THE ANTIMALARIAL POTENTIAL OF UGANDAN TRADITIONAL MEDICINES: A STUDY OF SIX PLANTS USED TO TREAT MALARIA SYMPTOMS

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Dedicated to Noah Matende, Catherine Matende and Joan Ziria Tibalwa for their selfless support

DECLARATION

'The antimalarial potential of Ugandan traditional medicines: a study of six plants used to treat malaria symptoms'

I declare that the work on which the above thesis is based is my original work, both in concept and execution. Normal guidance was received from my supervisors and any additional assistance is stated in the acknowledgements.

Neither the substance nor any part of the above thesis has been submitted in the past, or is being, or is to be submitted for another degree at this University or any other academic Institution.

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PREFACE

The work described in this thesis was carried out between February 2000 and June 2003. The work had two components, a field component which was carried out in Uganda, and a laboratory component most of which, was carried out in the laboratories of the Division of Clinical pharmacology, Department of Medicine at the University of Cape Town, South Africa. Some of the extractions were done in the Department of Chemistry at Makerere University in Uganda.

The thesis has got six chapters preceded by an abstract, table of contents, lists of tables and figures, a dedication and acknowledgements. A list of important references is attached at the end of chapter 6 followed by appendices. Chapter one reviews important literature on malaria with the aim of justifying this study. This chapter also covers the scope and objectives of the study. The second chapter details the methods used in the study. The results of the study are presented in chapters three, four and five, each followed with a discussion and conclusion. Chapter six presents the general discussion. In this chapter we review the study objectives, summarise essential conclusions from the study, point out the relevance of study findings and its limitations. We also look at research prospects that may arise from the study findings.

I have made a very effort to keep apace with contemporary literature and compare my results with existing information. This manuscript received inputs from my supervisors and a number of colleagues, and I have taken reasonable effort to rid it of any errors and redundancies. I am however fully accountable for the final version. I hope readers will find it valuable.

ACKNOWLEDGEMENTS

It gives me pleasure to acknowledge the guidance of my supervisors Professor Peter Folb and Associate Professor Peter Smith. The scope and liberty they entrusted to me in the development and execution of this project has taught me the discipline required of research. Moreover, the quality of work exhibited in this thesis would not be attained without their advice.

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ABSTRACT

The study investigates the antimalarial potential of six Ugandan traditional medicinal plants *Senecio discifolius* oliv, *Senecio stuhlmannii*, *Indigofera emarginella* steud. Ex A. Rich, *Aspilia africana* (Pers) C.D. Adams, *Cardiospermum halicacabum* L. and *Momordica foetida* Schumch. Et Thonn. Selection of the plants was based on ethnobotanical surveys of traditional treatment of malaria symptoms and reports from traditional healers practising in three different communities.

Extracts from the plants were evaluated for *in vitro* activity against both chloroquine-sensitive (D10) and chloroquine- and sulphonamide-resistant (K1) strains of *P. falciparum*. Extracts without *in vitro* antiplasmodial activity were tested for activity in a murine model of cerebral malaria. Bioassay-guided fractionation was carried out on active extracts. The antiplasmodial activity of one of the isolates was analysed geometrically to determine its interaction with chloroquine and artemisinin, respectively. *S. discifolius*, *S. stuhlmannii*, *I. emarginella* and *A. africana* were shown to have antiplasmodial activity, the 50% inhibitory concentration (IC_{50}) ranging 8.00 to 29.0 μg/ml against the two strains of *P. falciparum*. There was positive correlation between the activity of the extracts against the chloroquine-sensitive and -resistant strains ($r=0.967$, $p=0.05$). A sesquiterpene, referred to as **AA2**, with molecular weight 264.14 and molecular formula $C_{15}H_{20}O_4$ was isolated from *A. africana*. This isolate had antiplasmodial activity of IC_{50} 1.8 (1.6-2.0) μg/ml. Another isolate, **ACW1**, with molecular weight 213 and IC_{50} of 1.51(1.21-1.81) and 0.50 (0.26-0.86) μg/ml against D10 and K1, respectively, was isolated from another batch of *A. africana*. **ACW1** antagonised the antimalarial activity of artemisinin against both D10 and K1, and of chloroquine against K1, but was additive with chloroquine against D10. **ACW1** in concentrations of 150, 350 and 600ng/ml inhibited accumulation of ^{3}H-dihydroartemisinin by erythrocytes infected with K1, and enhanced its accumulation by D10. The water extract of *M. foetida*, a plant without *in vitro* antiplasmodial activity, delayed development of parasitaemia in mice and prolonged survival to 20 days.

This study provides evidence of *in vitro* antimalarial efficacy of *S. discifolius, S. Stuhlmannii*, I. *emarginella* and A. *africana*, and of *in vivo* activity of *M. foetida*. The results suggest that these Ugandan traditional medicines may have promising antimalarial potential for further development.

RESEARCH SUMMARY

Parasite resistance to existing antimalarial drugs is on the rise, extending to new geographical areas and affecting species other than *P. falciparum*, raising the need to develop new malaria remedies. In Uganda, malaria is responsible for over 20% of the national disease burden. Many communities use traditional medicines in the treatment of malaria. The potential of Uganda's traditional medicinal plants as sources of malaria remedies has not been explored to date. This study investigates the antimalarial potential of six Ugandan traditional medicinal plants: *Senecio discifolius* oliv, *Senecio stuhlmannii*, *Indigofera emarginella* steud. Ex A. Rich, *Aspilia africana* (Pers) C.D. Adams, *Cardiospermum halicacabum* L. and *Momordica foetida* Schumch. Et Thonn. The selection of these plants was based on ethnobotanical surveys on the traditional treatment of malaria related symptoms and reports from three traditional healers identified within the framework of the National Council for Traditional Healers Associations (NACOTHA) and practicing in three different communities.

The extracts from the plants were evaluated for *in vitro* efficacy using the lactate dehydrogenase antiplasmodial assay against both the chloroquine-sensitive (D10) and the chloroquine-and-sulphonamide resistant (K1) strain of *P. falciparum*. Extracts that did not show *in vitro* antiplasmodial activity were tested for antimalarial activity using a murine model of cerebral malaria. The study plants were collected in two batches, during the wet and dry season to determine seasonal variations in yields and antimalarial activity. Bioassay-guided fractionation was carried out on the active extracts. The antiplasmodial activity of one of the isolates from the study plants was determined in combination with chloroquine and artemisinin, and analysed geometrically to determine the nature of the interaction between the two. To determine the possible mechanism of action behind the interaction between artemisinin and the plant isolate, the accumulation of triated dihydroartemisinin (^{3}HDHA) by erythrocytes infected with the D10 strain and the K1 strain of *P. falciparum* was characterised. The effect of three extract doses on the uptake of ^{3}HDHA was determined.

The plants: *S. discifolius*, *S. stuhlmannii*, *I. emarginella* and *A. africana* had antiplasmodial activity of 50% inhibitory concentration (IC_{50}) ranging 8.00 to 29.0 μg/ml against the two

strains of *P. falciparum*. The ethyl acetate extract of *A. africana* was the most active from the two plant batches with IC_{50} of 9.3 (7.7-10.9) μg/ml against the D10 strain and 11.5 (8.7-14.3) μg/ml against K1 strain of *P. falciparum* during the wet season and 8.00 (5.30-10.70) μg/ml against the D10 strain during the dry season. There was a positive correlation between the activity of the extracts against the chloroquine-sensitive and the resistant strain (pearsons' coefficient r =0.967, p=0.05), with no significant difference between the activity of the extracts on both strains. The plant batch collected in the wet season had better yields than one collected during the dry season. Extracts from *S. Stuhlmannii*, and *I. emarginella* collected during the dry season had better antiplasmodial activity than those from the plant batch collected in the wet season. A sesquieterpene, referred to, as **AA2** in our laboratory with molecular weight of 264.14 and a molecular formula of $C_{15}H_{20}O_4$ was isolated from *A. africana*. This isolate had antiplasmodial activity of 1.8 (1.6-2.0) μg/ml. Another isolate from *A. Africana*, **ACW1** with molecular weight of 213, and antiplasmodial activity of IC_{50} 1.51(1.21-1.81) and 0.50 (0.26-0.86) μg/ml against the D10 strain and K1 respectively was isolated from another batch of *A. africa*. The isolate **ACW1** antagonised the antimalarial activity of artemisinin against both the D10 and K1 strains of *P. falciparum*. The same isolate antagonised the antimalarial activity of chloroquine against the K1 strain but had an additive effect with chloroquine against the D10 strain of *P. falciparum*. The accumulation of dihydroartemisinin ^{3}HDHA by erythrocytes infected by both the D10 and K1 strains of *P. falciparum* was characterised to be temperature dependent and saturable although the K1 strain accumulated ^{3}HDHA more efficiently than the D10 strain. The isolate, **ACW1** at the study doses: 150, 350 and 600ng/ml inhibited the accumulation of ^{3}HDHA by erythrocytes infected with the chloroquine-resistant K1 isolate of *P. falciparum*. On the other hand the same test doses caused enhanced accumulation of ^{3}HDHA by erythrocytes infected with the chloroquine-sensitive D10 strain of *P.falciparum*.

The water extract of *M. foetida* a plant whose extracts did not show any *in vitro* antiplasmodial activity was well tolerated in oral doses as high as 1000mg/kg/day by C57BL mice infected with *P. berghei* (Anka). The above dose delayed the development of parasitaemia in mice for 6 days and prolonged the survival of mice infected intraperitoneally with $1x10^7$-infected erythrocytes to 20 days. The mean survival infected mice increased with dose of the extract. The water extract of *C. halicacabum* was not tolerated, with all the study animals dying with in the first four days of the experiment.

This study provides evidence of antimalarial efficacy for the plants: *S. discifolius, S. Stuhlmannii*, I. *emarginella* and A. *Africana* based on *in vitro* findings and *M. foetida* basing on animal studies. There are seasonal variations in yields of active principles and antiplasmodial activity. There is no cross-resistance between the traditional medicines studied and chloroquine suggesting that remedies based on traditional medicines can be used to treat both chloroquine-sensitive and chloroquine-resistant malaria. Ugandan traditional medicines can also be sources of lead compounds for the development of antimalarial drugs. Some traditional medicines antagonise the antimalarial activity of antimalarial drugs a situation, which could contribute to treatment failure in many communities. It is our conclusion that Ugandan traditional medicines have a high antimalarial potential.

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LIST OF ABBREVIATIONS

^{3}H-DHA	Triated Dihydroartemisinin
AIDS	Acquired Immunodeficiency Syndrome
APAD	3-acetylpyridine adenine dinucleotide
APADH	Reduced 3-acetylpyridine adenine dinucleotide
BBC	British Broadcasting Corporation
BCE	Before Christian Era
C_{max}	Maximum Concentration
DDT	Dichlorodiphenyltrichloroethane
DHA	Dihydroartemisinin
DNA	Deoxyribonucleic acid
DPM	Disintegrations Per Minute
EDTA	Ethylenediaminetetra-acetic Acid
FC	Flash chromatography
FIC	Fractional Inhibitory Concentration
fmol	Femtomoles
HEPES	N-(2-hydroxyethyl)-piperazine–N-2-ethanesulphonic acid
HPLC	High Performance Liquid Chromatography
IC_{50}	50% Inhibitory Concentration
IFN-γ	Interferon-γ
IL-10	Interleukin-10
IL-12	Interleukin-12
Mc	Micro curie
ml	Millilitre

MS	Mass Spectrometry
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
NACOTHA	National Council for Traditional Healers Associations in Uganda
NAD	Nicotinamide Adenine Dinucleotide
NADH	Reduced Nicotinamide Adenine Dinucleotide
NAPRALERT	Natural Products Alert
NBT	Nitroblue tetrazolium
ng	Nanogramme
NMR	Nuclear Magnetic resonance
PBS	Phophate Buffered Saline
PCR	Polymerase Chain Reaction
PES	Phenazine ethosulfate
PLDH	Parasite Lactate dehydrogenase assay
RPM	Revolutions Per minute
RT	Retention Time
SP	Sulphadoxine/Pyrimethamine
SPE	Solid Phase Extraction
THETA	Traditional and Modern Health Practitioners Together Against AIDS
TNF-α	Tumour Necrosis Factor-α
US$	United States Dollar
UV	Ultra Violet
V3	Version 3
WHO	World Health Organisation

CHAPTER 1

Chapter I: Introduction, literature review and scope of the study

1.1 Introduction

Malaria, a disease perhaps as old as mankind, is caused by obligate intracellular protozoa of the genus *Plasmodium*. Records on deadly fevers that could have been due to malaria are found in the Indian Vedic writings of 1600 B.C (Desowitz, 1991). The origin of this disease is not certain but human movements are responsible for its spread. Today, people present with malaria in all parts of the world. This disease has a short natural history and may result in death if untreated.

In this chapter we give an overview of the current global malaria disease burden, transmission, pathogenesis and control measures. The Uganda experience with malaria is discussed, together with potential solutions to this problem. There is a section that discusses the status, organisation and practice of traditional medicine in Uganda. In the last part, a study that investigates the antimalarial potential of Ugandan traditional medicines is introduced.

1.2 Malaria disease burden, transmission, pathogenesis and control

1.2.1 The malaria disease burden

More than two-thirds of the world's population are living in malaria endemic areas. It is estimated that 200 million people show signs and symptoms of malaria every year of which one to two million people succumb annually to the disease (Wernsdorfer, 1998). Most of the malaria victims live in the developing part of the world, where the average yearly per capita income is between 200-600 United States dollars (US$) and government expenditure on health is extremely low. The exact economic impact of malaria in these countries is difficult to assess due to a lack of relevant data; however, it is estimated that the average cost of a malaria attack to a family in sub-Saharan Africa is close to 12 US$ (Ettling and Shepard, 1991). With the emergence of multi-drug resistant strains of *Plasmodium* and, despite widespread national efforts to combat malaria, the incidence of the disease is on the rise in many countries (Chattopadhyay and Sengupta, 2000; Accorsi *et al.*, 2001, and Ndiaye *et al.*, 2001) and the world is facing an increasing disease burden (WHO, 1998). This is attributed to population growth, increased global movements, changing agricultural practices, weakening of public health systems, long-term environmental changes and global warming. These factors

are compounded by the increasing incidence of parasite resistance to the most effective drugs and vector resistance to the affordable insecticides (Sachs and Malaney, 2002).

At the start of the 20th century, malaria was a problem in all continents; however, today it is becoming more of a tropical problem, where its distribution correlates closely with low household incomes. Changes in global malaria risk distribution between 1946 and 1994 are shown in figure 1.

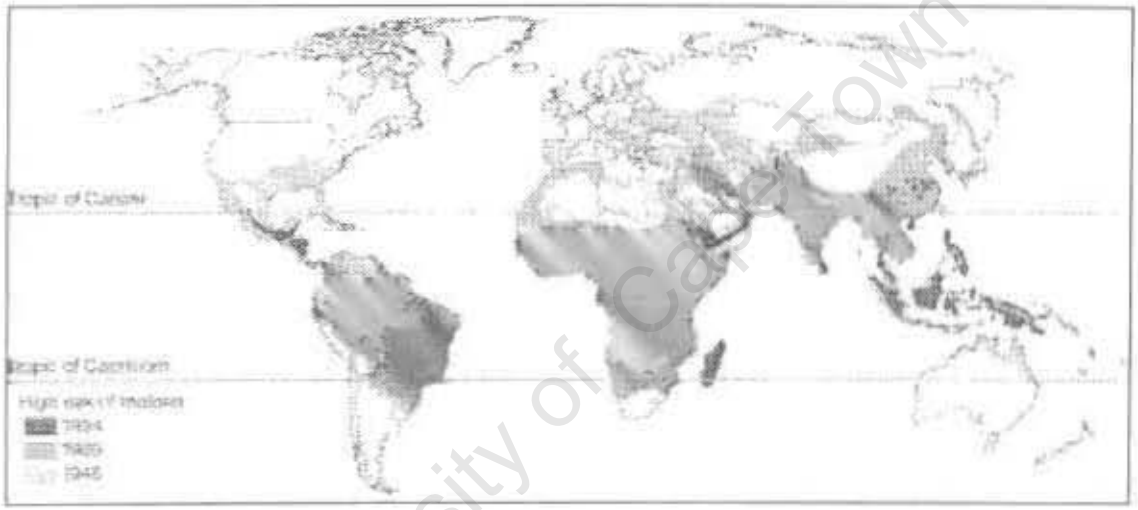

Figure 1.2.1 Global distribution of malaria. The changing global distribution of malaria risk from 1946 to1994 shows a disease burden that is increasingly confined to tropical regions (After Sachs and Malaney, 2002)

1.2.2 Transmission of Malaria

Human malaria is caused by parasites of the zoological order *Haemosporidia*, the family Plasmodiidae, genus *Plasmodium*. This genus is divided into two sub-genera: *Plasmodium* in the strict sense and *Laverania*. The most important human pathogen *Plasmodium falciparum* belongs to the *Laverania* sub-genera while *Plasmodium malariae, Plasmodium ovale* and *Plasmodium vivax* belong to *Plasmodium* subgenera. Other *Plasmodium* species that are mammalian pathogens include *P. berghei, P. chabaudi, P. yoelii and P. vinckei;* these generally affect lower mammals.

The *Plasmodium* species spends part of its life-cycle in both a vertebrate and invertebrate host. Man is the vertebrate host of the human species of *Plasmodium.* In the vertebrate host it is found both in the liver cells and outside liver cells, either inside or outside the erythrocytes. The insect vector for human malaria is the mosquito of the genus *Anopheles,* Order: Diptera, Family: Culicidae and Subfamily: Anophelinae. The species within the *Anopheles gambiae* complex that are responsible for the transmission of malaria in Africa are: *A. arabiensis, A. bwambae, A. gambiae s.s, A. melas, A. merus and A.quadriannulatus.* The variety of malaria vector species has ensured the existence of at least one malaria transmission vector in most tropical habitats (Rogers *et al.*, 2002).

1.2.3 Life-cycle of the malaria parasite

Human infection is initiated when the mosquito, through its bite, inoculates sporozoites into the blood stream. The sporozoites are carried to the liver parenchyma cells, which they invade and there develop into merozoites through a process of fission called schizogony. The infected liver cells burst to release merozoites about two weeks after the mosquito bite. The merozoites then invade red blood cells, where they develop into ring forms. These mature within hours to trophozoites and later to erythrocytic schizonts. The schizont-infected cells burst to release merozoites, which then invade other erythrocytes forming an erythrocytic cycle. Sexual stages form in some erythrocytes, which are then taken up into the mosquito when taking its blood meal. The female and male gametocytes meet and develop into an oocyst in the gut wall of a mosquito. The oocyst develops into sporozoites within 2 weeks and move to the mosquito salivary glands where they mature ready for the next part of the parasite life cycle.

1.2.4 Immunological response to malaria parasites

The malaria parasites go through several stages in the animal host, each stage exposing specific antigen characteristics. The human immune system responds differently to the pre-erythrocytic stage antigens and erythrocytic stage antigens. Both Th1 and Th2 responses play a role in the active immunological response to malaria. The response to the pre-erythrocytic stages is largely a Th1 response. The macrophage presents the parasitic antigen to the T-lymphocytes, which then release IFN-γ and IL-2; the latter are responsible for the proliferation of CD8 (+) T cells into cytotoxic cells and the release of nitric oxide by

macrophages, which kill the parasitised erythrocytes. Interferon-γ serves also to amplify and enhance the production of TNF-α (by macrophages), IL-10 and IL-12 (by $CD4^+$ cells), besides the malaria toxin directly causes release of TNF-α by macrophages (Kwiatkowski *et al.*, 1989). Although TNF-α causes proliferation of $CD8^+$ cells, it has also been implicated as the causative agent of several pathological changes in malaria. Severe forms of malaria are associated with raised levels of TNF-α and IL-10 (Shaffer *et al.*, 1991) and down-regulation of IL-12 activity (Peyron *et al.*, 1994).

Development of passive immunity to malaria in humans takes a number of years and it is not absolutely protective. It is possible that this is due to factors such as antigenic variation, antigenic polymorphism, and poor immunological responses to critical antigens. The immunological response to malaria is not fully understood but varies with infecting species, previous exposure to the host and severity of disease. These factors combined have made it difficult to develop antimalarial agents with immunomodulatory activity and put prospects of a malaria vaccine distant.

1.2.5 Clinical aspects of the malaria infection

Plasmodium falciparum accounts for most of the human malaria infections and is responsible for almost all severe forms of malaria. The species *P. malariae, P. ovale* and *P. vivax* are other important human pathogens. There have been some reports of accidental transmission of simian malaria to man by *P. cynomolgi* and *P. knowlesi* (Most, 1973, Cross *et al.*, 1973), but these are far less epidemiologically important.

The clinical features of human malaria include fever, anaemia, cerebral symptomatology and non-specific symptoms including headache, malaise, diarrhoea and dyspepsia. Cerebral malaria is the most life-threatening form of malaria, presenting with high parasitaemia, convulsions and respiratory distress. Cerebral malaria is commonly caused by *P. falciparum* but rare cases caused by *P. vivax* (Arora *et al.*, 1988, Sachdev and Mohan, 1985, Mishra and Singh, 1989) have been reported. Other important complications of falciparum malaria include renal failure, spontaneous bleeding, haemoglobinuria and algid malaria (Chishti *et al.*, 2000). While the malaria parasite can directly cause organ damage, the immunological response to the parasite is responsible for major symptoms such as fever (Richards *et al.*,

1997). Anaemia occurs in symptomatic and asymptomatic malaria and is mainly due to haemolysis unmatched by compensatory haematopoiesis (Burchard *et al.*, 1995, Burgmann *et al.*, 1996). Bone marrow suppression contributing to anaemia has been suggested by Kurtzhals and colleagues (Kurtzhals *et al.*, 1997, Kurtzhals *et al.*, 1999). The convulsions in cerebral malaria are due to hypoxia and possibly hypoglycaemia, while respiratory distress is due to lactic acid accumulation (English *et al.*, 1996) as a result of reduced delivery of oxygen to tissues (English *et al.*, 1997). Prostaglandins and cyclic AMP have been reported to be involved in the development of diarrhoea in malaria (Hertelendey *et al.*, 1979) and several cytokines are known to cause tissue injury.

1.2.6. Prognosis of malaria

The proportion of malaria infections that results in death is still high in many parts of the world (Webster, 2001). The prognosis depends on pathogen and host factors. Infections due to *P. falciparum* may present with high parasitaemia, have a high incidence of multi-drug resistance, and have been associated with poor prognosis (Mehta *et al.*, 1998). Other factors affecting prognosis include nutritional status, age and physiological state of the patient (Hess *et al.*, 1997, Olumese *et al.*, 1997, Murphy *et al.*, 2001 and Singh *et al.*, 1999). Despite advances in knowledge of the management of malaria in the previous decade, the prognosis of malaria remains poor, especially among young children and pregnant mothers. The treatment-seeking behaviour and poor access to effective and good quality drugs may contribute to the poor prognosis.

1.2.7 Control of malaria

The prospects of eradicating malaria by the mid-1950s raised optimism in the global control of malaria. The World Health Organisation (WHO) launched the first global malaria eradication campaign in 1955. This campaign has not been successful in tropical countries due to climatic, social and economic reasons (de Zulueta, 1998). The key elements in the malaria eradication campaign include modification of the vector habitat, eradication of the mosquito, prevention of mosquito bites, chemoprophylaxis and treatment of malaria-infected patients.

Modification of the mosquito habitat includes ensuring that there is no stagnant water and bushes near human residences, which serve as breeding grounds for the mosquito. While these were once appropriate methods of controlling malaria, current pressures to conserve the environment have reduced their application. Emphasis is now put on improving the quality of housing which has been found to reduce infection with malaria by close to 44% in Malawi (Wolff *et al.*, 2001). The adaptation of the mosquito vector to new environments that has been observed in some areas is a major threat to this method (el-Amine, 1995).

The parasite development of resistance to the major insecticides is a major setback to efforts aimed at eradicating the mosquito (Curtis, 2001). Insecticides affect the environment in many ways even when used in low concentrations (McLean *et al.*, 1975) and the price of many insecticides is prohibitively high. Even the cost of dichlorodiphenyltrichloroethane (DDT) is on the rise as a result of decreased production (Walker, 2000).

Mosquito nets and insect repellents are effective in the prevention of mosquito bites (Govere *et al.*, 2000). Insecticide impregnated nets are now considered to be a more effective way of preventing mosquito bites and field studies have shown that they reduce mortality due to malaria (Aikins *et al.*, 1998). The high cost and poor availability of the nets results in poor adherence, increasing the risk of selecting mosquitoes resistant to the commonly used insecticides (Vulule *et al.*, 1999, Santos, 1999).

Chemoprophylaxis is one of the most important ways of preventing malaria infection amongst people visiting malaria endemic areas. The commonly used drugs are doxycycline, mefloquine and a combination of chloroquine and proguanil. However these drugs are not free of side effects and have interactions with other drugs and certain diseases. Development of resistance to the commonly used chemotherapeutic agents limits the effectiveness of malaria chemoprophylaxis.

Early diagnosis and treatment of malaria is an important component of malaria control programmes. The first choice treatment of malaria varies from one region to the other, depending on the resistance pattern in the area and the resources available. It is, however, generally accepted that the treatment of choice for cerebral malaria is intravenous quinine, with doxycycline added for patients from non-endemic areas. The development of artemisinin

derivatives is a great contribution to malaria chemotherapy. They are effective against multi-drug resistant strains of *P. falciparum*, and have a quick onset of action with a favourable safety profile.

A malaria vaccine might be the most cost-effective way of preventing malaria (Graves, 1998, Goodman and Mills, 1999). By 1998, close to 40 phase 1 and 2 vaccine trials had been carried out on synthetic peptides or recombinant proteins based on the malaria antigen (Engers and Godal, 1998). However, only a few field trials have been conducted especially with the synthetic peptide SPf66. A meta-analysis of nine field trials of this candidate vaccine shows evidence of some protection against malaria in South America, but no efficacy in Africa (Graves and Gelbrand, 2003). Developing a vaccine against malaria presents formidable challenges. It requires substantial investment of resources and capacity to handle trials; above all, preliminary studies lack validated models that can reliably predict the protection a vaccine can give to humans (Richie and Saul, 2002). Nevertheless, we expect to see more candidate vaccines undergoing field trials in the near future.

1.2.8. Malaria in Uganda

Uganda is located between 4° N, 1° S, 30° W and 34° E in a highly malaria endemic area. Malaria accounts for 25-40% of all outpatient visits at health facilities and 20% of all hospital admissions (Uganda Ministry of Health, 2003). In Mulago hospital, Kampala, Uganda's largest university teaching hospital, about 500 patients are admitted monthly due to malaria and its complications (Medical records, 2002), making it the single most important reason for hospital admissions. The direct cost of treating a malaria episode in Uganda is estimated to be 4.1 US$ in the urban areas and slightly less than this in the rural areas. It is estimated that the country loses close to US$350 million a year due to malaria (Associated Press, 2002).

In Uganda, malaria is commonly caused by *P. falciparum,* whose transmission is on the rise due to increasing environmental modifications, many of which have created breeding sites for the anopheles mosquito. Malaria transmission is generally highest during the wet season, unfortunately the busiest time in this predominantly agricultural country. The problem of refugees and internally displaced people as a result of conflicts in the Great Lakes Region poses serious challenges to the malaria control programmes. The elements of the malaria

control programme include curative treatment and vector control using insecticides and mosquito nets. The first-line treatment of malaria is chloroquine in combination with sulfadoxine / pyrimethamine. While the potential of traditional medicines is acknowledged, nothing systematic has been done to explore this.

1.3 Malaria chemotherapy and drug discovery

1.3.1 Principles of malaria drug therapy

The current principles of malaria chemotherapy are based on Paul Ehrlich's concepts (Bruce-Chwat, 1988). He postulated that pathogenic organisms have chemoreceptors that differ from one another, some of which have no analogues on human cells. He further postulated that an alien chemical compound cannot be completely harmless to the human cells and tissues, but the more acceptable it is, the higher the dose that can be administered. Compounds acting against parasites are selected on the basis of their selective toxicity in relation to mammalian cells.

1.3.2 History of malaria chemotherapy

Malaria chemotherapy was first recognised in the seventeenth century when cinchona bark was used in the treatment of malaria fever in Latin America (Bruce-Chwat, 1988, Dobson, 1998). In 1820, quinine was isolated from the cinchona bark but it was only identified as a quinoline in 1908 (Gramiccia, 1998). Quinine served as the standard treatment for malaria to the end of World War I. The sanctions imposed on Germany after World War I led to increased research aimed at developing semi-synthetic analogues of quinine. A number of successful antimalarials have been developed as a result of these efforts.

1.3.3 Classification of antimalarial drugs

The drugs used in the treatment of malaria are classified either on the basis of the stage of the parasite cycle affected (biological classification) or on the chemical class to which they belong.

Biological classification

Antimalarial drugs are classified into tissue schizontocides, drugs acting on the hypnozoites, and blood schizonticides.

Tissue schizontocides: These affect pre-erythrocytic stages of the parasite in hepatocytes. Examples include proguanil and pyrimethamine. They are used in causal prophylaxis.

Drugs acting on the hypnozoites: These affect dormant liver stages. An example of such a drug is primaquine. These drugs are used to prevent relapse of *P. ovale* and *P. vivax* malaria.

Blood schizonticides: These act on the erythrocytic stages of the parasite and are generally the most widely used antimalarial agents. Examples include quinine, chloroquine, amodiaquine, mefloquine, pyronaridine, artemisinin derivatives and atovaquone.

Chemical classification

Antimalarials drugs are classified into the quinolines and related antimalarial compounds, artemisinin derivatives, antifolates, and other antimalarial drugs. Each of these classes has a characteristic chemical skeleton (Ridley R.G., 2002).

Quinoline and related antimalarials: These include quinine, chloroquine, amodiaquine, mefloquine, halofantrine and lumefantrine. Except for quinine, which is a plant derived natural product, the others are fully synthetic compounds based on the quinine structure. The site of action of these drugs is believed to be the food vacuole of the parasite.

Artemisinin derivatives: These are the semi-synthetic derivatives of artemisinin, an isolate from the Chinese herb *Artemisia annua* L. They include artemether, arteether, artesunate and their main active metabolite dihydroartemisinin. The exact site of action of the artemisinin derivatives has not been conclusively elucidated but most studies point to the food vacuole as the most probable site of action (Hong *et al.*, 1994, Pandey *et al.*, 1999). The antimalarial activity of these compounds is closely linked to the unstable peroxide bridge, which is confined in their structure.

Antifolates: The most widely used antifolates are pyrimethamine and sulphadoxine. They interfere with the enzymes dihydrofolate reductase and dihydroopteroate synthase, important enzymes in the folate synthesis pathway. The pathway is necessary for nucleic acid synthesis.

Other antimalarial drugs: These include atovaquone, proguanil and antibiotics like tetracyclines and clindamycin.

