Bioassay-guided fractionation of *Artemisia afra* for *in vitro* antimalarial activity against *Plasmodium falciparum*

MERYL A. ABRAHAMS

B.Sc. (UCT)

B.Sc. (Med) Hons. (UCT)

Dip Bus M (DMS)

Bioassay-guided fractionation of *Artemisia afra* for *in vitro* antimalarial activity against *Plasmodium falciparum*

MERYL A. ABRAHAMS

A project submitted to the Department of Pharmacology, University of Cape Town in fulfilment of the requirements for the degree

MASTER OF SCIENCE (MEDICINE)

SUPERVISORS

Prof. P.I. Folb

Dr. D.W. Gammon

December 1996

Declaration

I, ...MERYL A. ABRAHAMS....., hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

I empower the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

Signed: ..... Signature Removed .....

Date: ..... JANUARY 1997 .....

Acknowledgements

I wish to express my sincere thanks to the following people:

My supervisors, Prof. P.I. Folb (Pharmacology Department, U.C.T.) and Dr. D.W. Gammon (Chemistry Department, U.C.T.) for their time, interest and supervision of this project.

Dr. P.J. Smith for getting me started on the HPLC and for affording me the opportunity to undertake this project.

Mr. W.E. Campbell (Chemistry Department, U.C.T.) for his constant support, dedication and assistance throughout.

Dr. B.M. Sehlapelo (Chemistry Department, U.C.T.) for sharing his chromatographic skills with me and for characterizing the isolated pure compound.

Prof. K. Hostettmann and Drs. J-L.Wolfender and A. Marston at the Institute of Pharmacognosy and Phytochemistry, University of Lausanne, Switzerland, for analysis of artemisinin by thermospray LC-MS.

Prof. I. Parker (Medical Biochemistry Department, U.C.T.) for allowing me the use of the department's tissue culture facilities for cytotoxicity screening.

Mrs. H. Karjiker (Medical Biochemistry Department, U.C.T.) for maintaining the RAT-1 cell line.

Dr. S.J. Cridland and Mr. K. Saliba for their helpful suggestions and for their assistance in editing this thesis.

The Medical Research Council of South Africa for funding this project.

Mr. N. Jordaan for his tireless efforts in maintaining the sterile laboratory.

Mr. L. Heiberg, Mrs. J. van Dyk and Dr. S.J. Cridland, for all the late night lifts home.

My colleagues, K. Saliba, N. Davids, G. Matsabisa, B. Adams, J. Walden, G. Gabriels, M. Newmarch and J. Rawlings for their sharing of knowledge, encouragement and support whenever it was most needed.

A special friend, Joshna Govind, for seeing me through the best and worst of times. Thanks for *everything*.

My family in Cape Town and Johannesburg, in particular my sister, Desireé, for understanding and enduring so much, especially through some very difficult times.

Finally, to my best friend, teacher and guide, to whom I owe everything, sweet, sweet, sweetest Bapdada.

Abstract

With the increase in recent years in the prevalence of malaria, and in drug resistance of *Plasmodium falciparum*, there has been much interest in natural plant products for new antimalarials with novel modes of action against *Plasmodium*. Artemisinin or Qinghaosu is one such antimalarial isolated from a Chinese herb, *Artemisia annua* (Asteraceae) and it is currently undergoing phase I and II clinical trials. The Southern African species, *Artemisia afra* (African wormwood, wildeals, lengana) is commonly used by local traditional healers for symptoms of malaria, in particular fever. Thus it seemed appropriate to investigate this species for antimalarial activity.

Crude petroleum ether soxhlet extracts of *Artemisia afra* had demonstrated antimalarial activity against *Plasmodium falciparum*, FCR-3, cultured *in vitro*. The IC_{50} values ranged from 5-13μg/ml. The extract from leaves and flowers was then screened against D10 (chloroquine-sensitive) and FAC8 (chloroquine-resistant) *P. falciparum*, *in vitro*, with IC_{50} values of 1.03μg/ml and 1.5μg/ml respectively. This extract was fractionated by column chromatography using silica gel-60 and the fractions obtained were screened for antimalarial activity. The most active fraction had an IC_{50} of 0.5μg/ml against D10 and FAC8. Using TLC and HPLC-UV analysis with pure artemisinin as a standard, no artemisinin could be detected in this fraction. This result was confirmed by thermospray LC-MS analyses. Purification of this fraction yielded ultimately a single pure compound; a clear colourless oil identified by MS and NMR analyses as hydroxydavanone. The compound was screened against a variety of *P. falciparum* strains with varying degrees of sensitivity and resistance to both chloroquine and mefloquine. Their sensitivity against artemisinin was also established. IC_{50} values obtained for the isolated pure compound against *P. falciparum* ranged from 0.87 to 2.54μg/ml. The IC_{50} values obtained for general cytotoxicity of the crude extract

and isolated pure compound against RAT-1 fibroblast cells were 34.78 ± 8.23 and 6.29 ± 0.95 μg/ml (n=4) respectively. Thus the crude extract and isolated pure compound exhibited a greater antimalarial than cytotoxic effect. Hence, there are implications for *A. afra* to be used as a phytomedicine for the treatment of malaria. *In vivo* studies are recommended for hydroxydavanone in order to fully assess its potential for clinical use.

Table of Contents

Conference Presentations and Publications

IOCD (International Organization for Chemical Sciences in Development)

Working Group on Plant Chemistry

INTERNATIONAL SYMPOSIUM

Theme: Chemistry, Biological and Pharmacological Properties

of African Medicinal Plants

25-28 February 1996

VICTORIA FALLS, ZIMBABWE

1. Short Lecture:

In vitro antimalarial activity of *Artemisia afra* against *Plasmodium falciparum*.

In Press:

A manuscript of the short lecture delivered was submitted in July 1996.

The title of the book is to be the same as for the Symposium.

2. Poster Presentations

(i) Chemical composition of varieties of *Artemisia afra*.

M. Abrahams[#], W.E. Campbell[*], D.W. Gammon[*], P.I. Folb[#], P. Smith[#]

and B.M. Sehlapelo[*]

Departments of Chemistry[*] and Pharmacology[#], University of Cape Town, Rondebosch, 7700, Republic of South Africa.

(ii) Phytochemical research in the Traditional Medicines Programme at the University of Cape Town.

M. Abrahams[#], W.E. Campbell[*], D. Chen[*], D. Dodds[*], D.W. Gammon[*],

P.I. Folb[#], J.J. Nair[*], T.D. Purves[#], P. Smith[#] and B.M. Sehlapelo[*]

Departments of Chemistry[*] and Pharmacology[#], University of Cape Town, Rondebosch, 7700, Republic of South Africa.

33RD CONVENTION OF THE SOUTH AFRICAN CHEMICAL INSTITUTE

29 January - 2 February 1996

CAPE TOWN, SOUTH AFRICA

Poster Presentation

Chemical composition of varieties of *Artemisia afra*.

M. Abrahams[#], W.E. Campbell[*], D.W. Gammon[*], P.I. Folb[#], P. Smith[#]

and B.M. Sehlapelo[*]

Departments of Chemistry[*] and Pharmacology[#], University of Cape Town,
Rondebosch, 7700, Republic of South Africa.

In Preparation:

A detailed scientific paper of the work conducted on *A. afra*, between the Departments of Chemistry and Pharmacology.

List of Tables

List of Figures

Abbreviations

TLC	Thin Layer Chromatography
GLC	Gas Liquid Chromatography
HPLC	High Performance Liquid Chromatography
LC	Liquid Chromatography
UV	Ultra violet
IR	Infra-red
NMR	Nuclear Magnetic Resonance
MS	Mass Spectrometry
TSP	Themospray
m/z	mass/charge ratio
conc	concentration
IC_{50}	Inhibitory concentration at which 50% of parasite growth is inhibited
MTT	3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide
b.p.	boiling point
THF	Tetrahydrofuran
MeOH	Methanol
MeCN	Acetonitrile

U.C.T.	University of Cape Town
G.S.H.	Groote Schuur Hospital
RBC	Red blood cells
PRBC	Parasitized red blood cells
$NaHCO_3$	Sodium bicarbonate
PBS	Phosphate buffered saline
v/v	volume/volume ratio
no.	number
NaCl	Sodium chloride
CQ	Chloroquine
min	minutes
RT	Retention time
DPM	Disintegrations per minute
SD	Standard deviation
DMSO	Dimethyl sulphoxide
MW	Molecular weight

"We shall not cease from exploration

And the end of all our exploring

Will be to arrive where we started

And know the place for the first time."

T.S. Elliot

1. Introduction

Drug resistance is a major obstacle to the successful treatment and control of malaria. Chloroquine resistance, in particular, has become widespread in many parts of the world where *Plasmodium falciparum* is endemic. Resistance to other drugs such as amodiaquine, sulfadoxine-pyrimethamine, quinine, and mefloquine, exists to varying degrees in certain endemic regions (1,2). The limited therapeutic options for drug-resistant *P. falciparum*, and the development of resistance to drugs other than chloroquine, has led to the urgent need to search for new antimalarials. As a result, there has been renewed interest in plants either as a source of new antimalarials or in providing template molecules for novel structures with novel modes of action.

The recent chemical, biochemical and clinical studies of the Chinese antimalarial, artemisinin, has given considerable impetus to the search for new antimalarials from plants. Artemisinin was isolated in 1971 as the active principle from the Chinese herb *Artemisia annua* Linn. (Asteraceae) which had been used for more than 2 000 years to treat febrile illnesses (3). Artemisinin however, is poorly soluble in water or oil and hence a number of water-soluble derivatives (artesunate and artelinate) and oil-soluble derivatives (artemether and arteether) were synthesized. These drugs are currently undergoing clinical trials (1,4).

The Southern African variety of *Artemisia*, *Artemisia afra* Jacq. (Asteraceae) had been identified as a common plant used by local traditional healers for the treatment of fever and symptoms of malaria (5). This led to the investigation of *in vitro* antimalarial activity of *Artemisia afra* against *P. falciparum* in 1993 (6). Crude petroleum ether soxhlet extracts from the aerial parts of the plant were generated and screened for activity against the chloroquine-resistant strain of *P.*

falciparum, FCR-3, *in vitro*. The IC_{50} values that were obtained ranged from 5.4-13μg/ml. As a result, extracts of leaves and flowers were selected to be fractionated and purified in order to isolate and identify the active principle. It was also of interest to determine whether artemisinin was present in this crude extract and if so, to establish if it were responsible for this antimalarial effect.

2. Literature Review

The following topics are discussed in this review:

- The problem of malaria
- The potential of plants to produce new drugs
- The example of artemisinin in drug development
- The potential of *Artemisia afra* to produce new drugs
- Proposed strategy for the antimalarial investigation of *Artemisia afra*

Malaria - the world's most common tropical disease

Human malaria is considered to be the world's most common tropical disease (7). It is a key problem in public health care with respect to both morbidity and mortality. Presently, there is a rapid increase in the resistance of malaria parasites, in particular *Plasmodium falciparum*, to existing antimalarial drugs. Over 400 million people are estimated to be affected annually. At least 2.7 million deaths occur each year (8) of which 1-2 million are children between the ages of 1 and 5 (9,10). The World Health Organization (WHO) estimate that 85% of the acute and chronic infections of malaria each year are caused by *Plasmodium falciparum* (11). In sub-Saharan Africa alone, *P. falciparum* is responsible for at least 90% of world-wide morbidity and mortality (12). The other 3 species of *Plasmodium* known to cause human malaria are *P. vivax*, *P. malariae* and *P. ovale* (in decreasing order of importance).

Transmission and life cycle of *Plasmodium*

The complex life cycle of *Plasmodium* comprises four phases. The first phase is sexual but without multiplication. The following three phases are asexual with

multiplication. The sexual and first asexual phases occur only in female *Anopheles* mosquitoes. The second asexual phase occurs in the liver of an infected human (hepatic schizogony) and the third asexual phase takes place in the blood (erythrocytic schizogony) of the infected individual. The third phase can be repeated many times. Each phase ends when new invasive parasites appear (13). During the erythrocytic stage, differentiation occurs for some parasites, specifically the merozoites into male and female gametocytes or sex cells which do not develop further unless another female *Anopheles* mosquito feeds on the infected blood. Thus, the sexual phase of the life cycle of the malaria parasite takes place in the gut of the mosquito which results in the release of sporozoites (14). As soon as these sporozoites enter the bloodstream they pass quickly to the liver where they proliferate and sporulate for the next 7-10 days. No symptoms are experienced during this time. Parasites released from the hepatocytes, called merozoites, will invade erythrocytes. The merozoites soon develop into trophozoites which reproduce asexually to form schizonts. From these schizonts as many as 16-24 new parasites or merozoites are released which will infect more erythrocytes or red blood cells. As this cycle is repeated, fever is experienced each time merozoites are released (13).

The lethal form of malaria

Malaria caused by *P. falciparum* is called severe falciparum malaria or cerebral malaria which is a much more serious and progressive illness. It occurs in non-immune people such as travellers and in inhabitants of areas where epidemics of malaria break out from time to time. Irregular tertian fever (i.e. fever every alternate day) is experienced at first and then there is rapid deterioration of the patient into stupor, fits and coma (13). From coma, the end result is usually death, especially if the patient is not treated timeously.

Partial immunity

Adults in endemic areas, i.e. indigenous people are protected by a form of partial immunity called premunition (13). Immunity develops slowly and to a specific species of *Plasmodium* only in response to repeated infections of that species. Partial immunity is lost if there is no longer continuous exposure to infection (13). In such individuals parasites are commonly found in their blood without the experience of symptoms. This is known as asymptomatic parasitaemia.

Drug resistance

Drug resistance of malaria parasites has been defined as 'the ability of a parasite strain to multiply or to survive in the presence of concentrations of a drug that normally destroy parasites of the same species or prevent their multiplication' (2). This definition has been further qualified into relative resistance, i.e. yielding to increased doses of the drug tolerated by the host, or complete resistance, i.e. withstanding maximum doses tolerated by the host. Today, the common use of the term drug resistance relates to complete resistance and is based on parasitological features (2).

In the 1950s, it was thought that malaria would be completely eradicated with the use of synthetic drugs such as chloroquine, primaquine and pyrimethamine and with the use of DDT and other insecticides against the *Anopheles* mosquito vector (14,15). However, resistance to chloroquine by *Plasmodium* was recognized by 1960 (14) and has since worsened. Chloroquine resistant strains of *Plasmodium* have been confirmed in at least 73 countries (2). In South Africa, it was first reported in 1985 (16). This is not necessarily surprising since chloroquine has been used in increasing quantities over the last 40 years. Resistance has now been reported against mefloquine and pyrimethamine (17) and under laboratory conditions it is possible to produce resistance against

artemisinin, the Chinese antimalarial (11,14). Resistance to sulphonamide-pyrimethamine combinations is widespread in South-east Asia, western Oceania and South America, and it is on the increase in Central, East and West Africa. In the Thai/Cambodia and Thai/Myanmar border areas resistance to quinine and mefloquine has become a major problem (18). No drug resistance however, has been reported in *P. ovale* and *P. malariae* (18). It would appear that resistance to new drugs is likely, unless the use of these drugs are strictly controlled (i.e. there is rational use of these drugs). In addition, there is also increasing resistance of the vector mosquitoes to DDT and other insecticides (14).

The vector

Only mosquitoes of the genus *Anopheles* are able to transmit malaria to humans. The mosquito vector is said to be the greatest single source of variation in the epidemiology of malaria worldwide (19). In most malaria endemic areas the parasites are often transmitted by more than one species of mosquitoes. These species vary greatly in terms of their numbers, feeding habits and ability to transmit the parasite. So not all species of *Anopheles* are vectors and among those species known to be vectors, not all of their populations are involved, and among the vector populations not all the individual mosquitoes are infected with *Plasmodium* (19). This is a major factor to consider in biological control schemes. Biological control schemes have thus far failed to make a significant impact on mosquito populations or the diseases they transmit (8,19). The primary vector in sub-Saharan Africa is *A. gambiae* which is responsible for the high levels of human morbidity and mortality (19).

Traditional medicine and plants

Plants have formed the basis for the treatment of diseases throughout the ages and continue to be a major source of primary health care for about 80% of the

world's population, as estimated by the WHO (20). Sophisticated plant-based traditional medicine systems have been in existence for thousands of years in countries such as China and India. Medicinal plants are also used extensively in African traditional health systems (21). The first African pharmacopoeia under the auspices of the Organization of African Unity's Scientific and Technical Research Commission was released in 1985 (first volume) and 1986 (second volume). The first volume describes about 100 common medicinal plants and specifies the standards to be used in their quality control. The second volume describes the methods to be used in the quality control of these herbs (22). Numerous phytomedicines are registered and are extensively used in Europe. More than 600 botanical items have been officially recognized in various editions of the United States Pharmacopoeia even though current regulations prohibit most from being marketed as drugs. In Australia the traditional therapeutic use of native plants by the indigenous people was not recorded in a written format. The extent of their knowledge is only now becoming apparent (21).

The potential of plants to produce new drugs

In developed countries, which make up 20% of the world's population, more than 50% of all the drugs in clinical use are of natural product origin. Of the 25 best selling pharmaceutical agents in the world, 12 are natural product derived (21). It has also been reported that there are 119 drugs of known structure that are still being extracted from higher plants and are being used globally (20). Of these 119 plant-derived drugs, 74% were discovered as a result of chemical studies specifically designed to isolate the active principles responsible for the use of the plants in traditional medicine. These drugs were derived from only about 90 of the 250 000 known species of flowering plants throughout the world (20). Less than 10% of the estimated 250 000 flowering species in the world

have in fact, been examined scientifically for their potential in medicine. It is estimated that by the year 2050, 60 000 species of higher plants (i.e. 1 out of every 4 plants) will have become extinct (23). Sadly, there is little time remaining to explore this rapidly diminishing resource. Throughout the developing world there are more than 20 000 species of higher plants which are used in traditional medicines (24,25) and these together with the other 230 000 species of higher plants, form a vast reservoir of potential for new drugs.

Traditional medicines in South Africa

There is a genuine and increased interest in traditional medicine developing in South Africa. There is an increase too, in the understanding of and acceptance of practitioners of traditional, indigenous and alternative systems of medicine. There are an estimated 200 000 traditional healers (26) in a total population of about 45 million in South Africa. As many as 12 000 healers are known to be present in Soweto alone (27). Almost 75-80% of black South Africans consult these healers, often in addition to using western medicines (26,28). Medicinal plants are used extensively by these healers and are also commonly used by the public for self care. These medicines are generally used in a crude form. They are harvested from the wild by rural communities, traditional healers and professional herb gatherers. Approximately 3 000 plant species are being used as medicines, probably, because of South Africa's rich biodiversity. About 350 species are recognized to be commonly used and are traded throughout the country (26). In a recent survey, more than 500 species were found to be harvested and traded as medicinal plants in the Witwatersrand alone. Most of these were being sold as bark and roots, although the leaves, stems, whole plants and bulbs are also sold. The most commonly stocked plant was *Artemisia afra*, followed by *Scilla natalensis*, *Thesium pallidum* and *Knowltonia bracteata* (27).

Research performed in South Africa

In South Africa, Noristan Ltd. began a systematic natural product research project in 1974, with the aim of isolating and identifying pharmacologically useful compounds from the diverse and rich Southern African flora. South Africa alone has 24 000 species of flora. The success of their project has indeed been very encouraging. From a total of about 300 plants evaluated, 81% showed biological activity (29).

Advances in technology

Over the past three decades, there has been an enormous improvement in techniques for the isolation and structure determination of plant constituents. New, sensitive and sophisticated chromatographic techniques such as TLC, GLC and HPLC are being used for separation and isolation procedures and X-ray crystallography and spectroscopic techniques such as UV, IR, CD, ^{1}H and ^{13}C NMR are used for structure elucidation (25).

Until recently, gram quantities of a pure compound were required to undertake whole or isolated organ experiments in order to establish pharmacological activity. Now with the development of modern pharmacological assays that are highly sensitive and specific, minute amounts of compounds can be rapidly screened for a whole series of biological activities (25) and their structures determined (30). Industry has also established automated high throughput *in vitro* screens for biological activities which are capable of examining thousands of compounds or extracts in very short periods of time. In the field of malaria research, continuous *in vitro* culture of *P. falciparum* was not achieved until 1976 (31) and the first *in vitro* antimalarial screening assay was only made possible in 1979 by Desjardins *et al.* (32) where drug effects were monitored by the measurement of radiolabelled hypoxanthine incorporation into parasite nucleic acids. Hence, there is a race to locate new drug entities from natural

sources. Never before has there been such an opportunity for collaborative research amongst botanists, chemists, pharmacognosists, pharmacologists and toxicologists to isolate and produce novel drugs.

As a result of these new technologies it has become current fashion to use bioactive-guided fractionation of plant extracts in attempts to isolate biologically active compounds (25). Such studies offer a logical approach for new drug discovery and for the identification of template molecules for the design of new drug molecules (24). For example, morphine from the opium poppy, used as a highly effective analgesic for the relief of terminal pain, had served as a template molecule for the design of numerous drugs including analgesics such as pethidine and pentazocine and the cough suppressant dextromethorphan. Similarly, tubocurarine from *Chondrodendron tomeniosum* is the potent muscle relaxant of tube curare which has served as a template molecule for a number of synthetic analogues such as decamethonium, suxamethonium, pancuronium and more recently, atracurium (24).

During the past 25 years only a few drugs from higher plants have yielded clinical agents. The outstanding examples are taxol derived from *Taxus brevifolia* (the Pacific yew tree) (34), vinblastine and vincristine from the Madagascan periwinkle, *Catharanthus roseus* and etoposide, the semi-synthetic lignan from the May-apple *Podophyllum peltatum* (24). The isolation of artemisinin from *Artemisia annua* has once again focused on higher plants as potential sources of new drugs.

Scientific verification of plant use

The WHO estimate that 80% of the world population reside in developing countries and that 80% of these people use traditional medicines, which includes medicinal plants for their primary health care needs. This means that there are

more than 3.2 million people in the world who are currently using extracts of plants as drugs. Most of these plants have not been verified scientifically as being useful for the conditions that they are being used for (20).

Thus, another important reason for studying the use of medicinal plants is to validate scientifically their effects and side-effects so that they can be recommended for use in developing countries where they are culturally acceptable and provide cost-effective medicines (20).

The ethnobotanical approach

The pharmacology of herbal plants involves a number of disciplines. The study of the use of plants by various cultures is called ethnobotany. The study of how a culture uses herbs therapeutically is a branch of ethnomedicine. The study of the active principles in these plants is ethnopharmacology. In general, the chemistry, pharmacology and toxicology of even widely used plants have been poorly studied (33). The ethnobotanical approach to drug discovery (i.e. if a plant has been used by indigenous cultures over a long period of time, there should be valid drug potential in the plant), is likely to yield greater success than random screening of plants (33,34). The discovery of taxol is one notable exception. It was discovered in a random screening programme conducted by the National Cancer Institute (NCI). Taxol derived from *Taxus brevifolia* (the Pacific yew tree), was approved by the FDA (Food and Drug Administration) for the treatment of ovarian cancer and in 1994 it was approved for treating metastatic breast cancer unresponsive to any other therapy (34).

Food for pharmacological thought

Attention has to be drawn to the use of plant foods as a means of maintaining health. For example, the anti-inflammatory and antimicrobial effects of onion

(*Allium cepa* L.), garlic (*Allium sativum* L.), basil (*Ocimum basilicum* L.), fennel (*Foeniculum vulgare* Mill.) and ginger (*Zingiber officinale* Rosc.) are well known. Such foods may be used in small quantities but frequency of consumption may ensure sufficient exposure to effect both preventative and therapeutic action against illness (35).

The processing of some foods may involve grating, alkaline treatment and high temperature cooking in water or milk. Such treatment not only softens and renders the food more palatable but also increases the solubility of the plant constituents considerably which might only have been marginally available from medicines prepared with coarsely cut materials infused in small volumes of water, and particularly when medicines are prepared quickly for immediate use (35).

Thirdly, just as modern medicine recognizes both beneficial and adverse interactions between drugs and food, so too, combinations may influence physiological effects of plants. For example, synergistic and potentiating interactions occur between digitalis and high calcium foods, and among alcohol containing foods, interactions occur between metronidazole and narcotic drugs. Antagonism occurs between tetracyclines and high calcium or iron-rich foods. Similarly, in plant mixtures the oxytocic and vasopressive activities of serotonin and tyramine (e.g. in *Gossypium hirsutum* L., *Cystisus scoparius* (L.) Link and *Phoradadendron serotinium* (Raf) MC Johnst.) are protected by monoamine oxidase inhibitors such as myristicin and its congeners in, for example, dill, parsnips and parsley (*Athenum graveolens* L., *Pastinaca sativa* L., and *Petroselinum crispum* (Mill) Nym.).

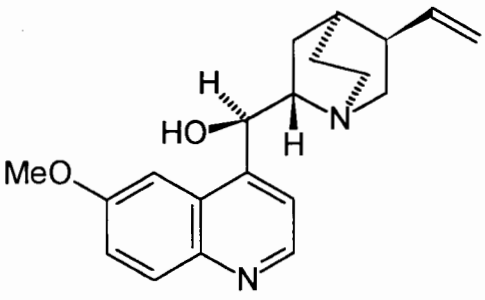
In Nigeria, studies of Hausa medicine have shown a mutually complementary relationship between diet and medicine. Many of the Hausa plants contain substantial amounts of phytates (especially in cereals and legumes) and tannins

(in most ligneous species) which serve as chelators and may have the potential to suppress the effect of malaria by sequestering iron. This action may be further potentiated by the oxidising action of other Hausa foods and medicines such as *Cassia tora* L., *C. occidentalis* L. (also recorded in the PHARMEL database; used in the treatment of malaria), *Guiera senegalensis* Lam., and *Acacia arabica* Willd. Alternatively, the antimalarial effect may be antagonised by antioxidants such as vitamins C and E, β-carotene and selenium which are major radical traps and which occur in high concentrations in some of the same plants such as tea leaf, *Camellia sinensis* (L.) Kuntze; tumeric, *Curcuma longa* L.; date palm, seed *Phoenix dactylifera* L.; *Tribulus terrestris* L.; *Amaranthus viridis* L.; *Amaranthus spinosus* L.; *Solanum nigrum* L.; *Euphorbia hirta*; *Moringa oleifera* Lam. Studies of a Hausa village noted high consumption of both foods and medicine that have antiplasmodial potential during the malaria season (35).

