

**Antimalarial activity and cytotoxicity of some South African
medicinal plants and their active constituents**

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DECLARATION

Investigation of antimalarial activity of South African medicinal plants and chemical identification of antiplasmodially active compounds

I, Mamello Sekhoacha hereby declare that the studies in this thesis are the result of my own investigations, except where acknowledgements indicate otherwise, and neither the substance nor any part of the above thesis has been, or is being submitted for another degree at this university or any other academic Institution.

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ABSTRACT

Background

Malaria continues to be a health threat in many parts of the world, claiming 2-3 million lives annually. Treatment and control of malaria have become more difficult with the spread of parasite resistance to modern antimalarial drugs and limited availability of effective and safe alternatives. This situation has highlighted the need for new effective antimalarials.

Traditional herbal remedies have been used to treat malaria for thousands of years and continue to be a source of treatment in areas where access to effective antimalarial drugs is difficult. In South Africa, over 3000 indigenous plant species are used for medicinal purposes. South African flora, by virtue of its biodiversity has a significant role to play in being able to provide new leads that can be developed into antimalarials. However, little work has been done to investigate the antimalarial potential of medicinal plants indigenous or endemic to South Africa. The few plants that have been shown to possess antiplasmodial activity have not been assessed for toxicity. Of some of the plants that have shown positive antiplasmodial activity, the constituents responsible for activity remain unidentified. In the search for antimalarial lead compounds, this thesis describes the investigation of *in vitro* and *in vivo* antiplasmodial activity and toxicity of five plants, and the isolation and characterisation of plant constituents that are responsible for activity.

Methods and Findings

In a small-scale ethnobotanical survey, 10 plants species endemic to South Africa were reported for use in treatment of malaria and fever. Based on the preliminary screening of the plants extracts, five plant species; *Tarnconanthus camphoratus*, *Eriocephelus africanus*, *Warburgia salutaris*, *Achillea millefolium* and *Agathosma betulina*, were selected for further investigation. The *in vitro* antiplasmodial activity of the plant extracts was assessed against cultured blood stage parasites of

Plasmodium falciparum using the parasite Lactate Dehydrogenase assay. Extracts which exhibited antiplasmodial activity of 10 μgml^{-1} and below were considered active. Cytotoxicity was tested on Chinese Hamster Ovarian mammalian cell line (CHO) in an *in vitro* 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay. Plant extracts that exhibited antiplasmodial activity were subjected to fractionation using a combination of chromatography techniques. Bioassay-guided fractionation led to the identification of compound(s) responsible for the antiplasmodial activity in each plant extract. The identity of the compounds was determined by two dimensional ^1H and ^{13}C nuclear magnetic resonance (NMR) spectroscopy, or Gas Chromatography Mass Spectrometer (GC-MS). The *in vivo* antimalarial activity of the extracts and selected fractions was determined using modified protocols of a 4-day suppressive test in *Plasmodium berghei* ANKA mouse model.

The dichloromethane (DCM) extract of *T. camphoratus* possessed *in vitro* antiplasmodial activity ($\text{IC}_{50} = 7.7 \mu\text{gml}^{-1}$) and low cytotoxicity ($\text{IC}_{50} > 100 \mu\text{gml}^{-1}$). Bio-assay guided fractionation using a combination of Solid Phase Extraction (SPE) and High Performance Liquid Chromatography (HPLC) led to isolation of 5,7 dihydroxy-flavanone, which showed antiplasmodial activity against CQ sensitive D10 ($\text{IC}_{50} = 2.2 \mu\text{gml}^{-1}$) and CQ resistant K1 ($\text{IC}_{50} = 2.4 \mu\text{gml}^{-1}$) strains of *P. falciparum*, and low *in vitro* cytotoxicity ($\text{IC}_{50} = 89.1 \mu\text{gml}^{-1}$). The DCM extract of *E. africanus* showed antiplasmodial activity against D10 ($\text{IC}_{50} = 8.5 \mu\text{gml}^{-1}$) and K1 ($\text{IC}_{50} = 8.7 \mu\text{gml}^{-1}$) and low cytotoxicity ($\text{IC}_{50} = 89.1 \mu\text{gml}^{-1}$). Fractionation of the extract led to the isolation of four compounds, three of which were identified as carnosol, Lup-20(29)-en-28-oic acid, and 5, 7-dimethoxy-4'-hydroxyflavone. The compounds had antiplasmodial activity with IC_{50} values 7.6 μgml^{-1} , 9.5 μgml^{-1} , and 6.5 μgml^{-1} against D10 respectively. The unidentified compound showed activity against D10 with IC_{50} of 6.9 μgml^{-1} . The identified compounds showed varying degrees of toxicity with IC_{50} values 4.4 μgml^{-1} , 9.6 μgml^{-1} , and 35.5 μgml^{-1} respectively. The ethanol (EtOH) leaf extract of *W. salutaris* possessed antiplasmodial activity against D10 ($\text{IC}_{50} = 2.5 \mu\text{gml}^{-1}$) and K1 ($\text{IC}_{50} = 2.8 \mu\text{gml}^{-1}$) strains. The DCM twig extract of this plant showed activity with IC_{50} values of 1.8 μgml^{-1} and 3.6 μgml^{-1} against D10 and K1 respectively. The active plant constituents were identified as cinnamolide ($\text{IC}_{50} = 1.4$

μgml^{-1}) and 3 β -acetoxy-cinnamolide ($\text{IC}_{50} = 0.84 \mu\text{gml}^{-1}$). Interestingly, cinnamolide showed less toxicity ($\text{IC}_{50} = 30.5 \mu\text{gml}^{-1}$) than its derivative ($\text{IC}_{50} = 3.6 \mu\text{gml}^{-1}$).

The DCM extract of *A. millefolium* exhibited similar antiplasmodial activity against both D10 ($\text{IC}_{50} = 3.9 \mu\text{gml}^{-1}$) and K1 ($\text{IC}_{50} = 3.8 \mu\text{gml}^{-1}$). A combination of flash chromatography and column chromatography led to semi-pure fractions with enhanced activity ($\text{IC}_{50} = 1.3- 2.8 \mu\text{gml}^{-1}$), and the isolation of an active constituent which possessed antiplasmodial activity with IC_{50} of $4.4 \mu\text{gml}^{-1}$ against D10 and low cytotoxicity ($\text{IC}_{50} > 100 \mu\text{gml}^{-1}$). Although the DCM leaf extract of *A. betulina* had a low antiplasmodial activity ($\text{IC}_{50} = 14.4 \mu\text{gml}^{-1}$) against D10, its SPE fractions showed enhanced activity ranging from 1.6 to $2.0 \mu\text{gml}^{-1}$, and low toxicity ($\text{IC}_{50} > 100 \mu\text{gml}^{-1}$). The active components within each fraction were identified by GC-MS. Some of the identified constituents have been reported previously as components of the essential oil and fatty acid composition of the plant.

The acute toxicity experiments performed in mice revealed that the DCM crude extracts of *A. millefolium*, *A. betulina*, *T. camphoratus* and EtOH extract of *W. salutaris* had no toxic effects when administered at a dose of 500mg/kg per day for 7 consecutive days. The DCM crude extract of *E. africanus* had effects of toxicity at oral doses of 500mg/kg and 250mg/kg per day. The antiplasmodial suppressive test showed that extracts of *A. millefolium*, *W. salutaris* and *A. betulina* possessed antimalarial activity with an average chemosuppression percentages of 46.5%, 95%, and 55.7% respectively. The selected active fraction of *W. salutaris* showed good suppressive and curative schizontocidal activity when administered both orally and subcutaneously. The fraction however, exhibited no prophylactic activity. The active fraction of *A. betulina* extract exhibited opposite results. The fraction possessed prophylactic activity when administered 72hours before infection, but showed no schizontocidal curative or suppressive antiplasmodial activity when administered at a dose of 250mg/kg per day.

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LIST OF ABBREVIATIONS

ACN	acetonitrile
APAD	3-acetylpyridine adenine dinucleotide
CHO	chinese hamster ovarian (cells)
COSY	proton-proton homonuclear shift correlated spectroscopy
CQ	chloroquine
DHFR	dihydrofolate reductase
DHPS	dihydropteroate synthetase
DMEM	dulbecco's Modified Eagles Medium
DMSO	dimethylsulphoxide
<i>et al.</i>	and all others
EtOAc	ethyl acetate
FCS	fetal calf serum
g	gram(g)
HMQC	heteronuclear multiple bond connectivity
HPLC	high pressure liquid chromatography
HREIMS	high resolution electron impact mass spectrometry
hrs	hours
HSQC	heteronuclear single quantum coherence
Hz	hertz
IC50	50% inhibitory concentration
J	coupling constant
Kg	kilogram(s)
MeOH	methanol
mg	milligram(s)
min	minutes
ml	millilitre
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
ND	not done
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser enhancement and exchange spectroscopy

NOESY	nuclear overhauser enhancement and exchange spectroscopy
PBS	phosphate buffered saline
PLDH	parasite lactate dehydrogenase
ppm	parts per million
rpm	revolutions per minute
SD	standard deviation
SI	selectivity index
SPE	solid phase extraction
TLC	thin layer chromatography
TMS	tetramethylsilane
UV	ultraviolet (radiation)
WHO	World Health Organisation
λ	Wavelength
μg	microgram(s)

Glossary of terms

Antiplasmodial activity: the ability of the test sample to kill the parasite.

Antimalarial activity: the ability of the test sample to kill or suppress parasite growth, and inhibit expression of the typical malaria symptoms.

Chemosuppression: the measure of activity of the test sample in suppressing parasite growth in comparison with the control group.

Chapter 1

University of Cape Town

1 Introduction and Literature Review

1.1 Malaria

Malaria is a parasitic infectious disease caused by protozoa belonging to the genus *Plasmodium*. Parasitic protozoa are single celled organisms known to cause a variety of diseases in many animals including humans.

Malaria is a serious infectious disease that kills more people than any other communicable disease, except tuberculosis and HIV/AIDS. It is estimated that there are 300-500 million cases of malaria annually across the world with a mortality of 2-3 million deaths, of which one million are children [WHO, 2003; WHO, 1997]. Children and pregnant women are at special risk to malaria [Desai *et al.*, 2007]. Over 80 percent of the reported cases occur in Africa, while one million deaths occur in sub-Saharan Africa alone. [WHO, 1989; WHO, 2000; Beiersman, 2007]. Malaria has now become a health threat in more than 90 countries, which are inhabited by 40 percent of the world's population [Sharp, 1993] (Figure 1.1.1.1).

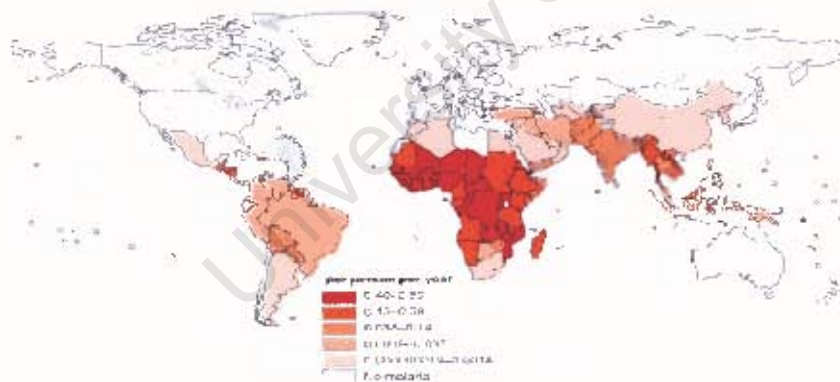


Figure 1.1.1.1 The estimated incidence of clinical malaria episodes, caused by any species of *Plasmodium*. Source: <http://www.rbm.who.int/wmr2005/html/map3.htm>

Malaria epidemics are more frequent in tropical and semi-arid areas, where the warm and humid climate favours increased vector breeding and survival [Abeku, 2007]. There is however a trend of increasing malaria transmission in highland areas in Africa. Malaria is geographically specific, but the mass movement of refugees, migrant workers and non-immune travellers across borders have facilitated the spread

of the disease across the globe. There has also been an increase in the number of reported cases of imported malaria in Europe and North America, with over 30 000 cases annually. France which has the largest numbers of migrants of African origin, United Kingdom, United States, Italy and Germany were top countries with the highest number of cases of imported malaria [Legros *et al.*, 2007].

1.1.1 Plasmodium species affecting mammals

There are four species of *Plasmodium* which are able to infect humans; *Plasmodium ovale*, *Plasmodium vivax*, *Plasmodium malariae* and *Plasmodium falciparum*. Of the four species, *P. falciparum* and *P. vivax* are the most common causative agents of severe malaria. *P. falciparum* is the most virulent and accounts for the majority of infections and deaths. *P. malariae* has a low prevalence even in malaria endemic areas, while *P. ovale* is rarely reported as a cause of morbidity [Mims *et al.*, 2004 and Mueller *et al.*, 2007]. Other *Plasmodium* species that are mammalian pathogens include *P. berghei*, *P. chabaudi*, *P. yoeli* and *P. vinckei*. These species generally affect lower mammals.

1.1.2 Transmission of Malaria

Malaria parasites are transmitted by the various species of female *Anopheles* mosquitoes. The parasite needs two hosts in order to complete its life cycle; the human host and the mosquitoes. All four *Plasmodium* species that affect humans have a similar life cycle [Khan and Waters, 2004].

1.1.3 The life cycle of the malaria parasite

The complex life cycle of *Plasmodium* comprises of four phases. The first phase is sexual but without multiplication while the following three phases are asexual with multiplication. The sexual and first asexual phases occur in the mosquitoes while the last two asexual phases occur in the infected human host (Figure 1.1.3.1).

When an infected mosquito bites an individual, a small amount of saliva containing an anticoagulant and sporozoites enters the bloodstream. Sporozoites migrate through the lymphatics and bloodstream to the liver and rapidly invade hepatocytes. In the liver,

the parasite undergoes a series of asexual replications. The parasite grows and undergoes several nuclear divisions without the cytoplasm dividing. Division of the cytoplasm then follows, and the infected hepatocytes rupture and release thousands of small offspring, merozoites into the blood stream to invade host erythrocytes. Every successful sporozoite can produce some 20,000 merozoites [Ménard, 2000].

In the intra-erythrocytic stage the parasites feed upon the host cell's haemoglobin and undergo developmental stages [Prescott, 1993]. Merozoites develop into trophozoites, which divide asexually to produce schizonts. Schizonts divide and rupture the erythrocyte to release 16-32 merozoites per schizont [Bannister *et al.*, 2002]. It is during the schizont rupture that a toxin ferriprotoporphyrin, also known as heam, is released. This intra-erythrocytic stage of the parasite is cyclic and is repeated every 48 or 72 hours depending on the species of the parasite.

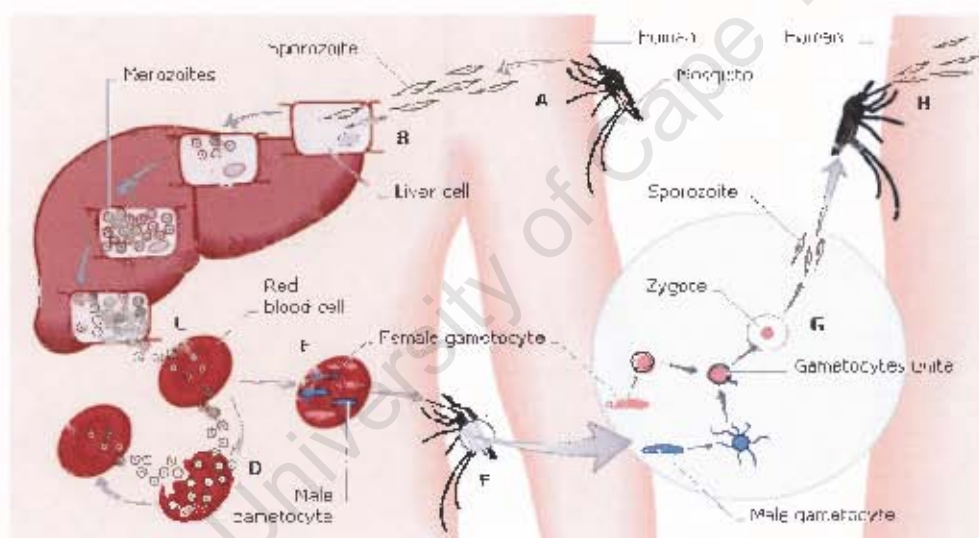


Figure 1.1.3.1 The life cycle of the malaria parasite, *Plasmodium*. Source: http://encarta.msn.com/media_461541582/Life_Cycle_of_the_Malaria_Parasite.html

While in the erythrocytes, merozoites can develop into male or female gametocytes that can be ingested by a mosquito during a blood feed. Once inside the mosquito gut, the gametocytes fuse to produce a zygote. The zygote asexually replicates and differentiates to form sporozoites that spread to the mosquito's salivary glands and can be inoculated into a mammalian host.

1.1.4 The Pathogenesis of Malaria

During development in the erythrocyte, the parasite depends on the uptake of nutrients and essential solutes such as hemoglobin from the host cell or from the extracellular environment for survival [Goldberg *et al.*, 1990]. The parasite feeds on the globin component of haemoglobin, and releases ferriprotoporphyrin (haem) toxin into the blood stream. The toxin causes a cytokine response that leads to the typical malaria symptoms. [Berman, 2004]. The haem is toxic to the parasite. However the parasite has developed mechanisms to detoxify the haem by polymerising it to form nontoxic haemozoin [Fitch *et al.*, 1999 and Pandey *et al.*, 2003].

Malaria caused by *P. falciparum* is called falciparum malaria. *P. falciparum* malaria is considered a serious illness. It is a much more progressive and lethal illness. The typical symptoms are high fever, weakness, chills, loss of appetite, diarrhea, vomiting, nausea, headache and malaise [Beiersmann *et al.*, 2007; Legros *et al.*, 2007; English *et al.*, 1996]. Irregular tertian fever is experienced at first, followed by dysfunction of main organs such as the brain, liver, spleen, kidneys and lungs. Then there is rapid deterioration of the patient into stupor, fits and coma. From coma, the end result is usually death, especially if the patient is not treated immediately [MacPherson *et al.*, 1985; Hearn *et al.*, 2000; Idro *et al.*, 2005; Heddini, 2002].

1.1.5 Partial Immunity to Malaria

The severity of the malaria illness depends mostly on the immunity of the person who is infected. Indigenous people and inhabitants in endemic areas have developed partial immunity called premonition [Soe-Soe *et al.*, 2001]. In such people, parasites are commonly found in their blood without the experience of malaria symptoms [Thomson, 1933]. Immunity to a specific species of *Plasmodium* is acquired slowly in response to repeated infections of that species. Partial immunity is short-lived and can be lost if there is no longer a continuous exposure to the infection. People who have acquired immunity may contribute to some extent to the spread of the parasites through blood transfusion and as carriers of parasites to help complete the life cycle.

Hereditary traits such as glucose-6-phosphate dehydrogenase deficiency, sickle cell anaemia, β -thalassaemia and haemoglobin E, absence of a duffy blood factor, are partially protective against malaria [Miller *et al.*, 1976; Hutagalung *et al.*, 1999; Edingto and Watson 1965; Yuthavon and Wilairat 1993; Gilles, 1967; Martin, 1994].

1.1.6 Prevention and Control of Malaria

The type and target of preventive interventions in a country depends on the cost-effectiveness, time, and availability of both financial and human resources [Abeku, 2007]. Preventive measures for malaria involve use of insecticide-treated bednets, repellents and protective clothing. Complete prevention of malaria has unfortunately been set back by a widespread resistance of the vector mosquitoes to insecticides such as DDT [Subbarao, 1988; Corbel *et al.*, 2007].

The interventions aimed at controlling and containing the epidemic include case management through prompt diagnosis, antimalaria therapy and vector control. Vector control encompasses indoor residual spraying with insecticides, and larvicide's, and proper environmental management to limit the number of mosquito breeding sites.

1.1.6.1 Vaccine for malaria

Vaccination is regarded as the most cost-effective and powerful prevention strategy for attacking the malaria problem [Graves, 1998]. The rapidly growing problem of drug resistance and resistance of the vector to insecticides have made vaccine development highly imperative. Unfortunately production of a safe, effective and affordable vaccine for malaria has not been successful. This is mainly due to the complexity of the malaria parasites and the difficulty of identifying and selecting the appropriate protective antigens from a parasite [Webster, 2003; Xin-zhuan *et al.*, 2003].

However, significant progress has been made in malarial vaccine research. While a number of potential targets in the parasite have been identified, recent research has proved that the whole organism strategy and strategies that combine many antigens to

induce responses are more successful than those based on single antigens [Pinzo-Charry *et al.*, 2006]. Several candidate malaria vaccines are being investigated, and some are in early clinical trials [Ghosh *et al.*, 2002, Schellenberg *et al.*, 2005 and Girard *et al.*, 2006]. Some of the milestones achieved include the launch of a phase I b candidate malaria vaccine trial by The African Malaria Network Trust [Wanga, 2007] and phase II b trials [Alonso *et al.*, 2004; Snounou *et al.*, 2005].

1.1.6.2 Treatment of malaria

In the absence of a vaccine for malaria, chemotherapy remains the major approach to combating the disease. Unfortunately, other than the artemisinin derivatives, there are few effective antimalarial drugs on the market, as the malaria parasite has grown resistant to most of them [Peters, 1998]. Meanwhile, more effort is directed towards improving on other crucial factors such as early diagnosis, correct dosing regime, and use of combination therapy, to influence the epidemiology of the disease.

1.1.6.2.1 Classes of antimalarial drugs

There are three main groups of antimalarial drugs in use; the quinoline based antimalarials, the antifolates, and artemisinin and its derivatives. *P. falciparum* has developed resistance to almost all drugs except artemisinin and its derivatives.

1.1.6.2.1.1 Quinoline-containing antimalarials

Quinolines have been the most important class of antimalarial drugs. These include quinine, quinidine, synthetic derivatives; chloroquine, mefloquine, primaquine, and amodiaquine (Figure 1.1.6.1).

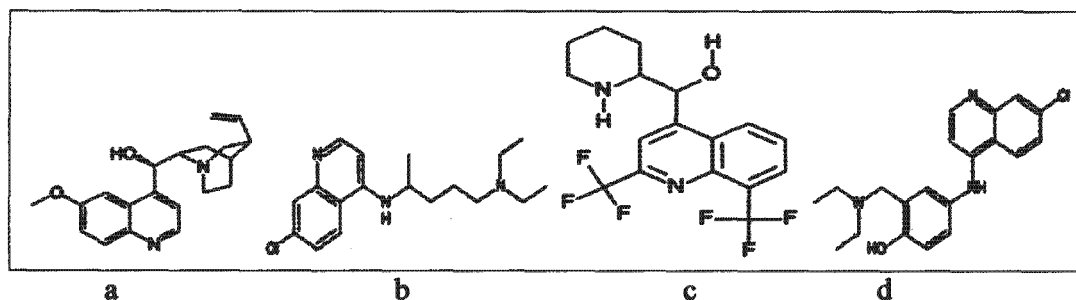


Figure 1.1.6.1 Chemical structures of the antimalarial drugs quinine (a), chloroquine (b), mefloquine (c), amodiaquine (d).

1.1.6.2.1.2 Quinine and Quinidine

Quinine and quinidine are two alkaloids isolated in 1820 from the bark of the Cinchona tree, which is native to South America [Foley and Tilley, 1998]. Quinine was one of the first effective treatment drugs for malaria until the emergence of parasite drug resistance in the 1940s. Resistance has since spread at varying degrees across the world [Gregson and Plowe, 2005]. Quinine is now used as a second or third line drug in Africa for treatment of severe cases of chloroquine resistant malaria [Wongsrichanalai *et al.*, 2002]. Quinidine, which can also be prepared from quinine, is used mostly as an antimalarial in the US but not in most African countries. Both quinine and quinidine have a gametocidal effect on *P. falciparum* and *P. vivax* [Skinner, 1996] and lethal effects on late rings, trophozoites and early schizonts stages of the parasite. Their mode of action is through inhibition of the formation of the malaria pigment, haemozoin.

1.1.6.2.1.3 Chloroquine

Chloroquine (CQ) is a synthetic analogue of quinine. CQ has played a major role in the malaria eradication campaigns of the 1950s and 1960s. The wide use of CQ is attributed to its low cost, safety and suitability for oral administration. Unfortunately, resistance to CQ emerged in the 1960s, and has now spread across almost all malaria endemic parts of Africa. CQ however, is still being used for economic reasons even though its efficacy is on the decline [Sweetny, 1981; Bruce-Chwaat *et al.*, 1986; Chauhan *et al.*, 2001].

Mode of action of Chloroquine

There are a number of hypotheses on the possible mode of action of CQ [Parker and Irvin, 1952; Vander *et al.*, 1986; Welles, 1992; Surolia *et al.*, 1991]. The most common hypothesis is that CQ acts primarily by interfering with haem polymerization in the intra-erythrocytic stage of the parasite life-cycle [Egan *et al.*, 1994]. Free haem is toxic to the parasite. The parasite has developed a mechanism of haem detoxification by polymerizing haem to form a non-toxic haemozoin polymer. In the

absence of CQ, haem is degraded by glutathione and polymerized. There has been evidence that CQ inhibits haem polymerisation by 'capping the growing polymer thereby terminating further growth. Also CQ binds to haem dimers to form haem-CQ complexes which can bind to growing haemozoin and inhibit polymerization. This results in accumulation of haem in the parasite membrane, which disrupts membrane barrier properties in the parasite and leads to parasite death [Dorn *et al.*, 1998].

1.1.6.2.1.3.1 Mode of chloroquine resistance

A few mechanisms of CQ resistance have been proposed. Evidence has shown that in resistant strains of the parasite, there is less accumulation of CQ than in sensitive strains. The actual mechanism of how the parasite attains the decrease in CQ accumulation is thought to be due to the high rate of CQ efflux, a lower rate of CQ uptake, or a varying combination of both of these mechanisms. CQ resistance reversal by drugs such as verapamil, desipramine and chlorpromazine support the hypothesis of an enhanced CQ efflux mechanism [Krogstad *et al.*, 1987; Martin *et al.*, 1987; Reed *et al.*, 2000]. The enhanced efflux rate has been linked to mutations found in the *P. falciparum* multidrug resistance gene (*pfmdr1*) [Wellems, 1990; Foote *et al.*, 1990] and in *P. falciparum* CQ related transporter gene (*pfcr1*). [Sidhu *et al.*, 2002].

1.1.6.2.1.4 Other quinoline- based antimalarial drugs

Amodiaquine is a CQ analogue. Following reports on resistance to CQ, amodiaquine remained effective against many CQ resistant strains of *Plasmodium* in many parts of Africa [Olliaro *et al.*, 1996], but resistance to amodiaquine has since escalated. The other class of quinolines, the aryl amino alcohol derivatives of quinine, includes primaquine and mefloquine. These are well tolerated but highly expensive. While primaquine is active only against the liver stages of the parasite, its analogue, bulaquine was shown to have gametocidal activity. The subclass of 9-phenanthrenemethanols such as halofantrine and bisquinoline derivatives are yet another group of antimalarials which proved to be effective against CQ resistant strains but have been restricted by reports of serious toxicity. Resistance to

mefloquine and halofantrine developed very quickly and has become a major problem [Chauhan *et al.*, 2003].

1.1.6.2.1.5 Antifolates antimalarials

Antifolates are synthetic group of drugs that target the parasite-specific enzymes. These drugs were designed through synthetic medicinal chemistry and understanding of the parasite biochemical pathways at a molecular level. Antifolates are divided in to two classes; the dihydropteroate synthase (DHPS) inhibitors (sulfadoxine and dapsone), and the dihydrofolate reductase (DHFR) inhibitors (Pyrimethamine and proguanil) (Figure 1.1.6.2).

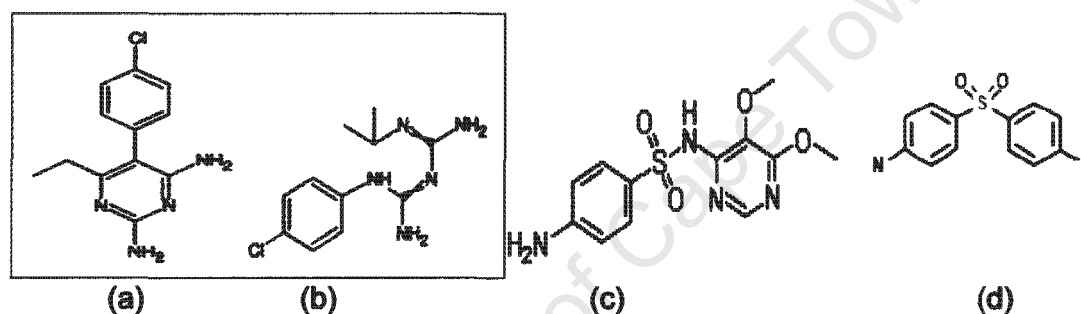


Figure 1.1.6.2 Chemical structures of pyrimethamine (a), proguanil (b), sulfadoxine (c), dapsone (d). Source: http://redpoll.Pharmacy.uslberta.ca/drugbank/drugbank/PC_IMAGE

1.1.6.2.1.5.1 Mechanism of action of antifolates

Antifolates act by inhibiting the synthesis of folic acid, by interfering with the folate synthesis pathway [John and Hyde, 2005]. Tetrahydrofolate is the essential co-factor for the synthesis of pyrimidines and purines, which are the bases needed for DNA replication [Schellenberg and Coatney, 1961; Gutteridge and Trigy, 1971]. The folate metabolic pathway is unique to the parasite. Humans cannot synthesize folic acid but obtain it from their diet. This makes the parasite distinguishably susceptible to folate synthesis inhibitors [Hyde, 2002].

Antifolate drugs target the enzymes that catalyze various steps of folate synthesis process. DHFR inhibitors competitively inhibit DHFR by mimicking the structure of its natural substrate, *p*-aminobenzoic acid [Zhang and Meshnick, 1991], while DHPS

inhibitors bind with high affinity to DHPS enzyme, thereby blocking the synthesis of folate [Diggens *et al.*, 1970]. Since the parasite is unable to take up pyrimidines from the host, inhibition of folate synthesis retards DNA replication in the parasite, leading to parasite death.

1.1.6.2.1.5.2 Mechanism of resistance

Resistance to DHPS and DHFR inhibitors is largely due to combinations of amino acid substitution mutations in the DHPS and DHFR enzymes [Delfino *et al.*, 2002]. These mutations possibly alter the structure (size or the shape) of the target site on the enzyme, which could impair proper binding and reduce the affinity of the inhibitors. Some degree of resistance is due to the use of an alternative pathway to salvage folate [Wang *et al.*, 1997; Olliaro and Yuthavong, 1999].

1.1.6.2.1.5.3 Antifolate-based combination therapy

Increasing resistance to antifolates prompted the use of drugs in combination, which proved to be more potent against resistant parasites. Usually two drugs with different mechanisms of action or a drug with short-half life and another with a long-half life are combined to minimize the occurrence of drug resistance [Trape, 2001].

Sulfadoxine-pyrimethamine, also known as Fansidar® produced a synergistic effect on inhibiting the folate synthesis. Although the failure rates of 45%-60% in trials have been reported [Winstanley, 2001; Winstanley *et al.*, 2002], a number of African countries still use Fansidar® as the first line treatment drug against uncomplicated malaria. Unfortunately, resistance to Fansidar has now spread worldwide [Sharma, 1990; Talisuna *et al.*, 2004; 2002; Nzila *et al.*, 2000; TaKecki *et al.*, 2001]. Chlorproguanil-dapsone (LAPDAP) is another low cost combination which proved to be effective against resistant parasites. Resistance to LAPDAP has been reported but is less compared to the resistance seen for Fansidar® [AHFS Drug Information, 2000; Nzila *et al.*, 2000]. Atovaquone-proguanil, also known as Malarone® is another combination first introduced in 2000. It has high efficacy to multi-drug resistant

strains. Resistance to Maleron® was reported only two years after its introduction, and has since escalated [Farnert *et al.*, 2003 and Fivelman *et al.*, 2002].

1.1.6.2.1.6 Artemisinin and derivatives

Artemisinin (Qinghaousin) is a sesquiterpene lactone peroxide, which was first isolated in 1971 from the aerial parts of *Artemisia annua* L., a Chinese herb used traditionally to treat fever and malaria [Klayman, 1985; Li and Rieckmann, 1992; Dhingra *et al.*, 2000]. Artemisinin and its derivatives (Figure 1.1.6.3) are known for their rapid activity and high efficacy against multi-drug resistant strains of *Plasmodium* [Hein and White, 1993; van Agtmael *et al.*, 1999].

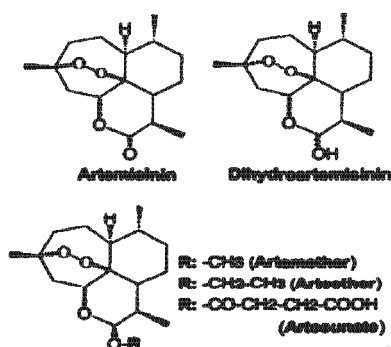


Figure 1.1.6.3 The structures of Artemisinin and its derivatives. Source: International Journal of Pharmaceuticals 2007.

The great therapeutic value of artemisinin is limited by a number of factors such as short half-life, neurotoxicity, and low solubility leading to low bioavailability [Balint *et al.*, 2001; Akamita, 2005]. As a result, attempts to improve the pharmacokinetic parameters of artemisinin have led to the development of several semi-synthetic derivatives; dihydroartemisinin, artemether, arteether and artesunate, which have greater stability, lower toxicity and greater potency [van Agtmael *et al.*, 1999].

1.1.6.2.1.6.1 Mode of action

Artemisinin and its derivatives contain an endoperoxide moiety, which is crucial for their antimalarial activity [Meshnick *et al.*, 1996 and O'Neill *et al.*, 2001]. The exact

mechanism is not thoroughly understood at present. However, there is evidence that the peroxide bridge is cleaved, consequently generating artemisinin-based free radicals, which alkylate parasite proteins through formation of covalent bonds [Jefford, 2001]. It has also been reported that artemisinin acts by inhibiting cytochrome oxidase, which occurs at the nuclear and food vacuole membrane as well as in the mitochondria of trophozoites of *Plasmodium* parasite [Zhao *et al.*, 1986]. Artemisinin and its derivatives kill the parasites at blood stages of their development.

1.1.6.2.1.7 Artemisinin-based Combination Therapy (ACT)

There is no evidence of sustained drug resistance to artemisinin and any of its derivatives up to date. However, their short half-lives often lead to recrudescence of parasites after treatment. As a result, artemisinin is used in combination with drugs with long half-lives [van Agtmael, 1999]. An example is artemether-lumefantrine combination, which is used as a first-line treatment of uncomplicated malaria [Olumese, 2006].

1.1.7 Malaria and drug resistance in South Africa

South Africa has had a significant increase of malaria cases since 1996. The disease reached epidemic levels in 2000. Malaria is distributed unevenly across the 10 provinces in the country. The disease is endemic in three provinces; Mpumalanga, Limpopo and KwaZulu-Natal. KwaZulu-Natal is at the top with over 50 000 cases of malaria and over 400 deaths reported in 2003 (DoH National Malaria Update, 2003). In the year 2005 the annual number of reported malaria cases was approximately 7 755 while in 2006 it went up to 12 098 (Department of Health, 2007).

In South Africa, malaria transmission is distinctively seasonal according to local climate. In KwaZulu-Natal and Mpumalanga provinces, malaria transmission occurs in the rainy season, while in Limpopo province, the transmission reaches the peak during the summer months [DoH, 2000, DoH National Malaria Update, 2003].

Containment of the disease has been challenged by the emergence of drug resistant parasites. In all three provinces CQ was used as the first-line drug, but had to be

The parasite strain is considered resistant when it is able to survive and multiply in the presence of concentrations of a drug that normally destroy parasites of the same species. But the common use of the term drug resistance usually relates to complete resistance, which means the ability of the parasite strain to withstand maximum doses tolerated by the host [Wernsdorfer, 1991].

The development of resistance of the malarial parasite to current antimalarials has emphasised the need to search for new antimalarial agents with alternative mechanisms of action. One of the approaches to antimalarial drug discovery is the investigation of medicinal plants used traditionally to treat malaria and malaria-like symptoms.

1.2 Traditional Medicine and Traditional Healing

Traditional medicine refers to the health practices, approach, knowledge and beliefs that usually incorporate plant-based medicine therapy, which are applied to treat, diagnose, prevent illness, and maintain well-being [Babb *et al.*, 2004]. Traditional healing is seen as a holistic approach to illness, which addresses treatment of symptoms, psychological, spiritual, and social aspects of a patient [Goldman, 2001]. It can be intertwined with religion, cultural beliefs and healing practices. These practices are usually administered by traditional practitioners.

1.2.1 Use of Traditional Medicine

Traditional medicine and traditional healing have been practised for thousands of years in countries such as China, India and Africa. Today multitudes of people, especially populations in rural areas of Africa, Asia and Latin America still consult traditional healers for health care. WHO estimates that 80% of the world's population, mostly people living in developing countries rely heavily upon traditional medicines for primary health care and as a source of remedies for treatment of diseases [WHO, 2002]. Over the past few decades, there has been an increasing interest in traditional medicines. The growing utilisation of traditional medicine could be attributed to the fact that traditional medicine is more easily accessible than modern medicine,

especially in most rural areas where modern health institutes are far and there is shortage of health professionals. Populations in urban areas also continue to consult traditional healers and use herbal medicine because it forms part of their culture [Beirsmann *et al.*, 2007]. Traditional medicine is more affordable and people can use other means of payment convenient to them. Reported records of adverse effects are less frequent in patients taking herbal medicines compared to recorded adverse effects from allopathic medicines [Willcox and Bodeker, 2000]. Furthermore, not many cases of organism resistance to plant extracts have been reported [Gilani *et al.*, 2005].

1.2.2 Traditional Medicine in South Africa

There is a strong history of utilization of traditional medicine in South Africa. It is estimated that almost 80% of the South Africa population consult traditional healers, and that there is 1:10 ratio of traditional healers to general practitioners in the country [Edinburg, 1998]. Although traditional practitioners are classified differently by various cultural communities in South Africa, the term traditional healer is used generally to describe a practitioner of indigenous medicine, which includes herbalists, diviners and faith healers.

The interest in traditional medicine as alternative treatment for ailments has increased in the last decade owing to the acceptance and understanding of practitioners of traditional, indigenous and alternative systems of medicine. The South African government recognizes traditional medicine as an important potential resource for primary health care. The ministry of Health has made advances in the process of reassessing traditional medicine and integrating it in the national health care system. This process encompasses the evaluation, registration, and regulation of traditional medicines, registration of qualified traditional healers, co-operation between western and traditional practitioners, and promotion of research for the validation of safety and efficacy of traditional medicines [Edinburg, 1998; Baleta, 1998].

1.2.3 Use of plants in traditional medicine and their potential as sources of new drugs

Medicinal plants form the principal component of traditional medicine. They have invariably been a rich source for new drugs and continue to provide effective treatments both in places where pharmaceuticals are unaffordable or not available, and where the disease is resistant to commonly prescribed drugs [Tran *et al.*, 2003]. The majority of people in the world today rely almost entirely on plant-derived medicines [WHO, 2002], causing an exponential increase in the market for plant-based products [Raskin *et al.*, 2002; Dubey *et al.*, 2004; Voigt, 2006].

A number of biologically active molecules such as antiviral, anti-parasitic, anti-microbial and anti-inflammatory compounds have been discovered in great diversity from plants. These compounds have been developed into commercial drugs, and some have provided chemical skeletons for synthesis of more drugs. In developed countries, which make up 20% of the world's population, more than 50% of all drugs in clinical use are of natural product origin [Simmonds, 2003; Newman and Cragg, 2007]. These drugs were derived from only about 90 of the 250 000 known flowering plant species in the world. Furthermore, there are more than 85 000 plant species throughout the developing world which are used in traditional medicines [Balunas and Kinghorn, 2005]. These, together with the other 23 000 species of higher plants, form a vast reservoir of potential for new drugs.

A number of commercial drugs including current antimalarials such as quinine and artemisinin have been derived from plants [Van Wyk *et al.*, 1997]. Table 1.2.3.1 lists a few examples of drugs that have been derived from plants [De Smet, 1997; Kinghorn, 2001].

Table 1.2.3.1 Examples of plant-derived drugs and plant species from which they were isolated

Name of the drug	Plant isolated from	Effect
Artemisinin	<i>Artemisia annua</i>	Antimalarial
Quinine	<i>Cinchona</i> species	Antimalarial
Morphine	<i>Opium poppy</i>	Relieve pain
Tubocurarine	<i>Chondrodendron tomentosum</i>	Muscle relaxant
Taxol	<i>Taxus brevifolia</i>	Anticancer
Vinblastine and vincristine	<i>Catharanthus roseus</i>	Anticancer
Atropine	<i>Atropa belladonna</i>	Anticholinergic
Reserpine	<i>Rauwolfia serpentina</i>	Antihypertensive
Salicylic acid (Aspirin)	<i>Salix</i> species	Pain relieve

As potential sources of new antimalarial drugs, plants continue to provide molecules that possess inhibitory activity against various specific stages of the life cycle of the parasite [Carraz *et al.*, 2006; Francois *et al.*, 1997]. A number of compounds with good antiplasmodial activity have been isolated from plants in great diversity, ranging from simple 8-carbon structures to larger and more complicated molecules. The classes of compounds that are more commonly isolated include terpenes, flavonoids, alkaloids, tannins, steroids and glycosides [Chauhan *et al.*, 2003; Son *et al.*, 2004; Schwikkard and Heerden, 2002; Wright *et al.*, 1992; Böhlke *et al.*, 1996; Yu *et al.*, 1994]. Schwikkard and Heerden reviewed over 170 structures of compounds with antiplasmodial activity. These compounds were isolated from different species in over 30 different families. A considerable number of compounds showed selective antiplasmodial activity, and a few compounds also showed good antimalarial activity *in vivo* [Ndifor *et al.*, 1992]. Some novel chemical structures isolated from plants have been proven to possess antimalarial activity exceeding that of current antimalarial drugs [Ishih *et al.*, 2000; Perez *et al.*, 1994], and possess more favourable selectivity indexes [Zhang *et al.*, 2002]. Quan *et al.*, 2003 and Saxena *et al.*, 2003 have reviewed a large number of plant extracts with antimalarial properties. Some of the plants have gained use as substitutes for chloroquine. A number of lead compounds isolated from these plants are undergoing further development as potential clinical antimalarials. Recent advances in antimalarial compounds are covered in detail by Mital, 2007.

Furthermore, alkaloids with chemosensitizing activity against CQ resistant strains have also been isolated from plants. While parasites acquired resistance by decreasing the accumulation of CQ in the parasite, these plant molecules proved to be able to increase the accumulation of CQ by preventing CQ efflux from, and stimulating CQ influx into CQ resistant parasites [Ramanitrahasimbola *et al.*, 2006].

1.2.4 The potential of South African medicinal plants as sources of antimalarials

In South Africa, medicinal plants play an important role in the African culture and tradition (Fennel *et al.*, 2002). The country boasts remarkable biodiversity of plant species. South Africa is considered to have the most diverse temperate flora in the world, with an estimated 30 000 indigenous species of higher plants, 3000 of which are utilized for medicinal purposes, and 300 are traded as medicines [van Wyk *et al.*, 1997]. A few South African plants have become popular across the globe and are highly commercialized. These include *Aloe ferox* (Cape aloes), *Agathosma betulina* (Buchu), *Aspalanthus linearis* (Rooibos tea), *Hypoxis hemerocallidea* (African potato), *Harpagophytum procumbens* (Devil's claw) and *Sutherlandia frutescens* (Hoodia) [van Wyk, 2002]. The pharmacological investigation of South African flora however, is still largely unexplored. South Africa contains 10% of the world plant species, yet little phytochemical work has been done on these medicinal plants. Of the 24,300 higher plant taxa recorded in the Flora of Southern Africa (FSA) region, only about 15% are reported to be used for medical purposes [Arnold *et al.*, 2002; van Wyk, 2002]. The Ethnobotanical survey carried out by Thring and Weitz, 2006 revealed that in the Western Cape Province only, 36 plant species from 19 families were used for medicinal purposes, and 58% of these plants are indigenous to South Africa. Hutchings *et al.*, 1996 has also reported on 1032 plant species, which make nearly 25% of the flora of KwaZulu-Natal province, to be in use in Zulu traditional medicine.

African medicinal plants studied in other African countries have demonstrated high potential as possible source of antimalarial compounds [Saidu *et al.*, 2000; Okokon *et al.*, 2006; 2007; Philippe *et al.*, 2007; Frederich *et al.*, 2008; Chauhan *et al.*, 2003; Addae-Kyereme *et al.*, 2001; Rukunga *et al.*, 2007; Odugbemi *et al.*, 2007].

Relatively, little work has been done to assess the antiplasmodial activity of similar or related species which are native to or naturalised in South Africa. Many plant preparations used in South Africa to treat malaria or fever are without any scientific evidence on efficacy. It is necessary therefore that the species that occur in South Africa be tested for activity, since the activity of plants within the same species can be affected by the soil composition, geographical and climatic conditions [Massotti *et al.*, 2003; Angioni *et al.*, 2006]. The *Croton* species for example, have gained use as antimalarials in many parts of the world. The species of *Croton* were reported toxic by Lewis and Elvin-Lewis, 1997, whereas Hutchings *et al.*, 1996 reported conflicting results on the toxicity of South African species of *Croton*.

Just over 100 plant extracts from South African flora have been screened for *in vitro* antiplasmodial activity [Campbell *et al.*, 1998; Nundkumar *et al.*, 2002; Clarkson *et al.*, 2004; Kamatou *et al.*, 2005; van Wyk *et al.*, 2002; Kamatou *et al.*, 2008]. A large number of reports on the antiplasmodial activity of South African plants extracts are however, without reference to toxicity [Clarkson *et al.*, 2003; 2004; Saxena *et al.*, 2003]. Other authors have reported on antiplasmodial activity of extracts of South African plants, but have reported toxicity on only a selected few of the list [van Zyl and Viljoen, 2002; Atindehou *et al.*, 2004; Prozesky *et al.*, 2001]. Since many cytotoxic compounds can also exhibit antiplasmodial properties under the conditions of *in vitro* testing, it is important that the selectivity of these compounds be assessed to distinguish general cellular toxicity from specific antiplasmodial activity.

Moreover, most of the research on *in vivo* antimalarial activity of plants has been done in other parts of Africa. Only a few *in vivo* studies have been done on South African flora [Waako *et al.*, 2005; Abosi *et al.*, 2006]. South African plant extracts that have been assessed scientifically for antiplasmodial activity were subjected to *in vitro* testing only, the *in vivo* results are still lacking.

Medicinal plants may serve as medicinally significant entities in their crude extract form. They also play an important role of providing lead structures for the development of therapeutic agents and modified derivatives that possess enhanced activity and/ or reduced toxicity [WHO, 2000]. Therefore fractionation of biologically active crude extracts and isolation of their active constituents remain crucial in drug

discovery. A handful of South African plants have been shown to be selective against the malaria parasite, but little is known about their phytochemistry. As a result, there are no reports on the *in vivo* antimalarial activities of isolated constituents of these plants. Table 1.2.5.1 lists several plants used in South Africa to treat malaria or malaria-like symptoms. Most of these plants have been evaluated for *in vitro* antiplasmodial activity, but information on their active components and *in vivo* activity is lacking.

In a search for new plant-derived biologically active compounds against malaria parasites, essential oils remain one of the unexplored resources. These volatile, natural complex mixtures of compounds have demonstrated numerous biological activities including antimicrobial, antifungal, antiviral, insecticidal, cytotoxicity, genotoxicity, and antiparasitical activities [Bakkali *et al.*, 2008; Carlton *et al.*, 1992; Piccaglia *et al.*, 1993; Buchbauer and Jiroverts, 1994; Aruna *et al.*, 1996; Jazet Dongmo *et al.*, 2002]. The antimalarial properties of essential oils have been little studied. The encouraging reports on the antiplasmodial activity of essential oils underline their potential as antimalarials [Valentin *et al.*, 1995; Lopes *et al.*, 1999; Fabrice *et al.*, 2003] and as insecticides against the mosquito vector [Moore *et al.*, 2002]. There are no reports on the antimalarial activity of essential oils from South African medicinal plants, which presents an opportunity for more research in this area.

1.2.5 Safety of traditional herbal remedies

Traditional medicines account for 15.8% of cases of acute poisoning. Poisoning with traditional medicines is often due to inappropriate use of traditional medicines, contamination with pesticides, and variation of toxins levels in the plants. Rarely, adverse drug-drug interactions are reported, as many patients consult both western doctors and traditional healers simultaneously, without revealing this to either of the doctors.

The significance of poisoning caused by traditional medicines in the South African population is minimal when compared to the 80% of acute poisonings due to orthodox medicines [Joubert and Mathibe, 1989]. Less than 10% of the endogenous plant species in South Africa are classified as poisonous to man. This low number of toxic

plants relative to the plants used medicinally increases the potential of South African plants as sources of new chemotherapeutic agents.

Table 1.2.5.1 Plants used traditionally in South Africa to treat malaria and fevers

Generic name	Family	Local name
<i>Acacia burkei</i> Benth	Fabaceae	Mkhaya
<i>Acokanthera venenata</i> G.Don.	Apocynaceae	Tsebedinthla/ intlungunyembe
<i>Bachylaena elliptica</i> Less.	Asteraceae	Uhlunguhlungu
<i>Barringtonia racemosa</i> Roxb	Lecythidaceae	Iboqo/ Umuluka
<i>Cinchona</i> species	Simaroubaceae	
<i>Dicoma anomala</i> Sond	Asteraceae	Hloenya, Nyongwana
<i>Gunnera perpensa</i> L	Haloragaceae	Qobo/Ugobo/Phalla
<i>Harpagophytum procumbens</i> DC. ex Meissn	Pedaliaceae	Sengaparile
<i>Harpephyllum caffrum</i> Bernk. ex Krauss	Anacardiaceae	Umgwenya
<i>Helichrysum caespitosum</i> Auct	Asteraceae	Phate ea ngaka
<i>Leonotis leonurus</i> (L.) R.Br.	Lamiaceae	Klipdagga
<i>Letononis eriantha</i> Berth.	Fabaceae	Molomo monate
<i>Mentha longifolia</i> (L) L subsp <i>Capensis</i> (Thumb.) Briq.	Lamiaceae	Koena
<i>Pentania pruneloides</i> Walp.	Rubiaceae	Setima-mollo
<i>Pittosporum viridiflorum</i> Sim	Pittosporaceae	Mfusamfu
<i>Sclerocarya birrea</i> subspecies <i>caffra</i> (Sond.) Kokwaro	Anacardiaceae	Morula/Umganu
<i>Siphonochilus aethiopicus</i> (Schweinf.) B.L. Burtt	Zingiberaceae	Serokolo/Sirugulu
<i>Strychnos henningsii</i> Gilc.	Loganiaceae	Mqaloti
<i>Typha capensis</i> Rohrb.	Typhaceae	Ibhuma
<i>Warbugia saluaris</i> Bertolf Chiov	Canellaceae	Sehaba
<i>Xysmalobium undulatum</i> R.Br	Asclepiadaceae	Shongwe/Leshokhwa
<i>Zanthoxylum capense</i> (Thunb.) Harv.	Rutaceae	Mnongwane/Knobwood

1.2.6 Depletion of medicinal plants in South African

The increased commercialisation of traditional medicine is leading to the depletion of many plant species. More than 700 species and 700 000 tones of plant material are harvested and traded through out South Africa and internationally annually [Lewu *et al.*, 2007; Mander, 1999]. Unrestricted collection and trading of medicinal plants has resulted in an over-exploitation of medicinal plant resources, such that most medicinally important species such as *Artemisia afra*, *Scilla natalensis*, *Thesium pallidum* and *Knowltonia bracteata*, *Ocotea bullata*, *Eucomis autumnalis* and *Warburgia salutaris*, are in danger of extinction [Zschocke *et al.*, 2000; Williams, 1996]. This has made management of natural medicinal plant resources a matter of urgency.

There has been an integrated effort from the National and Provincial Governments, and Non Governmental Organizations (NGO) to conserve the flora. The launch of Medicinal Flora Co-operative in 2000 has created a platform for developing policies and strategies aimed at conserving medicinal plants. Currently, the strategies that are enforced include; cultivation of medicinal plants on a large scale, minimization of destructive harvesting practice such as ring-barking and uprooting, restriction of unauthorised collection of plants, and selecting species for the plant trade. Additionally, traditional healers are being equipped with appropriate propagation and cultivation skills to enable them to grow medicinal plants in their home environment. Also, the healers are encouraged to use alternative parts of the plants e.g. the leaves and twigs, instead of the bark [Cunningham, 1998; Zschocke *et al.*, 2000; Crouch and Symmonds, 1999].

1.3 Intellectual property rights

The question of intellectual rights and remuneration of the local people remains a political and important issue when utilizing indigenous knowledge for drug discovery. South Africa has a well-established intellectual property framework under the Biological Diversity Act, aimed to protect and preserve indigenous knowledge [Wolson, 2001]. The National Research Foundation (NRF) has made research on indigenous knowledge, and intellectual property two of its nine key focus areas. The

NRF strongly enforces protection of indigenous property rights and continues to create awareness on intellectual property and its value in drug discovery research [NRF, 2003].

1.4 Drug discovery and development process

The process of drug discovery is divided into phases. The initial phase entails identification of the bioassay suitable for the pathogen at test, followed by initial screening process to identify a compound with the desired positive biological activity in a bioassay. Such a compound is called a hit. Once the hit compound has been identified, it can be chemically modified to improve its medicinal properties. It can also serve as a template for the synthesis of derivatives.

The hit compound is tested for its profile of ADMET properties (Absorption, distribution, metabolism, elimination and toxicity) [Lipiski *et al.*, 1997; Wess, 2001] before it is tested in pre-clinical animal models. The candidate hit goes through extreme evaluation, and ultimately enters the clinical safety and efficacy trials. If the compound is successful, it will then be registered as a drug and marketed.

There are different approaches to discovering a hit. A hit compound can be discovered through empirical screening methods, where compounds from synthetic chemical and combinatorial libraries and natural product libraries are screened for any biological activity [Harvey, 1999]. Rational drug design through use of combinatorial chemistry and computer-aided techniques, is another approach based on identifying a biological target and designing a compound to interact with the target. The third approach is through drug metabolism studies, where metabolites are screened for activity with the aim of identifying the active functional groups of the molecule.

1.4.1 Plant product based drug discovery process

The process of bioprospecting can be lengthy and costly. In this process, the selection of the plant species to be investigated for biological activity is a crucial factor. There are three approaches that may be used to choose plants for investigation, namely, the *chemotaxonomical approach*, where plants are selected according to a chemical class

in a genus or family, the *ethnobotanical approach*, where plants are selected based on folklore therapeutic reputation and use by indigenous people, and the *random approach*, where thousands of plant species are randomly studied for any therapeutic use [Gurin-Fakim, 2006].

The ethnobotanical and ethnopharmacology approaches to drug discovery were followed in this study. While the ethnobotanical approach gives information that helps to reduce the number of plants for investigation, ethnopharmacology (the study of the active principles in the plants) is important in identifying the constituents of extracts for development of single entity drugs.

Following proper botanical identification of the plant, plant constituents are extracted and screened to identify an extract that gives a positive outcome in a bioassay process. Through bio-activity guided fractionation, the active constituents of the extract are purified using a number of scientific techniques, then identified by spectroscopic methods. The compounds are then tested *in vitro* for selectivity. Successful compounds are screened for efficacy and toxicity in pre-clinical animal models. The efficacious, selective and non-toxic compound is then considered for clinical trials.

1.4.2 Evaluation of antimalarial agents

A number of techniques for detection and quantification of malaria parasite in cultures and body fluids are used to evaluate *in vitro* and *in vivo* efficacy of potential antimalarial agents [Makler *et al.*, 1993; Tamura and Waki, 1986; Druilhe *et al.*, 2001; Mazier *et al.*, 2004; Harald *et al.*, 2003]. The techniques include giesma-stained light microscopy, parasite lactate dehydrogenase assay, antigen capture, fluorometry based on the use of DNA staining dyes, and isotopic assays based on the use of radiolabelled precursors in nucleic acid synthesis by the parasite. Evaluation for safety *in vitro* requires determination and quantification of cell viability. The assays used include; MTT assay, which detects metabolic viability of the cells [Mosman, 1983], electron microscopy, which provides information on morphological changes, erythrocyte haemolytic assay based on quantification of haemoglobin in erythrocyte culture [[Fischer *et al.*, 2003; Winski *et al.*, 1997).

1.5 Scope of the study

1.5.1 Motivation for the study

The world-wide escalating problem of resistance of *Plasmodium* to existing antimalarial drugs has emphasized the need for new drugs with alternative mechanisms of action. Malaria is common in South Africa and endemic in three provinces. Failure of treatment due to drug resistance and shortage of drugs in many provinces led the South African population to rely heavily on traditional herbal remedies.

It is estimated that more than three million people in South Africa use traditional plant medicine from healers for various ailments [van Wyk and Gericke, 2000]. The hazard is that many herbal preparations used in South Africa to treat people are without scientific evidence on safety and efficacy. There is a lack of research output on the scientific information concerning the safety and efficacy of traditional medicine in *in vitro* and mostly *in vivo* laboratory setups [Gessler *et al.*, 1994].

South African flora by virtue of its diversity has great potential as source of chemotherapeutic antimalarial agents, yet it has not been fully explored [Duncan *et al.*, 1999]. There are 30 000 indigenous species of higher plants, 3000 of which are used medicinally. Of this reservoir, just over 100 plant extracts have been assessed for *in vitro* antiplasmodial activity [Clarkson *et al.*, 2004; van Dyk *et al.*, 2001]. Most of the reports on *in vitro* antiplasmodial activity of these extracts are without reference to toxicity. Of the few plants that have been assessed for selectivity for malaria parasites, little is known about their phytochemistry, and the active constituents. Furthermore, there are even fewer reports on the *in vivo* antimalaria activity of South African plants. Little work has been done on the potential antiplasmodial activity of essential oils of species occurring in South Africa. It remains an important factor to thoroughly study promising medicinal plants and identify the active antimalarial compounds within the plants.

