In vitro antimalarial activity of ethnobotanically selected indigenous plants and characterisation of a bioactive compound

by

Erwin Antoni Prozesky

submitted in partial fulfillment of the requirements of

the degree

MAGISTER SCIENTIAE (Plant Physiology)

in the Faculty of Biological and Agricultural Sciences

University of Pretoria

Pretoria

February 1999

Supervisor: Prof J.J.M. MEYER

Co-supervisor: Prof A.I. LOUW

Contents

List of abbreviations

DDT: Dichlorodiphenyltrichloroethane

EDTA: Ethylenediaminotetra-acetic acid

GC: Gas chromatography

HEPES: N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid

HPLC: High pressure liquid chromatography

IC_{50}: 50% inhibition concentration

ID_{50}: 50% inhibition dose

MEM: Minimum essential medium

MS: Mass spectrometry

NH: Natal herbarium, Durban

NMR: Nuclear magnetic resonance

PBS: Phosphate buffer saline

PRE: National herbarium, Pretoria

PRU: University of Pretoria herbarium, Pretoria

PTLC: Preparative thin layer chromatography

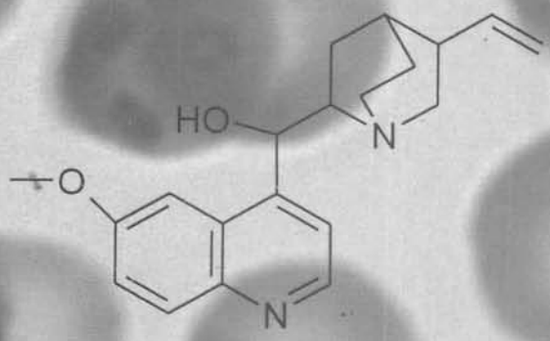
TLC: Thin layer chromatography

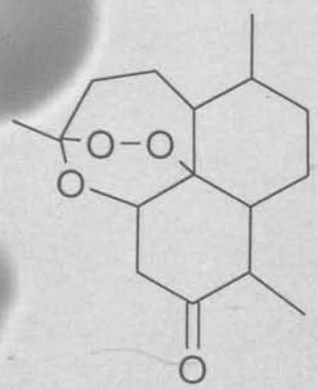
Tris: N-Tris (hydroxymethyl) aminomethane

UV: Ultra violet

Chapter 1

Introduction





1 Introduction

1.1 Background

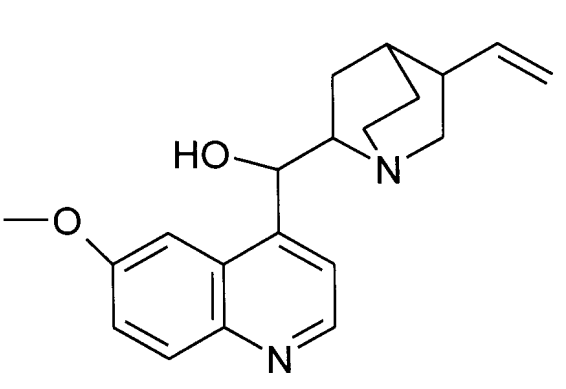
The use of medicinal plants as a source for relief from illness can be tracked back over five millennia to written documents of the early civilisations in China, India and the Near East, but it is doubtless an art as old as mankind (Hamburger & Hostettmann 1991). It has been estimated that 80% of people living in developing countries are almost completely dependent on traditional medical practices for their primary health care needs, and higher plants are known to be the main source of drug therapy in traditional medicine.

Although only about 119 plant-derived drugs of known structure are still extracted and used globally in allopathic medicine, they constitute about 25% of prescribed medicine in industrialised countries. About 74% of these were discovered by chemists who were attempting to identify the chemical substances in plants that were responsible for their medicinal uses by humans. These 119 plant derived drugs are produced commercially from less than 90 species of higher plants. Since there are at least 250 000 species of higher plants on earth, it is logical to presume that many more useful drugs will be found in the plant kingdom if the search for these entities is carried out in a logical and systematic manner. On the basis of current drug usage and the inestimable role that these drugs have played in the alleviation of human suffering, it seems justified that the search for new and improved plant-derived drugs should continue (Farnsworth 1990). South Africa is in the fortunate position of having 25 000 (10%) of the world's plants. This coupled with a rich ethnobotanical history makes South Africa an ideal place to search for new drugs.

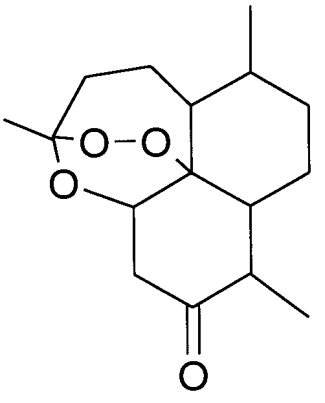
The alkaloid quinine (Figure 1), obtained from the bark of several species of *Cinchona* indigenous to South America, was the first effective antimalarial drug to be introduced early in the nineteenth century. Knowledge of its chemical structure led to the development of a series of antimalarial drugs that were synthesised using the quinine molecule as a template for new drug design. So successful were such drugs as the 4-amino- and the 8-aminoquinolines that quinine became almost superseded as a clinically useful antimalarial. In the 1950s, it was confidently expected that the disease would be eradicated because of effective drugs that would eliminate *Plasmodium falciparum* infections in man and potent insecticides, which would destroy the anopheline vector mosquitoes. Today, malaria has reached epidemic proportions on a worldwide scale due to resistance of *Plasmodium falciparum* to clinically useful drugs such as chloroquine and pyrimethamine as well as resistance of the vector mosquitoes to DDT and other insecticides. It has been estimated that in Africa alone some one million children die from the disease each year. The anticipated vaccine does not appear likely to be available in the near future and even if it were, new chemotherapeutic agents would still be necessary for complete management of the disease. There is obvious need for the development of new antimalarial drugs, preferably with novel modes of action against plasmodia (Phillipson & Wright 1991).

The isolation of the active principle, artemisinin, from a Chinese herb used traditionally in the treatment of malaria, *Artemisia annua* (Asteraceae), has done much to stimulate research into higher plants as potential sources of antimalarial drugs (Phillipson & Wright 1991). For about twenty centuries, Chinese herbalists have known about the medicinal value of the weed, *qing hao*, the source of the new antimalarial, artemisinin

(Figure 1). In the late 1960s, an effort was made in China to evaluate several traditional herbal remedies for their present day validity. Although *qing hao* was one of them, a hot tea made from the plant disappointingly lacked antimalarial activity. In 1971, a low temperature extraction with diethyl ether yielded an extract that gave positive antimalarial results in infected mice and monkeys. A biological activity-directed isolation of the specific constituent responsible for the antimalarial properties yielded artemisinin. Artemisinin, after testing in animals, was administered to humans infected with malaria and was found to be an effective blood schizontocide with virtually no toxicity. Chemically, artemisinin is unlike any previous antimalarial agent, because virtually all of the rest have a nitrogen-containing heterocycle, whereas artemisinin is a sesquiterpene lactone that incorporates an endoperoxide moiety. The latter is a group that is infrequently encountered in natural products (Klayman 1993).



quinine



artemisinin

Figure 1: Important antimalarial compounds isolated from plants

The recognition and validation of traditional medical practices and the search for plant-derived drugs could lead to new strategies in malaria control. Since many modern drugs originated from plants, the investigation of the chemical components of traditional medicinal plants could lead to the development of new antimalarial drugs. It is also necessary to obtain more scientific information concerning the efficacy and safety of the remedies in use, because many people in third world countries already use and depend on herbal medicines for the treatment of malaria. Health planners in developing countries also require such information for evaluating the position and effectiveness of traditional medicine (Gessler *et al.* 1994).

At present very little is known about the antimalarial activity of extracts from South African plant species. Habitat plays an important role in the chemical composition and bioactivity of plants. Various medicinal plants tested for antimalarial activity in other parts of Africa have shown potential as Western medicine and many related members of those plants also occur in South Africa. Very few studies on the antimalarial activity of these plants have been done in South Africa.

Most of the drugs derived from isolated compounds or originally isolated from higher plants were discovered in an ethnobotanical context. Three criteria are important when selecting plants for biotests. Firstly, medicinal plants used for many generations are more likely to have useful bioactivity. Secondly, plants should be selected from an area with a diverse flora – different plants should have been tried and the best ones selected. Thirdly, people should have lived in the area over a number of years, having enough time to explore local floral resources (Cox 1990).

1.2 Objectives of the study

- Ethnobotanical selection of plants used against malaria
- Preparing extracts of the collected material
- Testing *in vitro* antimalarial activity of extracts
- Determining the cytotoxicity of extracts with bioactivity
- Identifying non-toxic bioactive extracts with potential for further testing
- Purifying these extracts with activity guided fractionation
- Isolation and identification of active compounds

1.3 Scope of the dissertation

The selection and collection of indigenous plants as well as the *in vitro* antimalarial activity of 20 extracts from 14 plant species prepared by extraction in different solvents are described in Chapter 2. Chapter 3 describes the cytotoxicity of selected extracts with the best antimalarial activity tested with the luminescent bacteria cytotoxicity test and the monkey kidney cell cytotoxicity test. Purification of the dichloromethane extract from *Ozoroa engleri* is described in Chapter 4. Purified fractions tested for antimalarial activity showed only moderate antimalarial activity and no attempts were made to identify active compounds. The isolation and identification of kaurenoic acid from *Croton pseudopulchellus* is described in Chapter 5. This chapter also deals with the *in vitro* antimalarial activity and cytotoxicity of kaurenoic acid, while Chapter 6 consists of the general discussion and conclusions.

1.4 References

COX, P. A. 1990. Ethnopharmacology and the search for new drugs. In: Bioactive compounds from plants, ed. D. J. Chadwick and J. Marsh, pp. 40 – 55. Ciba Foundation.

FARNSWORTH, N. R. 1990. The role of ethnopharmacology in drug development. In: Bioactive compounds from plants, ed. D. J. Chadwick & J. Marsh, pp. 2 – 21. Ciba Foundation.

GESSLER, M. C., NKUNYA, M. H. H., MWASUMBI, L. B., HEINRICH, M. & TANNER, M. 1994. Screening Tanzanian medicinal-plants for anti-malarial activity. *Acta Trop*. 56: 65 – 77.

HAMBURGER, M. & HOSTETTMANN, K. 1991. Bioactivity in plants: the link between phytochemistry and medicine. *Phytochem.* 30: 3864 – 3874.

KLAYMAN, D. L. 1993. *Artemisia annua*: From Weed to Respectable Antimalarial Plant. In: Human Medicinal Agents from Plants, ed. A. D. Kinghorn & M. F. Balandrin, Ch 17, pp. 242 – 255. ACS Symposium Series 534.

NKUNYA, M. H. H., WEENEN, H., BRAY, D. H., MGANI, Q. A. & MWASUMBI, L. B. 1991. Antimalarial activity of Tanzanian plants. 3. Antimalarial activity of Tanzanian plants and their active constituents - the genus *Uvaria*. *Planta Med.* 57: 341 – 343.

PHILLIPSON, J. D. & WRIGHT, C. W. 1991. Can ethnopharmacology contribute to the development of antimalarial agents? *J. Ethnopharmacol*. 32: 155 – 165.

Chapter 2

In vitro antimalarial activity of plant extracts

2 *In vitro* antimalarial activity of plant extracts

2.1 Introduction

Although there exists other strategies for human malaria prevention, like the control and eradication of the vector (Baudon 1987), drugs constitute an obvious and convenient tool in the fight against the disease (Black 1981). Unfortunately, up to now, there have been only four standard antimalarial drugs available for unlimited clinical use: chloroquine, quinine, and more recently mefloquine and halofantrine. Since it has been determined to be a safe drug, artemisinin is now being launched via commercial channels. There is an urgent need for the development of totally new antimalarial drugs and for intensive research directed towards the discovery of drugs that may reverse chloroquine resistance in malaria (Wernsdorfer & Trigg 1988). Natural products will probably play an important role in the discovery of new antimalarial compounds (Nkunya 1991). The search for antimalarial drugs from plants involves the screening of crude or refined extracts against the malaria parasite. Before 1976 this was only done *in vivo*, making it a very expensive and difficult procedure, but since the development of the method for continuous *in vitro* culturing of human malaria parasites (Trager & Jensen 1976), extracts and compounds could be tested *in vitro*, making antimalarial testing much easier.

2.2 Selection and collection of plant material

Plants selected for this study were obtained by analysing ethnobotanical literature. Ethnobotanically described antimalarial South African plants were selected, as well as South African representatives of closely related antimalarial plants, found in other parts of the world (Table 1).

The plants analysed in this study were collected mainly from two areas in the malaria distribution region, namely Venda and Northern Kwazulu-Natal. At least two field trips to each of these regions were undertaken to collect plant material. Voucher specimens are preserved in the H. G. W. J. Schweickerdt herbarium at the University of Pretoria. Some of the material collected is shown in Figure 1.

Figure 1: Samples of plant material collected

Table 1: Plant species included in this study and their medicinal uses reported in literature

Family	Plant species and herbarium voucher number	Medicinal uses reported in literature	
		Against malaria	Other medicinal uses
Anacardiaceae	*Ozoroa engleri* R.A. Fernandes (**E. A. Prozesky 95** PRU[a])	Related species, *O. insignis* in Tanzania (Weenen *et al.* 1990).	Bark, leaves and roots are used medicinally and fruit is traditionally used to dress hair (Pooley 1993). The Vhavenda traditionally take bark decoctions for venereal diseases and also, in soft porridge, as a general cleanser for men.
Balanitaceae	*Balanites maughamii* Sprague (**E. A. Prozesky 89** PRU)	Related species, *B. aegyptica* in Tanzania (Weenen *et al.* 1990).	Bark decoctions are administered as emetics in unspecified parts of southern Africa (Palmer & Pitman 1972). Fruit is used as an arrow poison by the Vhavenda (Mabogo 1990). Fruit has molluscicidal properties (Pretorius *et al.* 1988).
Celastraceae	*Maytenus senegalensis* (Lam.) Excell (**E. A. Prozesky 79** PRU)	Stem bark used in Tanzania (Gessler *et al.* 1994). Included as a positive plant control.	Hot infusions of pounded bark are taken as emetics for chronic coughs (Bryant 1966). Leaf and bark infusions are also taken as emetics for respiratory ailments (Watt & Breyer-Brandwijk 1962).

Family	**Plant species and herbarium voucher number**	**Medicinal uses reported in literature**	
		Against malaria	Other medicinal uses
Combretaceae	*Combretum molle* R.Br. ex G.Don (**E. A. Prozesky 81** PRU)	Related species, *C. aff. psidioides* subsp. *psilophyllum* in Tanzania (Gessler *et al.* 1994).	Inner bark infusions are administered orally or as enemas for various stomach complaints. Unidentified parts are reported to be used for fevers (Pooley 1993). Decoctions of the inner bark are used for stomach complaints by the Luvale, who also use leaf decoctions for wounds. In Ghana, leaves are used as wound dressings, abortifacients and anthelmintics and also for stomach ache, colic and fevers (Irvine 1961). The Vhavenda use the bark for worms, roots for infertility, intestinal worms and as a laxative while leaves and roots are used for wounds, snakebite, stomach complaints and difficult confinements (Mabogo 1990). Roots are used for abdominal pain, diarrhoea , infertility, bleeding after childbirth, convulsions, as an aphrodisiac and against backache (Gelfand *et al.* 1985).

Family	**Plant species and herbarium voucher number**	**Medicinal uses reported in literature**	
		Against malaria	Other medicinal uses
Euphorbiaceae	*Croton pseudopulchellus* Pax (**E. A. Prozesky 91** PRU)	Related species, *C. megalobotrys* used by Vhavenda (Mabogo 1990).	Leaves and roots are used as antiviral and antitussive medicines in Ghana (Irvine 1961). The Nyamwezi use root decoctions for asthma and leaves and twigs with other ingredients in vapor steams for syphilitic sores (Watt & Breyer-Brandwijk 1962).
Fabaceae	*Erythrina lysistemon* Hutch. (**E. A. Prozesky 103** PRU)	Related species, *E. sacleuxii* in Tanzania (Weenen *et al.* 1990).	Bark is used as a poultice for swellings and abscesses (Pujol 1990). Crushed leaves are reported to clear wounds of maggots. Bark is used for toothache by the Vhavenda (Mabogo 1990).
Meliaceae	*Entandrophragma caudatum* (Sprague) Sprague (**E. A. Prozesky 100** PRU)	Related species *E. bussei* in Tanzania (Weenen *et al.* 1990).	None found.