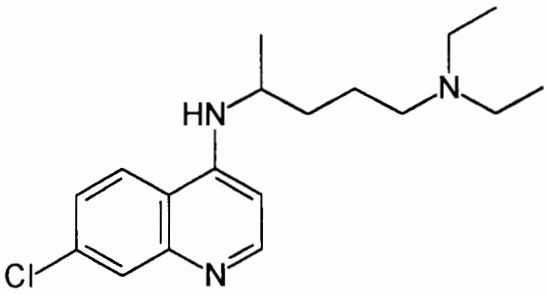
New antimalarials with novel modes of action

Since there are no effective vaccines and still no sign of a vaccine in the near future, new chemotherapeutic agents or antimalarial drugs, preferably with novel modes of action against *Plasmodium* are urgently being sought. Chemotherapy often produces serious adverse side effects e.g. Fansidar^{R} (pyrimethamine and sulphadoxine) may cause epidermal necrosis (14). Since traditional plants may provide a rich source of potential antimalarial drugs it made good sense to investigate medicinal plant preparations used by local traditional healers for symptoms of malaria. Plants may directly provide new antimalarials e.g. quinine from Cinchona bark. Alternatively, they could provide template molecules on which to base synthesis of further novel structures. Quinine led to the development of synthetic drugs such as chloroquine, amodiaquine, primaquine and more recently, mefloquine (14,36).

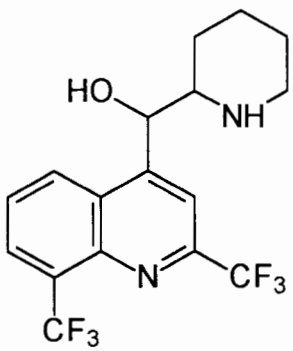
There is certainly great potential for novel synthetic leads offered by natural plant products.



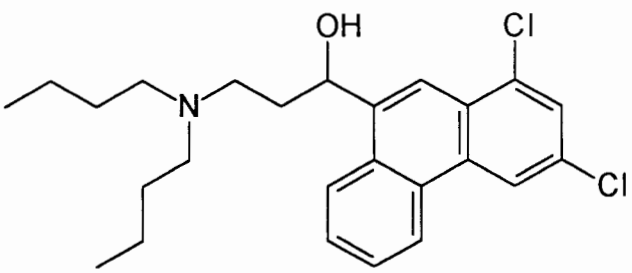
Quinine



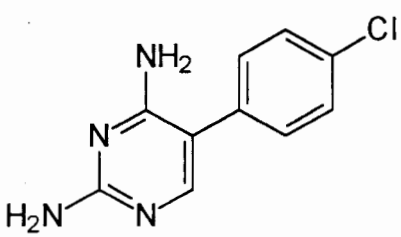
Chloroquine



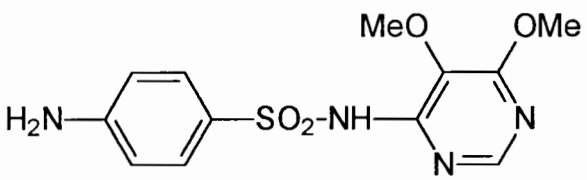
Mefloquine



Halofantrine



Pyrimethamine



Sulfadoxin

Fig. 1: Chemical structures of some antimalarial drugs in clinical use.

Artemisinin - the Chinese antimalarial

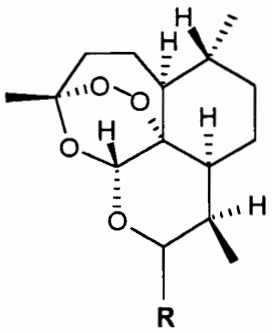
In China herbal medicines are as important as synthetic drugs and account for 30-50% of the total consumption of drugs (11,37). An extensive search for new antimalarials from traditional sources in China which started in 1967 led to the isolation of an active antimalarial fraction from *Artemisia annua* L. (sweet wormwood) (Asteraceae) in 1971. The active compound, Qinghaosu or Artemisinin was isolated and its structure was determined the following year (3,38). Qinghaosu literally means "extract of qinghao (green herb)". The herb has been used for more than 2 000 years as an antipyretic and for symptoms of malaria (3,11). The plant is widely distributed in Europe, North America, India and Eastern Asia (38).

Artemisinin and its derivatives

Artemisinin or Qinghaosu is a sesquiterpene lactone peroxide which belongs to a new class of antimalarials called the endoperoxides. Artemisinin is structurally different from other known antimalarial drugs (Fig. 2). It is not water-soluble, but decomposes in a number of protic solvents, which probably cause the lactone ring to open. The compound is stable in aprotic solvents up to 150°C (3). The unusual endoperoxide moiety appears to be essential for antimalarial activity because derivatives that lack the endoperoxide ring do not have antimalarial activity. Substitutions at the lactone carbonyl group results in an increase in potency (3,38,39).

The first-generation endoperoxides

Dihydroartemisinin, a borohydride-reduction product of artemisinin, is more potent than artemisinin (3). This compound was converted into a number of ether derivatives, namely, artemether (methyl ether of dihydroartemisinin) and arteether (an ethyl ether of dihydroartemisinin) which are both oil-soluble.



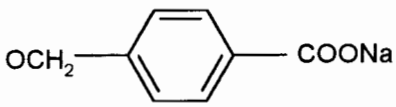
	R
Artemisinin	=O
Dihydroartemisinin	OH
Artemether	OMe
Arteether	OCH_2Me
Na^+ artesunate	$OCO(CH_2)_2COONa$
Na^+ artelinate	

Fig. 2: Chemical structure of artemisinin and its derivatives.

These derivatives represent the first generation endoperoxides. Artemether was initially prepared for intramuscular injection but it can now be taken orally too (4). Artmether injection (Paluther^R) is registered in Brazil, China, Burma, Thailand and in at least 9 African countries (40). Capsules are also produced. Phase II trials in adult patients with non-severe multidrug resistant falciparum malaria were completed in 1995 and the results are likely to be published soon. Meanwhile, clinical trials of intramuscular formulations in patients with severe falciparum malaria were initiated in 1995 and will continue for about 2 years (40). Artesunate tablets and injection are registered in Brazil, China, Ghana, Burma, Thailand and Vietnam (40). An oral formulation of sodium artelinate was found to be as effective as artesunate (40,41). Other derivatives included

carboxylic esters, carbonates and sulfonates. Carbonates are the most potent, followed by esters, ethers, and then artemisinin (3,11).

The second-generation endoperoxides

The total stereoselective synthesis of artemisinin has made it possible to synthesize the artemisinin derivatives other than the ethers or esters of the dihydroartemisinin lactol. The second generation endoperoxides are synthetic compounds. Literally hundreds have been synthesized. Many resemble artemisinin but others such as the trioxanes and tetraoxanes do not (40).

Cultivation

Only *A. annua* and *A. apiacea* have so far been reported to contain artemisinin (38). Artemisinin was either lacking or only found in trace amounts in about 100 other species of *Artemisia* (42). Artemisinin is obtained from the leaves of *A. annua*. Its yield is variable and it depends on a number of factors such as plant strain, stage of development, environmental and soil conditions. Maximum yields of artemisinin from cultivated *Artemisia* are about 2% of plant dry weight whereas, values in the wild are usually 0.01-0.5% (38,43) but higher concentrations have been reported from Chinese clones (44). The highest content is either just before flowering (38,45) or in the inflorescence itself at anthesis (42). In Vietnamese plants however, the highest possible yield of artemisinin was found at the early developmental stages i.e. 5 month old plants (46). This was probably as a result of the tropical climate which meant that the vegetation period of *A. annua* is longer than in countries with moderate climates. Therefore, plants can be harvested twice, instead of once a year. Drugs in current use are all derived from cultivated *A. annua*.

Plant biotechnology

Because chemical synthesis of artemisinin is complicated (although possible) and has provided poor yields, the plant remains the only viable source of supply for artemisinin and the subsequent synthesis of its derivatives (46,47). Numerous laboratories are therefore searching for strains of *A. annua* that contain the highest levels of artemisinin. Experiments have so far included field grown plants, plants grown in greenhouses, and tissue-cultured plants (42,43,44,46,47). A Chinese clone has been reported to produce more than 1% artemisinin which is considerably greater than the general range of 0.01-0.50% (44).

Antimalarial activity

Dihydroartemisinin, artemether and artesunate, first developed in China have been used to treat *P. falciparum* and *P. vivax* infections in countries such as China, Vietnam, Burma, Thailand, Gambia and Kenya (4). These compounds are particularly useful for the treatment of severe malaria. They are the most rapidly acting of all the antimalarials and have the broadest stage specificity (asexual and sexual) of antimalarial action. In contrast, quinine, chloroquine, hydroxychloroquine, amodiaquine and quinacrine, chlorguanide and pyrimethamine, sulfonamides and sulfones are sensitive only to the asexual erythrocytic stages (48). Primaquine is effective against the sexual erythrocytic stage (gametocidal) (48). Artemisinin and its derivatives produce a more rapid clinical and parasitological response as well as reduce fever more quickly than any of the other antimalarial drugs (38,49).

Pharmacological experiments have shown that artemisinin and its derivatives affect the erythrocytic stages of *Plasmodium* in nanomolar concentrations (45,50). The drugs have fast schizonticidal action (11). Clearance of parasitemia is achieved within 24 hours. The mature stages of the schizonts of *P. falciparum*

were found to be more susceptible to the drugs than the ring stages (11). In contrast to these findings, ter Kuile and co-workers found that the maximum effect of artemisinin and sodium artelinate was against the late ring and early trophozoite. Antimalarial action was rapid after a lag phase of 1-4 hours (51). The drug is also reported to have gametocidal activity which could be important for blocking transmission of the disease (45,49). The drug unfortunately cannot be used for prophylaxis because of its short half-life. It also lacks sporontocidal activity i.e. activity against the mosquito-stage sporozoites and causal prophylactic activity i.e. activity against the pre-erythrocytic stages. Hypnozoitocidal activity is also absent i.e. inactive against the liver stages of the parasite (49).

Resistance

Artemisinin and its derivatives are effective against *Plasmodium* species that have developed resistance to other antimalarial drugs, both *in vitro* and *in vivo* (11). They have not demonstrated any cross resistance with chloroquine in drug-sensitive and drug-resistant strains of *P. falciparum in vitro* (11). Although there is cross resistance of artemisinin with chloroquine in mice infected with *P. berghei* (11) to date, there is no cross resistance with other currently used antimalarial drugs (49). Interestingly, in a multidrug-resistant strain of *P. falciparum*, synergism has been found between arteether and mefloquine or quinine *in vitro* (45).

The mechanism of drug resistance is not clearly understood. It is clear that *P. falciparum* parasites that are resistant to chloroquine concentrate less drug in the food vacuole than do sensitive parasites which seems to suggest that the mode of action of the drug is independent of the mechanism of resistance (52). Decreased accumulation of chloroquine in resistant parasites could occur by two mechanisms: firstly, efflux of the drug from the food vacuole and secondly,

alterations that reduce the uptake of the drug in the parasite. There is also the possibility that both mechanisms could be operating together (52).

Pharmacokinetics and drug metabolism

Pharmacokinetic studies have been hampered by difficulties encountered in developing a reproducible assay for the parent drug and its metabolites even though a variety of methods such as HPLC using an electrochemical detector (53), packed-column supercritical fluid chromatography with electron-capture detection (54), and more recently HPLC with chemiluminescence detection (55), radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA) (56) are also available. It is hoped that the most recent attainment of the stereoselective total synthesis of radiolabelled (deuterated) artemisinin, dihydroartemisinin and arteether (57), will boost further studies into the pharmacokinetics and drug metabolism of this class of drugs.

Oral administration of artemisinin leads to low plasma levels of drug and rapid first pass metabolism to inactivate metabolites. However, the derivatives are hydrolysed mainly to the active derivative, dihydroartemisinin and to other more hydrophilic metabolites. Intramuscular and intravenous administration improves drug plasma levels and drug efficacy (49).

Artemisinin and its derivatives bind avidly and irreversibly to membranes including to those of normal erythrocytes. They may also bind covalently to plasma proteins. In plasma, arteether binds both to albumin and to α_1-acid glycoprotein. Binding is 20-fold greater for the glycoprotein. In plasma about 75% of the drug is bound. Intravenous artesunate is cleared very rapidly by biotransformation and is eliminated with a half-life of 45 minutes (38). Artemisinin is rapidly distributed in the tissues; it passes the erythocytic wall, the blood-brain barrier, the salivary glands and the placenta (45).

Recrudescence

The one major disadvantage of the artemisinin derivatives is the high rate of recrudescence i.e. recurrence of the disease after a brief intermission period (3,38). Recrudescence is greater than 10% which is unacceptably high (40). Interestingly, there are lower recrudescent rates reported when the artemisinin derivatives are combined with other antimalarial drugs, in particular mefloquine. As mentioned earlier, the artemisinin derivatives and mefloquine have been reported to be synergistic both *in vitro* and *in vivo* (49). The combination of artesunate and artemether with mefloquine in the treatment of falciparum malaria has substantially improved efficacy. Cure rates for 3-5 days of combined treatment still exceeds 85% even though the sensitivity to mefloquine is expected to continue to decline (58).

Mechanism of action

Changes of the ultrastructure of parasite membranes after exposure to the drug have been described. Meano *et al.* (59) reported ultrastructure changes induced by *P. falciparum in vitro* by artemisinin and dihydroartemisinin. The drugs accumulated in the food vacuole and mitochondria. An additional incubation period of 2-4 hours revealed changes in the parasite mitochondria, rough endoplasmic reticulum, nuclear envelope, nuclear and plasma membranes. There was also a disappearance of ribosomes and destruction of the food vacuole membranes (11,45,49). Artemisinin and its derivatives have also been shown to have the following effects in malaria parasites: membrane damage, alkylation and oxidation of proteins, oxidation of fats, inhibition of protein and nucleic acid synthesis as well as interaction with cytochrome oxidase and the glutamine transport system (45).

Since *Plasmodium* decompose host-cell haemoglobin to obtain essential amino acids in the food vacuole, it was suggested that artemisinin reacts with

haemozoin, thereby causing antimalarial activity. Evidence to support this hypothesis came from an *in vitro* experiment that demonstrated a reaction between haemozoin and artemisinin (39). Intraparasitic haeme-activated artemisinin irreversibly decomposed to generate free radicals that alkylate and oxidise proteins (haemozoin) and lipids (38,39). Since the action of these compounds are potentiated by oxygen and oxidant drugs such as miconazole and doxorubicin (and attenuated by reducing agents such as catalase, dithiothreitol and α-tocopherol) (3,45), the oxidative stress already imposed by the parasite on its erythrocyte host is augmented and surface recognition signals are presumably enhanced (38). This serves to promote cytotoxicity, phagocytosis and clearance by the host leucocytes (38). It was also found that when *P. falciparum* infected red blood cells were exposed to radiolabelled artemisinin, it was possible to isolate a haem-artemisinin adduct. It was suggested that the same occurs *in situ*. No such adduct was isolated when uninfected red blood cells were exposed to radiolabelled drug. This suggests that the drug does not react with haemoglobin-bound haemozoin. It could also account for the selective toxicity of the drug to the parasites (39). Further evidence for the role of haemozoin comes from an *in vivo* experiment of a chloroquine-resistant *P. berghei* strain which lacked haemozoin. Artemisinin was found to be more than 50 times less effective against the strain which indicated that the parasites were insensitive to the drug (39).

Furthermore, it has been suggested that the antimalarial activity of artemisinin in animal cell cultures is associated with the dramatic reduction in the concentration of polyamine putrescine (45). Antimalarial activity has been shown not to be mediated via intercalation with nucleotides as is the case of the aminoquinoline-type antimalarials (40,45).

Artemisinin and its derivatives have now been shown to affect parasites very differently from other oxidant drugs. Instead of reacting with oxygen and producing large quantities of oxygen-containing free radicals such as superoxide and $OH^{\bullet}$, artemisinin itself, becomes a free radical in a reaction catalysed by iron (40). *In vitro*, it was found that haem and iron catalyse the conversion of artemisinin and its derivatives into free radicals in a manner similar to the way that they catalyse the decomposition of hydrogen peroxide into free radicals. Using cyclic voltammetry it was shown that haem caused a 0.6-V shift in the reduction potential of artemisinin, which indicates that haem accelerates the decomposition of artemisinin by several orders of magnitude (39). The iron-catalysed generation of a free radical from artemisinin was confirmed by electron paramagnetic resonance spectroscopy and by studies of isolated erythrocyte membrane (40). The mechanism of iron-mediated decomposition of artemisinin and its derivatives *in vitro* has been determined by the structural elucidation of the decomposition products and by mechanistic studies on their rates of formation. The reductive cleavage of the endoperoxide bridge involves the transfer of an electron from ferrous iron (i.e. Fe[II]) and the formation of Fe[IV]=O (60). The endoperoxide bridge is cleaved first and then there is intramolecular electronic rearrangements which produce carbon-centred radicals. These carbon-centred radicals are important because artemisinin derivatives that do not form such radicals have no biological activity (40).

In summary, artemisinin and its derivatives are thought to exhibit a two step mode of action as proposed by Meshnick *et al.* (40,61). In the first step, artemisinin compounds are activated by haem or molecular iron to produce free radicals and electrophilic (alkylating) intermediates. In the second step, these reactive species react with and damage specific malaria membrane-associated proteins. Therefore, artemisinin derivatives are free radical generators. The resulting free radicals are however, carbon-centred. There are also specific

target proteins that are involved (40,61). Elucidation and identification of these target proteins are yet to be achieved.

Toxicity

From the available literature, artemisinin is remarkably non-toxic even at high doses (45). In a study by Meshnick *et al.* (45) mice were not adversely affected with doses as high as 2g/kg body weight. The therapeutic dose of artemisinin in mice is about 4mg/kg. In monkeys however, high doses have been shown to cause inhibition of haematopoiesis, as well as cardiac, renal and hepatic toxicity (45). In dogs and rats a selective and dose-related neurotoxicity in the central nervous system and mid-brain had been found (45). There is also toxicity reported of artemisinin derivatives to neuronal cells *in vitro* where protein synthesis is inhibited (62).

In humans, no significant toxicity has been reported. This is in stark contrast to the quinoline antimalarials. In clinical studies of over 4 000 patients there is no evidence of toxicity (38). However, it has been suggested that there is a possibility of cumulative toxicity due to a long-lived toxic metabolite, especially if the drugs are misused when given as prophylaxis or when patients are treated frequently (38). The China Cooperative Research Group on Qinghaosu and Its Derivatives as Antimalarials has stated that the artemisinin drugs can be safely used for malaria patients with cardiac, hepatic or renal disorders (11).

Distribution of *Artemisia*

The genus *Artemisia* (Asteraceae, previously Compositae) with more than 500 species, including the sometimes separated genus Seriphidium (63) is very widely distributed. It is predominantly found in the northern temperate regions of the world (i.e. North America, Europe, Asia and North Africa) with a

southward extension towards the tropics. *A. afra* grows at an altitude of 2 400m in Tanzania, Kenya and South Africa. The following Artemisia oils are commercially available: *A. absinthium* (wormwood); *A. annua*; *A. pallens* (davana oil) used to flavour tobacco; *A. dracunculus* (tarragon) used as a herb in cooking; and *A. herba alba* (armoise) which is used in the perfumery industry (64). *A. absinthium* was used until the 1920s to prepare the narcotic and now illegal (due to its neurotoxicity) drink called absinthe (3).

Reasons for investigating *A. afra*

Since *A. afra* is commonly used by traditional healers for symptoms and treatment of malaria, and because it belongs to the same family as *A. annua*, from which artemisinin was isolated (chemotaxanomic approach), it seemed appropriate that the South African variety be investigated for antimalarial activity. Furthermore, the Tanzanian variety demonstrated antimalarial activity with IC_{50} values of 10-49μg/ml but the active compound has as yet, not been identified (65). Also, although there have been a number of phytochemical studies conducted on *A. afra* (66,67,68) none of the isolated compounds had been screened for antimalarial activity.

Artemisia afra

A. afra Jacq. (African wormwood) is a bushy branched perennial shrub that grows up to 1 metre (68). It is the only species of *Artemisia* that occurs in South Africa (67). The plant can be found throughout the Amatola and Drakensberg mountain regions of South Africa. It can be propagated from seeds, cuttings and root pieces. Its grey-green leaves are aromatic when crushed and its essential oil has a similar fragrance to armoise. Other names for the plant include: wildeals (Afrikaans); lengana (Tswana); zengana (Southern Sotho); umhlonyane (Xhosa); and mhlonyane (Zulu).

Medicinal properties

A. afra is one of the most widely used medicinal plants in Southern Africa. It is applied to a large range of illnesses, which include the common cold, *diabetes mellitus*, respiratory complaints and gastric intestinal tract disorders, toothache and earache. The leaves are used as a tea, for a bath, as a leaf poultice or vapours from boiling leaves can be inhaled. It is also used as an insect repellant and as an antithelmintic (5,69). The essential oils of *A. afra* were shown to exhibit antibacterial, antifungal and anti-oxidant properties (68).

Toxicity

There has been only one toxicity experiment on the essential oil of *A. afra*, performed on rabbits (oral administration) which resulted in haemorrhagic nephritis, degenerative change in the liver and pulmonary oedema (5). These results may not necessarily be reflected in humans. Since the registration of the essential oil of *A. afra*, there have been no reports of toxicity and neither can any reports be found on toxicity of the plant when used medicinally in humans.

Proposed strategy for the investigation of *A. afra*

The proposed strategy for the antimalarial investigation of *A. afra* was adopted from a similar strategy reported by Hamburger and Hostettmann (70). The following steps were involved:

(i) positive identification of the plant by a botanist, collection and drying of the plant material;

(ii) preparation of crude extracts and preliminary chromatographic analysis by TLC;

(iii) bioassay of crude extracts;

(iv) several consecutive steps of chromatographic separation with each fraction obtained submitted for bioassay in order to follow the activity;

(v) verification of the purity and activity of the isolated compounds;

(vi) structure elucidation of isolated pure compounds;

(vii) toxicological testing of isolated pure compounds.

As mentioned earlier, medicinal plant research requires a multidisciplinary approach. Although the search process for pharmacologically active compounds from plants is very long, the benefits (economic, cultural and social) to be derived therefrom, are enormous.

3. Aims and Objectives

Aim

To fractionate a crude extract of *Artemisia afra* that has demonstrated antimalarial activity *in vitro* against *Plasmodium falciparum* in order to purify and identify the active antimalarial compound(s).

Objectives

1. Purification of *A. afra* crude extract in order to identify the active compound(s).

2. To compare antimalarial activity *in vitro* of the purified compound(s) against a variety of strains of *P. falciparum* which have varying degrees of sensitivity and resistance to both chloroquine and mefloquine.

3. To determine whether or not extracts of *A. afra* contain artemisinin.

4. To distinguish general cytotoxic properties of the compound(s) from *in vitro* antimalarial activity against *P. falciparum* using the MTT assay.

4. Methods

4.1. Plant material

Artemisia afra Jacq. was collected from the National Botanical Gardens (Kirstenbosch) in Cape Town. The aerial parts of the plant which were harvested on two occasions in 1996 were allowed to air-dry at room temperature away from direct sunlight. The first harvest took place in January before the flowering season. The second harvest was at the end of the flowering season and at the stage when seeds were developing, in May.

The material of the crude extract which was of leaves and flowers, active *in vitro* against *P. falciparum* had been collected in April 1993 during the flowering season.

4.2. Extraction

Initial plant extractions for *A. afra* were performed in a Soxhlet apparatus (Appendix A) which involved heating of the solvent to high temperatures. Even though this method is used to extract artemisinin from *A. annua* (3,71) there was concern that there may be compounds of interest that could be heat sensitive and were therefore, being degraded. As a result, cold extractions with dichloromethane and water at room temperature were also performed.

Dried aerial parts of *A. afra* were divided into 3 batches. The first batch was continuously extracted with petroleum ether (b.p. 40-60^{o}C) in a Soxhlet apparatus for 21 hours. The second batch was exhaustively extracted at room temperature in dichloromethane for 24 hours at a time. The third batch was extracted in distilled water for 3 × 24 hours, also at room temperature.

A sample of *A. afra* collected during the flowering season was continuously extracted with distilled water in a Soxhlet for up to 28 hours so that it could be compared to the cold water extract.

The crude extract prepared in 1993 which was of leaves and flowers had also been extracted with petroleum ether (b.p. 60-80°C) in a Soxhlet for 14 hours.

The extracts obtained were clarified by filtration and then concentrated *in vacuo* in a rotary evaporator (Buchi 461). Water extracts were dried in a freeze drier (Virtis Company Inc.). All the extracts were stored in sealed glass vials in a refrigerator until needed.

4.3. Column chromatography

The crude extract of leaves and flowers, 4.6g, was dissolved in 20ml dichloromethane and heated gently before being applied to a silica gel-60 (0.063-0.200mm) column (86.50×2.50cm). Components of the extract were initially eluted with 100% petroleum ether followed by petroleum ether with increasing amounts of ethyl acetate and finally with methanol. As fractions were collected they were analysed by TLC and the polarity of the solvent mixture was increased only when elution with a particular solvent composition was complete. A total of 120 fractions were collected which were then pooled on the basis of their TLC profiles. The resulting fractions, P1-P9, were screened for antimalarial activity.