There is clearly a need to look into traditional medicines with a record of use in treatment of malaria in South Africa, to scientifically validate their use and evaluate

their toxicity, but most importantly to isolate and structurally characterize the active constituents of the plants. It is encouraging to note that of the 119 plant-derived drugs present in the market, 74% were discovered as a result of studies directed at the isolation of the active substances from plants used in traditional medicine [Gurib-Fakim, 2006]. Furthermore, a few hit compounds with antiplasmodial activity have been isolated from South African medicinal plants [Pillay *et al.*, 2007a; Clarkson *et al.*, 2003]. The analogue of these compounds was used as scaffold to prepare more active and less cytotoxic derivatives through chemical modification [Clarkson *et al.*, 2003]. There is therefore supporting evidence that the methodology adopted in this study has potential to yield lead compounds that can be developed into antimalarials.

South Africa supports the Roll Back Malaria (RBM) movement, which aims at halving malaria deaths by 2010, and halving again by 2015 [Narasimhan and Attaran, 2003]. Along with other new strategies in malaria control, it is imperative that more efforts be directed towards the recognition and validation of traditional medicinal practices, and obtaining drugs with different structural features.

1.5.2 The aim of the study

The aim of this study was to investigate the antimalarial potential of five South African medicinal plants, by conducting *in vitro* and *in vivo* efficacy and safety studies, and to characterize their active constituents. The objectives of the study were:

- 1) To use an *in vitro* system to screen extracts of selected plants for antiplasmodial activity against CQ-sensitive and CQ-resistant strains of the malaria parasite, *P. falciparum*.
- 2) To use the *in vitro* system to determine the cytotoxicity of the extracts on a mammalian cell line.
- 3) To isolate the bioactive constituents of the extracts through bio-guided fractionation and application of various chromatographic techniques.
- 4) To determine chemical structures of the isolated compounds using spectroscopic methods such as mass spectrometry and NMR.
- 5) To use mice models to screen extracts that showed antiplasmodial activity *in vitro*, for efficacy and safety *in vivo*.

The study was expected to provide information on the efficacy and safety of selected South African medicinal plants. The information could be used in the development of herbal remedies or for sources of lead compounds that can be developed into more favourable compounds useful in treatment of malaria.

University of Cape Town

Chapter 2

University of Cape Town

2 Plants investigated

2.1 Introduction

Information on the use of plants for treatment of malaria and fever was gathered in 2003 from traditional healers in the Eastern Cape and Western Cape provinces which harbor 70% of South African flora. Although the healers lacked the scientific information on the lifecycle of the parasite and the pathogenesis of malaria, the malaria symptoms are however, well characterized. The typical symptoms of malaria were used as guiding information for the traditional healers during the interviews.

2.2 The criteria for selecting plants

The criteria for selection of plants were as follows:

1. Fresh plant material was readily available in abundance locally, or the plant could be easily harvested.
2. No references in the literature were found on the isolation and identification of active anti-malarial principles in the plant species naturalised in South Africa.
3. No reference in the literature on the *in vivo* antiplasmodial activity of the plant species.

For selection of plants for further investigation, it was required that in the initial screening, the crude extracts of the plants showed 50% inhibitory concentration (IC_{50}) value that is less than $10 \mu\text{gml}^{-1}$ against both the CQ-sensitive and CQ-resistant strains of *P. falciparum*, and that the plant extract showed low cytotoxicity on a mammalian cell-line.

Based on these criteria, a total of 10 plants were collected, extracted and screened for *in vitro* antiplasmodial activity against the CQ-sensitive strain of *P. falciparum*. Out of the 10 plants screened, 5 of them that showed relatively higher antiplasmodial activity were selected for investigation. These are; *Eriocephalus africanus*, *Achillea millefolium*, *Tarhonianthus camphoratus*, *Agathosma betulina* and *Warbugia salutaris*.

2.3 *Achillea millefolium* L.

Common vernacular names: Yarrow

2.3.1 Botanic Description and Distribution

Achillea millefolium belongs to the botanical family *Asteraceae*, which has numerous well-defined species and a number of subspecies [Kubelka *et al.*, 1999]. But in other parts of the world *A. millefolium* L. is defined as a single species [Gudaityte, 2007]. *A. millefolium* yields blooms in many colors. The species investigated in this study is *A. millefolium* L. ssp. *Millefolium*, which bears rosy white colored flowers (Figure 2.3.1.1). Yarrow is widely spread over the Northern Hemisphere [Mockutė *et al.*, 2002]. In South Africa it is found in almost all the provinces.



Figure 2.3.1.1 The picture of *A. millefolium* L. the plant (a) and at its flowering season (b).
Sources: <http://plants.usda.gov/java/profile?symbol=ACMI2>,
http://www.ppws.vt.edu/scott/weed_id/achmi.htm

2.3.2 Uses

A. millefolium has been used traditionally for treatment of gastro-intestinal disorders as a digestive tonic [Benedek *et al.*, 2007]. It has been reported to possess antiseptic and infection preventing properties [Mitich, 1990]. It is used to heal wounds, sores, rashes, fever, respiratory infection, urinary-genital infection, menstrual problems, and hemorrhoids and rheumatism [Jarić *et al.*, 2007; Willuhn, 2002; Mockute *et al.*, 2002]. The plant also gained use as an antiphlogistic drug [Benedek *et al.*, 2007], an

2.4 *Eriocephalus africanus*

Common vernacular names: kapokbos, wild roosmaryn (Afrikaans), wild rosemary (English)

2.4.1 Botanical Description and Distribution

E. africanus belongs to the family Labiatae. *E. africanus* is a small shrub of up to one metre in height. The plant is evergreen multi-branched with hairy, thin silvery grey leaves borne along the branches from which the flower heads emerge [Dhanaj S *et al.*, 2007]. The flower heads mature and bear the tufts of seed snow-white hairs (Figure 2.4.1.1). The Afrikaans vernacular name is derived from the resemblance to snow. The plant is found in all provinces in South Africa, but occurs mainly in the Western Cape, Eastern Cape and Namaqualand.



Figure 2.4.1.1 The picture of aerial parts of *Eriocephalus africanus*. Source: <http://plantzafrica.com/plantefg/eriocephfr.htm>

2.4.2 Uses

The parts of the plant that commonly used are the leaves and twigs. The leaves are extensively used in cooking to flavor foods as they contain a pepperish smell and taste. The related species *Rosmarinus officinalis*, commonly known as rosemary, is known for its potent antioxidant properties and has been used for many years as a preservative [Ahn *et al.*, 2008; Georgantelis *et al.*, 2007]. It exhibits its antioxidant characteristics by increasing free radical scavenging activity in the body [Atsumi *et*

et al., 2007]. Rosemary has been shown to have a protective effect against gamma 6 Gy γ -radiations in the liver [Soyal, 2007]. The antimicrobial properties of the plant [Govaris *et al.*, 2007], and antiplatelet activity of its major constituents have also been reported [Lee, 2006]. It exhibits diuretic, carminative, diaphoretic, anti-rheumatic, anti-epileptic, antimutagenic and antispasmodic properties. It has been used as a digestive tonic, and in the treatment of prostate disorders, colds and high blood pressure heart failure. It has been reported to strengthen the capillaries, relieve respiratory disorders, alleviate oedema, and inhibit HIV protease, an enzyme involved in the replication of the Human Immune Virus [Al-Sereiti *et al.*, 1999; El-Gadi and Bshina 1989].

2.4.3 Phytochemistry

Wild rosemary species contain apigenin, rosmanol, epirosmanol and bertulinic acid as some of the major constituents. A variety of bioflavonoids found in *E. africanus* and related species include: diosmin and luteolin. Organic acids include carnosic, oleanic, ursolic and rosmarinic acids. [van Wyk *et al.*, 1997]. Rosmarinic acid and rosmanol are potent antioxidants in the plant. The plant species also contain α -pinene, 1,8-cineole, and linalool as major compounds [Gachkar, 2007].

2.5 *Agathosma betulina*

Common vernacular names: Buchu, Bucco, (Khoi, English), Boegoe (Afrikaans), Ibuchu (Xhosa).

2.5.1 Botanical Description and Distribution

Agathosma is from the family Rutacea. It consists of about 150 species [Goldblatt and Manning, 2000]. *A. betulina* is a perennial shrub with woody branches and small leaves that vary in shape and size. There are three species used commercially: *A. betulina*, with broad round leaves that bear white or pink flower, *A. crenulata*, with oval leaves that display white flowers, and *A. serratifolia* which is a long leaf Buchu (Figure 2.5.1.1) [Spreeth, 1976; Pillans, 1950]. These species are usually confused. *A. betulina* is up to two metres in height. The leaves are of a pale green colour, leathery and glossy, with a blunt tip and round oil glands scattered through the leaf. They have a strong aromatic taste and a peppermint-like odour.



Figure 2.5.1.1 The figure of species of *Agathosma* species. Source: <http://www.spirit-web.com/Agathosma%20Betulina.asp>

A. betulina is indigenous to South Africa. The plant grows only in the narrow fynbos strip in the Western Cape Mountains [Spreeth, 1976]. The plant is particularly adapted to dry conditions and grows on sunny hillsides [Van Wyk and Gericke, 2000; Stander *et al.*, 2003].

2.5.2 Uses

A. betulina is mostly used to treat any infection of the genitourinary system, including inflammation of the urethra, nephritis, cystitis and catarrh of the bladder. The plant also exhibits diuretic, carminative and diaphoretic action. Other medicinal actions include; treatment of prostate disorders, colds and flu, relief of stomach complaints, and symptomatic relief of rheumatism [Moolla, 2006; Simpson, 1998], digestive tonic, uterine stimulant, diabetes and external application on wounds [Van Wyk *et al.*, 1997; Bruneton, 1995].

2.5.3 Phytochemistry

The principal constituents of the leaves are volatile oils, mucilage, camphor and diosphenol. Diosphenol is responsible for the plant's mild antiseptic properties. The plant also contains glycoside terpenes (terpineol), quinolinone alkaloids and bioflavonoids (diosmin, hesperidin, quercetin and derivatives) [Posthumus, 1996; Campbell *et al.*, 1990; Blommaert and Bartel, 1976]. Diosmin and its analog hesperidin are known for their phlebotonic and antioxidant properties and as vascular protectors, also as chemopreventive agents in urinary-bladder and colon carcinogenesis. These constituents have also demonstrated use in treatment of chronic hemorrhoids [El-Shafae and El-Domlaty, 2001].

2.6 *Tarchonanthus camphoratus*

Common vernacular names: Mohathla (Tswana), mofahla (Southern Sotho), mathola (Xhosa), Vaalbos, Veld-vaalbos (Afrikaans), Leleshua (Swahili) wild camphor bush (English).

2.6.1 Botanical Description and Distribution

T. camphoratus belongs to the family Asteraceae [Arnold *et al.*, 2002]. It is a small multi-stemmed tree of about six metres in height. The leaves are oblong in shape. The top of the leaves has a grey green color while the lower surface is yellow to crème and furry (Figure 2.6.1.1). The leaves are oval or lanceolate shaped, and have a strong camphorated aromatic smell [Hilliard, 1977]. The plant bears creamy white flowers on the terminal end of the branch.



Figure 2.6.1.1 The branch, leaves and flowers of *T. camphoratus*. Source: <http://www.plantzafrica.com/medmonographs/tarchonanthcamp.pdf>

T. camphoratus grows in east Africa and Arabia. It is distributed in Botswana, Swaziland, Namibia, Lesotho and is widespread across all provinces of South Africa [Arnold *et al.*, 1993; Grant and Thomas, 1998]. It is found in almost any part of the country. It grows in poor soils, even on water deficient sites. It is suited to tough conditions.

2.6.2 Uses

The leaves and the twigs are parts of the plant that are commonly used for medicinal purposes. The plant is used to treat stomach problems, abdominal pain, headaches, toothaches, asthma, bronchitis, rheumatism, blocked sinuses, gastritis, inflammation and fever produced by the bacterial endotoxin [Amabeoku *et al.*, 2000; Ben-Erik van Wyk *et al.*, 1997]. It is used to prevent swelling up of the legs by diminishing the lactic acid created in the muscle. It is also known to have insect repellent, anti-irritant, a muscular tone and cellular oxygenation diminishing action and was shown to antagonize the acetic acid induced writhes [Faleiro *et al.*, 2003]. For treatment of ailments, the plant is boiled and taken as a decoction. In the case of treatment of sinuses, the fresh leaves are inserted in the nostrils of a patient.

2.6.3 Phytochemistry

The volatile oil contains many constituents, which differ in abundance between localities [Mwangi *et al.*, 1994]. Principal constituents of *T. camphoratus* include 1,8-cineole, α -terpinol, α -fenchyl alcohol and a flavanone-pinocembrin [Bishay *et al.*, 2002]. The plant contains other numerous minor constituents including camphor [Mwangi *et al.*, 1994], and various flavones such as luteolin, apigenin, and nepetin. Sesquiterpene lactones and quaternary alkaloids such as tarchonanthaline have also been found in *T. camphoratus* [Bishay, 2002].

2.7 *Warburgia salutaris*

Common names: Manaka (Venda), shibaha (Tsonga), isibhaha (Zulu), pepper-bark tree (English), peperbusboom (Afrikaans).

2.7.1 Botanical Description and Distribution

Warburgia belongs to the family Canellaceae. There are three species of *Warburgia*: *W. ugandensis*, *W. stuhlmannii* and *W. salutaris* [Esterhuysen, 1996; Rabe, 2000]. *W. salutaris* is a medium sized tree of about 10 meters in height. The leaves are oblong, glossy and dark green on top and paler below Figure 2.7.1.1. It produces small greenish-yellow flowers of about 7mm in diameter, and round green fruit [Coates, 1977; Palmer and Pitman, 1973].



Figure 2.7.1.1 The leaves of *Warburgia salutaris*. Source: <http://www.plantzafrica.com/plantwxyz/warburg.htm>

W. salutaris occurs in Southern Africa. It is found in coastal forest in Zimbabwe, Mozambique, Swaziland, and South Africa. In South Africa it is mainly found in Kwazulu Natal, Mpumalanga and Northern Province [Hollmann and Van der Schijff, 1996]. It grows mostly in forests.

2.7.2 Uses

W. salutaris has been used extensively for medicinal purposes, with the stem bark as the most widely used part of the plant. The plant has been used for treatment of colds, influenza, headaches, sinus, respiratory complaints, malaria [Mander *et al.*, 1995], sores and skin irritations, and cases of inflammation [Hutchings *et al.*, 1996]. It has potent antibacterial activity [Rabe and van Staden, 2000; Mashimbye, 1993].

2.7.3 Phytochemistry

Major chemical constituents of *Warburgia* species include monoterpenoids, sesquiterpenoids, triterpenoids and bioflavonoids. A number of drimane sesquiterpenes have been isolated from the stem bark of *Warburgia* species [Mashimbye *et al.*, 1999]. These include: warburganal, polygodial, salutarisolide, muzigadial, ugandensidial, isopolygodial. These have a wide range of biological activity including antimicrobial, insect antifeedant, antifungal, cytotoxic, antiviral, haemolytic, molluscicidal and skin irritant effects [Kubo *et al.*, 1977; Jansen and de Groot, 1991; Treurnicht, 1997; Rabe *et al.*, 2000; Rabe and van Staden, 1997; Madikane *et al.*, 2006].

3 Materials and Methods

3.1 Collection and identification of plants

Terrestrial plants were collected in the field in the area of the study. The plants were identified by the Department of Botany, University of Cape Town and Kew Herbarium, London. The plants were identified with specimens that include a photograph and a small portion of the plant. The plants were identified by the Department of Botany, University of Cape Town.

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Chapter 3

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3.2 Preparation of plant extracts

The leaves and twigs of selected plants were left to dry at room temperature away from direct sunlight. The dry material was ground to a fine powder using a plant grinder (Warring, Connecticut, USA). The leaf material of each plant (100 g) was extracted separately with 500 ml of solvent and subjected to vigorous shaking on a horizontal orbit shaker (Labcon, California, USA) for 48 hours. Non sequential extractions were performed using the following solvents: deionised millipore water (H₂O), hexane (hex), methanol (MeOH) and dichloromethane (DCM). The mixtures obtained from extractions were filtered using filter paper (Whatman® Maidstone, England). The organic filtrates were concentrated under vacuum using a rotary evaporator (BÜCHI 461 Labortechnik AG, Flawil, Switzerland), while water extracts were concentrated by freeze-drying (Virtis, New York, USA) under a reduced pressure at -80°C. The concentrated extracts were dried under fume hood at room temperature and stored at 4°C until use. The plant extracts were weighed before and after the extraction to determine the percentage yield. The yields were calculated as a percentage of the plant material extracted.

3.3 Preparation of plant extracts solutions for antiplasmodial assay

The initial stock solution of 2 mgml⁻¹ of the crude extracts, fractions, and the active compounds was prepared. The weighed samples (2 mg) were dissolved in MeOH and diluted accordingly in deionised H₂O, then in complete medium to achieve a final stock solution of 200 µgml⁻¹ in 1% MeOH for the assay.

Fresh stock solutions were made up on the day of the experiment. CQ (Sigma) was used as a control to verify the *in vitro* susceptibility of the different strains of *P. falciparum*. A 1 mgml⁻¹ stock solution of CQ was made up in water and serially diluted in complete medium to a concentration of 200 µgml⁻¹.

3.4 Antiplasmodial activity testing

The experiments were carried out under sterile conditions in duplicate in 96-well microtitre plates (Greiner). The wells of the plate are arranged in a matrix of eight rows and 12 columns (1-12). Aliquots of 100 μ l of complete medium were dispensed to all the wells except in column 3. In column 3, aliquots of 200 μ l of drug stock solutions (200 μ gml⁻¹) were added in duplicate (in two adjacent rows), and two fold serial dilutions were carried across the plate using a multi-channel pipette.

Parasitized red blood cells (RBC) in the trophozoite stage were adjusted to a 2% parasitaemia and a 2% haematocrit in complete medium, and 100 μ l of this suspension was added to columns 2-12. The first column served as a blank and contained 100 μ l of complete medium and 100 μ l of unparasitised RBC at 2% haematocrit. Column 2 contained no drug and served as a positive control. The plates were covered with a sterile lid, put in a chamber, gassed with 4% CO₂, 3%O₂ and 93%N₂ and then incubated at 37°C for 48 hours.

3.4.1 Cultivation of malaria parasites

P. falciparum parasites were cultured as described by Trager and Jensen (1976), with minor modifications. The CQ-sensitive strain D10, derived from FCQ-27 from Papua New Guinea [Ekong *et al.*, 1993] and CQ-resistant strain K1 isolated in Kanchanaburi, Thailand [Thaithong *et al.*, 1981] and RSA11 isolated in Kwa-Zulu Natal, South Africa were donated by Mr D. Taylor of the Division of Pharmacology at UCT.

The parasites were maintained in RPMI 1640 (BioWhittaker) culture medium supplemented with phenol red, albumax II (bovine serum albumin) (Gibco) (25 g/L), HEPES (N-[2-hydroxyethyl]-piperazine-N'-[2-ethansulphonic acid]) (6 g/l), 4.25% of sodium bicarbonate and gentamycin (50 mg/l). The reagents were purchased from Sigma-Aldrich, South Africa. Washed O⁻ human RBC and human serum were added to the culture. RBC were washed twice with medium before use.

Human serum/ blood was donated by the Blood Transfusion Service and Hematology Department at Groote Schuur hospital, Cape Town. The parasites were cultured in sealed flat bottom flasks and maintained at 37°C in an atmosphere of 93% N₂, 4% CO₂, and 3% O₂. The haematocrit and parasitaemia were kept between 2-4% by the addition of RBC. Parasites were synchronized at the ring stage regularly by treatment with 5% D-sorbitol. The parasitaemia was determined microscopically using a giemsa stained thin blood smear of culture on the slide.

3.4.2 Parasite Lactate Dehydrogenase assay

The parasite lactate dehydrogenase (pLDH) assay was used to measure parasite viability as described by Makler *et al.*, 1993. This assay is based on the discovery that pLDH activity is distinguishable from host cell LDH using the 3-acetyl pyridine adenine dinucleotide (APAD¹), an analogue of NAD⁺. Lactate dehydrogenase is a terminal enzyme in the glycolysis pathway of malaria parasites. It catalyses conversion of L-lactate to pyruvate in the presence of NAD⁺ which gets reduced to NADH. pLDH has the ability to use APAD as a coenzyme and reduces it to APADH, but this process happens negligibly slowly in the host. As the APADH is released, it reduces the yellow tetrazolium salt (NBT/PES) to purple crystals of formazan salt. pLDH enzyme is used primarily as an indicator of parasite survival. Its production and accumulation is used as indices of parasite viability, whereby the levels of the enzyme correspond to the parasite density.

3.4.3 Measuring pLDH Activity

The pLDH activity was measured using, MalstatTM reagent (1ml/L) APAD (0.33 g/l) in millipore water), and 0.24mM phenazine ethosulphate (PES)/1.96mM nitro blue tetrazolium NBT (Sigma). At the end of a 48 hour incubation period of the test plate, the parasites were resuspended and 15 µl was transferred with a multi-channel dispenser to the corresponding wells in an empty microtitre 96-well plate. To this developing plate, 100 µl of Malstat and 25 µl of NBT/PES solution were added. The plate was placed in the dark for 5 minutes for colour development. Absorbance values were read at 620 nm using a 7250 microplate Reader (Cambridge Technology, Inc.). The parasite viability was calculated as a percentage of the

control absorbance. The dose response curves were plotted in Prism V.4.0 to obtain the 50% inhibitory concentration (IC₅₀) values.

3.5 Cytotoxicity assay

The cytotoxicity assay measures cell survival after exposure to test samples. The assay determines the relative toxicity and selectivity of the active compound against malaria parasites. The cytotoxicity was evaluated against the mammalian cell line, Chinese Ovarian Hamster (CHO). The cell-line stock was donated by Dr H. Hoppe, Department of Pharmacology, University of Cape Town.

3.5.1 Cell culturing

The cells were maintained in complete medium; 45% Dulbecco's Modified Eagle's Medium (DMEM) (13.3 g of DMEM, 3.7 g NaHCO₃ at pH 7.1, gentamycin-500 µl/L and 2 L of Millipore water), and 45% HAMS F-12 medium supplemented with 10% of heat inactivated Fetal calf serum and gentamycin (0.04 µgml⁻¹).

The cells were stored at -80°C and thawed for culturing. Cell growth was monitored under a light microscope. Cells were subcultured once they had reached confluency. Cells were rinsed twice with 5 ml of sterile phosphate buffered saline (PBS). Then 5 ml of 1% trypsin solution (37°C) was added into the flask and incubated at 37°C for 2 minutes to detach the cells from the flask. A 5 ml aliquot of complete medium was added to inhibit enzyme activity. Cells were centrifuged at 700 rpm for 5 min. The pellet was re-suspended in the 5 ml of medium, from which 20 µl was removed and diluted with 20 µl of crystal blue nuclear dye. Cell viability assessment and cell counting were done using a microscope. The stock culture with a cell density of 10⁵ cellsml⁻¹ was prepared for the assay.

3.5.2 Drug preparations and dilutions for cytotoxicity assay

Emetin (Sigma) was used as a positive control in the assay. An initial emetine stock of 2 mgml⁻¹ was prepared in H₂O and stored at -20°C until use. The stock solutions of

the test samples were prepared at 2 mgml^{-1} in 10% MeOH on the day of the experiment. Six 10-fold serial dilutions of the stock solutions were prepared in complete medium to give concentrations of $200 \text{ }\mu\text{gml}^{-1}$ to $0.002 \text{ }\mu\text{gml}^{-1}$.

The experiments were carried out under sterile conditions in 96-well plates. The cells were diluted to a concentration of $10^5 \text{ cellsml}^{-1}$, and $100 \text{ }\mu\text{l}$ of this cell suspension was dispensed in all the wells except column 1. An aliquot of $100 \text{ }\mu\text{l}$ of complete medium was added to all the wells. The plated cells were incubated for 24 hours at 37°C in a gas controlled environment of 5% CO_2 . After incubation, the medium was aspirated out of the wells, and cells were exposed to a drug concentration range of $100\text{-}0.0001 \text{ }\mu\text{gml}^{-1}$. Aliquots of $100 \text{ }\mu\text{l}$ of the drug concentrations were dispensed in to columns 3-8 in a decreasing order of concentration, then $100 \text{ }\mu\text{l}$ of complete medium was added to these wells. Column 1 served as blank and contained $200 \text{ }\mu\text{l}$ of complete medium only. Column 2 contained no drug and served as a positive control. The plate was then incubated at 37°C for 48 hours.

3.5.3 Measuring cell viability

The cell growth was measured using the MTT (3-[4, 5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay as described by Mosmann, 1983. Once the 48-hour incubation was complete, $25 \text{ }\mu\text{l}$ of sterile MTT (5 mg/ml in PBS) was added to each well and plates were incubated for 4 hours at 37°C . The plates were then centrifuged at 200 rcf for 10 minutes. The supernatant was aspirated from the wells, $100 \text{ }\mu\text{l}$ of 100% Dimethylsulphoxide (DMSO_4) was added to each well to dissolve the formazan crystals. The plates were gently shaken on a microtitre plate shaker for 5 minutes and then read immediately on a microtitre plate reader at 540 nm. For cell viability analysis, dose response curves were plotted using nonlinear dose response curve fitting analyses with GraphPad Prism V.4.0 software.

3.5.4 The MTT assay

The MTT assay is a non-radioactive quantitative colorimetric assay used for *in vitro* measurement of the cytotoxicity and metabolic activity of cell cultures subjected to

different culture conditions. It is based on the ability of viable cells to reduce the yellow water-soluble tetrazolium salt to a water-insoluble purple formazan product. Since this activity requires functional mitochondria, only viable cells can cleave the tetrazolium ring. The metabolic activity in the cells and the number of viable cells are directly proportional to the amount of formazan crystals formed.

3.6 Selectivity Index

The selectivity index is calculated to determine the selectivity of a test sample against malaria parasite. The selectivity index is the ratio of cytotoxicity to antiplasmodial activity, given that units used are the same. Higher selectivity index reflects less cytotoxicity, and is therefore more favorable. It is undesirable for the test sample to kill the mammalian cells at the same rate or concentration as it does to the parasites.

Selectivity index = Cytotoxicity IC_{50} / Antiplasmodial IC_{50} [Benoit-vial *et al.*, 1999].

3.7 Fractionation of plant extracts that showed *in vitro* antiplasmodial activity in bioassay

3.7.1 Solid Phase Extraction (SPE)

The crude extracts of *W. salutaris*, *A. betulina*, *T. camphoratus* and *E. africanus* were each fractionated using SPE on a C18 ISOLUTE cartridge of mass absorbent 10 g and 70 ml capacity (International Sorbet Technology, Glamorgan, UK). Samples were eluted with a step gradient of decreasing polarity of water: acetonitrile (H_2O : MeCN) mobile phase. All the extracts were weighed, dissolved in 100% MeCN and diluted with H_2O such that the final concentration was 5 mgml⁻¹ in 40% MeCN. The samples were then centrifuged at 500 rpm for 5 minutes. Prior to application of the samples on to the column, the cartridge was wetted with 50 ml of H_2O and conditioned with 50 ml of 8:2 H_2O : MeCN. The sample (10 ml) was applied onto the cartridge and eluted under small vacuum. The pressure from the vacuum pump was set to control the flow rate to 17 ml/min. The cartridge was then rinsed with 50 ml of H_2O to elute unretained material. The retained material on the cartridge was separated by eluting at a step gradient with 50 ml volumes of H_2O : MeCN at different increments for each plant extract. The final 100% MeCN elution was followed by 100 ml wash with acetone to

rinse any remaining material off the cartridge. The collected fractions were concentrated by rota-evaporation under vacuum (240 mbar) at 45°C and dried by freeze-drying. The samples were reconstituted in methanol, dried in pre-weighed glass vials in the fume hood and stored at 4°C. The collected fractions were then tested for *in vitro* antiplasmodial activity.

The crude extract of *W. salutaris* was eluted with a mobile phase system of increasing 20% increments of MeCN in H₂O starting at 10% MeCN. Eight fractions; wash (100% H₂O), 10%, 30%, 50%, 70%, 90%, 100% MeCN and Acetone wash, were collected.

The eluting system for crude extract of *E. africanus* was a step gradient of increasing 10% increments of MeCN in H₂O (from 0% to 100% MeCN), yielding a total of 12 fractions including the H₂O wash and acetone wash.

The crude extracts of *A. betulina* and *T. camphoratus* were each eluted at a gradient of increasing polarity of 20% increments of MeCN in H₂O. Seven fractions; H₂O wash, 20%, 40%, 60%, 80%, 100% MeCN, and acetone wash were collected.

3.7.2 High Pressure Liquid Chromatography (HPLC)

The instrument used was a Shimadzu LC-10AS that is connected to a flexi 486 DX/2-50 desktop PC running software via a Shimadzu CBM-10A communication module (Shimadzu Corporation, Kyoto, Japan). The samples were detected by a SPD-M10A Shimadzu diode array detector at wavelengths 210-359nm. The mobile phase was a mixture of HPLC grade MeCN and millipore H₂O (Milli-Q Water System). The flow rate and injection volume were adjusted for each fraction to maximize resolution. The samples were dissolved in 1:1 MeCN: H₂O, sonicated and centrifuged in a microcentrifuge (Abbott) at 10 000 rpm for 5 minutes to pellet any undissolved material. The supernatant was then run on HPLC.

The active constituents of *W. salutaris* and *T. camphoratus* extracts were identified using an HPLC based methodology. To identify the active constituents in each plant crude extract, the extracts were prepared in 10% MeCN in H₂O, and run on HPLC at a

shallow gradient of 10-100% MeCN over 40 minutes at a flow rate of 2 mlmin⁻¹, to view the full profile of the components of the extract. The HPLC profile was divided into fractions. Following multiple injections, eluting fractions were collected separately, prepared appropriately and tested for *in vitro* antiplasmodial activity against D10. The fractions that showed activity were run on HPLC and their profiles were further segmented into smaller fractions, which were collected and tested for activity. Bioassay guided fractionation was carried out in this manner until the peaks responsible for activity were identified. The amounts collected from HPLC were too little and could not be weighed. Thus a quantitative analysis was impracticable. However, a qualitative comparison of antiplasmodial activity was achieved. The concentrations of the fractions were not calculated, as at this stage the aim was to identify the active fraction, not to evaluate the IC₅₀ values. The results were plotted arbitrarily for comparison, whereby the highest sample concentration loaded onto the plates was arbitrarily set to 100 µgml⁻¹.

Once the active peak had been identified, SPE was used to semi-purify and concentrate fractions that exhibited activity. Preparative HPLC was then performed in order to obtain weighable amount of active constituents for determination of IC₅₀ values.

3.7.2.1 Preparative HPLC

Preparative HPLC was used to purify and obtained weighable amounts of constituents of selected fractions generated from SPE. The fractions were selected on the basis of their antiplasmodial activity. The preparative HPLC separations were achieved using a preparative Discovery® C₁₈ 5 µm (250 cm x 10 mm) column, protected by a pre-packed Bondesil C₁₈ 40 µm guard column (Anatech). The injection volume was set at 200 µl and the flow rate at 2.5 mlmin⁻¹. A 5 mgml⁻¹ stock solution of the selected samples was prepared and applied onto the column. Separation conditions were adjusted to achieve desired resolution for each fraction. The run conditions were optimised for each fraction. Fractions were then delineated for collection. Repeated injections of the samples were then run and the eluting fractions were collected, prepared appropriately (section 3.3) and tested for *in vitro* antiplasmodial activity.

Based on purity analysis, the collected fractions were either further purified using an analytical column, or were taken for NMR analysis for structure elucidation.

3.7.2.2 Analytic HPLC

For each plant extract fraction, the final purification of the active components active fractions was carried out on analytical HPLC. The injection volume was set to 100 μ l and the flow rate at 1.0 mlmin^{-1} or 1.5 mlmin^{-1} . To purify SPE fractions of *E. africanus*, *W. salutaris*, and HPLC fractions of *T. camphoratus*, a reverse phase C_{18} column (Discovery ® C_{18} , 150 x 4.6 mm, 5 μ m) and mobile phase of MeCN:H₂O at varying gradients were used.

The SPE fractions of *E. africanus* that showed antiplasmodial activity; R70, R80, R90 and R100, were run on HPLC at different gradients. The run time was set to 20 min. Fraction R70 was run at 40% to 90% MeCN, fraction R80 at 50-100% MeCN, while R90 and R100 were run at 70% to 100% MeCN.

The SPE fractions of *A. betulina* were purified on HPLC using a C_{16} amide column (Supelco Sigma-Aldrich C_{16} , 150 x 4.6 mm, 5 μ m), and mobile phase of MeCN and 1% TFA in H₂O, at varying gradients, at a flow rate of 2 mlmin^{-1} . The run time for all the fractions was 15 minutes. Fraction 60 was run on HPLC at a gradient of 30% - 80% MeCN, Fraction 80 was run at a gradient of 40%-100 while fraction 100 was run at a gradient of 50%-100%.

3.8 Fractionation of DCM extract of *A. millefolium*

3.8.1 Flash Chromatography

Flash Chromatography was used in the initial separation of the DCM extract of *A. millefolium*. The extract (2 g) was dissolved in 10 ml ethyl acetate (EtAc). The solution was transferred to a round bottom flask, to which silica (Type H Si gel 10- 4 μ g, Sigma) was added until a homogenous slurry was obtained. The EtAc was evaporated off (Buchi rota-evaporator), and the plant extract silica powder residue

was loaded into a flat-bottom funnel column (10.5 x 9.0 cm). The column was washed twice with 50 ml of hexane. The sample was eluted with a step gradient, with 150 ml increments of 10% increasing polarity from hexane to EtAc (100:0, 90:10, 80:20, 70:30, 60:40, 50:50, up to 0:100%) to afford 11 fractions. The column was then eluted with EtAc: MeOH at increasing concentrations of MeOH; (90:10, 70:30, 40:60 20:80 and 100% MeOH) at 150 ml per fraction.

The profile of each fraction was monitored using Thin Layer Chromatography (TLC). Fractions with similar profiles were pooled, concentrated and stored at 4°C in pre-weighed vials.

3.8.2 Column Chromatography

Bioassay detected the antiplasmodial activity in fractions; 60:40, 70:30, 80:20, 90:10 (hexane: EtAc), and 90:10, 70:30, 40:60 (EtAc: MeOH). The active fractions were pooled and further fractionated using column chromatography. Silica (0.063-0.200mm) was mixed with DCM to make slurry, which was poured in to a glass column (60 x 4 cm) and left to settle. The active fraction (20 mgml⁻¹) dissolved in 1ml MeOH was applied onto the column and washed with DCM to wash out MeOH. The wash was followed by a slow and constant flow of EtAc: hexane: MeOH (5:4:1) mobile phase, yielding a total of 110 fractions. Collected fractions of about 2 ml each were analyzed by TLC and were then pooled on the basis of the similarity of their TLC profiles.

3.8.3 Short column chromatography

Five of the fractions generated from column chromatography showed good antiplasmodial activity. These were subsequently combined and subjected to fractionation on silica hexane slurry using a short column (7 ml x 3.3 cm). The sample (1.1 g) was dissolved in 10 ml of EtAc and MeOH (1:1) and was slowly applied onto the column using a 200 µl pipette. The column was washed with 50 ml of hexane to wash out MeOH, and then eluted with a continuous flow of 10% DCM in EtAc at 0.5 mlmin⁻¹ to afford 140 fractions. Fractions were pooled to 7 fractions based on their profiles on a TLC plate.

3.8.4 Thin Layer Chromatography

TLC was performed on Whatman aluminium sheets coated with a 0.2 mm layer of silica (Si gel 60). TLC was used to determine the profiles of the eluted fractions generated from flash chromatography and column chromatography. The fractions were reconstituted in MeOH and spotted on the TLC sheets using 10 µl capillary tubes. For the first 8 fractions generated from flash chromatography (100% hexane to 6:4 EtAc: hexane), the TLC plates were developed in the mobile phase hexane: EtAc (6:4). The polarity of the mobile phase was increased to a hexane: EtAc: MeOH (5.5:4.0:0.5) solvent system for the more non-polar fractions and all the fractions generated from column chromatography. The TLC plates were viewed under 254 nm and 365 nm UV light.

3.8.5 Preparative Thin Layer Chromatography (PTLC)

Active compounds obtained from short column chromatography were in minute amounts and were inadequate for spectral analysis. The fractionation process was repeated under similar conditions to obtain more of the active compounds. The crude extract was subjected to flash chromatography followed by column chromatography of the active fractions. PTLC was used to isolate compounds present in the active fractions generated from column chromatography. Preparative TLC was performed on silica coated glass plates (1mm layer thickness). The fractions were reconstituted in MeOH, sonicated and applied on to the plate using 10µl capillary tubes and dried. The TLC plates were developed in 20 ml of mobile phase EtOAc: hex: MeOH (5:4:1) in a glass tank. The plates were then viewed under 254nm and 365nm light in the dark room. The bands were each scraped off the plate, reconstituted in MeOH. The suspension was clarified by filtration, prepared accordingly and tested for *in vitro* antiplasmodial activity. The fractionation process led to the isolation of a single compound that exhibited antiplasmodial activity.

The active band of *A. millefolium* was purified using a C₈ column (Spheri GROM-OCTYL, 250 x 4 mm, 5 µm) and mobile phase system of MeOH: H₂O, at a gradient

of 40-100% over 15 minutes at a flow rate of 1.5 mlmin⁻¹. The injection volume was at 50 µl.

3.9 Spectroscopy for isolated compounds

The mass of the isolated compounds was determined firstly by running samples on low resolution mass spectra APCI 2000 LC/MS/MS. (Applied Biosystems, California, USA). The masses were recorded in 1:1 MeCN: H₂O at 25°C. The High Resolution Mass Spectra (HRMS) were run on a Waters API Q-TOF Ultima (Waters, Milford, USA). The mass range scanned was between 100-1800 atomic mass units (amu). The run conditions were as follows: sample introduction: 300 µlmin⁻¹ Waters UPLC, injection: 5 µl, source: ESI+, capillary voltage: 3.5kV, cone voltage: 35, RF1: 40, source: 100 °C, desolvation temp: 350 °C, desolvation gas: 350 L/h, cone gas: 50 L/h.

The HPLC isolates of the SPE fractions of *A. betulina* were analysed on Gas Chromatography Mass Spectrometer, (Mass Spec Electron Impact Agilent 6890 GC, MSD Agilent 5973 mass selective detector). Samples were run on capillary column HP-1MS, 30M, ID 0.25mm, film 0.25µm, at a flow rate of 1 mlmin⁻¹. The carrier gas was helium. The initial temperature of the oven was 90°C while the injection port was at 250°C. The initial time was 1 minute, the rate was 35 degrees per minute to 340 degrees, and the final time was 8 minutes.

3.9.1 Nuclear Magnetic Resonance (NMR)

The proton (¹H) and carbon (¹³C) NMR data were recorded in CDCl₃ or DMSO₄ or acetone-*d*₆ at room temperature, at frequencies of 600MHz and 150MHz respectively on a Varian ^{UNIFY} Inova 600 spectrometer (Varian Inc., California, USA). The sample tube (2mm diameter) was used. All the chemical shifts (δ) are reported in parts per million (ppm) relative to TMS (δ = 0) as an internal standard. The 2-Dimensional NMR analysis included COSY, NOESY, HSQC, and HMQC experiments.

3.10 The *in vivo* antimalarial activity of plant extracts and active fractions

A murine model of cerebral malaria was used to investigate the antimalarial activity of plant extracts and selected active fractions. The model was further manipulated in order to investigate the protective and curative activity of the tested samples. The study was granted clearance from the Research and Ethics Committee of the University of Cape Town, under project number 061/1.

3.10.1 Animals

Wild strains of C57BL/6 mice of either sex, weighing between 20–30 g, 8–10 weeks old were obtained from the University of Cape Town (UCT) animal house. Animals were housed under standard environmental conditions (24°C room temperature) and fed with standard pellets and water. The animals had continuous free access to water and food throughout the experiments. The experiments were conducted under the surveillance of the ethical committee of animal care and use of UCT.

3.10.2 Acute toxicity

Acute toxicity of the crude extracts of the five plants was evaluated at a dose of 500mg/kg body weight twice a day. The extracts were administered orally for 7 consecutive days and the animals were monitored for a period of 30 days. Parameters such as survival, changes in body weight and general health indicators such as mobility, paralysis, and swollen abdomen were used to assess toxicity.

3.10.3 Parasite strain

The *P. berghei* (ANKA) strain was donated by the Swiss Tropical Institute, Basle Switzerland. The stock of the parasite was preserved in liquid nitrogen in our laboratories.

3.10.4 Parasite inoculation

The stock of CQ-sensitive *P. berghei* was stored in liquid nitrogen. The preserved parasites were thawed on ice, diluted with saline and introduced intraperitoneally into two mice, as parasite donors. Each animal received 0.2 ml of the inoculum. After 6 days, thin blood smears were prepared and the parasitemia was determined by light microscopy. The blood from the donor mice was collected through tail bleeding into heparinised bottles and diluted with saline such that 0.2 ml of blood contained approximately 1×10^6 infected red blood cells/ml. The animals (five of each group) were inoculated intraperitoneally with 0.2 ml of *Plasmodium berghei* ANKA parasitized blood. The donor mice were sacrificed by cervical dislocation. Parasite strains were maintained by serial passage of blood from mouse to mouse.

3.10.5 Treatment of animals

The test protocol was based on the 4-day suppressive test as described by Peters *et al.*, 1975. A total of five infected but untreated mice served as a negative control and received the solvent. Another group of five mice infected and treated with CQ served as positive control. The test samples were administered at different dosages. The animals were treated orally or subcutaneously for four consecutive days, with 0.2 ml of the test sample. The crude extracts were dissolved in 20% EtOH, 5% bicarbonate in H₂O, and given at a dose of 250mg/kg once a day. The animals were marked by a black permanent marker on the tail.

3.10.5.1 Suppressive test

The suppressive test was carried out to evaluate the schizontocidal activity of active fractions of *W. salutaris* and *A. betulina* on early infection, using the method described by Knight and Peters 1980. The mice were divided into four groups of five mice each. The day of inoculation was termed D₀. On the first day (D₀), mice were inoculated accordingly. Treatment with the fractions/drugs commenced 24 hours post-inoculation. The fractions were administered orally at 250mg/kg once a day for 4 consecutive days. CQ (10mg/kg) was given to the positive control and an equal volume of 5% bicarbonate was administered to the negative control group. Thin

blood smears were made on the fifth day (D₄), and used in assessing the activity of the test samples. The average chemosuppression of parasitemia was then calculated.

3.10.5.2 Evaluation of prophylactic activity

The test was performed to assess the prophylactic activity of fractions of *W. salutaris* and *A. betulina* using the method described by Peters 1965. The mice were randomly divided into four test groups of five mice each, two groups per each test fraction. The mice were administered orally with a 250mg/kg dose of the fractions for 3 consecutive days (D₀- D₂) before infection. On day 4 (D₃), the mice were inoculated with *Plasmodium berghei* (section 3.10.4). Following infection, one group for each test sample stopped treatment while the other group continued with treatment for further 4 days. Thin blood smears were made on day 4 post-infection in both groups and the parasitemia levels were assessed.

3.10.5.3 Curative test

The curative test evaluated the schizontocidal activity in established infection using a modified method similar to that described by Ryley and Peters 1970. On the first day (D₀), mice were infected as in section 3.10.4. In this case, oral treatment with the test fractions at a dose of 250mg/kg commenced on day 3 (72 hours after infection). CQ (10 mg/kg) was given to the positive control and an equal volume of 5% bicarbonate was given to the negative control group. Treatment continued daily until day 7 (4 days post-infection). Thin blood smears were made on the 4th day post-infection (D₇).

3.10.6 Evaluation of activity of test samples

The activity of test samples was evaluated by observation of survival, behavioural changes, typical signs of cerebral malaria, and parasitemia levels in mice. Survival of the mice was observed every day. The mean survival time for each group was determined arithmetically by finding the average survival time (days) of the mice (post-inoculation) in each group over a period of 20 days. Parasitized erythrocytes were counted in Giesma stained thin blood films. The films were made from the tail blood of each mouse, fixed with methanol, stained with 4% Giesma at pH 7.2 for 10

minutes and examined microscopically. The parasitemia level was determined by counting the number of parasitized erythrocytes out of total erythrocytes in random fields of the microscope. The percentage chemo suppression of parasitemia for each compound was calculated with the formula $(A-B/A) \times 100$, where A is the average percentage parasitemia in the negative control group and B is the average percentage parasitemia in the test group [Tona *et al.*, 2001]. Parasitemia was observed from the fourth day post- infection and every second or third day thereafter. The mean parasitemia, survival time and weight were determined and analysed graphically using the Microsoft excel XP and Prism vision 4.

3.11 Statistical analysis

Data obtained from the study were analyzed statistically using Student's test, and values of $P < 0.05$ were considered significant.

University of Cape Town

Chapter 4

University of Cape Town

4 Results

4.0 Introduction

This chapter reports on the results of the extraction of plants, antiplasmodial and cytotoxicity activities of the extracts and active fractions, bio-activity guided fractionation of extracts, and phytochemical analysis of active constituents of the extracts.

The yield of the extracts is expressed as a percentage of dry weight of the plant material. The procedure for antiplasmodial activity of extracts, fractions and compounds was followed as detailed in section 3.4.5. The antiplasmodial activity of test samples was determined by their 50% inhibitory concentration (IC_{50}). Extracts with antiplasmodial activity of 50% inhibitory concentration (IC_{50}) of $10\mu\text{gml}^{-1}$ or less were considered to have significant activity. CQ was used as a positive control in all the tests for antiplasmodial activity. CQ exhibits IC_{50} values of 7-15 ngml^{-1} against the CQ-sensitive strain D10, and 100-200 ngml^{-1} against the CQ-resistant strain K1 of *P. falciparum*. The experiments were done in duplicate on three different occasions. The plots shown in this study are a pooled combination of dose response curves used to determine the mean ($n=3$) IC_{50} value.

The extracts, fractions and compounds that showed antiplasmodial activity against D10 were screened for cytotoxicity on Chinese Hamster Ovarian (CHO) Cell line. Emetine was used as a positive control. The IC_{50} of emetine is in the range 0.04-0.06 μgml^{-1} . The solvent control, 20% MeOH did not have any effect ($IC_{50} > 100\mu\text{gml}^{-1}$) on the antiplasmodial activity or cytotoxicity results of the test samples. Plant extracts, fractions or compounds that showed selectivity indices above 15 were considered to have specific antiplasmodial activity as opposed to general cytotoxicity. The dose response curves of positive control drugs and solvent control are shown in appendix A (page 188).

4.1 *Tarconanthus camphoratus*

4.1.1 Introduction

The leaf material of *T. camphoratus* was extracted using H₂O, MeOH and DCM. The crude extracts were prepared appropriately and tested for antiplasmodial activity *in vitro* against the D10 strain of *P. falciparum*. The active crude extract was subjected to bio-guided fractionation using a combination of HPLC, SPE and TLC techniques.

4.1.2 The *in vitro* antiplasmodial activity of the leaf extracts of *T. camphoratus*

The DCM leaf extract of *T. camphoratus* showed antiplasmodial activity against the D10 strain with an IC₅₀ value $7.60 \pm 0.6 \mu\text{gml}^{-1}$ (Figure 4.1.2.1). The MeOH extract showed moderate activity (IC₅₀ = $10.4 \pm 1.2 \mu\text{gml}^{-1}$), while the water extract showed little activity (IC₅₀ > $25 \mu\text{gml}^{-1}$) (Table 4.1.3.1).

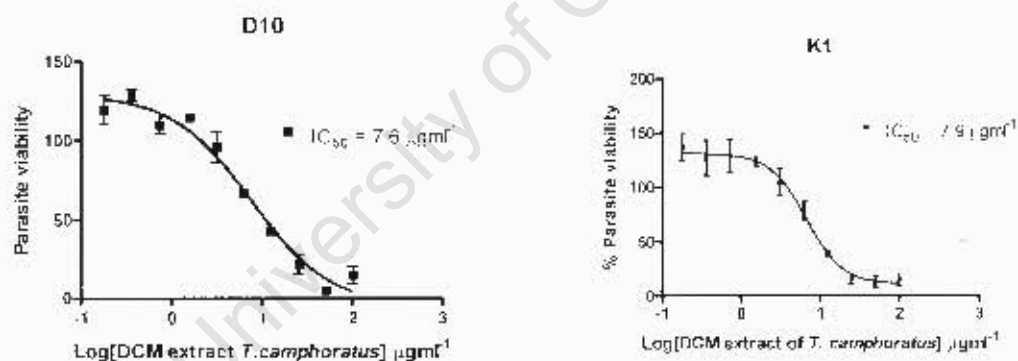


Figure 4.1.2.1 Dose response curves of the DCM crude extract of *T. camphoratus* against the D10 and K1 strains of *P. falciparum*.

4.1.3 Identification of the active principles of the DCM crude extract of *T. camphoratus*

An aliquot of the crude extract was run on HPLC at a shallow gradient to view all the components of the extract. The gradient was adjusted accordingly to improve separation between the components. Four fractions (1-4) were collected separately as delineated on the HPLC chromatograph in Figure 4.1.3.1.

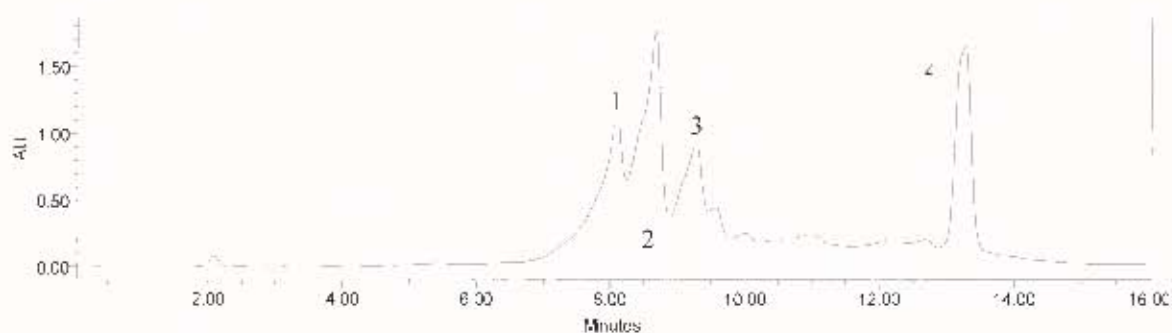


Figure 4.1.3.1 The chromatogram of the full HPLC profile of the DCM extract of *T. camphoratus* run on a C₁₈ column at a gradient of 10-100% MeCN in H₂O over 40 minutes, at flow rate of 1 mlmin⁻¹. No constituents eluted after 16 minutes.

The yields of the fractions collected by HPLC were low and could not be weighed, therefore a qualitative comparison rather than a quantitative analysis of antiplasmodial activity was carried out. Fractions 1 and 2 showed equal antiplasmodial activity, fraction 3 showed no activity while fraction 4 showed the highest activity. Following identification of the active fractions, SPE (details see 3.7.1) was performed to concentrate the active fractions using the eluting system of increasing 20% increments of MeCN in H₂O. SPE fractions 40, 60 and 80 (%MeCN) showed activity with IC₅₀ values 4.6 ± 0.6 µgml⁻¹, 3.4 ± 1.2 µgml⁻¹ and 2.2 ± 0.2 µgml⁻¹ respectively, as shown in Table 4.1.3.1 and on their respective dose response curves in Figure 4.1.3.2.

Table 4.1.3.1 The yields and antiplasmodial activity of the leaf crude extracts and SPE fractions of the DCM extract of *T. camphoratus*.

Plant extract	Yield in percentage (%)	IC ₅₀ mean ± S.D (µgml ⁻¹) D10 strain
DCM	0.9	7.70
MeOH	2.4	10.4
H ₂ O	1.8	>25
SPE fractions (MeCN %)		
40	2.04	4.6 ± 0.6
60	1.1	3.4 ± 1.2
80	0.8	2.2 ± 0.2

In vitro antiplasmodial activity of the SPE fractions

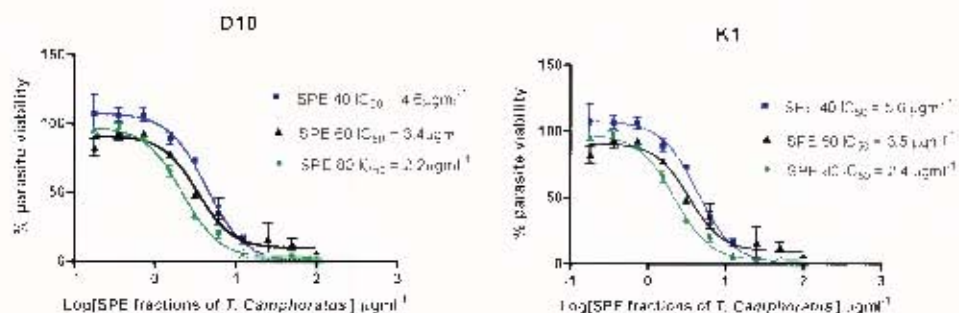


Figure 4.1.3.2 The antiplasmodial activity of the SPE fractions of DCM extract of *T. camphoratus* against the D10 and K1 strains.

4.1.4 Purification of Fraction 4 (T-60)

Fraction 4 (T-60), as identified on HPLC, was present in both SPE fractions 60 and 80. T-60 eluted at a retention time of 3.94 minutes at specified chromatographic conditions (Figure 4.1.4.1). The purified peak (T-60) showed antiplasmodial activity of $2.2 \pm 0.3 \mu\text{gml}^{-1}$ and $2.4 \pm 0.3 \mu\text{gml}^{-1}$ against the D10 and K1 strains of *P. falciparum* respectively. The elucidated chemical structure of compound T-60 is shown in Figure 4.1.9.1.

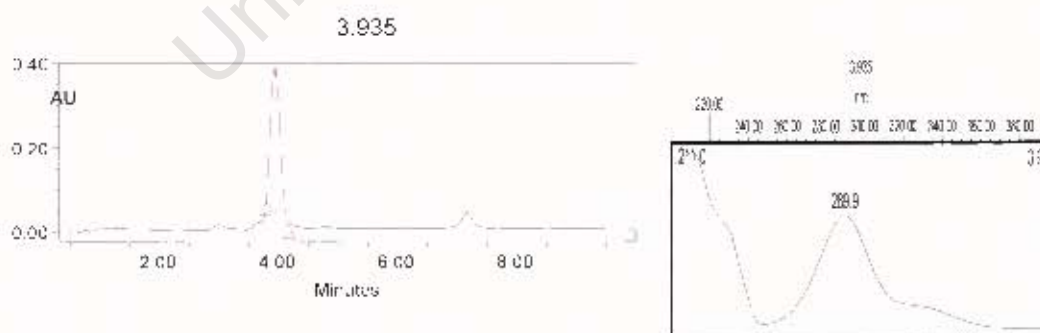


Figure 4.1.4.1 The HPLC chromatogram and UV spectrum of compound T-60 present in the SPE 60 fraction, run at a gradient of 40-80% MeCN over 15 minutes at 1.5 mlmin^{-1} flow rate.

4.1.5 Purification of fraction 1 and fraction 2 of the DCM crude extract of *T. camphoratus*

Fractions 1 and 2 appeared as single peaks on the HPLC chromatogram of the crude extract. The two fractions were collected and run separately on HPLC at the same gradient as the crude. The chromatogram of each peak appeared to have three extra peaks. These results suggested that each peak collected from the crude extract could be a combination of three compounds with close retention times. It was also possible that the compounds represented by the original peaks in the crude were unstable, and could have decomposed into two other compounds.

In recollecting fractions 1 and 2 on HPLC, several attempts were made to improve separation between the fractions. The concentration of the sample run on HPLC was lowered to avoid overloading of the column. The gradient was made more shallow (20%-40% MeCN over 40 minutes, flow rate 1.5 ml min^{-1}) to improve resolution and to avoid any overlap or carryover. The sample was dissolved in a more polar mobile phase (7:3 H₂O: MeCN) to improve separation. The connecting tube from the detector to the collecting end was shortened to reduce possibilities of cross contamination.

The peaks were fairly spread out from each other, with a resolution factor of 2.59. However, when the collected peaks were run individually on HPLC, each peak still yielded two other peaks (Figure 4.1.5.1). Peak 1 and peak 2 were separately run isocratically at 35% MeCN to investigate if the compounds were not co-eluting when run at a gradient. The results were similar to the ones obtained previously.

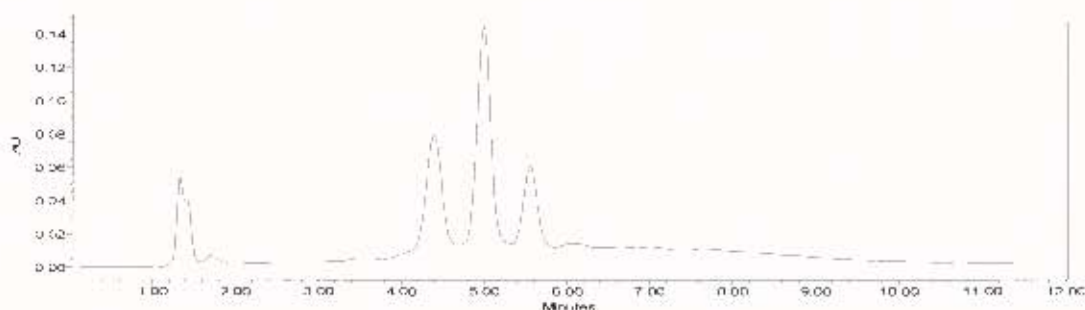


Figure 4.1.5.1 The chromatogram of "peak 1" fraction (similar to that of "peak 2") isolated from the crude extract of *T. camphoratus*. The peak was run on C₁₈ at a gradient of 10-60% MeCN over 15 min at a flow rate of 1.5 ml min^{-1} . The two "peaks" showed similar decomposition patterns.

The suggestion that the compound represented by one of the peaks was unstable and decomposed into two other compounds seemed to be a sensible interpretation of the results from HPLC. To investigate this suggestion further, the TLC separation method was applied as an attempt to purify fraction 1 and fraction 2.

4.1.5.1 Thin-layer chromatography

Fractions 'peak1' and 'peak2' were separated on a TLC plate as described in section 3.8.4 using the mobile phase, petroleum ether: DCM: EtAC: MeOH (1:1:8:0.2). The results revealed that the TLC profiles of the two fractions were similar, with the presence of three bands with R_f values of 0.36 cm, 0.27cm and 0.24 cm. The bands were scraped from the plates, reconstituted in mobile phase and run on HPLC. Unfortunately the HPLC chromatogram results obtained for each band still showed the presence of three peaks with similar UV spectra Figure 4.1.5.2 (a) and (b).

