ANTIMALARIAL COMPOUNDS FROM

CRINUM BULBISPERMUM

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SUMMARY

Malaria is caused by protozoan parasites of the genus *Plasmodium*, of which *Plasmodium falciparum* is the most widespread and dangerous. Around 800 000 children under the age of five die from malaria every year. An increase in resistance to previously effective drugs is also evident. This disease therefore has social and economical consequences. The isolation of antimalarial compounds from medicinal plants may provide the solution to an ever increasing demand for new effective antimalarial agents. Compounds with antimalarial activity also tend to have antimicrobial activity, thus when testing plants for antimalarial activity, it must be considered that they may also provide effective antimicrobial agents.

Six plants were selected and 62 extracts of the different morphological plant parts were prepared, using Soxhlet extraction with petroleum ether, dichloromethane, ethyl acetate and ethanol consecutively. The antimalarial activity was assessed by employing the [^{3}H] - hypoxanthine incorporation assay against the chloroquine-resistant Gambian FCR-3 strain of *P. falciparum.* The dichloromethane and ethyl acetate extracts of *Crinum bulbispermum* exhibited the most promising activity, with IC_{50} values of 0.379 ± 0.126 and 0.08 ± 0.004 µg/ml respectively, and were selected for further study.

Two acids, namely linoleic acid **(24)**, oleic acid **(25)** and an alkaloid, namely lycorine **(26)** was isolated with column and thin layer chromatography and structures were elucidated by using nuclear magnetic resonance, mass and infrared spectrometry.

The antimalarial activity of the isolated compounds **(24 – 26)** were assessed. The IC_{50} value of the isolated compound lycorine **(26)** (0.0291 ± 0.01 µg/ml) compares well to that of chloroquine **(1)** and quinine **(2)** (IC_{50} values of 0.04 ± 0.01 and 0.17 ± 0.02 µg/ml, respectively). These compounds **(24 – 26)** were found to be relatively non-toxic as determined by an *in vitro* cellular toxicity assay. IC_{50} values for toxicity were determined for the respective compounds **(24 – 26)** and lycorine **(26)** had the best toxicity index of > 15 000. Since this compound had such a high toxicity index it was regarded as suitable for further investigation as an antimalarial drug.

Antimicrobial activity was assessed with the direct plate method and minimum inhibitory concentration values were determined. The best activity was observed for the alkaloid lycorine **(26)** against *B. subtilis.*

The isolated alkaloid lycorine **(26)** is not structurally related to any other antimalarial drug currently in use and could therefore be used as a lead compound for a new class of antimalarial drugs. The diverse chemistry of medicinal plants affords a viable source in the search for biologically active compounds.

OPSOMMING

Malaria word veroorsaak deur 'n protosoale parasiet van die genus *Plasmodium,* waarvan *Plasmodium falciparum* die wydste verpreid en gevaarlikste is. Na beraming sterf 800 000 kinders onder die ouderdom van vyf jaarliks as gevolg van malaria. Toename in weerstand teen voorheen effektiewe geneesmiddels vererger die situasie. Malaria het ook sosiale en ekonomiese gevolge. Dus kan die isolering van antimalariaverbindings vanuit medisinale plante 'n oplossing bied vir die toenemende dringendheid in die soektog na nuwer effektiewe antimalaria middels. Verbindings met antimalaria aktiwiteit toon meestal ook antimikrobiese aktiwiteit, wat beteken dat wanneer plante vir antimalaria aktiwiteit getoets word, in gedagte gehou moet word dat hul ook as effektiewe antimikrobiese middels kan dien.

Ses geselekteerde plante is versamel en verdeel in verskillende morfologiese plant dele. Twee en sestig ekstrakte is berei deur middel van Soxhlet ekstraksie. Petroleumeter, dichloormetaan, etielasetaat en etanol is as oplosmiddels gebruik. Die antimalaria aktiwiteit van die ekstrakte is bepaal deur die meting van die opname van radio-aktiewe hipoxantien deur die chlorokienresistente FCR-3 stam van *P. falciparum.* Aktiwiteit van die ekstrakte het gewissel, met die dichloormetaan- en etielasetaatekstrakte van *Crinum bulbispermum* as die mees belowende met IC_{50} waardes van 0.379 ± 0.126 en 0.08 ± 0.004 µg/ml respektiewelik. Bogenoemde twee ekstrakte is gebruik vir verdere studies.

Twee sure en 'n alkaloïed, naamlik linoleensuur **(24)**, oleïensuur **(25)** en likorien **(26)** is geïsoleer vanuit die dichloormetaanekstrak deur kolom- en dunlaagchromatografie en struktuur opklaring is gedoen deur kernmagnetiese resonans-, massa- en infrarooispektrometrie.

Die IC_{50} waarde vir antimalaria aktiwiteit van likorien **(26)** (0.0291 ± 0.01 µg/ml) vergelyk goed met die van chlorokien **(1)** en kinien **(2)** (IC_{50} waardes van 0.04 ± 0.01 en 0.17 ± 0.02 µg/ml, respektiewelik). Die verbindings **(24 – 26)** is relatief nie-toksies *in vitro* soos gevind met 'n toets vir sellulêre toksisiteit. IC_{50} waardes is *in vitro* bepaal en likorien **(26)** het die beste toksisiteitsindeks van >15 000 getoon. Die hoë toksisiteitsindeks maak dit bruikbaar vir verdere ondersoek as 'n antimalariamiddel.

Antimikrobiese aktiwiteit is bepaal deur die mikroplaat- en tetrasoliumsoutmetode. Die beste antimikrobiese aktiwiteit is waargeneem vir die alkaloïed likorien **(26)** teenoor *B. subtilis.*

Die alkaloïed is struktureel nie verwant aan enige van die bestaande geneesmiddels wat tans teen malaria gebruik word nie en kan moontlik as 'n leidraadverbinding vir 'n nuwe klas antimalaria middels dien. Die diverse chemie van medisinale plante is dus steeds 'n belangrike bron in die soektog na nuwe biologies aktiewe verbindings.

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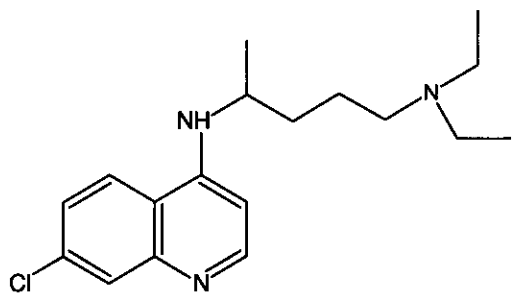
CHAPTER 1

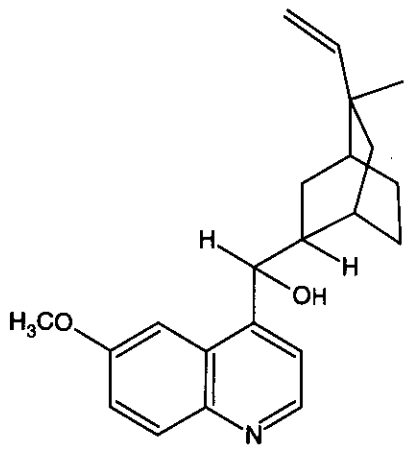
INTRODUCTION AND RESEARCH STATEMENTS

1.1 Introduction

Malaria remains one of the most serious diseases globally with an estimated 500 million cases occurring each year. It is endemic in 92 countries, with 41% of the world population being at risk of contracting the disease. More than one million deaths per year are attributed to malaria, the mortality in African children being the highest (Breman, 2001). Chloroquine (**1**), a 4-aminoquinoline introduced in 1945, gave us a very efficient tool to combat malaria. Chloroquine having a long half-life could be used as a prophylactic drug. It was cheap, well tolerated and effective against all strains of plasmodia. However 12 years after its introduction the first cases of chloroquine resistant falciparum malaria were reported (Wongsrichanalai *et al.*, 2002). Since then resistance to the most common antimalarial drugs has spread to almost every part of the world contributing to the emergency in the development of new compounds for malaria therapy (Meek *et al.*, 1986; WHO, 1999; Winstanley, 2000). Furthermore, the financial burden of the disease falls heavily on those who can least afford it. There is thus an urgent need for new, inexpensive drugs or a vaccine that is both effective and suitable for mass production.



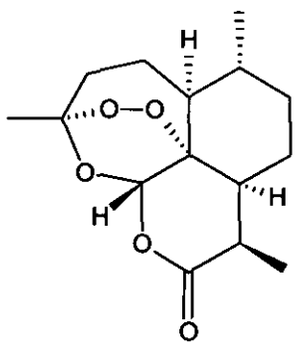
(1)



(2)

Figure 1.1: Structures of chloroquine **(1)** and quinine **(2)**.

Some of the earlier natural products used as antimalarials include the bark of the *Cinchona* tree and extracts of *Artemisia annua* (wormwood plant). The respective compounds derived from these plants, quinine (**2**) and artemisinin (**10**) play a very important role in the search for derivatives against multidrug resistant malaria and has focused attention on plants as potential sources of antimalarial drugs (Willcox & Bodeker, 2000).



(3)

Figure 1.2: Structure of artemisinin.

The use of plants as medicine dates back to the ancient civilisations. The earliest drugs were plant extracts, followed by natural compounds of known chemical structure and by inorganic substances. Since the beginning of synthetic organic chemistry, synthesis of compounds has become the most popular means of drug discovery. However plants still play a very important role in medicine today and are used by many different cultures for various ailments. Thus further investigations into the active compounds are of utmost importance.

1.2 Aim and objectives of this study

The aim of this study was to identify and screen specific plants with perceived antimalarial activity and then to isolate and characterise the active compounds responsible for this activity. Compounds with antimalarial activity also tend to have antimicrobial activity, thus it was also decided to determine the antimicrobial activity.

After initial antimalarial screening of 62 different extracts from the six plant species tested, *Crinum bulbispermum* was selected for further investigation. The study then focused on the biological evaluation of the extracts and fractions of *Crinum bulbispermum* and the isolation and characterisation of compounds with possible antimalarial activity from this plant species. The chloroquine resistant strain of *P. falciparum* was used in this study because of its high prevalence, especially in South Africa, and difficulty in treating this form of malaria.

To reach the aim of this study the following objectives were proposed:

- Thorough discriminative literature screening to select South African plants with described ethnopharmacological use as antimalarials or similar activity from species available in the Potchefstroom area (table 2.1).
- Fractionation and biological evaluation of extracts from the selected species for antimalarial and antibacterial activity.
- Selection of the most promising species and isolation and characterisation of the compounds responsible for the antimalarial and antibacterial activity.
- Determination of the *in vitro* activity and toxicity of the isolated compounds.

CHAPTER 2

BACKGROUND

2.1 Malaria

2.1.1 The lifecycle of *Plasmodium* sp.

Malaria has been a cause for considerable concern throughout the history of man. With probable origin in Africa, malarial parasites from fossils of mosquitoes have been dated back to 30 million years ago. These unique protozoal parasites and causative agents of malaria belong to the *Plasmodium* genus consisting of four species of obligate intracellular sporozoans; *P. malariae, P. vivax, P. ovale and P. falciparum.* With the exception of *P. malariae*, these plasmodium species are exclusive parasites of humans (Viswanathan, 1998). *P. falciparum* is the deadliest of all the species due to its widespread resistance to chloroquine and are thus the biggest threat to mankind.

The life cycle of *P. falciparum* consists of two cycles of asexual reproduction; firstly sporozoites enter the bloodstream as the female *Anopheles* mosquito takes its blood meal as seen in figure 2.1. These sporozoites are rapidly transported to the liver, where they penetrate hepatocytes. Disease occurs only as a result of the asexual blood stage after the parasite leaves the liver and begins to invade and grow inside red blood cells. Here they usually develop into exoerythrocytic schizonts that may contain many thousands of merozoites. The merozoites infect the red blood cells (erythrocytic schizont) and undergo schizogony which produces either asexual trophozoites or sexual gametocytes in the red blood cells. Trophozoites multiply until the red blood cells eventually burst releasing more merozoites into the blood stream to infect more red blood cells (Quast, 1999).

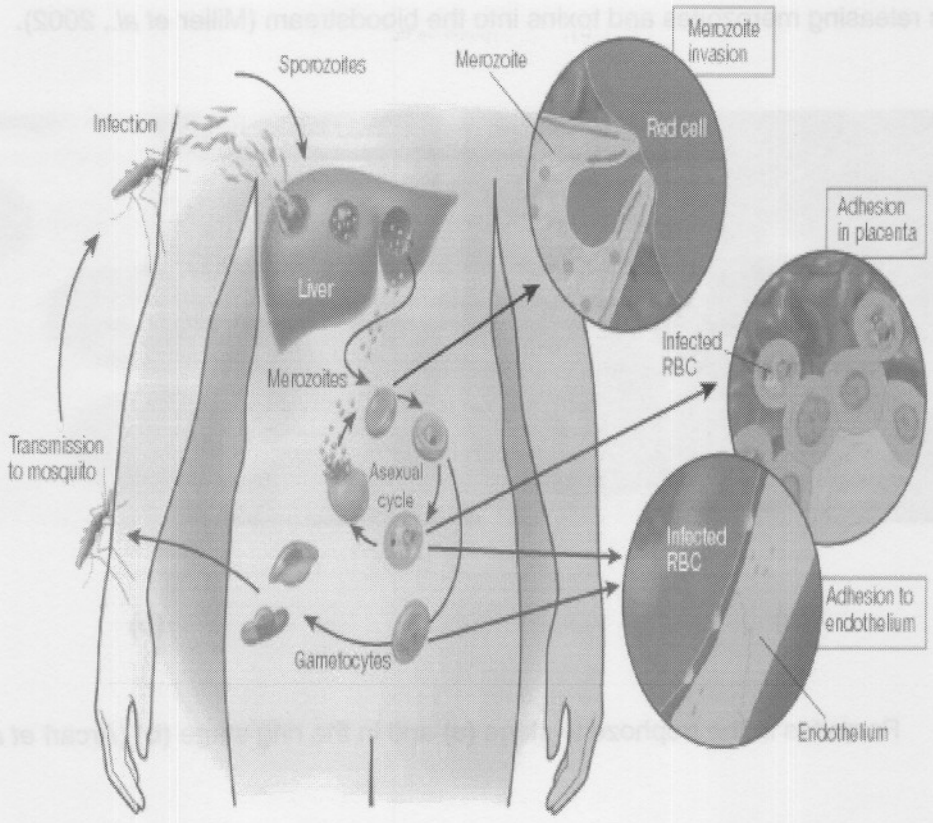
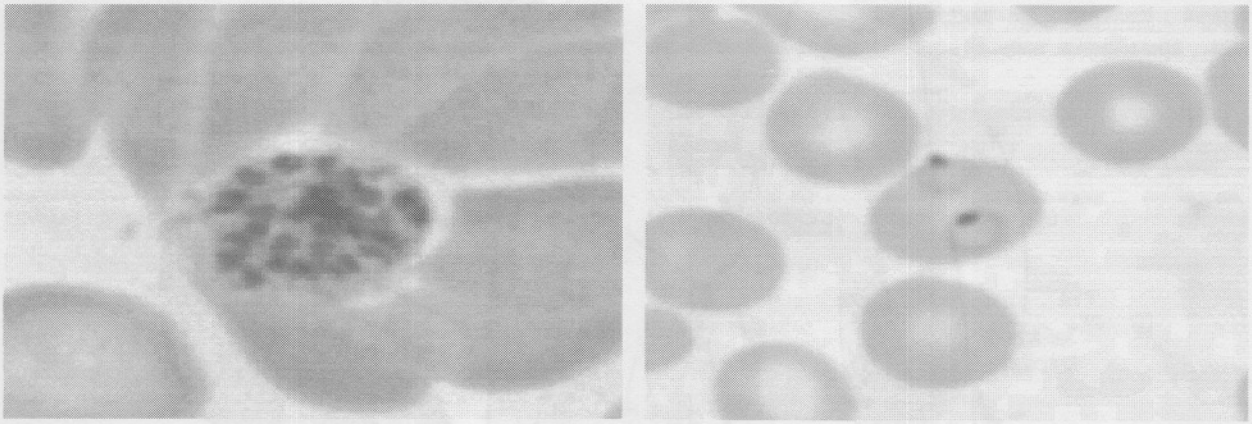


Figure 2.1: Parasite life cycle and pathogenesis of *P. falciparum* malaria (Miller *et al.*, 2002).

In contrast to the asexual pathway, instead of forming trophozoites the parasites may develop into immature sexual gametocytes. For this pathway to continue, the male and female gametocytes must be taken up in the blood meal of a mosquito, to initiate the stages within the intermediate host. The gametocytes are stimulated to mature to micro- and macrogametes. The fertilized female macrogamete forms a zygote, which goes on to form an ookinete that penetrates the midgut wall of the mosquito, forming an oocyst. Within the oocycst a cycle of reproduction takes place, with the formation of numerous sporozoites. When mature, the oocyst bursts open releasing these sporozoites, which then migrate to the insect's salivary glands. From here they may enter the bloodstream of a new host, thus completing the parasite's lifecycle.

Generally the parasite's lifecycle stages are highly synchronised, such that at any one time all the parasites are at the trophozoite stage as seen in figure 2.2a, or all are at the ring stage as seen in figure 2.2b. Episodes of fever are associated with rupture of the mature schizont infected

erythrocytes releasing merozoites and toxins into the bloodstream (Miller *et al.*, 2002).



(a) **(b)**

Figure 2.2: Parasites in the trophozoite stage **(a)** and in the ring stage **(b)** (Arcari *et al.*, 2003).

In some species such as *P. vivax* the sporozoite on invasion of the hepatocyte, develops into a hipnozoite, a "resting" stage of the parasite in which the development of the schizont is retarded. This stage may last months or sometimes years before it continues through the rest of the parasites lifecycle, and is responsible for recrudescence of the parasitaemia, after supposed chemotherapeutic cure and clearance of bloodstream forms of the parasite. There are in fact usually two cycles of schizogony in the liver. Namely the primary tissue schizont (absent in the most important of the human malarias, *P. falciparum*), and the formed merozoites derived from the primary tissue schizont. Both of these schizont stages release numerous merozoites, capable of infecting erythrocytes and generating the bloodstream forms of the parasite. Secondly the bloodstream form, of the malaria parasite consists of a number of forms, seen in the peripheral blood.

2.1.2 Pathology of *P. falciparum*

The molecular and cellular events during the life cycle of the parasite influence the severity of the disease. All human *Plasmodium sp.* invade by the same mechanism, but *P. falciparum* reaches high parasitaemia because of greater flexibility in the receptor pathways it uses to invade red

blood cells. Red blood cells infected with *P. falciparum* must bind to endothelium or placenta for the parasite to avoid spleen-dependent killing mechanisms, but this binding also leads to much of the pathology (Miller *et al*, 2002). In *P. falciparum* malaria the surface membrane of the infected erythrocyte becomes 'sticky', and can adhere to the surface epithelium of blood vessels of the internal organs such as the heart, lung, brain, liver, kidney, subcutaneous tissues and placenta. The various endothelial cells in these organs and syncytiotrophoblasts in placenta express different and variable amounts of host receptors.

To successfully adhere to these cells, the parasite can bind to several receptors as shown in figure 2.3 (Baruch, 1999). The adhesion phenotype is not homogenous, and different parasites can bind to variable numbers and combinations of host receptors. The variant antigen family of *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) is central to host–parasite interaction and pathogenesis. PfEMP1 expressed on the surface of mature red blood cells infected with *P. falciparum* is involved with clonal antigenic variation and can bind to many host receptors through its multiple adhesion domains. The different properties of PfEMP1 — sequestration for evading spleen-dependent killing and antigenic variation for evading antibody-dependent killing — contribute to the virulence and pathogenesis of *P. falciparum* and are essential for the survival of the parasite. Parasite sequestration in the brain and placenta contribute to the complications of cerebral malaria and placental malaria, respectively. Simultaneous binding to several receptors, binding of uninfected erythrocytes (rosetting), and clumping of infected erythrocytes through platelets are associated with the pathogenesis of malaria. The binding of parasite-infected red blood cells to dendritic cells down regulates the host's immune response.