Family	Plant species and herbarium voucher number	Medicinal uses reported in literature	
		Against malaria	Other medicinal uses
Meliaceae	*Trichilia emetica* Vahl **(E. A. Prozesky 76** PRU)	*Trichilia glabra* tested by MacKinnon *et al.* (1997).	Bark decoctions are administered as enemas for stomach and intestinal complaints (Bryant 1966). Infusions of the bark or leaf are used for lumbago, rectal ulceration in children and dysentery (Watt & Breyer-Brandwijk 1962). Enemas made from bark are administered for kidney ailments, as stomach and blood cleansers and also for intestinal worms by the Vhavenda (Mabogo 1990). Bark is used in enemas for dysentery by the Rhonga, (Watt & Breyer-Brandwijk 1962). Roots are used for fever and as purgatives, bark for indigestion and leaves and bark for dysentery in various parts of Africa (Gelfand *et al.* 1985).
Mimosaceae	*Acacia xanthoploea* Benth. **(E. A. Prozesky 96** PRU)	Powdered bark and roots are used by Zulus in emetics for malaria and are also taken prophylactically (Watt & Breyer-Brandwijk 1962).	Bark decoctions are taken for sickle cell anemia in Tanzania (Chhabra *et al.* 1984).

Family	**Plant species and herbarium voucher number**	**Medicinal uses reported in literature**	
		Against malaria	Other medicinal uses
Rhamnaceae	*Rhamnus prinoides* L'Hérit. (**E. A. Prozesky 104** PRU)	Used in Kenia (Prof. A.E. van Wyk personal communication).	Embrocations from unspecified parts are used for sprains (Bryant 1966). Decorticated root decoctions are used as blood purifiers (Watt & Breyer-Brandwijk 1962).
Rhamnaceae	*Ziziphus mucronata* Willd. (**E. A. Prozesky 70A** PRU)	Selected as closely related genus to *Rhamnus*.	Hot infusions of pounded bark are taken as emetics for chronic coughs (Bryant 1966). Leaf and bark infusions are also taken as emetics for respiratory aliments (Watt & Breyer-Brandwijk 1962). Leaves and roots are used for pain, while roots are used for infertility and for purification by the Vhavenda (Mabogo 1990). Decoctions from leaves and shoots are inhaled and gargled for measles and scarlet fever. Bark decoctions are used for chest complaints, leaf poultices for boils and carbuncles and root infusions for dysentery. Unspecified parts have been used for gonorrhea (Hutchings *et al.* 1996). In East Africa, roots are used for treating snakebite and bark decoctions are used for rheumatism and stomach ailments (Kokwaro 1976).

Family	Plant species and herbarium voucher number	Medicinal uses reported in literature	
		Against malaria	Other medicinal uses
Rubiaceae	*Catunaregam spinosa* (Thunb.) Tirvengadum subsp. *spinosa* (**E. A. Prozesky 82** PRU)	Used by Zulus for fever (Gerstner 1941).	Infusions from unspecified parts are administered as emetics for fevers (Gerstner 1941). Roots are reported to have well known antifebrile properties. Used as an aphrodisiac and also for gonorrhea in East Africa (Verdcourt & Trump 1969). Seed decoctions are used for headaches by the Kuledi (Watt & Breyer-Brandwijk 1962). In Zimbabwe, roots are used for many ailments including snakebite, nausea, various respiratory, febrile and gynecological ailments, epilepsy and dizziness (Gelfand *et al.* 1985).

Family	**Plant species and herbarium voucher number**	**Medicinal uses reported in literature**	
		Against malaria	Other medicinal uses
Solanaceae	*Solanum panduriforme* E. Mey. (**E. A. Prozesky 94** PRU)	Leaf infusions of a related species *S. nigrum* are used by the Vhavenda (Mabogo 1990) and is also used in Zimbabwe (Watt & Breyer-Brandwijk 1962).	Sap from unspecified parts [probably fruit] is used for skin infections (**A.R. Bain 4145** PRE[b]). Fruit is used as traditional medicine (**Cunningham 2441** NH[c]). Roots are used for toothache and root decoctions are taken for hemorrhoids by the Sotho (Watt & Breyer-Brandwijk 1962). Fruit is used for wounds and toothache and, with other plants, for ulcers by the Vhavenda (Mabogo 1990).

Family	Plant species and herbarium voucher number	Medicinal uses reported in literature	
		Against malaria	Other medicinal uses
Verbenaceae	*Lippia javanica* (Burm. f.) Spreng. **(E. A. Prozesky 78** PRU)	Used by Vhavenda (Mabogo 1990).	Hot leaf infusions are widely used for coughs and colds, most frequently as inhalants but also taken orally (Hutchings *et al.* 1996). Leaves are also used to treat febrile rashes (Doke & Vilakazi 1972). They are also used in washes and poultices for chest ailments (Roberts 1990). Cold leaf infusions are taken for a condition referred to as gangrenous rectitis (Bryant 1966). Weak leaf and stem infusions are taken for coughs, colds and bronchial ailments and, with the addition of *Artemisia afra* Jacg. Ex Willd., are also used for fevers and measles by the Xhosa (Smith 1895; Watt & Breyer-Brandwijk 1962). Leaves are used for a variety of ailments including asthma, headaches, febrile and respiratory complaints, convulsions, weak joints, cataracts and sore eyes in Zimbabwe (Gelfand *et al.* 1985). Roots are used for headaches, scabies and backache, and in infusions used to bathe marasmic infants. The Vhavenda use leaf infusions as anthelmintics, for respiratory

Family	Plant species and herbarium voucher number	Medicinal uses reported in literature	
		Against malaria	Other medicinal uses
Verbenaceae	*Lippia javanica* (continued)		and febrile ailments and as prophylactics against dysentery, diarrhea and malaria (Mabogo 1990). In West Africa, leaves and roots are used for fevers, headaches and skin diseases (Dalziel 1937; Irvine 1961).

[a]University of Pretoria Herbarium, Pretoria; [b]National Herbarium, Pretoria; [c]Natal Herbarium, Durban

2.3 Materials and methods

2.3.1 Preparation of extracts

Samples of stem bark and leaves were collected and left at room temperature for two weeks to dry. Samples were chopped into smaller pieces and then ground into powder with an IKA dry mill. The samples were then stored in jars at room temperature until needed for extraction. For each extraction, 5 - 20 g of powdered leaves or stem bark were put in a round bottomed flask and 150 ml of solvent (acetone, ethanol, dichloromethane, chloroform or petroleum ether) added. The extracts were then stirred with a magnetic stirrer for 24 hours, filtered and dried by rotary vacuum evaporation. The mass of the extracts was determined, re-dissolved in ethanol or dimethyl sulfoxide at concentrations of 100 or 200 mg/ml and stored at 4°C.

Extracts for the visual test were tested in triplicate at 200, 100, 50 and 25 µg/ml and in duplicate at 50 µg/ml with the microscopic test. For the flow cytometric analysis, extracts were tested in duplicate at 50 µg/ml and serial dilutions made from this concentration for the determination of the IC_{50} values. One extract of each species with an inhibition of more than 70% at 50 µg/ml was selected for determination of the IC_{50} values. Through previous experiences in our laboratory with extraction solvents influencing biotests, solvent controls were also tested for antimalarial activity. Extraction solvents were concentrated to the same degree as the extracts and dissolved in the same solvents as were the case with the extracts. These concentrated solvents were then diluted equivalently

to the highest extract dilutions (500 times and 1000 times) giving 0.2% and 0.1% solvent percentages and included in the microscopic and flow cytometric tests.

2.3.2 *In vitro* culturing of malaria parasites

The South African isolate (PfUP1) of the malaria parasite *Plasmodium falciparum*, was used in the bioassays. For continuous *in vitro* culturing a slightly modified version of the Trager and Jensen method was employed (Trager & Jensen 1976; Hoppe 1993). The wash medium consisted of 10.4 g RPMI 1640 L-glutamine, 5.94 g HEPES buffer, 4.0 g D-glucose, 44 mg hypoxanthine, 5% sodium hydrogen carbonate and 4 mg of gentamycin dissolved in 900 ml deionised sterile water. For use as culture medium, this wash medium was supplemented with 10% human serum of a positive blood group after heat inactivation at 56°C for 20 minutes. The parasite culture was then suspended in 10 ml of this culture medium, in a 75 ml culture flask (Sterilin). The culture, consisting of parasites and culture medium was then further supplemented with fresh, uninfected human erythrocytes with an O^+ blood group.

Erythrocytes for maintenance of the culture were obtained from whole blood which was centrifuged in a Hermle Z 320 bench centrifuge at 500 g for 5 minutes. The plasma portion as well as the leukocyte buffy coat was removed. The erythrocytes were then suspended in the wash medium and centrifuged at 500 g for 5 minutes. The supernatant was removed and the procedure repeated three times. Washed erythrocytes were then stored in 10 ml wash medium, at 4°C, for up to 2 weeks.

The culture's hematocrit was adjusted to about 5% by adding washed erythrocytes to the parasite-culture medium. The culture flask was then filled with a special gas mixture consisting of 5% oxygen, 5% carbon dioxide and 90% nitrogen before being incubated at a constant temperature of 37°C. Culture medium was exchanged daily while the hematocrit was continually maintained at 5% by adding fresh cells at least every 2 - 3 days.

2.3.3 Giemsa-stained blood smear preparation

A drop of parasite culture was placed on a microscope slide close to the frosted edge. This drop was then smeared across the length of the slide with a second slide held at an angle and allowed to dry. Methanol (analytical grade) was used to fixate the blood smear and allowed to stand for ca. 1 minute, after which the methanol was removed by decanting. The DNA intercalator Giemsa was used to stain the parasite DNA. Giemsa stain was formulated in glycerol and methanol as indicated by the supplier. A phosphate buffer, containing 9.5 g/L sodium dihydrogen phosphate and 9.2 g/L disodium hydrogen phosphate at a pH of between 6.8 and 7.2, was used to dilute the Giemsa solution. Two drops of Giemsa solution were added for every 1 ml of phosphate buffer. The slide was covered with this solution for ca. 5 minutes and then allowed to air dry. A drop of microscope immersion oil was placed on the slide and it was viewed under the oil immersion 100x objective of a Nikon microscope.

2.3.4 *In vitro* synchronisation of malaria parasites

The method described by Vernes *et al.* (1984) and modified by Hoppe (1993) was used for

the *in vitro* synchronisation of malaria parasites. Synchronisations were performed on malaria cultures consisting of ca. 80% ring-phase parasites. The cultures were transferred from the culture flasks to 50 ml centrifuge tubes, centrifuged, at 500 g for 5 minutes and the supernatant removed. The pellet volume was about 1 ml when 10 ml of a parasite culture (5% hematocrit) was centrifuged. Hereafter 4 ml of a 15% D-sorbitol solution was added to every 1 ml of parasite pellet. After careful mixing, by tube inversion, the solution was incubated at 37°C for 5 minutes. After incubation, 8 ml of a 0.1% D-glucose solution was added per 1 ml of parasite pellet, followed by mixing through tube inversion. The parasite solution was again incubated at 37°C for 5 minutes, centrifuged at 500 g for 5 minutes and the supernatant, containing lysed erythrocytes, was removed. The pelleted ring-phase-infected and uninfected erythrocytes were resuspended in 10 ml of culture medium, returned to the culture flasks, filled with the special gas mixture as described in 2.3.2 and returned to the incubator.

2.3.5 Preparation of microculture plates

Microculture plates were prepared in the same way for all the antimalarial bioassays. 20 µl extract from the different concentrations was added to the flat-bottomed wells of a 96-well microculture plate (Flow), as well as 80 µl of a 5% suspension of 0.5 - 1% parasitised cells (95 - 100% rings) in supplemented RPMI 1640 medium. Microcultures were incubated for 48 hours in a modular incubation chamber (Flow) at 37°C in a gas mixture of 5% oxygen, 5% carbon dioxide and 90% nitrogen for assessment of antimalarial activity. After 24 hours, 50 µl of medium from each well was removed and replaced by 10 µl of extract and 40 µl of fresh culture medium.

2.3.6 Visual test

Preliminary bioassays were done by modification of the visual *in vitro* test (Kotecka & Rieckmann 1992) to screen extracts for their antimalarial activity. The test relies on the formation of dark pigment precipitates after an alkaline solution is added to synchronous cultures of *Plasmodium falciparum*. Pigment is produced when the maturation of rings to schizonts during incubation is not prevented by a bioactive extract. However, in the presence of effective concentrations of an extract, maturation is inhibited and, consequently, pigment and precipitation are not observed. This method was evaluated as a possible less expensive means of excluding inactive or less active extracts from assessment by the more expensive microscopic and flow cytometric methods.

2.3.6.1 Visual analysis of microculture plates

After incubation of the microcultures for 48 hours, 25 µl of a freshly mixed preparation of equal parts of 1M sodium hydroxide and 1M sodium chloride was added to the sides of each well of the microculture plate. The contents of each well then turned to a greenish-yellow color and a turbid coagulum was formed. The loose coagulum, probably due to the denaturation of serum and cell proteins, was broken up by tapping the sides of the plate for about 5 minutes until pigment precipitation formed in the control wells. The plate was allowed to stand for 20 minutes and then tapped again gently to resuspend the precipitate. The wells were examined for the presence or absence of dark precipitates against a light background with a magnifier. The dark precipitates observed in the wells containing no extract or ineffective concentrations of the extract were differentiated from the faint, light

precipitates observed in the wells in which parasite growth had been inhibited by effective concentrations of the extracts.

2.3.7 Microscopic test

For a more accurate estimate of the inhibition of the extracts against the parasites the microscopic method was used. The percentage parasitemia of a culture was determined by counting the number of parasitised erythrocytes, in a given microscope field, expressed as a percentage of the total number of erythrocytes counted in that field. A minimum of 5 000 erythrocytes were counted per sample when determining parasitemia (Whaun 1983). For all parasitemia determinations 500 erythrocytes were counted over at least 10 different fields per microscope slide, to get an estimated value of the parasitemia.

2.3.8 Flow cytometric analysis

To determine the activity of extracts against *P. falciparum* in an accurate *in vitro* assay, the flow cytometric method of (Schulze *et al.* 1997) was used. Samples of cultures with extracts as well as controls were stained using thiazole orange. The flow cytometer was programmed to have three electronic gates, each of which counted the erythrocytes of a different fluorescence intensity. All uninfected erythrocytes were counted in gate 1, which covered the region near zero fluorescence intensity. Gate 2 counted ring-infected erythrocytes, which had a fluorescence intensity lower than that of the later-phase parasites and gate 3 counted trophozoite- and schizont-phase infected erythrocytes,

which showed the highest fluorescence intensity. The percentage of parasites present in the ring-phase or later phases could then be determined.

2.3.8.1 Flow cytometric analysis of fixed parasite cultures

Parasites in the 96-well plates were pre-fixed by adding fixing solution to them in a 1:1 ratio after which they were incubated at 4°C for at least 18 hours. The fixing solution consisted of 10% formaldehyde and 4% D-glucose formulated in a Tris-saline buffer (10 mM Tris, 150 mM sodium chloride and 10 mM sodium azide). The final pH was adjusted to 7.3 using sodium hydroxide. The adjustment of the pH was important in preventing lysis of the erythrocytes. After incubation at 4°C for 18 hours or longer, 50 µl fixed parasite culture was added to 1 ml phosphate buffer saline (PBS) containing 0.25 µg thiazole orange, in plastic tubes (Corning). This amount of thiazole orange was used to ensure that there was sufficient DNA intercalating dye available, even at higher parasitemias. The parasite-PBS-thiazole orange solution was mixed carefully, by inverting the tube 2 - 3 times, and incubated at ambient temperature, in the dark, for 1 hour. The samples were then placed on ice to inhibit further staining of the parasite DNA prior to flow cytometric analysis. A volume of 200 µl of prepared parasite sample was analysed by the flow cytometer and a total of 100 000 erythrocytes were counted in each sample.

2.4 Results and discussion

2.4.1 Visual test

The visual test was initially used to exclude the inactive extracts from the more expensive microscopic and flow cytometric tests. The ethanol extract of *Maytenus senegalensis* was used to compare the results of the visual and microscopic test. Results obtained with the visual test were 4 times lower than results obtained with the microscopic method (Table 2).

Table 2: *In vitro* antimalarial activity of an ethanol extract of *Maytenus senegalensis* as determined with the visual test and microscopic test.

Concentration	Visual test	Microscopic test
200 µg/ml	+	+
100 µg/ml	-	+
50 µg/ml	-	+
25 µg/ml	-	+

+ activity

- no activity

The accuracy of the visual method compared to the microscopic method was not good enough and all extracts were tested via the microscopic method as a first screening in determining antimalarial activity.