The UV spectra of peaks present in the HPLC profile of crude extract and of HPLC fractions of *T. camphoratus*.

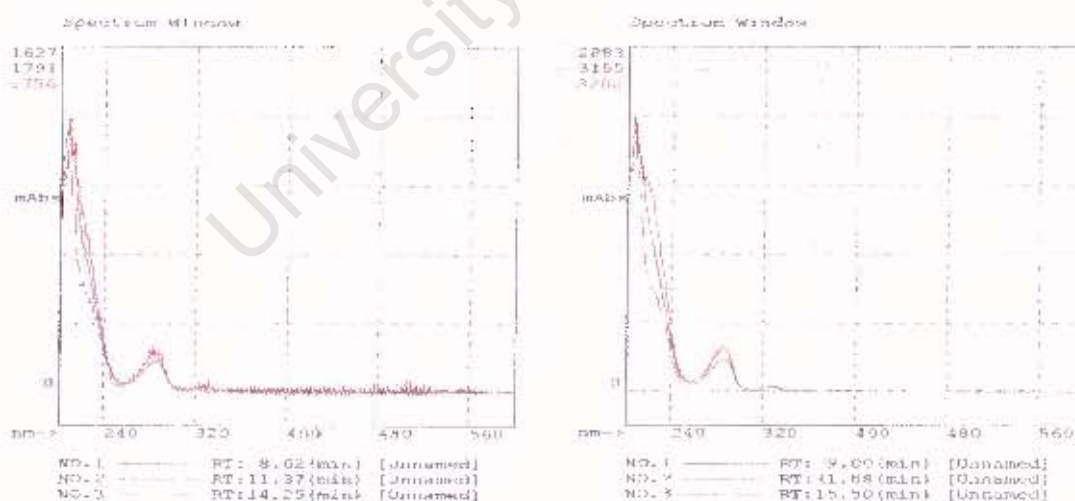
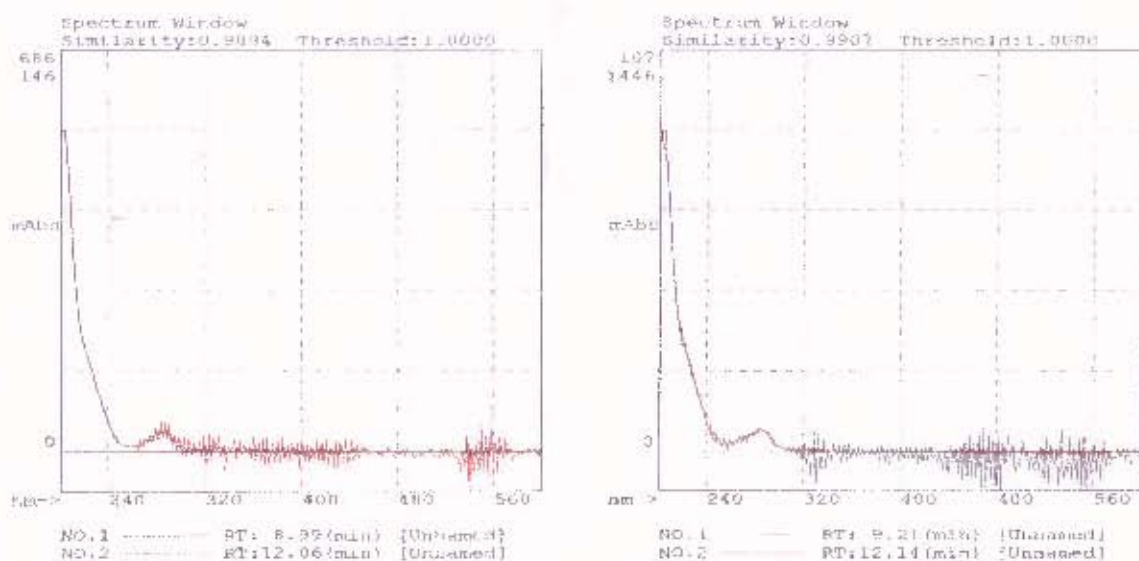


Figure 4.1.5.2 (a) Comparison of the UV spectra of the three original peaks in the crude extract of *T. camphoratus* (left) and the UV spectra of the three peaks present in the isolated samples (right).



(b) The comparison of the UV spectra of peak 1 and 2 from the “peak 1” fraction (left) and the UV spectra of peak 1 and peak 2 in the “peak 2” fraction, after separation with TLC.

4.1.6 The *in vitro* antiplasmodial activity of “peak 1” and “peak 2” fractions isolated from the crude extract of *T. camphoratus*

Although the antiplasmodial activity testing was performed using samples that were not absolutely pure, there was an observable enrichment of each peak of interest, which accounted for the major part of the activity. Fractions 1 (“peak 1”) showed activity with an IC_{50} of $6.1 \mu\text{gml}^{-1}$ and $6.2 \mu\text{gml}^{-1}$ against D10 and K1 strains respectively, while fraction 2 (“peak 2”) had an IC_{50} of $4.2 \mu\text{gml}^{-1}$ and $4.12 \mu\text{gml}^{-1}$ against D10 and K1 strains respectively. The respective dose response curves are shown in Figure 4.1.6.1.

The crude extract showed no toxicity effects at tested concentrations ($IC_{50} > 100 \mu\text{gml}^{-1}$). The isolated compound, T-60, and fractions “peak 1” and “peak 2”, exhibited less cytotoxicity on the C110 cell line with IC_{50} values $79.4 \mu\text{gml}^{-1}$, $63.1 \mu\text{gml}^{-1}$ and $60.3 \mu\text{gml}^{-1}$ respectively (Figure 4.1.6.2).

In vitro antiplasmodial activity and cytotoxicity of HPLC fractions and isolates of DCM extract of *T. camphoratus*.

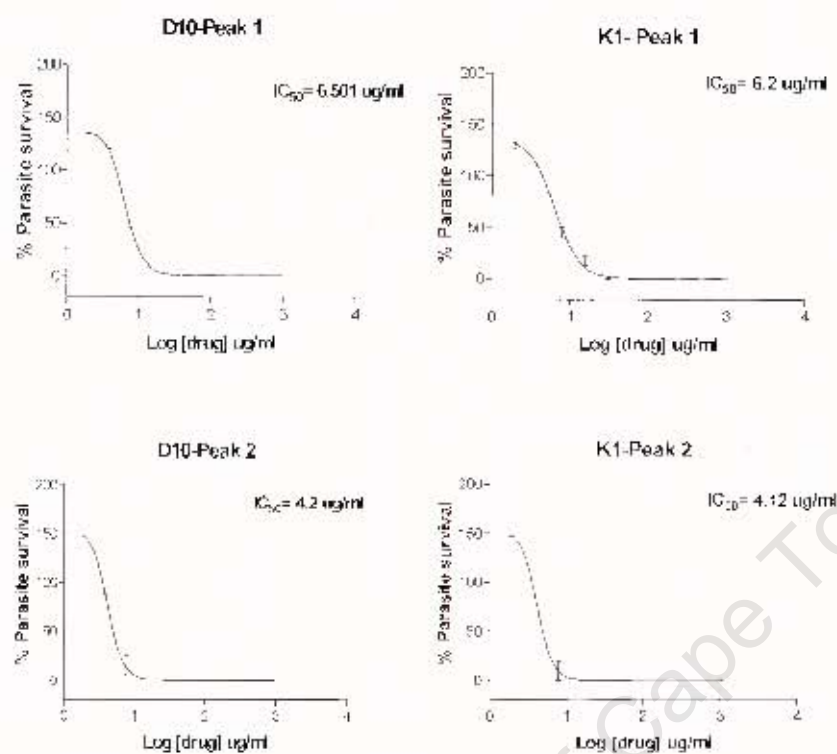


Figure 4.1.6.1 Dose response curves of enriched "peak1" and "peak2" fractions of *T. camphoratus* against the D10 and K1 strains of *P. falciparum*.

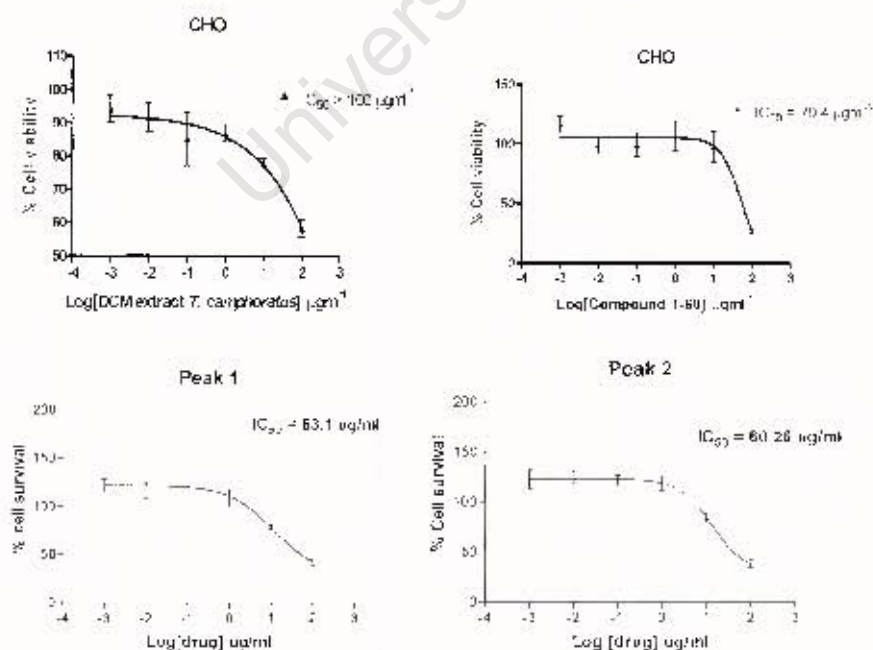


Figure 4.1.6.2 Dose response curves showing toxicity effect of the DCM crude extract, fractions and isolates of *T. camphoratus* on the CHO cell line.

4.1.7 Mass spectrometry

The isolated peak samples contained more than one compound. Their purity was not sufficient to obtain NMR and molecular formula data, however their molecular mass was determined using low resolution mass spectrometry. Peak 1 had a molecular weight of 386.26 while peak 2 had a molecular weight of 408.27.

4.1.8 NMR of compound T-60

Assignment of the molecular formula $C_{15}H_{13}O_4$ was based on the IR-FIS-MS data ($m/z = 257.0821$ [M⁺], calculated 257.0814). The 1H , ^{13}C , and 2D-NMR; HSQC, HMQC and COSY NMR are summarised in Table 4.1.8.1. Spectra are in appendix B (page 189). The chemical structure of the compound is shown in Figure 4.1.9.1.

Table 4.1.8.1 The summary of the 1H , ^{13}C , COSY and HSQC spectra of compound T-60 isolated from SPE 60 and 80 fractions of *T. camphoratus*.

Position	δ 1H (J Hz)	δ ^{13}C	HMQC	COSY
1				
2	5.46 dd (13.2, 3.0Hz)	79.0d		H-3a
3a	3.09 dd (15.9, 12.6Hz)	42.8t	C-1', C-4, C-4a	H-2, H-3b
3b	2.78 dd (15.9, 3.0Hz)		C-1', C-2, C-4	H-2, H-3a
4		194.9s		
4a		102.0s		
5		164.1s		
6	5.89 d (2.1Hz)	95.7d	C-7, C-8	
7		167.0s		
8	5.93 d (2.1 Hz)	94.8d	C-7, C-8a	
8a		163.2s		
1'		139.0s		
2'	7.49 d (7.8Hz)	125.9d	C-2, C-3'	H-3'
3'	7.41 t (7.5Hz)	128.3d	C-1', C-4', C-5'	
4'	7.36 t (7.2Hz)	128.2s	C-5'	
5'	7.41 t (7.5Hz)	128.3d	C-1', C-3'	
6'	7.49 d (7.8Hz)	125.9	C-2', C-5'	H-2'

4.1.9 The chemical structure of T-60

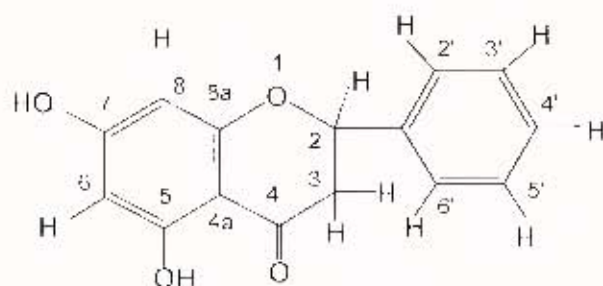


Figure 4.1.9.1 The chemical structure of T-60

4.1.9.1 Assignment of ^{13}C and ^1H NMR

The absence of a NOESY spectrum indicated that T-60 is a planar molecule.

The molecular mass of $m/z = 256$ suggested a 15-carbon molecule. The UV spectrum of the molecule was highly similar to that of flavonoids as it consisted of two major absorption maxima at approximately 270 nm and 320 nm, which originate from the A-ring and B-ring of flavonoids. The two meta-coupled doublets at δ 5.89 and δ 5.93 ($J = 2.1$ Hz) are typical of protons on the A-ring of flavonoids with O-group substitution at C-7 and C-5 positions.

The resonances of the B-ring overlapped due to the symmetrical nature of the ring at C-1' and C-4'. The symmetry caused C-2' signals to overlap with that of C-6', and similarly signals of C-3' overlapped with that of C-5'. A pair of ortho-coupled doublets ($J = 7.8$ Hz) at δ 7.49 integrated for two protons which were identified as H-2' and H-6'. H-2' and H-6' protons were linked to carbon at δ 125.9 through correlations observed in HSQC spectrum. A pair of ortho-coupled triplets ($J = 7.5$ Hz) at δ 7.41 integrated for two protons identified as H-3' and H-5'. HSQC correlations linked both H-3' and H-5' to a carbon at δ 128.3. H-3' and H-5' protons were distinguished from H-2', 6' protons as they always resonate up-field, and resonate as triplets due to the additional splitting by H-4'. The magnitude of the coupling constants and correlations in COSY and HMQC spectra resolved the positioning of protons H-3', H-5', and H-2', H-6' protons. Proton H-2' interacted with the H-3', H-5' pair in the COSY spectrum, and correlated to C-3' in HMQC. Furthermore, H-3', H-5' showed correlation to C-4'.

A double doublet at δ 5.46 was assigned to H-2. This signal is highly characteristic of flavanones. HSQC linked H-2 to C-2 at δ 79.0. The COSY spectrum revealed that H-2 coupled with two double doublets at δ 3.09 and δ 2.78, assigned to H-3a and H-3b respectively and positioned at C-3 at δ 48.2 through correlations in the HSQC spectrum. Their positioning was confirmed by their double doublet splitting pattern due to spin-spin interaction with each other, and with H-2. This information was highly supportive of a flavanone. Correlations from the H-3 protons to C-2, and C-1' were observed in the HMQC spectrum.

The carbonyl at δ 194.9 and two highly deshielded quaternary carbons at δ 102.0, and 163.2 were assigned to C-4, C-4a and C-8a respectively. Two and three bond correlations from the H-3 protons to C-4 and from H-3a to C-4a observed in the HMQC spectrum resolved the assignment of the quaternary carbons. HMQC contours from H-6 and H-8 to a carbon at δ 167.0 (C-7) and from H-6 to a carbon at δ 164.1 (C-5) resolved the positioning of C-7 and C-5. The assigning of δ 163.2 to C-8a was also supported by the HMQC correlation observed between H-8 and C-8a. The spectroscopic data supported the characterization of compound T-60 as 5,7-dihydroxyflavanone.

4.1.10 Discussion

The DCM extract of *T. camphoratus* showed antiplasmodial activity against D10 and K1 strains without any evidence of chloroquine cross resistance between the two strains. The results of antiplasmodial activity of the extract are in agreement with activity reported by Clarkson *et al.*, 2004. The reported activity was however, without reference to toxicity, and the constituents responsible for activity remained unidentified. The results in this study have indicated that the extract has relatively less cytotoxicity against the mammalian CHO cell line. The selectivity of the antiplasmodial activity was high by a magnitude of over 12 for the crude, and 14 for purified fractions.

The resolution factor for 'peak 1' and 'peak 2' fractions was found to be 2.59. Resolution values greater than 2 are regarded as good resolution. Therefore it was highly unlikely that there was any overlap or carryover between the fractions. Instead there is a high possibility that the compounds in 'peak 1' and 2 have similar chemical structures with identical carbon backbone. The UV spectra of 'peak1' and 'peak2' were similar. Furthermore, the results also revealed that the UV spectra of the three peaks present in the collected samples were identical to the UV spectra of the first three peaks in the crude extract.

T-60, one of the active constituents of the extract was identified as 5,7-dihydroxyflavanone. The compound 5,7-dihydroxyflavanone has been isolated from medicinal plants before, and has been reported to possess molluscicidal and fungicidal biological activities [Zhanglan *et al.*, 2002; Kehau *et al.*, 2001]. This is the first report of the isolation of 5,7-dihydroxyflavanone from *T. camphoratus*, and its antiplasmodial activity. The structure of 5,7-dihydroxyflavanone is not related and has no similarities to the chemical structures of current antimalarial drugs. This could be an indication of the different mode of action exerted by this compound to kill the malaria parasite.

In conclusion, the "peak 1" and "peak 2" fractions, and the isolated compound (T-60), showed good antiplasmodial activity without chloroquine cross resistance and low cytotoxicity. The activity is however, considered moderate when compared to the activity of current antimalarial drugs, which is in the range of nanograms. In malaria endemic areas where chloroquine resistance is prevalent and there is a shortage of antimalarial drugs, extracts of *T. camphoratus* could be useful. The effectiveness of the extracts however, still needs to be determined in *in vivo* systems.

4.2 *Eriocephalus africanus*

4.2.1 Introduction

The leaf material of *E. africanus* was extracted with MeOH and DCM. The extracts were screened for *in vitro* antiplasmodial activity against the D10 strain. The active crude extract was subjected to bio-activity guided fractionation using SPE and HPLC.

4.2.2 *In vitro* antiplasmodial activity of the crude extract of *E. africanus*

The DCM and MeOH leaf extracts of *E. africanus* showed antiplasmodial activity against D10 with IC_{50} values of $8.5 \pm 0.5 \mu\text{gml}^{-1}$ and $9.2 \pm 1.2 \mu\text{gml}^{-1}$ respectively. The DCM extract also showed antiplasmodial activity against the resistant strain K1 with an IC_{50} value of $8.7 \pm 0.5 \mu\text{gml}^{-1}$. The DCM extract showed slightly more antiplasmodial activity than the MeOH extract, and less cytotoxicity ($IC_{50} = 89.1 \pm 5.2 \mu\text{gml}^{-1}$) (Figure 4.2.2.1), and was selected for further fractionation.

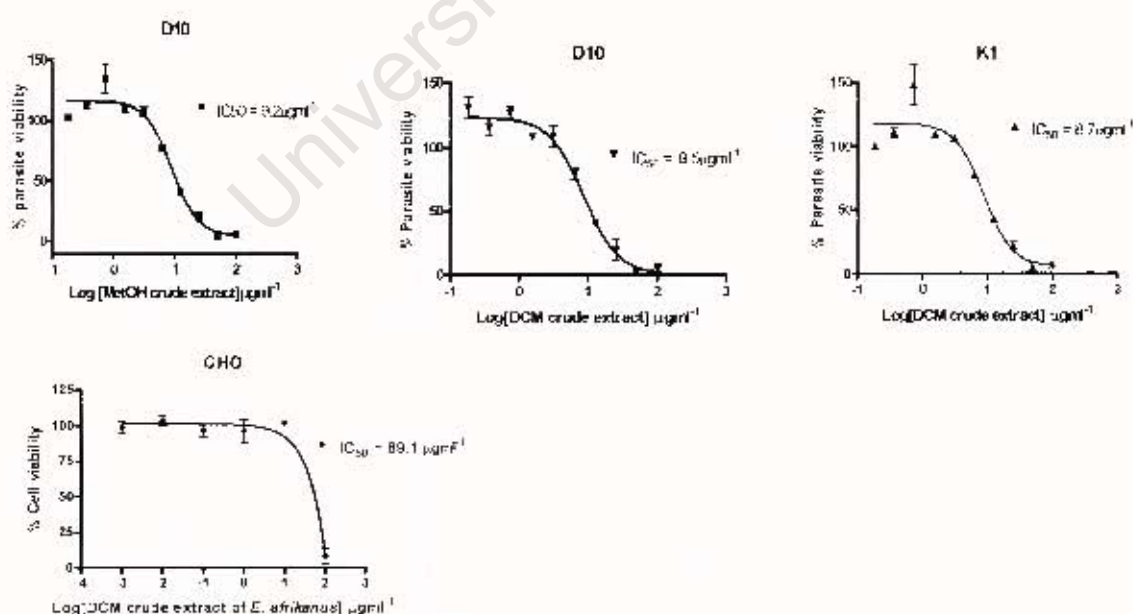


Figure 4.2.2.1 The *in vitro* antiplasmodial activity and cytotoxicity of the crude MeOH and DCM extracts of *E. africanus*.

4.2.3 Fractionation of DCM extract of *E. africanus* by SPE

The DCM extract of *E. africanus* was fractionated using SPE following the procedure stipulated in section 3.7.1. The fractionation process yielded 10 fractions, which were prepared appropriately and tested for *in vitro* antiplasmodial activity against the D10. Four fractions; 70:30, 80:20, 90:10 and 100:0 (MeCN: H₂O) exhibited positive antiplasmodial activity. The yields and IC₅₀ values of the crude and the active fractions are shown in Table 4.2.4.1. The active fractions were selected and further fractionated on HPLC in order to purify their active constituents.

4.2.4 Cytotoxicity of crude extract and active fractions from SPE

The four active fractions; R70, R80, R90 and R100, were tested for cytotoxicity on the CHIO cell line. Fractions R70 and R80 had IC₅₀ values of 2.8 – 0.6 µgml⁻¹ and 14.8 ± 1.1 µgml⁻¹ respectively while R90, and R100 had IC₅₀ values of 35.5 ± 0.5 µgml⁻¹ and 13.5 ± 0.5 µgml⁻¹ respectively. The selectivity indexes of the crude and active fractions were calculated (section 3.6) and are shown in Table 4.2.4.1.

Table 4.2.4.1 The percentage yields, *in vitro* antiplasmodial activity and selectivity indexes of the DCM leaf extract of *E. africanus*, and fractions generated from SPE.

Extract or Fraction	Percentage yield (%)	IC ₅₀ D10 mean ± S.D. (µgml ⁻¹)	Selectivity Index
DCM extract	2.6	8.5 ± 0.5	10.4
R70 (70:30)	3.2	8.5 ± 0.4	0.3
R80 (80:20)	0.6	6.5 ± 1.2	2.3
R90 (90:10)	1.1	6.7 ± 0.4	5.3
R100 (100:0)	2.3	4.5 ± 0.2	3.0

4.2.5 Purification of active SPE fractions using HPLC

The active fractions generated from the SPE were run separately on HPLC under the different chromatographic conditions stipulated in section 3.7.2.2, using a C₁₈ column. Peaks on the chromatogram were collected individually, quantified and tested for activity.

4.2.5.1 Fraction R70

The HPLC chromatogram revealed that the fraction contained three clusters of peaks, which were grouped into three fractions (Figure 4.2.5.1). Only the second fraction (70-2), eluting at about 7 minutes showed antiplasmodial activity. This sub-fraction contained a single major peak. The peak exhibited an antiplasmodial activity of IC₅₀ value $7.6 \pm 0.5 \mu\text{gml}^{-1}$. The peak was purified and the chemical structure of the compound was elucidated.

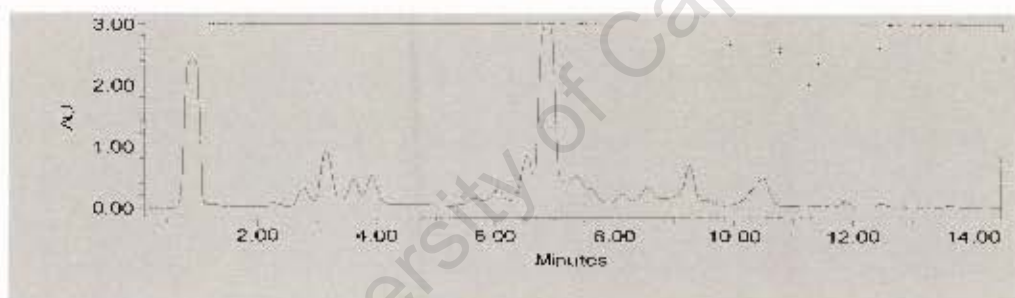


Figure 4.2.5.1 The HPLC chromatogram of the SPE fraction R70 when run at a gradient of 45-100% MeCN over 15 minutes at a flow rate of 2 ml min⁻¹. The peak eluting at about 1 min is a solvent peak.

4.2.5.2 Fraction R80

The chromatogram of the R80 fraction showed one prominent peak and three small peaks. The major peak accounted for 95% of the total area under the peak of the entire chromatogram (Figure 4.2.5.2). The major peak possessed antiplasmodial activity with an IC₅₀ value of $6.5 \pm 0.4 \mu\text{gml}^{-1}$, while the three small peaks showed negligible activity (IC₅₀ > 20 μgml^{-1}). This compound (R90-1) was also present in the R90 fraction. The compound was collected as it eluted from HPLC and its chemical

structure was determined. The structure elucidation and the assignment of ^{13}C and ^1H NMR are discussed in section 4.2.7.3.1.

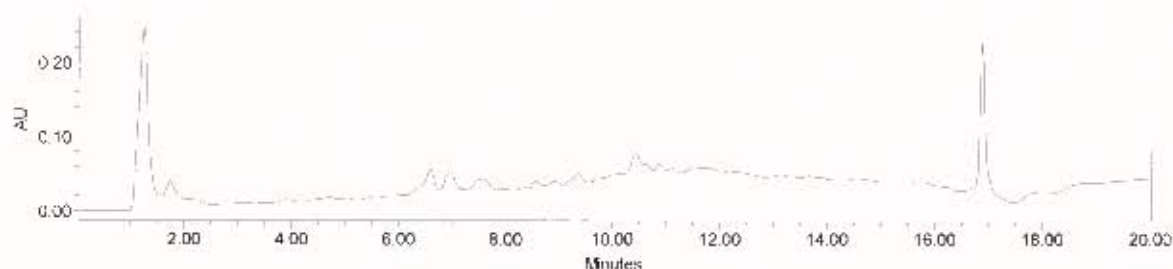


Figure 4.2.5.2 The chromatograph of fraction 80 when run at a gradient of 40-100% over 20 min at a flow rate of 1.5 ml min^{-1} . The fraction contained compound R90-1 eluting at 16.8 min.

4.2.5.3 Fraction 90

The profile of the fraction 90 contained two main peaks, R90-2 and R90-3, which showed antiplasmodial activity with IC_{50} values of $9.5 \pm 0.5 \mu\text{gml}^{-1}$ and $6.9 \pm 0.3 \mu\text{gml}^{-1}$ respectively. Both compounds were also present in the R100 fraction.

4.2.6 Cytotoxicity of the isolated compounds

The yields of the isolated compounds R90-1 and R90-3 were low. Compounds R90-1 and R90-3 could not be tested for cytotoxicity due to insufficient amounts. Compounds R70-2 and R90-2 exhibited toxicity with IC_{50} values of $4.4 \pm 0.4 \mu\text{gml}^{-1}$ and $9.6 \pm 0.6 \mu\text{gml}^{-1}$ respectively.

Table 4.2.6.1 Summary of the antiplasmodial activity and cytotoxicity of the compounds isolated from the DCM extract of *E. africanus*. ND indicates that the test was not done.

Compounds	IC_{50} D10 mean \pm S.D (μgml^{-1})	Selectivity index
R70-2	7.6 ± 0.5	0.6
R90-1	6.5 ± 0.4	ND
R90-2	9.5 ± 0.5	1.01
R90-3	6.9 ± 0.3	ND

Activity of SPE fractions and isolated compounds

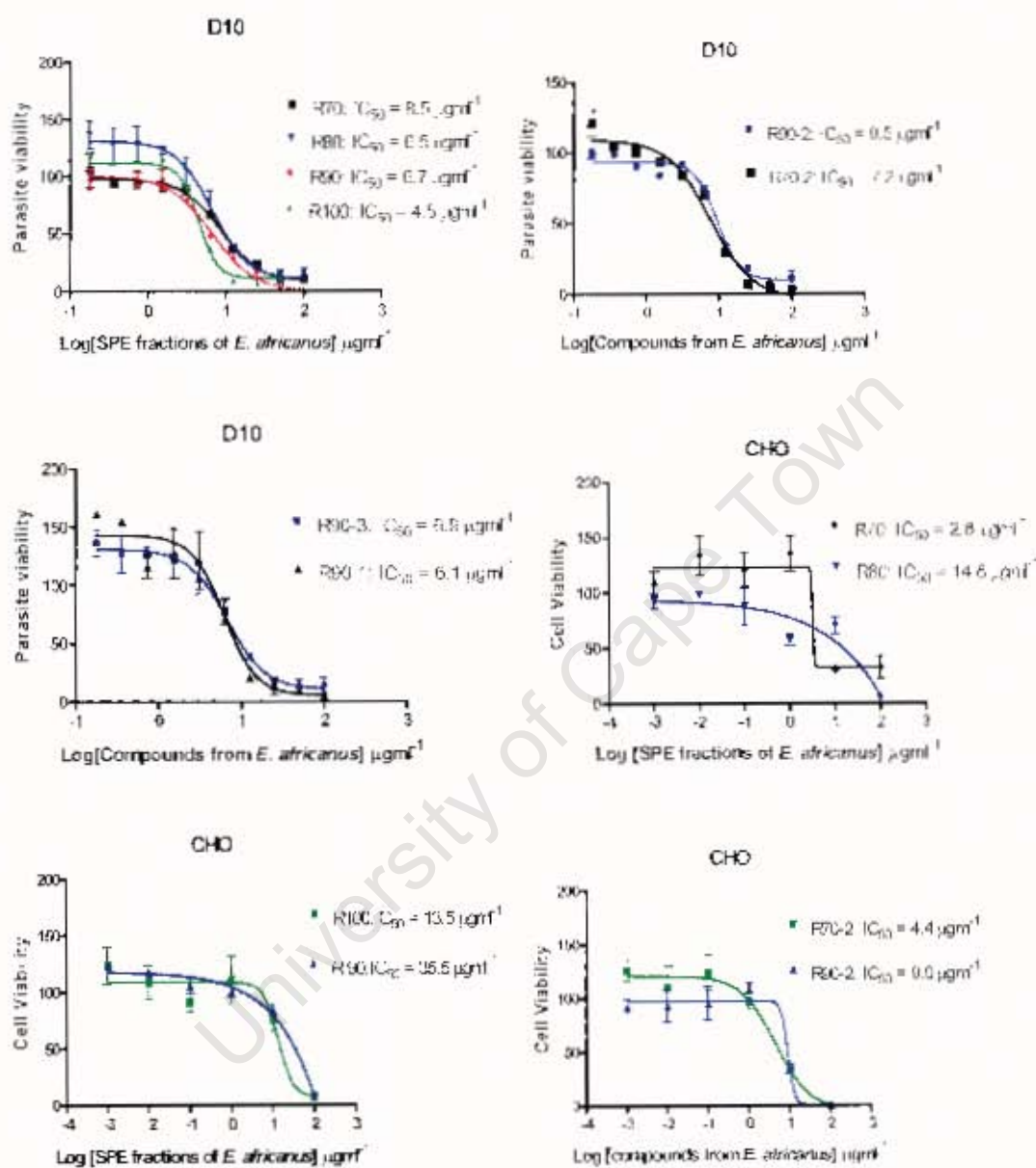


Figure 4.2.6.1 The *in vitro* antiplasmodial activity and cytotoxicity effect of SPE fractions and selected compounds isolated from the DCM extract of *E. africanus*.

4.2.7 Structure elucidation of isolated compounds

4.2.7.1 NMR of compound R70-2

The ^1H and ^{13}C NMR spectra were recorded in CDCl_3 and in DMSO_d as some of the resonances on the ^1H spectrum were not clear in CDCl_3 due to overlapping signals. The ^1H and ^{13}C chemical shifts, and the HMQC, HMQC and COSY correlations are shown in Table 4.2.7.1 and Table 4.2.7.2 below. The spectra are shown in appendix B (page 193). The chemical structure of compound R70-2 is shown in Figure 4.2.7.3.

Table 4.2.7.1 R70-2 in CDCl_3

Position	$\delta^1\text{H}$ (J Hz)	$\delta^{13}\text{C}$	HMQC	COSY	NOESY
1	α 2.56 ddd (13.8, 7.1, 4.8 Hz) β 2.82 dd (14.4, 1.8 Hz)	28.9 t	C-2, C-3, C-9, C-10, C-20	H-1 β , H-2 β H-1 α	
2	α 1.59 quintet (13.8, 3.5 Hz) β 1.91 qt (13.8, 3.3 Hz)	18.8 t		H-2 β H-1 α , H-2 α , H-3 α	
3	α 1.31 ddd (13.5, 13.5, 3.6 Hz) β 1.51 dd (13.2, 1.8 Hz)	41.0 t		H-2 β , H-3 β H-3 α	
4		34.3 s			
5	α 1.69 dd (10.5, 6.0 Hz)	45.8 d	C-1, C-4, C-9, C-10, C-18, C-19, C-20	H-6 α , H-6 β	
6	α 1.83 dd (10.8, 1.2 Hz) β 2.19 ddd (9.3, 9.3, 4.7 Hz)	29.7 t		H-5 α , H-6 β H-5 α , H-6 α , H-7 α	
7	α 5.42 dd (3.9, 1.5 Hz)	78.5 d	C-5, C-8, C-9, C-14, C-20	H-6 β	H-14
8		132.1 s			
9		121.8 s			
10		48.6 s			
11		143.5 s			
12		143.0 s			
13		134.9 s			
14	6.68 s	111.3 d		CH3-16, CH3-17	
15	3.24 septet (6.9 Hz)	26.8 d	C-12, C-13, C-14, C-16, C-17		H-7
CH3-16	1.20 d (7.1 Hz)	23.4 q	C-13, C-15, C-17	H-15	
CH3-17	1.19 d (7.1 Hz)	23.3 q	C-13, C-15, C-16	H-15	H-2 β , H-3 β , H-6 β , H-5
CH3-18	0.87 s	32.0 q	C-3, C-4, C-5, C-19		
CH3-19	0.86 s	20.1 q	C-3, C-4, C-5, C-18		
20		178.2 s			

Table 4.2.7.2 Compound R70-2 run in DMSO_d

Position	$\delta^1\text{H}$ (J Hz)	$\delta^{13}\text{C}$	HMBC	COSY	NOESY
1	α 2.42 ddd (14.0, 13.8, 4.2 Hz) β 2.64 dd (14.0, 1.5 Hz)	29.4 t	C-2, C-3, C-9, C-10, C-20	H-1 β , H-2 β H-1 α	
2	α 1.51 dquintet (13.2, 3.6 Hz) β 1.75 m	19.2 t	C-10	H-2 β H-1 α , H-2 α , H-3 α	
3	α 1.21 ddd (13.3, 13.5, 3.3 Hz) β 1.42 dd (13.3, 1.4 Hz)	41.2 t	C-4	H-2 β , H-3 β H-3 α	
4		34.8 s			
5	α 1.59 dd (10.8, 6.0 Hz)	45.6 d	C-4, C-6, C-9, C-10, C-18, C-19, C-20	H-6, H-6 β	
6	α 1.75 m β 2.07 m	29.9 t	C-10 C-4, C-5, C-7, C-8	H-5 α , H-6 β H-5 α , H-6 α , H-7 α H-6 β	
7	α 5.44 dd (4.2, 1.2 Hz)	77.6 d	C-5, C-8, C-9, C-20,		H-14
8		132.2 s			
9		122.6 s			
10		48.5 s			
11		143.9 s			
12		143.7 s			
13		134.9 s			
14	6.68 s	111.9 d	C-7, C-9, C-12, C-15	CH3-16, CH3-17	H-7
15	3.24 septet (7.1 Hz)	26.9 d	C-12, C-13, C-14, C-16, C-17		
CH3-16	1.20 d (7.1 Hz)	23.4 q	C-13, C-15, C-17	H-15	
CH3-17	1.19 d (7.1 Hz)	23.3 q	C-13, C-15, C-16	H-15	
CH3-18	0.87 s	32.0 q	C-3, C-4, C-5, C-19		H-2 β , H-3 β , H-6 β , H-5
CH3-19	0.86 s	20.1 q	C-3, C-4, C-5, C-18		
20		176.2 s			

Assignment of the molecular formula of as $C_{20}H_{27}O_4$ was based on the HR-ESI-MS data ($m/z = 331.1923 [M^+]$, calculated 331.1909).

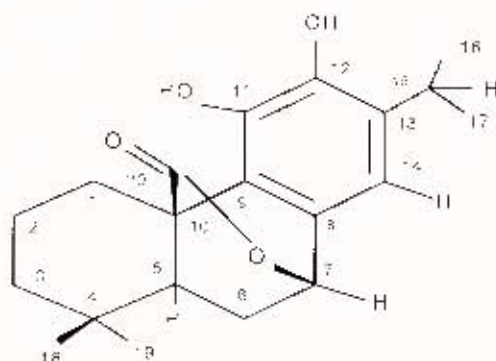


Figure 4.2.7.3 The chemical structure of Compound R70-2.

4.2.7.2 The Proton and Carbon assignments of NMR spectra of R70-2

The $DMSO_d_6$ run results were used for assignment of the proton and carbon NMR. The ^{13}C spectrum revealed the presence of 20 carbons, a typical carbonyl resonance at δ 176.2, oxygen attached aromatic carbons resonances at δ 143.7, 143.9 and 122.6, and three other aromatic carbons. The DEPT spectrum revealed presence of four CH_3 , four CH_2 , four CH and eight quaternary carbons.

The 1H spectrum showed four three-proton signals at δ 1.20, 1.19, 0.87 and 0.86, and one aromatic proton at δ 6.68 ppm. The presence of only one aromatic proton at δ 6.68 resonating as a singlet, suggested substitutions on the other five carbons on the ring. The singlet was identified as H-14. H-14 correlated to the carbon at δ 111.9 (C-14) as observed in the HSQC spectrum. The HMBC spectra showed long-range correlations from H-14 to quaternary carbons at δ 122.6, δ 143.9 and δ 143.7 which were identified as C-9, C-12 and C-11 respectively. The downfield resonances of C-12 and C-11 suggested that they are directly attached to an O-group. The deshielded signal of quaternary carbons at δ 134.9 and δ 132.2 linked in the HMQC to CH_3 -16 and H-14 respectively were assigned C-13 and C-8. The HSQC linked a septet resonating at δ 3.24 to a carbon at δ 26.9, identified as C-15. The integration of a septet (H-15) revealed that it was one proton. The multiplicity pattern indicated that H-15 was split by 6 other protons. The COSY spectrum showed vicinal coupling ($J = 7.1$ Hz) between H-15 and two methyl signals at δ 1.20 and δ 1.19, identified as CH_3 -

16 and CH3-17 through their HSQC correlations to carbons δ 23.4 and δ 23.3 respectively. The COSY correlations between H-15 and the two methyl groups positioned H-15 in the middle. Correlations from H-15 to CH3-16, CH3-17, C-13, C-14 and C-12 observed in the HMBC spectrum confirmed its positioning. Further correlations from CH3-16 and CH3-17 to C-15 and C-13 were also observed.

The carbon resonance of δ 77.6 is highly indicative of a carbon attached to an O-group. Carbon δ 77.6 was identified as C-7. The HSQC spectrum linked a double doublet at δ 5.44 to C-7. The downfield resonance of δ 5.44 was supportive of an O-group substitution at C-7. H-7 also showed correlation to C-8 and C-9 through HMBC contours. The double doublet splitting pattern indicated that H-7 was interacting with two other protons. The COSY spectrum revealed H-7 coupled with two protons resonating as multiplets at δ 1.75 and δ 2.07. The protons interacted through vicinal coupling with each other and both correlated to the carbon at δ 29.9 in the HSQC, and were consequently identified as the H-6 protons. The H-6 multiplets further coupled with a double doublet at δ 1.59, identified as H-5. HSQC linked H-5 to C-5 at δ 45.6. Correlations from H-6 to C-5, C-7, and C-8, and from H-5 to C-6 and C-9 were observed in the HMBC spectrum.

More correlations in HMBC from H-6 and H-5 to two quaternary carbons at δ 48.5 and δ 34.8 together with two bond correlations from CH3-18 and CH3-19 to carbon δ 34.8 helped assign the two quaternary carbons to C-10 and C-4 respectively. Two of the four methyl singlets at δ 0.87 and δ 0.86 were positioned on carbons δ 32.0 and δ 20.1 by HSQC correlations, and assigned as CH3-18 and CH3-19 respectively. Further correlations from H-5 to CH3-18 and CH3-19 confirmed their position at C-4. The two methyl groups showed three-bond correlation in the HMBC to carbon at δ 41.2, which was identified as C-3. The HSQC spectrum revealed that two protons resonating as a double double doublet (ddd) and a double doublet (dd) at δ 1.21 and δ 1.42 respectively were attached to C-3. The protons showed geminal coupling (J = 13.3 Hz) in the COSY and also showed correlation to C-4 in the HMBC spectrum. The space orientation of the protons was resolved by interactions in the NOESY spectrum. H-7 was assigned an α -orientation, as the molecule would be most stable with H-7 in this orientation. NOESY spectrum showed that H-3 at δ 1.42 interacted with H-6 β and was therefore assigned a β -orientation. The COSY spectrum showed

that H-3 α coupled with H-3 β and with a multiplet at δ 1.75 which was identified as H-2 β . In addition to H-3 β , H-2 β also showed geminal coupling ($J= 13.8$ Hz) to a double quintet which was linked to C-2 at δ 19.2 through HMQC correlation. The double quintet was identified as H-2 α . H-2 β also showed COSY coupling with another proton resonating as a double double doublet at δ 2.42, identified as H-1 α . H-1 α and another double doublet (H-1 β) showed HSQC correlation to a carbon δ 29.4 which was then identified as C-1. Protons H-2 α and H-2 β exhibited vicinal coupling ($J= 13.2$ Hz) with H-3 protons and geminal coupling ($J= 3.6$ Hz) with each other as revealed in the COSY. Furthermore, H-1 β showed three bond coupling with H-3 β ($J= 1.5$ Hz). Proton H-1 α confirmed that it was positioned two or three bonds from C-2, C-3, C-9, C-10 and C-20 carbons through correlations in the HMBC spectrum.

By comparison of the spectroscopic data with compounds previously isolated from related plant species, the NMR data of compound R70-2 was similar to that of carnosol. It was concluded that the molecule isolated is carnosol. There are no reports in the literature on the isolation of carnosol from *E. africanus* species. This is the first report on the isolation of carnosol from *E. africanus*. There are however, reports on the isolation of a derivative of carnosol, which has an oxygen atom attached to carbon 6 (C-6) instead of C-7.

4.2.7.3 Structure elucidation of compound R90-1

The molecular formula of compound R90-1 was found to be $C_{17}H_{14}O_5$ from the M^+ peak in the HRMS at m/z 299.0921 (calculated 299.0924). The 1H and ^{13}C -NMR spectra were recorded in $CDCl_3$. The structure of the compound is shown in Figure 4.2.7.5, while the 1H , ^{13}C , HMBC, and COSY spectra are summarized in Table 4.2.7.4. The spectra are in appendix B (page 192).

Table 4.2.7.4. The calculated chemical shifts and 2D-NMR correlations of R90-1 Compound, 5,7-dimethoxy-4'-hydroxy flavone

Position	δ^1H (J Hz)	$\delta^{13}C$	HMBC	COSY
2				
3	6.69 s	103.4 d	C-1', C-4	
4		182.0 s		
4a		164.6 s		
5				
6	6.36 d (2.4Hz)	98.1 d		H-8
7				
8	6.68 d (2.4Hz)	92.4 d	C-8a	H-6
8a				
1'		78.2 s		
2'	7.98 d (8.71Hz)	128.2 d	C-1'	H-3'
3'	7.10 d (4.21Hz)	114.5 d	C-4'	H-2'
4'		163.2		
5'	7.10 d (4.21Hz)	114.5 d		H-6'
6'	7.98 d (8.7Hz)	128.2 d	C-1'	H-5'
5-OMe		55.3		
7-OMe		54.9		
4'-OH	9.49 s			

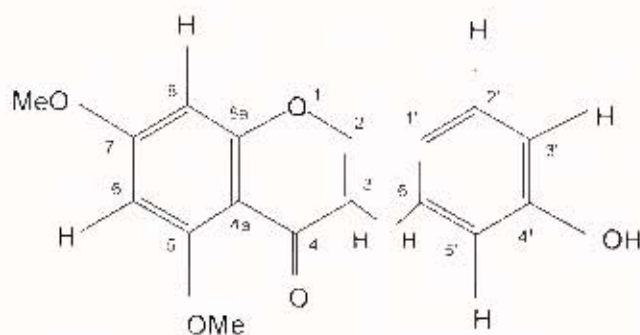


Figure 4.2.7.5 The structure of Compound R90-1, which was characterized as 5,7-dimethoxy-4'-hydroxyflavone.

4.2.7.3.1 Proton and Carbon assignments for compound R90-1

The absence of a NOESY spectrum suggested a planar aromatic molecule. The presence of two meta-coupled doublets at δ 6.68 and δ 6.36, and further two ortho-coupled doublets at δ 7.98 and δ 7.10 are strongly indicative of a flavanoid. The singlet at δ 6.69 is highly characteristic of the H-3 proton of a flavone. H-3 positioning was confirmed by a correlation to a quaternary carbon at C-4 as observed in the HMQC spectrum. The presence of only two doublets in the B-ring indicated substitution at C-4'. There was an overlap of signals from H-2' and H-6' at δ 7.98, and overlap from H-3' and H-5' at δ 7.10 due to the symmetrical nature and the free rotation of the B-ring. H-2', H-6' and H-3', H-5' protons appeared as two pairs of ortho-coupled doublets ($J = 8.7$ Hz) at δ 7.98 and 7.10. The H-3', 5' doublet occurred upfield (δ 7.10) from the H-2', 6' doublet (δ 7.98) due to the deshielding by the O-group substitution at C-4'. The increased electron density is due to O-group at C-4' feeding electrons to the carbon at C-3' and C-5'. Also, a HMQC correlation from H-2', H-6' to C-1' helped position protons H-2' and H-6'. The range of the resonance of these two doublets are typical of that of flavones. HSQC correlations linked H-2', H-6' and H-3', H-5' pairs to carbons at 128.2 (C-2', C-6') and 114.5 (C-3', C-5') respectively. A resonance downfield at δ 9.49 suggested a hydroxyl group, and HSQC correlation from δ 9.46 to carbon δ 163.2 positioned the OH-group at C-4'.

The ^1H spectrum showed two signals at 4.0 and 3.9 ppm, which are typical of methoxy groups. The doublet splitting of H-8 and H-6, and the meta-coupling between the two protons ($J = 2.4$ Hz) suggested O-group substitutions at positions C-7

and C-5. The chemical shifts of δ 6.68 (H-8) and δ 6.36 (H-6) of the A-ring, suggested that the methoxy groups are positioned at positions C-5 and C-7. The methoxy group at C-5 is slightly downfield due to its proximity to the ketone at C-4. HMBC correlation from the methoxy at C-5 to C-4 further confirmed its position. H-8 (δ 6.68), is more deshielded due to its close proximity to an O-group at position C-1, which then left proton δ 6.36 at position C-6.

From the spectroscopic data, compound R90-1 was characterized as 5,7-dimethoxy-4'-hydroxyflavone. This compound is the derivative of apigenin, which is present in *Rosemarinus* species. In the literature, there has been a report on the isolation of 5,7-dimethoxy-4-hydroxy flavone from this species. To the best of our knowledge, this is the first report of the isolation of this derivative from *E. africanus*.

4.2.7.4 Structure elucidation of compound R90-2

Compound R90-2 was obtained as a white amorphous powder. The HR-ESI-MS spectrum showed a molecular ion peak at $m/z - M^+$ 456.2102, calculated (456.2110), corresponding to the molecular formula $C_{30}H_{24}O_7$. The 1H and ^{13}C chemical shifts and HMBC, COSY and NOESY NMR are shown Table 4.2.7.6. The spectra in appendix B (page 194). The chemical structure of R100 is shown in Figure 4.2.7.7.

Table 4.2.7.6. The 1H , ^{13}C , HMBC, COSY and NOESY NMR spectra of compound R90-2.

Position	δ 1H (J Hz)	δ ^{13}C	HMBC	COSY	NOESY
1	α 0.92 m (1Hz) β 1.69 m	38.9 t	C-3, C-5 C-3, C-5, C-25	H-1 β H-1 α	H-3 α
2	α 1.55 β 1.6	26.9 t	C-1	H-3 α	CH3-24 CH3-23, CH3-25
3	α 3.12 dd (12.0, 4.8 Hz)	78.5 d	C-23, C-24	H-2 α , H-2 β	H-1 α , H-5 α , CH3-24
4		38.8 s			
5	α 0.71 d (9.6Hz)	55.7 d	C-6	H-6 α , H-6 β	H-3 α , H-7 α , H-9 α , CH3-24
6	α 1.37 m β 1.53 m	18.3 t		H-5 α , H-7 α H-6 α	CH3-24 CH3-25, CH3-26
7	α 1.41 m β 1.45 m	34.4 t		H-6 α H-6 α , H-6 β	H-5 α , H-9 α , CH3-27
8		40.7 s			

9	α 1.33 dd (12.6, 24Hz)	50.9 d	H-11a	H-11a, H-5a, H-12a, CH3-27
10	α 1.43 m	37.1 s	H-11b	H-11b
11	β 1.24 dddd (12.9, 12.9, 12.6, 6.4)	20.9 t	C-8, C-9	H-9a, H-11a
12	α 1.05 m	25.7 t	H-12b	H-9a, H-18a, CH3-27
13	β 1.71 dddd β 2.50 ddd (12.3, 11.7, 3.3Hz)	38.5 d	H-18a	H-11b, H-15b, H-19b, CH3-26
14	1.16 dt 1.54 m	42.4 s	H-15b	H-13b, CH3-26
15a	β 1.41 m	29.6 t	H-16a	H-18a, H-22a, CH3-27
16	α 2.22 dt (12.6, 3.3Hz)	32.3 t	H-15b, H-16b, H-22b	H-15b, H-16b, H-22b
17	β 1.63	56.2 s	H-16a	H-12a, H-21a, CH3-27
18	α 3.02 ddd (11.1, 10.9, 4.6Hz)	49.2 d	H-18a	H-13a, H-22b, CH3-27
19b		47.4 d	C-29	H-18a
20	α 1.41 m	130.8 s	H-21b, H-19b, H-21a	H-19b, H-21a
21	β 1.9 m	30.6 t	H-21b, H-19b, H-21a	H-19b, H-21a
22	α 1.44 β 1.63	37.0 t	H-21a	H-16a, H-16b, H-19b, H-21a, H-21b, H-6b, H-19b, CH3-25
CH3-23	0.74 s	14.9 q	C-3, C-4, C-5, C-24	CH3-24
CH3-24	0.94 s	27.4 q	C-3, C-4, C-5, C-24	CH3-23
CH3-25	0.85 s	15.5 q	C-1, C-5, C-9, C-23	H-1a
CH3-26	0.96 s	15.4 q	C-7, C-8, C-9, C-10	H-6b, H-11b, H-13b, H-15b, CH3-26
CH3-27	1.00 s	13.9 q	C-8, C-13, C-14, C-15	H-7a, H-9a, H-12a, H-16a, H-18a, CH3-25
28		178.9 s	C-15	H-29b, CH3-18a
29 a	4.58 d (1.81Hz)	108.9 t	C-30	H-29a, CH3-30
29 b	4.70 d (1.8Hz)		C-30	H-29a, CH3-30
CH3-30	1.69 s	18.6 q	C-20	H-29b, H-29a, CH3-30

4.2.7.4.1 The ^1H and ^{13}C -spectra Assignment for compound R100 (R90-2)

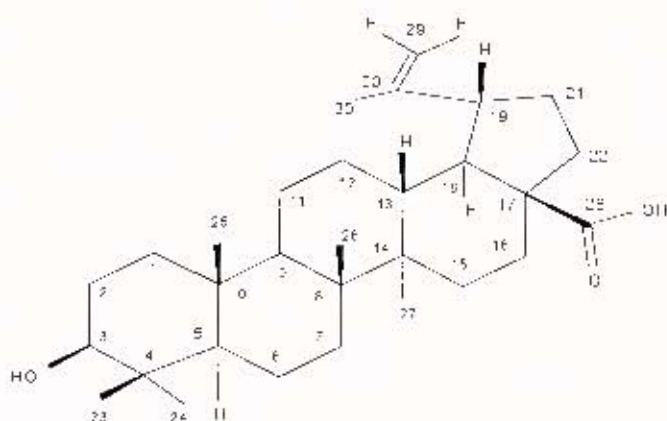


Figure 4.2.7.7 The chemical structure of compound R90-2.

The high molecular weight of the compound suggested a C-30 compound, possibly a triterpenoid or dimeric sesquiterpenoid. The ^{13}C -NMR revealed the presence of 30 carbons; 6 methyls, 12 methylenes, 5 methines and 7 quaternary carbons including a carbonyl group at δ 178.9. The ^1H spectrum displayed 6 singlets integrating for the 6 methyls (δ = 0.74, 0.94, 0.85, 0.96, 1.01, and 1.69). The above spectral data suggested a triterpenoid. Typically, from the biosynthetic pathway, triterpenoids bear 2 methyl groups at C-4 and a methyl group at every ring junction of the molecule (C-8, C-10 and C-14).

The 5 methyl singlets were assigned to their respective carbons through HSQC correlations and their positioning confirmed through their correlations to adjacent carbons in the HMQC spectrum. CH₃-23 and CH₃-24 both showed correlation to C-4 at δ 38.8 and to each other in the HMQC spectrum. Correlation from a double doublet signal at δ 3.12 to both CH₃-23 and CH₃-24 was observed in the HMQC spectrum. A deshielded upfield signal of δ 3.12 suggested a proton in close proximity to an O-group. The signal was linked to C-3 at δ 78.5 through HSQC correlation. The chemical shift of C-3 supported a link to an O-group substitution. The presence of two methyl groups at C-4 and the O-R functional group on C-3 of the A-ring are characteristic of terpenoids from their biosynthetic pathway. The OH group at an equatorial position would be highly favourable for the stability of the molecule. The β CH₃ at C-4 would be more deshielded. CH₃-23 assumed an α -orientation due to its

resonance up field. CH₃-24 was consequently assigned β -orientation. H-3 α also coupled with two protons at δ 1.55 and 1.6 in the COSY, positioning the protons adjacent to C-3. Both protons correlated to C-2 at δ 26.9 in HSQC and were identified as H-2 α and H-2 β respectively. C-1 (δ 38.9) was identified through correlation from H-2 α using HMBC contours. Two protons resonating as multiplets at δ 0.92 and δ 1.69 showed correlation to C-1 in the HSQC. No coupling between H-1 protons and H-2 protons was observed in COSY. The positioning of the H-1 protons was confirmed by their correlation to C-3 and C-5 as observed in the HMQC spectrum. The positioning of CH₃-25 at C-10 was confirmed also by a correlation from H-1 β . The H-1 protons correlated to C-5 (δ 55.7) to which a doublet at δ 0.71 identified as H-5, was attached. H-5 correlated to a carbon at δ 18.3 assigned as C-6, and coupled in the COSY with two protons resonating as multiplets at δ 1.37 and 1.53. The multiplets correlated to C-6 in the HSQC and were identified as H-6 protons. The multiplicity is due to splitting by H-5, two H-7 protons as well as interaction with each other. Apart from H-5, H-6 β further coupled with a multiplet at δ 1.41 identified as H-7 α . Another multiplet at δ 1.45 showed correlation to C-7 in the HSQC, and also showed interaction with both H-6 protons in the COSY spectrum. The multiplet was identified as H-7 β . COSY coupling placed the following protons adjacent to each other; H-5 α to H-6 α , H-6 α to H-7 α , H-7 β to H-6 α and H-6 β , H-9 α to H-11 α , H-15 β to H-16 α , H-13 β to H-18 α , H-18 α to H-19 β , H-19 β to H-21 β and H-1 α to CH₃-25 β .

The other three methyl groups CH₃-25, CH₃-26 and CH₃-27 were positioned on ring junctures at quaternary carbons C-10, C-8 and C-14 respectively. The relative stereochemistry was determined by analysis of the NOESY spectrum. The NOESY correlations of CH₃-25 and CH₃-24, CH₃-25 and CH₃-26, indicated that the rings have a chair configuration with trans-fused ring junctions and β -axial orientations of CH₃-25, 26 and H-13, while CH₃-27 at C-14 projects below the plane of the ring. The chair configuration is more stable. This configuration was confirmed by NOESY correlation between CH₃-24 and H-6 α , and, CH₃-23 and CH₃-25. The NOESY spectrum also revealed spacial correlations between the following proton signals; H-1 α /H-3 α , H-2 α /CH₃-24, H-5 α /H-3 α /H-7 α /H-9 α /CH₃-24, CH₃-25/CH₃-26/H-6 β , H-11 β /H-13 β /H-15 β , and between H-16 β /H-22 β /H-19 β .

Commonly, triterpenoids are tetracyclic or pentacyclic. The 5th ring of the pentacyclic triterpenoids is always a pentagon, with a number of functional groups attached, commonly alkenyl and carboxyl groups.

C-17 at δ 56.2 appeared to be deshielded due to its attachment to the carboxyl group. C-20 was highly deshielded as expected of a carbon with no protons attached within a typical CH₃-C-CH₂ structure. C-20 was identified as a carbon at δ 130.8 on which H-29a and H-29b are attached (HSQC). Further confirmation of the structure was obtained from HMQC correlation from CH₃-30 to C-20, and from H-19 β to C-18, C-20 and C-21. H-29a and H-29b protons also correlated to C-30.

The structure of the R90-2 molecule was confirmed as 3 β -hydroxylup-20(29)-en-28-oic acid (betulinic acid).

4.2.7.5 Compound R90-3

Compound R90-3 was isolated from fraction R90 and fraction R100. The compound was in small quantities in the fraction. The NMR data of the compound was obtained. Due to the minute amounts of the purified sample, the quality of the ¹H spectrum was poor. The upfield proton resonances were not clear, and there was a lot of overlap of resonances, which made the spectrum complicated and impossible to interpret. As a result the chemical structure of compound R90-3 could not be elucidated.

