

A PHARMACOLOGICAL STUDY OF SOME NIGERIAN MEDICINAL PLANTS

by

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“Recall the face of the poorest and weakest man whom you have seen, and ask yourself, if the steps you contemplate are going to be any use to him, will he gain anything by it? Will it restore to him control over his own life and destiny?” Mahatma Ghandi

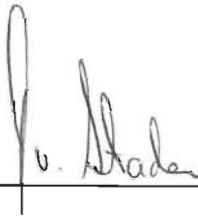
DECLARATION

I hereby declare that this thesis, unless otherwise acknowledged to the contrary in the text, is the result of my own investigation, under the supervision of Professor J. van Staden, in the Research Centre for Plant Growth and Development, School of Biological and Conservation Sciences, University of KwaZulu-Natal, Pietermaritzburg.



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I certify that the above statement is correct.



Supervisor: Professor J. van Staden

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ABSTRACT

Petroleum ether, dichloromethane, and 80% ethanol extracts of 15 plant species collected in Nigeria were screened for *in vitro* antibacterial, anti-inflammatory and antimalarial activities. Antibacterial activity was tested using the agar diffusion method, while the minimum inhibitory concentrations (MIC) of the active extracts were determined using the microtitre serial dilution method. Most antibacterial activity detected was against Gram-positive bacteria with *Staphylococcus aureus* being the most susceptible. The highest activity was found in petroleum ether and dichloromethane leaf extracts of *Mallotus oppositifolius*; petroleum ether, dichloromethane and ethanolic root extracts of *Newbouldia laevis*; and ethanolic root extracts of *Morinda lucida* and *Canthium subcordatum*. Against the Gram-negative bacterium *Escherichia coli*, the highest activity was found in dichloromethane leaf extracts of *Newbouldia laevis*, ethanolic root extracts of *Phyllanthus amarus*, *Mallotus oppositifolius*, and *Canthium subcordatum*. A total of 60 plant extracts were screened for antiplasmodial activity. A chloroquine sensitive strain of *Plasmodium falciparum* (D10) was used. In the assay, the parasite lactate dehydrogenase (pLDH) activity was used to measure parasite viability. About 11 extracts showed promising activity with an IC₅₀ ranging from 2.5 to 13.4 µg/ml. The petroleum ether leaf extract of *Hyptis suaveolens* had the highest activity (IC₅₀ = 2.5 µg/ml). The cyclooxygenase (COX-1 and COX-2) assays were used to test for anti-inflammatory activity. All the plant species, with the exception of *Hedranthera barteri* and *Picralima nitida* showed anti-inflammatory activity. Apart for a few ethanolic extracts, all the activities were

recorded with petroleum ether and dichloromethane extracts. Employing bioassay-guided activity fractionation, an antibacterial anthraquinone identified as emodin was isolated from ethanolic root extract of *Senna occidentalis*. Although this compound had been isolated from other sources, this was the first report of isolation from *Senna occidentalis*. Using a similar approach a novel antimalarial diterpenoid was isolated from the petroleum ether leaves extract of *Hyptis suaveolens*. It had IC_{50} of 0.1 $\mu\text{g/ml}$. This new compound is worthy of further investigation and may act as an important lead compound for future antimalarial drugs.

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LIST OF ABBREVIATIONS

AIDS.....	Acquired Immune Deficiency Syndrome
CAM.....	Complimentary Alternative Medicine
HIV.....	Human Immunodeficiency Virus
INT.....	<i>p</i> -iodonitrotetrazolium Violet
ISIS.....	Institute of Science in Society
MBC.....	Minimum Bactericidal Concentration
MH.....	Mueller-Hinton
MIC.....	Minimum Inhibitory Concentration
NMR.....	Nuclear Magnetic Resonance
PHC.....	Primary Health Care
R _f	mobility relative to front
TLC.....	Thin Layer Chromatography
UKZN.....	University of KwaZulu-Natal
UNESCO.....	United Nations Educational, Scientific and Cultural Organisation
UV.....	ultraviolet
VLC.....	Vacuum Liquid Chromatography
WHO.....	World Health Organisation

CHAPTER 1

LITERATURE REVIEW

1.1. Introduction

Nature has always been the mother of all treatments, which provides therapies for all sorts of illnesses and diseases. Medicinal plants, since times immemorial have been used in virtually all cultures as a source of medicine. Then their enormous usefulness in the primary health care system cannot be overemphasized (BALANDRIN *et al.*, 1993). The widespread use of medicinal plant preparations obtained from commonly used traditional herbs and medicinal plants, has been traced to the occurrence of natural products with medicinal properties (HOAREAU and DA SILVA, 1999). The use of traditional medicine and medicinal plants in most developing countries, as normative basis for the maintenance of good health, has been widely reported (UNESCO, 1996). There has been an increasing reliance on the use of medicinal plants in Western Societies, which has been traced to the extraction and development of several drugs from these plants as well as from traditionally used rural herbal remedies (UNESCO, 1998). Evidence of the therapeutic effects of medicinal plants is seen in their continued use. Well-known examples are India's traditional medical system, Ayurveda, and the traditional medicine developed by Native American, African, Chinese and South American Cultures. It is estimated that about 25% of all modern prescription medicines are directly or indirectly derived from plants. Such drugs include quinine, reserpine, ephedrine, ipecac and morphine that have been in widespread use for a long time, and

more recently adopted compounds such as the antimalarial artemisinin (BALANDRIN *et al.*, 1993)

In 2001, the House of Lords in the United Kingdom sanctioned the regulation of acupuncture and herbal medicine. As from August 2002, 25 of WHO's 191 member states have developed a National Traditional and Complimentary Alternative Medicine known as TM/CAM (ISIS, 2002). About 70% of the population in Canada, 48% in Australia, 42% in USA, and 38% in France use CAM. The global market for TM stands at US\$60 billion, with the United Kingdom's expenditure at US\$2.3 billion per year (ISIS, 2002).

Natural products are typically secondary metabolites, produced by organisms in response to external stimuli such as nutritional changes, infection and competition (COTTON, 1996). Natural products produced by plants and fungi have been isolated as biologically active compounds using bio-activity guided separation methods. About 25% of modern medicines in the world today are higher plant natural products, or are from their derivatives, often with an ethnopharmacological background (ISIS, 2002). They are widely recognized in the pharmaceutical industry for their broad structural diversity as well as their wide range of pharmacological activities. Less than 10% of the estimated 250,000 flowering plant species on earth have been examined for biological activity (VINCENT and FURNHAM, 1997). It can thus be assumed that natural products will continue to offer new leads for novel therapeutic agents once the untapped source of

biodiversity are available for screening. Thus, the future of plants as sources of drugs seems secure (VINCENT and FURNHAM, 1997).

1.2. Traditional Medicine: A Global View

Traditional medicine can be seen as diverse health practices, approaches, knowledge and beliefs incorporating plant and, animal, and/or mineral based medicines, spiritual therapies, manual techniques and exercises applied singularly or in combination to maintain well-being, as well as to treat, diagnose or prevent illness (WHO, 2003). These approaches to health form part of the traditions of each country, and have been handed down from generation to generation (WHO, 1996).

Over the years, the World Health Assembly has adopted a number of resolutions drawing attention to the fact that most of the populations in developing countries around the world depends on traditional medicine for primary health care. The work force represented by the practitioners of traditional medicine is a potentially important resource for the delivery of health care as medicinal plants are of great importance to the health of individuals and communities. This is being supported by the fact that about 80% of the developing world's population depends on traditional medicine for their primary health care (ABELSON, 1990; CHAUDHURY, 1995). The huge reliance on traditional medicine by most of the developing world's population has been partly attributed to the high cost of modern drugs. However, the use of traditional medicine over the years has gone beyond the borders of the developing world. Western cultures are gradually embracing many aspects of traditional medicine. This can be attributed to

the proven efficacy of some remedies used in traditional medicine. In Europe, North America and other industrialized regions, over 50% of the population have used complementary or alternative medicine at least once (WHO, 2003). Yet, there are still criticism about several aspects of traditional medicine such as the practice, safety, and efficacy of remedies. WHO, through its Traditional Medicine Programme supports member states in their efforts to formulate national policies on traditional medicine, to study the potential usefulness of traditional medicine including evaluation of practices and examination of the safety and efficacy of remedies, to upgrade the knowledge of traditional and modern health practitioners, as well as to educate and inform the general public about proven traditional health practices.

Traditional medicine is more holistic in its approach than western medicine, which has been accused of emphasizing the cure of symptoms rather than underlying causes, and of dividing illness too rigorously into 'physical' and 'mental' categories. Other positive features accredited to traditional medicine include diversity and flexibility, accessibility and affordability in many parts of the world, broad acceptance among many populations in developing countries, increasing popularity in developed countries, comparatively low cost, low levels of technological input, and growing economic importance (WHO, 2001).

Although modern and traditional health care may exist side by side, they seldom cooperate. Herbal medicine no doubt makes obvious and important contributions to primary health care, however it continues to be frowned upon, especially by the majority of our western medical practitioners. The argument being that herbal medicine has no

scientific basis due to the secrecy of most traditional doctors with regards to methods, techniques, medicines and even training. In addition they also make highly contentious claims (ADDAE-MENSAH, 1992). It is frequently argued that herbalists are not competent to diagnose, especially diagnosing chronic illness in time; no data on long-term toxicity of herbs have been documented, absence of standard dosages can lead to overdosage or underdosage: herbal preparations are generally unhygienic and poorly packaged, and dosages are usually large and difficult to cope with (ADDAE-MENSAH, 1992). SIDINGA (1995) indicated that the rational use of traditional medicine has not been well defined as it relies on mysticism and intangible forces, for example witchcraft.

Herbal medicine has existed for a long time and continues to grow in popularity. According to VINCENT and FURNHAM (1997) the upsurge of interest in plant-based medicines has several explanations that include the success of plant derived anti-cancer drugs and other bioactive compounds. Plants, while not clinically effective in themselves, can provide molecular templates for the design of more effective drugs. Within contemporary rural Africa, there is no doubt about the efficacy of herbal medicine. Many Africans, especially rural people and the urban poor, rely on the use of herbal medicine when they are ill. In fact, many rural communities in Africa still have areas where traditional herbal medicine is the major, and in some cases, the only source of health care available.

Ethnobotanical researches have proved the efficacy of many herbal medicines.

Recently a Chinese herbal remedy *Artemisia annua* was found to be efficacious against

strains of drug-resistant malaria (ISIS, 2002). The fact that many valuable drugs such as quinine, codeine and some anti-cancer drugs originate from herbal medicine gives some credence to the efficacy of some herbal medicines. However, in as much as some herbal medicines are effective, one should not turn a blind eye to some dangers associated with some herbal remedies. Like drugs, some herbal preparations clearly have effects on the body and so have a potential for harm as well as therapeutic benefit (VINCENT and FURNHAM, 1997). Herbs are potentially dangerous if misused. The particular danger of herbal preparations amongst its users stems from the belief that they are natural and therefore necessarily safe because they act in harmony with the body's own functions (VINCENT and FURNHAM, 1997).

Besides efficacy, accessibility plays a huge role in the use of traditional medicine in developing Africa. The relative ratios of traditional practitioners and orthodox doctors in Africa are revealing. In Ghana, for example, there are 224 people for every traditional practitioner, compared to nearly 21,000 people for one orthodox doctor. The same applies to Swaziland where the ratio is 110 people for every traditional doctor and 10,000 people for every orthodox doctor (RUKANGIRA, 2002). In Tanzania, Uganda, and Zambia, researchers have also found a ratio of traditional doctors to population of 1:200 - 1:400. This contrasts starkly with the availability of orthodox doctors where the ratio is typically 1:20,000 or less (WHO, 2002).

1.3. Traditional Medicine and Primary Health Care (PHC)

The importance of traditional medicine as a source of Primary Health Care (PHC) was first officially recognized by WHO in the PHC Declaration of Alma Ata 1978 and has been globally addressed since 1976 by the Traditional Medicine Programme of the World Health Organisation. Since then many researchers in this field have been advocating the promotion and integration of traditional medicine in the PHC system. According to DAVIES (1995), PHC is health care based on practical, scientific, and socially acceptable methods and technology. Its main priorities include making health care accessible, acceptable, and affordable; gaining maximum self-reliance; and encouraging community participation. The traditional medical practitioners' approach to health care is, in general, holistic, considering environmental, social, psychological, and spiritual factors. Like PHC, traditional medicine sees poor health as not simply resulting from biological malfunctions, but also relating to living conditions (DAVIES, 1995). On the basis of the trust they engender, their well-established customers, and cultural acceptance, traditional healers could contribute to PHC programs if recognized as legitimate caregivers and are trained in the use of the modern instruments of PHC.

In Africa, up to 80% of all health care is traditional medicine, and 60% of children in the region with malaria are treated with herbal preparations at home (WHO, 2003). This highlights the need for reliable, accessible, and affordable herbal medicines that are locally available. Greater accessibility to traditional medicine and confidence in their ability to manage debilitating and incurable diseases probably explain why most

Africans living with HIV/AIDS use traditional herbal medicine to obtain symptomatic relief and to manage opportunistic infections (WHO, 2002).

For a better healthcare, the integration of traditional medicine into the PHC system is desirable. Unlike ethnomedicine, biomedicine is inaccessible both in physical distance and cost, shortage of drugs and equipment, inability to treat and cure certain diseases and impersonal care like distance between medical staff and patients (SIDINGA, 1995). Its numerous benefits notwithstanding, traditional medicine can be dangerous. For its full integration into the PHC, a lot of work needs to be done. Governments need to establish the necessary institutional financial support to promote the potential role of herbal medicine in PHC delivery. In doing this, priority should be given to listing and documenting the various medicinal plants and herbs, which are used to treat common diseases in each country. Moreover, establishing local botanical gardens for the preservation of essential medicinal herbal plants, and most importantly setting up of testing laboratories with adequate facilities for the assessment of the efficacy of medicinal herbs. Essential also is investigating possible long-term deleterious effects, and establishing dosage norms for the most efficacious of herbal extracts. Finally, laying a more pragmatic and scientific basis for the practice is paramount (ADDE-MENSAH, 1992).

For too long, traditional and modern medicine have followed their separate paths in mutual antipathy. Their aims are surely identical - the improvement of human health and, hence, improvement of quality of life. Therefore there is room for all practitioners to

participate and contribute to health, well-being and healing. Each area of knowledge and expertise has something to offer, and healing potential is expanded by the synergy of all methods joining in a common interest determined to serve a healing purpose. It has a greater effect when people work together, rather than competing.

1.4. Medicinal Plants and Malaria

Malaria is a disease caused by Apicomplex protozoans, represented by 150 species of *Plasmodium*, transmitted by the bites of mosquito vectors to man, simians, rodents, birds, and reptiles (KRETTLI *et al.*, 2001). Over 90 % of cases occur in sub-Saharan Africa, causing over two million deaths each year with high mortality among children (see KRETTLI *et al.*, 2001). Typically, malaria produces fever, headache, vomiting and other flu-like symptoms (RBM, 2001). Malaria, together with HIV/AIDS and TB, is one of the major public health challenges undermining development in the poorest countries of the world (RBM, 2001). Employing medicinal plants, African traditional healers have been trying the much they can to combat malaria. However, resistance of *Plasmodium falciparum* to currently used antimalarials have rendered these drugs almost ineffective. Increases in the incidences of malaria due to drug-resistant parasites and the need for more effective and safer drugs have necessitated the search for new malaria agents. With the isolation of artemisinin from *Artemisia annua* a compound very active against drug resistant malaria parasites (KLAYMANN, 1985), there has been growing interest in medicinal plants as a source of new

antimalarials. By screening some selected medicinal plants for antiplasmodial activity, one would be able to advise the traditional healers on the plants to use. This process will also afford us the opportunity to select plants for further studies.

1.5. Drug Discovery and Development

Historically, plants have provided a source of inspiration for novel drug compounds, as plant derived medicine have made large contributions to human health and well-being. Cardiac glycosides from some *Digitalis* species are almost certainly the only major discovery of the 18th Century, followed later by morphine from *Papaver somniferum*, quinine from *Cinchona* species, atropine from *Atropa belladonna* (NJAU, 1991). Natural products from plants may become the base for the development of a medicine, a natural blueprint or template for the development of new drugs, or a phytomedicine to be used for the treatment of diseases. It is estimated that plant materials are present in, or have provided the models for, 50% of Western drugs (ROBBERS *et al.*, 1996). Many commercially proven drugs used in modern medicine were initially used in crude form in traditional or folk healing practices, or for other purposes that suggested potentially useful biological activity (IWU *et al.*, 1999). An estimated 250,000 higher plants have been described and only a small number have been exhaustively studied for their potential value as a source of drugs. In other words, tested for several bioactivities instead of only one bioactivity (FARNSWORTH, 1988). There are at least 120 distinct chemical substances derived from plants that are considered as important drugs currently in use in one or more countries of the world (TAYLOR, 2000). Several of the drugs sold today are simple synthetic modifications or copies of the naturally obtained

substances. Some of these drugs/chemicals include antibacterials, antiinflammatories, and antimalarials (Table 1).

The objectives of research in the field of drug development are the identification of the bioactive agents in medicinal plants, and investigation of the extracts to ensure that they are safe, effective, and of constant activity. Ultimately the isolation of the bioactive agents, and determination of their structure in order that they may be synthesized, structurally modified or simply extracted more efficiently are of major concern (CAVE, 1986). There are many approaches to the search for new biologically active principles in higher plants, but experimental evidence has proved the 'ethnopharmacological approach' the best (PEI SHENG, 2001). Other approaches include the 'chemotaxonomical approach' that relies on correlations between plant taxonomy and the occurrence of specific chemical constituents (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). In the 'random selection approach', a variety of plants (and plant parts) are subjected to routine extraction and bioassay without preconceived selection on the basis of ethnobotanical knowledge or chemotaxonomical data (RASOANAIVO and RATSIMAMANGA-URVERG, 1993). Ethnopharmacological studies have been used for the discovery of new drugs and new drug development (PEI SHENG, 2001). Therefore the role of ethnopharmacology in drug discovery cannot be questioned (Table 1). According to CANIGUERAL (1999) 75% of drugs used in pharmacies that were obtained from higher plants had originally been isolated from species used in traditional medicine.

Table 1. Some drugs discovered from ethnobotanical leads (COX, 1994)

Drug	Medical use	Plant Source
Ajmaline	For heart arrhythmia	<i>Rauvolfia spp</i>
Aspirin	Analgesic,antiinflammatory	<i>Filipendula ulmaria</i>
Atropine	Pupil dilator	<i>Atropa belladonna</i>
Benzoin	Oral disinfectant	<i>Styrax tokinensis</i>
Caffeine	Stimulant	<i>Camellia sinensis</i>
Camphor	For rheumatic pain	<i>Cinnamomum camphora</i>
Cascara	Purgative	<i>Rhamnus purshiana</i>
Cocaine	Ophthalmic anaesthetic	<i>Erythoxylum coca</i>
Codeine	Analgesic, antitussive	<i>Papaver somniferum</i>
Colchicine	For gout	<i>Colchicium autumnale</i>
Demecolcine	For leukaemia, lymphomata	<i>Colchicium autumnale</i>
Deserpidine	Antihypertensive	<i>Rauvolfia canescens</i>
Dicoumarol	Antithrombotic	<i>Melilotus officinalis</i>
Digoxin, Digitoxin	For atrial fibrillation	<i>Digitalis purpurea</i>
Emetine	For amoebic dysentery	<i>Psychotria ipecacuanha</i>
Ephedrine	Bronchodilator	<i>Ephedra sinica</i>
Eugenol	For toothache	<i>Syzygium aromaticum</i>
Gallotanins	Haemorrhoid suppository	<i>Hamamelis virginia</i>
Hyoscyamine	Anticholinergic	<i>Hyoscyamus niger</i>
Ipecac	Emetic	<i>Psychotria ipecacuanha</i>
Ipratropium	Bronchodilator	<i>Hyoscyamus niger</i>
Morphine	Analgesic	<i>Papaver somniferum</i>
Noscapine	Antitussive	<i>Papaver somniferum</i>
Papain	Attenuator of mucus	<i>Carica papaya</i>
Papaverine	Antispasmodic	<i>Papayer somniferum</i>
Physostigmine	For glaucoma	<i>Physostigma venenosum</i>

Table 1. continued

Drug	Medical use	Plant source
Picrotoxin	Barbiturate antidote	<i>Anamirta cocculus</i>
Pilocarpine	For glaucoma	<i>Pilocarpus jaborandi</i>
Podophyllotoxin	For condyloma acuminatum	<i>Podophyllum peltatum</i>
Proscillaridin	For cardiac malfunction	<i>Drimia maritima</i>
Protoveratrine	Antihypertensive	<i>Veratrum album</i>
Pseudoephedrine	For rhinitis	<i>Ephedra sinica</i>
Psoralen	For vitiligo	<i>Psoralea corylifolia</i>
Quinine	For malaria prophylaxis	<i>Cinchona pubescens</i>
Quinidine	For cardiac arrhythmia	<i>Cinchona pubescens</i>
Rescinnamine	Antihypersentive	<i>Rauvolfia serpentina</i>
Reserpine	Antihypersentive	<i>Rauvolfia serpentina</i>
Sennoside A,B	Laxative	<i>Cassia angustifolia</i>
Scopolamine	For motion sickness	<i>Datura stramonium</i>
Sigmasterol	Steroidal precursor	<i>Physostigma venenosum</i>
Strophanthin	For congestive heart failure	<i>Strophanthus gratus</i>
Tubocurarine	Muscle relaxant	<i>Chondrodendron tomentosum</i>
Teniposide	For bladder neoplasms	<i>Podophyllum peltatum</i>
Tetrahydrocannabinol	Antiemetic	<i>Cannabis sativa</i>
Theophylline	Diuretic, antiasthmatic	<i>Camellia sinensis</i>
Toxiferine	Relaxant in surgery	<i>Strychnos guianensis</i>
Vinblastine	For Hodgkin's disease	<i>Catharanthus roseus</i>
Vincristine	For paediatric leukaemia	<i>Catharanthus roseus</i>
Xanthotoxin	For vitiligo	<i>Ammi majus</i>

Several studies have also shown that the rate of discovery of active compounds was higher when plants were selected according to traditional use rather than at random (CANIGUERAL, 1999). In the field of anticancer activity, a correlation between biological activity and use in traditional medicine has been demonstrated (HOSTETTMANN *et al.*, 1996).