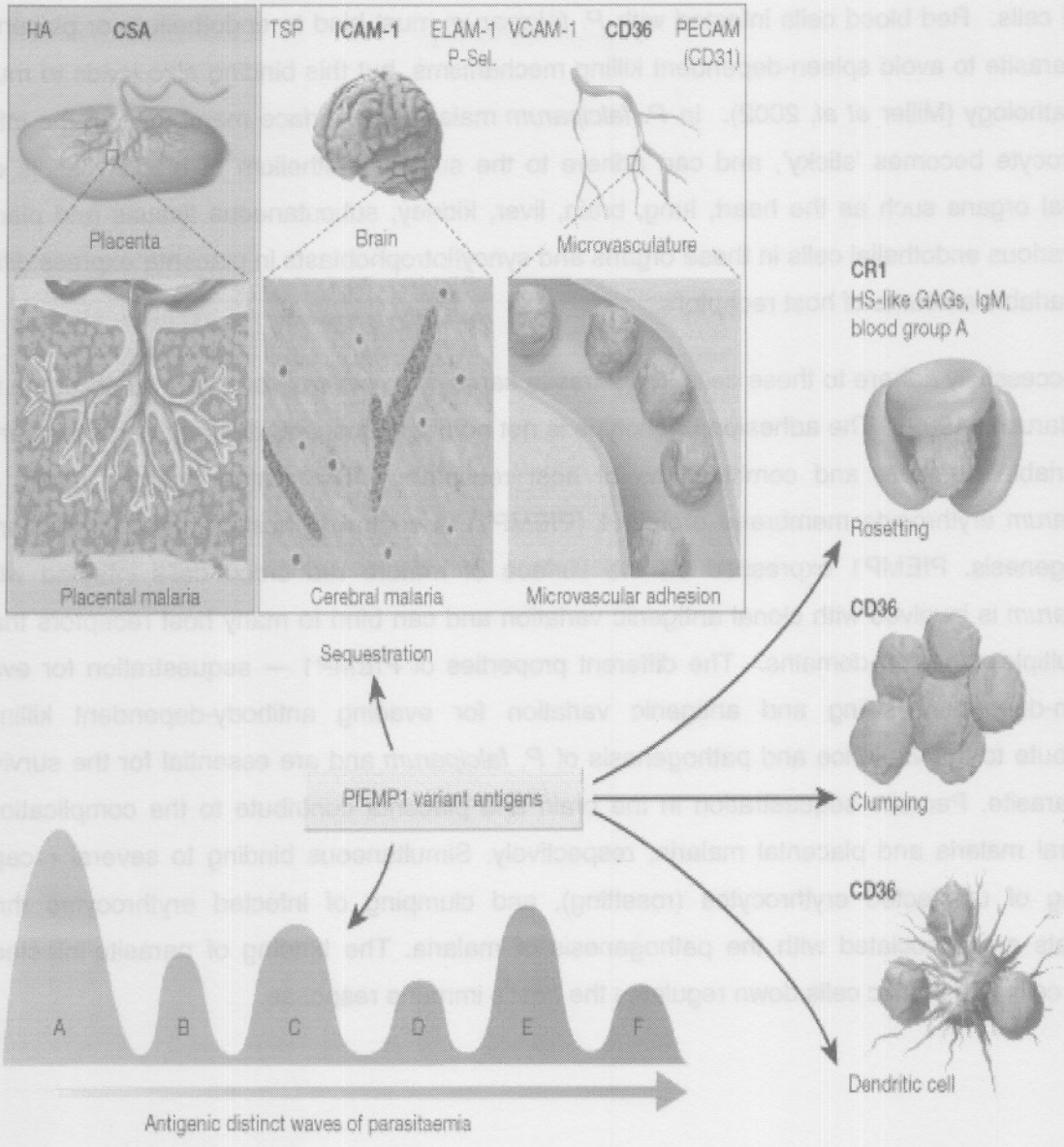


Figure 2.3: Adhesion phenotypes of *P. falciparum* (Miller *et al.*, 2002).

2.1.3 Symptoms of malaria

In the early stages of malaria the symptoms can be similar to those of many other illnesses caused by bacterial, viral, or parasitic infections and are characteristically similar to flu. The symptoms may include fever (periodically), chills, headache, sweats, fatigue, nausea and vomiting.

The symptoms may appear in cycles and present at different intensities and for different lengths of time. However, especially at the beginning of the illness, the symptoms may not follow this typical cyclic pattern. This pattern is related to the life cycle of the malaria parasites, their development and reproduction. Symptoms of malignant tertian malaria include anaemia along with chills and fever alternating at 72 hour intervals. This cyclic appearance of symptoms is diagnostic of malaria.

Infection with *P. falciparum* is usually life threatening with some of the following complications: cerebral malaria, pulmonary oedema, renal failure and severe anaemia (Goldsmith, 1998a).

2.1.4 Prevalence of the disease

"Malaria disaster in Africa" heads the letter from Kevin Marsh to the Lancet in September 1998. A disaster, he states, "which is not just on its way but is already happening". The global burden of malaria is enormous, amounting to approximately 300 to 500 million new infections and an estimated 2 to 3 million deaths annually. Each minute, 3 to 5 children die of malaria! Each hour, malaria kills more people than the 1995 EBOLA epidemic in Zaire. Unlike AIDS, EBOLA and other major hardships, malaria is not recognised in the developed world as a disaster (Nason-Burchenal, 2002).

Such is the situation more than 100 years after two key discoveries; one that the infection is caused by a blood-dwelling complex parasite belonging to the genus *Plasmodium* and two that the parasites are transmitted by the blood-feeding female *Anopheles* mosquito. This was followed by many remarkable discoveries, especially during the last 50 years (Kumar, 2002). Malaria is perceived as the world's worst health problem, but the endemic areas have the least developed health systems and annual reporting of the incidence of malaria cases and fatalities are at best guesses of the actual numbers (Snow *et al*, 1999). Almost 10% of the world's population will suffer a clinical attack of malaria each year. Fortunately, most will survive after an illness lasting 10 to 20 days, but during a clinical illness, they will be unable to attend school or work, diminishing educational attainment and productivity.

Malaria is considered a re-emerging disease, largely due to the rapid spread of drug-resistant parasite strains. Other factors include armed conflicts which lead to migration to and from high risk

malaria areas, changes in rainfall patterns, socio-economical conditions and an increase in the susceptible population (Nchinda, 1998). New breeding sites for the vector are created by road building, deforestation, mining (especially open cast mining), irrigation projects and new agricultural practices. All of the previous, environmental changes which might be expected to be of economic benefit.

South Africa is not exempt from the potential ravages of malaria with its debilitating effects on communities and development. The areas affected can be seen from the distribution of the disease as illustrated in figure 2.4. The red areas in figure 2.4 indicate a climate suitable for *Anopheles* breeding and probably endemic malaria; the blue and white areas indicate an unsuitable climate for the *Anopheles* species with relatively few cases of malaria (Gallup & Sachs, 1998).

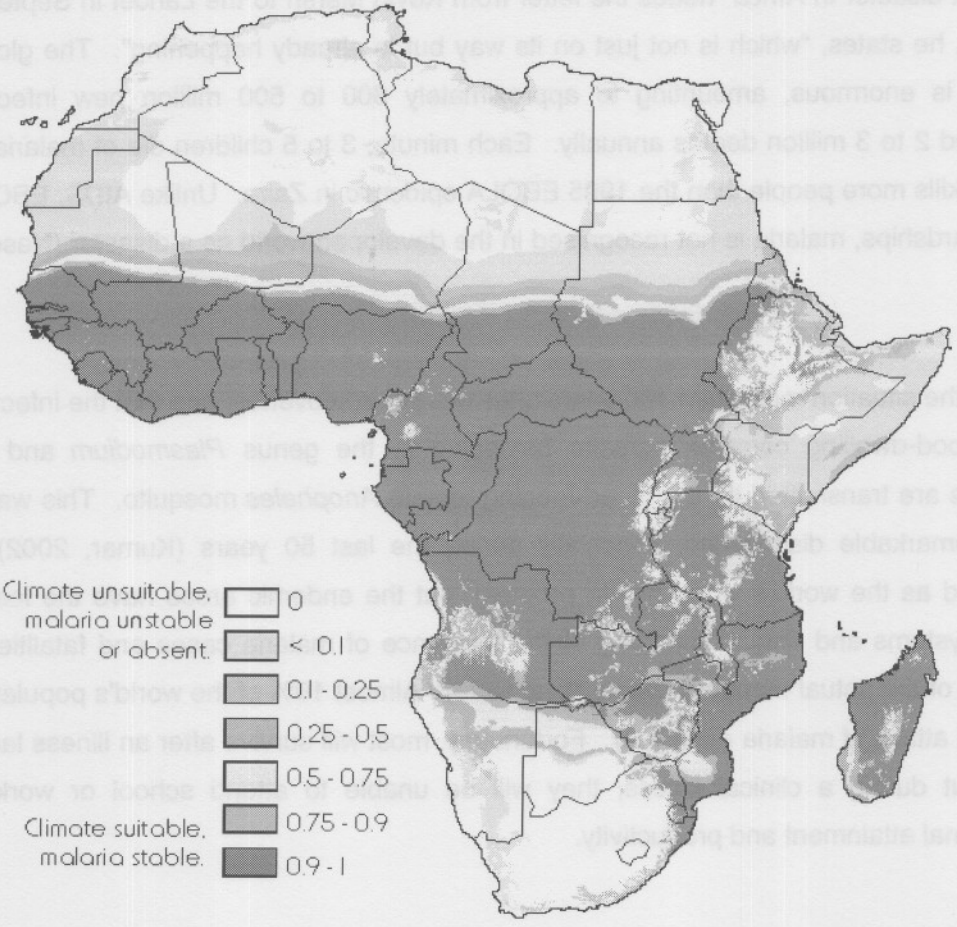


Figure 2.4: Continental distribution of malaria (MARA/ARMA, 2002).

Malaria transmission is distinctly seasonal in South Africa with notifications generally increasing from November onwards and declining by June, corresponding to seasonal rainfall patterns and climate changes.

There has been a remarkable increase in malaria transmission in South Africa since 1996 (figure 2.5).

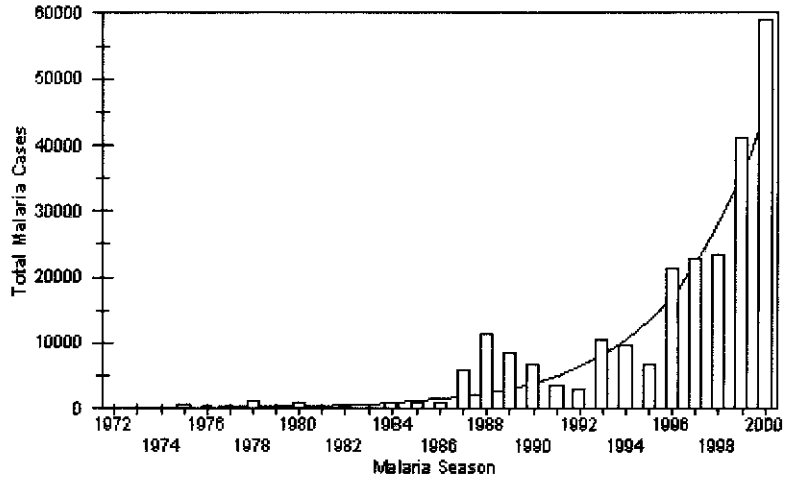


Figure 2.5: Malaria season case totals for South Africa (MARA/ARMA, 2002).

The underlying reasons for this increase are difficult to quantify. The rediscovery of *Anopheles fenustus* in sprayed houses in malaria areas may be a factor. This mosquito has been shown to be resistant to synthetic pyrethroids (the insecticide used to spray houses). The problem with insecticide resistance has been addressed in Kwazulu-Natal by a prompt reversion to the use of DDT for intra-domiciliary spraying during the winter of 2000 (MARA/ARMA, 2002).

Another factor is the high levels of resistance to first line malaria treatment (sulphadoxine/pyrimethamine) in Kwazulu-Natal by the malaria parasite *P. falciparum* (figure 2.6). The high level of resistance to malaria treatment is an ongoing problem. This encourages the ongoing investigation for newer and more effective antimalarial drugs.

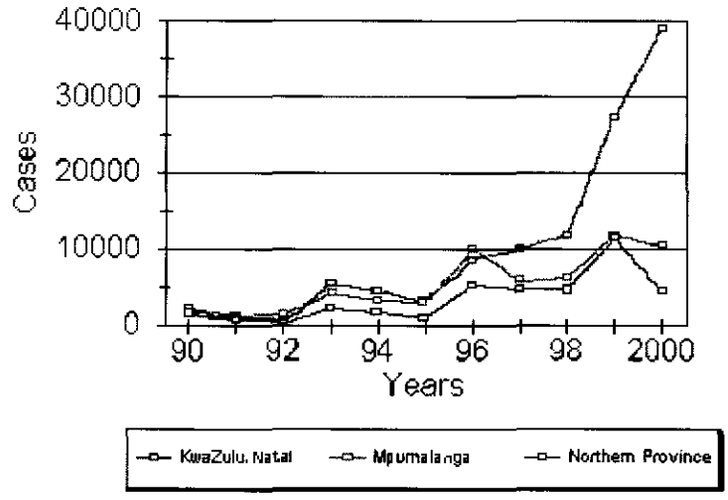


Figure 2.6: Notified malaria cases from the 3 malarious provinces of South Africa (MARA/ARMA, 2002).

2.1.5 Malaria vaccine development

No malaria vaccines are currently available, although extensive research is being done in this area to prevent malaria cases. Malaria parasites have complex life cycles and distinct developmental stages, each of which has multiple antigens that could serve as targets for an immune response. A pre-erythrocytic vaccine would protect against the infectious form injected by a mosquito (sporozoite) and/or inhibit parasite development in the liver. In a previously unexposed individual, that has now been infected there might be a few parasites that can escape the immune defences induced by a pre-erythrocytic vaccine. These parasites could eventually multiply and then result in full-blown malaria. An erythrocytic or blood stage vaccine would protect against parasite multiplication in the red blood cells, thus preventing (or diminishing) severe disease during the blood infection. A sexual stage vaccine does not protect the person being vaccinated. Instead it interrupts the cycle of transmission by inhibiting the further development of parasites once they, along with antibodies produced in response to the vaccine, are ingested by the mosquito. Transmission blocking vaccines could play a role as part of a multi-faceted strategy. This is directed at elimination of parasites from low-transmission or drug directed at pre-erythrocytic or erythrocytic stages (James & Miller, 2001).

An optimum vaccine would have the ability to elicit protective immunity that blocks infection as well as prevents pathology and interrupts transmission of parasites. Such a vaccine would most likely be a combination vaccine comprising of subunits from different parasite stages. There is thus a need to identify the right antigenic components for a vaccine, but also to find presentation and delivery methods that induce appropriate immune responses. To date, no pattern of immune response fully predictive of protection has been identified or validated. Naturally occurring immunity wanes rapidly in the absence of ongoing parasite exposure, and protection has been similarly short-lived in those few subunit vaccine trails that has demonstrated measurable efficacy (James & Miller, 2001).

2.1.6 Resistance to and side effects of existing drugs

Resistance to antimalarial drugs is proving to be a challenging problem in malaria control in most parts of the world. Drug resistance being the ability of a parasite species to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended but within the limit of tolerance. Since the early 1960s the sensitivity of parasites to chloroquine **(1)**, the best and most widely used drug for treating malaria, has been on the decline (figure 2.7). Newer antimalarials were developed in an effort to tackle this problem, but all of these drugs are either expensive or have undesirable side effects. Moreover, after a variable length of time, the parasites, especially *P. falciparum*, started showing resistance to these drugs as well.

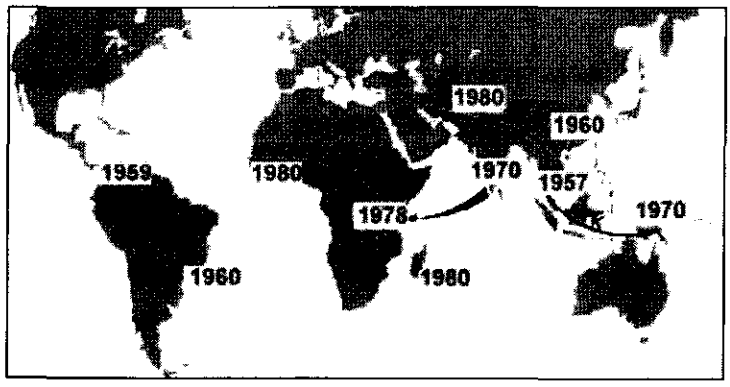


Figure 2.7: Spread of chloroquine resistant *Plasmodium falciparum.*

Quinine and Chloroquine

Chloroquine **(1)** resistance has brought quinine **(2)** back into the limelight. Quinine **(2)** remains quite effective even after extensive use. Reports of resistance to quinine are rare, but cases have been reported from Thailand and East Africa. A high degree of resistance to quinine **(2)** is not common and it is difficult to induce quinine **(2)** resistance under experimental conditions. The efficacy of quinine **(2)** can be improved by combining it with a tetracycline. However, poor compliance is a major drawback of this drug (White, 1992).

Quinine **(2)** is a naturally occurring compound of relatively low potency and narrow therapeutic range and is specifically used in the treatment of malaria. The concurrent use of mefloquine or beta-blockers with quinine, may result in bradycardia or other cardiac disorders. Use of quinine with mefloquine may also result in an increased risk of convulsions. Chinchonism, a symptom complex characterised by tinnitus, hearing impairment, and sometimes vertigo or dizziness, occurs in a high proportion of treated patients. Dose-related cardiovascular, gastrointestinal and central nervous system effects may arise following excessive infusion or from accumulation following oral administration. Severe hypotension may develop if the drug is injected too rapidly (Supanaranond, 1993).

The discovery of chloroquine **(1)** revolutionised the treatment of malaria, pushing quinine to the sidelines. However, the alarming increase in resistance in eastern and southern Africa requires the replacement of chloroquine (Peters, 1998).

Chloroquine **(1)** is a blood schizontozide and is highly effective, but controversy exists as to its mechanism of action. One hypothesis is that chloroquine **(1)** being a weak base is driven by a pH gradient and acts by accumulating in the food vacuole, which leads to the temporary alkalisation of this acidic compartment. This is counteracted by a proton pump. Resistant strains are able to efflux the drug by an active pump mechanism and release the drug at least 40 times faster than sensitive strains, thereby rendering the drug ineffective. Non-specific inhibitors like calcium channel blockers or antagonists of calmodulin (e.g. verapamil), cyproheptadine, chlorpheniramine and hydroxyzine have been shown to suppress the efflux pump mechanism. In practice these drugs have not shown any benefit of reversing chloroquine resistance and it is too early to say anything about the utility of these agents in the management of chloroquine resistant *P. falciparum*

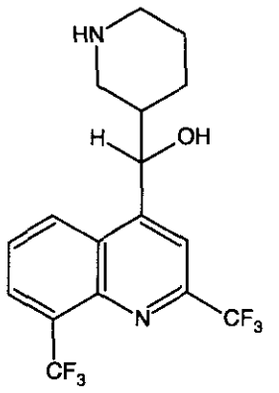
malaria. There is an increase in the surface area of the resistant parasites, permitting more efficient pinocytosis. Binding of chloroquine **(1)** with haemoglobin breakdown products to form toxic complexes is also prevented. Chloroquine **(1)** resistance is maintained throughout the whole life cycle and is transferred to the progeny. Cross-resistance has been demonstrated with other 4-amino quinolines and mepacrine, but not to quinine, mefloquine, proguanil, (para-amino benzoic acid blockers) or pyrimethamine (antifolates) (Dollery, 1999).

Serious adverse reactions to chloroquine **(1)** are rare at the usual antimalarial dosage, but pruritus, which may be intolerable, is common among dark-skinned people. Transient headache, nausea, vomiting, gastrointestinal symptoms and "blurred vision" may also be experienced following chloroquine **(1)** administration. Attacks of acute porphyria and psoriasis may be precipitated in susceptible individuals. Very rarely, adverse events include leucopoenia, bleaching of hair and extremely rarely, aplastic blood and neurological disorders, such as polyneuritis, ototoxicity, seizures and neuromyopathy (WHO, 1998 & 1999).

Mefloquine

Mefloquine **(3)** is structurally closely related to quinine and hence cross-resistance with quinine is common. When combined with sulphadoxine/pyrimethamine there is a reduced emergence of resistance. To prevent development of resistance to this valuable drug, it has been suggested that mefloquine should always be used in combination with other antimalarials, like pyrimethamine/sulphadoxine.

Mefloquine **(3)** is used only for uncomplicated malaria in richer countries with multidrug resistance; it is unaffordable for general use throughout tropical Africa. This drug has the potential for inducing neuropsychiatric adverse reactions. There have also been concerns that other adverse effects, such as dizziness, may impair the ability of patients performing activities that require a high level of precision. Vomiting may affect efficacy and the use of the drug during pregnancy and in patients taking cardio-active drugs may lead to an increased risk of adverse events (Ter Kuile, 1995).

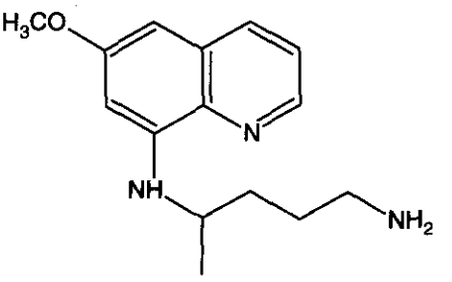


(3)

Figure 2.8: Structure of mefloquine **(3)** (Foley & Tilley, 1998).

Primaquine

Primaquine **(4)** is the only drug effective against the pre-erythrocytic stages (hypnozoites) of *P. vivax* and *P. ovale* which is not eradicated by any of the other drugs mentioned above, and which may cause a late relapse (Baird, 1995).