4.2.8 Discussion

The leaf DCM extract of *E. africanus* showed antiplasmodial activity against the D10 and K1 strains of *P. falciparum*, and low cytotoxicity on the CHIO mammalian cell line. Through bio-guided fractionation using SPE and HPLC, three compounds were isolated and identified as carnosol, 5,7-dimethoxy-4'-hydroxyflavone, and betulinic acid, and were shown to have positive activity against *Plasmodium falciparum*.

Carnosol is one of the potent phenolic antioxidants present in extracts of the herb rosemary (*Rosmarinus officinalis*) [Inatani *et al.*, 1983; Wei *et al.*, 2005]. This diterpenoid was isolated for the first time by White and Jenkins from *Salvia carnososa*

in 1942, and from *R. officinalis* 20 years later [Gajhede *et al.*, 1990]. It is the first time that carnosol has been isolated from the *E. africanus* species.

Carnosol has been reported to possess antiseptic, anti-inflammatory, antioxidant, anticarcinogenic and weak antimicrobial properties [Moujir *et al.*, 1993; Horiuchi *et al.*, 2007; Haung *et al.*, 1994; Chan *et al.*, 1995]. It has been reported to inhibit tumour formation, induce apoptosis, and cause cell cycle arrest [Singletary *et al.*, 1996; Chan *et al.*, 1995]. These properties could account for its high cytotoxicity effect on CHO as observed in this study. There is a high possibility that carnosol acquires its antiplasmodial activity by exerting similar mechanisms to inhibit the growth of the parasite. Therefore, the activity observed here might not be specific to the parasite, but be due to its general cytotoxic effect to any biological cell, including the host cells [Offord *et al.*, 1995]. Based on these results, carnosol can therefore not be considered as an antiplasmodial agent.

The problem encountered when isolating this compound is its instability. The peak of carnosol decomposed into two other unknown products. Literature reports that carnosol can easily be converted into rosmanol and other intermediates [Gonzalez *et al.*, 1992; Ho *et al.*, 1994].

Betulinic acid, also known as lup-20(29)-en-28-oic acid derivative, was isolated from fraction R90. Betulinic acid is a naturally occurring pentacyclic lupane-type triterpene, a derivative of betulin. This compound was first isolated from the bark of *Ziziphus mauritiana* and has since been identified in many other plants. It can be chemically derived from betulin, a substance found in large quantities in the bark of white birch trees, especially in *Betula alba* [Pisha *et al.*, 1995]. Betulinic acid has been reported to possess anti-inflammatory, anti-HIV, anti-malarial and anti-tumour properties [Tan *et al.*, 2003]. Betulinic acid was originally thought to selectively induces apoptosis in human melanoma cells, but has been found to be active even against non-melanoma tumor cell types including neuroectodermal and malignant brain tumors [Zuco *et al.*, 2002; Selzer *et al.*, 2000; Schmidt *et al.*, 1997]. Betulinic acid is currently undergoing preclinical development to increase its pharmacological properties as a potential anticancer agent [Gauthier *et al.*, 2006; Tan *et al.*, 2003].

Betulinic acid has been found to exhibit moderate *in vitro* antimalarial activity against asexual erythrocytic stages of the human malaria parasite *Plasmodium falciparum* [Bringmann *et al.*, 1997; Steele *et al.*, 1999]. The results from this study are in agreement with these findings. Betulinic acid however, exerted a considerable level of toxicity on CHO cells. The selectivity index of this compound is low, which indicates that the mode of action of betulinic acid is not selective for the parasite. This could be attributed to its antitumour properties. Based on the results obtained in this study, this compound can not be considered a hit as an antimalarial agent.

The third compound isolated from fraction 90 is 5,7-dimethoxy-4'-hydroxyflavone. It is also known as pinocembrin. This compound has been previously isolated from a few plants such as *Alinia galangal*, *Flourensia retinophylla*, *Populus species*, and sunflower species [Kumar *et al.*, 2006; Jasso de Rodriguez *et al.*, 2007; Zhou *et al.*, 1999; Bruce *et al.*, 2001]. It has been shown to possess antifungal and antitumour properties. It has also been shown to trigger mitochondrial-independent cell death [Kumar *et al.*, 2006]. In this study pinocembrin has shown moderate antiplasmodial activity and less cytotoxicity on the CHO cell line.

In conclusion, *E. africanus* has high a composition of bioflavanoids. Bioflavanoids exhibit multiple biological activities including inhibition of proliferation or induction of apoptosis in tumor cells. The antiplasmodial activity and cytotoxicity observed in this study correlate with the antitumor and antioxidant properties of these molecules.

4.3 *Warburgia salutaris*

4.3.1 Introduction

The initial screening was aimed at identifying medicinally active parts of the plant. EtOH, H₂O, hexane, and DCM were used to extract the bark, leaves and twigs of the plant. The extracts were prepared appropriately and tested for *in vitro* antiplasmodial activity against the D10 strain of *P. falciparum*.

4.3.2 The yields and antiplasmodial activity of crude extracts of *W. salutaris*

Both the ethanol and hexane leaf extracts showed good *in vitro* antiplasmodial activity, with IC₅₀ values of $2.5 \pm 1.0 \mu\text{gml}^{-1}$ and $2.5 \pm 0.6 \mu\text{gml}^{-1}$ respectively. The hexane extract of the bark and the DCM extract of the twigs also showed significant antiplasmodial activity with IC₅₀ of $2.9 \pm 0.8 \mu\text{gml}^{-1}$ and $3.0 \pm 0.10 \mu\text{gml}^{-1}$ respectively. The H₂O extract did not show significant antiplasmodial activity. The yields and antiplasmodial activities (IC₅₀ values) of the extracts obtained from each solvent are summarized in Table 4.3.2.1.

The hexane extract of the bark had good antiplasmodial activity, but due to the limited amount of the bark material, only the leaf and twig extracts were pursued. Also, due to small yields of hexane leaf and twig extracts, only the EtOH leaf extract and DCM twig extract were assessed further and subjected to bio-activity guided fractionation. The EtOH leaf extract was tested against K1 and RSA11, and exhibited antiplasmodial activity with IC₅₀ values of $1.9 \pm 0.5 \mu\text{gml}^{-1}$ and $2.9 \pm 0.2 \mu\text{gml}^{-1}$ respectively. The respective dose response curves are shown in Figure 4.3.2.1.

Table 4.3.2.1 The yields and antiplasmodial activity of the extracts of *W. salutaris* on the D10 strain of *P. falciparum*.

Extract	Plant part	% Yield	IC ₅₀ mean ± SD (µgml ⁻¹)
Hexane	Leaves	1.5	2.5 ± 0.5
	Twigs	0.4	6.2 ± 0.1
	Bark	0.42	2.9 ± 0.8
Ethanol	Leaves	4.2	2.5 ± 0.8
	Twigs	2.05	6.8 ± 0.2
	Bark	2.2	4.2 ± 0.2
Dichloromethane	Twigs	0.8	3.0 ± 0.2
Water	Twigs	2.6	13.8 ± 0.5
	Bark	2.5	6.5

In vitro antiplasmodial activity of the EtOH leaf extract of *W. salutaris*

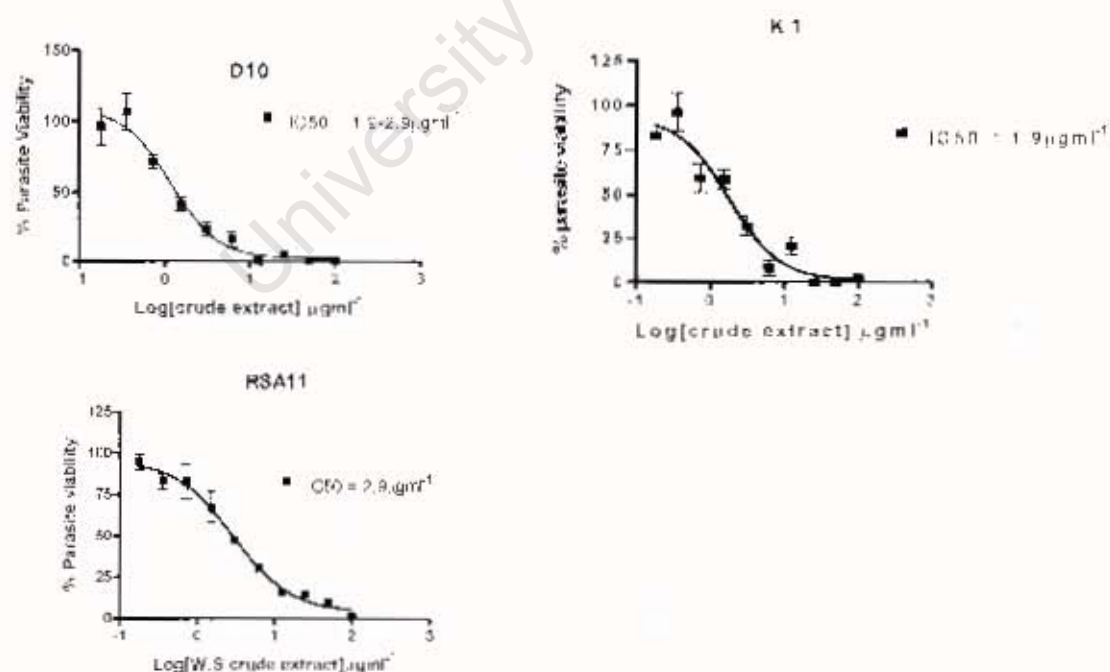


Figure 4.3.2.1 The *in vitro* antiplasmodial activity of the EtOH leaf extract of *W. salutaris* against the D10 and the resistant strains of *P. falciparum*, K1 and RSA11.

4.3.3 Bioassay-guided fractionation of the active extracts of *W. salutaris*

The EtOH leaf extract was fractionated using the HPLC based methodology as described in section 3.7.2. The HPLC profile of the crude was divided into four fractions (Figure 4.3.3.1), which following multiple injections, were collected separately, prepared appropriately and tested for antiplasmodial activity against D10. Fractions 2, 3 and 4 showed activity. Through further fractionation of each fraction, peaks responsible for activity were identified. The active regions were then concentrated using SPE.

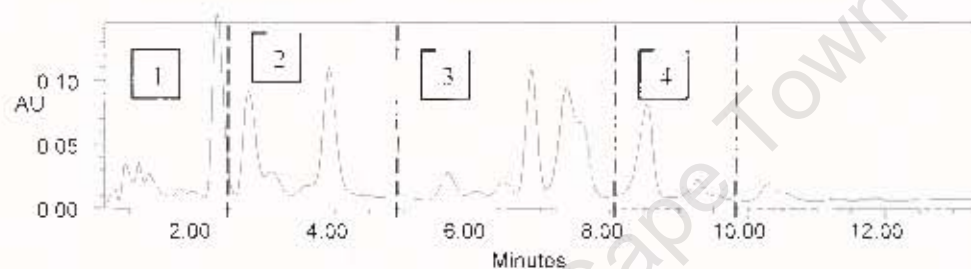


Figure 4.3.3.1 The HPLC chromatogram of EtOH crude extract of *W. salutaris* showing the four fractions demarcated and labeled 1-4. Fractions were collected separately and tested for activity. The crude was run on a C_{18} column at a gradient of 20-100% MeCN over 15 min at 1.5 ml min^{-1} .

4.3.4 The *in vitro* antiplasmodial activity of the SPE fractions of the EtOH crude extracts of *W. salutaris*

Subsequent to identifying the three regions responsible for activity on the crude extract HPLC profile, SPE was used to concentrate and semi-purify the identified active fractions. The SPE process yielded a total of eight fractions, which were prepared appropriately and tested for antiplasmodial activity against D10.

Three fractions; W.S50, W.S70 and W.S90 showed good antiplasmodial activity with IC_{50} values $1.6 \pm 0.2 \mu\text{gml}^{-1}$, $0.84 \pm 0.2 \mu\text{gml}^{-1}$ and $2.4 \pm 0.6 \mu\text{gml}^{-1}$ respectively.

4.3.5 Purification of the active SPE fractions of the EtOH leaf extract by HPLC

Fractions W.S70, W.S50 and W.S90 were run separately on HPLC. The profile of W.S70 was subdivided into four peaks, which were collected individually and tested for antiplasmodial activity against D10. Peak four, 70(2-4) shown in Figure 4.3.5.1a, was found to be responsible for the antiplasmodial activity with the IC_{50} value of $0.9 \pm 0.5 \mu\text{gml}^{-1}$. Fraction W.S50 contained three peaks. The third peak (WS50-3) (Figure 4.3.5.1b) was found to be responsible for the activity with an IC_{50} of $1.6 \pm 0.2 \mu\text{gml}^{-1}$.

The HPLC profile of fraction W.S90 showed two peaks with close retention times (figure not shown). Together the two peaks exhibited *in vitro* antiplasmodial activity with IC_{50} values of $1.5 \pm 0.25 \mu\text{gml}^{-1}$.

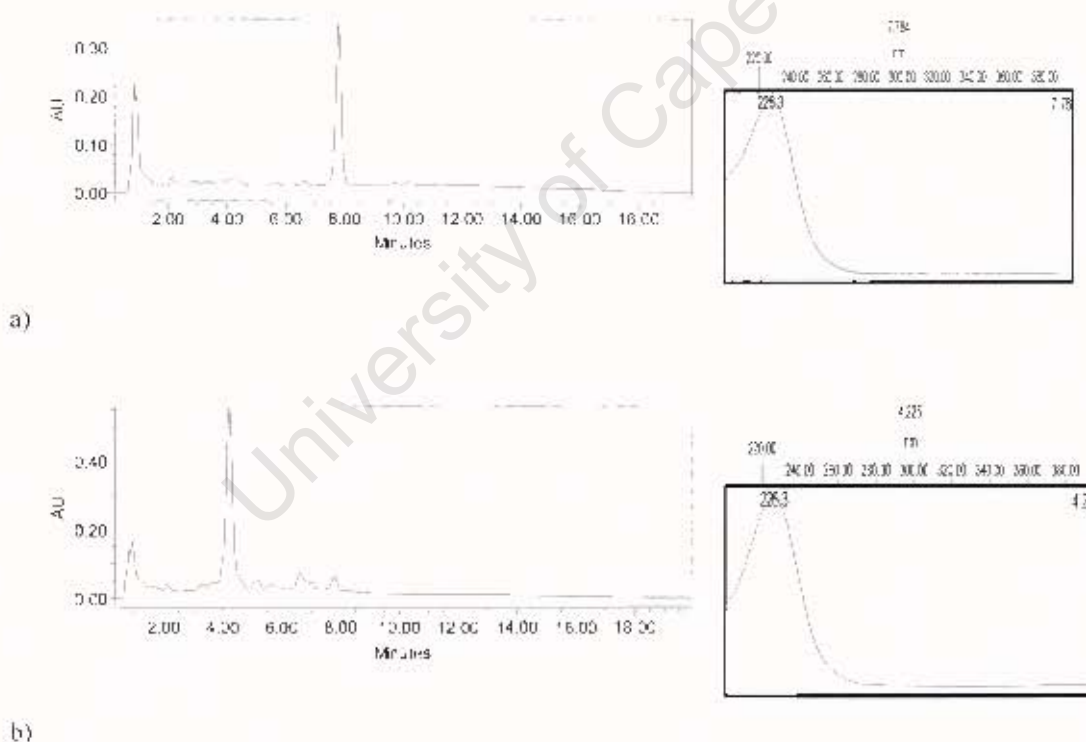


Figure 4.3.5.1 HPLC Chromatograms of semi-pure PK-2-4(a) and W.S50-3 (b), and their respective UV spectra. Samples were run on a C_{18} column under similar chromatographic conditions, a gradient of 35-100% over 15 min at flow rate of 1.5 mlmin^{-1} .

4.3.6 Decomposition of peak 70-2-4

Following repeated multiple injections of the SPE W.S70 fraction on to the HPLC, peak 70(2-4) was collected and stored at -4°C . The collections were done on three different occasions. Unfortunately, when sample 70(2-4) was re-run on HPLC, the HPLC chromatogram revealed the presence of two extra peaks, and the original 70(2-4) peak had also decreased in size. These findings suggested that the collected 70(2-4) peak had decomposed. Furthermore, the antiplasmodial activity of the decomposed sample had decreased ($\text{IC}_{50} 25 \pm 5.0 \mu\text{gml}^{-1}$).

4.3.7 *In vitro* antiplasmodial activity of batch II of the leaves of *W. salutaris*

In an attempt to obtain larger quantities of the active constituents of the plant, additional plant material was required. 10 kilograms of leaves of the plant were obtained. The leaves were dried and extracted the same way as the first batch. Unfortunately the extract had reduced antiplasmodial activity. The IC_{50} was $8.7 \pm 0.9 \mu\text{gml}^{-1}$. The profile of the crude extract on HPLC showed that the active peaks were present, but in minute amounts. As a result, the active peak could not be isolated from this batch. A second attempt was made to source more plant material or leaf extract of this plant from other research groups.

4.3.8 *In vitro* antiplasmodial activity of *W. salutaris* batch III

The dry EtOH leave extract of *W. salutaris* was obtained from Oxford University, a kind donation by Dr Madikane. The extract had an antiplasmodial activity of $\text{IC}_{50} 3.5 \mu\text{gml}^{-1}$ against D10. The activity of the extract was good, and the amount of the active peak as revealed by the size of the peak on the chromatogram was high enough and could be collected. The isolation of both 70-2-4 and 50-3 peaks was performed as before, using SPE to concentrate the fractions, and HPLC to purify the peaks of interest. The purified compounds were identified. The structure elucidation of these compounds is discussed in section 4.3.11.1.

In vitro antiplasmodial activity of crude extracts from batch II and batch III, the SPE fractions and isolated compounds

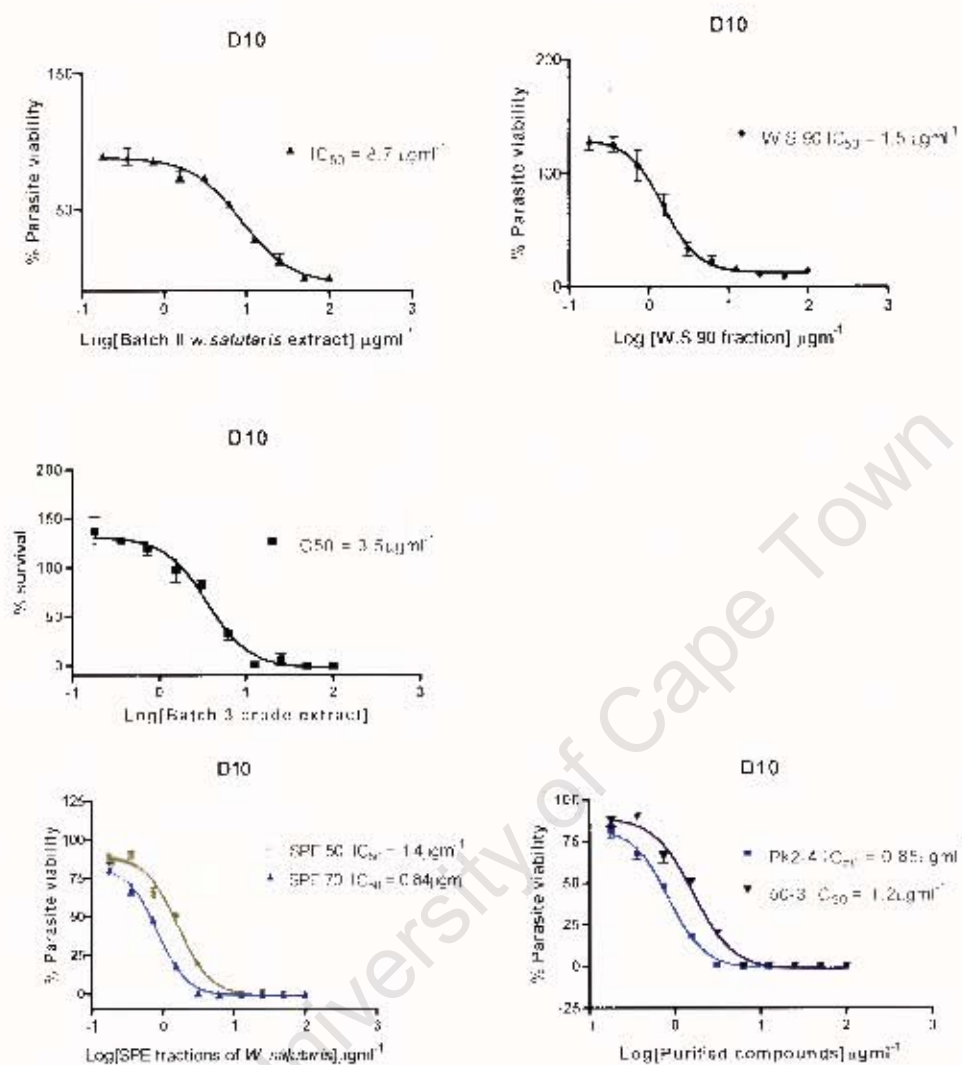


Figure 4.3.8.1 The antiplasmodial activity (against D10) of the EtOH leaf extracts of batches of *W. salutaris* and the active fractions generated from SPE. The activity of the second and third of the crude extract is also shown.

4.3.9 Purification of the active SPE fractions of the DCM twig extract of *W. salutaris*

The DCM, hexane and EtOH twig extract showed antiplasmodial activity with IC₅₀ values of 1.8 µgml⁻¹, 2.9 µgml⁻¹ and 6.9 µgml⁻¹ against the D10 strain, and 3.6 µgml⁻¹, 3.8 µgml⁻¹ and 12.9 µgml⁻¹ against the K1 strain respectively. The DCM twig extract was fractionated using SPE and eluting with increasing 20% increment of MeCN in

H₂O. From the six fractions generated from fractionation by SPE, three fractions: TW60, TW80 and TW100 exhibited antiplasmodial activity of $1.1 \pm 0.1 \mu\text{gml}^{-1}$, $0.6 \pm 0.1 \mu\text{gml}^{-1}$ and $0.8 \pm 0.09 \mu\text{gml}^{-1}$ respectively. The active SPE fractions of the DCM twig crude extract of *W. salutaris*: TW60, TW80 and TW100 were purified on HPLC.

4.3.9.1 Fraction TW60

The HPLC profile of fraction TW60 had two prominent peaks eluting at retention times 8.2 and 9.2 minutes (Figure 4.3.9.1). Peaks TW60-1 and TW60-2 possessed antiplasmodial activity, with IC₅₀ values of $0.86 \pm 0.1 \mu\text{gml}^{-1}$ and $1.4 \pm 0.2 \mu\text{gml}^{-1}$ respectively. The UV spectrum, carbon and proton spectra of TW60-1 were similar to that of PK2-4 isolated from the leaf extract. Enough quantities of TW60-2 were purified for the purpose of structure identification. The Assignment of ¹H and ¹³C NMR and structure elucidation are discussed in section 4.3.12.

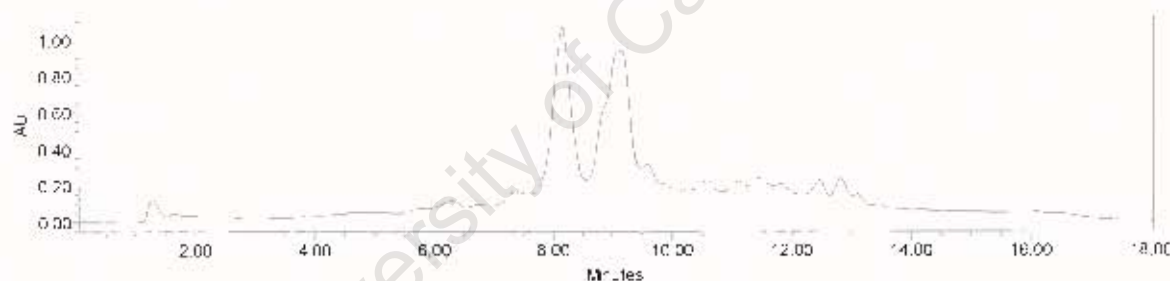


Figure 4.3.9.1 The chromatogram of SPE fraction TW60 run at a gradient of 25-90% MeCN over 15 min at a flow rate of 1.5 mlmin^{-1} . The profile displays TW60-1 eluting at 8.2 min and TW60-2 eluting at 9.2 min.

4.3.9.2 Fraction TW80

The HPLC profile of fraction TW80 composed of four peaks, all of which had good antiplasmodial activity. The chromatograms of TW60 and TW80 were superimposed. The first two peaks in TW80 were the same compounds in TW60-1 and TW60-2. The mass spectrum of TW80-2 was identical to that of TW60-2. The last two peaks on the TW80 fraction chromatogram TW80-3 ($R_T = 11.0 \text{ min}$) and TW80-4 ($R_T = 11.4 \text{ min}$) in Figure 4.3.9.2 had antiplasmodial activity of $1.2 \mu\text{gml}^{-1}$ and $0.88 \mu\text{gml}^{-1}$

respectively. The two peaks were isolated, but their chemical structures could not be elucidated due to their rapid decomposition and limited amounts.

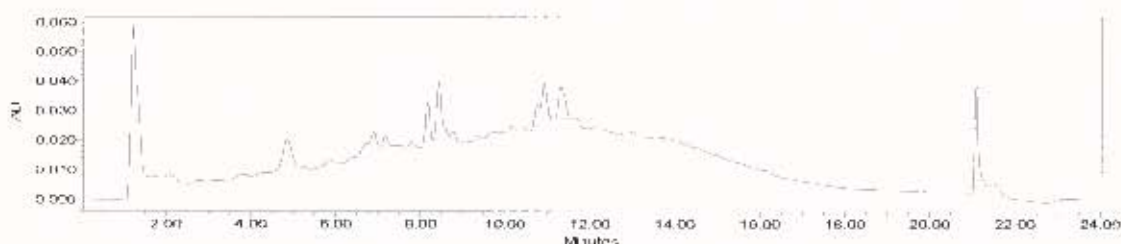


Figure 4.3.9.2 The chromatogram of SPE fraction TW80 run on a C_{14} column at a gradient of 25-90% MeCN over 15 min at a flow rate of 1.5 ml min^{-1} . The profile of the fraction shows TW80-1 and TW80-2 which similarly eluted at 8.2 and about 9.0 minutes as did TW60-1 and TW60-2 respectively. TW80-3 and TW80-4 eluted at retention times 11.0 and 11.4 minutes respectively.

4.3.9.3 Fraction TW100

The chromatogram of fraction 100 had two small peaks eluting at 11.0 and 11.4 minutes. The chromatogram was superimposed on that of TW80. The two peaks present in TW100 eluted at the same retention times as peaks TW80-3 and TW80-4. The UV spectra of corresponding peaks were identical. The peaks were however, in small quantities and could therefore not be collected from this fraction.

4.3.10 Cytotoxicity of the crude extracts and SPE fractions

The leaf and twig crude extracts of *W. salutaris*, and their respective active fractions generated from SPE were tested for cytotoxicity. The IC_{50} values of the EtOH leaf crude extract, SPE70 and SPE50 on CHO cells were $4.8 \pm 1.9 \mu\text{gml}^{-1}$, $3.4 \pm 0.5 \mu\text{gml}^{-1}$ and $30.2 \pm 3.5 \mu\text{gml}^{-1}$ respectively. The crude extract and SPE70 had an IC_{50} of $18.2 \pm 2.0 \mu\text{gml}^{-1}$ and $32.1 \pm 2.4 \mu\text{gml}^{-1}$ respectively against the HEpA cell line. The respective dose response curves are shown in Figure 4.3.10.2.

The twig DCM crude extract, TW60, and TW80 showed IC_{50} values of $28.4 \pm 0.6 \mu\text{gml}^{-1}$, $2.4 \pm 0.4 \mu\text{gml}^{-1}$ and $6.7 \pm 0.6 \mu\text{gml}^{-1}$ respectively. The selectivity indexes of the crude extracts and fractions are shown in Table 4.3.10.1. The respective dose response curves of the cytotoxicity results are shown in Figure 4.3.10.2.

The antiplasmodial activity of the twig crude extracts and SPE fractions generated from fractionation of the DCM extract.

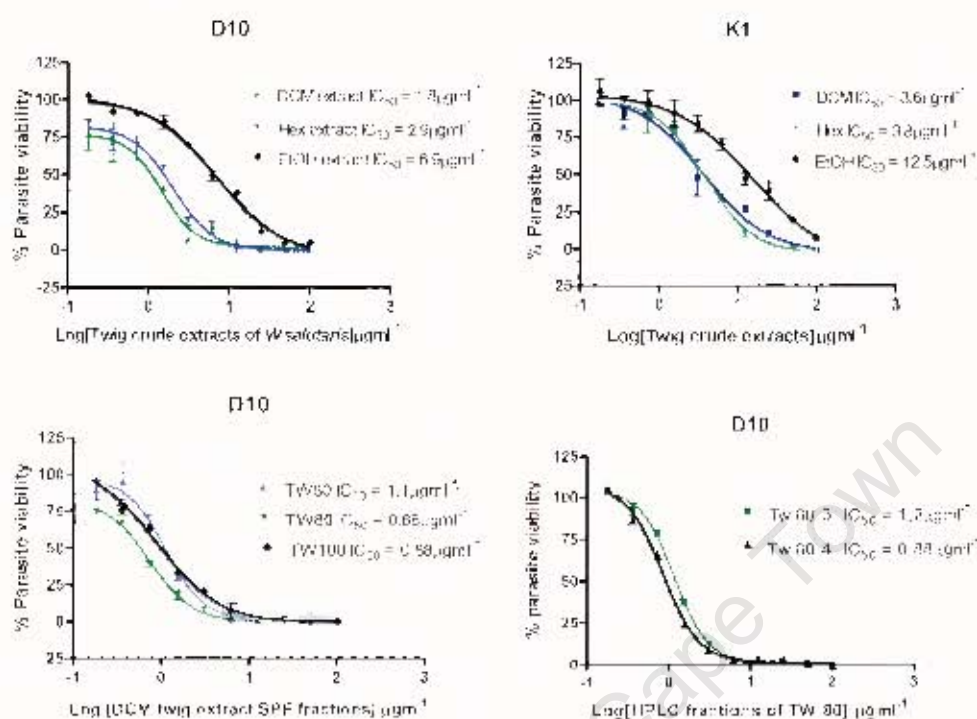


Figure 4.3.10.1 The antiplasmodial activity of twig crude extracts, SPE fractions of the DCM extract and of constituents of the SPE TW80 fraction (TW80-3 and TW80-4), as shown on their respective dose response curves.

Table 4.3.10.1. Summary of the results on the antiplasmodial activity and cytotoxicity of leaf and twig crude extracts of *W. salutaris*, and their respective SPE fractions.

Sample	IC_{50} mean \pm SD ($\mu\text{g/ml}$) D10 strain	Selectivity index	IC_{50} mean \pm SD ($\mu\text{g/ml}$) K1 strain
EtOH leaf crude extract	2.5 ± 0.8	1.92	1.9 ± 0.5
SPE 50	1.6 ± 0.2	18.88	1.4 ± 0.25
SPE 70	0.84 ± 0.06	4.2	0.9 ± 0.06
SPE90	1.5 ± 0.6	ND	
DCM twig crude extract	3.0 ± 0.2	15.78	3.6 ± 0.08
TW60	1.1 ± 0.1	2.2	1.2 ± 0.1
TW80	0.6 ± 0.1	9.8	0.82 ± 0.06
TW100	0.8 ± 0.09	ND	0.82 ± 0.06

The evaluation of cytotoxicity of the crude extracts, and selected SPE fractions

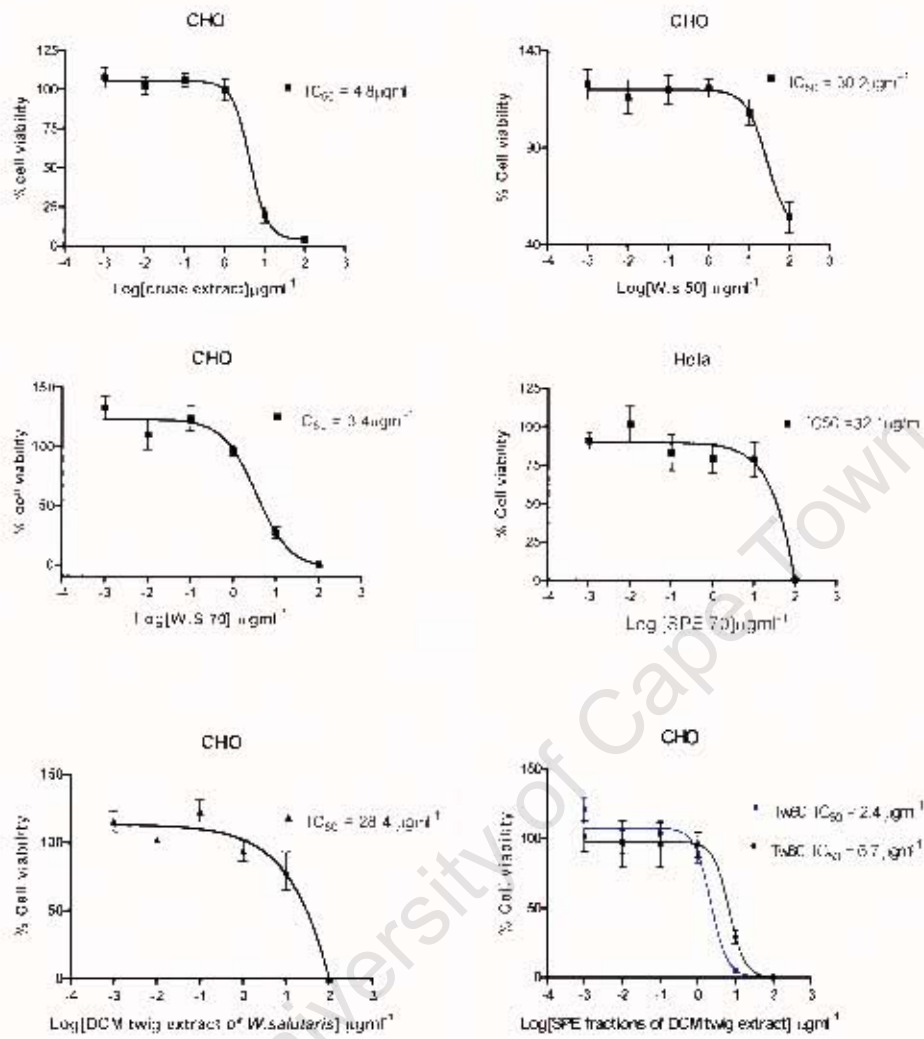


Figure 4.3.10.2 The cytotoxicity dose response curves of the twig and leaf crude extracts of *W. salutaris* and their respective SPE fractions. The SPE 70 fraction was also tested on the HELA cell line.

4.3.11 Structural identification of compound 70-2-4.

Table 4.3.11.1 The ^1H , ^{13}C , HSQC, HMQC, COSY and NOESY data of compound 70-2-4, which was isolated from SPE 70 fraction of the EtOH leaf extract of *H. salutaris*.

Position	$\delta^1\text{H}$ (J Hz)	$\delta^{13}\text{C}$	HMBC	COSY	NOESY
1	α 1.44 β 1.68 dt	36.7t	C-3, C-10 C-5, C-10		H-2 β
2	α 1.47 m β 1.7 dddd	23.5t	C-3 C-2	H-3 α , H-2 β H-2 α	H-1 β , CH3-13 H-5, H-1 α , CH3-15
3	α 4.54, dd (9.0 Hz)	80.7d			
4		37.5 s			
5	α 1.55 dd (11.4, 5.7, 2.7 Hz)	48.2d	C-9, C-4, C-10, CH3-15, C-6, CH3-13		H-3 α , H-9 α , H-1 α , CH3-15
6	α 2.44 dd β 2.3 dddd (11.4, 4.9, 3.3 Hz)	24.5t	C-10	H5, H6 β H5	CH3-15 CH3-14
7	6.84, dd (7.2, 3.6 Hz)	136.3t	C-12	H-6 α H-6 β	H-5
8		127.4 s			
9	α 2.92 m (9.2, 9.2, 4.9, 3.8 Hz)	59.4d	C-10, C-11	H-11 β , H-11 α	H-5
10		33.9s			
11	α 4.41 t (9.3 Hz) β 4.43 t (9.3 Hz)	67.5t	C-12, C-8, C-9 C-10, C-9	H-11 β , H-9 H-11 α , H-9	H-5
12		171.1s			
13	0.85 s	12.4q	C-1, C-9, C-5, C-10		H-11 β , H-2 β , H-6 β , CH3-14
14	1.03 s	15.1q	C-3, C-5, C-4, CH3-15		H-6 β , H-2 β , CH3-13
15	0.93 s	27.0q	C-3, C-5, C-4, CH3-14		H-6 α , H-5, H-5
16		171.5s			
17	2.04	19.9q	C-16, C-3		

4.3.11.1 NMR assignment of compound 70-2-4

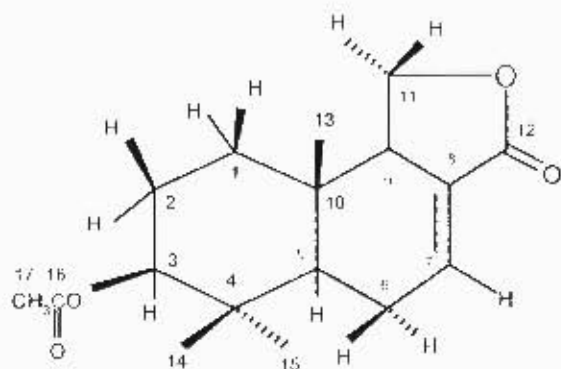


Figure 4.3.11.1 The chemical structure of compound 70-2-4, elucidated as 3 β -Acetoxycinamolide.

Compound 70-2-4 was obtained as colorless needles. The HREI-MS spectrum showed a prominent peak at m/z : measured 293.1722 $[M+H]^+$, calculated for $C_{17}H_{20}O_4$: 293.1764), which corresponded to a molecular formula $C_{17}H_{20}O_4$. The 1H , ^{13}C , HSQC, HMQC, COSY and NOESY NMR of compound 70-2-4 are summarized in Table 4.3.11.1. The spectra are shown in appendix B (page 198).

The presence of fifteen carbon atoms suggested a sesquiterpene. Sesquiterpenes isolated from *Warburgia* species are characterized by α , β -unsaturated carbonyl chromophores assembled around a trans-decalin ring system. An absorption maximum at about 234nm in the UV spectrum was indicative of an α , β -unsaturated lactone.

The 1H spectrum revealed the presence of four three proton signals at δ 0.85, 1.03, 0.93 and 2.04. The methyl singlets were linked to their respective carbons at δ 12.4, 15.1, 27.0 and 19.9 based on correlations observed in the HSQC, and were assigned as CH3-13, CH3-14, CH3-15 and CH3-17 respectively. Methyl groups CH3-14 and CH3-15 were positioned at C-4 through HMQC correlations to a quaternary carbon at δ 37.5, identified as C-4. It is also characteristic of some sesquiterpenes to have an O-group substitution at position C-3, two methyl groups at C-4, and another methyl group at ring juncture (C-10). Both methyl groups, CH3-14 and CH3-15 showed HMQC correlation to a carbon at δ 80.7, identified as C-3. The chemical shift of C-3 suggested an O-group substituted carbon.

The two downfield signals of carbonyl carbons at δ 171.5 and 171.1 were assigned to C-16 and C-12 respectively. An acetyl group was identified through correlation from CH3-17 to C-16 in the HMQC spectrum, which explained the downfield resonance of the methyl signal (δ 2.02). The positioning of the acetyl group at C-3 was further confirmed by HMQC correlations from CH3-17 to C-3.

The double doublet resonating at δ 4.54, was linked to carbon δ 80.7 through HSQC correlation and was identified as H-3. The multiplicity of H-3 suggested a splitting by only two neighboring protons at C-2, and this confirmed the O-group substitution at C-3. The molecule would be more stable with an acetyl group at C-3 at β -orientation, which would consequently leave H-3 at an axial orientation. H-3 α showed correlation in HMQC to carbon at δ 23.5, assigned to C-2. Two protons, a multiplet at δ 1.47 and a double triplet at δ 1.70 coupled in the COSY and both correlated to C-2 in the HSQC spectrum were assigned to H-2 α and H-2 β respectively. Coupling between H-2 α and H-3 α ($J = 4.2$ Hz) observed in the COSY confirmed the positioning of protons on C-2 adjacent to C-3. The HSQC experiment indicated correlations from proton signals at δ 1.68 and δ 1.44 to a carbon at δ 36.7. Correlations from the two protons to C-3, C-10, and C-5 in the HMQC spectrum led to their identification as H-1 protons. Correlation between δ 1.68 (H-1 β) and H-2 β in NOESY was observed, leaving δ 1.44 at an α -orientation. A long range correlation from H-1 α to C-3 in the HMQC spectrum was also observed. The third methyl group CH3-13, showed a correlation to C-9 in the HMQC spectrum, and was thus positioned at the juncture at C-10.

A double doublet resonating at δ 1.55 was identified as H-5. H-5 correlated to C-5 at δ 48.2 in the HSQC spectrum. Positioning of H-5 was confirmed by its correlation to C-10, C-4, C-9 and to CH3-15 and CH3-13 as observed in the HMQC. The splitting pattern of H-5 suggested that it was interacting with two protons. The COSY spectrum revealed that H-5 coupled with a double double triplet (ddt) at δ 2.44 ($J = 5.7$ Hz) and a double double double doublet (dddd) at δ 2.3, identified as H-6 α and H-6 β respectively. HSQC linked H-6 protons to C-6 resonating at δ 24.5. Correlations in the HMQC from H-6 β and H-6 α to C-5 and C-4 were observed. NOE interactions between H-6 α and CH3-15, and between H-6 β and CH3-14, gave the H-6 protons their respective orientations. The correlation between H-5 and CH3-15 in the NOESY

spectrum positioned H-5 at an α -orientation. Furthermore, H-5 also showed correlation in NOESY with H-1 α , and H-3 α . H-6 α and H-6 β coupled with a signal at δ 6.84 ($J = 3.5$ Hz) in the COSY. The downfield resonance of this deshielded double doublet at δ 6.84 identified an α - β -unsaturated carbonyl group. The signal was assigned to H-7. The HMQC spectrum revealed long range correlation from H-7 to a carbonyl at δ 171.1 (C-12).

In the ^{13}C NMR spectrum, double bond carbon resonances at δ 127.4 (C-8) and 136.3 (C-7) and a carbonyl carbon C-12 confirmed the presence of an α , β -unsaturated lactone ring. Considering the general structure of known sesquiterpenoid lactones isolated from *W. salutaris*, the lactone carbonyl group was placed at position C-12. The HSQC experiments showed correlation from a multiplet at δ 2.92 to C-9 at δ 50.4. H-9 coupled with two triplet signals at δ 4.41 and δ 4.43, identified as H-11 α and H-11 β respectively. The protons were more deshielded due to the close proximity to a lactone group and its resulting inductive effect. H-11 protons coupled with each other ($J = 9.3$ Hz) and with H-9 ($J = 9.2$ Hz). Positioning of H-9 was confirmed by correlation observed in the HMQC spectrum from H-9 to C-11 and to a quaternary carbon at δ 33.9 (C-10). H-9 further interacted with H-5 α in space, and was assigned an α -orientation. The spacial interactions between CH₃-13 and CH₃-14, CH₃-13 and H-11 β , H-2 β , and H-6 β observed in the NOESY helped resolve the stereochemistry of the H-11 protons. H-9 α and H-11 α also showed NOE correlation to H-5 α .

On the basis of these observations, compound PK2-4 was identified as 3 β -acetoxy cinnamolide. The NMR data was similar to that of cinnamolide -3 β -acetate reported by Kioy *et al.*, 1990 and Ying *et al.*, 1995.

The ^1H and ^{13}C NMR data for W.S50-3 showed similar signals to those observed for PK2-4. The chemical shifts were similar to that of PK2-4 with some carbon and proton signals differing by 0.02 units. The COSY, HSQC and HMQC experiments results were the same as those in PK2-4. The HRFI-MS ion patterns of the two compounds were similar, showing an ion at 279, indicating a loss of a CH₃ of an acetyl group, and m/z 235 indicating a loss of an acetyl group. According to the NMR data obtained and our interpretation, the difference between the two compounds could not be identified. However, it is suspected that the two molecules might differ by

different spacial orientation of functional groups. In W.S50-3 compound, the acetate group and C-3 could have possibly assumed an α -orientation.

4.3.12 The structural identification of TW60-2

The ^1H , ^{13}C , HSQC, HMBC, COSY and NOESY NMR of compound TW60-2 are summarized in Table 4.3.12.1. The spectra are shown in appendix B (page 204).

Table 4.3.12.1 The ^1H , ^{13}C , HSQC, HMBC, COSY and NOESY NMR of compound TW60-2

Position	δ H (J Hz)	δ ^{13}C	HMBC	COSY	NOESY
1	α 1.44dd β 1.24m	39.1	C-9, C-3 C-9, C-10, C-3	H-1 β , H-2 β	H-2 β
2	α 1.66m β 1.48m	18.2	C-3, C-1, C-3, C-4	H-1 β , H-3 β	H-1 β , CH3-13
3	α 1.52 dd(2.7, 3 Hz) β 1.28	42.2	C-2, C-14	H-3 β H-2 α	H-5, H-1 α , CH3-15
4		37.0			
5	α 1.46 dd (11.6, 5.3 Hz)	49.7 24.9	C-6, C-14, C-13, C-9, C-3, C-1, C-10, C-15	H-6 α , H-6 β	3 α , 9 α , 1 α , CH3-15
6	α 2.45 ddddd (9, 3.6, 1.8, 1.2 Hz) β 2.17m		C-7, C-8, C-5, C-4 C-7, C-8, C-5 C-7	H-7, H-6 β , H-5 H-6 α	CH3-15 CH3-14
7	6.83dd(7.2, 3.6Hz)	136.7	C-12, C-6, C-5, C-9		H-5
8		127.5			
9	α 2.9m (9.2, 3.6Hz)	50.7	C-7, C-11, C-10, C-1, C-13, C-9	H-11 α , H-11 β	
10		34.2			
11	α 4.42t (9.3 Hz) β 4.08t (9.3 Hz)	67.8	C-12, C-8, C-9 C-9, C-10	H-11 β , H-9 H-9, H-11 α	
12		171.4			
13	0.84s	12.4			H-11 β , H-2 β , H-6 β , CH3-14
14	0.97s	20.5		CH3-15	H-6 β , H-2 β , CH3-13
15	0.93s	32.4		CH3-14	H-6 α , H-3 α , H-5 α

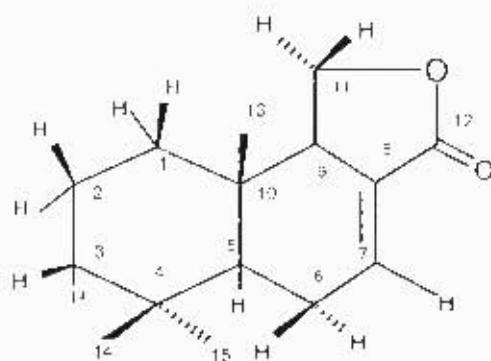


Figure 4.3.12.1. The chemical structure of compound TW60-2

The HRMS of TW60-2 showed a molecular ion[M+H]⁺ at m/z 235.5214 corresponding to a molecular formula of C₁₅H₁₈O₂, calculated for m/z 235.5201 [M+H]⁺.

The ¹H NMR and ¹³C NMR spectra of compound TW60-2 closely resembled that of compound 70-2-4. A comparison of the ¹³C-NMR spectrum revealed that the number of carbon resonances were similar except for the absence of an acetoxy group in the ¹³C spectrum of TW60-2, which appeared in the ¹³C NMR of PK-2-4 at δ 171.5 (C-17) and δ 19.9 (C-16). This indicated a loss of an acetyl group in compound TW60-2. This was further confirmed by the absence of a C-3 at δ 80.7 in the ¹³C spectrum of TW60-2. The indication of a loss of an acetyl group was also supported by the difference of 58 in the molecular weight of the two compounds, which is equal to that of an acetyl group.

The similarity in the COSY, HSQC, HMBC and NOESY spectra of the two compounds suggested that their main skeletons were identical. While the majority of chemical shifts and splitting patterns in their ¹H spectra were similar, a methyl singlet resonating at δ 2.04 in the ¹H spectrum of PK-2-4 was absent in the ¹H spectrum of TW60-2. In the HSQC, the proton at δ 2.04 correlated with C-17 at 19.9 (CH₃-17), and with a carbonyl carbon C-16 in the HMBC. The second noticeable difference in the ¹H spectrum of TW60-2 was the absence of a triplet resonating at δ 4.43, corresponding to H-3 in the ¹H spectrum of PK-2-4. Instead, there were additional proton resonances in the ¹H spectrum of TW60-2, a multiplet at δ 1.28 and a double

doublet at δ 1.52, which were linked to C-3 at δ 42.2 by HSQC correlations. Using HMBC contours, the H-3 protons correlated to C-2, C-1, C-4 and C-14. Furthermore, COSY coupling was observed between H-3 (δ 1.52) and H-2 α , which then positioned C-3 adjacent to C-2. Protons resonating as multiplets at δ 1.66 and δ 1.48 both correlated to a carbon at δ 18.2, assigned C-2 and were identified as H-2 α and H-2 β respectively. H-2 α showed coupling with H-3 β and another multiplet at δ 1.24. Protons at δ 1.24 and 1.44 correlated to carbon at δ 39.1 in HSQC, and were consequently identified as H-1 β and H-1 α respectively. COSY correlation between H-1 α and H-1 β and H-2 β , and between H-2 β and H-3 β and H-1 β were observed. The HMBC spectra showed that H-1 α correlated to C-3, H-1 β to C-2, C-9, C-10 and C-13. The H-2 protons showed correlation to C-3, and further correlation from H-2 β to C-1 was observed.

In the NOESY spectrum, H-3 at δ 1.52 interacted with H-5 α , H-1 α , CH3-15, indicating that it assumed an α -orientation. H-3 at δ 1.28 was assigned a β -orientation. H-2 β showed spatial interactions with CH3-13 and H-1 β as revealed in the NOESY. More interactions are summarized in Table 4.3.12.1.

The double doublet resonating at δ 1.46 was assigned to H-5. The position of H-5 was confirmed by COSY correlations with protons at δ 2.45 and δ 2.17 identified as H-6 β and H-6 α respectively, and further HMBC correlations to C-1, C-3, C-6, C-9, C-10, C-14 and C-15. H-6 α coupled with H-6 β , and H-5 in the COSY. H-6 α further coupled with a deshielded double doublet at δ 6.83, H-7. Also, two bond correlations from H-6 protons to C-7 and C-5, and a three bond correlation to C-8 were observed in the HMBC spectrum. H-7 positioned on C-7 at δ 136.7 by HSQC showed correlation to C-6, C-5, C-9, and C-12. The mutually coupled protons at δ 4.08 and δ 4.42 ($J = 9.2, 9.2$ Hz) were identified as H-11 β and H-11 α respectively. H-9 was positioned through its COSY correlation with the H-11 protons and through carbon correlations to C-7, C-10, C-11, C-1, and C-13. The three methyl groups were similar to that in the ^1H spectrum of PK-2-4 and were positioned accordingly.

4.3.13 Discussion

The crude extracts of both the leaves and twigs of *W. salutaris* showed positive antiplasmodial activity against both D10 and K1 strains of *P. falciparum*. The results are not surprising as this plant has been reported for use in the treatment of malaria. The *in vitro* antiplasmodial activity of the hexane and water extracts of this plant have been reported by Gumede B., 2003 and Clarkson *et al.*, 2002]. The reports were however, without reference to toxicity, and most importantly, the active molecules responsible for antiplasmodial activity of the plant were never isolated or chemically characterized. This work reports for the first time, the isolation and identification of chemical structures of compounds responsible for the antiplasmodial activity of leaf and twig extracts of *W. salutaris*.

A large number of sesquiterpenoids isolated from *Warburgia* species have shown various biological activities such as anti-bacterial, anti-molluscicidal, anti-cancer, and anti-fungal properties [Kubo *et al.*, 1983; 1988; Rabe and van Staden., 2000; Brooks *et al.*, 1969; Manguro *et al.*, 2003; Kioy *et al.*, 1990]. Although few sesquiterpenoids with a characteristic chemical skeleton as the ones reported here have been isolated from *W. salutaris* species [Mashimbye *et al.*, 1999; Rabe and van Staden., 2000; Clarkson *et al.*, 2007], none of the isolated sesquiterpenoids have been reported to possess antiplasmodial activity. The two sesquiterpenoids isolated in this study, cinnamolide and 3 β -acetoxy-cinnamolide, have been isolated previously from other plants and from related plant species [Canonica *et al.*, 1967; Kubo *et al.*, 1976; Kioy *et al.*, 1990; Wube *et al.*, 2005], but there were no reports on their antiplasmodial activity.

Only two compounds were characterized in this study. Other compounds which also showed good antiplasmodial activity could not be characterized because of their instability and limited amounts. The isolated compounds represented by individual peaks on the HPLC chromatogram, decomposed and yielded one or two more peaks.

Both compounds exhibited good *in vitro* antiplasmodial activity. The purified compounds were in limited amounts and were inadequate for cytotoxicity testing. However, the activity of the W.S70 and W.S50 SPE fractions was used as an

indication of the activity of each compound since the compounds were in large amounts (>95%) in their respective SPE fractions, and could have accounted for activity in the fractions. Cinnamolide-3 β -acetate showed relatively high toxicity against the CHO cells. The compound however, showed reduced toxicity to the HeLa cells by a magnitude factor of 4.2 when compared to toxicity against the CHO cell line. Cinnamolide showed similar antiplasmodial activity but exhibited low toxicity on the CHO cells with a selectivity index of 18. This compound can be regarded as promising and can be considered for further development.

W. salutaris is one of the medicinally important plants in South Africa. It is widely traded both locally and across the countries borders. The high demand for the plant has contributed greatly to depletion of the plant species. Sadly, this plant has now become one of the endangered plant species. Since the bark is the part of the plant that is used for medicinal purposes, the debarking method is used to harvest the plants. This destructive method of harvesting is of great concern as the debarked tree has no means of survival.

It was interesting to discover that the profile of the constituents in the leaves is similar to the chemical profile in the twigs. This would allow substitution of bark with the leaves. Healers can be encouraged to collect alternative plant parts such as leaves and twigs instead of bark, since leaf harvesting poses less damage than debarking [Cunningham, 1988].

4.4 *Achillea millefolium* L.

4.4.1 Introduction

The plant leaf material was extracted with DCM and MeOH solvents. The DCM extract was first fractionated using SPE. The yields from SPE were however, extremely small. As a result, flash chromatography was selected as an alternative method of fractionation. The profile of each fraction generated was monitored using TLC (See section 3.8 for details). Fractions that had similar band profiles were pooled, prepared appropriately and tested for antiplasmodial activity against the D10 strain of *P. falciparum*. Active fractions were combined and further fractionated using column chromatography. Fractions generated from column chromatography were tested for activity. Active fractions were separately purified using short column chromatography. Final purification was performed on HPLC.

4.4.2 Antiplasmodial activity and bio-activity guided fractionation of the DCM extract of *A. millefolium*

The DCM extract of *A. millefolium* showed the highest activity with IC_{50} values of $3.9 \pm 0.3 \mu\text{gml}^{-1}$ and $3.1 \pm 0.4 \mu\text{gml}^{-1}$ against D10 and K1 respectively.

4.4.2.1 Flash chromatography

Column preparation and sample application were done as stipulated in section 3.7.3. A total of sixteen fractions were collected from eluting the extract with a step gradient of increasing polarity with 10% increments from hexane to EtOAc, and EtOAc to MeOH. Seven fractions; 40:60, 30:70, 20:80, 90:10 (hex: EtOAc) and 90:10, 70:30 and 60:40 (EtOAc: MeOH), were identified as active. The IC_{50} values of the fractions are summarized in Table 4.4.2.1 and the respective dose response curves are shown in Figure 4.4.2.1. The active fractions were selected for further fractionation by column chromatography.

4.4.2.2 Column chromatography

A total of 64 fractions were collected from eluting the sample with a slow and constant flow of mobile phase, EtOAc: hexane: MeOH (5:4:1). Fractions with similar profiles on the TLC plate were pooled, yielding a total of 15 fractions. The IC_{50} values of each fraction are listed in Table 4.4.2.1 while the respective dose response curves are shown in Figure 4.4.2.1.

Table 4.4.2.1 The yields and antiplasmodial activity of DCM and MeOH extracts of *A. millefolium*, and fractions of the DCM extract generated from flash chromatography and column chromatography.

Extract	Extraction Yield (%)	Antiplasmodial activity D10 IC_{50} mean \pm SD (μgml^{-1})
DCM extract	3.2	3.9 \pm 0.3
MeOH extract	4.1	4.6 \pm 0.5
Flash Chromatography fractions		
EtOAc: hex (%)		
60:40	4.6	3.1 \pm 0.32
70:30	4.0	2.5 \pm 0.3
80:20	2.4	1.9 \pm 0.15
90:10	1.6	1.4 \pm 0.2
EtOAc: MeOH (%)		
90:10	1.4	2.9 \pm 0.4
70:30	2.6	2.4 \pm 0.2
60:40	2.8	3.1 \pm 0.6
Column chromatography fractions		
Fraction 5	1.8	2.6 \pm 0.4
Fraction 6 and 8	1.6	1.7 \pm 0.25
Fraction 7	0.51	4.9 \pm 0.4
Fraction 9	1.4	1.3 \pm 0.16
Fractions 10 and 11	1.6	2.8 \pm 0.12
Fraction 12	0.54	5.7 \pm 0.22

Antiplasmodial activity of the DCM crude extract and fractions of *A. millefolium*.