Of all the antimalarials in use, chloroquine has in the past been the most universally successful. Although at the time of its discovery it was considered to be toxic, it is now generally known to be the safest antimalarial in children and pregnant women (Phillips-Howard and Wood, 1996). The potential teratogenicity that has been demonstrated *in vitro* (Ambroso and Harris, 1994, Landauer, 1978) has never been observed clinically. Its favourable pharmacokinetic profile (Krishna and White, 1996) and low cost are responsible for its widespread use. Intravenous administration can however lead to fatal hypotension and overdose causes hypokalemia, hypotension, nuerotoxicity with convulsions, and ventricular tachyarrhythmias (Jordan *et al.*, 1999). Even now that *P. falciparum* strains have become resistant to chloroquine, its use is still promoted in many countries (Rab, 2001). Other commonly used antimalarials have important side effects. Amodiaquine is associated with agranulocytosis and hepatic toxicity when used for prophylaxis. Quinine is associated with blackwater fever and cardiac toxicity, and mefloquine may cause convulsions, depression, psychotic episodes, and toxic encephalopathy (Bem *et al.*, 1992). Methaemoglobinaemia and haemolysis commonly in people with glucose-6-phosphate dehydrogenase deficiency, have limited the use of primaquine in malaria prophylaxis.

1.3.4 Parasite resistance to antimalarial drugs

One of the greatest challenges to malaria chemotherapy is the development of drug-resistant strains of *Plasmodium.* As early as 1968 cases of chloroquine-resistant *P. falciparum* had been reported (Modell, 1968). Since then, several strains of *Plasmodium* resistant to various drugs have been reported and recently multi-drug resistant malaria has been reported in several parts of the world (Mutanda, 1999). The emergence of drug-resistant *P. falciparum* has increased the incidence of severe malaria and its mortality (Trape *et al.*, 1998; Trape, 2001). Many countries in Africa have changed their first-line drug from chloroquine to pyrimethamine-sulfadoxine, and some countries are using both drugs. This has raised the cost of treating malaria and increased the incidence of pyrimethamine-sulfadoxine resistant strains of *P. falciparum* (Ronn *et al.*, 1996). Drug resistance has mostly been reported in *P. falciparum* species; however, there are also reports of multi-drug resistance in *P.vivax*

(Kshirsagar *et al.*, 2000; Alecrim *et al.*, 1999), indicating that drug resistance is spreading to plasmodium species other than *P. falciparum*.

The problem of parasite resistance is being approached on three fronts: (i) reversing chloroloquine resistance; (ii) combination therapy; and (iii) discovery of new antimalarial compounds (White *et al.*, 1999).

1.3.5 Reversal of chloroquine resistance

Since the advent of chloroquine resistance, much research has been done in the field of resistance reversal. The mechanism of resistance reversal is not clear, but many agents that reverse chloroquine resistance potentiate chloroquine accumulation in the parasite vacuole (Van Schalkwyk *et al.*, 2001). Compounds reported to reverse chloroquine resistance *in vitro* include calcium channel blockers, tricyclic antidepressants, antihistamines and phenothiazine derivatives (Bhattacharjee *et al.*, 2001, Ye and Van Dyke, 1994). Although a study of seven compounds: verapamil, chlorpromazine, prochlorperazine, cyproheptadine, ketotifen, a tiapamil analog (Ro 11-2933) and a chlorpromazine, have shown a correlation between *in vitro* and *in vivo* chloroquine resistance reversal activity (Kyle *et al.*, 1993), there is need for this to be supported by clinical trials. Attempts to identify compounds from traditional medicinal plants that might reverse chloroquine resistance, have lead to isolation of malagashanine, a parent compound for a number of indole alkaloids from *Malagasy strychnos*, which reverses *chloroquine* resistance *in vitro* and *in vivo* (Rafatro *et al.*, 2000).

1.3.6. Use of combination therapy

A number of countries have turned to combination therapy for first-line treatment of malaria. Drug combinations presently being used around the world include sulfadoxine-pyrimethamine (SP) with the 4-aminoquinolines, artemisinin derivatives and SP, and artemisinin derivatives and mefloquine. Drug combinations have a double effect, clearing the parasites completely and thus reducing transmission, and delaying emergence of resistance. There is a need for clinical trials and field trials to demonstrate these advantages and to show that these drug combinations are safe.

1.3.7. Antimalarial drug discovery

Drug discovery is part of a long process of drug development that traditionally includes drug design, pre-clinical studies, clinical studies, field studies and post-marketing surveillance. A successful drug discovery programme would result in identification of a lead compound. The lead compound might be a drug already in clinical use or a molecule that has never been used as a drug before. New drugs could be generated by computational methods based on a specific validated drug target, or isolated from an organism. Knowledge of the molecular targets in the parasite of existing antimalarial drugs is important if it is to be ensured that new drugs are developed to avoid cross-resistance.

While the orthodox and systematic way of drug development is well known, the development of traditional medicines for use as herbal remedies is not well established. The WHO has issued guidelines for the development of herbal medicines (WHO, 2000). The important component is the recognition of traditional experience as evidence for efficacy. The other important component is the recognition of phytomedicines as a combination of more that one principle and could be used as such.

1.3.8. Potential antimalarial drug targets

A potential target for a chemotherapeutic agent is normally an essential component of the parasite structure or metabolic pathway that is rate limiting and has no alternative pathway. Heme metabolism in the food vacuole of *Plasmodium* is an example of an antimalarial drug target. The detoxification of haem and antioxidant defence mechanisms that occur in the vacuole are critical to parasite survival. During haemoglobin digestion, an aspartic haemoglobinase is responsible for the breakdown of haemoglobin (Francis *et al.*, 1994). The presence of a peptidomimetic inhibitor that selectively blocks aspartic haemoglobinase provides a good starting point for the development of new antimalarial drugs at this target.

The cytoplasm and cell nucleus are the site for nucleic acid metabolism, phospholipid metabolism, glycolysis, and tubulin assembly. These can act as potential targets for new antimalarial drugs. Recently, some attention has been paid to the fatty acid biosynthetic pathway as a potential target; this is as a result of the discovery of sphingomyelin synthase in the Golgi apparatus of human *P. falciparum*. This enzyme is also found in the tubovesicular

membranes in the cytoplasm of infected erythrocytes and is considered important in the development of the tubular network in the erythrocytes that transports nutrients to the parasite. It has already been reported that inhibitors of this enzyme block parasite proliferation in culture (Lauer *et al.*, 1995).

Another potential target is the *P. falciparum* plasma membrane. The integrity of the plasma membrane is critical for the intracellular survival of *P. falciparum*. Pathways for the trafficking of nutrients start at this point and it also mediates intracellular signalling. These pathways are critical for the intracellular survival of *Plasmodium*, which makes it a good target for *novo* antimalarial agents (Olliaro *et al.*, 1999). Logically in the search for *novo* antimalarial agents, a combination of inhibitors acting on pathways that converge to produce a final product, could be more effective than a single inhibitor, and reduces the rate of evolution of drug resistance.

1.3.9 Plants as sources of antimalarial drugs

Plants pioneered malaria chemotherapy when cinchona bark was used successfully to treat Cardinal Juan di Luigi in 1632. Many authors recount the story of the cinchona bark powder, which had historically been used to treat fever and later found to contain quinine (Sharma and Sharma, 1998, Dobson, 1998, Bruce-Chatt, 1988). Quinine, an alkaloid, was first extracted by French chemist Pelletier in 1834, and has remained an effective drug even with the prevalence of multi-drug resistant parasites. Since the elucidation of the quinine structure, it has been used as a template for some of the most widely used drugs in malaria treatment and prophylaxis. Another landmark in malaria chemotherapy is the isolation of artemisinin from *Artemesia annua L*, a Chinese herb, whose use in Chinese traditional medicine is traced as far back as 186 BC. Artemisinin and its derivatives (artemether, artesunate and arteether) are now being used to treat infections caused by chloroquine-resistant *P. falciparum* and cerebral malaria. There are several compounds that have been isolated from plants that possess significant antiplasmodial activity that can be followed up. Triterpenoids and β-carboline alkaloid glycosides derived from the stems of *Brucea javanica* have shown inhibition of chloroquine-resistant *P. falciparum* strain *in vitro* (Kitagawa *et al.*, 1994). A naphthylisoquinoline alkaloid, named ancistroheynine A, from the Indian liana *Ancistrocladus heyneanus* has also been shown to display pronounced *in vitro* activity against

P.falciparum (Bringmann *et al.*, 1996). Another indole alkaloid cadambine, a major constituent of *A. chinensis*, was shown to exhibit moderate growth-inhibitory activity against the malarial parasite *P. falciparum* (Kitagawa *et al.*, 1996). Naphthylisoquinoline alkaloid-containing extracts from the plants *A. barteri*, *A. heyneanus*, *A. robertsoniorum*, and *A. tectorius*, used to treat fevers in several tropical countries, have shown high antiplasmodial activity against asexual erythrocytic forms of *P. falciparum* (NF 54, clone A1A9) and *P. berghei* (Anka) *in-vitro* (Francois *et al.*, 1997). Plant derived naphthaquinones have been used as starting molecules for the synthesis of atovaquine, an antimalarial that inhibits mitochondrial electron transport (Ridley R.G, 2002). In Africa, *Cryptolepis sanguinolenta* a plant widely used in West Africa for the treatment of many ailments has both *in vitro* and *in vivo* antiplasmodial activity (Paulo *et al.*, 2000; Cimanga *et al.*, 1997). The list of plants whose extracts have antimalarial potential is long, yet there are many plant species that have not been investigated at all. Plants have a high potential to produce antimalarial drugs and they are arguably the single most important source of antimalarial lead compounds.

1.3.10. Evaluation of antimalarial agents

Potential antimalarial agents are evaluated for both efficacy and safety during the process of drug discovery. In both cases, in *vitro* and *in vivo* studies are carried out. Evaluation for efficacy either in *vitro* or *in vivo* requires techniques for detection and quantification of *Plasmodium* in cultures and body fluids. Evaluation of safety *in vitro* requires detection and quantification of cellular chemical injury or death (cytotoxicty). In experimental animals safety is studied by observation and laboratory monitoring of body function parameters.

Detection and quantification of plasmodium

The techniques for detection and quantification of malaria parasites have improved tremendously over the past three decades. Evaluation of *in vitro* activity of potential antimalarial agents requires an assay that is fast, reliable and easy to interpret.

Light microscopy: Light microscopy is the oldest method of detecting Plasmodium. The first case of malaria was linked to haemoparasites by light microscopy of unstained blood smears. The development of giemsa-stained light microscopy made detection of plasmodium easier and is widely used in the diagnosis of malaria. While giemsa-stained microscopy is very

sensitive and used as a standard in the validation of most modern tests, it is time-consuming and a high level of sensitivity is attained only with experienced hands.

Fluorometry: Methods based on fluorometry have been developed; they involve use of DNA staining dyes such as ethidium bromide, acridine orange and benzothiocarboxypurine. Their sensitivity parallels that of isotopic studies (Tamura *et al.*, 1986) and geimsa-stained microscopy. Based on DNA staining, the detection of DNA fragments from dead parasites limits the specificity of the tests and utilises carcinogenic dyes.

Antigen capture: A number of techniques based on antigen capture have been developed. The key antigens include histidine-rich protein 2 surface antigen and the parasite lactate dehydrogenase enzyme. These tests are very sensitive with the latter comparable to Polymerase Chain reaction (PCR) (Druilhe, *et al.*, 2001). They however use monoclonal antibodies that render them expensive.

Isotopic assays: Isotopic assays are one of the most sensitive methods used for the detection of plasmodium. They are based on the utilisation of hypoxanthine and adenosine in nucleic acid synthesis by *P. falciparum*. One of these is normally used as radiolabelled precursor. These two precursors produce virtually identical results (Ye, *et al.*, 1987). The isotopic assays are sensitive, reproducible and fast, but they require specific precautions for the handling of radioactive material.

Parasite lactate dehydrogenase assay: This is based on the principle that the amount of (pLDH) in a sample correlates with the degree of parasitemia. (Makler and Hinrichs, 1993). The fact that pLDH utilizes 3-acetyl pyridine NAD (APAD) to APADH, that has the ability to reduce a yellow nitroblue tetrazolium (NBT) salt to a blue formazan product whose absorbance can be measured by microplate reader, makes it possible to measure pLDH. When used to evaluate drug sensitivity, this assay is reproducible, easy to interpret, rapid and inexpensive to perform and does not involve handling of radioactive material (Makler et al., 1993).

In vitro cytotoxicity

Most of the assays used in cytotoxicity studies are based on determination of cell viability.

One of the most used assays is the 3-(4,5-dimethylthiazol-2-yl)-2,5-

diphenyltetrazoliumbromide (MTT), an assay that detects metabolic viability of the cells in an *in vitro* system (Mosman, 1983). Assays based on lactate dehydrogenase quantification have been used to assess cellular injury to mammalian cells (Fischer *et al.*, 2003). These and other cytotoxicity assays require the maintenance of cell lines in culture, and are carried out in separate experiments from those used to determine antiplasmodial activity, increasing the cost and time of investigation. The quantification of haemoglobin in erythrocyte culture media is the basis of the erythrocyte haemolytic assay that can be used to investigate cytotoxicity of xenobiotics (Winski *et al.*, 1997). Results from this assay could be extrapolated to other cells, since all cells have lipids in their cell membrane structures. This assay has the advantage that it can be carried out using the same reagents and conditions as the antiplasmodial assay. Further more, erythrocyte morphological changes on exposure to a potential chemotherapeutic agent can provide qualitative information on possible cellular injury (Bessis, 1973). While electron microscopy provides detailed information on morphological changes, simple giemsa-staining can provide important basic information.

In vivo evaluation

The screening of compounds for *in vivo* antimalarial activity requires an animal disease model akin to human malaria. The animal host should absorb, metabolise and excrete the test antimalarial agent in the same way as humans. Animal studies have the advantage of providing safety information in addition to antimalarial properties. Safety information is obtained by observing animal survival, behavioural changes and, changes in biochemical and haematological indices. Animals used in experimental malaria models include primates, birds and rodents.

Primates: Primates are phylogenically close to humans and share most of the human physiological processes. They are receptive to adapted human plasmodium strains that are passaged several times in splenectomised animals. Despite these advantages their use has reduced tremendously over the past thirty years due to limited availability. They are difficult to handle, take a long time to breed, and most species have been listed as endangered.

Birds: The big phylogenetic gap between birds and humans, together with interspecies variations in response to the same plasmodium species, limits the use of birds as models of

experimental malaria. The plasmodium species affecting birds are very different from the mammalian species.

Rodents: Rodents are the most common hosts of experimental malaria because of their close proximity to humans and ease in laboratory handling. Rodent malaria species include *P. berghei*, *P. chabaudi*, *P. yoelii* and *P. vinckei*. Genetic modifications have been carried out on rodent strains to suite a wide range of investigations. Examples of transgenic mice used to understand malaria include the interferon-γ (IFN-γ) gene knock-out mice that have been used to study the role of interferon-γ in the progress of cerebral malaria (Senaldi *et al.*, 1999), and the T- and B-cell depleted mice (SCID) that have been used as a host of human erythrocytes in a rodent model of *P. falciparum* malaria (Moreno *et al.*, 2001).

1.4 Traditional medicines

1.4.1 Definition

Traditional medicinal practices are used in the prevention, diagnosis and treatment of physical and mental illnesses based on the theory, beliefs and experiences indigenous to the culture of a community. The terms 'traditional medicine', 'indigenous medicine' and 'folk medicine' can be used interchangeably. Communities around the world have traditional medicine systems that are considered alternative to western medicine. Some of the popular forms of alternative medicines are Ayurveda, Homoeopathy, Unani, Siddha, Naturopathy, Yoga therapy, Acupuncture, Acupressure, Magnetotherapy, Shiatsu, Herbalism, Meditation, Aromatherapy, Bach Flower Remedies, Gem therapy, Chromotherapy, Hydropathy, Diet Therapy and Reiki.

1.4.2 Traditional medicines in health care

The use of traditional medicines around the world is on the rise (Chou, 2001). In sub-Saharan Africa, more than 80 percent of the population relies on traditional medicines as their primary source of health care (WHO, 2002). The cost and poor access to allopathic medicines contributes to the popularity of traditional medicines. In many communities culture links people to traditional medicines making people perceive them as readily accessible, understandable and safe, with an acceptable mode of payment.

Plants by far are the most important sources of traditional medicines. It is estimated that at least 80% of countries where malaria is endemic have well-established antimalarial herbs (Sesay, 2000). Traditional medicines are administered both as self-medicaments and by traditional healers. The knowledge about the therapeutic usefulness of plants is usually passed on from generation to generation.

The major challenge to the use and development of traditional medicines is the lack of evidence of efficacy, safety and quality control measures. Guidelines have been developed on methods of evaluating the safety and efficacy of traditional medicines (WHO, 2000). These are aimed at strengthening the position of traditional medicines in health care.

1.4.3 Traditional medicine practices in Uganda

Traditional medicines are a very important component of the health delivery system in Uganda. The strength of traditional medicines is based on the fact that there is one traditional healer for every 200-400 people compared to one trained western health personnel to every 20,000 people (Hibler, 2001). The indigenous traditional healers in Uganda include Herbalists, Spiritual healers, Bone Setters, Traditional Birth Attendants, Hydrotherapists and Traditional Dentists. There are non-indigenous practices that have been introduced recently and their popularity is on the rise. These include Chinese traditional medicines and practices like Acupuncture, Ayurvedic practices from India, Reiki, Chiropractice, Homeopathy and Reflexology.

The indigenous traditional practitioners have been organized into several associations with registered members at the sub-county and district levels, coordinated by Cultural Officers. These associations are controlled by the National Council for Traditional Healers Associations (NACOTHA). This structure arose out of the need to make traditional medicines safe and weed out unacceptable practices like child sacrifice. Through the council, government and non-government agencies are reaching out to the traditional healers to improve the quality of traditional medicines (Medlinks, 2003). The practitioners of non-indigenous traditional medicines are generally not affiliated to any of these associations.

There is a difference in the understanding of disease between the traditional healing system and the western system. In the traditional system most disease entities are referred to symptomatically (Tabuti *et al.*, 2003). Although the disease entity malaria is well known, it is referred to by the same word as other fevers of different etiology. The plants used in the treatment of malaria are more or less designated to symptoms like fever, convulsions, headache abdominal pain and general weakness. Plant products more than animal products are used in the treatment of malaria. It is common to find a small medicine garden in each homestead although the majority of herbs are collected from the wild. A survey carried out in Bwindi impenetrable forests indicated that leaf material is the most widely used of all plant parts (Adjanohoun *et al.*, 1993). Traditional healers, collect some of the commonly used plants, dry them and preserve them. When collecting the plants several precautions are taken in terms of season, time of the day, location of the plant and the plant part collected. People come to buy these herbs and traditional healers at times dispense them to people who present with health complaints. This service is extended to markets in urban areas, although commercial trade in traditional medicines is still at a low level in this country (Cunningham, 1992).

The treatment of particular conditions varies extensively from community to community and even amongst traditional healers in the same community. The methods of administration of traditional medicines are perhaps as many as those used in western medicine. In the treatment of fevers the most common method is oral intake of plant decoction or maceration. The other important methods of administration include bathing with the extract, steam bath and inhalation of smoke from a burning plant part (Nalwoga, 2001). The parts used may be from one plant and in some cases a mixture of more than one plant is used, a practice that is similar to other communities in Tanzania (Gessler *et al.*, 1995).

The major challenges facing traditional medicine practices in Uganda is the lack of information on effectiveness and safety of the medicines. The lack of standardised methods of preparing and administering traditional medicines makes it more difficult to investigate these two important aspects of traditional medicines. There are researchers who have directed their efforts to help traditional healers prepare better, safer, and cheaper remedies (Hibler, 2001). Many traditional healers don't record relevant information that would help them monitor patients. There are no detailed studies to demonstrate the treatment outcome among patients

who visit traditional healers, but one minor study showed evidence of clinical and parasitological cure among malaria patients who took a traditional remedy (Bitawha *et al.*, 1997). The continued existence of the traditional healers and growing use of traditional medicines in Uganda could be argued to be an indication of their effectiveness. Some traditional healers are still involved in practices like human sacrifice; these taint the image of other healers who are working to cure legitimate ailments like malaria. There is presently a nation-wide campaign against such practices (Nampala, 2001).

Even with the current level of modern medicine in Uganda, people continue to seek traditional health care as before. Modern medical practitioners are continuing to recognise the traditional healers as a group that can fill gaps in their discipline (BBC news archives, 1999). By 1992, the two types of care providers had come together and established an organisation called THETA (Traditional and Modern Health Practitioners Together Against AIDS) that aimed at creating a mutually respectful collaboration between traditional healers and conventional health practitioners (Engle, 1998). This initiative intends working against other diseases like malaria. Traditional healing systems are dynamic and inclusive, and therefore we expect to see more incorporation of western elements into traditional medicine as the two systems continue to work together (Whyte, 1982).

1.4.4 Adverse events associated with traditional medicines

The notion that traditional medicines are natural and therefore safe is widely held around the world. This has led to increased use of traditional medicines without paying close attention to their safety. The incidence of adverse events in the use of traditional medicines is largely unknown. A few reports are now emerging with the increased marketing of herbal remedies based on traditional medicinal plants. Some herbal preparations have been reported to cause hypersensitivity reactions that range from a transient dermatitis to anaphylactic shock (Perharic *et al.*, 1993). *Agnus castus,* a plant with estrogen-like properties and taken commonly for the treatment of a variety of gynaecological problems, has been observed to cause increased uterine stimulation and predisposes mothers to miscarriages (Cahill *et al.*, 1995). St John's wort, a plant whose extracts are widely used in the treatment of depression, has been found to be an enzyme inducer of the CYP450-3A4, reducing plasma levels of drugs metabolised by this enzyme system and consequently the efficacy of many drugs (Roby *et al.*, 2000, Mai *et al.*, 2000).

The multi-system nature of the malaria infection and the short disease course reduces the chances of detecting adverse effects of the traditional medicines when used. There is need to develop simple laboratory assays that can reliably predict possible adverse events and interactions of traditional medicines with other drugs. Some of the toxic effects of traditional medicines are predictable if the chemical constituents are known. The report of deaths due to cardiac glycoside poisoning from traditional medicines in some parts of South Africa (McVann *et al.*, 1992) calls for increased investigation into traditional medicines.

1.5 Scope of the study

1.5.1 Background of the study

The rising incidence of *Plasmodium* strains resistant to the existing antimalarial drugs has emphasized the need for alternative malaria remedies. Almost all communities in malaria endemic areas have plants that are used to treat malaria symptoms. The potential of these plants as sources of antimalarial herbal remedies or lead compounds for antimalarial drugs has not been comprehensively assessed. In order for a traditional medicinal plant to have a high antimalarial potential, it requires evidence of efficacy and safety. With the current trend of combination therapy, favourable pharmacodynamic interactions with already developed antimalarial drugs like chloroquine and artemisinin enhances the antimalarial potential of a medicinal plant.

The search for new antimalarial remedies has generally stimulated interest in traditional medicines and the WHO has declared it a priority area. The development of traditional medicines requires information on the uses and methods of preparation of these medicines. In Uganda an ethnobotanical survey was carried out in which various plants were reported to be used in the treatment of malaria symptoms (Mubiru *et al.*, 1993). Most of these plants have never been investigated for efficacy and safety and their potential as sources of malaria remedies is not established.

1.5.2 Study objectives

The main objective of this study was to investigate the antimalarial potential of some of the traditional medicinal plants pointed out both in previous literature and the recent

ethnobotanical survey. This involved looking for information on the efficacy, safety and any potential interactions with other medicines. To achieve this objective the study focused on the following specific objectives:

(1) To determine the antiplasmodial activity of extracts from the six study plants.

(2) To study potential interactions between traditional medicines and some of the already established drugs, and establish the possible mechanisms behind these interactions.

(3) To determine *in vivo* antimalarial activity of plants that may not show any in *vitro* antiplasmodial activity.

(4) To carry out phytochemical analysis and characterisation of any principles from plants that possess antiplasmodial activity.

(5) To establish the relevancy of our results to the development of Ugandan traditional medicinal plants as sources of malaria remedies.

1.5.3 Rationale of study approach

The investigation of a number of plants for antimalarial potential required simple and reliable high throughput assays that could screen a number of extracts in a short time. In this study, information on efficacy, safety and potential interactions was required for six plants. While information from an *in vivo* system would give a high predictive value, setting up *in vivo* models is expensive, time consuming and has ethical issues. In this study, we decided to use an *in vitro* system and only the extracts from plants that did not show evidence of efficacy *in vitro* were screened in an animal model.

1.5.4 Expected study outcomes

This study was expected to provide information on the efficacy and safety of extracts from traditional malaria medicinal plants, which could be used in the development of herbal remedies or sources of lead compounds for antimalarial drugs. The information on possible interactions between traditional medicines and some of the drugs already in use for the treatment of malaria opens up a new area in terms of research and policy on the use of traditional medicines in malaria control.

1.5.5 Originality of the work

In this study six plants that have never been investigated for antimalarial properties, based on the NAPRALERT (natural products alert) database, were selected from the plants that have been reported in ethnobotanical surveys of malaria treatment in Uganda. These were further confirmed in a meeting between the investigators and three traditional healers practising in three different communities in Uganda. The plants selected include *Senecio discifolius oliv, Senecio stuhlmannii, Indigofera emarginella* steud. Ex A. Rich, *Aspilia africana* (Pers) C.D. Adams, *Cardiospermum halicacabum* L and *Momordica foetida* Schumch. Et Thonn. These plants belong to four plant families: *Asteraceae, Fabaceae* (Leguminoseae), *Sapindaceae* and *Cucurbitaceae.*

1.5.6 Description of study plants

The plants *S. discifolius, S. stuhlmannii* and *A. africana* belong to the family of *Asteraceae.* The plant *S. discifolius* oliv is a common weed in gardens in parts of southern, central and eastern Uganda. It generally flourishes during the rain season and is scarce during the dry season. It is used in the treatment of abdominal pain and jaundice, both important symptoms of malaria. In the treatment of abdominal pain, leaves are squeezed in salted water and drunk, while in the treatment of jaundice, 250 ml of a leaf decoction is drunk once daily (Neuwinger, 2000). This plant is also used a galactagogue and an antifungal (Adjanohoun *et al.*, 1993).

The other plant of the *Senecio* genus, *S. stuhlmannii*, is also called *S. cydoniifolius* O. It is a shrub with hairy leaves and fleshy stems. It is commonly found in homesteads where it is domestically grown because of its medicinal value. In the treatment of fever, pounded leaves are added to bath water, while 250 ml of leaf decoction is drunk once daily for the treatment of jaundice. This plant has been reported in the treatment of diarrhoea (Meikere-Faniyo *et al.*, 1989) and convulsions, both of which are common malaria symptoms.

The plant *A. africana* is a shrub that grows wild in many grassland areas of Uganda. It is used in the treatment of wounds, acne, gonorrhoea, syphilis, pneumonia, headache, malaria and liver inflammation in parts of Central Africa (Rwangabo, 1993). A mixture of the root decoction with extracts from *Pseudoarthria hookeri, Flueggea virosa* and *Vernonia*

amygdalina has been reported in the treatment of fever. In the treatment of malaria and fevers as mentioned above, decoctions are taken orally (Adjanohoun *et al.*, 1993).

The *Aspilia* genus is perhaps the best studied of all the plants selected in this study. This has been as a result of widespread medicinal use and also the observation that wild primates feed on plants of this genus in a special way. Studies on the phytochemistry and biological activity of leaf extracts from *Aspilia mossambicensis* demonstrated the presence of oxytoccic diterpenes (Page *et al.*, 1992). Oils rich in monoterpenes and limonene have been extracted from the leaves of West African *A. africana* (Pervez, 1993). Some of the already reported pharmacological effects of *A. africana* derivatives include anticoagulant properties (Hanna and Niemetz, 1987) and antibiotic properties, which are attributed to the presence of thiarubrine A (Rodriguez, 1985).

A number of biologically active molecules have been isolated from the *Asteraceae* family, the most successful of which is the sesquiterpene endoperoxide lactone, artemisinin, isolated from *Artemisia annua* L (Klayman, 1985, Hien *et al.*, 1993), from which a number of semi-synthetic antimalarial compounds have been derived. The other compounds with antiplasmodial activity derived from these plants include the sesquieterpenes, zingiberene-3,6-β-endoperoxide and zingiberene-3,6-α-endoperoxides (Ruecker et al., 1996). The compound Thiarubrine A, isolated from *A. africana,* has antimicrobial properties (Page *et al.*, 1997). A number of compounds have been isolated from the *Asteraceae* family, but their biological activity is not reported (South African Traditional Medicines Research Unit database, 2003).

The plant *Indigofera emarginella* steud. Ex A. Rich. belongs to the family *Fabaceae (Leguminoseae).* This plant has been reported to be used in the treatment of rectal prolapse, swollen legs, abdominal pain and convulsions in children. In the treatment of convulsions (a common sign of childhood malaria) a root infusion is drunk together with an extract from *Hoslundia opposita* (Kokwaro, 1976). There are no chemical compounds that have been isolated from this particular plant species neither has the biological activity of the extracts from this plant been investigated, but benzofurans with antimicrobial activity has been isolated from *Indigofera microcarpa,* a plant from the same genus (de moraes e Souza *et al.*, 1991).

Momordica foetida Schumch. Et Thonn is a climber that is commonly found in swampy areas in parts of central Uganda. This plant belongs to the family *Cucurbitaceae.* It has medicinal uses ranging from spiritual and psychiatric conditions to physical diseases. The drinking of aqueous leaf extracts of this plant for malaria treatment has been reported widely in parts of east and central Africa (Hakizamungu *et al.,* 1992, Rwangabo, 1993). The other uses of extracts from this plant include the treatment of hypertension, ulcers, and diabetes, and as a purgative. These uses have not been investigated.

Cardiospermum halicacabum L is a climber that grows near tall trees. It is found in tropical forests in central and southern Uganda. This plant belongs to the family of *Sapindaceae.* The plant is used in the treatment of cough, hyperthermia, rheumatism, lumbago, nervous illnesses and amenorrhoea (Neuwinger, 2000). Two glasses of a 12-hour maceration of aerial parts of the plant are drunk or used for bathing in the treatment of hyperthermia. In some areas, seed water extracts are drunk for the treatment of fever (Neuwinger, 2000). These traditional uses have not been investigated and there is no scientific evidence yet to prove that extracts from this plant are effective for these conditions.

While traditional uses of many Ugandan plants are well known, there is hardly any information on their biological activity, safety and interactions with other medicaments. This study is expected to provide information relevant to the development of antimalarial remedies from these plants.

