The therapeutic effectiveness of some local Nigerian plants used in the treatment of malaria

by

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DECLARATION

The therapeutic effectiveness of some local Nigerian plants used in the treatment of malaria

I, Paula E. Melariri hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the substance nor any part of this work has been, is being, or is to be submitted for another degree at this University or at any other University.

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ABSTRACT

In Nigeria most of the populace relies heavily on medicinal plants for the treatment of malaria. This thesis describes the investigation of the antiplasmodial properties of seven plants used in the traditional treatment of malaria in Nigeria. The seven plants include *Mangifera indica* L., *Citrus limon* L. Burm.f, *Musa sapientum* L., *Psidium guajava* L., *Carica papaya* L., *Cymbopogon citratus* Staph, and *Vernonia amygdalina* Delile. These plants are widely distributed in Nigeria and are used in Nigerian folk medicine to treat malaria and febrile illnesses. After the initial screening of the antiplasmodial properties of these plants, two out of the seven plants were screened out due to poor activity. In this study, investigation of plants which showed antiplasmodial activities ≥10µg/ml was not taken further. The two plants screened out were *Mangifera indica* and *Musa sapientum* while the five plants selected include *Carica papaya*, *Citrus limon*, *Cymbopogon citratus*, *Psidium guajava*, and *Vernonia amygdalina.* The *in vitro* antiplasmodial screening of these plants with selected solvents showed equipotent activity against the chloroquine sensitive (D10) and the chloroquine resistant (DD2) strains of *P. falciparum* and no *in vitro* cytotoxicity against the Chinese Hamster Ovarian cell line (CHO). The selected five plants were further tested singly and in combination. Bioassay-guided fractionation of *C. papaya* ethyl acetate fraction using a combination of solid phase extraction and HPLC yielded two polyunsaturated fatty acids. GC-MS and NMR spectroscopic methods identified and characterized these compounds as 9,12,15-Octadecatrienoic acid (Compound 1) and 9,12-Octadecadienoic acid (Compound 2). Both compounds showed IC_{50} <10 µg/ml against the D10 and DD2 strains of *P. falciparum,* but were not as potent as the parent ethyl acetate crude extract.

Oral administration of 9,12,15-Octadecatrienoic acid (linolenic acid) and 9,12-Octadecadienoic acid (linoleic acid) *in vivo* to mice infected with *P. berghei* using the 4-day suppressive test showed an enhancement in parasite suppression when the two compounds were combined. 9,12,15- Octadecatrienoic acid was more potent (70% growth inhibition) but showed rapid recrudescence post treatment. A bioavailability study using the methyl ester of 9,12,15- Octadecatrienoic acid (which has identical *in vitro* and

in vivo antimalarial activity) was performed to investigate its elimination rate from plasma. The results showed poor oral bioavailability and rapid elimination or metabolism. Evaluation of the elimination rate of 9, 12,15-Octadecatrienoic acid from plasma using the intravenous data showed a fast elimination from plasma with a half life of approximately 1 hour. Determination of *in vivo* antimalarial activity after intravenous administration of the compound is consistent with this finding. *In vivo* experiments using dichloromethane extracts of *C. citratus* and *V. amygdalina* gave parasite inhibition of 87.2% and 95.8%, respectively on day four post treatment. A combination of these two extracts cured mice infected with *P. berghei*.

It is apparent that experimental evaluation of herbal drugs for the treatment of malaria is rather impressive, however, only few are in the market and very few have reached clinical trials. For instance quinine and artemisinin are products of medicinal plants used in the treatment of malaria. Researchers need to take more active interest in the investigation and standardization of herbal drugs with potent antimalarial activity so as to be clinically effective and globally competitive. This study provided information on the efficacy and safety of selected Nigerian medicinal plants used in the traditional treatment of malaria. Since majority of the African populace patronize the traditional healers, the information from this study could serve as a guide to the antiplasmodial and toxicity properties of these plants. The information can also be used in the development of herbal remedies or for sources of lead compounds that can be developed into more favourable antimalarial compounds. Studies using the murine model of PbA in (BALB/C x 57BL/6) have shown several features in common with human cerebral malaria. However findings from the use of these models may not be directly extrapolated to humans since the ideal animal model that could display precise clinical characteristics of human malaria is not yet available. The results can only be predictive due to major differences which exist between the small mammals and humans.

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LIST OF ABBREVIATIONS

ACN	Acetonitrile
APAD	3-Acetylpyridine adenine dinucleotide
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
^{13}C	Carbon-13
CDC	Centres for Disease Control and Prevention
CHO	Chinese hamster ovarian
CLA	Conjugated Linoleic acid
CLnA	Conjugated Linolenic acid
CM	Complete medium
COSY	Correlation spectroscopy
CQ	Chloroquine
CQR	Chloroquine-resistant
CQS	Chloroquine-sensitive
1D	One-dimensional
2D	Two-dimensional
DCM	Dichloromethane
DDT	Dichloro-diphenyl-trichloroethane
dH_2O	Distilled water
DHFR	Dihydrofolate reductase
DHPS	Dihydropteroate synthetase
DMEM	Dulbecos modified Eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EA	Ethyl acetate
EFA	Essential fatty acid
***et al*.**	And all others
ESI	Electrospray Ionisation
FA	Fatty acid
FAME	Fatty acid methyl ester
FCS	Foetal calf serum
g	Gram(s)
^{1}H	Proton
HAMS	HAM's Nutrient mixture F-12
HEPES	*N*-[2-Hydroxyethyl]-piperazine-*N'*-[2-Ethanesulphonic acid]
Hex	Hexane
HIV	Human immunodeficiency virus
HMQC	Heteronuclear multiple quantum coherence
HPLC	High performance liquid chromatography

Hrs	Hours
HSQC	Heteronuclear single quantum coherence
Hz	Hertz
IC_{50}	Concentration inhibiting 50% of growth
J	Coupling constant
i.v	Intravenous
Kg	Kilogram(s)
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography mass spectrometry/mass spectrometry
LDH	Lactate dehydrogenase
LLOQ	Lower limit of quantification
MeOH	Methanol
Mg	Milligram
mH_2O	Millipore water
MIC	Minimum inhibitory concentration
Ml	Millilitre
MP	Mobile phase
MQ	Mefloquine
MRM	Multi reaction monitoring
MS	Mass spectrometry
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
NAD	Nicotinamide adenine dinucleotide
NBT	Nitro blue tetrazolium
ND	Not determined
NHLS	National Health Laboratory Service
NMR	Nuclear magnetic resonance
PBS	Phosphate buffered saline
PET	Petroleum ether
pLDH	Parasite lactate dehydrogenase
ppm	Parts per million
pRBC	Parasitized red blood cells
RBC	Red blood cell
RI	Resistance index
SD	Standard deviation
SI	Selectivity index
SMEDDS	Self microemulsifying drug delivery system
SPE	Solid phase extraction
TIC	Total ion current
WHO	World Health Organisation
µ	Micro

µg	Microgram(s)
µM	Micromolar
λ	Wavelength (nm)

CHAPTER 1

MALARIA, TRADITIONAL MEDICINES, AND THE ROLE OF NATURAL PRODUCTS IN MALARIA TREATMENT

1.1 Malaria

Malaria is one of the major infectious diseases responsible for the high rate of mortality and morbidity in developing countries. Malaria is caused by apicomplexan protozoan parasites which belong to the genus *Plasmodium* (Miller *et al.,* 1994), and are known to contain apicoplast which is an indispensable organelle for the parasites' metabolic processes. The double membranous wall of this organelle evolved from a cyanobacterium through a secondary endosymbiotic process (McFadden *et al*., 1996). Four species of Plasmodium parasites are known to cause infection in humans; *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale* and *Plasmodium malariae*. *Plasmodium knowlesi,* previously known to infect monkeys has been implicated in human infection as well (Singh *et al*., 2004). *Plasmodium falciparum* is the most virulent parasite, and is highly incriminated in the global mortality rate. It is the most common human parasite in Africa, and is seen in all malaria endemic regions of the world (WHO, 2005). *P. vivax* is not common in Africa, and is mostly seen outside Africa (Mendis *et al*., 2001). The prevalence of *P. malariae* is low, even in malaria endemic areas, while morbidity due to *P. ovale* is rarely reported (Mims *et al*., 2004, Mueller *et al*., 2007).

Clinical manifestations of this disease include anaemia, and numerous complications, such as hypoglycaemia, cerebral malaria, metabolic acidosis, and respiratory distress. Common symptoms and complaints include fever, chills, convulsion, headache, nausea, loss of appetite, diarrhea, weakness, vomiting and malaise (English *et. al.,* 1996, Biersmann *et al*., 2007, Legros *et al*., 2007). An annual global estimate of 300-500 million cases of malaria has been reported with a mortality of 2-3 million deaths, and children account for 1 million of these deaths (WHO, 1997; WHO, 2003). Children especially those less than five years old, and pregnant women, are at high risk to malaria (Desai *et al*., 2007). Malaria during pregnancy can result in low birth weight thus affecting the chances of survival (Rowe and Kyes, 2004). Anaemia and jaundice induced by malaria may

occur as a result of the rupture and lyses of red blood cells. The disease can be fatal if not adequately treated in good time, and may also create room for opportunistic infections (Snow *et al*., 2005; WHO, 2005).

1.1.1 History of Malaria

Malaria has been noted for more than 4,000 years and it is believed that this disease has greatly influenced human populations and history (CDC, 2010).
An earlier assumption was that malaria arose from fetid marshes, suggesting 'mal aria' which means bad air. This belief that malaria fevers were caused by miasmas rising from swamps persisted, and it is widely held that the word malaria comes from the Italian mal'aria meaning bad air. The Italians used this term to describe the relationship between intermittent fever and exposure to marsh air (CDC, 2010). The name is traceable to the belief that the accumulation of bad stagnant water initiates infection (Kondrasen *et. al*., 2004). Prior to this time, the disease was thought to be of supernatural origin, as those infected were said to have offended their gods. The first person to correct this erroneous assumption was Hippocrates (CDC, 2010). He related the infection and its manifestations to seasonal and geographical differences (CDC, 2010).

Malaria incidences date back to 6000 years in Egypt and China and 1600 BC in India (WHO, 1986). Malaria is geographically specific, however, the mass movements of migrant workers, refugees and non-immune travellers across borders have largely contributed to the global spread of the disease (Rooth, 1992). Available records stated that between 2,500 and 2,000 years ago, malaria reached the shores of the Mediterranean sea, while its presence in northern Europe was about 1,000 to 500 years ago (Carter and Mendis 2002). It is believed that the Europeans and the West Africans were incriminated for introducing malaria into the new world at the end of the 15th century AD. *P. falciparum* was likely to have reached the Americans through the African slaves who were brought in by the Spanish colonial masters (Carter and Mendis 2002). More

than one-half of the world's population was at risk of malaria by the 19th century, and the mortality rate was also increasing at an alarming rate.

The search for the cause of malaria was however heightened by the discovery of bacteria by Antoni van Leeuwenhoek in 1676. This search was further intensified by the development of the germ theory of infection, by Louis Pasteur and Robert Koch in 1878-1879, and the incrimination of microorganisms as the causative agent of infectious diseases (Cox, 2010). Laveran, a military doctor, in his work in Algeria in 1889, identified the etiologic agent as a protozoan parasite (CDC, 2004). Eight years later, the female *Anopheles* mosquito was identified as the vector of these protozoan parasites by Ross (CDC, 2004; CDC, 2010). In 1898 the Italian malariologists, Giovanni Battista Grassi, Amico Bignami, Giuseppe Bastianelli, Angelo Celli, Camillo Golgi and Ettore Marchiafava demonstrated conclusively that human malaria was also transmitted by anopheline mosquitoes (CDC, 2010). Two years after, Giovanni Batista Grassi and Raimondo Filetti were the first to introduce the names of two human malaria parasites as *Plasmodium vivax* and *P. malariae* (CDC, 2010). In 1948 Henry Shortt and Cyril Garnham discovered that malaria parasites undergo pre-erythrocytic stages in the liver before entering the blood stream. Wojciech Krotoski in 1982 reported the presence of the dormant stages in the liver as the final stage in the life cycle of the parasite (Cox, 2010**;** CDC, 2010)

At this juncture, a clearer picture of the causative agent and transmission pattern started unfolding. This understanding enhanced the development and implementation of malaria control measures. Efforts were made to control the vectors by draining the swamps and water bodies where they breed, and also by using kerosene or oil to destroy their developmental stages (Burton, 1967). Spreading kerosene on mosquito breeding sites was one the first strategy adopted in the control of malaria (Burton, 1967). Preventive measures were advocated such as the use of insecticide-treated bednets, repellents and protective clothing. Unfortunately, total prevention of malaria has been set back, due to the

development of resistance by vector species to insecticides such as dichlorodiphenyltrichloroethane (DDT) (Subbarao, 1988; Baird, 2000; Corbel *et al*., 2007).

The earliest recorded treatment for malaria dates back to 1600. This was traceable to the native Peruvian Indians who used the bitter bark of the Cinchona tree to treat fever (CDC, 2010). The quinine from Cinchona tree extracts was the mainstay of treatment until the Chinese developed another potent drug from the worm wood *Artemisia annua* L. (CDC, 2010). Artemisinin (Qinghaousin) was first isolated in 1971 from the aerial parts of *Artemisia annua* L., which is a Chinese herb used in the traditional treatment of fever and malaria (Klayman, 1985; Li and Rieckmann, 1992).

The therapeutic effectiveness of artemisinin is limited by a number of factors such as short half-life, neurotoxicity, and low solubility which affects its bioavailability (Balint, 2001). However, attempts to improve the pharmacokinetic parameters of artemisinin have led to the development of several semi-synthetic derivatives; dihydroartemisinin, artemether, arteether and artesunate, which are less toxic and have shown greater potency with enhanced stability (Van Agtmael *et al*., 1999).

There was a recorded success in eradication of malaria in Europe and North America (WHO, 2005). However, the issue of 'imported malaria' from migrant workers and tourists from a number of endemic regions is still an issue of concern. The emergence of drug resistant parasites has further heightened this fear. Resistance of parasites to chloroquine was first reported in East Africa in 1978 (Fogh *et al*., 1979). Huong *et al*., (2001) reported a decrease in the sensitivity to artemisinin. WHO recommended the use of artemisinin combination therapies as a way to fight parasite resistance to already existing antimalarials (WHO, 2006).