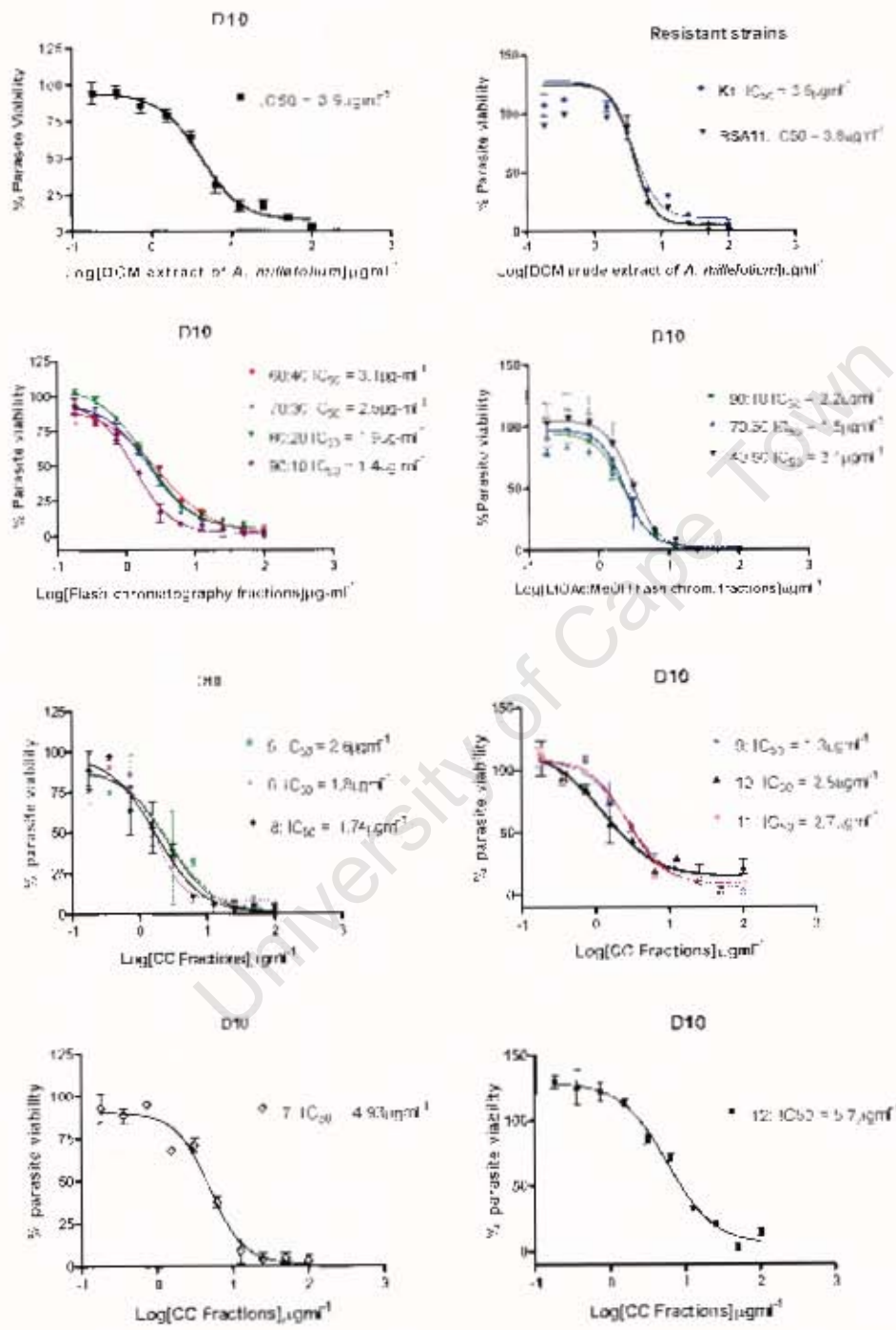


Figure 4.4.2.1 The dose response curves of the crude DCM extract of *A. millefolium*, fractions resulting from bio-guided fractionation using flash chromatography followed by column chromatography (CC).

The first four fractions generated from column chromatography showed no antiplasmodial activity. Fractions 5, 6, 8-11 showed the highest antiplasmodial activity with IC_{50} values less than $2.8 \mu\text{gml}^{-1}$. A decreased activity was observed in fractions 7 and 12, while the last three fractions showed no considerable antiplasmodial activity with IC_{50} values $>10 \mu\text{gml}^{-1}$.

4.4.2.3 Short column chromatography

The active fractions generated from column chromatography, fractions 5-8 and fractions 9-11 were combined and were each further fractionated separately on a silica hexane slurry using short column chromatography. The samples were eluted with a slow and constant flow of 10% DCM in EtOAc. A total of 140 fractions of about 1.5ml each were collected from each run. Based on the similarity of their profiles on TLC plates, fractions were pooled to yield four fractions. The yields from each fraction were low and the IC_{50} values could not be obtained. However, a qualitative analysis revealed that fractions 20-80 had the highest activity, while fractions 81-140 had decreased antiplasmodial activity.

4.4.2.4 Preparative TLC

The fractionation of the extract was repeated in order to generate more samples of active semi-pure fractions. Following fractionation by flash chromatography and column chromatography, active fractions were fractionated on TLC plates (mobile phase; 5:4:1 EtOAc: hexane: MeOH), as opposed to using short column chromatography. The profile of the samples on TLC plates displayed 9 bands. The bands were each scraped off the TLC plate, prepared appropriately (section 3.8.5) and tested for antiplasmodial activity. Four bands (R_f values; 1.7, 1.61, 1.36 and 1.25 cm) were found to be responsible for the activity. Unfortunately only one of these bands ($R_f = 1.61$ cm) was in sufficient quantities for structure elucidation. The band however, exhibited decreased antiplasmodial activity ($IC_{50} = 4.8 \mu\text{gml}^{-1}$), compared to the activity of the fractions. The band ($R_f = 1.61$ cm) will henceforth be referred to as the Yar Lit compound.

4.4.2.5 Purification of compound Yar Lit on HPLC

The active band obtained from preparative TLC was purified on HPLC. Samples were run on a C₈ column, at a gradient of 30%-100% MeOH: H₂O over 20 minutes. The chromatogram revealed the presence of small impurities and an enhanced peak corresponding to the compound of interest. The peak was collected.

4.4.3 Cytotoxicity

The crude extract and active fractions generated from flash chromatography were tested for toxicity on the CHO cell line. The cytotoxicity effect of the DCM crude extract was low with an IC₅₀ value of $69.6 \pm 3.5 \mu\text{gml}^{-1}$. All the seven active fractions generated from flash chromatography showed less cytotoxicity with IC₅₀ values > $100 \mu\text{gml}^{-1}$ (Figure 4.4.3.1).

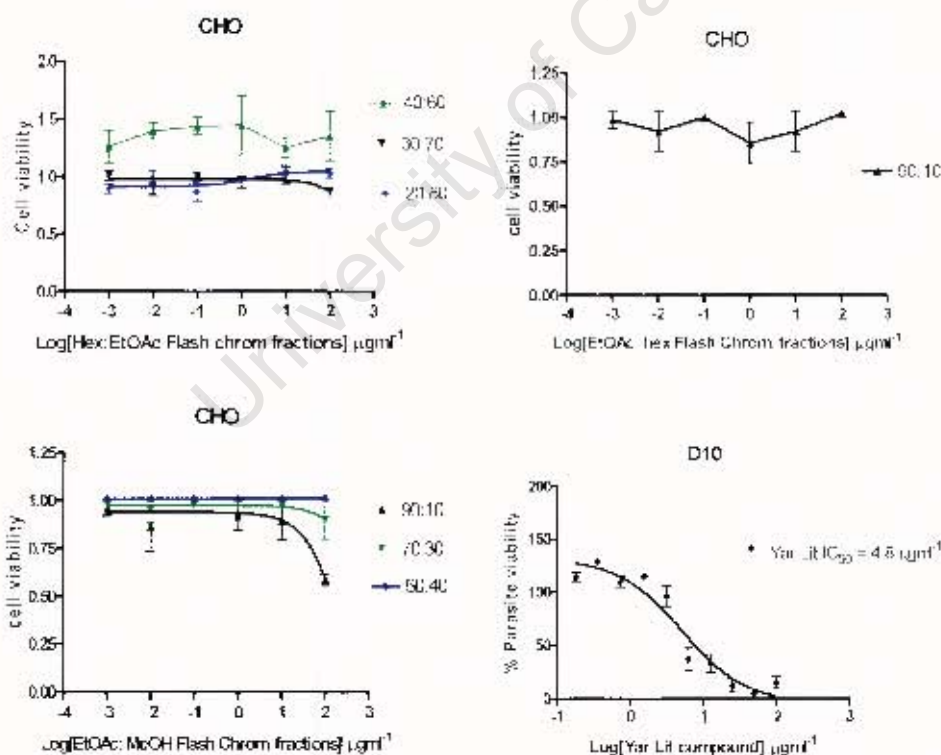


Figure 4.4.3.1 The cytotoxicity effect of the active flash chromatography fractions of *A. millefolium* on CHO cell line, and the antiplasmodial activity of the isolated compound, Yar Lit.

4.4.4 Structure elucidation of the isolated compound, Yar-Lit

The LC-MS results showed that the sample was a mixture of two compounds with molecular weight 338.35 and 340.36. The molecular weight and NMR interpretation agree with molecular formulae $C_{17}H_{22}O_7$ and $C_{17}H_{24}O_7$ for the compounds respectively. The compounds appear to have a similar carbon skeleton. The difference of 2 AMU indicates an addition or loss of protons on one of the compound. The 1H , HSQC, HMBC and COSY NMR of compound Yar-Lit are summarized in Table 4.4.4.1. The spectra are shown in appendix B (page 209).

Table 4.4.4.1 The 1H , ^{13}C , COSY and HMQC spectra of compound Yar-Lit isolated from the DCM extract of *A. millefolium*.

δ 1H (J Hz)	δ ^{13}C	COSY	HMQC
	131.2		
3.64 dd (5.7, 4.5 Hz)	72.7 t	3.58, 3.51	63.2
3.58 dd (11.1, 4.8 Hz)	63.2 t	3.51, 3.64	72.7
3.51 dd (11.1, 6.0 Hz)		3.58, 3.64	
	29.2		
	128.7		
1.28 q	31.5	1.58	C-29.7
1.58 m	26.6	1.28, 2.14	C-31.5
2.14 dd	38.2 d	1.58	C-29.7, C-31.5
	131.5		
	181.9		
1.31	29.7		C-29.7
0.89 d (7.2 Hz)	31.5	1.28	
1.28 s	29.5	CH3-0.90	
0.90 d (7.2 Hz)	13.2		
	169.2		
1.88 s	22.5		C-169.2

The ^{13}C carbon spectrum of compound Yar-Lit revealed the presence of 17 carbons including two carbonyl resonances at δ 181.9 and δ 169.2, and the double bond quaternary alkene carbons at δ 128.7 and 131.5. The proton spectrum showed four methyl signals, one methylene and 5 methines.

The presence of an α -methyl- γ -lactone ring and an acetate group was evident. The acetate and the lactone carbonyls were assigned from their chemical shifts. The HSQC and HMBC spectra placed a doublet at δ 1.31 and a CH_3 at δ 1.28 as part of the γ -lactone ring. This assignment became evident from the HMBC correlations observed from δ_{H} 1.31 to δ_{C} 181.9. The determination of an acetate group was achieved through HMBC correlations from CH_3 at δ 1.88 to δ_{C} 169.2.

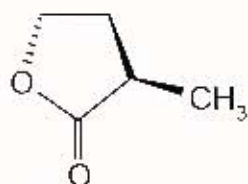


Figure 4.4.4.1 The γ -lactone group present in compound Yar-Lit

There was evidence of a methylene adjacent to a methine with an O-group substituted carbon. The methylene double doublets resonating at δ 3.51 and 3.58 showed geminal coupling ($J = 11.1$ Hz) in the COSY and correlation to δ_{C} 63.2 through HSQC experiments. These doublets further showed vicinal coupling ($J = 4.6$ Hz) to a double doublet at δ 3.64 linked to a carbon at δ 72.7. The multiplicity of the methylene protons, and of δ_{H} 3.64, and the downfield chemical shift of carbon 72.7, supported an O-group substitution. The positioning of the protons on adjacent carbons was further supported by correlations observed in the HMQC spectrum.

Two methyl signals resonated as doublets at δ 0.89 and 9.0 ppm, indicating a splitting by one proton in a typical H-C- CH_3 arrangement. One of the methyl groups showed correlation in the HMQC to a carbon at δ 31.5, to which a quartet resonating at δ 1.28 was also attached. The quartet coupled in the COSY with a multiplet at δ 1.58, which also displayed further coupling with a double doublet at δ 2.14. Unfortunately, the impurity of the sample and unclear NMR data did not allow complete elucidation of

the full structure of the compounds. However, comparison of the chemical shifts with that of compounds isolated from *Achillea millefolium* species gave an indication of the possible carbon skeleton of the compound.

A large number of sesquiterpenes including α -peroxyachifolid, proazulenes, and other peroxides have been isolated in great variety from *A. millefolium* species (Figure 4.4.4.2). The sesquiterpenes from this species are subdivided into different classes based on their carbon skeletons; guaianolides, eudesmanolides, longipinenes and germacranes. It is suspected that Yar-Lit falls under the guaianolides class as it is the most dominant of the four classes (Figure 4.4.4.2a). The variety within this class is limited by the narrow set of substituents which is restricted to the acyl-residues of acetic, tiglic and angelic acid [Schroder et al., 1994; Kubelka et al., 1999; Glasl et al., 2001].

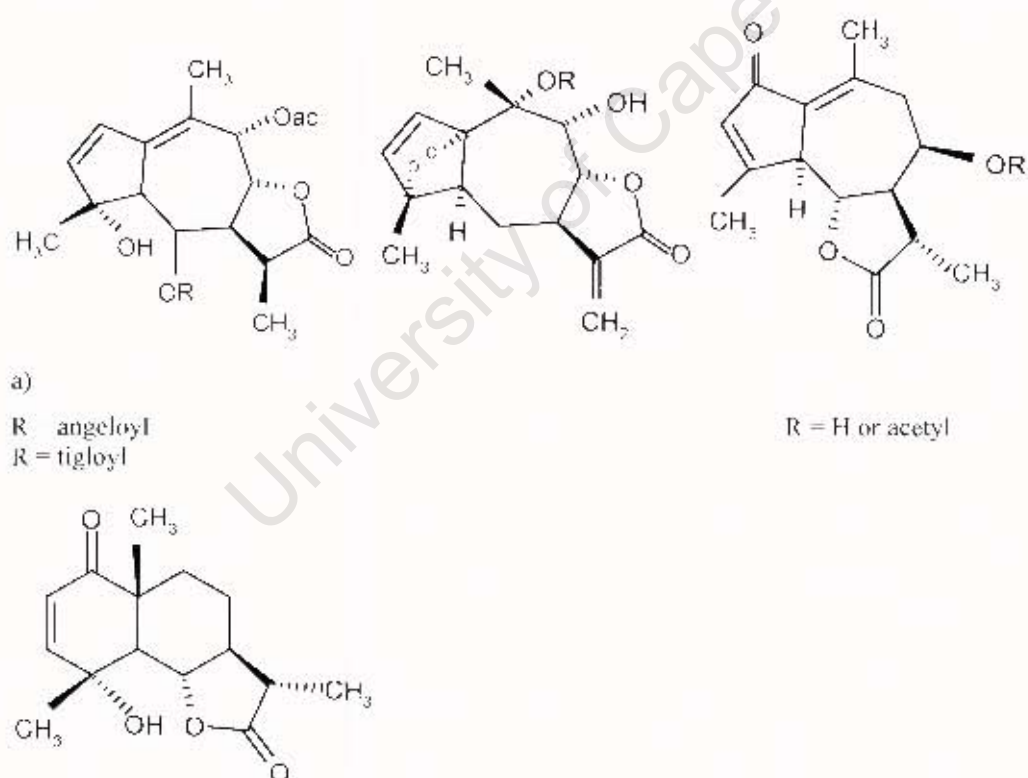


Figure 4.4.4.2 The chemical structures of examples of sesquiterpenes that have been isolated from *A. millefolium* species.

The sesquiterpenes from *Achillea millefolium* species have shown different effects and properties. The α -peroxyachifolid and some peroxides have shown antiplasmodial

activity [Rücker *et al.*, 1991]. Interestingly, the overall structure of these guaianolides, which is composed of a 7-membered ring, a 6 or 5-membered ring and a lactone ring, is related to that of a current antimalarial drug, artemisinin. The presence of a peroxide bridge is characteristic of artemisinin and its derivatives, and the potent antiplasmodial activity of these drugs is attributed to this structural feature [Li and Rieckmann, 1992]. Sesquiterpenes with a peroxide bridge have also been isolated from *A. millefolium* species [Kubelka *et al.*, 1999]. However, the peroxide bridge in the *A. millefolium* sesquiterpenes has not been linked to any biological activity. The noticeable difference is that while artemisinin and its derivatives have the peroxide bridge in the 7-membered ring, sesquiterpenes isolated from *A. millefolium* have it in their 5-membered ring.

4.4.5 Discussion

The DCM extract of *A. millefolium* showed positive antiplasmodial activity against D10 and K1 strains of *P. falciparum* ($IC_{50} = 3.9 \mu\text{gml}^{-1}$) and less cytotoxicity on the CHO cell line. The selectivity was roughly 20 times higher towards the malaria parasites. Bio-activity guided fractionation of the crude extract using column chromatography led to five active semi-pure fractions. The active fractions exhibited better antiplasmodial activity than the purified compound(s), which suggests a synergistic antiplasmodial activity of the compounds within a fraction. The cytotoxicity results indicated that the fractions were highly selective for the malaria parasite. Although the active compound was not identified, the NMR revealed that the isolated compound (s) showed similarity and possess structural features found in the sesquiterpenes isolated previously from this species.

The water extract of *A. millefolium* from Indonesian flora has been reported to have *in vitro* antiplasmodial and *antigibsoni* activity [Murnigshi *et al.*, 2005]. This report was however, without reference to cytotoxicity, and the concentrations used to test the extracts in the report were ten-fold higher than the concentration reported here. Furthermore, the crude extracts tested in the report were not fractionated and the active constituents of the extract remained unidentified.

4.5 *Agathosma betulina*

4.5.1 Introduction

The leaf material of *A. betulina* was extracted with DCM and MeOH. The extracts were prepared appropriately and tested for antiplasmodial activity against D10. The DCM extract showed enhanced antiplasmodial activity and was subjected to bio-activity guided fractionation using SPE and HPLC. Fractionation by SPE yielded three fractions that possessed good antiplasmodial activity. The three fractions were purified on HPLC, with the aim of identifying the active constituents. Due to complicated NMR data and the impurity of the isolated peaks, the final analysis of the fractions was achieved using gas chromatography-mass spectroscopy (GC-MS).

4.5.2 Fractionation and antiplasmodial activity of the DCM crude extracts of *A. betulina* and the fractions generated from SPE.

The DCM extract of *A. betulina* showed low activity against D10 ($IC_{50} = 14.4 \mu\text{gml}^{-1}$). Fractionation of the extract by SPE yielded a total of seven fractions, which were tested for antiplasmodial activity. Three fractions (60%, 80% and 100% MeCN) showed antiplasmodial activity with IC_{50} values $1.7\mu\text{gml}^{-1}$, $2.0\mu\text{gml}^{-1}$ and $1.6\mu\text{gml}^{-1}$ respectively.

4.5.3 Purification of SPE fractions of DCM extract of *A. betulina* on HPLC

The fractions generated from fractionation of the DCM extract of *A. betulina* by SPE were purified on HPLC using a C_{16} amide column. The HPLC run conditions for each SPE fraction were manipulated accordingly to produce maximum resolution between the components of each fraction. The details on the run conditions for each fraction are stipulated in section 3.7.2.2.

4.5.3.1 Fraction SPE 60

The chromatogram showed the presence of three prominent peaks (Figure 4.5.3.1); B60-1 (RT = 6.9 min), B60-2 (RT = 9.2 min) and B60-3 (RT = 10.35 min) which were collected separately and tested for activity. The peaks exhibited antiplasmodial activity with IC_{50} values of $2.8 \mu\text{gml}^{-1}$, $2.7 \mu\text{gml}^{-1}$ and $2.0 \mu\text{gml}^{-1}$ respectively. When subjected to purification, B60-1 appeared as a cluster of two peaks (RT = 6.9 and 7.5 min). The level of purity and quantity of B60-1 and B60-3 did not allow further purification on HPLC.

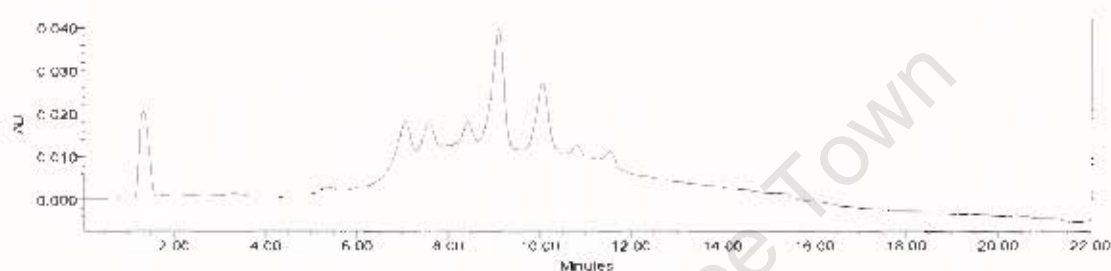


Figure 4.5.3.1 The HPLC chromatogram of SPE 60 fraction of DCM extract of *A. betulina*, run on a C_{16} amide column at 25-90% MeCN over 15 min gradient at 1.5 ml/min.

4.5.3.2 Fraction SPE 80

The chromatogram revealed the presence of three peaks; B80-1 (RT = 12.6 min), B80-2 (RT = 13.4 min), and B80-3 (RT = 14.83 min) which were collected separately and tested for antiplasmodial activity. The three peaks exhibited antiplasmodial activity with IC_{50} values $5.5 \mu\text{gml}^{-1}$, $3.4 \mu\text{gml}^{-1}$ and $3.4 \mu\text{gml}^{-1}$ respectively. The chromatogram in figure 4.5.3.2 shows a run of a more diluted sample, whereas the peaks were collected from a preparative scale run of a more concentrated sample.

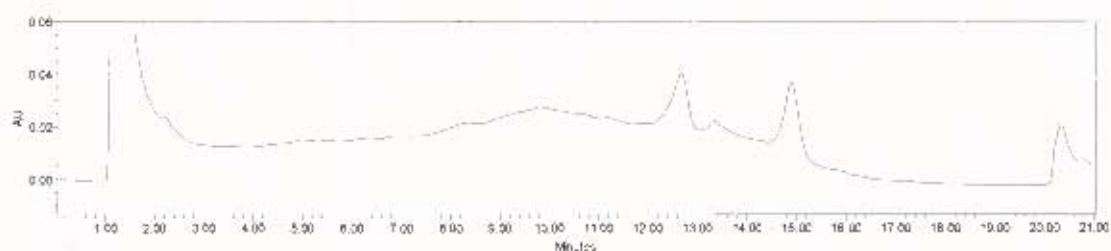


Figure 4.5.3.2 The HPLC chromatogram of SPE 80 fraction of DCM extract of *A. betulina*, run on a C_{16} amide column at 25-90% MeCN over 15 min gradient at 1.5ml/min.

4.5.3.3 Fraction 100

The chromatogram revealed the presence of one major broad peak (RT = 16 min) and other smaller peaks. The major peak showed antiplasmodial activity of IC_{50} $1.8 \pm 0.4 \mu\text{gml}^{-1}$ against the D10.

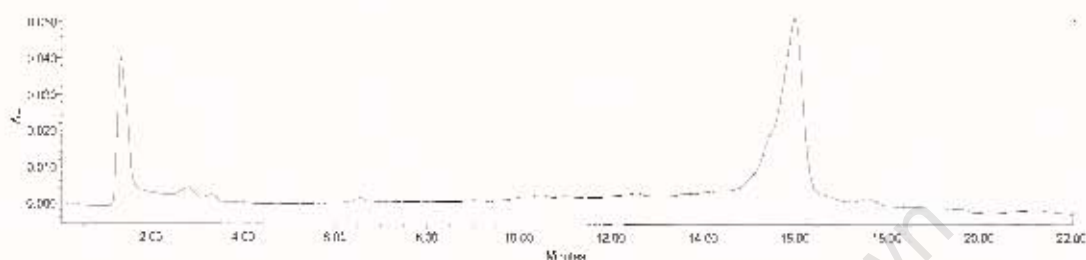


Figure 4.5.3.3 The HPLC chromatogram of SPE 100 fraction of DCM extract of *A. betulina*, run on a C_{16} amide column at 25-90% MeCN over 15 min gradient at 1.5ml/min.

4.5.4 Identification of constituents of SPE fractions of *A. betulina* by GC-MS

The NMR data for the compounds B-60-2, B-80-2 and B80-3, were obtained. Unfortunately the proton spectra were highly complicated due to signal overlap, and could not be interpreted. As a result, the components of the three SPE fractions, SPE60, 80 and 100 were analysed by GC-MS. The spectra are shown in Figure 4.5.5.2. The conditions of the run are stipulated in section 3.9.2. The identification of compounds in each fraction was assigned on the basis of comparison of their mass spectra with those given in the literature [Adams, 1989; Jennings and Shibamoto, 1980]. The components of each fraction and their area percentages are tabulated in Table 4.5.4.1.

Table 4.5.4.1 Chemical composition of SPE fractions of *A. betulina*, analysed by GC-MS. The area percentages reflect the concentration of each compound in the fraction.

Fraction 60

Compound	Area %
Pulegone	7.48
Cyclopropanoic acid	3.16
Carvacrol	15.13
Benzene sulphonamide	1.81
Hexadecanoic acid (palmitic)	7.27
4-(2-propenyl) phenol	6.70
Amyl-10-undecenoate	2.77
Phosphoric acid triphenyl ester	8.65
p-methane-8-thiol-3-one	1.61

Fraction 80

Compound	Area %
Pulegone	7.36
Cyclopropanoic acid	1.38
Cyclopropanonanoic acid	0.6
Terpineol	3.11
Acoradiene	3.30
Spathulenol	2.51
Diosphenol	2.88
Piperitone oxide	3.56
Phosphoric acid triphenyl ester	1.14
Dibutyl phthalate	2.36
Piperonyl butoxide	3.16

Fraction 100

Compound	Area %
Hexadecanoic acid (palmitic)	10.72
14- methyl-methylhexadecanoate	6.41
Hexanedioic acid	5.12
Ethyl-1- hydroxymethyl-2- hydroxyhexadecanoate	28
Heptadecanoic acid	3.97
Methyl-heptadecanoic acid	1.04
9-Octadecenoic acid	2.93
Octadecenoic acid methyl ester	4.03
9, 12-Octadecadienoic acid (linoleic)	3.64

4.5.5 The cytotoxicity of fractions and isolated compounds

The crude extract and fractions generated from SPE were subjected to cytotoxicity testing against the CHO cell line. The crude extract, SPE fractions and compounds showed high selectivity for the malaria parasite. The IC_{50} of the crude extract was greater than $100 \mu\text{gml}^{-1}$. When tested at double the concentration, an IC_{50} of $170 \mu\text{gml}^{-1}$ was obtained. The IC_{50} values of the SPE fractions and peaks collected from their respective HPLC profiles were greater than $100 \mu\text{gml}^{-1}$. The results of the antiplasmodial activity and cytotoxicity of all the tested samples are summarised in Table 4.5.5.1. The respective dose response curves are shown in Figure 4.5.5.1.

Table 4.5.5.1 The summary of antiplasmodial activity and cytotoxicity of DCM crude extract of *A. betulina*, SPE fractions and isolated compounds.

Sample	$IC_{50} \pm S.D \mu\text{gml}^{-1}$ D10 strain	Cytotoxicity $IC_{50} \mu\text{gml}^{-1}$
Crude extract	14.4	> 100
SPE 60	1.7	> 100
B60-1	2.8	89.1
B60-2	2.7	> 100
B60-3	2.0	> 100
SPE 80	2.0	> 100
B80-1	5.5	>100
B80-2	3.4	ND
B80-3	3.4	> 100
SPE 100	1.6	> 100
B 100	1.8	>100

The *in vitro* antiplasmodial activity and cytotoxicity effects of samples

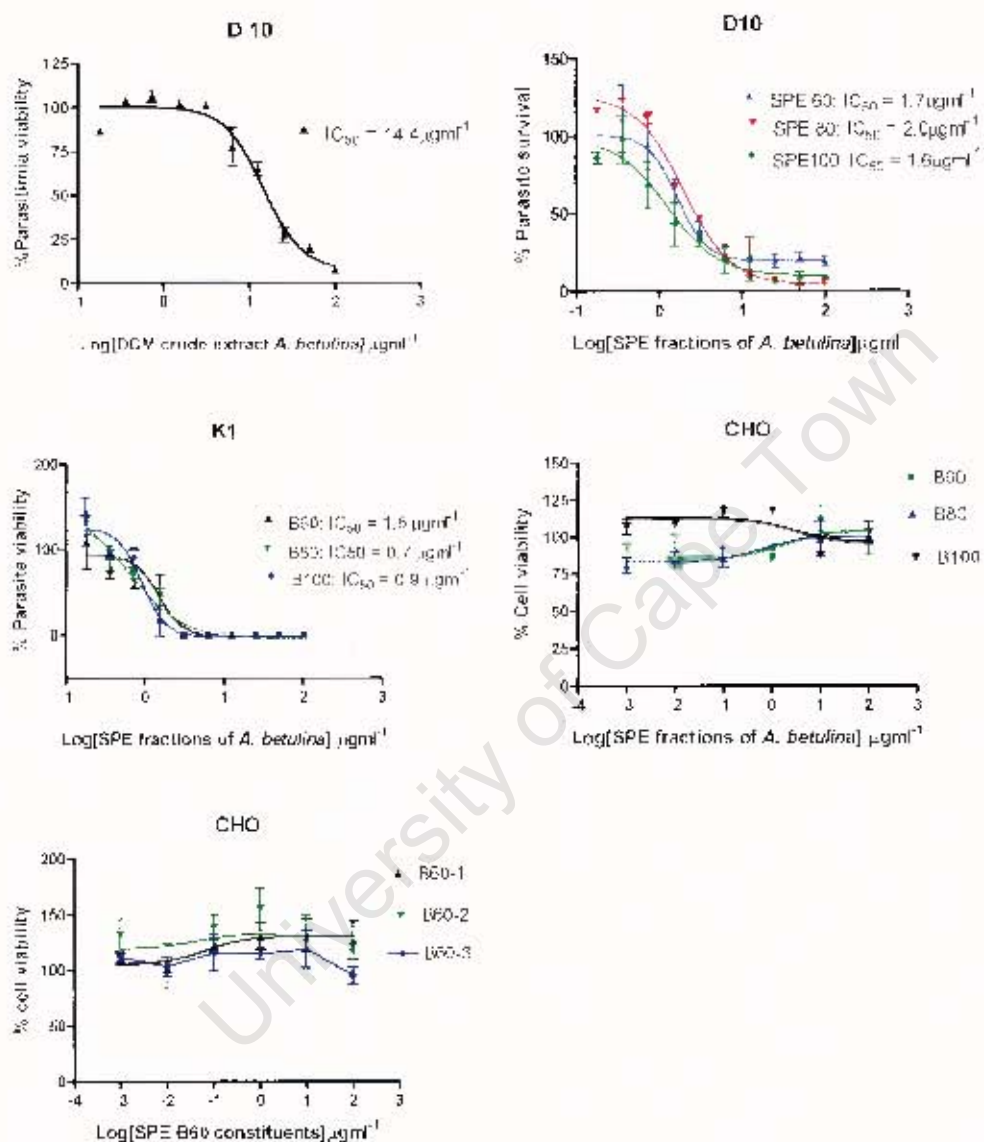


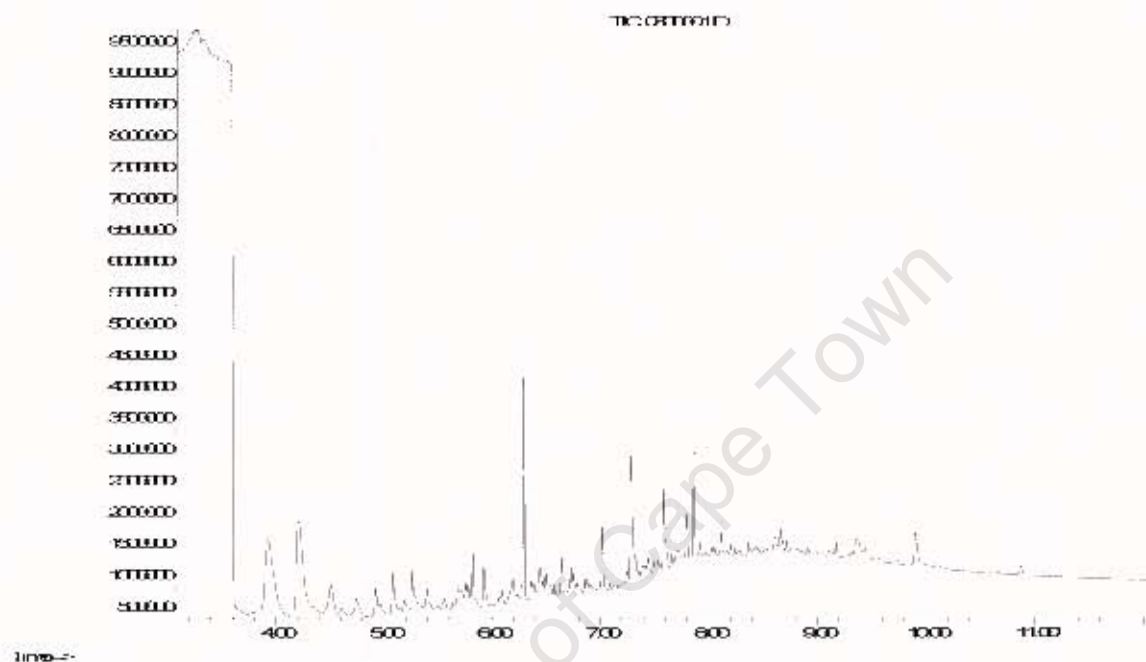
Figure 4.5.5.1 The dose response curves of the antiplasmodial activity and cytotoxicity effects of DCM crude extract of *A. betulina*, fractions generated from fractionation by SPE, and constituents of the SPE 60 fraction.

The Gas chromatography-Mass spectrometry spectra

Figure 4.5.5.2 The Gas Chromatography-Mass spectrometry spectra of HPLC isolates of the SPE fractions of the DCM extract of *A. betulina*.

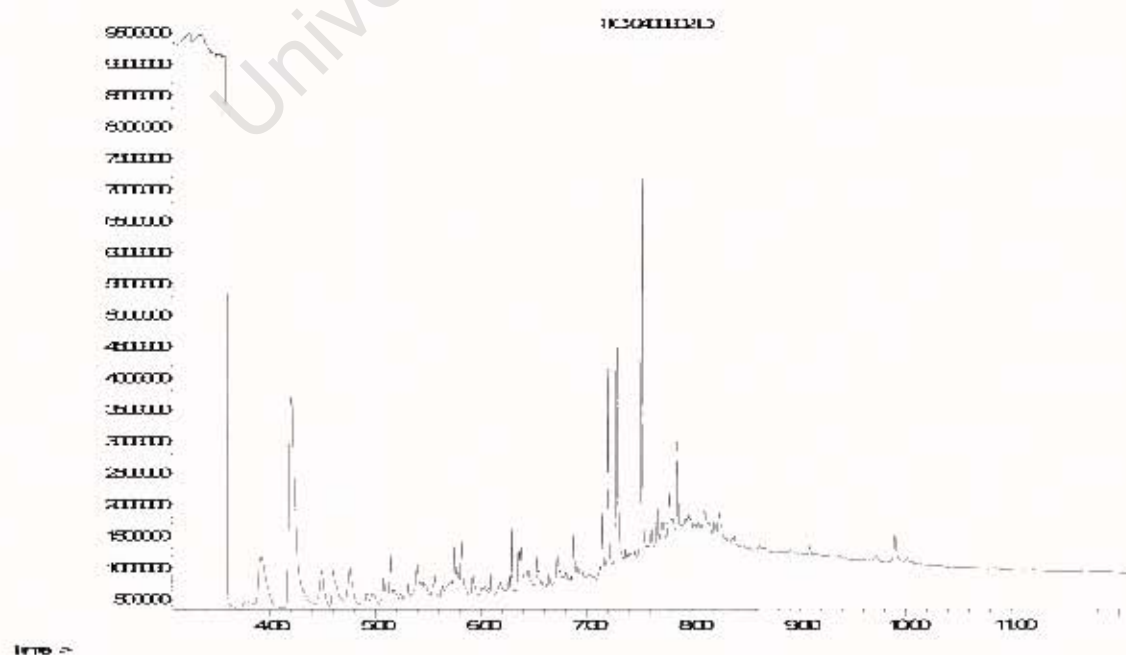
Bu 60-2

Abundance



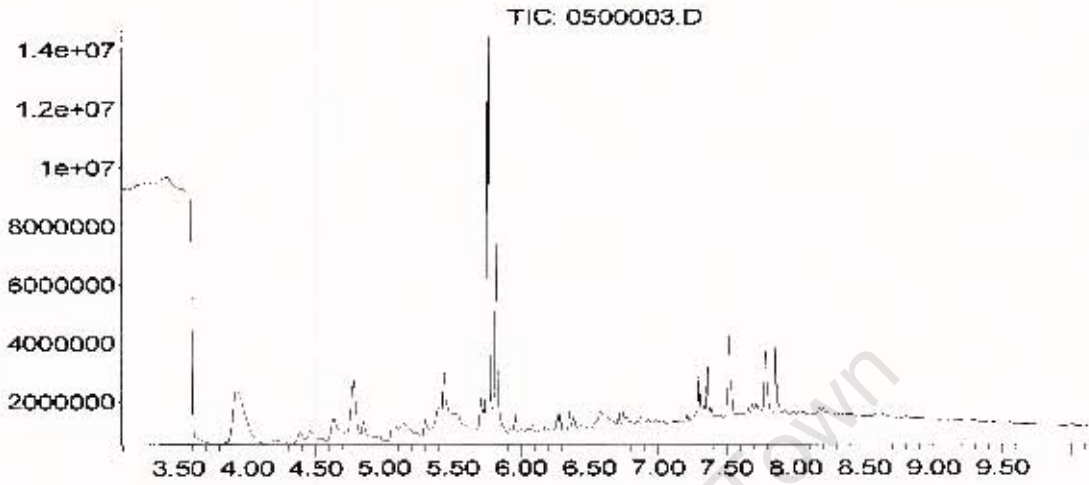
Bu 60-3

Abundance



Bu 80-1

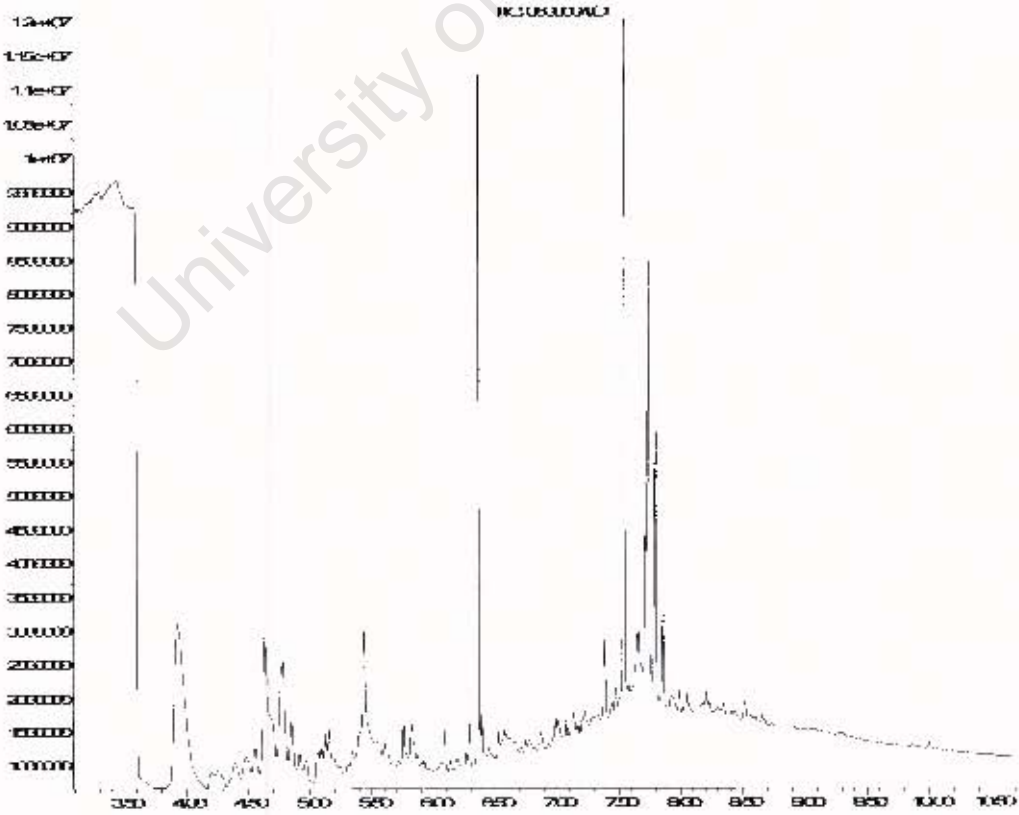
Abundance



Time →

Bu 80-2

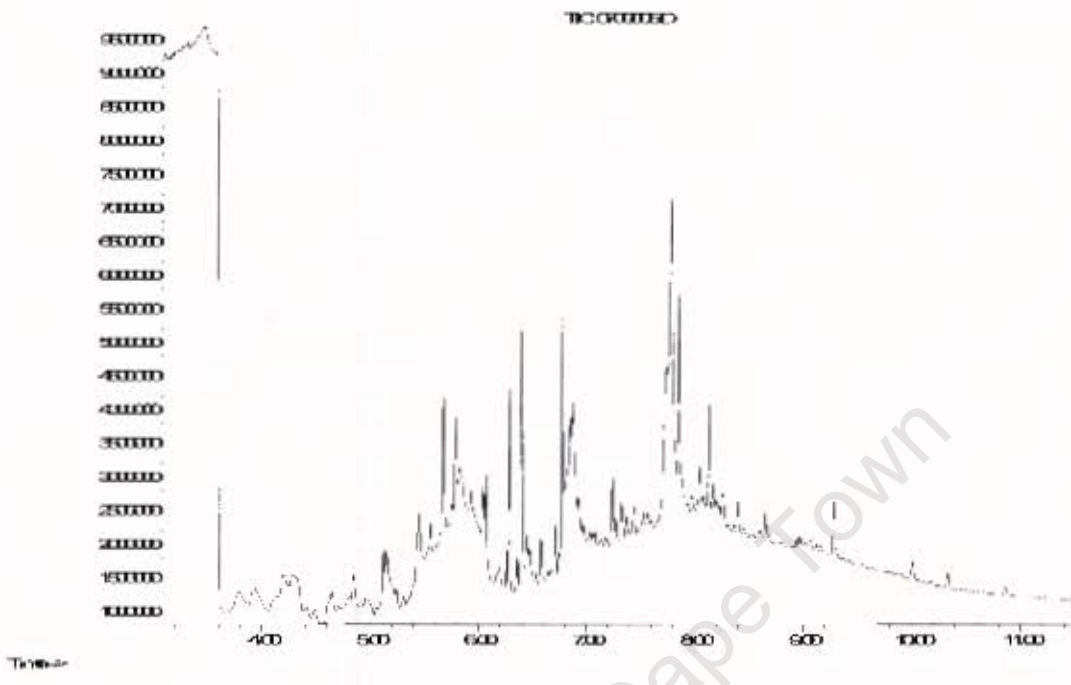
Abundance



Time →

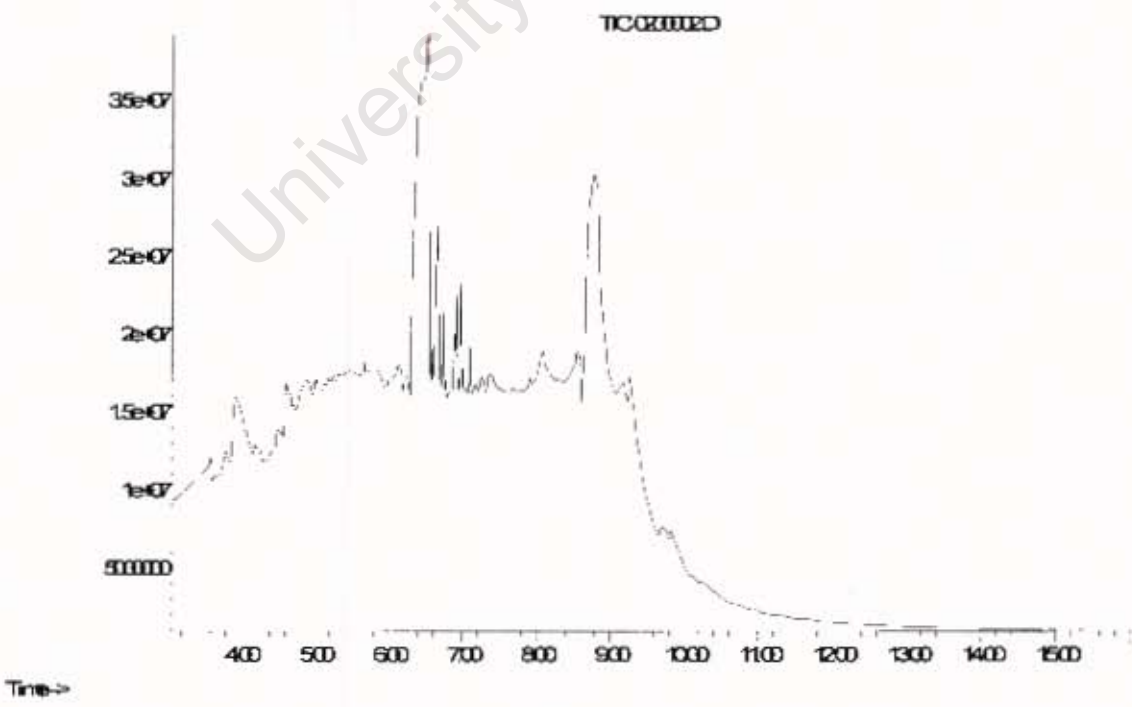
Bu 80-3

Abundance



Bu 100

Abundance



4.5.6 Discussion

The DCM crude extract of the leaves of *A. betulina* showed moderate antiplasmodial activity ($IC_{50} = 14.4 \mu\text{gml}^{-1}$) and low cytotoxicity ($IC_{50} > 100 \mu\text{gml}^{-1}$) on CHO cells. The fractions yielded from fractionation by SPE showed enhanced activity with IC_{50} values in the range of 1.6- 2.0 μgml^{-1} . Furthermore, the fractions showed high selectivity for the malaria parasite with selectivity indices of over 50. Further purification of the SPE fractions yielded single constituents and semi pure constituents which exhibited decreased antiplasmodial activity compared to their respective SPE fractions from which they were isolated. This highlighted the possibility of synergistic activity of compounds within each fraction.

Analysis of the SPE fractions by GC-MS revealed that each fraction contained many constituents (Table 4.5.4.1). Although the specific components of the fractions that elicit antiplasmodial activity remain unclear, our results show that the active constituents possess good and selective activity against the malaria parasite. The difference in the activity of the crude extract and the SPE fractions provide evidence that the active principles are in small quantities. In addition, the loss of activity when the SPE fractions are fractionated shows that the synergistic activity arises from a number of compounds. A few of the identified compounds such as pulegone, terpineol, diosphenol, and piperitone oxide (fraction 60 and 80) have been identified from the essential oils of *Agathosma* species. However, these compounds have not been linked to any biological activity. There are no reports on the antiplasmodial activity of *A. betulina* or essential oils of *Agathosma* species.

The large number of compounds within one fraction make it difficult to attribute the activity to specific compounds. However, the results show that fraction SPE 100 contains high concentrations of hexadecanoic acid, heptadecanoic acid, octadecanoic acid, and their derivatives. Most components of the SPE 100 fraction are major fatty acids more commonly found in plants [Bell and Charlwood, 1980]. In fraction 60 for instance, pulegone (7.48%), carvacrol (15.13%), hexadecanoic acid (7.27%) and phosphoric acid triphenyl ester (8.65%) are in higher concentrations.

Chapter 5

University of Cape Town

5 *In vivo* antimalarial activity of plants traditionally used to treat malaria in South Africa

5.1 Introduction

Despite the emphasis on the urgent need for new and affordable chemotherapeutic compounds, no new discoveries from plants have made it on to market in the last decade. While many extracts may possess *in vitro* activity, only a few show activity *in vivo*. This might be attributed to low solubility of extracts in non-toxic vehicles, low absorption and rapid elimination of active ingredients. Since many antimalarial drugs such as quinine and artemisinin originate from plants, it is still essential that medicinal plants that have traditional folklore reputation for antimalarial properties be investigated *in vivo* to establish their safety and efficacy, thus determining their potential as sources of new-antimalarial drugs.

The results presented in this thesis are of an investigation conducted on five medicinal plants used to treat malaria and fever in South Africa. The crude extract of each plant and the concentrated active fractions of selected plants were evaluated for antimalarial activity using a mouse model. The purified compounds that showed good *in vitro* antiparasmodial activity could not be tested *in vivo* due to limited amounts available.

5.1.1 Animal models in experimental malaria

There are a number of different animal models that are currently being used for experimental malaria. These include birds, rodents, and primates. Primates offer advantages of being genetically similar to humans, and their reaction can be stipulated to that of human beings. The Aotus monkey (*Aotus nancymae*) is an example of a promising experimental model for *P. falciparum* infections [Osamu *et al.*, 1999]. Rodents, particularly mice, remain the widely used animals in research [Herrera *et al.*, 2002].

In this study, the primates were not available and so the choice of experimental animal was the mouse. Mice are advantageous in that they have relatively low

maintenance cost, they are easy to handle, and are genetically similar to man [Malakoff *et al.*, 2000]. Also, mice need smaller doses of test samples. There are several different in-bred strains of mice with varied genetic modification, which have been helpful in gaining the better understanding of pathogenesis of cerebral malaria and the immune response.

There is no single mice model that reflects the exact immunopathogenesis in humans. However, different models provide valuable information. There are four rodent malaria species that provide, in their different host association, opportunity to investigate diverse aspects of malaria disease. These are *Plasmodium berghei*, *Plasmodium chabaudi*, *Plasmodium yoelii* and *Plasmodium vinckei*. The two species, *P. berghei* ANKA and *P. vinckei vinckei* are lethal to mice and are better suited for studies on the development of cerebral malaria, and examining efficacy and safety of remedies. On the contrary, *P. chabaudi*, *P. vinckei petteri* and *P. yoelii* cause infection which resolve after a few days. The infection can either be cleared completely, or there can be recrudescence over a long period [Taylor-Robinson *et al.*, 1998]. These non-lethal infections have been used to investigate mechanisms of acquired immunity and immunological responses including production of antibodies, after infection with malaria parasites. For this study *P. berghei* ANKA was used for investigation of efficacy of plant extracts and their active fractions.

Routes of infection and administration of treatment

The parasites can be delivered into the body by an intravenous injection or intraperitoneally. The latter is more common, as it is easy and quick to perform whereas intravenous injection for instance, can be both complicated and strenuous to the animal as it involves putting the animal into a restraining device or under anesthetic. In this study parasite inoculations were done intraperitoneally.

The test sample can be delivered to animals intravenously, intraperitoneally, subcutaneously, orally, intramuscularly or rectally. The choice of route of administration is influenced by the physical and chemical properties of the drug/extract at test. Oral administration is relatively easy to perform and appropriate for dosing large numbers of mice. Furthermore, plant extracts often do not completely

dissolve in delivery solutions and are preferably administered orally as suspensions. For this study, oral gavage and subcutaneous injection were the preferred routes of administration of extracts, fractions and drugs.

5.2 Choosing the appropriate strain of mice

This study was conducted to assess susceptibility levels of different strains of mice to *P. berghei* ANKA. For the purpose of testing the efficacy of plant extracts, a suitable strain of mice would show gradual progression of parasitemia for at least a period of 10 days and die thereafter. It is important that the strain is not highly sensitive or resistant to infection. Highly sensitive strains die shortly after infection, while resistant strains resolve infection and clear parasites. In both cases the monitoring of parasitemia progression becomes difficult and the effect of the treatment sample on the suppression of parasitemia could easily be missed.

The three strains of mice; C57/BL6, BalbC and DBA strains were tested for susceptibility to *P. berghei* ANKA. Mice (10 in a group) were infected with *P. berghei*, section 3.9.4. The parasitemia and body weight and survival were monitored daily and recorded. The C57/BL6 strain of mice proved to be the appropriate strain for testing efficacy of extracts for antimalarial activity. Mortality was first observed on the seventh day post-infection and all the infected and untreated animals died by the tenth day post-infection.

5.3 Optimizing Infection

The level of parasitemia and the concentration of cells in an inoculation stock affect the rate of development and level of parasitemia in the test animal, the animal survival period, and therefore the outcome of analysis of the activity of the sample at test. This experiment aimed to find the optimum concentration of cells that will allow a slow progression of parasitemia, and survival of infected animals up to at least 10 days post infection. This would allow time for a 4-day or 7-day treatment, and accurate analysis of activity of the extract. To find the optimum cell concentration, the

test animals were infected with different cell concentrations; 1×10^5 , 1×10^6 and 1×10^7 parasitized erythrocyte/ml with similar parasitemia.

5.3.1 Discussion

The development of parasitemia was slow in mice infected with a low cell concentration of 1×10^5 cells/ml. The advantage is that the animals can be studied over a long period of time before they develop cerebral malaria and anaemia. Disadvantageously, the slow progression of parasitemia reduces the accuracy of counted parasitemia percentages. Also, since treatment starts 24 hours post infection, and continues for only four days, the effect of the extracts on the suppression of parasitemia could easily be missed. It also prolongs the experiment. The high dosage (10^7 cells/ml) on the other hand exhibited an exponential progression of parasitemia in the five days post infection. Although the animals developed cerebral malaria faster and died sooner, thereby shortening the period over which the experiment runs, there is a high risk of missing the effect of moderately active extracts. The above results informed the choice of cell concentration of 1×10^6 cells/ml as the optimal cell concentration for infection. For all the efficacy studies performed, the cell concentration for infection was maintained at 10^6 cells/ml.

5.4 Acute toxicity of the crude extracts

Healthy mice (5 in a group) were orally administered a dose of 500mg/kg of DCM extracts of *A. betulina*, *T. camphoratus*, *A. millefolium*, *E. africanus* and the EtOH extract of *W. salutaris*. The extracts were administered twice a day for 7 consecutive days. The mice were monitored for a further 30 days following treatment with the extracts. The negative control group was given equal volumes of the vehicle only (5% sodium bicarbonate in water). The results on survival are shown in Figure 5.3.1.1

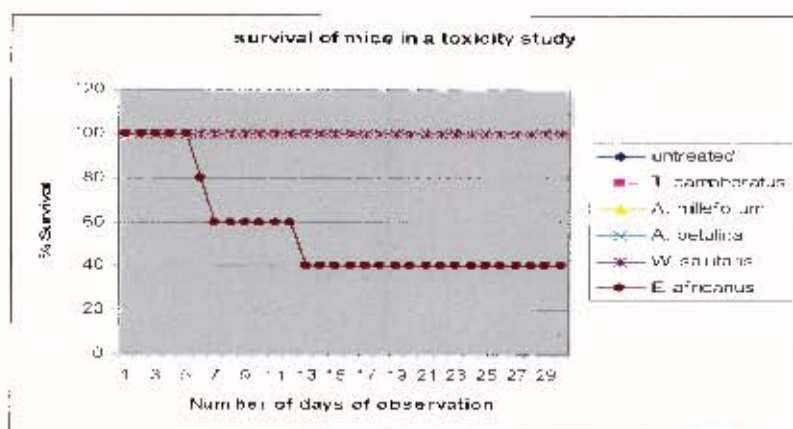


Figure 5.3.1.1 The survival of mice following dosage with different plant extracts at a dose of 500mg/kg twice a day for 7 consecutive days.

5.4.1 Discussion

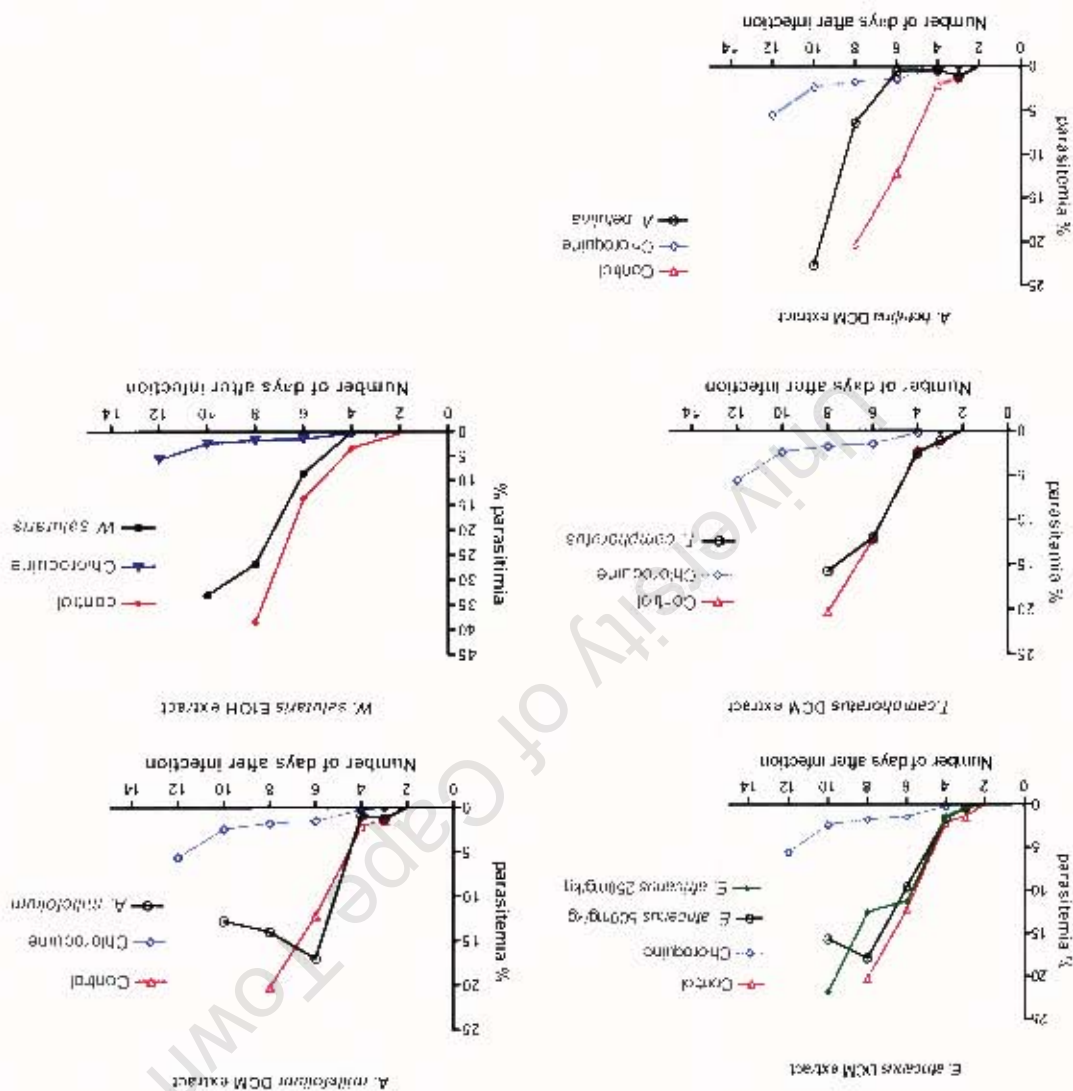
Mortality, physical symptoms of abnormalities (paralysis, lack of mobility), and weight loss were used as indicators for toxicity. For all crude extracts except *E. africanus*, no mortality or toxic effect or any signs of unhealthiness or body weight loss were observed. Based on the results obtained in this study, the median lethal dose is taken to be greater than the total dose of 1000mg/kg/day used in this study. At this dose, the extracts are considered safe. However, the full examination of the major body organs such the brain, liver and kidneys, is necessary before a conclusion on the safety on of these extracts could be made.