Drug discovery and development is a time-consuming and lengthy process and it takes about 12 years at a cost of around US\$231 million to develop a drug (FARNSWORTH, 1994). The sequence usually begins with the identification of active lead molecules, detailed biological assays, and formulation of dosage forms in that order. This is followed by several phases of clinical studies designed to establish safety, efficacy and the pharmacokinetic profile of the new drug (IWU *et al.*, 1999).

The use of ethnobotanical data provides a valuable short cut by indicating plants with specific folk-medicinal uses which might be likely sources of biologically active chemicals (GENTRY, 1993). Historically, ethnobotanical leads have resulted in three different types of drug discovery: unmodified natural plant products where ethnomedical use suggested clinical efficacy (e.g. digitalis); unmodified natural products of which the therapeutic efficacy was only remotely suggested by indigenous plant use (e.g. vincristine); and modified natural or synthetic substances based on a natural product used in folk medicine (e.g. aspirin) (COX, 1994). In as much as this approach has a great potential for discovering potent new compounds, it may be limited in the type of drugs it is most likely to provide (COX, 1994).

Crucial to any investigation of plants with biological activities is the availability of suitable bioassays for monitoring the required effects (HOSTETTMANN *et al.*, 1996). This has led to the development and introduction of high-throughput tests for biological activity (HOUGHTON, 1999). The test systems should ideally be simple, rapid, reproducible and inexpensive, and the number of false positives should be reduced to a minimum. These tests have the advantage that a large number of samples can be investigated in a relatively short time and the amount of sample required is usually quite small. The process lends itself, not only to the investigation of a large number of plants obtained, either through random collection or selected according to ethnopharmacological information, but also to fractions from individual plant extracts so that the chemical compounds responsible can be determined (HOUGHTON, 1999).

In as much as drug development requires an enormous time frame and money - higher plants still remain chemical factories that are capable of synthesizing significant numbers of highly complex and unusual chemical substances whose structures could escape the imagination of synthetic chemists (FARNSWORTH, 1988). Tropical forests represent nature's main storehouse of raw materials for modern medicine. There is a need for continued search for new drugs or blueprints for new drugs in plants for better treatment of diseases that has plagued mankind for centuries.

1.6. Aims and Objectives

Infectious diseases account for approximately one-half of all deaths in tropical countries (IWU *et al.*, 1999). Malaria is the most widespread insect-borne disease. It kills between one and two million people every year, with as many as 300-500 million people being infected (SAXENA *et al.*, 2003). Therefore the aims when embarking on this project were to screen a number of medicinal plants collected, for antiplasmodial activity. The infection leads to loss of resistance and greater susceptibility to bacteria, fungi and other disease causing microorganisms. For this reason attention was also given to screening for antibacterial and anti-inflammatory activity. Attempts were also made towards isolating the most bioactive constituent(s). Validation of the traditional use of these plants was a prime consideration.

CHAPTER 2

ANTIBACTERIAL SCREENING

2.1. Introduction

Bacteria have been on the earth for millions of years yet it was not until the late 1600s that scientists discovered them. They are prokaryotic and they come in a variety of sizes and shapes that include spherical, rodlike and spiral forms. Most bacterial cells have a rigid cell wall made of peptidoglycan. Since they do not possess a nucleus, the genetic material simply floats around in the cytoplasm (BLACK, 2002). Besides being pathogenic, bacteria play a very vital role in our ecosystem. The actinomycetes produce antibiotics such as streptomycin and nocardicin; others live symbiotically in the gut of animals including humans, or on the roots of some legumes converting nitrogen into a usable form. They also help to break down dead organic matter and form the base of the food web in our environment (BLACK, 2002).

2.2. Disease Causing Bacteria

Infectious diseases account for approximately one-half of all deaths in tropical countries (IWU *et al.*, 1999). Mortality rates due to infectious diseases are actually increasing in developed countries such as the United States (IWU *et al.*, 1999). Death from infectious diseases, which ranked fifth in 1981, has become the third leading cause of death in 1992, an increase of 58% (PINNER *et al.*, 1996). As examples a number of bacteria that can cause major health problems are discussed.

2.2.1. *Escherichia coli*

This is a Gram-negative, rod-shaped bacterium. It belongs to a group of bacteria known as enterobacteria, so called because they inhabit the intestines of humans and animals. It is a very common, but certainly not the most abundant bacterium. Some strains of this bacterium cause enteritis in young infants and the young of farm animals, where it can cause diarrhoea and fatal dehydration. It is a common infectant of the urinary tract and bladder in humans (HUGO, 1992).

2.2.2. *Klebsiella pneumonia*

Klebsiella pneumonia is a Gram-negative, facultative anaerobic, rod-shaped and non-motile bacterium. It is a member of the bacteria family known as Enterobacteriaceae. It is commonly found in water, soil, and occasionally food and can form part of the intestinal flora of humans and animals. Distinguished by the presence of a capsular polysaccharide, *Klebsiella pneumonia* colonies are larger than those of other bacteria and are highly mucoid. Although it can cause severe pneumonia, it is most commonly the cause of hospital-acquired urinary tract infections or burn wound infections. The autoimmune disease, ankylosing spondylitis is thought to be a possible sequel of *Klebsiella* infection (HUGO, 1992). The virulence of *Klebsiella* is not well understood, but its antiphagocytic capsule plays a role in the lung infections by preventing phagocytosis (HUGO, 1992).

2.2.3. *Staphylococcus aureus*

It is a Gram-positive, spherical bacterium that occurs in microscopic clusters resembling grapes. *Staphylococcus aureus* forms a fairly large yellow colony on rich medium, often hemolytic on blood agar. It can grow at a temperature range of 15 to 45°C and at NaCl concentrations as high as 15%. *Staphylococcus aureus* is a normal pathogen of humans, found in nasal passages, on skin and mucous membranes. Some strains are capable of producing a highly heat-stable protein toxin that causes illness in humans. Enterotoxins produced by some strains of this bacterium cause staphylococcal food poisoning. It also causes a wide range of suppurative (pus-forming) infections and a toxic shock syndrome. It is also a major cause of hospital acquired (nosocomial) infections of surgical wounds and infections associated with indwelling medical devices. Today in excess of 95% of this bacterium worldwide is resistant to penicillin, ampicillin, and the antipseudomonas penicillin (NEU, 1992).

2.2.4. *Bacillus subtilis*

It belongs to the family Bacillaceae. *Bacillus subtilis* is a Gram-positive rod, endospore-forming, motile and obligate aerobe. It is common in soil and dust. Harmless as it might be, *Bacillus subtilis* may occasionally cause human eye infections. *Bacillus subtilis* is a source of the antibiotic bacitracin. The only species within this family Bacillaceae that regularly causes serious infection is *Bacillus anthracis*. It causes a disease called anthrax in farm animals and humans (FUERST, 1978).

2.2.5. *Micrococcus luteus*

This is an aerobic Gram-positive coccus and a pigment (yellow to cream-white) producer. It is a member of the family Micrococcaceae. *Micrococcus luteus* is a common inhabitant of human skin, meat, and dairy products. It is non-pathogenic, but may be opportunistic in immunosuppressed individuals (FUERST, 1978).

2.3. Antibacterial Agents

An antibiotic was originally defined as a substance produced by one microorganism, which kills or inhibits the growth of other microorganisms (RUSSELL, 1992). This has since being modified considering the fact that microorganisms are not the only source of antibiotics. Most people now define it as drugs that help the host fight bacteria, viruses and other microorganisms, either by directly killing the causative microorganism, inhibiting its growth, or weakening it so that the host's immune system can fight and kill it more easily (RUSSELL, 1992). Besides microorganisms, plants are also good sources of antibiotics. An ideal antibiotic must display selective toxicity. In other words, it is harmful to the microbe without being harmful to the host.

As early as the 1940s, the quest for chemotherapeutic agents active against pathogenic microbes had already begun. This was when Howard Florey and his colleagues seized upon Alexander Fleming's penicillin and turned it into a major therapeutic compound (GREENWOOD, 1995a). Ever since then many more antibiotics have emerged. Most antibiotics over the years have been discovered by screening of soil samples for such

natural products that kill bacteria including known pathogens, first on culture plates and then in animal systems (WALSH, 2000).

There are three proven targets for the main antibacterial drugs - bacterial cell wall biosynthesis; bacterial protein synthesis; and bacterial DNA replication and repair. The fact that mammalian cells lack cell walls makes the bacterial cell wall a good target. Although not all the bacterial cell walls are the same, in general they all possess a crossed-linked chain of peptidoglycan, which gives the cell its strength. Cell-wall-active antibiotics act by interfering with the biosynthesis of this structure (GREENWOOD, 1995b and c). Despite the fact that the general mechanism of protein synthesis is thought to be universal, the process as it occurs in bacterial cells is sufficiently different from mammalian protein synthesis to offer scope for the selective toxicity required of therapeutically useful antibacterial agents. Besides, some antibacterial agents kill bacteria by targeting the enzyme DNA gyrase (SHEN, 1993). This is the enzyme responsible for uncoiling the intertwined circles of double-stranded bacterial DNA that arise after each round of DNA replication (WALSH, 2000).

2.3.1. Resistance to Antibacteria Agents

Resistance of bacterial strains to antibiotics is one of the biggest problems faced when combating infectious diseases. According to WALSH (2000), once an antibiotic is proven to be effective and enters widespread human therapeutic use, its days are numbered. A strain of bacteria becomes resistant to an antibiotic by making a protein that its susceptible ancestors could not make. This protein, usually an enzyme, may

inactivate incoming molecules of the antibiotic, as β -lactamases do. Alternatively, it may somehow protect the bacterial function targeted by the molecules for sabotage, as does a methylase found in many erythromycin-resistant bacteria (O'BRIEN, 1992). The enzyme methylates, and thus, shields from erythromycin, the specific site on the bacterial ribosome to which the drug would bind to stop protein synthesis and the growth of the bacteria (WEISBLUM, 1984). The resistance of some bacterial strains to antibiotics has contributed substantially to the high mortality rates recorded with infectious diseases. According to FAUCI (1998), a multi-pronged approach that includes prevention, improves monitoring, and allows for the development of new treatments should be employed.

2.4. Antibacterial Assays

Antibacterial sensitivity testing is usually employed to establish the degree and spectrum of *in vitro* activity of new antibacterial agents or to screen for a new antibacterial agent. This can be done in a variety of ways, and most methods fall into one of three main categories.

2.4.1. Diffusion Method

This was originally designed to monitor the amount of antibiotic substances in crude extracts (RIOS *et al.*, 1988). Agar diffusion tests, in which the sample to be tested is allowed to diffuse from a point source (disc, hole, or cylinder), commonly in the form of an impregnated filter paper disc, into an agar medium that has been seeded with the test organism were developed. After incubation, the diameter of the clear zone around

the filter paper disc (inhibition diameter) is measured. In most studies, inhibition zones or diameters are compared with those obtained for antibiotics that are normally used as the positive control. This is useful in establishing the sensitivity of the test organism, but the comparison of the antibacterial potency of the samples and antibiotics (positive control) cannot be determined from this (RIOS *et al.*, 1988). The size of the inhibition zone or diameter may be profoundly influenced by the physio-chemical characteristics of the sample such as solubility, ionic charge, and molecular size. Growth rate of the bacterial cell, for example slow-growing organisms give rise to large zones (GREENWOOD, 1995d). It is widely used for reasons of speed, simplicity, cost, as up to six samples can be tested on one culture plate (RIOS *et al.*, 1988; GREENWOOD, 1995d). In addition, it is also well suited for preliminary screening of pure substances (RIOS *et al.*, 1988).

2.4.2. Microdilution Method

Dilution techniques are those, which require a homogenous dispersion of the sample in water (RIOS, *et al.*, 1988). They are used when only a few strains of bacteria need to be tested or when accurate minimum inhibition concentration (MIC) values are estimated for an extract, essential oil or pure compound(s) (RIOS *et al.*, 1988; GREENWOOD, 1995d). They can also be used in the preliminary screening of antimicrobial activity (RIOS *et al.*, 1988). A series of two-fold dilutions of the sample under study is prepared in a suitable broth medium and a standard inoculum of the test strain (commonly 10^5 bacteria) is introduced into each tube. The test is incubated at 37°C overnight and the end point is read as that concentration of the sample in which turbidity can be seen.

Uninoculated tubes containing broth (or water) plus antibiotic and broth alone act as the sterility controls. An antibiotic free tube inoculated with the test organism serves to indicate that the organism is alive and well in case the end point is missed (RIOS *et al.*, 1988; GREENWOOD, 1995d). This is the only method for determining minimum bactericidal concentrations (MBC). It is done by subculture of the tube with inhibition in an agar plate or liquid medium. When the bacterium does not grow, the sample is a microbicide (RIOS *et al.*, 1988; GREENWOOD, 1995d).

Microdilution method is a dilution method using a 96-well microtitre plate developed by ELOFF (1998). In this method, a two-fold serial dilution of sample to be tested is prepared in the wells of the microplate, and bacterial culture added afterwards. It is incubated overnight at 37°C like other dilution methods. After incubation, *p*-iodonitrotetrazolium violet is added, and bacterial growth is indicated by a deep red colour, while wells with antibacterial activity stay clear. This technique requires only a small amount of test sample, and is quick, sensitive and reproducible (ELOFF, 1998).

2.4.3. Bioautographic Method

This is the most important detection method for new or unidentified antimicrobial compounds (RIOS *et al.*, 1988). It is based on the biological (eg. antibacterial) effects of the substances under study. The method makes it possible to localize antimicrobial activity on a chromatogram. Inhibition zones are visualised by a dehydrogenase-activity-detecting reagent (RIOS *et al.*, 1988).

However, since some factors like inoculum size, composition of culture medium, extraction method, pH and solubility of the sample in the culture medium can influence results, it is difficult using these methods, to standardize a procedure for the study of antimicrobial plants (RIOS *et al.*, 1988; GREENWOOD, 1995d).

2.5. Materials and Methods

2.5.1. Plant Collection

After consultation with various traditional healers who advised as to the uses of various plants, plant material was collected between May and June 2002 from Oraifite in Anambra state of southeastern Nigeria. Prof. Jonathan G. Okafor of Tree Crops and Tropical Ecology Consultancy, 3 Kingsway Road, Enugu, Nigeria confirmed their identity. Voucher specimens were deposited in the Enugu State Herbarium (Table 2).

2.5.2. Plant Extract Preparation

Plant parts were air-dried for two weeks and then powdered. Plant material (10 g) was extracted sequentially with 150 ml each of petroleum ether (PE), dichloromethane (DCM) and then with 80% ethanol (ET) using a sonication bath for 1 h. Crude extracts obtained were filtered and dried under reduced pressure at 30°C.

2.5.3. Test Organisms

Test organisms used were from The American Type Culture Collection (ATCC): *Staphylococcus aureus* (ATCC 12600), *Micrococcus luteus* (ATCC 4698), *Escherichia*

Table 2. Selected medicinal plants used and collected in Nigeria investigated for antibacterial, anti-inflammatory and antiplasmodial activities

Family	Scientific name and voucher specimen	Local name	Plant parts used	Medicinal uses	Reference
Apocynaceae	<i>Hedranthera barteri</i> (Hook. f.) Pichon EFH9002	Utumbekwu	Leaves and roots	Malaria, hiccups	Traditional healers*
Apocynaceae	<i>Holarrhena floribunda</i> (G.Don) EFH9001	Mba	Roots	Dysentery, fever and malaria	Traditional healers*
Apocynaceae	<i>Picralima nitida</i> (Stapf) EFH9008	Agbudugbu	Seeds, stems and roots	Malaria, sleeping sickness, local anagesic, arthritis, cough, aphrodisiac and diabetes	Iwu (1993), Traditional healers*
Apocynaceae	<i>Rauvolfia vomitoria</i> (Afz) EFH9015	Akanta	Roots, stem and leaves	Fever, sedative in maniac syndromes, emetic	Iwu (1993), Traditional healers*
Apocynaceae	<i>Strophanthus hispidus</i> (D.C.) EFH9011	Agbulugbu	Seeds and roots	Cardiac insufficiency, arrow poison	Iwu (1993), Traditional healers*
Bignoniaceae	<i>Newbouldia laevis</i> (SEEM.) EFH9006	Ogilisi	Leaves, stems bark, roots and fruits	Malaria and fever, scorpion and snake bite, febrifuge, wound dressing, stomach ache, coughs, stds, aphrodisiac, migraine, ear-ache, eye-disease, breast cancer, dysentery, rheumatoid arthritis, epilepsy and skin infections	Iwu (1993), Gormann <i>et al.</i> (2003), Houghton and Osibogun (1993), Traditional healers*
Caesalpinaceae	<i>Senna occidentalis</i> L. EFH9009	Nsiwu muo	Leaves	Malaria and fever	Traditional healers*

Table 2. Continued

Family	Scientific name and voucher specimen	Local name	Plant parts used	Medicinal uses	Reference
Euphorbiaceae	<i>Mallotus oppositifolius</i> (Geiseler) Müll.Arg EFH9016	Okpo-biriba	Leaves, fruits and roots	Dysentery, hemorrhage, stomach-ache and chest pains	Traditional healers*
Euphorbiaceae	<i>Phyllanthus amarus</i> (Schum and Thonn) EFH9014	Nke kwo nwa na azu	Leaves and roots	Viral hepatitis, jaundice, gonorrhea, frequent menstruation, diabetes, stomachache, cold, malaria, skin sores, swelling and itchiness	Iwu (1993), Okafor and Ham (1999), Traditional healers*
Labiataea	<i>Hyptis suaveolens</i> (L.) POIT EFH9010	Nsiwu muo	Leaves and stem	Respiratory track infections, cold, skin diseases, female hygiene	Iwu (1993), Traditional healers*
Loganiaceae	<i>Anthocleista vogelii</i> (Planch.) EFH9013	Odudummiri	Stem bark and leaves	Inflammation, diabetes, wound dressing, venereal diseases	Iwu (1993), Traditional healers*
Polygalaceae	<i>Carpolobia alba</i> (G.Don) EFH9012	Okokpa ofia	Roots and fruits	General tonic, cough, indigestion, externally for pains, aphrodisiac	Iwu (1993), Traditional healers*
Rubiaceae	<i>Canthium subcordatum</i> (D.C.) EFH9007	Okpu- aruru	Roots and stem bark	Malaria and fever	Traditional healers*

Table 2. Continued

Family	Scientific name and Voucher specimen	Local name	Plant parts used	Medicinal uses	Reference
Rubiaceae	<i>Morinda lucida</i> (Benth syn.) EFH9005	Isi-mkpi, Njisi, Ezeogwu	Roots stem bark, and leaves	Fever, malaria and pains, Ulcerating abscess, Helminthiasis, trypanosomiasis	Iwu (1993), Awe and Makinde (1998), Okpekon <i>et al.</i> , (2004), Traditional healers*
Rubiaceae	<i>Nauclea latifolia</i> (Smith) EFH9004	Ubulu inu	Roots and stem bark	Malaria, fever teas, inflammation, stomach ache	Iwu (1993), Traditional healers*
Rubiaceae	<i>Uvaria chamae</i> (P. Beauv.) EFH9003	Uda-ani	Leaves, roots and stem bark	Haemorrhoids, malaria, coughs and liver, kidney and bladder diseases. As a febrifuge and puragative, peptic ulcers, celic, dysentery, gastric and abdominal pain	Traditional healers*

* Information obtained by personal communication.

coli (ATCC 11775), *Klebsiella pneumoniae* (ATCC 13883), and *Bacillus subtilis* (ATCC 6051). They were maintained at 4°C on nutrient agar plates.