1.1.2 Transmission of malaria

Transmission of the parasite to man is achieved by the intravenous inoculation of sporozoites from an infected female anopheles mosquito. These sporozoites are themselves not pathogenic, but are rather a transient phase, whose survival and development are necessary for the completion of the parasite's life cycle and subsequent transmission. The sporozoites invade the hepatocytes where they multiply rapidly for about a week or two, releasing thousands of merozoites. Every successful sporozoite has the potential of producing about 20,000 merozoites (Ménard, 2000). The merozoites rupture the hepatic cells and invade the erythrocytes where they undergo proliferation and invade more erythrocytes. This process of replication asexually continues, but some differentiate into gametocytes. These gametocytes are the stages ingested by the female anopheles mosquito in the course of its blood meal. The parasite develops in the mosquito vector for about 2 weeks. The ingested gametocytes form gametes which fuse in the midgut giving rise to zygotes. The zygotes in turn develop into ookinetes which penetrate the gut wall to develop into a form known as the oocysts. The oocytes burst and releases sporozoites which migrate to the salivary gland of the mosquito vector. As the mosquito takes a blood meal which is necessary for its reproductive process, the sporozoites are injected into the new host thereby maintaining transmission.

An effective and efficient transmission requires the presence of the parasite and human host, and an anthropophylic (bite humans) vector. The species *A. gambiae* have a unique anthropophilic tendency. Effective transmission requires endophagic or exophagic (indoor and outdoor biting) and an exophilic (rest outside) female Anopheles mosquito (WHO, 2004). Female mosquitoes need a blood meal to produce eggs (Moorthy *et al*., 2004). Occasionally, malaria can be transmitted congenitally or through blood transfusion, organ transplantation, or through shared needles and these transmission routes do not require a vector since the transmission is direct. The parasite extrinsic cycle shortens in a

warmer environment, while temperatures below 16ºC adversely affect the extrinsic cycle of malaria parasites and the biting activity of vector species (Colluzi, 1999). This may partly explain the fact that the disease is endemic in tropical and warmer regions of the world. The concern about global warming and its consequences as regards malaria prevalence and distribution may not be unconnected to the observed temperature effects. Hot weather could also cause people to sleep outdoors, without bed nets, and even to expose their skin to mosquitoes (CDC, 2010). The rate of transmission in humans could be determined by genetic composition of the populace, for instance carriers of sickle cell anaemia are usually not prone to malaria attack (Carter and Mendis 2002). Some innate, inborn or acquired immunity may also cause variations in a given population. The vectoral capacity and preferences for biting and resting are also contributing factors to these variations (WHO, 2004; CDC, 2010).

1.1.3 Life Cycle of malaria parasite

The life cycle of malarial parasites involves the asexual and the sexual stages of development. The parasites have obligatory phases in the human host and the mosquito vector (Stanley, 1997). The pre-erythroctic and the erythrocytic stages are basically the two phases in the parasites life cycle (Fig: 1.1.3.1)

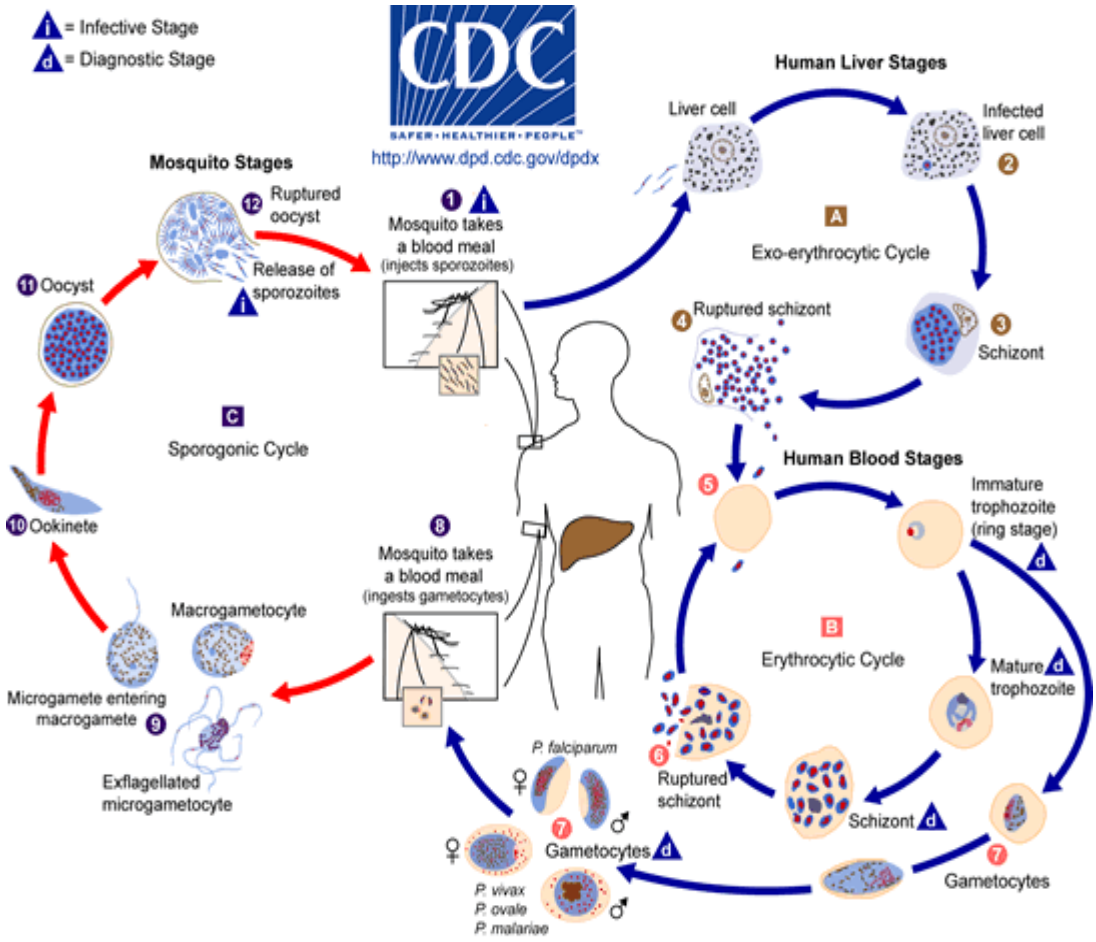


Fig 1.1.3.1 Life cycle of malaria parasite
(Source:http//www.dpd.cdc.gov/dpdx)

1.2 Malaria Epidemiology

Epidemiology may be defined as that branch of science which studies the state of health or health related incidence and distribution in a given population and the application of the unraveled information to control health problems (Last, 2001). Malaria epidemiology is geared towards an in-depth understanding of the disease distribution and transmission patterns which are relevant to the control of malaria. Malaria is said to be endemic in Africa due to the observed consistency in the pattern of *Plasmodium* transmission in humans over a

number of years. The global epidemiology spectrum of malaria as recorded in several studies showed varying transmission intensities and patterns (Snow *et al*., 2005). It is evident from these studies that the intensity of transmission differs within continents and within countries (Snow *et al*., 2005, Guerra *et al*., 2006). Stable malaria, as is the case in sub-tropical and tropical regions is characterized by a genetically diverse parasite surface antigen (Bull *et al*., 1998).

The rate at which parasites develop resistance to the already existing antimalarial drugs is daunting. The genetic diversity and mutations of these parasite populations could be some factors contributing to parasite resistance (Jianbing *et al*., 2003). Genetic diversity is inevitable in regions of high *Plasmodium* transmission such as Africa and Papua New Guinea (PNG) where multiple infections are the order of the day (Babiker *et al*., 1994; Paul *et al*., 1995). This provides more avenues for the spread of alleles which favour the parasite's population (Babiker *et al*., 1994; Paul *et al*., 1995). Areas such as PNG experience local variations in malaria epidemiology between villages and even between households within the same village (Cattani *et al*., 1986). The varying patterns of malaria epidemiology across the globe could be attributed to the complex interplay of the environmental, vectorial, human factors and parasite species.

1.3 Malaria Pathogenesis

The human hosts, as well as the parasite species, have evolved various physiological characteristics and behavioural traits that seem to favour the disease pathology (CDC, 2010,). An in-depth understanding of these variations in man and parasite is vital for the control and subsequently eradication of malaria. The parasite's life cycle gives a good idea of the transmission process in the human host. The exo-erythrocytic stage is not responsible for infection

(Miller *et al*., 1994). The mosquito infective stage is a product of the erythrocytic cycle. The size of the parasite population is to a large extent amplified, resulting in differentiation into gametocytes. Over 20 merozoites are produced by a single mature parasite of *P*. *falciparum* within a time frame of 48 hours (Miller *et al*., 2002). This *Plasmodium* parasite, as an apicomplexan parasite, possesses three organelles; the rhoptries, micronemes and dense granules on the apical or invasive end of the parasite. The secretions from the apical organelles, post contact with the red blood cells, induce their exposure to antibodies (Miller *et al*., 2002). The receptors located in the micronemes, rhoptries and cell surface initiate the invasion of the liver by the sporozoites and red blood cells by the merozoites (Adams *et al*., 1990). Some complications associated with *P*. *falciparum* include anaemia, hypoglycaemia, cerebral malaria, renal failure, and noncardiac pulmonary edema (Miller *et al*., 1994). The most frequent complaints and symptoms include fever, chills, nausea, headache, and weakness, loss of appetite, diarrhea, vomiting and malaise (English *et al*., 1996., Biersmann *et al*., 2007., Legros *et al*., 2007). In various organs, like the heart, lungs, liver, kidney, subcutaneous tissues, brain and placenta, parasite sequestrations have been observed, and cerebral malaria could occur as a result of the parasites sequestered in the brain, which may involve the intracellular adhesion molecule 1(ICAM-1) receptor (Newbold *et al*., 1999)

1.4 Global Malaria situation

Malaria has been one of the world's most devastating parasitic diseases, and the parasite incriminated is *Plasmodium falciparum* (Fisher and Bialek, 2002). Malaria is an issue of global public health concern. More than 40% of the world's population lives in malaria endemic regions. An estimated 300-500 million new clinical cases are reported annually (Snow *et. al*., 2005). Those at risk of malaria each year are about 3.2 billion people.

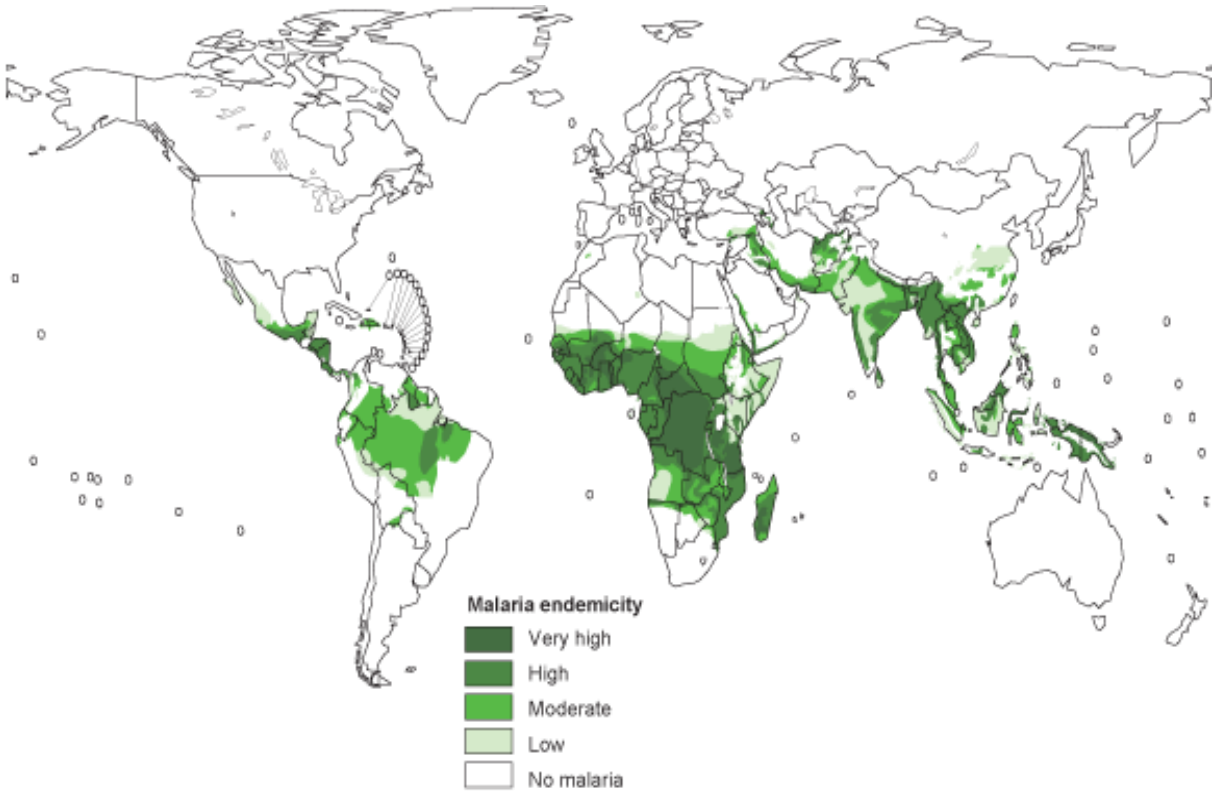


Fig 1.4.1 Map showing the malaria risk countries. World malaria report 2005: Global distribution of malaria transmission risk.
Source: http://www.rollbackmalaria.org/wmr2005/html/map1.htm

The devastation caused by this disease is alarming, particularly in sub-Saharan Africa where more than 90% of deaths occur (WHO, 2005). Globally, the two regions with the highest malaria transmission intensity are Oceania and sub Saharan Africa. In sub-Saharan Africa, a child dies of malaria every second (Snow *et al*., 2005). This is a death toll which exceeds the mortality rate from AIDS. It also constitutes a severe threat in some regions of South–East Asia, Oceania, Haiti, the Amazon basin of South America, and the Dominican Republic (CDC, 2010). *P*. *vivax* is mostly prevalent in Central America, the Middle East, and India (CDC, 2010).