2.4.2 Microscopic test

Results obtained from the screening of extracts with the microscopic test are shown in Table 3. Ten of the 12 species tested with the first screening had an inhibition percentage of 70% or more at 50 µg/ml. This high number of active extracts is probably due to very careful ethnobotanical selection of the plant species included in the study.

Table 3: *In vitro* antimalarial activity of plant extracts as determined with the microscopic test.

Plant species	Plant part	Minimum inhibition % at 50µg/ml			
		PE[a]	DM[b]	A[c]	E[d]
1. *Acacia xanthoploea*	Stem Bark	-[e]	-	70 (±5.6)[f]	50 (±9.4)
2. *Balanites maughamii*	Stem Bark	-	80 (±12.1)	-	-
3. *Catunaregam spinosa*	Stem Bark	-	-	65 (±9.4)	-
4. *Combretum molle*	Stem Bark	-	-	90 (±8.3)	60 (±9.2)
5. *Croton pseudopulchellus*	Stem Bark	-	-	95 (±9.5)	-
6. *Entandrophragma caudatum*	Stem Bark	80 (±13.8)	60 (±14.1)	-	-
7. *Erythrina lysistemon*	Stem Bark	-	-	95 (±15.7)	-
8. *Lippia javanica*	Leaves	-	-	95 (±11.3)	-
9. *Maytenus senegalensis*	Stem Bark	-	-	90 (±12.1)	80 (±9.3)
10. *Ozoroa engleri*	Stem Bark	70 (±9.7)	85 (±8.3)	-	-
11. *Rhamnus prinoides*	Leaves	-	-	60 (±7.6)	-
12. *Solanum panduriforme*	Leaves	-	-	80 (±8.8)	-

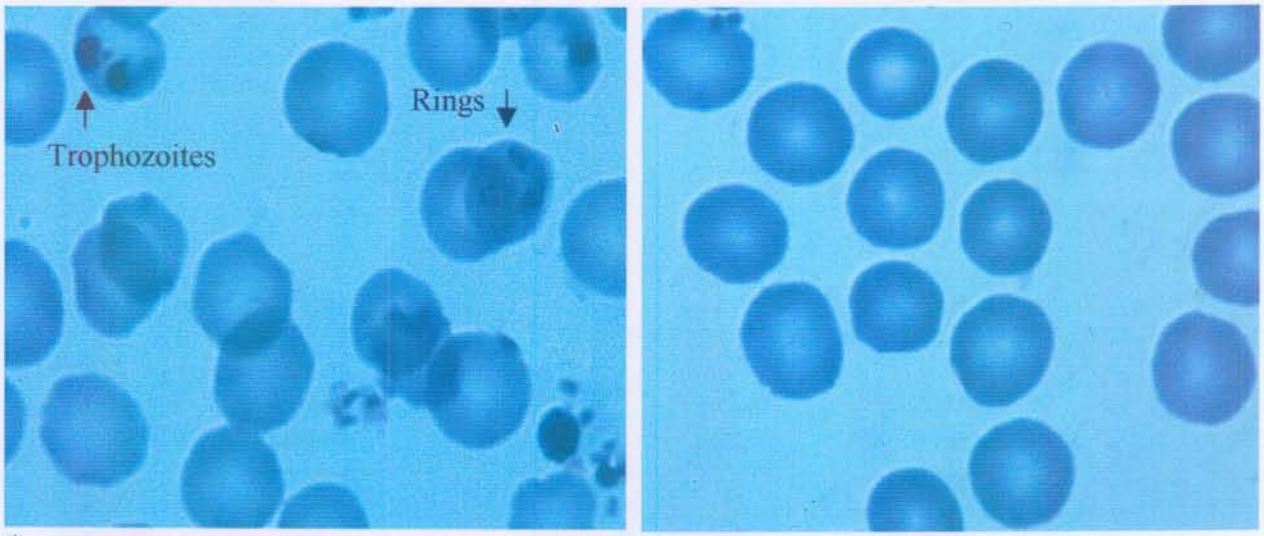
[a]petroleum ether, [b]dichloromethane, [c]acetone, [d]ethanol, [e]not tested, [f]standard error

Results from the solvent controls are shown in Table 4. Concentrated acetone had the most inhibition (10%) at 0.1%, while all other concentrated extraction solvents had no effect at 0.1%. A typical result with the microscopic method is shown in Figure 2.

Table 4: *In vitro* antimalarial activity of solvents as determined with the microscopic test.

Solvent	Inhibition % at 0.2%	Inhibition % at 0.1%
Concentrated acetone	20 (± 5.7)[a]	10 (± 7.9)
Concentrated dichloromethane	10 (± 6.8)	0 (± 3.4)
Concentrated ethanol	0 (± 7.3)	0 (± 4.6)
Concentrated petroleum ether	10 (± 5.4)	0 (± 5.1)
Dimethyl sulfoxide	0 (± 4.8)	0 (± 2.8)
Ethanol	0 (± 5.2)	0 (± 3.8)

[a]standard error



A B

Figure 2: A typical result with the microscopic method with (A) representing the control and (B) after incubation with the extract. (A) has a lot more parasite infected erythrocytes than (B).

2.4.3 Flow cytometric analysis

A typical flow cytometric histogram is shown in Figure 3. Results obtained from the flow cytometric analysis are shown in Table 5. Most extracts had more than 50% inhibition at 50 µg/ml. One extract per species with an inhibition of more than 70% at 50 µg/ml was selected for determination of IC_{50} values. Results from this experiment are shown in Table 6.

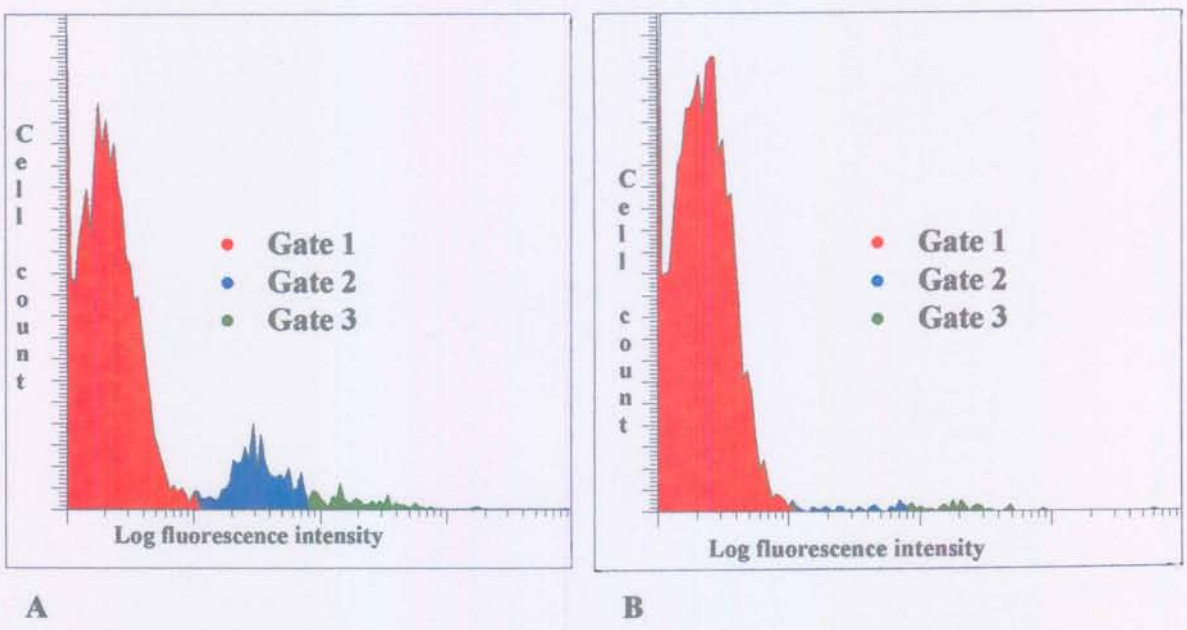


Figure 3: A flow cytometric histogram of (A) a parasite control with a high parasitemia and (B) an extract with a low parasitemia. Gate 1 depicts uninfected erythrocytes, gate 2 early (ring) stage-infected erythrocytes, and gate 3 later (trophozoite) stage-infected erythrocytes.

Table 5: *In vitro* antimalarial activity of plant extracts as determined with the flow cytometric test. Concentrated extraction solvent and solvent controls had no inhibition at 0.1% except the concentrated acetone which had a 5% inhibition at 0.1%.

Plant Species	Plant Part (Starting mass)	Minimum inhibition % at 50µg/ml				
		PE[a]	DM[b]	CF[c]	A[d]	E[e]
1. *Acacia xanthoploea*	Stem Bark (5.0g)	-[f]	-	-	75 (±4.6)[g]	60 (±9.1)
2. *Balanites maughamii*	Stem Bark (20.0g)	-	70 (±5.6)	-	-	-
3. *Catunaregam spinosa*	Stem Bark (5.0g)	-	-	-	40 (±9.4)	-
4. *Combretum molle*	Stem Bark (5.0g)	-	-	-	53 (±6.3)	62 (±7.5)
5. *Croton pseudopulchellus*	Stem Bark (5.0g)	-	-	82 (±4.9)	82 (±6.1)	-
6. *Entandophragma caudatum*	Stem Bark (10.0g)	80 (±12.4)	82 (±11.7)	-	-	-
7. *Erythrina lysistemon*	Stem Bark (5.0g)	-	-	-	82 (±13.5)	-
8. *Lippia javanica*	Leaves (5.0g)	-	-	-	78 (±12.1)	-
9. *Maytenus senegalensis*	Stem Bark (5.0g)	-	-	-	60 (±8.7)	50 (±6.8)
10. *Ozoroa engleri*	Stem Bark (10.0g)	80 (±6.7)	75 (±8.3)	-	-	-
11. *Rhamnus prinoides*	Leaves (5.0g)	-	-	-	48 (±9.2)	-
12. *Solanum panduriforme*	Leaves (5.0g)	-	-	-	75 (±5.7)	-
13. *Trichilia emetica*	Stem Bark (5.0g)	-	82 (±8.3)	-	-	-
14. *Ziziphus mucronata*	Stem Bark (5.0g)	-	-	-	73 (±8.9)	-

[a]petroleum ether, [b]dichloromethane, [c]chloroform, [d]acetone, [e]ethanol, [f]not tested, [g]standard error

Among the plant species tested, the strongest antimalarial activity was found in the dichloromethane extract of *Balanites maughamii* with an IC_{50} of 965 ng/ml. This plant, together with *Croton pseudopulchellus, Entandrophragma caudatum, Ozoroa engleri, Solanum panduriforme* and *Ziziphus mucronata* belonged to the category of plants with the highest antimalarial activity (IC_{50} values < 1 µg/ml). The extracts of *Erythrina lysistemon, Lippia javanica* and *Trichilia emetica* had IC_{50} values between 1 - 2 µg/ml, while *Acacia xanthoploea* was the only one of the selected plants with an IC_{50} above 10 µg/ml.

Table 6: IC_{50} values of extracts with more than 70% inhibition at 50 µg/ml.

Species	Plant Part	Solvent	IC_{50} ng/ml	95% confidence interval[d]
Acacia xanthoploea	**Stem Bark**	**A**[a]	10091	7512 - 12670
Balanites maughamii	**Stem Bark**	**DM**[b]	965	917 - 1013
Croton pseudopulchellus	**Stem Bark**	**CF**[c]	998	991 - 1004
Entandrophragma caudatum	**Stem Bark**	**DM**	976	920 - 1032
Erythrina lysistemon	**Stem Bark**	**A**	1004	1000 - 1009
Lippia javanica	**Leaves**	**A**	1011	997 - 1025
Ozoroa engleri	**Stem Bark**	**DM**	977	930 - 1023
Solanum panduriforme	**Leaves**	**A**	998	990 - 1006
Trichilia emetica	**Stem Bark**	**DM**	1000	995 - 1006
Ziziphus mucronata	**Stem Bark**	**A**	997	975 - 1019
Chloroquine			2	1 - 5

[a]acetone, [b]dichloromethane, [c]chloroform

[d]95% confidence intervals were determined with the Student's t-test (Snedecor & Cochran 1980)

Results from the microscopic screening were obtained with material collected in April, while results from the flow cytometric method were obtained with material collected in July. Differences in results obtained with these two methods might be due to seasonal variation as well as more accurate values obtained with the flow cytometric method.

Little is known about the chemistry of *B. maughamii* but a related species *B. aegyptiaca* (L.) Del. did show antimalarial activity between 10 - 50 µg/ml (Weenen *et al.* 1990). Bark from *B. aegyptiaca* is reported to be toxic to fish, but not to man (Lewis & Elvin-Lewis 1977), while seeds have shown significant anthelmintic activity (Ibrahim 1992). Antimalarial activity of *B. maughamii* in this study was ten times higher than the antimalarial activity from *B. aegyptiaca*. This could be due to different antimalarial strains used or variation in the amount of active principle(s) present in the different species. Several species of *Croton* are known to be toxic (Lewis & Elvin-Lewis 1977), but conflicting reports on the toxicity of South African *Croton* species are reported (Hutchings *et al.* 1996). Various *Croton* species are used as antimalarials throughout the world and results obtained with *C. pseudopulchellus* warrants further investigation into the antimalarial activity of *C. megalobotrys* used traditionally against malaria by the Vhavenda (Mabogo 1990).

Various species of Meliaceae are used traditionally against malaria and the highly active antimalarial compound gedunin has been isolated from some species (MacKinnon *et al.* 1997). Very little information is available about the traditional uses of *E. caudatum* and

this coupled with its good antimalarial activity, might warrant further investigation. Little is known about *O. engleri*'s medicinal activity, but *O. insignis* tested in Tanzania did show some antimalarial activity between 10 - 49 µg/ml (Gessler *et al.* 1994). This is lower than the result obtained with *O. engleri* in this study and might be due to a different parasite strain used or active compounds present in different concentrations in these two species.

The genus, *Solanum* contains some very poisonous species (Hutchings *et al.* 1996). Despite this, *S. nigrum* is used as a traditional antimalarial (Mabogo 1990). Our results obtained with *S. panduriforme* confirm the antimalarial activity in some species of the genus and further investigation into the use of *S. nigrum* might be worthwhile. Various *Trichilia* species have shown antimalarial activity (MacKinnon *et al.* 1997) and results form *T. emetica* confirms the antimalarial activity of the genus. Although *Rhamnus prinoides* is used as a traditional antimalarial in Kenia, no reports on the antimalarial use of *Z. mucronata* could be found. Investigation of the genus might lead to interesting findings.

Erythrina species are widely used as antimalarials in Madagascar and East Africa (Gessler *et al.* 1994). Results from *E. lysistemon* in this study confirm the antimalarial activity of the genus. *Lippia* species are widely used as traditional medicine including against malaria (Valentin *et al.* 1995). The antimalarial activity found in *L. javanica* supports its use as a traditional medicine in South Africa. The antimalarial activity found in the acetone extract of *A. xanthoploea* supports the use of this plant as a traditional antimalarial remedy.

2.5 References

BAUDON, D., CARNEVALE, P., AMBROISE-THOMAS, P. & ROUX, J. 1987. La lutte antipaludique en Afrique: de l'éradication du paludisme au contrôle des paludismes. *Revue d'Epidémiology et de Santé Publique.* 35: 401.

BLACK, R. H., CANFIELD, C. J., CLYDE, D. F., PETERS, W. & WERSDORFER, W. H. 1981. Chemotherapy of malaria. WHO Monograph Series N°27, 2nd edn, Geneva.

BRYANT, A. T. 1966. Zulu Medicine and medicine-men. C. Struik, Cape Town.

CHHABRA, S. C., UISO, F. C. & MSHIU, E. N. 1984. Phytochemical screening of Tanzanian medicinal plants. *J. Ethnopharmacol.* 11: 157 – 179.

DALZIEL, J. M. 1937. The Useful Plants of West Tropical Africa. Crown agents, London.

DOKE, C. M. & VILAKAZI, B. W. 1972. Zulu-English Dictionary. 2nd edn, Witwatersrand University Press, Johannesburg.

GELFAND, M., MAVI, S., DRUMMOND, R. B. & NDEMERA, B. 1985. The Traditional Medical Practitioner in Zimbabwe. Mambo Press, Gweru, Zimbabwe.

GERSTNER, J. 1941. A preliminary checklist of Zulu names of plants with short notes. *Bantu Stud.* 12(3): 215 – 236.

GESSLER, M. C., NKUNYA, M. H. H., MWASUMBI, L. B., HEINRICH, M. & TANNER, M. 1994. Screening Tanzanian medicinal-plants for antimalarial activity. *Acta Trop.* 56: 65 – 77.