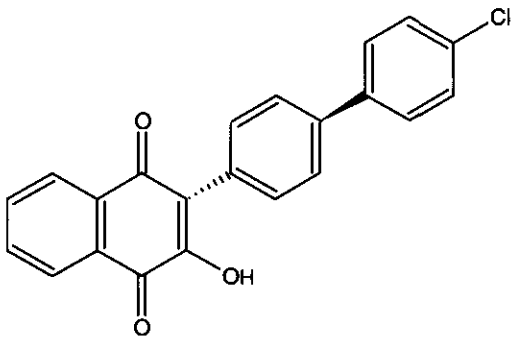


(4)

Figure 2.9: Structure of primaquine **(4)** (Foley & Tilley, 1998).

Atovaquone

Atovaquone **(5)** alone has weak antimalarial activity and recrudescence of parasitaemia occurs in one-third of patients with *P. falciparum* when used alone and are thus combined with proguanil. Adverse effects include abdominal pain, nausea, vomiting, diarrhoea, headache, anorexia and coughing. Atovaquone **(5)** is new on the South African market, but is expensive to produce. Atovaquone-proguanil might be unaffordable for most African nations (Goldsmith, 1998b).



(5)

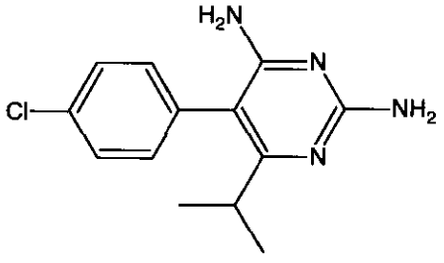
Figure 2.10 Structure of atovaquone **(5)**.

Sulphas and their combinations

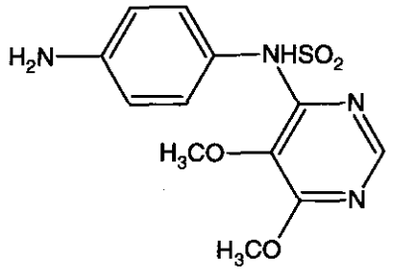
Proguanil (PABA blocker) and pyrimethamine **(6)** (antifolate) acts by sequential inhibition of the enzymes of the folate metabolism. Resistance to these drugs has developed over the past 30 years and is now wide spread. Resistance to these drugs develops very rapidly and remains stable due to a single point mutation. The mechanism of resistance to these drugs involves modification of drug transport systems, increased synthesis of blocked enzymes, increase in drug inactivating enzymes and the use of alternative pathways. Resistance is seen for *P. falciparum* and *P. vivax*. Hence these drugs may not be of any benefit in complicated malaria.

Pyrimethamine **(6)** is formulated in a fixed combination with sulphadoxine **(7)** or dapsone **(8)** illustrated in figure 2.9. Sulphadoxine/pyrimethamine, the most widely used combination, is cheap

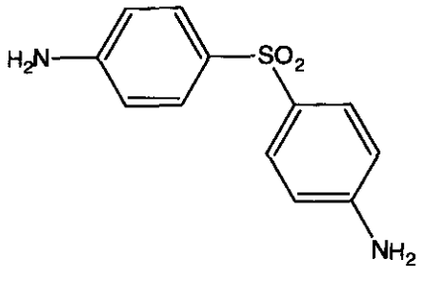
and practicable, since only one dose is needed because of slow elimination from the body. Sulpha-pyrimethamine combinations are generally well tolerated when used at the recommended doses for malaria therapy. The most serious events are associated with hypersensitivity to the sulpha component, involving the skin and mucous membranes and normally occurring after repeated administration (Winstanley, 2000).



(6)



(7)

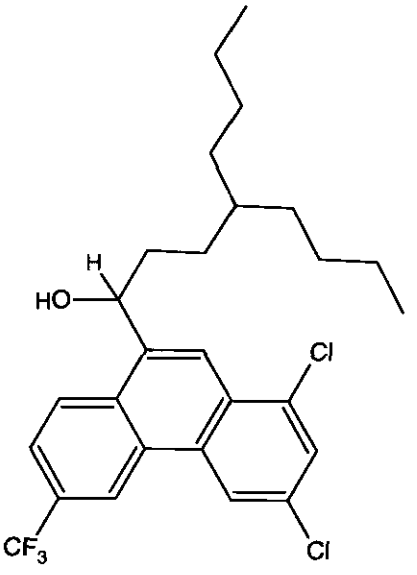


(8)

Figure 2.11: Structures of pyrimethamine **(6)** (Goldsmith, 1998b), sulphadoxine **(7)** (Winstanley, 2000) and dapsone **(8)** (Goldsmith, 1998b).

Halofantrine

Halofantrine **(9)** like mefloquine is an expensive drug without a parental formulation. Adverse effects include nausea, abdominal pain, diarrhoea, pruritus and skin rashes. Prolongation of the QTc interval and rare cases of serious, sometimes fatal, ventricular dysrhythmias, have also been reported (Malvey *et al*, 2000).



(9)

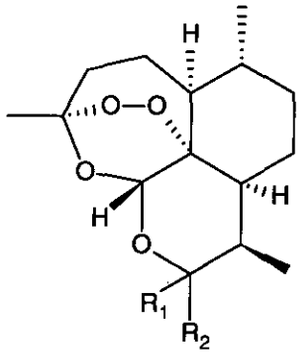
Figure 2.12: Structure of halofantrine **(9)**.

Artemisinin derivatives and lumefantrine

Artemisinin is a peroxide antimalarial which releases carbon-centred free radicals when it comes into contact with heme. True stable resistance to artemisinin has not been observed so far, but cannot be precluded (Kakkilaya, 2002; Tracy & Webster, 1996).

Artemisinin **(10)** is a pharmacologically active molecule discovered in the Chinese herb *Artemisia annua* illustrated in figure 2.8. Many derivatives have been synthesized from dehydroartemisinin

(14), namely arteether **(11),** artemether **(12)** and sodium artesunate **(13)** currently in use. There is some concern about cerebellar dysfunction (Davis, 1997) with the use of artemisinin. Prolonged or repetitive treatment with artemisinin and its derivatives **(10-14),** in areas of high transmission, must be viewed with caution. Monitoring of subtle neurological changes and hearing loss are required, especially in patients undergoing repetitive treatment. Arthemether and lumefantrine **(15)** is currently used against *P.falciparum* in Kwazulu-Natal due to the development of resistance against pyrimethamine/sulphadoxine (Fansider®). Resistance is developing in Limpopo at such a rate that arthemether is to be combined with Fansider® in the very near future.



Artemisinin **(10)**: $R_1 = R_2 = O$

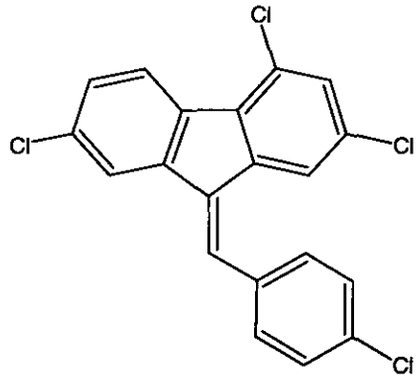
Arteether **(11)**: $R_1 = H$, $R_2 = OEt$

Artemether **(12)**: $R_1 = H$, $R_2 = OMe$

Sodium artesunate **(13)**: $R_1 = H$

$R_2 = OCO(CH_2)_2CO_2Na$

Dehydroartemisinin **(14)**: $R_1 = H$, $R_2 = OH$



(15)

Figure 2.13: Structures of artemisinin its derivatives and lumefantrine (van Agtmael *et al*, 1999).

2.2 Plants and medicine

A considerable number of definitions have been proposed for the term "medicinal plant". According to the World Health Organization, "a medicinal plant is any plant which, in one or more of its organs, contains substances that can be used for therapeutic purposes, or which are precursors for chemo-pharmaceutical semi-synthesis". The fascination with natural products, mostly used as a preparation from a plant with known medicinal properties, goes back to ancient times. The discovery of pure compounds as active principles in plants was first described at the beginning of the 19th century, and the art of exploiting natural products has become part of the molecular sciences. In the past decades natural products have attracted renewed interest, especially with bacteria and fungi as important sources of biologically active compounds (Kayser, *et al.*, 2004).

The use of medicinal plants (Phytotherapy) for healing purposes is a practice pursued since ancient times, as herbs were the first medicines with which people came into contact. This information was carried over from generation to generation and developed as new healing properties were discovered and new experiences in use and management was attained. At the end of the 19th and the beginning of the 20th centuries, chemistry had developed so far that it seemed that within a few years it could offer mankind immortality (Zentrich, 2001). But years passed and somehow immortality failed to make an appearance. Instead the unwanted side effects of chemical medicines began to show up on an increasing scale. Nowadays, Phytotherapy is widely used throughout the world and a great number of products are produced from plants (Zentrich, 2001).

It is estimated that between 25 000 and 75 000 plant species are used as traditional medicine. Only 1% is known to scientists and accepted for commercial purposes. Much of the world's population depend on traditional medicine to meet daily health requirements, especially in developing countries. The use of plant-based remedies is also widespread in many industrialized countries and numerous pharmaceuticals are based on or derived from plant compounds (Rajasekharan, 2002). Over 120 pharmaceutical products currently in use are plant derived, and some 75% of these were discovered by examining the use of these plants in traditional medicine (Farnsworth, *et al.*, 1985). Single entity plant drugs, which mostly treat serious medical conditions, include atropine, digoxin, morphine, paclitaxel, pilocarpine, reserpine, scopolamine, topotecan and vincristine among many others (Rajasekharan, 2002).

Until the early 1970s, there was a strong interest in looking at plants as sources of new pharmaceutical agents. In fact, many modern pharmaceutical companies can trace their origins to products originating from plants. However, advances in molecular biology, genetic engineering, and computational chemistry in the late 1970s and 1980s and even more recently, advances in combinatorial chemistry (Borman, 1996 & Baum, 1996) created much promise for the pharmaceutical industry without the need to explore nature's chemical diversity.

2.2.1 Plants and malaria

Drugs presently in use have become ineffective against malaria because of parasites developing resistance to most of them (Peters, 1998). The success of artemisinin **(10)**, has stimulated the search for new antimalarial drugs from traditional remedies (Qinghaosu Antimalarial Coordinating Research Group, 1979). 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. South Africa with its rich floral resources and ethnobotanical history is an ideal place to screen plants for antiplasmodial activity. 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 (Gessler *et al.*, 1994). At present very little is known about the antiplasmodial activity of extracts of South African plant species.

In 1630, a great discovery was made by the Spanish when they found quinine (**2**) as a remedy for malaria. Throughout the 1600s to the mid-1800s, quinine was the most widely used treatment for malaria, proving to be the first chemical compound used successfully to treat an infectious disease. Of the 36 alkaloids found in the cinchona bark, only four possessed antimalarial properties, with quinine (**2**) being the most effective.

Medicinal plant research has become more important, especially after the studies of the Chinese antimalarial drug artemisinin (**10**), isolated from *Artemisia annua* (Lee *et al.* 1989). In 1972, a crystalline compound was extracted from the qinghaosu plant, known in western countries as artemisinin (**10**). In 1979, chemists successfully determined the structure of artemisinin using X-ray crystallographic analysis.

The investigation of plant species for antimalarial and antimicrobial activity as well as its toxicity is of utmost importance. The information obtained through these studies can save millions of lives.

2.2.1.1 Plant families with antimalarial activity

A few examples of plant families that contain antimalarial compounds are the, Amaryllidaceae, Anacardiaceae, Celasteraceae, Combretaceae, Lilaceae, and Rubiaceae.

It is important to note that species of the same genera may contain the same active constituents and are often used in the treatment of the same disease. Table 2.1 lists the plants with known antimalarial activity of importance to this study (see Chapter 1). In this table the traditional use and active compounds found in previous studies, of the different plant species of each family used in this study, are discussed.

An in depth description of the species *Crinum bulbispermum* is given as this species was selected for further investigation based on the initial screening of sixty two plant extracts (chapter 4).

Table 2.1: Antimalarial activity reported in plant families and species of importance to this study.

Family	Plant species	Traditional uses	Activity against malaria
Amaryllidaceae	*Brunsvigia littoralis* *Crinum amabile* *Crinum bulbispermum* *Crinum delagoense* *Crinum latifolium* *Crinum macowanii*	The listed species is used by the Zulu, Sotho and Tswana people to treat rheumatism, aching joints, septic sores, varicose veins and kidney and bladder infections (Roberts, 1990).	Cold ethanol extracts of the bulb of *B. littoralis* exhibited antimalarial activity against two strains of *P. falciparum* (Campbell, *et al.* 1997). A preliminary biological evaluation of an ethanol extract of the bulbs of *C. amabile* revealed both cytotoxic and antimalarial potential for the plant (Likhitwitayawuid, *et al.* 1993)
Anacardiaceae	*Rhus aromatica* *Rhus glabra* *Rhus retinorrhoea* *Rhus succedenea*	Some of the listed species of the genus *Rhus* are used in traditional medicine either as antimicrobial concoctions (Saxena *et al.* 1994) or for their cytotoxic properties (Lin *et al.* 1989).	The biflavanone isolated from the leaves of *Rhus retinorrhoea* exhibited moderate antimalarial activity with an IC_{50} of 0.98 µg/ml (Ahmed *et al.* 2001).

Family	Plant species	Traditional uses	Activity against malaria
Combretaceae	*Combretum fragrans* *Combretum micranthum* *Combretum molle* *Terminalia sambesiaca* *Terminalia belerica*	These listed species are traditionally used in Africa against malaria (Benoit, *et al.*, 1996)	Four lignin's (termilignan, thannilignan, hydroxy-3',4'-[methylenedioxy] flavan, and anolignan B) isolated from *Comretum micranthum* possesses demonstrable *in vitro* antimalarial activity (Valsaraj *et al.*, 1997).
Celasteraceae	*Maytenus heterophylla* *Maytenus pyria* *Maytenus senegalensis*	These listed species are used by people in rural areas to treat infectious diseases and the recurrent fever typical of malaria (Tahir *et al.*, 1999).	*Maytenus senegalensis* showed activity with IC_{50} values of 3.9 µg/ml against chloroquine sensitive strains and 10 µg/ml against chloroquine resistant strains of *P. falciparum* (Tahir *et al.*, 1999).

Family	*Plant species*	Traditional uses	Activity against malaria
Liliaceae	*Aloe andorgensis* *Aloe bulbilifera* *Aloe excelsa* *Aloe greatheadii* *Aloe marlothii*	Traditionally used for a wide range of therapeutic purposes including antimalarial, antibacterial, antifungal, antimicrobial and antiviral benefits.	Antiplasmodial activity and toxicity of 34 *Aloe* species and their main constituents were determined, and a number of methanol extracts possessed antimalarial activity (van Zyl & Viljoen, 2002).
Rubiaceae	*Pavetta coffeoides* *Pavetta crassipens* *Pavetta gardeniifolia* *Pavetta zeyheri* *Mitragyna inermis* *Mitragyna stipulosa*	Traditionally these listed species are used as an antimalarial (Bruce, 1998).	Crude hot water extracts of *Pavetta crassipes* are capable of 100% inhibition of *P. falciparum* (Gbeassor *et al.*, 1989).

2.2.2 The genus, *Crinum* – bioactivity and chemistry.

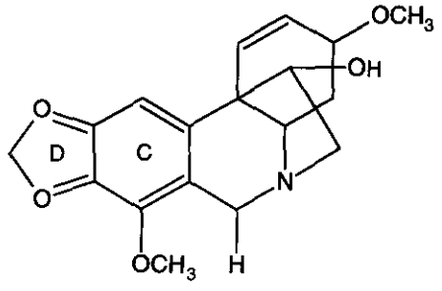
Figure 2.14: *Crinum bulbispermum*

The genus *Crinum* belongs to the family Amaryllidaceae and comprises approximately 160 species distributed throughout the tropics and warm regions of the world in Asia, Australia, Africa and America (Mabberly, 1990).

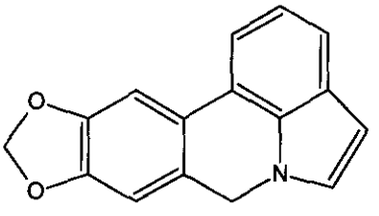
The *C. bulbispermum* plant (subject of this study) is most commonly revered to as the Orange River Lily and occurs in South Africa around marshes and the banks of rivers. It is a bulbous perennial with long, strap-like leaves. White and pink tubular flowers with 6-parted leaves are variously clustered on a long, naked stem (Tram *et al.*, 2002).

Only 30 of approximately 160 *Crinum* species have been investigated for chemical composition. Attention has particularly been given to the study of alkaloids, and very little to other constituents (Tram *et al.* 2002). The ease with which these plants hybridise however makes comparisons difficult.

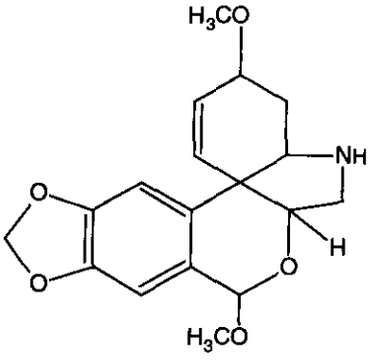
The most numerous group of alkaloids as shown in figure 2.15 were isolated after 1985 comprises crinane type compounds for example 11-O-acetylambelline **(16)**, where the main source is usually the bulbs. Structural variations in ring C predominate (double bond, oxiran ring substituents). This group has recently attracted significant attention due to the valuable biological activity of some of its representatives. Another important group of alkaloids found in *Crinum* species belong to the lycorine type for example 4,5-dehydroanhydrolycorine **(17)**. They have been isolated predominantly from the fruits and bulbs. The newly-isolated compounds differ mainly in the number and position of double bonds in rings C and D and in the type, position and stereochemistry of substituents on ring C. Some quaternary salts have also been isolated as well. Other types of alkaloids isolated from different *Crinum* species include tazettine **(18)**, phenanthridine **(19)**, lycorenine **(20)**, galanthamine **(21)**, ryllistine **(22)**, and cherylline **(23)** (Tahir, *et al.*, 1999).



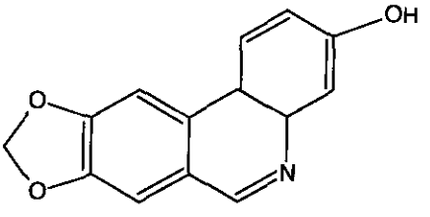
(16) 11-*O*-acetylambelline



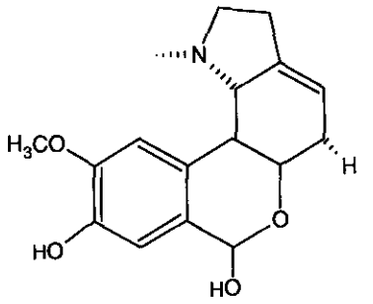
(17) 4,5-dehydroanhydrolycorine



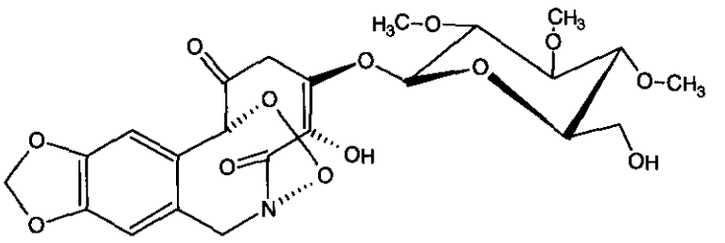
(18) *N*-demethyl-8α-ethoxypretazettine



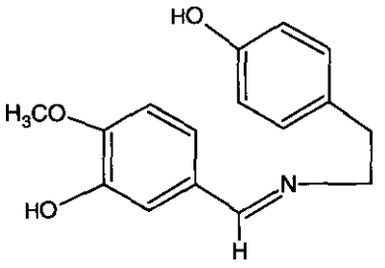
(19) 3-hydroxy-8,9-methylene-dioxyphenantridine



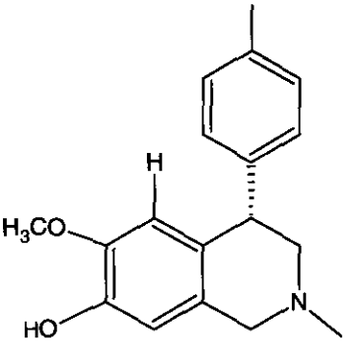
(20) 9-*O*-demethylhomolycorine



(21) cripowellin B



(22) a ryllistine type alkaloid



(23) cherylline

Figure 2.15: Alkaloids from *Crinum* species (Tahir, *et al.*, 1999).

Other non-alkaloidal compounds isolated from this genus include flavonoids, chalcones, chromones, terpenoids and sterols. Long chain aliphatic alkenes, alcohols, hydroxyketone fatty acids and their esters, as well as carbohydrates were also isolated from species of *Crinum* (Tahir,*et al.*, 1999).

Crinamine from *Crinum yagus* possessed strong antibacterial activity. Hamayne and 6-hydroxycrinamine were inactive against a range of bacteria tested.