Among the high risk individuals are children less than 5 years old (Breman, 2001). They are chronic victims of this pandemic. The debilitating effect of

malaria in the very young populace is disheartening. That many children die from the disease is not questionable, but it is also important to realize that even in those surviving, a lot may have been damaged, altered or malfunctioning due to hypovitaminosis arising from the several bouts of infection in this populace. It has also been observed that malaria infection causes poor school attendance and impairs the physical and intellectual development of affected individuals (Sachs and Malaney 2002). Though a host of other factors contribute to the endemicity of this disease, the regions mostly affected are the poorer regions of the world. It is also important to realize that the low immunity of this age group to infection, as well as the compromised immunity of pregnant women, further aggravates the situation. Pregnant women, especially those in their first trimester or the primigravidae and the unborn foetus are adversely affected as parasites are sequestered in their placenta (Rowe and Kyes, 2004). Another group of individuals at high risk are non-immune travelers, refugees and migrant workers entering endemic regions (Rooth, 1992). The movements of refugees, migrant workers and non-immune travellers across borders have also encouraged the global spread of the disease (Rooth, 1992).

The global transmission pattern of malaria some decades ago highlighted the success of antimalarial control measures in the temperate regions of the world and the increased prevalence of the disease burden in the tropics. However, the current global warming is threatening the stability of these temperate regions.

1.5 State of the pandemic in Nigeria

Malaria is endemic in Nigeria. Malaria is common usually in malnourished populations, especially children and low class populations (Sanjoaquin and Molyneux 2009). It is recorded that the dominant parasite incriminated is *P. falciparum*, and on rare occasions *P. vivax* (Mendis *et al*., 2001; Carter and Mendis 2002). The dominant vector is the *Anopheles gambiae,* and to a less

extent *A*. *funestus* (WHO, 2004*)*. According to WHO (2007) statistical data, the Nigerian population is about 149,000,000, and the number at risk is about 144,600,000. About 97% of the population lives in areas at risk of malaria. Nigeria records an annual death rate of 300,000 due to malaria, and of this number 25% are infant-related mortality, 30% child-related mortality and 11% maternal mortality. In health facilities, about 60% of the outpatient cases are due to malaria (WHO statistics, 2007). It was estimated that an individual can suffer from as many as four bouts of malaria annually (WHO, 1995). The frequency is rapidly increasing due to onset of resistance by parasite species to existing antimalarial drugs (WHO, 2000). Nigeria recorded a progressive increase in the incidence throughout the country, ranging from 1.12 million in 1990 and 2.25 million by 2000 and 2.61 million in 2003 (WHO, 2005). The economic burden of the disease in Nigeria is disheartening. Work performance, effectiveness and commitment are compromised. School attendance records a high level of truancy due to malaria. Increase in medical expenditures and bills are alarming. This generally leaves the populace illiterate, poorer, weaker and less productive.

1.6 Traditional medicinal practices in Nigeria

Nigeria is made up of diverse cultural entities that embody different ethnic groups. These locations are geographically described as the north; where the Hausas and the Fulanis concentrate, the southwest is made up of the Yorubas and the southeast the Igbos. There are also other sizable groups that live in these locations. They include the Kanuri in the north, the Edo, Efik, and Urhobo in the south, and the Tiv in the center. The oldest component of the health system in Nigeria is made up of traditional healers and birth attendants. They are the major providers of primary health care. The practices of these healers culturally meet the needs and the expectations of the patients (Iwu, 1994). A good number of Nigerians hold on tenaciously to the belief that the

remedies used by the traditional healers are safe and more tolerated by the body (De Smet, 1991). They emphasize the availability, affordability, effectiveness and reproducibility of these herbal remedies.

Traditional medicine, as it is popularly called, is a cultural heritage (Kaou *et al*., 2008) of various communities in most countries of the world. It encompasses different kinds of folk medicine, unconventional medicine, and indeed any kind of treatment regimen that is acknowledged and practiced by a community or ethnic group. It embraces the totality of indigenous knowledge, attitudes, beliefs and practices, of a locality or region which they apply in diagnosis, prevention and treatment of diseases. This depends absolutely on practical experiences and observations which run across the generations, whether acquired, learnt, or by instinct.

Nigeria is a multi-cultural country with diverse flora, fauna, and traditions to take care of its teeming population. Herbal remedies and preparations are used by traditional healers in the treatment of disease conditions, including malaria (Farnsworth and Morris, 1976). The traditional healers and birth attendants in Nigeria have a great diversity of plants to explore in their practice. Nigeria has two major seasonal variations; the rainy and dry season. The distribution of plants in Nigeria vary because of the diverse ethnic groups with their diverse geographical locations and because of seasonal variations. Differences also exist in the method of using or applying these herbal remedies. Such diversities, however, are traceable to the cultures and traditions of the various locations. The diversity of plants used in traditional medicine varies with culture and tradition (Farnsworth and Morris, 1976).

The traditional medicinal practitioners use plants singly or in combination in their formulations (Igoli *et al*., 2005). Decoctions, infusions, macerations and inhalations are some of the ways these herbal remedies are taken (Igoli *et al*., 2005). Public health education and community enlightenment programmes

could help to reduce the level of illiteracy in Nigeria and may, in the long run convince the traditional healers on proper dosage formulations. It could be possible to reduce the number of individuals who are carriers of the disease, but who are asymptomatic, through the synergistic application of control methods.

1.7 Malarial Control Strategies

1.7.1 Targets to vectors

Vector control is an effective way to prevent malaria transmission (WHO, 2004). Indoor residual spraying (IRS), insecticide treated nets (ITN), ultra-low volume sprays and the screening of houses are ways to fight the vectors. Combination of IRS and ITN could be of immense help in the management of insecticide resistance. However, effective application of IRS depends on adequate professional vector control services, good planning and a good understanding of the activities of the vector species (Rowland, 1999; WHO, 2006). There is no doubt that a certain degree of success has been recorded through these control mechanisms. However, this requires that the vectors feed and rest indoors. The disadvantages inherent in these approaches include the fact that exophagic vectors are selectively excluded, spraying may adversely affect humans, since adequate information on the best insecticide is insufficient. Moreso, there availability of human and financial resources to sustain control effort is limited (WHO, 2004). Significant increase in parasite resurgence could be linked to a decrease in the indoor residual spraying for vector control.

Larvicides can be effective by killing larvae, or blocking their breathing trumpets as is observed when kerosene is poured on the stagnant water surfaces (CDC, 2010). The challenge here is all too obvious since the kerosene is not supplied free of charge. There is a limit to which method could be sustained. This is also the situation with using DDT, insecticide treated nets (ITNs), pyrethroids and

organophosphates (WHO, 2004). Most of the populace often resorts to domestic sanitation, water drainage and naturally-induced biological control measures which, though necessary, are grossly inadequate to control vectors. The oil extracted from *Citrus limon* L. Burm.f. has been shown to be effective against mosquito larva (Zayed *et al*., 2009). This is in line with the recommendations in favour of plants as alternative sources to control mosquito vector due to their bioactive chemical composition (Park *et al*., 2002). The introduction of genetically- modified mosquitoes has been proposed, but the speed of replication and distribution may be inadequate to counterbalance the naturally existing vector species (WHO, 2004). The speed of replication could be hindered by adaptation and survival of the fittest. A new population will have to develop a special means of survival for sustainability. This more often than not is met with challenges. Health education and community involvement are indispensable tools for an effective use and application of existing control measures.

1.7.2 Targets to Humans

This approach is aimed at reducing human vector contact (Rowland *et al*., 2004a). Use of insecticide treated nets (ITNs), repellents, protective clothing, house screening or larval control are some of the ways to achieve this. Insect repellants such as DEET (*N*,*N*-diethyl-3-toluamide) are also effective (Rowland *et al*., 2004a, Rowland *et al*., 2004b). Public health education, community involvement and enlightenment campaigns are vital tools to achieving the set objective. The difficulties observed in such a method include the issue of keeping individuals indoors, due to hot weather and since these are people engaged in work which necessitates leaving the temporary comfort of their sprayed homes (CDC, 2010).

1.7.3 Targets to parasites

This aims at destroying or killing the parasites. One major way of doing this is by the use of chemotherapeutic agents (Dorsey, 2000). In the case of malaria, antimalarial or antiplasmodial agents are administered. Most of the populace in Nigeria and Sub-Saharan Africa depend solely on the herbal preparations from the traditional healers in the treatment of various ailments, including malaria (Igoli *et al*., 2005). Only very few of the populace visit a local health centre for treatment.

1.8 Challenges to Malaria Control Efforts

1.8.1 Environmental factors

There are inherent factors prevalent in a community or region. Such factors include climatic factors and geographical locations or locality, the type of soil, the pattern of rainfall, speed of wind, temperature, altitude, water salinity and vegetation. These, in one way or the other, affect control measures and are natural factors which humans are unable to alter, for instance temperature variation which is a factor in explaining the geographical distribution of malaria (Sachs and Malaney 2002).

1.8.2 Factors attributable to vectors

This refers to the vectoral capacities and their longevity. *Plasmodium falciparum* sporogony fails to occur at low temperatures, while high temperatures favour their transmission (Abeku *et al*., 2003). Optimal temperature conditions between 20ºC-30ºC are required for effective transmission of the *Plasmodium* parasites. Below this temperature, transmission is delayed or altered, as parasites do not develop at temperatures below 16ºC (Sachs and Malaney 2002). Some vector species suspend biting activity at very low temperatures (Colluzi, 1999) while others have, over time, developed some adaptations to survival. Some are endophagic, while others are exophagic (WHO, 2004). Endophilic and endophagic habits are genetically induced measures to vectoral survival. The

success of indoor residual spraying (IRS) relies heavily on the mosquitoes feeding and resting indoors (WHO, 2004). However, this is not the habit of most mosquitoes and the pyrethroid used in spraying sooner or later wards them off. Factors that are favourable to vectors encourage transmission of parasites to humans. Vectoral resistance and behaviour adversely affect control measures (WHO, 2004; CDC, 2010). Resistance may be due to detoxification of insecticide by enzymes or by mutation on its target site; sodium channels for DDT and pyrethroids and acetylcholinesterase for organophosphates (CDC, 2010). Similarly, the exophilic species which prefer to rest outdoors are less likely to acquire lethal doses of insecticides sprayed on the walls, when comared to the endophillic species which rest inside (CDC, 2010).

1.8.3 Factors attributable to Humans

Human activities in a given area are also important factors that oppose control efforts, e.g. deforestation, reforestation. Agricultural practices, such as dams, canals and irrigation contribute to the heavy burden of malaria in affected regions. Land cover and land use play a great role in malaria transmission (Hay *et al*., 2000). Global warming, urbanization and its consequences, especially in developing countries, pose challenges to control mechanisms. Knowledge, attitudes and perceptions of the populace could make control feasible or difficult. Non-compliance with adequate treatment modalities sustains prevalence and encourages resistance. It heightens the epidemiology and pathogenicity of infection. Lack of an adequate and sustained supply of DDT and ITN to the populace poses its hindrance (CDC, 2010). Poverty is a major factor, and that could be why malaria is often referred as the ‘disease of the poor’.

1.8.4 Factors attributable to parasites

The major concern in this regard is the resistance of parasites to existing antimalarial drugs (Jianbing *et al*., 2003). . The rate at which these parasites develop resistance is alarming. Chloroquine, which was the mainstay of treatment, has met with parasite resistance (Jianbing *et al*., 2003). The relief from artemisinin, its derivatives and combinations are seriously threatened by several treatment failures recorded in some regions. The first case of treatment failure of artemether-lumefantrine has been documented in a Japanese traveler (Mizuno *et al*., 2009). Reduced sensitivity to artemisinin has been reported in China and Vietnam (Huong *et al*., 2001, Yang *et al*., 2003). There is therefore an urgent need for new and effective antimalarials.

1.8.5 The urgent need for new antimalarials

The need to employ new, effective and sustainable strategies in the development of new antimalarials is of utmost importance. Chloroquine resistance was first documented in East Africa as far back as 1978 among non-immune travelers (Fogh *et al*., 1979). Subsequently, similar reports emanated from the rest of tropical Africa (Trape, 2001) where chloroquine has been the first line treatment for malaria (Trape *et al*., 2002). Sulphadoxinepyrimithamine (SP) is another widely used antimalarial. It is relatively cheap and available, but has, unfortunately, been met with resistance. There have been cases reported on the therapeutic failures of SP in many areas in Asia, South America and Africa (Plowe, 2003). Treatment failures have been recorded with artemisinin combination treatments (ACTS) in various regions (Jambou *et al*., 2005; Rogers *et al*., 2009).

The conditions that favour the development of drug resistance by parasites include the absence of a variety of drug treatment options, high migration levels of population, significant under dosing, the presence and usage of adulterated drugs, prescription delivery by non-qualified personnel and non- compliance with treatment. Little wonder that the endemicity of malaria, and the resistance to existing drugs, affects the poorer, underdeveloped, and illiterate regions of the world. There is a need to identify effective antimalarials to which the parasites are unlikely to develop resistance. One of the best ways of achieving this is by the application of combination therapy for malaria.

1.9 Malaria combination therapy

The use of combination therapy to fight drug resistance was proposed in 1955 (Covell *et al*., 1955). This was further advocated and emphasized by Peters in 1970, and was also reinforced by White and Olliaro (1996). Recently, the World Health Organization (WHO, 2001) has recommended the use of artemisinin combination treatments (ACTs) to fight the issue of resistance, and

as a first line treatment in countries where parasite resistance has severely compromised the therapeutic efficacy of drugs already in use (WHO, 2007). The combination of artesunate (AS) + amodiaquine (AQ) has currently been adopted as a first line treatment in 15 African countries (WHO, 2006). Excellent clinical responses and tolerability were recorded in Senegal through the use of AS+AQ, AS+ mefloquine (MQ) and AQ+SP in the treatment of uncomplicated falciparum malaria (Faye *et al*., 2007). However, a further study in children from Burkina Faso reported the onset of minor discomforts which disappeared at the completion of dosage (Barennes *et al*., 2004). Combination therapy is an effective means to the optimal control of malaria in developing countries (Guerin *et al*., 2002). Further studies recommended the use of combination treatment with rapid elimination rates to reduce drug resistance pressure in Africa (Watkins *et al*., 1988, Watkins and Mosobom, 1993).