HOPPE, H. C. 1993. Identification and characterisation of selected merozoite-stage antigens in southern African isolates of *Plasmodium falciparum*. PhD thesis, University of Pretoria, Pretoria.

HUTCHINGS, A., SCOTT, A. H., LEWIS, G. & CUNNINGHAM, A. B. 1996. Zulu Medicinal Plants. University of Natal Press, Pietermaritzburg.

IBRAHIM, A. M. 1992. Anthelmintic activity of some Sudanese medicinal plants. *Phytother. Res.* 7: 348 – 151.

IRVINE, F. R. 1961. Woody Plants of Ghana. Oxford University Press, London.

KOKWARO, J. O. 1976. Medicinal plants of East Africa. East African Literature Bureau.

KOTECKA, B. & RIECKMANN, K. H. 1992. An inexpensive and simple method for screening antimalarial drugs. *J. Tropical medicine and parasitology* 43: 9 – 12.

LEWIS, W. W. H. & ELVIN-LEWIS, M. P. F. 1977. Medical Botany. John Wiley & Sons, New York.

MABOGO, D. E. N. 1990. The Ethnobotany of the Vhavenda. Unpublished Master of Science Thesis, University of Pretoria, Pretoria.

MACKINNON, S., DURST, T. & ARNASON, J. T. 1997. Antimalarial Activity of Tropical Meliaceae Extracts and Gedunin Derivatives. *J.Nat. Prod.* 60: 336 – 341.

NKUNYA, M. H. H. 1991. Progress in the search for antimalarials from plants, *NAPRECA Monograph series No 4* published by NAPRECA, Addis Ababa University, Ethiopia.

PALMER, E. & PITMAN, N. 1972. Trees of Southern Africa Vol. 2. Balkema, Cape Town.

PRETORIUS, S. J., JOUBERT, P. H. & EVANS, A. C. 1988. A re-evaluation of the molluscicidal properties of the torchwood tree, *Balanites maughamii* Sprague. *S. Afr. J. Sci.* 84: 201 – 202.

POOLEY, E. 1993. The complete Field Guide to Trees of Natal, Zululand and Transkei. Natal Flora Publications Trust, Natal Herbarium, Durban.

PUJOL, J. 1990. Natur Africa: The herbalist handbook. Jean Pujol Natural Healers Foundation, Durban.

ROBERTS, M. 1990. Indigenous Healing Plants. Southern Book Publishers, Halfway House.

SCHULZE, D. L. C., MAKGATHO, E. M., COETZER, T. L., LOUW, A. I., VAN RENSBURG, C. E. J. & VISSER, L. 1997. Development and application of a modified flow cytometric procedure for rapid *in vitro* quantitation of malaria parasitaemia. *S. Afr. J. Sci.* 93: 156 – 158.

SMITH, A. 1895. A contribution to South African Materia Medica, chiefly from plants in use among the natives. Juta, Cape Town.

SNEDECOR, G. W. & COCHRAN, W. G. 1980. Statistical methods. 7th ed. Iowa State University Press.

TRAGER, W. & JENSEN, J. B. 1976. Human malaria parasites in continuous culture. *Science* 193: 674.

VALENTIN, A., PELISSIER, Y., BENOIT, F., MARION, C., KONE, D., MALLIE, M., BASTIDE, J-M. & BESSIERE, J-M. 1995. Composition and antimalarial activity *in vitro* of volatile components of *Lippia multiflora*. *Phytochem.* 40(5): 1439.

VERNES, A., HAYNES, J. D., TAPCHAIRISRI, P., WILLIAMS, J. L. DU TOIT, E. & DIGGS, C. L. 1984. *Plasmodium falciparum* strain-specific human antibody inhibits merozoite invasion of erythrocytes. *Am. J. Trop. Med. Hyg.* 33: 197 – 203.

VERDCOURT, B. & TRUMP, E. C. 1969. Common poisonous plants of East Africa. Collins , London.

WATT, J. M. & BREYER-BRANDWIJK, M. G. 1962. The Medicinal and Poisonous plants of Southern and Eastern Africa. 2nd edn. Livingstone, London.

WEENEN, H., NKUNYA, M. H. H., BRAY, D. H. MWASUMBI, L. B., KINABO, L. S. & KILIMALI, V. A. E. B. 1990. Antimalarial activity of Tanzanian medicinal plants. *Planta Med.* 56: 369.

WERNSDORFER, W. H. & TRIGG, P. I. 1988. Recent progress of malaria research: chemotherapy, In: Malaria, Principles and Practice of Malarialogy, ed. W.H. Wernsdorfer and I. McGregor, Vol I, p. 1569. Churchill Livingstone, U.K.

WHAUN, M. J., RITTERHAUS, C. & IP, S. H. C. 1983. Rapid identification and detection of parasitised human red blood cells by automated flow cytometry. *Cytometry* 14: 117 – 122.

Chapter 3

Cytotoxicity of extracts with antimalarial activity

3 Cytotoxicity of extracts with antimalarial activity

3.1 Introduction

Validation of traditional medicines constitutes not only the recording of biological activity, but also the cytotoxicity of the extracts used by traditional healers. It is important to give accurate data on the cytotoxicity of the extracts as well, especially in the light of incorporation of traditional medicine with Western medicine in South Africa. Various cytotoxicity methods have been used to determine the cytotoxicity of extracts from plants used traditionally as medicines.

A very simple method to determine cytotoxicity is the brine shrimp cytotoxicity assay in microwells. Brine shrimp larvae (nauplii) are easily obtainable after 48 hours by hatching the eggs in seawater in a warm room. The nauplii are then added to microwells, together with serial dilutions of extracts or compounds. After 24 hours the ID_{50} can be determined by counting the dead and alive nauplii (Solis *et al.* 1992). In this paper by Solis *et al.*, 21 pharmacologically active agents were tested. Results showed that there were no clear correlation between the cytotoxicity against the brine shrimp and a cytotoxicity test against human cell lines. This method is sometimes also used to determine biological activity (Massele & Nshimo 1995).

The value of this test seems limited due to the difficulty to distinguish between general cytotoxicity and biological activity and may be better applied in specific cases in stead as a general indicator of cytotoxicity or biological activity.

A very quick method to determine cytotoxicity is by using a modified version of a biotoxicity test kit (BioOrbit 1243-500 Biotox™ Kit) normally used for the determination of cytotoxicity of water soluble samples (Bondesson *et al.* 1994). The inhibitory effect of the sample on the light emission of luminescent bacteria, *Vibrio fischeri*, is measured with a luminometer. The inhibition of the luminescence is determined by combining different concentrations of the test sample with luminescent bacteria. The decrease of light intensity is measured after a contact time of 20 minutes. The inhibitory effect of the samples to be analysed is compared to a toxin free control to give the percentage inhibition. The value is plotted against a dilution factor and the resultant curve is used to calculate the ID_{50} of the sample. This method provides a very quick way to determine the cytotoxicity of an extract.

A standard method of determining cytotoxicity of extracts is against cell lines (usually primate cells). This method determines the cytotoxicity of the extracts by measuring the effect of the extract on the cells over a time span of at least 2 days. By using this method one can determine a therapeutic index (comparison of the bioactivity and cytotoxicity ratios). A value better than 1000 is normally considered as a good therapeutic value. In the case of the popular antimalarials like chloroquine this value is in the region of about 300 depending on the cell line used (Gessler *et al.* 1995).

In this study we initially tested some of the plant extracts with antimalarial activity by means of the luminescent bacteria cytotoxicity test. Monolayer monkey kidney cells in microtitre plates were used to determine the cytotoxicity of the five extracts with the highest antimalarial activity at 50 µg/ml with the flow cytometric method.

3.2 Materials and Methods

3.2.1 Luminescent bacteria cytotoxicity test

The salinity of the extract was firstly adjusted to be equivalent to 2% NaCl solution. This was done by adding 100 µl of extract (100 mg/ml stock) to 900 µl of a 2% NaCl solution (dilution 1). From this dilution 10 times dilutions were made. Bacterial suspension (0.2 ml) was pipetted into luminescence tubes and left to stabilise for at least 20 minutes at 15°C. The luminescence intensity (I_0) was measured from cuvette number one immediately after 0.8 ml of extract was added to the cuvette containing bacterial suspension. Extract dilutions were then incubated at 15°C for 20 minutes and the luminescence intensity (I_1) from cuvette number one was again determined.

The inhibition was determined as follows:

Correction factor (CF) = IC_{20}/IC_0

Inhibition % = $100 - LI_{20} / CF / LI_0 \times 100$

Where:

IC_0 = Initial luminescence intensity of control

IC_{20} = Luminescence intensity of control after 20 minutes contact time

LI_0 = Initial luminescence intensity of the extract

LI_{20} = Luminescence intensity of extract after 20 minutes contact time

3.2.2 Monkey kidney cell cytotoxicity test

Microtitre plates with vervet monkey kidney cells were used for testing the plant extracts for cytotoxicity. Multilayer cells present in the tissue culture plates were first rinsed three times with 10% phosphate buffer saline (PBS), and then 3 ml of 0.1% Trypsin EDTA were added to them. This helped to loosen the cells adhered to the bottom surface of the plates. The plates were then incubated for 5 minutes at 37°C. Fresh maintenance medium (7 - 8 ml) was poured in the tissue culture plates and the content was transferred to a test tube. The test tube was then centrifuged for 5 minutes at 3000 rpm.

After centrifugation, the cells settled at the bottom of the test tube and the supernatant was discarded. Fresh minimal essential medium (MEM) was mixed thoroughly with the cells. This medium consisted of 97 g of Eagle's MEM with Earle's salts (Highveld Biological), 47.06 g nitrogen-2-hydroxyethylpiperasinenitrogen-2-ethanesulfonic acid (Hepes BSS), 8.5 g sodium hydroxide made up in 10 l sterile distilled water. 100 µl from these freshly mixed cells in MEM was transferred to microtitre plates and incubated at 37°C for 24 hours. The monolayer cells were then formed in the microtitre plates. The MEM was poured off and replaced with 200 µl of plant extract diluted to the desired concentration with MEM.

Plant extracts were made up to a concentration of 100 mg/ml. From this stock solution 5 µl were then diluted 1000 times with MEM to a final volume of 5 ml. Serial dilutions from this concentration were made by adding 1000 µl extract to 1000 µl MEM. Three controls; a negative control (just the media), dimethyl sulfoxide 0.1% and ethanol

0.1% were used. Cells were incubated for 6 days and a visual estimate of the inhibition compared to the control was taken on day 2, 4 and 6.

3.3 Results & discussion

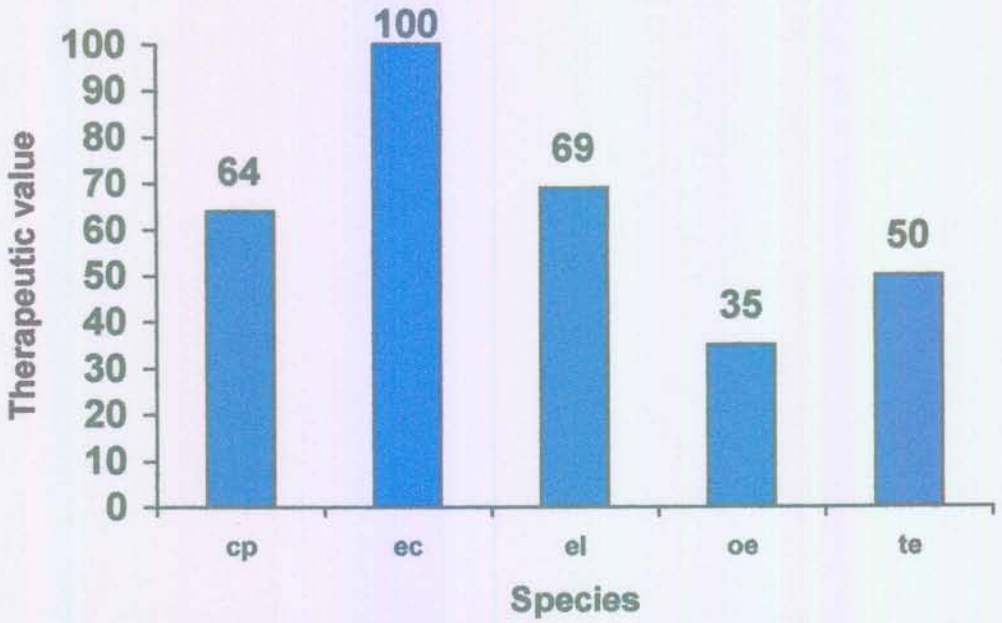
Results from the cytotoxicity tests are shown in Table 1.

Table 1: *In vitro* cytotoxicity of plant extracts tested on monkey kidney cells and luminescent bacteria.

Extract	ID_{50} µg/ml monkey kidney cells	ID_{50} µg/ml bacteria test
Croton pseudopulchellus **(chloroform)**	64	500
Entandrophragma caudatum **(dichloromethane)**	100	2000
Erythrina lysistemon **(acetone)**	69	120
Ozoroa engleri **(dichloromethane)**	35	100
Trichilia emetica **(dichloromethane)**	50	not tested

The highest cytotoxicity in the monkey kidney cell test was found with the dichloromethane extract of *Ozoroa engleri* with an ID_{50} of 35 µg/ml. The lowest cytotoxicity was found with the dichloromethane extract of *Entandrophragma caudatum* with an ID_{50} 100 µg/ml. Results obtained with the bacteria test showed the same trend, but values were much higher in some instances. These two methods cannot be compared directly because duration of the experiment as well as the biological test material used, was not the same.

The therapeutic index is expressed as the antimalarial activity (flow cytometric test) to cytotoxicity (monkey kidney cell test) ratio. These index values of the extracts was in the range of 35 - 100 and therefore have a poor selectivity index (Figure 1). The best ratios were obtained with the dichloromethane extract of *E. caudatum* (100) and the acetone extract of *E. lysistemon* (69).



cp = *Croton pseudopulchellus*, ec = *Entandrophragma caudatum*,

el = *Erythrina lysistemon*, oe = *Ozoroa engleri*, te = *Trichilia emetica*

Figure 1: Therapeutic index of extracts tested against a monkey kidney cell line and malaria parasites with the flow cytometric test. A value greater than a 1000 is normally considered as a good therapeutic value for a clinically useful drug.

It has been proposed that the ratio for a good therapeutic remedy should be > 1000, as it is for example for quinine (Likhitwitayawuid *et al.* 1993). *In vitro* cytotoxicity as was determined in this study, is not always a clear indication of the cytotoxicity *in vivo*. Kirby *et al.* (1993) describe the extreme cytotoxicity *in vitro* of highly active antimalarial quassinoids isolated from *Brucea* species, although human clinical studies using crude preparations of *Brucea* fruits have shown antimalarial efficiency without toxicity. It is therefore possible that the extracts investigated in this study will have less cytotoxicity *in vivo*, as well.

3.4 References

BONDESSON, I., EKWALL, B., HELBERG, S., ROMBER, L., STENBERG, K. & WALUM, E. 1994. MEIC - A New International Multicenter Project to Evaluate the Relavance to Human Toxicity of *in vitro* Cytotoxicity Tests. *Cell Biol. Toxicol.* 5: 331 – 348.

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.

KIRBY, G. C., WARHURST, D. C. & PHILLIPSON J. D. 1993. Plants as a source of novel antimalarial drugs. *Trans. R. Soc. Trop. Med. Hyg.* 87 (4): 370.

LIKHITWITAYAWUID, K., ANGERHOFER, C. K., CORDELL, G. A. & PEZZUTO, J. M. 1993. Cytotoxic and antimalarial bizbenzyl-isoquinoline alkaloids from *Stephania erecta*. *J. Nat. Prod.* 56 (1): 30 – 38.

MASSELE, A.Y. & NSHIMO, C. M. 1995. Brine shrimp bioassay for biological activity of medicinal plants used in traditional medicines in Tanzania. *E. Afr. Med. J.* 72 (10): 661.

SOLIS, P. N., WRIGHT, C. W., ANDERSON, M. M., GUPTA, M. P. & PHILLIPSON, J. D. 1993. A microwell cytotoxicity assay using *Artemia salina* (Brine shrimp). *Planta Med.* 59: 250.