Crinamidin, undulatin, macowine and 4a-dehydroxycrinamabine showed no antimalarial activity (Tahir, *et al.*, 1999).

Establishing the types of compounds that has previously been isolated from various species and tested for antimalarial and antimicrobial activity, was done through a thorough literature research

CHAPTER 3

EXPERIMENTAL AND RESULTS

The selection, collection and identification of plant material to be studied are the first steps in a phytochemical investigation (Silva *et al.* 1998).

All collected plant material must be disease free, since products of microbial synthesis may be detected and wrongly attributed to the plant and unexpected products may be formed due to plant metabolism that has been altered due to an infection. Care should also be taken to avoid the gathering of mixtures of plants, since many similar species grow side by side (Harborne, 1984).

Conditions under which plant materials are dried should be controlled to avoid occurrence of chemical changes. Materials should be kept from direct sunlight, and not be dried at temperatures higher than 30°C, as this may lead to degradation of specific compounds. Good ventilation and homogeneous distribution of plant material are important to avoid fungal infestation (Harborne, 1984).

Solvents used in the extraction of plant material must be inert, easy to remove and of high quality. Extraction is usually started with solvents of lower polarity, such as petroleum ether and dichloromethane and then with more polar solvents such as ethanol.

3.1 Phytochemical preparation of plant material

3.1.1. Selection of plants

Following an extensive ethnobotanical literature study, 6 genera with described antimalarial activity were identified. The selected genera were as follows: *Aloe, Combretum, Crinum, Maytenus, Pavetta* and *Rhus.* A species from each genus was selected keeping the following factors in mind;

- Reported antimalarial activity of plants of the same genus;
- Availability in the Potchefstroom area and no previous reports of antimalarial testing on the chosen species.

3.1.2 Collection and storage of plant material

Plants were collected from the area around Potchefstroom between March and April 2003. Mr. Bert Ubinck of the Department of Botany of the North West University positively identified plant specimens. Plants were separated into different morphological parts to determine in which part(s) the antimalarial and antimicrobial compounds were localized. Separating plants into different morphological parts rendered extracts less complex and eliminated the possibility of contamination of extracts from other plant parts (Cannell, 1998).

The leaves and stems were dried at room temperature for 5 days. The roots and bulbs were frozen, to prevent the plant material from rotting as it contained a high concentration of water. The dried plant parts were ground and the frozen plant parts were cut into smaller pieces to obtain smaller particle sizes, thus ensuring more efficient extraction.

3.1.3 Preparation of extracts

In this study Soxhlet extraction was used as it is a convenient way to prepare plant extracts. A range of pure solvents was employed, starting with petroleum ether, and dichloromethane (to separate lipids and terpenoids), followed by ethyl acetate and alcohol for more polar compounds.

Soxhlet extraction is automatic, continuous and saves solvent by recycling it over the sample. Disadvantages associated with this method include the fact that thermally labile components may decompose as a result of the heating during the extraction process, it is only useful when working with several grams of plant material and complete separation of constituents is rarely achieved. The same compounds may therefore be recovered in varying proportions in several fractions (Silva *et al.* 1998). Fractionation of crude extracts is desirable in order to separate the main classes of constituents from each other, prior to chromatographic analysis.

Approximately 10 g of the ground plant material was extracted using the Soxhlet extraction method with petroleum ether (Pet.Et.), dichloromethane (DCM), ethyl acetate (EtOAc) and ethanol (EtOH) as solvents in order of increasing polarity. By using a wide range of solvents, it was ensured that all possible plant constituents with different polarities were present in the screened extracts. After

the extraction was completed, the solvents were evaporated by rotary vacuum evaporation.

The extract was then air-dried for another 48 hours at room temperature. Percentages obtained are shown in table 3.1.

Table 3.1 Description of the extracts obtained.

Plant part	Mass of plant material (grams)	Solvent	Mass of extract (grams)	Yield (%)	Description
Aloe greatheadia - leaves	8.16	Pet.Et	0.0022	0.03%	Yellow-brown sticky substance
		DCM	0.2007	2.46%	Red-brown deposit
		EtoAc	0.1831	2.24%	Brown-green deposit
		EtOH	0.2173	2.66%	dark green, sticky substance
Combretum erythrophyllum - leaves	4.72	Pet.Et	0.3009	6.38%	light brown, sticky substance
		DCM	0.1760	3.73%	Black-green deposit
		EtoAc	0.1302	2.76%	Black-green deposit
		EtOH	0.3425	7.26%	Brown, sticky substance

Plant part	Mass of plant material (grams)	Solvent	Mass of extract (grams)	Yield (%)	Description
Combretum erythrophyllum - stems	12.71	Pet.Et	0.2529	1.99%	light brown, sticky substance
		DCM	0.0736	0.58%	Black–green, deposit
		EtoAc	0.1454	1.14%	Black–green, deposit
		EtOH	0.6147	4.84%	light brown, sticky substance
Crinum bulbispermum - leaves	6.02	Pet.Et	0.0724	1.20%	Yellow–green, powder
		DCM	0.0254	0.42%	light green, deposit
		EtoAc	0.1077	1.79%	dark green, crystals
		EtOH	1.0429	17.3%	dark green, substance with translucent crystals
Crinum bulbispermum - roots	6.98	Pet.Et	0.0086	0.12%	red-brown, sticky substance
		DCM	0.6297	9.02%	translucent brownish, sticky substance
		EtoAc	0.3855	5.52%	red-brown, sticky substance
		EtOH	1.5518	22.2%	brown sticky substance

Plant part	Mass of plant material (grams)	Solvent	Mass of extract (grams)	Yield (%)	Description
Crinum bulbispermum - bulb	7.19	Pet.Et	0.0322	0.45%	Mustard-yellow, sticky substance
		DCM	2.0293	28.2%	dark brown, sticky substance
		EtoAc	0.486	6.76%	brown sticky substance
		EtOH	1.0129	14.1%	brown sticky substance
Maytenis heterophylla - seeds	6.89	Pet.Et	0.2549	3.69%	bright yellow, crystals
		DCM	0.1202	1.74%	Mustard-yellow, sticky substance
		EtoAc	0.0465	0.67%	green deposit
		EtOH	0.5515	8.00%	light brown, deposit
Maytenis heterophylla - leaves	5.65	Pet.Et	0.315	5.58%	White-yellow, crystals
		DCM	0.0456	0.81%	dark green, powder
		EtoAc	0.0767	1.36%	Black-green, deposit
		EtOH	0.3474	6.15%	green crystals

Plant part	Mass of plant material (grams)	Solvent	Mass of extract (grams)	Yield %	Description
Maytenis heterophylla stems	9.99	Pet.Et	0.0876	0.88%	White-yellow, crystals
		DCM	0.0332	0.33%	Green-yellow, deposit
		EtoAc	0.0465	0.47%	green deposit
		EtOH	0.1001	1.00%	green deposit
Pavetta gardeniifolia - seeds	8.05	Pet.Et	0.0222	0.28%	White-yellow, powder
		DCM	0.0325	0.40%	White-yellow, powder
		EtoAc	0.2365	2.94%	translucent yellowish oil
		EtOH	0.8818	10.9%	translucent colorless oil
Pavetta gardeniifolia - leaves	10.17	Pet.Et	0.0895	0.88%	green crystals
		DCM	0.2443	2.40%	green powder
		EtoAc	0.5333	5.24%	greenish powder
		EtOH	1.8549	18.2%	Yellow-green, powder
Pavetta gardeniifolia - stems	9.84	Pet.Et	0.0674	0.68%	Yellow-green, deposit
		DCM	0.0361	0.37%	light green, deposit
		EtoAc	0.0721	0.73%	light green, powder
		EtOH	0.1754	1.78%	light greenish, powder

Plant part	Mass of plant material (grams)	Solvent	Mass of extract (grams)	Yield (%)	Description
Rhus pyroides - seeds	6.63	Pet.Et	0.0444	0.67%	White-green, crystals
		DCM	0.0146	0.22%	translucent green, sticky substance
		EtoAc	0.1642	2.48%	green deposit
		EtOH	0.3814	5.75%	light green, powder
Rhus pyroides - leaves	5.76	Pet.Et	0.1237	2.15%	green powder
		DCM	0.0909	1.58%	yellow-green, powder
		EtoAc	0.2426	4.21%	light green, powder
		EtOH	0.3808	6.61%	greenish deposit
Rhus pyroides - stems	8.57	Pet.Et	0.0301	0.35%	yellow-green, deposit
		DCM	0.0166	0.19%	yellowish deposit
		EtoAc	0.0356	0.42%	light yellow, deposit
		EtOH	0.0888	1.04%	White-yellow, deposit

3.2 Biological testing

The concentrated, air-dried extracts were reconstituted in 100 % dimethyl sulphoxide (DMSO, Saarchem) to the desired initial concentration. Dilutions were then made so that the final concentration of DMSO did not exceed 1%. This concentration has been shown not to affect parasite or bacterial growth. Extracts were screened for antimalarial, antibacterial and antifungal activity and for toxicity. This was done to obtain the most active extract. After selection of the most active extract, isolation of the active principles commenced and the isolated compounds (discussed in chapter 3.3) were then also tested for antimalarial activity and their toxicity determined.

3.2.1 Antimalarial activity

Parasite susceptibility to antimalarial compounds can be measured by the following methods: Giemsa microscopy (Rieckman *et al.* 1978; Makler *et al.* 1993); parasite lactate dehydrogenase enzyme assays (Makler *et al.* 1993); the use of flow cytometry (Schulze *et al.* 1997) and by tritiated hypoxanthine uptake (Desjardins *et al.* 1979).

The hypoxantine uptake method was selected because quantitative measurements of the antimalarial activity of large numbers of compounds can be obtained. Hypoxanthine a major purine base is used by *P. falciparum* for the synthesis of adenosine and guanosine nucleotides and nucleic acids (Webster & Whaun, 1981). Since the parasite is incapable of *de novo* purine synthesis, exogenous hypoxanthine is necessary for parasite survival. Thus [^{3}H]-hypoxanthine can be used as a tool for assessing *in vitro* parasite growth. Radioisotope incorporation can be measured after the addition of [^{3}H]-hypoxanthine to the micro cultures. Synchronised ring- staged cultures are used in this assay.

During the harvesting method, molecules such as DNA and RNA are bound to glass fiber mats. The radioactivity measured therefore represents primarily [^{3}H]-hypoxanthine incorporated into parasite nucleic acids. Background [^{3}H]-hypoxanthine incorporated by uninfected erythrocytes is low, since mature red blood cells do not synthesis DNA (Chulay *et al.*, 1983). It is partially automated to ensure rapid analysis and prevent human error.

3.2.1.1 *In vitro* culturing of malaria parasites

The chloroquine resistant strain (FCR-3) was used in the determination of antimalarial activity and was cultured according to the method of Jensen & Trager (1977).

Preparation of media

The media used for the culturing of *P. falciparum* parasites consisted of complete culture media and erythrocytes.

Complete culture media

A modified version of the method used by Jensen and Trager (1977) was employed for *in vitro* culturing. Culture media consisted of:

- 10.4 g RPMI-1640,
- 5.9 g HEPES (N-2-hydroxyethyl-piperazine-N'-2-ethane-sulfonic acid),
- 4.0 g glucose,
- 44 mg hypoxanthine and 50 mg of gentamycin in 1 liter millipore water.

The media was stirred for approximately 1 hour before being sterilized by filtration with a Sterilin filter unit and then stored at 4°C. The achievement of the complete culture media was done just before addition to the culture, when 10% (v/v) plasma and 0.21% (w/v) $NaHCO_3$ were added to the incomplete culture media.

Plasma

Pooled sterile human plasma was thawed and inactivated at 56 °C for 2 hours, centrifuged in a

Sorvall T6000D centrifuge at 400 x g, aliquotted and stored at – 20 °C until needed.

Erythrocytes

Erythrocytes were obtained from whole blood, which could be stored for up to 3 weeks at 4 °C. Whole blood was centrifuged for 5 minutes at 400 x g, where after the plasma portion and the leukocyte buffy coat were removed. The remaining erythrocyte pellet was resuspended in phosphate buffer saline (PBS) and centrifuged for 5 minutes at 400 x g. The supernatant was aspirated and this procedure was repeated 3 times. An equal volume of complete media was added to the washed red blood cell pellet, and stored for up to a week.

PBS consisted of: 8 g NaCl, 0.3 g KCl and 0.73 g Na_2HPO_4 in 1 liter of millipore water and was autoclaved for sterilisation.

Culture maintenance

Daily culturing

Aseptic technique and flaming was used to maintain sterility. The parasite culture was suspended in complete culture media in 100 ml flat bottomed flasks. After the daily preparation of a blood smear (see preparation of blood smear), spent media was aspirated and replaced with pre-warmed, fresh complete culture media. The culture flask was gassed with a mixture containing 3 % oxygen, 5 % carbon dioxide and 92 % nitrogen before incubated at 37 °C. Washed erythrocytes were added when parasites were in the trophozoite stage, where the volume of erythrocytes never exceeded 5 % of the total culture volume. Every second day, when cultures were in the ring stage, the culture was sorbitolled to maintain a synchronised culture (see synchronisation of culture).

Preparation of blood smear

The percentage parasitaemia and parasite stage of the culture was determined daily by preparing a thin blood smear. Blood smears were air dried, fixed in methanol and stained for 15 minutes with filtered, diluted Giemsa stain (neat Giemsa stain : Giemsa buffer (1:5)). Ten fields of the smear

were examined under oil immersion. The following equation was used to determine the percentage parasitaemia:

$$\% \text{ Parasitaemia} = \frac{\text{Number of infected cells x 100}}{\text{Total number infected cells + uninfected cells}} \qquad \text{Equation 3.1}$$

Giemsa buffer consisted of 3.5 g/l KH_2PO_4 and 14.5 g/l $NaHPO_4.12H_2O$.

Synchronisation of the culture

For the [^{3}H]-hypoxanthine incorporation assay a synchronised ring-staged culture had to be maintained. Synchronisation of the culture is achieved by a selective destruction of the trophozoite-schizont stages of the parasite, which are more osmotically fragile than the ring-stage parasites. This was done by treating the culture with 5 % (w/v) D-sorbitol for 20 minutes at room temperature (Lambros & Vandenberg, 1979).

3.2.1.2 [^{3}H]-hypoxanthine incorporation assay

Preparation of extracts and isolated compounds

Preparation of a known concentration of the extracts and isolated compounds in a form suitable for addition to the microtitre plate requires that aqueous solubility and sterility be considered. Both the plant extracts and isolated compounds were reconstituted in 100 % DMSO. Sterility was obtained by using 100 % DMSO which is bactericidal. Dilutions were then prepared insuring that the DMSO concentration did not exceed 1 %. Chloroquine diphosphate (Sigma) and quinine sulphate (Fluka) were both dissolved in autoclaved millipore water and sterilised using 0.22 nm filter unit. Dilutions were made with hypoxanthine-negative culture media, consisting of 10.4 g/l RPMI-1640, 5.9 g/l HEPES and 4.0 g/l glucose. Corresponding dilutions of DMSO were also made to assess the effect of DMSO on the parasites. Chloroquine dilutions were made in the following ranges: 1 µg/ml, 0.1

μg/ml, 0.01 μg/ml, 0.001 μg/ml and 0.0001 μg/ml.

Preparation of parasite suspension

After the preparation and examination of a thin blood smear, the percentage parasitaemia and percentage haematocrit were adjusted to 0.5% and 1.0% respectively as determined by a GWbasic program (Havlink, 2003) to calculate the required volume of washed erythrocytes, complete hypoxanthine-negative culture media and parasite erythrocytes.

The erythrocyte suspension was prepared by adding the appropriate volume of washed erythrocytes to the complete hypoxanthine-negative culture media. The parasite suspension was then prepared by adding the required volume of parasitised erythrocytes to the latter erythrocyte suspension.

Preparation of the microtitre plate

The microtitre plate (Nunc) consisting of 96 flat-bottomed wells was arranged in a matrix of eight rows (A-H) and 12 columns (1-12) as seen in figure 3.1.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H	Red blood cell control				Parasite control							

Figure 3.1: Representation of the 96 well microtitre plate.

When prepared as described, wells 1-4 of row H served as the erythrocyte control (no compound and no parasites) and wells 5-12 of row H served as the parasite control (no compound). Each dilution was plated in triplicate in adjacent wells.

Drug dilutions were prepared in the following ranges: 1000 µg/ml, 100 µg/ml, 10 µg/ml, 1 µg/ml, 0.1 µg/ml, 0.01 µg/ml, 0.001 µg/ml and 0.0001 µg/ml. For a more precise determination of the estimated concentrations other dilutions were added in between to minimise the standard error. For example, if a concentration were found to be in the range of 0.0025 µg/ml, the dilution range was adapted as follows. 0.0 1 µg/ml, 0.005µg/ml, 0.0025µg/ml, 0.001 µg/ml and 0.0005 µg/ml.

25 µl of the extract/compound/drug solution was pipetted in triplicate under strict aseptic conditions to wells in rows A to G. To the control row, 25 µl of hypoxanthine-negative media was pipetted per well. The plates were then placed in a humidified candle jar and sealed once the candles had been lit to remove excess oxygen, and then placed in the 37 °C incubator for 24 hours.

After incubation 25 µl of a 1.48% (v/v) tritated hypoxanthine solution (5 mCi, AEC-Amershem, UK) was added to each well of the plate. The plates were then returned to the humidified candle jar as before and incubated for a further 24 hours at 37°C.

Harvesting parasites and scintillation counting

At the end of the second incubation period, each plate was harvested on a semi-automated cell harvester (Flow laboratories) and the nucleic acids deposited onto glass fibre filter mat (Wallac). Each filter mat was dried and placed in a plastic bag, along with 10 ml of scintillation fluid (Wallac) and sealed. Radioactivity of all the rows, corresponding to the rows of the microtitre plate were measured in counts/minute (cpm) in a Wallac beta plate liquid scintillation counter.

Data analysis

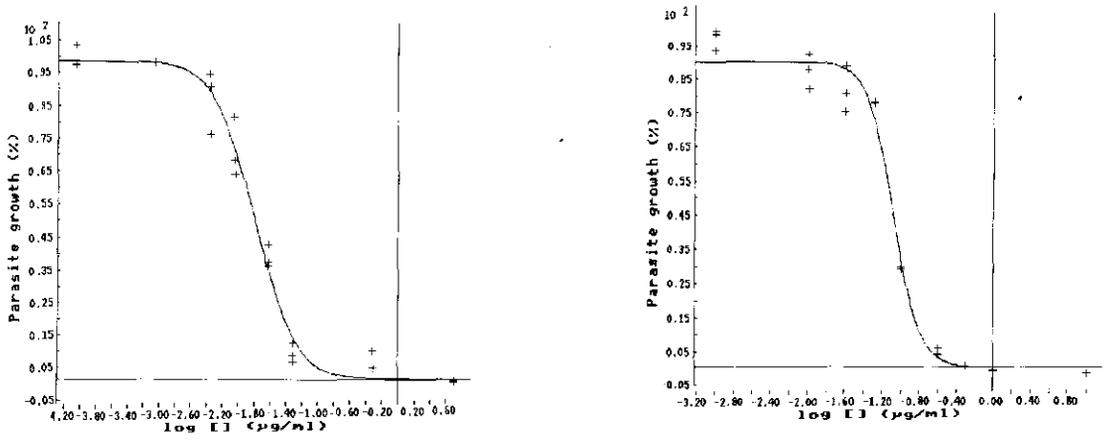
The counts for each well were recorded by the Wallac Genterm computer program and converted

to corrected counts per minute (ccpm) for each well. The mean ccpm for the erythrocyte and parasite control were calculated from row H, wells 1-4 and wells 5-12, respectively. The percentage parasite growth in each well was calculated using equation 3.2.

$$\% \text{ Parasite growth} = \frac{\text{ccpm for well - mean ccpm of the erythrocyte controls x 100}}{\text{mean ccpm for parasite controls - mean ccpm of erythrocyte controls}}$$

Equation 3.2

The percentages obtained from these calculations were plotted against the respective concentration of the isolated compound or extract using the Enzfitter® program. After logarithmic transformation of the concentration, the concentration response curves were characteristically sigmoidal (Figure 3.2).



(a) (b)

Figure 3.2: The dose response curves illustrating the inhibitory activity of the dichloromethane and ethyl acetate extracts of the bulbs of *Crinum bulbispermum* against chloroquine-resistant *P. falciparum*. The IC_{50} values of the respective curves were 0.379 ± 0.126 and 0.081 ± 0.004µg/ml.

Results of the *in vitro* antimalarial activity of the plant extracts are shown in table 3.2.

Table 3.2 Calculated IC_{50} values obtained for the different extracts.