Combination treatment has several advantages over monotherapies. It is a good anti-plasmodial strategy and is effective in preventing or delaying resistance (White et. al., 1996; White, 1998) and it effectively slows down selection pressure since each component is target specific. Combination therapy extends the useful therapeutic life of the component agents and reduces treatment times. It has shown proven efficacy in treating tuberculosis and HIV/AIDS (Nyunt and Plowe, 2007). Combination therapy also has the advantage of increasing patient compliance with treatment, (Nosten *et al*., 1994). It lowers the total cost of production, transport and dispensing, especially in a fixed dose regimen. Importantly, also, it reduces the risk of patients taking the wrong dosage especially when prescribed by the unqualified drug dealers seen in most underdeveloped countries. It also plays a vital role in simplifying patient counseling and education. It prevents and delays the progression of resistance and augments cure rates (Price *et al*., 1997). The concept of drug combination treatment is based on the premise that parasite resistance is delayed when it has to mutate in several sites with independent mechanism of action. The essence of a good drug combination relies on the

fact that at least one of the agents in the combination will be clinically active even in the onset of resistance (White, 1998). Since our environment is richly blessed with diverse natural products, they should be utilized where possible, to address our health problems and challenges. There is need for new, integrated, and multivalent treatment strategies to delay the progression of resistance, especially now that treatment failures, even with ACTs, have been recorded in the Thai-Cambodian border and Thailand (Jambou *et al*., 2005; Vijaykadga *et al*., 2006, Wongsrichanalai and Meshnick, 2008, Rogers *et al*., 2009). This has challenged scientists to search for new drug combinations by exploring further our natural resources.

1.9.1 ROLE OF NATURAL PRODUCTS IN MALARIA TREATMENT

1.9.1.1 Antiplasmodial agents from natural products

Nature continues to be an ever-evolving source for substances and compounds of great medicinal value. Diverse groups of natural products have offered tremendous help in the treatment of various illnesses. Most of these natural products are taken in their natural states in the traditional medicinal practices. The extraction, purification, isolation and characterization of compounds from natural sources with good medicinal values have caught the interest of industrialized societies and scientists to explore the flora and fauna of our rich ecosystem to identify their medicinal uses and properties. Natural products can offer new and novel scaffolds for development of new antimalarial drugs. They contain several classes of antimalarial compounds including; alkaloids, terpenoids, flavonoids, chalcones, peptides xanthones, quinones, coumarins and fatty acids.

1.9.1.2 Antiplasmodial agents from plants

Plants have been good sources of antimalarial agents (Farnsworth and Morris, 1976). The activities of herbal remedies and extracts from plants are traceable to the medicinal properties of natural products in them. The quinolines and artemisinins are antimalarial drugs from plant products which have contributed immensely to the fight against malaria (CDC, 2010). Various classes of compounds with antimalarial activity have been isolated from plants and other natural sources. These are discussed in detail below (section 1.9.2).

Unsaturated hydrocarbons are also a class of compounds which could be derived from plant products and food supplements. The antimalarial activities of this class of hydrocarbons have been documented. Kurmaratilake *et al*., (1992) demonstrated the antiplasmodial activity of Omega 3 and Omega 6 fatty acids.

1.9.1.3 Antiplasmodial agents from marine organisms and microorganisms

More than 70% of the earth's surface is occupied by the marine environment. The marine body is a potential source of diverse medicinal products, new compounds, nutritional food supplements, cosmetics and agrochemicals (Blunt *et al*., 2006). The diverse medicinal potential of the marine body is not unconnected to the wealth of diverse resources, both biological and chemical that abound in it. Diverse marine life forms such as algae, sponges, corals, ascidians and fungi which inhabit the marine environment have been studied to reveal their medicinal values (Faulkner, 2001, 2002). Such explorations of the flora and fauna of the marine environment have revealed that the marine environments are rich sources of potent compounds of diverse medicinal importance, however no isolated or synthesized drug from the marine

environment has yet made its way to clinical application (Newman and Cragg 2004). Their antimicrobial, anti- inflammatory, antitumur, antibiotic and antimalarial properties have been documented (Hill, 2006; Laurent *et al*., 2006; Nakao and Fusetani, 2007; Carbelleira, 2008; Loset, 2008). A survey of different marine invertebrates by Mendiola *et al*., (2006) showed the *in vitro* and *in vivo* antiplasmodial activity of seven extracts from chordates, porifera, echinoderms and cnidarians. The chordates antimalarial activity was traceable to the major metabolites like alkaloids and polypeptides. Fresh water organisms, e.g. cyanobacteria, with promising antiplasmodial properties have been found in the marine environment (Gademann and Kobylinska 2009). They have been washed out into the oceans from their fresh water habitats e.g. streams, and rivers (Gademann and Kobylinska 2009). Recently, six pure metabolites and some complex mixtures of fatty acids which exhibited promising PfFabI inhibitory and antiplasmodial activity were isolated from the Turkish marine sponge *Agelas oroides* (Tasdemir *et al*., 2007).

1.9.2 CLASSES OF NATURAL PRODUCTS IN MALARIA TREATMENT

1.9.2.1 Alkaloids

Alkaloids are one of the most important groups of natural products. This class of natural products has been of great medicinal value. Alkaloids have been used in the treatment of parasitic infections. The popular antimalarial drug quinine is an indole alkaloid from *Cinchona succirubra* Pav. ex Klotzsch of the family Rubiaceae. Quinine was the mainstay of malaria treatment for more than three centuries. Indole alkaloids isolated from natural sources has shown promising antiplasmodial activity in both *in vitro* and *in vivo* systems (Frederich *et al*., 2008) Alkaloids showing antimalarial activity could be further classified as; naphthylisoquinolines, bisbenzylisoquinolines, protoberberines, aporphines, indoles, and manzamines. These, have been isolated from extracts of natural products and have exhibited promising anti-plasmodial activity against different strains of malaria parasites.

Extracts of *Triphyophyllum peltatum* (Hutch. and Dalziel) Airy Shaw, of the family Dioncophyllaceae which were identified as containing naphthylisoquinoline alkaloids, exhibited high antiplasmodial activity in *P. berghei* infected mice (Francois *et al*., 1997). Korupensamine a monomeric naphthylisoquinoline alkaloid (IC_{50}=2.0 µg/ml) (Hallock *et al*., 1997) and korundamine A, a heterodimeric naphthylisoquinoline alkaloid with antiplasmodial activity (IC_{50}=1.1 µg/ml) (Hallock *et al*., 1998) were obtained from *Ancistrocladus korupensis* D.W. Thomas and Gereau, of the family Ancistrocladaceae. The antiplasmodial and cytotoxic activities of 53 bisbenzylisoquinoline alkaloids were recorded by Angerhofer *et al*. (1999). These alkaloids showed activity against the D6 clone and W2 clone of *P. falciparum* in the IC_{50} range of 29-1500nM and 59-4030nM respectively. Antiplasmodial activity of compounds from the stem bark of *Isolona*

qhesquierenina of the family Annonaceae has been reported by Mambu *et al.* (2000). The promising antiplasmodial activities of the protoberberine alkaloids were shown by Wright *et al*. (2000). Recently, the antiplasmodial activities from three ascidians have been reported by Mendiol *et al*. (2006). Rao *et al*. (2006) reported the antimalarial activities of manzamine alkaloids isolated from an Indonesian *Acantho strongylophia* sponge. Previous studies also documented the antimalarial activity of manzamine alkaloids (Ang *et al*., 2000, Roa *et al*., 2004)

1.9.2.2 Terpenoids

This class comprises a large group of structurally diverse natural products (Zwenger and Basu, 2008). They are further divided into other groups which include sesquiterpenes, diterpenes, triterpenes, and miscellaneous terpenes. Sesquiterpene lactones abound in the genus *Vernonia*. Numerous researchers have also recorded the antimlarial activity of the sesquiterpenes, from *Distephanus angulifolius* (DC.) H.Rob. & B.Kahn of the family Asteraceae (Pedersen *et al*., 2009), *Tithoma diversifolia* A.Gray of the family Asteraceae (Goffin *et al*., 2002), *Drechslera dematioidea* (Bubák & Wróbl.) Subram. & B.L. Jain of the family Pleosporaceae (Osterhage *et al*., 2002), and the Red algae *Laurencia obtusa* (Topcu *et al*., 2003). Novel cembradiene diterpenoids with antimalarial activity (IC_{50} 15.0 µg/ml) were isolated from the Caribbean gorgonian octocoral *Eunice* species (Wei *et al*., 2004). Five antimalarial labdane diterpenoids from *Aframomum zambesiacum* K.Schum. seeds of the family Zingiberaceae were isolated and tested against an FCBI chloroquine resistant strain (Kenmogne *et al*., 2006). Moderate antiplasmodial activity was recorded from novel furanoterpenoids isolated from the ethyl acetate fraction of *Siphonochilus aethiopicus* (Schweinf.) B.L.Burtt of the family Zingiberaceae (Lategan *et al*., 2009). Artemisinin and its derivatives are sesquiterpene trioxane lactones and their antimalarial activity is traceable to the endoperoxide bridge present in this group (Martinelli *et al*., 2008).

1.9.2.3 Quassinoids and limonoids

The activities of two quassinoids reported by Okunade A.L., (2003) include ailanthone (IC_{50}=0.03 µg/ml) and 6α-tigloloxychaparrinone (IC_{50}=0.06 µg/ml). These were isolated from *Ailanthus altissima* (Mill.) Swingle, in the family Simaroubaceae. Pasakbumin B, pasakbumin C and eurycomanone with antimalarial activity of (IC_{50} 22.6 ng/ml, 93.3 ng/ml and 40.0 ng/ml) respectively were isolated from *Eucalyptus longifolia* Link of the family Myrtaceae (Kuo *et al*., 2004; Chan *et al*., 2004). Quassinoids from the roots of *Simaba orinocensis* Kunth of the family Simaroubaceae showed antiplasmodial activity (IC_{50} 3.0 and 3.67 ng/ml vs. 3.2 and 8.5 ng/ml respectively) (Muhammad *et al*., 2004) against the D6 and W2 strains. However, quassinoids have shown *in vivo* toxicity which could be attributed to protein synthesis inhibition and efforts to develop selective inhibitors of parasite and host cell ribosomes has been faced with the challenge of differentiating between the two. This could possibly suggest that the recorded antimalarial activity of the quassinoids may be due to its toxicity to the host cells. Limonoids with *in vitro* antimalarial activity have been reported from *Cedrela odorata* L. of the family Meliaceae (Bray *et al*., 1990), *Khaya senegalensis* A.Juss. of the family Meliaceae (Khalid *et al*., 1998) and *Khaya grandifoliola* C.DC. of the family Meliaceae (Bickii *et al*., 2000). *Azadirachta indica* A.Juss. (Neem) belonging to the family Meliaceae which has been widely used as an antiplasmodial agent belongs to this group.

1.9.2.4 Flavonoids and Chalcones

Promising antimalarial activities have been recorded for flavonoids. A good number of biflavones have been documented to possess moderate to good antiplasmodial activity as shown in *Wikstroema indica* (L.) C.A.Mey. of the family Thymelaeaceae, cikokianin B and C (IC_{50} 0.53 and 0.56 µg/ml) (Nunome *et al*., 2004) respectively. Isolates from *Ochna integerrima* (Lour.) Merr. of the

family Ochnaceae (IC_{50} 80 ng/ml) (Ichino *et al*., 2006) and *Garcinia livingstonei* T. Anderson of the family Clusiaceae (IC_{50} 6.7 µM) (Mbwambo *et al*., 2006). Licochalcone A, isolated from *Glycyrrhiza inflata* Batalin belonging to the family Leguminosae has shown good antiplasmodial activity. This is traceable to its ability to inhibit the protease activities of Plasmodium (Chen *et al*., 1994). Nyasol a chalcone, isolated from *Asparagus africanus* Lam. Of the family Asparagaceae showed weak antiplasmodial activity with IC_{50} = 49 1M (Oketch-Rabah et. al., 1997).

1.9.2.5 Peptides

A number of compounds with antiplasmodial activities have been identified from this group. Two antiplasmodial compounds exhibiting moderate activity against K1 strain (EC_{50}=1.60 and 12.0 µg/ml) were isolated from an insect pathogenic fungus *Paecilomyces tenuipes.* These compounds, beuvericin and beuvericin A, belong to the group known as the cyclodepsipeptides (Nilanonta *et al*., 2000). A cyclic peptide from a marine sponge showed good antiplasmodial activity against *P. falciparum.* The recorded activity is traceable to the apical protrusion which seems to interfere with the invasion of the erythrocytes by the merozoites (Mizuno *et al*., 2002). The antimalarial activity of a peptide microtubule inhibitor, dolastatin 10 isolated from the sea hare *D*. *auricularia* was reported by Fennel *et al*., (2003). Venturamide A and venturamide B (IC_{50}=8.2 and 5.6 µM, respectively) are two cyclic hexapeptides isolated from the marine *Cyanobacterium oscillatoria* with antimalarial activity against the W2 strain and mild toxicity against mammalian Vero cells (Linington *et al*., 2007).

1.9.2.6 Xanthones, quinones and coumarins

Antiplasmodial activities have been recorded for cowaxanthone (IC_{50}=1.5 µg/ml), calothwaitesixanthone (IC_{50} 2.7 µg/ml) and mangostin (IC_{50}= 17.0 µM) which were isolated from *Garcinia cowa* Roxb. of the family Clusiaceae, *Calophyllum caledonicum* Vieill. ex Planch. and Triana of the family Clusiaceae, and *Garcinia mangostana* L. of the family Clusiaceae respectively (Hay *et al*., 2004, Mahabusarakam *et al*., 2006). Isolates from the roots of *Salasia kraussii* Harv. of the family Celastraceae known as quinone methides A and B showed high antiplasmodial activity (IC_{50}= 94.0 and 27.6 ng/ml respectively) (Figueiredo *et al*., 1998). Antiplasmodial activity of compounds isolated from *Vernonia brachycalyx* O.Hoffm. of the family asteraceae, 2´-epicycloisobrachy-coumarinone epoxide (IC50=54 µM) against the Dd2 strain has been reported. Clausarin and dentatin isolated from *Clausena harmandiana* Pierre ex Guillaumin of the family Rutaceae showed antiplasmodial activity (IC_{50}=0.1 and 8.5 µg/ml), respectively (Yenjai *et al*., 2000). The phenylcoumarins isolated from the ethyl acetate extract of *Hintonia latiflora* Sessé and Moc. ex DC. of the family Rubiaceae stem bark showed complete suppression of parasitemia and the development of *P. berghei* schizonts in infected mice (Argotte-Ramos *et al*., 2006).