The DCM extract of *E. africanus* produced physical signs of toxicity 1 hour after administration. The animals appeared generally unhealthy. The signs of toxicity included writhing, decreased motor activity, gasping, decreased respiratory and then death. About 10% of the animals appeared bloated around the abdominal area. There was 60% mortality on day 13. For the period of 30 days from the first day of administration of the extract, only 40% of the animals survived.

5.5 Antimalarial efficacy studies

Mice were randomly allotted to five groups, and were inoculated intraperitoneally with 10^6 parasite infected red blood cells/ml *P. berghei* ANKA following procedures outlined in section 3.10.4. The mice were treated orally 24-hours post infection with the plant extracts which showed positive antiplasmodial activity *in vitro*. Mice were

Figure 5.5 The time course changes of parasitemia in mice in response to treatment with CQ and with plant crude extracts (250mg/kg, and 500mg/kg for *L. africanus*) for four days after infection. The control group received an equivalent volume of the vehicle (20% EtOH in 5% sodium bicarbonate in H₂O).

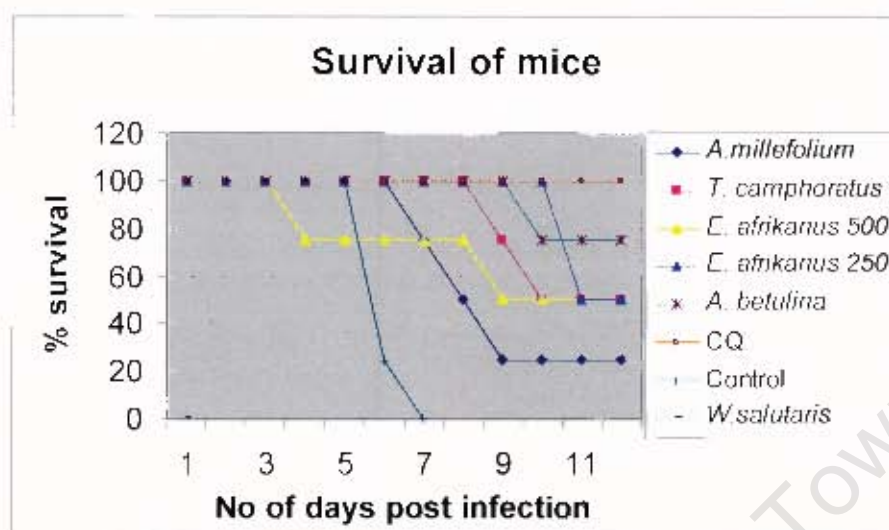


Results

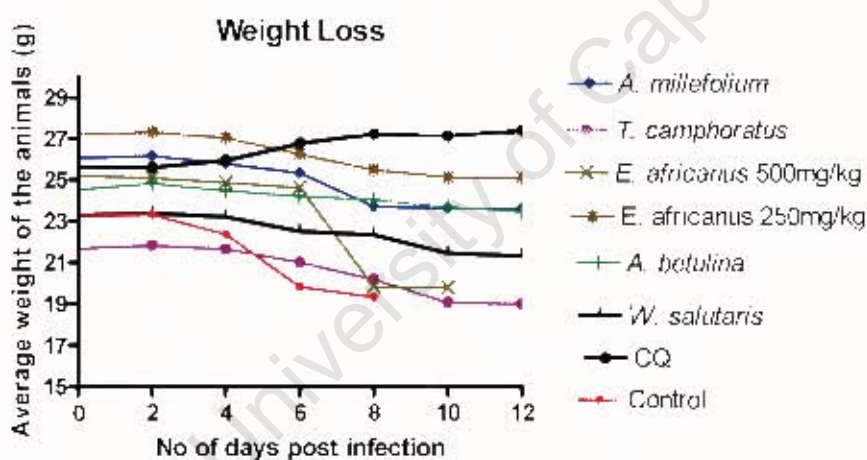
Figure 5.5. 2.

given extracts of *A. millefolium*, *T. camphoratus*, *W. salutaris*, and *A. helvum*, at a dose of 250mg/kg/day, and the extract of *E. africanus* at doses of 250mg/kg and 500mg/kg/day. CQ, given at 10mg/kg/day was used as a positive control. The negative control group was given 5% sodium bicarbonate in water. The study indicators; parasitemia, weight change, convulsions, respiratory distress and mortality [English *et al.*, 1996] were compared with those observed in the negative control. The results of antimalarial activity of the crude extracts are shown in Figure 5.5.1 and

Survival of Mice



a)



b)

Figure 5.5.2 The time course changes of survival rate (a) and weight loss (b) of mice in response to treatment with different extracts, or CQ. The experiment was terminated on day 13.

5.5.1 Discussion

The level of parasitemia in the untreated control group increased throughout the period of observation. In the positive control group treated with CQ, parasitemia was

suppressed to below 2% up to day 7. Recrudescence was observed from day 10, and parasitemia had reached about 8% at the time the experiment was terminated.

The crude extracts of *T. camphoratus*, *A. millefolium* and *E. africanus* did not show any considerable parasitemia suppression when compared with the negative control. The extract of *W. salutaris* showed 42.9% parasitemia suppression on day 6, while *A. betulina* caused reduction in parasitemia by 76.9% (calculation details are in section 3.10.6). It was also noted that although the group treated with *A. betulina* showed rapid increase in the parasitemia levels after day 8, the animals tolerate high parasitemia levels of up to 40%. The animals appeared healthy, no visible signs of unhealthiness or typical symptoms of cerebral malaria were observed. The animals maintained their body weight for a longer period, and survived longer than all other groups except the CQ group.

All the mice in the negative control group died on day 7, while the CQ control group maintained 100% survival until termination of the experiment. Mice in a group that was treated with *E. africanus* at a dose of 500mg/kg died early (day 4). It is suspected that this was due to the toxicity of the extract since the group given the lower dose maintained 100% survival up to day 10. The group that received *A. millefolium* extract had only 25% survival by day 9. The group treated with *T. camphoratus* had 50% survival. The results show that the extracts of *A. betulina* and *W. salutaris* prolonged the lives of mice at a relatively higher survival rate when compared with the negative control group and other test groups.

The body weight of the animals was monitored daily. Generally, the results showed that there was a gradual continuous body weight loss in all the groups except in the CQ-treated group (Figure 5.5.2 b).

5.6 The antimalarial activity of fractions of selected extracts

The active fractions of *W. salutaris*, *A. betulina* and *A. millefolium* (sections 4.3.4, 4.5.2 and 4.4.2.1 respectively) were selected for antimalarial activity testing *in vivo*, since they showed good antiplasmodial activity *in vitro* and their respective crude

extracts showed no toxicity *in vivo*. CQ positive control was done in every experiment. For clarity purposes, the activity of CQ is not shown on each graph.

5.6.1 Suppressive test

The active fractions of *W. salutaris*, *A. betulina* and *A. millefolium* were concentrated and prepared accordingly (Section 3.10.5.1). The treatment was administered orally at a dose of 250 mg/kg once a day for 4 consecutive days, 24-hours post-infection. The results are shown in respective graphs in figure 5.6.1.1.

Results

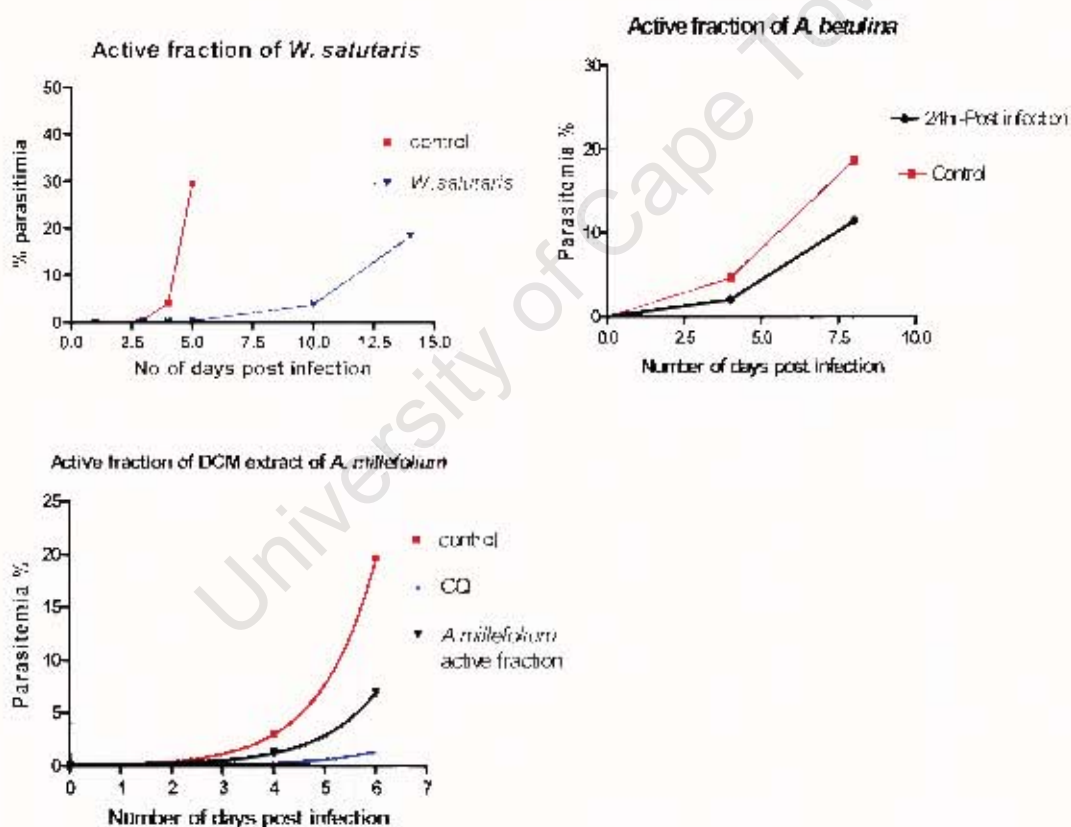


Figure 5.6.1.1 The graphs showing the percentage parasitemia, in groups treated with active fractions of *W. salutaris*, *A. betulina* and *A. millefolium*, in comparison to the control group and CQ-treated group. The experiments were done separately on different occasions.

5.6.1.1 Discussion

The results revealed that the active fraction of *A. millefolium* suppressed the parasitemia by 48% and 67% on day 4 and 6 respectively. The fraction was effective in suppressing the parasitemia only during the administration period (up to day 5), but the parasitemia levels escalated upon termination of treatment. The survival period was not prolonged. On day 7, the mice were anemic and showed the same signs of sickness such as ruffled hair, lack of mobility, and convulsions, as the control group.

By comparison to the control group, the active fraction of *W. salutaris* was effective in suppressing the parasitemia, and extending the survival period of the treated group by 8 days. The fraction exhibited 92% and 98% parasitemia suppression on days 4 and 6 respectively. The control group died on day 8 while the treated group displayed the signs of sickness on day 12 and mortality was first observed on day 16.

The results also showed that the active fraction of *A. betulina* had antimalarial activity. The fraction exhibited parasitemia suppression of 46% and 63.3% on day 4 and day 6 respectively. This group survived longer than both the control group and the group treated with the active fraction of *W. salutaris*. Interestingly, the animals in this group tolerated high parasitemia without showing typical signs of cerebral malaria. They still survived longer even after the signs of sickness were observable. The animals were sacrificed after 18 days to avoid suffering.

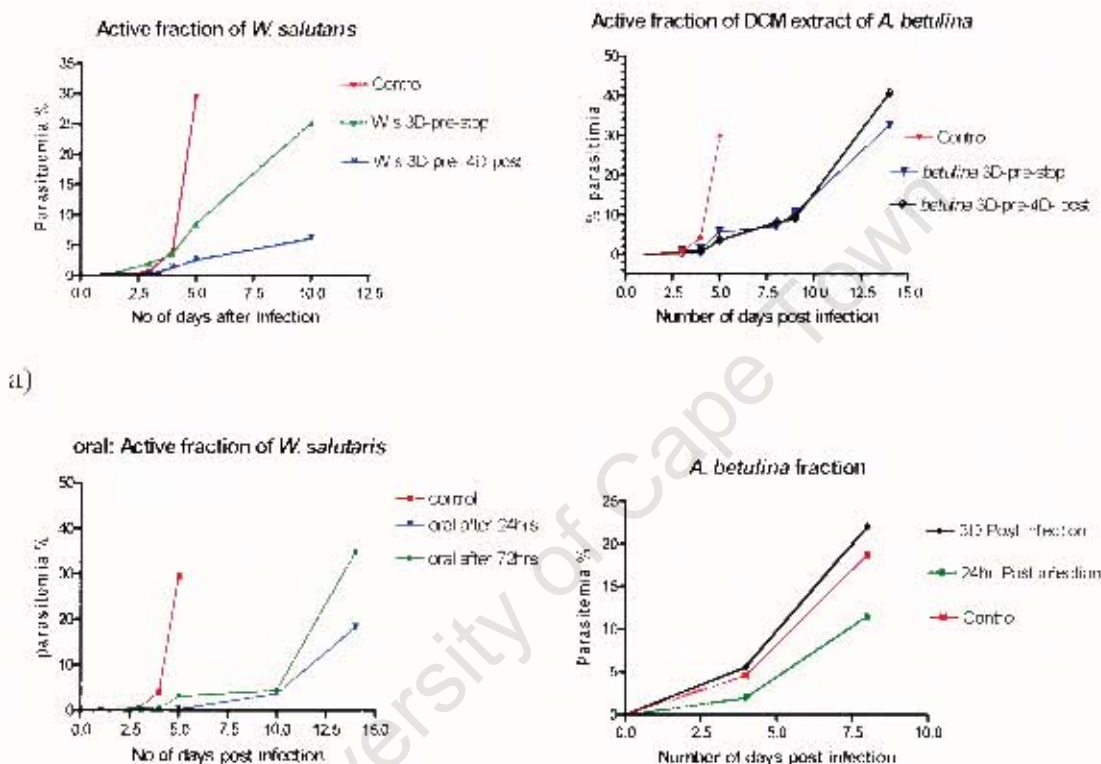
The active fractions of *W. salutaris* and *A. betulina* showed parasite suppression and prolonged the survival period of the treated animals. These fractions were taken for further investigation

5.6.2 Prophylaxis and curative properties of fractions

The aim of this experiment was to assess the preventive or prophylactic and curative effects of the selected fractions against malaria. To assess the potential prophylaxis properties of fractions, the test fractions were administered orally three days prior to infection. In one group, administration of the test fraction was stopped immediately after infection, while in the second group, treatment with the test fraction was

continued for further 4 days after infection. To assess curative properties of the fractions, fractions were administered orally 72 hours after infection. The detailed methodology is outlined in sections 3.10.5.2 and 3.10.5.3. Figure 5.6.2.1 shows the results.

Results



b)

Figure 5.6.2.1 The time course changes in parasitemia to demonstrate the preventive (a) and curative (b) properties of fractions of *W. salutaris* and *A. betulina* when administered orally at a dose of 250mg/kg body weight per day.

5.6.2.1 Prophylaxis potential of fractions

The group that received the SPE fraction of *W. salutaris* for 3-days before infection and treatment stopped after infection, showed no significant suppression of parasitemia on day 4 when compared to the negative control group. Whereas the group that was given the fraction 3-days before infection and for a further 4-days after infection showed lower parasitemia on day 4 with inhibition activity of 52%, and

continued to exhibit lower parasitemia levels on day 5 (85% inhibition) and day 10 relative to the negative control. When comparing the two test groups, the group that received treatment for further 4 days after infection maintained relatively low parasitemia levels of 7% up to day 10, while the other group had 25% parasitemia. The mean survival period for the control group was 6 days, while the survival period of mice in both test groups was extended for a further 3 days. The results suggest that the fraction of *W. salutaris* did not have significant prophylactic effect when parasitemia was observed on day 4, but somehow maintained lower parasitemia levels relative to the negative control. Continuing treatment after infection dramatically decreased parasitemia levels.

The fraction of *A. betulina* extract showed interesting results. There was a small difference in the parasitemia levels in the negative control group and in test groups on day 4. The test groups however, maintained low parasitemia levels of roughly under 10% up to day 9 post-infection, while the control group had reached parasitemia levels of 48%. On day 5 the groups exhibited parasitemia reduction of about 75%. It is also worth noting that the parasitemia levels in the two test groups were similar up to 11 days ($P < 0.2$). It is interesting to note that further administration of the fraction for 4 days after infection had no suppressive impact on parasitemia. The results show that administration of the fraction 3 consecutive days before infection had a protective or prophylactic effect, which led to low levels of parasitemia for up to 10 days. The fraction seemed to impede parasite growth or the rate of parasite multiplication. The similarity in the pattern of levels of parasitemia in the two test groups could suggest that the fraction did not have antiplasmodial activity on the blood stage parasites.

5.6.2.2 Clearance or curative test

The results on the curative schizontocidal activity of the fractions of *W. salutaris* and *A. betulina* are shown in figure 5.6.2.1b. The results from treatment with the fraction of *W. salutaris* revealed the significant difference between the treated groups and the negative control group. When comparing the two test groups, the group that received treatment 72 hours post-infection showed high parasitemia levels (4.6%) on day 5, while the group that received treatment 24hrs post-infection showed almost undetectable levels of less than 1%. This difference was expected since at 72hrs post-

infection, the infection is established and parasitemia should be detectable in the absence of treatment. Introduction of the test sample on day 3 led to a significant decrease in parasitemia, such that the two groups (treated 24hr or 72hr post-infection) had similar parasitemia of about 3.8% on day 10. The results indicate that the fraction has schizontocidal activity, and has the potential to reduce parasitemia whether given at the beginning of an infection or when the infection is already established. Thus demonstrating considerable antiplasmodial activity.

The test group that received the fraction of *A. betulina* 72 hrs post-infection showed no major difference in parasitemia levels when compared to the control group. Throughout the experiment the group maintained higher parasitemia than that observed in the control group. The fraction failed to show antiplasmodial activity in established infection. The test group that was treated with the fraction 24 hrs post-infection showed parasitemia reductions of 57% on day 4 and of 45% on day 8. The fraction showed moderate degree of parasite reduction. All the results are summarized in Table 5.6.2.1. Chloroquine was used as a positive control in suppressive testing only, since it has been reported to possess no prophylaxis activity [Ridley *et al.*, 1997].

Table 5.6.2.1 *In vivo* antiplasmodial activity of fractions of *W. salutaris*, *A. betulina* and *A. millefolium* when administered to infected mice at a dose of 250mg/kg. The average percentage values were taken for day 4 and day 7.

Drug/extract	Suppressive test	Prophylaxis test	Curative test
	Average (%) parasitemia suppression	Average (%) parasitemia suppression	Average (%) parasitemia suppression
<i>A. betulina</i>	51.7	75	5
<i>W. salutaris</i>	95	<2	75.6
<i>W. salutaris</i> (subcutaneous)	99	ND	94
<i>A. millefolium</i>	57.5	ND	ND
CQ	99.1	ND	99

*ND – test not done.

5.6.3 Subcutaneous administration of the active fraction of *W. salutaris*.

Factors such as incomplete absorption and elimination can reduce the bioavailability of active constituents, which leads to reduction in their overall activity. This study aimed to establish whether incomplete absorption and or rapid elimination of the active constituents from the gut (metabolism or excretion) played any role in the level of bioavailability of the active constituents.

The animals were infected and treated with 250mg/kg body-weight of active fractions of *W. salutaris*. The fractions were administered subcutaneously. One group was treated 24 hrs post infection while the other group received treatment 72 hrs post infection. The results are shown in Figure 5.6.3.1.

Results

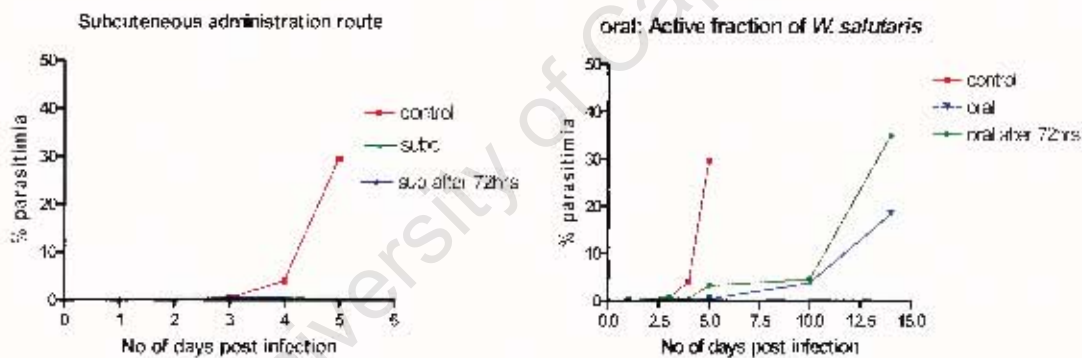


Figure 5.6.3.1 The investigation of the effect of absorption or first pass metabolism by comparing the parasitemia suppression activity of the fraction when administered orally or subcutaneously at the same dose of 250mg/kg once a day for 4 days.

The two groups that received treatment subcutaneously maintained almost undetectable levels of parasitemia as observed on day 3, 4 and day 5, while the negative control had parasitemia percentage of 29% on day 5. Comparatively, the two test groups showed similar parasitemia patterns.

The experiment could not be monitored for longer than 6 days due to the fatal effects of the test fraction on the animals. The sample was sticky. Following administration,

it was observed that the skin became glued onto the back of the neck. This could have made blood circulation difficult and led to the death. However, it could be deduced from the results that the subcutaneous route of administration was more effective than the oral route in suppressing the parasitemia.

5.6.4 Concluding discussion

The crude extract of *A. betulina* possesses moderate *in vitro* antiplasmodial activity ($IC_{50} = 13.5\mu\text{gml}^{-1}$) and poor curative antimalaria activity *in vivo*. The extract however, proved to have preventive or prophylactic activity *in vivo*. This suggest that the extract is poorly active against the blood stage parasites. In our observations, in groups treated for 3 consecutive days before infection and 24 hrs after infection, the blood stage infection and its associated clinical manifestations were delayed and highly minimised.

In order to determine whether protection from infection might be in part due to an additional effect of the fraction on the blood stage parasite, mice were infected on day 0 and treated with the fraction 3 days post infection. No significant inhibition of the erythrocytic multiplication cycle could be detected. This could be an indication that the protective properties were due to either an inhibition of the hepatic stages of the parasite or increased immune response. The fraction of *A. betulina* might be exerting antimalarial effects by mechanism(s) other than a direct antiplasmodial action. The fraction could be exerting a natural boosting of immune responses against malaria parasites. Stimulating the immune response prior to infection would activate the macrophage mechanism of immunity [Webster, 1989], and improve the robustness of the response and the efficiency of the body in fighting the infection, thereby resulting in relatively decreased parasitemia levels and delayed manifestation of clinical symptoms of malaria.

While many extracts show good *in vitro* antiplasmodial activity, most of them show decreased suppressive activity *in vivo* and no curative activity at all [Saidu *et al.*, 2000]. It was interesting to note that the fraction of *W. salutaris* possesses blood schizontocidal activity observed during the suppressive test and curative test. The

observation that the active fraction was highly effective even in established infection highlights the potential of the fraction in treatment of malaria. The plant however, did not show any prophylactic activity. It was disappointing that the extract showed toxicity when administered subcutaneously. However, it is common that extracts that have been proven safe when administered orally exhibit toxicity when given intraperitoneally, intravenously or subcutaneously [Benoit-Vical *et al.*, 2008]. The significance of the toxicity of the fraction when administered subcutaneously is reduced by the fact that African traditional medicine uses exclusively the oral route of administration.

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Chapter 6

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6.1.1 The extent to which objectives have been met

The plant material of selected plants was extracted with various solvents and screened for antiplasmodial activity against D10. Extracts that showed antiplasmodial activity less than or equal to $10 \mu\text{gml}^{-1}$ were considered active. The most highly active and abundant extract for each plant was tested against the resistant strains, assessed for cytotoxicity on a mammalian cell line, and then subjected to bio-guided fractionation using a combination of various chromatography techniques.

Isolation of active constituents

The fractionation process for each of the selected plant extracts led to isolation of a number of active compounds. In total, 7 compounds have been isolated and their chemical structures determined. There were, however many active compounds which were isolated but could not be identified due to the small quantities or their instability. A total of 27 compounds present in the active fractions of DCM extract of *A. betulina* were identified by GC-MS.

Two compounds from the DCM extract of *T. camphoratus* could not be identified due to their instability. One compound successfully identified as 5'7-dihydroxy flavanone, showed good antiplasmodial activity ($\text{IC}_{50} = 2.2 \pm 0.2 \mu\text{gml}^{-1}$), a high selectivity index of 20 and a low resistance index of 0.98. The antiplasmodial activity of this compound was selective for the malaria parasite and similar against the CQ sensitive and resistance strains.

Four compounds were isolated from the DCM extract of *E. africanus*. The level of purity and quantity of compound R90-3 did not allow further purification for structural elucidation. The other three compounds were successfully identified as carnosol, betulinic acid and 5,7-dimethoxy-4'-hydroxyflavone. The compounds showed moderate activity ($\text{IC}_{50} = 7.6 \pm 0.6 \mu\text{gml}^{-1}$, $9.5 \pm 0.5 \mu\text{gml}^{-1}$ and $6.5 \pm 0.4 \mu\text{gml}^{-1}$) and low selectivity indexes (0.3 to 1.1). The low selectivity indexes of the compounds indicated a narrow safety margin, which hampers their potential as lead compounds.

Two sesquiterpenoids identified as cinnamolide ($IC_{50} = 0.9 \pm 0.2 \mu\text{gml}^{-1}$) and 3- β -acetoxy cinnamolide ($IC_{50} = 1.4 \pm 0.2 \mu\text{gml}^{-1}$) were isolated from the EtOH extract of *W. salutaris*. Although the compounds showed close antiplasmodial activity, there was a great difference in their cytotoxicity on CHO cells. Cinnamolide was about 8 times less toxic than its derivative. Based on these observations, it is possible that structural changes in both compounds could lead to derivatives that have improved activity and less cytotoxicity. Cinnamolide has a potential as a lead compound.

The fractions of the DCM extract of *A. millefolium* showed enhanced activity with IC_{50} values ranging from 1.6 to 2.8 μgml^{-1} . The isolated compound however, showed decreased activity ($IC_{50} = 4.8 \pm 0.2 \mu\text{gml}^{-1}$). This demonstrated the synergistic activity of the compounds within a fraction. The fractions were highly selective for the malaria parasite (CHO $IC_{50} > 100 \mu\text{gml}^{-1}$). The compound also showed high selectivity for the parasite ($IC_{50} > 100 \mu\text{gml}^{-1}$). The components of *A. millefolium* have showed good antiplasmodial activity, high selectivity and low resistance index, and could therefore be considered for further development for use against malaria.

SPE fractions of the DCM extract of *A. betulina* had antiplasmodial activity with IC_{50} values ranging from 1.6 μgml^{-1} to 2.0 μgml^{-1} against both the D10 and K1 strains. These fractions showed good activity and were highly selective for the malarial parasite. The cytotoxicity IC_{50} values proved to be greater than 100 μgml^{-1} . The GC-MS results revealed that each fraction contained a few constituents, some of which have been reported previously as components of the essential oil, and fatty acid composition of the plant. Due to the large number of compounds within each fraction, it was difficult to attribute the observed antiplasmodial activity to particular compound(s).

The safety and *in vivo* antimalarial activity of extracts and selected fractions

The safety of the tested extracts and fractions was evaluated by looking at survival, weight loss, typical symptoms of cerebral malaria such as convulsions, lack of mobility, and general physical signs of unhealthiness of the animals.

The crude extracts of *W. salutaris*, *A. millefolium*, *A. betulina* and *T. camphoratus*, showed no effect of toxicity when administered orally at 500mg/kg twice a day for 7 consecutive days. The extracts were well tolerated by the animals at tested dosages. On the contrary, the crude extract of *E. africanus* showed toxicity to mice at oral doses of 250mg/kg and 500mg/kg with no evidence of protection against *Plasmodium berghei* malaria.

The crude extracts of *E. africanus*, *A. millefolium* and *T. camphoratus* did not show antimalarial activity, while extracts of *W. salutaris* and *A. betulina* showed considerable antimalarial activity at a dose of 250mg/kg in a 4-day suppressive test. The selected fractions of *A. millefolium* that had good *in vitro* antiplasmodial effect exhibited antiplasmodial activity *in vivo* with 40% chemo suppression of parasitemia, but showed no antimalarial effect. The fraction of *W. salutaris* showed good suppressive and curative antimalarial effect, but showed no prophylactic effect. The fraction of *A. betulina* exhibited a prophylactic effect against malaria and induced tolerance to high parasitemia for a prolonged period of time. This antimalarial activity observed could be as a result of stimulation of the immune response. The fractions of *A. millefolium*, *W. salutaris* and *A. betulina* have the potential of being developed into a herbal remedy that is useful in malaria treatment.

6.2 Cross resistance between plant extracts and CQ

There was no major difference between the antiplasmodial activities of the plant extracts against the CQ-sensitive and CQ-resistant strains of *P. falciparum*. Instead, the activity of each extract against both strains was comparable. The absence of cross resistance between plant extracts and CQ suggest that malarial remedies derived from medicinal plants could be effective in treating malaria caused by CQ-resistant parasites. In the absence of drug-drug interactions, the extracts can be co-administered with current antimalarial drugs to synergistically improve their activity especially against the resistant strains of the parasite.

6.3 Limitations and challenges encountered during the study

One of the main limitations in working with plant extracts is the low yields of extracts, fractions and isolated compounds. Most of the highly active semi-pure fractions, B60-1 for example, could not be investigated and purified further due to limited quantities. In other fractions, the decomposed compounds could not be recollected.

The variability of the level of chemical constituents in plants due to geographic and seasonal conditions proved to be a problem. This was observed with *W. salutaris*, where the difference in the quantity of the active constituents between batches was large. In cases where recollection of plant material is necessary, the time and place of collection should be maintained. This can be a long wait depending on the season in which such a need arises and the season in which the initial collection was made.

The loss of activity in bio-guided fractionation posed a challenge. Some plant constituents work synergistically to exhibit antiplasmodial activity. When these compounds are separated during fractionation, activity is lost. This was observed from SPE fractions of *A. betulina*, and with *A. millefolium*, where fractions generated from column chromatography were more active than purer fractions from short column chromatography, and the isolated compound. Loss of activity complicated isolation of the compounds responsible for the activity.

The other challenge encountered was the use of an appropriate HPLC column for each extract. The extracts from three plants were separated using a C₁₈ column, while extracts of *A. millefolium* and *A. betulina* were separated using C₈ and C₁₆ amide columns respectively. A number of columns had to be tested before a suitable column could be found.

The approach used in this study to assess safety of plant extracts only evaluates the acute toxicity and not the chronic effects that the extracts/fractions might have on the body. It does not provide the detailed assessment of the effect of the extracts or fractions on the body tissues and on important organs such as the brain, liver, kidneys,

in the body. Additional and more detailed assessments such as full blood count, size, shape and functionality of main organs should be done. Autopsy should also be performed on dead animals to identify the exact cause of death.

The antimalarial activity of the crude extracts and fractions was evaluated by counting the blood smear slides microscopically. While this method is time consuming, the accuracy of the parasitemia counted from slides is compromised, firstly by the genetic variation between animals, and secondly by the complexity of the unsynchronised blood smear slides, and lack of standardization when making the smears.

While the use of a murine model increases the predictive value of the results obtained, there are major differences between rodents and humans. Small mammals have a faster metabolism than humans. Small animals require 5-10 times per body weight the amount of drug taken by humans to produce the same effect [Freireich *et al.*, 1966]. Furthermore, a non-human species of *Plasmodium* was used to assess the *in vivo* antimalarial activity. While this model provides valuable information, the results can not be directly extrapolated to represent the situation in humans.

The process of selecting extracts for antimalarial activity assessment is limiting. The plant extracts are first screened using *in vitro* assays. *In vitro* systems have shortfalls particularly as they do not take into account any pro-drug effect or the role of the immune system in controlling the infection. As was observed with a crude extract of *A. betulina*, a negative *in vitro* result does not necessarily exclude *in vivo* antimalarial activity. Plants might possess other activities that have a generally protective effect against malaria or that help minimise the symptoms of malaria.

6.4 The relevance of the study

Many people already use herbal medicines for the treatment of malaria in South Africa, and therefore more scientific information concerning the efficacy and safety of medicinal plants can help discourage the use and educate about those herbal medicines that are toxic. From this study in particular, traditional Healers can learn to reduce the doses or limit the use of *E. africanus* extracts.

In traditional medicine in South Africa, fevers are poorly defined and they tend to be broadly attributed to malaria. Some plants reported for use to treat malaria lack activity against and selectivity for the malaria parasite [Addae-Kyereme *et al.*, 2001; Carvalho *et al.*, 1991]. Since plants that have antipyretic, analgesic activity, and are stimulants of the immune system would provide considerable relief to malaria patients [Younos *et al.*, 1990; Kirby, 1997], they are all classified as antimalarial. Scientific investigation of plants would positively impact on correct categorisation of plants. The example is *A. betulina* extract, which from this study proved to be more of an immune modulator than an antimalarial remedy.

The government of South Africa recognises traditional medicines and has made the effort of incorporating traditional medicine in the health system. This work will contribute towards informing the health planners to accurately evaluate the usefulness and effectiveness of traditional medicine, which will bring about the support for research on the plant-derived drugs, and recognising it as one of the strategies in malaria control.

6.5 Future work

- Active fractions of *W. salutaris*, *A. betulina* and *A. millefolium* showed positive *in vitro* antiplasmodial activity and selectivity to the malaria parasite. The fractions showed no toxicity effects and considerable antimalarial activity *in vivo* at tested concentrations. Furthermore, the major active components in each fraction have been identified. This information warrants further investigation to evaluate the effectiveness and usefulness of the fractions in combination, as a potential herbal remedy to use in treatment of malaria.

More research on the fractions will include:

- Evaluating the toxicology of the fractions in combination on a monkey model using a 90-day sub-chronic test.

- Evaluating the prophylaxis, suppressive and curative effects of combined fractions on the vetvet monkey model.
- Possibly, work towards developing this potential herbal remedy as a product for commercial purposes.

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Bibliography

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Abeku, A.T., 2007. Response to malaria epidemics in Africa. *Emerging infectious disease*. www.cdc.gov/eld 13 (5), 681-686.

Abosi, A.O., Mbukwa, E., Majinda, R.T., Raseroka, B.H., 2006. *Vangueria infausta* root bark: *in vivo* and *in vitro* antiplasmodial activity, *British Journal of Biomedical Science* 63, 129-133.

Adams, P.R., 1989. Identification of essential oils by ION Trap Mass Spectroscopy. Academic Press INC. Harcourt Brace Jovanovich Publishers. San Diego, California.

Addae-Kyereme, J., Croft, L.S., Kendrick, H., Wright, W.C., 2001. Antiplasmodial activities of some Ghanaian plants traditionally used for fever/malaria treatment and of some alkaloids isolated from *Pleiocarpa mutica*; *in vivo* antimalarial activity of pleiocarpine. *Journal of Ethnopharmacology* 76, 99-103.

AHFS Drug Information., 2000. American Society of Health-System Pharmacists, USA.

Ahn, J., Kim, Y., Seo, E., Choi, Y., Kim, H., 2008. Antioxidant effect of natural extracts on the microencapsulated high oleic sunflower oil. *Journal of Food Engineering* 84 (2), 327-334.

Al-Sereiti, M.R., Abu-Amer, K.M., Sen, P., 1999. Pharmacology of rosemary *Rosemarinus officinalis* Linn. and its therapeutic potentials, *Indian J. Exp. Biol.* 37, 124-130.

Alonso, P.L., Sacarlal, R., Aponte, M., Leach, A., Macete, E., 2004. Efficacy of the RTSS/AS02A vaccine against *Plasmodium falciparum* infection and disease in young African children; Randomized controlled trial. *Lancet* 364, 1411-1420.

Amabeoku, G.J., Green, I., Eagles, P., Benjeddou, M., 2000. Effects of *Tarhonanthus camphoratus* and *Eriocephalus africanus* on nociception in mice and pyrexia in rats. *Phytomedicine* 7(6), 517-522.

Angioni, A., Barra, A., Coroneo, V., Dessi, S., Cabras, A., 2006. Chemical composition, seasonal variability and antifungal activity of *Lavandula stoechas* L. ssp *stoechas* essential oils from stem/leaves and flowers. *Journal of Agriculture Food and Chemistry* 54 (12), 4364-70.

Arnold, N., 1993. Morphologisch-anatomische und chemische Untersuchungen an der Untergattung *Telamonia* (*Cortinarius*, Agaricales). – Libri Botanici 7, 1–214.

Arnold, T.H., Prentice, C.A., Hawker, L.C., Snyman, E.E., Tomalin, M., Crouch, N.R., Pottas-Bircher, C., 2002. Medicinal and Magical Plants of Southern Africa: An Annotated Checklist. *Strelitzia* 13. National Botanical Institute, Pretoria, pp. 1–2.

Aruna, K., Sivaramakrishnan, V.M., 1996. Anticarcinogenic effects of the essential oils from cumin, poppy and basil. *Phytotherapy Research* 10, 577-580.

Atindehou, K.K., Schmid, C., Brun, R., Koné, M.W., Traore, D. 2004. Antitrypanosomal and antiplasmodial activity of medicinal plants from Côte d'Ivoire. *Journal of Ethnopharmacology* 90, 221-227.

Atsumi, T., Tonosaki, K., 2007. Smelling lavender and rosemary increases free radical scavenging activity and decreases cortisol in saliva. *Psychiatry Research* 150 (1), 89-96.

Babb, D.A., Pemba, L., Seatlanyane, P., Charalambous, S., Curchyard, G., Grand, A.D., 2004. Use of traditional medicine in the era of the antiretroviral therapy; experience from South Africa. *Med Gen Med* 11, 1.

Bakkali, F., Averbeck, S., Averbeck, D., Idaomar, M., 2008. Biological effects of essential oils. A review. *Food and Chemical Toxicology* **46** (2), 46-475.

Baleta, A., 1998. South Africa to bring traditional healers into mainstream medicine. *The Lancet* **352**, 554.

Balint, G.A., 2001. Artemisinin and its derivatives: an important new class of antimalarial agents. *Pharmacology and Therapeutics* **90**: 261-265.

Balunas, M.J., Kinghorn, A.D., 2005. Drug discovery from medicinal plants, *Life Sciences* **78**, 431-441.

Banh-Nuh, C., Gacs-Baitz, E., Radics, L., Tamas, J., Ujszaszt, K., Verzar-Petri, G., 1979. Achillicin, the first proazulene from *Achillea millefolium*. *Phytochemistry*. **18** (2), 331-332.

Bannister, L.H., Hopkins, V.M., Fowler, R.E., Krishna., Mitchell, G.H., 2002. A brief illustrated guide to the ultra structure of *P. falciparum* asexual blood stages. *Parasitology Today* **16** (10), 427-433.

Bell, E.A., Charlwood, B.V., 1980. Secondary plant products. *Encyclopedia of Plant Physiology* **8**, 55-539.

Beiersmann, C., Sanou, A., Wladarsch, E., Allegri, M., Kouyate, B., Muller, O., 2007. Malaria in rural Burkina Faso: local illness concepts, patterns of traditional treatment and influence on health-seeking behaviour. *Malaria Journal* **6**, 106.

Benedek, B., Geisz, N., Jager, W., Thalhammer, T., Kopp, B., 2006. Choloretic effects of yarrow (*Achillea millefolium* s.l.) in the isolated perfused rat liver. *Phytomedicine* **13**, 702-706.

Benedick, B., Kopp, B., Matthias, F., Melzig., 2007. *Achillea millefolium* L. s.l.- Is the anti-inflammatory activity mediated by protease inhibition? *Journal of Ethnopharmacology*. **113**, 312-317.

- Benedek, B., Geisz, N., Jäger, W., Thalhammer, T., Kopp, B., 2006. Choleric effects of yarrow (*Achillea millefolium* s.l.) in the isolated perfused rat liver . *Phytomedicine* 13 (9-10), 702-706.
- Benoit-Vical, F., Njomnang S.P., Sallery, M., Harguem, L., Poupat, C., Nongonierma, R., 2008. Evaluation of Senegalese plants used in malaria treatment: Focus on *Chrozophora senegalensis*. *Journal of Ethnopharmacology* 116, 43-48.
- Berman, J., 2004. Toxicity of commonly-used antimalarial drugs. *Travel Medicine and infectious Diseases* 2: 171-184.
- Bishay, D.W., Attia, A.A., Fayed, M.A., 2002. Flavones and quaternary alkaloid from *Tarchonanthus camphoratus* L. *Bulletin Pharm. Sci. Assiut Univ.* 25 (1), 1-6.
- Blommaert and Bartel., 1976. Chemotaxonomic aspects of the buchu species *Agathosma betulina* (Pillans) and *Agathosma crenulata* (Pillans) from local plantings. *Journal of South Africa Botany* 42, 121-126.
- Bohlke, M., Guinaudeau, H., Angerhofer, C.K., Wongpanich, V., Soejarto, D.D., Fransworth, N.R., 1996. *Journal of Natural Products* 59, 576.
- Bringmann, G., Saeb, W., Assi, L.A., François, G., Sankara Narayanan, A.S., Peters, K., Peters, E.M., 1997. Betulinic acid: isolation from *Triphyophyllum peltatum* and *Ancistrocladus heyneanus*, antimalarial activity, and crystal structure of the benzyl ester. *Planta medica* 63, 255-257.
- Brooks, C.J.W., Draffan, G.H., 1969. Sesquiterpenoids of *Warburgia* species, Ugandensolide and ugsndensidial. *Tetrahedron* 25, 2887-2898.

- Bruce, R. D., 2001. Antioxidants in Australian Floral Honeys–Identification of health-enhancing nutrient components. *Rural Industries Research and Development Corporation 2*, 1-7.
- Bruce-Chwatt, L., Black, R.H., Cnfield, C.J., Clyde, D.F., Peters, W., Wernsdorfer, W.H., 1981. “*Chemotherapy of Malaria*” Eds. 2nd Edition No. 27 p205-210 WHO, Geneva.
- Bruneton, J., 1995. Pharmacognosy, Phytochemistry, Medicinal Plants. Andover, Hampshire. England, Intercept, Ltd. Pp 909.
- Buchbauer, G., Jirovets, L., 1994. Aromatherapy-uses of fragrances and essential oils as medicaments. *Flavour and Fragrance Journal 9*, 217-222.
- Bulletin 175 of the World Organization 2001. *The Lancet 72* (2), 55.
- Campbell, W.E., Davidowitz, B., Jackson, G.E., 1990. Quinolinone alkaloids from an *Agathosma* species. *Phytochemistry 29*, 1303-1306.
- Campbell, W.E., Nair, J.J., Gammon, D.W., Codina, C., Bastida, J., Viladomat, F., Smith, P.J., Albrecht, C.F., 1998. Cytotoxic and antimalarial alkaloids from *Brunsvigia littoralis*. *Planta Medica 64*, 91-93.
- Canonica, L., Corbella, A., Garibaldi, P., Jami, G., Krepinasky, J., Ferrari, G., Casagrande, C., 1967. The structure of cinnamolide, cinnamosmolide and cinnamodial, sesquiterpenes with drimane skeleton from *Cinnamosma fragrans* baillon. *Tetrahedron Letters*, pp 2137.
- Carazz, M., Joasng, A., Franetich, J., Siau, A., Ciceron, L., Hannoun, L., Sauerwein, R., Frappier, F., Rasoanaivo, P., Snounou, G., Mazier, D., 2006. A plant-derived morphinan as a novel lead compound active against malaria liver stages. *PloS Medicine 3* (12), 2392-2402.

Carlton, R.R., Waterman, P.G., Gray, A.L., Deans, S.G., 1992. The antifungal activity of the leaf gland volatile oil of sweet gale (*Myrica gale*) (Myrtaceae). *Chemoecology* **3**, 55-59.

Carvalho, L.H., Brandao, M.G.L., Santos-Filho, D., Lopes, J.L.H., Krettli, A.U., 1991. Antimalarial activity of crude extracts from Brazilian plants studied *in vivo* in *Plasmodium berghei*-infected mice and *in vitro* against *Plasmodium falciparum* in culture. *Brazilian Journal of medical and Biological Research* **24**, 1113-1123.

Cavalcanti, A.M., Baggio, C.H., Freitas, C.S., Rieck, L., de Sousa, R.S., Da Silva-Santos, J.E., Mesia-Vela, S., Marques, M.C., 2006. Safety and antiulcer efficacy studies of *Achillea millefolium* L. After chronic treatment in Wistar rats. *Journal of Ethnopharmacology* **107**, 277-284.

Chan, M.M., Ho, C.T., Huang, H.I., 1995. Effects of three dietary phytochemicals from tea, rosemary, and turmeric on inflammation-induced nitrite production. *Cancer Letters* **96**, 23-29.

Chauhan, P.M.S., Srivastava, S.K., 2001. Present trends and future strategies in chemotherapy of Malaria. *Current Medicinal Chemistry* **8**, 1535-1542.

Chauhan, P.M.S., Kumar, A., Katiyar, B.S., Agarwal, A., 2003. *Perspective in Antimalaria Chemotherapy* **10**, 1137-1150.

Clarkson, C., Chitalu, C., Musonda, Chibale, K., Campbell, W.E., Smith, P., 2003. Synthesis of Totarol amino alcohol derivatives and their antiplasmodial activity and cytotoxicity. *Bioorganic and Medicinal chemistry* **11**, 4417-4422.

Clarkson, C., Maharaj, V.J., Crouch, N.R., Grace, O.M., Pillay, P., Matsabisa, M.G., Bhagwandin, N., Smith, P.J., Folb, P.I., 2004. In vitro antiplasmodial activity of medicinal plants native to or naturalized in South Africa. *Journal of Ethnopharmacology* **92**, 177-191.

Clarkson, C., Madikane, E., Hansen, S., Smith, P., Jaroszewski, J., 2007. HPLC-SPE-NMR Characterization of Sesquiterpenes in an antimycobacterial fraction from *Warbugia salutaris*. *Planta Medica* **73**, 503-614.

Clarkson, C., Campbell, W.E., Smith, P., 2003. *In vitro* antiplasmodial activity of abietane and totarane diterpenes isolated from *Harpagophytum procumbens* (Devil's Claw), *Planta Medica* **8** pp. 720-724.

Coates Palgrave, K., 1977. Trees of southern Africa, edition 2. Struik, Cape Town.

Corbel, V., N'Guessan, R., Brengues, C., Chandre, F., Djogbenou, L., Martin, T., Akogbeto, M., Hougard, J.M., Rowland, M., 2007. Multiple insecticide resistance mechanisms in *Anopheles gambiae* and *Culex quinquefasciatus* from Benin, West Africa. *Acta Tropica* **101** (3), 207-216.

Crouch, N., Symmonds, R., 1999. The traditional value and propagation of *Ornithogalum longibracteatum* (Hyacinthaceae). *Plant Life* **2**, 27-29.

Cunningham, A.B., 1988. An investigation of the herbal medicine trade in Natal/KwaZulu. Investigational Report No. 29, Institution of Natural Resources, University of Natal.

Dalsenter, R.P., Cavalcanti, M.A., Andrade, M.J., Araulo, L.S., Marques, C.M., 2004. Reproductive evaluation of aqueous crude extract of *Achillea millefolium* L. (Asteraceae) in Wistar rats. *Reproductive Toxicology* **18** (6), 819-823.

Delfino, R., Santos-Filho, O., Figueroa-Villar, J., 2002. Molecular modeling of wild-type and antifolate resistant mutant *Plasmodium falciparum* DHFR. *Biophysical chemistry* **98** (3), 287-300.

Della Loggia, R., Sosa, S., Tubaro, A., Kastner, U., Jurentschi, J., 1992. Anti-inflammatory principles from *Achillea asplenifolia* and *Achillea pratensis*. *Planta Medica* **58** (1), 641.

Department of Health (DoH), June 2000. Statistical Notes 2 (17).
<http://www.doh.gov.za/facts/stats-notes/2000>.

Department of Health (DoH) Report., 2001/2002. Malaria pp 45-46.

Department of Health (DoH), December 2003. National Malaria update, South Africa. <http://www.doh.gov.za>.

Department of Health (DoH), 2007. National Malaria cases in South Africa 1999-2007. <http://www.doh.gov.za/facts/index.html>

Desai, M., Kuile, F., Nosten, F., McGready, R., Asamoah, K., Brabin, B., Newman, R.D., 2007. Epidemiology and burden of malaria in pregnancy. *The Lancet* 7(2), 93-104.

De Smet., 1997. The role of plant-derived drugs and herbal medicines in healthcare. *Drugs* 54, 801-840.

Diggens, S.M., Gutteridge, W.E., Trigg, P.I., 1970. An altered dihydrofolate reductase associated with pyrimethamine resistant *P. berghei* produced in a single step. *Nature* 228, 579-580.

Dorn, A., Vippagunta, S.R., Matile, H., Jaquet, C., *et al.*, 1998. An Assessment of Drug-Haematin binding as a mechanism for inhibition of haematin polymerization by Quinoline antimalarials. *Biochemical Pharmacology* 55: 727-736.

Druilhe, P., Moreno, A., Blanc, C., Brasseur, P.H., Jacquier, P., 2001. A colorimetric *in vitro* assay drug sensitivity assay for *Plasmodium* based in a highly sensitive double-site lactate dehydrogenase antigen-capture enzyme-linked immunosorbent assay. *Am. Trop. Med. Hyg.* 64 (5-6), 233-241.

Dubey, N. K., Kumar, R., Tripathi, P., 2004. Global promotion of herbal medicine: India's opportunity. *Current Science* **86** (1) 37-42.

Duncan, A.C., Jaer, A.K., Van Standen, J., 1999. Screening of Zulu medicinal plants for angiotensin converting enzyme (ACE) inhibitors. *Journal of Ethnopharmacology* **68** (1-3), 63-70.

Edinburg, T.L., 1998. Traditional medicines in South Africa. *The Pharmaceutical Journal* **261**, 242-261.

Edingto, G.M., Watson-Williams, E.J., 1965. Sickling, Haemoglobin C, Glucose-6-phosphate dehydrogenase Deficiency and malaria in Western Nigeria, Abnormal Haemoglobins in Africa. *Blackwell Scientific Publications Oxford*, 393-401.

Ekong, R.M., Robson, K.J., Baker, D.A., Warhurst, D.C., 1993. Transcripts of the multidrug resistance genes in chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum*. *Parasitology*, **106** (2), 107-115.

El-Feraly, F.S., McPhail, A.T., Onan, K.D., 1978. *Journal of the Chemical Society, Chemical Communications* **1**, 75-76.

El- Gadi, A., Bshina, S.M., 1989. Usage of some plants in Libyan folk medicine. Part I Dar Alkutob Alwatania. Benghazi.

El-Shafae, A.M., El-Domiaty, M.M., 2001. Improved LC methods for the determination of diosmin and/or hesperidin in plant extracts and pharmaceutical formulations. *Journal of Pharmaceutical and Biomedical analysis*. **26**, 539-545.

English, M.C., Waruiru, C., Lightowler, C., Murphy, S.A., Kirigha, G., Marsh, K., 1996. Hyponatraemia and dehydration in severe malaria. *Archives of Disease in Childhood* **74**, 201-205.

Esterhuysen, C.J., 1996. *Trees of the Year*. Issued by the Forestry Chief Directorate of the Department of Water Affairs and Forestry, Pretoria, South Africa.

Fabrice, F. B., Vincent N., Amvam. Z., Chantal. M., Jean, M.B., Jiri G., Philip J. R., 2003. Composition and anti-plasmodial activities of essential oils from some Cameroonian medicinal plants *Phytochemistry* **64** (7), 1269-1275.

Faleiro, M.L., *et al.*, 2003. Antimicrobial activity of essential oils isolated from Portuguese endemic species of *Thymus*. *Letters in applied Microbiology* **36** (1), 35-40.

Farnert, A., Lindberg, J., Gil, P., Swedberg, G., *et al.*, 2003. Evidence of *Plasmodium falciparum* malaria resistant to atovaquone and proguanil hydrochloride: case reports. *British Medical Journal* **326**: 628-629.

Fennel, C.W., Lindsey, K.L., McGraw, L.J., Sprag, S.G., Stafford, G.I., Elogarashi, E.E., Grace, O.M., Van Staden, J., 2002. Assessing African medicinal plants for efficacy and safety; pharmacological screening and toxicity. *Journal of Ethnopharmacology* **94**, 205-217.

Fischer, D., Li, Y., Ahlemeyer, B., Krieglstein, J., Kissel, T., 2003. *In vitro* cytotoxicity testing of polycations: influence of polymer structure on cell viability and hemolysis. *Biomaterials* **24** (7), 1121-1131.

Fitch, C.D., Cai, G.Z., Chen, Y.F., Shoemaker, J.D., 1999. Involvement of lipids in ferriprotoporphyrin IX polymerization in malaria. *Biochimica et Biophysica Acta* **1454**: 31-37.

Fiume, M.Z., 2001. Final report on the safety assessment of Yarrow (*Achillea millefolium*) extract. *International Journal of Toxicology* **20** (2), 79-84.

Fivelman, Q.L., Butcher, G.A., Adagu, I.S., Warhurst, D. C., Pasvol, G., 2002. Malarone treatment failure and in vitro confirmation of resistance of *Plasmodium falciparum* isolate from Lagos, Nigeria. *Malaria Journal* 1:1.

Folley, M., Tilley, L., 1998. Quinoline antimalarials: mechanisms of action and resistance and prospects for new agents. *Pharmacology and Therapeutics* 1: 55-87.

Foote, S. J., Kyle, D. E., Martin, R. K., et al., 1990. Several alleles of the multidrug-resistance gene are closely linked to chloroquine resistance in *Plasmodium falciparum*. *Nature* 345, 255-258.

Francois, G., Timperman, G., Steenackers, T., Ake Assi, L., Holenz, J., Bringmann, G., 1997. *Parasitology Research* 83, 673.

Frederich, M., Tits, M., Angenot, L., 2008. Potential antimalarial activity of indole alkaloids. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 102, 11-19.

Freireich, E.J., Gehan, E.A., Rall, D.P., Schmidt, L.H., Skipper, H.E., 1966. Quantitative comparison of toxicity of anticancer agents in mouse, rat, hamster, dog, monkey and man. *Cancer Chemotherapy Reports* 50, 219-244.

Gachkar, L., Yadegari, D., Rezaei, M., Taghizadeh, M., Astaneh, S., Rasooli, I., 2007. Chemical and biological characteristics of *Cuminum cyminum* and *Rosmarinus officinalis* essential oils. *Food Chemistry* 102 (3), 898-904.

Gajhede, M., Anthoni, U., Nielsen, P.H., Pedersen, E.J., Christophersen, C.J., 1990. Carnasol, crystal structure, absolute configuration and spectroscopic properties of a diterpene. *Crystallography and Spectroscopy Research* 20, 165-171.

Gauthier, C., Legault, J., Lebrun, M., Dufour, P., Pichette, A., 2006. Glycosidation of lupane-type triterpenoids as potent *in vitro* cytotoxic agents. *Bioorg. Med. Chem.* **14**, 6713-6725.

Georgantelis, D., Ambrosiadis, I., Katikou, P., Blekas, G., Georgakis, A.A., 2007. Effect of rosemary extract, chitosan and α -tocopherol on microbiological parameters and lipid oxidation of fresh pork sausages stored at 4°C. *Meat Science* **76** (1), 172-181.

Gesler, M.C., Nkunya, M.H.H., Mwasumbi, L.B., Heinrich, M., Tanner, M., 1994. Screening Tanzanian medicinal-plants for antimalarial activity. *Acta Tropica* **56**, 65-77.

Gericke, N., Van Wyk, B. E., 2000. People's Plants. A Guide to Useful Plants of Southern Africa. Briza Publications, Pretoria

Gilani, A.H., Rahman, A., 2005. Trends in ethnopharmacology. *Journal of Ethnopharmacology*. **100**, 43-49.

Gilles, H.M., 1967. Glucose-6-phosphate Dehydrogenase Deficiency, sickling and malaria in African children in South Western Nigeria. *Lancet* **1**, 138-140.

Girard, M.P., Reed, Z.H., Friede, M., Kieny, M.P., 2006. A review of human vaccine research and development: *Malaria* . Article in press

Glasl, S., Kastner, U., Jurenitsch, J., Kubelka, W., 1999. Qualitative and quantitative determination of sesquiterpenoids in *Achillea* species by reversed-phase high-performance liquid chromatography, mass-spectrometry and thin-layer chromatography. *Journal of Chromatography B* **729**, 361-368.

Glasl, S., Presser, A., Gunbilig, D., Werner, I., Narantuya, S., Haslinger, E., Jurenitsch, J., Kubelka, W., 2001. Highly hydroxylated quaianolides of *Achillea asistrica* and Middle European *Achillea* species. *Phytochemistry* **58**, 1189-1194.

Goldberg, D.E., Slater, A.F., Cerami, A., Henderson, G.B., 1990. Haemoglobin degradation in the malaria parasite *Plasmodium falciparum*: an ordered process in a unique organelle. *Proc. Natn. Acad. Sci. U.S.A.* **87**, 2931.