2.5.4. Diffusion Method: Disc-diffusion Assay

The disc-diffusion assay (RASOANAIVO and RATSIMAMANGA-URVERG, 1993) was employed to investigate the inhibition of bacterial growth by plant extracts. Plants assayed for antibacterial activity and what they are used for traditionally are listed in Table 2. Plant extracts were resuspended in extracting solvent at a concentration of 100 mg/ml. Base plates were prepared by pouring 10 ml Mueller-Hinton (MH) agar into sterile 90 mm Petri dishes. MH agar maintained at 48°C was inoculated with a MH broth (10^6 - 10^8 bacteria per ml) of each of the test organisms and poured over the base plates to form a homogenous layer. Filter paper discs (6 mm diameter, Whatman no. 3) were sterilized by autoclaving. Plant extract (10 µl) was dispensed on each filter paper disc. This gave a final concentration of 1 mg plant extract per disc. Discs with plant extracts were air-dried and each placed onto the seeded MH agar plates. Each extract was tested in quadruplicate (4 discs per plate) with a neomycin disc (500 µg) as a positive control. Air-dried solvent (80% ethanol, petroleum ether, and dichloromethane) discs were used as negative controls. The plates were incubated overnight at 37°C. Antibacterial activity was expressed as a ratio of the inhibition zone produced by the plant extract and the inhibition zone produced by the positive control (neomycin).

2.5.5. Dilution Method: Microdilution Assay

The microtitre bioassay (ELOFF, 1998) was used to determine the Minimal Inhibitory Concentration (MIC) for the plant extracts that inhibited bacterial growth or showed a bacteriostatic effect in the disc-diffusion assay. Plant extracts (both polar and non-polar) were made up to 50 mg/ml with 25% ethanol. Plant extracts (100 µl) were two-fold serially diluted with distilled water in 96-well microplates to give concentrations from 12.5 – 0.098 mg/ml. Overnight MH broth cultures (grown at 37°C in a water bath with continuous shaking) of the test organisms were diluted 100 fold with MH broth, and 100 µl of the resulting bacterial culture were added to each well. Neomycin (100 µg/ml) was used as a positive control for each bacterium, with solvent and bacteria free wells being included as negative controls. Microplates were covered and incubated overnight at 37°C. To indicate bacterial growth, 40 µl of 0.2 mg/ml p-indonitritetrazolium violet (INT) were added to each well and incubated at 37 °C for 30 min. The colourless tetrazolium salts act as an electron acceptor and it is reduced to a red-coloured formazan product by biologically active organisms (ELOFF, 1998). Clear wells with INT after incubation indicate inhibition of bacterial growth. MIC values were recorded as the lowest concentration of extract that completely inhibited bacterial growth.

2.6. Results and Discussion

Antibacterial activity detected using the disc diffusion assay and MIC values of crude extracts of different plant parts of different species are presented in Table 3. Plant extracts with high antibacterial activity are highlighted in bold. Most activity was detected in 80% ethanol extracts, followed by dichloromethane extracts. Very little

activity was detected in the petroleum ether extracts. Of the 57 extracts screened, 27 were active against *E. coli*; 22 against *M. luteus*; 14 against *K. pneumoniae*; 32 against *S. aureus* and 29 against *B. subtilis*. The Gram-positive bacteria, especially *S. aureus*, were the most susceptible. Few extracts showed activity against the Gram-negative bacteria used. Gram-negative bacteria are generally more difficult to inhibit due to the presence of the thick murin layer that tends to prevent the entry of inhibitors (MARTIN, 1995; VLIETINCK *et al.*, 1995).

As earlier stated, the microtitre bioassay (ELOFF, 1998) was used to determine the MIC for the plant extracts that inhibited bacterial growth or showed a bacteriostatic effect in the disc-diffusion assay. In as much as this assay records the minimum inhibitory concentration, it can also indicate a bacteriocidal effect. Since there were no further tests carried out to actually determine if it was inhibition of the bacterial growth or a bacteriocidal effect detected, results are recorded as MIC. One would expect those extracts that had high inhibition zones when compared to the inhibition zones of the reference (neomycin) ratio in the disc diffusion assay to show much lower MIC values, but this was not the case. Some did exhibit much lower MIC values (dichloromethane leaf extract of *M. oppositifolius* against *S. aureus*) while some did not (dichloromethane root extract of *N. laevis* against *S. aureus*).

In the disc-diffusion assay, petroleum ether, dichloromethane and 80 % ethanol extracts of *N. laevis* all exhibited broad-spectrum activity with the dichloromethane extract

Table 3. Determination of antibacterial activity^a of some medicinal plants used and collected in Nigeria using the disc-diffusion (Dif) and microdilution assays (MIC expressed in mg/ml). Values are the mean \pm S.E.M. of results obtained from two assays

Plant species	Plant ^b part	Extract	Bacteria tested ^c									
			E.c.		M.l.		K.p.		S.a.		B.s.	
			D.Dif.	MIC	D.Dif.	MIC	D.Dif.	MIC	D.Dif.	MIC	D.Dif.	MIC
<i>H. barteri</i>	L	PE	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		DCM	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		ET	0.00	-	0.00	-	0.12 \pm 0.2	3.125	0.12 \pm 0.00	1.560	0.00	-
<i>H. floribunda</i>	R	PE	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		DCM	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		ET	0.00	-	0.48 \pm 0.01	0.780	0.00	-	<0.10	1.560	<0.10	1.560
<i>P. nitida</i>	R	PE	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		DCM	0.00	-	0.71 \pm 0.17	<0.098	0.00	-	<0.10	1.560	<0.10	3.125
		ET	Bst.	3.125	0.68 \pm 0.00	0.195	0.00	-	0.25 \pm 0.01	0.780	0.16 \pm 0.08	3.125
<i>R. vomitoria</i>	R	PE	Bst.	3.125	0.00	-	0.00	-	0.00	-	0.00	-
		DCM	0.25 \pm 0.02	1.560	0.85 \pm 0.01	6.250	0.00	-	<0.10	1.560	0.84 \pm 0.04	3.125
		ET	0.00	-	0.18 \pm 0.00	0.195	Bst	3.125	0.14 \pm 0.01	1.560	0.25 \pm 0.00	0.780
	L	PE	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		DCM	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		ET	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
	R	PE	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		DCM	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		ET	0.71 \pm 0.07	0.780	<0.10	1.560	0.00	-	0.29 \pm 0.00	0.780	<0.10	0.780
<i>H. hispidus</i>	L	PE	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		DCM	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		ET	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
	R	PE	<0.10	6.250	0.53 \pm 0.01	6.250	0.00	-	1.02 \pm 0.03	1.560	0.67 \pm 0.00	1.560
		DCM	1.29 \pm 0.14	6.250	0.51 \pm 0.07	3.125	0.00	-	1.96 \pm 0.02	0.780	0.68 0.01	6.250
<i>N. laevis</i>	R	ET	0.27 \pm 0.03	0.390	0.00	-	0.00	-	1.00 \pm 0.01	1.560	0.23 \pm 0.00	1.560

Table 3. Continued

Plant species	Plant ^b part	Extract	Bacteria tested ^c									
			E.c.		M.l.		K.p.		S.a.		B.s.	
			D.Dif	MIC	D.Dif	MIC	D.Dif	MIC	D.Dif	MIC	D.Dif	MIC
<i>S. occidentalis</i>	R	PE	0.00	-	0.00	-	<0.10	3.125	0.56 ± 0.00	3.125	0.00	-
		DCM	0.50 ± 0.04	6.250	0.27 ± 0.12	1.560	0.00	-	<0.10	3.125	0.33 ± 0.01	3.125
		ET	0.00	-	0.00	-	0.00	-	0.13 ± 0.02	<0.098	0.16 ± 0.00	0.780
	L	PE	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		DCM	Bst.	3.125	<0.10	3.125	0.00	-	0.00	-	0.00	-
		ET	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
<i>M. oppositifolius</i>	R	PE	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		DCM	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		ET	0.80 ± 0.01	0.390	0.00	-	0.00	-	<0.10	1.560	0.0	-
	L	PE	0.00	-	0.16 ± 0.00	0.195	0.00	-	1.89 ± 0.27	<0.098	0.40 ± 0.01	1.560
		DCM	Bst.	3.125	0.36 ± 0.01	6.250	0.00	-	2.36 ± 0.01	<0.098	0.43 ± 0.01	0.780
		ET	Bst.	1.560	0.48 ± 0.03	0.780	0.00	-	0.50 ± 0.00	0.390	0.36 ± 0.02	0.780
<i>P. amarus</i>	R	PE	0.00	-	0.00	-	0.14 ± 0.00	>12.500	0.32 ± 0.00	6.250	0.00	-
		DCM	Bst.	1.560	0.00	-	0.00	-	0.57 ± 0.03	1.560	0.00	-
		ET	0.83 ± 0.02	0.780	0.61 ± 0.00	0.195	0.00	-	0.50 ± 0.2	0.195	<0.10	0.780
	L	PE	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		DCM	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		ET	Bst.	0.195	0.00	-	Bst.	0.780	0.21 ± 0.12	0.390	<0.10	1.560
<i>H. suaveolens</i>	R	PE	Bst.	3.125	0.00	-	<0.10	>12.500	<0.10	1.560	<0.10	3.125
		DCM	0.27 ± 0.01	0.390	0.00	-	0.00	-	<0.10	0.780	0.19 ± 0.02	6.250
		ET	0.00	-	0.00	-	Bst.	1.560	0.00	-	<0.10	3.125
	L	PE	Bst.	0.195	0.14 ± 0.01	6.250	Bst.	>12.500	0.34 ± 0.01	1.560	0.23 ± 0.00	6.250
		DCM	Bst.	0.390	0.00	-	0.00	-	0.14 ± 0.01	1.560	<0.10	6.250
		ET	Bst.	0.390	0.53 ± 0.00	0.780	0.00	-	0.00	-	Bst.	1.560
<i>C. subcordatum</i>	R	PE	0.00	-	0.00	-	<0.10	6.250	0.00	-	0.00	-
		DCM	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		ET	0.83 ± 0.00	0.390	<0.10	0.390	0.17 ± 0.03	0.780	0.67 ± 0.00	0.195	<0.10	0.780

Table 3. Continued

Plant species	Plant ^b part	Extract	Bacteria tested ^c									
			E.c.	M.l.		K.p		S.a.		B.s.		
			D.Dif	MIC	D.Dif	MIC	D.Dif	MIC	D.Dif	MIC	D.Dif	MIC
<i>M. lucida</i>	R	PE	0.00	-	0.00	-	0.00	-	0.33 ± 0.02	0.780	0.16 ± 0.2	1.560
		DCM	Bst.	0.195	0.29 ± 0.01	0.780	Bst.	1.560	0.17 ± 0.01	0.195	0.19 ± 0.00	0.780
		ET	Bst.	0.390	0.25 ± 0.00	0.780	0.00	-	0.64 ± 0.00	0.390	0.13 ± 0.01	0.195
<i>N. latifolia</i>	R	PE	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		DCM	Bst.	3.125	<0.10	>12.500	<0.10	12.500	Bst.	0.780	<0.10	12.500
		ET	Bst.	0.195	<0.10	0.195	Bst.	0.390	0.00	-	0.10	0.780
<i>U. chamae</i>	R	PE	0.00	-	0.00	-	0.00	-	0.00	-	0.00	-
		DCM	Bst.	3.125	0.00	-	0.00	-	Bst.	3.125	Bst.	3.125
		ET	Bst.	0.098	<0.10	0.195	<0.10	3.125	0.28 ± 0.01	0.390	0.00	-
Neomycin				0.062		0.032		0.018		0.032		0.018

^a The antibacterial activity is expressed as the ratio of the inhibition zone of the extract (100 mg ml⁻¹) to the inhibition zone of the reference (neomycin, 500 µg/ml).

^b Plant part: R = Roots; L = leaves; PE, petroleum ether; DCM, dichloromethane; ET, 80% ethanol

^c Bacteria: E.c., *Escherichia coli*; M.l., *Micrococcus luteus*; K.p., *Klebsiella pneumoniae*; S.a., *Staphylococcus aureus*; B.s., *Bacillus subtilis*.

Bst., Bacteriostatic effect; -, extract not tested.

showing the highest activity. Best activity was against *S. aureus* (1.96) followed by *E. coli* (1.29).

Naphthoquinones, isolated from a dichloromethane extract of *N. laevis* roots were shown to have antibacterial activity against *B. subtilis* and *E. coli* (GAFNER *et al.*, 1996). In the present study, the antibacterial activity against *M. luteus* and *S. aureus* may be due to the presence of such compounds in the root extracts. Ethanol (80%) extracts of *P. amarus*, *M. oppositifolius*, *S. hispidus*, and *C. subcordatum* roots also exhibited high broad-spectrum activity against *E. coli* (Table 3). *M. oppositifolius* had the highest overall activity of 2.36 against *S. aureus*. This extract also had one of the lowest MIC values (0.098 mg/ml). There has never been any report on the anti-bacterial activity nor phytochemical studies for *M. oppositifolius*. Considering the broad-spectrum activity shown by this plant, the isolation of the active agent would be a worthwhile effort. There is also no report of antibacterial activity of *C. subcordatum*, but phytochemical studies have lead to the isolation of iridoid glycosides from its stem bark (ACHENBACH *et al.*, 1981). Iridoids from the stem bark of *Kigelia africana* have previously been shown to have antimicrobial activity (AKUNYILI *et al.*, 1991), and the presence of these phytochemicals may be responsible for the antibacterial activity demonstrated by *C. subcordatum* roots.

Previous studies showed mostly polar extracts of *P. amarus* to posses anti-viral (VENKATESWARAN *et al.*, 1987; THYAGARAJAN *et al.*, 1988; BLUMBERG *et al.*, 1989; NIU *et al.*, 1990; UNANDER *et al.*, 1990; THAMLIKITKUL *et al.*, 1991; LEE *et al.*,

1996; OTT *et al.*, 1997; NOTKA *et al.*, 2003), antimutagenic, antitumour and anticarcinogenic properties (SRIPANIDKULCHAI *et al.*, 2002; RAJESHKUMAR *et al.*, 2002). Anti-inflammatory properties were also reported (KIEMER *et al.*, 2003; RAPHAEL and KUTTAN, 2003) as well as contraceptive effects (RAO and ALICE, 2001). In the present study, both the leaf and root extracts of *P. amarus* were screened for antibacterial activity. The 80% ethanol, dichloromethane, and petroleum ether root extracts, and 80% ethanol leaf extracts were active against one or more of the bacteria screened.

Most extracts that showed high antibacterial activity are petroleum ether and dichloromethane extracts. These extracts may have contained fatty acids as petroleum ether and dichloromethane are ordinarily used for the removal of fatty acids (CORDELL, 1981). Antibacterial properties of fatty acids are well known (HENRY *et al.*, 2002; MCGAW *et al.*, 2002).

2.7. Conclusions

In the present study, a number of plants exhibited promising activity against a variety of bacteria. The antibacterial activity of some of the plant extracts studied against Gram-negative bacteria is very promising considering the fact that Gram-negative enteropathogenic bacteria pose a greater threat by causing more serious, life threatening diarrhoea and dysenteric diseases. This study has also to a considerable extent validated the traditional use of some of the plants studied. In doing so it has also proved to be irrelevant in terms of validating the use of some of the plants studied in

traditional medicine with regards to solvent choice as most petroleum ether and dichloromethane extracts exhibited high antibacterial activity. However, further investigation is needed to establish if the activities of these non polar solvent extracts are due to fatty acids or not especially for those plants for which the specific activity is reported for the first time. Toxicity studies are also needed to establish the safety of the relevant extracts.

CHAPTER 3

ANTI-INFLAMMATORY SCREENING

3.1. Introduction

Inflammation is a primary cellular response to an injury from microbial infection, chemical or physical agent. One of the commonest causes of inflammation is microbial infection. Bacteria release specific exotoxins - chemicals synthesized by them, which specifically initiate inflammation or endotoxins, which are associated with their cell walls. Additionally, some organisms cause immunologically-mediated inflammation through hypersensitivity reactions. Parasitic infections and tuberculosis inflammation are instances where hypersensitivity is important. Tissue damage leading to inflammation may occur through physical trauma, ultraviolet or other ionizing radiation, burns or excessive cooling. Corrosive chemicals (acids, alkalis, oxidising agents) provoke inflammation through gross tissue damage. However, infecting agents may release specific chemical irritants, which lead directly to inflammation. Death of tissues from lack of oxygen or nutrients resulting from inadequate blood flow is a potent inflammatory stimulus. Inflammation is often characterized by fever, pain, often localized to the inflamed area, and a rapid pulse rate. These inflammatory responses are triggered by the biosynthesis from arachidonic acid of a group of compounds known as prostaglandins. These reactions are catalysed by the

enzyme cyclooxygenase. (VANE, 1971; DEWITT and SMITH, 1988; RAZ *et al.*, 1988; SALMON and HIGGS, 1994).

3.2. Cyclooxygenase Isoenzymes (COX-1 and COX-2)

Cyclooxygenase (COX), the enzyme that catalyses the synthesis of cyclic endoperoxide from arachidonic acid to form prostaglandins (PG), was isolated in 1976 and cloned in 1988 (DEWITT and SMITH, 1988). In the 1990s, biological studies demonstrated increased COX activity in a variety of cells after exposure to endotoxin, pro-inflammatory cytokines, growth factors, hormones and tumour promoters. This observation gave rise to the concept of constitutive COX activity referred to as COX-1, and an inducible one subsequently referred to as COX-2 (O'BANNION *et al.*, 1991). They are bifunctional haemoproteins that both catalyse the biooxygenation of arachidonic acid to form prostaglandins G_2 (PGG_2) and the peroxidative reduction of PGG_2 to form prostaglandin H_2 (PGH_2) (KULKARNI *et al.*, 2000). The two isoforms of COX are almost identical in structure but have important differences in substrate and inhibitor selectivity and in their cellular locations (JOUZEAU *et al.*, 1997; VANE and BOTTING, 1998). The COX-1 isoform synthesises prostaglandins that are required for normal physiologic function like gastrointestinal cytoprotection and platelet activity (SMITH, 1998; FOSSLIEN, 1998). Inhibition of COX-1 may have an important role in non-steroidal anti-inflammatory drugs (NSAIDs) induced toxicity in humans, such as gastric ulcer formation (SUBONGKOT *et al.*, 2003). The second isoform COX-2, is not detectable in most normal tissues but induced at sites of inflammation by cytokines, growth factors, tumour promoters, and other

agents (SMITH *et al.*, 1996; 2000). The major differences between COX-1 and COX-2 are represented in Table 4.

Besides COX-1 and COX-2, there exists another form of cyclooxygenase enzyme known as cyclooxygenase 3 (COX-3). COX-3, a new acetaminophen-sensitive isoform of the COX family has recently been cloned from canine tissues (CHANDRASEKHARAN *et al.*, 2002). It is a variant of the COX-1 gene but retains intron 1 in its mRNA. This enzyme is expressed in the canine cerebral cortex. In humans, COX-3 mRNA is expressed as a >5.2 –kb transcript and is most abundant in the cerebral cortex and heart (CHANDRASEKHARAN *et al.*, 2002). In insect cells, COX-3 is efficiently expressed as membrane-bound proteins. It possesses glycosylation-dependent cyclooxygenase activity. Comparison of canine COX-3 activity with murine COX-1 and –2 by CHANDRASEKHARAN *et al.*, (2002) demonstrated that this enzyme is selectively inhibited by analgesic/antipyretic drugs such as acetaminophen that have low anti-inflammatory activity. In studies carried out by KIS *et al.*, (2003) they investigated the expression of the putative rat COX-3 mRNA in primary cultures of neurons, astrocytes, endothelial cells, pericytes, and choroidal epithelial cells from the rat brain and found that the mRNA of this enzyme is constitutively and differentially expressed in all of the cells except neurons. SHAFTEL *et al.*, (2003) in their studies also detected COX-3 in the mouse central nervous system that is identical in sequence to COX-1 except for the in-frame retention of intron 1. Furthermore, they found that COX-3, like its counterpart

Table 4. Differences between COX-1 and COX-2 Isoenzymes (KULKARNI *et al.*, 2000)

COX-1	COX-2
Physiology <ul style="list-style-type: none"> Constitute form of COX "housekeeping gene" produce PG that regulate normal kidney and stomach function and vascular homeostasis 	<ul style="list-style-type: none"> Inducible form of COX "inflammation response gene" induced during inflammation, produce PG involved in inflammation, "immediate early gene" thought to control mitogenesis, may produce PG involved in cell growth
Localization <ul style="list-style-type: none"> present in platelets, endothelial cells, stomach, kidney, smooth muscle, most tissues lumen of endoplasmic reticulum 	<ul style="list-style-type: none"> present in brain – control and limbic neurons, activated monocytes of fibroblasts and synoviocytes during inflammation and in follicles preceeding ovulation endoplasmic reticulum and nuclear envelope
Amino acids <ul style="list-style-type: none"> 599 amino acids a cassette of 17 amino acid sequence near the N-terminal that is absent in COX-2 N-terminal sequence begins with ADPGA 	<ul style="list-style-type: none"> 604 amino acids a cassette of 18 amino acid sequence near the C-terminal that is absent in COX-1 N-terminal begins with ANPCC
Molecular weight <ul style="list-style-type: none"> 73000 	<ul style="list-style-type: none"> 74000
Regulation of expression <ul style="list-style-type: none"> gene is 22 Kb, with 11 exons gene located on chromosome 9 mRNA transcript is 2.8 to 3.0 Kb mRNA transcript is not degraded fast promoter region of gene has poor inducibility post-transcriptional addition of 3 high mannose oligosaccharides not inhibited by glucocorticoids 	<ul style="list-style-type: none"> gene is 8.3, with 10 axons gene located on chromosome 10 mRNA transcript is 4.0 to 4.5 Kb mRNA transcript is degraded quickly promoter region contains many transcriptional factors which can be upregulated by proinflammatory cytokines post-transcriptional addition of 5 high mannose oligosaccharides inhibited by glucocorticoids
Active sites <ul style="list-style-type: none"> smaller active size may be because isoeucine in COX-1 is replaced by smaller valine on COX-2 	<ul style="list-style-type: none"> larger active size
Substrate <ul style="list-style-type: none"> only C20 carboxylic acids 	<ul style="list-style-type: none"> Both C18 and C20 carboxylic acids
Phase of inflammation <ul style="list-style-type: none"> main source of PG in chronic inflammation phase 	<ul style="list-style-type: none"> Main source of PG in acute inflammation phase
Acetylation by aspirin <ul style="list-style-type: none"> acetylation of Ser530 complete inhibition of COX activity 	<ul style="list-style-type: none"> acetylation of Ser516 modification of enzyme to produce 15-hydroxyeicosatetraenoic acid (15 HETE)

COX-1, does not generally appear to be induced by acute inflammatory stimulation.