Clearly plants still hold considerable promise for producing new antimalarial lead compounds, and plant extracts themselves, used either singly or in combination may be effective against the parasites. In this regard, the antiplasmodial activities of seven Nigerian plants were investigated in this study. The details of these plants and the justification for their study are discussed in the next chapter.

CHAPTER TWO

LITERATURE REVIEW OF PLANTS INVESTIGATED AND STUDY OBJECTIVES

2.0 INTRODUCTION

The seven plants used in this study were collected based on their traditional medicinal uses in the treatment of febrile conditions and other related ailments in Nigeria. The traditional healers in Nigeria make use of medicinal plants in the management of several body disturbances (Igoli *et al*. 2005). Of interest is the observation that the fruits and the leaves of the plants investigated are used as food for humans and livestock in Nigeria and other tropical parts of the world.

2.1 Description and medicinal uses of *Vernonia amygdalina*

Fig 2.1.1 *V. amygdalina* plant

Source: **http://www.thetaug.org/vernoniaamygdalina.html**

Vernonia amygdalina Delile, commonly known as bitter leaf, is a shrub or small tree belonging to the family Asteraceae. It grows to a height of about 3 meters

(Gresham *et al*., 2008). *V. amygdalina* is a popular African vegetable which grows in several parts of tropical and subtropical Africa (Oleszek *et al*., 1995; Farombi 2003; Erasto *et al*., 2006). In southwestern Uganda it is locally known as “omubirizi”, and is traditionally used for analgesia and in the treatment of malaria infections (Anoka *et al*., 2008). The greenish powder formulations of this species are sold as AM (anti-malaria) by the Medical Traditional Healers Association in Rukararwe, Bushenyi district, Uganda (Anoka *et al*., 2008). In Nigeria, it is locally known as “ewuru” in Yoruba, “onugbu /olugbu” in Igbo, and “shiwaka/chukwuaka” in Hausa (Ajebesone and Aina, 2004; Igoli *et al*. 2005).

The genus *Vernonia* consists of about 200 species in Brazil, where some are ingested to treat gastrointestinal disorders (Monteiro *et al*, 2001). *Vernonia* species are found to thrive well at temperatures slightly below 30ºC and altitudes at of between 600 m-3000 m. It enjoys loose and moist soil rich in humus and a rainfall range between 750-2000 mm annually. Despite its characteristic bitter taste, *V*. *amygdalina* is used as a vegetable in the western and central parts of Africa (Ajebesone and Aina, 2004; Igile *et al*., 1995). For instance, in Nigeria it is used as a vegetable and herb for soup. It is used to a large extent in tropical Africa for its culinary and medicinal purposes, it is also used in the traditional treatment of malaria, diabetes, diarrhea, veneral disease, hepatitis, gastrointestinal problems, skin disorders, cough, constipation and in the treatment of wounds (Bullogh and Leary, 1982; Aka and Ekekwe, 1995; Igile *et al*., 1995; John *et al*., 1995; Hamil *et al*., 2000; Otshudi *et al*., 2000; Kambizi and Afolayan, 2001; Ajebesone and Aina, 2004). Traditionally, in Ethiopia the decoctions of *V. amygdalina* are used to control tick in cattle (Regassa, 2000). It is also used as an anthelmintic by a good percentage of livestock farmers in Nigeria (Nwude and Ibrahim, 1980; Kudi and Myint 1999).

Most of the livestock farmers in Nigeria use herbal remedies to treat their animals. Further studies documented its use in various folk medicines as an anthelmintic, antiprotozoal and antibacterial agent (Burkill, 1985; Tadesse, *et*

al., 1993; Huffman *et al*., 1996a). The bitter pith from young shoots of *V. amygdalina* helps in the control of intestinal nematode infection when ingested by chimpanzees (Huffman, 2003). Several investigations have reported the nutritional composition of the bitter pith from the young shoots to contain high levels of vitamin A and C, as well as a high level of protein (Ajebesone and Aina, 2004; Igile *et al*., 2005). Phytochemical analysis of *V. amygdalina* has shown the presence of several bioactive principles, which include bitter sesquiterpene lactones, vernolide, vernolepin, vernodalin and vernomydin (Kupchan *et al*., 1969).

The antitumour and antimicrobial properties of *V. amygdalina* have been traced to its bioactive principles (Kupchan *et al*., 1969, Jisaka, *et al*., 1993 Koshimizu, *et al*., 1994). *V. amygdalina* is known to contain steroids, saponins and flavonoids (Rwangabo *et al*., 1986; Ohigashi *et al*., 1991; Igile *et al*., 1994). Flavonoids, sesquiterpenes and triterpenes isolated from *V. amygdalina* have been shown to possess anti-ulcer properties (Arrieta *et. al*., 2003, Giordano *et. al*., 1990, Havsteen, 2002). The lipid composition of *V. amygdalina* was shown to contain 12 fatty acids and amounts to 74.1% of the lipid content (Erasto *et al*., 2007a; Erasto *et al*., 2007b). The most abundant is palmitic acid, (22.2%). The essential fatty acids (EFA) α-linolenic and linoleic acid were present at 21.55 and 15.8%, respectively. Dietary foods remain a good source of EFA since they cannot be manufactured in the body. *V. amygdalina* has been reported by some researchers to show antioxidant activity (Erasto *et al*., 2007a, 2007b), hypoglycemic, antineoplastic, antibacterial and antioxidant properties (Akah and Okafor, 1992; Izevbigie *et al*., 2004; Taiwo and Lee, 1999; Iwalewa *et al*., 2003). *V. amygdalina* has shown antiplasmodial properties, which has been attributed to the sequesterpene lactones isolated from the leaves (Masaba, 2000; Abosi and Raseroka, 2003; Tona *et al*., 2004). Previous studies have shown that *V. amygdalina* may possess anticancer activity (Opata *et al*., 2006; Izevbigie *et al*., 2004).

2.2 Description and medicinal uses of *Psidium guajava*

Fig 2.2.1 *P. guajava* plant

Source: http://www.institutohorus.org.br/download/fotos_guajava/ps guava.htm

Psidium guajava L. belongs to the family Myrtaceae and is a native to tropical America (Begum *et al*., 2002). It grows in the central and northern parts of America, several European and African countries and has long been naturalized in southeast Asia (Begum *et al*., 2002). *P. guajava* is a small tree that grows to a height of about 10 m and has a smooth, but patchy, peeling

bark (Stone, 1970). The leaves are opposite and have a short petiole with prominent veins in the blade (Stone, 1970). *Psidium guajava* thrives in tropical and subtropical regions of the world, however it thrives well in dry climates (Stone, 1970). The common name is guava. It is a popular plant, and most of the local names are very close to its common name in pronunciation or spelling, for instance guave, goyave, or goyavier in French, guavenbaum, guayave in German, goiaba, goiabeiro in Portuguese, guiaba in Brazil, guayaba in Spanish and guava in English. It is also known as ugova, ugwuoba, gurofa and gwaabaa in Nigeria. Its ethnomedical use is wide (Killion, 2000).

A previous report documented the use of guava leaf decoction to treat digestive disorders due to diarrhea (Heinrich *et al*., 1998). Further investigation into its traditional uses showed the different ways in which the leaves are used. They are applied topically on wounds and ulcers, used to treat rheumatic pain, and could be chewed as a palliative measure for toothache (Heinrich *et al*., 1998). A decoction of the leaves and bark given to pregnant women after delivery helps to expel the placenta and serves as a vaginal and uterine wash, especially in leucorrhoea (Conway, 2002). In South Africa, infusions or decoctions of these species are used traditionally to control or treat ailments such as diabetes mellitus and hypertension (Oh *et al*., 2005; Ojewole, 2005). The anti-inflammatory and analgesic effects of this species have been reported (Ojewole, 2006; Murugananda *et al*., 2001). In Brazil, the ripe fruit, flowers and leaves are traditionally used as a decoction to treat anorexia, cholera, diarrhea, digestive problems, dysentery, gastric insufficiency, skin problems, sore throat, ulcers, vaginal discharges and laryngitis (Holetz *et al*., 2002; Cybele *et al* 1995). In West Africa, Latin America and the Caribbean, decoctions of these species are used in the treatment of diarrhea and problems associated with indigestion (Mitchell and Ahmad, 2006b). The decoctions which serve as a diuretic are also used in the treatment of sore throat, laryngitis and mouth swelling. The ethanol and aqueous extracts of *P. guajava* at a concentration of 80 µg/ml showed significant antidiarrhoeal activity (using morphine as the

reference standard) due to their ability to inhibit 70% of the solution-induced contractions of isolated guinea pig ileum (Lutterodt, 1992; Tona *et al*., 1999). *P. guajava* species have also shown antibacterial, antimicrobial (Coutino *et al*., 2001, Arima and Danno, 2002; Chah *et al*., 2006), and antifungal activity against *Arthrinium sacchari* M001 and *Chaetomiu funicola* M002 strains (Sato *et al*., 2000). Its potency against *Propionibacterium acnes* has also been reported (Qadan *et al*., 2005).

The aqueous extract of *P. guajava* showed-dose dependent *in vitro* cytotoxic activity (at a dose of 1.0 mg/ml) against the cancer cell line D4-145 (Chen *et al*., 2007). It was noted that the viability of the D4-145 cell lines after incubating them with the extract for 48 and 72 hours was reduced to 36.1% and 3.6%, respectively (Chen *et al*., 2007). It has further been shown that the essential oils from the leaves of these species showed activity against human epidermal carcinoma of the nasopharynx (KB) and the murine leukemia (P388) cell lines at a concentration range of 0.019 mg/ml to 4.96 mg/ml. It was documented in this report that the IC_{50} value recorded on P388 cell lines was four times greater than the potency of vincristine (Manosroi *et al*., 2006). This effect was attributed to the presence of monoterpenes in the essential oil of *P. guajava* (Cito` *et al*., 2003). Subsequently, *P. guajava* was reported to show significant activity on myocardial injury (Conde *et. al*., 2003). In India, the leaves of this species are used to treat cough (Khan and Ahmad, 1985), and extracts from the leaves have also shown good hepatoprotective effects at a dose of 500mg/kg (Roy *et. al*., 2006). Guava leaf extracts are good sources of natural antioxidants (Ojan and Nihorimbere, 2004). A further study documented the anti-allergic activity of the leaf extract on T cell immunity in mice (Seo *et al*., 2005). The work of Mukhtar *et al*., (2004) has shown the potent hypoglycaemic activity of the water extract at a dose of 250 mg/kg. Anti-diabetic activity of ethanol fractions of this species has been reported in a further study (Mukhtar *et al*., 2006). Other researchers previously reported the hypoglycaemic activity in mice and humans (Cheng and Yang, 1983). An anti-hyperglycemic effect of

the butanol-soluble fraction from *P. guajava* leaves was shown in mouse models with type 2 diabetes (Oh *et al*., 2005). It showed an analgesic effect against thermally and chemically induced nociceptive pain (Ojewole, 2006). Guava leaf extract significantly reduced dysmenorrhea in a double-blind and randomized clinical trial, and proved more effective when compared to the group which received conventional treatment and placebo (Vldislavona *et al*., 2007; Svetlana *et al*., 2007). The species has shown antiplasmodial properties (Nundkumar and Ojewole, 2002).

The bioactivity guided isolation of compounds showed the presence of quercetin, quercetin-3–arabinoside (Lutterodt, 1989) and asiatic acid (Conde *et al*., 2003). Other compounds including flavonoids, saponins, and oleanolic acids were shown to be present in the leaves (Arima and Danno, 2002). A further study reported the presence of nerolidol, ß-sitosterol and ursolic, crategolic and guayavolic acids. Triterpenoids were isolated from the leaves (Begum *et al*., 2002). Ascorbic acid is the most abundant constituent of the skin of *P. guajava* fruit; it is also present in the fleshy part and central pulp (Charles *et al*., 2006). The unripe fruit is reported to have a high concentration of tannins, and when consumed tends to induce constipation (Jain *et al*., 2003).

2.3 **Description and medicinal uses of *Carica papaya***

Fig 2.3.1 *C. papaya* plant

Source: http://www.drosera.pl/papaja-p-42.html

Carica papaya L. of the family Caricaceae is a soft-stemmed perennial plant. It is usually unbranched and can grow to a height of about 20m (De Maria *et al*., 2006). It is believed to originate from the Caribbean coast of Central America and over the years has found its way into many tropical and subtropical climates (Ei Moussaoui *et al*., 2001). *C. papaya* can grow in male, female or hermaphrodite forms. It is found growing wild in many parts of the tropics, and is cultivated because of its sweet juicy fruit which serves as a nutritious food with rich medicinal value, and also because of the ease with which it digests. It

commonly features in breakfasts, cooked in diverse ways, and as ingredients in jellies beverage and juice (Oloyede, 2005)

The fruits, leaves and latex of this species are traditionally used in different parts of the world to treat diverse disease conditions. It is used various places in the treatment of asthma, rheumatism, fever, diarrhea, boils, hypertension and to increase the production of milk in lactating individuals (Zakaria *et. al*., 2006). The fruit and seed extracts of *C. papaya* have shown significant bactericidal activity against *Staphylococcus aureus*, *Bacillus cereus*, *Escherischia coli*, *Pseudomonas aeruginosa* and *Shigella flexneri* (Emeruwa, 1982). The unripe fruit contains glycine, phenylalanine and tryptophan, which have shown antisickling properties (Igbal and Kazi, 1980). The compounds isolated from the methanol and ethanol fractions of *C. papaya* seeds (MCP1 and ECPI), respectively have shown contraceptive activities in male albino rats (Nirmal *et al*., 2005). A dose-dependent antimalarial activity of the petroleum ether fractions of the pulp and rind of *C. papaya* (IC_{50}=18.09 and 15.19 µg/ml, respectively) delayed the development of the ring stages of the parasite after 24 hours incubation with the extract, and a noticeable morphological distortion of the trophozoites was reported (Bhat and Surolia, 2001). Further studies have shown that this species has promising antifungal (Giordani *et al*., 1991), antibacterial (Osato *et al*., 1993) and anthelminthic (Satrija *et al*., 1994) properties. *C. papaya* ameliorates vaginal disturbances due to *Trichomonas vaginalis* (Calzada *et al*., 2007). It has been reported to show anti-inflammatory and immunomodulatory properties (Mojica-Henshaw *et al*., 2003). The anthelminthic activity of *C. papaya* is traceable to the presence of carpain (alkaloid), carpasemine (benzylthiourea) and benzylisothiocyanate (Kermanshai *et al*., 2001). The latex of *C. papaya* at a dose of 8g/kg has been found to be 84.5% effective against *Heligmosomoides polygyrus* (in mice) and *Ascaris suum* in pigs (Satrija *et al*., 1994). These researchers reported a dose response activity of papaya latex and stated that the calculated ED_{100} of papaya latex against adult *Heligmosomoides polygyrus* was 12g/kg using probit

analysis (Satrija *et al*., 1994). Papain which is a major component of this species, is used in tenderizing meat and production of bubble gum. *C. papaya* has also shown hypoglycaemic effects (Olagunju *et al*., 1995). It has been reported as accessible, non-toxic, prophylactic and to be a promising monotherapy against intestinal parasitosis in tropical countries (Okeniyi, 2007).