1.6 Summary

Malaria is a major world disease problem that is yet to be satisfactorily controlled. At present, chemotherapy is the mainstay of malaria control. Parasite resistance to existing antimalarial drugs is on the rise, extending to new geographical areas and affecting species other than *P. falciparum.* This raises the need for ongoing discovery of new, safe and effective drugs/herbal remedies for the treatment of malaria. Historically, traditional medicines have been the source of most successful antimalarial agents namely the quinolines and the endoperoxides/artemisinin derivatives. Although Africa has rich flora and fauna, it is yet to be tapped as a source of medicine. This study investigates the antimalarial potential of *S. discifolius, S. stuhlmannii, I. emarginella, A. africana, C. halicacabum and M. foetida* all of

which have been reported in ethnobotanical surveys and confirmed by traditional healers practising in three different Ugandan communities.

CHAPTER 2

Materials and methods

2.1 Introduction

The fact that traditional medicinal plants have a long history of use makes clinical studies the most logical approach to the investigation of their efficacy and safety. This approach is however costly, time consuming and has ethical complications that make it unsuitable for large-scale screening. Preclinical studies therefore remain important in the early screening of potential antimalarial plants.

In this chapter we describe the methods used in the selection, collection, identification, phytochemical analysis, determination of efficacy and safety of six Ugandan traditional medicinal plants. *In vitro* assays were used to investigate antiplasmodial efficacy and interactions between traditional medicines and drugs commonly used in malaria treatment. A selection of plants that did not show *in vitro* antiplasmodial activity was screened for antimalarial activity using a murine model of cerebral malaria.

2.2 Collection and identification of plant materials

Three traditional healers registered with the National Council for Traditional Healers Associations in Uganda (NACOTHA) were identified around the towns of Masaka, Iganga and Mbale. A map of Uganda showing the location of these towns is shown in Appendix I. These towns have different tribal communities and were selected so as to include medicinal plants from at least three different communities. In a meeting with each of the traditional healers, the list of antimalarial traditional medicinal plants reported in the ethnobotanical surveys was confirmed. Plants, which from literature search had previously been investigated for antimalarial properties, were excluded from the list. The plants *Momordica foetida* Schumch. Et Thonn, *Aspilia africana* (Pers) C.D. Adams and *Cardiospermum halicacabum* L., were selected from Masaka, *Senecio discifolius oliv* and *Senecio stuhlmannii* from the Iganga area and Indigofera *emarginella* steud. Ex A. Rich *from* the Mbale area.

During collection only plants judged as mature by the investigators and the traditional healer who acted as a guide were harvested, and this was done during daytime. The first batch was

collected between May and June 2000; during this period most parts of Uganda received a lot of rainfall. The second batch was collected in December 2000

and January 2001, a period that is generally dry and hot. For each specimen a shoot with leaves and flowers was taken to the Department of Botany of Makerere University for identification. The investigator with the help of a botanist discarded plants that showed evidence of viral, bacterial or fungal infection. Voucher specimens were air dried, allocated voucher numbers and preserved in the Makerere University Herbarium.

2.3 Phytochemical analysis

2.3.1 Preparation and extraction of plant material

Traditional healers preserve plant specimens by sun drying. In this study it was preferred to air-dry the plant material at room temperature as it is more convenient and produces cleaner plant materials with less risk of contamination. The dry material was later crushed to powder using pestle and mortar. While water is the most widely used solvent by traditional healers, it is generally difficult to eliminate from the sample/extract. This has made water extraction less popular in plant studies. Soxhlet extraction with alcohol tends to extract exhaustively but extracts only polar compounds, and involves boiling the extract with the solvent over time. Sustained boiling may not be desirable if one is uncertain of the stability of the active principles. Alternatively, extraction may be by successive extractions with solvents of increasing polarity. This is based on the solubility principle of '*similia similibus solvuntur*' (similar dissolves similar). This method does extraction and fractionation concurrently, and was used for all extractions in this study.

Materials and solvents

The solvents used for the extractions were hexane, ethyl acetate (Merck-Aldrich), methanol (Scharlau Chemicals, South Africa) and Millipore® water. A mechanical shaker (Labcon Inc:), Rotary evaporator (BUCHI Scientific Equipments) and a freeze drier (FTS Systems, Stone Ridge, New York, USA), were used.

Experimental system

To determine the yields, 15g of the plant powder were placed in 200 ml Millipore water in a 1-litre flask, shaken for 24 hrs using a mechanical shaker at 125 revolutions per minute (RPM), and filtered using a 125mm filter paper. The extract was then frozen at –80 degrees for 6hrs in a 1liter flask. The frozen extract was then freeze-dried at a vacuum of 50mmHg and temperature of –70 °C. The dried extract was weighed and the yield expressed as the weight of water extract as a percentage of dry plant powder. The freeze-dried extracts were kept at room temperature and subsequently reconstituted and screened for antiplasmodial activity.

An additional 35g of plant powder was weighed and solvent extraction performed as above, this time with organic solvents. For each portion of plant powder, hexane 300 ml was added and shaken for 24 hrs. The remaining plant material from hexane extraction was then subjected to ethyl acetate extraction and finally to methanol extraction. After 24 hours of extraction, extracts were concentrated by rotary evaporation using the Buchi rotary evaporator at 40°C and a vacuum of 335 mbars for hexane, 240 mbars for ethyl acetate, and 337 mbars for methanol. The yield was determined, as described above, and the extract stored at room temperature for subsequent antiplasmodial screening.

A second batch of the plants *A. africana,* S. *discifolius,* S. *stuhlmannii* and *I. emarginella* was collected from the same areas as the first batch in the months of December 2000 and January 2001. The plant material was processed as described above and rescreened for antiplasmodial activity following the same procedures. For bioassay-guided fractionation, 1000 g of dry plant material of the most active batch was extracted. During collection of the third batch, only *A. africana* was collected during June-July 2002.

2.3.2 Bioassay-guided fractionation

Fractionation of plant extracts before screening for biological activity improves antimalarial activity (Statz and Coon, 1976). Commonly used methods of fractionation in plant work include solvent partitioning and chromatography. In solvent partitioning the extract is placed in two immiscible solvents in a separating funnel. The different constituents in the extract partition according to their respective solubilities in the two solvents. This method is simple

and rapid but produces at most three fractions. Chromatography involves the separation of compounds basing on their difference in binding to the stationary phase. Chromatography techniques where used in the fractionation of extracts in this study.

2.3.2.1 Solid phase extraction of the crude extracts

Materials and reagents

Solid Phase Extraction (SPE) was done using hexane and ethyl acetate (Merck-Aldrich). Methanol and acetonitrile was supplied by Scharlau Chemicals, South Africa. Silica and C-18 SPE columns both of sorbent mass 10g and a volume of 70mls were supplied by Anatech Instruments, South Africa.

Experimental system

Fractionation of the methanol extract of *S. discifolius* and ethyl acetate extracts from *S. stuhlmannii, I. emarginella* and *A. africana* was done by SPE. Stock solutions were prepared by dissolving 400 mg of the extract in 65 ml ethyl acetate. The solution was sonicated for about 3 minutes to dissolve as much of the extract as possible, and then filtered using Whatman filter paper with 125 µm pores. The residue was allowed to dry and redissolved in ethyl acetate. The filtrate was then topped up with 130ml of hexane to make a stock solution of 7:3 ethyl acetate: hexane, and transferred into 50ml centrifuge tubes and spun at 13000 RPM for 10 minutes. The supernatant was used as stock solution for SPE while the pellet was kept for the antiplasmodial assay. The mobile phase for all extracts was made up of hexane and ethyl acetate. Six sets of 800ml eluting solvent were prepared with the following hexane: ethyl acetate ratios by volume: 100:0, 75:25, 50:50, 25:75 and 0:100, together with 100% acetone to wash the column after elution with each solvent ratio.

A silica column was first conditioned by elution with 100ml ethyl acetate followed by 100mls of hexane. The column was then loaded with 50ml extract solution (about 100mg, 1% of the column sorbent mass) and the extract was eluted with a low vacuum. The percolate was collected and concentrated by rotary evaporation and subsequently tested for *in vitro* antimalarial activity. Using a vacuum of 0.5 Kpa, 200ml of the mobile phase at each ratio was used for eluting. The elutions were in increasing ratio of ethyl acetate. All the fractions were collected, concentrated by rotary evaporation and tested for antiplasmodial activity. The same

process was followed for all crude extracts, except with *S. stuhlmannii* in which preliminary separation was not satisfactory and a C-18 column was used with increasing ratio of acetonitrile: water as the mobile phase.

In the fractionationation of *S. stuhlmannii,* 25% increments of acetonitrile to water were used, followed by 10% increments. A stock solution of the extract was prepared by dissolving 400mg of crude extract in 65 ml of acetonitrile, and sonicated for 5 minutes. The solution was filtered and the filterate supplemented with 130ml of Millipore water, to make a stock solution of 7:3 water to acetonitrile. This was then centrifuged and the pellet prepared for antiplasmodial screening. Each time 50ml (about 100mg of extract, 1% of cartilage sorbent mass) of the extract were loaded in the column and eluted with 200 ml mobile phase at each ratio, with the same vacuum as previously. The fractions were collected and concentrated by rotary evaporation to remove the acetonitrile. The water portion was then removed using a freeze-drier, as described previously.

2.3.2.2 Further SPE on the crude ethyl acetate fraction of *A. africana*

In order to isolate the active principle in the ethyl acetate fraction of *A. africana,* SPE was done on a large scale with crude ethyl acetate extract. The silica column was used with ethyl acetate and hexane as the mobile phase. The ratios of ethyl acetate to hexane were 0:100, 10:90, 20:80, and by progressive increments to 100:0. Pure acetone was used to wash the column as before. The extracts were prepared and the elutions were carried out as previously. The fractions were concentrated by rotary evaporation, weighed, and tested for antiplasmodial activity. The most active fraction was subjected to analytical High Performance Liquid Chromatography (HPLC) to check the purity of the extract.

The 50% ethyl acetate fraction was subjected to more refined SPE, the mobile phase this time being prepared with 1% increments in the ratio ethyl acetate: hexane. The mobile phase combination ratio started from ethyl acetate: hexane: 41:59, 42:58, 43:57, and by serial increments to 50:50 and followed by an acetone wash. The stock extract solution and method of extract preparation were as previously. The elutions were carried out similarly and the extracts concentrated by rotary evaporation. The dried extract was tested for antiplasmodial activity and the most active fraction analysed for purity using analytical HPLC.

2.3.2.3 Preparatory high performance liquid chromatography

The 1:1 ethyl acetate: hexane fraction of *A. africana* from the third batch was fractionated by preparatory HPLC.

Materials and solvents

A pre-packed semi-preparatory C-18 column (Haisal, 100 C-18, S/N 943692, Higgins Analytical Inc:) of dimensions 250x10 mm and a Shimadzu LC-10AS with a diode array detector SPD-M10A and a communications bas module, CBM-10A, a guard column packed with C-18, 40µm (Bondesil guard column) were used. Acetonitrile of HPLC grade supplied by the Merck-Aldrich and Millipore® water were used in the mobile phase. Purity test runs were carried out using a pre-packed C-18 analytical column 150 x 4.6mm (Phenomenex, St. Torrance, USA).

Experiment

The 1:1 ethyl acetate/hexane fraction of the third batch of plant material was fractionated using preparatory HPLC. A stock solution was prepared by dissolving 20mg of the extract in 1000µl acetonitrile. This was then topped up to 3000µl with Millipore water to make a stock solution of 30% acetonitrile in water. Trial runs were conducted and an injection volume of 200µl selected with a flow rate of 4 ml/minute and isocratic run of 2:3 acetonitrile to water for 8 minutes. The column was washed for an additional 4 minutes, during which the proportion of acetonitrile was raised to 100 % in the first 2 minutes and reduced to 0% in the last two minutes. The column was conditioned by having two runs, the first with 100µl water injection followed by 100µl acetonitrile. These runs were under the same conditions described above. The UV spectra of the various extracts were determined and the fractions collected in flat-bottomed flasks. Four fractions F_1, F_2, F_3, and F_4, were collected between retention times, 2.5 to 3.2, 3.2 to 4.3, 4.3 to 5.2 and after 5.2 minutes. The acetonitrile was removed with a rotary evaporator. Water was eliminated as described previously. The fractions were weighed, and stored at room temperature for later antiplasmodial activity testing and mass spectrometry.

2.3.3 Characterisation of plant principles

Isolates from extracts were characterized by mass spectrometry (MS) and Ultra violet (UV) absorbance. Mass spectrometry provided their molecular weights, which were used to determine the molecular formula. The UV spectra were determined using the diode array detector of the HPLC machine. Mass spectrometry was done on about 1 mg of the isolate using high-resolution mass spectrometer or low-resolution mass spectrometer.

2.4 Antiplasmodial activity of plant extracts

The parasite lactate dehydrogenase (pLDH) assay was used in the determination of antiplasmodial activity of extracts in this study. The principle behind this assay is described in chapter one.

Materials and reagents

Culture medium used was made up of RPMI 1640 (Bio Whittaker) medium supplemented with albumax II (lipid rich bovine serum albumin) 25g/L, hypoxanthine (44 mg/l), N-(2-hydroxyethyl)-piperazine–N-2-ethanesulphonic acid (HEPES) (6gm/L), sodium bicarbonate (2.1 g/L) and gentamycin (50 mg/L). All the reagents were supplied by Sigma-Aldrich, South Africa. The reagents used for the lactate dehydrogenase assay included: Nitroblue tetrazolium (NBT) (1.96 mM) and phenazine ethosulfate (PES) (0.24 mM) solution in Millipore water. The malstat reagent was made from triton (1 ml/L), APAD (0.33 g/L) and TRIS buffer (3.3 g/L) in Millipore water (Sigma-Aldrich, South Africa). Sigma-Aldrich, South Africa also supplied chloroquine diphosphate, artemisinin and D-sorbitol. Group O+ blood was obtained from the Groote Schuur Hospital Blood Transfusion Services Department. A 7520 Plate reader (Cambridge Technology, Inc:) was used.

Two strains of *P. falciparum* were used. The chloroquine-sensitive D10 was derived from the FCQ-27, an isolate from a patient in Papua New Guinea (donated by Dr A. Cowman, Walter and Eliza Hall Institute of Research, Melbourne, Australia). The other, chloroquine-and sulphonamide-resistant K1; was isolated from a patient at Kanchanaburi, Thailand (donated by Dr D. Wallaker, University of Edinburgh, Scotland). These strains are kept in liquid

nitrogen in the laboratory of the Division of Clinical Pharmacology, University of Cape Town.

Thawing of parasites.

Erythrocytes parasitised by the two strains D10 and K1, respectively were transferred from liquid nitrogen to a 10ml centrifuge tube and washed three times with sterile 12% sodium chloride. Using a pipette 100µl of fresh red blood cells were added followed by 50 ml of culture media. The mixture was then transferred to a 250 ml culture flask and gassed for 2 minutes with 93% nitrogen, 3% oxygen and 4% carbon dioxide. The two strains were maintained in continuous culture (Trager and Jensen, 1976). Culture media was changed every 24 hours and whenever the parasites were in trophozoite stage they were fed with fresh red blood cells to maintain the parasitaemia below 10%. The cultures were synchronized regularly, and before each drug sensitivity experiment.

Synchronizing of parasite cultures

To each volume of infected erythrocyte pellet, 5 volumes of 5% D-sorbitol at 37° C was added, and allowed to stand at room temperature for 10 minutes. The culture was then spun at 600g for 5 minutes. The level of parasitaemia of the red blood cells was adjusted to 2% using normal human group O Rhesus +ve red blood cells and the haematocrit adjusted to 2% using tissue culture medium.

Preparation of the plant extracts and drugs

Experience from our laboratory has shown that crude extracts with significant antiplasmodial activity have a 50% inhibitory concentration (IC_{50}) between 2 and 25 µg/ml. Stock solutions of the crude extracts were made so as to have a dosing range of 1000 to 2.0 µg/ml on the 96-well plate. Stock solutions with concentrations ranging from 1000 to 20µg/ml of plant extracts were prepared by dissolving the extracts in culture medium. For those extracts that did not dissolve readily, a solvent of 1% methanol in culture medium was used. Solid phase extraction fractions and pure plant isolates required lower dosing ranges. The stock solutions were kept in sterile conditions at 4°C for subsequent testing. For chloroquine and artemisinin the initial concentration of 1µg/ml was achieved by dissolving the drug in culture media for chloroquine, and methanol then topped up with culture media for artemisinin.

Lactate dehydrogenase assay

A 96-well plate was used for the lactate dehydrogenase assay. To each well, 100 µl of culture media were added, except in column three. To the third column, 200 µl of the stock extract solution was added, using a multi-pipette dispenser 100µl of the extract was transferred and mixed with culture medium in the fourth column, then from fourth to fifth leading to double dilution across the plate to the last column. To the first column, 100 µl of non-parasitised red blood cells the haematocrit of which had been adjusted to 2% was added. This acted as the negative control. To the other wells of the plate 100µl suspension of human red blood cells of 2% parasitaemia adjusted to 2% haematocrit with culture medium was added. This allowed two fold dilutions to be studied, concentrations ranging from 1000 to 2.0µg/ml, and sometimes lower, depending on the expected activity of the extract. Column two contained parasitised red blood cells without test drug, and served as the positive control. The plates were covered with a sterile lid and placed in an airtight cabinet, gassed with 93% N, 3% O_2 and 4% CO_2 for at least 1 minute and then placed in a 37°C incubator for 48hrs.

The plates were incubated for 48 hr to complete one erythrocytic cycle, which exposed all parasite stages to the drug. After incubation, 15µl of the blood suspension from each well was transferred to a 96-well, flat-bottomed microtitre plate containing 100 µl of malstat reagent. Then 25 µl of NBT were added to all plates and allowed to develop for 10 minutes away from direct light. The plates were read at 640nm. The optical density of the first column (blank) was subtracted from the other readings.

Chemical injury to erythrocytes

To assess any chemical injury to erythrocytes that could be attributed to the extract, 100 µl of erythrocytes was incubated with 100 µl of the extracts at a dose equal to the highest dose used in the antiplasmodial assay. The conditions of the experiment remained as for the antiplasmodial assay. After 48 hrs of incubation thin blood smears were giemsa-stained and observed for any morphological changes using a light microscope at a magnification of 100 times. The morphological findings were compared with those observed in erythrocytes from the blank described above (incubated uninfected erythrocytes without extract)

Data analysis

The percentage parasite survival at each concentration was determined by expressing absorbance at that concentration as a percentage of the absorbance of the corresponding wells in column 2 (Infected erythrocytes incubated without drug, representing normal parasite growth). Results were compared using the non-parametric Mann-Whitney U-test. Percentage survival was plotted against the logarithm of concentration to generate a dose-response curve from which the IC_{50}, was determined being the concentration at which there was 50% parasite inhibition. Calculation was done using the prism graph pad V3. Each extract was tested in duplicate and the experiment repeated 2 or 3 times.

2.5 Interactions between traditional medicines and antimalarial drugs

Patients often take traditional medicines together with the already established antimalarials. There is hardly any information on possible interactions between traditional medicines and established drugs. A traditional medicine with high antimalarial potential should not impair the antimalarial activity of other drugs that might be administered concurrently. This was investigated in an *in vitro* system.

An interaction might be investigated in two ways. One would be to determine the antiplasmodial activity of the combination of a known drug with active extract from the plant, and compare it with the activity of the individual drug. The other is to determine how an extract from the plant might affect the accumulation of the known drug in infected erythrocytes. In this study both methods were applied.

2.5.1 Antiplasmodial activity of extract-drug combinations

The lactate dehydrogenase assay was used to determine the *in vitro* antiplasmodial activity of the combination of a plant extract and the two drugs. To extrapolate the results to the clinical situation would require that the experimental dose range should correspond with therapeutic concentrations of the drugs. The ratios of the drug combinations should be as close as possible to those that occur *in vivo*. The normal levels of active ingredients from plants in patients taking traditional medicines are not known and neither are the concentration ratios of

traditional medicines and the established drugs. Investigators working on drug combination studies have chosen starting concentrations basing on the IC_{50}. Ringwald *et al.*, (1999) and Gupta *et al.*, (2002) used starting concentrations 10- to 100-times fold the IC_{50}. In this study starting concentrations of 7-times fold the in *vitro* IC_{50} of *A. africana* isolate was used, and the combination ratios were selected arbitrarily, between 4 and 40 (ACW1 to chloroquine or artemisinin, respectively.

Materials and reagents

The materials used in this assay were the same as those described before.

Experiment

A stock solution of the most active isolate from the third batch of *A. Africana*, **ACW1**, was prepared to a concentration of 25 µg/ml. Stock solutions of chloroquine and artemisinin were prepared at a concentration of 2 µg/ml. The chloroquine-sensitive D10 and the chloroquine- and sulphonamide-resistant K1 strain of *P. falciparum* were made up to 2% haematocrit and a parasitaemia of 2%. The wells of the first three rows in the third column contained 150 µl of **ACW1** stock solution and were topped up with 50 µl of chloroquine or artemisinin stock solution. In the second triplicate, 100µl of **ACW1** were topped up with 100 µl of the standard drugs, in the third row 80µl of the **ACW1** and 120µl of drug, and in the last, 50µl of the **ACW1** were combined with 150 µl of the standard drugs. Double dilutions and the rest of experimental procedures were carried out as in the screening of plant extracts described earlier. The experiment was repeated twice for all combination ratios against the D10 and K1 strains of *P. falciparum* and for the two drugs investigated.

Data analysis and interpretation

The interaction between two chemotherapeutic agents is described by comparing the chemotherapeutic effect achieved by the agents alone with that in combination. The 50% inhibitory concentration (IC_{50}) was used in the comparison of the chemotherapeutic effects. The concentration of each of the agents at the IC_{50} of the combination was determined from a plot of percentage parasite survival against the base 10 logarithms of the concentrations using the prism Graph Pad V3. Two methods are used to analyse drug combination data, one is algebraical and the other is geometrical. The algebraic method involves expressing the drug concentration at the IC_{50} of the combination as a fraction of its IC_{50}, the fractional inhibitory

concentration (FIC). The sum of the FICs of the two agents in a combination describes the nature of the interaction. In this study, we used the geometrical method in which concentrations of the partner agents at the combination IC_{50} are plotted on each axis and a line joining their IC_{50s} (Isobole) included. Both methods give similar information, if the sum of the FIC is above 1, in which case the data point will be above the isobole on the graph, the agents will be antagonistic. If the sum is 1, the point will be on the line or close to it, and the interaction will be additive. If below 1, the agents will be synergistic, and the point will be below the line.

2.5.2 Effect of extract on uptake of ^{3}H dihydroartemisinin (^{3}H-DHA)

Artemisinin and its active derivatives are sesquiterpene endoperoxide lactones that bind to heme in the parasite (Kamchonwongpaisan and Meshnick, 1996). The drug has to cross the red blood cell membrane and pass through the cytoplasm, along specific pathways into the parasite food vacuole. Interactions with other drugs might occur at any stage from uptake to the heme-binding site. To investigate drug interactions at cellular level radiolabeled artemisinin was used. The use of radiolabeled drugs assumes that disintegrations per minute are proportional to the amount of the drug taken up. This study investigated the effect of a plant extract on the uptake of ^{3}H-dihydroartemisinin by erythrocytes infected by two strains of *P. falciparum*.

Materials and reagents

^{3}H-dihydroartemisinin (concentration, 714 nm, molecular weight, 284.3 and specific activity, 1 mC/ml) was kindly provided by Professor Michael Ashton, University of Gothenburg, Sweden. Dibutylphthalate, Solubable and Scintillation fluid were obtained from Packard Bioscience, BV, Groningen, The Netherlands. A Tri-Carb 2100 TR Liquid Scintillation Analyser (Packard Bioscience Company) was used. Ethylenediaminetetra-acetic Acid (EDTA) was obtained from Sigma-Aldrich, South Africa.

2.5.2.1 Specific activity of ^{3}H-DHA

To determine the specific activity, 10 µl of ^{3}H-DHA was made up to 100 µl with methanol. To three scintillation vials 10 µl of this solution was added, followed by 2ml of scintillation

fluid. To another three vials 2 ml of scintillation fluid was added, as a negative control. The disintegrations per minute (DPM) of the samples was determined using a liquid scintillation analyzer. The mean DPM was determined for the three experiments and the disintegrations per unit concentration of drug determined.

2.5.2.2 Effect of drug dose on the uptake of ^{3}H-DHA

This study aimed to use doses close to the therapeutic dose of dihydroartemisinin. *In vitro* systems however lack key components of the *in vivo* system; drug protein binding, drug metabolism and elimination. This posed a challenge in choosing *in vitro* working dose that could give clinically significant results. A study among children reported parasite clearance of 97-100% after 24 hours of treatment with artesunate. In this study, the mean maximum blood concentration (C_{max}) of DHA was 0.18 μg/ml (Halpaap et al., 1998); this would be equivalent to an exposure of 2.44 x 10^{-4} femtomoles per erythrocyte (Assuming a haematocrit of 50% and that, 1 μl of packed erythrocytes has 5.2 x 10^{6} cells). Another study done by Na Bangchang et al., 1994, indicated steady state concentrations of DHA ranging from 36 to 60 ng/ml, in six volunteers who taking 200mg of artemether orally, 100mg after 12 hrs and 100mg daily for days. This would be equivalent to a steady state exposure of 4.9 x 10^{-5} to 8.1 x 10^{-5} femtomoles/per erythrocyte considering the assumptions above. Consideration the effect of protein and cellular binding on *in vivo* drug availability to erythrocytes, this study worked with drug ranges below those observed in the above studies (0.68-6.83 x 10^{-6} femtomoles/erythrocyte).

Experiment

To determine the effect of dose on drug uptake per erythrocyte, increasing drug concentrations were incubated with erythrocytes at 37°C. A stock solution of ^{3}H-dihydroartemisinin was prepared at a concentration of 0.001ng/μl and stored at 4°C. Erythrocytes infected with the D10 trophozoites that had been synchronized 12 hours previously were made up to a 5% parasitaemia and haematocrit of 1%. (These parameters had been used successfully in studies on the uptake of chloroquine and mefloquine in our laboratory). To six sets of triplicate appendorff vials, 1 ml of infected erythrocytes suspension were added, controlled with uninfected erythrocytes of haematocrit 1 %. The infected and uninfected erythrocytes were then exposed to 1μl, 2 μl, 3 μl, 5 μl, 8 μl and 10 μl of

radiolabeled dihydroartemisinin. The vials were incubated in a water bath at 37 ° C for 1 hour.

At the end of the incubation time, 100 µl of dibutylphthalate was added to each vial and the vial spun for 1 minute at 13,000 RPM in a centrifuge. A corresponding set of sterile scintillation vials was prepared in triplicates. The tips of the vials were cut and dropped into the scintillation tubes. To each scintillation vial, solubable was added directly to the blood pellet. After 30 minutes, 50µl of EDTA were added and 5 minutes later, 100 µl of hydrogen peroxide added. After another 30 minutes of standing, 2 ml of scintillation fluid were added. The scintillation vials were allowed 1 hr after which they were closed, labelled and placed in a scintillation counter. This experiment was repeated twice for each of the D10 strain and K1 isolate of *P. falciparum*.

Data analysis

The mean DPM of the two experiments was determined and assuming a linear correlation between DPM and drug concentrations, the specific activity determined above was used to calculate the amount of drug taken into an erythrocyte at a particular drug dose. For the infected erythrocytes, the mean DPM of the 95% of the uninfected erythrocyte pellet exposed to the same drug dose was subtracted from the mean DPM of the pellet; to obtain the DPM due to 5% infected erythrocytes. The percentage drug uptake was then determined as the average amount of drug taken up by an erythrocyte as a percentage of the amount of drug exposed to the erythrocyte. The data was analyzed using the Microsoft Excel and Statistica soft wares.

2.5.2.3 Effect of lower temperature on the uptake of ^{3}H-DHA

To investigate the effect of temperature on the uptake of ^{3}H-DHA infected and uninfected erythrocytes, an experiment was set up to determine the uptake of ^{3}H-DHA at 4°C. A set of 5 centrifuge vials was filled with 1ml of uninfected erythrocytes suspension adjusted using culture media to a haematocrit of 1%. Parallel to this experiment another set of 5 vials were filled with 1 ml suspension of erythrocytes infected with the D10 strain of *P. falciparum* (Parasitaemia adjusted to 5% and a haematocrit of 1% as in the earlier experiment). To these vials 3µl of ^{3}H-DHA from the stock solution was added. The vials were incubated at 4°C for

1 hour. After stopping the experiment the vials were processed as described above. The percentage uptake of ^{3}H-dihydroartemisinin by erythrocytes was calculated and the results compared with those of the earlier experiment carried out at 37°C.

2.5.2.4 Effect of extract on uptake of ^{3}H-DHA

To investigate the effect of an isolate from *A. africana* on the uptake of ^{3}HDHA a dose of 2.04 x 10^{-6} fmols/erythrocyte was selected, as it could allow detection of any increase or decrease in percentage uptake. Three extract doses; 150ng, 350ng and 600ng were chosen basing on the range of extract concentration found at the IC_{50} of the combination with artemisinin. To 4 sets of 3 appendorf vials, 1 ml of erythrocyte suspension infected with the D10 strain of *P. falciparum* (haematocrit 1%, parasitaemia, 5%) was added. To the four sets of vials, 0, 150ng, 350ng and 600ng of *A. africana* isolate was added respectively. This was followed by 3µl of ^{3}H-dihydroartemisinin from the stock solution. The vials were incubated at 37°C for 2 hours. The vials were then prepared as already described above for reading using the liquid scintillation counter. The experiment was repeated 3 times for each of the D10 and K1 strain of *P. falciparum*. The data was analysed using the Microsoft excel programme and percentage uptake worked out as described earlier. The non-parametric Mann-whitney U-test was used to compare the effects of different extract dose on ^{3}H-DHA uptake.

2.6 Studying the *in vivo* antimalarial activity

A murine model of cerebral malaria was used to investigate the effect of two plant extracts on disease progress. The study was granted clearance from the Research and Ethics Committee of the University of Cape Town as shown in appendix 8.

Materials and reagents

Wild strains of C57BL mice, 6-8 weeks old were obtained from colonies in the animal unit of the University of Cape Town, South Africa. The P. *berghei* (Anka) strain cryopreserved in our laboratory originated from the Swiss Tropical Institute, Basle. Switzerland. Diethyl ether, chloroquine diphosphate and Giemsa stain were from Merck-Aldrich, Steinheim, German. An automated cell counter ADVIA 120 supplied by Bayer was used for the haematological indices.

Experiment

The experiments were carried out in the animal unit of the Faculty of Health Sciences of the University of Cape Town, South Africa. The animals were handled in accordance with the regulations for the handling of laboratory animals approved by the Research and Ethics Committee of the University of Cape town. The animals were housed in groups of five per cage at 22 °C and relative humidity of 80%. The diet was made up of standard pellet diet and continuous clean drinking water.

2.6.1. The natural history of rodent *P. berghei* malaria

To study the natural history of rodent *P. berghei* (Anka) in the wild strains of C57BL mice, 23 animals were obtained from the animal unit. The animals were put into 5 cages labelled A to E. Cage E had 3 animals only, the rest of the cages had 5 animals each. Animals in cage A and B were used as the control (uninfected) while animals in cage C and D were infected with *P. berghei* (Anka).