Plant	Solvent	IC50 (µg/ml)	Standard error (±µg/ml)
Aloe greatheadii - leaves	Pet.Et	4.8986	2.9769
	DCM	32.45355	6.5776
	EtOAc	43.182	21.837
	EtOH	27.036	12.889
Combretum erythrophyllum – leaves	Pet.Et	1.1830	0.4862
	DCM	0.7015	0.2505
	EtoAc	1.0415	0.3616
	EtOH	0.8749	0.2842
Combretum erythrophyllum - stems	Pet.Et	1.4701	0.3786
	DCM	0.7022	0.2115
	EtoAc	10.681	0.0278
	EtOH	4.4092	1.6477
Crinum bulbispermum – leaves	Pet.Et	0.8869	0.1935

	DCM	1.9596	1.1014
	EtoAc	15.175	3.2089
	EtOH	62.294	16.859

Plant	Solvent	IC50 (µg/ml)	Standard error (±µg/ml)
Crinum bulbispermum - roots	Pet.Et	3.5410	1.4849
	DCM	1.0306	0.0819
	EtoAc	0.0237	0.0071
	EtOH	18.231	0.5580
Crinum bulbispermum - bulbs	Pet.Et	0.3971	0.0853
	DCM	0.3793	0.0276
	EtoAc	0.0836	0.0077
	EtOH	0.3213	0.1276
Maytenis heterophulla – seeds	Pet.Et	23.086	7.5781
	DCM	20.065	2.4158
	EtoAc	2.9948	0.4638
	EtOH	27.836	6.5026
Maytenis heterophulla - leaves	Pet.Et	12.577	4.4532
	DCM	2.1043	0.5715
	EtoAc	3.8395	0.9600
	EtOH	89.473	46.649

Plant	Solvent	IC50 (µg/ml)	Standard error (±µg/ml)
Maytenis heterophulla - stems	Pet.Et	6.3612	0.3197
	DCM	1.2035	0.9324
	EtoAc	1.3574	0.5354
	EtOH	10.558	0.5339
Pavetta gardeniifolia – seeds	Pet.Et	3.3901	1.4269
	DCM	1.3008	0.4944
	EtoAc	35.423	14.639
	EtOH	4.2959	0.6066
Pavetta gardeniifolia - leaves	Pet.Et	5.5004	0.5756
	DCM	12.908	1.2407
	EtoAc	7.2823	4.1357
	EtOH	21.153	1.3527
Pavetta gardeniifolia - stems	Pet.Et	1.8306	0.1994
	DCM	1.4731	0.3928
	EtoAc	3.3166	0.5419
	EtOH	19.921	0.3637

Plant	Solvent	IC50 (µg/ml)	Standard error (±µg/ml)
Rhus pyroides – seeds	Pet.Et	9.6707	0.4258
	DCM	1.5968	0.9499
	EtoAc	21.097	0.2355
	EtOH	24.297	4.3311
Rhus pyroides - leaves	Pet.Et	1.6826	0.1111
	DCM	1.0039	0.4085
	EtoAc	1.3166	0.3123
	EtOH	9.3037	1.8442
Rhus pyroides - stems	Pet.Et	1.1057	0.4695
	DCM	3.0504	0.5957
	EtoAc	1.3510	0.2933
	EtOH	9.2941	0.4643

Results of the in vitro antimalarial activity of compounds and fractions isolated (as described in section 3.3) from *Crinum bulbispermum* are shown in table 3.3.

Table 3.3 Antimalarial activities of the different isolated compounds and fractions.

Compound	IC_{50} (µg/ml)	Standard error (µg/ml)
24	104.64	1.1387
25	123.84	15.041
26	0.0291	0.0131
Fractions	**IC_{50} (µg/ml)**	**Standard error (µg/ml)**
1	132.62	28.815
2	165.17	56.894
3	135.73	31.600
4	13.513	3.8115
5	21.404	9.7954

3.2.2 Toxicity testing

It is important to assess the safety of medicinal plants and the active compounds isolated from them, before encouraging the use thereof (Milliken, 1997). The MTT assay (Mosmann, 1983), a quantitative colorimetric assay, was used to determine the toxicity of extracts and isolated compounds against mammalian cell survival and proliferation.

In this assay, a tetrazolium salt, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) is used to measure the viability of cells. The pale yellow substance (MTT) is transformed to an insoluble formazan product by the NADH-generating dehydrogenase enzyme found in the mitochondria of metabolically active cells. The amount of formazan formed can be measured spectrophotometrically and is directly proportional to the number of living cells. This method is thus an indication of cytotoxicity, proliferation or activation. Results are measured on a multiwell

scanning spectrophotometer that insures a high degree of precision. There are no washing or removal steps required, as the medium does not interfere with the measurements.

Preparation of the cells

Graham (Human kidney epithelial) cells were cultured in Ham F10 containing 5% (v/v) heat inactivated fetal calf serum (FCS) and 0.1% gentamycin. The media was replaced every second day. The cells were trypsinised weekly and then allowed to reach confluency before being used in the toxicity assays. After trypsinisation, a single cell suspension was obtained. Cell density was determined by mixing an equal volume of the single cell suspension and trypan blue stain, and calculating the density on a haemacytometer, and ensuring that at least 95% of the cells were viable. The stock cell solution was then adjusted to 0.5 million cells per milliliter, by diluting with the culture media containing only 5% FCS.

Preparations of the compounds

The compounds were dissolved in DMSO in concentrations of ca. 10 mg/ml. Appropriate dilutions and DMSO controls were made. Dilution ranges were as follows: 1000 µg/ml; 100 µg/ml; 10 µg/ml; 1 µg/ml; 0.1 µg/ml; 0.01 µg/ml; 0.001 µg/ml and 0.0001 µg/ml.

Preparation of the microtitre plate

96 well microtitre plates were used in the assay. 10 µl of each drug dilution was plated out in at least quadruplicate, in column 2 through to 11. Column 12 was used for DMSO controls and column 1 as the positive growth indicator; 10 µl media was added to all the wells in column 1 to obtain the same volume as the rest of the wells. Cell suspension (90 µl) was added to all the wells except for well one of row H, were 90 µl of 5 % FCS/media was added and which served as a blank. The plates were incubated under humidified conditions at 37 °C and 5 % CO_2 for 48 hours.

Preparation and addition of MTT

MTT stock solution (5 mg/ml PBS) was filter sterilised and stored at 4°C until required. After 44 hours of incubation, 20 µl of the prepared MTT solution was added to all wells and the plates were incubated for another 4 hours for MTT cleavage. Thereafter, 80 µl of the supernatant was removed from each well. 200 µl of DMSO was then added to each well to stop the reaction and solubilise the formazan crystals (Carmichael *et al.* 1987). The plates were shaken at 400 x g for 4 minutes before the absorbance of each well was measured at a test wavelength of 540 nm and a reference wavelength of 690 nm using a microplate reader (Labsystems iEMS reader MF). Using equation 3.3, results were expressed as the percentage cellular viability of the controls.

$$\% \text{ Cellular viability} = \frac{(\text{absorbance}_{540} - \text{absorbance}_{690}) - (\text{mean blank}_{540} - \text{mean blank}_{690}) \times 100}{(\text{cell control}_{540} - \text{cell control}_{690}) - (\text{mean blank}_{540} - \text{mean blank}_{690})}$$

Equation 3.3

Percentage cellular viability was plotted against concentration by using the Enzfitter® program. The LD_{50} values of the different extracts and compounds were determined and the mean of the different experiments calculated as shown in figure 3.3. The concentration response curves were sigmiodal after logarithmic transformation of the concentration as was characteristically expected.

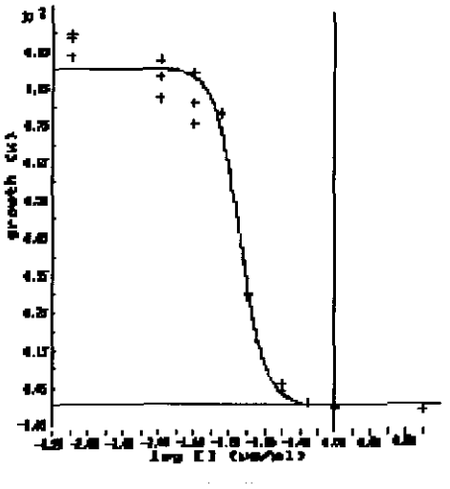


Figure 3.3: Percentage cellular viability plotted for one of the *Crinum* extracts.

The IC_{50} values of the different extracts and isolated compounds and fractions (section 3.3) are shown in table 3.4 and in table 3.5.

Table 3.4 Toxicity results of the different plant extracts.

Plant	Solvent	LD_{50} (µg/ml)	Standard error (µg/ml)
Aloe greatheadii – leaves	Pet.Et	4.7403	0.7776
	DCM	392.96	83.888
	EtoAc	144.51	60.372
	EtOH	94.299	38.901
Combretum erythrophyllum – leaves	Pet.Et	66.036	7.5785
	DCM	42.780	7.2379
	EtoAc	32.919	2.6509
	EtOH	925.72	20.381
Combretum erythrophyllum - stems	Pet.Et	2.7437	0.2333
	DCM	70.509	6.5853
	EtoAc	20.852	7.6439
	EtOH	66.968	4.3775
Crinum bulbispermum – leaves	Pet.Et	45.168	6.6748
	DCM	11.075	2.0573
	EtoAc	20.555	1.7236
	EtOH	11.691	1.7255

Plant	Solvent	LD_{50} (µg/ml)	Standard error (µg/ml)
Crinum bulbispermum -roots	Pet.Et	16.076	9.9852
	DCM	2.3614	0.6459
	EtoAc	9.6020	5.0188
	EtOH	171.08	56.732
Crinum bulbispermum - bulb	Pet.Et	177.57	45.568
	DCM	9.7096	8.9419
	EtoAc	176.25	12.583
	EtOH	8.7099	0.3105
Maytenus heterophylla – seeds	Pet.Et	5.4425	0.2934
	DCM	90.188	41.711
	EtoAc	47.005	4.3569
	EtOH	6259.3	45.568
Maytenus heterophylla - leaves	Pet.Et	36.665	3.6128
	DCM	7.3749	1.5732
	EtoAc	26.173	0.1059
	EtOH	64.406	11.522
Maytenus heterophylla - stems	Pet.Et	50.605	5.1779
	DCM	34.207	5.2493
	EtoAc	58.841	2.4106
	EtOH	230.82	5.4009

Plant	Solvent	LD50 (µg/ml)	Standard error (µg/ml)
Pavetta gardeniifolia – seeds	Pet.Et	225.47	37.368
	DCM	153.43	61.082
	EtoAc	495.94	161.32
	EtOH	384.31	171.10
Pavetta gardeniifolia – leaves	Pet.Et	25.927	2.3734
	DCM	238.95	8.1395
	EtoAc	236.86	143.28
	EtOH	729.71	245.39
Pavetta gardeniifolia - stems	Pet.Et	50.748	14.729
	DCM	1065.9	1610.6
	EtoAc	87.224	8.2569
	EtOH	540.15	219.48
Rhus pyroides – seeds	Pet.Et	82.854	17.126
	DCM	22.908	6.6735
	EtoAc	230.19	60.559
	EtOH	96221	135500

Plant	Solvent	LD50 (µg/ml)	Standard error (µg/ml)
Rhus pyroides - leaves	Pet.Et	50.316	4.1708
	DCM	7.1719	0.9334
	EtoAc	84.107	1.8469
	EtOH	942.53	800.10
Rhus pyroides - stems	Pet.Et	10.636	0.9749
	DCM	5.3345	1.5892
	EtoAc	34.374	12.755
	EtOH	112.79	19.226

Table 3.5 Toxicity of the isolated compounds and fractions

Compound	IC_{50} (µg/ml)	Standard error (µg/ml)
24	363.76	60.83502
25	447.86	92.45693
26	445.47	18.07609
Fractions	**IC_{50} (µg/ml)**	**Standard error (µg/ml)**
1	310.65	78.87647
2	166.83	2.1016
3	127.14	2.4472
4	196.11	10.932
5	7.4185	1.7371

3.2.3 Toxicity index

The toxicity index is used to determine the toxicity of a compound. A toxicity index greater than 100 is an indication of a low toxicity versus the activity of the compound and is determined as shown in Equation 3.4.

$$\text{Toxicity index} = \frac{\text{Cytotoxicity } (IC_{50} \text{ ug/ml})}{\text{Antimalarial activity } (IC_{50} \text{ ug/ml})}$$

Equation 3.4

Table 3.6: Toxicity index calculated for extracts.

Plant	Solvent	Tocicty index
Aloe greatheadii – leaves	Pet.Et	5.5585
	DCM	12.109
	EtoAc	3.3465
	EtOH	3.3488
Combretum erythrophyllum – leaves	Pet.Et	55.819
	DCM	60.984
	EtoAc	31.632
	EtOH	1057.97
Combretum erythrophyllum - stems	Pet.Et	1.8664
	DCM	100.41
	EtoAc	1.9486
	EtOH	15.188
Crinum bulbispermum – leaves	Pet.Et	50.927
	DCM	5.6517
	EtoAc	1.3545
	EtOH	0.1877
Crinum bulbispermum - roots	Pet.Et	4.5399
	DCM	2.2913
	EtoAc	405.611
	EtOH	9.185

Plant	Solvent	Tocicty index
Crinum bulbispermum - bulb	Pet.Et	185.770
	DCM	25.601
	EtoAc	2107.62
	EtOH	27.106
Maytenus heterophylla – seeds	Pet.Et	0.2357
	DCM	4.4948
	EtoAc	15.696
	EtOH	224.865
Maytenus heterophylla - leaves	Pet.Et	2.91533
	DCM	3.5047
	EtoAc	6.8166
	EtOH	0.7199
Maytenus heterophylla - stems	Pet.Et	7.955
	DCM	28.421
	EtoAc	43.349
	EtOH	21.862
Pavetta gardeniifolia – seeds	Pet.Et	66.496
	DCM	117.955
	EtoAc	14.000
	EtOH	89.459

Plant	Solvent	Tocicty index
Pavetta gardeniifolia – leaves	Pet.Et	4.713
	DCM	18.511
	EtoAc	32.525
	EtOH	34.497
Pavetta gardeniifolia - stems	Pet.Et	27.722
	DCM	723.579
	EtoAc	26.299
	EtOH	27.469
Rhus pyroides – seeds	Pet.Et	8.567
	DCM	14.345
	EtoAc	10.910
	EtOH	3960.22
Rhus pyroides - leaves	Pet.Et	29.904
	DCM	7.144
	EtoAc	63.884
	EtOH	101.307
Rhus pyroides - stems	Pet.Et	9.619
	DCM	1.748
	EtoAc	25.442
	EtOH	12.136

Table 3.7: Toxicity index's determined for the isolated compounds and fractions.

Compound	Toxicity index
24	3.4763
25	3.6164
26	15308.3
Fractions	**Toxicity index**
1	2.3424
2	1.0082
3	0.9367
4	14.513
5	0.3466

3.2.4 Antimicrobial activity

The antimicrobial activity of the plant extracts was determined by using agar diffusion techniques as well as the minimum inhibitory concentration technique. The disc diffusion technique however does not make it possible to distinguish between bactericidal and bacteristatic effects and the minimum inhibitory concentration cannot be determined. The size of the inhibition zones are not only influenced by the concentration of the extract but also by the diffusion rate of the extract from the filter paper into the agar plate. This test was therefore only used as a qualitative indication of antimicrobial activity (Eloff, 1998).

Disc diffusion assay

To determine the antimicrobial spectrum of the prepared plant extracts, the extracts were screened against several bacteria and a yeast. The range of microorganisms that were used included gram (+): *Bacillus subtilis* and *Staphylococcus aureus.* Gram *(–) bacilli, Escherichia colli and Pseudomonas aeruginosa.* Cultures of the various microorganisms were obtained from the Department of Microbiology of the North-West University.

The antimicrobial properties of the crude extracts were established by using the direct agar diffusion method employed by Van der Vijver and Lotter (1979), with slight modifications. Sterile agar plates were prepared with Biolab bacteriological agar (Biomed-Merck). 1 ml of a 24-hour nutrient broth (Biomed-Merck) culture of the test organism was spread evenly over the solid agar surface.

Extracts were dissolved in approximately 2 ml dichloromethane and after soaking small filter paper disks in these solutions for a few minutes, the discs were removed with tweezers and left to dry for approximately one hour. This ensured that all solvent evaporated before using the disks in the assay. Two disks were placed on each agar plate and the plate was then incubated for 24 hours at 37°C. After incubation the plates were then examined for zones of growth inhibition which is shown in table 3.8 as a (-) and positive growth as a (+).

Table 3.8: Disc diffusion growth indication

Plant	Extract	B. subtilis	S. aureus	E. coli	P. aeruginosa
Aloe greatheadii leaves	Pet.Et	+	+	+	+
	DCM	+	-	+	+
	EtOAc	+	-	-	-
	EtOH	-	+	-	-
Crinum bulbispermum - leaves	Pet.Et	+	+	-	-
	DCM	-	-	-	-
	EtOAc	+	-	-	-
	EtOH	+	+	-	+
Crinum bulbispermum - roots	Pet.Et	-	-	-	-
	DCM	-	+	+	-
	EtOAc	+	+	-	+
	EtOH	+	+	+	+
Crinum bulbispermum - bulbs	Pet.Et	-	-	-	-
	DCM	+	+	+	+
	EtOAc	-	+	-	-
	EtOH	+	+	+	+

Plant	Extract	B. subtilis	S. aureus	E. coli	P. aeruginosa
Combretum erythrophyllum - leaves	Pet.Et	-	+	-	-
	DCM	-	-	-	-
	EtOAc	-	-	-	-
	EtOH	-	+	-	-
Combretum erythrophyllum - stems	Pet.Et	-	+	-	-
	DCM	-	-	-	-
	EtOAc	-	-	+	-
	EtOH	+	+	-	+
Maytenus heterophylla – leaves	Pet.Et	+	+	-	+
	DCM	-	-	-	-
	EtOAc	+	-	-	-
	EtOH	-	-	-	-
Maytenus heterophylla - seeds	Pet.Et	+	+	-	+
	DCM	+	+	-	-
	EtOAc	-	-	-	+
	EtOH	+	+	-	+

Plant	Extract	B. subtilis	S. aureus	E. coli	P. aeruginosa
Maytenus heterophylla - stems	Pet.Et	-	+	-	-
	DCM	-	-	-	-
	EtOAc	+	+	-	+
	EtOH	+	-	-	+
Rhus pyroides - leaves	Pet.Et	-	+	-	-
	DCM	-	+	-	-
	EtOAc	+	+	-	-
	EtOH	-	-	-	+
Rhus pyroides - seeds	Pet.Et	-	-	-	-
	DCM	-	-	-	-
	EtOAc	-	+	-	+
	EtOH	-	-	-	-
Rhus pyroides - stems	Pet.Et	-	-	-	-
	DCM	-	-	-	+
	EtOAc	-	-	-	-
	EtOH	-	-	-	-

Plant	Extract	B. subtilis	S. aureus	E. coli	P. aeruginosa
Pavetta gardeniifolia - leaves	Pet.Et	-	-	-	-
	DCM	-	+	+	+
	EtOAc	-	-	-	-
	EtOH	-	-	-	+
Pavetta gardeniifolia - seeds	Pet.Et	-	-	-	-
	DCM	+	+	-	+
	EtOAc	+	+	-	+
	EtOH	-	+	-	+
Pavetta gardeniifolia - stems	Pet.Et	+	-	-	-
	DCM	+	-	-	-
	EtOAc	+	-	-	-
	EtOH	+	+	+	+

Minimum inhibitory concentration

To obtain quantitive values for the microbial growth inhibition, the MIC values for the extracts and compounds were calculated. These results are shown in table 3.10.

Preparation of compound solutions

Stock solutions of the extracts were prepared by reconstituting the extracts with 1 ml DMSO.

Preparation of the microorganisms

The same range of microorganism were used in the MIC test as in the disc diffusion test. Microorganisms were prepared as described by A. Uys *et al*, 2001.

Table 3.9: Absorbance values of different microorganisms at 600 nm used to prepare stock cultures.

Micro-organism	Absorbance
B. subtilis	0.111
S. aureus	0.166
E. coli	0.115
P. aeruginosa	0.042

Preparation of the 96 well microtitre plates

Twofold dilutions were prepared in columns 1 through 11 using 100 µl of Müller-Hinton broth (Fluka) and 100 µl of the stock test solution. Column 11 served as a 0 % growth control, which gave an indication of contamination. In contrast column 12 served as the 100 % bacterial growth control. After preparation of the plates, they were incubated at 37 °C for 24 hours. Each compound was tested in triplicate against the six above-mentioned microorganisms.

After incubation, to indicate bacterial growth, 20 µl of a 0.2 mg/ml 3-(4-iodophenyl)-2-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (Merck) was added to the microtitre plate and incubated at 37 °C for approximately 30 minutes.

The color intensity of the samples was compared to the color intensity of the 0% and 100% controls. The concentration of the compound in the well in which 100% growth occurred was taken as the minimum inhibitory concentration. An example of color changes is indicated in figure 3.4.