Chapter 4

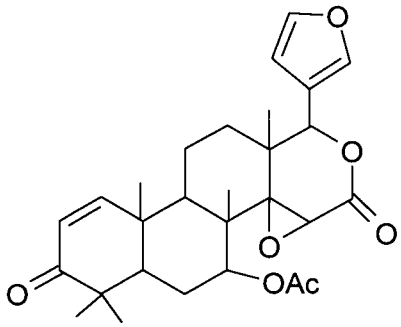
Antimalarial activity of purified fractions from *Ozoroa engleri*

4 Antimalarial activity of purified fractions from *Ozoroa engleri*

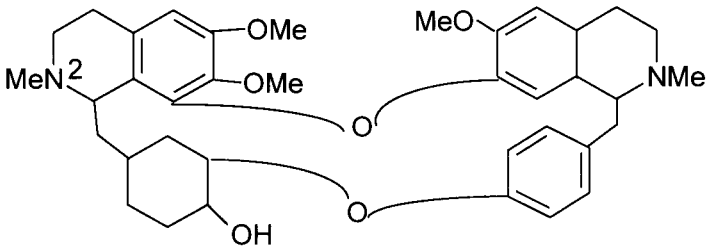
4.1 Introduction

Various approaches to identify new compounds that might serve as therapeutic agents have been fruitful since the inception of medicinal chemistry. Natural products always formed an important source for the isolation of new drugs. The rich diversity of structural types provided by natural products also add to their attractiveness as bioactive compounds (Cordell 1995). Several active antimalarial compounds with different structural types have been isolated from higher plants in the last few decades (Phillipson & Wright 1991). This includes the terpene gedunin, the alkaloid picnamine and the quinone uvaretin (Figure 1).

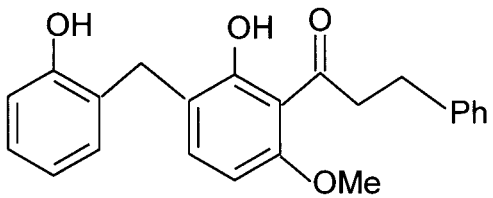
Four extracts with good antimalarial activity were considered for further purification, namely *Croton pseudopulchellus, Erythrina lysistemon, Entandrophragma caudatum* and *Ozoroa engleri. Entandrophragma caudatum* was not selected because it possibly contained the known antimalarial compound, gedunin, isolated from other species of the Meliaceae. The dichloromethane extract of *Ozoroa engleri* was selected for further purification because it had an IC_{50} of 977 ng/ml as determined with the flow cytometric method and also had the lowest cytotoxicity as indicated with a preliminary ATP inhibition cytotoxicity test.



gedunin



picnamine



uvaretin

Figure 1: Antimalarial compounds with different structural features

In this study, *Ozoroa engleri* was extracted with dichloromethane, separated into fractions through the application of silica gel column chromatography, Sephadex column chromatography, high pressure liquid chromatography and the fractions analysed for antimalarial activity with the microscopic method described in 2.3.7.

4.2 Materials & methods

4.2.1 Extract preparation

Stem bark of *Ozoroa engleri* was ground into a powder with an IKA dry mill and extracted with dichloromethane. After agitation for 24 hours on a magnetic stirrer, it was filtered and concentrated to dryness under reduced pressure and the dry mass determined (2.358 g).

4.2.2 Separation and purification

The separation of one or more antimalarial compounds from a crude extract, can be a long and expensive process. Obtaining a pure compound from a crude extract often requires a number of separation steps involving various techniques.

Chromatography, the separation process in which the sample mixture is distributed between two phases in the chromatographic bed was used to separate and purify the crude extract. One phase is stationary whilst the other passes through the chromatographic bed.

In the 90 years since the discovery of chromatography, tremendous advances have been realised. In low-pressure column chromatography, the chromatographic packing material consists of a densely packed adsorbent through which a mobile phase is "flowed". Depending on the choice of the packing material and mobile phase, several separation mechanisms are available (Salituro & Dufresne 1998).

Silica gel is perhaps the most widely used stationary phase for the separation of natural products. The chemical nature of the surface of silica gel consists of exposed silanol groups. These hydroxyl groups are the active centers and potentially can form strong hydrogen bonds with compounds being chromatographed. Thus, in general, the stronger the hydrogen-bonding potential of a compound, the stronger it will be retained by silica gel. For example, polar compounds containing carboxylic acids, amines, or amides are strongly adsorbed by silica gel. Nonpolar compounds, such as terpenes or other compounds lacking polar functional groups contains few hydrogen-bonding sites and are thus poorly retained on silica gel (Salituro & Dufresne 1998).

How strongly a given compound is retained depends equally on the polarity of the mobile phase. The stronger the hydrogen-bonding potential of a solvent, the faster it will elute polar compounds adsorbed on a silica gel column. Similarly, very nonpolar solvents would be used to chromatograph nonpolar compounds. Examples of nonpolar solvents useful for silica gel chromatography include hexane and dichloromethane, while polar solvents would include ethyl acetate and methanol. Column development usually involves increasing the percentage of the polar solvent during a chromatography run (Salituro & Dufresne 1998).

4.2.2.1 Silica gel column chromatography

Packing material for a silica gel column is introduced into the column either dry or as a slurry in a solvent. Columns used for low-pressure chromatography are usually made out of glass. Glass is resistant to solvents, and thick-walled glass can tolerate the low-to-

medium pressures created during column development. A glass frit is usually placed at the bottom of the column to support the packing material. The amount of adsorbent to use depends on sample loading. This varies depending on the difficulty of the separation and the adsorbent being used. In general, 100 - 500 gram of packing material is used per gram of crude sample (Salituro & Dufresne 1998).

For the fractionation of the extract a glass column (30 cm length, 2 cm diameter) was loaded by means of slurry packing. A beaker was half-filled with silica (silica gel 60, size 0.08 mm, Merck, Darmstadt) and dichloromethane added to obtain a pourable slurry. This slurry must not be so thick that air bubbles are trapped in the column or so thin that the column cannot be packed in "one pour" (Salituro & Dufresne 1998). The column was left to settle for at least an hour before the extract was added.

The extract dissolved in a small amount of dichloromethane was added to the column and the fractions collected. The polarity of the solvent was gradually increased by adding ethyl acetate to dichloromethane in different ratios ranging from dichloromethane: ethyl acetate (20:1) to (1:1). Fractions containing the same compounds as determined by thin layer chromatography (TLC), were combined and each of the pooled fractions concentrated to dryness under reduced pressure. Fractions were spotted on a TLC plate and then developed with chloroform: ethyl acetate (6:1). TLC plates were viewed under UV light (254 and 366 nm) after development and also dipped in vanillin reagent (15 g vanillin, 500 ml ethanol and 10 ml concentrated 98% sulfuric acid) and heated to detect compounds not absorbing UV light. Each pooled fraction was tested for antimalarial activity.

4.2.2.2 Sephadex column chromatography

Sephadex LH-20 is a hydroxypropylated form of Sephadex G-25. This derivatisation adds lipophilicity to the gel and at the same time retains its hydrophilicity. The gel swells in polar solvents such as water and methanol, to about four times its dry volume. The added lipophilicity that allows this gel to swell adequately in organic solvents makes LH-20 the preferred gel for the fractionation of organic-soluble natural products. In gel filtration mode, compounds are separated based on their size as shown in Table 1 (Salituro & Dufresne 1998).

Fraction 6 and 7 collected from the silica gel column had good antimalarial activity and fraction 7 was further purified by Sephadex column chromatography. A glass column (25cm length, 1.25cm diameter) was loaded with Sephadex LH-20 by means of slurry packing with ethanol as eluent. The column was left to settle for at least an hour before fraction 7 was added. Fractions collected were spotted on TLC and developed with chloroform: methanol (5:1). Similar fractions were pooled together, dried under reduced pressure and tested for antimalarial activity.

Table 1: Fractionation range of Sephadex LH-20 and G-series (Salituro & Dufresne 1998)

Sephadex type	Useful fractionation range, molecular weight	Approximate swollen volume in water, ml/g dry beads
G-10	0 - 700	2.5
G-15	0 - 1500	3.0
G-25	100 - 5 000	5.0
G-50	500 - 10 000	10.0
G-100	1000 - 100 000	15.0
LH-20	100 - 4 000	4.0

4.2.2.3 Rechromatography on silica gel column

Fraction 7.1 from the Sephadex column had antimalarial activity and was purified further on a silica gel column (25 cm length, 1.25 cm diameter) with chloroform: methanol (10:1) as eluent. Fractions collected were subjected to TLC, similar fractions pooled together and tested for antimalarial activity.

Fraction 6 collected from the first silica gel column had good antimalarial activity and was purified further on another silica gel column (25 cm length, 1.25 cm diameter) with chloroform as eluent. Fractions collected were subjected to TLC and similar fractions pooled together, dried and tested for antimalarial activity.

4.2.2.4 High pressure liquid chromatography (HPLC)

HPLC is a versatile, robust, and widely used technique for the isolation of natural products. The main difference between HPLC and other forms of column chromatography is that the diameter of stationary phase particles is comparatively low (3 - 10 μm), and these particles are tightly packed to give a very uniform column bed structure. The low particle diameter means that high pressure is needed to drive the chromatographic solvent through the bed. However, because of the very high total surface area available for the interaction with solutes and the uniformity of the column bed structure, the resolving power of HPLC is very high (Stead 1998).

Fraction 7.1.1 was further purified on HPLC (Varian 9012 pump and UV 6000 LP diode array detector) with ethanol: water (1:1) as eluent on a silica reverse phase column (Luna 5µ C18(2) 250 mm x 4.6 mm). The resulting fractions were collected and tested for antimalarial activity.

4.2.2.5 Antibacterial testing

Isolating antibacterial compounds can be simplified dramatically by direct bioassay on TLC (Cordell 1995). This approach was repeated with the most active antimalarial fractions, with the hypothesis that there might be a correlation between the antibacterial and antimalarial activity.

The extract as well as the most active antimalarial fractions (1, 2, 6 and 7) was tested for antibacterial activity by direct bioautography on TLC plates. The extract and fractions (± 5 µl) were applied to silica gel 60 plates and developed in chloroform:ethyl acetate (6:1). The TLC plate was observed under UV light (254 and 366 nm) after development, left overnight for the solvent to evaporate completely and sprayed with the bacterial suspension prepared as described below.

Staphylococcus aureus was collected from the Department of Microbiology and Plant Pathology, University of Pretoria. The bacteria were maintained on nutrient agar slant and were recovered for testing by growth in nutrient broth (No.2, Biolab) for 24 hours at 37°C. The culture was then centrifuged at 3000 rpm for 20 minutes. The supernatant was discarded and the sedimented bacteria resuspended in fresh nutrient broth to an

absorbance of 0.84 at 560 nm with a spectrofotometer (Lund and Lyon, 1975). The bacterium suspension was sprayed with a fine spray onto the TLC plates. These plates were then dried for a few minutes until they appeared translucent and incubated at 25°C for 24 hours in humid conditions. The plates were then sprayed with an aqueous solution of 2.0 mg/ml *p*-iodonitrotetrazolium violet and reincubated at 25°C for 3 hours. Any inhibition of bacterial growth could clearly be seen as white spots on a red background.

4.3 Results and discussion

A summary of the results is shown in Figure 2. From the first silica gel column 10 pooled fractions were tested against the malaria parasite and only four fractions showed some antimalarial activity. Fractions 1 and 2 had moderate antimalarial activity with an estimated IC_{50} of 10 µg/ml, while fractions 6 and 7 showed good antimalarial activity with an estimated IC_{50} of 1 µg/ml, similar to that of the crude extract.

Three fractions were collected from fraction 7 on the Sephadex column. Fraction 7.1 and 7.2 showed good antimalarial activity (estimated IC_{50} = 5 µg/ml) while fraction 7.3 had less activity (IC_{50} > 50 µg/ml).

From fraction 7.1 (mass = 80 mg), 7 fractions were collected from the second silica gel column. All these fractions were active but had only moderate antimalarial activity (IC_{50} > 20 µg/ml). From fraction 7.1.1 the resulting two fractions had only moderate antimalarial activity (IC_{50} > 30 µg/ml). Seven pooled fractions were collected from fraction 6 and all fractions had moderate antimalarial activity (IC_{50} > 20 µg/ml).

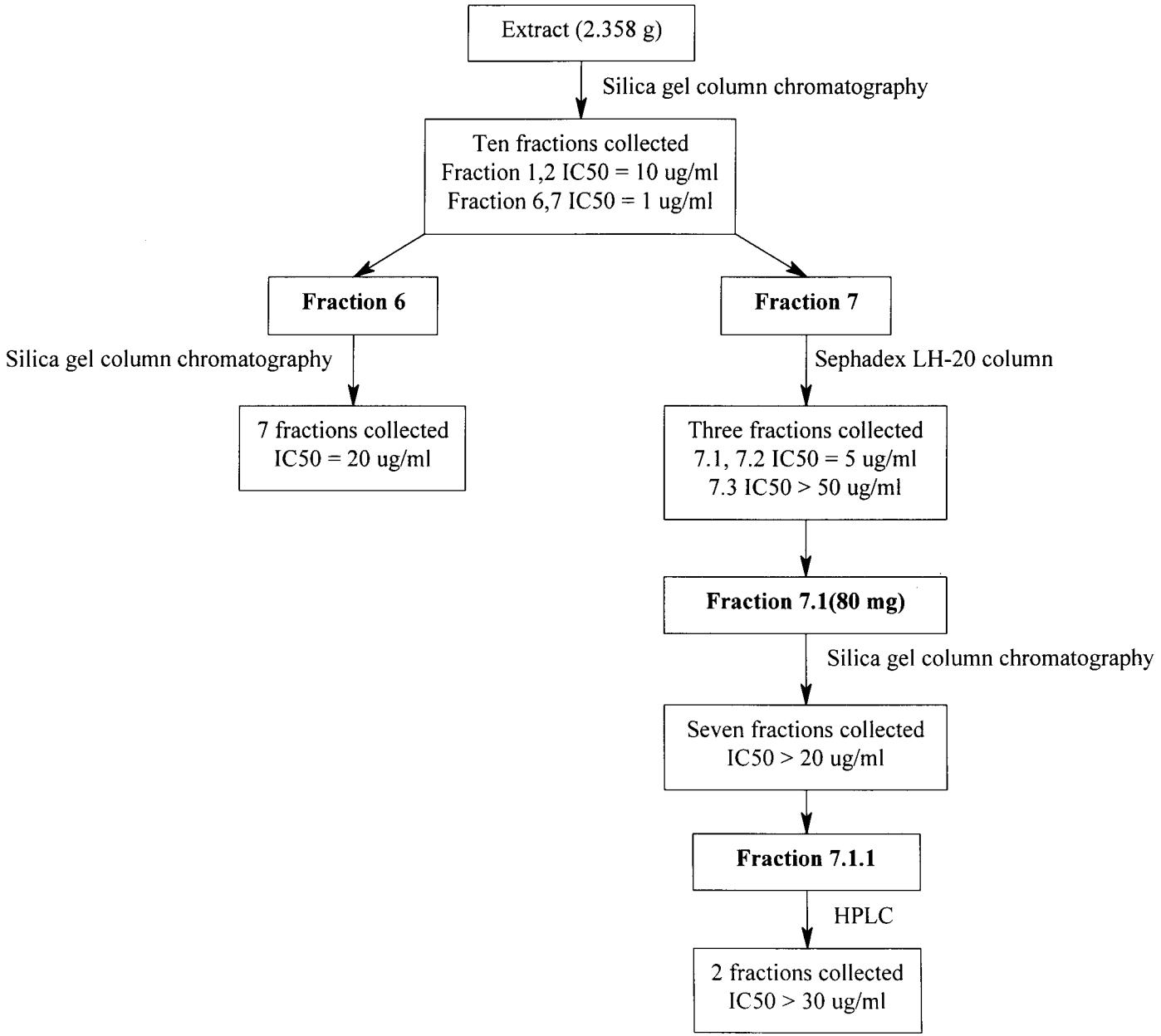
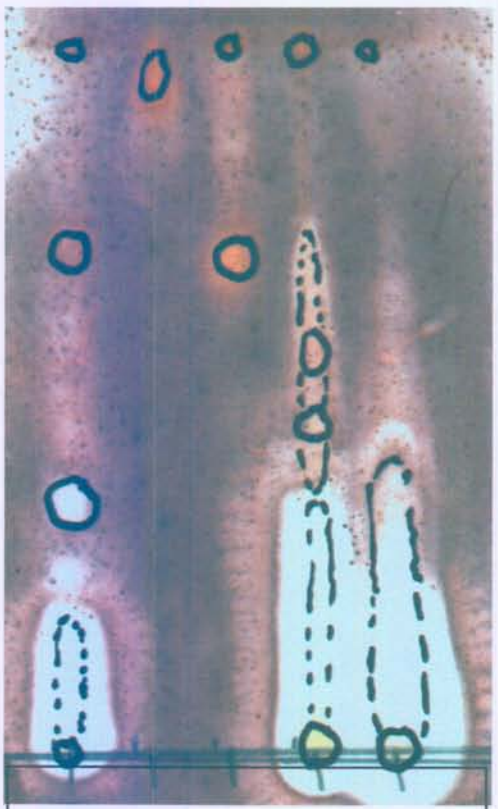


Figure 2: Summary of results from the fractionation of the dichloromethane extract from *Ozoroa engleri*

There was no clear correlation between the antimalarial activity and the antibacterial activity from the most active fractions 1, 2, 6 and 7 collected from the first silica gel column (Figure 3). Antimalarial activity was mainly found in the top half of fractions 6 and 7 while antibacterial activity was mainly found in the bottom half (as indicated by white areas on the TLC plate) of the fractions seen in Figure 3. In the case of this extract, this method didn't help in identifying antimalarial compounds from purified fractions.