Goldblatt., Manning, J., 2000. Cape Plants: A conspectus of the Cape Flora of South Africa, National Botanical Institute of South Africa. Pretoria 2000. South Africa.

Goldman, P., 2001. Herbal medicines today and the roots of modern pharmacology, *Annals of Internal Medicine* **135** pp. 594–600.

Gonzalez A. G., Luis, J. G., Andres, L. S., Mederos, S., 1992. Diterpenes from in vitro-grown *Salvia canariensis*. *Phytochemistry* **31** (9), 3272-3273.

Govaris, A., Floro-Paneri, P., Botsoglou, E., Giannenas, I., Amvrosiadis, I., Botsoglou, N., 2007. The inhibitory potential of feed supplementation with rosemary and/or α -tocopheryl acetate on microbial growth and lipid oxidation of turkey breast during refrigerated storage. *LWT-Food Science and Technology* **40** (2), 331-337.

Grant, R., Thomas, V., 1998. Sappi Treee spotting Kwazulu-Natal. Jacana Publishers. Johannesburg.

Graves, P.M., 1998. Comparisom of the cost effectiveness of vaccines and insecticide impregnation of mosquito nets for the prevention of malaria. *Annual Tropical Medical Parasitology*. **92**, 399-410.

Gregson, A., Plowe, C., 2005. Mechanisms of resistance of malaria parasites to antifolates. *Pharmacological Reviews* **57** (1), 117-145.

Gudaityte, O., Venskutonis, R.P., 2007. Chemotypes of *Achillea millefolium* transferred from 14 different locations in Lithuania to the controlled environment. *Biochemical Systematic and Ecology* 35 (9), 582-592.

Gurib-Fakim, A., 2006. Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular aspects of Medicine* 27, 1-93.

Gutteridge, W.E., Trigg, P.I., 1971. Action of pyrimethamine and related drugs against *Plasmodium knowlesi* in vitro. *Parasitology* 62, 431-444.

Harald, N., Chansuda, W., Walther, H., Wernsdorfer., 2003 Malaria drug-sensitivity testing: new assays, new perspectives. *Trends in Parasitology* 19 (4), 227-232.

Harvey, A.L., 1999. Medicines from nature: are natural products still relevant to drug discovery? *Trends in Pharmacological Sciences* 20: 196-198.

Hearn, J., Rayment, N., Landon, D., Katz, D.R., DE Souza, J.B., 2000. Immunopathology of Cerebral Malaria: Morphological evidence of Parasite sequestration in murine brain microvasculature. *Infection and Immunity* 68 (9), 5364-5376.

Heddini, A., 2002. Malaria pathogenesis: a jigsaw with an increasing number of pieces. *International Journal for Parasitology* 32(13), 1587-1598.

Hein, T.T., White, N.J., 1993. Drug Profiles-Qinghaosu. *The Lancet* 341, 603-608.

Herrera, P. L., 2002. Defining the cell lineages of the islets of langerhans using transgenic mice. *Int. J. Dev. Biol.* 46: 97-103.

Hilliard, O.M., 1977. *Compositae in Natal*. University of Natal Press, Pietermaritzburg, 110-112.

- Ho, C.T., Ferraro, T., Chen, Q., Rosen, R.T., Huang, M.T., 1994. Phytochemicals in tea and rosemary and their cancer-preventive properties. *Food phytochemicals for cancer prevention II. Tea, spices, and herbs, ACS symposium series American Chemical Society* 547, 1–19.
- Hofmann, L., Fritz, D., Nitz, S., Kollamannsberger, H., Drawert, F., 1991. Essential oil composition of three polyploids in the *Achillea millefolium* 'complex'. *Phytochemistry*. 31 (2), 537-542.
- Hollmann, J., Van der Schijff, M., 1996. Portrait of a medicinal tree. *Veld and Flora* 82 pp 115–116
- Horiuchi, K., Shiota, S., Kuroda, T., Hatano, T., Yoshida, T., Tsuchiya, T., Potentiation of Antimicrobial Activity of Aminoglycosides by Carnosol from *Salvia officinalis*. *Biological & Pharmaceutical Bulletin* 30 (2), 287.
- Huang, M.T., Ho, C.T., Wang, Z.Y., Ferraro, T., Lou, Y.R., Stauber, K., Ma, W., Georgiadis, C., Laskin, J.D., Conney, A.H., 1994. Inhibition of skin tumorigenesis by rosemary and its constituents carnosol and ursolic acid. *Cancer Research* 54, 701–708.
- Hutagalung, R., Wilairatana, P., Looreeesu-wan, S., Brttenham, G.M., Aikawa, M., Gordeuk, V.R., 1999. Influence of Haemoglobin E trait on severity of *falciparum* malaria. *Journal of infectious diseases* 179 (1), 283-286.
- Hutchings, A., Scott, A.H., Lewis, G., Cunningham, A., 1996. Zulu Medicinal Plants. University of Natal Press, Pietermaritzburg.
- Hyde, J.E., 2002. Mechanism of resistance of *Plasmodium falciparum* to antimalarial drugs. *Microbes and Infection* 4 (2): 165-174.

Idro, D.R., Jenkins, N.E., Newton, C.R., 2005. Pathogenesis, clinical features, and neurological outcome of cerebral malaria. *The Lancet* 4 (12), 827-840.

Inatani, R., Nakatani, N., Fuwa, H., 1983. Antioxidant effect of constituents of rosemary (*Rosmarinus officinalis* L) and the derivatives. *Agricultural Biological Chemistry* 47, 521-528.

Ishih, A., Ikeya, C., Yanoh, M., Takezoe, H., Miyase, T., Terada, M., 2000. A potent antimalarial activity of *Hydrangea macrophylla* var. *Otaksa* leaf extract against *Plasmodium yoelii* 17XL in mice. *Parasitology International* 50, 33-39.

Jansen, B.M.J., de Groot, A., 1991. The occurrence and biological activity of drimane sesquiterpenoids. *Natural Products Reports* 8, 309-318.

Jaric, S., Popovic, Z., et al., 2007. An ethnobotanical study on the usage of wild medicinal herbs from Kopaonik Mountain (Central Serbia). *Journal of Ethnopharmacology* 111 (1), 160-175.

Jazet Dongmo, P.M., Kuate, J., Fekam Boyom, F., Ducleier, D., Damesse, F., Amvam Zollo, H.P., Menut, C., Bessiere, J.M., 2002. Composition chimique et activité antifongique *in vitro* des huiles essentielles de Citrus sur la croissance mycélienne de *Phaeoramularia angolensis*. *Fruits* 57, 98-104.

Jefford, C.W., 2001. *Current Medicinal Chemistry* 8, 1803-1826.

Jennings, W., Shibamoto, T., 1980. Qualitative Analysis of Flavor and Fragrance Volatiles by Glass Capillary Gas Chromatography. Academic Press Rapid Manuscript. Harcourt Brace Jovanovich Publishers. New York.

John, E., Hyde., 2005. Exploring the folate pathway in *Plasmodium falciparum*. *Acta Tropica* 94 (3), 191-206.

Joubert, P.H., Mathibe, L., 1989. Acute poisoning in developing countries. *Adverse Drug Reactions and Acute Poisoning Reviews* 8, 165-178.

Jovanoiv, Z., Kostic, M., Popovic, Z., 2007. Grain-protective properties of herbal extracts against the bean weevil *Acanthoscelides obtectus* Say. *Industrial Crops and Products*. **26** (1), 100-104.

Kamatou, G.P.P., Viljoen, A.M., ono-Bwalya, A.B., van Zyl, R.L., van Vuuren, S.F., Lourens, A.C.U, Baser, K.H.C., Demirci, B., Lindsey, K.L., van Staden, J, Steenkamp, P., 2005. The *in vitro* pharmacological activities and a chemical investigation of three South African *Salvia* species. *Journal of Ethnopharmacology* **102**, 382-390.

Kamatou, G.P.P., Van Zyl, R.L., Davids, H., Van Heerden, F.F., Lourens, A.C.U., Viljoen, A.M., 2008. Antimalarial and anticancer activities of selected South African *Salvia* species and isolated compounds from *S. radula*. *South African Journal of Botany* **74**, 238-243.

Kastner, U., Sosa, S., Tubaro, A., Breuer, J., Rücker, G., Della Loggia, R., Jurenitsch, J., 1993. Anti-edematous activity of sesquiterpene lactones from different taxa of the *Achillea millefolium* group. *Planta Medica* **59** (Suppl.), 669.

Kehua, P., Yunjie, W., Zhanglan, Q., 2001. Synthesis and fungicidal activity of 5,7-dihydroxyflavanone derivatives. *Nongyaoxue Xuebao* **3**(1), 76-78.

Kirby, G.C., 1997. Plants as sources of antimalarial drugs. *Tropical Doctor* **27**, 7-11.

Khan, S.M., Waters, P.A., 2004. Malaria parasite transmission stages: an update. *Trends in Parasitology* **20** (12), 575-580.

Klayman, D.L., 1985. Qinghaosu (artemisinin), an antimalarial drug from China. *Science* **228**, 1049-1055.

Kinghorn, A.D., 2001. Pharmacognosy in the 21st century, *Journal of Pharmacy and Pharmacology* **53** pp. 135–148.

Kioy, D., Gray, A.I., Waterman, P.G., 1990. A comparative study of the stem bark trimane sesquiterpenes and leaf volatile oils of *Warbugia ugandensis* and *W. stuhlmannii*. *Phytochemistry* **29**, 3535-3538.

Klayman, D.L., 1985. Qinghaosu (Artemisinin): an antimalarial drug from China. *Science* **228**: 1049-1055.

Knight, D.J., Peters, W., 1980. The antimalarial action of *N*-benzyloxy-dihydrotriazines. The action of Clociguanil (BRL50216) against rodent malaria and studies on its mode of action. *Annals of Tropical Medicine and Parasitology* **74**, 393-404.

Krogstad, D.J., Gluzman, Y., Kyle, D.E., Odula, A.M., Martin, S.K., Milhous, W.K., Schlesinger, P.H., 1987. Efflux of chloroquine from *Plasmodium falciparum*: mechanism of chloroquine resistance. *Science* **238**, 1283.

Kubelka, W., Kastner, U., Glasl, S., Saukel, J., Jurenitsch, J., 1999. Chemotaxonomic relevance of sesquiterpenes within the *Achillea millefolium* group. *Biochemical systematics and Ecology* **27**, 437-444.

Kubo, I., Lee, Y-W., Pettei, M., Pilkiewicz, F., Nakanishi, K., 1976. Potent army worm antifeedants from the East African *Warburgia* plants. *Journal of the Chemical Society, Chemical Communications* **24**, 1013-1014.

Kubo, I., Miura, I., Lee, Y-W., Pettei, M., Pilkiewicz, F., Nakanishi, K., 1977. Muzigadial and warburganal, potent antifungal, anti-yeast and African worm antifeedant agents. *Tetrahedron Letters* **52**, 4553-4556.

Kubo, I., Matsomoto, T., Kakooko, A.B., Mubiru, N.K., 1983. Structure of mukaadial, a molluscicide from *Warburgia* plants. *Chemistry Letters* **7**, 979-980.

Kubo, I., Taniguchi, M., 1988. Polygodial, an antifungal potentiator. *Journal of Natural Products* **51**, 22-29.

Kumar, M.A., Nair, M., Hema, P.S., Mohan, J., Santhoshkumar, T.R., 2006. Pinocembrin triggers Bax-dependent mitochondrial apoptosis in colon cancer cells. *Molecular Carcinogenesis*. 46(3), 231-241.

Lancet Editorial., 1994. Antimalarial Drug Development, great potential few funds. *Lancet* 343 (891), 1513-1515.

Lans, C., Tuener, N., Khan, T., Brauer, G., 2007. Ethnoveterinary medicines used to treat endoparasites and stomach problems in pigs and pets in British Columbia, Canada. *Veterinary Parasitology* 148 (3-4), 325-340.

Lee, J., Jin, Y., Hong, J., Kim, T., Chug, J., Yun, Y., 2007. Antiplatelet activity of carnosol is mediated by the inhibition of TZA2 receptor and cytosolic calcium mobilization. *Vascular Pharmacology* 45 (3), 148-153.

Legros, F., Bouchaud, O., Ancelle, T., Arnaud, A., Cojean, S., Le Bras, J., Danis, M., Fontanet, A., Durand, R., 2007. Risk factors for imported fatal *Plasmodium falciparum* malaria, France 1996-2003. *Emerging infectious disease*. www.cdc.gov/eld. 13 (6), 883-888.

Lemmens-Gruber, R., Marchart, E., Rawnduzi, P., Engel, N., Benedek, B., Kopp, B., 2006. Investigation of the spasmolytic activity of the flavonoid fraction of *Achillea millefolium* s.l on isolated guinea-pig ilea. *Drug Research* 56, 582-588.

Lewis, W.W.H., Elvin-Lewis, M.P.F, 1997. *Medical Botany*. Wiley, New York.

Lewu, F.B., Adebola, P.O., Afolayan, A.J., 2007. Commercial harvesting of *Pelargonium sidoides* in the Eastern Cape, South Africa: String a balance between resource conservation and rural livelihoods. *Journal of Arid Environments* 70, 380-388.

Li, X., and Rieckmann, K., 1992. A biassay for derivatives of qinghaosu (artemisinin). *Tropical Medicine and Parasitology* 43: 195-196.

Li, F., Xiong, Z., Lu, X., Win, F., Li, X., 2006. Strategy of quality control for traditional Chinese medicines and chromatographic technology, *Chinese Journal of Chromatography* **24**, 537–544.

Lipinski, C., Lombardo, F., Dominy, B., Feeney, P., 1997. Experimental and computational approaches to estimate solubility and permeability in drug discovery and development settings. *Adv. Drug Deliv. Rev.* **23**, 3–25.

Lopes, N.P., Kato, M.J., Andrade, E.H.A., Maia, J.G.S., Yoshida, M., Planchart, A.R., Kartz, A.M., 1999. Antimalarial use of volatile oil from leaves of *Virola surinamensis* (Rol) Warb. By Waiãpi Amazon Indians. *Journal of Ethnopharmacology* **67**, 313-319.

MacPherson, G.G., Warrell, M.J., White, S., Looareesuwan, S., Warrell, D.A., 1985. Human cerebral malaria: a quantitative ultra structural analysis of parasitized erythrocyte sequestration. *American Journal of Pathology* **119**, 385-401.

Madikane, V., Bhakta, S., Russell, A. J., Campbell, W. E., Claridge, T.W., Elisha, B. G., Davies, S. G., Smith, P., Sim, E., 2006. Inhibition of mycobacterial arylamine *N*-acetyltransferase contributes to anti-mycobacterial activity of *Warburgia salutaris*. *Bioorganic & Medicinal Chemistry* **15** (10), 3579-3586.

Makler, M.T., Hinrichs, D.J., 1993. Measurement of the lactate dehydrogenase activity of *Plasmodium falciparum* as an assessment of parasitemia. *American Journal of Tropical Medicine and Hygiene* **48** (2), 205-210.

Makler, M.T., Ries, J.M., Williams, J.A., Bancroft, J.E., Piper, R.C., Gibbins, B.L., Hinrichs, D.J., 1993. Parasite lactate dehydrogenase as an assay for *Plasmodium falciparum* drug sensitivity. *American Journal of Trop. Med. Hyg* **48** (6), 739-741.

Malaria Research Programme of the Medical Research Council 2001, South Africa. Malaria in Southern Africa. <http://www.malaria.org.za>.

Mander, M., 1999. Marketing of indigenous medicinal plants in South Africa. A case study in KwaZulu-Natal. *FAO of the UN*, Rome.

Mander, W., Mander, J., Crouch, N., McKean, S. and Nichols, G., 1995. *Catchment Action: Growing and Knowing Muthi Plants*, Institute of Natural Resources, Scottsville, South Africa ShareNet Booklet.

Manguro, L.O.A., Ugi, I., Hermann, R., Lemmen, P., 2003. Flavonol and drimane-type sesquiterpene glycosides of *Warburgia stuhlmannii* leaves, *Phytochemistry* **63** (2003), pp. 497–502.

Mashimbye, M.J., Drewes, S.E., Appleton, C.C., Cunningham, A.B., 1992. Observations on the molluscicidal properties of (-)-warburganal on the South African *Bulinus africanus* (Planorbidae). *Journal of Medical and Applied Malacology* **4**, 37-40.

Mashimbye, M.J., 1993. PhD thesis, University of Natal. South Africa.

Mashimbye, M.J., Maumela, M.C., Drewes, S.E., 1999. A drimane sesquiterpinoid lactone from *Warburgia salutaris*. *Phytochemistry* **51**, 435-438.

Masotti, V., Juteau, F., Bassiere, J.M., Viano, J., 2003. Seasonal and phenological variations of the essential oil from narrow endemic species *Artemisia molinieri* and biological activities. *Journal of Agric. Food. Chem.* **51** (24), 7115-21.

Martin, S.K., Oduola, A.M., Milhous, W.K., 1987. *Science* **235**, 899-901.

Martin, S.K., 1994. The malaria glucose-6-phosphate hypothesis revisited. *Parasitology Today* **10** (2), 251-252.

Mazier, D., Franetich, J.F., Carraz, M., Silvie, O., Pino, P., 2004. Models for studying the effects of herbal antimalarials at different stages of the *Plasmodium*

life cycle. Traditional medicines for modern times. Boca Raton (Florida): GRG Press, 271-278.

Ménard R., 2000. The journey of the malaria sporozoite through its hosts: two parasite proteins lead the way. *Microbes and Infection* 2 (6), 633-642.

Meshnick, S.R., Taylor, T.E., Kamchonwongpaisan, S., 1996. Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbiological Reviews* 60 (2): 301-315.

Mitich, L. W., 1990. Yarrow the herb of Achilles. *Weed Technology* 4, 451-453.

Moolla, A., 2006. A phytochemical and pharmacological investigation of indigenous *Agathosma* species. MSc dissertation, University of the Witwatersrand. South Africa.

Miller, L.H., Mason, S.J., Clyde, D.F., McGinniss, M.H., 1976. The resistance factor of *Plasmodium vivax* in blacks. *The New England Journal of Medicine* 295 (6), 302-304.

Mims, C., Dockrell, H.M., Goering, R.V., Roitt, I., Goering, V., 2004. Medical Microbiology, 3rd edition, Mosby Co. Publishers. pp 201-237.

Mital, A., 2007. Recent advances in Antimalarial compounds and their patents. *Current Medical Chemistry* 14, 759-773.

Mockutė, D., Judžentienė, A., 2002. Chemical composition of the essential oils of *Achillea millefolium* L. ass millefolium(yarrow) growing wild in Vilnius. *Chemija*. 13 (2), 97-102.

Montanari, T., Ernesto de Carvalho, J., Dolder, H., 1998. Antispermatic effect of *Achillea millefolium* L. in mice. *Contraception*. 58 (5), 309-313.

Moore, S.J., Lenglet, A., Hill, N., 2002. Field evaluation of three plant-based insect repellents against malaria vectors in Vaca Diez Province, the Bolivian Amazon. *Journal of American Mosquito Control Association* **18**, 107-110.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods* **65**, 55-63.

Moujir, L., Navarro, G.A., Andres, L.S., Luis, J.G., 1993. Antiseptic and anti-inflammatory activity. *Phytochemistry* **34**, 1493-1495.

Mueller, I., Zimmerman, P.A., Reeder, J.C., 2007. *Plasmodium malariae* and *Plasmodium ovale*- the 'bashful' malaria parasites. *Trends in Parasitology* **23** (6), 278-283.

Murnigsih, T., Subeki., Matsuura, H., Takahashi, K., Yamasaki, M., Yamato, O., Maede, Y., Katakura, K., Suzuku, M., Kobayashi, C., Yoshihara, T., 2005. Evaluation of the inhibitory activities of the extracts of Indonesian Traditional Medicinal Plants against *Plasmodium falciparum* and *Babesia gibsoni*. *Journal of Veterinary Medicinal Science* **67** (8), 829-831.

Mwangi, J.W., Achola, K.J. *et al.* 1994. Volatile constituents of the essential oil of *Tarchonanthus camphoratus* L. *Journal of Essential Oil Research* **6**, 183-185.

Narasimhan, V., Attaran, A., 2003. Roll Back Malaria? The scarcity of international aid for malaria control. *Malaria Journal* **2**, 8-12.

Ndifor, A.M., Howells, R.E., Ward, S.A., 1992. Chloroquine sensitivity of *Plasmodium falciparum* *in vivo* in Savannah town in Cameroon. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **86**, 479-482.

Newman, D.J., Cragg, G.M., 2007. Natural products as sources of new drugs over the last 25 years, *Journal of Natural Products* **70** pp. 461-477.

NRF, 2003. People's Science Voice. NRF newsletter, April-May issue.
<http://www.nrf.ac.za/focusareas/iks/>.

Nundkumar, N., Ojewale, J.A.O., 2002. Studies on the antiplasmodial properties of some South African medicinal plants used as antimalarial remedies in Zulu folk medicine. *Methods and Findings in Experimental and Clinical Pharmacology* 24 (7), 397-401.

Nzila, A.M., Nduati, E., Mberu, E.K., 2001. Molecular evidence of greater selective pressure for drug resistance exerted by the long-acting antifolate pyrimethamine/sulphadoxine compared with the shorter-acting chlorproguanil/dapsone on Kenyan *Plasmodium falciparum*. *Journal of Infectious Diseases* 181: 2023-2028.

Odugbemi, T.O., Akinsulire, R.O., Aibinu, E.I., Fabeku, P.O., 2007. Medicinal plants useful for malaria therapy in Okeigbo, Ondo State, Southwest Nigeria. *African Journal of Traditional, Complementary and Alternative Medicines* 4 (2), 191-198.

Offord, E.A., Mace', K., Ruffieux, C., Malnoe, A., Pfeifer, A.M., 1995. Rosemary components inhibit benzo[a]pyrene-induced genotoxicity in human bronchial cells. *Carcinogenesis* 16, 2057-2062.

Okokon, E.J., Ita, N.B., Udokpoh, E.A., 2006. Antiplasmodial activity of *Cylicodiscus gabunensis*. *Journal of Ethnopharmacology* 107, 175-178.

Okokon, E.J., Udokpoh, E.A., Antia, S.B., 2007. Antimalaria activity of ethanolic extract of *Tetrapleura tetraptera* fruit. *Journal of Ethnopharmacology* 111, 537-540.

Olliaro, P., Nevill, C., Lebras, J., Ringwald, P., Mussano, P., Garner, P., Brasseur, P., 1996. Systematic review of amodiaquine treatment in uncomplicated malaria. *Lancet* 348, 1196.

Olliaro, P., Yuthavong, Y., 1999. Chemotherapeutic target for antimalarial drug discovery. *Pharmacology and Therapeutics* **81**, 91-110.

Olumese, P., 2006. Antimalarial treatment policies for *P. falciparum* and *P. vivax* by country in WHO Africa and Eastern Mediterranean region [June 2006 Update].

O'Neill, P.M., Pugh, M., Davies, J., Ward, S.A., Park, B.K., 2001. Regioselective hydroperoxysilylation of 2-alkyl- or 2-aryl-prop-2-en-1-ols: application to a new synthesis of 1,2,4-trioxanes. *Tetrahedron Letters* **42** (27): 4569-4571.

Osamu, K., Sandrine, C., Soubes, L., H. Miller., 1999. *Plasmodium falciparum*: Invasion of *Aotus* Monkey Red Blood Cells and Adaptation to *Aotus* Monkeys *Experimental Parasitology* **93** (2), 116-119.

Palmer, E., Pitman, N., 1973. Trees of southern Africa 3. Balkema, Cape Town.

Pandey, A.V., Babbarwal, V.K., Okoyeh, J.N., Joshi, R.M., *et al.*, 2003. Hemozoin formation in malaria: a two-step process involving histidine-rich proteins and lipids. *Biochemical and Biophysical Research Communications* **308**: 736-743.

Perez, H.A., De La Rosa, M., Apitz, R., 1994. *Antimicrobial Agents Chemotherapy* **38**, 337.

Peter. W., 1975. The chemotherapy of rodent malaria, XXII. The value of drug-resistant strains of *P. berghei* in screening for blood schizontocidal activity. *Ann Trop Med Parasitol* **69**:155-171.

Peters, W., 1998. Drug resistance in malaria parasites of animals and man. *Advances in Parasitology* **41**, 1-4.

Peters, W., 1970. *Chemotherapy and Drug Resistance in Malaria*. Academic Press, New York, pp 876.

- Peters, W., Portus, J.H., Robinson, B.L., 1975. The chemotherapy of rodent malaria XX11. The value of drug resistance strains of *Plasmodium berghei* in screening for blood schizonticidal activity, *Ann. Trop. Med. Parasitol.* **69** pp. 155–165.
- Philippe, G., De Mol, P., Angenot, L., Tits, M., Frederich, M., 2007. *In vivo* antimalarial activity of isosungucine, an inolomonoterpenic alkaloid from *Strychnos icaja*. *Planta Medica* **73**, 478-479.
- Piccaglia, M., Marotti, E., Giovanelli, E., Deans, S.G., Eaglesham, E., 1993. Antibacterial and antioxidant properties of Mediterranean aromatic plants. *Industrial crops and Products* **2**, 47-50.
- Pillans, N., 1950. A revision of the genus *Agathosma* (Rutaceae). *Journal of South Africa Botany* **16**, 55-117.
- Pillay, P., Vlegaar, R., Maharaj, V.J., Smith, P.J., Lategan, C.A., Chouteau, F., Chibale, K., 2007a. Antiplasmodial hirsutinolides from *Vernonia staehelinoides* and their utilisation towards a simplified pharmacophore. *Phytochemistry* **68**, 1200-1205.
- Pillay, P., Vlegaar, R., Maharaj, V.J., Smith, P.J., Lategan, C.A., 2007b. Isolation and identification of antiplasmodial sesquiterpene lactones from *Oncosiphon piluliferum*. *Journal of Ethnopharmacology* **112**, 71-76.
- Pinzon-Charry, A., Vivian, A., McPhun, V., Wykes, M., Good, M.F., 2006. Malaria vaccines: New hope in old ideas. *Drug Discovery Today* **3** (2), 167-172.
- Pisha, E., Chai, H., Ik-Soo Lee, T.E., Chagwedera, N.R., Farnsworth, G.A., Cordell, C.W., Beecher, W., Fong, H.H.S., Kinghorn, A.D., Brown, D.M., Wani, M.C., Wall, M.E., Hieken, T.J., Das Gupta, T.K., Pezzuto, J.M., 1995.

Discovery of betulinic acid as a selective inhibitor of human melanoma that functions by induction of apoptosis. *Nature Medicine* 1 (10), 1046-1051 .

Posthumus, M.A., 1996. Chemical composition of the essential oils of *Agathosma betulina*, *A. crenulata* and *A. betulina A. crenulata* hybrid (buchu). *Journal of Essential oils Research* 8, 223-228.

Prescott, L.M., 1993. Microbiology, second edition. Dubugue; Wm.C. Brown, pp 793-795.

Prozesky, E.A., Meyer, J.J.M, Louw, A.I., 2001. *In vitro* antiplasmodial activity and cytotoxicity of ethnobotanically selected South African plants. *Journal of Ethnopharmacology* 76, 239-245.

Quan, L.T., Yasuhiro, T., Jun-ya, U., Nhan T.N., Yukiko, M., Khurshida, B-., Hye-Sook, K., Yusuke, W., Qui, K.T., Shigetoshi, K., 2003. *In vitro* antiplasmodial activity of malaria medicinal plants used in Vietnamese traditional medicine. *Journal of Ethnopharmacology*, 85 (2-3), 231-235.

Rabe, T., van Staden, J., 1996. Antibacterial activity of South African plants used for medicinal purposes. *Journal of Ethnopharmacology* 56, 81-87.

Rabe, T., van Staden, J., 2000. Isolation of an antibacterial sesquiterpenoid from *Warburgia salutaris*. *Journal of Ethnopharmacology* 73, 171-174.

Ramanitrahasimbola, D., Rasoanaivo, P., Ratsimamanga, S., Vial, H., 2006. Malagashanine potentiates chloroquine antimalarial activity in drug resistant *Plasmodium malaria* by modifying both its efflux and influx. *Molecular and Biochemical Parasitology* 146 (1), 58-67.

Raskin, I., Ribnicky, D.M., Komarnytsky, S., Ilic, N., Poulev, A., Borisjuk, N., Brinker, A., Moreno, D.A., Ripoll, C., Yakoby, N., O'Neal, J.M., Cornwell, T.,

Pastor, I., Fridlender, B., 2002. Plants and human health in the twenty-first century, *Trends in Biotechnology* **20** pp. 522–531.

Reed, M.B., Saliba, K.J., Caruana, S.R., Kirk, K., Cowman, A.F., 2000. Pgh1 modulates sensitivity and resistance to multiple antimalarials in *Plasmodium falciparum*. *Nature* **403**: 906-909.

Ridley, R.G., 2002. Medical need, scientific opportunity and the drive for antimalarial drugs. *Nature* **415**, 686-693.

Ridley, R.G., Matile, H., Jaquet, C., Dorn, A., Hofheinz, W., Leupin, W., Peters, W., *et al.*, 1997. Antimalarial activity of the bisquinoline trans-N1, N2-bis (7-chloroquinolin-4-yl) cyclohexane-1,2-diamine: comparison of two stereoisomers and detailed evaluation of the S,S enantiomer, Ro 47-7737. *Antimicrob Agents Chemother* **41**(3), 677–686.

Rodríguez-García, R., Jasso de Rodríguez, D., Gil-Marín, J.A., Angulo-Sánchez, L., Lira-Saldivar, R.H., 2007. Growth, stomata resistance, and transpiration of *Aloe vera* under different soil water potentials. *Industrial Crops and Products* **25**(2), 123-128.

Rooth, I., Bjorkmna, A., 1992. Fever episodes in holoendemic malaria area of Tanzania. Parasitological and clinical finding and diagnostic aspects related to malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **86**, 479-482.

Rücker, G., Walter, R.D., Manns, D., Mayer, R., 1991. *Planta Medica* **57**, 295.

Rukungu, G.M., Murgi, F.W., Tolo, F.M., Omar, S.A., Mwitari, P., *et al.*, 2007. The antiplasmodial activity of spermine alkaloids isolated from *Albizia gummifera*. *Fitoterapia* **78**, 455-459.

Ryley, J.F., Peters, W., 1970. The antimalarial activity of some quinolone esters. *Annals of Tropical Medicine and Parasitology* **84**, 209-222.

Saidu, K., Onah, J., Orisadipe, A., Olusola, A., Wambebe, C., Gamaniel, K., 2000. Antiplasmodial, analgesic, and anti-inflammatory activities of the aqueous extract of the stem bark of *Erythrina senegalensis*. *Journal of Ethnopharmacology* **71**, 275-280.

Santoro, G.F., Cardoso, M.G., Guimarães, L.L., Mendonça, L.Z., Soares, M.J., 2007. Trypanosoma cruzi: Activity of essential oils from *Achillea millefolium* L., *Syzygium aromaticum* L. and *Ocimum basilicum* L. on epimastigotes and trypomastigotes. *Experimental Parasitology* **116** (3), 283-290.

Saxena, S., Pant, N., Jain, D.C., Bhakuni, R.S., 2003. Antimalarial agents from plant sources. *Current Sciences* **85** (9), 1314-1329.

Schellenberg, D., Menendez, C., Aponte, J.J., Kahigwa, E., Tanner, M., Mshinda, H., Alonso, P., *et al.*, 2005. Intermittent preventive antimalarial treatment for Tanzanian infants: follow-up to age 2 years of a randomized, placebo-controlled trial. *Lancet* **365**, 1481-1483.

Schellenberg, K.A., Coatney, G.R., 1961. The influence of antimalarial drugs on nucleic acid synthesis in *Plasmodium gallinaceum* and *Plasmodium berghei*. *Biochem Pharmacol* **6**, 143-152.

Schmidt, M.L., Kuzmanoff, K.L., Ling-Indeck, L., Pezzuto J.M., 1997. Betulinic acid induces apoptosis in human neuroblastoma cell lines. *European Journal of Cancer* **33** (12), 7-10.

Schroder, H., Kastner, U., Gargula, K., Budesinsky, M., Haslinger, E., Jurenitsch, J., Kubelka, W., 1994. Artabsin and 3-oxa-artabsin derivatives from *Achillea millefolium* species. *Phytochemistry* **36** (6), 1449-1451.

Schuster, B.G., Milhous, W.K., 1993. Reduced resources applied to antimalarial drug development. *Parasitology Today* 9 (5), 167-174.

Schwikkard, S., van Heerden, R.R., 2002. Antimalarial activity of plant metabolites. *The Royal Society of Chemistry* 19, 675-692.

Selzer, E., Pimentel, E., Wacheck, V., Schlegel, W., Pehamberger, H., Jansen, B., Kodym, R., 2000. Effects of betulinic acid alone and in combination with irradiation in human melanoma cells. *Journal of Invest Dermatol* 114 (5), 935-940.

Sharp, B.L, Malaria update 1993, Medicines information center, pp: 1-6.

Sharma, S., 1990. *Prog. Drug. Res* 35, 365-485.

Sidhu, A.B., Verdier-Pinard, D., Fidock, D.A., 2002. Chloroquine resistance in *Plasmodium falciparum* malaria parasites conferred by pfcrt mutations. *Science* 298: 210-213.

Simmonds, M., 2003. Novel drugs from botanical sources, *Drug Discovery Today* 8 pp. 721-722.

Simpson, D., 1998. Buchu-South Africa's amazing herbal remedy, *Scottish Medical Journal* 43, 189-191.

Singletary, K., MacDonald, C., Wallig, M., 1996. Inhibition by rosemary and carnosol of 7,12-dimethylbenz[a]anthracene (DMBA)-induced rat mammary tumorigenesis and *in vivo* DMBA-DNA adduct formation. *Cancer Letter* 104, 43-48.

Skinner, T. S., Manning, L.S., Johnston, W.A., Davis, T.M.E., 1996. *In vitro* stage-specificity of *Plasmodium falciparum* to quinine and artemisinin drugs. *International Journal for Parasitology* 26: 519-525.

Slatter, A.F., Cerami A., 1992. Inhibition by chloroquine of a novel haem polymerase enzyme activity in malaria trophozoites. *Nature* **355**, 167-169.

Soe-Soe., Khin-Saw-Aye., Htay-Aung., Nay-Win., Tin-Aung., Than-Swe., Roussilhon, C., Pérignon, J., Druilhe, P., 2001. Premunition against *Plasmodium falciparum* in a malaria hyperendemic village in Myanmar. *Transaction of the Royal Society of Tropical Medicine and Hygiene* **95** (1), 81-84.

Sohn, H. Y., Son, K.H., Kwon, C. S., Kwon, G. S., Kang, S.S., 2004. Antimicrobial and cytotoxic activity of 18 prenylated flavonoids isolated from medicinal plants: *Morus alba* L., *Morus mongolica* Schneider, *Broussonetia papyrifera* (L.) Vent, *Sophora flavescens* Ait and *Echinosophora koreensis* Nakai. *Phytomedicine* **11** (7-8), 666-672.

Soyal, D., Jindal, A., Singh, I., Goyal, P.K., 2007. Modulation of radiation-induced biochemical alterations in mice by rosemary (*Rosemarinus officinalis*) extract. *Phytomedicine* **14** (10), 701-705.

Stander S., 2003. Natural medicine Buchu. *The SA Journal of Natural Medicine*. <http://www.naturalmedicine.co.za>

Spreeth A.D., 1976. A revision of the commercially important *Agathosma* species. *Journal of South African Botanists*. **42**, 109-119.

Snounou, G., Gruner, A.C., Muller-Gra, C.D., Mazier, D., Renia, L., 2005. The *Plasmodium* sporozoite survives RTS,S vaccination. *Trends in Parasitology* **21**, 456-461.

Steele, J. C. P., Warhurst, D. C., Kirby, G. C., Simmonds, M. S. J., 1999. *In vitro* and *In vivo* evaluation of betulinic acid as an antimalarial. *Phytotherapy Research* **13** (2), 115 – 119.

Subbarao, S.K., 1988. The *Anopheles culicifacies* complex and control of malaria. *Parasitology Today* 4 (3), 72-75.

Takecki, M., Matsuo, M., Ziba, C., *et al.*, 2001. Therapeutic efficacy of sulphadoxine/pyrimethamine and susceptibility in vitro of *Plasmodium falciparum* isolates to sulphadoxine-pyrimethamine and other antimalarial drugs in Malawian children. *Tropical Medicine and International Health* 6: 429-434.

Talisuna, A.O., Langi, P., Bakyaite, N., *et al.*, 2002. Intensity of malaria transmission, antimalaria-drug use and resistance in Uganda: what is the relationship between these three factors? *Transactions of the Royal Society of Tropical Medicine and Hygiene* 96: 310-317.

Tan, Y., Yu, R., Pezzuto, J.M., 2003. Betulinic acid-induced programmed cell death in human melanoma cells involves mitogen-activated protein kinase activation. *Clinical Cancer Research* 9 (7), 2866-75.

Tamura, J., Waki, S., 1986. Assessment of the *in vitro* growth of *Plasmodium falciparum* using fluorometry. *Parasitology* 72 (5), 595-597.

Taylor, R.L., Towers, G.H.N., 1998. Antibacterial constituents of the Nepalese medicinal herb, *Centipeda minima*. *Phytochemistry* 47 (4): 631-634.

Thaithong, S., Beale, G.H., 1981. Resistance of 10 Thai isolates of *Plasmodium falciparum* to chloroquine and pyrimethamine by *in vitro* tests. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 75, 271-273.

Thomson, J.G., 1933. Immunity in malaria. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 26 (6), 483-514.

Thring, T.S., Weitz, F.M., 2006. Medicinal plant use in the Bredasdorp/Elim region of the Southern Overberg in the Western Cape Province of South Africa. *Journal of Ethnopharmacology* **103**, 261-275.

Tona, L., Mesia, K., Ngimbi, N.P., Chrimwami, B., Okond'ahoka, K. Cimanga, T. De Bruyne, S. Apers, Hermans, N., Totte, J., Peters, L., Vlietinck, A.J., 2001. *In vivo* antimalarial activity of *Cassia occidentalis*, *Morinda morindoides* and *Phyllanthus niruri*, *Ann. Trop. Med. Parasitol.* **95**, 47-57.

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

Trape, J.F., 2002. The public health impact of chloroquine resistance in Africa. *American Journal of Tropical Medicine and Hygiene* **64** (suppl.): 12-17.

Tran, Q.L., Tezuka, Y., Ueda, J., Nguyen, N.T., *et al.*, 2003. *In vitro* antiplasmodial activity of antimalarial medicinal plants used in Vietnamese traditional medicine. *Journal of Ethnopharmacology* **86**: 249-252.

Treurnicht, F.T., 1997. An evaluation of the toxic and potential antiviral effects of some plants used by South Africans for medicinal purposes. MSc thesis, University of Stellenbosch.

Valentin, A., Pelissier, Y., Benoit, F., Marion, C., Kone, D., Mallie, M., Bastide, J.M., Bessiere, J.M., 1995. Composition and antimalarial activity *in vitro* of volatile components of *Lippia multiflora*. *Phytochemistry* **40**, 1439-1442.

Van Agtmael, M.A., Eggelte, T.A., van Boxlel, C.J., 1999. Artemisinin drugs in the treatment of malaria: from medicinal herb to registered medication. *Trend in Pharmacological Science* **20**, 199-205.

Van Wyk, B.E., 2002. A review of ethnobotanical research in Southern Africa. *South African Journal of Botany* **68**, 1-13.

Van Wyk, B., van Oudtshoorn, B., Gericke, N., 1997. Medicinal Plants of South Africa. Briza Publications. Briza Publications: Pretoria, South Africa.

Van Wyk, B.E., Gericke, N., 2000. People's plants. Briza Publications, Pretoria, South Africa.

Van Zyl, R.L., Viljoen, A.M. 2002. *In vitro* activity of *Aloe* extracts against *Plasmodium falciparum*. *South Africa Journal of Botany* **68**, 106-110.

Voigt, K., 2006. Our look at global burgeoning Asian herbal industry, *The Wall Street Journal* **11**: 9-12.

Waako, P.J., Gumede, B., Smith, P., Folb, P.I., 2005. The *in vitro* and *in vivo* antimalarial activity of *Cardiospermum halicacabum* L. and *Momordica foetida* Schumch. Et Thonn. *Journal of Ethnopharmacology* **99**, 137-143.

Wang, P., Lee, C.S., Bayoumi, R., Djimde, A., Doumbo, O., Swedberg, G., Dao, L.D., Mshinda, H., Tanner, M., Watkins, W.M., Sims, P.F., Hyde, J.E., 1997. Resistance to antimalarials in *Plasmodium falciparum* monitored by sequence analysis of dihydropteroate synthetase and dihydrofolate reductase alleles in a large number of field samples of diverse origins. *Mol Biochem Parasitol.* **89** (2), 161-177.

Wang, P., Read, M., Sims, P.F.G., Hyde, J.E., 1997. Sulfadoxine resistance in the human malaria parasite *Plasmodium falciparum* is determined by mutations in dihydropteroate synthetase and an additional factor associated with folate utilization. *Molecular Microbiology* **23**, 979-986.

Wanga, C.L., 2007. AMANET launches Clinical Trial of candidate Malaria candidate in Mali. Press Release. African Malaria Network Trust. Tanzania.

Warhust, D.C., 1995. Haemozoin and the mode of action of blood schizonticides: more controversy. *Parasitology Today* 11 (6), 204-205.

Webster, H.K., 1989. Immunology of human malaria. A cellular perspective. *Parasite Immunology* 11, 105-116.

Webster, D., Hill, A.V., 2003. Progress with new malaria vaccines. *Bulletin World Health Organization*. 81, 902-909.

Wei, G. J., Ho, C. T., 2005. A stable quinone identified in the reaction of carnosol, a major antioxidant in rosemary, with 2,2-diphenyl-1-picrylhydrazyl radical. *Food Chemistry* 96 (3), 471-476.

Wellems, T.E., 1992. How chloroquine works. *Nature* 355, 108-110.

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

Wess, G., Urmann, M., Sickenberger, B., 2001. Medicinal Chemistry: Challenges and Opportunities. *Angew. Chem. Int. Ed.* 40 (18), 3341-3349.

White, A. I., Jenkins, G. L., 1942. *Journal of American Pharmaceutical Association*. Sci. Ed, 33-43.

WHO., 1989. *Weekly Epidemiological Record* 64 (32), 241.

WHO., 1997. World malaria situation in 1994. *Weekly Epidemiological Records* 72: 269-292.

WHO., 2000. "General guidelines for methodologies on research and evaluation of Traditional Medicine" WHO/EDM/TRM/2000.1; Geneva

WHO., 2002. WHO Traditional Medicine Strategy 2002-2005. WHO, Geneva.

WHO., 2003. World Health Organisation Fact Sheet No. 94. WHO information. <http://www.int/inf~fs/en/fact094.html>

Williams, V.L., 1996. The Witwatersrand muti trade. *Journal of the Botanical Society of South Africa, veld and Flora* 4, 12-14.

Willcox, M.L., and Bodeker, G., 2000. Plant-based malaria control: Research initiative on traditional antimalarial methods. *Parasitology Today* 16 (6): 220-221.

Willcox, M., Bodeker G., 2004. Traditional herbal medicines for malaria. *BMJ* 329, 1156-1159.

Willuhn, G., Willuhn., 2002. Millefolii herba. *Teedrogen und Phytopharmaka*, pp. 399-403.

Winski, S.L., Barber, D.S., Rael, L.T., Carter, D.E., 1997. Sequence of toxic events in arsine-induced hemolysis *in vitro*: implications for the mechanism of toxicity in human erythrocytes. *Fundamental and Applied Toxicology* 38 (2), 123-128.

Winstanley, P.A., 2000. Chemotherapy for *falciparum* malaria: the armoury, the problems and the prospects. *Parasitology Today* 16, 146-153.

Winstanely, P.A., 2001. Modern chemotherapeutic options for malaria. *The Lancet Infectious Diseases* 1: 242-250.

Winstanely, P.A., Ward, S.A., Snow, R.A., 2002. Clinical status and implications of antimalarial drug resistance. *Microbes and Infection* 4: 157-164.

Wolson, R., 2001. Towards TRIPS compliance: South Africa's experience and legislative reforms. In: Eastern and Southern Africa Multi-Stakeholder Dialogue on Trade, Intellectual Property Rights and Biological Resources in Eastern and Southern Africa, <http://www.ictsd.org/dlogue/2001-07-30/wolson.pdf>

Wongsrichanalai, C., Pickard, A.L., Wernsdorfer, W.H., Meshnick, S.R., 2002. Epidemiology of drug resistant malaria. *Lancet Infectious Diseases* 2: 209-218.

Wright, W.C., Allen, D., Cai, Y., Phillipson, D.J., Said, M.I., Kirby, G.C., Warhurst, D.C., 1992. *Phytotherapeutic Research* 6, 121.

Wube, A.A., Bucar, F., Gibbons, S., Asres, K., 2005. Sesquiterpenes from *Warburgia ugandensis* and their antimycobacterial activity. *Phytochemistry* 66, 2309-2315.

Xin-zhuan, S., Jianbing, M., Deirdre, J.A., 2003. The Malaria's Eve hypothesis and the debate concerning the origin of the human malaria parasite *Plasmodium falciparum*. *Microbes and Infection* 5, 891-896.

Ye, Z.G., Van Dyke, K., Wimmer, M., 1987. Effect of artemisinin (qinghaosu) and chloroquine on drug-sensitive and drug-resistant strains of *Plasmodium falciparum* malaria: use of [2,8-3H] adenosine as an alternative to [G-3H] hypoxanthine in the assessment of *in vitro* antimalarial activity. *Experimental Parasitology* 64 (3), 418-423.

Younos, C., Rolland, A., Fleurentin, J., Lanhers, M.C., Misslin, R., Mortier, F., 1990. Analgesic and behavioural effects of *Morinda citrifolia*. *Planta Medica* 56, 430-434.

Yu, W.H., Wright, W., Cai, Y., Phillipson, J.D., Kirby, G.C., Warhurst, D.C., 1994. *Phytotherapeutic Research* 8, 436.

Yuthavong, Y., Wilairat, P., 1993. Protection against malaria by Thalassaemic and Haemoglobin variants. *Parasitology Today* 9 (7), 241-245.

Zhanglan, Q., Lihong, H., Kehua, P., Yunjie, W., 2002. Synthesis and molluscicidal activity of flavanone derivatives. *Huaxue Yanjiu Yu Yingyong* 14(4), 475-476, 483.

Zhang, Y., Meshnick, S.R., 1991. Inhibition of *Plasmodium falciparum* dihydropteroate synthetase and growth *in vitro* by sulfa drugs. *Antimicrobial Agents Chemotherapy* **35**, 267-271.

Zhang, Q J., Wang., Wang, Z., 2005. Systems biology and traditional Chinese medicine (TCM) modernization, *China Journal of Chinese Materia Medica* **30** pp. 1725–1727.

Zhao, Y., Hanton, W.K., Lee, K.H., 1986. Antimalaria agents: Artesunate, an inhibitor of cytochrome oxidase activity in *Plasmodium berghei*. *Journal of Natural Products* **49**, 139-142.

Zhou, M., Liu, P., Jia, W., Zhao, A., Qiu, M., Qiu, Y., Su, M., 2006. Evaluation of therapeutic effects of TCM based on metabolic variations, *World Science and Technology – Modernization of Traditional Chinese Medicine and Materia Medica* **8** pp. 113–119.

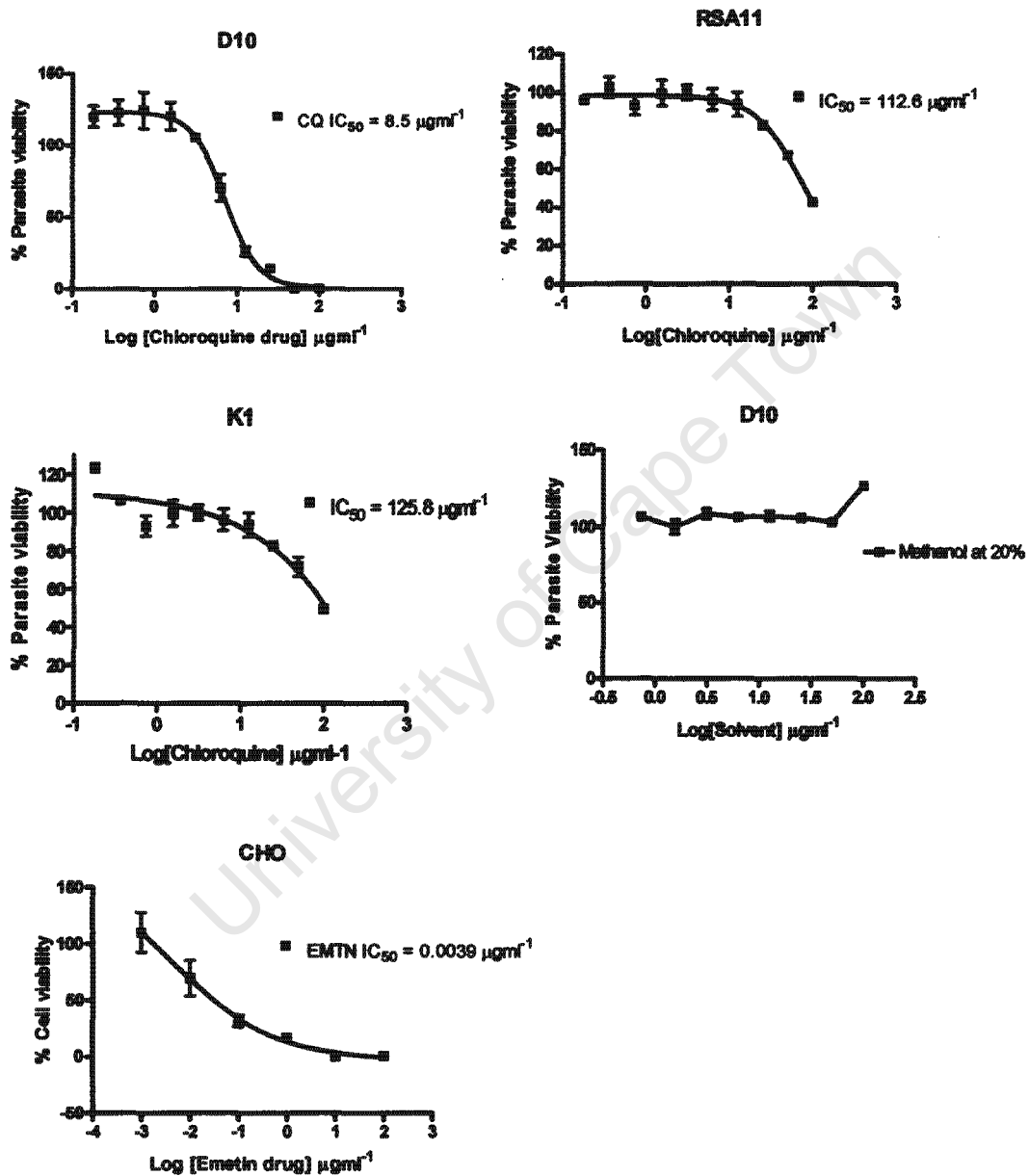
Zhou, L., Guo, J., Yu, J., 1999. Flavonoids from Beijing propolis. *China Journal of Chinese material medica* **24**(3), 162-164.

Zschocke, S., Rabe, T., Taylor, J.S.L., Jager, A.K., van Staden, J., 2000. Plant part substitution – a way to conserve endangered medicinal plants? *Journal of Ethnopharmacology* **71**, 281-292.

Zuco, V., Supino, R., Righetti, S.C., Cleris, L., Marchesi, E., Gambacorti-Passerini, C., Formelli, F., 2002. Selective cytotoxicity of betulinic acid on tumor cell lines, but not on normal cells. *Cancer Letter* **175** (1), 17-25.

Appendix A

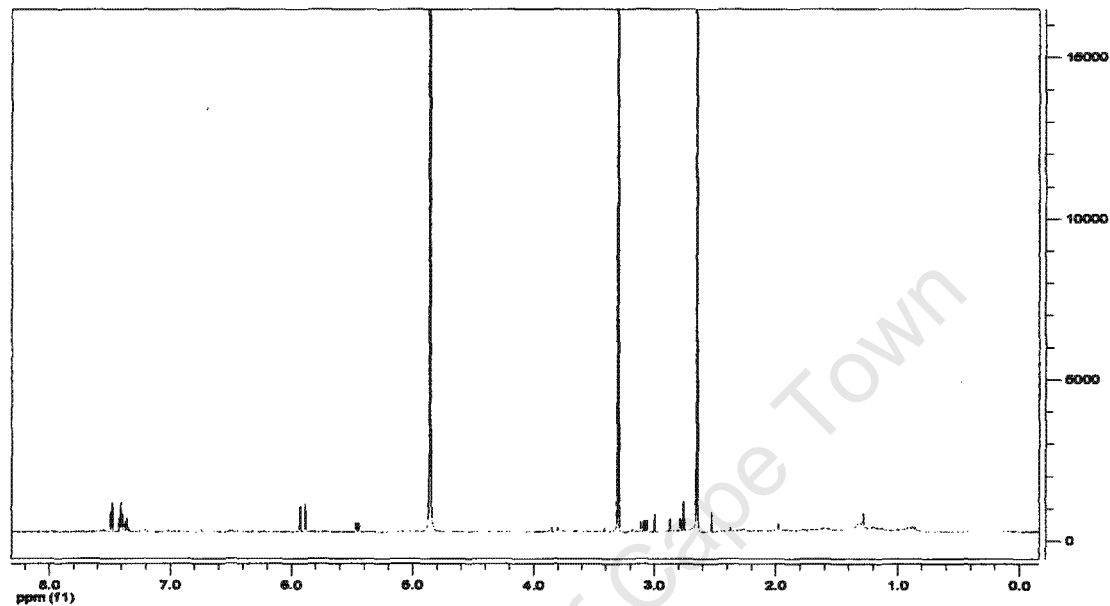
The activity of the control drugs on the CHO cell-line, CQ-sensitive and CQ-resistance strains of *P. falciparum*, and the effect of the solvent on parasite growth.