The *P. berghei* (Anka) strain of rodent malaria, cryopreserved in our laboratory was thawed as earlier described for *P. falciparum*; human erythrocytes were not added this time. The cell suspension was instead introduced intraperitoneally into 3 study animals each animal receiving 0.2 mls. After five days the animals were anaesthetised using diethyl ether and blood from the animals was collected into heparinised bottles by cardiac puncture, the animals were subsequently sacrificed by cervical dislocation. Thin smears of the blood were prepared and stained using the Giemsa stain and the parasitaemia determined by light microscopy. Studies done indicate that normal C57BL mice of about 8 weeks have about 9.3×10^9 red blood cells per ml, together with the parasitaemia determined on giemsa stained microscopy, the number of infected erythrocytes per millitre was determined. Using Phosphate Buffered Saline (PBS) the blood was diluted to make a suspension of 5×10^7 parasitised erythrocytes per millilitre.

An infecting dose of 1×10^7 parasitised cells was then used by introducing 0.2mls of blood suspension intraperitoneally into the mice in cages C and D. To obtain the changes in parasitaemia and haematological indices, one animal was sacrificed from cage B and D on days 0, 2, 4, 6 and 8 and 2ml of blood obtained by the method similar to the one used by

SaiRam et al., 1997. Thin smears, 2 from each sample, were prepared from the blood samples and the rest of the blood transferred in a heparinised bottle for measurement of haematological parameters, which included: haemoglobin concentration, total white cell count, total platelet count and differential white cell count. The slides were stained using the giemsa stain and the parasitaemia determined by counting the number of parasitised erythrocytes per 1000 erythrocytes under light microscopy. The mice in groups A and C were counted and weighed, to determine the average weight and percentage survival on the corresponding days. The mean parasitaemia, average weight and mean survival time of mice in cages A (uninfected) and C (infected) was calculated using Microsoft Excel 2000.

2.6.2. Antimalarial activity of *M. foetida* and *C. halicacabum*

Investigation of the water extracts of *M. foetida* and *C. halicacabum* was carried out in two stages. The experimental design was similar to the four-day suppressive test, used by Peters et al., 1993. The first stage was a preliminary investigation in which 15 mice divided into 3 groups; A, B, and C of 5 mice each were used. The mice were handled as described in the first part of experiment. All the animals in the 3 groups were infected with the *P. berghei* (Anka) as described earlier. Starting from day 0 (day of infection) the animals in group A were treated with chloroquine 10mg/kg twice a day, the animals in group B and C were treated with 250 mg/kg of the water extracts of *M. foetida* and *C. halicacabum* twice a day respectively. The drug and extracts were administered in a volume of 0.2 mls at each time by gastric lavage. The mice were observed for 14 days, during which, animals were observed for survival, weight change and development of parasitaemia. The mice were tail bled and two thin smears geimsa-stained to determine parasitaemia. The parameters taken on day eight were used in assessing the effect of the extracts on disease progress. The results were compared with those in the first animal experiment.

The water extract of *M. foetida* was investigated further. To do this 20 mice were divided into 4 groups of 5 mice each and labelled A to D. The mice were handled as described earlier. The mice were transferred from the Animal unit to the Department of Pharmacology animal unit were they were kept for at least a week before the experiment was started to allow acclimatisation. The mice in all the groups were infected with *P. berghei* anka as described above. The water extract of *mormodica foetida* was dissolved in PBS to make a stock solution of 150 mg/ml. The animals were weighed and basing on their weights doses of 10mg/kg,

100mg/kg, 200mg/kg and 500mg/kg were administered twice a day on days: 0, 1, 2 and 3 after infection. The animals were monitored for three weeks, during which, a record of the weights, dying animals and parasitaemia were taken. The blood for the determination of parasitaemia was collected by tail bleeding and preparing two smears, which were giemsa-stained. The animals were also observed for any special characteristics during the time of the experiment. The mean parasitaemia, survival time and weight were determined using the Microsoft excel 2000 and analysed graphically.

CHAPTER 3

The in vitro antiplasmodial properties and phytochemical analysis of six Ugandan traditional medicinal plants

3.1 Introduction

This study investigated the antimalarial potential of medicinal plants of Uganda. To achieve this objective six plants *Senecio discifolius* oliv, *Senecio stuhlmannii*, *Indigofera emarginella* steud. Ex A. Rich, *Aspilia africana* (Pers) C.D. Adams, *Cardiospermum halicacabum* L and *Momordica foetida* Schumch. Et Thonn (voucher numbers WP1/2000 to WP6/2000 respectively) were selected for investigation with the help of traditional healers. In this chapter we report the results of the first part of the study, which involved collection, identification, extraction, screening for antiplasmodial activity and phytochemical analysis of extracts from the study plants.

3.2 Collection and identification of the plants

Shoots of the plants, *S. discifolius* and *S. stuhlmanni* were collected from the eastern region of Uganda, 30km on Jinja –Iganga road. The plant *I. emarginella* was collected near Mbale town on the eastern border of Uganda. The plants *M. foetida*, A. *africana* and *C. halicacabum* were collected 20 Km on Masaka-Mbarara road. All plants were collected during the month of June 2000 (wet season). The second batch of potentially active plants was recollected in December 2000 and part of January 2001, during this period, most parts of Uganda experienced the dry season. The plants were identified by a botanist* and voucher samples were deposited at the Makerere University Herbarium.

3.3 Antiplasmodial activity of the study plants

The yields and antiplasmodial activity of extracts from the first batch of plants are shown in Table 3.3.1a and 3.3.1b. Extracts with antiplasmodial activity of 50% inhibitory concentration (IC_{50}) of 25 µg/ml or less were considered to have significant activity. The yields of the extracts, expressed as a percentage of dry weight of the plant material ranged from 0.8% to

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18.7%. For all the study plants water produced the highest extract yields. The most active extract was the ethyl acetate extract of *A. africana*, with activity of IC_{50}, 9.3(7.7-10.9)µg/ml against the chloroquine-sensitive D10 strain, and 11.5(8.7-14.3) µg/ml against the chloroquine-resistant K1 strain of *P. falciparum*. Except for *A. africana*, water extracts from all other plants did not show significant antiplasmodial activity. There was a positive correlation between the antiplasmodial activity of the extracts against the D10 and K1 strains of *P. falciparum* (Pearsons' coefficient, r = 0.9691, p=0.05) as shown on a scatter diagram in figure 3.3.1. This suggests similar antiplasmodial activity of extracts against the chloroquine-sensitive and -resistant strains of *P. falciparum*. The methanol extract of *I. emarginella* and the ethyl acetate extracts of both *S. stuhlmannii and S. discifolius* showed significant activity against the two study strains. The second plant batch comprised of plants I. *emarginella*, *S. stuhlmannii*, *S. discifolius* and *A. africana*, whose extracts had shown significant antiplasmodial activity.

Microscopic observation of uninfected erythrocytes incubated with the methanol extract of *I. emarginella*, and the ethyl acetate extracts of *S. stuhlmannii*, *S. discifolius* and *A. africana*, and uninfected erythrocytes from the blank column of the 96-well plate showed no morphological differences after 48 hours of incubation.

3.3.1 Yields and antiplasmodial activity of plants collected in the dry season

The four plants collected during the dry season were extracted in exactly the same way as the first batch of plant material. Attention was paid to extracts that had shown antiplasmodial activity in the first batch. The antiplasmodial activity for the four extracts ranged from IC_{50} values of 8.00 to 29.0 µg/ml as shown in Table 3.3.2. The ethyl acetate extract of *A. africana* showed the highest antiplasmodial activity with IC_{50} of 8.0 (5.30-10.70) µg/ml. The yields of extracts from these plants ranged from 1.2 to 2.3% and were generally lower than those of plants collected during the wet season, as shown in figure 3.3.2.

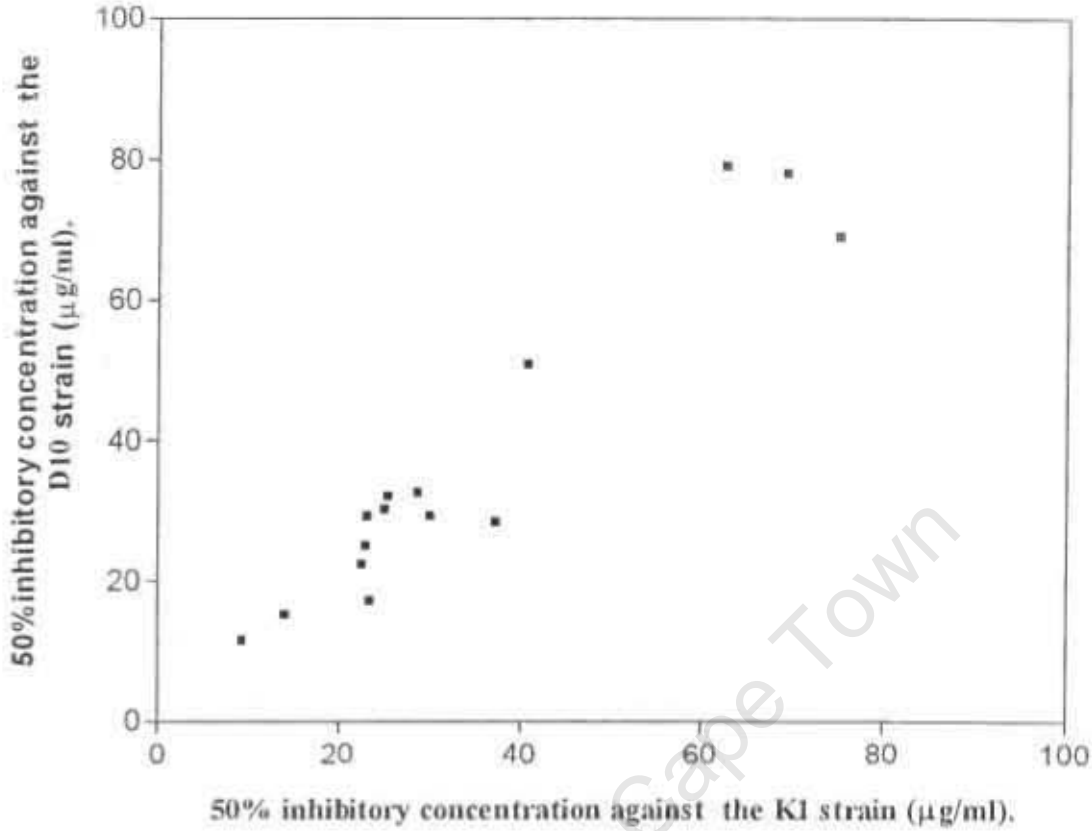

Fig 3.3.1. Correlation between the antiplasmodial activity of 15 crude extracts from the plants: *S. discifolius, S. stuhlmannii, I. emarginella, A. africana, C. halicacabum* and *M. foetida* against the chloroquine-sensitive D10 and the chloroquine- and sulfonamide-resistant K1 strains of *P. falciparum*. (Pearsons' coefficient; r=0.967, p=0.05, Values are based on 3 experiments carried out in duplicate. The study plants were collected during the wet season.

Table 3.3.2. Yields and antiplasmodial activity of extracts from *I. emarginella*, *S. stuhlmannii*, *S. discifolius* and *A. africana* collected during the dry season. The antiplasmodial activity was tested against the D10 strain of *P. falciparum*.

Extracts	Yields[1] (%)	IC_{50} (µg/ml)[2]
I. emarginella (M)[a]	1.80	10.00 ± 2.8
S. stuhlmannii (E)[b]	1.50	9.50 ± 1.6
S. discifolius (E)[b]	1.20	21.80 ± 13.5
A. africana (E)[b]	2.30	8.00 ± 2.7

[a]Methanol extract, [b]Ethyl acetate extract. [1]Values of yields are based on one experiment. [2]Values of IC_{50} are based on three experiments carried out in duplicate and standard error of the mean worked out at 95% confidence interval.

The methanol extract of *I. emarginella* and the ethyl acetate extract of *S. stuhlmanni* (S.S) from plants collected in the dry season had better antiplasmodial activity than extracts from plants collected during the wet season. The ethyl acetate extracts of *S. discifolius* and *A. africana* had comparable activity as shown in figure 3.3.3.

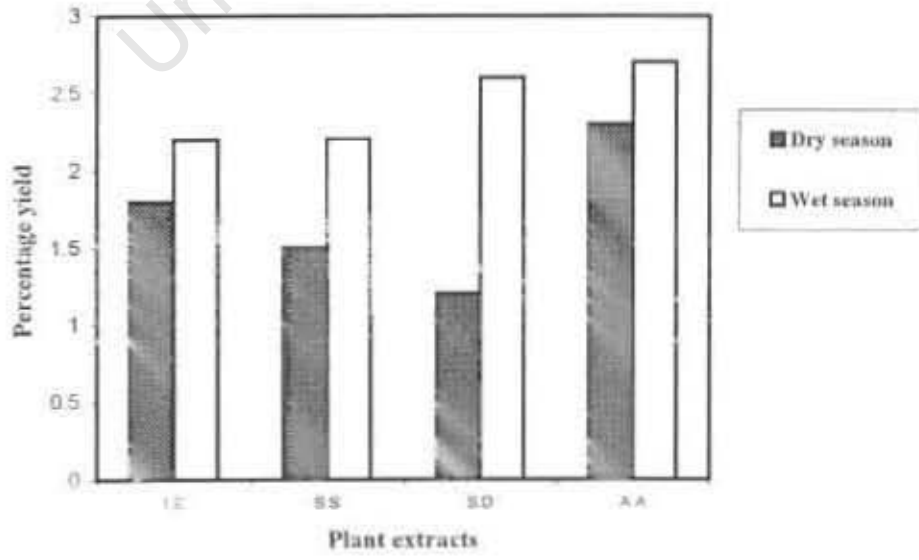

Fig 3.3.2 Yields of extracts from *I. emarginella* (I.E), S. *stuhlmanni* (S.S), S. *discifolius* (S.D) and *A. africana* (A.A) collected during the dry and wet seasons. Values are based on one experiment.

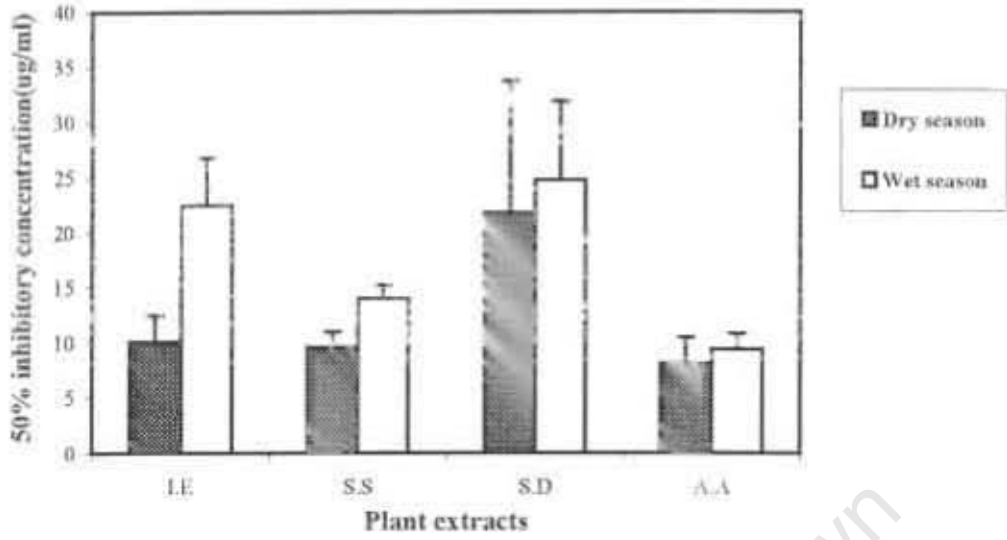

Fig 3.3.3. Antiplasmodial activity of extracts from *I. emarginella* (I.E), S. *stuhlmannii* (S.S), S. *discifolius* (S.D) and *A. africana* (A.A) collected during the dry and wet seasons against the D10 strain of *P. falciparum*. Values are based on 3 experiments carried out in triplicate and standard error of the mean worked out at 95% confidence interval.

3.4 Bioassay-guided fractionation

Fractionation of crude extracts is generally expected to improve biological activity and result into the isolation of principles responsible for the biological activity. The ethyl acetate extracts of the plants S. *stuhlmanni, S. discifolius and A. africana,* together with the methanol extract of I. *Emarginella,* all of plants collected in the dry season, were fractionated using solid phase extraction (SPE). Fractions were tested for antiplasmodial activity against the D10 strain of *P.falciparum.*

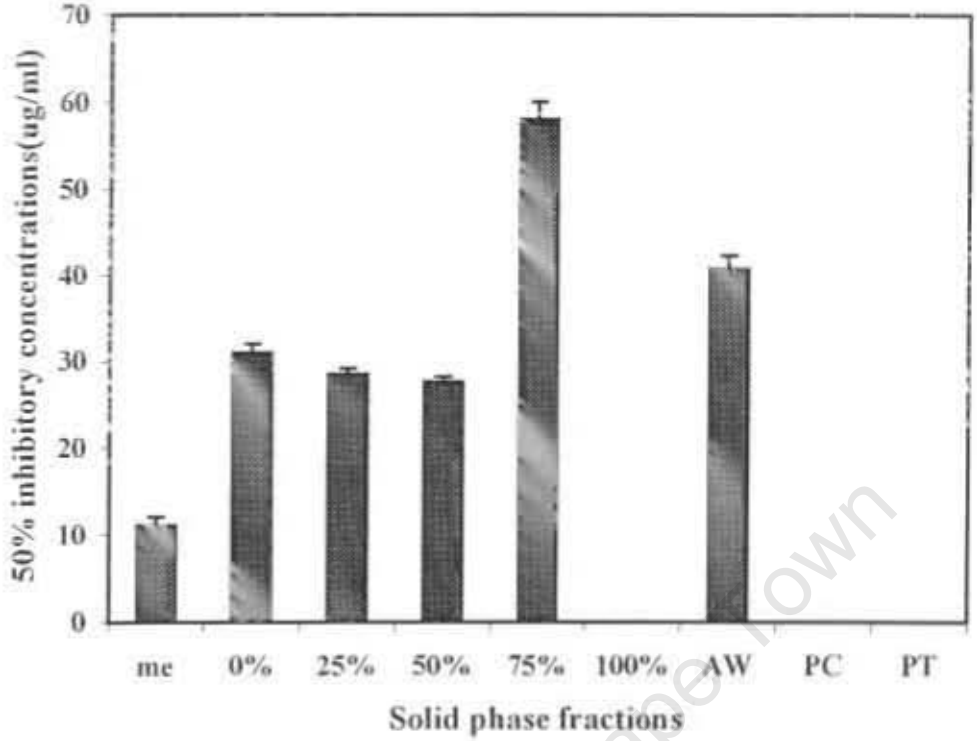

Fig 3.4.1 Antiplasmodial activity of fractions from the methanol extract of *I. emarginella* against the D10 strain of *P. falciparum*. Values are based on two experiments carried out in duplicate and standard error of the mean worked out at 95% confidence interval. The 100% fraction did not show antiplasmodial activity. AW denotes acetone wash. The percolate (PC) and pellet (PT) did not show antiplasmodial activity.

The solid phase fractions from the methanol extract of I. *emarginella* were generally less active than the parent extract on preliminary fractionation. The 100% ethyl acetate fraction, the percholate and the pellet showed no activity at all as shown in fig. 3.4.1. Fractions from this experiment where not subjected to further study. Preliminary fractionation of the ethyl acetate extract of *S. discifolius* similarly did not show improved antiplasmodial activity, instead there was a steady reduction in antiplasmodial activity with increasing proportion of ethyl acetate in the mobile phase. The pure ethyl acetate fraction, acetone wash, percolate and pellet showed no activity, these results are shown in figure 3.4.2.

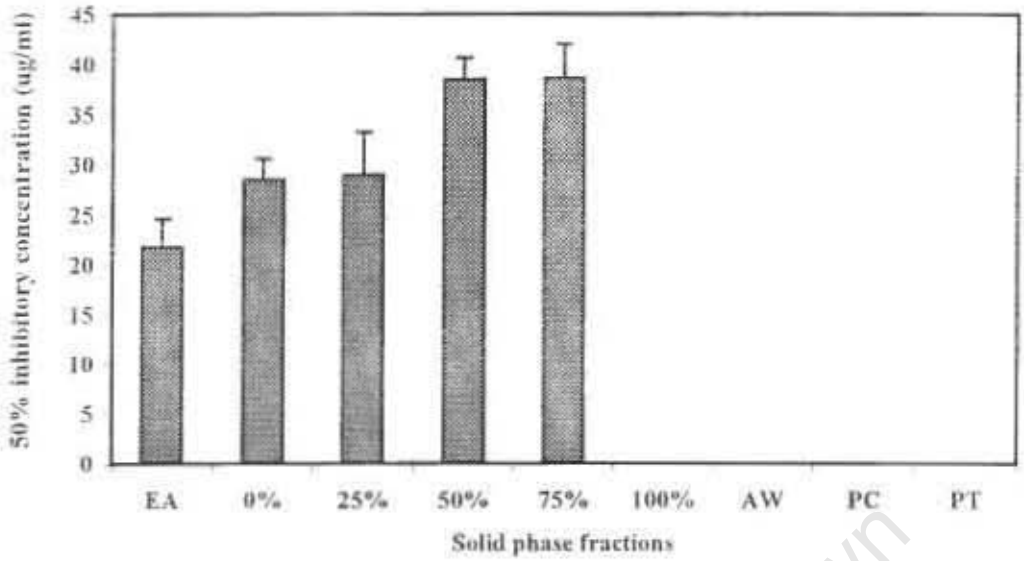

Fig 3.4.2 Antiplasmodial activity of fractions from the ethyl acetate extract of S. *discifolius* against the D10 strain of *P. falciparum*. Values are based on two experiments carried out in duplicate and standard error of the mean worked out at 95% confidence interval. EA denotes the parent extract. The 100% fraction did not show activity. The acetone wash (AW), percolate (PC) and pellet (PT) did not show antiplasmodial activity.

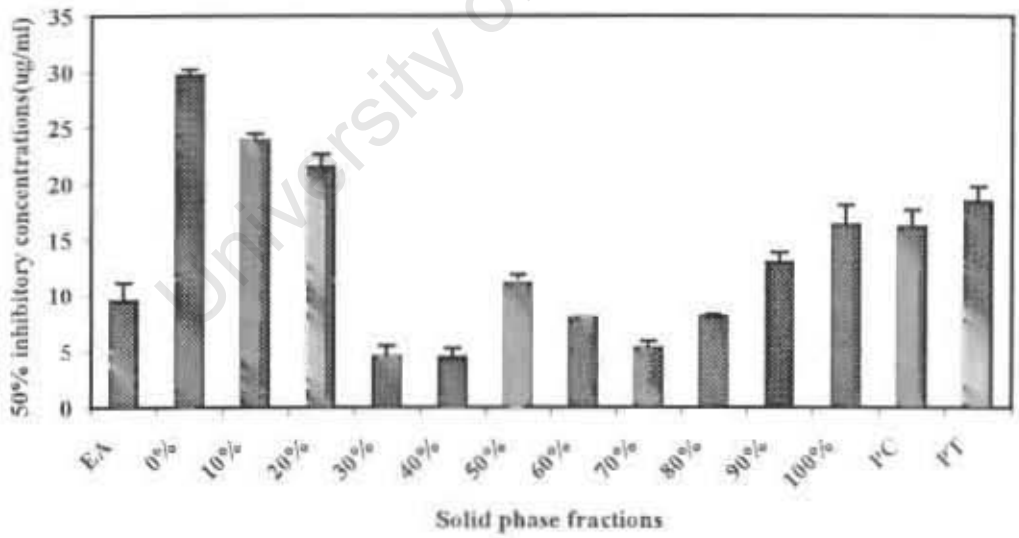

Fig 3.4.3 Antiplasmodial activity of the solid phase fractions of *S. stuhlmannii* against the D10 strain of *P. falciparum*. Values are based on two experiments carried out in duplicate and standard error of the mean worked out at 95% confidence interval. EA refers to the parent extract. PC and PT denote percolate and Pellet respectively.

The preliminary fractionation of the ethyl acetate extract of *S. stuhlmannii* indicated an improvement in the antiplasmodial activity of the fractions. A more refined fractionation of the extract was carried out with 10% increasing proportion of acetonitrile to water by volume.

The fractions between 20% to 80% acetonitrile to water had better antiplasmodial activity than the parent extract. The 30% and 40% acetonitrile fractions were the most active with IC_{50} value of 5.0 µg/ml. Most of the fractions had better antiplasmodial activity than the parent crude ethyl acetate fraction as shown in figure 3.4.3. These two fractions however, lead to extensive haemolysis when incubated with uninfected erythrocytes over the 48-hour period, with no visible erythrocytes on giemsa-stained light microscopy. These findings suggest that the two extracts are toxic to erythrocytes, and most likely to other mammalian cells.

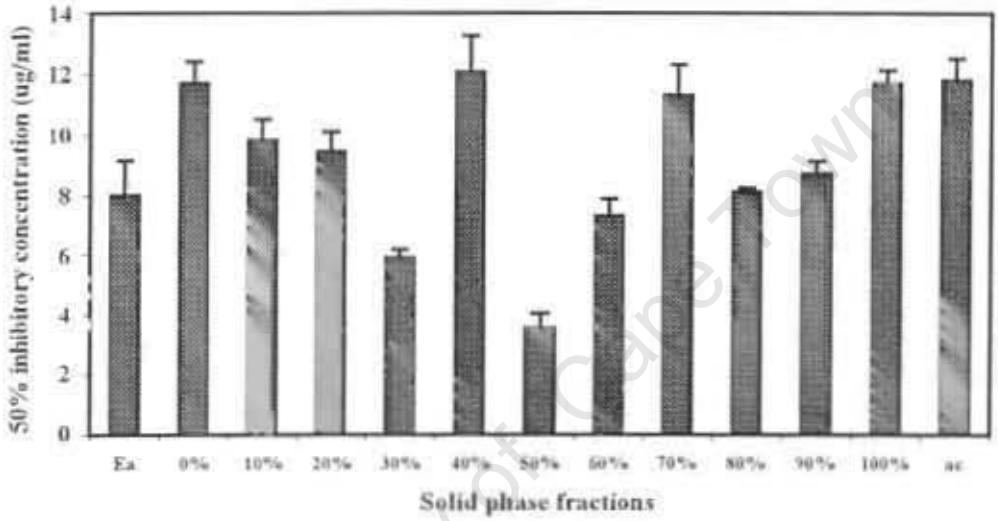

Fig. 3.4.4 Antiplasmodial activity of the solid phase fractions of *A. africana* against the D10 strain of *P. falciparum*. Values are based on two experiments carried out in duplicate and standard error of the mean worked out at 95% confidence interval. Ea is the parent fraction while ac is the acetone wash.

The SPE fractionations from the ethyl acetate extract of *A. africana* were more active than the parent fractions. A more refined SPE was carried out with 10% increments of ethyl acetate to hexane in the mobile phase. The 1:1 ethyl acetate to hexane fractionation was the most active with IC_{50} of 3.6 µg/ml. The other fractions had antiplasmodial activity ranging from IC_{50}, 5 to 12 µg/ml. The pellet and percolate did not show antiplasmodial activity and are not indicated in figure 3.4.4. To get an idea on the number of compounds that are present in the most active fraction, 1 mg of the fraction was dissolved in 400 µl of HPLC grade acetonitrile; 20 µl of this was run on an HPLC analytical column. Figure 3.4.5 shows a chromatogram with 4 main peaks corresponding to compounds that have Ultra Violet (UV) absorbance at the wavelength of 210 nm and eluted at retention times (RT) of 10.903, 12.930, 14.439 and 18.255 minutes.

This study focused on demonstrating that Ugandan traditional medicines can be a source of lead compounds for modern antimalarial drugs. To achieve this objective, we selected the 1:1 fraction of *A. africana* for further fractionation. This was done using SPE but this time with 1% increase in ethyl acetate to hexane proportion in the mobile phase. The 42% ethyl acetate fraction to hexane was the most active with antiplasmodial activity of 1.8 ±0.2 µg/ml as shown in table 3.4.1. Microscopic observation of uninfected erythrocytes after incubation with this fraction over the 48-hour period did not show changes in morphology.

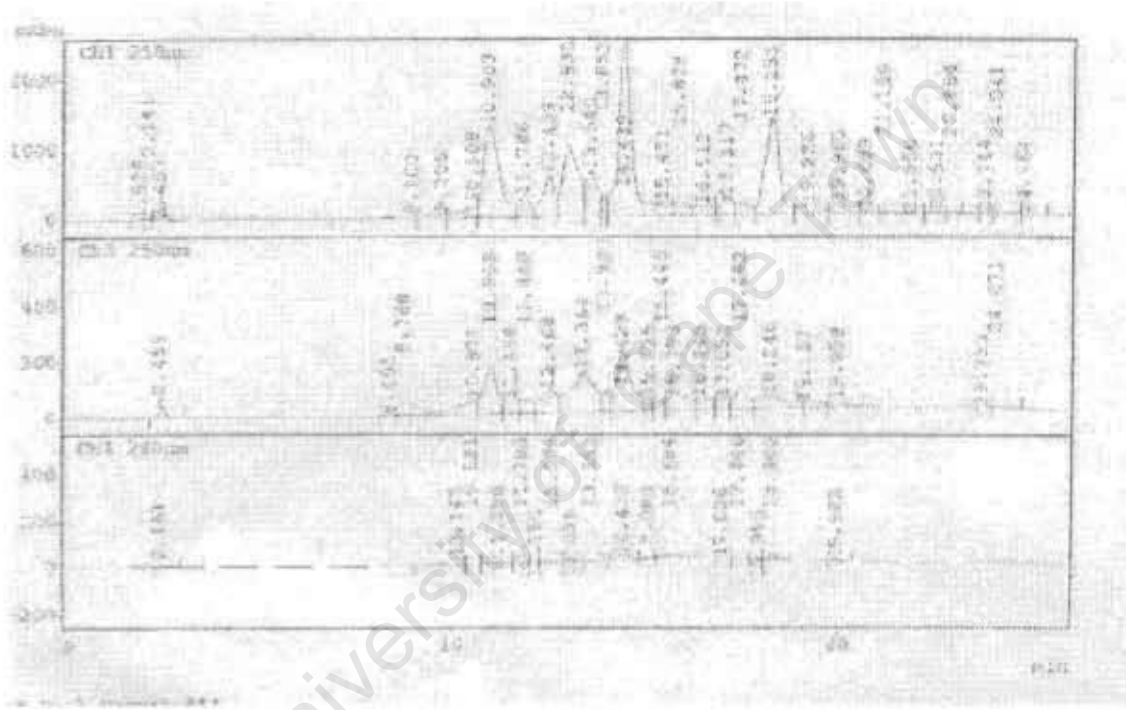

Fig 3.4.5 The HPLC chromatogram of the 1:1 ethyl acetate/hexane SPE fraction of *A. africana*. About 50µg of the fraction was injected on a C-18 pre-packed analytical column. A gradient run of acetonitrile: water from 20% to 100% at a rate of 1ml/min. for 25 minutes was used. A Shimadzu LC-10AS with a diode array detector was used.