E = extract, 1, 2, 6, 7 - fraction numbers

Figure 3: Antibacterial activity on TLC of the most active antimalarial fractions.

It seems that the high antimalarial activity present in the crude extract of *Ozoroa engleri* is a combination of at least 5 to 6 compounds all with moderate antimalarial activity (IC_{50} > 10 µg/ml). It has been suggested that if refined extracts have IC_{50} values > 5 µg/ml they should be considered non-effective (Rasoanaivo & Ratsimamanga-Urverg 1993). No further efforts were undertaken to identify these compounds, especially with extracts from other plants with similar activity and better cytotoxicity values and *Croton pseudopulchellus* was therefore selected for further purification of active principles.

4.4 References

CORDELL, G. A. 1995. Changing strategies in natural products chemistry. *Phytochem.* 40 (6): 1598 – 1612.

LUND, B. M. & LYON, G. D. 1975. Detection of inhibitors of *Erwina carotovora* and *E. herbicola* on thin layer chromatograms. *Journal of Chromatography* 110: 193 – 196.

PHILLIPSON, J. D. & WRIGHT, C. W. 1991. Can ethnopharmacology contribute to the development of antimalarial agents? *J. Ethnopharmacol.* 32: 155 – 165.

RASOANAIVO, P. & RATSIMAMANGA-URVERG, S. 1993. Biological evaluations of plants with reference to the malagasy flora. p.55. Monograph prepared for the IFS-NAPRECA Workshop on Bioassays held in Antanarivo, Madagascar.

SALITURO, G. M. & DUFRESNE, C. 1998. Natural products isolation. In: Methods in biotechnology, ed. R. J. P. Cannell Vol. IV, Ch. 4, p. 111 – 125. Humana Press, Totowa.

STEAD, P. 1998. Natural products isolation. In: Methods in biotechnology, ed. R. J. P. Cannell Vol. IV, Ch. 6, p. 165. Humana Press, Totowa.

Chapter 5

Antimalarial activity of kaurenoic acid isolated from *Croton pseudopulchellus*

5 Antimalarial activity of kaurenoic acid isolated from *Croton pseudopulchellus*

5.1 Introduction

The acetone extract of *Croton pseudopulchellus* showed promising results in the flow cytometric antimalarial activity test with an IC_{50} of 998 ng/ml and a low cytotoxicity (ID_{50} = 500 µg/ml) with the luminescent bacteria method. It was therefore selected for isolation of the active principle(s). It is possible that the isolated active compound(s) may be more active than the crude extract, because the extract consists of a mixture of compounds.

5.2 Materials and methods

5.2.1 Extract preparation

Powdered stem bark from *C. pseudopulchellus* was extracted in acetone for 24 hours and dried under reduced pressure. The dried extract (3.067 g) was dissolved in a small amount of solvent and subjected to different chromatographic techniques.

5.2.2 Isolation of active compound

Fractions collected were tested for antimalarial activity with the microscopic method described in 2.3.7.

5.2.2.1 Chromatography

a) Silica gel column chromatography

A silica gel column (30 cm length, 2 cm diameter) was loaded by means of slurry packing. A beaker was half filled with silica (silica gel 60, size 0.08 mm, Merck, Darmstadt) and chloroform added to obtain a pourable slurry. The column was left to settle for a least an hour before the extract was added. The eluent was made more polar by adding ethyl acetate: chloroform in a ratio from (20:1) to (1:1). The different fractions were collected and dried under reduced pressure. Fractions containing the same compounds as determined by TLC, were combined and each of the pooled fractions concentrated to dryness under reduced pressure. Fractions were spotted on a TLC plate and developed with chloroform. Each pooled fraction was tested for antimalarial activity.

b) Sephadex column chromatography

Fraction 1, the most active fraction from the silica gel column, was further purified on a Sephadex LH-20 column with ethanol as eluent. The resulting fractions were dried

under reduced pressure, tested for antimalarial activity and active fractions further purified by preparative thin layer chromatography.

c) Preparative thin layer chromatography (PTLC)

The purified active fractions were further purified by PTLC (silica gel 60 F_{254} 0.25 mm), using chloroform as developing solvent. The isolated compound (5 mg) was analysed by gas chromatography-mass spectroscopy and proton nuclear magnetic resonance spectroscopy.

5.2.3 Identification of active compound

a) Gas chromatography (GC)

Gas chromatography is essentially a method for separating volatile mixtures of substances of analytical interest. Although the equipment can vary from very simple to very complex and sophisticated assemblies, the principle is a simple one and is analogous to other forms of chromatography (Leach 1960).

The basic instrument consists of a tubular column containing a packing of finely powdered material which, for gas-liquid chromatography, has been coated with a suitable substance. This substance, a liquid at operating temperatures, is the stationary phase of the system. At the entrance to the column is a port through which the sample can be introduced and at the exit is a device, which either may be chemical or electrical, to

detect substances emerging from the column. The column, and often the detector, is enclosed in a thermostatically controlled oven. If a stream of gas is passed through the column the components of the sample mixture travel along the column in the gas stream. The speed of their progress is dependent on the absorption and adsorption of the components by the very large surface area of the stationary phase. By choice of a suitable stationary phase the partition between gas and stationary phase will be different for each component of the sample mixture so that by the time the sample is beginning to reach the end of the column the components will be separated and emerge into the detector as individual compounds. The response of the detector to the emerging compounds is amplified and the resulting signal fed to a chart recorder (Leach 1960).

The procedure is therefore similar to other forms of chromatography except that the mobile phase and the sample are in the vapor state. The gas used as the mobile phase contributes little to the efficiency of the chromatographic process although it is of importance from the point of view of the detector efficiency. It is the column which determines the efficiency of the separation and the correct choice of both support material and stationary phase is of prime importance (Leach 1960).

b) Mass Spectrometry (MS)

At its simplest, mass spectrometry is a technique for measuring the mass, and therefore the molecular weight, of a molecule. In addition, it's often possible to gain information about an unknown structure by measuring the masses of fragments produced when high energy molecules are scattered when bombarded by electrons. There are several different kinds of

mass spectrometers available, but one of the most common is the electron-ionization, magnetic sector instrument shown schematically in Figure 1 (McMurry 1995a).

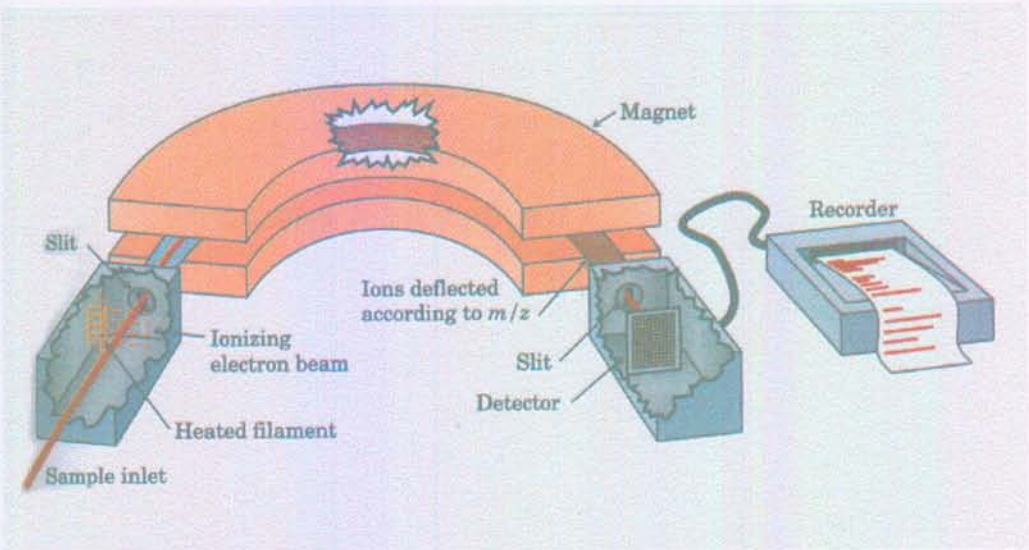
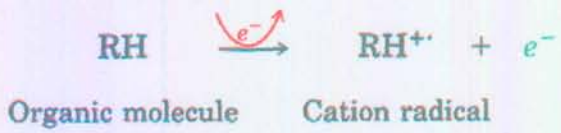


Figure 1: Schematic representation of an electron-ionisation, magnetic-sector mass spectrometer. Molecules are ionised by collision with high energy electrons, causing some of the molecules to fragment. Passage of the charged fragments through a magnetic field then sorts them according to their mass (McMurry 1995a).

A small amount of sample is introduced into the mass spectrometer, where it is bombarded by a stream of high-energy electrons. The energy of the electron beam can be varied but is commonly around 70 electron volts. When a high-energy electron strikes the organic molecule, it dislodges a valence electron from the molecule, producing a cation radical (McMurry 1995a).



Electron bombardment transfers such a large amount of energy to the molecules that most of the cation radicals fragment after formation: They scatter into smaller pieces, some of which retain the positive charge, and some of which are neutral. The fragments then flow through a curved pipe in a strong magnetic field, which deflects them according to their mass-to-charge ratio (*m/z*). Neutral fragments are not deflected and are lost on the walls of the pipe, but positively charged fragments are sorted by the mass spectrometer onto a detector, which record them as peaks at the various *m/z* ratios. Since the number of charges *z* on each ion is usually 1, the value of *m/z* for each ion is simply its mass *m* (McMurry 1995a).

The mass spectrum of a compound is usually presented as a bar graph with masses (*m/z* values) on the x-axis, and intensity on the y-axis. The tallest peak, called the base peak, is arbitrarily assigned an intensity of 100% (McMurry 1995a).

c) Nuclear magnetic resonance (NMR) spectroscopy

Many kinds of nuclei behave as if they were spinning about an axis, much as the earth spins daily. Since they're positively charged, these spinning nuclei act like tiny magnets and therefore interact with an external magnetic field (denoted $\mathbf{H}_0$). Not all nuclei act this way, but fortunately for organic chemists, both the proton (^{1}H) and the ^{13}C nucleus do have spins (Figure 2) (McMurry 1995b).

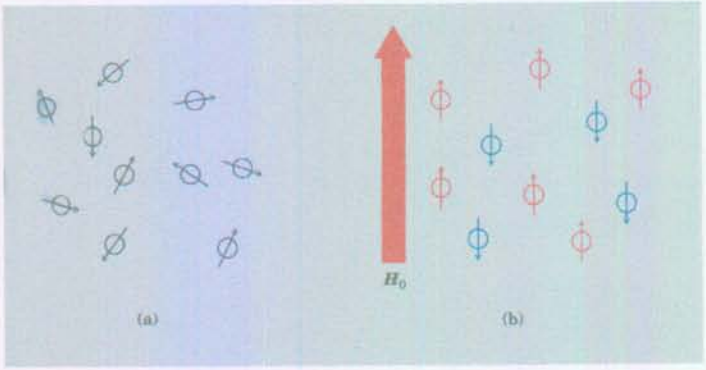


Figure 2: Nuclear spins are oriented randomly in the Abs ence of an external magnetic field (a), but have a specific orientation in the presence of an external field, H_0 (b) (McMurry 1995b).

If the oriented nuclei are now irradiated with electromagnetic radiation of the right frequency, energy absorption occurs and the lower-energy state "spin-flips" to the higher-energy state. When this spin-flip occurs, the nuclei are said to be in resonance with the applied radiation, hence the name nuclear magnetic resonance (McMurry 1995b).

All nuclei in molecules are surrounded by electrons. When an external magnetic field is applied to a molecule, the electrons set up tiny local magnetic fields of their own. These local magnetic fields act in opposition to the applied field so that the effective field actually felt by the nucleus is a bit smaller than the applied field (McMurry 1995b).

In describing this effect, it is said that nuclei are shielded from the full effect of the applied field by the circulating electrons that surround them. Since each specific nucleus in a molecule is in a slightly different electronic environment, each nucleus is shielded to a slightly different extent, and the effective magnetic field is not the same for each nucleus.

If the NMR instrument is sensitive enough, the tiny differences in the effective magnetic fields experienced by different nuclei can be detected, and a distinct NMR signal for each chemically distinct carbon or hydrogen nucleus in a molecule can be seen. Thus, the NMR spectrum of an organic compound effectively maps the carbon-hydrogen framework. With practice, it is possible to read the map and thereby derive structural information about an unknown molecule (McMurry 1995b).

The antimalarial compound from the acetone extract of *Croton pseudopulchellus* was identified by means of proton NMR and GC-MS. Results from the GC-MS database suggested that it might be kaurenoic acid. Subsequent comparisons with published data confirmed this.

5.2.4 Antimalarial and cytotoxicity assays of the isolated compound

An antimalarial assay on pure kaurenoic acid was done with the flow cytometric method described in 2.3.8 and the IC_{50} determined. Cytotoxicity ID_{50} was determined with the monkey kidney cell cytotoxicity test described in 3.2.2.

5.3 Results and discussion

A summary of the results is shown in Figure 3. From the first silica gel column 10 pooled fractions were collected and tested for antimalarial activity. From these the most active fractions were fraction 1 and fraction 4. Fraction 1 was selected for further purification on a Sephadex column and 8 pooled fractions were collected and tested for antimalarial

activity with estimated IC_{50} values between 1 and 10 µg/ml. A pattern was detected with the presence of two spots correlating with the highest antimalarial activity (Figure 4) and one of these two spots were further purified on PTLC, giving only one spot on TLC after two separations on PTLC. This compound (5 mg) was subjected to NMR and GC-MS (Figure 5). Results from the GC-MS showed a mixture of two compounds and one was identified by the GC-MS database as kaurenoic acid (Figure 6).

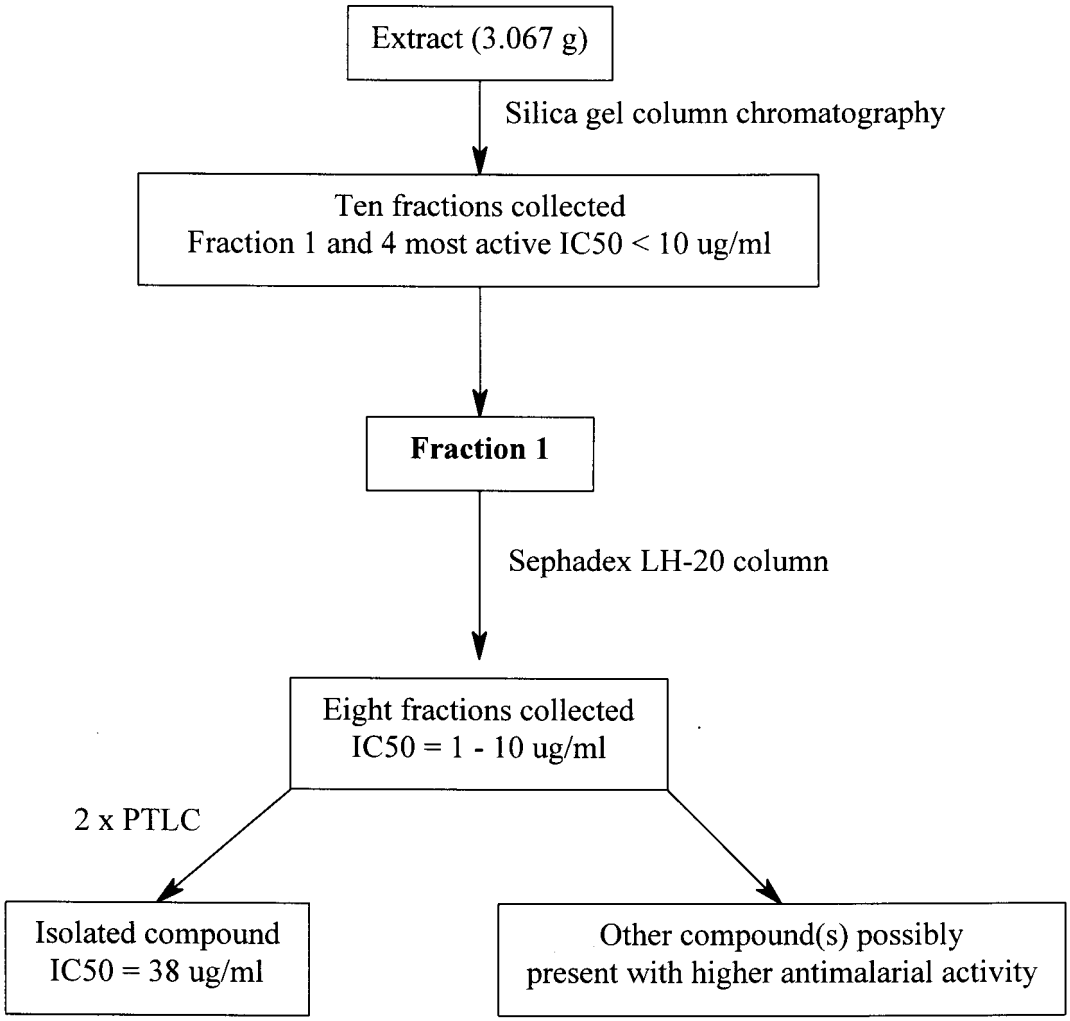


Figure 3: Summary of results from fractionation of the acetone extract from *C. pseudopluchellus*

Comparison with published data from Enriquez *et al.* (1997), shown in Table 1 confirmed this. Only comparative values are shown in Table 1. Some extra peaks from the ^{1}H-NMR data (Figure 7) were due to another compound separated by GC and are not shown.