Scientific studies have shown the potency and cost effectiveness of the fruit when applied topically in the treatment of chronic ulcers in Jamaica (Hewitt *et al*., 2002). A further study attributed this mechanism of action to the activity of the proteolytic enzymes chymopapain and papain, as well as the antioxidant and antimicrobial activities of the species (Anuar *et al*., 2008). Another study reported the use of the fruit from this species in the treatment of burns, as investigated using a mouse model (Gurung and Škalko-Basnet, 2009).

2.4 Description and medicinal uses of *Cymbopogon citratus*

Fig 2.4.1 *C. citratus* plant

Source: http://www.floridata.com/ref/c/images/cymb-ci10.jpg

Cymbopogon citratus Staph is a tall perennial grass which belongs to the family Poaceae. It is a genus of about 55 species. It originated from the warm temperate and tropical regions of the world. It is commonly known as lemon grass. It has a simple leaf with a central margin, very distinct from its parallel venation running through the entire length of the leaf. Its traditional applications in Nigeria include use in the treatment of fever, jaundice, hypertension, as analgesic, in soap making and as an insect repellant (Onabanjo *et al*., 1993). This study also reported the presence of alkaloids, saponins, tannins and simple sugars in the aqueous extracts from the leaves. It has a wide application in the traditional medicinal practices in tropical Africa (Tortoriello and Romero, 1992). A further study also indicated its traditional medicinal values in tropical and subtropical countries and in producing a pleasant aroma

in their herbal teas (Cheel *et al*., 2005). *C. citratus* has a characteristic pleasant flavour, which may explain the wide usage of its essential oils in the production of perfumes, cosmetics and as a flavouring ingredient in a good number of food beverages and confectionaries (Guynot *et al*., 2003, Pandey *et al*., 2003). The essential oil of *C. citratus* is documented to have antibacterial, as well as antifungal, activity (Suhr and Nielsen, 2003; Wilkinson and Cavanagh, 2005).

Cymbopogon citratus is widely used in Brazilian traditional medicine to ameliorate nervous and gastrointestinal abnormalities (Melo *et al*., 2001). A further study documented its use in Brazilian folk medicine as a tea to relieve anxiety, and this was shown by the activity of the essential oils in a mouse model (Blanco *et al*., 2009). Its sedative and anticonvulsant properties as well as its use as an anxiolytic agent has been documented (Blanco *et al*., 2009). This study compared the anxiolytic activity of the essential oils from this species to that of diazepam in mice. The oils were shown to have significant activities in mice. Some researchers have proposed herbal extracts with recorded activity in the central nervous system as a better option in the treatment of anxiety, depression, headache or epilepsy than the conventional drugs (Phillipson, 2001; Carlini, 2003). Further studies recorded the antinociceptive activity of the essential oils from *C. citratus* (Viana, 2000). The extracts from the tea have been reported to show analgesic, diuretic, sedative, antispasmodic and anti-inflammatory properties (Carlini *et al*., 1986). Five *C*-glycosylflavonoids from; orientin, isoorientin, isoscoparin, swertiajaponin and isoorientin 2″-*O*-rhamnoside have been isolated from *C. citratus* and have shown potent antioxidant activity by significantly inhibiting lipid peroxidation in erythrocytic membranes (Orrego *et al*., 2009). Citral, myrcene and limonene have been isolated from *C. citratus* (Ha et. al., 2008). Previous studies have also isolated and identified cymbopogonol and cymbopogone (Hanson et. al., 1976, Yakoyama et. al., 1980).

2.5 Description and medicinal uses of *Citrus limon*

Fig 2.5.1 *C. limon* plant

Source: http://www.bambusarium.de/exoten/citrus/citrus.htm

Citrus limon L. Burm.f. plants are small trees which belong to the family Rutaceae. They have a characteristic spiny shoot and conspicuously green leaves. The species has a wide usage of its fruit in the commercial production of lemonade or to garnish drinks. Several drinks are served with a slice of lemon decoratively placed on the rim of the cup or in the glass of juice. In Nigeria it is used to tenderize meat and the fruit juice is used to wash and clean molluscs before cooking (Igoli *et al*., 2005). Some useful components such as

citric acid, ascorbic acid, minerals and flavonoids are contained in this species (Rio *et al*., 2004). Some researchers have attributed the activity of this species to the presence of flavonoids which have diverse biological and health-related functions (Middleton and Kandaswami, 1992, Elangovan and Govindasamy, 1994). Flavonoids have shown antiviral, antimutagenic (Iwase *et al*., 2001) and antimicrobial properties (Cushinie and Lamb, 2005). The hexane extract of *C. limon* was active against *Trichophyton mentagrophytes* and *Microsporum canis* (Johann *et al*., 2007).

Further studies on the flavonoids from *Citrus* species have described their anti-hemorroidal, anti-oxidant, anti-inflammatory and anti-lipid peroxidation properties (Galley and Thillet, 1993; Da Silva *et al*., 1994). The oil extracted from *C. limon* effectively killed mosquito larvae (Zayed *et al*., 2009). This suggests that plants could be used to control the mosquito vector due to their bioactive chemical composition (Park *et al*., 2002). Limonoids are a group of triterpene derivatives found in the Rutaceae and Meliaceae families (Bennett and Hasegawa, 1982; Hashinaga *et al*., 1990). It has been recorded that most of the natural limonoids are from *citrus* plants of the family Rutaceae (Ohta *et al*., 1992). Further studies reported that limonin and nomilin are the most abundant *Citrus* limonoids and have shown anti-carcinogenic properties in rodent models (Karim and Hashinaga, 2002; Kelly *et al*., 2003).

2.6 Description and medicinal uses of *Musa sapientum*

Fig 2.6.1 *M. sapientum* plant

Source: http://www.stripes.com/08/may08/GTMO

Musa sapientum L. belongs to the family Musaceae. This family comprises bananas, plantains, and ornamental bananas. Plantain leaves are similar to banana leaves but are larger and stronger. Plantains tend to be firmer and lower in sugar content than desert bananas and are used either when green or under ripe (starchy) or ripe (sweet). Plantains are a staple food in the tropical region of the world for instance in Nigeria (Igoli *et al*. 2005), it could be processed in the same way as potatoes. Propagation is by suckers.

The juice of the *Musa sapientum* flowers has been reported to show hypoglycemic effects (Pari and Maheswari, 1999). The methanolic extract of banana has shown anti-oxidant effect *in vitro* (Goel *et al*., 2001). Researchers

have reported the anti-ulcerogenic activity of dried powder of banana pulp against ulcers in guinea pigs and in rats (Sanyal *et al*., 1963). Further work confirmed the anti-ulcerogenic activity of banana against histamine induced gastric ulcers in mice (Elliot and Heward, 1976) and aspirin-induced gastric ulcers in rats (Best *et al*., 1984). The anti-ulcerogenic activity of sterylaclyglycosides, sitoindosides I-IV isolated from *Musa paradisiaca* Linn. has been demonstrated in rats and humans (Ghosal and Saini, 1984; Ghosal, 1985; Bhattacharya and Ghosal, 1987). The antiulcer activity of a flavanoid, leucocyanidin isolated from unripe plantain banana pulp has been reported (lewis *et al*., 1999).

2.7 Description and medicinal uses of *Mangifera indica*

Fig 2.7.1 *M. indica* plant

Source: Djatmiko W.A., (Wie146)

Mangifera indica L. of the family Anacardiaceae is a perennial tree with evergreen leaves. The leaves are simple with alternate arrangement.. The fruit is a drupe, and hangs from the tree on long stems when it matures. It has various shapes and sizes. The ripe fruit is variably coloured yellow, orange and red, green colour usually indicates that the fruit is not yet ripe, however, this depends on the species (Igoli *et al*. 2005). In the center of the fruit is a single flat, oblong seed. This seed depending on the species can be fibrous or hairless on the surface.

Native from tropical Asia, and thrives well in many parts of tropics and subtropics (Ross, 1999). *M*. *indica* has naturalised in most tropical biotopes in India, Southeast Asia, Malaysia, Himalayan regions, Sri Lanka, Africa, America and Australia (Kirtika and Basu 1993; Sahni, 1998). It is common in different parts of the world where it is given vernacular names such as in Brazil: Manga, Mango, Mangueira, Skin Mango; Canary Islands: Mango; China: An Lo Kuo, Mangguo, Mango; Egypt: Mango; Fiji: Aam, Mango; Germany: Mango; Mango; Guatemala: Mango; Sudan: Mango; Tanzania: Embe, Mango, Mwembe; Tonga: Mango; United States: Bowen mango; Venezuela: Mango (Kirtikar and Basu 1993; Ross, 1999). *M. indica* has hundreds of cultivars which are distributed throughout the world.

Different parts of *Mangifera indica* are used in the traditional herbal remedies across the globe (Ross, 1999). The aqueous extract of *M*. *indica* L. is reported to have antioxidant, anti-inflammatory, analgesic and immunomodulatory properties, which helps to prevent disease progress and dermatological disorders, AIDS, cancer and asthma (Guha *et al*., 1996, Nuñez-Selles, 2005).

Ross (1999) reported the chemical constituents of the different organs of *M*. *indica* L. Mangiferin, is a major C-glucosylxanthone isolated from various parts of *M. indica*. Mangiferin is widely distributed in the Anacardiaceae and Gentianaceae families, especially in the leaves and the bark (Yoshimi *et al*.,

2001). The natural C-glucoside xanthone mangiferin [2-C-β-Dgluco- pyranosyl-1,3,6,7- tetrahydroxyxanthone; C19H18O11; Mw, 422.35; melting point, anhydrous 271°C (Muruganandan *et al*. 2002). Researchers have reported the presence of this compound in various parts of *M. indica*: leaves (Desai *et al*. 1966), fruits (El Ansari *et al*., 1971), stem bark (Bhatia *et al*., 1967), heartwood (Ramanathan and Seshadri 1960) and roots (Nigam and Mitra 1964).

Mangiferin is a vital ingredient of a natural extract from *Mangifera indica* known as Vimang and has shown diverse pharmacological uses such as, antioxidant (Muruganandan *et al*. 2002; Leiro *et al*., 2003), immunomodulatory (Garcia *et al*., 2002, 2003), lipolytic (Yoshikawa *et al*. 2002), anti-allergic (Rivera *et al*., 2006), anti-inflammatory and anti-nociceptive (Beltran *et al*. 2004; Garrido *et al*., 2004), antitumor (Yoshimi *et al*., 2001), antidiabetic (Muruganandan *et al*. 2005), and antiparasitic activities (Perrucci *et al*. 2006). Mangiferin showed *In vitro* activity against 7 bacterial species, *Bacillus pumilus*, *B*. *cereus*, *Staphylococcus aureus*, *S*. *citreus*, *Escherichia coli*, *Salmonella agona*, *Klebsiella pneumoniae*, 1 yeast (*Saccharomyces cerevisiae*) and 4 fungi (*Thermoascus aurantiacus*, *Trichoderma reesei*, *Aspergillus flavus* and *A*. *fumigatus*) (Stoilova *et al*., 2005).

2.8 Scope of this study

Researchers have shown keen interest to investigate African medicinal plants to identify possible potential sources of antimalarial compounds (Saidu, *et al*., 2000; Orafidiya *et al*., 2001; Orafidiya, *et al*., 2002; Noor *et al*., 2007; Odugbemi, *et al*., 2007; Frederich *et al*., 2008). This study aims to investigate scientifically, the antiplasmodial activities of five Nigerian plants singly or in combination. The escalating challenge posed by parasite resistance, to existing antimalarial drugs emphasized the urgent need for drugs with different mode of action (Ridley, 2002). Several reports stated the advantages of combination treatment (White and Olliaro, 1996; White, 1998; 1999; White *et al*., 1999) It

became necessary therefore to explore the antiplasmodial activities of these plants singly or in combination.

2.8.1 Why Nigerian plants?

Nigeria is a tropical rain forest country in an endemic malaria region with a great diversity of natural products yet to be explored. The tropical rain forest natural products are potential sources of biologically active compounds (Noor *et al*., 2007). Traditionally these plants are used in treating malaria in Nigeria. Majority of the African populace depend on these plants used by traditional healers. They are accessible, cheap, affordable, available and sustainable. African medicinal plants have demonstrated diverse potentials as possible sources of promising antiplasmodial compounds (Saidu, *et al*., 2000; Okokon *et al*., 2006; 2007; Philippe *et al*., 2007). However, little work has been done on the antiplasmodial and toxicity properties of similar or related species naturalized in Nigeria. Moreso, most of the herbal preparations used to treat malaria in Nigeria are without scientific evidence on efficacy.

2.8.2 Rationale for plant collection

- Used in the Nigerian traditional medicinal practices to treat malaria.
- Availability all through the year, hence supply will not be interrupted, since it is not seasonal.
- They are readily grown, accessible and quite easy to maintain since they are also found growing in the wild, thus it poses little or no difficulty in propagation.
- Their leaves are used as food in Nigeria.
- Lack of research output on the scientific information concerning the safety, and efficacy of these plants naturalized in Nigeria, in the traditional treatment of malaria, using *in vitro* and *in vivo* sytems.