4.4. Thin layer chromatography

Samples were dissolved in dichloromethane and applied to TLC aluminium sheets pre-coated with silica gel-60 (F_{254}) with 0.2mm layer thickness (Merck). Pure artemisinin (Sigma) (1mg/ml) was prepared in the same way and applied as a standard. The plates were dried using a hair dryer, after which they were

transferred to a tank containing the developing solvent system (mixtures of petroleum ether, dichloromethane and ethyl acetate). As soon as the plates were developed (± 5 minutes) the solvent front was marked and the plates were blown dry. To visualize the spots, the plates were immersed in an anisaldehyde stain reagent. Excess anisaldehyde stain was blotted off with paper towel and the plate was then heated gently on a hot plate until the colour reaction was complete.

The anisaldehyde reagent was prepared in the following order with thorough cooling with liquid nitrogen before addition of glacial acetic acid and concentrated sulphuric acid: 6.5ml p-anisaldehyde (Riedel-de Häen) in 232ml 95% ethanol; 2.5ml glacial acetic acid and 7.5ml concentrated sulphuric acid. Sulphuric acid needed to be added slowly.

4.5. Flash Chromatography

Fraction P6, 372mg, was chromatographed under pressure on a silica gel-60 (230-400 mesh) column (60.0×1.7cm) to yield 5 sub-fractions labelled 1 to 5 (Fig.3). The initial solvent composition was petroleum ether/ethyl acetate [3:1] and followed by petroleum ether/ethyl acetate [3:2] for complete elution of the sample components.

4.6. Centrifugal Thin Layer Chromatography

Fraction P6.2, the second sub-fraction of fraction P6, appeared to contain the major compound. This fraction (199mg) was chromatographed on a chromatotron, which is a preparative, centrifugally accelerated, radial, thin-layer chromatograph (Appendix B). The absorbent layer containing 70g silica gel-60 PF_{254} with gypsum for preparative layer chromatography (Merck), 5g calcium sulphate ($CaSO_4.½H_2O$) and 132ml H_2O at 0-5°C was cast onto the

rotors, allowed to set (25min) and then dried overnight in an oven at 70°C before being scraped to 2mm thickness with a rotating scraping tool. A gradient solution of petroleum ether/ethyl acetate [90:10], petroleum ether/ethyl acetate [80:20] and petroleum ether/ethyl acetate [60:40] was used to elute the compounds. Three fractions labelled A, B and C were generated, of which sub-fraction B was the major compound.

The second sub-fraction, P6.2B was repeatedly chromatographed on the chromatotron using petroleum ether with increasing amounts of ethyl acetate. A mixture of petroleum ether/dichloromethane/ethyl acetate [25:3:10] was finally used as eluent and the fraction was then purified by flash chromatography (42.0×1.5cm) using the same procedure. The total yield of purified P6.2B from fraction P6 was 113.5mg of which 26mg was submitted for NMR analysis. Fig. 3. below summarizes the fractionation sequence for the crude *A. afra* extract.

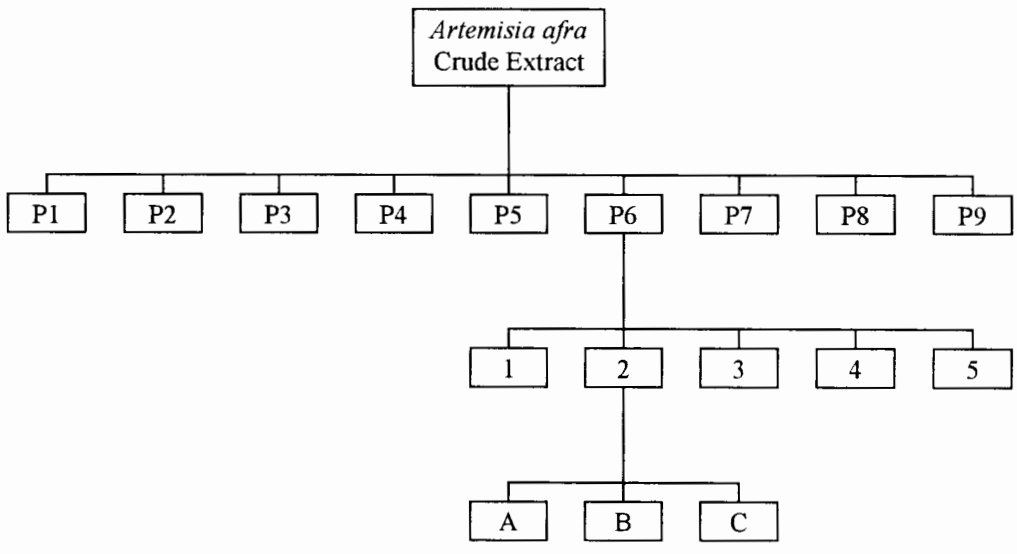


Fig. 3: Fractionation Sequence.

4.7. High pressure liquid chromatography

Reverse-phase high pressure liquid chromatography (HPLC) (Shimadzu LC-10AS, Shimadzu Corp., Kyoto, Japan) linked to a diode array detector (Shimadzu SPD-M10A) and two Shimadzu LC-10 AS liquid chromatographic pumps, a 486 DX2-50 personal computer and a Hewlett Packard Deskjet 600 printer was performed using an analytical C_{18} Prodigy ODS-2 column with dimensions: 150×4.60mm 5 micron (Phenomenex, California USA). The mobile phase was a gradient of MeCN (HPLC-grade) and distilled, deionized water (Millipore). A guard column was used throughout experiments to retain particulate matter, especially from crude plant extracts. The flow rate was 1ml/min.

HPLC profiles (45min) of artemisinin, crude *A. afra* extracts and the isolated pure compound, P6.2B, were obtained.

4.8. Detection of artemisinin by thermospray LC-MS

Three samples of crude *A. afra* extracts were sent to Prof. K. Hostettmann at the Institute of Pharmacognosy and Phytochemistry, University of Lausanne, Switzerland, for an accurate detection of artemisinin using thermospray LC-MS (72). The samples were: the crude petroleum ether soxhlet extract of leaves and flowers; the cold dichloromethane extract of leaves; and the petroleum ether soxhlet extract of leaves.

Each extract (100mg) was dissolved in 1ml of a THF/MeOH (50:50) solution of which 10μl was injected onto the HPLC. Effectively, 1mg of extract was injected onto the column.

The crude extracts were separated on a C_{18} column (Novapak, 4μm, 150×3.9mm) using MeCN-H_2O gradient (40:60 to 60:40) in 20 min (1ml/min). The LC-MS analysis was carried out with a thermospray (TSP) interface

mounted on a triple quadrupole MS instrument (Finnigan MAT). Optimisation of the TSP parameters was performed by loop injection of pure artemisinin.

The TSP LC-MS analyses were carried out in the positive ion mode with post column addition of buffer (ammonium acetate 0.5M, 0.2ml/min). The best sensitivity was found for a source temperature of 280°C and the vaporizer at 90°C. The TSP spectra were recorded from 150-500 μ every 2 seconds.

To test the detection limit of the method, one of the extracts, the cold dichloromethane extract of *A. afra* leaves, was spiked with 1μg of artemisinin before being injected onto the HPLC column.

4.9. Cultivation of parasite cultures

Seven strains of *P. falciparum* were cultured routinely. They were D10, FAC8, W_2mef and W_2mef^{CQ} from Dr. Alan Cowman, Walter Eliza Hall Institute, Melbourne, Australia; and RSA3, RSA11 and RSA15 obtained from Dr. Janet Freese, Medical Research Council, National Malaria Programme, Durban, South Africa.

D10 originated from FCQ-27 in Papua New Guinea (73). FAC8 was regenerated from the ItG2 Fb strain in Brazil (74). W_2mef was from W_2 which had been drug-pressurized with mefloquine. The W_2 strain originated from the hybridization of Indochina and Sierra Leone strains (75). W_2mef^{CQ} was drug pressurized with chloroquine (76). There were 3 South African strains: RSA3, originated from North East Transvaal; RSA11 and RSA15 originated from KwaZulu-Natal (16).

Type O^+ human erythrocytes (obtained from Western Province Blood Transfusion Services and Haematology Department, U.C.T./G.S.H.) with *Plasmodium falciparum* i.e. parasitized red blood cells (PRBC) were cultured in

HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) (6g/l) buffered RPMI 1640 (BioWhittaker) culture medium supplemented with glutamine (10.4g/l), glucose (4g/l), hypoxanthine (44mg/l), gentamycin (40mg/ml), 0.2% $NaHCO_3$ and 10% human A^+ serum (obtained from Western Province Blood Transfusion Services and Haematology Department, G.S.H.) in tissue culture flasks (Corning) and incubated at 38°C.

4.9.1. Changing medium

Medium was changed daily. Cells were suspended at 5% hematocrit and with parasitaemia usually not more than 5%. PRBC were centrifuged at 600g for 5 minutes. The supernatant was removed and 1-2μl of PRBC were aspirated to prepare a smear. Fresh medium was added to the cell pellet and gently swirled. Uninfected type O^+ RBC were added every 3-4 days, or when parasitemia was greater than 10%. The cell suspension was transferred to a tissue culture flask and flushed with filtered gas containing 3% O_2, 4% CO_2 and 93% N_2 for 20 seconds. The lid was tightened and the flask was returned to the incubator.

4. 9.2. Identification of parasites - staining of slides

The blood smear was fixed with methanol and then covered with Giemsa stain (1 part Giemsa to 10 parts phosphate buffered saline (PBS)) for 5 minutes. The microslide was gently rinsed with water and allowed to air dry, before viewing under the microscope.

4.9.3. Cryostorage and thawing

Glycerol (28%) was prepared in PBS and filter sterilized using a 0.22μm filter (Millipore Millex-GS filter unit). This solution was then mixed with PRBC containing more than 1% rings in a ratio 1:1 (v/v). The suspension mixture (1ml) was transferred into cryotubes and dropped into liquid nitrogen.

Frozen parasites were thawed quickly at 37°C and transferred to a 10ml tube. Filter sterilized 3.5% NaCl (1ml/0.5ml cells) was added to the cell suspension, mixed well and allowed to stand for 1 minute. The cell mixture was centrifuged at 600g for 2 minutes and the supernatant discarded. The cell mixture was washed twice more with NaCl, and each time centrifuged and the supernatant discarded. Uninfected RBC and medium were added to the cell pellet. The cells were flushed with gas and placed in the incubator.

4.10. Antimalarial screening

This rapid, semi-automated microdilution method measures the activity of potential antimalarial drugs against cultured intraerythrocytic asexual forms of *P. falciparum* (32). Microtitration plates allow for quantitative measurement of the antimalarial activity of a number of compounds simultaneously. The assay is based on the inhibition of uptake of a radiolabelled nucleic acid precursor, hypoxanthine, by parasites. To avoid competition between the radiolabelled hypoxanthine and unlabelled hypoxanthine in culture medium for uptake by the parasites, hypoxanthine was excluded from the culture medium.

4.10.1. Preparation of drugs and plant extracts

Chloroquine (CQ) obtained from Sigma (diphosphate salt), was soluble in culture medium. Stock solution, 1mg/ml was prepared (filter sterilized) and diluted in culture medium to yield the following final drug concentrations: 400, 200, 100, 50, 10, 1, 0.1 and 0.01ng/ml.

Artemisinin and mefloquine were each dissolved in MeCN (HPLC grade), 1mg/ml. Of this stock solution 18μl was transferred to an Eppendorf vial with medium so that the total volume was 1ml. From this sample further dilutions were prepared with the following final drug concentrations 100, 50, 10, 1, 0.1

and 0.01ng/ml. The highest final concentration of MeCN that was used was 0.002% in the parasite culture.

Stock solutions of 2mg/ml were prepared for the crude extracts, initially dissolved in MeCN and then diluted in culture medium to yield the following final concentrations: 100, 10, 1, and 0.1μg/ml. The highest final concentration of MeCN was 0.56%.

For all the fractions, stock solutions of 1mg/ml in MeCN were diluted in culture medium to achieve final concentrations of 30, 10, 1, 0.5, 0.1, 0.01, 0.001 and 0.0001 μg/ml with the highest final concentration of MeCN of 3% which had no adverse effect against D10 and FAC8.

P6.2B (2mg/ml stock) also dissolved in MeCN was diluted in culture medium to yield final concentrations of 100, 10, 1, 0.1μg/ml, with the highest MeCN concentration of 0.56%.

For all the crude H_2O extracts, 100mg/ml stock solutions in culture medium were prepared and diluted so that final concentrations of 10 000, 1 000, 500, 200, 100, 10, 1 and 0.1μg/ml were achieved.

4.10.2. Preparation of microtitration plates

The microtitration plates used were 96 well flat-bottomed tissue culture plates (Bibby, Sterilin). First, 200μl of the parasite culture (haematocrit 1.5%, parasitemia 1%) was dispensed into each of the test wells using an Eppendorf dispenser and 200μl of 1.5% of uninfected RBC, suspended in culture medium, was aliquotted to the control wells. Each drug concentration (25μl) was then added to each of the test wells in triplicate by an Eppendorf dispenser. Culture medium (25μl) was added to the control wells. All tests were conducted in triplicate.

A control experiment was set up to investigate the effects of MeCN against the *P. falciparum* strains. MeCN (25μl) was added to wells containing 200μl uninfected RBC (haematocrit 1.5%) and to wells containing PRBC (parasitemia 0.5%, haematocrit 1.5%) so that the final MeCN concentrations were: 3.0, 1.11, 0.56, 0.28, and 0.06 %.

The plates were then placed in a desiccator and flushed with the gas mixture (described in section 4.8.1.) for 2 minutes. The desiccator was sealed tightly and incubated for 24 hours at 38°C. After the 24 hour incubation period, the plates were removed from the desiccator and 25μl of the labelled hypoxanthine in culture medium, prepared as described below, was added to each well. The plates were then returned to the desiccator, flushed with the gas mixture for 2 minutes, sealed and incubated (38°C) for a further 21 hours.

4.10.3. Preparation of isotope

[G-^{3}H]-Hypoxanthine (Amersham) was used as an indicator of parasite growth. The isotope, supplied as a lyophylate (1 000 mCi/mmol) in ampoules containing 1.0mCi, was dissolved in 2.0ml of 50% ethanol (filter sterilized) to provide a stock solution which was stored at 4°C.

The ethanol of 0.1ml stock solution was evaporated using N_2 gas. To the remaining 0.05ml of the isotope in water, 2.45ml of culture medium was added. Thus, the final isotope solution consisted of 20μCi of [G-^{3}H]-hypoxanthine per ml culture medium.

4.10.4. Harvesting of parasites

At the end of the 21 hour incubation period, the plates were harvested using a PHD cell harvester (Cambridge Technology, Inc.). This instrument aspirated and deposited the particulate contents of each of the wells onto small discs of

glass fiber filter strips (Macherey-Nagel: retention 0.5μm, 0.4mm thickness) which were then washed 5 times with distilled water. Each disc was dried and placed in a plastic scintillation vial containing 4ml of scintillation cocktail (Packard Emulsifier Scintillator 299 for aqueous and non aqueous samples) and left on a shaker overnight. The vials were then counted (DPM) in a Packard Tri-Carb 1900 CA liquid scintillation analyzer for 1 minute each.

4.10.5. Analysis of data

The mean DPM (disintegrations per minute) values for each drug or extract concentration and control wells were calculated. The mean DPM was then converted to a percentage of total [G-^{3}H] hypoxanthine as follows:

$$\frac{\text{DPM of drug or extract} \pm \text{SD} - \text{DPM of RBC control} \pm \text{SD}}{\text{DPM of PRBC control} \pm \text{SD} - \text{DPM of RBC control} \pm \text{SD}}$$

The percentage of [G-^{3}H]-hypoxanthine incorporated (expressed as a percentage of the control) was then plotted against the logarithmic values of the corresponding drug concentrations (77). The inhibitory concentration of the drug or extract which results in 50% [G-^{3}H]-hypoxanthine incorporation into the parasites is known as the IC_{50} value. IC_{50} values were calculated as follows:

$$\log (IC_{50}) = \log (x_1) + \frac{y_1 - y_0/2}{y_1 - y_2} \; [\log (x_2) - \log (x_1)]$$

where two concentrations, x_1 and x_2 are such that the percentage [G-^{3}H]-hypoxanthine incorporation, y_1, at concentration x_1 (and lower concentrations) is more than half of the percentage in the control, y_0, and the percentage [G-^{3}H]-hypoxanthine incorporation found at x_2 (and all higher concentrations) is less

than half of y_0. The IC_{50} is then found by linear interpolation between x_1 and x_2 (Fig. 4) below.

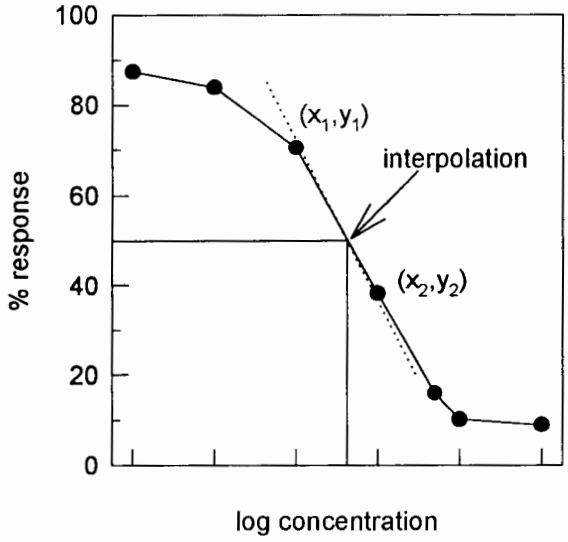


Fig. 4: Schematic diagram demonstrating the interpolation method for calculating IC_{50} values.

4.11. Cytotoxicity screening

There was a need to distinguish a general cytotoxic effect from an antimalarial effect of the crude plant extract and its isolated pure compound. Hence the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was set up (78). Viable cells are able to reduce the water-soluble yellow coloured MTT to a water-insoluble purple coloured formazan product. The amount of coloured formazan product formed, determined spectroscopically after dissolving the formazan crystals in DMSO, is proportional to the metabolic activity and the number of cells in the test sample.

4.11.1. Preparation of cell cultures

A mammalian cell line, normal rat fibroblast cells, RAT-1, maintained in the Department of Medical Biochemistry, U.C.T., was used to screen for general cytotoxicity. The stock cultures, cultivated in sterile $75cm^3$ tissue culture flasks (NUNC) were sub-cultured every 5-7 days and kept at 37°C in an incubator that was continually flushed with 5% CO_2. Rat-1 cells were cultured in Dulbecco's Modified Earle's Medium (DMEM) containing 1% of a mixture of penicillin (100 units/ml) and streptomycin (100μg/ml) and supplemented with 10% heat inactivated (55°C for 30 minutes) fetal calf serum (Highveld Biological).

When cells were 90% confluent, a trypsin solution consisting of 0.25% trypsin and 0.1% EDTA in PBS, 5ml (BioWhittaker) was added and incubated for 5-10 minutes at 37°C. An equal volume of culture medium was then added. Fetal calf serum, present in the medium inhibits further tryptic activity which could cause cell damage.

To determine the cell density of the stock culture a Nebauer haemocytometer was used. Cells were also stained with trypan blue (Merck) which distinguished viable cells (which actively exclude trypan blue) from non-viable cells. From the stock culture a cell density of 10 000 cells/ml was prepared for addition to 96-well microtitration plates.

4.11.2. Preparation of drugs and plant extracts

Colchicine was used as a standard. The antimalarial drugs, chloroquine, artemisinin and mefloquine were also tested so as to compare their toxicities with that of the crude plant extract and the isolated pure compound. Colchicine and chloroquine were dissolved in culture medium while the other samples to be tested were dissolved in HPLC grade MeCN and then serially diluted in culture medium.

For colchicine and chloroquine 1mg/ml stock solutions were prepared and diluted to achieve the following concentrations: 1000, 500, 100, 10, 1, 0.1 μg/ml. Stock solutions of 1mg/ml in MeCN for artemisinin and mefloquine were also prepared and the following concentrations prepared: 100, 10, 1, 0.1 μg/ml. The same concentrations: 100, 10, 1, 0.1 μg/ml were also prepared for the crude extract and P6.2B (stock solutions were however, 10mg/ml as opposed to 1mg/ml).

A control experiment was set up to investigate the effect of MeCN against the Rat-1 cell-line. A 10% stock solution was prepared and diluted 2-fold to yield the following concentrations: 10, 5, 2.5, 1.25, 0.625, and 0.3125 %.

Each of the stock samples prepared above, were filter sterilized using a 0.22μm filter (Millipore Millex-GS filter unit) before being diluted.

4.11.3. Preparation of microtitration plates

To each well of a 96-well flat-bottom microtitration plate, with the exception of the wells in row 1, 200μl of the cell suspension (10 000 cells/ml) was added so that each well contained 2 000 cells. The 8 wells of row 1, contained 200μl of culture medium which served as a blank. The plates were then placed overnight in the incubator flushed with 5% CO_2 at 37°C to allow for cell adherance to the surface of each well in the plate.

Following the overnight incubation period, medium was aspirated from each well and replaced with 200μl of the prepared drug or plant extract samples, with the exception of wells in rows 1 and 2. Medium only (200μl) was added to the wells in rows 1 and 2. Since the wells of row 2 contained cells too, these wells served as the positive cell control while the wells of row 1 served as a blank. The plates were then incubated at 37°C for 68 hours.

4.11.4. Addition of MTT

After the 68 hour incubation period, 50μl of MTT in PBS (1mg/ml) was added to each well. The plates were returned to the incubator for an additional 4 hours so that the total incubation time was 72 hours. The plates were then centrifuged at 600g for 10 minutes. Medium was carefully aspirated from each well without disturbing the formazan crystals. DMSO (100μl) was then added to each well and gently shaken for 10 minutes to dissolve the crystals completely before being read at 540nm on a 7520 microplate reader (Cambridge Technology, Inc.).

4.11.5. Analysis of data

Cell viability at each concentration was determined. The mean absorbance and corresponding standard deviation for each concentration and positive cell controls were calculated. To determine the percentage cell viability for each concentration the following equation was used:

$$\% \text{ cell viability} = \frac{\text{average absorbance at a particular conc.} \pm \text{SD}}{\text{average absorbance of the cell control} \pm \text{SD}}$$

Percentage cell viability was plotted against log concentration and IC_{50} values were calculated using the linear interpolation method (Fig. 4).

4.12. Statistical analysis

Statistical differences (p values) between IC_{50} values were obtained using the non-parametric two-tailed t-test for two independent means. The software package, Instat, was used.

5. Results

5.1. Fractionation and purification of crude extract

The number and mass of all the fractions produced from the fractionation process of the crude petroleum ether soxhlet extract of leaves and flowers from *A. afra* are recorded in Tables 1-3 below.

Table 1: Yield of fractions obtained from 4.6g of crude *A. afra* as a result of column chromatography.

Fraction	Yield (mass in mg)	% Yield
P1	219.2	4.77
P2	171.2	3.72
P3	308.0	6.70
P4	312.2	6.79
P5	171.5	3.73
P6	400.2	8.70
P7	290.0	6.30
P8	1 020	22.17
P9	1 100	23.91

Table 2: Yield of fractions obtained from the most active antimalarial, fraction P6, 372mg, as a result of flash chromatography.

Fraction	Yield (mass in mg)	% Yield
P6.1	40	10.75
P6.2	199	53.49
P6.3	71	19.09
P6.4	<15	<4.03
P6.5	15	4.03

Table 3: Yield of fractions obtained as a result of further fractionation of 199mg of P6.2, the most abundant sub-fraction.

Fraction	Yield (mass in mg)	% Yield
P6.2A	16	8.04
P6.2B	113.5	57.04
P6.2C	31	15.58

5.2. Thin layer chromatography

No artemisinin could be detected in any of the fractions obtained, in particular the most active fraction, P6. Fig. 5 shows the thin layer chromatographic profile of pure artemisinin used as a standard, and of *A. afra*.

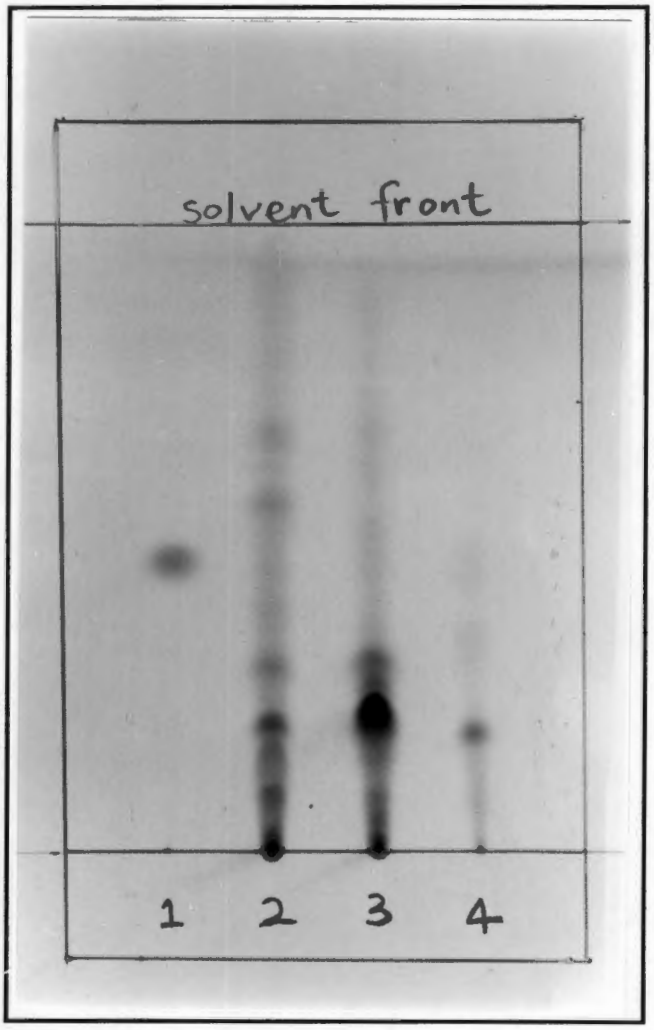


Fig. 5: Thin layer chromatogram of artemisinin (lane 1); crude extract of *A. afra* (lane 2); Fraction P6 (lane 3); and Fraction P6.2B (lane 4). The plate was developed twice in petroleum ether/ethyl acetate [3:1] and stained with anisaldehyde.