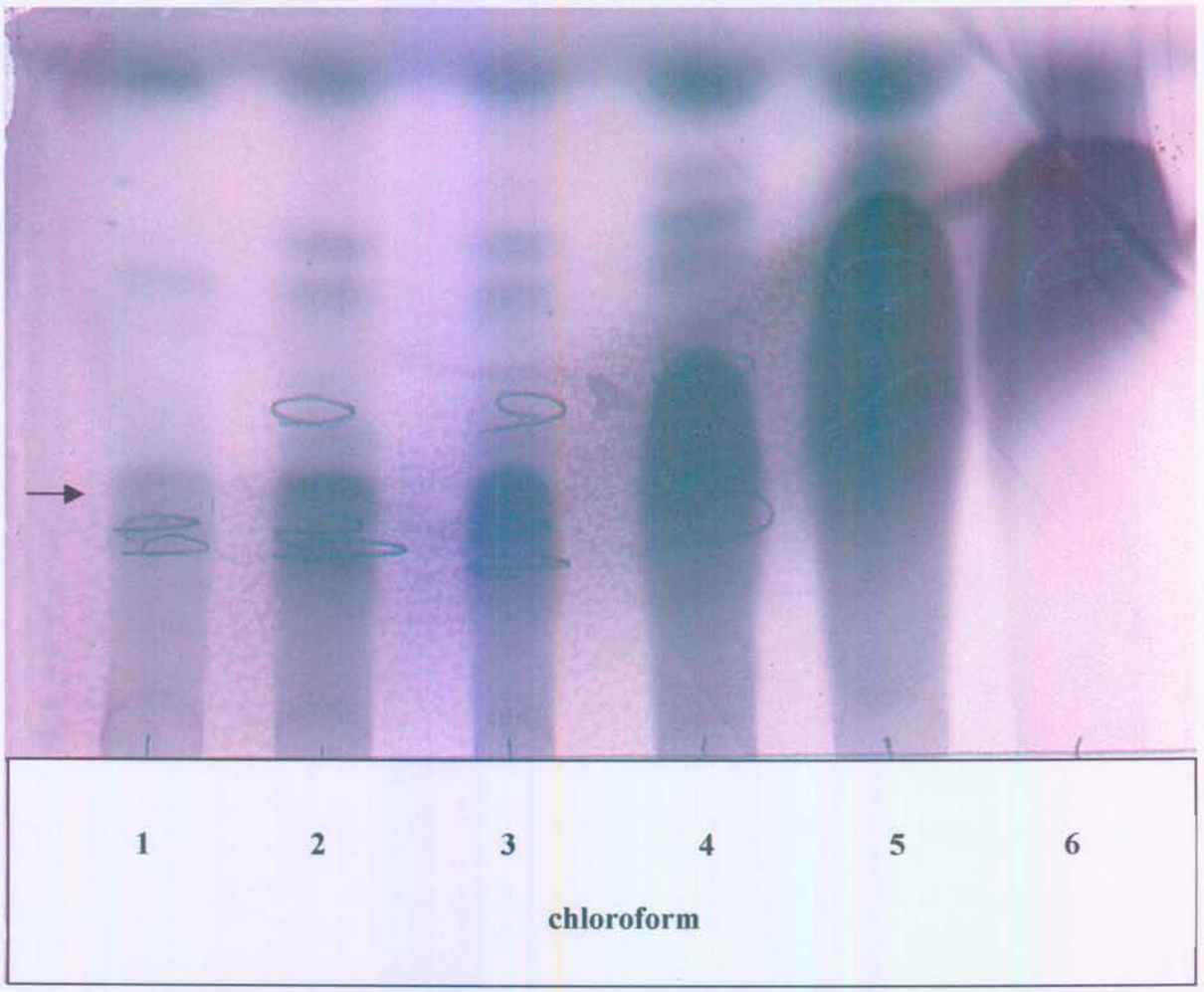


Figure 4: TLC plate developed with chloroform and sprayed with vanillin reagent showing some fractions collected from the Sephadex column.

Table 1: ^{1}H-NMR data from kaurenoic acid (Enriquez *et al.* 1997) and the isolated compound.

	Kaurenoic acid		Isolated compound	
Carbon	$\delta_{H\alpha}$	$\delta_{H\beta}$	$\delta_{H\alpha}$	$\delta_{H\beta}$
1	1.88	0.82	1.88	0.83
2	1.86	1.42	1.84	1.41
3	2.16	1.02	2.11	1.02
4	-	-	-	-
5	-	1.06	-	1.07
6	1.82		1.83	
7	1.47	1.62	1.47	1.60
8	-	-	-	-
9	-	1.04	-	1.04
10	-	-	-	-
11	1.56	1.60	1.56	1.58
12	1.52	1.45	1.52	1.44
13	2.63	-	2.62	-
14	1.99	1.13	1.99	1.13
15	2.05		2.04	
16	-	-	-	-
17	4.80	4.73	4.78	4.72
18	-	-	-	-
19	-	1.24	-	1.23
20	0.95	-	0.93	-

After identifying the isolated compound as kaurenoic acid (Figure 8), the ^{13}C-NMR spectrum of kaurenoic acid, previously isolated in our laboratory, was compared with published data and is summarised in Table 2.

From the comparison with the published data all the peaks present in kaurenoic acid correlated with peaks present in the isolated compound. This together with MS data and comparison on TLC with kaurenoic acid confirmed the isolated compound as kaurenoic acid.

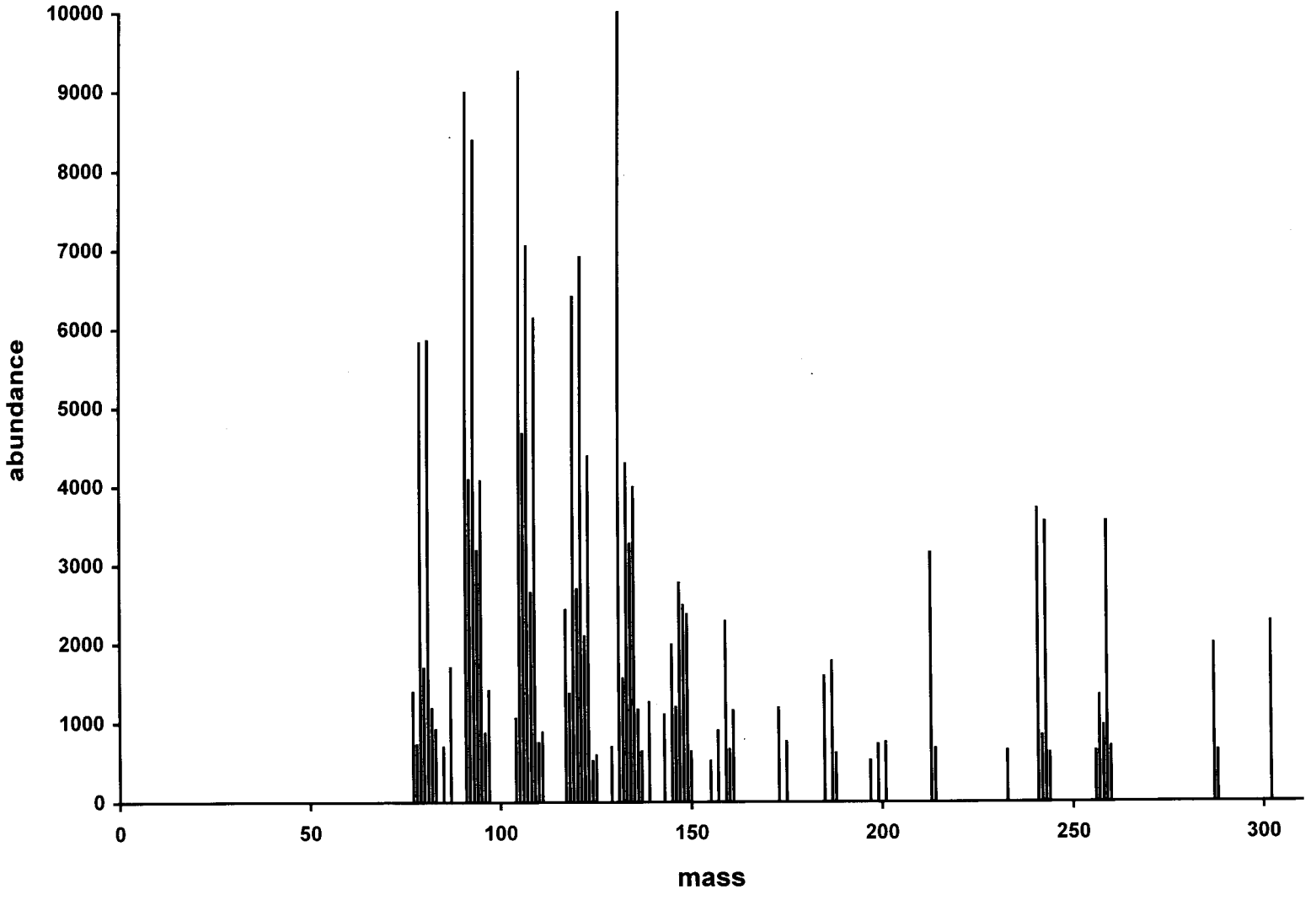


Figure 5: MS-data of kaurenoic acid isolated from *Croton pseudopluchellus*

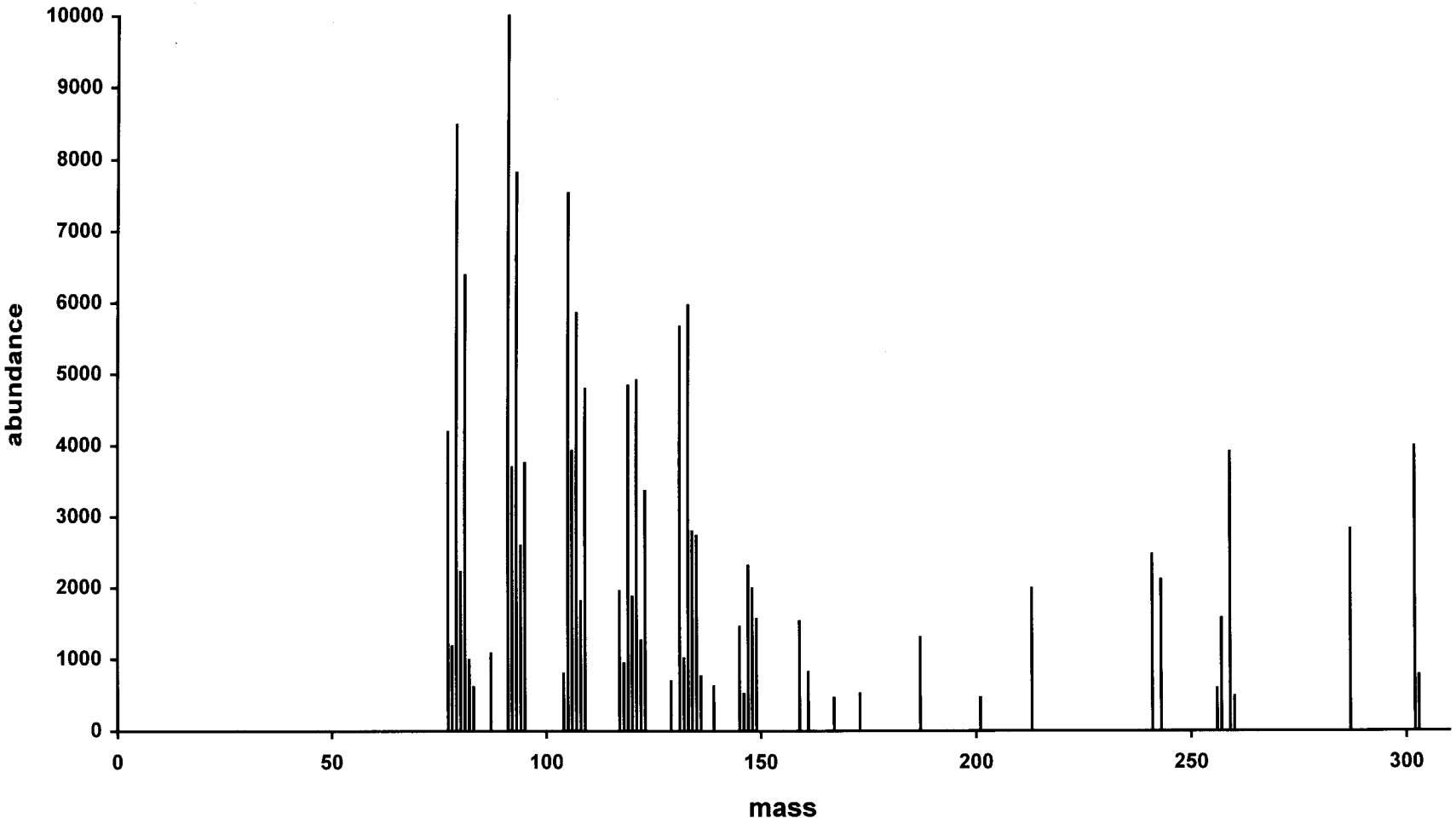


Figure 6: MS-data of kaurenoic acid from the MS-database

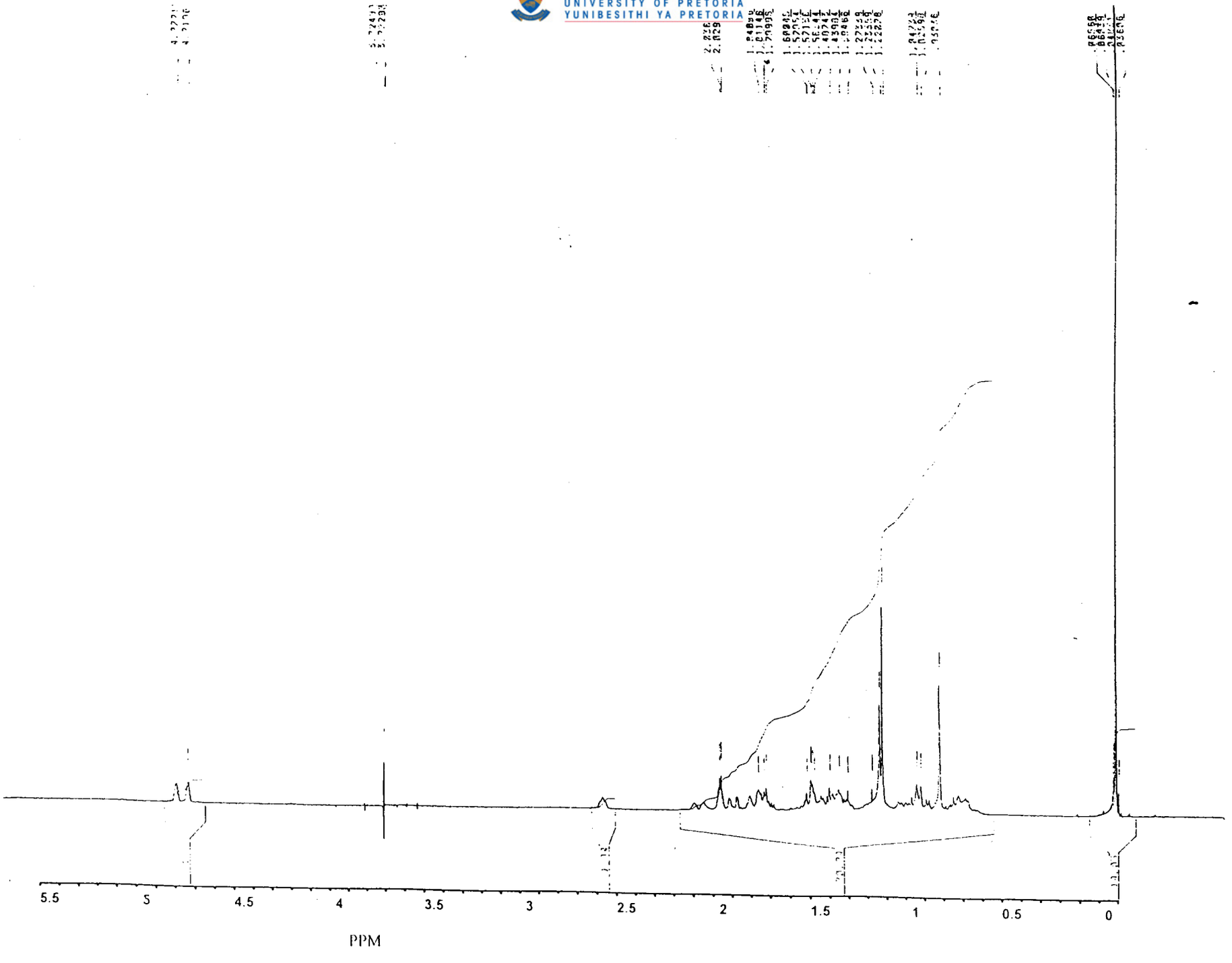


Figure 7: ^{1}H-NMR spectrum from isolated compound

Table 2: ^{13}C-NMR of kaurenoic acid (Enriquez *et al.* 1997) and isolated kaurenoic acid.