3.3. Biosynthesis of Prostaglandins and their Functions

Prostaglandins also known as eicosanoids were discovered in 1935 when a Swedish physiologist and Nobel laureate, Ulf von Euler (see ROBERTS and NEWTON, 1982), and other investigators found that extracts of seminal vesicles of human semen lowered blood pressure and caused contraction of strips of uterine tissue (ROBERTS and NEWTON, 1982; CAMPBELL and HALUSHKA, 2001). Eicosanoids are 20-carbon fatty acids that are produced in a variety of tissues that mediate an array of physiologic and pathologic processes (CAMPBELL and HALUSHKA, 2001). Prostaglandins are highly potent short-lived substances that act as local hormones. They are not stored but are produced as needed by cell membranes in virtually every body tissue (CAMPBELL and HALUSHKA, 2001). Prostaglandins consist of the prostaglandins PGA through PGH, which are present in nearly all mammalian tissues, where they regulate function; the related thromboxanes, which are found in blood platelets; and the leukotrienes, whose biological effects include respiratory, vascular, and intestinal activities (VANE, 1971; ROBERTS and NEWTON, 1982).

The key precursor in the eicosanoid biosynthetic pathways is arachidonic acid that is formed from linolenic acid through reactions catalysed by a series of

enzymes that dehydrate fatty acids (FOEGH *et al.*, 1998; CAMPBELL and HALUSHKA, 2001). Cells store arachidonic acid as a component of membrane phospholipids such as phosphoinositol. In response to an appropriate stimulus, arachidonic acid is liberated from the storage lipid by an enzymatic reaction catalysed by phospholipase A₂ (PLA₂) (FOEGH *et al.*, 1998; CAMPBELL and HALUSHKA, 2001). The principal pathways of arachidonic acid metabolism are – the 5 – lipoxygenase pathway, which produces a collection of leukotrienes (LT) and the cyclooxygenase pathway, which produces prostaglandin H₂ (PH₂) (FOEGH *et al.*, 1998). PH₂ serves as the substrate for two enzymatic pathways: one leading to the production of several prostaglandins (PG); the other leading to the production of thromboxanes (Tx) (Figure 1). Cyclooxygenase stereospecifically adds two molecules of oxygen to arachidonic acid to form the unique bicyclic endoperoxide PGG₂. The hydroperoxide group of PGG₂ is then reduced by the cyclooxygenase (PGH – synthase) to yield the single 15 (S)-alcohol PGH₂ (MARNETT *et al.*, 1999). The role of prostaglandins in inflammation was brought into focus with the discovery by VANE (see MARKENSON, 1999) that non-steroidal anti-inflammatory drugs (NSAID), like aspirin, inhibit the enzyme (cyclooxygenase). Anti-inflammatory steroids also inhibit the formation of prostaglandins by blocking the release of arachidonic acid from the phospholipids. PGH₂ serves as a branch point for specific enzymes leading to the formation of prostacyclin (PGI₂), the various prostaglandins (Figure 1) as well as the thromboxanes (MARNETT, *et al.*, 1999).

The lipooxygenase pathway of arachidonic acid metabolism produces a variety of acyclic lipid peroxides (hydroperoxyeicosatetraenoic acids or HPETEs), which can be reduced to the corresponding alcohols (hydroxyeicosatetraenoic acids or HETEs). The HPETEs can yield oxirane (epoxide) LTA₄ that may be hydrolysed to LTB₄ or conjugated with glutathione to yield LTC₄.

Modification of the glutathione conjugate amino acids by hydrolysis yields the other leukotrienes LTD₄, LTE₄ and LTF₄. Different functions of different prostaglandins and other eicosanoids are presented in Table 5. In arachidonic acid metabolism that leads to biosynthesis of different prostaglandins, inhibition of COX-1 is not necessary to achieve the anti-inflammatory and analgesic effects of NSAIDs, but doing so significantly contributes to the risk of gastro-intestinal ulceration, bleeding, inhibition of renal blood flow, and inhibition of platelet aggregation. Inhibition of COX-2 with new selective agents yields equal anti-inflammatory and analgesic effects without the above-mentioned side effects (MEADE *et al.*, 1993; LANEUVILLE *et al.*, 1994).

3.4. Aims and Objectives

The aim of this part of the research was to screen the target plant extracts for anti-inflammatory activity. Due to the adverse effects associated with the inhibition of COX-1 in the inflammatory processes, plant extracts was first screened for COX-2 inhibitory activity. Plant extracts that exhibited high ($\geq 70\%$) COX-2 inhibitory activity was then screened for COX-1. Bearing in mind that

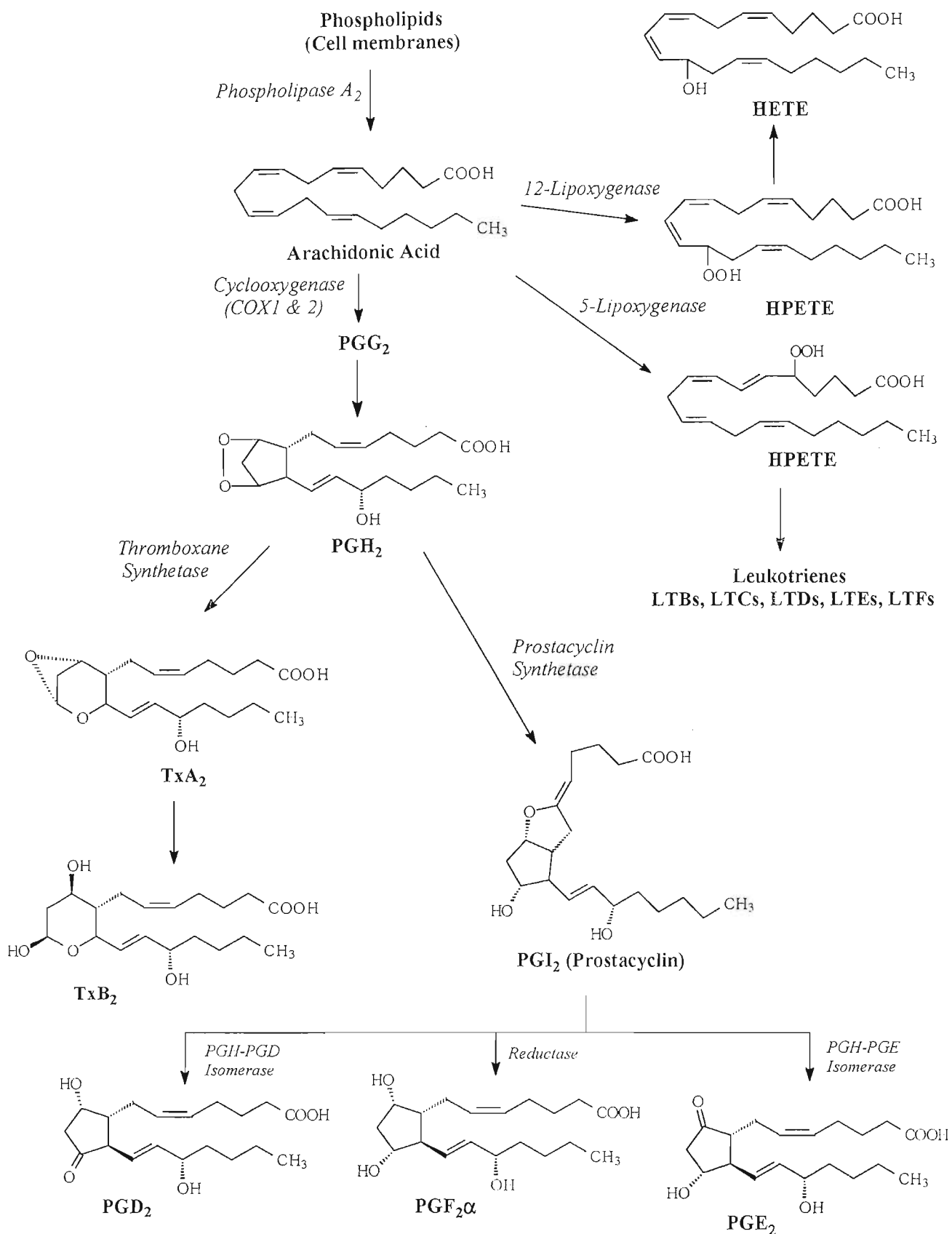


Figure 1. Biosynthesis of eicosanoids (DeRUITER, 2002)

Table 5. Summary of the physiological actions of the eicosanoids (DeRUITER, 2002)

Eicosanoid	Biochemical and Physiologic actions
PGD ₂	Weak inhibitor of platelet aggregation
PGE ₁ and PGE ₂	Bronchial Vasodilation Inhibitor of lipolysis Inhibitor of platelet aggregation Contraction of GI smooth muscle Stimulates hyperalgesic response (sensitize to pain) Renal and bronchial vasodilation Inhibitor of platelet aggregation Stimulates uterine smooth muscle relaxation Cytoprotection: Protects GI epithelial cells from acid degradation Reduces gastric acid secretion Elevates thermoregulatory set-point in anterior hypothalamus (fever) Promotes inflammation
PGF ₂	Stimulates breakdown on corpus luteum (luteolysis) Stimulates uterine smooth muscle contraction Bronchial constrictor
PGI ₂	Potent inhibitor of platelet aggregation Potent transient CV vasodilator, then vasodilator Bronchial dilator Uterine relaxant Sensitize/amplify nerve pain response
TXA ₂	Potent inducer of platelet aggregation Potent vasoconstrictor (bronchioles, renal) Decreases camp levels in platelets Stimulates the release of ADP and 5-HT from platelets
LTB ₄	Increases leukocyte chemotaxis and aggregation
LTC/D ₄	Slow-reacting substance of anaphylaxis Potent and prolonged contraction of ileal smooth muscle Contraction of lung parenchymal strips Bronchoconstriction in humans Increased vascular permeability in skin
5- or 12-HPETE	Vasodilation of gastric circulation
5- or 12-PETE	Aggregates human leukocytes Promotes leukocyte chemotaxis

these plants are used in traditional medicine, validation of their traditional use is important.

3.5. Materials and Methods

3.5.1. Plant Collection and Extraction

As described in Sections 2.5.1. and 2.5.2 in Chapter 2.

3.5.2. COX-2 Bioassay

The COX-2 assay was carried out as described by NOREEN *et al.* (1998).

Inhibition was measured by the reduction of prostaglandin formation in comparison to the control. Human recombinant COX-2 with 70% purity, isolated from *Baculovirus* overexpression system, was purchased from Sigma – Aldrich, (USA). Each aliquot (10 µl) of the enzyme containing 3 units were prepared and stored at -70°C. Enzyme (10 µl) was activated with 50 µl cofactor solution (0.6 mg/ml L-epinephrine, 0.3 mg/ml reduced glutathione and 1 µM hematin in 0.1 M Tris buffer, at pH of 8) on ice for 5 min. The enzyme/co-factor solution (60 µl) was added to sample solutions (20 µl of aqueous plant extracts, 2.5 µl of ethanolic plant extract + 17.5 µl of distilled water; final concentration of 250 µg/ml of plant extract) and then incubated at room temperature for 5 min. The reaction was started by adding 20 µl of [¹⁴C] arachidonic acid (16 Ci/mol, 30 µM) to each of the samples. Samples were then incubated at 37°C for 10 min and the reaction terminated by adding 10 µl 2 M HCl. Unlabelled prostaglandin (PGE₂:PGF₂ 1:1 v/v) carrier solution (4 µl per sample) was added to the reaction mixture, and ¹⁴C-prostaglandins synthesized in the assay were separated from unmetabolized

arachidonic acid by column chromatography using silica columns. Silica gel, (Kieselgel 60, Korngroesse 0.063-0.2 mm, 70-230 mesh ASTM) in eluent 1 (hexane:1,4-dioxan:acetic acid, 350:150:1 v/v/v) was packed to a height of 3 cm in Pasteur pipettes stoppered with glass wool. The assay mixture was applied to the column with 1 ml eluent 1. This was followed by an additional 4 ml eluent 1 to elute the unreacted arachidonic acid, which was then discarded. The prostaglandins were then eluted into scintillation vials using 3 ml eluent 2 (ethyl acetate:methanol, 85:15 v/v). Scintillation fluid (4 ml) was added and the radioactivity was counted after 1 h in the dark, using a Beckman LS 6000LL scintillation counter.

3.5.3. COX-1 Bioassay

The COX-1 assay was carried out as described by JÄGER *et al.* (1996). It follows the same protocol as the COX-2. This allows for a comparison of the activities of the crude plant extracts on the isoenzymes. Inhibition was measured by the reduction of prostaglandin formation in comparison to control. COX-1 with 95% purity, isolated from ram seminal vesicles was also purchased from Sigma – Aldrich, (USA). Each aliquot (10 µl) of both enzymes containing 3 units were prepared and stored at -70°C. COX-1 enzyme (10 µl) was activated with 50 µl of co-factor solution (0.3 mg/ml L-epinephrine, 0.3 mg/ml reduced glutathione and 1 µM hematin in 0.1 M Tris buffer, at pH of 8.2) on ice for 5 min. Enzyme (60 µl) and sample solutions (20 µl of aqueous plant extracts, 2.5 µl of ethanolic plant extract + 17.5 µl of distilled water; final concentration of 250 µg/ml of plant

extract) were incubated at room temperature for 5 min. The reaction was started by adding 20 μl of [^{14}C] arachidonic acid (16 Ci/mol, 30 μM). Samples were then incubated at 37°C for 10 min and the reaction terminated by adding 10 μl 2 M HCl. Results are the mean of two experiments (each experiment in duplicate).

3.5.4. Controls

In each assay three controls were run (2.5 μl ethanol + 17.5 μl water). One was the background in which the enzyme was inactivated with HCl before the addition of ^{14}C -arachidonic acid, and the second was a solvent blank. Indomethacin was used as a positive control at a concentration of 20 μM for COX-1 and 200 μM for COX-2 to determine the efficiency of the assay-system.

3.5.5. Calculation of Inhibition

The percentage inhibition of the extracts was obtained by measuring the amount of radioactivity in the solutions relative to that of the solvent blank. Inhibition refers to the reduction of PGE_2 formation with reference to an untreated sample (solvent blank). All samples were tested in duplicate and the test repeated twice. The means and the standard error of the two repeats were calculated using Microsoft Excel.

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

3.5.6. Determination of IC₅₀

The IC₅₀ of plant extracts that exhibited high COX-2 and low COX-1 inhibitory activity were determined. Samples were assayed at three different final concentrations (250 µg/ml, 125 µg/ml, and 65 µg/ml) and results were recorded. Employing regression analysis IC₅₀ were determined using Microsoft Excel.

3.6. Results and Discussion

The effects of different plant extracts on the inhibition of prostaglandin synthesis by inhibiting COX-1 and COX-2 activity are presented in Figure 2. Results were recorded as the mean percentage inhibition of the isoenzymes by the extracts. Of the 66 extracts tested, 18 petroleum ether extracts, 16 dichloromethane extracts, and one 80% ethanol extract showed high inhibitory activity (>70%) against COX-2. These active extracts were subsequently screened for COX-1 inhibitory activity. Many of them showed less than 60% inhibitory activity against COX-1. Researchers have established that selective COX-2 inhibition by NSAIDs is a therapeutically desirable goal (KULKARNI, *et al.*, 2000). To assess the comparative COX selectivity, the IC₅₀ of promising plant extracts with regards to COX-2 selective inhibition was determined (Table 6). The higher the ratio value the more desirable the extract with regards to COX-2 selectivity. Extracts that exhibited more specific inhibitory activity against COX-2 were mostly petroleum ether and dichloromethane extracts. An exception was the 80% ethanol root extract of *C. occidentalis*. Prominent amongst these more specific COX-2

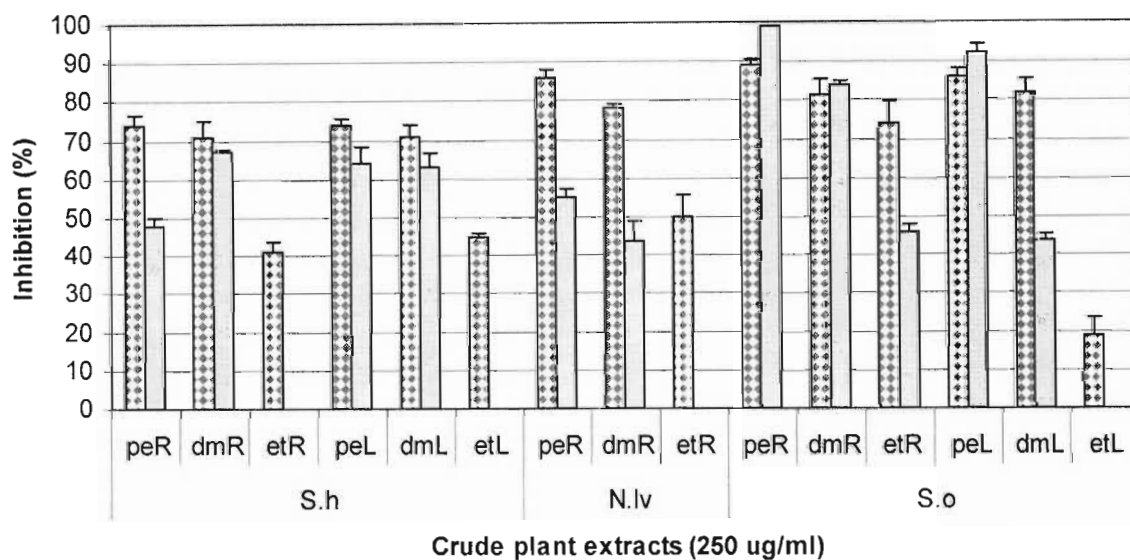
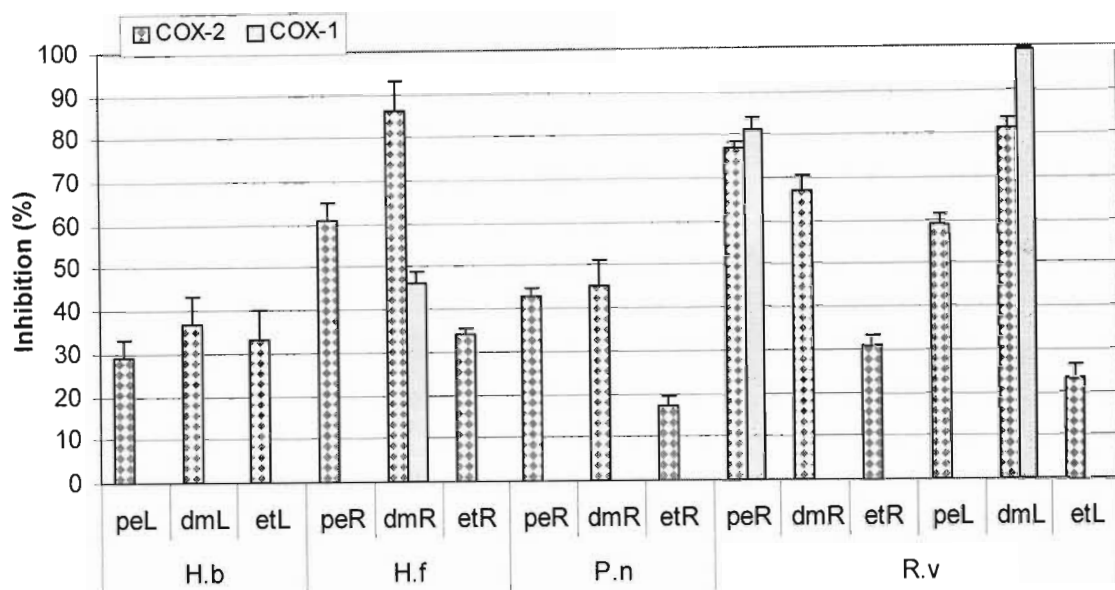


Figure 2a. Inhibition (%) of COX-2 and COX-1 by some crude plant extracts

H.b = *H. barteri*, H.f = *H. floribunda*, P.n = *P. nitida*,
R.v = *R. vomitoria*, S.h = *S. hispidus*, N.lv = *N. laevis*, S.o = *S. occidentalis*;
Pe = petroleum ether, dm = dichloromethane, et=80% ethanol; R=roots,
L=leaves