The rest of the fractions had lower antiplasmodial activity than the parent fraction.

A small amount (1mg) of the 42% ethyl acetate fraction was run on an analytical column. Figure 3.4.6 shows the chromatogram of the 42% ethyl acetate fraction with a major peak at retention time (RT) of 8.102 minutes and a shoulder at RT of 8.369 minutes are shown.

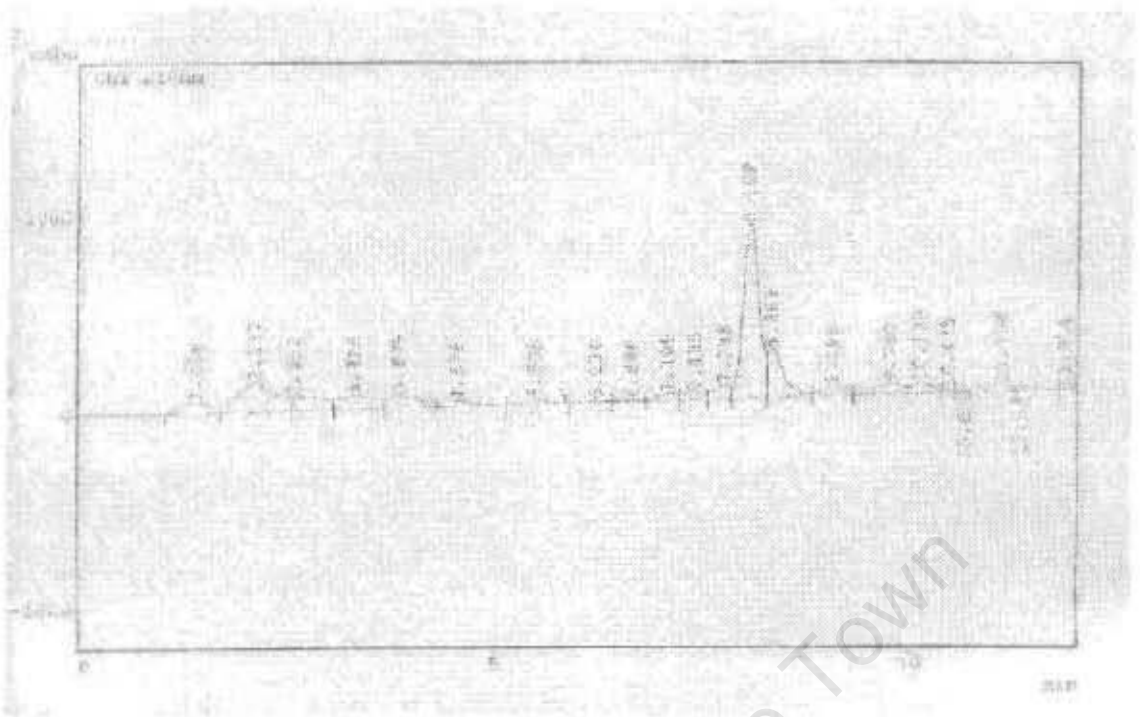

Fig 3.4.6. The HPLC chromatogram of the 42% ethyl acetate: hexane fraction of *A. africana*. About 50µg of the fraction was injected for this run. A gradient run of increasing acetonitrile: water from 10% to 100% for 12 minutes on a pre-packed C-18 analytical column was used at a rate of 1ml/min. A Shimadzu LC-10AS with a diode array detector was used.

The weight of the 42% ethyl acetate fraction was 6.3 mg. At least 7.73mg of the parent fraction was not recovered.

3.5 Characterisation of isolates from *A. africana*

The isolates were characterised using mass spectrometry and UV spectroscopy. The mass spectrum of the 42% ethyl acetate isolate **AA2,** showed a molecular ion peak at m/z ratio of 264.13571. The mass spectra are shown in appendix 2 and 3, and the molecular formulae of the ions in appendix 4. This corresponded to the molecular weight of the isolate and the molecular formula of the isolate was worked out to be $C_{15}H_{20}O_4$. The presence of fifteen carbon atoms is typical of sesqiuterpenes. The UV spectrum of this compound is shown in Appendix 5. The level of purity and quantity of isolate recovered did not allow further purification for final structure elucidation using Nuclear Magnetic Resonance (NMR).

Table 3.4.1. Antiplasmodial activity and yields of fractions from SPE of the 1:1 ethyl acetate: hexane fraction of *A. africana* on further partitioning using 1% increments of ethyl acetate: hexane in the mobile phase. The fractions were tested against the D10 strain of *P. falciparum*.

Solid phase fraction (%)	Amount recovered (mg)	IC_{50} (µg/ml)
Percolate	2.80	≥100
41	3.40	11.1 ±2.4
42	6.30	1.8 ±0.2
43	6.41	7.8 ±1.1
44	2.80	9.8 ±0.9
45	3.20	11.8 ±1.1
46	4.10	14.3 ±1.6
47	3.00	≥100
48	2.30	≥100
49	4.50	≥100
50	2.10	8.8 ± 1.7
Parent fraction	48.64	3.6 ± 0.7

The fractions are percentages of ethyl acetate to hexane in mobile phase. The weights are values of one experiment. The IC_{50} values are based on two experiments carried out in duplicate, the standard error of the mean was worked out at 95% confidence interval.

In order to obtain more of the isolate **AA2,** another batch of plant material was collected from the same location in the month of June 2002. The material was processed as above and to fasten the separation, the 50% ethyl acetate/hexane fraction was this time subjected to semi-preparatory HPLC. The antiplasmodial activity of the 50% fraction from the third batch was 4.9 ±0.8 µg/ml. An HPLC purity test was run on a semi-preparatory column using an isocratic ratio of 2:3 acetonitrile to water before collection runs where done. Two major peaks corresponding to compounds with retention time of 2.883 and 4.692 minutes were observed as shown on the chromatogram in figure 3.5.1. There were other minor peaks corresponding to compounds with retention time of 4.136 and 5.489 minutes. Four fractions: F_1, F_2, F_3 and F_4 were collected in between the times marked on figure 3.5.1. The fractions were tested for antiplasmodial activity against the D10 and K1 strain of *P. falciparum*. The fraction F_1, later called **ACW1** in our laboratory and F_3, later called **ACW2,** had high antiplasmodial activity against the chloroquine-sensitive D10 and the chloroquine-and sulphonamide-resistant K1 strains of *P. falciparum*. There was no evidence of toxicity against uninfected erythrocytes.

The two isolates were interestingly more effective against the chloroquine-resistant strain as shown in table 3.5.1. The isolate **ACW1** particularly is about 3 times more active against the resistant strain than the chloroquine-sensitive strain. Comparing with chloroquine, the isolate is effective in a narrower dose range as shown in figure 3.5.3.

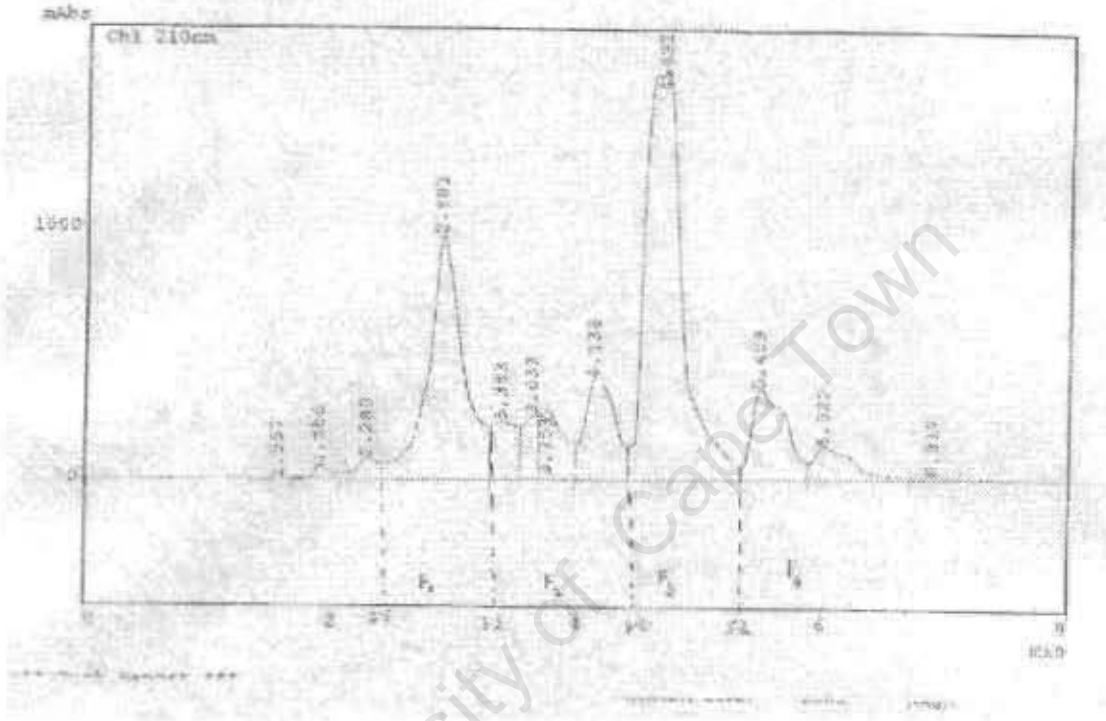

Fig. 3.5.1. The HPLC chromatogram of the 50% ethyl acetate: hexane fraction of *A. africana* from the third batch of plant material. About 1340μg in 200μl was injected and an isocratic run of 2:3 acetonitrile to water at 4ml/min for 8 minutes. A C-18 pre-packed semi-preparatory column and Shimadzu LC-10AS with a diode array detector were used.

Table 3.5.1 Antiplasmodial activity and yields of fractions F_1 (ACW1), F_2, F_3 (ACW2), F_4 (isolates from *A. africana*), artemisinin and chloroquine against the chloroquine-sensitive D10 and the chloroquine- and sulfonamide-resistant K1 strains of *P. falciparum*.

Extract/Drug	Percentage Yield	IC_{50} against D10 strain (µg/ml)	IC_{50} against K1 strain (µg/ml)
ACW1 (F_1)	0.00036	1.51 (1.21-1.81)	0.56 (0.26-0.86)
ACW2 (F_3)	0.00049	1.50 (1.33-1.67)	1.12 (0.52-1.72)
F_2	0.00042	11.55 (9.1-14.0)	14.35 (11.70-17.00)
F_4	0.00058	≥100	≥100
Artemisinin	0.40-0.60*	0.043 (0.042-.044)	0.017 (0.016-0.017)
Chloroquine	N/A	0.102 (0.032-0.172)	0.334 (0.104-.564)
Parent fraction	0.00360	4.90 (4.1-5.7)	N/D

N/D indicates that the experiment was not done while N/A denotes measure not applicable. Yields are based on one experiment. *Yield of artemisinin from the leaves of Chinese *Artimisia annua* L. by hexane extraction (Jansen, 2002). Values of IC_{50} are based on two experiments done in duplicate, standard error of the mean has been worked out at 95% confidence interval.

To check the purity of the fractions F_1 **(ACW1)** and F_3 **(ACW2),** a purity run was carried out under the same conditions as used in semi-preparatory chromatography. The figures 3.5.2 and 3.5.3 show relatively pure isolates eluting at RT of 2.548 minutes for **ACW1** and 4.642 minutes for **ACW2.** The other fractions were not tested for purity as they showed lower antiplasmodial activity. Low-resolution mass spectrometry of **ACW1** indicated a molecular ion of mass/charge ratio of 213 as shown on the mass spectrum in Appendix 6. The UV spectrum of **ACW1** is attached in appendix 7. The compound **ACW1** was different from **AA2** earlier isolated.

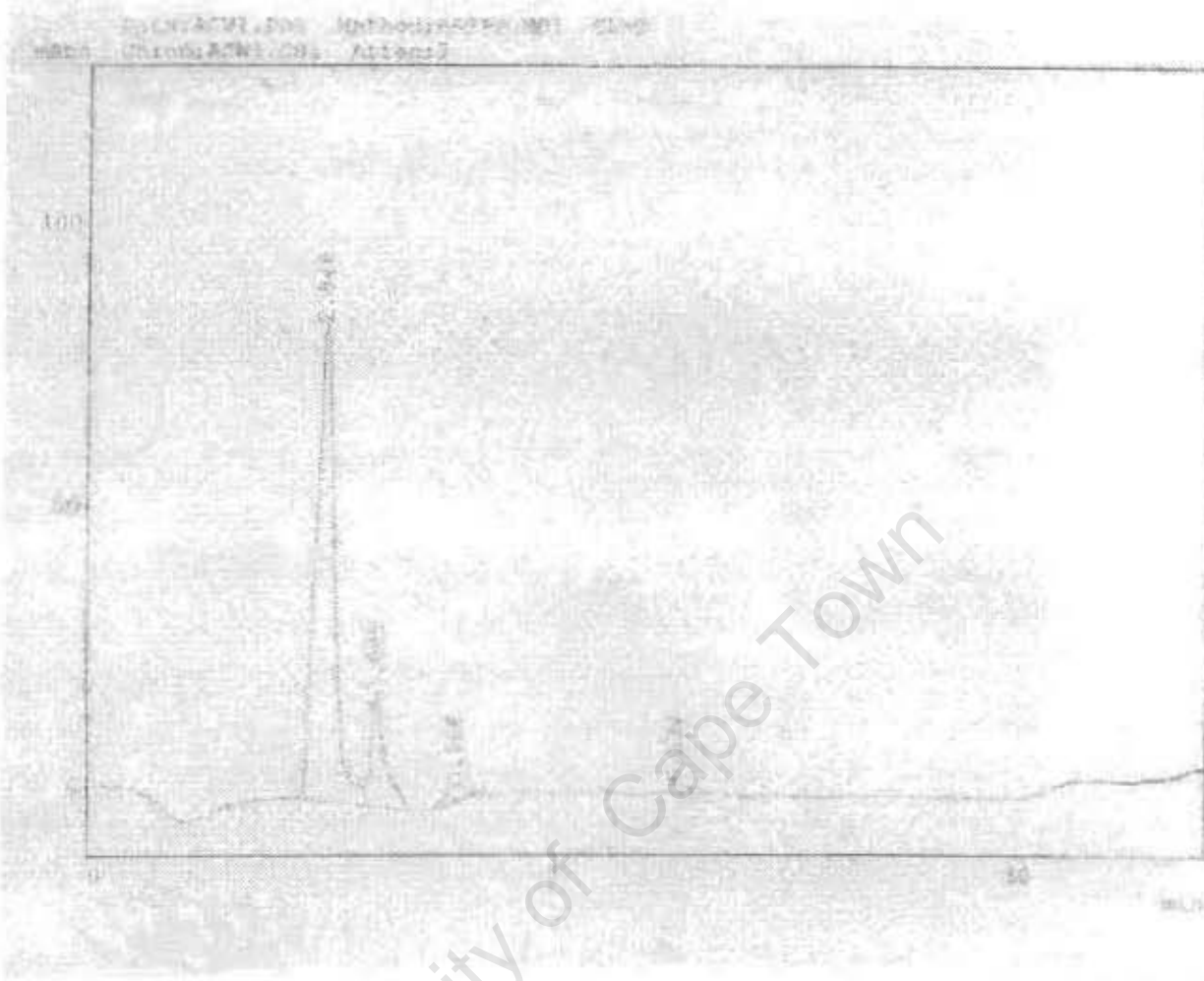

Fig 3.5.2 The HPLC chromatogram of ACW1, an isolate from the third batch of *A. africana*. About 50µg in 200µl was injected and eluted using an isocratic run of 2:3 acetonitrile to water at 4ml/min for 8 minutes, raising the proportion of acetonitrile to 100% in the next 2 minutes and dropping it to 0% in the last 2 minutes. A C-18 pre-packed semi-preparatory column and Shimadzu LC-10AS with a diode array detector were used.

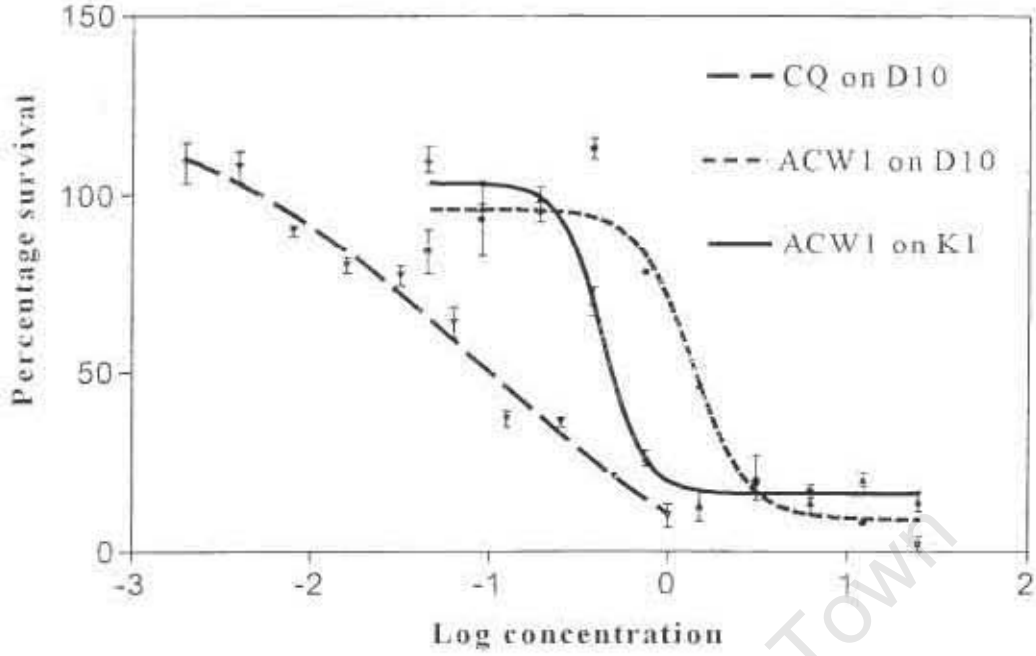

Fig 3.5.3. Antiplasmodial activity of ACW1 against the chloroquine-senstive D10 and the chloroquine- and sulphonamide-resistant K1 strain of *P. falciparum*, compared with that of chloroquine (CQ) against the D10 strain. Values are based on two experiments done in duplicate.

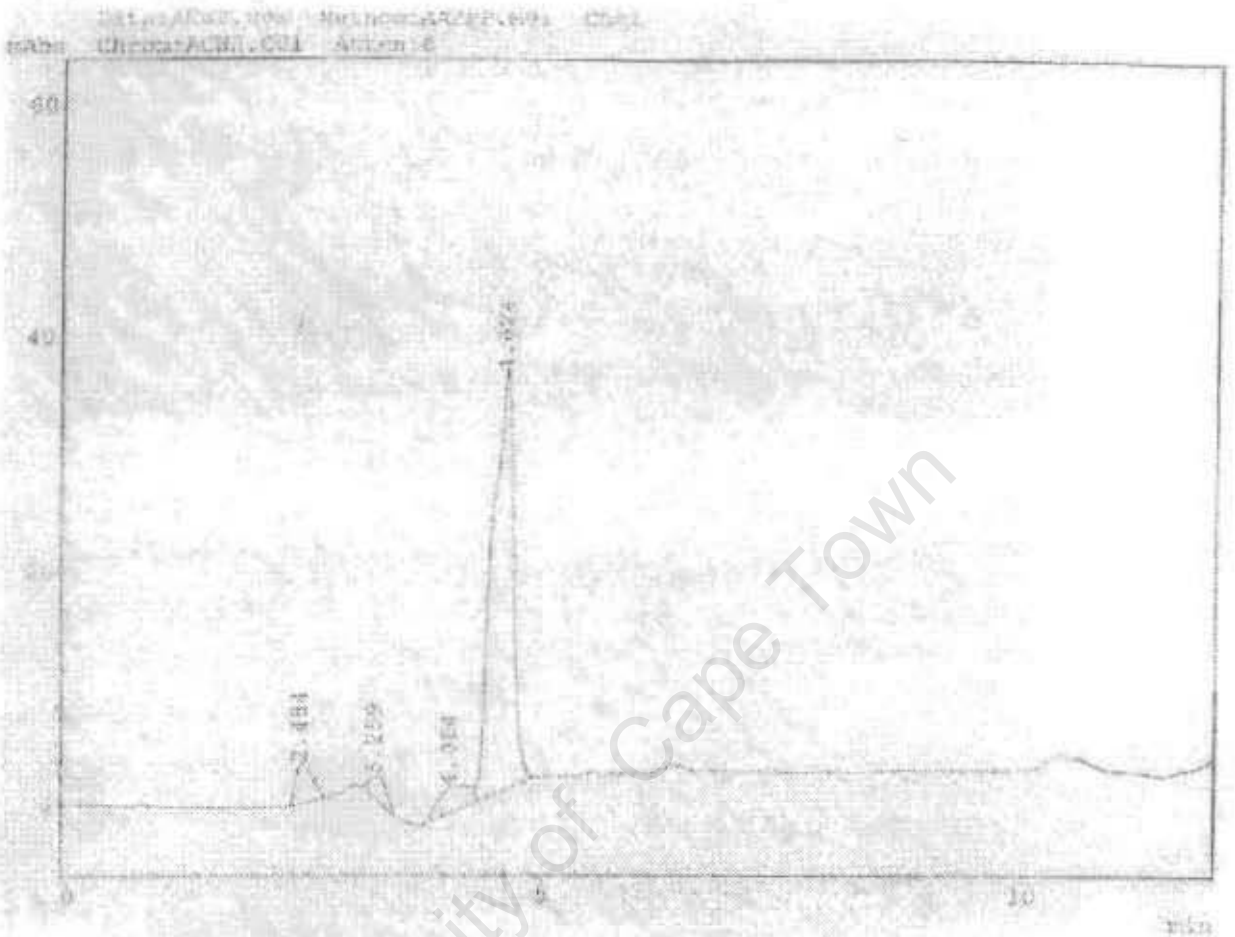

Fig 3.5.4. The HPLC chromatogram of ACW2, an isolate from the third batch of *A. africana*. About 50µg in 200µl was injected and eluted using an isocratic run of 2:3 acetonitrile to water at 4ml/min for 8 minutes, raising the proportion of acetonitrile to 100% in the next 2 minutes and dropping it to 0% in the last 2 minutes. A C-18 pre-packed semi-preparatory column and Shimadzu LC-10AS with a diode array detector were used.

3.6. Discussion

This study shows that extracts from the plants S. *discifolius, S. stuhlmannii, I. emarginella,* and *A. africana* have significant antiplasmodial activity. For all the four plants significant activity was observed with ethyl acetate and methanol extracts. Going by the solubility principle of 'similar dissolves similar' this finding suggests that constituents of these plants responsible for the antiplasmodial activity are generally polar compounds. The aqueous and

methanol extracts of *A. africana*, the plant with the most active antiplasmodial extract have been reported to possess antibacterial activity (Macfoy and Cline, 1990), suggesting that constituents of this plant have broad-spectrum antimicrobial properties.

Surprisingly, water extracts were generally less active, yet traditional healers reported using water decoctions and macerations in the treatment of malaria symptoms. It is however apparent, from the yields of the water extracts in this study that water tends to extract more compounds, this could have a diluting effect on the antiplasmodial activity of the extracts. A possibility of *in vitro* antagonism amongst the compounds cannot be ruled out as well. If this were the case then the possibility of selective absorption could lead to *in vivo* efficacy when water decoctions and macerations are used in traditional medicine.

Study plants were screened against the chloroquine-sensitive D10 and the chloroquine- and resistant-resistant K1 strain. A positive correlation was observed between the activity of plant extracts against the two strains (Pearsons' coefficient; $r=0.967$, $p=0.05$) and no significant difference in antiplasmodial activity of all the extracts against the two strains. This finding shows that cross-resistance between chloroquine and traditional medicines does not exist, a phenomenon, which can allow herbal remedies based on traditional medicines to be used in the treatment of both chloroquine-sensitive and -resistant malaria.

It was observed that plants collected during the wet season had better yields than those collected during the dry season. Extracts from the plants *I. emarginella* and *S. stuhlmannii* had better antiplasmodial activity during the dry season than the wet season. Since chemical constituents are responsible for the antiplasmodial activity of plant extracts, it is logical to conclude that the chemical composition of these plants varied during the two seasons. This implies there are variations in plant metabolism with seasons, which also explains the existence of **AA2, ACW1** and **ACW2** in different plant batches. This is in agreement with the earlier observations, that plants down regulate metabolism in response to stress (Hoekstra, *et al.*, 2001, Howe and Schilmiller, 2002). The variations in yields has also been observed with artemisinin, an antimalarial isolate from Artimisia *annua* L, whose yields vary with seasons and even after one night of stressful weather (Wallaart *et al.*, 1999). Incidentally the variations in artemisinin yields have been observed even under highly controlled growth conditions of *A. annua* L. (Liu *et al.*, 2003). These observations raise the need for consideration of season when collecting traditional medicinal plants. It is probable that more

predictable yields and antiplasmodial activity could be obtained from plants grown under controlled conditions than those growing in the wild.

Fractionation of extracts from the plants, *I. emarginella* and *S. discifolius* did not result into any increase in antiplasmodial activity. This suggests that the observed antiplasmodial activity is due to a combination of plant constituents. The concept of synergy in treatment of malaria is exemplified by the widely used sulphadoxine/pyrimethamine combination, together with combinations like sulfamonomethoxine and 5-fluoroorotate, which are still experimental (Kim, *et al.*, 1998). The existence of synergy among traditional medicines is however easy to imagine going by the wide spread use of plant combinations in malaria treatment (Gessler *et al.*, 1995). There is yet no experimental evidence of synergy among antimalarial traditional medicinal plants and this study provides indirect evidence. The fact that the antiplasmodial activity of some plants cannot be improved by fractionation supports the possibility of developing herbal remedies for treatment of malaria instead of the widely targeted isolation of lead compounds.

The plants *A. africana* and *S. stuhlmannii* belong to family *Asteraceae*. This is the family to which *artemisia annua L* belongs, a plant from which the endoperoxide sesquiterpene lactone, artemisinin was isolated. Fractions from both these plants had high antiplasmodial activity. Fractions from *S. stuhlmannii* however, caused haemolysis of uninfected erythrocytes at a dose of 100µg/ml. Bessis, 1973, reported a number of chemical compounds that can cause haemolysis, these include: saponins, bile salts, fatty acids and lecithins. This could be attributed to the presence one or more of these compounds in the fraction. Since the mechanism of haemolysis *in vitro* involves damage of the erythrocyte cell membrane, it is possible that this fraction could as well be toxic to other mammalian cells.

More refined separation of the *A. africana* fraction from the second plant batch lead to the isolation of **AA2**, characterised to be a sesquiterpene. The third plant batch of *A. africana* also yielded equally active compounds, **ACW1** and **ACW2** that could be sesquiterpenes. These compounds are effective against both the chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* although they act within a narrower dose range compared to chloroquine. Structural modifications can improve the antimalarial activity of these compounds. Since the isolation of artemisinin, sesquiterpenes are continuing to be recognised

as a compounds with antimalarial potential. Lopes *et al.*, 1999, has also attributed the *in vitro* antiplasmodial activity of the volatile oil from *Virola Surinamensis* to a sesquiterpene, nerolidol, adding to the list of antimalarial sesquiterpenes.

The plants, *C. halicacabum* and *M. foetida* did not show any *in vitro* antimalarial activity. There are a number of possibilities that explain this result. Extracts from these plants may be inactive, in which case the plant does not have any antimalarial activity. The extracts may be acting as pro-drugs in which case they are effective only in an *in vivo* system or the extracts may act by enhancing the immune system. The wide spread use of these plants calls for further investigation of their antimalarial properties using an *in vivo* system, which is reported in one of the next chapters of this report.

While the isolation of active principles was successful, the final elucidation of the structures was not particularly complete due to the low yields of the isolates. The variations in plant principles overtime contributed to this as well, as the collection, extraction and fractionation of plant material from the third batch isolated different compounds from those isolated earlier using the same methods. The extractions in this study were done by water extraction and sequential organic extraction. These methods are not exactly the same as those used by traditional healers. This study therefore does not fully replicate traditional healers practices but uses a scientific approach to interpret the practices of traditional healers.

The yields reported in this study are due to a single extraction. The results would have given a better picture if repeated extractions were carried out on the same batch. Some aspects of the plant microhabitats may not have been put into consideration during plant collection such as ridges and uplands, these might have contributed to the variations in yields and antiplasmodial activity.

An *in vitro* antiplasmodial assay was used to test for antimalarial efficacy. While it is known that all known antimalarials have *in vitro* activity, many compounds with *in vitro* activity may not be effective in malaria treatment. This could be a result of unfavourable pharmacokinetic properties such as: poor absorption of the extract, restricted distribution, rapid metabolism and rapid elimination of the principles. The *in vitro* antimalarial assay may therefore not be a perfect predictor of antimalarial potential.

In this study we assessed the cytotoxicity of extracts qualitatively, by simple light microscopic observation of uninfected erythrocytes that had been incubated with the extracts. This approach has been used to assess the toxicity of other compounds (Fischer, *et al.*, 2003; Winski *et al.*, 1997) but not in the discovery of malaria remedies. The changes in erythrocyte morphology under giemsa-stained light microscopy and electron microscopy following chemical injury are described by Bessis, 1973. This method has the advantage that the uninfected erythrocytes can be incubated with the extract on the same 96-well plate as the antiplasmodial assay. In the search for simple and accurate methods of assessing cytotoxicity of antimalarial plant extracts, this method can be developed further and validated for quantitative assessment.

3.7 Conclusions

Extracts from shoots of the plants *S. discifolius*, *S. stuhlmannii*, *I. emarginella*, and *A. africana* have a significant *in vitro* antiplasmodial activity. The extracts from the shoots of the plants *C. halicacabum* and *M. foetida* have no *in vitro* antimalarial activity. Generally extracts from all the plants screened did not show cross-resistance with chloroquine. The ethyl acetate extract from the plant *A. africana that* belongs to the family *Asteraceae* had the best antiplasmodial activity, which is due to the presence of antimalarial sesquiterpenes. This plant can serve as a source of lead compounds for the development of new antimalarial plants. The study also revealed that yields of extracts and their antiplasmodial activity varied with seasons in some plant species, a situation that requires the optimisation of the conditions of harvest and extraction to have good yields. This study indicates that Ugandan traditional medicines have a high potential for the production of lead compounds for the development of antimalarials and herbal remedies.

CHAPTER 4

Interactions between traditional medicines and antimalarial drugs: combination of Aspilia africana extract with chloroquine and artemisinin

4.1 Introduction

Some of the patients who take traditional medicines for the treatment of malaria end up in hospitals where antimalarial drugs are prescribed. These patients may end up taking the two medications concurrently. Interactions between traditional medicines and some of the commonly used antimalarial drugs have not been reported before. The chemical principles responsible for the antimalarial effect in traditional medicines are in most cases unknown, making it impossible to predict pharmacokinetic and pharmacodynamic interactions with other medicines.