5.3. HPLC analysis

The HPLC chromatograms of artemisinin, P6.2B and the crude petroleum ether soxhlet extract of leaves and flowers are shown in Fig. 6-8. The retention times of artemisinin and P6.2B are 3.95 and 21.11 minutes respectively. Their UV spectra are also different, see Fig. 9.

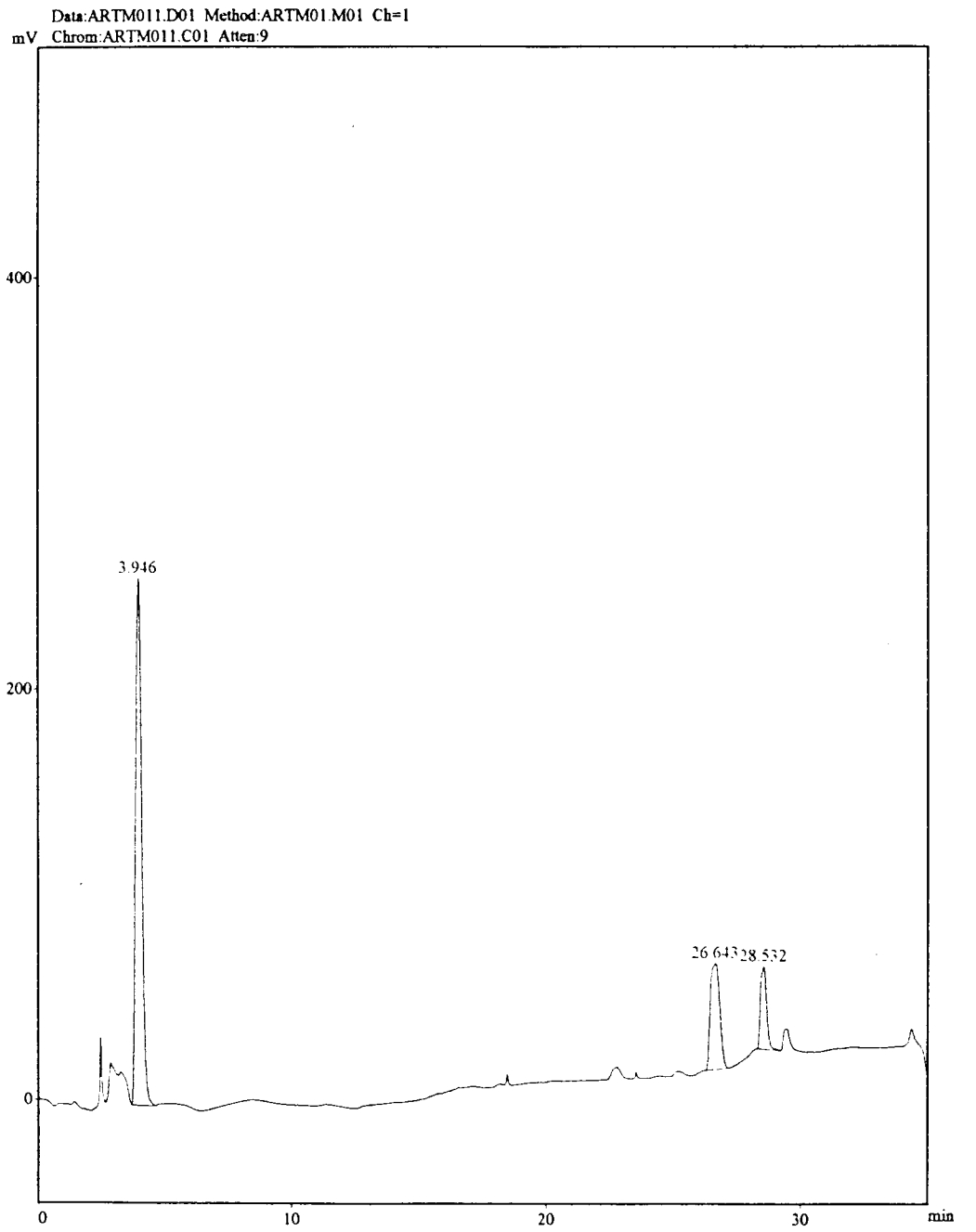


Fig. 6: HPLC chromatogram of artemisinin.

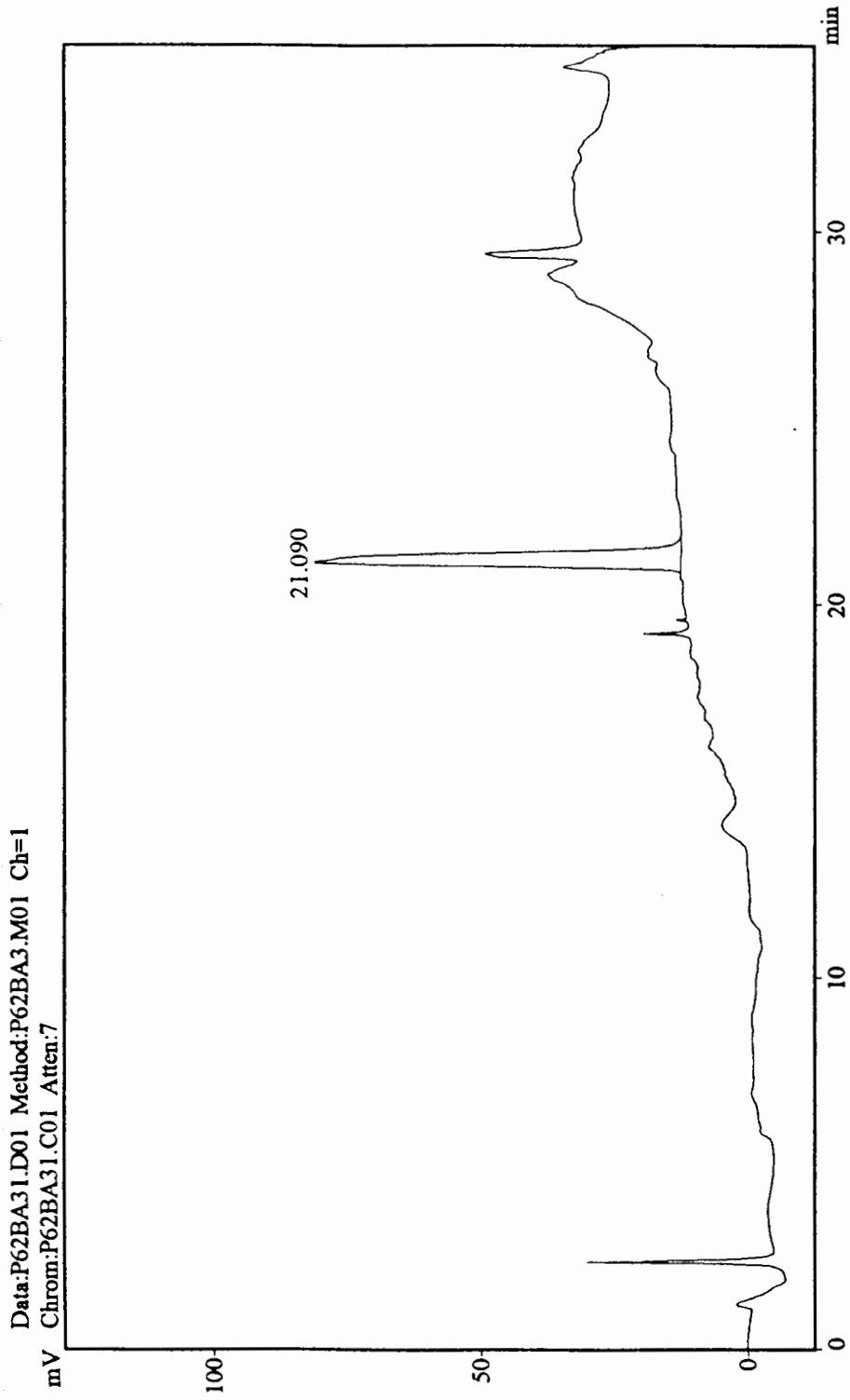


Fig. 7: HPLC chromatogram of P6.2B.

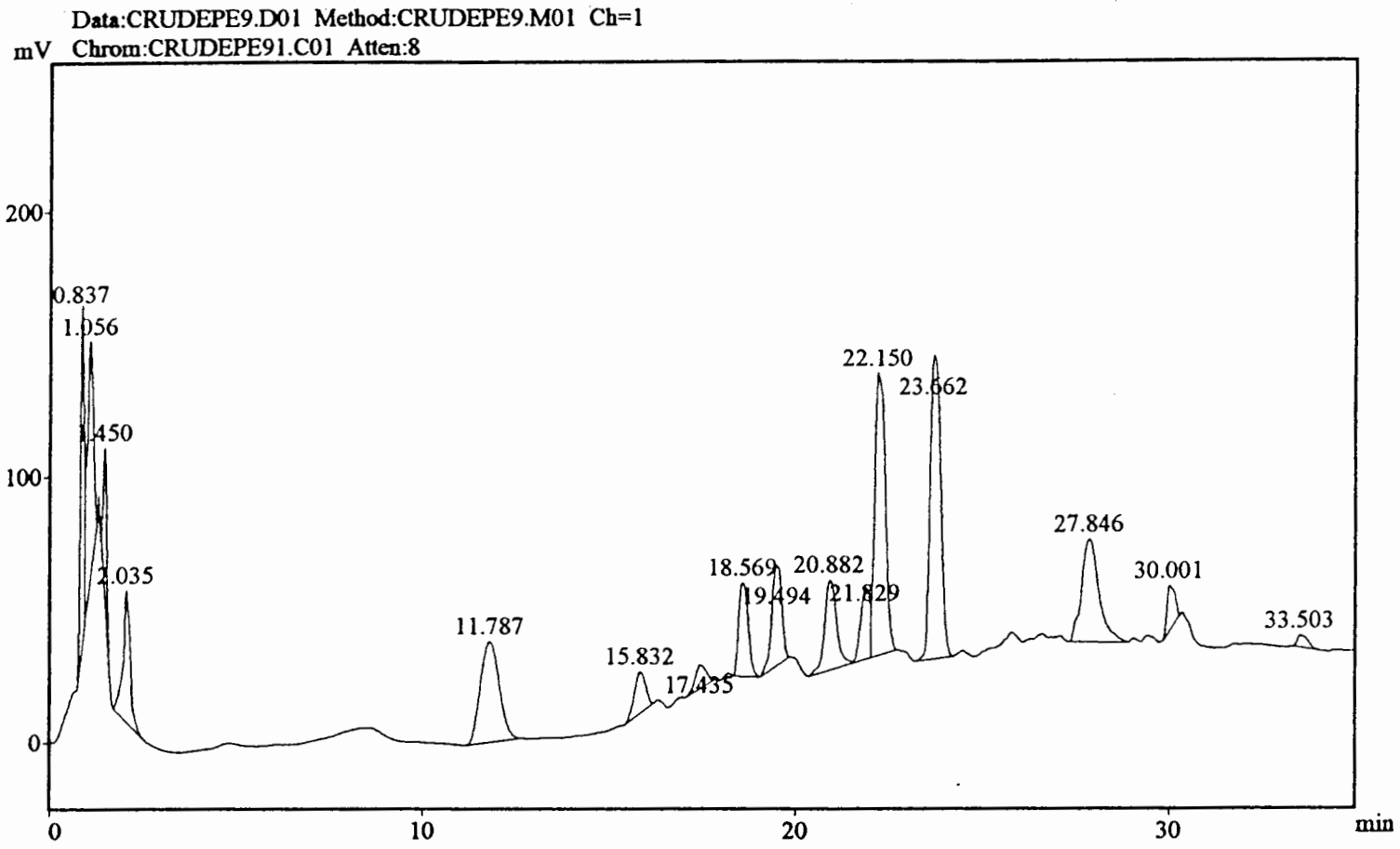


Fig. 8: HPLC chromatogram of the crude petroleum ether soxhlet extract of leaves and flowers from *A. afra*.

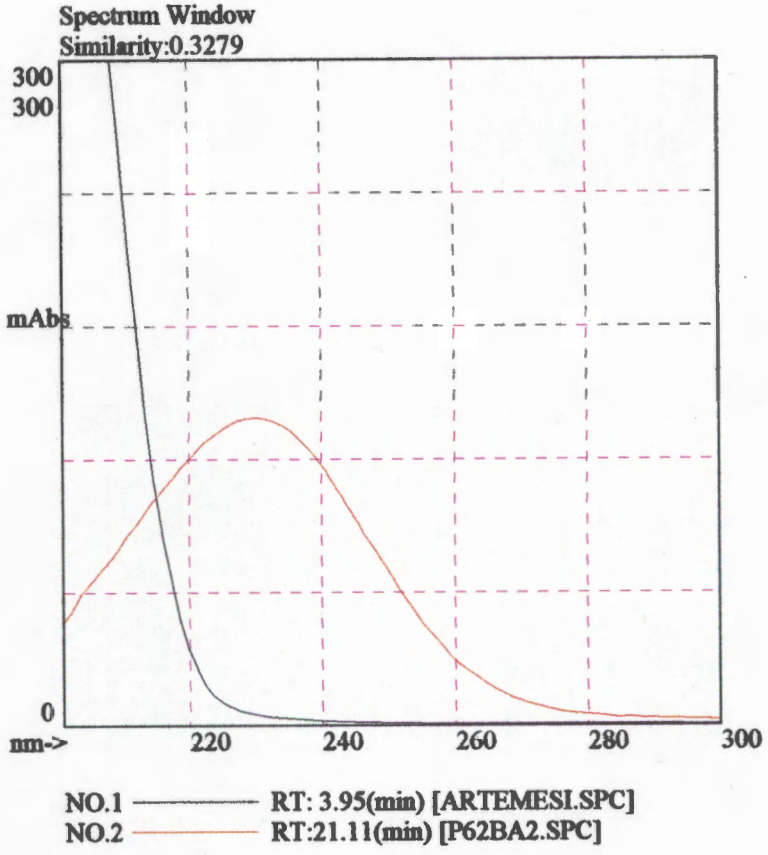


Fig. 9: UV Spectra of artemisinin (black) and P6.2B (red).

In the chromatogram of the crude extract (Fig. 8) there are no peaks between 2.5 and 11.5 minutes where artemisinin is likely to elute if present in the extract. The peak at 20.88 min is P6.2B. Its UV spectrum is similar to that of pure P6.2B. Their retention times are also similar.

HPLC chromatograms of all the crude extracts from *A. afra* in 1996 were also obtained. Their chromatographic profiles were compared to investigate differences, if any, between the two extraction techniques (i.e. cold extraction versus extraction in a Soxhlet apparatus), and between the different solvents used. Significantly, no artemisinin or P6.2B could be detected in any of the crude *A. afra* extracts of leaves or flowers, whereas P6.2B was isolated from the very first extract, which had been obtained from leaves and flowers. The absence of P6.2B in the more recent extracts, suggested that the compound could have been an artefact which had arisen from the extraction and/or fractionation of the crude extract. Alternatively, occurrence of P6.2B could be dependent on seasonal factors or developmental stages of the plant.

Comparing the petroleum ether soxhlet extract of leaves to the cold dichloromethane extract of leaves (Fig. 10) it can be seen that the two solvents extracted the same compounds but in different amounts (peak heights were different between the two extracts). There were also a few additional peaks present only in the dichloromethane extract (± 20 min and 22 min). The cold water extract of leaves contained only a few minor compounds (Fig. 10) compared to the organic extracts.

HPLC chromatograms of the petroleum ether soxhlet extract and the cold dichloromethane extract of flowers are shown in Fig. 11. The two solvents appear to have extracted the same number and quantity of compounds from *A. afra*. The chromatograms are not very different except at about 10 min where a few major peaks appear in the petroleum ether soxhlet extract only. The cold *A. afra* water extract of flowers also did not contain any major peaks (Fig. 11).

The cold dichloromethane extracts between the leaves (Fig. 10) and flowers (Fig. 11) of *A. afra* were also compared. In the extract from leaves, compounds that elute between 18 and 30 min appear to be in larger amounts than in the

extract from flowers. However, between 8 and 9 min there are 2 peaks present in the extract from flowers that appear to be in larger quantities.

The HPLC chromatograms of the petroleum ether soxhlet extracts of *A. afra* leaves (Fig. 10) and flowers (Fig. 11) were similar, except between 8.5 and about 10 min where there were a few major peaks present in the flowers extract, but not in the extract from leaves.

The soxhlet water and cold water extracts from flowers of *A. afra* were compared (Fig. 12). There were a few peaks present in the cold extract only at 2-10 min and at ± 38 min. The peak at about 13 min occurs as a minor peak in the cold extract but as a major peak in the soxhlet water extract. The chromatogram of the cold petroleum ether extract of *A. afra* flowers is similar to the cold water extract of flowers (Fig. 12) than to the petroleum ether soxhlet extract of *A. afra* flowers (Fig. 11).

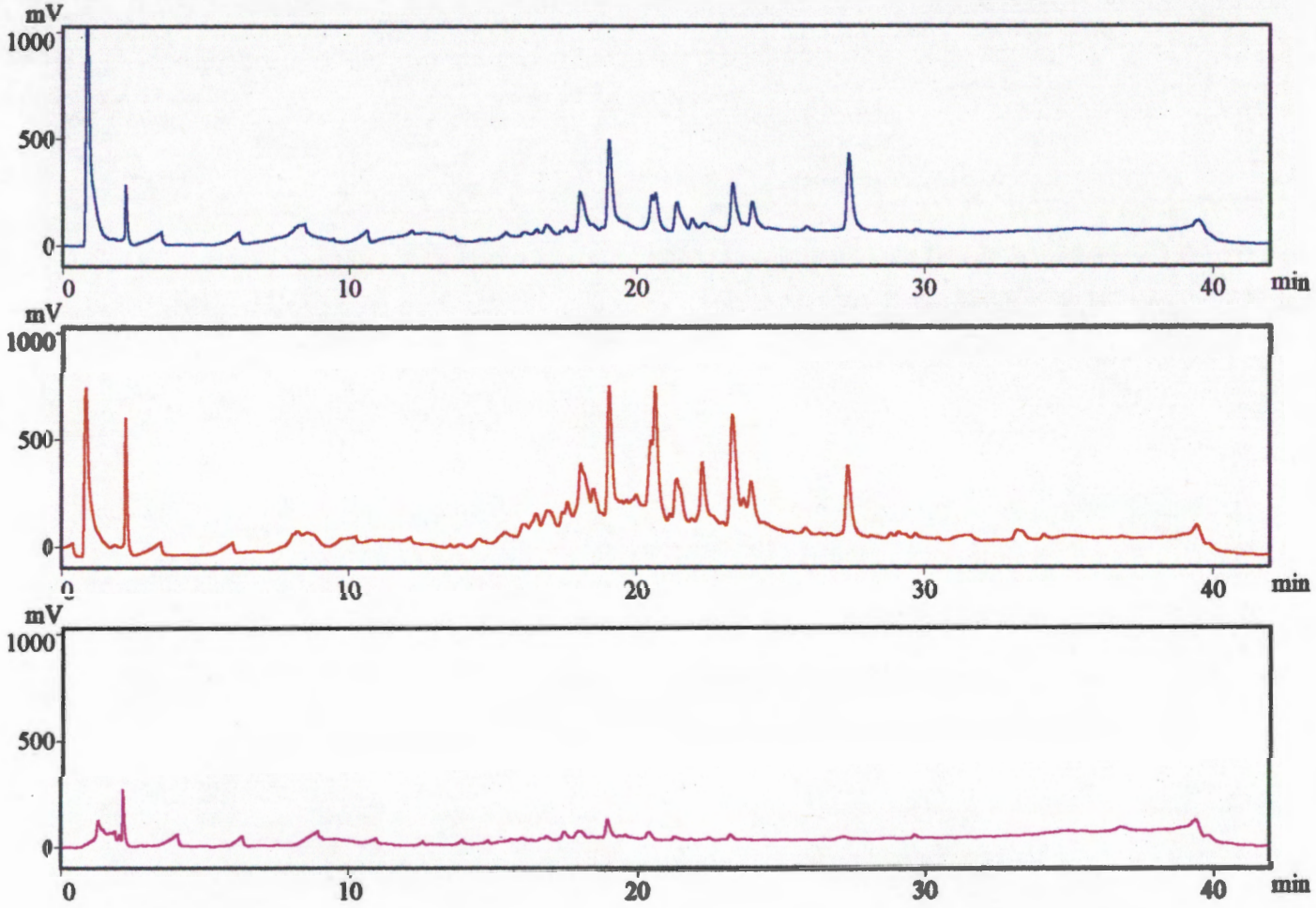


Fig. 10: HPLC chromatograms of the crude petroleum ether soxhlet extract (blue), cold dichloromethane extract (red) and cold water extract (magenta) of leaves from *A. afra*.

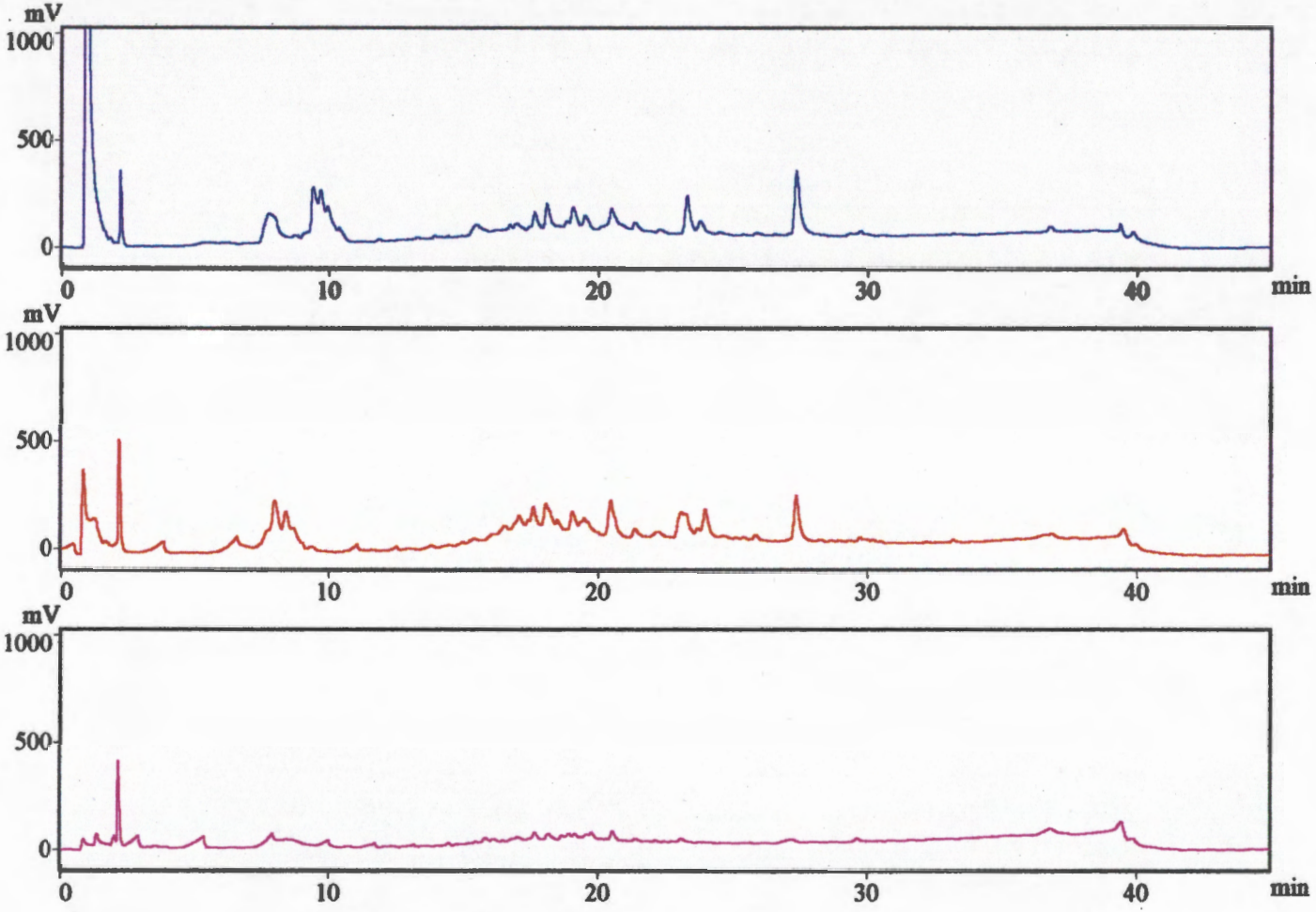


Fig. 11: HPLC chromatograms of the crude petroleum ether soxhlet extract (blue), cold dichloromethane extract (red) and cold water extract (magenta) of flowers from *A. afra*.