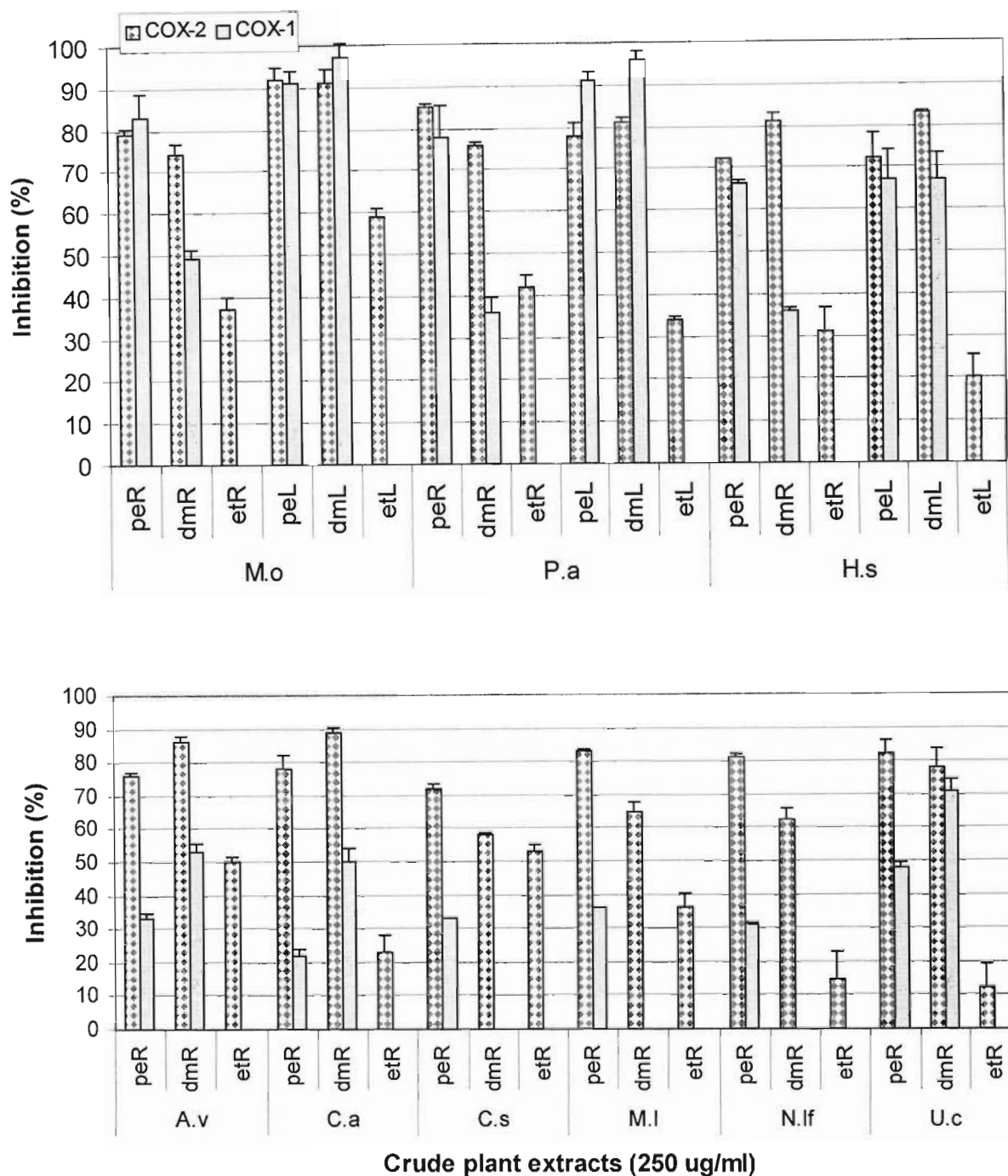


Figure 2b. Inhibition (%) of COX-2 and COX-1 by some crude plant extracts

M.o = *M.oppositifolius*, **P.a** = *P.amarus*, **H.s** = *H.suaveolens*, **A.v** = *A.vogelii*,
C.a = *C.alba*, **C.s** = *C.subcordatum*, **M.l** = *M.lucida*, **N.lf** = *N.latifolia*,
U.c = *U.chamae*, **Pe** = petroleum ether, **dm** = dichloromethane, **et**=80% ethanol;
R=roots, **L**=leaves

inhibitors were petroleum ether root extracts of *N. latifolia*, *M. lucida*, *C. subcordatum*, *C. lutea*, *A. vogelii* and dichloromethane leaf extracts of *P. amarus*, *H. suaveolens*, *N. laevis* roots and *S. occidentalis*. Since an *in vitro* bioassay is employed in this study, the results may not actually predict the actual amount of enzyme inhibition *in vivo*, nor does it directly translate into a measure of clinical efficacy. According to MANDELL (1999), the usefulness of IC₅₀ is to compare, under *in vitro* circumstances, the potency of inhibition, not the actual clinical effect. This is the first time anti-inflammatory activity has been reported for *C. subcordatum*, *C. lutea*, *N. latifolia*, *N. laevis*, *S. occidentalis* and *H. suaveolens*.

Table 6. COX selectivity of some plant extracts

Plant name	Plant part	Extract	COX-1 IC ₅₀ (µg/ml)	COX-2 IC ₅₀ (µg/ml)	COX-2 selectivity (1IC ₅₀ [COX-1]/IC ₅₀ [COX-2])
<i>H. floribunda</i>	R	DM	302	123	0.4
<i>U. chamae</i>	R	PE	266	149	0.6
<i>N. latifolia</i>	R	PE	195	42	0.2
<i>M. lucida</i>	R	PE	487	28	0.05
<i>N. laevis</i>	R	PE	182	147	0.8
<i>N. laevis</i>	R	DM	296	160	0.5
<i>C. subcordatum</i>	R	PE	306	40	0.1
<i>S. occidentalis</i>	L	DM	173	54	0.3
<i>C. occidentalis</i>	R	ET	282	182	0.6
<i>H. suaveolens</i>	R	DM	256	48	0.2
<i>S. hispidus</i>	R	PE	258	195	0.8
<i>M. oppositifolius</i>	R	DM	220	138	0.6
<i>C. alba</i>	R	PE	210	74	0.4
<i>C. alba</i>	R	DM	199	120	0.6
<i>A. vogelii</i>	R	PE	376	74	0.2
<i>A. vogelii</i>	R	DM	196	96	0.5
<i>P. amarus</i>	R	DM	324	169	0.5

R = Roots; L = Leaves; PE = petroleum ether; DCM = dichloromethane; ET = 80% ethanol

N. laevis is a common tropical plant with many therapeutic uses in traditional medicine in Nigeria (AZUINE *et al.*, 1996). A decoction of the bark is given to

children in the Ivory Coast and Nigeria for epilepsy and convulsions (AZUINE *et al.*, 1996). A pulp of the bark is used in Senegal for rheumatism, especially painful arthritis (DALZIEL, 1937). In Ghana and Nigeria, the bark is used for the treatment of breast tumors (BURKILL, 1985). Ethanol leaf extracts of this plant was found to reduce the paw edema induced by fresh egg albumin (AZUINE *et al.*, 1996). In the present study, the non-polar (PE and DCM) root extracts were found to exert better COX-2 inhibitory activity (>70%) than the polar (80% ethanol) extract (50%). The differences in percentage activity could be due to the variation in the distribution of the active agent within the plant and differences in the test technique used. However, the most important thing is that the plants do posses anti-inflammatory properties. The present study also provides evidence of COX-2 selective inhibitory effect of *N. laevis*. Chemical studies of the roots of *N. laevis* have revealed the presence of 2,3-dehydrofuranonaphthoquinones (HOUGHTON *et al.*, 1994; GAFNER *et al.*, 1996, 1998), pyrazole alkaloids (ADESANYA *et al.*, 1994; HOUGHTON *et al.*, 1994; ALADESANMI *et al.*, 1998), and phenylpropanoid glycosides (GAFNER *et al.*, 1997). Researchers have also isolated naphthoquinones from the stem bark (GORMANN *et al.*, 2003). Roots of *N. laevis* are known to contain lapachol – a naphthaquinone (HOUGHTON *et al.*, 1994) that has also been isolated from other species (*Tabebuia heptaphylla* and *Tecoma stans*) belonging to the same family (Bignoniaceae) (GUERRA *et al.*, 2001). Lapachol possesses anti-inflammatory activity (ALMEIDA, 1988) and this could explain the anti-inflammatory activity displayed by the *N. laevis* root extract.

Petroleum ether root extract of *M. lucida* exhibited significant COX-2 selectivity (Table 6). Anti-inflammatory activity of the methanol leaf extract has been reported (AWE *et al.*, 1998). Chemical studies on *M. lucida* had lead to the isolation of anthroquinones from the stem bark and roots of *M. lucida* (KOUMAGLO *et al.*, 1992). Anthroquinones are well known to possess pharmacological activities, and the anti-inflammatory activity demonstrated by *M. lucida* might well be attributed to the presence of anthroquinones. Another extract that showed significant COX-2 selectivity was the PE root extract of *C. subcordatum*. There is no report on the anti-inflammatory activity of *C. subcordatum*. However, phytochemical studies have shown *C. subcordatum* stem bark to possess iridoid glycosides (ACHENBACH, 1981).

Plants of the genus *Cassia* are mainly tropical or subtropical trees, shrubs or very rarely herbs or scramblers (OKOLI *et al.*, 2003). In traditional medicine, the leaves are used as dressings for ulcers, swellings or inflammatory conditions (ABATAN, 1990). In the present study both the DCM leaf extract and 80% ethanol root extract of *S. occidentalis* displayed COX-2 selectivity (Table 6). Ethanol leaf extracts of related species, *C. sieberiana*, *C. spectabilis*, *C. siamea*, *C. alata* and *C. nodosa* have been reported to inhibit increase in paw volume induced by carrageenan (ABATAN, 1990). The DCM root extract of *M. oppositifolius* also exhibited COX-2 selectivity. Related species of *M. oppositifolius* have been shown to possess anti-inflammatory and analgesic activities in many animal models. These include leaf extracts from *M. peltatus*

(CHATTOPADHYAY *et al.*, 2002), aqueous acetone extracts obtained from the pericarps of *M. japonicus* and chloroform root extracts of *M. spodicarpus* (INTAHPHUAK *et al.*, 2004).

3.7. Conclusions

The screening of medicinal plants for any pharmacological activity is mostly inspired by the possible discovery of either new drugs or blueprints for new drugs. Due to the efficacy of some of the plants screened as evident in the results obtained, these plants will serve as alternative and complementary therapies in the herbal treatment of inflammatory disease conditions. Not only have some of these plants showed high anti-inflammatory activity, but also exhibited selective COX-2 inhibitory activity. Isolation of the anti-inflammatory agent(s) from plants where anti-inflammatory activities are reported for the first time in this study is important. Toxicology studies of biologically active extracts are also urgently needed. There is no doubt that medicinal plants will continue to serve as reservoir for the development of potent drugs with less serious and life-threatening adverse effects.

CHAPTER 4

ANTIPLASMODIAL SCREENING

4.1. Introduction

Malaria is a life-threatening parasitic disease transmitted by mosquitoes. It is caused by a unicellular parasite called *Plasmodium*. The parasite is transmitted from person to person through the bite of a female Anopheles mosquito, which requires blood to nurture her eggs (KRETTLI *et al.*, 2001; RBM, 2001). Typically, malaria produces fever, headache, vomiting and other flu-like symptoms. In absence of treatment, the infection can progress rapidly to become life-threatening. Malaria can kill by infecting and destroying red blood cells (anaemia) and by clogging the capillaries that carry blood to the brain (cerebral malaria) or other vital organs. Approximately 40% of the world's population mostly those living in the world's poorest countries are at risk of malaria. It is found throughout the tropical and sub-tropical regions of the world and causes more than 300 million acute illnesses and at least one million deaths annually (RBM, 2001). Africa is the worst hit by malaria. About 90% of the deaths due to malaria occur in Africa, south of the Sahara mostly among young children. Malaria kills a child in Africa every 30 seconds (RBM, 2001).

Plasmodium is represented by 150 species of which only four (*P.falciparum*, *P.vivax*, *P.malariae* and *P. ovale*) infect humans. *P. falciparum* and *P.vivax* are the most common and *P. falciparum* the most deadly type of malarial infection. *P.falciparum* malaria is the most common in Africa, south of the Sahara, accounting for a large part of the extremely high mortality in this region (RBM, 2001; KRETTLI *et al.*, 2001).

4.2. Drug Resistance against Malaria

One of the greatest challenges facing Africa and other malaria-endemic regions in the fight against malaria is drug resistance (RBM, 2001). For several years, the most widely used drug for the treatment of malaria was chloroquine, a 4-aminoquinoline that was previously characterised by its efficacy, low toxicity and affordability (WHITE, 1996). Chloroquine acts by binding to haem moieties produced from proteolytically processed haemoglobin inside infected erythrocytes, thereby interfering with haem detoxification (PAGOLA *et al.*, 2000; URSOS and ROEPE, 2002). From the first report of chloroquine resistance strains of *Plasmodium falciparum* till date, chloroquine resistance has spread to the majority of malaria-endemic areas, rendering this drug increasingly ineffective (FIDOCK *et al.*, 2004). Resistance of *Plasmodium vivax* to chloroquine and mefloquine has also been reported (ALECRIM *et al.*, 1999). Sulfadoxine-pyrimethamine (SP) is the only other widely used inexpensive antimalarial, but resistance is also leading to unacceptable levels of therapeutic failure in many malaria endemic regions (PLOWE, 2003). Owing to this multiple drug resistance, the malaria-endemic regions of the world are faced with an unprecedented situation in which the only affordable treatment options are rapidly losing therapeutic efficacy.

4.3. Plants as Sources of Antimalarial Drugs

Over the years, plants have either provided antimalarial drugs or leads for synthetic antimalarial drugs. Most of the drugs currently available to treat malaria are quinoline derivatives developed on the quinine molecule found in the bark of the *Cinchona officinalis*, *C. succirubra*, *C. ledgeriana* and other *Cinchona* species. These are trees

found at high altitude in South America (KRETTLI *et al.*, 2001). The quinine molecule stimulates the synthesis of chloroquine and it became the principal replacement for quinine. Chloroquine has been used for years due to its high efficacy against all species of malaria parasites, its low toxicity, and low cost and high tolerance. It is still broadly used to treat malaria in areas where significant drug resistance has not yet emerged (KRETTLI *et al.*, 2001; SAXENA *et al.*, 2003).

Amongst the modern antimalarial compounds isolated from plants, artemisinin is the most important one at present. It was isolated from the Chinese plant *Artemisia annua* L., a plant which has been used for thousands years to treat malaria (KRETTLI *et al.*, 2001; SAXENA *et al.*, 2003). In view of the success with these two important chemotherapeutic agents, quinine and artemisinin, both of which are derived from plants, plants may well prove to be an important source of new antimalarial drugs.

4.4. Aims

To develop new antimalarial drugs, the ethnobotanical investigation of traditional medicine can be an important source of new leads. The aim of this part of the project was to screen extracts of the medicinal plants collected for antiplasmodial activity and to identify plants with good antiplasmodial activity for further investigation.

4.5. Materials and Methods

4.5.1. Plant Collection and Extraction

As described in Sections 2.5.1 and 2.5.2 in Chapter two.

4.5.2. Antiplasmodial Assay

All experiments were performed in duplicate on a single occasion using a chloroquine-sensitive strain of *Plasmodium falciparum* (D10). The parasites were continuously cultured according to the methods described by TRAGER and JENSEN (1976) and the parasite lactate dehydrogenase (pLDH) activity was used as a measure of parasite viability as described by MAKLER *et al.* (1993). The results are expressed as the average percentage parasite viability at three different concentrations of the plant extracts. A full dose response experiment (concentration range of 100 – 0.20 µg/ml) was performed on extracts that showed 50% parasite inhibition at 6.25 µg/ml. The 50% inhibitory concentration (IC₅₀) values for these extracts were obtained from the dose-response curves, using non-linear dose-response curve fitting analysis with GraphPad Prism v.3.00 software.

4.6. Results and Discussion

The parasite viability at three different concentrations of the extracts, and the IC₅₀ values of the most active extracts, are presented in Table 7. Out of the 60 extracts that were tested, 11 showed ≥50% parasite inhibition at 6.25 µg/ml. The IC₅₀ values of these active extracts were then determined.

Table 7. *In vitro* antiplasmodial activity of some extracts from Nigerian medicinal plants against *P. falciparum* D10 (CQ^s); L, Leaves; R, Roots; PE, Petroleum ether; DM, Dichloromethane; ET, 80% ethanol

Plant name	Plant part	Solvent	Parasite Viability (%) at different concentrations			
			25 µg/ml	12.5 µg/ml	6.3 µg/ml	IC ₅₀ µg/ml
<i>H. barteri</i>	L	PE	21.86	58.79	72.36	-
		DM	5.75	41.26	80.72	-
		ET	48.31	87.37	91.11	-
<i>P. nitida</i>	R	PE	89.60	104.06	88.80	-
		DM	6.35	78.06	82.99	-
		ET	0	0	42.12	6.29
<i>R. vomitoria</i>	R	PE	68.90	97.01	99.78	-
		DM	0.84	5.18	13.50	4.78
		ET	0	6.79	36.66	5.33
	L	PE	15.94	80.43	77.05	-
		DM	23.44	81.94	89.69	-
		ET	79.31	88.53	83.72	-
<i>S. hispidus</i>	R	PE	8.23	22.87	71.56	-
		DM	15.79	67.55	92.11	-
		ET	82.68	110.77	97.74	-
	L	PE	5.28	12.70	47.70	-
		DM	49.86	89.22	91.46	-
		ET	77.30	95.95	104.90	-
<i>N. laevis</i>	R	PE	19.30	82.73	77.60	-
		DM	4.64	11.83	11.03	5.00
		ET	37.14	104.97	97.96	-
<i>C. occidentalis</i>	R	PE	74.48	95.24	107.43	-
		DM	58.34	87.80	102.61	-
		ET	19.54	76.25	101.53	-
	L	PE	9.83	33.77	62.97	-
		DM	34.22	71.32	91.33	-
		ET	64.22	69.16	76.96	-
<i>M. oppositifolius</i>	L	PE	10.74	42.13	90.75	-
		DM	9.86	13.99	83.36	-
		ET	93.49	91.99	99.04	-
	R	PE	44.01	71.36	89.19	-
		DM	75.71	80.77	89.17	-
		ET	13.15	46.49	86.99	-
<i>P. amarus</i>	R	PE	19.37	59.39	77.87	-
		DM	78.74	86.98	99.43	-
		ET	78.16	102.18	105.24	-
	L	PE	0	10.67	30.63	4.99
		DM	12.20	44.06	53.53	-
		ET	23.17	65.51	93.30	-

Table 7. Continued

Plant name	Plant part	Extract	Parasite Viability (%) at different concentrations			
			25 µg/ml	12.5 µg/ml	6.3 µg/ml	IC ₅₀ µg/ml
<i>H. suaveolens</i>	R	PE	9.48	34.59	99.74	-
		DM	9.01	24.16	82.82	-
		ET	90.73	99.81	104.91	-
	L	PE	0	0.47	7.26	2.54
		DM	0	7.79	40.76	6.22
		ET	42.86	71.00	80.41	-
<i>A. vogelii</i>	R	PE	3.84	14.29	23.28	9.59
		DM	30.22	75.23	100.59	-
		ET	96.00	101.03	105.57	-
<i>C. alba</i>	R	PE	13.75	64.70	76.10	-
		DM	0	0	1.13	7.10
		ET	14.85	61.77	81.89	-
<i>M. lucida</i>	R	PE	79.41	84.81	98.53	-
		DM	19.05	36.55	48.13	13.37
		ET	61.27	96.27	82.46	-
<i>N. latifolia</i>	R	PE	96.07	103.31	104.57	-
		DM	59.84	95.28	104.70	-
		ET	78.33	96.75	110.64	-
<i>U. chamea</i>	R	PE	77.99	96.84	99.10	-
		DM	54.27	78.90	72.62	-
		ET	95.88	102.46	85.21	-

The majority of the IC₅₀ values were less than 6.3 µg/ml, except for the petroleum ether root extract of *A. vogelii* and dichloromethane root extracts of *M. lucida* and *C. alba*. The petroleum ether extract of *A. vogelii* roots and dichloromethane extract of *M. lucida* roots were only partially soluble. This probably contributed to the non-reproducible results obtained with these extracts. There have been extensive reports on the antiplasmodial activity of *M. lucida* (MAKINDE and OBIH, 1985; OBIH *et al.*, 1985). Anthroquinones with antiplasmodial activity against *P. falciparum* have been isolated

from the stem bark and roots of this plant (KOUMAGLO *et al.*, 1992; SITTIE *et al.*, 1999). This study supports the findings of these previous researchers that this plant does possess antimalarial activity. Traditional healers boil the roots in water and use the filtrate to treat malaria. However the 80% ethanol extract did not exhibit antiplasmodial activity. According to AWE *et al.* (1998), a methanolic extract of the leaves displayed antipyretic activity. This could mean that the local peoples actually use it to treat fever, which is one of the symptoms of malaria. AWE and MAKINDE (1998) also reported antiplasmodial activity of the petroleum ether extract of *M. lucida* leaves against *P. falciparum*. In the present study, the petroleum ether extract of the roots did not exhibit antiplasmodial activity. This could be due to a number of factors such as variation in the distribution of the active compound within the plant and seasonal variation.