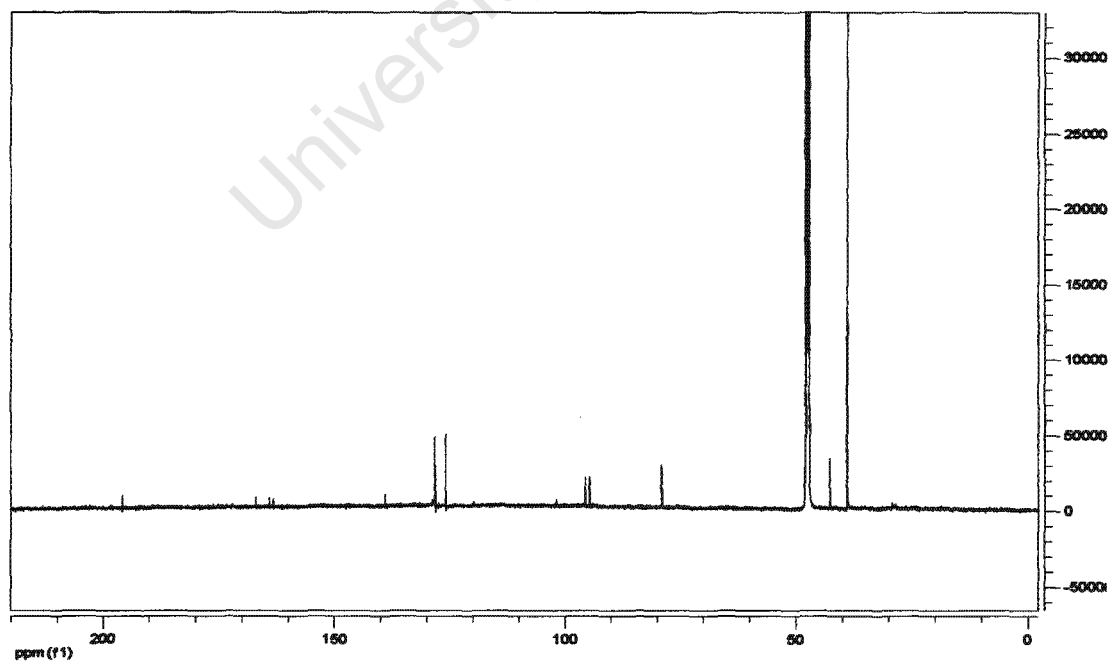
Appendix B

NMR of Compound T-60 identified as 5,7-dihydroxyflavone. Compound extracted from DCM leaf extract of *T. camphoratus*

T60 ^1H spectrum

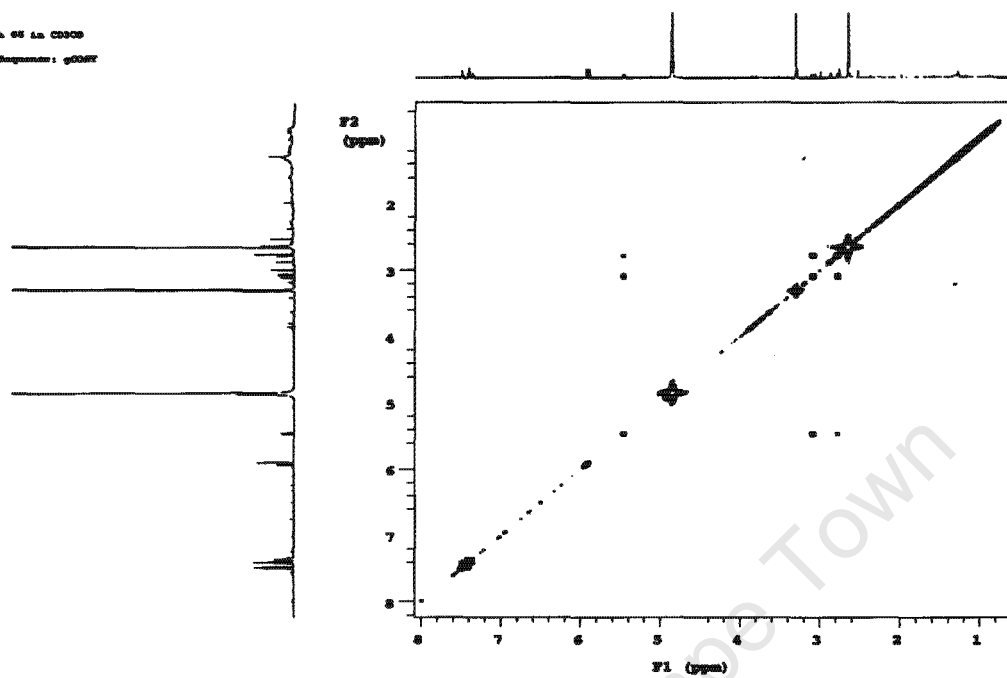


^{13}C spectrum



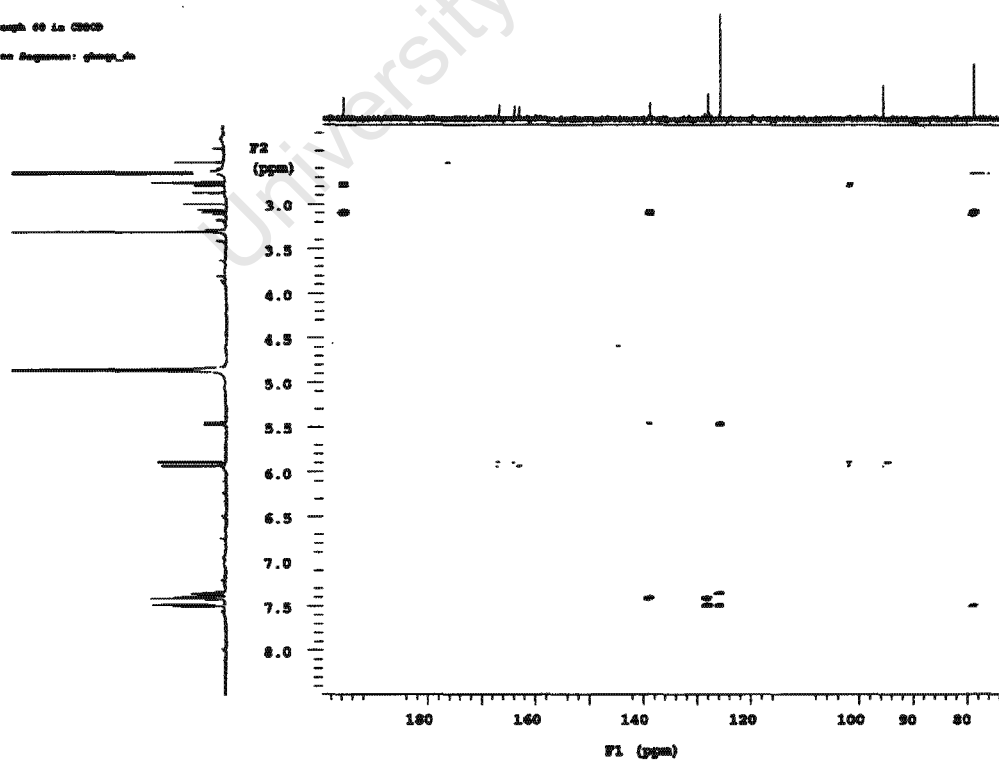
COSY

F2-range: 0.0 to 8.000
Pulse Sequence: gcosy



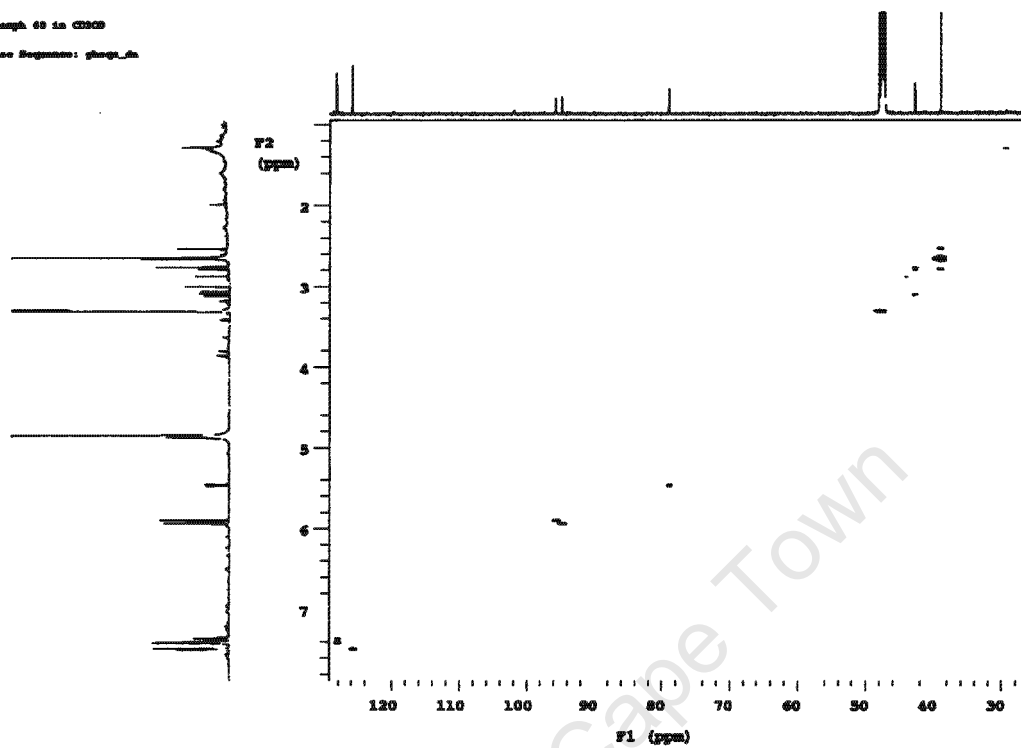
HSQC

F2-range: 0.0 to 8.000
Pulse Sequence: ghsqc_4h

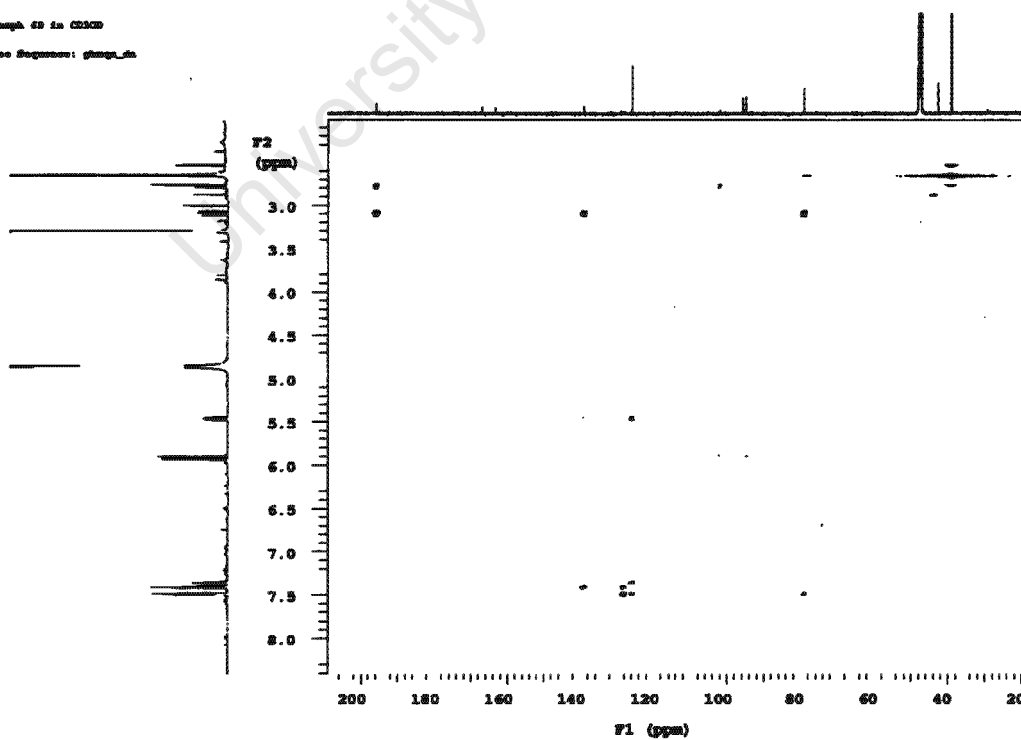


HMQC

T-sample 03 in CDCl3
Pulse Sequence: ghmqz_3h

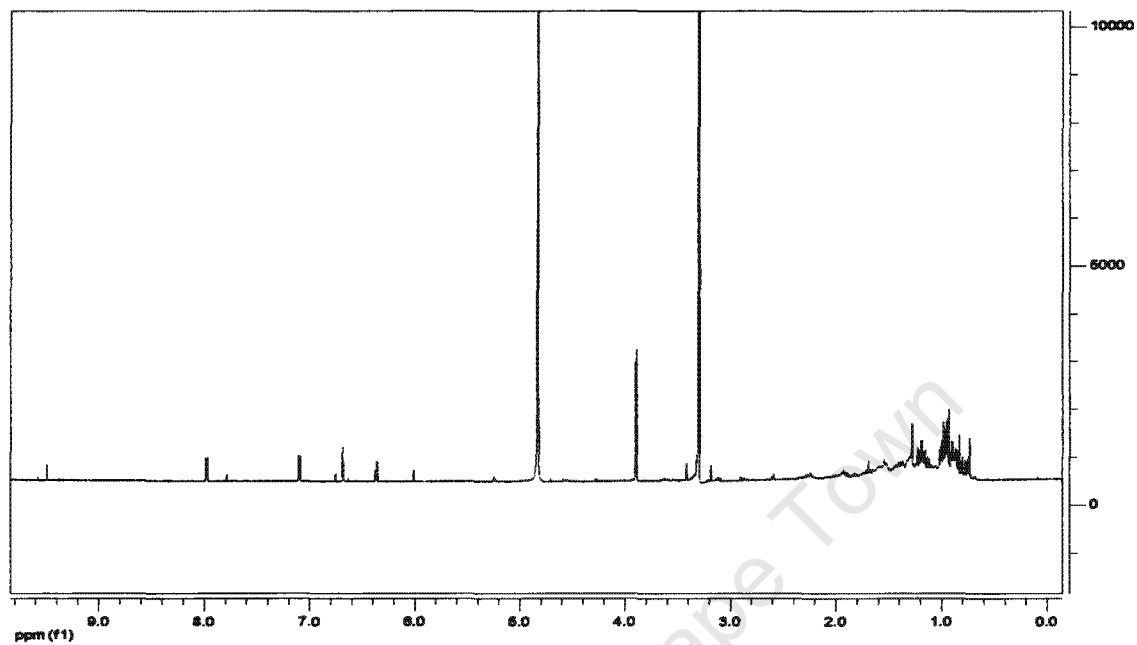


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Pulse Sequence: ghmqz_3h

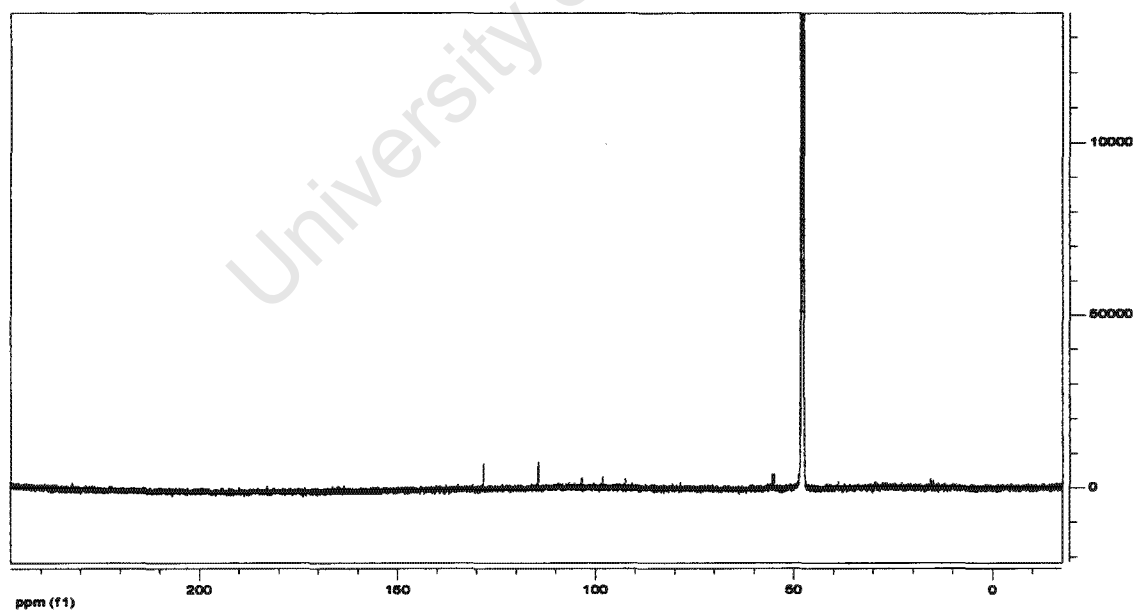


NMR of compound R 90-1, isolated from DCM leaf extract of *E. afrikanus*

R90-1 ^1H spectrum

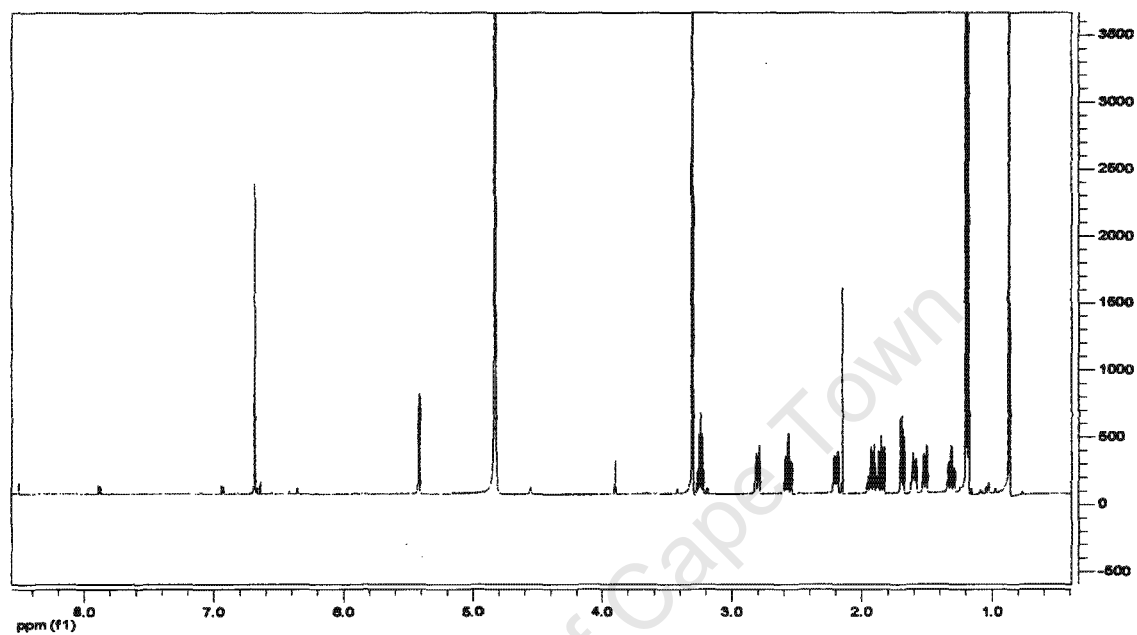


R 90-1 ^{13}C Spectrum

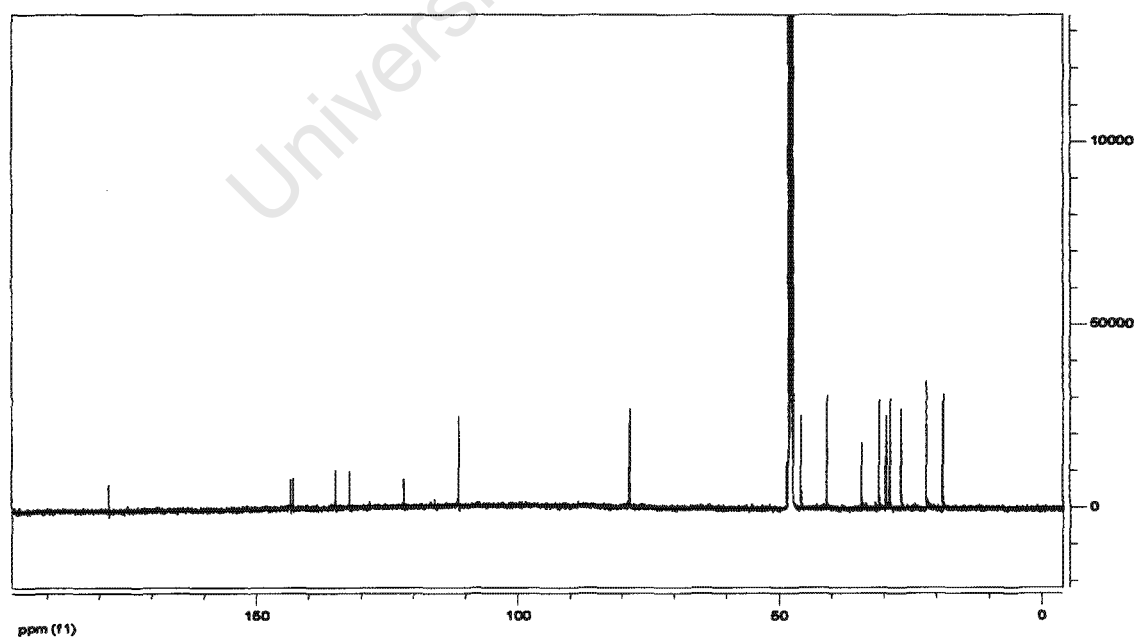


NMR spectra of Compound R70, identified as carnosol. Compound isolated from DCM extract of *E. afrikanus*.

R70 ^1H spectrum in M+OH

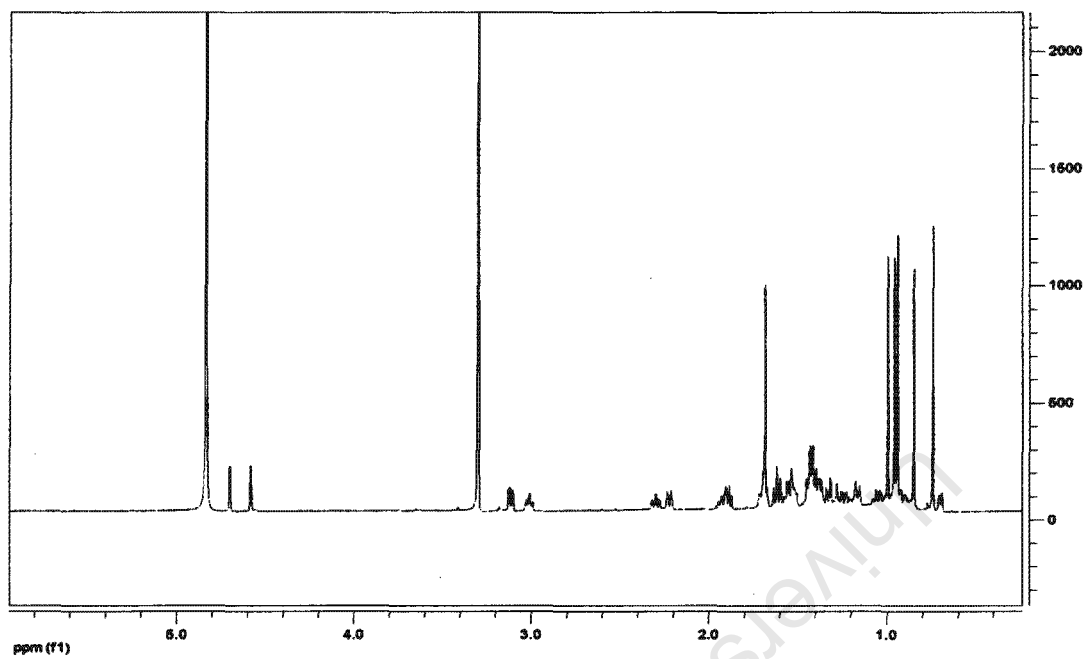


R70 ^{13}C spectrum

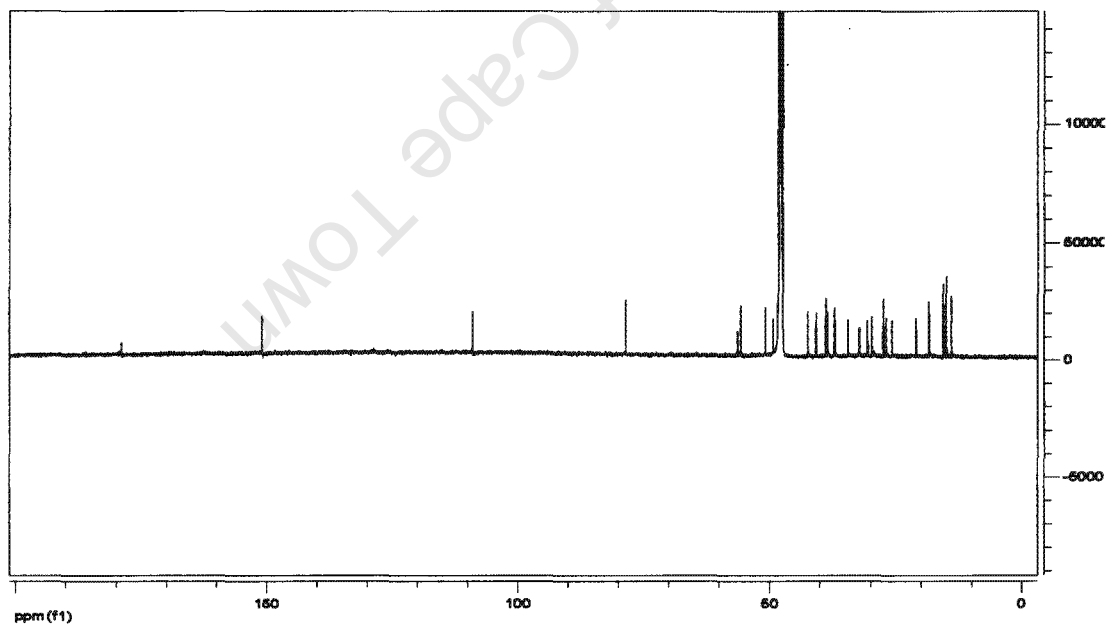


NMR spectra of R90-2 (R100), identified as Betulinic acid.

R100 ^1H spectrum

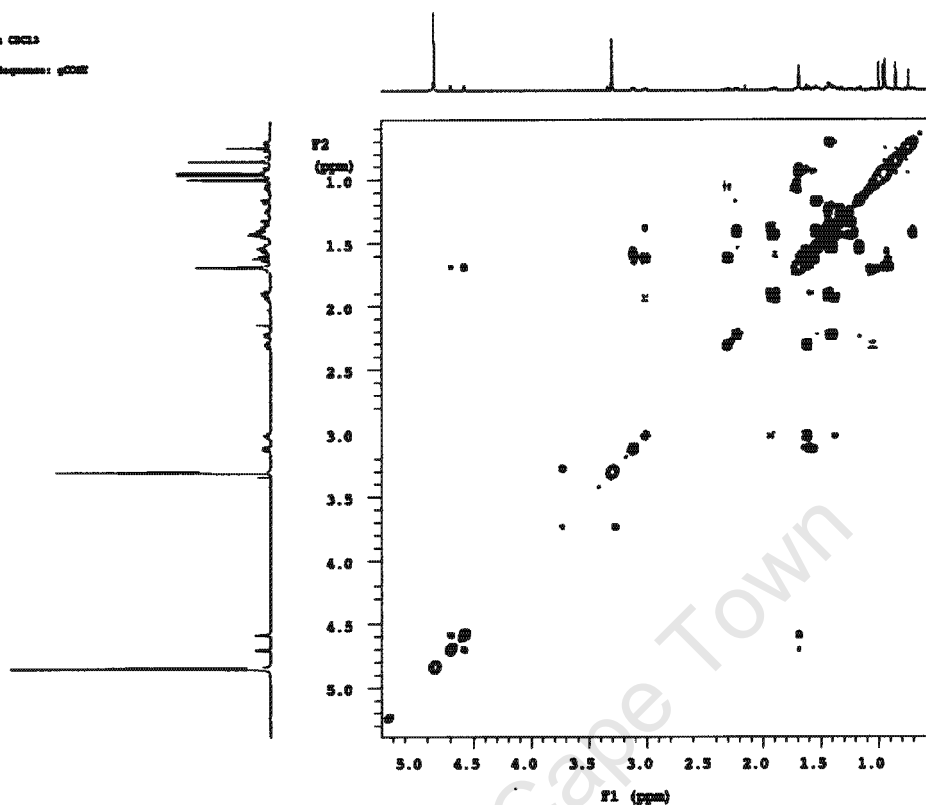


^{13}C spectrum



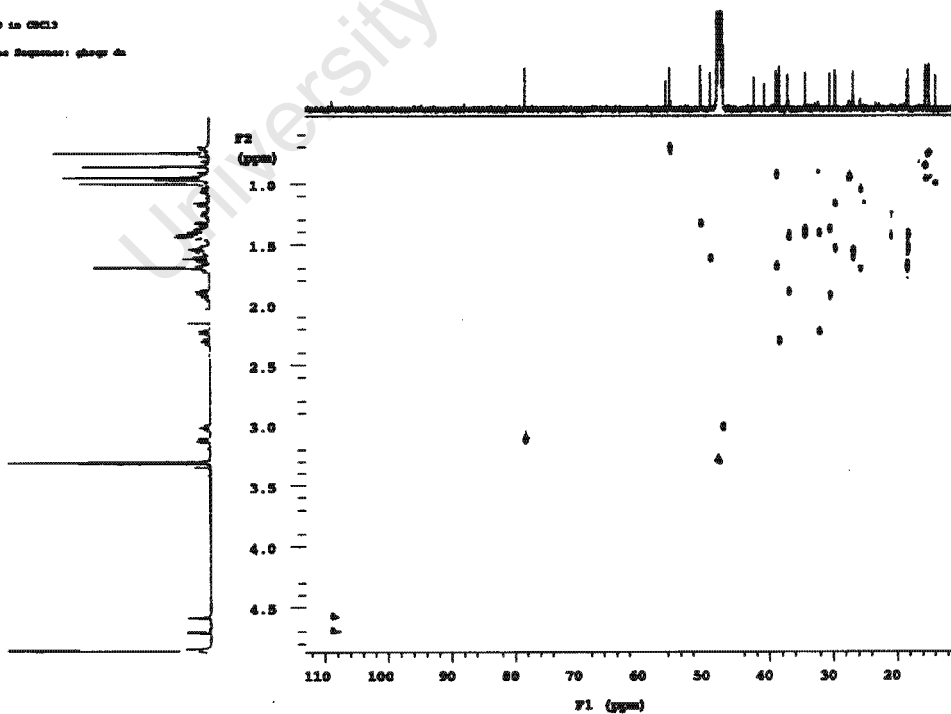
COSY

1100 in CDCl3
Pulse Sequence: gCOSY



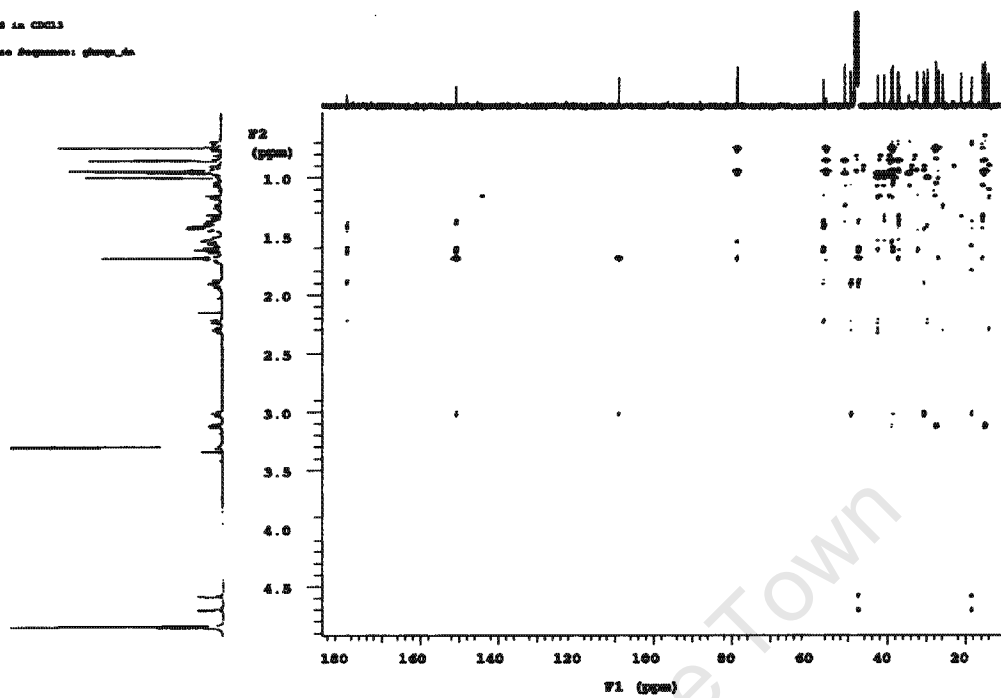
HSQC

1100 in CDCl3
Pulse Sequence: ghsqc de



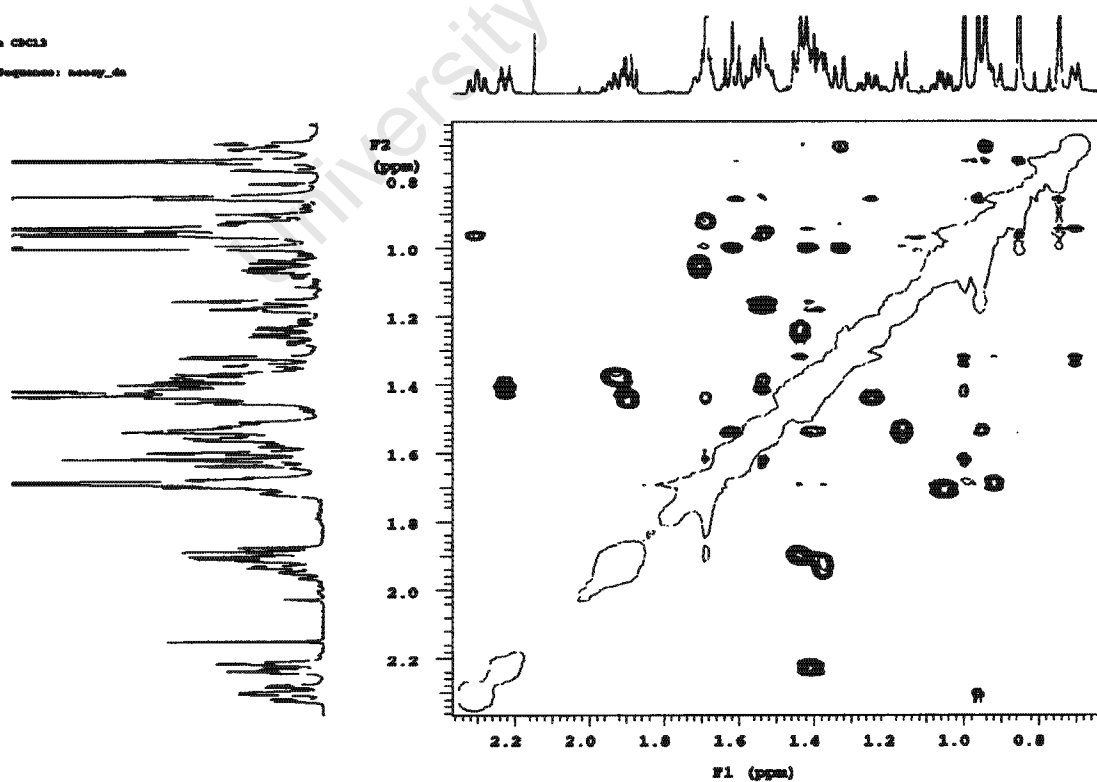
HMQC

H100 in CDCl3
Pulse Sequence: ghmqz_fm



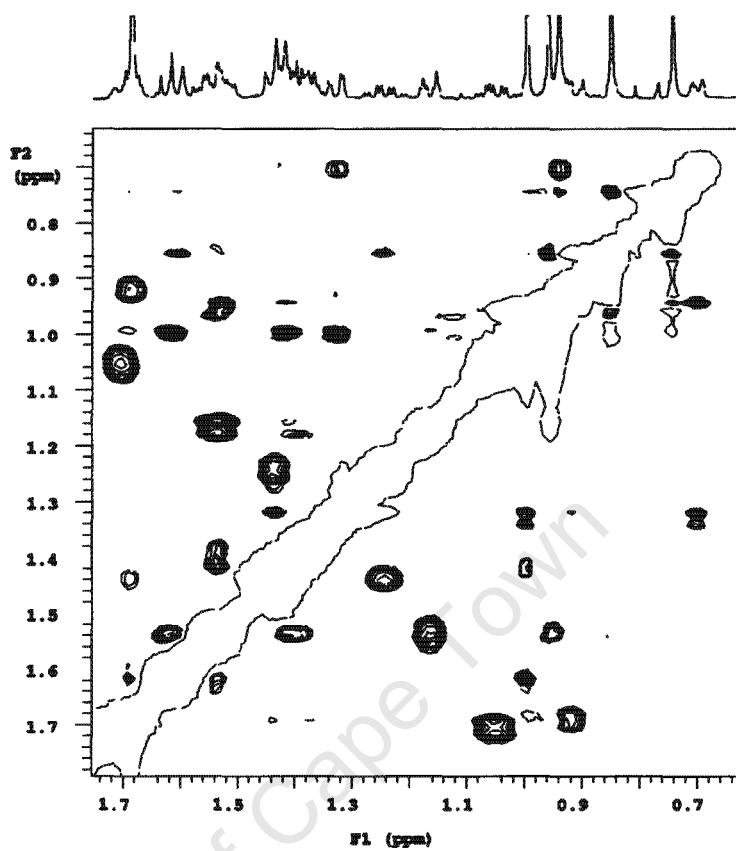
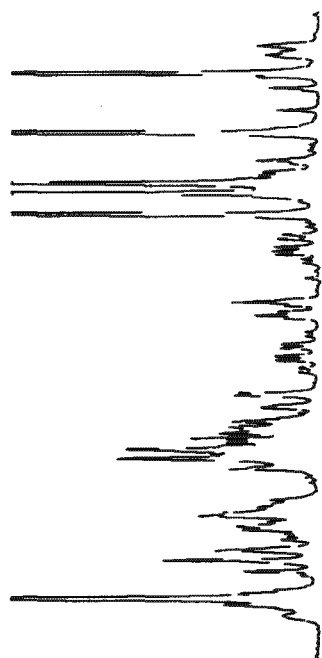
NOESY

H100 in CDCl3
Pulse Sequence: noesy_fm



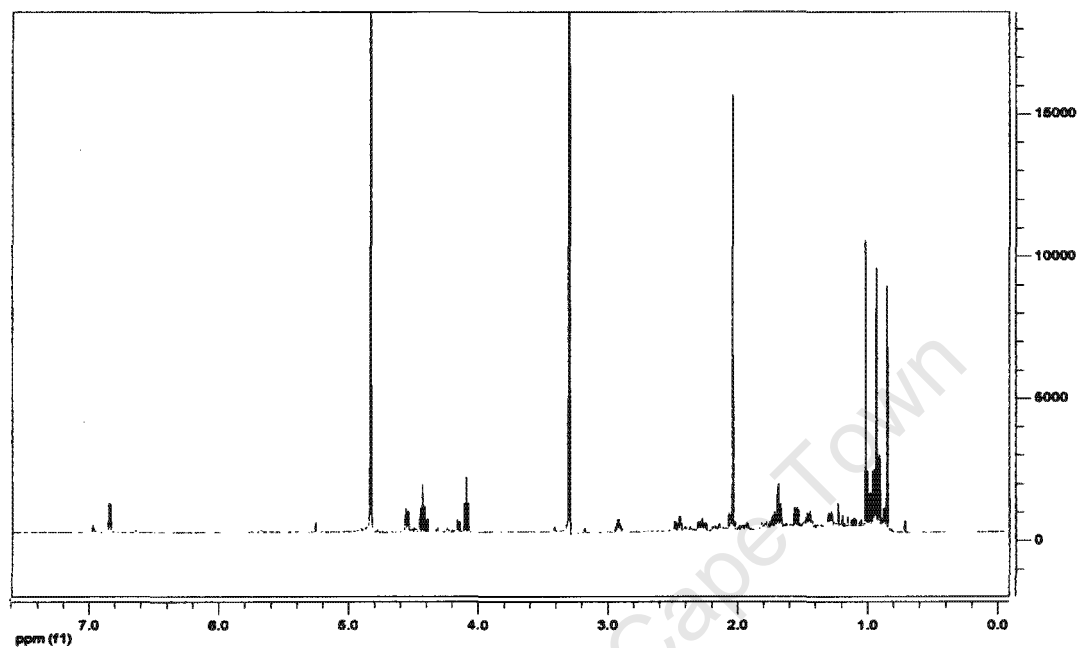
R100 in CDCl3

Pulse Sequence: mecyg_4h

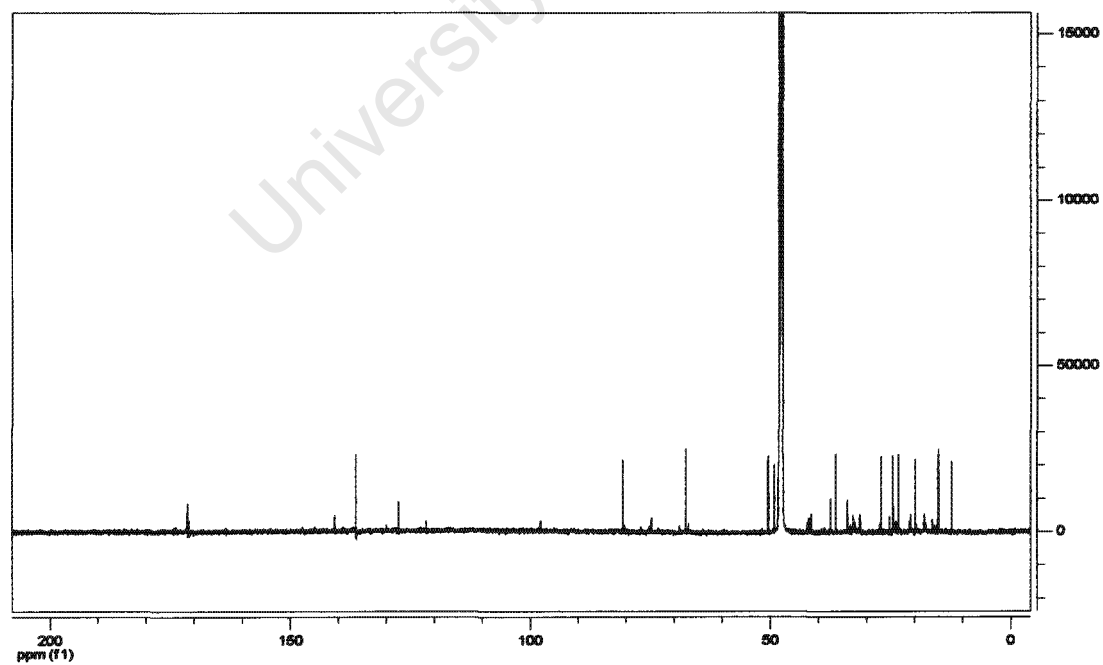


^1H and ^{13}C NMR spectra of compound PK2-4, identified as 3 β -acetoxy cinnamolide.
Compound isolated from EtOH leaf extract of *W. salutaris*.

^1H spectrum

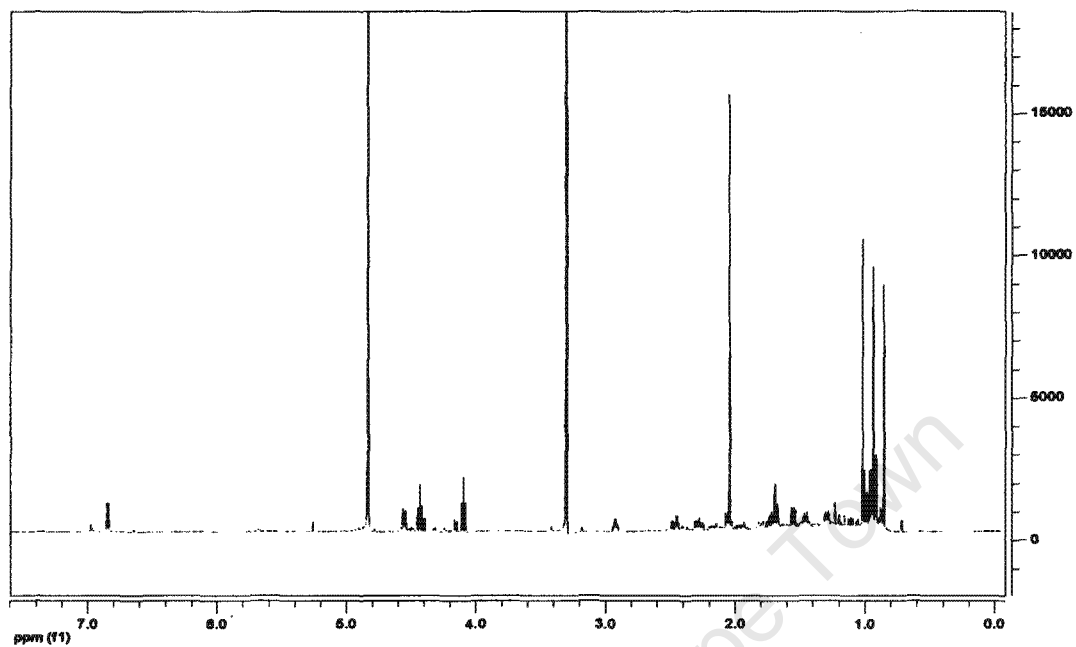


PK2-4 ^{13}C spectrum

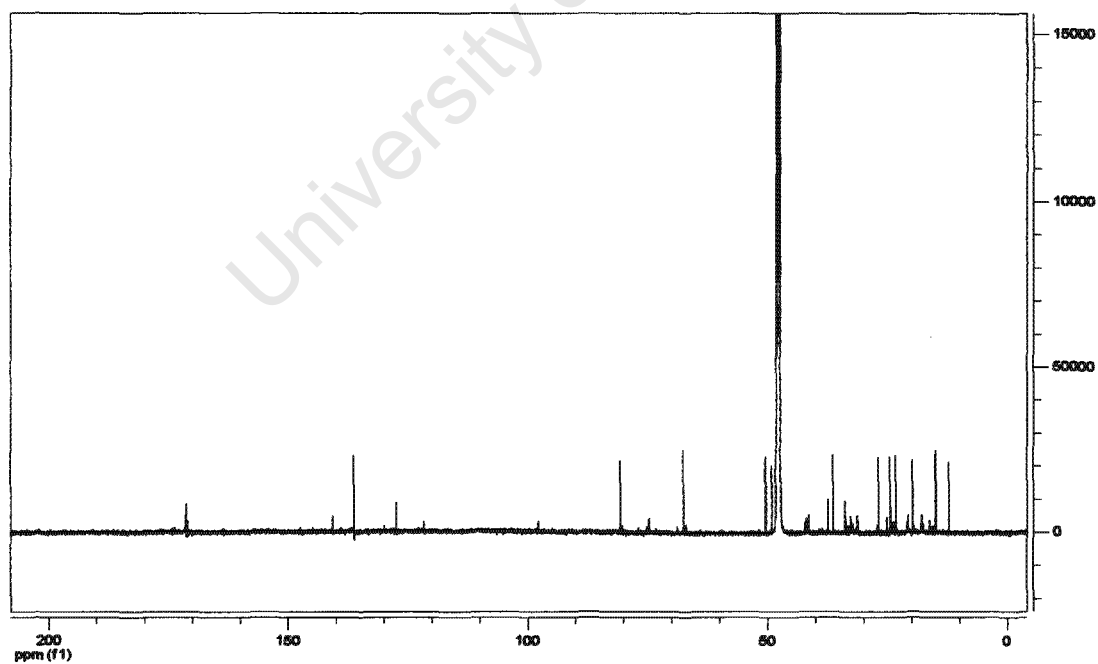


NMR of compound W.S 50-3

^1H spectrum

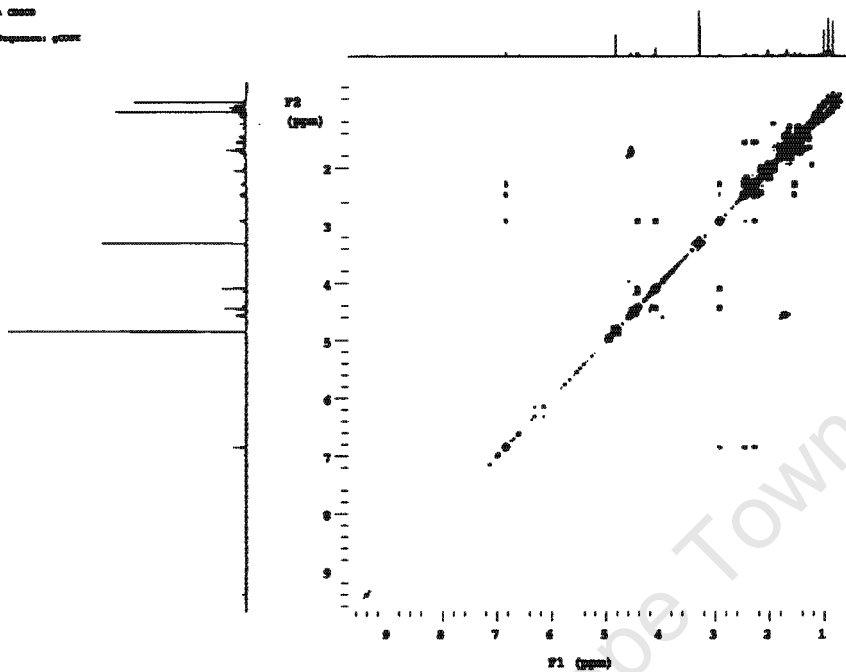


W.S50-3 ^{13}C spectrum

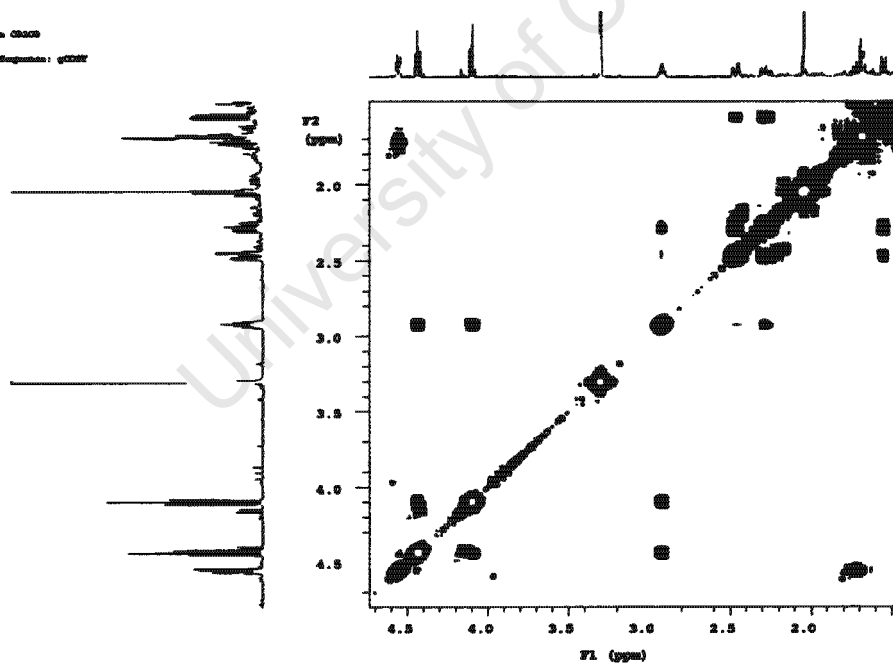


COSY

NAME: 1a.CH20
Pulse Program: gCOSY

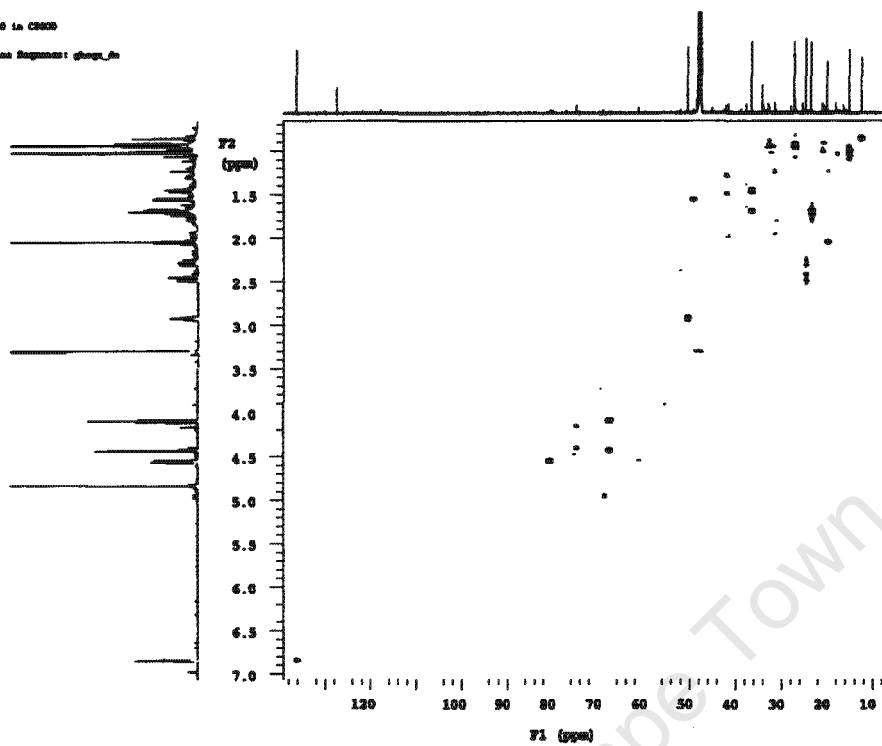


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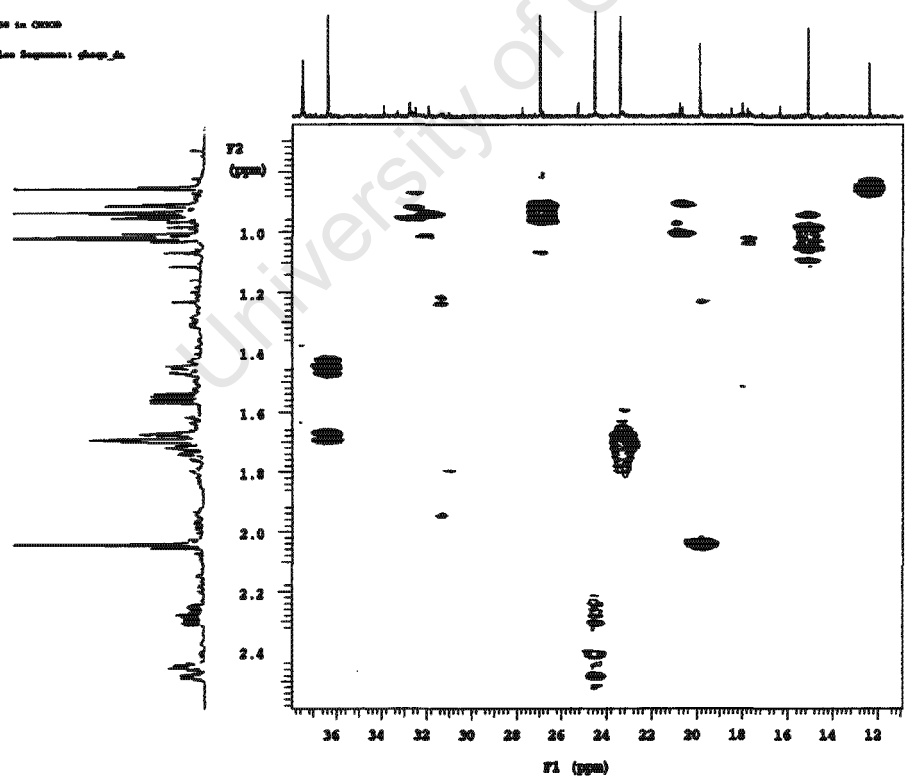


HSQC

W50 in CDCl₃
Pulse Program: ghsqz_4h

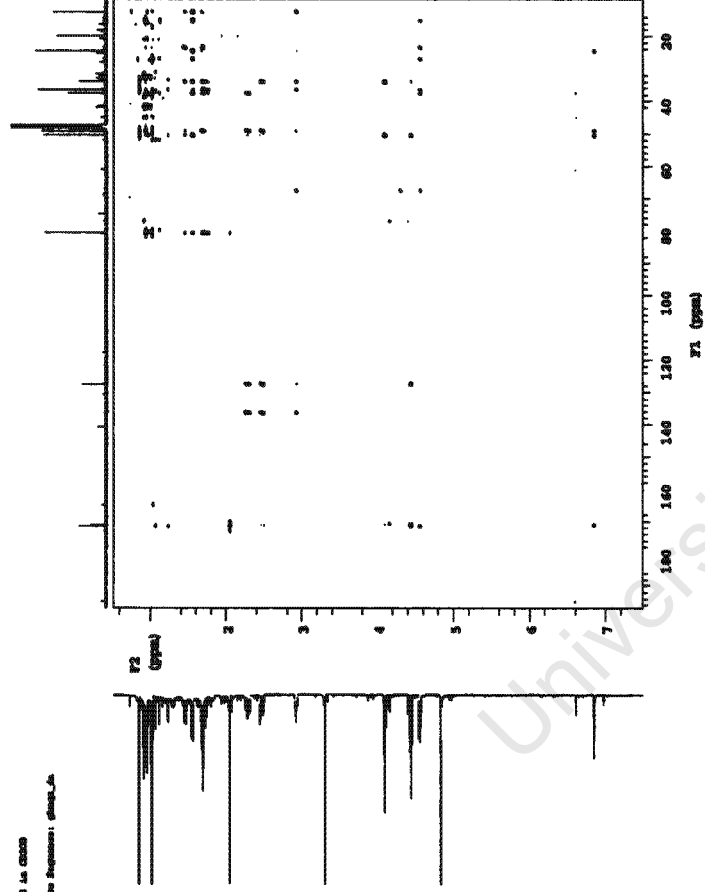


W50 in CDCl₃
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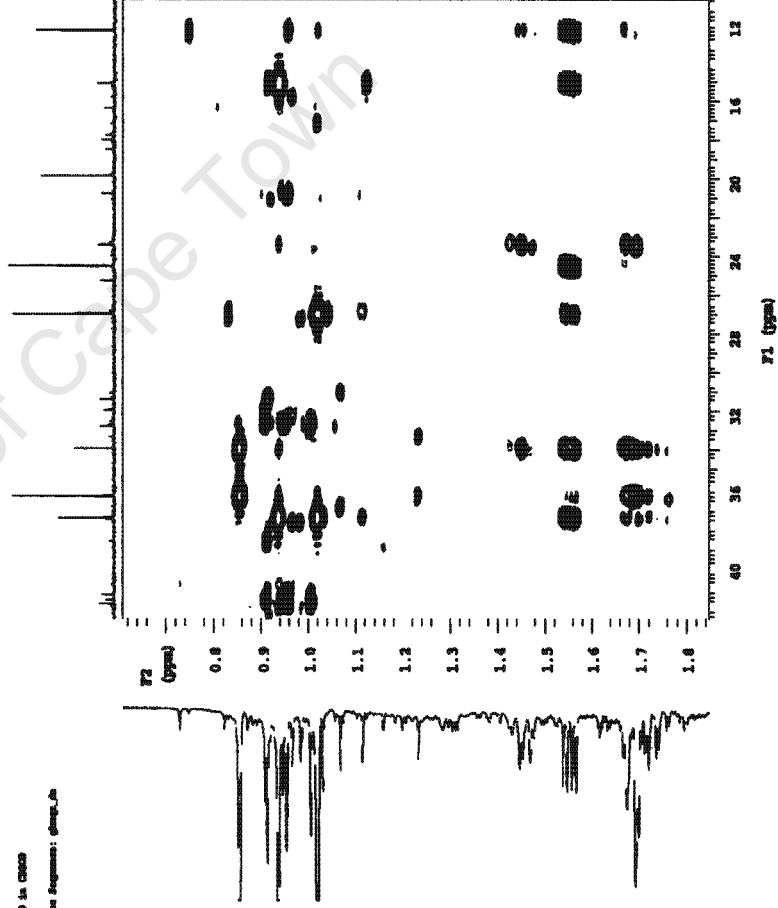


HMQC

HM90 J4 C000
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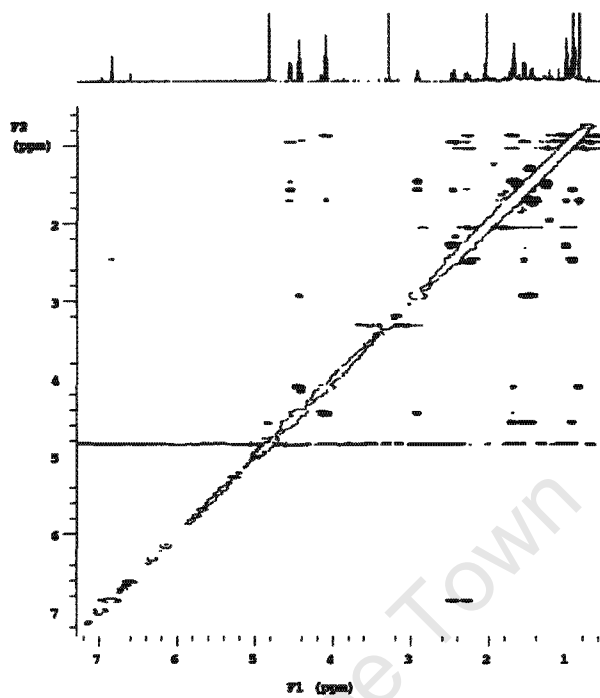
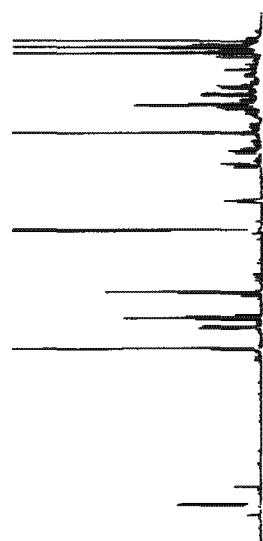


HM90 J4 C000
Pulse Program: gmgp_4a



NOESY

0000 In CDCl3
Folios Segment: noesy_00



0000 In CDCl3
Folios Segment: noesy_00