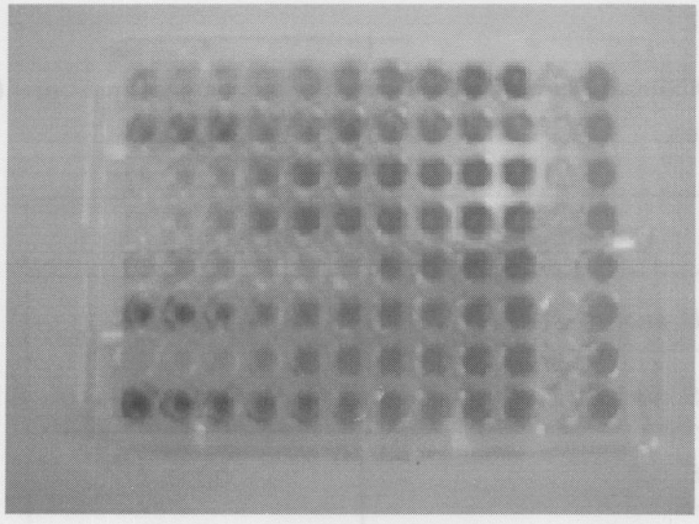


Figure 3.4: Example to indicate the color change that takes place while testing some of the extracts against *Pseudomonas* sp.

After the initial determination of MIC's of the crude extracts it was determined that several of the extracts exhibited activity against a variety of organisms. Results are presented in table 3.10.

Table 3.10: Minimum inhibitory concentration (MIC) of the crude petroleum ether (Pet.Et), dichloromethane (DCM), ethyl acetate (EtOAc) and ethanol (EtOH) plant extracts against various bacteria.

Plant	Extract	B. subtilis	S.aureus	E. coli	P. aeruginosa
Aloe greatheadii leaves	Pet.Et	29.65	495.70	123.93	61.96
	DCM	39.75	9.81	19.63	19.63
	EtOAc	13.75	6.88	3.44	6.88
	EtOH	9.28	37.13	0.58	9.28
Crinum bulbispermum leaves	Pet.Et	29.65	29.65	3.71	0.46
	DCM	1.90	0.24	0.24	1.90
	EtOAc	19.25	4.81	0.15	4.81
	EtOH	57.49	229.98	7.19	28.75
Roots	Pet.Et	0.29	2.35	0.04	1.18
	DCM	2.00	128.15	16.02	1.00
	EtOAc	42.90	10.73	10.73	42.90
	EtOH	76.73	38.37	19.18	76.73
bulbs	Pet.Et	4.25	4.25	2.13	0.53
	DCM	890.75	445.38	13.92	111.34
	EtOAc	8.88	17.76	8.88	8.88
	EtOH	166.8	166.8	41.70	41.70

Plant	Extract	B. subtilis	S.aureus	E. coli	P. aeruginosa
Combretum erythrophyllum leaves	Pet.Et	4.01	64.22	8.03	1.00
	DCM	4.32	8.63	8.64	2.16
	EtOAc	1.56	3.12	3.12	3.12
	EtOH	8.92	8.92	2.23	1.11
stems	Pet.Et	7.14	57.15	1.79	3.57
	DCM	0.87	3.49	0.22	0.44
	EtOAc	7.18	3.59	14.36	3.59
	EtOH	72.79	18.20	9.10	36.39
Maytenus heterophylla leaves	Pet.Et	35.25	70.63	8.83	35.25
	DCM	0.13	2.04	0.26	2.04
	EtOAc	25.35	1.58	0.20	6.34
	EtOH	2.48	9.91	0.62	4.95
seeds	Pet.Et	28.41	56.83	7.12	14.21
	DCM	13.355	26.70	3.34	6.68
	EtOAc	7.05	7.05	3.53	28.2
	EtOH	13.18	13.18	6.59	13.18

Plant	Extract	B. subtilis	S.aureus	E. coli	P. aeruginosa
stems	Pet.Et	8.39	33.55	1.05	8.39
	DCM	5.34	5.34	0.17	2.66
	EtOAc	34.40	34.40	4.30	34.40
	EtOH	21.28	2.66	2.66	10.64
Rhus pyroides leaves	Pet.Et	3.11	12.45	0.19	0.40
	DCM	1.28	10.20	1.28	0.32
	EtOAc	23.20	23.20	5.80	0.73
	EtOH	0.70	1.40	1.40	10.62
seeds	Pet.Et	0.28	8.93	0.14	4.46
	DCM	0.12	1.85	0.12	0.12
	EtOAc	4.30	68.85	4.30	17.21
	EtOH	9.70	4.85	2.43	2.43
stems	Pet.Et	4.55	4.55	1.14	4.55
	DCM	1.55	3.10	1.55	12.40
	EtOAc	0.04	2.70	1.35	0.08
	EtOH	4.60	9.21	2.30	4.60

Plant	Extract	B. subtilis	S.aureus	E. coli	P. aeruginosa
Pavetta gardeniifolia leaves	Pet.Et	4.71	9.41	9.41	4.71
	DCM	6.84	27.36	27.36	54.73
	EtOAc	3.70	1.85	0.92	0.92
	EtOH	3.22	6.44	3.22	12.89
seeds	Pet.Et	8.00	4.00	4.00	8.00
	DCM	23.10	11.55	2.89	11.55
	EtOAc	52.18	13.04	3.26	52.18
	EtOH	3.03	12.10	6.05	48.42
stems	Pet.Et	22.55	0.18	0.09	5.64
	DCM	10.40	5.20	2.60	5.20
	EtOAc	27.65	6.91	3.46	6.91
	EtOH	22.03	22.03	11.01	22.03

3.3 Isolation and characterisation of compounds from *Crinum bulbispermum*

The different compounds were isolated using bioguided teqniques, including:

- Antimalarial and antimicrobial testing of crude extracts;
- Selecting the most active extract;
- Extraction and eventual isolation of pure compounds.

3.3.1 Instrumentation

The ^{13}C and ^{1}H spectra were obtained on a Varian Gemini-300 spectrometer in a 7 Tesla magnetic field at a frequency of 300.075 MHz for ^{1}H and 75.462 MHz for ^{13}C spectra. All the chemical shifts are reported in parts per million (ppm) relative to tetramethylsilane (TMS; $\delta = 0$). A Nicolet Nexus 470 FT-IR spectrometer was used to record IR spectra (in cm^{-1}) with the use of KBr pellets. The oils were painted onto the KBr pellets and left to dry before their IR spectra were determined. The mass spectra were recorded on an analytical Varian VG 7070E mass spectrometer using electron impact at 70 eV as ionisation technique. The following abbreviations are used to describe multiplicity of ^{1}H signals: s = singulet, d = doublet, m = multiplet. Combinations of these abbreviations indicate corresponding multiplicity of the signals. All Rf values were determined in a mobile phase that consisted of petroleum ether: dichloromethane: ethyl acetate (4:8:1).

3.3.2 Thin layer chromatography

Thin layer chromatography was employed in the selection of suitable mobile phases for the isolation of compounds using aluminum silica gel sheets (Alufolien 60 F_{254}, Merck). Since the dichloromethane extract of the bulb of *Crinum bulbispermum* showed promising antimalarial activity, thin layer chromatograms of this extract were run (figure 3.3).

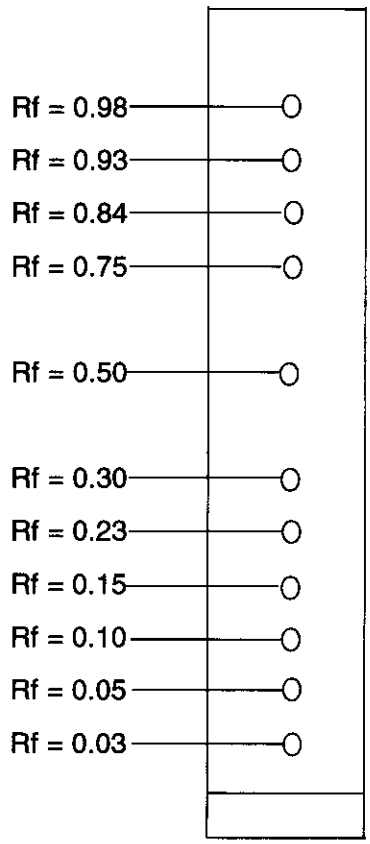


Figure 3.5: Representation of the thin layer chromatogram.

A mobile phase consisting of petroleum ether: dichloromethane: ethyl acetate (4:8:1) was used.

3.3.3 Silica gel column chromatography

A thick walled glass column, resistant to solvents and tolerant to low and medium pressures, was used during column development (Salituro & Dufrensne, 1998). The silica gel (size 0.063-0.2 mm, Macherey-Nagel) was introduced as a suspended slurry in the mobile phase. The plant extract were dissolved in small amounts of the mobile phase and after being filtered, applied to the column bed with a pasteur pipette.

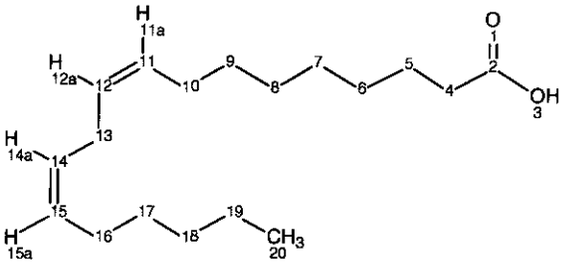
Initial separation with the mobile phase mentioned above yielded 5 fractions that were then further purified using the same mobile phase to give eight purer fractions in total. All compounds obtained were further purified by recrystallisation from ethanol. Not all the fractions isolated were of

sufficient quantity to obtain all the data required for their identification. 3 Compounds **(24 - 26)** could be identified and were tested for antimalarial activity and toxicity while the other 5 fractions were tested for antimalarial activity and toxicity only.

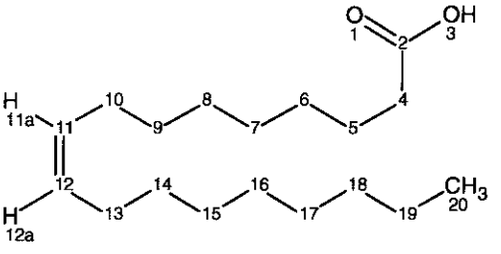
3.3.4 Characterisation of the isolated compounds

3.3.4.1 Physical data

Compound **(24)** was established as 9,12-octadecadienoic acid (oleic acid). Physical data (spectrums 1 – 2) corresponds to that reported in literature (Keller, 2000 and Pouchert & Behnke, 2001). Compound **(25)** was established as 9-octadecadienoic acid (linoleic acid). Physical data(spectrums 3 – 6) corresponds to that reported in literature (Keller, 2000 and Pouchert and Behnke, 2001).



(24)



(25)

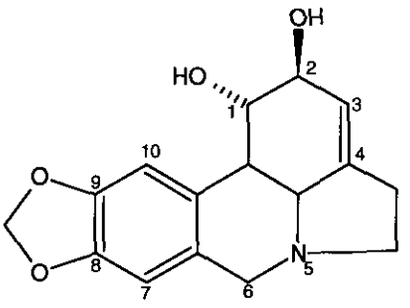
Compound **(26)** was identified as lycorine, an amorphous powder, with the following empiric formula $C_{16}H_{19}NO4$;. The mass was determined as EI-MS 287 M/Z; (spectrum 7): 287 M^+; 226; 147; 57; 41. The IR data was established as ν_{max} (spectrum 8): 3423,36; 2842,67; 1501,00; 1480,51; 1255,19; 1234,71; 1034,99.

Table 3.11: ^{1}H- and ^{13}C-nmr spectral assignment of compound **26** in comparrison to data found in the literature by Likhitwitayawuid *et al.*, (1993).

Position	^{1}H	$^{1}H^{b}$	^{13}C	$^{13}C^{b}$
1	4.28 (s)	4.27 (br s)	71.657	70.21
2	3.98 (s)	3.97 (br s)	70.139	71.72
3	5.35 (s)	5.37 (br s)		118.48
4			141.539	141.68
4a	2.61 (d)	2.60 (d)	60.721	60.83
6α	3.34 (d)	3.32 (d)	56.587	56.73
β	4.05 (d)	4.02 (d)		
6a			129.555	129.75
7	6.65 (s)	6.68	106.956	107.01
8			145.156	145.20
9			145.520	145.65
10	6.80 (s)	6.81	105.007	105.06
10a			129.6	129.57
10b	2.48 (m)	2.50 (m)	42.0	40.18
11α,β	2.44 (m)	2.44 (m)	28.098	28.13
12α	2.20 (ddd)	2.19 (ddd)	53.241	53.31
β	3.20 (dd)	3.19 (dd)		
OCH_2O	5.92 (s)	5.94 (s)	100.508	100.57

[a] Chemical shifts are reported in ppm (δ) in DMSO -d_6 fir ^{1}H and ^{13}C.

[b] Data obtained from Likhitwitayawuid *et al.*, (1993).



(26)

All of the isolated compounds were previously isolated from various *Crinum* species and all the data corresponded to that previously reported in literature.

CHAPTER 4

Discussion and Conclusion

The aim of the study was to identify a plant extract with antimalarial activity and to isolate and identify the compounds responsible for this activity, in the hope to provide some sort of a solution to the increasing demand for new effective antimalarial agents.

4.1 Screening of plant extracts

With initial antimalarial screening it was determined that the 62 extracts of the selected plant species namely, *A. greatheadii, C. bulbispermum, C. erythtrophyllum, M. heterophylla, R. pyroides* and *P. gardeniifolia* had varying antimalarial activity against chloroquine-resistant *P. falciparum.* The activity varied from 0.02 μg/ml, the lowest IC_{50} value obtained from the ethyl acetate root extract of *C. bulbispermum* to 56.49 μg/ml, the highest IC_{50} value obtained from the ethanol leaf extract of *M. heterophylla.* These values were compared to the IC_{50} values of chloroquine and quinine, which are 0.04 μg/ml and 0.13 μg/ml respectively. All extracts with IC_{50}'s higher than 10 ug/ml were not considered for further investigation. The extracts of *A. greatheadii var davyana* showed the least antimalarial activity while *C. bulbispermum* and *C. erythrophyllum* extracts exhibited the most promising antimalarial activity over a single cycle of parasite growth. The ethyl acetate and dichloromethane extracts of the bulbs of *C. bulbispermum* had the lowest IC_{50} values ranging from 0.023673 ± 0.007066 μg/ml to 0.081 ±0.004 μg/ml. *C. bulbispermum* bulbs and root extracts were more active than the leaf extracts in all of the cases. These extracts were selected for further investigation for antimalarial activity and toxicity.

The overall toxicity index (section 3.2.3) for the different extracts varied between 0.18 and 3960.22 as determined in Graham kidney cells. Extracts with a toxicity index value of greater than 100 as determined in section 3.2.3 was considered for further investigation. For *A. greatheadii* the toxicity values were lower than the IC_{50} values obtained for the antimalarial activity. This indicates that toxicity will appear before the malaria parasites are affected by the antimalarial activity of these

extracts. The toxicity indexes obtained for *C. bulbispermum* varied with the ethanol leaf extract being the most toxic with a toxicity index of 0.1877 and an IC_{50} value of 50.4 µg/ml. The toxicity index calculated for the ethyl acetate root extract was, 405.611 while the IC_{50} value was 0.02 µg/ml. The large toxicity index and low IC_{50} value for antimalarial activity makes this extract a good choice for further investigation. The toxicity index of *C. erythrophyllum* extracts varied but was always the same concentration or higher than the concentration at which antimalarial activity was found. When evaluating the toxicity index values of *M. heterophyllum, R. pyroides* and *P. gardeniifolia* it was clear that most of these extract were toxic, with a few exclusions. The toxicity index for the dichloromethane seed extract of *P. gardeniifolia* is 117.955 and the IC_{50} value was estimated at 1.30 µg/ml, this big difference then also indicated that the extract had antimalarial properties with low toxicity.

The antimicrobial activity of the prepared extracts was tested against *B. subtilis, S. aureus, E. coli* and *P. aeruginosa.* The activity of the different extracts varied, with the ethyl acetate and ethanol leaf extracts of *A. greatheadii* being the most active against *E. coli.* The *C. bulbispermum* leaf ethyl acetate extract showed the best activity against *E. coli* and the least activity was found for the dichloromethane bulb extract against *B. subtilis.* For *C. erythrophyllum* the best antimicrobial activity was found for the dichloromethane stem extract against *E. coli* and the least activity was found for the petroleum ether stem extract against *S. aureus.* For *M. heterophylla* the best activity was found for the dichloromethane leaf and stem extracts against *B. subtilis* and *E. coli* respectively, the lowest activity was for the petroleum ether leaf extract against *S. aureas. R. pyroides* showed an overall good antimicrobial activity with especially the dichloromethane seed extracts against *P. aeruginosa, B. subtilis* and *E. coli* as well as for the ethyl acetate stem extracts against both *P. aeruginosa* and *B. subtilis.* The least activity was found for the ethyl acetate seed extract against *S. aureus. P. gardeniifolia* ethyl acetate leaf extracts were the most active against *P. aeruginosa* and *E.coli* while both the dichloromethane leaf and ethanol seed extracts were the least active against *P. aeruginosa.*

4.2 Isolation of compounds from *Crinum bulbispermum*

Compounds **(24), (25)** and **(26)** were isolated from the dichloromethane extract and the biological activity was determined. The quantities of the fractions isolated were much less when compared to the quantity of crude extracts, but their activity increased as the active compounds were purified.

9,12-Octadecadienoic acid **(24)** and 9-octadecadienoic acid **(25)** were isolated from the dichloromethane extract of *C. bulbispermum*. A broad band at 3441.82 cm^{-1} on the infrared spectrum (spectrum 1) showed the presence of the carboxylic hydroxyl group indicating an acid, while a weak absorption near 1650 cm^{-1} indicated the of C=C double bonds which are confirmed by an aliphatic CH absorption to the right of 3000 cm^{-1}. The ^{1}H-NMR (spectrum 2) indicated the presence of two double bonds in a carbon chain at δ_H 5.32 ppm and a methyl group at δ_H 0.89 ppm. Compound **24** was identified as 9, 12-octadecadienoic acid (linoleic acid); this corresponds to the data reported by Keller (2000).

EI-MS (spectrum 3) of compound **25** exhibited a major $[M]^+$ peak at *m/z* 452. A strong broad acid band is visible at 3436.70 cm^{-1} and a double bond at 1623.90 cm^{-1} on the IR-spectrum (spectrum 4). The ^{13}C-NMR (spectrum 6) indicated only one double bond that presents at Cδ 31.918 (C11) and an acid group (C2) at Cδ 129.81. These groups were also present in the ^{1}H-NMR at Hδ 2.58 and Hδ 2.25 respectively. This compound was identified as 9-octadenoic acid (oleic acid), which corresponds to the data reported by Keller, (2000).

An alkaloid, compound **26** exhibited a major $[M]^+$ peak at *m/z* 287 on the EI-MS (spectrum 7). In the IR-spectrum the nitrogen group was seen as two strong absorptions at 1600-1500 cm^{-1} and 1390 – 1300 cm^{-1}. This structure was identified, by observing the ^{1}H-NMR (spectrum 9) and the ^{13}C-NMR (spectrum 10) indicated in table 3.9. Compound **26** was identified as lycorine and its physical chemical data corresponds to that reported in literature (Likhitwitayawuid *et al.*, 1993).

Although fraction **(1)** was not obtained in sufficient quantity to obtain the structure it could be identified as an alkaloid. This fraction showed no antimalarial activity and was found to be toxic. All the other fractions were also relatively toxic with no antimalarial activity expect for fraction **(4)** which showed poor antimalarial activity and high toxicity.

4.3 *In vitro* antimalarial activity of compounds isolated from *Crinum bulbispermum*

The antimalarial activities of compound **24** (C24), and **25** (C25) were found to be very poor, the IC_{50} values were all greater than than 100 µg/ml. Compound **26** (C26), had the best activity and were compared to chloroquine (CQ), quinine (QN) and the initial dichloromethane (DCM) plant extracts from the bulb of *C. bulbispermum,* as presented in figure 4.1.