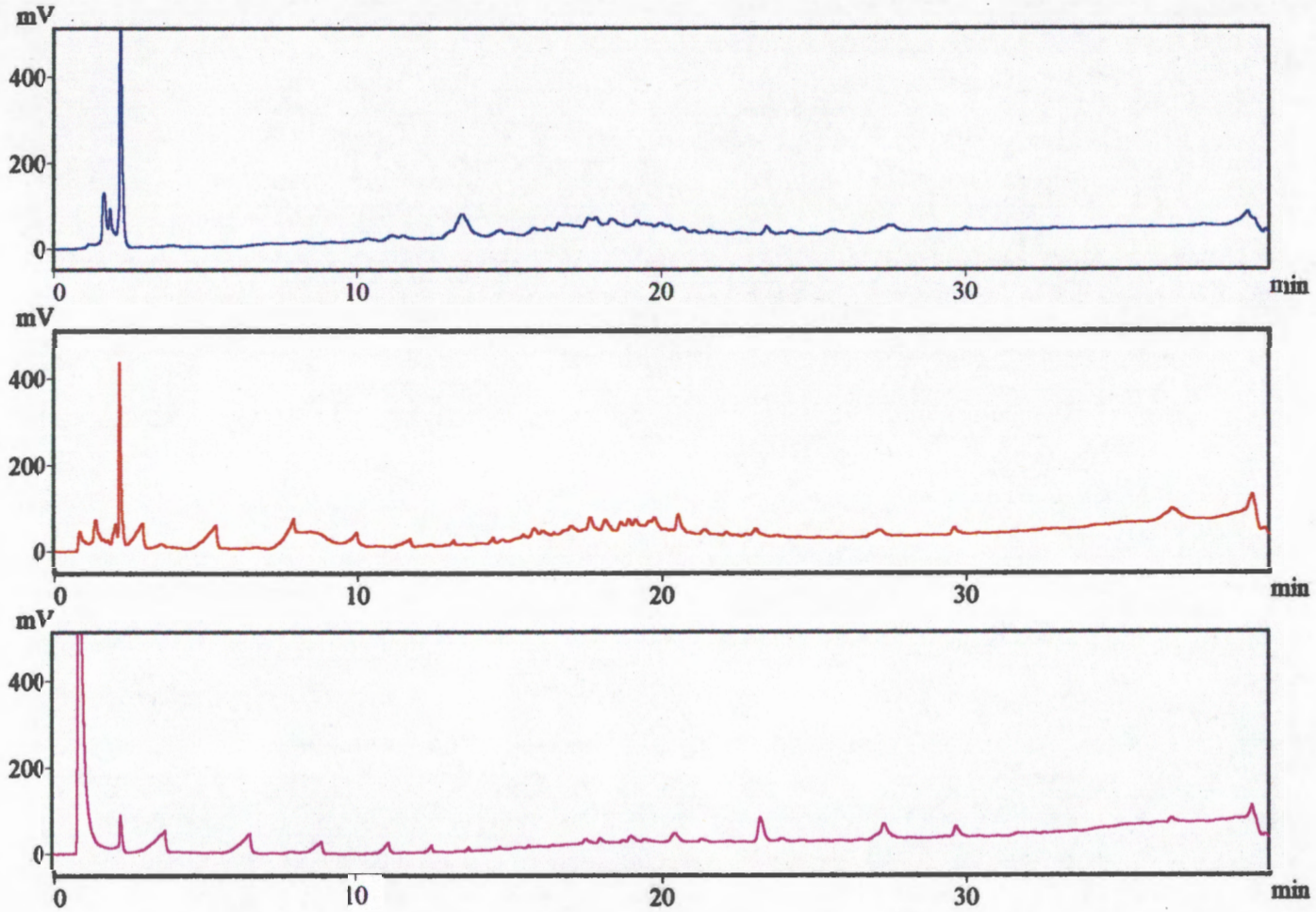


Fig. 12: HPLC chromatograms of the water soxhlet extract (blue), cold water extract (red) and cold petroleum ether extract (magenta) of flowers from *A. afra*.

5.4. Thermospray LC-MS analysis

Under the given conditions for the TSP LC-MS analyses, artemisinin (0.5μg) eluted at 10 min (Fig. 13). The TSP LC-MS spectrum of this compound showed intense peaks at m/z 283 and m/z 300 corresponding respectively to the $[M+H]^+$ and $[M+NH4]^+$ pseudomolecular ions of artemisinin (MW 282) in the different samples, thus allowing for very sensitive detection.

In all three extracts, no artemisinin could be detected, i.e. no presence of the three ions m/z 237, 283 and 300 at 10 min or 290 scans (Fig. 14-16).

The detection limit of the method showed that much less than 1μg of artemisinin could easily be detected in the extract as there was an intense response for this compound in the TSP LC-MS trace (Fig. 17). This means that considerably less than 0.1% of artemisinin could easily be detected in an extract.

In each of the TSP LC-MS traces (Fig. 13-17) the reconstructed ion trace (RIC), the UV trace at 210nm (USER) and the m/z 237, 283 and 300 ion traces are displayed. TSP LC-MS spectrum of pure artemisinin standard is provided (Fig. 13).

For each analysis 0-600 scans (i.e. 20 min) a full chromatogram is given together with an extension where artemisinin is to be detected at 250-350 scans.

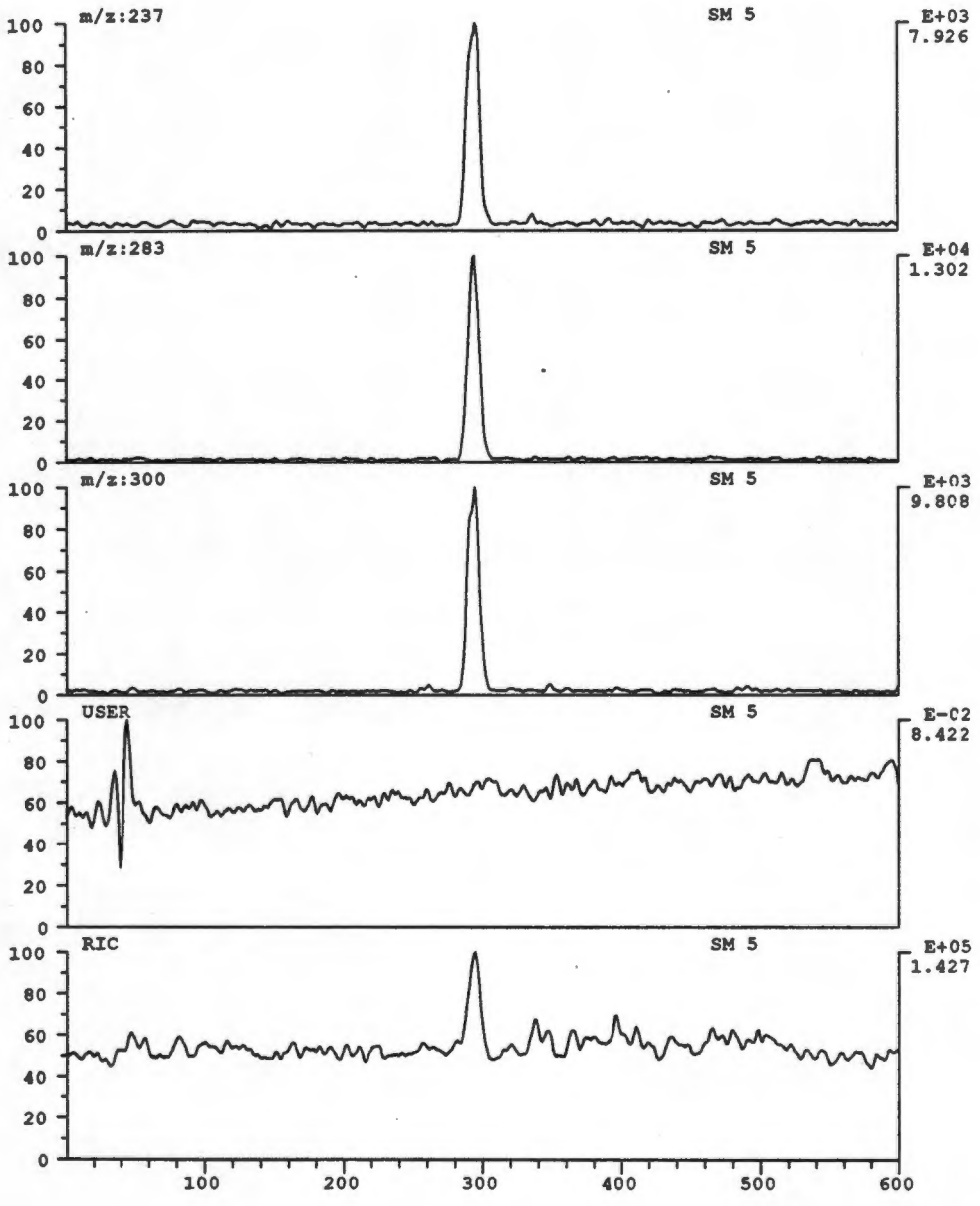


Fig. 13: TSP LC-MS spectrum of artemisinin. Top to bottom: m/z 237, 283 and 300 ion traces; USER: UV trace at 210nm; RIC: reconstructed ion trace.

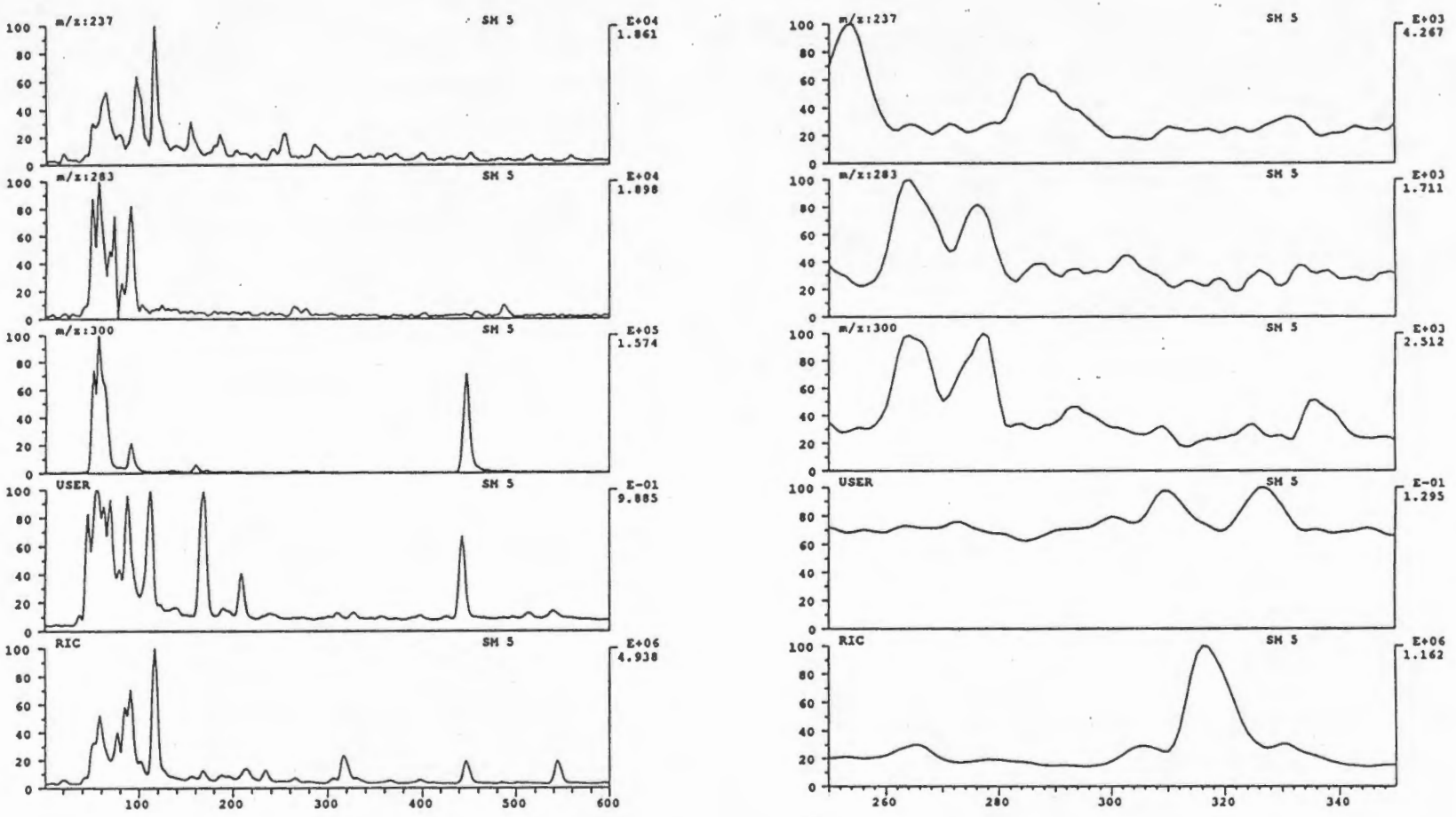


Fig. 14: TSP LC-MS traces of the crude petroleum ether soxhlet extract of *A. afra* leaves and flowers. Top to bottom: m/z 237, 283 and 300 ion traces; USER: UV trace at 210nm; RIC: reconstructed ion trace.

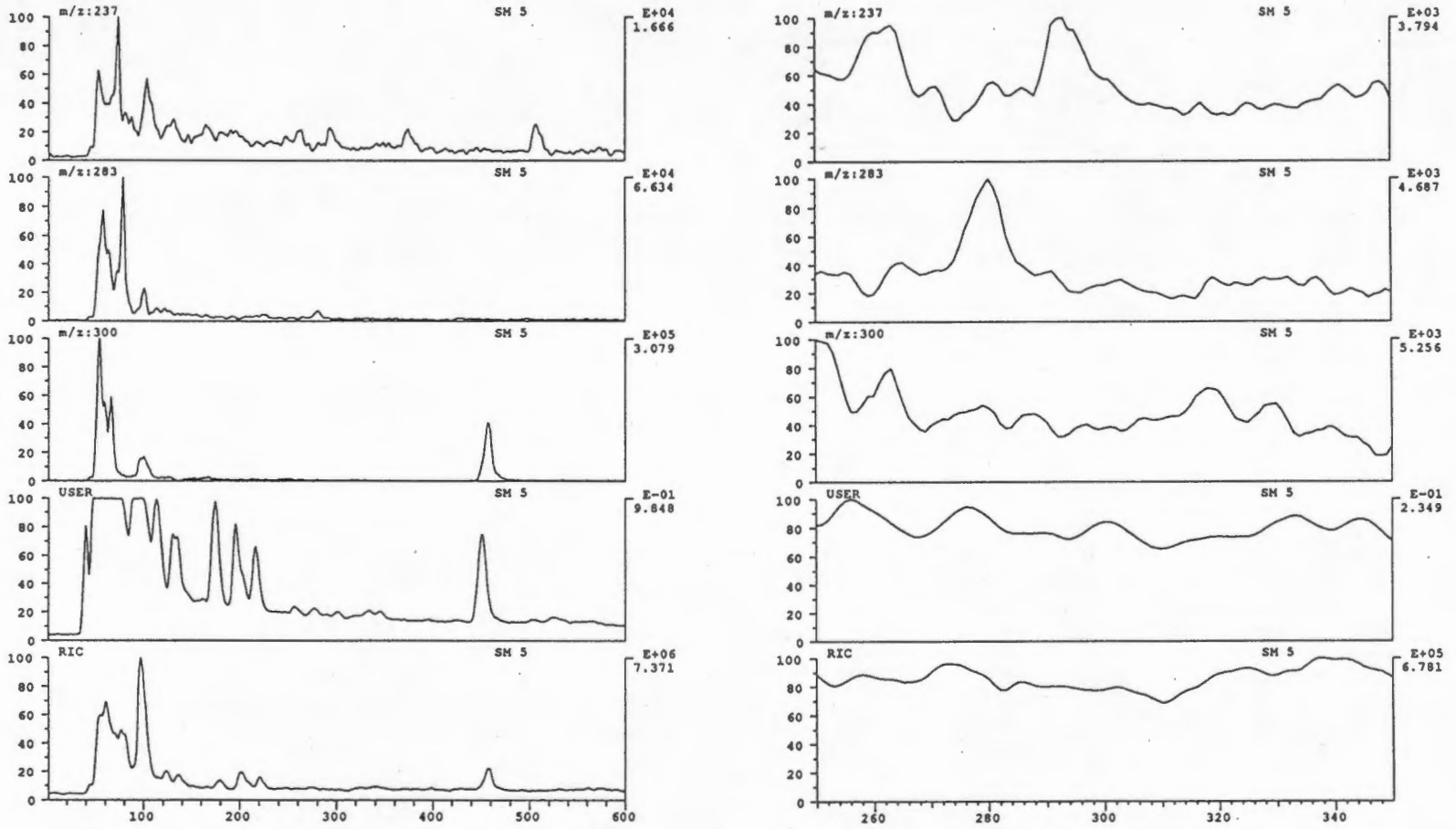


Fig. 15: TSP LC-MS traces of the cold dichloromethane extract from leaves of *A. afra*. Top to bottom: m/z 237, 283 and 300 ion traces; USER: UV trace at 210nm; RIC: reconstructed ion trace.

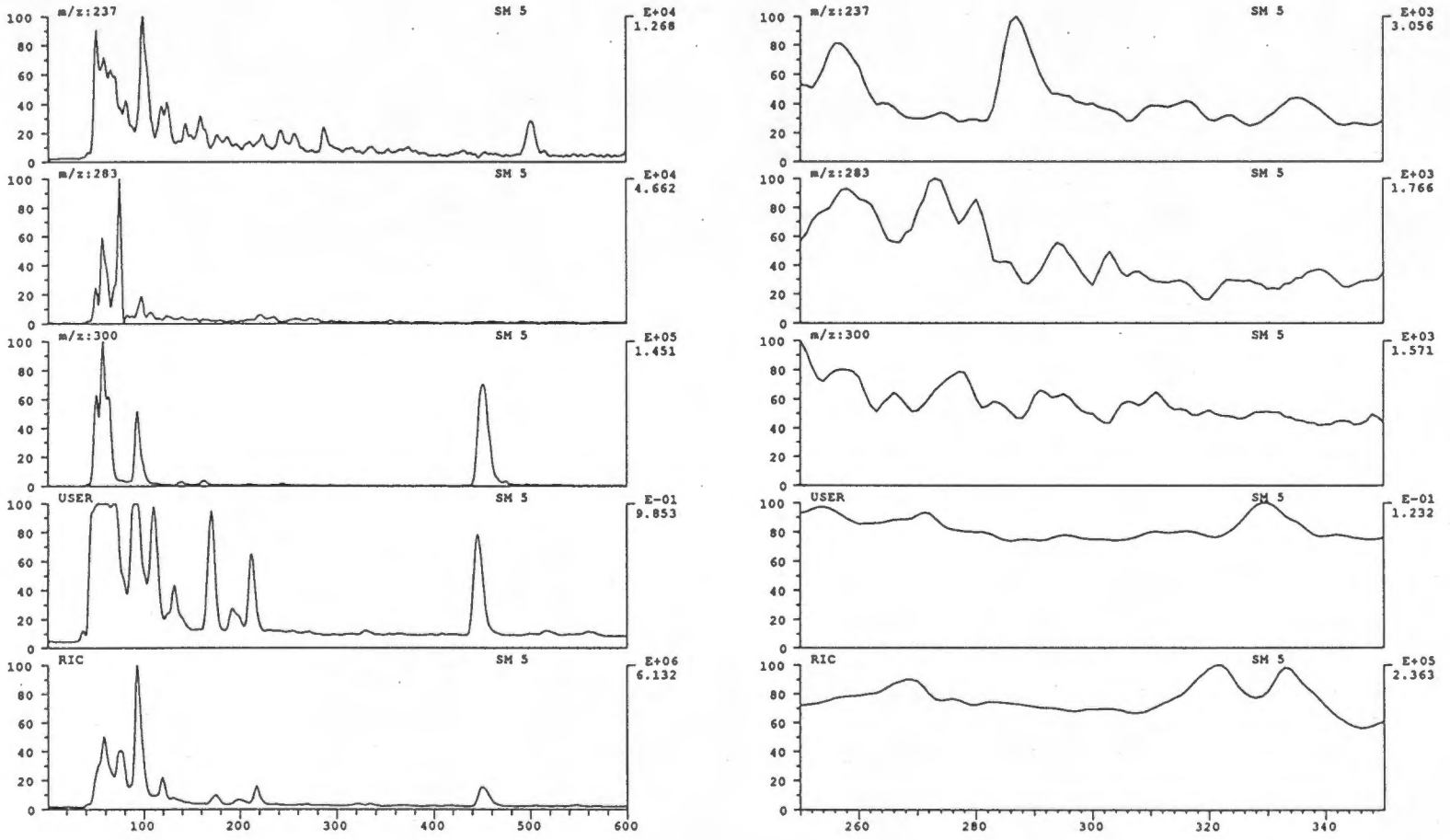


Fig. 16: TSP LC-MS traces of the crude petroleum ether soxhlet extract of *A. afra* leaves. Top to bottom: m/z 237, 283 and 300 ion traces; USER: UV trace at 210nm; RIC: reconstructed ion trace.

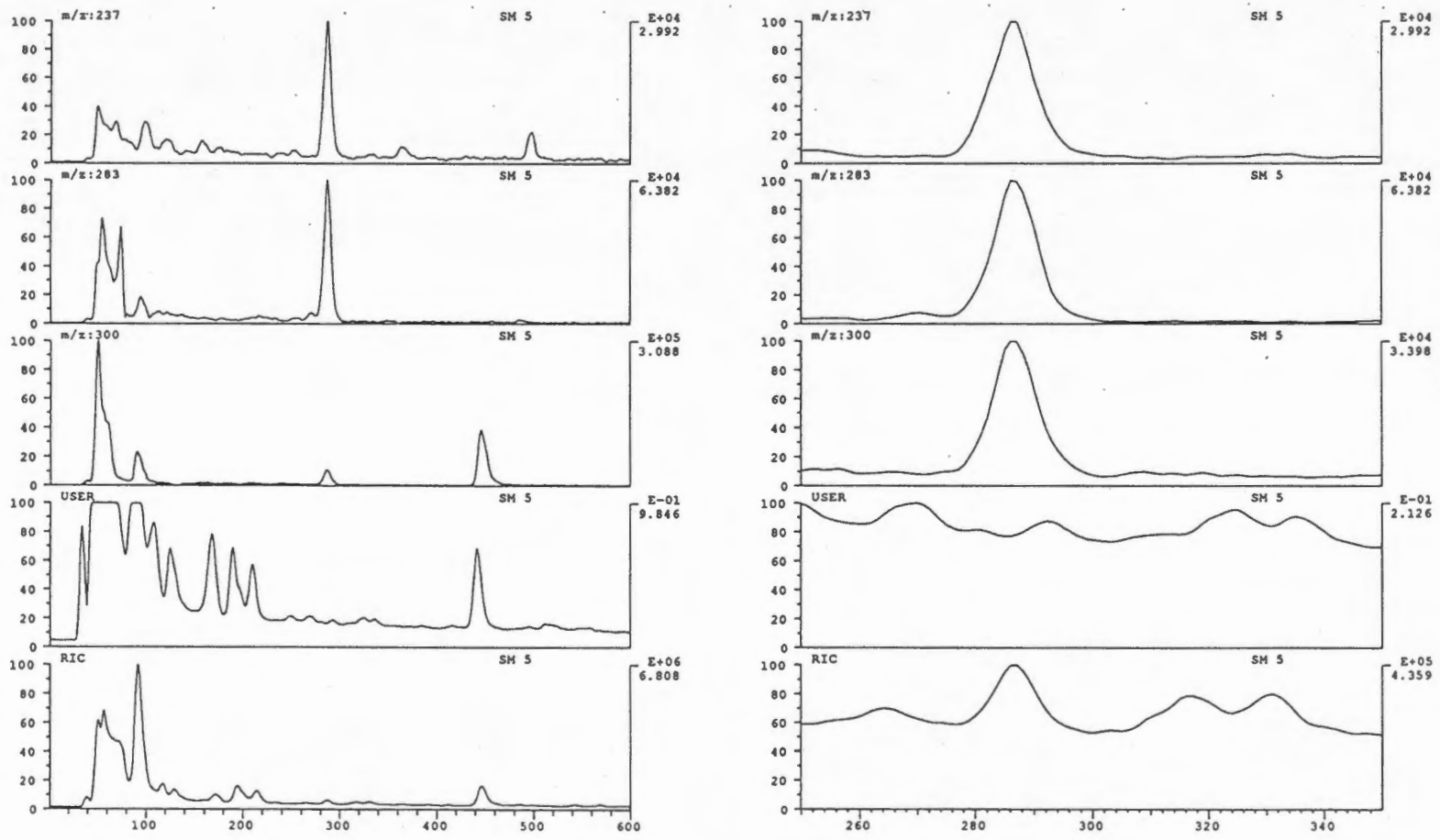


Fig. 17: TSP LC-MS traces of the cold dichloromethane extract from *A. afra* leaves spiked with 1μg of artemisinin to detect the limit of sensitivity. Top to bottom: m/z 237, 283 and 300 ion traces; USER: UV trace at 210nm; RIC: reconstructed ion trace.

5.5. Chemical identification of isolated compound

Characterization (MS and NMR analyses) of the isolated pure compound, P6.2B was performed by Drs. B.M. Sehlapelo, D.W. Gammon and W.E. Campbell (Chemistry Department, U.C.T.). The compound was identified as hydroxydavanone (2,6,10-trimethyl-7,10-oxido-dodeca-3,11E-dien-2-ol-5-one) which had been isolated previously from *A. maritima* L. spp. *maritima* (79), and *A. herba alba* Asso. from Morocco and Tunisia (80). In *A. inculta* Del. from Saudi Arabia (81) a highly oxygenated sesquiterpene, the hydroperoxide was isolated and could be transformed to the corresponding alcohol, hydroxydavanone. However, none of these compounds isolated had been tested for antimalarial activity. The molecular formula of hydroxydavanone is $C_{15}H_{24}O_3$ with MW 252.1860.

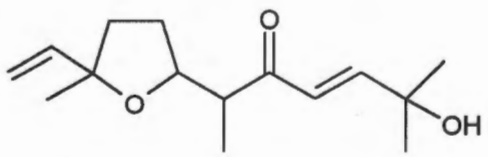


Fig. 18: Chemical structure of hydroxydavanone.