This study used an isolate **ACW1** from *A. africana* to investigate possible pharmacodynamic interactions between chloroquine and artemisinin. This isolate had been found to have antiplasmodial activity of 50% inhibitory concentration (IC_{50}) value of 1510 ng/ml and 560 ng/ml against the chloroquine-sensitive D10 and chloroquine- and sulphonamide-resistant K1 strains of *P. falciparum* respectively. Two drugs, artemisinin and chloroquine were used against the two strains. In this chapter we report the antiplasmodial activity of chloroquine and artemisinin in combination with the extract **ACW1** and the analysis to determine the nature of the interaction between the two drugs and the extract. The second part of this chapter reports the characteristics of cellular uptake of triated dihydroartemisinin (^{3}H-DHA) by *P. falciparum* infected erythrocytes and how it is affected by the isolate, **ACW1.** In both studies efforts were made to use clinically relevant doses. Drug protein and cellular binding, drug metabolism, and elimination are key components *in vivo* that are not reproduced fully in *in vitro* systems. *In vitro* models generally use low haematocrit and controlled parasitaemia that require the use of lower doses to obtain clinically relevant results.

A combination study of antimalarial drugs by Ringwald *et al.*, (1999) used starting doses ranging from 5 to 20 times the IC_{50}. This was used in selection of the working dose range in this study. The combination ratios used in this study were selected arbitrarily as there is no information on the possible concentration ratios achieved in blood when plant extracts are used together with antimalarial drugs in the treatment of malaria. Details of materials and methods used have been described in chapter two.

4.2 Antiplasmodial activity of extract-drug combinations

The dose and combination ratios of extract **ACW1,** and the two drugs: artemisinin and chloroquine are shown in Table 4.2.1. The highest dose of **ACW1** used was 6 times its IC_{50} against the D10 strain and 17 times the IC_{50} against the K1 strain. For both chloroquine and artemisinin, the highest dose ranged between 2 and 20 times their respective IC_{50} against the *Plasmodium* strains used in the study.

Table 4.2.1 Concentrations and combination ratios of the extract, and the two antimalarial drugs (chloroquine and artemisinin) used in the study.

	Concentration of ACW1 (ng/ml)	Concentration of chloroquine/ artemisinin (ng/ml)	Combination ratio*
1	9400	250	37.6
2	6250	500	12.5
3	5000	600	8.3
4	3100	750	4.1

*The concentration ratio ion of **ACW1:** Chloroquine or Artemisinin in a study combination.

Table 4.2.2 Antiplasmodial activity of ACW1 in combination with artemisinin against the D10 strain of *P. falciparum*.

Combination ratio (ACW1:Artemisinin)	Concentration of ACW1 at IC_{50} of combination (ng/ml)	Concentration of artemisinin at IC_{50} of combination (ng/ml)
37.6	1404.5 ±18.6	37.4 ±0.4
12.5	635.0 ±9.8	50.8 ±0.8
8.3	600.0 ±78.4	72.0 ±9.4
4.1	280.0 ±19.60	67.7 ±4.7

The IC_{50} values are based on two experiments carried out in duplicate and standard error of the mean calculated at 95% confidence interval.

The IC_{50} of **ACW1** and artemisinin have been worked out to be 1510 and 43 ng/ml respectively against the D10 strain of *P. falciparum*. The concentrations of **ACW1** at the IC_{50}

of all study combinations are lower than its IC_{50} as shown in table 4.2.2. The concentrations of artemisinin at the IC_{50} are generally higher except at 37.6.

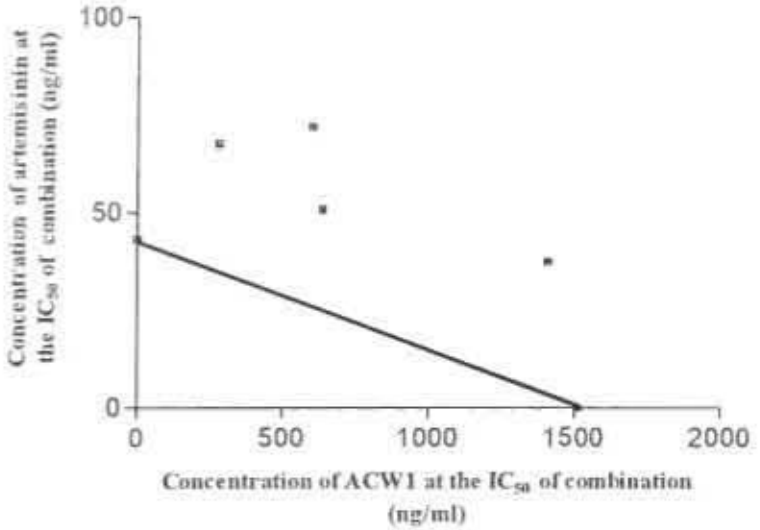

Fig 4.2.1. Isobologram of the interaction between ACW1 and artemisinin against the D10 strain of *P. falciparum* at the IC_{50}. The line joining the IC_{50} of the pure extract (1510 ng/ml) and pure artemisinin (43 ng/ml), the isobole, represents the additive effect. Datum points above the line denote antagonism. The IC_{50} values are based on two experiments carried out in duplicate.

Geometrical analysis shows datum points for the four combination ratios above the isobole as shown in figure 4.2.1. This suggests that the extract **ACW1**, from *A. africana* antagonises the antimalarial activity of artemisinin against the D10 strain of *P. falciparum*.

Table 4.2.3 Antiplasmodial activity of ACW1 in combination with artemisinin against the K1 strain of *P. falciparum*.

Combination ratio (ACW1/Artemisinin)	Concentration of ACW1 at IC_{50} of combination (ng/ml)	Concentration of artemisinin at IC_{50} of combination (ng/ml)
37.6	444.8 ± 0.6	12.3 ±1.0
12.5	349.5 ±10.0	28.0 ±0.7
8.3	355.0 ± 49.0	42.6 ±5.8
4.1	240.0 ± 58.0	58.1 ±9.5

The IC_{50} values are based on two experiments carried out in duplicate and standard error of the mean calculated at 95% confidence interval.

The IC_{50} of **ACW1** and artemisinin have been worked out to be 560 and 17 ng/ml respectively against the K1 strain of *P. falciparum*. The concentration of **ACW1** at the IC_{50} decreases as

the proportion of artemisinin increases in the combination, with values below the IC_{50}. The concentration of artemisinin generally increases.

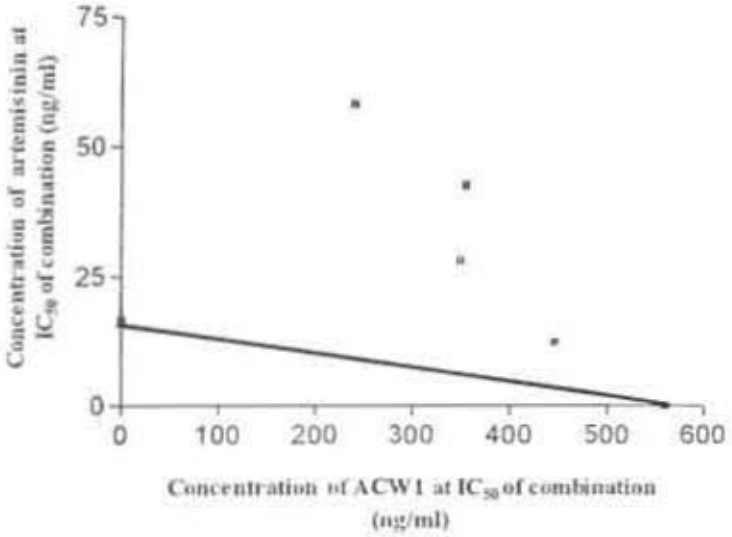

Fig 4.2.2 Isobologram of the interaction between ACW1 and artemisinin against the K1 strain of *P. falciparum* at the IC_{50}. The line joining the IC_{50} of the pure extract (560 ng/ml) and pure artemisinin (17 ng/ml), the isobole, represents the additive effect. Datum points above the line denote antagonism. The IC_{50} values are based on two experiments carried out in duplicate.

Similar to the interaction observed with the D10 strain of *P. falciparum*, figure 4.2.2 shows datum points that lie above the isobole, suggesting an antagonistic relationship between the extract and artemisinin against the K1 strain. Data points for combination ratios of 12.5, 8.3, 4.1 show a high level of antagonism than that of the 37.6 combination ratio suggesting a reduction in antagonism with increasing proportion of **ACW1** in the combination.

Table 4.2.4. Antiplasmodial activity of ACW1 in combination with chloroquine against the D10 strain of *P. falciparum*.

Combination ratio (ACW1/chloroquine)	Concentration of ACW1 at IC_{50} of combination (ng/ml)	Concentration of chloroquine at IC_{50} of combination (ng/ml)
37.6	1861.0 ±11.8	49.5 ±1.0
12.5	776.5 ±144	62.0 ±11.7
8.3	390.0 ± 19.6	46.8 ±2.4
4.1	369.5 ±14.7	89.5 ±2.9

The IC_{50} values are based on two experiments carried out in duplicate and standard error of the mean calculated at 95% confidence interval.

The concentration of **ACW1** at the IC_{50} of combination against the chloroquine-sensitive D10 strain decreases with increasing proportion of chloroquine in the combination as shown in table 4.2.4. Geometrical analysis of the relationship between **ACW1** and chloroquine against the D10 strain of *P. falciparum* shows a predominantly additive relationship for most of the combination ratios investigated as shown in figure 4.2.3.

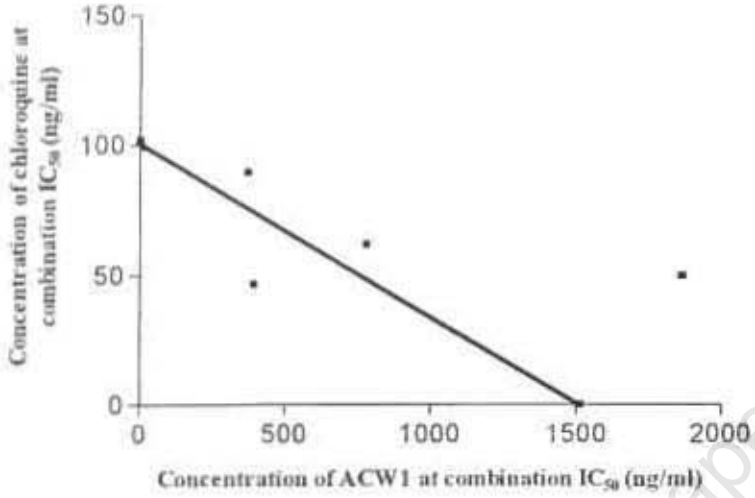

Fig 4.2.3. Isobologram of the interaction between ACW1 and chloroquine against the D10 strain of *P. falciparum* at the IC_{50}. The line joining the IC_{50} of the pure extract (1510 ng/ml) and pure chloroquine (102 ng/ml), the isobole, represents the additive effect. Datum points close to the isobole denote an additive relationship. The IC_{50} values are based on two experiments carried out in duplicate.

Table 4.2.5 Antiplasmodial activity of ACW1 in combination with chloroquine against the K1 strain of *P. falciparum*

Combination ratio (ACW1/chloroquine)	Concentration of ACW1 at IC_{50} of combination (ng/ml)	Concentration of chloroquine at IC_{50} of combination (ng/ml)
37.6	2200.0 ± 254.8	58.5 ±6.9
12.5	1758.5 ± 12.7	142.1 ±1.8
8.3	1265.0 ±127.4	152.0 ±15.7
4.1	661.0 ± 84.0	160.0 ±19.6

The IC_{50} values are based on two experiments carried out in duplicate and standard error of the mean calculated at 95% confidence interval.

The concentration of **ACW1** at the IC_{50} of the combination, against the chloroquine-resistant K1 strain, increases with the proportion of chloroquine as shown in table 4.2.5.

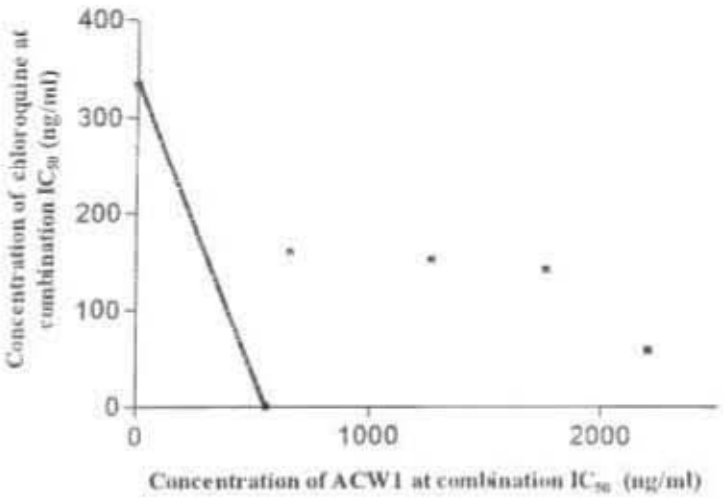

Fig 4.2.4 Isobologram of the interaction between ACW1 and chloroquine against the K1 strain of *P. falciparum* at the IC_{50}. The line joining the IC_{50} of the pure extract (560 ng/ml) and pure chloroquine (334 ng/ml), the isobole, represents the additive effect. Datum points above the isobole denote antagonism The IC_{50} values are based on two experiments carried out in duplicate.

Unlike the relationship observed between **ACW1** and chloroquine against the chloroquuine-sensitive D10 strain of *P. falciparum*, datum points in figure 4.2.4 lie above the isobole suggesting a strong antagonistic relationship between **ACW1** and chloroquine against the chloroquine-resistant K1 strain. The level of antagonism increases with the proportion of **ACW1** in the combination. In order to investigate the mechanism behind this interaction, this project investigated the effects of this extract on the uptake of dihydroartemisinin, the active metabolite of the artemisinin derivatives.

4.3 Effect of plant isolate on accumulation of ^{3}H-DHA by erythrocytes

The working dose range was selected on the basis of two clinical studies with the aim of making the study results clinically relevant. One of the studies was done among children, and reported a parasite clearance of 97-100% after 24 hours of treatment with artesunate. In this study the mean maximum blood concentration (C_{max}) of DHA was 0.18 µg/ml (Halpaap *et al.*, 1998), this is equivalent to an exposure of 2.61×10^{-4} fmols per erythrocyte, assuming a haematocrit of 50% and that 1µl of packed normal erythrocytes contains 5.2×10^6 cells (Diem and Lentner, 1970). The other study done by Na Bangchang *et al.*, (1994), found steady state

concentrations of DHA ranging from 36 to 60 ng/ml in the therapeutic use of artesunate this would it would mean that each erythrocyte was exposed to $5.3x\ 10^{-5}$ to 9×10^{-5} fmols under steady state conditions. Putting into consideration the effect of protein and cellular binding on in *vivo* drug availability to erythrocytes the actual drug exposure is much less than computed above. In this study we used a dose range of $0.68\text{-}6.83 \times 10^{-6}$ fmols/erythrocyte, below the calculated exposure in the above studies.

4.3.1 Specific activity of ^{3}H-DHA

A linear relationship between the concentration and disintegrations per minute (DPM) was assumed in all the experiments. The original drug stock was diluted to a 71.4nm solution and from this 10µl was used to determine the specific activity. The disintegrations per minute from three samples with 714 fmols of ^{3}H-DHA are shown in table 4.3.1.

Table 4.3.1 The activity of radiolabeled dihydroartemisinin

Experiment	Drug amount (fmols)	Disintegrations per minute	Activity (DPM/fmol)
1	714	2297345	3217.5
2	714	2294642	3213.7
3	714	2274673	3185.8

Values of disintegrations per minute are based on one experiment.

The mean activity was 3205.7 ±19.6 DPM per fmole. This was used to determine the amount of ^{3}H-DHA taken up by erythrocytes after an incubation period. This study focused on the amount of ^{3}H-DHA exposed to each erythrocyte and what percentage of it is taken up by the infected and uninfected erythrocyte. Important assumptions were made to achieve this, that the drug bound to albumin and the walls of the vials is available to the erythrocytes. The study used a haematocrit of 1% implying that each ml of erythrocyte suspension had 10 µl of erythrocytes. The dose of ^{3}H-DHA used in the study and the corresponding amount of drug exposed to each erythrocyte is shown in table 4.3.2.

Table 4.3.2. **Dose of ^{3}H-DHA used in the study expressed as amount of the drug exposed to each erythrocyte.**

Experiment	Dose of DHA (fmols/ml)	Amount of drug /erythrocyte[1] (fmols x10^{-6})
1	35.5	0.68
2	71.0	1.37
3	106.5	2.04
4	177.5	3.41
5	284.0	5.46
6	355.0	6.83

[1] One millitre of erythrocyte suspension was used in the study with a haematocrit of 1%. It was assumed that 1µl of packed erythrocytes contains 5.2 x 10^{6} erythrocytes (Diem and Lentner, 1970).

4.3.2. Factors that influence the uptake of ^{3}H-DHA

The effect of dose and temperature on the amount of drug taken by erythrocytes was investigated. Table 4.3.3 shows that uninfected erythrocytes take in 0.07-0.22% of the DHA exposed to the erythrocytes over the study dose range. Increase in DHA dose has little effect on drug uptake.

Table 4.3.3. The uptake of ^{3}H-DHA by uninfected erythrocytes

Dose fmols/ RBC x10^{-6}	Mean DPMI for RBC pellet	Drug taken per RBC fmols x 10^{-10}	Uptake of DHA (%)
0.68	82.30 ± 161	4.9	0.07 ± 0.14
1.37	353 ± 306.7	21.2	0.15 ± 1.9
2.04	594 ± 446	35.6	0.17 ± 0.13
3.41	1291 ± 633	77.4	0.22 ± 0.23
5.46	1773 ± 936	106.3	0.19 ± 0.27
6.83	2506 ± 947	150.3	0.22 ± 0.23

Values are based on two experiments carried out in triplicate at room temperature. Standard error of the mean has been worked out at 95% confidence interval.

The amount of drug associated with uninfected erythrocytes at the different doses in table 4.3.3, was used to calculate the proportion of the drug associated with the infected erythrocytes, putting into consideration the parasitaemia of 5% used in the study. The DPM due to 9.5µl of the uninfected erythrocytes was determined in each set of experiment and deducted from the recorded count.

Table 4.3.4. The uptake of DHA by erythrocytes infected by the D10 strain of *P.falciparum*.

Dose fmols/RBC ($x 10^{-6}$)	Mean DPM of pellet.	DPM due to 0.5 µl of pRBCs [1]	Drug per RBC. (fmols $x 10^{-7}$)	Uptake of DHA (%)
0.68	3374 ±162.8	3295.7	3.95	58.1 ±15.2
1.37	3748 ±1146	3412.8	4.09	29.9 ±9.3
2.04	5255 ±1348	4690.7	5.35	26.3 ±3.6
3.41	6155 ±2966	4928.7	5.91	17.3 ±2.5
5.46	8732 ±2005	7048.0	8.45	15.4 ±8.1
6.83	8567 ±1919	6187.0	7.42	10.8 ±5.1

[1]Values obtained by subtracting 95% of the DPM count of uninfected erythrocytes exposed to the same drug dose from the mean DPM obtained in this experiment. Values are based on two experiments set up in triplicate and standard error of the mean calculated at 95% confidence interval.

Erythrocytes infected with the chloroquine-sensitive D10 strain of *P. falciparum* took up 10.8 to 58.1% of the drug exposed to each erythrocyte. Increasing the external drug concentration reduces the percentage of DHA taken up by the erythrocytes. The trend is shown in table 4.3.4. A similar trend was observed with the chloroquine-and sulfonamide-resistant K1 strain of *P. falciparum* in table 4.3.5. The K1 strain however took in a higher proportion of the exposed drug at each concentration than the D10 strain.

Table 4.3.5. The uptake of DHA by erythrocytes infected by the K1 strain of *P.falciparum*.

Dose fmols/RBC (x 10^{-6})	Mean DPM of RBC pellet.	DPM due to 0.5 µl of pRBCs [1]	Drug taken per pRBC (fmols x 10^{-7})	Uptake of DHA (%)
0.68	4553 ±447	4474.6	5.37	78.9
1.37	5316 ±679	4980.5	5.97	43.6
2.04	6829 ± 826	6828.9	8.19	36.8
3.41	8654 ±1130	7427.7	8.91	26.1
5.46	13530 ±694	12836.7	15.39	26.0
6.83	11693 ±2108	9312.5	11.17	16.4

1. Value obtained by subtracting 95% of the DPM count of uninfected erythrocytes exposed to the same drug dose from the mean DPM obtained in this experiment. Values are based on two experiments set up in triplicate and standard error of the mean calculated at 95% confidence interval.

Table 4.3.6 shows the effect of external drug concentration on the amount of ^{3}H-DHA taken up by erythrocytes infected by the D10 and K1 strains of *P. falciparum* together with uninfected erythrocytes.

Table 4.3.6. The uptake of dihydroartemisinin by parasitised and unparasitised erythrocytes.

Dose (fmol /RBC x10^{-6})	Uptake by uninfected RBC (%)	Uptake by D10 pRBC (%)	Uptake by K1 pRBC (%)
0.68	0.07 ± 0.14	58.1 ±15.2	78.9 ±15.3
1.37	0.15 ± 1.9	29.9 ±9.3	43.6 ±8.2
2.04	0.17 ± 0.13	26.3 ±3.6	36.8 ±6.3
3.41	0.22 ± 0.23	17.3 ±2.5	26.1 ±4.0
5.46	0.19 ± 0.27	15.4 ±8.1	26.0 ±1.4
6.83	0.22 ± 0.23	10.8 ±5.1	16.4 ±2.0

Values are based on two experiments carried out in triplicate. The standard error of the mean was calculated at 95% confidence interval.

The accumulation of ^{3}H-DHA by erythrocytes infected with D10 and K1 strains of *P. falciparum* is dose dependent.

Table 4.3.7. The uptake of ^{3}H-DHA by both parasitised and unparasitised erythrocytes exposed to a dose of 2.04 x 10^{-6} fmols/erthrocyte at 4°C and 37°C.

Temperature (°C)	Percentage uptake by Uninfected erythrocytes	Percentage uptake by infected erythrocytes
4	0.07 ± 0.06	3.70 ± 0.60
37	0.17 ± 0.04	26.30 ± 7.80

Values are based on 4 experiments done in triplicate and standard error of the mean worked out at 95% confidence interval.

The accumulation of ^{3}H-DHA in both the infected and uninfected erythrocytes is temperature dependent. An increase of temperature from 4°C to 37° C increases the uptake of ^{3}H-DHA by uninfected erythrocytes two fold while the uptake by infected erythrocytes is increased at least seven fold.

4.3.3. Effect of ACW1 on the uptake of ^{3}H-DHA

A dose of 106.5 fmols/ml was used in this study to be able to detect any rise or reduction in amount of ^{3}H-DHA taken up by the erythrocytes. The incubation time of 2 hrs was used to allow as much time as possible for the equilibrium to be established. The experiment was carried out at 37 °C. The mean DPM for the uninfected erythrocytes exposed to 106.5 femtomoles/ml for 2 hrs was 1830. This was used in the calculation of the uptake of ^{3}H-DHA by infected erythrocytes. The percentage uptake was worked out as done before. The plant isolate **ACW1** significantly increases the uptake of ^{3}H-DHA by erythrocytes infected with the D10 strain of *P. falciparum* at the concentrations of 150, 350 and 600 ng/ml (Mann-Whitney U-test, $p < 0.05$). These results are shown in figure 4.3.2. The same doses of **ACW1** significantly reduced the uptake of ^{3}H-DHA in erythrocytes infected with the chloroquine- and sulphonamide-resistant K1 strain of *P. falciparum* as shown in figure 4.3.3.

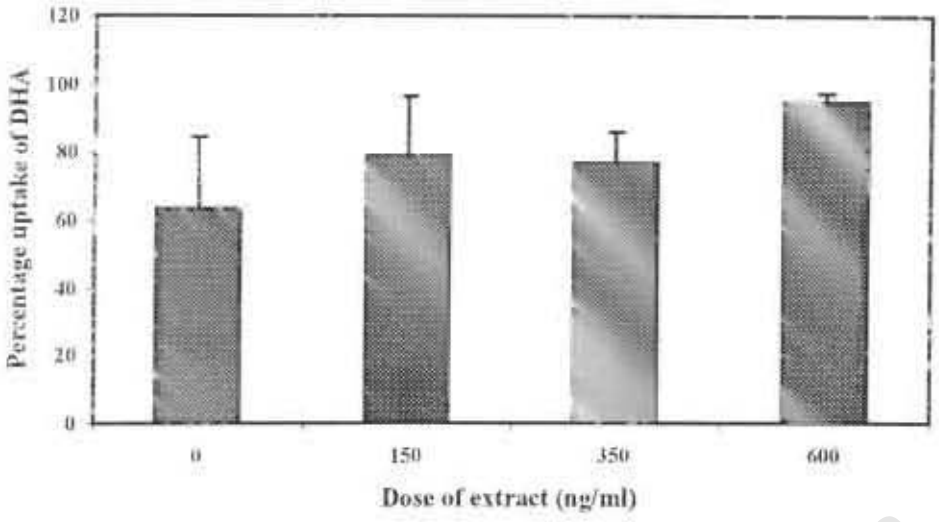

Fig 4.3.2. Effect of *A. africana* extract on the uptake of ^{3}H-DHA by erythrocytes infected with the D10 strain of P. *falciparum* on incubation with 106.5 femtomoles of ^{3}H-DHA for 2 hours. Values are based on 2 experiments carried out in quadruplicate. Standard error of the mean was worked out at 95% confidence interval.

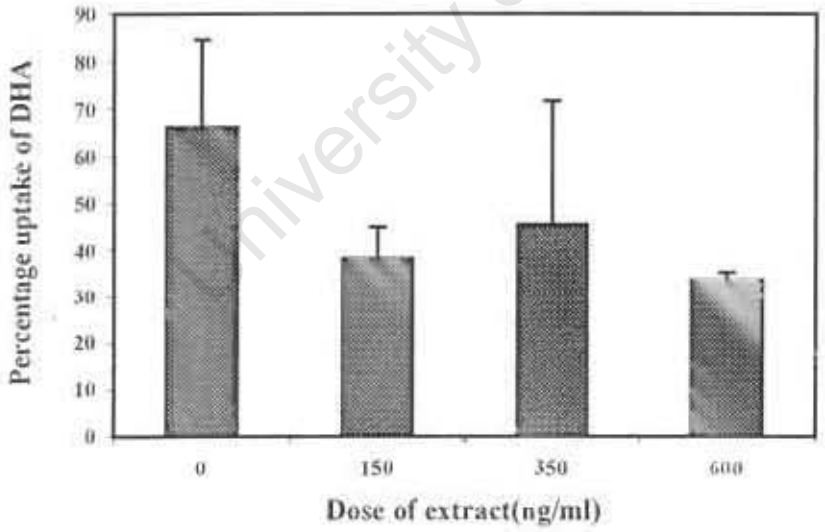

Fig 4.3.3. Effect of A. *africana* extract on the uptake of ^{3}H-DHA by erythrocytes infected with the K1 strain of P. *falciparum* on incubation with 106.5 femtomoles of ^{3}H-DHA for 2 hours. Values are based on two experiments carried out in quadruplicate and standard error of the mean calculated at 95% confidence interval.

4.4 Discussion

Ugandan communities use traditional medicines as self-medications for malaria, some of these people end up in health units where antimalarial drugs are prescribed. This results into concurrent use of traditional medicines and antimalarial drugs. This study investigated potential interactions between traditional medicines and two antimalarial drugs: chloroquine and artemisinin. An isolate from *A. africana*, **ACW1**, with antiplasmodial activity was preferred to the crude extract to reduce the possibility of extra-cellular interactions. The first part of the study investigated the antiplasmodial activity of extract-drug combinations against the chloroquine-sensitive D10, and the chloroquine-and sulphonamide-resistant K1 strains of *P. falciparum*. The second part characterised the accumulation of dihydroartemisinin (DHA) by *P. falciparum* infected erythrocytes, and how it is affected by **ACW1.** The possibility of competition between the isolate and DHA at cellular level was born out of the fact that *A. africana* belongs to the same family as Artimisia *annua L, Asteraceae*. *A. annua* L. is the source of the antimalarial sesqueterpene lactone, artemisinin whose derivatives are now widely used in the treatment of chloroquine-resistant malaria (Klayman, 1985, Hien *et al.*, 1993).

This study showed that **ACW1** antagonised artemisinin at the IC_{50} in combination ratios (**ACW1**: artemisinin) ranging from 4 to 37.6 and dose range of 3100 to 9400ng/ml for ACW1, and 250 to 750ng/ml for artemisinin. This relationship was observed with both the chloroquine-sensitive D10, and the chloroquine-and sulphonamide-resistant K1 strain of *P. falciparum*, which is more susceptible to both drugs. While blood concentrations of the active principles attained during therapeutic use of traditional medicines are not known, combination studies that used starting concentrations a few multiples the IC_{50} have produced clinically relevant findings.

A combination of **ACW1** and chloroquine produced similar results against the chloroquine-resistant K1 strain. The combination however exhibited an additive relationship against the chloroquine-sensitive D10 strain of *P. falciparum*. These findings suggest that extracts from *A. africana* could antagonise the antimalarial effect of chroloquine or artemisinin when used concurrently.

The interaction observed during isolate-drug combination studies could occur extra-cellular, at the erythrocyte membrane, in drug transport pathways or at the intracellular drug target. An extra-cellular interaction would generally involve the formation of an extract-drug complex. This phenomenon has not been widely discussed in reference to *in vitro* drug combination studies, yet it could explain some of the observed interactions. The effect of the plant extract on the uptake of dihydroartemisinin by *P. falciparum* infected erythrocytes provided more evidence on the possible site of interaction.

In this study, we observed that erythrocytes infected with the D10 and K1 strains of *P. falciparum* accumulated dihydroaretmisinin more than uninfected erythrocytes. Similar findings were observed by Gu *et al.*, 1984, and Meshnick *et al.*, 1991 investigated the mechanisms behind these observations. The uptake of ^{3}H-DHA was found to be dose dependant, saturable and temperature dependant, findings in agreement with the work of Gu *et al.*, 1984 and Kamchonwongpaisan *et al.*, 1994.

Previous studies, suggested that artemisinin and its derivatives are transported through the tubovesicular network (extending from the parasitorous vacuolar membrane to the periphery of the red cell) with help of an energy dependent carrier protein (Akompong *et al.*, 1999; Nehal *et al.*, 2002). The probable target areas for these drugs are the parasite membranes, the parasite food vacuole and hemozoin (Ellis *et al.*, 1985). These drugs are also distributed extensively into the cytosolic compartment and erythrocytic cell membranes (Vattanaviboon *et al.*, 1998). The ultimate cytotoxic effect of artemisinin and its derivatives has been attributed to the oxidation of cellular membranes, and denaturation of parasite proteins by oxygen-linked free radicals that are generated at the cleavage of the peroxide ring (Hong *et al.*, 1994).