Details of the *in vitro* cytotoxicity and *in vivo* toxicity properties of these Nigerian plants, as well as their activity against *Plasmodium* parasites are either scanty or lacking.

This information is vital so as to confirm if their antiplasmodial activity is specific to *Plasmodium* parasites or due to cytotoxicity of the components. It is necessary therefore, that the species that occur in Nigeria be tested for activity since the activity of same species of plants could vary due to soil composition, geographical and climatic conditions (Massotti *et al*., 2003; Angioni *et al*., 2006).

The traditional healers in Nigeria make use of these plants singly or in combination to treat malaria. These plants have been collected to scientifically investigate their antiplasmodial properties singly and when combined. Several studies have shown that the challenges posed by the resistance of parasites to already existing drugs could be averted by drug combination treatments (White, 1998; White *et al*., 1999; Guerin, 2002; Faye *et al*., 2007). The WHO recommended the use of Artemisinin combination therapies (ACT's) for the treatment of malaria (WHO, 2005; WHO, 2007).

These plants have been used singly or in combination with other plants in the traditional treatment of malaria in Nigeria. They are used in the form of decoctions, infusions, tinctures or macerations (Igoli *et al*., (2005). The majority of the populace makes use of plant remedies, and anecdotal reports suggest that combining the plants is more effective than using them singly. The traditional recipes of combinations of five of these plants as used in Nigeria have been documented as shown (Table 2.8.1).

Table 2.8.1 Tabular representation of some Nigerian medicinal plants that are used in combination in the traditional treatment of malaria

Group A	Group B	Group C	Group D	Group E
Carica papaya **(leaves)+**	***Cymbopogon citratus*** **(leaves)+**	***Vernonia amygdalina*** **(leaves)+**	***Cymbopogon citratus*** **(leaves) +**	***Vernonia amydalina+*** **(leaves) +**
Psidium guajava**((leaves)+**	***Curcuma longa*** **(rhizome)+**	***Ocimum gratissimum*** **(leaves) +**	***Carica papaya*** **(root) + *Citrus limon***	***Amaranthus spinosus*** **(leaves) +**
Mangifera indica **(leaves) +**	***Citrus aurantifolia*** **(leaves) +**	***Azardirachta indica*** **(bark) +**	***Vernonia. Amygdalina*** **(leaves) +**	***Xylopia aethiopica*** **(seed) +**
Alstonia boonei **(bark)**	***Carica papaya*****(leaves) + *Psidium guajava*** **(leaves)**	***Cymbopogon citratus*** **(leaves)**	***Citrus aurantium*** **(fruit)**	***Telfairea occidetalis*** **(leaves)**

Source: http://www.bioline.org.br/showimage?tc/photo/tc07026t2.jpg and Igoli *et al*. (2005)

2.9 General objectives

- To scientifically establish the *in vitro* and *in vivo* effectiveness of these plants singly and in combination against the plasmodium parasites and the bioavailability study of an active compound.

2.9.1 Specific objectives

- To use an *in vitro* system to determine the antiplasmodial activity of extracts from selected plants against the chloroquine sensitive and chloroquine resistant strains of *Plasmodium falciparium*.

- To determine the *in vitro* cytotoxicity properties of these plants singly and in combination using the CHO cell line.
- To identify and trace the activity of the most active extract *in vitro* through a bioactivity guided fractionation.
- To investigate the bioavailability of an isolated compound with antimalarial activity in an *in vivo* mouse model.
- To use mice models to screen extracts that showed promising antiplasmodial activity *in vitro* for efficacy and safety *in vivo*.
- To identify the synergistic combination of the plant extracts that will cure malaria in a mouse model.

The methods involved in the collection of these plants, and the details of the experimental processes are described in the next chapter.

CHAPTER THREE

MATERIALS AND METHODS

3.1 *In vitro* antiplasmodial experiment

The *in vitro* antiplasmodial studies involved a continuous culturing of both strains of the parasite. The parasites received in their frozen state were thawed and continuously cultured as described below.

3.1.1 Cultivation of *P. falciparum* parasites

The strains used for this experiment have adapted to *in vitro* experimental conditions. The D10 strain of the parasites, which was donated by Dr A. Cowman, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia was derived from FCQ-27 Papua New Guinea (Ekong *et al*., 1993). The Chloroquine resistance (CQR) DD2 strain was derived from Indochina (Wiesner *et al*., 2001). The asexual erythrocytic stages of these parasites were maintained in a continuous culture using a modified method of Trager and Jensen (1976). The *in vitro* assay procedure was done in the hood under sterile conditions. Continuous culturing of the erythrocytic stage necessitates the addition of red blood cells in the *in vitro* set up. The O^+ human blood was obtained from the Groote Schuur Hospital, Cape Town and washed before use, as described below.

3.1.2 Washing of blood

Blood samples usually at normal state contain both red blood cells (RBC) and the white blood cells (WBC). Washing gets rid of a proportion of the WBC. The RBC remaining are used to culture the malarial parasites *in vitro*. The washing process was started by transferring an aliquot of blood into sterile tube. An equal volume of complete medium (CM) was added to it. This was centrifuged at 1200rcf for 5 minutes. After spinning, the supernatant was aspirated. An equal volume of CM as used previously was added and the procedure was repeated. The supernatant was aspirated and the pellet containing RBC was stored in the fridge at 4ºC for eventual usage. The medium used in the *in vitro*

continuous culturing of parasites and washing of blood were prepared as described in section 3.1.3.

3.1.3 Preparation of medium

Three media were used for the *in vitro* culture of the parasites: complete medium (CM), incomplete medium, and wash medium.

3.1.3.1 Complete medium

This medium is used to maintain the parasites in culture. It is made up of 10.4 g/l RPMI 1640 with gluthamine (but no $NaHCO_3$**),** (Bio Whittaker), 4 g/L glucose, 6 g/lHepes (*N*-[2-hydroxyethyl]-Piperazine-*N*1-[2-ethnsulphric acid]) (Sigma-Aldrich), 0.088g/L Hypoxanthine enriched with 5 g/L Albumax and 1.2 ml/L (0.05 g/L) gentamycin (Sigma-Aldrich). At the desired P^H the colour indicator in the RPMI appears orange. The medium was filtered twice. The first is a pre-filtration under pressure using 0.45 µm to remove impurities or contaminants. The second stage was filter sterilization through 0.22 µm filter. The addition of 5% sodium bicarbonate (8.4 ml) to 200 ml medium gave rise to a complete medium (CM).

3.1.3.2 Incomplete medium

This is the same as the complete medium, but does not contain 5% sodium bicarbonate.

3.1.3.3 Wash medium

This is prepared and filtered the same way as CM, but without albumax. It contains the same amount of sodium bicarbonate solution. The complete medium, incomplete medium, and wash medium were stored at 4ºC.

3.1.4 Feeding or synchronization of parasites in continuous culture.

Parasites were grown and maintained in tissue culture flasks (Greiner Bio-One). The continuous culturing of the parasites involved three steps which included changing of the medium, feeding the parasites with RBC in the late trophozoite stage, and synchronization of the parasites with sorbitol in the early trophozoite stage (ring stage). Feeding and synchronization was usually not done on the same day since the phases of the parasites alternate every 24 hours. The medium was changed by centrifuging the entire content of the flask (CM, RBC, PRBC) at 750 rpm for 5 minutes and the supernatant was removed. Smears are made from the pellets to ascertain the diagnostic stage of the parasite and the parasitemia. The blood smear was allowed to air dry, fixed with methanol and stained with 10% Giemsa for about 10 minutes before viewing under the light microscope. It was at this juncture that the decision to feed or synchronize or to change the medium was taken. The parasites at the ring stage were synchronized by the addition of 20 ml of sorbitol to the pellets. This was allowed to stand for 10 minutes in a water bath (37ºC). Thereafter, it was centrifuged at 700 rpm for 3 minutes. The supernatant was aspirated and 10 ml of CM was added to the desiccant and placed back in culture. After the routine feeding or synchronization of the parasites, the culture flask was flushed with gas (3%O_2 4% CO_2), for about 1 minute and incubated at 37ºC for 24 hours. The parasitemia was maintained at 1% for the antiplasmodial assay.

3.1.5 Cryostorage and thawing of parasites

3.1.5.1 Cryostorage of parasites

Cryostorage involves freezing and storing the parasites in cryotubes. At one stage it became necessary to replenish the parasite stock which had been used up due to continuous culturing of the parasites. At a desired parasitemia and phase, the parasites were frozen and transferred to cryotubes which were stored in liquid nitrogen. Parasites were frozen in their ring stages since their

membranes are more robust than that of the later stages. The freezing medium was made up of 1.6 g sodium lactate, 30 mg potassium chloride, 1.38 g sodium dihydrogen phosphate and 57 g glycerol in 100 ml Millipore water (mH_2O). Glycerol disrupts crystal formation of the water which can damage the parasites. The freezing medium was filtered using a 0.22 µm filter. The filtrate was slowly added (5 volumes to 3 volumes PRBC) and allowed to stand for 5 minutes. They were transferred to cryotubes and kept at -80ºC overnight, and subsequently transferred to liquid nitrogen the next day.

3.1.5.2 Thawing of parasites

Three thawing solutions were used to thaw the parasites. Solution A contained 12% sodium chloride (NaCl), solution B contained 1.8% NaCl, while solution C contained 0.9% NaCl plus 0.2% glucose. Thawing of parasites using these 3 solutions was done step-wise and as fast as possible. The cryotubes taken from liquid nitrogen were placed in a water bath at 37ºC to thaw. Solutions A and B were added sequentially and were centrifuged at 400 rcf for 5 minutes. The supernatant was removed, and solution C was added to the pellet and allowed to stand for 5 minutes. Thereafter the PRBC was cultured as already described (3.1.4).

3.1.6 Preparation of samples for antiplasmodial assay

The preparation was the same for all of the extracts and fractions tested. Initially, a stock solution of 2 mg/ml of sample was prepared in 10% methanol (MeOH) (in deionised water). This was further diluted in complete medium to attain a final concentration of 200 µg/ml in 1% MeOH which is the desired concentration for the antiplasmodial assay. The stock solutions were prepared on the assay day. Chloroquine (CQ) (Sigma) was used as the standard reference drug (control). Two mg/ml stock of CQ (Sigma) was constituted in deionised water and further diluted in complete medium to a concentration of 200 µg/ml. DL-α- Dipalmitoylphosphatidylcholine (DPPC) (Sigma) served as a

vehicle for the unsaturated hydrocarbons investigated in this study. Fatty acids were dissolved in chloroform (2 mg/ml). Mixed fatty acids and DPPC micelles were prepared by adding DPPC four-fold the amount of fatty acid. Solvents were evaporated under nitrogen and 1 ml of complete medium (RPMI 1640) was added to the mixture. The mixture was sonicated for 2 minutes and thereafter used for assay.

3.1.7 *In vitro* experimental plate set up and procedure

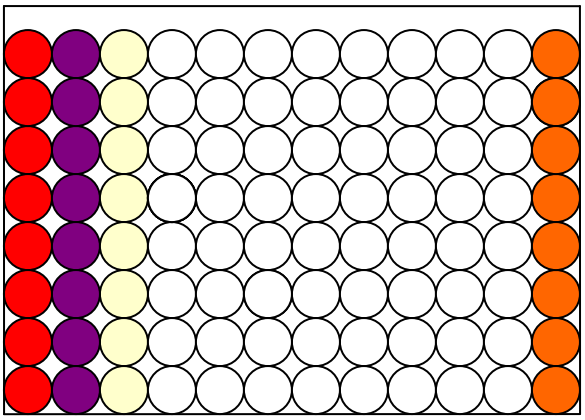


Fig 3.1.7 A diagrammatic representation of the 96-well microtitre plate set up used in this study.

KEY: Red = Column 1(Blank), Violet = Column 2(negative control), Light yellow = Column 3 (Positive control + drugs in duplicate), Orange =Column 12

A flat-bottomed, 96-well microtitre plate (Greiner Bio-One) was used for this assay as shown in fig 3.1.7. The suspension were dispensed into the wells in this order; column 1 was used as blank (100 µl 1% hematocrit + 100 µl complete medium (CM)), and column 2 was the negative control (100 µl 1% parasite suspension + complete medium) while columns 3-12 contained the different concentrations of plant samples (100 µg/ml serially diluted two-fold in complete medium up to 0.195 + 100µl 1% parasite suspension in each well). Columns 3 and 12 had the highest (100 µg/ml) and the lowest (0.195 µg/ml) concentrations, respectively. A volume of 100 µl CM was added to all the wells

except those in column 3 containing 200 µl of drugs which had already been diluted in CM. CQ (Sigma) which is the positive control compound was tested at a starting concentration of 100 ng/ml or 1000 ng/ml for the sensitive (D10) and resistant (DD2) strains of *P*. *falciparum* respectively. Each column has eight wells. The drugs in column 3 were arranged in duplicate and could contain a maximum of 4 drugs (CQ inclusive) in one plate. A two fold-serial dilution of the initial or starting concentration was done using the multi-channel pipette from columns 3-12 and the last 100 µl after column 12 was squirted out. This leaves each row in columns 3-12 with 100 µl of different drug concentrations in CM. Unparasitised erythrocyte (RBC) was added to column 1 (blank) which had no drugs, while parasitized red blood cells (pRBC) were added to columns 2-12. Column 2 (negative control) had the parasitized red blood cells but no drugs. Columns 3-12 had parasites and drugs at different concentrations. The plate was covered with a lid (Greiner Bio-One) and placed in a gassing chamber, gassed for 2 minutes (93% N_2, 4% CO_2 and 3% O_2) and incubated for 48 hours. A final hematocrit and parasitemia of 2% was used for all experiments. The IC_{50} recorded in this study is the mean of at least two independent experiments.

3.1.8 Parasite lactate dehydrogenase (PLDH) assay

Quantitative assessment of *in vitro* antiplasmodial activity in this study was determined by using a modified method of parasite lactate dehydrogenase assay as described by Makler *et al*.(1993). This is a colorimetric enzymatic method. A unique feature of this technique is its ability to distinguish parasite pLDH activity from the host LDH activity by making use of 3-acetyl pyridine adenine dinucleotide (NAD). Lactate is converted to pyruvate by pLDH using APAD as a co-enzyme. In the presence of the reduced APADH, the yellow coloured nitro blue tetrazolium (NBT) is converted to a purple formazan salt. The percentage of the parasites that survived and the concentration causing 50% growth inhibition (IC_{50}) was determined by measuring the conversion of

NBT by *P*. *falciparum.* This procedure is reliable (Basco *et al*., 1995) and does not consume time.