The dichloromethane root extract of *C. alba* showed high antiplasmodial activity initially, although the IC₅₀ value was greater than what would be expected. A possible explanation for this is that the active compounds are not stable in solution and a fresh stock solution needs to be made up on the day of the experiment. Due to the limited supply of plant material, this was not feasible. This is the first report on the antimalarial properties of this plant.

The petroleum ether leaf extract of *H. suaveolens* had the lowest IC₅₀ value (2.54 µg/ml). A dichloromethane extracts of its leaves also displayed good antiplasmodial activity. Both dichloromethane and petroleum ether root extracts exhibited much less antiplasmodial activity at 6.3 µg/ml concentration. This may be due to variation in the

distribution of the active compound(s) within the whole plant. The traditional healers use the leaves topically by soaking them in water and then bath with it. This finding will be duly communicated back to the traditional healers that identified the plant. Both dichloromethane and 80% ethanol root extracts of *R. vomitoria* displayed good antiplasmodial activity with IC₅₀ values of 4.78 and 5.33 µg/ml respectively. This plant has been investigated for its anti-inflammatory (KWEIFIOOKAI *et al.*, 1995) and antipsychotic properties (OBEMBE *et al.*, 1994), its effect in the treatment of haemorrhoids and anal prolapse (OLAPADE, 1993). In the literature, there is only one report on antimalarial and antipyretic properties of *R. vomitoria* (AMOLE *et al.*, 1998).

The petroleum ether leaf extract of *P. amarus* was another extract that showed significant antiplasmodial activity. As earlier mentioned, most ethnopharmacological studies on this plant have focused on its antiviral, antitumour, anticarcinogenic, antimutagenic and anti-inflammatory properties. *P. amarus* is also used to treat malaria in southeastern Nigeria where the plant collection was done (OKAFOR and HAM, 1999). Roots are soaked in gin (alcoholic spirit distilled from grain or malt) and taken to treat malaria. All the root extracts, including the 80% ethanol extract, did not exhibit significant antiplasmodial activity. This is the first time that parts of *P. amarus* is reported to possess antimalarial properties. Despite the antimalarial activity of *P. amarus*, the traditional use of this plant does not agree with the finding. Traditionally, roots are extracted with a polar solvent.

Another plant species that exhibited significant antiplasmodial activity was *N. laevis*. Dichloromethane root extracts of its showed antiplasmodial activity with an IC₅₀ of 5 µg/ml. Roots of *N. laevis* is known to contain lapachol – a naphthaquinone (HOUGHTON *et al.*, 1994) that has also been isolated from other species (*Tabebuia heptaphylla* and *Tecoma stans*) belonging to the same family Bignoniaceae (GUERRA *et al.*, 2001). Lapachol possesses antimalarial activity (CARVALHO *et al.*, 1988). This could explain the antiplasmodial activity displayed by *N. laevis*. This is the first time *N. laevis* is reported to possess antiplasmodial activity. Further investigation is needed to determine if lapachol is the active compound or not. The 80% ethanol extract of *P. nitida* roots also showed antiplasmodial activity with an IC₅₀ of 6.29 µg/ml. Antimalarial properties of different parts of *P. nitida* has been studied extensively (IWU and KLAYMAN, 1992; EZEAMUZIE *et al.*, 1994; FRANCOIS *et al.*, 1996). However, the IC₅₀ of an 80% ethanol extract of the roots recorded in this study is much higher than that reported by previous researchers. FRANCOIS *et al.* (1996) reported an IC₅₀ of 0.188 µg/ml for the root extract. The dissimilarity in IC₅₀ could be attributed to the difference in the strain of the parasite used as was evident in the work of IWU and KLAYMAN, (1992) where different IC₅₀ values were reported for the same extract using different clones of *P. falciparum*. Some compounds especially alkaloids (akuammidine, akuamine, akuammicine, akuammigine and pseudoakuammigine) with pharmacological activity have been isolated from *P. nitida* seeds (MENZIES *et al.*, 1998; DUWIEJUA *et al.*, 2002). However, none showed antimalarial activity although various solvent extracts from this plant exhibited significant antimalarial activity. Further investigation is needed towards isolating the antimalarial compound(s).

4.7. Conclusions

About 57% of the plant species studied exhibited high ($IC_{50} < 14 \mu\text{g/ml}$) antiplasmodial activity. Besides bioactive extracts that have been extensively studied, this part of the project has highlighted promising species like *H. sauveolens*, *C. alba*, and *A. vogelii* for further antimalarial investigation. The study has also validated the use of some of the plant species in traditional medicine.

CHAPTER 5

ISOLATION AND IDENTIFICATION OF ANTIMALARIAL COMPOUNDS FROM *Hyptis suaveolens* (L.) Poit.

5.1. Introduction

5.1.1. Description and Traditional Medicinal Uses



Figure 3. A picture of *Hyptis suaveolens* in the wild

Hyptis suaveolens is a perennial herb found in dense clumps along roadsides, over-grazed pastures and around stockyards across the tropics. It is an erect, fast-growing herb with branched, semi-woody stems up to 2 m tall with quadrate hairy stems. When crushed, the plant gives off a characteristic minty smell.

The broad leaves are in opposite pairs up the stem, with small mauve flowers in clusters in the upper leaf axils. The persistent spiny calyx enclosing the seeds assists with their dispersal by adhering to clothing, fur and wool. It flowers and fruits in autumn and winter. It is native to tropical America but is now widespread as a weed (MILLER and SCHULTZ, 1997).

H. suaveolens is used traditionally for the treatment of respiratory track infections, cold, pain, cramps and skin diseases (IWU, 1993). In Nigeria where the plant was collected, the leaves, when soaked in water, are used topically for the treatment of fever.

5.1.2. Chemical Constituents and Biological Activity

Some publications dealing with the composition and antifungal (PANDEY *et al.*, 1982; SINGH *et al.*, 1992; ZOLLO *et al.*, 1998), antibacterial (IWU *et al.*, 1990; ASEKUN *et al.*, 1999), and anticonvulsant (AKAH and NWAMBIE, 1993) activities of *H. suaveolens* leaf oil have been reported. Previous phytochemical screening of the aerial parts of *H. suaveolens* had revealed the occurrence of essential oils with the major constituents being β -phellandrene, 1,8-cineole, sabinene, limonene, bicyclogermacrene. Traces of p-mentha-2,4(8)-diene and γ -terpinene were also present (AZEVEDO *et al.*, 2001). ASPINALL *et al.* (1991) isolated an acidic polysaccharide (L-fuco-4-O-methyl-D-xylan) from the seed-coat mucilage of *H. suaveolens*. The occurrence of a triterpenoid (hyptadienic acid) from the aerial parts has been reported (RAJA RAO *et al.*, 1990).

5.2. Aims

The aim of this part of the project was to study the antimalarial activity of petroleum ether leaf extract of *H. suaveolens* and attempt to isolate the bioactive compound using bioassay-guided fractionation.

5.3. Material and Methods

5.3.1. Plant Extraction

Leaves of *H. suaveolens* were collected and dried as described in Sections 2.5.1. and 2.5.2 in Chapter 2. The dry powdered leaves (554g) were extracted with 1.5 L of petroleum ether by sonication for 1 h, and left overnight on a magnetic stirrer. The extract was filtered through a Buchner funnel and Whatman No. 1 filter paper, and the leaf residue extracted three times more with 0.5 L petroleum ether. The filtration was repeated, the extracts combined and the solvent evaporated under reduced pressure at 30°C in a cooled flash rotary evaporator. The dried residue achieved was 23 g.

5.3.2. Antiplasmodial Assay

Antiplasmodial assay was performed as described in Section 4.5.2. of Chapter 4.

5.3.3. Bioassay-Guided Fractionation for Isolation of Active Compound(s)

From this stage, all solvents used were redistilled using a rotary evaporator.

A flow diagram outlines the steps of isolation followed (Figure 5).

5.3.3.1. Vacuum Liquid Chromatography (VLC)

The petroleum ether crude extract (23 g) was first redissolved in dichloromethane, mixed with silica gel (15 g) and allowed to dry. The dried mixture was applied to the top of the column and then fractionated by Vacuum Liquid Chromatography (VLC) on silica gel 0.04-0.063 mm (Merck). A column 30 cm in length and with an internal diameter of 6 cm was packed with 150 g silica. Starting with 100% hexane, a hexane:dichloromethane gradient solvent system (9:1 to 0:1) was first used. The same dichloromethane:methanol gradient solvent system (9:1 to 0:1) was subsequently used for all VLC fractionation. A volume of 400 ml of each solvent mixture was used for elution of each fraction. The VLC fractionation yielded 30 Fractions. These fractions were spotted on TLC plate and were developed with hexane:dichloromethane (10:1) solvent system. Similar fractions were pooled together. Using the antiplasmodial bioassay it was established that the most active fractions were eluted with 1:1, 2:3 and 3:7 hexane:dichloromethane solvent mixtures (744 mg).

5.3.3.2. Gravity – Assisted Column Chromatography

Column chromatography was carried out on the most bioactive Fractions. These Fractions, 6, 7 and 8 were combined and are subsequently referred to as F4 (744 mg). A column of 73 cm length and 2.5 cm internal diameter packed with 127 g silica gel 0.063–0.200 mm (Merck) was used. One hundred ml of n-hexane was first passed through the column, followed by the dichloromethane:methanol (1:0 to 0:1) gradient solvent system. A volume of 500 ml of each solvent mixture was

used. A fraction collector (Gilson FC 203B) was used to collect fractions of approximately 10 ml each. TLC profiles were used to pool fractions with similar chromatographic patterns. These fractions were again tested for antiplasmodial activity.

5.3.3.4. Preparative Thin layer Chromatography

About 20 mg of the most bioactive Fractions were applied to each of three TLC plates (Merck glass plates, 20 x 20 cm, Silica gel 60 F₂₅₄, 0.25 mm thick). The solvent system used was dichloromethane:methanol (40:1). Plates were developed to a height of 18 cm. Three different R_f bands were scraped off the TLC plates individually and eluted from the silica with absolute ethanol. The ethanolic fractions were filtered individually through Millipore filters (0.45 µm (HVLP) and 0.22 µm (GVWP)) to remove the silica. The purity of the isolated compounds was checked by TLC using various solvent systems. Isolated compounds were again tested for antiplasmodial activity.

5.3.4. Identification of Purified Active Compound(s)

Nuclear Magnetic Resonance Spectroscopy (¹H, ¹³C, COSY, HMBC, HMQC and NOESY) was performed at the School of Chemical and Physical Sciences, University of KwaZulu Natal, Pietermaritzburg, to authenticate the structure of the isolated compounds. A Varian Unity Inova

500 MHz spectrometer was used. The NMR spectra were analyzed with the help of Professor D. A. Mulholland of the School of Pure and Applied Chemistry, University of KwaZulu-Natal, Durban. The isolated compound was spotted on TLC plate, developed with CH₂Cl₂:MeOH (40:1) and stained with anisaldehyde solution.

5.4. Results and Discussion

5.4.1. Plant Extraction

The dried and powdered leaves (554 g) of *H. suaveolens* yielded 23 g (4.15 %) of crude extract.

5.4.2. Bioassay-Guided Fractionation for Isolation of Active

Compound(s)

The VLC of the petroleum ether crude extract yielded 30 Fractions. These fractions were pooled according to TLC profile into 14 Fractions (Figure 5) and subjected to the antiplasmodial bioassay. Fraction F4, which eluted with hexane:CH₂Cl₂ (1:1, 2:3 and 3:7), was found to be the most active. The results of the antiplasmodial bioassay of the 14 Fractions indicating their effect on percentage parasite viability is presented in Table 8. Fraction F4 was further separated using Gravity-assisted Column Chromatography. This procedure yielded 163 eluants that were grouped into 8 Fractions according to their TLC profiles. They were again assayed for antiplasmodial activity. The results are

shown in Table 9. Subsequent chromatographic purification of the bioactive Fraction F4-3 by preparative TLC yielded two active pure compounds F4-3₂ and F4-3₃ (Table 10). Unfortunately only F4-3₃ (4 mg) was sufficient for identification.

Table 8. *In vitro* antiplasmodial activity (%) of a fractionated petroleum ether leaf extract of *H. suaveolens* against *P. falciparum* strain D10

Fractions collected	Parasite Viability (%) at different Fraction concentrations		
	10 µg/ml	5 µg/ml	2 µg/ml
F1	95.93	106.93	103.48
F2	69.65	93.01	97.64
F3	19.08	51.10	73.20
F4	19.77	22.58	45.29
F5	102.38	106.36	105.92
F6	93.90	98.96	93.12
F7	98.34	89.28	92.66
F8	103.70	93.84	98.21
F9	100.36	97.68	89.47
F10	77.52	88.85	87.22
F11	16.17	52.59	74.49
F12	32.63	75.77	93.48
F13	66.02	79.97	88.01
F14	70.84	83.78	98.15

Table 9. *In vitro* antiplasmodial activity (% parasite viability) of F₄ against *P. falciparum* strain D10

Fractions collected	Parasite Viability (%) at different Fraction concentrations			
	10 µg/ml	5 µg/ml	2.5 µg/ml	IC ₅₀ (µg/ml)
F4-1	98.31	108.64	97.62	
F4-2	43.91	62.36	76.03	
F4-3	4.80	0.00	0.00	0.7
F4-4	10.48	3.24	17.37	3.4
F4-5	11.49	17.37	38.44	
F4-6	77.31	89.07	57.18	
F4-7	91.70	96.46	57.41	
F4-8	91.54	84.96	68.53	

Table 10. Antiplasmodial activity of isolated compounds

Pure compounds	IC ₅₀ (µg/ml)
F4-3 ₃	0.11
F4-3 ₂	0.24
Chloroquine	12.1 ng/ml

5.4.3 Identification of the Pure Compound (“hyptimisin”)

The isolated compound (Figure 4(2)) was faintly visible under UV₂₅₄ and appeared light bluish on a TLC plate (Merck 5554) when stained with anisaldehyde solution. It had an R_f of 0.36 when developed with CH₂Cl₂:MeOH (40:1).

Inspection of the ¹H and ¹³C NMR spectra (Figures 6 and 7) of compound F4-3₃ (2) revealed it to possess 20 carbon atoms. The signals of an *iso*-propyl group (δ_{H} 0.94, 0.96 (each 3H, *d*, *J* = 2.62 Hz), 1.94 (1H, *m*); δ_{C} 17.1, 17.4 (each CH₃), 32.1 (CH)), two further quaternary methyl groups (δ_{H} 0.90, 1.10 (each 3H, *s*); δ_{C} 17.8, 19.4 (each CH₃)) and a trisubstituted double bond (δ_{H} 6.19 (1H, *s*); (δ_{C} 126.6 (CH), 144.6 (C)) were observed, together with those of an oxymethylene group ((δ_{H} 3.20, 3.32 (each 1H, *d*, *J* = 10.87 Hz); δ_{C} 72.5 (CH₂)) and two further oxygenated, fully substituted carbon atoms (δ_{C} 79.2, 80.9).

Correlations were observed in the HMBC spectrum between the signals of both the *iso*-propyl methyl groups, and the olefinic proton at δ_{H} 6.19, and the fully oxygenated carbon atom resonance at δ_{C} 79.2; between the quaternary methyl

group signal at δ_H 1.10 and that at δ_C 80.9; and between the final methyl group signal at δ_H 0.90 and the oxymethylene carbon resonance at δ_C 72.5.

An inspection of the literature on this basis suggested that compound, named "hyptimisin" (**2**) might be the methylenehydroxy analogue of an abietane-type diterpene acid 9,13-endoperoxide such as 4-*epipalustric acid*-9,13-endoperoxide (Figure 4(1)) from *Juniperus sabina* L. (SAN FELICIANO *et al.*, 1990). Although all of the various derivatives reported (3 β -hydroxy-9 α ,13 α -endoperoxy, from *Salvia oxyodon* Webb et Heldreich (ESCUDERO *et al.*, 1983); both of the 9 α ,13 α - and 9 β ,13 β -endoperoxy diepimers, from *Elodea canadensis* L.C.H.Richard (MONACO *et al.*, 1987); and the corresponding 4-*epi*-15,16-dehydro and 4-*epi*-16-hydroxy analogues, from *Illicium angustasepalum* A.C.Smith (SY and BROWN, 1998) possess a carboxyl group at C-18/C-19, preventing direct comparison of the ^{13}C NMR data, the values for C-8, C-9, C-13 and C-14 in hyptimisin (**2**) are virtually identical to those reported for **1**.

No molecular ion peak in agreement with the proposed formula of $C_{20}H_{32}O_3$ was observed in the HRMS of hyptimisin (**2**). However, SAN FELICIANO *et al.* (1990), in their elucidation of **1**, reported only a peak corresponding to the fragment $[M-O_2]^+$; the base peak of hyptimisin (**2**), at m/z 288.2444, corresponds well to the calculated value for $C_{20}H_{32}O$ of m/z 288.2453.

Determination of the placement of the hydroxymethylene group at C-18/19 and the stereochemistry of the 9,13-endoperoxy linkage was not possible in the absence of a NOESY spectrum. In previous cases this was accomplished by comparison of minute differences in the chemical shifts and coupling constants of

the possible epimeric forms with established literature values. As no data for comparison is available in this case, the hydroxymethylene group placement and 9,13-endoperoxy linkage stereochemistry cannot be ascertained. Irrespective of the stereochemistry, however, compound – “hyptimisin”, with structure **2**, is novel, as it is the first reported abietane possessing both the 9 ξ ,13 ξ -endoperoxide linkage and hydroxymethylene group at C-18/19.

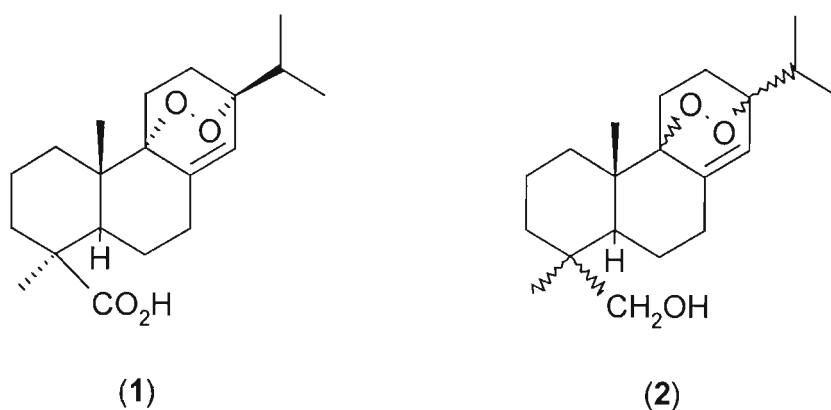


Figure 4. Chemical structures of (1) 4-epipalustric acid-9 α ,13 α -endoperoxide, (2) isolated compound – hyptimisin (F4-3₃)

5.5. Conclusions

Due to insufficient quantity of the isolated compound, a toxicity test was not done. However, mutagenic/antimutagenic tests done by REID *et al.* (2005, in press), on the dichloromethane and 90% methanolic leaf extracts of *H. suaveolens* revealed no activity. The mode of action of the isolated novel

compound is not yet known, but being a more hydrophobic compound; it is desirable in terms of absorption across the lipid bilayer of the cell membrane. Although the isolated compound is about ten times less active than chloroquine when comparing both IC_{50} values, it does possess a remarkable antiparasitic activity and could possibly lead to synthesis of more active new drugs.