The plant isolate **ACW1**, reduced the amount of ^{3}H-DHA taken up by K1 infected erythrocytes. On the other hand the isolate enhanced the uptake of ^{3}H-DHA by D10 infected erythrocytes. In both cases, antagonism had been observed in the combination studies. We had also observed earlier that the K1 strain accumulated ^{3}H-DHA more efficiently than the D10 strain. These findings suggest that the mechanism of accumulation of DHA into erythrocytes infected with the chloroquine-sensitive D10 strain is different from that of erythrocytes infected with the chloroquine- and sulphonamide-resistant K1 strain. The K1 strain seems to have an uptake mechanism that is more efficient and is readily blocked by

ACW1, a mechanism that may be lacking in the chloroquine-sensitive D10 strain of *P. falciparum*. Other researchers in our laboratory have also observed differences in accumulation of pyronaridine between the D10 and K1 strain.

The *in vitro* antagonism between **ACW1** and artemisinin against the D10 strain of *P. falciparum* is associated with increased accumulation of ^{3}H-DHA by the erythrocytes. This suggests a possible intracellular neutralisation of the cytotoxic free radicals, generated by the cleavage of the peroxide bond as a possible cause of the antagonism. It has already been postulated that compounds with antioxidant properties could antagonise the antimalarial activity of artemisinin and its derivatives by neutralising the free radicals that are responsible for parasite death (Krungkrai and Yuthavong, 1987; Meshnick *et al.*, 1989). These findings together, suggest that the *A. africana* isolate acts intracellarly.

The plant extract had an additive and antagonistic relationship with chloroquine against the chloroquine-sensitive D10 strain and the chloroquine-resistant K1 strain respectively. The mechanism of resistance to chloroquine is not clearly established, chloroquine resistance is however associated with reduced accumulation of chloroquine into the parasite food vacuole. The effect of the plant extract on the uptake of chloroquine was not studied, since the isolate antagonises the effect of chloroquine in the resistant strain and not the sensitive strain, we postulate that it accentuates the chloroquine resistance mechanisms; reduces the accumulation of chloroquine in the parasite food vacuole.

It is generally difficult to replicate the *in vivo* dynamism in an in *vitro* system, to study drug interactions. *In vitro* systems lack protein and cellular binding, metabolism, and elimination. To try and attain clinical relevancy, this study used doses of ^{3}H-DHA that are close to those attained *in vivo* under therapeutic conditions. The blood levels of active principles attained during the traditional use of *A. africana* are not known making it difficult to work with clinically relevant dose. This study only brings out the phenomenon, which can be investigated in clinical studies. There is need for studies to establish the effect of this phenomenon on treatment outcome in malaria patients using extracts from *A. africana* together with antimalarial drugs.

4.5 Conclusion

The plant isolate **ACW1** from *A. africana*, a plant traditionally used in the treatment of malaria in east and central Africa antagonises the antimalarial activity of chloroquine and artemisinin at some combination ratios against the chloroquine-sensitive D10 and the chloroquine-and sulphonamide-resistant K1 strains of *P. falciparum*. For artemisinin, the possible mechanism of antagonism is the inhibition of drug accumulation by infected erythrocytes or neutralisation of the intracellular cytotoxic free radicals. The isolate probably accentuates the resistance mechanisms in chloroquine-resistant strains. This study brings out the need to establish the extent to which this phenomenon contributes to treatment failure among patients taking traditional medicines together with chloroquine and artemisinin. This calls for increased investigation into interactions between drugs and traditional medicines and the need to take caution while prescribing drugs to patients who are taking traditional medicines even when they are used to treat the same condition.

CHAPTER 5

The effect of extracts from Cardiospermum halicacabum and Momordica foetida on disease progress in a murine model of cerebral malaria

5.1 Introduction

The two plants *Momordica foetida* Schumch. Et Thonn and *Cardiospermum halicacabum* are climbers that grow wild in forests of East, Central and Southern Africa. Leaf decoctions from these plants are used in the treatment of malaria related symptoms in addition to other ailments (Hakizamungu *et al.*, 1992, Rwangabo, 1993). Extracts from aerial shoots of these plants did not show significant antiplasmodial activity against the chloroquine-sensitive D10 and the chloroquine- and sulphonamide-resistant K1 strains of *P. falciparum* in the first part of this study, reported in chapter three.

In this chapter we report the effect of water extracts from the two plants on the development of murine cerebral malaria. This study was limited to water extracts on the basis of ethnopractices reported by traditional healers, and ethnobotanical surveys. The water extracts were obtained as reported earlier for the *in vitro* studies. The extracts were dissolved in phosphate buffered saline solution (PBS) in preparation for administration. A murine model of cerebral malaria was set up to determine the natural history of the disease. Intervention into the disease course was by oral administration of water extracts. The C57BL mice and the *P. berghei* (Anka) strain of rodent malaria were used in this study. The mice were infected by intraperitoneal administration of $1x10^7$ infected erythrocytes. The parasitaemia, weight changes, survival time and haematological parameters were studied and used to monitor disease progress. Details of materials and methods used are described in chapter two.

5.2 The natural history of *P. berghei* (Anka) malaria in the C57BL mice

Table 5.2.1 shows the development of parasitaemia, and the survival of C57BL mice infected with *P. berghei (Anka)*. Death was first observed on the sixth day post-infection and all the infected animals died by the eighth day post-infection. Death was associated with convulsions, rapid breathing and high parasitaemia.

Table 5.2.1 Survival and development of parasitaemia during *P. berghei* (Anka) infection of *C57BL* mice.

Days post-infection	Average parasitaemia (Percentage)	Mortality (Percentage)
0	0	0
2	0.33 ± 0.65	0
4	5.33 ± 1.53	0
6	25.0 ± 3.4	60 (6/10)
8	33.7 ± 5.1	100 (10/10)

Values are based on three slides prepared from one animal, the standard error of the mean was computed at 95% confidence interval. Percentage mortality was determined by observing 10 mice.

Parasitaemia was evident on the second day after infection; the highest parasitaemia attained was 34% on day eight.

Table 5.2.2 Changes in haemoglobin concentration during *P. berghei* (Anka) infection of *C57BL* mice.

Day post-infection	Haemoglobin concentration of uninfected mice gm/dl	Haemoglobin concentration of infected mice (gm/dl)
0	14.7	14.7
2	13.2	14.2
4	13.1	11.7
6	ND	10.4
7	ND	6.5
8	14.7	7.0

Values are based on one experiment. ND denotes sample was not analysed.

There was a progressive reduction in haemoglobin concentration during the course of infection. The trends are shown in table 5.2.2. The lowest haemoglobin concentration recorded was 6.5 gm/dl on day seven. The haemoglobin concentration did not show any significant changes in uninfected mice.

Table 5.2.3. Changes in total leukocyte count during *P. berghei* (Anka) infection of C57BL mice.

Day post-infection	Total leucocyte count in uninfected mice ($x10^9$/l)	Total leucocyte count in infected mice ($x10^9$/l)
0	4.9	4.9
2	3.0	2.3
4	5.1	5.8
6	ND	7.7
7	ND	17.7
8	4.1	16.3

Values are based on one experiment. ND indicates that the sample was not analysed.

The total leukocyte count increased through the time of infection. The highest total count recorded was 17.7 x 10^9 /l as shown in table 5.2.3. Leukocyte differential counts showed progressive reduction in the proportion of lymphocytes and a rise in the proportion of monocytes and nuetrophils during the course of infection. The total platelet count reduced during the course of murine malaria infection as shown in table 5.2.4.

Table 5.2.4. Changes in total platelet count during *P. berghei* (Anka) infection of C57BL mice.

Day post-infection	Platelet count in uninfected mice (x109/l)	Platelet count in infected mice ($x10^9$/l)
0	819	819
2	579	686
4	757	173
6	ND	160
7	ND	ND
8	589	287

Values are based on one experiment. ND indicates the sample was not analysed.

Figure 5.2.1 shows that the mean group weight of mice increased normally in the first four days of infection, which was followed by a steady weight reduction up to the demise of the study animals. The uninfected group of animals had steady increase in weight.

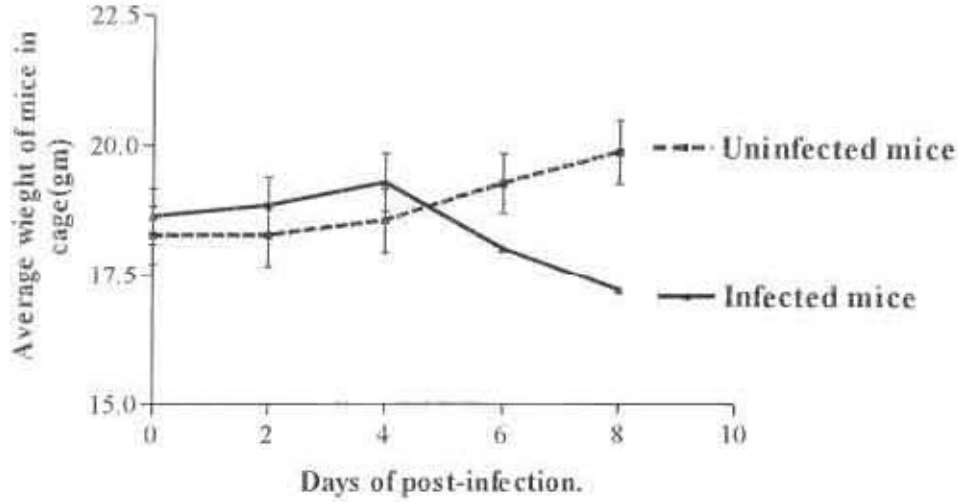

Fig 5.2.1 Weight changes in *P.berghei* (Anka) infected C57BL mice. Values are based on 10 animals at commencement of experiment. The standard error of the mean was calculated at 95% confidence interval.

The findings in this malaria model show a disease course akin to human cerebral malaria, whose symptoms have already been described in the first chapter. One difference, however, is the high differential increase in the number of monocytes during the course of infection. This model was used to study the effect of water extracts of *M. foetida* and *C. halicacabum* on disease progress.

5.3 Preliminary test on water extracts of *M. foetida* and C. *halicacabum*

Preliminary screening was carried out by administering 250 mg/kg of the water extracts, by gastric lavage, twice a day for four consecutive days to *P. berghei* (Anka) infected C57BL mice. The study animals were monitored for parasitaemia, weight change and mortality. A positive control experiment was set up in which the mice were given chloroquine (10 mg/kg) twice a day by gastric lavage. The study parameters were compared with those observed in untreated animals.

The mice that received the extract of *M. foetida* lived up to the 13th day after infection, beyond the maximum survival time observed with the untreated animals. Parasites were detectable in blood on day eight of infection. The animals however, showed weight loss much more than the untreated mice (figure 5.3.1). All the mice treated with the water extract of *C.*

halicacabum died in first four days of infection and treatment. The mice treated with chloroquine had no evidence of malaria for the 13 days of experimental observation, on the other hand they showed normal weight gain throughout the study period, as shown in figure 5.3.1.

Table 5.3.1 Survival and parasitaemia on the eighth day after treatment of *P. berghei* (Anka) infected C57BL mice with oral chloroquine (20mg/kg/day), water extracts of *M. foetida* (500 mg/kg /day) and *C. halicacabum* (500 mg/kg/day) in two divided doses for 4 consecutive days starting on the day of infection.

Drug/Plant extract	Percentage survival	Parasitaemia on day 8-post infection.	Percentage parasitaemia inhibition
Chloroquine	100	0	100
M. foetida	100	2	94.1
C. halicacabum	NA	NA	NA
Untreated mice	20	34	NA

Values are based on five study animals at the commencement of experiment. N/A (not applicable) shows groups where all the animals had died.

Analysis of some of the study parameters on day eight shows that the water extract of *M. foetida* was well tolerated and had a 94.1 percent inhibition at a dose of 500 mg/kg/day administered in two divided doses as shown in table 5.3.1. The positive control, chloroquine, in a dose of 20mg/kg/day in two divided doses, completely protected C57BL mice from developing parasitaemia up to day eight of infection. The mice that were treated with the water extract of *C. halicacabum* died by the fourth day of infection. One of the last animals to die in this group had a parasitaemia of 4.4%, close to the observed parasitaemia of 5.33% in untreated animals. Most death occurred with in 12 hours of administering the extract. For both extracts the mice generally lost weight as shown in figure 5.3.1. The mice treated with chloroquine gained weight steadily and did not develop parasitaemia.

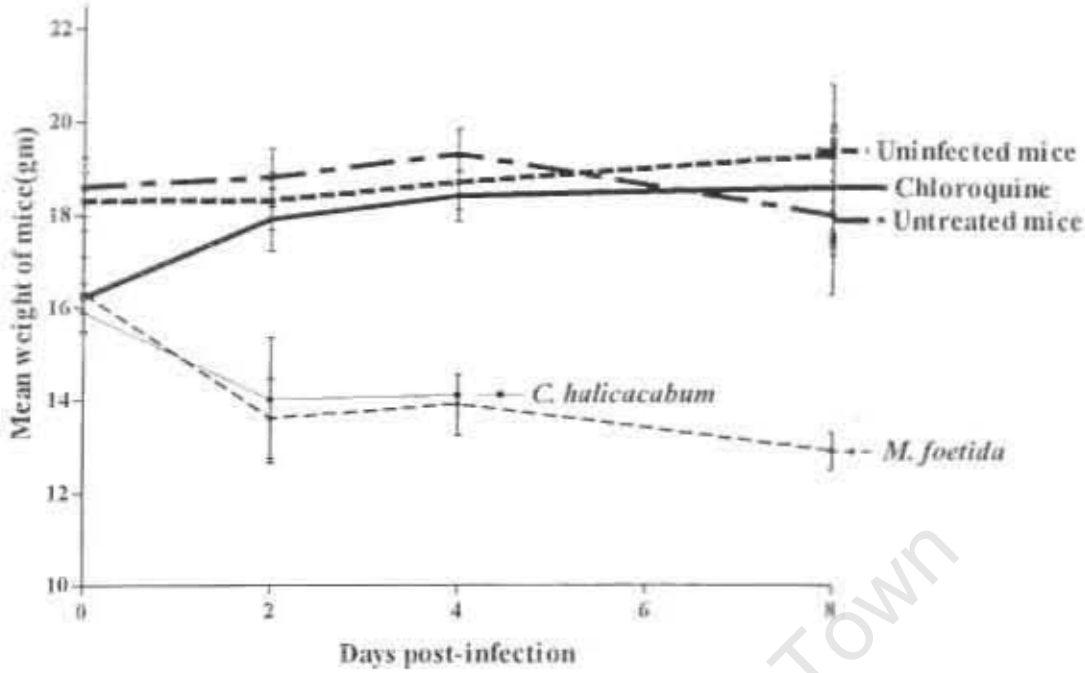

Fig 5.3.1 Weight changes in *P. berghei* (Anka) infected C57BL mice during treatment with oral chloroquine 20mg/kg/day, water extracts of *M. foetida* 500mg/kg/day and *C. halicacabum* 500mg/kg/day in two divided doses for 4 consecutive days starting on the day of infection. The results are compared with the weight changes in infected and uninfected mice in which older mice were used (n=10, at commencement of experiment).

5.4 Effect of extract dose on disease progress

The water extract of *M. foetida* was investigated further to determine how different doses are tolerated and the corresponding antimalarial activity. The preliminary experiment had shown that the highest dose that can be administered in a volume less than 0.2 mls was 500mg/kg due to solubility constraints. To be able to achieve a dose of 1000mg/kg/day, the extract was given in two divided doses 12 hours a part. The development of parasitaemia, mean survival time and weight changes were used to monitor disease progress.

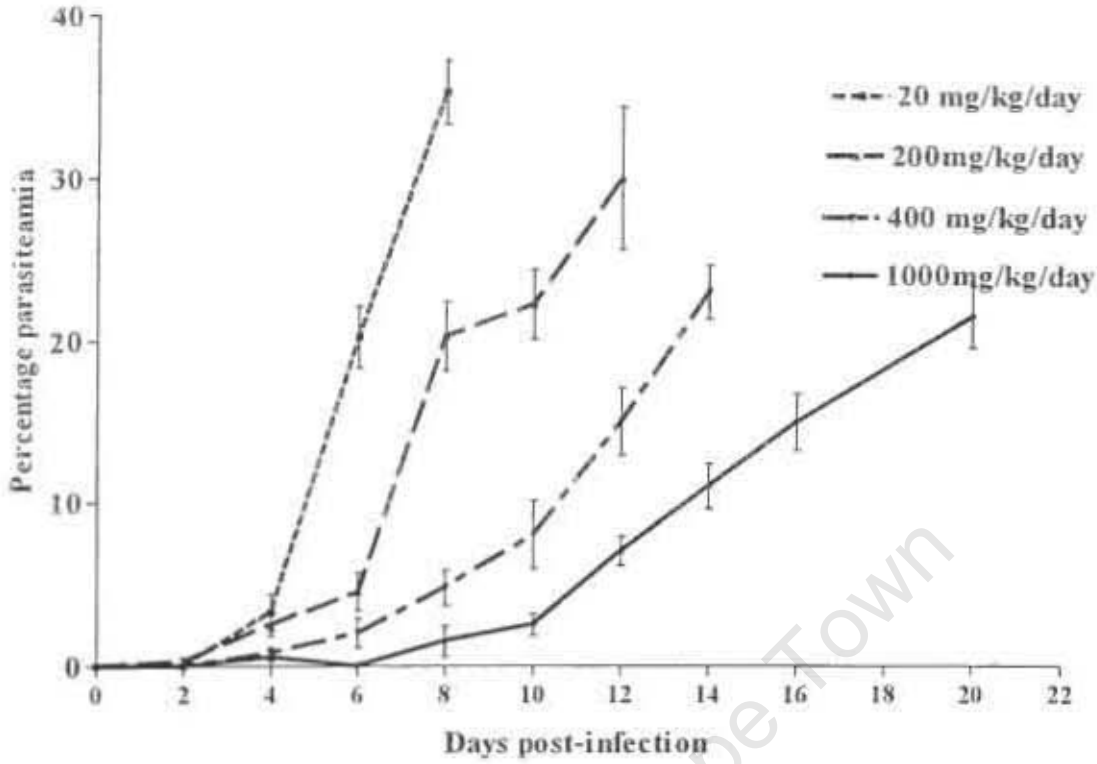

Fig 5.4.1. The development of *P.berghei* (Anka) parasitaemia in C57BL mice during treatment with four doses 20, 200, 400 and 1000mg/kg/day of the water extract of M. *foetida* given in two divided doses for 4 consecutive days starting with the day of infection. Values are based on four slides prepared from two study animals and computed at 95% confidence interval.

A dose of 1000 mg/kg/day of the water extract of *M. foetida* given in two divided doses delayed the development of parasitaemia by 6 days. The lower doses of 200mg/kg/day and 400mg/kg/day also delayed the development of *P.berghei* (Anka) parasitaemia in C57BL mice as shown in figure 5.4.1. All extract doses where well tolerated by the study animals. The mean survival time of *P.berghei* (Anka) infected C57BL mice increased with increasing dose of extract administered. The mice that received a dose of 1000 mg/kg/day had a mean survival time of 17.9(16.1-19.7) days with one of the mice in the group surviving up to 20 days as shown in table 5.4.1. The mice did not show signs of cerebral malaria at death and generally died with lower parasitaemia than the untreated mice.

Table 5.4.1. Survival *of P.berghei* (Anka) infected C57BL mice during treatment with four doses; 20, 200, 400 and 1000mg/kg/day of the water extract of *M. foetida*. The extract was administered orally in two divided doses for 4 consecutive days starting on the day of infection.

Experiment	Dose of extract (mg/kg/day)	Mean survival time[1] (days)
1	0	7.0 ± 0.40
2	20	7.2 ± 0.39
3	200	10.2 ± 2.0
4	400	13.1 ± 1.5
5	1000	17.9 ±1.8

Values are based on 10 mice at the commencement of experiment. Ten mice were studied in two separate experiments and the standard error of the mean calculated at 95% confidence interval

The mean group weights of mice increased normally in the first 2 days of the experiment as shown in figure 5.4.2. This finding is different from what was observed in the preliminary study in which young mice were used and less time for acclimatisation was given resulting into weight reduction from the first day of the experiment. The weight increase was followed by a steady reduction as the disease progressed. Animals that received smaller doses of the extract lost more weight than those treated with higher extract dose.

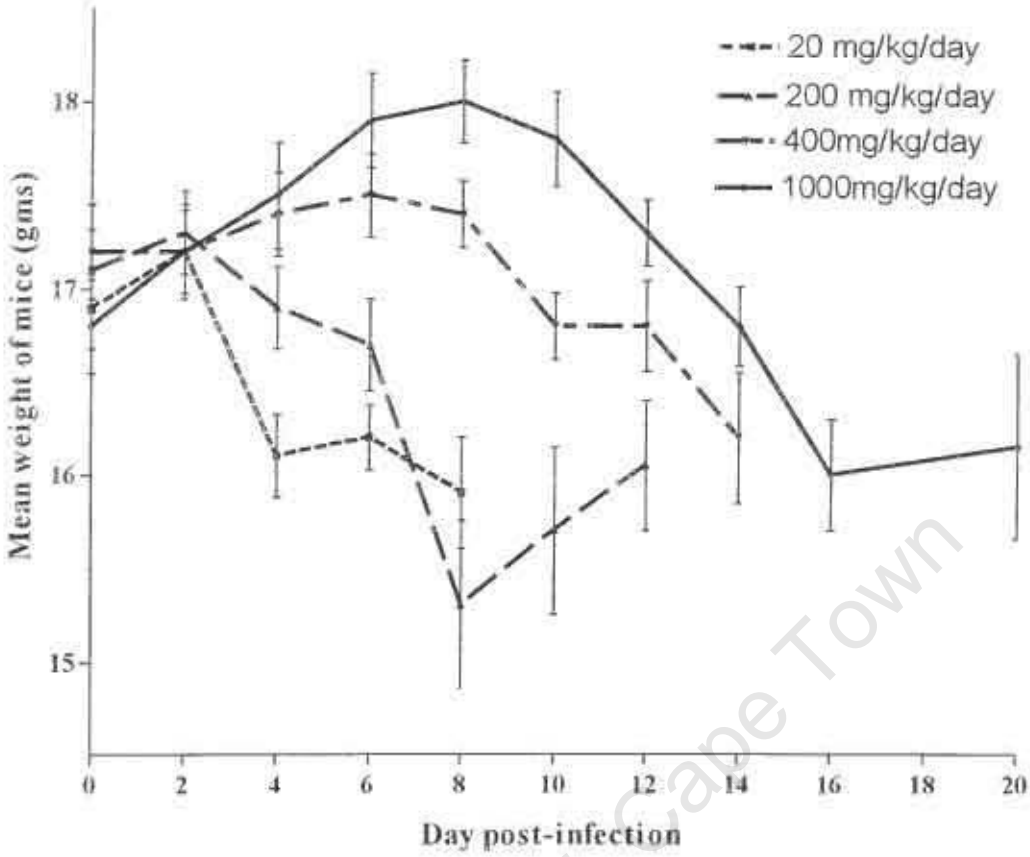

Fig 5.4.2. Weight changes in *P.berghei* (Anka) infected C57BL mice treated with four doses: 20, 200, 400 and 1000mg/kg/day of the water extract of M. *foetida*. The extract was administered in two divided doses for four consecutive days starting on the day of infection. Values are based on 10 animals at the commencement of the experiment. The standard error of the mean was computed at 95% confidence interval.

5.5 Discussion

This study set out to establish a murine model of cerebral malaria that would then be used to investigate the antimalarial properties of extracts from *M. foetida* Schumch. Et Thonn and *C. halicacabum*. Infection of C57BL mice with *P. berghei* (Anka) resulted into a malaria disease model with the key signs of high parasitaemia, convulsions and respiratory distress. Other disease signs observed included: anaemia, leucocytosis, low platelet count and weight loss. These signs are similar to those observed in human cerebral malaria (English *et al.*, 1996).

In the preliminary study, the water extract of *M. foetida* was well tolerated and delayed the development of *P. berghei* (Anka) infection in C57BL mice. On the other hand, the water

extract of *C. halicacabum* L. caused death to all the study animals with in the first four days of infection, with no evidence of protection against *P. berghei* malaria. No autopsy was done but most of the study animals died within 12 hours of extract administration, raising a possibility of an anaphylactic reaction as the possible cause of death. The positive control, chloroquine, offered absolute protection of C57BL mice against murine malaria.

On further investigation, the four doses 20, 200, 400 and 1000 mg/kg/day of M. *feotida* on were well tolerated. Survival time of mice increased with extract dose. The extract did not, however, provide total protection as all the treated animals eventually died. It has been reported that this extract has no significant *in vitro* antiplasmodial activity. The *in vivo* effect could be due to metabolic activation of the plant constituents, or the constituents require the immune system so as to have an effect. The possibility that the *in vitro* assay could not detect the antimalarial activity, for several reasons may not be ruled out. Most chemical constituents of *M. foetida* remain unknown, and their characterisation would help predict the pharmacological properties of its extracts. The extract suppressed the development of *P. berghei* (Anka) parasitaemia in the first days of infection, this suggests it acts on the blood stages of the malaria parasite. The subsequent development of infection in all the study animals points to short extract duration of action, probably due to rapid metabolism or elimination.

The water extract of *C. halicacabum* was less tolerated than that of *M. foetida*. Ethnobotanical surveys reported the use of a 12-hour maceration of the aerial parts of this plant both orally or for bathing in the treatment of fever (Adjanohoun *et al.*, 1989). The use of two routes of administration could arguably be further evidence of toxicity, in which case the oral route, which results in toxicity is being abandoned in favour of the safe topical route. Exact metric doses of these plant extracts used in traditional medicine are not known; it is possible that plant extracts in lower doses are safer. The possibility that mice died due to anaphylactic shock is worrying, and raises need for further investigations into the immunological effects of extracts from this plant. Hypersensitivity reactions with herbs based on traditional medicines are not unknown, they range from mild dermatitis to anaphylactic reactions (Perharic *et al.*, 1993).

The malaria disease model used in this study lacks the insect vector, and utilises laboratory inoculation doses, these result into rapid infection of erythrocytes without going through the

liver stages, generally higher parasite loads are achieved than during natural infection. This would reduce the effectiveness of test drugs under investigation. Small mammals are known to metabolise drugs faster and use different metabolic pathways from humans (Anonymous, 1984), this could result into reduced effectiveness of the extract under investigation. In fact small mammals require about five times the amount of drug taken by humans to produce the same effect (Freireich *et al.*, 1966). The *M. foetida* extract could actually possess better antimalarial activity in humans than observed in the murine model. The different metabolic pathways could also result into a different toxicity profile in humans.

The murine cerebral malaria disease model has a strong role of monocyte leucocytes in disease pathogenesis compared to the human disease (Neill *et al.*, 1992; Sein *et al.*, 1993). These serve to block the cerebral microvasculature, a role played by erythrocytes in human cerebral malaria. It is not obvious how this difference in pathogenesis could affect treatment outcome but it deserves mention. It is also important to note that the plant *M. foetida* is used in malaria treatment in endemic areas, and yet our model utilised naïve mice with no previous exposure to malaria, this could also contribute to the level of extract antimalarial activity observed in this study. It is expected that this extract has better antimalarial activity in humans who have been exposed to malaria, than in an experimental system using immunologically naïve animals.

The pathogenesis of cerebral malaria is different from uncomplicated malaria. It is possible that the extract could have shown different results if tested in a model of uncomplicated malaria. The duration of malaria treatment with the leaf decoction from *M. foetida* in the traditional treatment of malaria is not defined. In this experiment the extract was administered for four days, there is need to investigate the outcome of this study if the extract was administered for longer periods.

5.6 Conclusion

This study has shown that oral administration of the water extract from *M. foetida* delays the development of murine cerebral malaria. This extract was well tolerated in mice up to a dose as high as 1000mg/kg/day. The protection was not absolute however, with the experimental

animals dying after 3 weeks of infection. The water extract of *C. halicacabum* was found to be lethal to mice; animals died within ours of receiving the extract, from possibly anaphylactic reaction. While more studies are necessary to confirm the toxicity of *C. halicacabum*, extracts from this plant should be avoided. There is need for further studies on the water extract of *M. foetida*, especially observational field studies to document its efficacy and safety in humans before it promoted for use as a herbal remedy in malaria treatment.

CHAPTER 6

General discussion and conclusions

6.1 Study background and objectives

The antimalarial potential of Uganda's traditional medicinal plants has not been explored despite their widespread use by communities. There is no information on their efficacy, safety and interactions with other antimalarial medicines. New malaria remedies need to be developed as parasite drug resistance spreads to cover species other than *P. falciparum* and new geographical areas. The main objective of this study was to investigate the antimalarial potential of some of Uganda's traditional medicinal plants. To achieve this, six plants were selected based on the literature, ethnobotanical survey reports and the experience of three traditional healers practising in this country. Antimalarial potential was assessed with reference to efficacy and safety. Safety was looked at in the broader perspective to include negative interactions with other medicines. The study used *in vitro* and *in vivo* experimental systems that were set up specifically to:

(1) determine any variations in yields and antiplasmodial activity from these plants;

(2) investigate the antiplasmodial activity of extracts from the study plants and compare the activity on both chloroquine sensitive and resistant strains of *P. falciparum*;

(3) determine the *in vivo* antimalarial activity of extracts from plants that did not show any *in vitro* antiplasmodial activity; and

(4) investigate *in vitro* interaction between some of the plant extracts with drugs commonly used to treat malaria.

6.2 Essential conclusions from the study

Seasonal variation in yields and antiplasmodial activity

The yields of the plant extracts varied between the wet and dry seasons. Plants collected in the wet season had higher yields than those collected during the dry season. The extracts from the plants *S. discifolius, S. stuhlmannii, I. emarginella,* and *A. africana* had significant antiplasmodial activity; i.e. below the arbitrarily set 50% inhibitory concentration (IC_{50}) of 25 μg/ml. Ethyl acetate and methanol extracts generally had better antiplasmodial activity than the rest of the extracts, indicating that the principles responsible for antiplasmodial activity were polar compounds. The ethyl acetate extract of *A. africana* had the highest antiplasmodial activity. The extracts of *S. discifolius* and *I. emarginella,* collected during the dry season, had significantly better activity than those collected during the wet season.

Is there cross- resistance between plant extracts and chloroquine?

There was no significant difference between the antiplasmodial activity of the plant extracts against the chlororoquine- and sulphonamide-resistant K1 and the chloroquine-sensitive D10 strain of *P. falciparum*. There was, in fact, a positive correlation between the activity of the plant extracts against both strains of *P. falciparum* (Pearson's coefficient; r = 0.9691, p=0.05). This suggests that malaria remedies derived from these plants could be effective against malaria caused by both chloroquine-sensitive and -resistant parasites.