5.6. Antimalarial activity

All of the fractions were screened for *in vitro* antimalarial activity against *Plasmodium falciparum* using tritiated hypoxanthine. IC_{50} values of one experiment are reported in Tables 4 and 5 below.

Table 4: Calculated IC_{50} values (μg/ml) of *A. afra* against *P. falciparum in vitro*.

Sample	D10	FAC8
Crude	1.03	1.50
P1	5.4	>100
P2	1.3	3.7
P3	3.1	6.4
P4	1.7	2.9
P5	3.2	3.1
P6	0.5	0.5
P7	1.3	1.9
P8	7.1	4.8
P9	4.2	23.7
CQ	0.023	0.142

Table 5: Calculated IC_{50} values (μg/ml) of *A. afra* against D10.

Sample	D10
P6.1	21.0
P6.2A	16.0
P6.2B*	0.95
P6.2C	17.4
P6.3	5.9
P6.4	7.2
P6.5	15.3
CQ	0.023

P6.2B*: isolated pure compound

FAC8 unfortunately was not available for screening at this time. Although the assay against FAC8 was performed at a later date, the results were negative, probably due to deterioration of the samples which had been stored in acetonitrile at -18°C.

The two *P. falciparum* strains used thus far, D10 and FAC8, were characterized with respect to chloroquine, artemisinin and mefloquine. An additional 5 strains, of which 3 were South African, were also included in this investigation.

Table 6: Mean IC_{50} values (ng/ml) of chloroquine against *P. falciparum in vitro*, where n is the no. of experiments performed.

Strain	Chloroquine	n	sensitive or resistant
D10	23.23 ± 1.72	3	sensitive
RSA3	21.03 ± 1.93	3	sensitive
FAC8	142.4 ± 34.69	3	resistant
W_2mef	215.50 ± 50.16	3	resistant
W_2mef^{CQ}	252.40 ± 8.91	2	resistant
RSA11	212.23 ± 47.68	3	resistant
RSA15	265.50 ± 104.38	3	resistant

D10 and RSA3 was not significantly different ($p=0.2145$). They were classified as chloroquine-sensitive. D10 and FAC8 were significantly different ($p=0.0040$). FAC8 was not significantly different to W_2mef, $p=0.1065$ and W_2mef^{CQ}, RSA11 and RSA15 ($p>0.1065$). W_2mef was not significantly different to W_2mef^{CQ} ($p=0.3997$); the same applied to RSA11 and RSA15 ($p=0.4664$). These strains were classified as chloroquine-resistant.

Table 7: Mean IC_{50} values (ng/ml) of artemisinin against *P. falciparum in vitro*, where n is the no. of experiments performed.

Strain	Artemisinin	n	sensitive or resistant
D10	11.30 ± 6.59	3	resistant
RSA3	13.67 ± 7.96	3	resistant
FAC8	2.67 ± 0.50	3	sensitive
W_2mef	3.13 ± 0.15	3	sensitive
W_2mef^{CQ}	3.50 ± 0.96	3	sensitive
RSA11	3.80 ± 0.80	3	sensitive
RSA15	2.33 ± 1.00	3	sensitive

With respect to artemisinin, D10 and RSA3 could be classified as artemisinin-resistant. No significant difference was found between these two strains ($p=0.7115$). D10 was not significantly different to FAC8 ($p=0.0865$). Again, FAC8 was not significantly different to W_2mef ($p=0.2016$); W_2mef was not significantly different to W_2mef^{CQ} ($p=0.5456$); and RSA11 was not significantly different to RSA15 ($p=0.1177$). These strains could be classified as artemisinin-sensitive.

This trend of resistance was similar for mefloquine (Table 8) where D10 and RSA3 were not significantly different, $p=0.0962$. D10 and FAC8 were however, significantly different with $p=0.0435$. No significant difference was found for W_2mef and RSA11 ($p=0.3695$). D10 and RSA3 could be classified as

resistant although D10 confers greater sensitivity. FAC8, W_2mef, W_2mef^{CQ}, RSA11 and RSA15 were classified as mefloquine-sensitive.

Table 8: Mean IC_{50} values (ng/ml) of mefloquine against *P. falciparum in vitro*, where n is the no. of experiments performed.

Strain	Mefloquine	n	sensitive or resistant
D10	24.30 ± 0.42	2	resistant
RSA3	32.23 ± 4.43	3	resistant
FAC8	10.77 ± 5.38	3	sensitive
W_2mef	14.90 ± 6.34	3	sensitive
W_2mef^{CQ}	14.90 ± 8.92	3	sensitive
RSA11	10.30 ± 4.69	3	sensitive
RSA15	10.77 ± 5.34	3	sensitive

With respect to the isolated pure compound from *A. afra*, P6.2B (Table 9), D10 and RSA3 was found to be significantly different with p=0.0375, while D10 and FAC8 were not significantly different (p=0.9801). The highest activity was recorded against D10 and FAC8 with IC_{50} of 0.87 ± 0.04 and 0.88 ± 0.20 μg/ml respectively. P6.2B had the lowest activity against RSA3; IC_{50}: 2.65 ± 0.50 μg/ml.

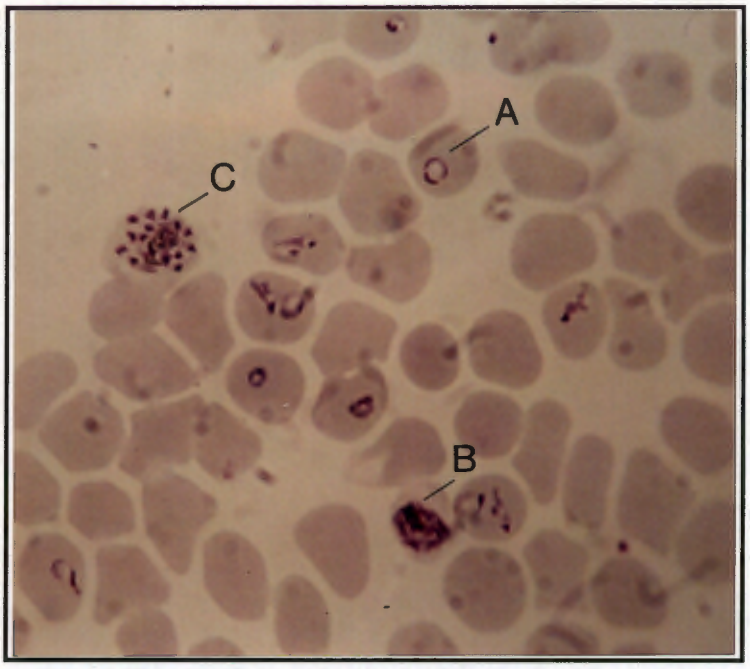
Table 9: Mean IC_{50} values (μg/ml) of P6.2B against *P. falciparum in vitro*, where n is the no. of experiments performed.

Strain	P6.2B	n
D10	0.87 ± 0.04	2
RSA3	2.65 ± 0.50	2
FAC8	0.88 ± 0.20	2
W_2mef	2.00	1
W_2mef^{CQ}	1.90	1
RSA11	2.54	1
RSA15	1.60	1

Fig. 19 is a photograph of the various erythrocytic stages of the life cycle of *P. falciparum* cultured *in vitro* as observed under the microscope with magnification ×1600.

Fig. 20-24 show the dose response curves of the crude petroleum ether soxhlet extract of *A. afra*, chloroquine, artemisinin, mefloquine and P6.2B against *P. falciparum*.

Appendix C shows the dose response curves for the concentrations of MeCN used for the *in vitro* experiments against *P. falciparum* strains. Clearly, the concentrations used to dissolve artemisinin, mefloquine, P6.2B and crude extracts of *A. afra* had no negative effect against the parasite strains.



A: Ring

B: Trophozoite

C: Schizont

Fig. 19: Erythrocytic stages of the life cycle of *Plasmodium falciparum*, cultured *in vitro*. Magnification ×1600.

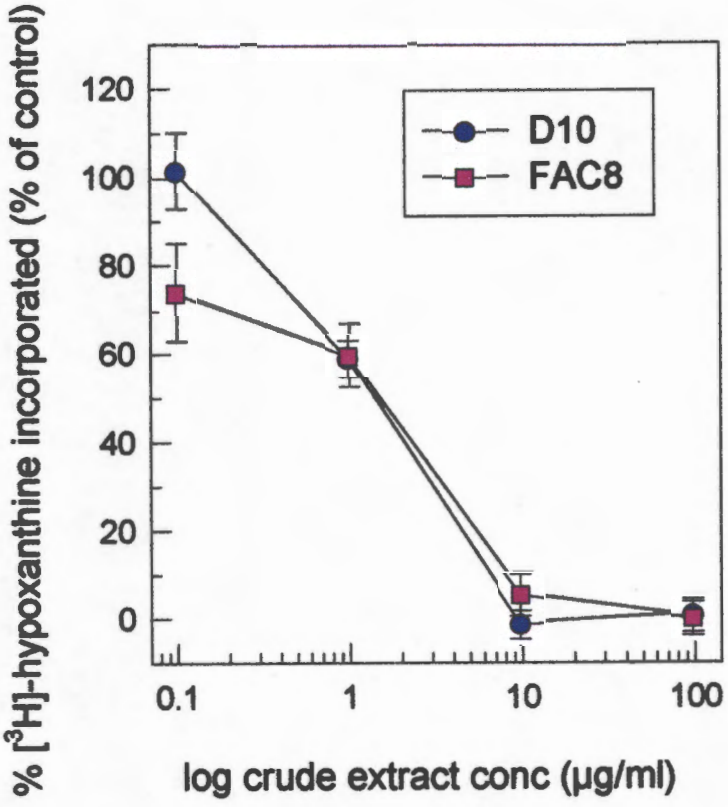


Fig. 20: The effect of the crude petroleum ether soxhlet extract of *A. afra* leaves and flowers on the incorporation of hypoxanthine into *P. falciparum*. Data indicate means ± SD (n=3).

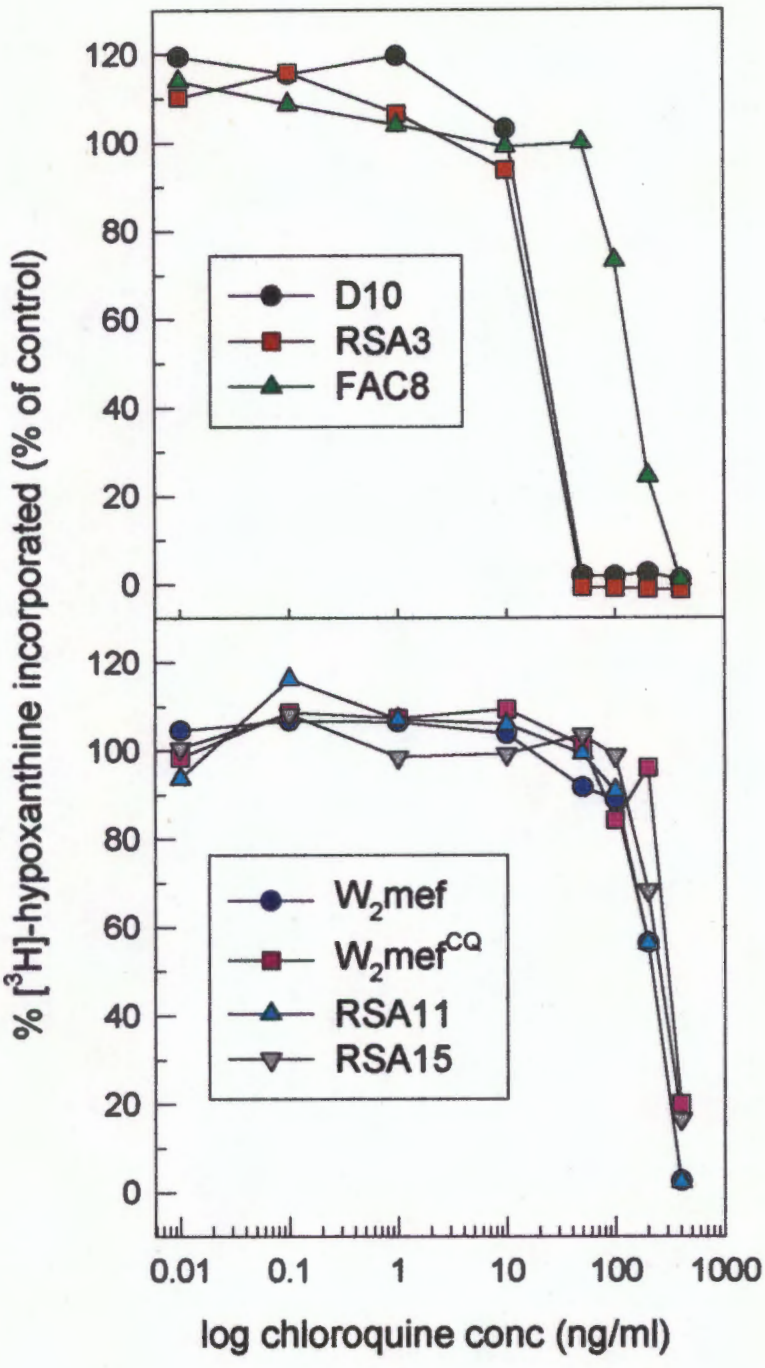


Fig. 21: The effect of chloroquine on the incorporation of hypoxanthine into *P. falciparum*. Data indicate means ± SD (n=3).

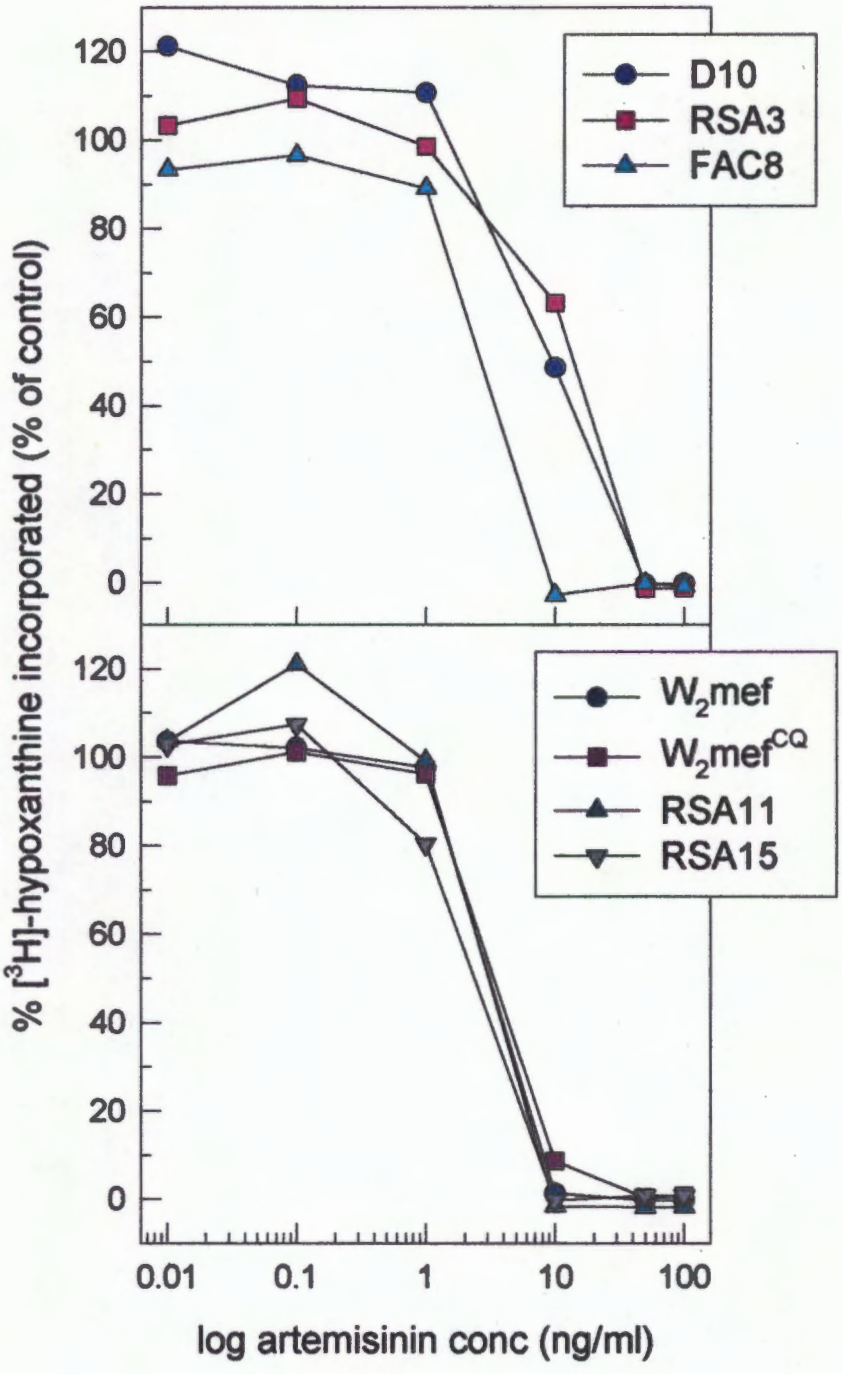


Fig. 22: The effect of artemisinin on the incorporation of hypoxanthine into *P. falciparum*. Data indicate means ± SD (n=3).

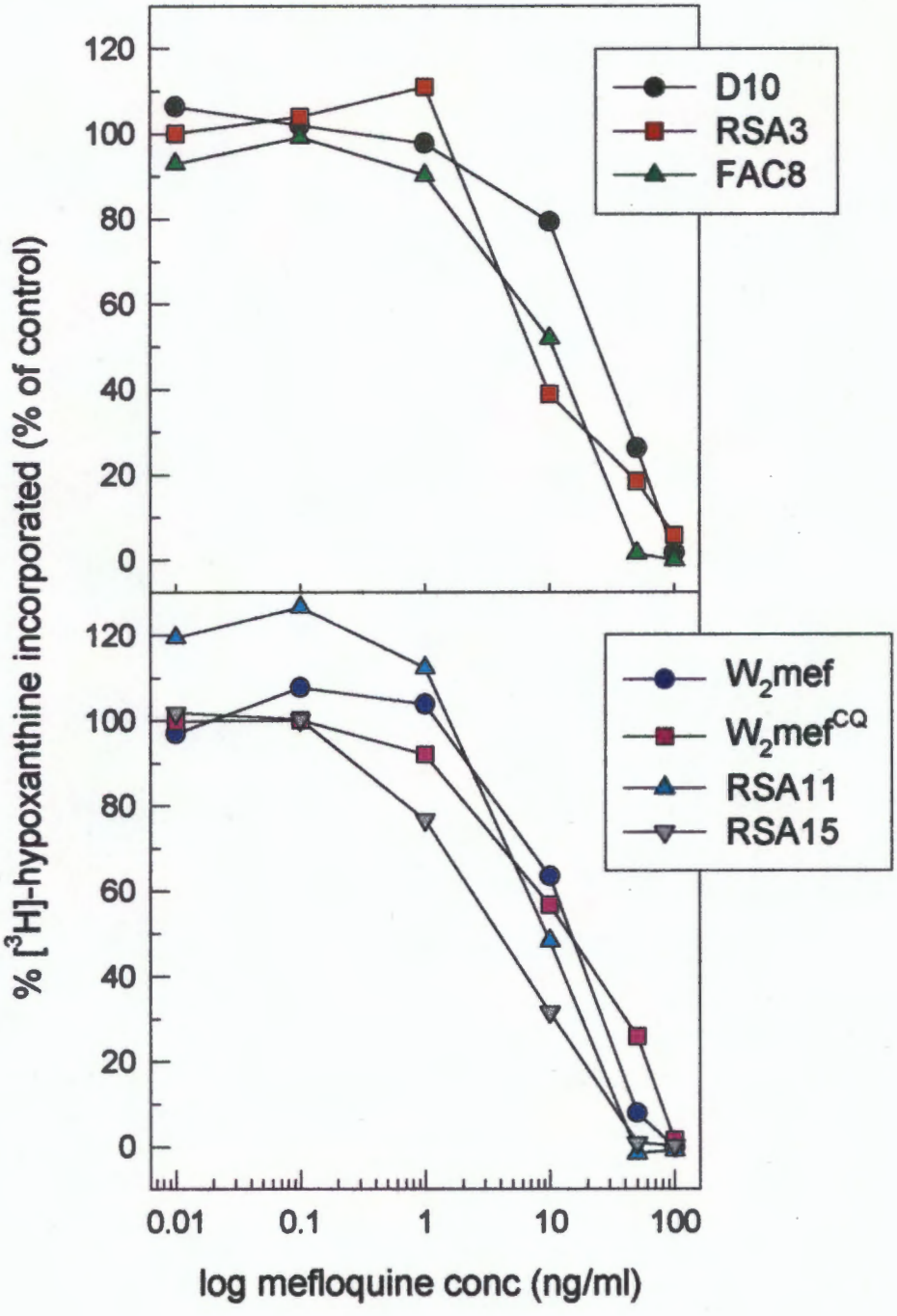


Fig. 23: The effect of mefloquine on the incorporation of hypoxanthine into *P. falciparum*. Data indicate means ± SD (n=3).

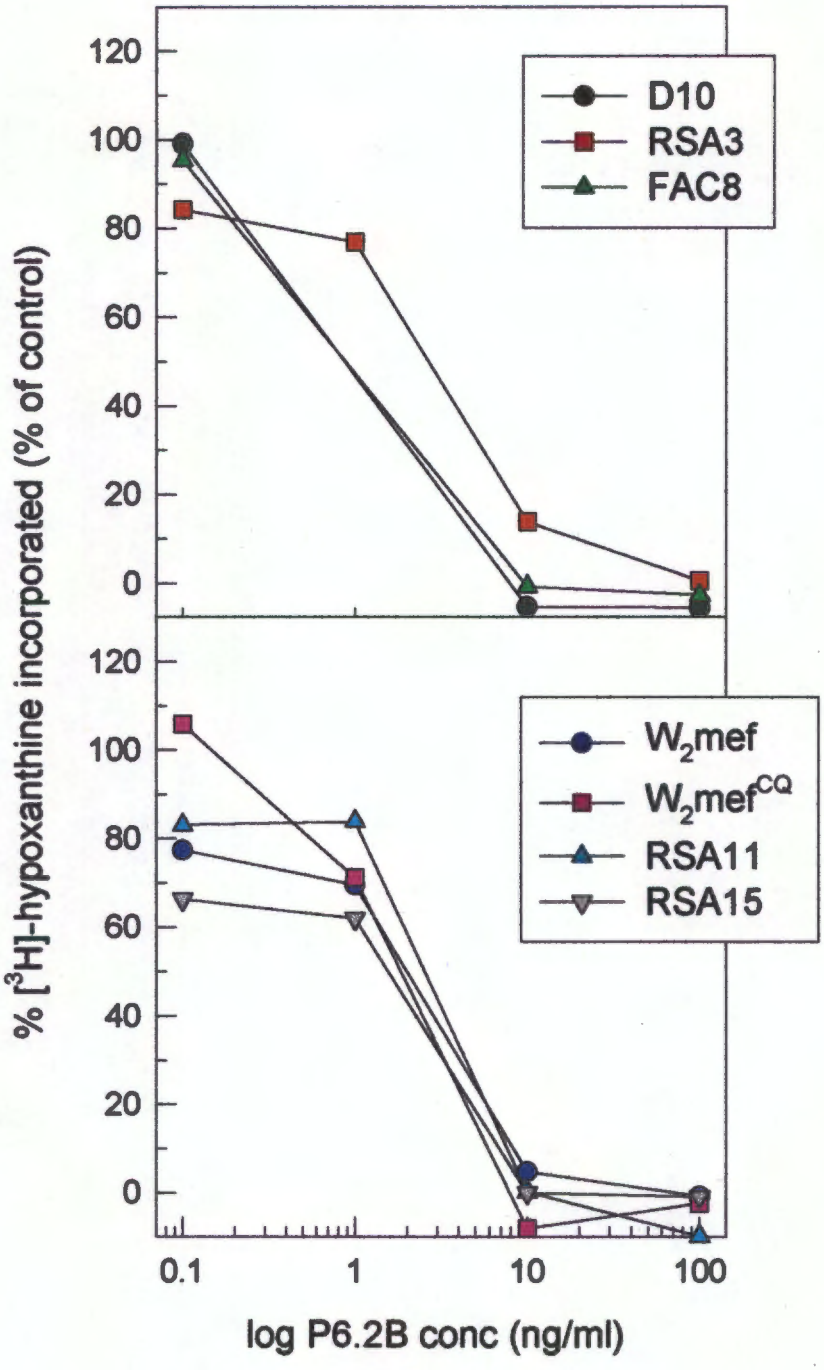


Fig. 24: The effect of P6.2B on the incorporation of hypoxanthine into *P. falciparum*. Data indicate means ± SD (n=2).

Table 10: Mean IC_{50} values (μg/ml) of 2 experiments of crude *A. afra* extracts against *P. falciparum in vitro*.

Part of plant used	Extract	D10	FAC8
Leaves	DCM cold	4.31 ± 0.45	2.91 ± 0.37
	P.E. soxhlet	6.26 ± 0.37	6.22 ± i.12
	H_2O cold	645.02 ± 18.65	420.24 ± 14.28
Flowers and Seedlings	DCM cold	9.79 ± 1.00	6.63 ± 0.81
	P.E. cold	29.25 ± 0.04	31.65 ± 0.35
	P.E. soxhlet	15.78 ± 2.09	17.99 ± 0.63
	H_2O cold	1 400*	1 740*

DCM: dichloromethane; P.E. petroleum ether; H_2O: water; *: one experiment only.

The antimalarial activities of the various crude extracts generated from *A. afra* are reported in Table 10. Statistical differences were determined. There was a significant difference between the cold dichloromethane and petroleum ether soxhlet extracts of leaves and flowers. When the cold dichloromethane and petroleum ether soxhlet extracts from leaves were tested against D10 a p value of 0.0419 was obtained (therefore significantly different) and when tested against FAC8, a p value of 0.0580 was obtained (not significantly different).