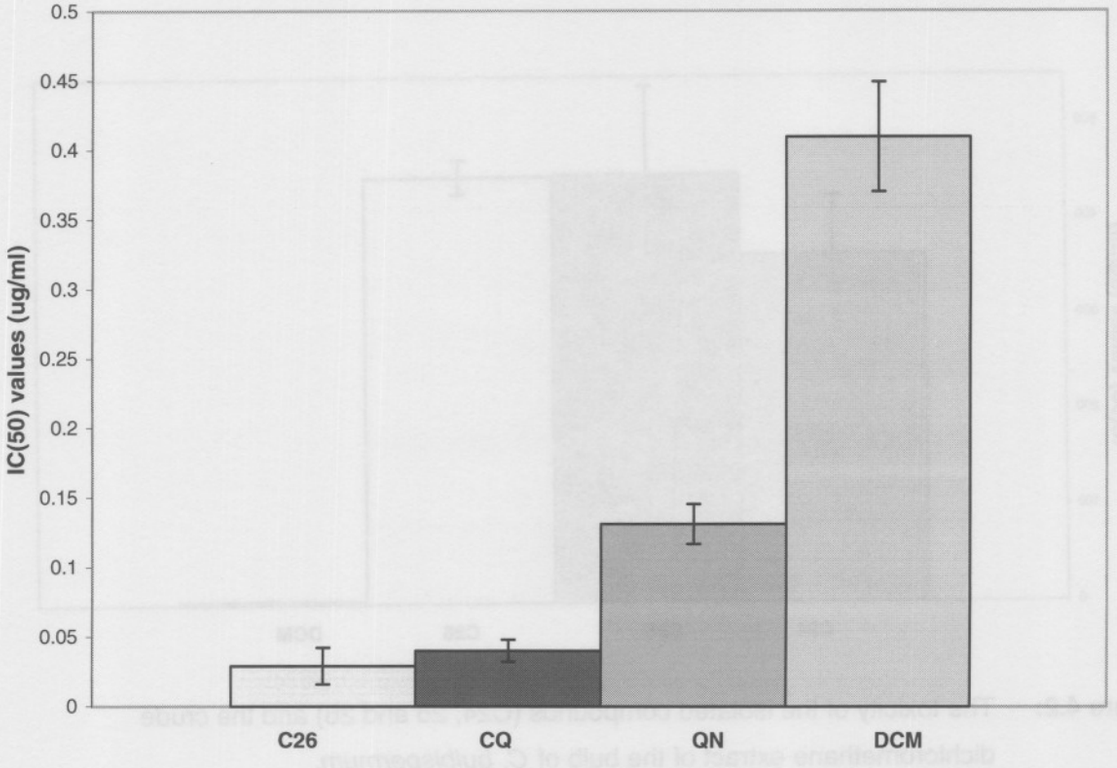


Figure 4.1 Comparison between compound **(26)** and other antimalarials.

The isolated compounds **(24 – 26)** showed variable *in vitro* antimalarial activity ranging between 0.02 and 130 µg/ml. The IC_{50} values for the two acids **(24, 25)** linoleic acid and oleic acid were higher than 100 µg/ml. Compound **26**, lycorine was the most active with an IC_{50} value of 0.0291 ± 0.01 µg/ml. Compound **26** had a lower IC_{50} value than chloroquine (0.04 µg/ml) and quinine (0.13 µg/ml). The antimalarial activity of compound **26** was thus very promising.

Lycorine was previously isolated from *Brunsvigia littoralis* and tested against the cultured D10 and FAC8 strains of *P.falciparum.* The IC_{50} values reported were 0.62 and 0.7 µg/ml respectively (W. E. Campbell, *et al.,* 2000).

4.4 Toxicity of compounds 24 – 26

The toxicity of isolated compounds **(24 - 26)** was determined with the MTT assay as described in paragraph 3.2.3. IC_{50} values for the isolated compounds **(24 - 26)** are shown in figure 4.2.

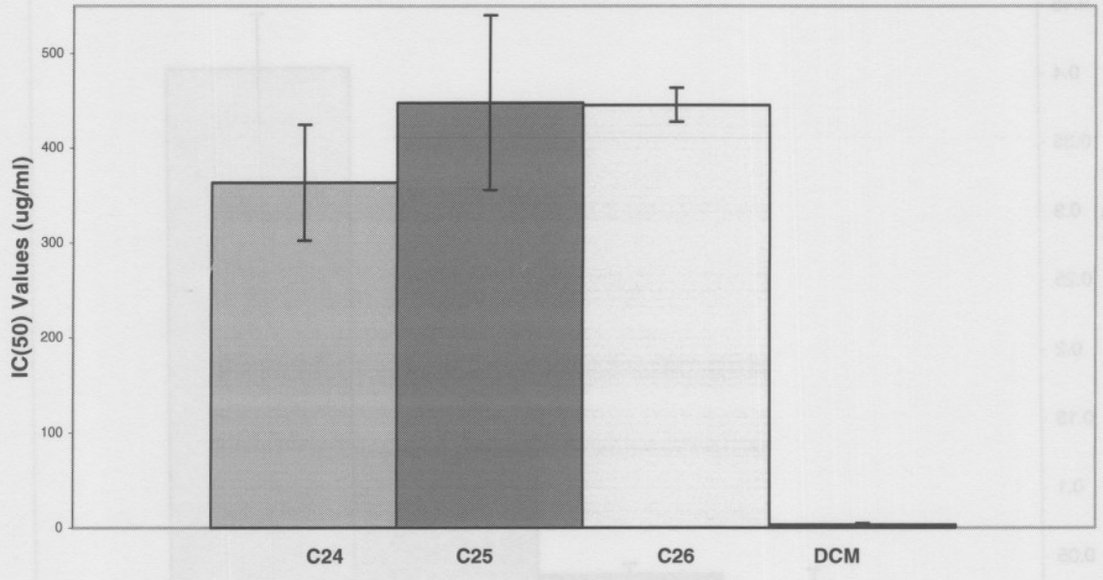


Figure 4.2: The toxicity of the isolated compounds (C24, 25 and 26) and the crude dichloromethane extract of the bulb of *C. bulbispermum.*

It is encouraging that some of the compounds that exhibited inhibitory effects on the intra-erythrocytic growth of the malaria parasite are relatively non-toxic on the tested human cell line. The *in vitro* toxicity of the isolated compounds **(24 – 26)** varied with **C26** showing the best toxicity index of 15 308.18 as calculated in section 3.2.3. As natural molecules such as quinine and artemisinin have acted as templates in the development of antimalarial agents, the results of this

study are encouraging for further investigation into this class of compounds in the search for potent antiplasmodial agents.

4.5 Antimicrobial activity of compounds 24 – 26

The antimicrobial activities of the compounds were determined by using the minimum inhibitory concentration (MIC) technique. Results obtained for the antimicrobial testing are shown in figure 4.3. Four different organisms were used in these test namely *Bacillus subtilis, Staphylococcus aureus, E. coli and Pseudomonas aeruginosa.*

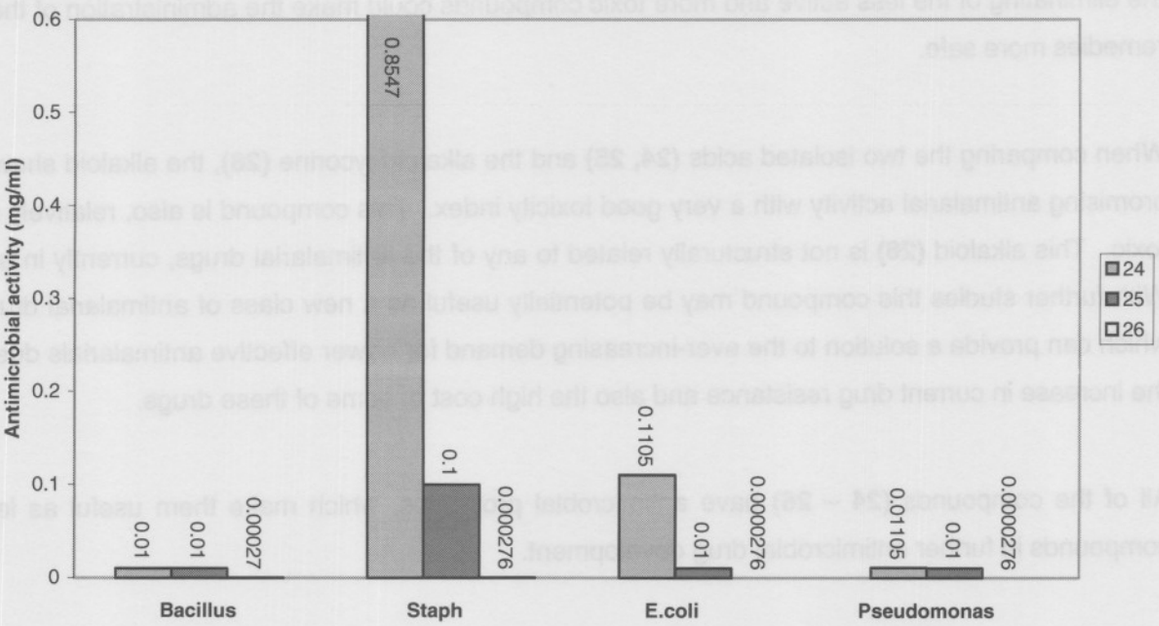


Figure 4.3: Antimicrobial activity of the isolated compounds.

Several of the initial plant extracts as well as compounds **24 – 26** isolated from *C. bulbispermum* showed antimicrobial activity and the MIC values were determined with the tetrazolium salt method. Compound **26** was the most active against all the organisms used, with the best activity against *B. sutilis* (IC_{50} 0.000276µg/ml).

4.6 Conclusion

In this study 6 plants were screened for antimalarial activity, cytotoxicity and antimicrobial activity used by traditional healers for various ailments including the treatment of malaria symptoms. Sixty-two different extracts were prepared and tested. Compounds were isolated from the extract showing the best antimalarial activity and with the least toxicity. The bulb extract of *C. bulbispermum* was selected for further study. Although the isolated compound lycorine was active against *P. falciparum* and had a low toxicity value it must be taken into account that most of the isolated compounds were toxic and had very little antimalarial activity. Traditional healers use the whole bulb when preparing remedies for their patients. These contain various compounds, some of which could be fatal to people using these remedies. Isolated antimalarial active compounds and the eliminating of the less active and more toxic compounds could make the administration of these remedies more safe.

When comparing the two isolated acids **(24, 25)** and the alkaloid lycorine **(26)**, the alkaloid showed promising antimalarial activity with a very good toxicity index. This compound is also, relatively not toxic. This alkaloid **(26)** is not structurally related to any of the antimalarial drugs, currently in use. With further studies this compound may be potentially useful as a new class of antimalarial drugs, which can provide a solution to the ever-increasing demand for newer effective antimalarials due to the increase in current drug resistance and also the high cost of some of these drugs.

All of the compounds **(24 – 26)** have antimicrobial properties, which make them useful as lead compounds in further antimicrobial drug development.

Medicinal plant research becomes more important, especially if one looks at the biological activities of the compounds **(24 – 26)** isolated from *C. bulbispermum*. The fact that the diverse chemistry of medicinal plants is very important and the fact that they are a viable source of novel biologically active and lead compounds are stressed.

Who knows what cures, are beheld in our diverse plant families.

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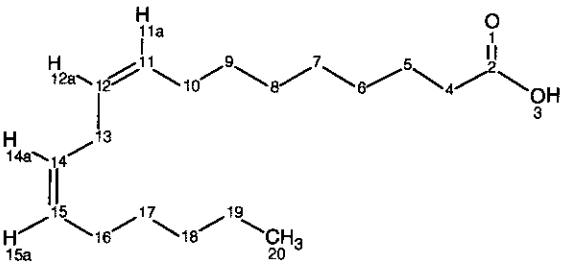
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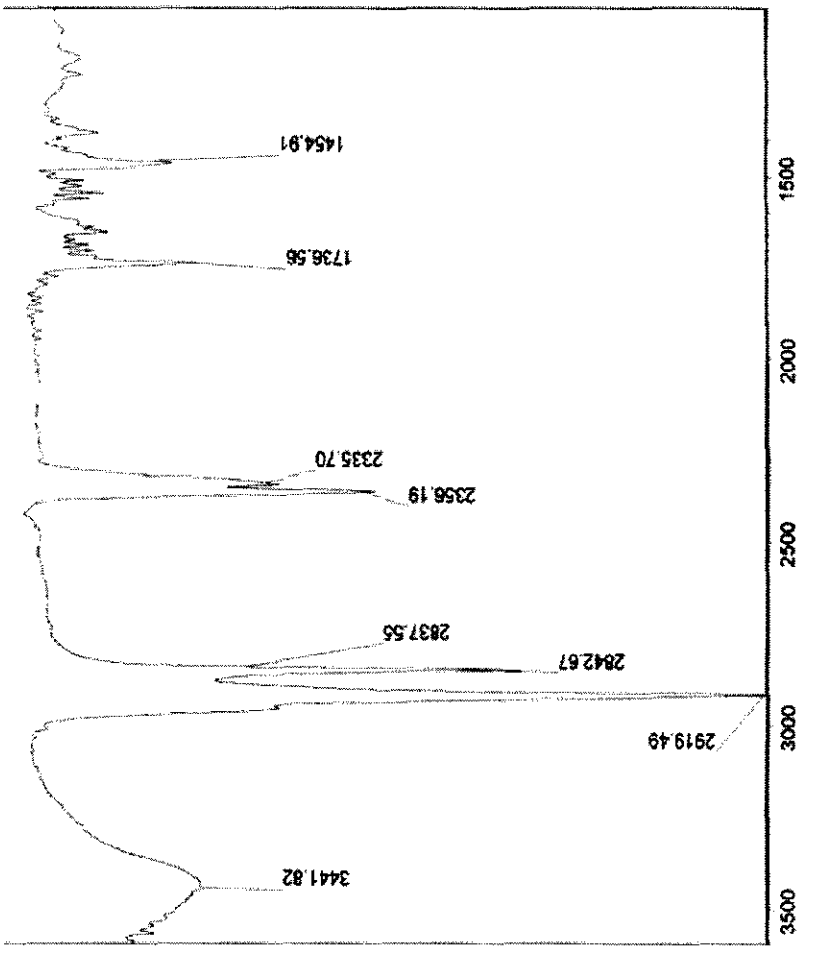
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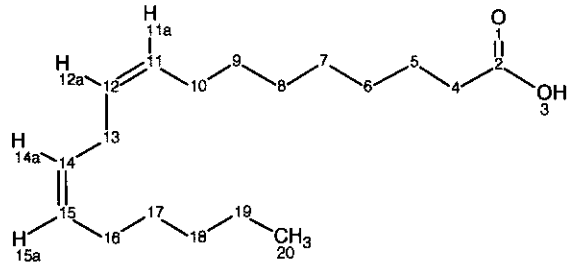
SPECTRUM 2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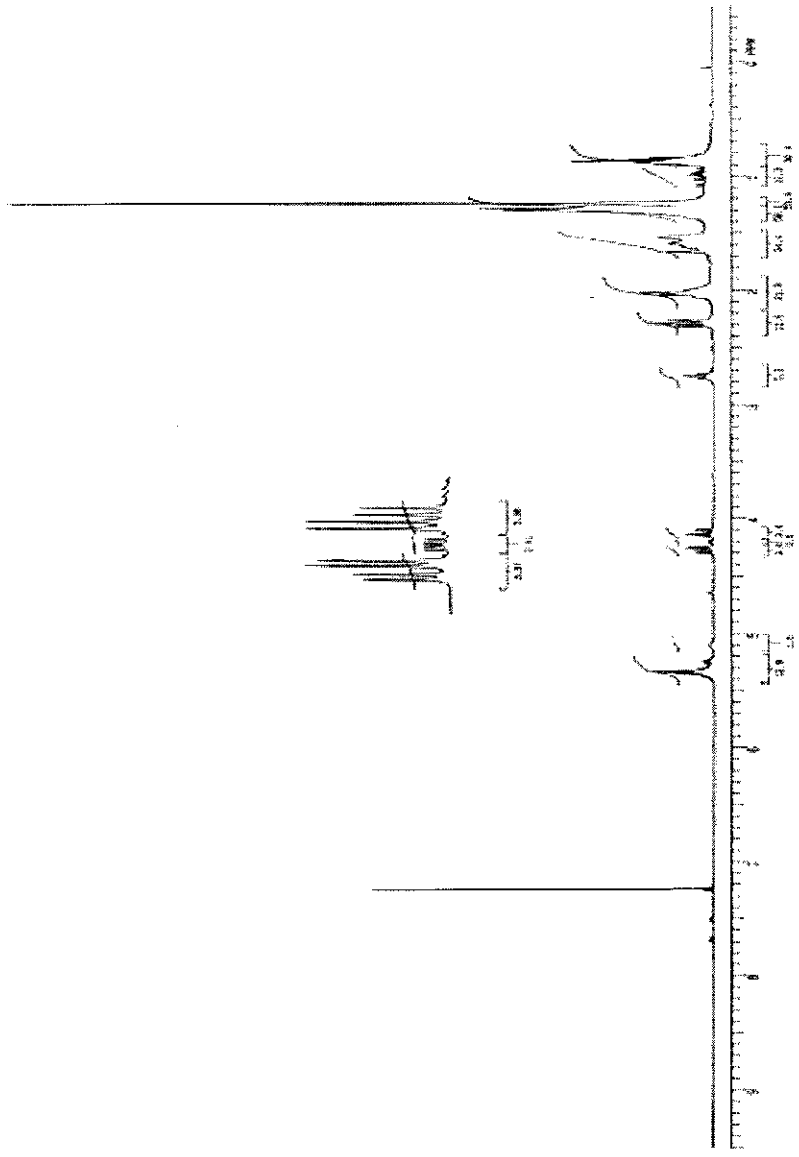