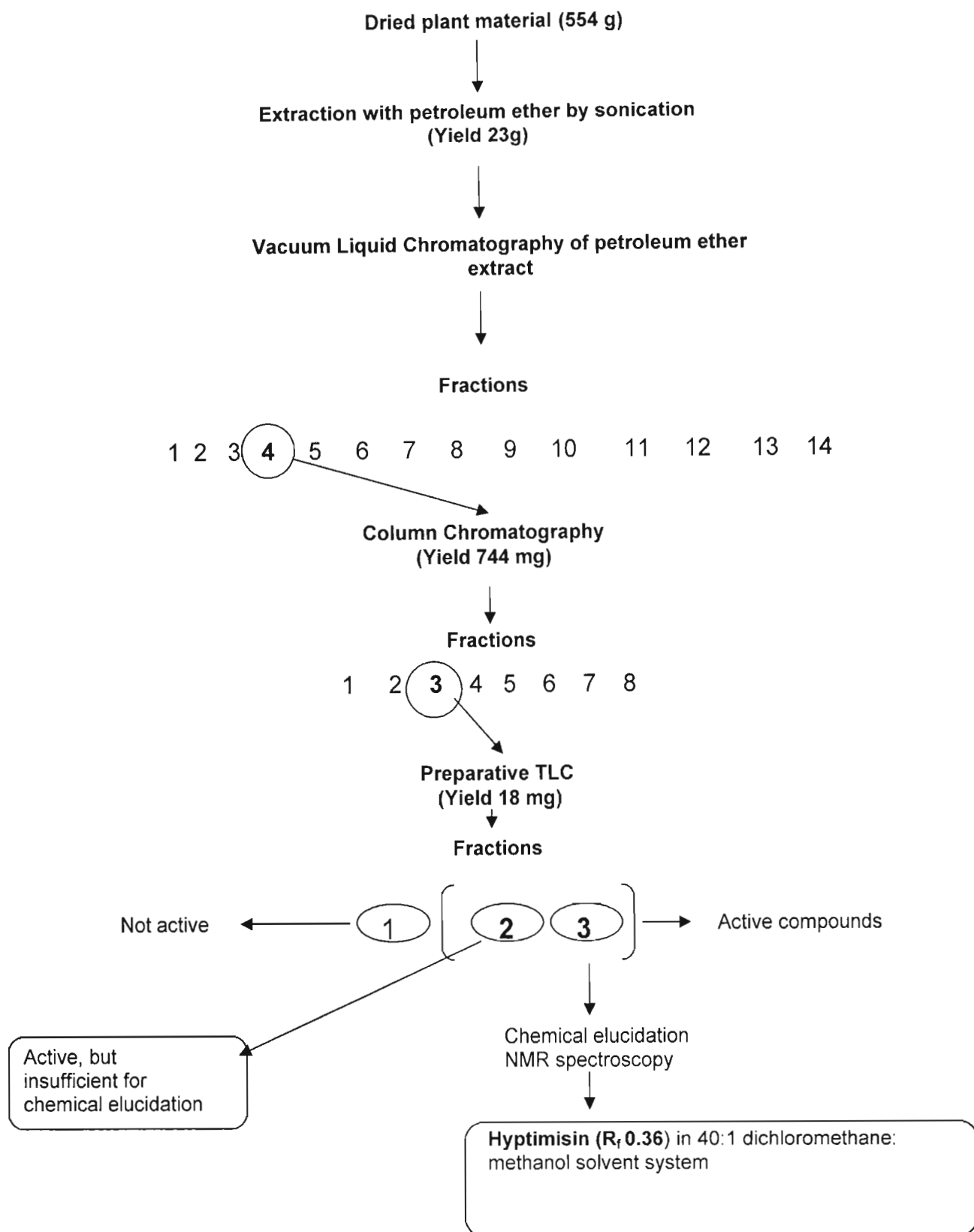


Figure 5. Flow diagram of the isolation steps for antiplasmodial compound(s) using dried *H. suaveolens* leaves

F4/33 in CDCl₃
Pulse Sequence: s2pu1

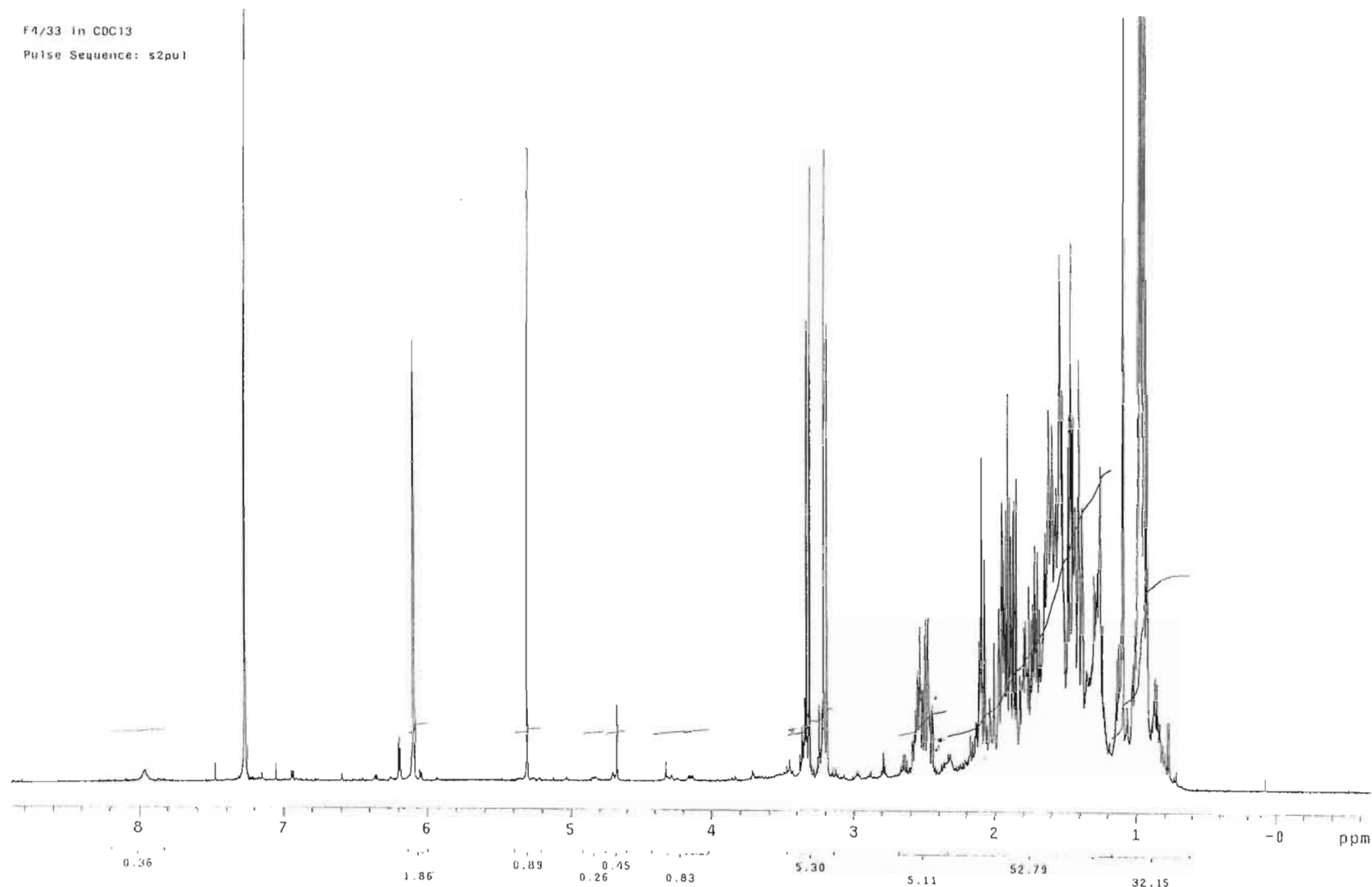


Figure 6. ¹H NMR Spectrum of antimalarial compound (F4-3₃) isolated from *H. suaveolens* leaves

F4/33 in CDCl₃
Pulse Sequence: s2pu1

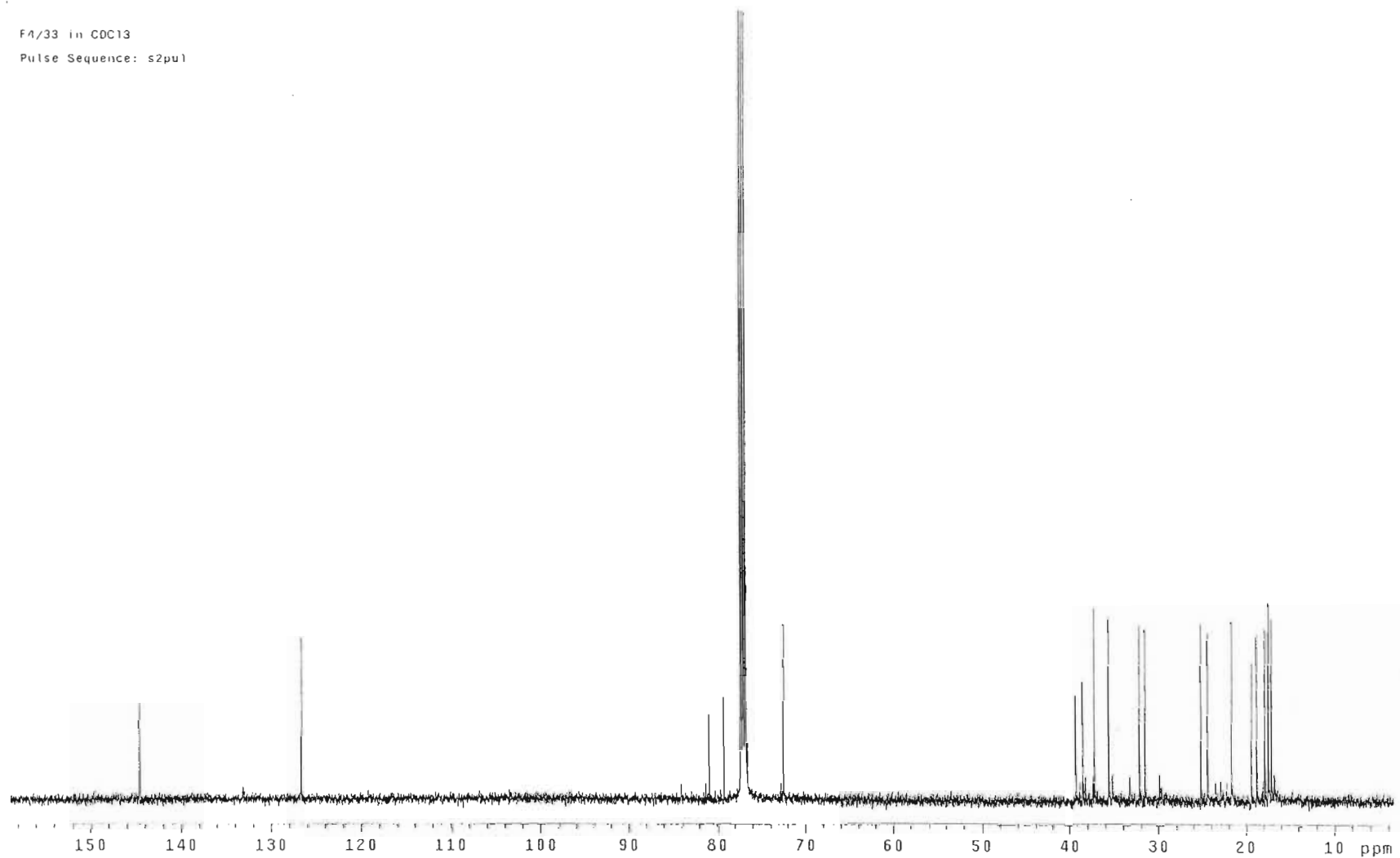


Figure 7. ¹³C NMR Spectrum of antimalarial compound (F4-3₃) isolated from *H. suaveolens* leaves

CHAPTER 6

ISOLATION AND IDENTIFICATION OF EMODIN, AN ANTIBACTERIAL AGENT FROM *Senna occidentalis*

6.1. Introduction

6.1.1. Description and Traditional Medicinal Uses

The genus *Cassia* belongs to the family Leguminosae, subfamily Caesalpinioideae. The subfamily comprises approximately 500 species of trees, shrubs, vines and herbs with numerous species growing in the South American rainforests and tropics. Many species are used medicinally and these tropical plants have a rich history in natural medicine.



Figure 8. *Senna occidentalis* L.

S. occidentalis, is a small tree growing 5 to 8 meters in height. It is found in many tropical areas of South America including the Amazon. Indigenous to Brazil, it is

also found in warmer climates and tropical areas of South, Central and North America. *S. occidentalis* has been used for natural medicine in the rainforest and other tropical areas. Its roots, leaves, flowers and seeds are used in herbal medicine around the world. In Nigeria, the leaves are boiled with yams and eaten for the treatment of fever. In Peru, the roots are considered a diuretic and a decoction is made for fevers (SOUKUP, 1970). The seeds are brewed into a coffee-like beverage for asthma and a flower infusion is used for bronchitis in the Peruvian Amazon (RUTTER, 1990). In Brazil, the roots are considered a tonic, febrifuge and diuretic and it is used for fevers, tuberculosis, anemia, liver complaints, and as a reconstituant for general weakness and illness (COIMBRA, 1994). The leaves and roots are also used in Brazil for gonorrhea, urinary tract disorders, hydropsy, erysclapias, and dysmenorrhoea (CRUZ, 1995). The Miskito Indians of Nicaragua use a fresh plant decoction for general pain, menstrual and uterine pain and for constipation in babies (DENNIS, 1988). *S. occidentalis* has a long history of use in India by indigenous people and tribes for fever, malaria, liver problems, scabies, and skin disorders (NAGARAJU and RAO, 1990).

6.1.2. Chemical Constituents and Biological Activity

Some of the chemical constituents isolated from *Senna occidentalis* include anthraquinone glycosides, C-glycosidic flavonoids, fatty oils, flavonoids, gallactomannan, polysaccharides and tannins (LAL and GUPTA, 1973, 1974; KUDAV and KULKARNI, 1974; SINGH and SINGH, 1985; HATANO *et al.*, 1999; CHAUHAN *et al.*, 2001; PURWAR *et al.*, 2003). In clinical research, S.

occidentalis leaves have shown *in vitro* antibacterial, anti-fungal, anti-parasitic, insecticidal, and anti-malarial properties (CACERES *et al.*, 1991; SCHMEDA-HIRSCHMANN and ROJAS DE ARIAS, 1992). In animal studies, *S. occidentalis* leaves have demonstrated anti-inflammatory, anti-hepatotoxic, hypotensive, smooth muscle relaxing, spasmogenic, weak uterine stimulative, vasoconstricting, hemolysis inhibition, and lipid peroxide formation inhibition activities (FENG *et al.*, 1962; SADIQUE *et al.*, 1987).

6.2. Aims

The aims of this part of the project was to study the antibacterial activity of ethanolic root extract of *Senna occidentalis* and attempt to isolate the bioactive compound(s) using bioassay-guided fractionation.

6.3. Materials and Methods

6.3.1. Plant Extraction

Roots of *S. occidentalis* were collected and dried as described in Sections **2.5.1.** and **2.5.2** in Chapter 2. The dry powdered roots (190 g) were extracted with 1 L of 80% ethanol by sonication for 1 h, at room temperature and left overnight on a magnetic stirrer. The extract was filtered through a Buchner funnel and Whatman No. 1 filter paper. The root residue was extracted three times more with 0.5 L 80% ethanol. The filtration was repeated, the solvent evaporated under reduced pressure at 30°C in a cooled flash evaporator and the extract combined.

6.3.2. Antibacterial Assay

The antibacterial activity of the crude extract was first reconfirmed using the direct bioautographic assay (HAMBURGER and CORDELL, 1987). A developed TLC plate was allowed to dry before being sprayed with a mixture of the test bacteria and liquid nutrient medium. The plate was incubated in a humid atmosphere, resulting in inhibition zone where growth was prevented by the active components. The inhibition zone (Figure 10) was visualized by the use of iodinitrotetrazolium chloride (INT), a tetrazolium salt. All 16 fractions obtained from the fractionation of the crude extract using VLC (Figure 11) were tested for antibacterial activity using the bioautographic assay. The inhibition of bacterial growth by compounds separated on the TLC plate was visible as white spots against a deep red background. The test organism was Gram-positive *Staphylococcus aureus* (ATCC 12600).

6.3.3. Bioassay-Guided Fractionation for Isolation of Active Compound(s)

From this stage, all solvents used were redistilled using a rotary evaporator. A flow diagram summarizes the isolation procedures used (Figure 11).

6.3.3.1. Vacuum Liquid Chromatography (VLC)

The ethanolic crude extract (15g) was first redissolved in ethanol and then mixed with silica gel (10 g). The mixture (extract and silica gel) was allowed to dry before a fan. The dried mixture was applied to the top of the column, and

fractionated by Vacuum Liquid Chromatography (VLC) on silica gel 0.04-0.063 mm (Merck). A column 30 cm in length and with an internal diameter of 6 cm was packed with 150 g silica. Starting with 100% dichloromethane, a dichloromethane:methanol gradient solvent system (100:0; 95:5; 90:10; 85:15; 80:20; 75:25; 70:30; 65:35; 60:40; 55:45; 50:50; 0:100) was employed. A volume of 400 ml of each solvent combination was used for each elution. Bioautography established that the most active fractions eluted with 75:25 (Fraction 6) and 70:30 (Fraction 7) (367 mg) dichloromethane:methanol solvent systems.

6.3.3.2. Gravity – Assisted Column Chromatography

Column chromatography was carried out on the most bioactive Fractions, 6 and 7, which were combined (367 mg) (Figure 11). A column of 73 cm length and 2.5 cm internal diameter packed with 127 g silica gel 0.063–0.200 mm (Merck) was used. Dichloromethane:methanol gradient system (100:0, 95:5, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 0:100) was again used with increasing amounts of methanol up to 100 %. Exactly 500 ml of each solvent mixture was used. The column was washed with 500 ml methanol. A fraction collector (Gilson FC 203B) was set to collect fractions of approximately 10 ml each. From this fractionation, 218 fractions were collected. Fractions were subsequently spotted on TLC plate and developed with CH_2Cl_2 :MeOH (15:1) solvent system. Similar fractions were pooled together and tested for antibacterial activity.

6.3.3.3. Preparative Thin Layer Chromatography

The active compound was identified using bioautographic assay as described in Section 6.3.2. About 20 mg of the most active fractions (Group 2: fractions 31-59, 81 mg) were applied to each TLC plate (Merck glass plates, 20 x 20 cm, Silica gel 60 F₂₅₄, 0.25 mm thickness). The solvent system was dichloromethane:methanol (15:1). Plates were developed to a height of 18 cm. The active band (identified as a dark brown band under UV₃₆₆) was scraped off the TLC plates separately and eluted from the silica with absolute ethanol. The compounds in ethanol were filtered independently through Millipore filters (0.45 µm (HVLP) and 0.22 µm (GVWP)) to remove the silica. The active compound collected was 14 mg in weight. The purity of the isolated compound was confirmed by TLC using various solvent systems. The isolated compound was again tested for antibacterial activity.

6.3.4. Identification of Purified Active Compound

Nuclear Magnetic Resonance Spectroscopy (¹H, ¹³C, COSY, HMBC, HMQC and NOESY) at the School of Chemical and Physical Sciences, University of KwaZulu-Natal, Pietermaritzburg, was performed to authenticate the structure of the isolated compound. A Varian Unity Inova 500 MHz spectrometer was used. The NMR spectra were analyzed with the help of Professor D. A. Mulholland and Dr. P. Coombes of the School of Pure and Applied Chemistry, University of KwaZulu-Natal, Durban.

6.3.5. MIC Determination

The MIC of the isolated compound was determined using the microdilution bioassay described in Section 2.5.5, Chapter 2. The compound was dissolved in 25 % ethanol at a concentration of 2 mg/ml (giving a starting concentration of 0.5 mg /ml). Due to A limited amount of pure compound, it was tested against *S. aureus* (ATCC12600), *E. coli* (ATCC 11775), *K. pneumoniae* (ATCC 13883) and *B. subtilis* (ATCC 6051) only.

6.4. Results and Discussion

6.4.1. Plant Extraction

The dried and powdered roots (190 g) of *S. occidentalis* yielded 15 g of crude extract.

6.4.2. Bioassay-Guided Fractionation for Isolation of Active Compound(s)

The result of antibacterial assay of the crude extract is presented in Figure 9. The VLC of the ethanolic crude extract yielded 16 fractions that were subsequently subjected to antibacterial bioassay. The results of the antibacterial bioassays showed Fractions 6 (198 mg) and 7 (169) to be the most active. Fractions 6 and 7 were combined and further separated using Gravity–assisted Column Chromatography. This process yielded 218 eluants that were grouped into 10 Groups according to their TLC patterns. They were again assayed for

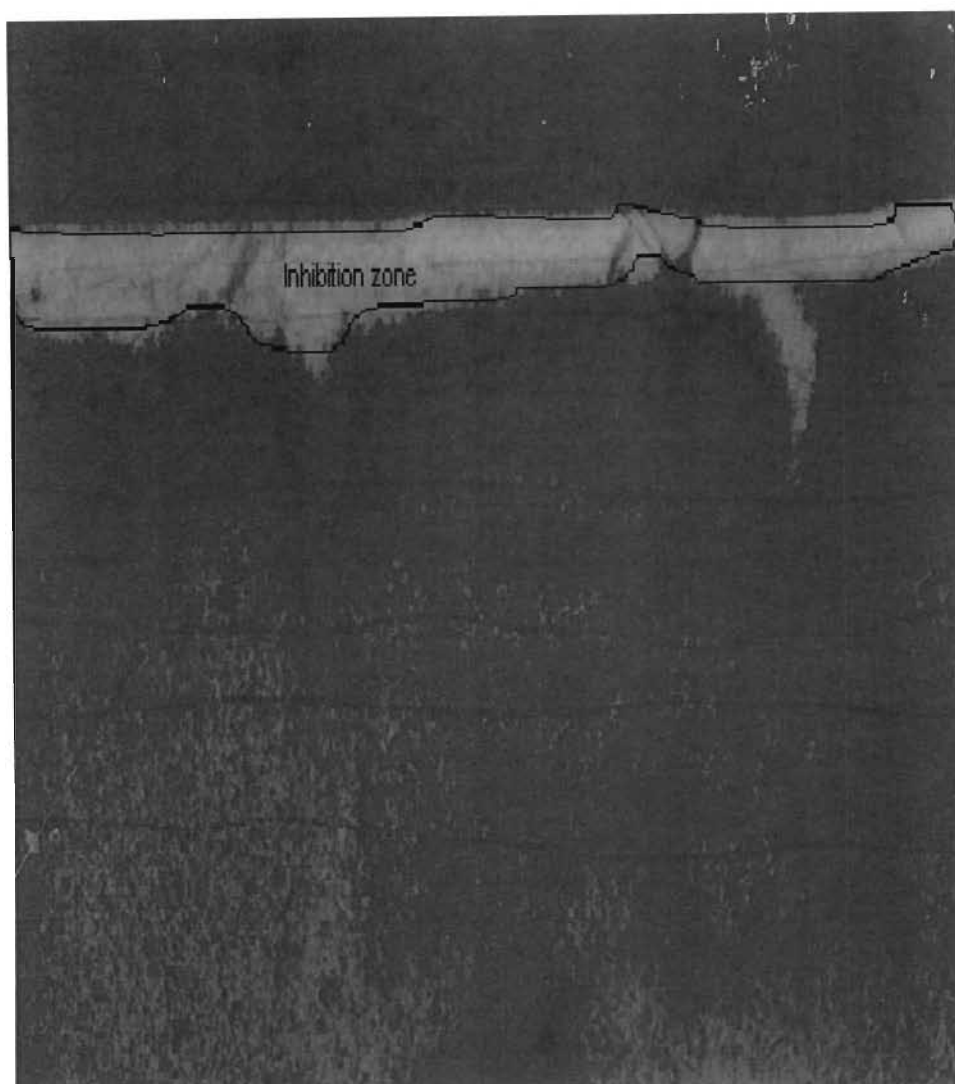


Figure 9. Chromatogram of ethanolic root extract of *Senna occidentalis* showing antibacterial activity (Inhibition zone) of the active component

antibacterial activity and the results showed Group 2 (Fractions 31-59) as the most active group. Group 2 was separated further using preparative TLC and a 15:1 (dichloromethane:methanol) solvent system. This yielded the active compound. The active compound (14 mg), visibly yellow on the TLC plate, was readily soluble in ethanol and deep red in solution. It appeared as a dark brown band under UV_{366} , and had an R_f of 0.9.

