATION ENVIRONMENTAL PROGRAMME (UNEP). 2000. New Ozone-Depleting Substance-Information On Hexachlorobutadiene. Item 3 (j) of the provisional agenda. Twelfth Meeting Of The Parties To The Montreal Protococol On Substances That Deplete The Ozone Layer. Ouagadougou.

VAN WYK, B-E., VAN OUDTSHOORN, B., GERICKE, N. 1997. Medicinal Plants of South Africa. 1st ed. Pretoria, Briza Puplications. ISBN 1875093095.

VAN WYK,B., VAN WyK, P., VAN WYK, B-E. 2000. Photographic Guide to Trees of Southern Africa.1st ed. Briza Publications, Pretoria. ISBN 1-87509-324-9.

VAN WYK, B-E., VAN HEERDEN, F., VAN OUDTSHOWN, B. 2002. Poisonous Plants of South Africa. Briza Publications, Pretoria. ISBN 1-87509.

VENTER, F., VENTER, J-A. 1996. Making The Most of Indigenous Trees. Briza Publications, Pretoria. ISBN 1875093052.

VERSCHAEVE, L., KESTENS, V., TAYLOR, J. L. S., ELGORASHI, E. E., MAES, A., VAN PUYVELDE, L., De KIMPE, N., VAN STADEN, J. 2004. Investigation of antimutagenic effects of selected South African medicinal plant extracts. Toxicology *in Vitro* 18, 29-35.

VON BREITENBACH, F. 1986. National List of Indigenous Trees. 3rd ed. Dendrological Foundation, Pretoria. ISBN 0-620-08717.

WALLACE, J. L., CHIN, B. C. 1997. New generation NSAIDS: The benefits without the risks ? Drugs Today 33, 371-378.

WATT, J. M., BREYER-BRANDWIJK, M.G. 1962. The Medicinal and Poisonous Plants of Southern and Eastern Africa. E and S Livingstone Ltd., London. ISBN 76004803B.

WHITE, H.L., GLASSMAN, A.T. 1974. A simple radiochemical assay for prostaglandin synthetase. Prostaglandins 7, 123-129.

WITT, K. L., BISHOP, J. B. 1996. Mutagenicity of anticancer drugs in mammalian germ cells. Mutation Research 355, 209-234.

WORLD HEALTH ORGANIZATION. 1977. Expert Committee on Antibiotic Information. No. 26, Technical report series No. 610, Anexe 5 WHO, Geneva, Switzerland 106-137.

WORLD HEALTH ORGANIZATION . 2004. New guidelines on the proper use of traditional, complementary and alternative medicines. Media centre. WHO library cataloguing-in-publication data. NLM classification: WB 890. ISBN. 9241591706. Web reference: www.who.int/mediacentre/news/releases/2004/pr44/en/index.html

WU, Q., WANG, M., SIMON, J.E. 2004. Analytical methods to determine phytoestrogenic compounds. Journal of Chromatography B 812, 325-355. Web reference: www.umanitoba.ca/faculties/dentistry/dentistry/oral_biology/research

ZSCHOCKE, S., VAN STADEN, J. 2000. *Cryptocarya* species substitute plants for *Ocotea bullata*? A pharmacological investigation in terms of cyclooxygenase-1 and -2 inhibition. Journal of Ethnopharmacology 71, 473-478.