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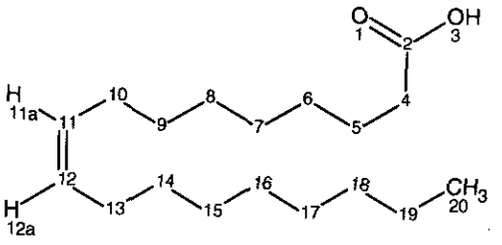
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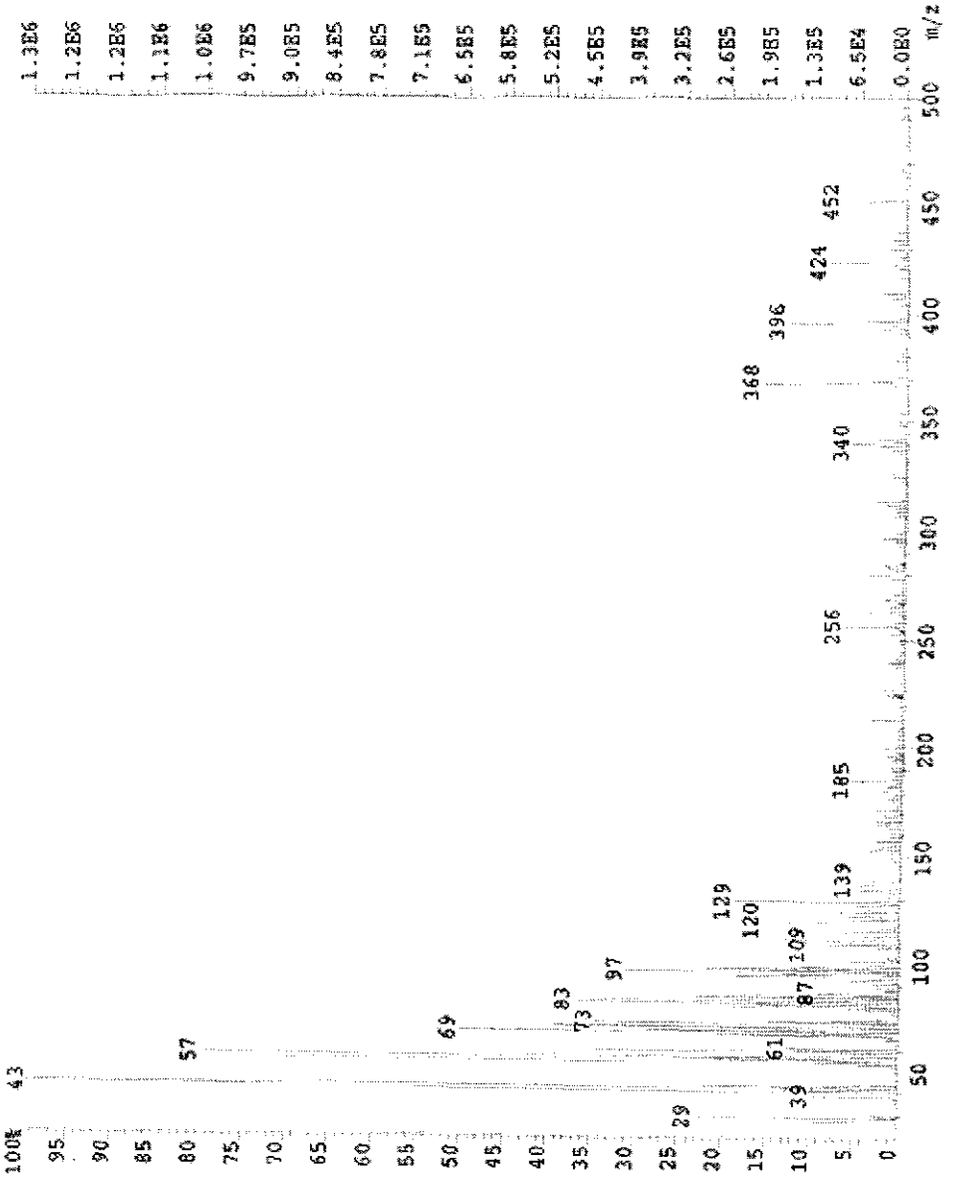
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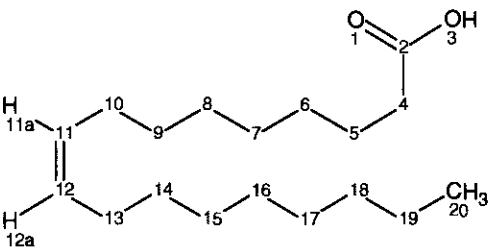
SPECTRUM 4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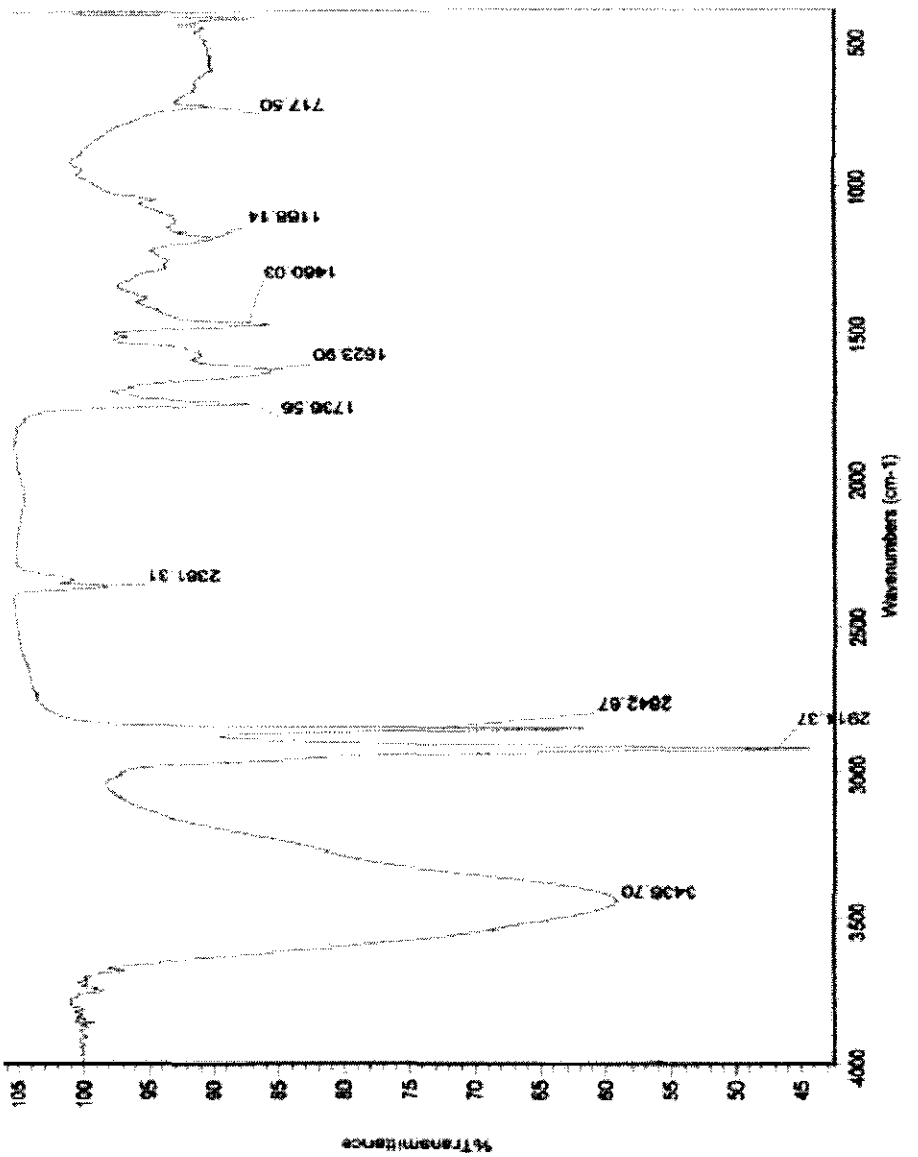
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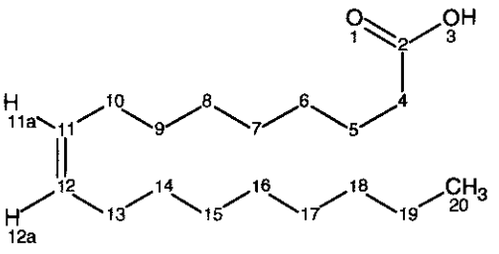
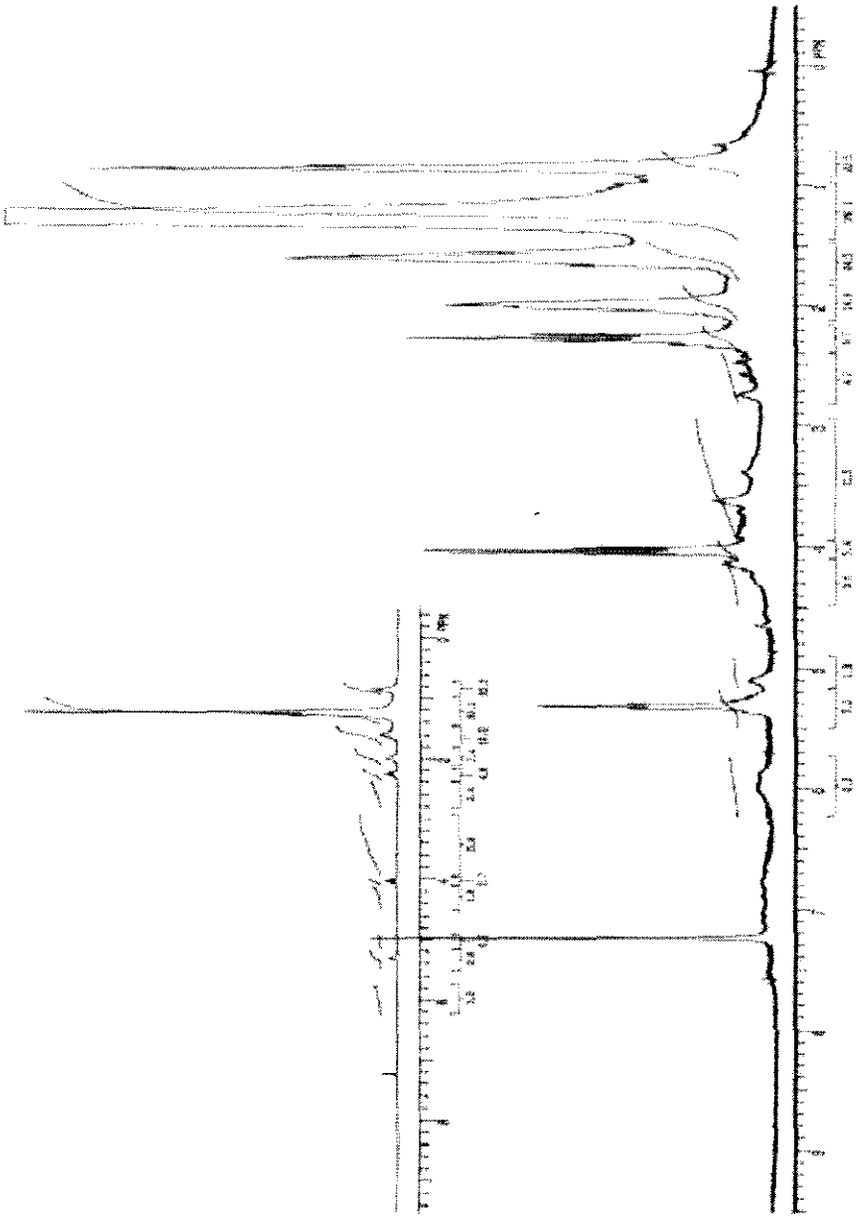
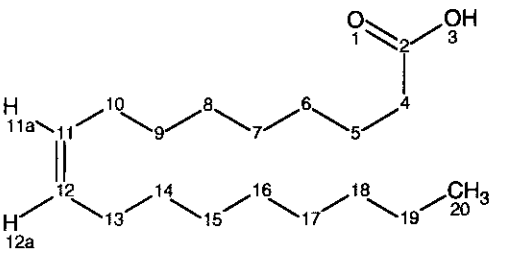
%Transmittance
Wavenumbers (cm-1)

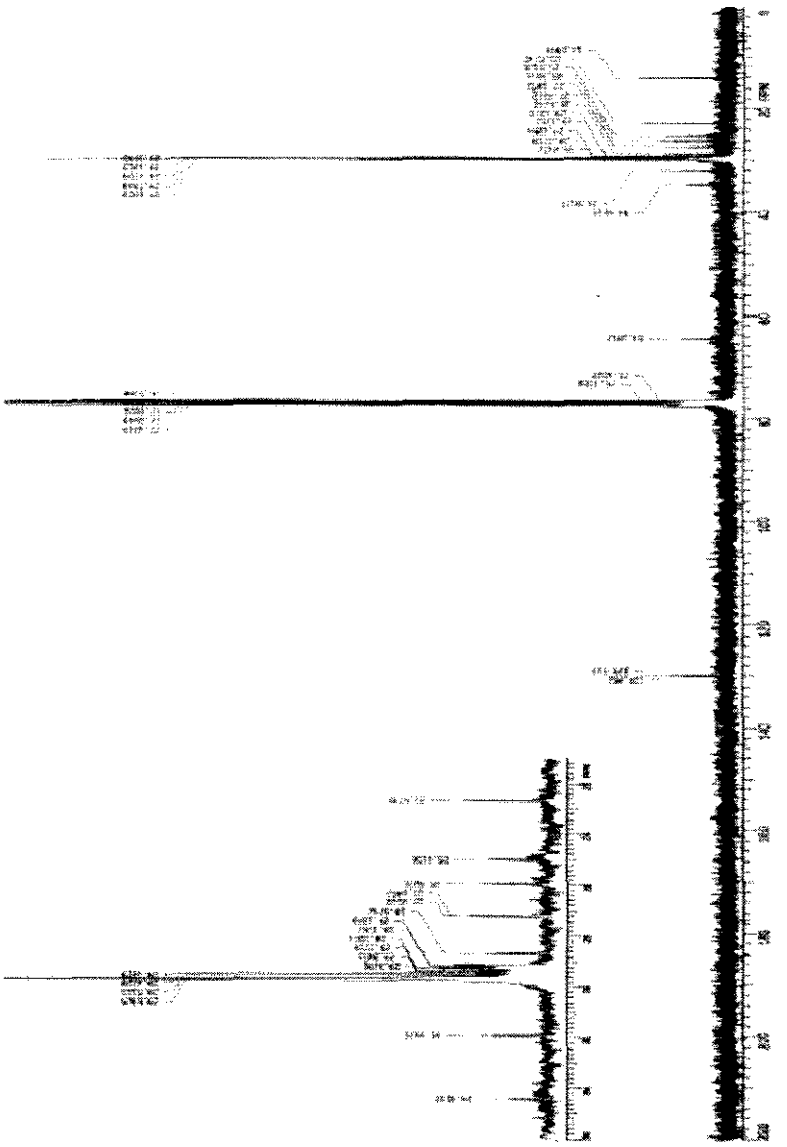
SPECTRUM 5



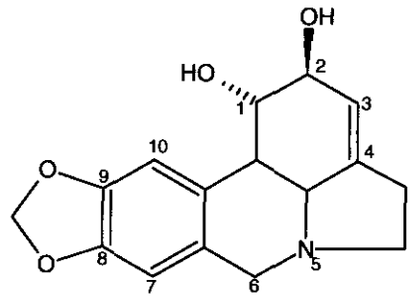


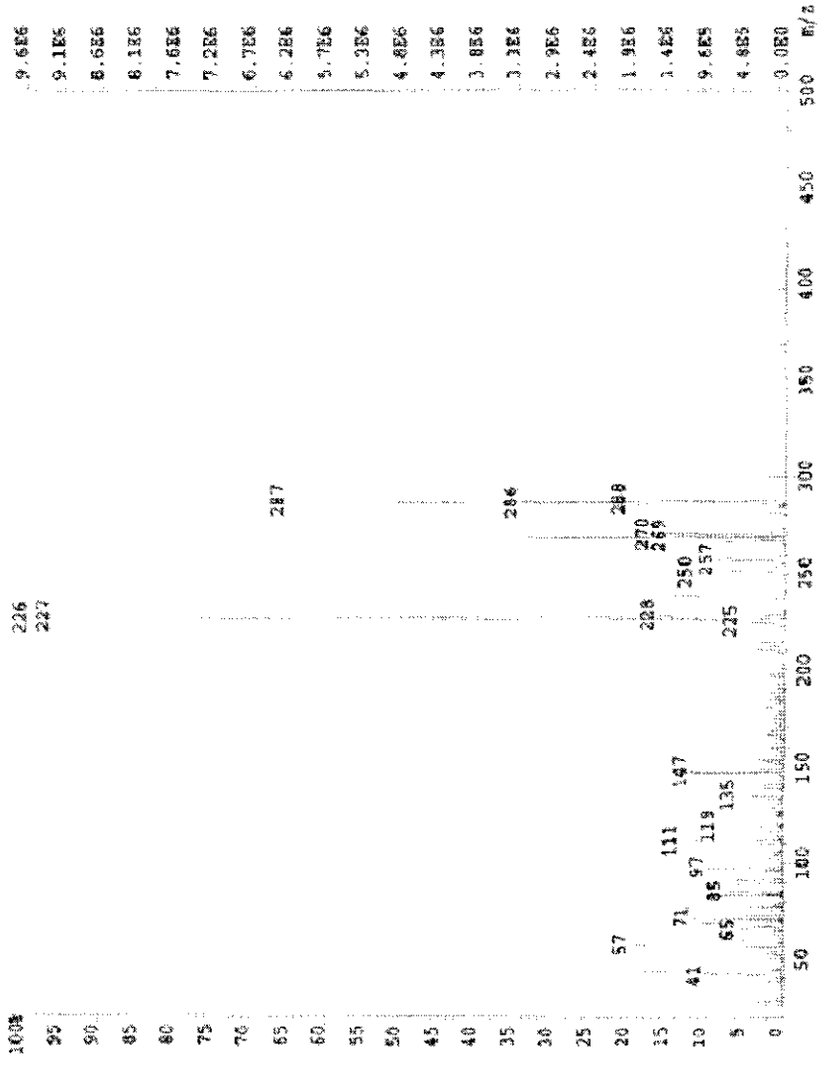
SPECTRUM 6



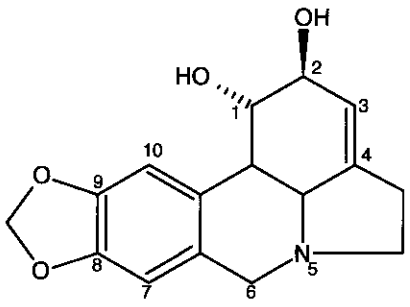


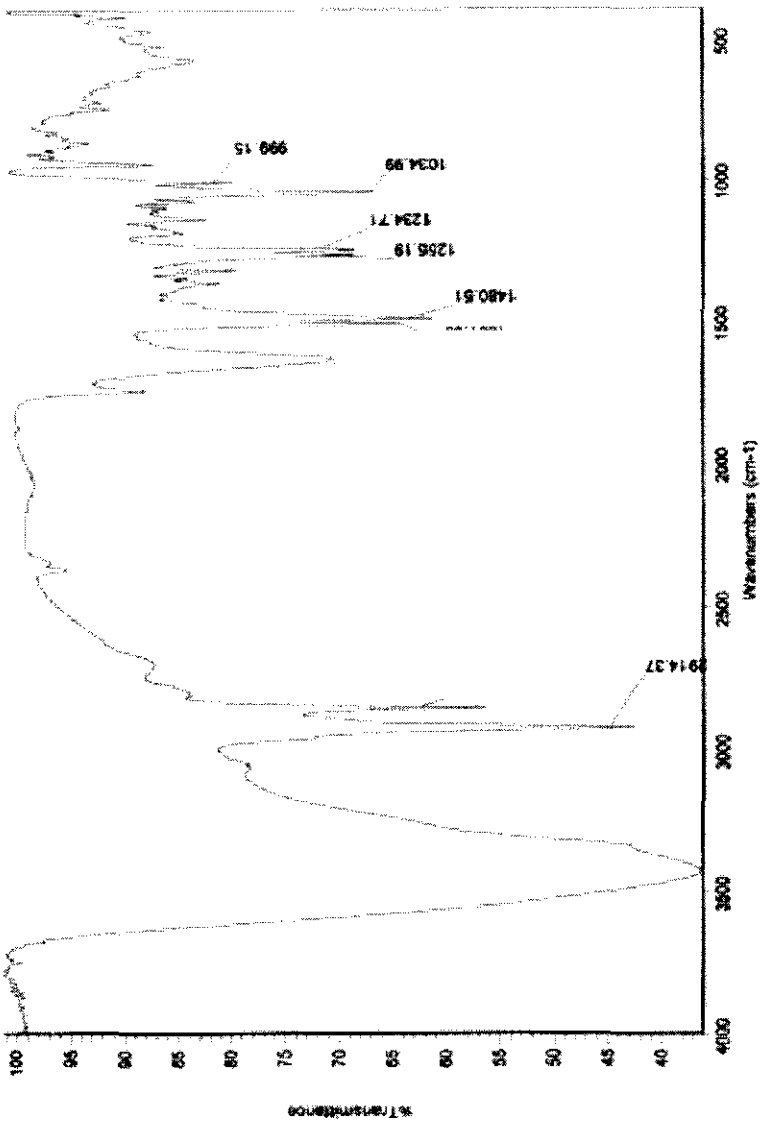
SPECTRUM 7


OH
HO
1
2
3
4
5
6
7
8
9
10
O
O
N

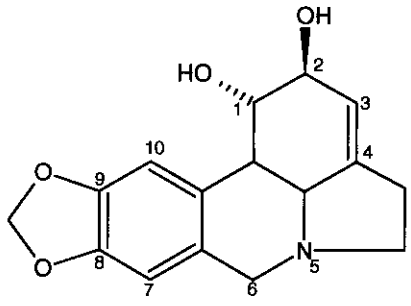

m/z
287
286
288
270
269
250
257
226
227
228
225
147
135
111
119
97
85
71
65
57
41

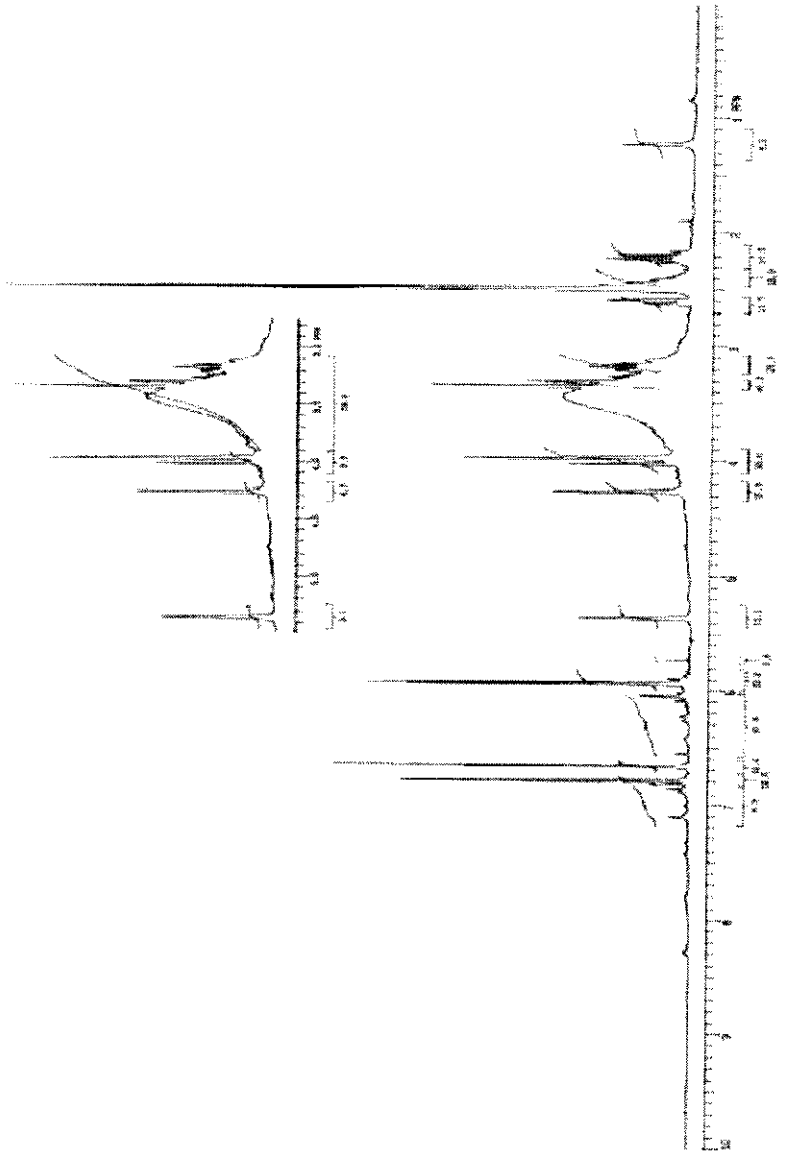
SPECTRUM 8


OH
HO
1
2
3
4
5
6
7
8
9
10
O
O
N


999.15
1034.99
1234.71
1266.19
1480.51
2914.37
Wavenumbers (cm-1)

SPECTRUM 9





SPECTRUM 10