The cold dichloromethane and cold petroleum ether extracts of flowers were compared and were found to be very significantly different with p values of 0.0013 against D10 and 0.0006 against FAC8. Dichloromethane, therefore extracted compound(s) with greater antimalarial activity.

When the cold petroleum ether extract and the petroleum ether soxhlet extract were compared for statistical difference, p values of 0.0118 and 0.0014 were obtained for D10 and FAC8 respectively. The difference was very significant with the extract obtained from the Soxhlet exhibiting a greater antimalarial effect.

5.7. Cytotoxicity

Colchicine was used as a standard (Fig. 25). Excluding colchicine, mefloquine was the most toxic against RAT-1 cells, followed by P6.2B, the crude *A. afra* extract, artemisinin and chloroquine. Statistical differences were also determined. There was no statistical difference between chloroquine and artemisinin ($p=0.7547$). The statistical difference for mefloquine and artemisinin was extremely high with $p=0.0004$. There was a significant difference between mefloquine and the crude *A. afra* extract ($p=0.0012$) and with the crude *A. afra* extract and artemisinin ($p=0.0489$). The statistical difference between the crude extract and P6.2B was extremely significant with a p value of 0.0005. Throughout these experiments the highest percentage of MeCN used did not exceed 1% which had no adverse effect against the RAT-1 cell-line (Appendix D).

Table 11: Mean IC_{50} values (μg/ml) of P6.2B against RAT-1 cells, *in vitro*, where n is the no. of experiments performed.

Sample	Mean $IC_{50} \pm$ SD (μg/ml)	n
Chloroquine	53.58 ± 13.33	3
Artemisinin	50.61 ± 7.65	3
Crude extract of *A.afra*	34.78 ± 8.23	4
P6.2B	6.29 ± 0.95	4
Mefloquine	2.55 ± 0.12	3
Colchicine	0.60 ± 0.47	3

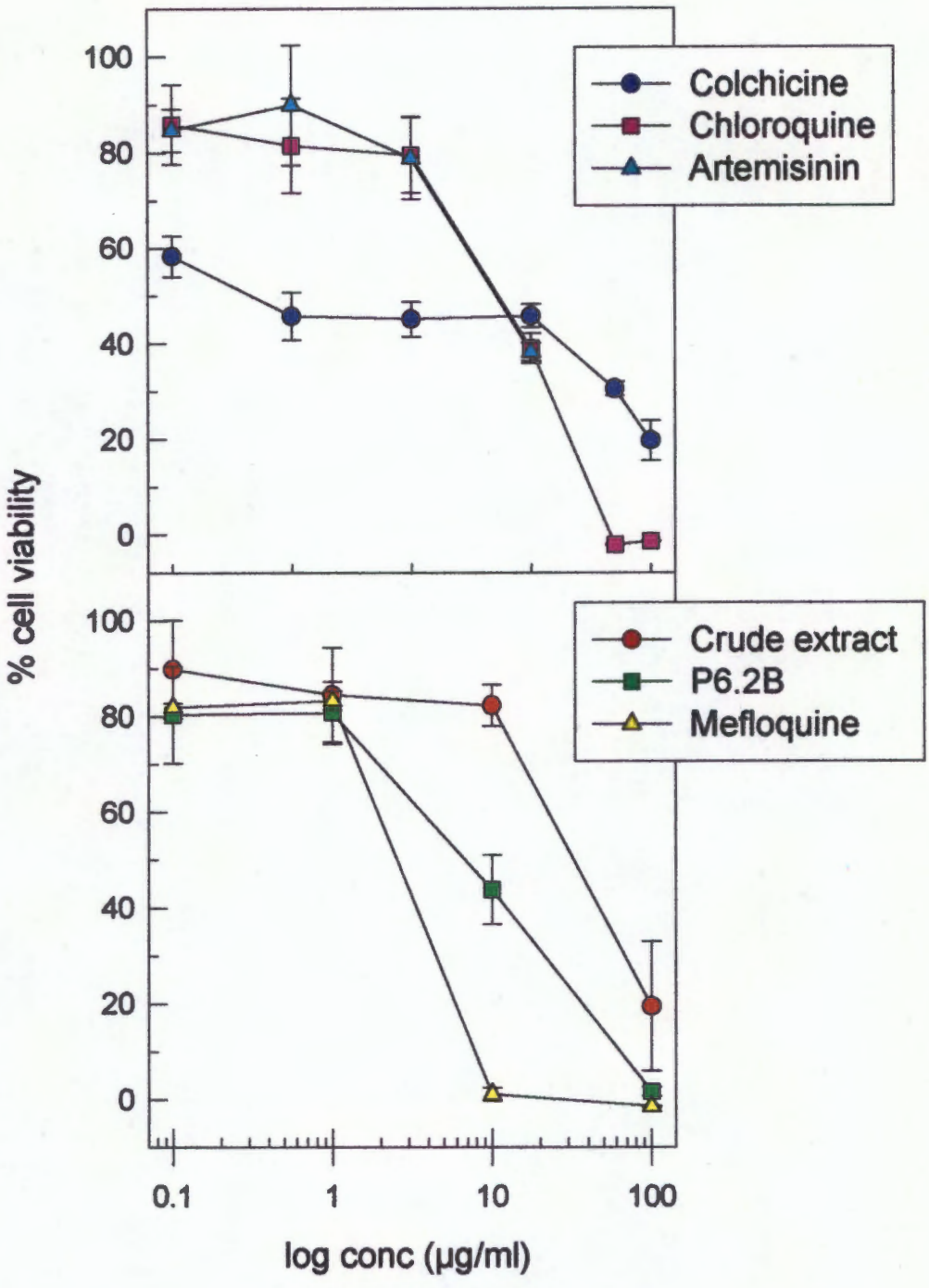


Fig. 25: Cytotoxic effects of P6.2B against RAT-1 fibroblast cells. Data indicate means ± SD (n=4). The y-axis represents cell viability as a percentage of the control.

5.8. Selectivity Index

The selectivity index (i.e. ratio of cytotoxicity to antimalarial activity) was calculated for P6.2B, the crude *A. afra* extract, chloroquine, artemisinin and mefloquine. Ratios < 1 indicate higher cytotoxicity than antimalarial activity (82).

Table 12: Selectivity indices i.e. mean IC_{50} for cytotoxicity/mean IC_{50} for antimalarial activity.

Strain	CQ	QHS	MEF	P6.2B	Crude
D10	2 307	4 479	105	7.2	33.8
RSA3	2 548	3 702	79	2.4	23.2
FAC8	376	18 955	237	7.1	nd
W_2mef	249	16 169	171	3.1	nd
W_2mef^{CQ}	212	14 460	171	3.3	nd
RSA11	253	13 318	248	2.5	nd
RSA15	202	21 721	237	3.9	nd

CQ: chloroquine; QHS: artemisinin; MEF: mefloquine; Crude: *A. afra* extract; nd: not determined.

The highest selectivity indices were recorded for artemisinin, which ranged from 3 702 to 18 955. The selectivity index for chloroquine, was higher for the chloroquine-sensitive strains, D10 and RSA3 (2 307 and 2 548 respectively), than for the chloroquine-resistant strains, which ranged from 202 to 376. The selectivity index for mefloquine ranged from 79 to 248 for the *P. falciparum*

strains tested. The crude extract from *A. afra*, from which P6.2B was isolated, had ratios of 33.8 and 23.2 against D10 and FAC8 respectively. P6.2B had the lowest selectivity indices recorded; range: 2.4 - 7.2.

6. Discussion

A crude petroleum ether soxhlet extract of leaves and flowers from *A. afra* had previously been shown to exhibit antimalarial activity against *P. falciparum in vitro* (6). The main objective of this project was to undertake a bioassay-guided fractionation process of this crude extract in order to identify the active principle and to determine whether artemisinin could be responsible for the antimalarial effect.

The crude extract (4.6g) was chromatographed on a silica gel column to yield 120 fractions. The column was initially washed with petroleum ether, which eluted most of the plant waxes. The petroleum ether fractions solidified at room temperature and were insoluble in a number of organic solvents, including acetonitrile. Consequently, they were excluded from the antimalarial screening. After petroleum ether was used, a small percentage of ethyl acetate was introduced into the eluant mixture and gradually increased until the eluant mixture was 100% ethyl acetate. The column was finally washed with 100% methanol. The early fractions collected would have contained most of the non-polar compounds and the later fractions would contain the more polar compounds. These fractions were pooled on the basis of their TLC profiles which resulted in a total of 9 fractions, labelled P1 to P9. Fractions P8 and P9 were the methanol fractions. The percentage yields of fractions P1-P9 are summarized in Table 1.

Each fraction was then screened for *in vitro* antimalarial activity against chloroquine-sensitive (D10) and chloroquine-resistant (FAC8) strains of *P. falciparum*. Their IC_{50} values are reported in Table 4. Fraction P6 had the highest antimalarial activity i.e. lowest IC_{50} value of 0.5μg/ml against both strains. Its activity had also increased from that of the crude extract which was 1.03 and 1.50 against D10 and FAC8 respectively, whereas the other IC_{50}

values obtained which ranged from 1.3 to 23.7μg/ml had increased from that of the crude extract. Interestingly, fraction P1 had an IC_{50} value of 5.4μg/ml against D10 but an IC_{50} value greater than 100μg/ml against FAC8. It is possible that there is a compound(s) present in this fraction that could be transported into the parasite in a similar way as for chloroquine, such that there is less accumulation in the chloroquine-resistant strain, FAC8. This fraction warrants further investigation.

Following a bioassay-guided fractionation approach, the most active fraction, fraction P6 was to be further fractionated. It was therefore subjected to flash chromatography and another 5 fractions were produced, denoted P6.1 to P6.5. Their masses and percentage yields are recorded in Table 2. The largest of these fractions, P6.2 was subjected to further chromatography to yield 3 sub-fractions (Table 3) of which one, P6.2B, a clear colourless oil, appeared to be a single pure compound from TLC analysis. This compound was the major constituent of fraction P6.2.

At this point all the sub-fractions were screened against D10 to investigate their antimalarial activity and to determine which sub-fraction contained the active principle (Table 5). All the sub-fractions were active against D10 with IC_{50} values ranging from 0.95 to 21.0μg/ml. Fraction P6.2B had the highest antimalarial activity with an IC_{50} of 0.95μg/ml. Of the sub-fractions generated from fraction P6.2 it was clear that P6.2B was the most active. The decrease in antimalarial activity of fraction P6 from 0.5 μg/ml to 0.95μg/ml in fraction P6.2B suggests that components in the extract, including compound P6.2B, could be acting in synergy to be effective against *P. falciparum*. It would be of interest to reconstitute the fractions and then screen for antimalarial activity to see if the activity is the same as before.

When fraction P6.2B was run on HPLC-UV it became apparent that P6.2B was a single pure compound as it exhibited a single, sharp peak (Fig. 7). P6.2B also migrated as a single spot on TLC in different solvent mixtures of petroleum ether, dichloromethane, ethyl acetate and methanol (results not shown). High resolution mass spectrometry revealed a mass of 252.1860, consistent with a molecular formula of $C_{15}H_{24}O_3$. The compound was identified by MS and NMR analyses as hydroxydavanone (Fig. 18) in the Chemistry Department at U.C.T. Hydroxydavanone had been isolated previously from two other species of *Artemisia*. It was first identified in *A.maritima* L. spp. *maritima* (79) and then later isolated from *A. herba alba* (80). This compound had not been tested previously for antimalarial activity. In *A. inculta* and *A. herba alba* the hydroperoxide analoque of hydroxydavanone was found (80,81) as well as a number of davanone-like derivatives in *A. herba-alba* (80).

The antimalarial activity of the most active fraction, P6, was not due to artemisinin since hydroxydavanone was identified as the antimalarial compound. This was confirmed by a TLC comparison of pure artemisinin, *A. afra* crude extract, fraction P6 and fraction P6.2B (Fig. 5) which showed the absence of artemisinin in fractions P6 and P6.2B. Different UV spectra were obtained for artemisinin and P6.2B by HPLC-UV analysis (Fig. 9). There was however, still the question of artemisinin occurring in crude extracts of *A. afra*.

In the study thus far, neither TLC nor HPLC analyses had detected artemisinin in any of the crude *A. afra* extracts. To confirm this result, 3 samples of crude *A. afra* extracts (i.e. the crude petroleum ether soxhlet extract of leaves and flowers from which hydroxydavanone was isolated, crude petroleum ether soxhlet extract of leaves and the cold dichloromethane extract of leaves) were sent to Prof. K. Hostettmann at the University of Lausanne, Switzerland, for an accurate detection of artemisinin using thermospray LC-MS. The results showed

that no artemisinin could be detected in any of the three samples (Fig. 14-16). The detection limit showed that much less than 1μg (i.e. less than 0.1%) of artemisinin could be detected easily in the extract (Fig. 17). Although it was deemed possible that there could be trace amounts of artemisinin in the extracts, this however, seemed unlikely.

It therefore appears that there is no artemisinin in *A. afra*, although two factors cannot be discounted as explanations for its absence from the crude extracts. Firstly, it had been reported that the highest amount of artemisinin in *A. annua* was isolated just before flowering of the plant (3,4). *A. afra* in these experiments had been harvested before the flowering stage or whilst in the flowering stage. Verification of these results would require a systematic study of extracts prepared from *A. afra* at various stages of growth. Secondly, it was thought that if artemisinin was indeed present in *A. afra* extracts, then the possibility exists that it could be decomposed or degraded as a result of the extraction process which involved heating of the solvent up to 80°C in the Soxhlet apparatus. This theory however, seemed unlikely as artemisinin was, and continues to be regularly isolated from *A. annua* using a Soxhlet apparatus and it is known to be stable in non-polar solvents (3,38,71).

In an attempt to collect more plant material in order to isolate more of compound P6.2B, *A. afra* was collected in January 1996, which was before the flowering season. The material was dried and divided into 3 portions. These portions were extracted with hot petroleum ether (Soxhlet apparatus) and cold (room temperature) extractions with dichloromethane and water were performed. Surprisingly, hydroxydavanone could not be detected in any of the 3 extracts. A further sample of *A. afra* was harvested from the same bush at Kirstenbosch in May 1996, at the end of the flowering season. The material collected had few flowers left and seeds were already developing. The material

was nevertheless dried and portions extracted with hot petroleum ether (Soxhlet) and cold petroleum ether, dichloromethane and water. Using HPLC-UV, no P6.2B could be detected in any of these extracts either. This leads to the conclusion that hydroxydavanone could be present only for a short while, at the beginning of the flowering of the plant. The possibility also exists that the compound could be an intermediate secondary metabolite such that it is present only for a short while, at a particular stage of plant development. Finally, there is the possibility of hydroxydavanone being an artefact of the original soxhlet extraction procedure.

Samples of all the crude *A. afra* extracts generated in 1996 were injected (200μg) on a reverse phase C_{18} column. Their HPLC chromatograms are shown in Fig. 10-12. The chromatographic profiles were compared to determine any differences between the soxhlet and cold extraction techniques, and the different solvents used. *In vitro* antimalarial activities of these crude extracts were also determined against two strains of *P. falciparum*, namely, D10 and FAC8 (Table 10). The organic solvents, excluding the cold extraction with petroleum ether, demonstrated greater efficiency in extracting the larger number and quantity of antimalarial compounds. None of the 3 extracts from leaves of *A. afra* were as active (IC_{50}s ranged from 2.91 ± 0.37 to 645.02 ± 18.65 μg/ml against D10 and FAC8) (Table 10) as the first extract from leaves and flowers (IC_{50}s of 1.03 and 1.50 μg/ml against D10 and FAC8 respectively) (Table 4). The H_2O extract of *A. afra* leaves had very low activity (IC_{50} of 645.02 ± 18.65 μg/ml against D10) compared to the organic extracts (IC_{50}s of 4.31 ± 0.45 and 6.26 ± 0.37 μg/ml against D10 for the cold dichloromethane and petroleum ether soxhlet extracts respectively) (Table 10). The cold dichloromethane extraction technique appeared to have the greater extraction efficiency as it extracted compounds in larger amounts than the petroleum ether soxhlet extract of leaves (Fig. 10).

Comparing the two extraction techniques with petroleum ether for flowers of *A. afra* (Fig. 11 and 12), it is clear that the soxhlet extraction technique proved to be more effective in extracting the antimalarial compound(s) as the petroleum ether soxhlet extract had greater antimalarial activity (IC_{50}s of 15.78 ± 2.09 and 17.99 ± 0.63 μg/ml against D10 and FAC8 respectively) than the cold petroleum ether extract (IC_{50}s of 29.25 ± 0.04 and 31.65 ± 0.35 μg/ml against D10 and FAC8 respectively). The cold dichloromethane extract of flowers also had greater antimalarial activity (IC_{50}s of 9.79 ± 1.00 and 6.63 ± 0.81 μg/ml against D10 and FAC8 respectively) than the cold petroleum ether extract of flowers. This result was not surprising since the cold dichloromethane extract contained larger amounts of compounds compared to that of the cold petroleum ether extract of *A. afra* flowers (Fig. 12).

The cold dichloromethane and water extracts of *A. afra* flowers had less activity against *P. falciparum* compared to the activities obtained for the extracts of leaves (Table 10). Antimalarial activity was therefore, found to vary according to the developmental stage of the plant. The leaves of *A. afra* give way to flowers as they are formed. The crude petroleum ether soxhlet extract of *A. afra* leaves and flowers, from which hydroxydavanone was isolated, had the highest antimalarial activity against D10 and FAC8 (IC_{50}s were 1.03 and 1.50 μg/ml respectively). The plant material had been harvested at the start of the flowering season. Therefore, there may also be seasonal variations that could influence antimalarial activity of the plant.

The antimalarial activity of hydroxydavanone or P6.2B was investigated against a variety of *P. falciparum* strains with varying degrees of sensitivity against chloroquine and mefloquine. These included 3 South African strains also with varying sensitivity against chloroquine. Artemisinin was also screened against these strains. The results are summarized in Tables 6-9 and figures 21-24.

The *P. falciparum* strains D10 and RSA3 could be classified as chloroquine-sensitive and FAC8, W_2mef, W_2mef^{CQ}, RSA11 and RSA15 could be classified as chloroquine-resistant. The IC_{50} values obtained were in good agreement with literature values (16,76,83). FAC8 had the mildest resistance against chloroquine with IC_{50} of 142.4 ± 34.69ng/ml as compared to RSA15, the most chloroquine-resistant strain: IC_{50} of 265.50 ± 104.38ng/ml. W_2mef^{CQ} was more resistant to chloroquine than W_2mef. The chloroquine-sensitive strains, D10 and RSA3 had similar IC_{50} values of 23.23 ± 1.72 and 21.03 ± 1.93 ng/ml respectively.

The trend of resistance of chloroquine was different for mefloquine. The chloroquine-sensitive strains, D10 and RSA3 were mefloquine resistant whereas the chloroquine-resistant strains, FAC8, W_2mef, W_2mef^{CQ}, RSA11, and RSA15 were more sensitive to mefloquine. This is also in good agreement with literature values (1). D10 and RSA3 had IC_{50} values of 24.30 ± 0.42 and 32.23 ± 4.43 ng/ml respectively. The range of IC_{50} values for FAC8, W_2mef, W_2mef^{CQ}, RSA11 and RSA15 ranged from 10.30 ± 4.69 to 14.90 ± 8.92 ng/ml.

A similar trend as for mefloquine could be observed for artemisinin. However, all the strains were more sensitive to artemisinin than to mefloquine. D10 and RSA3 had IC_{50}s of 11.30 ± 6.59 and 13.67 ± 7.96 ng/ml respectively. The IC_{50} values for FAC8, W_2mef, W_2mef^{CQ}, RSA11 and RSA15 ranged from 2.67 ± 0.50 to 3.80 ± 0.80 ng/ml.

P6.2B was active against all 7 strains of *P. falciparum* but in the μg/ml concentration range. The highest activity was against D10 and FAC8 with IC_{50}s of 0.87 ± 0.04 and 0.88 ± 0.20 μg/ml respectively. Note that when previously tested against D10, an IC_{50} value of 0.95μg/ml was obtained. Therefore, there is a good indication of the stability of the compound and reliability and reproducibility of the antimalarial assay. The IC_{50} obtained for RSA3 was

higher at 2.65 ± 0.50μg/ml. As for mefloquine, there was a statistical difference between D10 and RSA3. However, D10 and FAC8 were not significantly different. There was, however, a difference between the IC_{50} values of D10 and the remaining chloroquine-resistant *P. falciparum* strains which ranged from 1.60 to 2.54 μg/ml.

With the antimalarial activity of P6.2B confirmed, it was important to establish its cytotoxicity profile to investigate its potential for clinical use. To provide some perspective, the cytotoxic profiles of chloroquine, artemisinin and mefloquine in addition to colchicine which was used as a standard, were also investigated. The crude *A. afra* extract of leaves and flowers was also screened. The cytotoxic activities against RAT-1 cells are reported in Table 11 and illustrated in Fig. 25. Colchicine had the highest cytotoxicity with IC_{50}: 0.60 ± 0.47 μg/ml. Mefloquine had the second highest activity followed by P6.2B, the crude *A. afra* extract, artemisinin and chloroquine. Throughout these experiments the highest percentage of acetonitrile that was used did not exceed 1% which had negligible effect against RAT-1 cells (Appendix D). From the above data, it is clear that both the crude *A. afra* extract and P6.2B have greater antimalarial activity (IC_{50} of ±1μg/ml for both the crude extract and P6.2B) than cytotoxic activity (34.78 ± 8.23 and 6.29 ± 0.95 μg/ml for the crude *A. afra* extract and P6.2B respectively). The cytotoxic activity for P6.2B is higher than the crude *A. afra* extract. This probably accounts for the higher antimalarial activity of P6.2B (IC_{50}s of 0.87 and 0.88 μg/ml against D10 and FAC8 respectively) over the crude extract of *A. afra* (IC_{50}s of 1.03 and 1.50 μg/ml against D10 and FAC8 respectively).

P6.2B had the lowest selectivity indices which ranged from 2.4 to 7.2 compared to the other antimalarials tested (Table 12). The selectivity indices for the crude *A. afra* extract was also low (33.8 and 23.2 against D10 and FAC8

respectively). It has been proposed that the ratio for a good therapeutic remedy be ≥ 1 000, as for quinine (82). Artemisinin clearly satisfies this criterion with selectivity indices ranging from 3 702 to 18 955 for the parasite strains tested. The selectivity indices for chloroquine against the chloroquine-sensitive strains was considerably higher (≥ 2 307) than for the chloroquine-resistant strains of *P. falciparum* which ranged from 202 to 376. Selectivity indices for mefloquine, a drug in clinical use, were much less than 1 000 although greater than 1.

It is important to note that it is difficult to correlate results of this study directly with the effects of a traditional treatment, since there are a number of factors that need to be considered, for example, the methods of preparation, the effects of a combination of plant extracts, as well as storage conditions. These factors could also potentially affect reproducibility of the data. In this study, these factors have not been considered and may be significant. Although a soxhlet extraction with water of flowers from *A. afra* was prepared, there is doubt that its activity would have been as high as for the organic extractions based on the following observations. Early attempts to confirm antimalarial activity of a hot-water (Soxhlet) extract of *A. annua* had been disappointing (3). Results were more encouraging with a low-temperature extraction of the plant with an organic solvent such as ethyl ether (3).

In the laboratory it is also difficult to study the effects of a combination of extracts. Together with the age of the plant and seasonal variations, there are additional factors that could affect reproducibility of data, such as regional and even geographic variations. In fact, distinct chemical differences by both TLC and HPLC-UV analyses, was observed between crude petroleum ether soxhlet extracts of two *A. afra* bushes at Kirstenbosch which were less than 5 metres apart (results not shown). It is also possible that many traditional healers

prescribe plants for their antipyretic properties as opposed to direct antimalarial effects (65). It is recognized that no attempt was made in this study to investigate this type of activity in *A. afra* so that its possible antipyretic therapeutic value is not recognized.

Since aerial parts of the plant are harvested as opposed to the roots, harvesting does not lead to plant death. Greater antimalarial activity has been reported for the aerial parts of the plant than roots (65). Thus, demonstration of activity in the aerial parts has implications for the sustainable agriculture and harvesting of plants.

In spite of the selectivity indices for P6.2B being low, there is still potential for this compound to be used clinically. Animal studies e.g. mice infected with *P. berghei* are recommended to assess its clinical potential. Structure modifications of hydroxydavanone could result in new chemical structures with increased potency against strains of *P. falciparum*, both *in vitro* and *in vivo*. There is also scope for this compound to be studied in combination with other antimalarials. For example, hydroxydavanone could enhance the activity of chloroquine, especially against chloroquine-resistant strains of *P. falciparum*. Also, just as synergistic effects were observed between artemisinin and mefloquine, similar synergy could be observed between hydroxydavanone and artemisinin or mefloquine. On the other hand, it is possible that the effect(s) of a combination of antimalarials, together with hydroxydavanone could be additive.

7. Conclusion

Crude extracts of *A. afra* exhibit *in vitro* antimalarial activity against chloroquine-sensitive (D10) and chloroquine-resistant (FAC8) strains of *P. falciparum*. The crude petroleum ether soxhlet extract of leaves and flowers had been successfully fractionated, resulting in the isolation of a single pure compound which was active against a variety of *P. falciparum* strains, including all three South African strains tested. This compound, a clear colourless oil was identified as hydroxydavonone. Cytotoxic screening was performed against a RAT-1 fibroblast cell-line which revealed greater antimalarial activity than cytotoxic activity for both the crude extract and the pure compound. Although selectivity indices for the crude petroleum ether soxhlet extract of leaves and flowers from *A. afra* were higher than those of the isolated pure compound, there are implications for this compound to be used clinically as an antimalarial but further studies are required to assess its potential. This suggests a role for *A. afra* as a phytomedicine in malaria. Furthermore, the demonstration of activity in the aerial parts of the plant has implications for the sustainable agriculture and harvesting of plants.