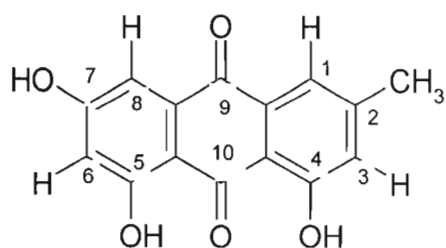
6.4.3. NMR Spectroscopy

Inspection of the 1H and ^{13}C NMR (Figures 12 and 13) of the isolated compound (Figure 10) revealed that it possesses 15 carbon atoms. Signals attributable to two conjugated carbonyl carbons (δ_C 188.3 (s), 185.3 (s)), three singlet oxygenated aromatic carbons (δ_C 163.0, 167.9, 180.3), four doublet aromatic carbons (δ_C 110.4, 117.3, 120.9 and 124.7), and an aromatic methyl group (δ_H 2.38 (s), 3H) collectively suggest the isolated compound to be a trihydroxymethylantraquinone.

A correlation in the HMQC spectrum between the more upfield of the carbonyl resonances at δ_C 185.3, ascribed to C-9, and the most downfield of the 1H aromatic proton doublet signals at δ_H 7.47 (*d*, $J = 1.9$ Hz), assigned this as H-1. A correlation in the NOESY spectrum between H-1 and the aromatic methyl signal at δ_H 2.38 placed this group at C-2, and a further correlation in the NOESY spectrum between this methyl resonance and that of a two proton, superimposed, multiplet signal at δ_H 6.97 assigned one of these as H-3.

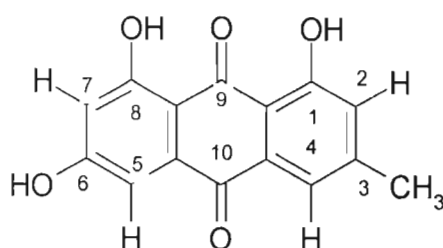
A further correlation observed in the HMQC spectrum between C-9 and the multiplet signal at δ_H 6.97, as it cannot be between C-9 and H-3, must be between C-9 and the second aromatic proton, which must be H-8; the fourth and the final aromatic proton doublet signal at δ_H 6.15 (*d*, *J* = 2.2 Hz) can then be assigned to H-6. The three hydroxyl groups are placed at C-4, C-5 and C-7; the placements at C-5 and C-7 are supported on biosynthetic grounds, and those at C-4 and C-5 by the absence of correlations in the HMQC spectrum between any of the aromatic proton resonances and the second singlet carbonyl carbon signal at δ_C 188.3, assigned to C-10.

A literature search revealed the isolated compound, as 4,5,7-trihydroxy-2-methylantraquinone, to be equivalent to 1,6,8-trihydroxy-3-methylantraquinone, or emodin, which has previously been reported from a wide variety of botanical and fungal sources (TURNER, 1971; TURNER and ALDRIDGE, 1983; WANG *et al.*, 1996; FUJIMOTO *et al.*, 1998), including other species of the genus *Cassia* (KELLY *et al.*, 1993) excluding *Cassia occidentalis*.



(a) Isolated compound

4,5,7-trihydroxy-2-methylantraquinone



(b) emodin

1,6,8-trihydroxy-3-methylantraquinone

Figure 10. Structure of emodin and the antibacterial isolated from *Senna occidentalis*

6.4.4. MIC Determination

The MIC (mg/ml) results obtained for the pure isolated compound are presented in Table 8. Due to a limited amount of the isolated compound, it was dissolved at a concentration of 2 mg/ml. At this concentration, there was no bacteriostatic effect on the Gram-negative bacteria. This was not surprising since the crude extract did not exhibit any bacteriostatic effect on these Gram-negative bacteria (Table 3). However, the pure compound exhibited a remarkable bacteriostatic effect on the Gram-positive bacteria tested; especially *S. aureus* and it had significantly higher activity when compared with the results obtained for the crude extract. The isolated compound also exhibited a higher bacteriostatic effect against *S. aureus* when compared to the standard (neomycin).

Table 11. Minimum Inhibition Concentrations (mg/ml) at which the isolated compound from *Senna occidentalis* roots showed a bacteriostatic effect against test bacteria (average of two experiments)

Compound tested	Bacterium ^a			
	<i>K.p</i>	<i>E.c</i>	<i>B.s</i>	<i>S.a</i>
4,5,7-Trihydroxy-2-methylantraquinone	>0.5	>0.5	7.8 x 10 ⁻³	3.9 x 10 ⁻³
Neomycin standard	1.6 x 10 ⁻³	1.6 x 10 ⁻³	7.8 x 10 ⁻⁴	6.3 x10 ⁻³

Abbreviations: ^a *K.p.* – *K. pneumoniae*; *E.c.* – *E. coli*; *B.s.* – *B. subtilis*; *S.a.* - *S. aureus*.

6.5. Conclusions

One bioactive agent responsible for part of the antibacterial activity of *Senna occidentalis* ethanolic crude extract was isolated. Although it is not a new compound, it is the first time it is reported from *Senna occidentalis* roots. The antibacterial activity exhibited by the pure compound was remarkably higher than that of the crude extract.

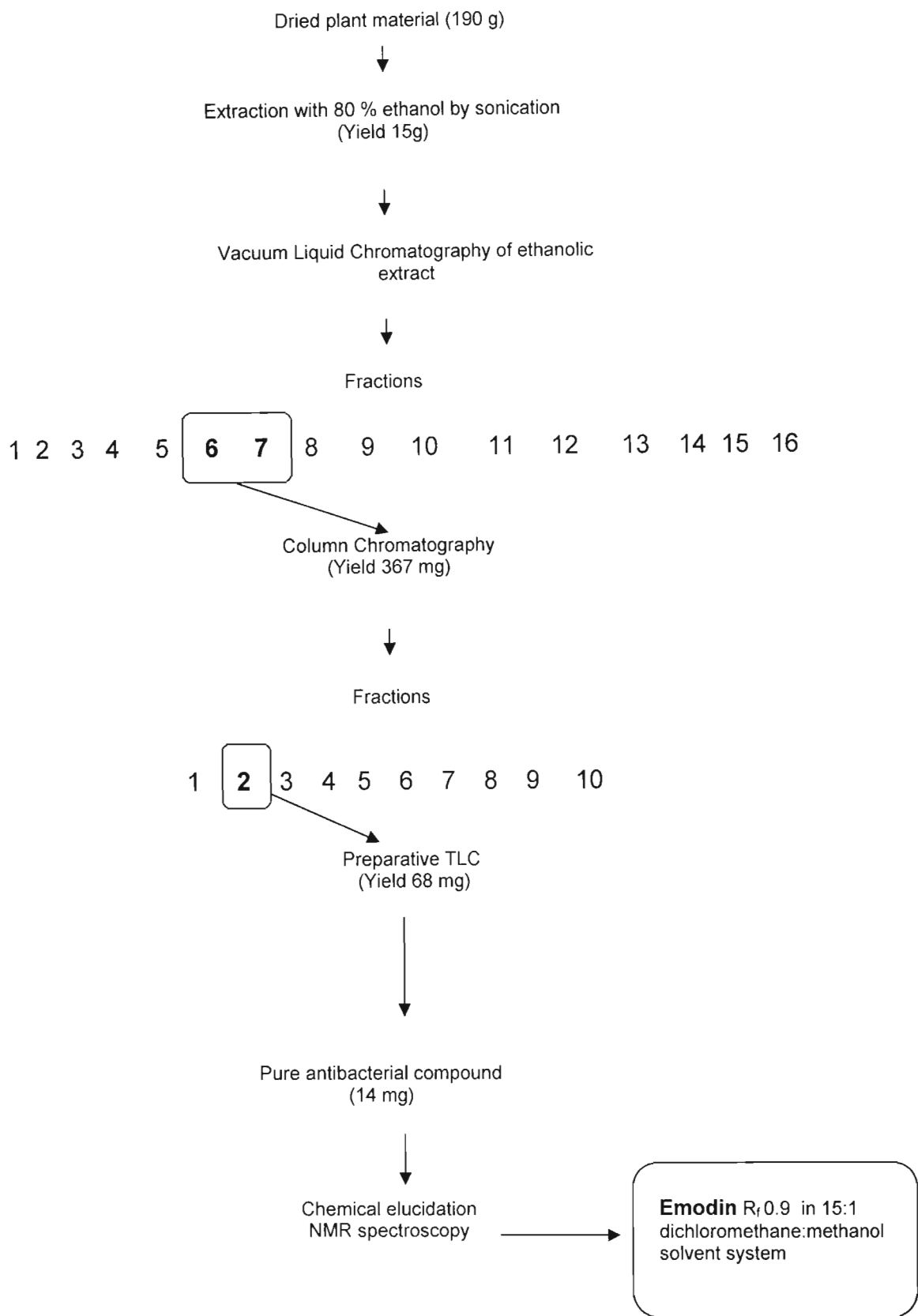


Figure 11. Flow diagram of the isolation steps for antibacterial compound(s) using dried *S. occidentalis* roots

9FG in CD300
Pulse Sequence: s2pul

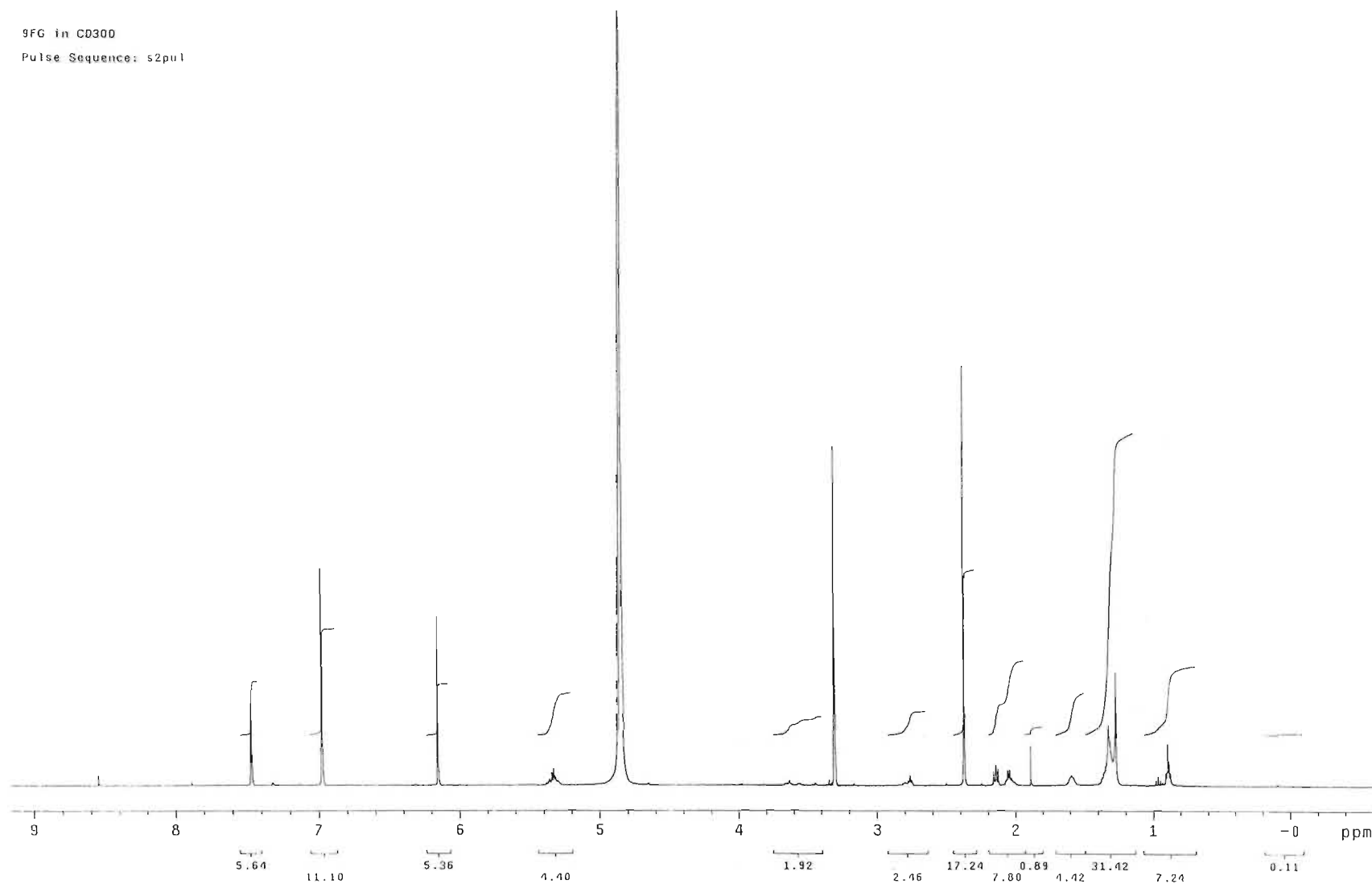


Figure 12. ^1H NMR Spectrum of antibacterial compound isolated from *S. occidentalis* roots

9FG in CD300

Pulse Sequence: s2pu1

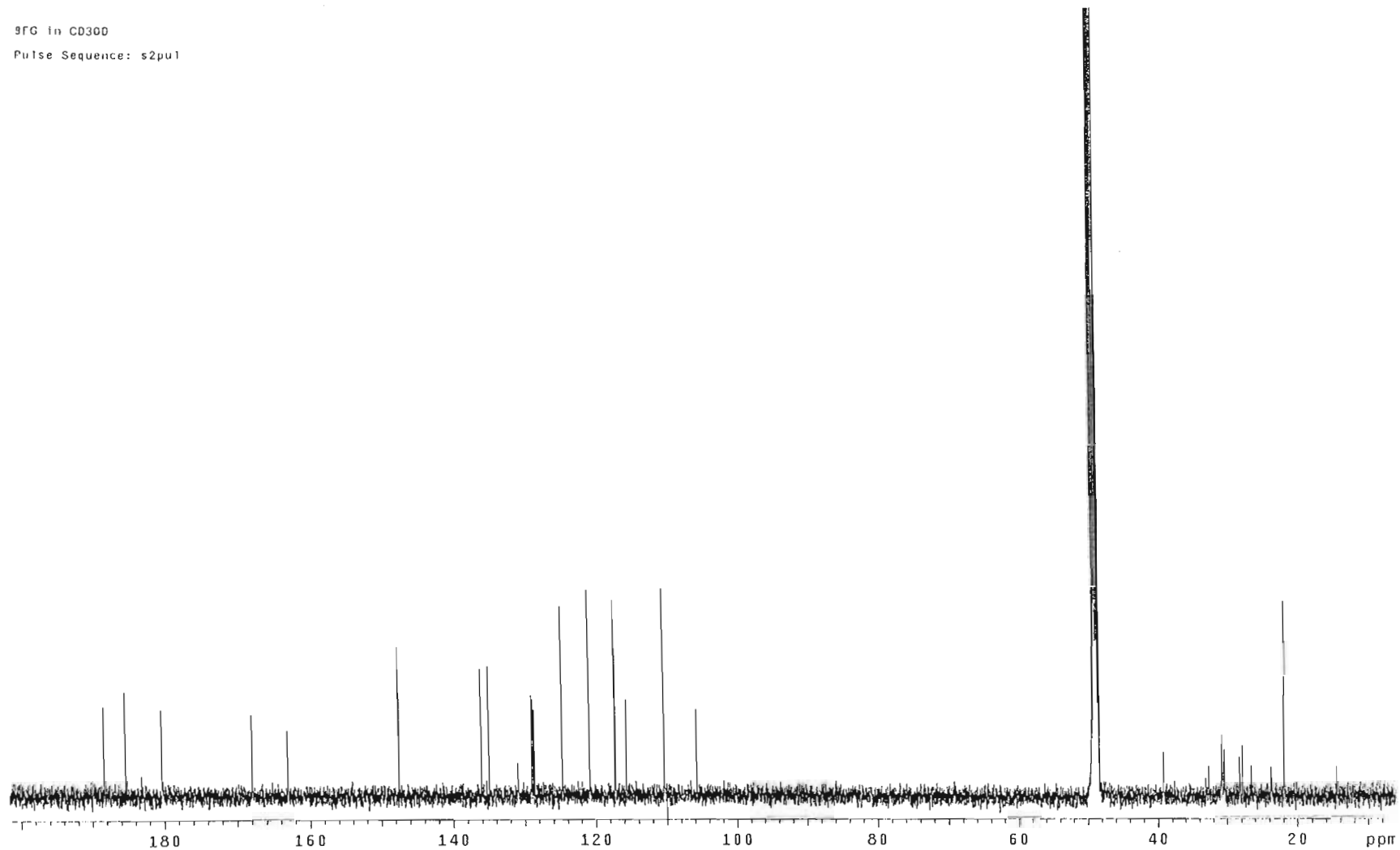


Figure 13. ^{13}C NMR Spectrum of antibacterial compound isolated from *S. occidentalis* roots

CHAPTER 7

GENERAL CONCLUSIONS

7.1. Introduction

Before the introduction of western medicine in Africa, Nigeria in particular, traditional healers have already been in existence, taking care of people's health problems. This has been possible due to the availability of large numbers of medicinal plants. Today most traditional healers operate in the rural communities where access to Western medical practitioners is inadequate. Because of a long history of traditional healing and the confidence the communities have in the healers, rural populations still depend on them for some of their medical problems. Diseases treated by traditional healers include bacterial infections, malaria and inflammations. This is possible due to the long experience of these healers in recognizing the symptoms of these illnesses.

The results presented in this thesis deals with the general screening of various medicinal plants used by Nigerian traditional healers for treating bacterial infections, inflammatory cases and malaria. Antibacterial, anti-inflammatory and antiplasmodial bioassays were employed in analysing different activities possessed by the medicinal plants analysed. After the initial screening, the investigation was taken further towards isolating the active agent(s) from some of the medicinal plants that showed promising activity in various bioassays used during initial screening.

7.2. General Screening of Plants for Biological Activity

As earlier highlighted in Chapter 1, the importance of traditional healers in the primary healthcare system, mainly in developing countries is now taken seriously by The World Health Organisation. Because of the role they play in our everyday health life, The World Health Organisation has been encouraging Countries to incorporate the traditional healers in their primary healthcare systems. The results of the general screening of medicinal plants studied presented in Chapters 2, 3 and 4 showed that a considerable number of the plants used by the traditional healers for treating bacterial infections, inflammatory cases and malaria are effective in *in vitro* tests. *In vivo* experiments and toxicity tests are needed to ascertain the total effectiveness and safety of the relevant plant preparations.

With regards to antibacterial screening, a number of plants exhibited promising activity against a variety of bacteria. The antibacterial activity of some of the plant extracts studied against Gram-negative bacteria is very promising considering the fact that Gram-negative enteropathogenic bacteria pose a greater threat by causing more serious, life threatening diarrhoea and dysenteric diseases. In the anti-inflammatory screening, some of the plants showed high activity, and also exhibited selective COX-2 inhibitory activity while the antimalarial screening provided us with new plants with promising antimalarial activity.

7.3. Isolation and Identification of Active Compound(s) in Plants

The screening of medicinal plants for any pharmacological activity is mostly inspired by the possible discovery of either new drugs or blueprints for new drugs. In Chapters 5 and 6, the isolation of a novel antiplasmodial diterpenoid from leaves of *Hyptis suaveolens* and the antibacterial emodin from roots of *Senna occidentalis* were presented. Although emodin is not a new compound, it is the first time it is reported from *Senna occidentalis* roots. The isolation of the antiplasmodial diterpenoid from *Hyptis suaveolens* is no doubt the major achievement in this project. However, the mode of action and toxicity of the compound need to be urgently ascertained in further research.

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