Ugandan traditional medicinal plants as sources of antimalarial lead compounds

The isolates **ACW1** and **AA2** from *A. africana* had *in vitro* antiplasmodial activity with IC_{50} of 1.51 (1.21-1.81) μg/ml and 1.50 (1.33-1.67) μg/ml against the D10 strain respectively. These isolates have even better activity against the chloroquine- and sulfonamide-resistant K1 strain. With this level of antiplasmodial activity, structural modifications can produce compounds with higher activity. The yields of these isolates was 0.00036% and 0.00049%, very low compared to the yield of artemisinin from Chinese *Artimisia annua L* by hexane extraction of 0.4-0.6% (Jansen, 2002). It is necessary to determine the conditions of growth, harvest and extraction of *A. africana* that would result in better yields to enable further studies of its isolates. The final structural elucidation of the isolates would also determine the possibility of producing synthetic analogues for further studies.

In vitro interactions between isolates from plants with existing drugs

The isolate **ACW1** from *A. africana* antagonised the antiplasmodial activity of artemisinin against both the K1 and D10 strains of *P. falciparum*, and chloroquine against the D10 strain. An additive effect was observed between the isolate and chloroquine against the K1 strain of *P. falciparum*. These findings predict potential antagonism between *A. africana* and the two drugs *in vivo*, and suggest that concurrent use of traditional medicines based on *A. africana* with either chloroquine or artemisinin could result in treatment failure. This study adds concurrent use of unstudied traditional medicines with antimalarial drugs to the list of factors affecting malaria treatment outcome. There is a need for more studies focusing on this concept, especially field studies, to determine its incidence and extent.

Effect of plant extracts on uptake of 3H-dihydroartemisinin (^{3}H-DHA)

This study demonstrated that *P. falciparum*-infected erythrocytes accumulate ^{3}H-DHA more than uninfected erythrocytes. The uptake of ^{3}H-DHA by infected erythrocytes was characterised to be dose, temperature dependent, and saturable, suggesting involvement of a carrier protein. On the other hand, uninfected erythrocytes take up a small percentage of ^{3}H-DHA erratically, but generally more at 37°C than at 4°C. The K1 isolate of *P. falciparum* was shown to have a more efficient mechanism of accumulating ^{3}H-DHA than the D10 strain of *P. falciparum*. The isolate of *A. africana*, **ACW1**, inhibited the accumulation of ^{3}H-DHA by the K1 strain and enhanced the accumulation by the D10 strain of *P. falciparum*. Combination studies have shown an antagonistic relationship between the isolate and artemisinin with both strains of *P. falciparum*. These findings suggest that two strains, K1 and D10, handle ^{3}H-DHA differently and that the antagonism observed *in vitro* could be due to two mechanisms: inhibition of ^{3}H-DHA accumulation and neutralising intracellular 'second messengers' of the artemisinin derivatives (the singlet oxygen radicals).

The in vivo antimalarial activity

The C57BL mice infected with the *P. berghei* (Anka) strain of rodent plasmodium produced a cerebral malaria disease model akin to the human disease. The difference observed was a high proportion of leukocyte monocytes. The mice did not tolerate the water extract of *C. halicacabum* in the study dose. The water extract of *M. foetida* was well tolerated and protected the C57BL mice against cerebral malaria. This extract did not show significant *in vitro* antiplasmodial activity. The antimalarial activity observed *in vivo* could therefore be a result of active secondary metabolites *in situ*, or immune modulation. Although the mice were protected at different doses, they later developed malaria and died within 3 weeks. This extract has the potential of being developed into a herbal remedy that is useful in malaria treatment.

The safety of the plant extracts

The water extract of *M. foetida* was well tolerated in mice at doses as high as 1000 mg/kg/day. The death of animals that received 500mg/kg/day of the water extract of *C. halicacabum* indicated a serious adverse event. Though no autopsy was done on the dead animals, the death with in 6 hours of extract administration suggests anaphylaxis as possible cause of death. This finding suggests that the water extract of *M. foetida* is safe while that of *C. halicacabum* could be toxic.

6.3 The relevance of the study findings

Traditional healers reported that collecting plant material during the dry season was desirable because of the advantage of drying from sun exposure. This study however revealed that yields and antiplasmodial activity of the study plants vary with season. Extracts from two of the study plants had better antiplasmodial activity during the dry season. The studies of Howe *et al.*, 2002 and Hoerkstra *et al.*, 2001, suggest that there are metabolic changes in plants during conditions of stress, as in the dry season. These changes generally result into changes in chemical composition of plants. The variations in chemical composition with season have never been investigated for any of the plants involved in this study. The study on yields of artemisinin in *Artimisia Annua L.* confirms the existence of extensive variations in chemical composition of plants with season (Wallaart *et al.*, 2000).

To develop traditional medicines with antimalarial properties as sources of lead antimalarial compounds or herbal remedies for malaria, it is necessary to carry out studies to optimise conditions of growth, harvest and preparation so as to make the venture economically viable. The use of traditional medicines in self-medication is quite extensive and education of communities on the conditions of harvest that provide better yields and activity, would go a long way in improving malaria treatment outcome.

Chloroquine-resistance is one of the greatest challenges facing malaria control programmes. New malaria remedies should generally not possess cross-resistance with chloroquine. The absence of cross-resistance between plant extracts and chloroquine increases the potential of Ugandan traditional medicines as a source of new antimalarial remedies. In fact the chloroquine- and sulfonamide-resistant K1 strain of *P. falciparum* was more vulnerable to one of the isolates from *A. africana* **ACW1** than the chloroquine-sensitive D10 strain. This situation can however be sustained only if traditional medicines continue to be used in the traditional way, and modifications in preparation and administration are accompanied by guidelines for rational use.

The isolates **AA2** and **ACW1** could act as lead compounds to new antimalarial drugs that require structural modifications to improve their antiplasmodial activity. Characterisation of these compounds showed they were sesquiterpenes (existence of a peroxide bond was not elucidated). Artemisinin, a plant derived compound, whose derivatives are now leading antimalarial drugs, belongs to the same class of compounds (Hien and White, 1993). It is also interesting to note that

the plant *A. annua* L, from which artemisinin was isolated, belongs to the *Asteraceae* family as does *A. africana.* Sesquiterpene peroxides with antiplasmodial activity have also been isolated from *Senecio selloi* and *Eupatorium rufescens,* both plants of the *Asteraceae* family (Ruecker *et al.,* 1996). This makes the plant family of *Asteraceae* a potential source of antimalarial sesquiterpene peroxide compounds.

The existence of synergy among constituents of the methanol extract of *I. emarginella* and the ethyl acetate extract of *S. discifolius* is easy to imagine based on the ethnopractice of using plant combinations as reported in neighbouring Tanzania (Gessler *et al.,* 1995). The findings of this study indicate that some plant principles act in synergy to produce the antimalarial activity. One could conclude that these traditional medicinal plants can be used only in crude form as anti-malarial herbal remedies.

There are no prior reports of interactions between chloroquine or artemisinin, and traditional medicines. There are however examples of interactions between herbal extracts and some of the drugs as discussed in the first chapter of this report (Roby *et al.,* 2000, Mai *et al.,* 2000). While interactions observed in this study could be classified as adverse events, they do not undermine the potential of traditional medicines. Like other remedies, they call for extra caution and establishment of proper guidelines. The level of antimalarial activity shown by the plant *M. foetida* reveals a high antimalarial potential for this plant. This finding has not been reported before and calls for clinical studies to determine its effectiveness in the treatment of human malaria.

6.4 Extent to which objectives have been met

This study provided evidence of antimalarial efficacy in all the plants studied with the exception of *C. halicacabum,* suggesting a high antimalarial potential that Ugandan traditional medicinal plants have. The study also demonstrated that there is no cross-resistance between plant extracts with chloroquine. It also reveals extensive variations in extract yields, which is associated with time of collection. There is the need for optimisation of conditions of growth, harvest and preparation so as to make the production of malaria remedies from these plants commercially viable. The isolation of **AA2, ACW1** and **ACW2** compounds with significant *in vitro* antiplasmodial activity, indicate that *A. africana* can be a source of lead antimalarial compounds.

6.5 Limitations and problems encountered during the study

While the plant material used in this study was collected in the same way as traditional healers do, the methods of extraction were different in order to make the handling of extracts easier, as explained in the methodology. The selection and collection of plant material were done following the methods used by traditional healers, but the methods of extraction were different. The study also utilised *in vitro* systems and a murine model of cerebral malaria to determine the efficacy and safety of the study plants. These systems, however, differ from the situation observed in humans. The key components- absorption, metabolism and cellular binding- are lacking in *in vitro* systems. These must be taken into consideration in any attempt to extrapolate the results to humans.

While the use of a murine malaria model increases the predictive value of our results, rodents do not handle drugs in exactly the same way as humans and, moreover, non-human species of *Plasmodium* are used (Anonymous, 1984). The disease model used in this study also lacks the insect vector, implying that it is not exactly representative of the pathogenesis of human malaria. Nevertheless, this model has been extensively used in the evaluation of antimalaria compounds and provides results that can be relied on in preliminary studies (WHO, 1973; Peters, 1975).

6.6 Research prospects

The findings of this study raise the need for conservation of the plant species that have been found to possess antimalarial potential. For all the plants with antimalarial potential, studies on the distribution of active principles with in different parts of the plant are necessary to ensure better harvesting. It is necessary to carry out agro-research aimed at improving the conditions of growth and harvest of these plants to achieve commercially viable yields. Furthermore, there is need to carry out clinical studies on plants like *M. foetida,* to determine their efficacy and safety in human use. Plants with *in vitro* antiplasmodial activity need to be investigated for *in vivo* antimalarial activity and safety before these studies are done.

There is need to carry out complete structural elucidation not only on the compounds isolated in this study but on several other isolates so as to establish their biosynthetic pathways and

structure-activity relationship. These could be used in any efforts to synthesize these compounds. With this information structural modifications can be done with the aim of improving their antimalarial activity. Information on plant biosynthetic pathways is necessary for any efforts towards the production of some of the antimalarial compounds in cell or tissue culture systems.

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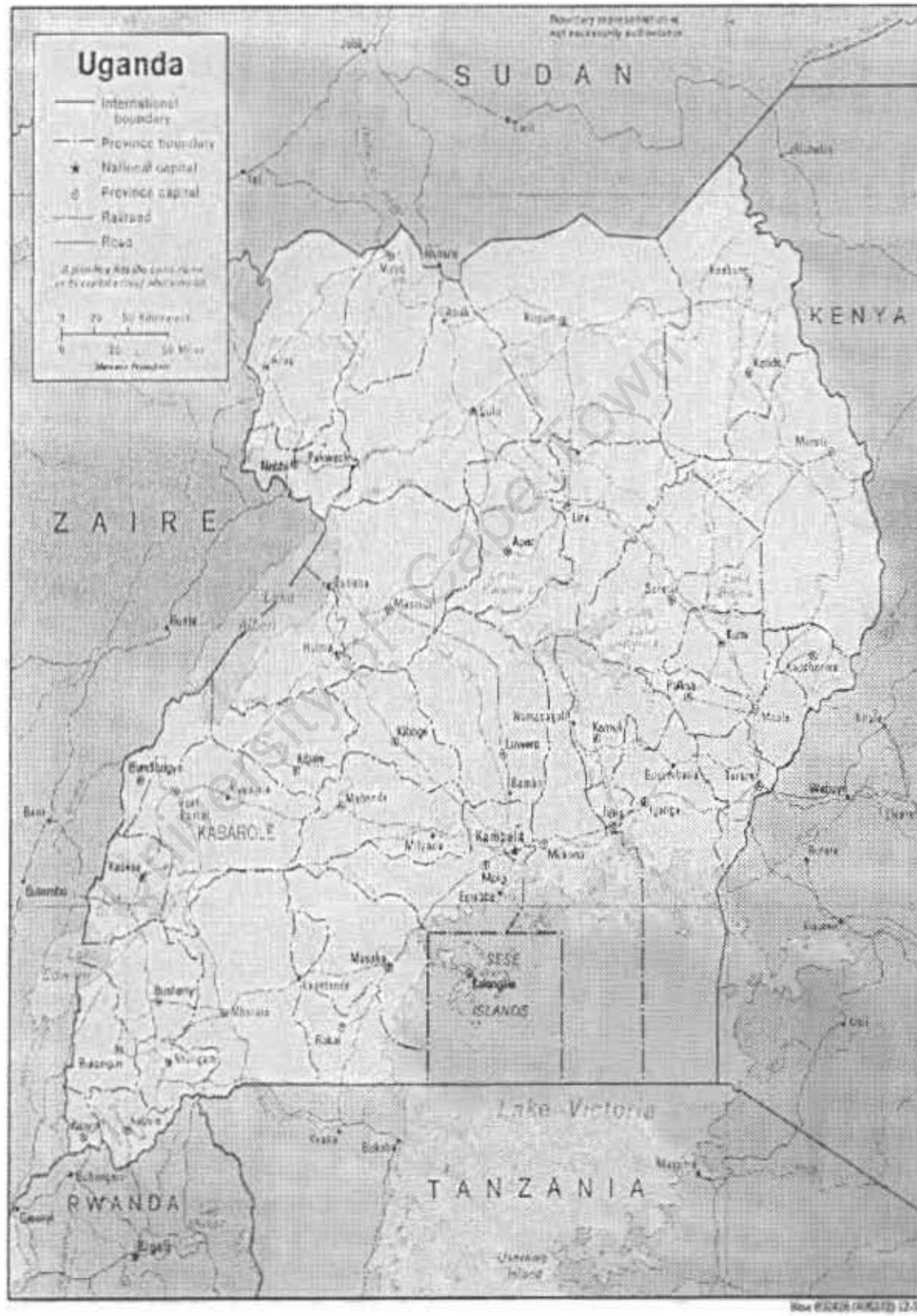
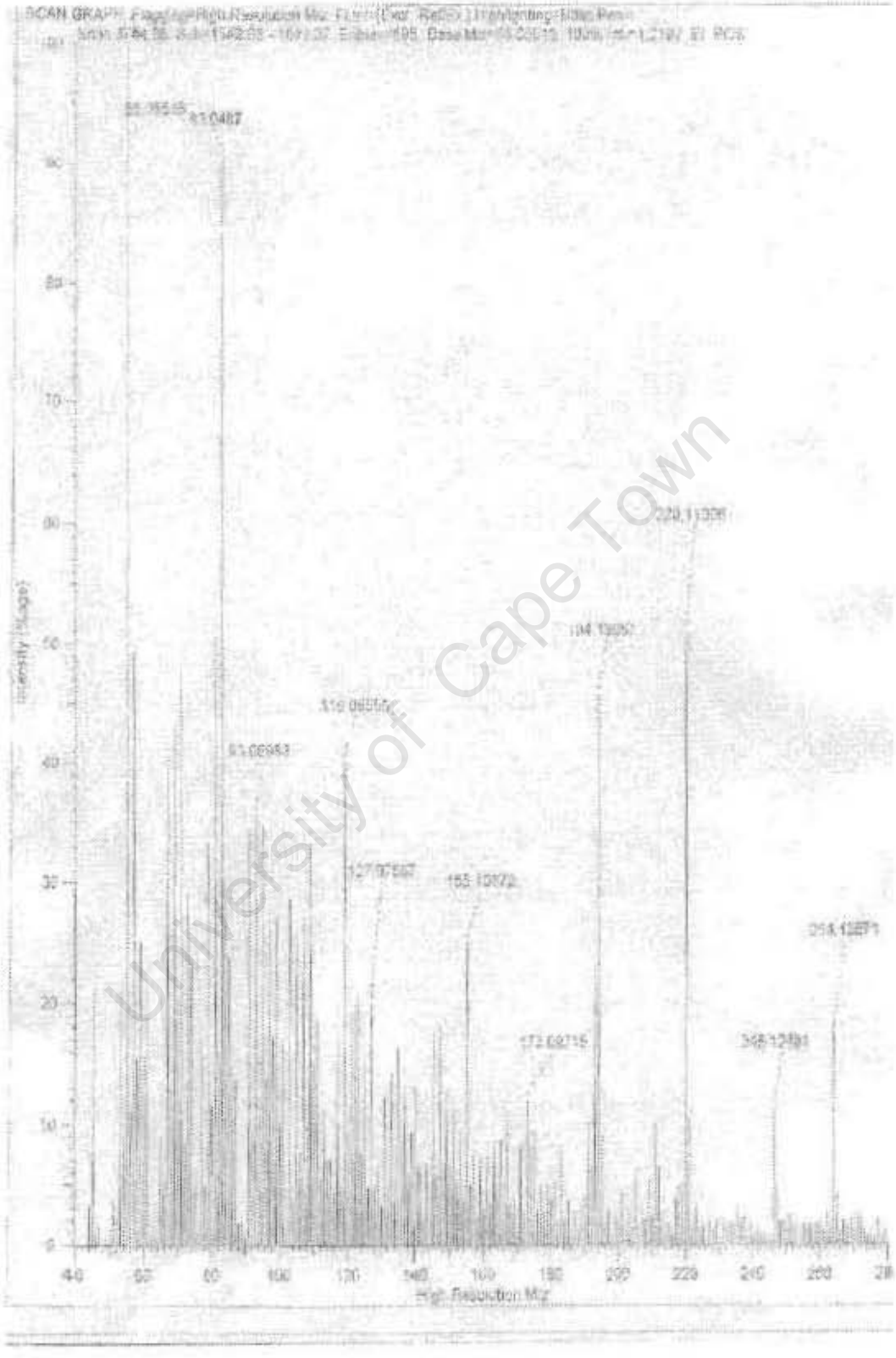
Appendices

Appendix 1: A map of Uganda showing the study areas (The three study towns are circled). "Maps courtesy of www.theodora.com/maps used with permission"



Appendix 2: The mass spectra of AA2



Appendix 3: The mass spectra of AA2 (Horizontal scale extended)

Appendix 4: Molecular formulae of AA2 ions

ATOMIC COMPOSITION REPORT (MANUAL)

Selected isotopes:

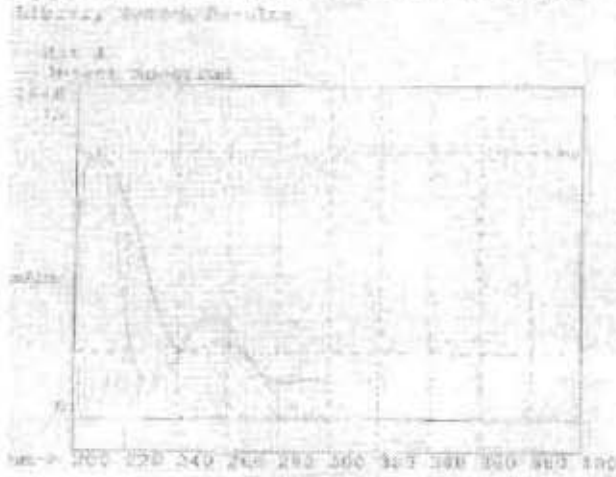
Symbol	Min	Max	Val	Name
C	0	24	4	Carbon-12
H	0	36	1	Hydrogen-1
O	0	6	2	Oxygen-16

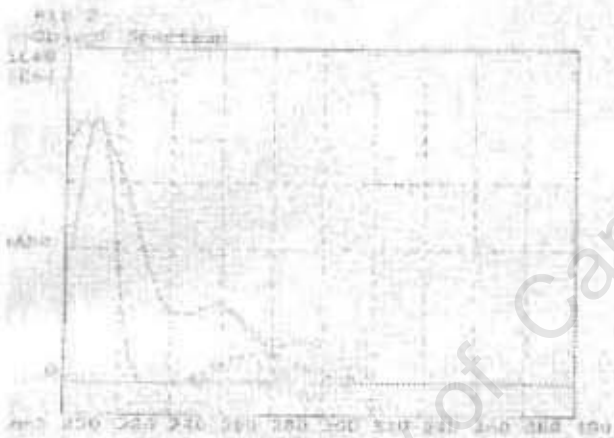
Allowable error = minimum of 20.0 ppm, 5.0 mmu
Ring/Double Bond limits = [0.5 : 100.0]

Mass	Calculated	ppm	mmu	RDB	Formula
264.13571	264.13616	1.7	0.4	8.0	$C_{16}H_{20}O_4$
246.12461	246.12559	3.2	0.8	7.0	$C_{15}H_{18}O_3$
220.11006	220.10994	-0.5	-0.1	8.0	$C_{13}H_{16}O_2$
194.13057	194.13059	0.6	0.1	4.0	$C_{12}H_{18}O_2$
193.12327	193.12285	-2.1	-0.4	4.5	$C_{12}H_{17}O_2$
165.10672	165.10720	3.1	0.5	2.5	$C_9H_{15}O_2$
83.08492	83.08608	15.1	1.3	1.5	C_6H_{11}
55.05510	55.05478	7.0	0.4	1.5	C_4H_7

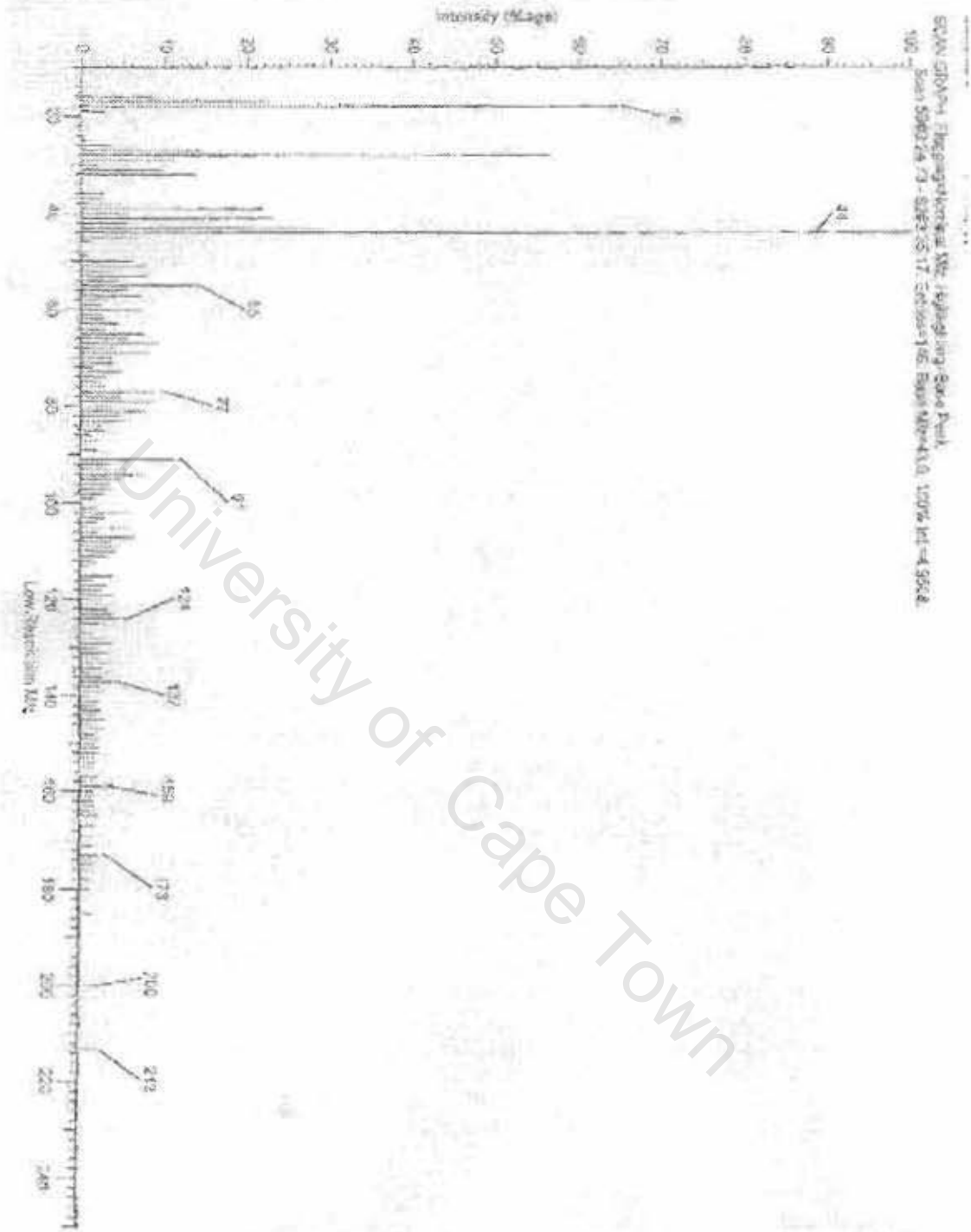
***** End of Atomic Composition Report *****

Appendix 5: UV spectra of AA2 with spectra of three related compounds

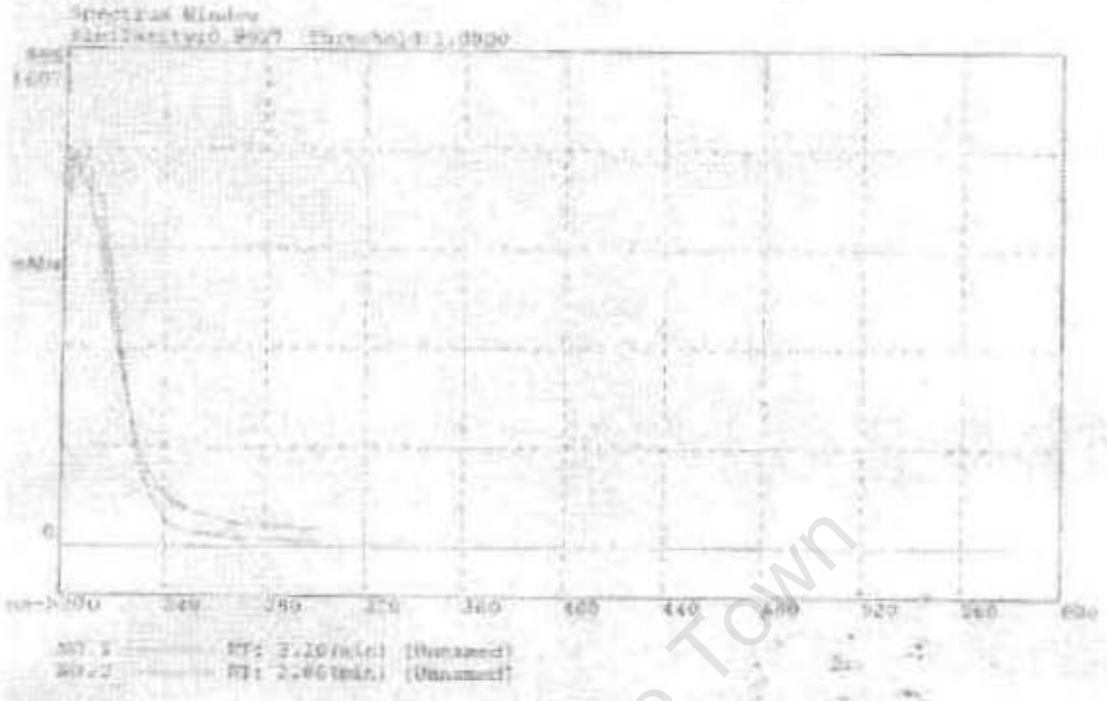




Appendix 6: The mass spectra of ACW1



Appendix 7: UV spectra of acw1 and an impurity eruting at 3.10 minutes.



Appendix 8: Research Ethics Committee clearance

UNIVERSITY OF CAPE TOWN

Research Ethics Committee
Faculty of Medicine
Anzio Road, Observatory, 7925
Queries : Xolile Fula
Tel : (021) 406-6492 Fax: 406-6390
E-mail : Xfula@curie.uct.ac.za

08 August 2001

REC REF: 01/038

Dr P. Waako
Pharmacology

Dear Dr. Waako

THE STUDY OF ANTIPLASMODIAL, IMMUNOMODULATORY AND TOXICITY OF SOME PLANT WATER EXTRACTS IN THE MOUSE MODEL

Thank you very much for your letter to the Research Ethics Committee dated 02 August 2001.

It is a pleasure to inform you that your study was formally approved by the Committee on the 03 August 2001.

Please quote above REC reference number in all correspondence.

Yours sincerely

PROF.D.KAHN
CHAIRPERSON

Table 3.3.1a. Yields and antiplasmodial activity of extracts from the plants *M. foetida*, *A. africana* and *C. halicacabuh* against the chloroquine-sensitive D10 and the chloroquine-and sulphonamide-resistant K1 strains of *P. falciparum*. The plants were collected during the wet season.

Plant species	Solvent	Yield[1] (%)	IC_{50} on D10 strain (µg/ml)[2]	IC_{50} on K1 strain (µg/ml)[2]
M. foetida	Water	18.7	40.7 ± 11.2	50.8 ± 3.3
	Hexane	3.4	≥100.0	≥100.0
	E/acetate	0.84	30.0 ± 1.7	29.3 ± 1.47
	Methanol	1.35	75.4 ± 17.5	68.8 ± 5.4
A. africana	Water	19.3	22.7 ± 7.5	25.2 ± 1.1
	Hexane	2.1	≥100.0	≥100.0
	E/acetate	2.7	9.3 ± 1.6	11.5 ± 2.8
	Methanol	2.3	23.1 ± 7.5	29.2 ± 3.05
C. halicacabuh	Water	16.1	≥100.0	≥100.0
	Hexane	1.8	≥100.0	≥100.0
	E/acetate	1.4	28.6 ± 4.2	32.6 ± 2.6
	Methanol	1.9	62.6 ± 9.4	79.0 ± 5.2

[1]Values for yields are results of a single experiment. [2]Values for the IC_{50} are means of three experiments carried out in duplicate. The standard error of the mean was calculated at 95% confidence interval. E/acetate denotes ethyl acetate.

Table 3.3.1b. Yields and antiplasmodial activity of extracts from the plants *I. emarginella* , *S. stuhlmannii* and *S. discifolius* against the chloroquine-sensitive D10 and the chloroquine-and sulphonamide-resistant K1 strains of *P. falciparum*. The plants were collected during the wet season.

Plant species	Solvent	Yield[1] (%)	IC_{50} on D10 strain (µg/ml)[2]	IC_{50} on K1 strain (µg/ml)[2]
I. emarginella	Water	15.4	≥100.0	≥100.0
	Hexane	2.4	≥100.0	≥100.0
	E/acetate	1.7	≥100.0	≥100.0
	Methanol	2.2	22.5 ± 4.9	22.4 ± .4
S. stuhlmannii	Water	17.4	69.3 ± 1.3	77.6 ± .7
	Hexane	2.4	≥100.0	≥100.0
	E/acetate	2.2	14.0 ± 1.4	15.2 ± 1.9
	Methanol	2.8	≥100.0	≥100.0
S. discifolius	Water	14.9	≥100.0	≥100.0
	Hexane	1.2	≥100.0	≥100.0
	E/acetate	2.6	24.7 ± 8.1	30.2 ± 2.7
	Methanol	2.0	≥100.0	≥100.0

[1]Values for yields are results of a single experiment. [2]Values for the IC_{50} are means of three experiments carried out in duplicate. The standard error of the mean was calculated at 95% confidence interval. E/acetate denotes ethyl acetate.