8. References

1. Basco, L.K., Le Bras, J. (1993) *In vitro* activity of artemisinin derivatives against African isolates and clones of *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene* **49:**301-307.

2. Wernsdorfer, W.H. (1991) The development and spread of drug-resistant malaria. *Parasitology Today* **7:**297-303.

3. Klayman, D. (1985) Qinghoasu (Artemisinin): An antimalarial drug from China. *Science* **228:**1049-1055.

4. White, N.J. (1994) Artemisinin: current status. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88** (Supplement 1): 3-4.

5. Watt, J.M., Breyer-Brandwijk, M.G. (1962). *Medicinal and Poisonous Plants of Southern Africa and Eastern Africa*. E & S Livingstone Ltd.

6. Abrahams, M.A. *In vitro antimalarial activity of extracts of Artemisia afra on Plasmodium falciparum*. Honours Thesis, University of Cape Town, 1993.

7. O' Neill, M.J., Bray, D.H., Boardman, P., Phillipson, J.D., Warhurst, D.C. (1985) Plants as sources of antimalarial drugs. Part. 1. *In vitro* test method for the evaluation of crude extracts from plants. *Planta medica*: 394-398.

8. Dame, J.B. *et al.* (1996) Current status of the *Plasmodium falciparum* genome project. *Molecular and Biochemical Parasitology* **79:**1-12.

9. Nkunya, M.H.H. (1992). *Progress in the search for antimalarials*. Addis Ababa, NAPRECA, Addis Ababa University.

10. Miller, L.H., Good, M.F., Milon, G. (1994) Malaria pathogenesis. *Science* **264:**1878-1883.

11. Woerdenbag, H.J., Lugt, C.B., Pras, N. (1990) *Artemisia annua* L.: a source of novel antimalarial drugs. *Pharmaceutisch Weekblad Scientific edition* **12:**169-181.

12. Marsh, K. (1992) Malaria - a neglected disease? *Parasitology* **104:**S53-S69.

13. Knell, A.J. (1991). *Malaria: a publication of the Tropical Programme of the Wellcome Trust*. Oxford, Oxford University Press.

14. Phillipson, J.D. (1991) Assays for antimalarial and amoebicidal activities. *Methods in Plant Biochemistry* **6:**136-151.

15. Phillipson, J.D., Wright, C.W. (1991) Can ethnopharmacology contribute to the development of antimalarial agents? *Journal of Pharmacology* **32:**155-165.

16. Freese, J.A., Markus, M.B., Golenser, J. (1991) *In vitro* sensitivity of Southern African reference isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine. *Bulletin of the World Health Organization* **69:**707-712.

17. Karbwang, J., Thanavibul, A., Chongsuphajaisiddhi, T., Na Bangchang, K., Bunnag, D. and Harinasuta, D. (1992) Comparison of oral artemether and mefloquine in acute uncomplicated falciparum malaria. *Lancet* **340:**1245-1248.

18. Wernsdorfer, W.H. (1994) Epidemiology of drug resistance in malaria. *Acta Tropica* **56:**143-156.

19. Gwadz, R.W. (1994) Genetic approaches to malaria control: how long the road. *American Journal of Tropical Medicine and Hygiene* **50** (Supplement): 116-125.

20. Farnsworth, N.R. (1993) Ethnopharmacology and future drug development: the North American experience. *Journal of Ethnopharmacology* **38:**145-152.

21. Baker, J.T. *et al.* (1995) Natural product drug discovery and development: New perspectives on international collaboration. *Journal of Natural Products* **58:**1325-1357.

22. Sofowora, A. (1993) Recent trends in research into African medicinal plants. *Journal of Ethnopharmacology* **38:**209-214.

23. Editorial. (1994) Pharmaceuticals from plants: great potential, few funds. *Lancet* **343:**1513-1515.

24. Phillipson, J.D. (1994) Natural products as drugs. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88** (Supplement 1): 17-19.

25. Phillipson, J.D. (1995) A matter of some sensitivity. *Phytochemistry* **38:**1319-1343.

26. Gericke, N. Traditional herbal medicines. Modern perspectives for South Africa. *Journal of the SA Druggists Group, Health Trends*, May 1996, 14-18.

27. Williams, V.L. The Witwatersrand muti trade. *Journal of the Botanical Society of South Africa, Veld and Flora*, March 1996, 12-14.

28. Kanhema, N. (14 Feb.1992) *Traditional doctors winning Western respect.* In: Sunday Star, Johannesburg.

29. Fourie, T.G., Swart, I., Snyckers, F. (1992) Folk Medicine: a viable starting point for pharmaceutical research. *South African Journal of Science* **88:**190-192.

30. Salemink, C.A. (1980) Problems involved in structure determination of active principles of plants used in traditional medicine: extraction, separation and determination of characteristics of active principles. *Journal of Ethnopharmacology* **2:**135-143.

31. Trager, W., Jensen, J.B. (1976) Human malaria parasites in continuous culture. *Science* **193:**673-675.

32. Desjardins, R.E., Canfield, C.J., Haynes, D., Chulay, J.D. (1979) Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrobial Agents and Chemotherapy* **16:**710-718.

33. Huxtable, R.J. (1992) The pharmacology of extinction. *Journal of Ethnopharmacology* **37:**1-11.

34. Cox, P.A., Balick, M.J. (1994) The ethnobotanical approach to drug discovery. *Scientific American*, June: 60-65.

35. Etkin, N.L., P.J. Ross. (1991) Should we set a place for diet in ethnopharmacology. *Journal of Ethnopharmacology* **32:**25-36.

36. Phillipson, J.D., O'Neil, M.J. (1986) Novel antimalarial drugs from plants. *Parasitology Today* **2:**355.

37. Zhu, Y., Woerdenbag, H.J. (1995) Traditional Chinese herbal medicine. *Pharmacy World & Science* **17:**103-111.

38. Hien, T.T., White, N.J. (1993) Qinghaosu. *Lancet* **341:**603-608.

39. Zhang, F., Gosser, D.K., Meshnick, S.R. (1992) Hemin-catalysed decomposition of Artemisinin (Qinghaosu). *Biochemical Pharmacology* **43:**1805-1809.

40. Meshnick, S.R., T.E. Taylor, Kamchonwongpaisan S. (1996) Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbiological Reviews* **60:**301-315.

41. Webster, H.K., Lehnert, E.K. (1994) Chemistry of artemisinin: an overview. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88** (Supplement 1): 27-29.

42. Ferreira, J.F.S., Simon, J.E., Janick, J. (1994) Developmental studies of *Artemisia annua*: Flowering and artemisinin production under greenhouse and field conditions. *Planta Medica* **61:**167-170.

43. Laughlin, J.C. (1994) Agricultural production of artemisinin - a review. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88** (Supplement 1): 21-22.

44. Ferreira, J.F.S., Simon, J.E., Janick, J. (1995) Relationship of artemisinin content of tissue-cultured, greenhouse-grown, and field-grown plants of *Artemisia annua. Planta Medica* **61:**351-355.

45. Woerdenbag, H.J. *et al.* (1994) Progress in the research of artemisinin-related antimalarials: an update. *Pharmacy World and Science* **16:**169-180.

46. Woerdenbag, H.J. *et al.* (1994) Artemisinin, related sesquiterpenes, and essential oil in *Artemisia annua* during a vegetation period in Vietnam. *Planta Medica* **60:**272-275.

47. Chan, K.L., Teo, C.K.H., Jinadasa, S., Yuen, K.H. (1995) Selection of high artemisinin yielding *Artemisia annua. Planta Medica* **61:**285-287.

48. Markell, E.K., Voge, M. (1981) *Medical Parisitology*. 5th Ed., W.B. Saunders Company, pp. 74-95.

49. Kamchonwongpaisan, S., Meshnick, S.R. (1996) The mode of action of the antimalarial artemisinin and its derivatives. *General Pharmacology* **27:**587-592.

50. Xiao, P.G., Fu, S.L. (1986) Traditional antiparasitic drugs in China. *Parasitology Today* **2:**353-355.

51. ter Kuile, F., White, N.J., Holloway, P., Pasvol, G. and Krisna, S. (1993) *Plasmodium falciparum*: *in vitro* studies of the pharmacodynamic properties of drugs used for the treatment of servere malaria. *Experimental Parasitology (United States)* **76:**85-95.

52. Cowman, A.F. (1995) Mechanisms of drug resistance in malaria. *Australian New Zealand Journal of Medicine* **25:**837-844.

53. Acton, N., Klayman, D.L., Rollman, I.J. (1985) Reductive electrochemical HPLC assay for artemisinin (Qinghaosu). *Planta Medica* **51:**455-456.

54. Mount, D.L., Todd, G.D. Navaratnam, V. (1995) Packed-column supercritical fluid chromatography of artemisinin (qinghaosu) with electron-capture detection. *Journal of Chromatography B* **666:**183-187.

55. Green, M.D., Mount, D.L., Todd, G.D., Capomacchia, A.C. (1995) Chemiluminescent detection of artemisinin novel endoperoxide analysis using luminol without hydrogen peroxide. *Journal of Chromatography A* **695:**237-242.

56. Ferreira, J.F.S., Janick, J. (1996) Immunoquantitative analysis of artemisinin from *Artemisia annua* using polyclonal antibodies. *Phytochemistry* **41:**97-104.

57. Avery, M.A., Bonk, J.D., Mehrotra, S. (1996) Deuterated antimalarials: synthesis of trideutero- artemisinin, dihydroartemisinin, and arteether. *Journal of Labelled Compounds and Radiopharmaceuticals* **38:**249-254.

58. Price, R.N. *et al.* (1996) Effects of artemisinin derivatives on malaria transmissibility. *Lancet* **347:**1654-1658.

59. Maeno, Y *et al.* (1993) Morphologic effects of artemisinin in *Plasmodium falciparum*. *American Journal of Tropical Medicine and Hygiene* **49:**485-491.

60. Posner, G.H., Cumming, J.N. (1995) Evidence for Fe(IV)=O in the molecular mechanism of action of the trioxane antimalarial artemisinin. *Journal of the American Chemical Society* **117:**5885-5886.

61. Meshnick, S.R. (1994) The mode of action of antimalarial endoperoxides. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **88** (Supplement 1): 31-32.

62. Wesche, D.L., DeCoster, M.A., Tortella, F.C., Brewer T.G. (1994) Neurotoxicity of artemisinin analogs *in vitro*. *Antimicrobial Agents and Chemotherapy* **38:**1813-1819.

63. Valant-Vetschera, K.M., Wollenweber, E. (1995) Flavonoid aglycones from the leaf surfaces of some *Artemisia* spp. (Compositae-Anthemideae). *Verlag der Zeitschrift fur Naturforschung* **50c:**353-357.

64. Buchbauer, G., Silbernagel, E (1989) Der Sudafrikanische Wermut: Artemisia afra JACQ. *Deutsche Apotheker Zeitung* **129:**2173-2177.

65. Weenen, H., Nkunya, H.H., Bray, D.H., Mwasumbi, L.B., Kinabo, L.S., Kilimali, V.A.E.B. (1990) Antimalarial activity of Tanzanian medicinal plants. *Planta Medica* **56:**368-369.

66. Worku, T. Rubjiolo, P. (1996) Major constituents of *Artemisia afra* oil. *Journal of Essential Oil Research* **7:**355-357.

67. Jakupovic, J., Klemeyer, H., Bohlmann, F., Graven, E.H. (1988) Glaucolides and guaianolides from *Artemisia afra. Phytochemistry* **27:**1129-1133.

68. Graven, E.H., Deans, S.G., Svoboda, K.P., Mavi, S., Gundidza, M.G. (1992) Antimicrobial and antioxidative properties of the volatile (essential) oil of *Artemisia afra* Jacq. *Flavour and Fragrance Journal* **7:**121-123.

69. Roberts, M. (1990) *Indigenous healing plants.* Southern Book Publishers (Pty) Ltd., pp. 226-227

70. Hamburger, M., Hostettmann, K., (1991) Bioactivity in plants: The link between phytochemistry and medicine. *Phytochemistry* **30:**3864-74.

71. ElSohly, H.N. (1990) A large-scale extraction technique of artemisinin from *Artemisia annua. Journal of Natural Products* **53:**1560-1564.

72. Wolfender, J., Maillard M., Hostettmann K. (1994) Thermospray liquid chromatography-mass spectrometry in phytochemical analysis. *Phytochemical Analysis* **5:**153-182.

73. Ekong, R.M., Robson, K.J.H., Baker, D.A., Warhurst, D.C. (1993) Transcripts of the multidrug resistance genes in chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum. Parasitology* **106:**107-115.

74. Biggs, B.A., Kemp, D.S., Brown, G.V. (1989) Subtelomeric chromosome deletions in field isolates of *Plasmodium falciparum* and their relationship to loss of cytoadherance *in vitro. Proceedings of the National Academy of Science (USA)* **86:**2428-2432.

75. Oduola, A.M.J. *et al. Plasmodium falciparum*: induction of resistance to mefloquine in cloned strains by continuous drug exposure *in vitro. Experimental Parasitology* **67:**354-360.

76. Barnes, D.A., Foote, S.J., Galatis, D., Kemp, D.J., Cowman, A.F. (1992) Selection for high level chloroquine resistance results in deamplification of the pfmdr gene and increased sensitivity to mefloquine in *Plasmodium falciparum*. *EMBO Journal* **11:**3067-3075.

77. Huber, W., Koella, J.C. (1993) A comparison of three methods of estimating EC_{50} in studies of drug resistance of malaria parasites. *Acta Tropica* **55:**257-261.

78. Twentyman, P.R. Luscombe, M. (1987) A study of some variables in a tetrazolium dye (MTT) assay for cell growth and chemosensitivity. *British Journal of Cancer* **56:**279-285.

79. Jork, H., Nachtrab, M. (1976) Neue substanzen aus ·therischen ··len verschiedener Artemisia-species, 4. Mitt. Hydroxy-davanone, ein furanoider sesquiterpenalkohol. *Arch. Pharm. (Weinheim)* **312:**923-932.

80. Marco, J.A., Sanz-Cervera, J.F., Ocete, G. (1994) New germacranolides and eudesmanolides from North African *Artemisia herba-alba*. *Journal of Natural Products* **57:**939-946.

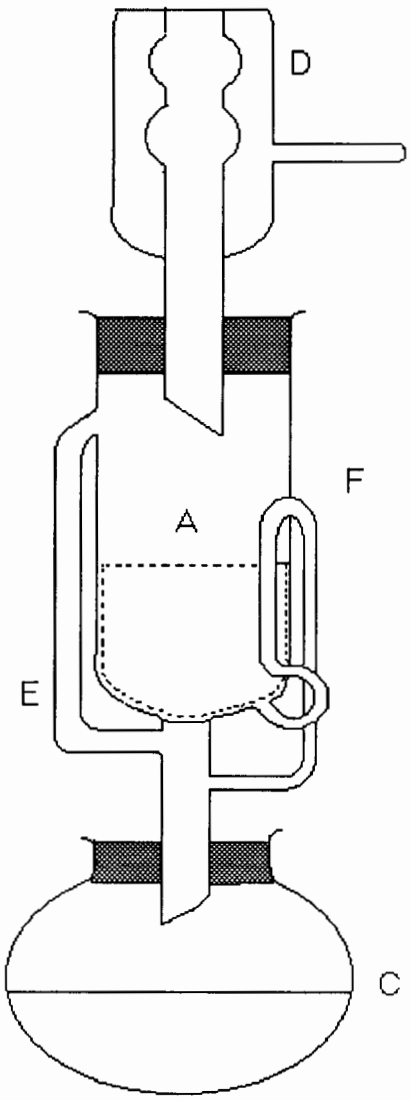
81. Khafagy, S.M., Al-Yahya, M.A., Ziesche, J., Bohlmann, F. (1983) An unusual nerolidol derivative from *Artemisia inculta*. *Phytochemistry* **22:**1821-1822.

82. Gessler, M.C., Tanner, M., Chollet, J., Nkunya, M.H.H. Heinrich, M. (1995) Tanzanian medicinal plants used traditionally for the treatment of malaria: *In vivo* antimalarial and *in vitro* cytotoxic activities. *Phytotherapy Research* **9:**504-508.

83. Foote, S.J., Thompson, J.K., Cowman, A.F., Kemp, D.J. (1989) Amplification of the multidrug resistance gene in some chloroquine resistant isolates of *Plasmodium falciparum*. *Cell* **57:**921-930.

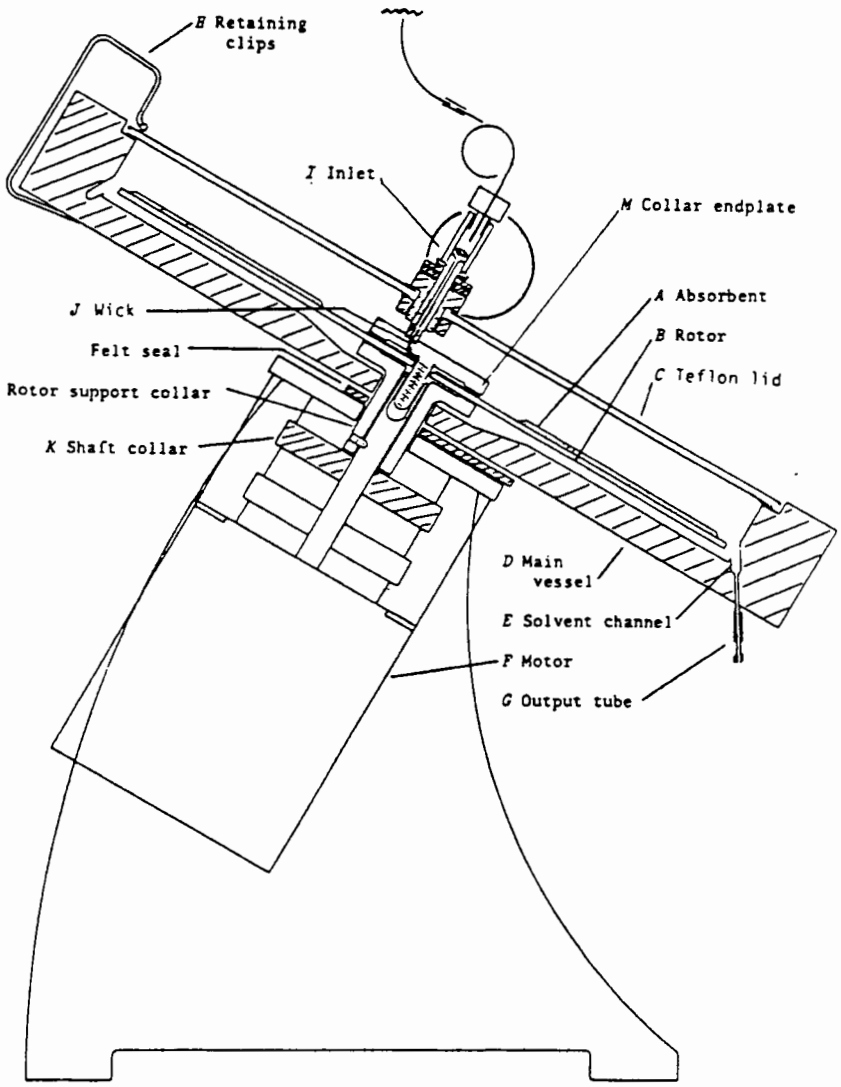
84. Vogel, A.I. (1956) Experimental Technique. In: *Practical Organic Chemistry Including Quantitative Organic Analysis*. 3rd Ed., Longman, pp. 153-154.

Appendix A



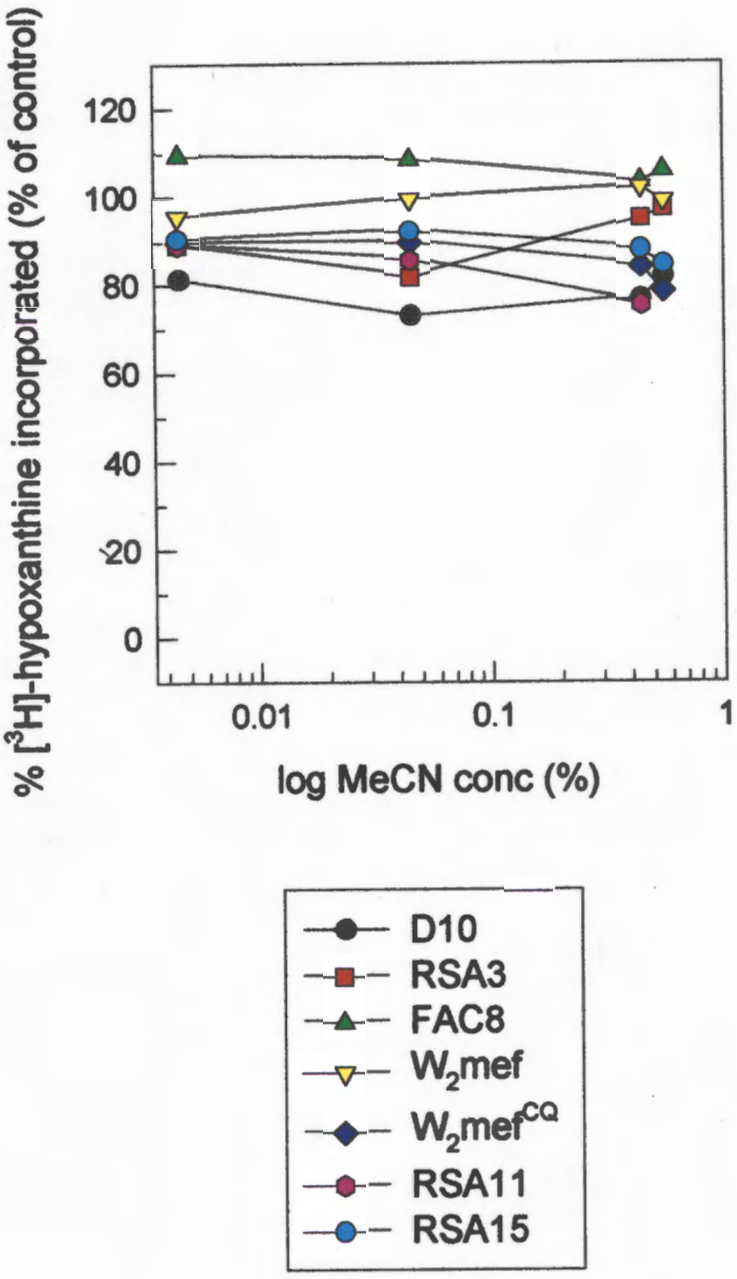
Principle of the Soxhlet Extraction: The plant material is placed in **A**, the Soxhlet apparatus. The apparatus is then fitted onto flask **C**, which contains the solvent. The solvent is boiled gently. Vapour passes up through the tube **E** which is condensed by the condenser **D**. The condensed solvent falls into **A** and slowly fills the body of the Soxhlet. When the solvent reaches the top of tube **F**, it siphons over into flask **C** and thus removes that portion of the substance which it has extracted in **A**. This process is repeated automatically until the extraction is complete, usually over several hours. The extracted compound is then isolated from **C** by evaporating the solvent (84).

Appendix B



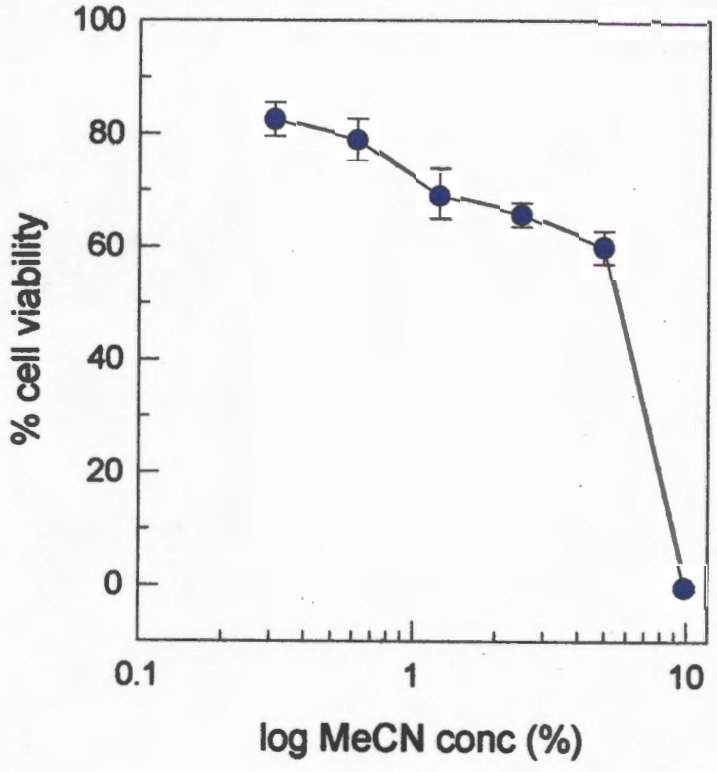
Principle of the Chromatotron: Chromatography is performed in a thin layer of absorbent **A** on a rotor **B**. A motor **F** drives the rotor at a constant speed by a shaft passing through a hole in the centre of the main vessel **D**. Solutions of compounds to be separated are applied to the absorbent via the inlet **I** and wick **J**. Elution by solvent forms concentric bands of separated substances which leave the edge of the rotor together with solvent. A channel **E** collects the eluate and brings it to the output tube **G**. The Teflon lid **C** is transparent to UV light, allowing detection of UV absorbing bands. Eight retaining clips **H** hold the Teflon lid on the main vessel.

Appendix C



The effect of acetonitrile on the incorporation of hypoxanthine into *P. falciparum* strains. Data indicate means of 2 experiments.

Appendix D



The effect of acetonitrile against RAT-1 fibroblast cells. Data indicate means ± SD (n=2). IC_{50} value: 5.63 ± 0.15%. For all experiments conducted the amount of acetonitrile used did not exceed 1%.