Carbon	Kaurenoic acid (published data)	Kaurenoic acid isolated
	δ_C	δ_C
1	40.70	41.61
2	19.09	20.01
3	37.83	38.89
4	43.71	44.08
5	57.04	57.64
6	21.83	22.76
7	33.10	33.87
8	44.23	45.02
9	55.11	56.00
10	39.66	40.42
11	18.43	19.10
12	41.27	42.10
13	43.85	44.72
14	39.70	40.42
15	48.96	49.78
16	155.90	156.33
17	102.98	103.57
18	183.74	179.14
19	28.96	29.29
20	15.60	16.22

Kaurenoic acid was tested for its antimalarial activity with the flow cytometric test and its cytotoxicity determined in monkey kidney cells. Results are shown in Figure 9.

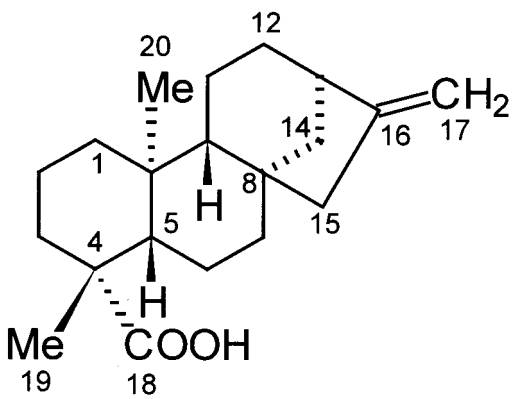


Figure 8: Kaurenoic acid

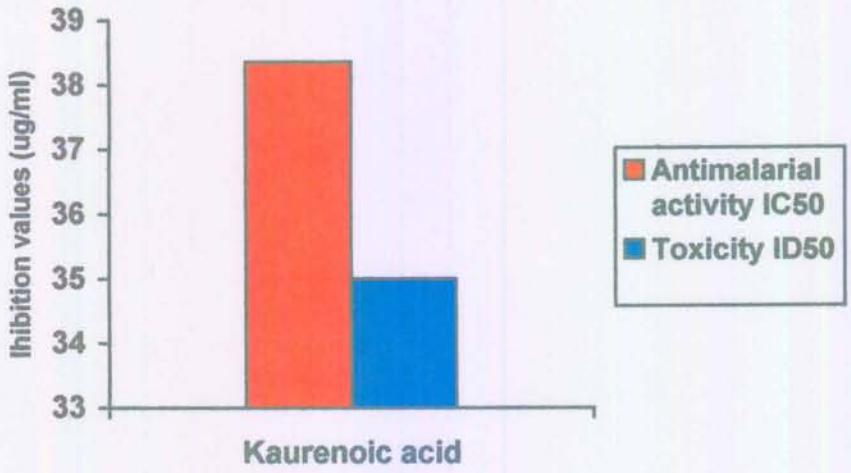


Figure 9: Antimalarial activity and cytotoxicity of kaurenoic acid

Antimalarial tests on kaurenoic acid confirmed moderate antimalarial activity with an IC_{50} of 38.36 µg/ml (35.76 - 40.65 95% confidence interval) while the cytotoxicity results showed an ID_{50} of 35 µg/ml. The therapeutic index of kaurenoic acid is 0.84 and it therefore has a poor selectivity index. It probably reacts more like a general toxin with no specific selectivity towards *Plasmodium*. *In vitro* antimalarial activity and cytotoxicity is not always comparable to activity found in an *in vivo* model. Gessler *et al.* (1995) reported that although the root bark of *Maytenus senegalensis* had high cytotoxicity *in vitro* against cell lines no toxic signs were observed in mice given the extract orally. The same extract also had a better *in vivo* antimalarial activity than the *in vitro* antimalarial activity. Kaurenoic acid's antimalarial activity and cytotoxicity might also be different in an *in vivo* model.

It is sometimes more effective to use the crude extract of a plant for treatment of an illness than the pure compound. Kirby *et al.* (1993) describe the extreme *in vitro* cytotoxicity of quassinoids isolated from *Brucea* species, although human clinical studies using crude preparations of *Brucea* fruits have shown no toxicity. The activity represented by kaurenoic acid is only a small percentage of the activity that was found in the purified fraction collected from the Sephadex column and the other compound(s) present in this fraction may have a much lower IC_{50} than kaurenoic acid.

5.4 References

ENRIQUEZ, R. G., BARAJAS, J., ORTIZ, B., LOUGH, A. J., REYNOLDS, W. F., YU, M., LEON, I. & GNECCO, D. 1997. Comparison of crystal and solution structures and ^{1}H and ^{13}C chemical shifts for grandiflorenic acid, kaurenoic acid, and monoginoic acid. *Can. J. Chem.* 75: 342 – 347.

KIRBY, G. C., WARHURST, D. C. & PHILLIPSON J. D. 1993. Plants as a source of novel antimalarial drugs. *Trans. R. Soc. Trop. Med. Hyg.* 87 (4): 370.

LEACH, H. 1960. Gas chromatography. In: Isolation and identification of drugs. Ed. E. G. C. Clarke, p. 59. The Pharmaceutical Press, London.

MCMURRY, J. 1995a. Organic chemistry. 4ed. Ch 12. pp. 424 – 435. Brooks/Cole Publishing, USA.

MCMURRY, J. 1995b. Organic chemistry. 4ed. Ch 13. pp. 454 – 475. Brooks/Cole Publishing, USA.

Chapter 6

General discussion and conclusion

6 General discussion and conclusion

Attempts to control the spread of malaria in African countries south of the Sahara with insecticides or synthetical antimalarials have so far failed (Grammiccia & Beales 1988). Presently used drugs have become ineffective because the parasites are developing resistance to most of these drugs. Plants have always proved to be a rich source for remedies of diseases and also of new drugs. At present, plants play an important role in the development of alternative substances for the treatment of disease in the light of growing cases of resistance to present chemicals and antibiotics. One option favored is to look for remedies used in traditional medicine (Irobi *et al.* 1994).

The success of artemisinin isolated from a Chinese traditional medicine with a different chemical structure has stimulated the search for new antimalarial drugs from traditional remedies. The results obtained by Gessler *et al.* (1994) showed that various plant extracts exerted strong antimalarial activity *in vitro*. Efforts are now being directed in obtaining drugs with different structural features from plants. Nkunya *et al.* (1991) found antimalarial activities in the bark extracts of various *Uvaria spp.* at comparable concentrations to currently used drugs.

6.1 *In vitro* antimalarial activity of plant extracts

Antimalarial tests on the plant extracts showed a high number of extracts with good antimalarial activity. The highest antimalarial activity was found in the dichloromethane and petroleum ether extracts and also in some acetone extracts. Solvent controls showed

little or no effect on the malaria parasites. The only exception was the concentrated acetone solvent, which had a 10% inhibition on parasite growth at 0.1% (1000 times dilution) with the microscopic method and 5% inhibition at 0.1% with the flow cytometric method. IC_{50} values obtained from the flow cytometric test with the 10 most active fractions were very good with 9 extracts having IC_{50} values below 2 µg/ml.

6.2 Cytotoxicity of extracts with antimalarial activity

Two cytotoxicity methods were used to determine the IC_{50} values of the most active extracts. Results obtained with the monkey kidney cell cytotoxicity test were lower than the results obtained with the bacteria cytotoxicity test. This was probably due to differences in the duration time of the two tests and the test material (bacteria and monkey kidney cells) used. The monkey kidney cell cytotoxicity test has got the advantage of testing extracts over the same time span as the antimalarial test and therapeutic values can be determined from these results. Therapeutic values determined for the extracts were between 35 and 100. This is not that high but it shows some selectivity of the extracts against *Plasmodium*. *In vitro* cytotoxicity is not always the same *in vivo* because toxic principles might be broken down or activated by metabolic processes and can therefore only be used as an indication of toxicity. *In vivo* toxicity tests will give much more information on the effects of extracts on a living system.

An ATP inhibition test was used to determine cytotoxicity of some extracts, but it was found that the medium used to dilute the extracts had an influence on the values obtained and this method wasn't used to determine the cytotoxicity of the extracts.

6.3 Antimalarial activity of purified fractions from *Ozoroa engleri*

Results from the fractionation of *Ozoroa engleri* showed four fractions with antimalarial activity. Further purification of these fractions showed antimalarial activity scattered through most of the fractions. The antimalarial activity of an extract is sometimes attributed to one or two main principles, as is the case with *Cinchona* sp. where quinine and quinidine are the main principles responsible for the plant's antimalarial activity. In some instances compounds act synergistically to give a higher extract activity than any individual compound. In other cases a number of compounds with moderate activity are responsible for the extract's activity in an additive effect. The dichloromethane extract of *O. engleri* has got at least five compounds responsible for its antimalarial activity and it is that likely that the extract's activity are the sum of these compounds.

6.4 Antimalarial activity of kaurenoic acid isolated from *Croton pseudopulchellus*

The active antimalarial compound(s) were separated mainly by silica gel column chromatography and Sephadex column chromatography. Good activity was found in some of the fractions from the Sephadex column and two spots detected by vanillin reagent correlated with the highest antimalarial activity. These two spots did not absorb UV light at 254 and 366 nm. They were then further purified on PTLC and 5 mg of one compound was isolated. The isolated compound was identified by ^{1}H-NMR and GC-MS as kaurenoic acid. Kaurenoic acid is classified as a diterpene. A literature survey showed no diterpenes with antimalarial activity, but some terpenes have been found with antimalarial activity.

These include the endoperoxide sesquiterpene lactone, artemisinin, the sesquiterpene lactone, parthenin and gedunin. Phillipson and Wright (1991) reported that many sequiterpene lactones, although active antimalarials are too toxic for clinical use. Chemical structures needed for activity and toxicity might be different and chemical manipulation might improve the pharmacokinetics of kaurenoic acid. The other active compound(s) present in *C. pseudopulchellus* might show better activity and less toxicity and future studies will include the isolation and identification of these principles. The mode of action of active compounds could give more insight into their effectiveness as antimalarial drugs, and studies to determine the mechanism of action against *Plasmodium* are anticipated.

6.5 References

GESSLER, M. C., NKUNYA, M. H. H., MWASUMBI, L. B., HEINRICH, M. & TANNER, M. 1994. Screening Tanzanian medicinal-plants for anti-malarial activity. *Acta Trop.* 56: 65 – 77.

GRAMMICCIA, G. & BEALES, P. F. 1988. The recent history of malaria control and eradication. In, *Malaria Principles and Practice of Malariology,* Vol.2, ed. by W.H.Wernsdorfer and I. McGregor, pp. 1335 – 1378. Churchill Livingstone, Edinburgh.

IROBI, O. N., MOOYOUNG, M., ANDERSON, W. A. & DARAMOLA, S. O. 1994. Antimicrobial activity of bark extracts of *Bridelia ferruginea* (Euphorbiaceae). *J. Ethnopharmacol.* 43: 185 – 190.

NKUNYA, M. H. H., WEENEN, H., BRAY, D. H., MGANI, Q. A. and MWASUMBI, L. B. 1991. Antimalarial activity of Tanzanian plants 3. Antimalarial activity of Tanzanian plants and their active constituents - the genus *Uvaria*. *Planta Med* 57: 341 – 343.

PHILLIPSON, J. D. & WRIGHT, C. W. 1991. Can ethnopharmacology contribute to the development of antimalarial agents? *J. Ethnopharmacol*. 32: 155 – 165.

Chapter 7

Summary

***In vitro* antimalarial activity of ethnobotanically selected indigenous plants and characterisation of a bioactive compound**

by

Erwin Antoni Prozesky

Supervisor: Prof J.J.M. MEYER

Co-supervisor: Prof A.I. LOUW

Department of Botany

MAGISTER SCIENTIAE (Plant Physiology)

Malaria still remains one of the world's biggest killers with more than two million people dying from the disease each year. Present drugs have become ineffective because parasites are developing resistance to most of them. Efforts are now being directed in obtaining drugs with different structural features. Plants have provided most of the antimalarial drugs so far and efforts now concentrate on screening plants for new antimalarial drugs. South Africa with its rich floral resources and ethnobotanical history is an ideal place to screen plants for antimalarial activity.

The antimalarial activity of 20 extracts from 14 ethnobotanically selected South African plants was screened for antimalarial activity *in vitro*. Results obtained showed that most of the plants had strong antimalarial activity. IC_{50} values obtained with the flow cytometric method were between 0.9 and 2 µg/ml for 9 of the 10 selected extracts. This represents a very high number of extracts with very good antimalarial activity.

Cytotoxicity of the most active extracts were determined against monkey kidney cells as well as a luminescent bacteria method. Results obtained had a ID_{50} between 35 and 100 µg/ml with the monkey kidney cell test and between 100 and 2000 µg/ml with the bacteria test. Therapeutic values ranged between 35 and 100. Extracts therefore have a poor selectivity towards *Plasmodium*.

The dichloromethane extract from *Ozoroa engleri* was further purified with silica gel column chromatography, Sephadex column chromatography and HPLC. Results obtained showed at least five or six compounds responsible for the antimalarial activity of the extract, all with moderate antimalarial activity and no further efforts were undertaken to identify them.

The acetone extract of *Croton pseudopulchellus* was then selected for isolation of active principles and was purified by silica gel column chromatography, Sephadex column chromatography and PTLC. Kaurenoic acid was isolated as one of the active principles and identified by NMR and GC-MS. Kaurenoic acid was found to have an antimalarial IC_{50} of 38 µg/ml, while its cytotoxicity ID_{50} was 35 µg/ml. Kaurenoic acid was responsible for only some of the activity found in the purified fraction and other compound(s) in the extract might have much better antimalarial activity.

Chapter 8

Acknowledgements

I would like to thank the following people and institutions,

Prof J. J. M. Meyer & Prof A. I. Louw for their guidance, comments and suggestions in the course of the research.

The Foundation for Research Development, the Medical Research Council and the University of Pretoria for financial support.

Prof W. du Plooy, Department of Pharmacology and Therapeutics, Medunsa for cytotoxicity tests.

Prof A. E. van Wyk for his taxonomic advice, comments and suggestions.

Eric Palmer, Department of Chemistry for assistance with NMR spectra.

Tony Hassett, Department of Chemistry for assistance with GC-MS.

Department of Virology for monkey kidney cell toxicity tests.

Staff at Tembe Elephant Park for assistance in the collection of plant material.

Deborah Schulze for assistance with malaria culturing and maintenance, advice, comments, suggestions and moral support.

All my other supportive relatives and friends.