3.1.9 Developing plates

The assay plate (plate A) was incubated for 48 hours. After the incubation, the plate was resuspended and 15 µl of the suspension was transferred to corresponding wells in another plate (plate B) already containing 100 µl Malstat. This was followed by the addition of 25 µl of NBT into each well of plate B. A hair dryer was used to remove any air bubbles that might be present in the plate. This was placed in a dark cupboard to develop, since NBT is light sensitive. The absorbance of each well was read after 5 minutes or as soon as a colour change from yellow to purple was observed, using a microplate reader at 590 nm.

3.1.9.1 Details of reagents used to develop plates

Malstat is made up of a solution of 400 µl Triton X 100, 4g of L-lactate, 1.32 g Tris- buffer and 22 mg APAD in 200 µl of mH_2O. Malstat helps to disrupt the red blood cell membrane, exposing the parasites to NBT. NBT consists of a solution of 160 mg NBT and 8 mg phenazine ethosulphate in 100 ml of mH_2O. NBT changes from yellow to purple in the presence of living parasites.

3.1.9.2 Preparation and storage of reagents used to develop plates

To prepare Malstat, 400 µl Triton X 100 was heated gently to dissolve, 4 g L-lactate and 1.32 g Tris buffer in 200 µl of mH_2O was added. The resultant mixture was stirred to dissolve and allowed to cool. Then 22 mg of APAD was added and the pH adjusted to 9. The solution was transferred into a bottle and covered with foil to reduce exposure to light since NBT is light sensitive. All the reagents were stored at 4ºC.

3.1.10 Data analysis

The absorbance of each well was read when the colour changes from yellow to purple, using a microplate reader at 590 nm. The percentage parasite survival and the concentration that inhibits the growth of parasites by 50% were determined by measuring the conversion of NBT by *P. falciparum*. This was achieved by analyzing the readings from the microplate reader using Microsoft Excel® 2002, and the IC_{50} value which is the concentration at which the growth of the parasite was inhibited by 50% was determined using a non-linear dose response curve fitting analysis in Graph Pad Prism version 4. In this study the IC_{50} values are mean of 2 or more independent experiments.

3.2 *In vitro* Cytotoxicity experiment

Cytotoxicity testing of samples was carried out using the following media as listed below.

Complete medium: This is made up of 10% FCS, 45% DMEM and 45% HAMS

Thawing medium: This solution consists of 30% FCS, 35% DMEN and 35% HAMS

Freezing medium: Solution of 90% FCS, 10% DMSO (filter sterilized)

3.2.1 Preparation of medium and reagents for cell culture

Dulbecos Modified Eagles Medium (DMEM) (Highveld Biological, South Africa) is made up of 13.53 g/L DMEM and 3.7 g/L $NaHCO_3$ in an acid clean volumetric flask and pH of 7.1. The solution was stirred for about 30 minutes, pre-filtered and supplemented with gentamycin (500 µl/L or 0.05 g/L) into sterile bottles and stored in the fridge at 4ºC.

Nutrient Mixture F-12 HAM (HAMS) (Sigma) contains 10.7 g/L HAMS and 1.17 g/L $NaHCO_3$ in an acid-clean flask at a pH of 7.1. This is filter sterilized, decanted into sterile bottles, and stored in the fridge at 4ºC.

Foetal Calf Serum (Highveld Biological, South Africa) was heat inactivated and stored at -20ºC. The cells were cultured and maintained in complete medium (CM), thawed in a thawing medium and frozen in a freezing medium.

Trypsin (Highveld Biological, South Africa) contains 0.10 g in 100 ml PBS. This was stirred for about 30 minutes, filter sterilized and stored at 4ºC.

MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) salt contains 5 mg/ml in Millipore water. The aliquot is filter sterilized before use and stored in a bottle at 4ºC.

Emetine was used as the positive control for the cytotoxicity assay (Clarkson *et al***.,** 2004, Chukwugekwu *et al*., 2009, Lategan *et al*., 2009).. The stock was made up with 2 mg/ml Millipore water and stored at 4ºC.

3.2.2 Subculturing Cells

The cells were cultured in the flask with vented caps (Greiner Bio-one) and incubated at 37ºC. They were maintained as a monolayer and subcultured as soon as they became confluent. To subculture the cells in the flask, all the medium used in culturing them earlier was removed and the cells rinsed twice with 10 ml each of sterile phosphate buffer saline (PBS). This was followed by the addition of 5 ml of 10% sterile trypsin (Highveld Biological, South Africa). It was placed in an incubator for 2 minutes and agitated slightly to lift cells. Long exposure to trypsin was avoided. To inhibit the activity of the trypsin, 5 ml of complete medium (CM) was added. The cell suspension was transferred to a sterile tube and centrifuged at 750 rpm for 5 minutes. The supernatant was poured off completely. The pellet was resuspended in 5 ml CM, and 1 ml of the cell suspension was transferred into a new flask containing 9 ml CM. This was

placed back into the incubator at 37ºC. However, if the cells were to be frozen, the method below was applied.

3.2.3 Freezing and cryostorage of cells

The freezing medium consist of 90% FCS and 10% DMSO (filter sterilized).The confluent cells were harvested and resuspended in 5ml CM as described above. The resultant cell suspension was slowly mixed with freezing medium (2 x volume of cells) while swirling on ice. They were decanted into cryotubes, swabbed thoroughly with webcol, sealed and stored in liquid nitrogen at -80ºC. Thawing the cells involved the procedure described below.

3.2.4 Thawing cells

The thawing solution was made up of 30% FCS, 35% DMEN and 35% HAMS. The frozen cells in the cryotubes were placed in a beaker with ice to thaw. They were allowed to thaw completely in a cold thawing medium. The contents were centrifuged at 750 rpm for 5 minutes and the supernatant was poured off. The pellets were resuspended in thawing medium and 1 ml of this suspension was transferred to flasks containing 9 ml of thawing medium. This was incubated at 37ºC (5% CO_2) and left to attach for 48 hours. If the cells had not reached confluency, then the medium was changed as stated in section 3.2.5.

3.2.5 Changing medium

The flasks containing the cells were viewed under the microscope to confirm if they were confluent or not. Half of the medium (5 ml) was changed and replaced with a fresh CM (5 ml) if they were not confluent. They were placed back into the incubator. These procedures were performed under sterile conditions.

3.2.6 Cytotoxicity assay

The mammalian Chinese hamster ovarian (CHO) cell line was used to investigate the cytotoxicity activity of all extracts, fractions and compounds. The CHO cell line was obtained from S. Schwager in the Department of Medical Biochemistry, University of Cape Town, South Africa. This cell line was chosen because of its wide acceptance and the little or no difficulty experienced in culturing and maintaining it *in vitro* (Clarkson *et al*., 2004). The cytotoxicity assay used in this study was the modified method described by Mossman *et al.* (1983). This is a rapid colorimetric assay method for determining cellular growth and chemosensitivity. It makes use of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) salt. There is a cleavage of the tetrazolium ring in active mitochondria showing that only viable cells are able to reduce the yellow coloured, water-soluble MTT to purple coloured, water-insoluble formazan (Sieuwerts *et al*., 1995).

3.2.7 Preparation of samples for cytotoxicity assay

The preparation was the same for all of the extracts and fractions tested as described earlier (3.1.6). Briefly, a stock solution of 2 mg/ml of sample was prepared in 10% MeOH (in deionised water). This was further diluted in complete medium to attain a final concentration of 200µg/ml in 1% MeOH. Owing to the high lipophilic nature of compounds investigated in this study, DL-α-dipalmitoylphosphatidylcholine (DPPC) (Sigma) served as a vehicle for these compounds. Fatty acids were dissolved in chloroform (2 mg/ml). Mixed fatty acids and DPPC micelles were prepared by adding DPPC four-fold the amount of fatty acid. Solvents were evaporated under nitrogen and 1 ml of complete medium (RPMI 1640) was added to the mixture. The mixture was sonicated for 2 minutes and thereafter used for assay. The stock solutions were prepared on the assay day. Emetine (Sigma) was used as the standard reference drug (control) to establish the cytotoxicity of the sample against the CHO cell lines (Clarkson *et al*., 2004; Chukwugekwu *et al*., 2009; Lategan *et al*., 2009). Two

mg/ml stock of emetine was constituted in deionised water and stored at 4ºC. A 10% dilution of stock solution in CM was made in complete medium to give the highest concentration of 200 µg/ml and the lowest concentration of 0.002 µg/ml. These concentrations were tested to determine the cytotoxicity of test samples against the CHO cells, as well as the IC_{50} by comparing with the reference drug emetine.

3.2.8 Cytotoxicity assay

This assay method could be grouped into two phases; the first is plating the cells and the second is addition of drugs.

3.2.8.1 Plating cells

A 96-well microtitre plate (Greiner-Bio one) was used. The cell suspension was prepared as explained during subculturing (3.2.2). Row H was used as blank and contained 200 µl CM only (with no cell suspension), while 100 µl of cell suspension + 100 µl CM were added from rows G-A. The plate was incubated for 24 hours. After 24 hours, cells became exponential and drugs were added at this juncture.

3.2.8.2 Addition of drug

After 24 hours, the CM was aspirated off. Row H was the blank (200 µl CM) Row G which served as the negative control received 200 µl CM with no drug, while rows F-A contained 100 µl of drug concentrations in triplicate + 100 µl CM. Thus each well had a final volume of 200 µl. The plate was covered with a sterile lid (Greiner Bio-One) and incubated for 48 hours at 37ºC (5%CO_2). After 48 hours the plate was developed as stated below.

3.3 Developing plates

After incubation for 48hours, 25µl of MTT was added to each well and incubated for a further 4 hours. The plate was centrifuged at 200 rpm for 10 minutes. The medium was carefully aspirated off and 100 µl DMSO was added to dissolve the resultant purple formazan crystals. The cell survival was determined using a microplate reader at 540 nm wavelength. The data was analyzed using Microsoft Excel® 2002 and Graph Pad Prism, version 4 was used for the non-linear dose response curve analysis. The IC_{50} values were given as a mean of 2 or more independent experiments.

3.3.1 Selectivity index (SI)

The selectivity index, which is the cytotoxicity: antiplasmodial ratio, was calculated to determine if the recorded activity was due to the antiplasmodial activity of the test samples or due to cytotoxicity to CHO cells. The formula below was used to calculate selectivity index (Attioua *et al*., 2007; Kaou *et al*., 2008; Chukwujekwu *et al*., 2009,).

SI= IC50 cytotoxicity/IC50 antiplasmodial activity.

3.4 *In vitro* drug combination experiment

3.4.1 Combination therapy

3.4.1.1 Plate Preparation for drug combination assay

The plate set up was the same as described in 3.1.7. The checkerboard method used in this study was adapted from Berenbaum (1978). The *in vitro* combination studies involved two extracts a and b. One extract (component a) with a predetermined concentration needed to inhibit parasite growth by 50% (IC_{50}) was kept constant, while the other extract also with a pre-determined IC_{50} (component b) was added at different concentrations to determine the antiplasmodial effect of the two extracts in combination. The same experiment was repeated, this time with component b constant while component a was varied. The combination experiment used a 96-well microtitration plate. The first column was the blank with complete medium and unparasitised erythrocytes (100 µl complete medium (CM) +100 µl RBC), while the second column contained complete medium with parasitized erythrocytes (100 µl CM+100 µl pRBC). The wells in columns 4-12 contained 100 µl of complete medium. Wells in column 3 contained 100 µl of [50 µg] concentration of component a in CM, except for the first duplicate rows (3A and 3B) which had the concentration of component a alone (200 µl). The rest of the rows in duplicate (rows C to H) containing 100 µl of CM received additional 100 µl aliquots of 50%, 25% and 5% of the IC_{50} of component b, respectively. Serial dilutions of these concentrations were made from columns 3 to 12 using a multichannel pipette with a volume of 100 µl transferred after resuspending thoroughly. The last 100 µl from column 12 was discarded. A volume of 100 µl pRBC was added to wells in columns 3-12 halving the drug concentrations. The final volume in each well was 200 µl. Plates were gassed for 2 minutes (93%N_2, 4% CO_2 and 3% O_2) and incubated for 48 hours, as carried out in the *in vitro* monotherapy experiment. The plates were developed using the pLDH assay method (3.1.8), and read using the microplate reader at 590 nm. The

results were analyzed using Microsoft Excel® 2002 and the Graph pad prism, version 4. This method was used initially for studying drug interactions with bacteria (Hall *et al*., 1993). However, the principles are easily applied to *P. falciparum* (Fivelman *et al*., 2004).

3.4.1.2 Plate preparation for drug combination cytotoxicity test

The combined drugs were tested for cytotoxicity, as already described, using the CHO cell lines. This was done to determine if the activity of the combined drugs was due to cytotoxicity to the CHO cell lines or to the selectivity for *Plasmodium falciparum.* The plates were analyzed in the same way, as explained in the monotherapy experiments.

3.4.1.3 Method used to measure synergism, additivity or antagonism of combined extracts

Fractional inhibitory concentrations (FIC) as described by Berenbaum (1978) were used to determine the interactions between the plants in combination. This method has been used by other researchers including Canfield *et al*. (1995) and Fivelman *et al*. (2004)).

The formula used in calculating the sum of the fractional inhibitory concentrations (FIC) is:

$$\frac{IC_{50}\ (\mu g/ml) \text{ of extract (a) in combination}}{IC_{50}\ (\mu g/ml) \text{ of extract (a) alone}} + \frac{IC_{50}\ (\mu g/ml) \text{ of extract (b) in combination}}{IC_{50}\ (\mu g/ml) \text{ of extract (b) alone}}$$

3.5 Procedures for the bioassay guided fractionation, isolation and identification of compounds from *C. papaya* ethyl acetate fraction.

3.5.1 Solid phase extraction (SPE)

The solid phase extraction (SPE) procedure was carried out with reverse phase octadecyl C18 Isolute cartridges (2.6x13.0 cm, 10 g sorbent, IST Ltd, Anatech, South Africa). Samples of the ethyl acetate extract from the leaves of *C. papaya* were dissolved in methanol. This mixture was diluted to a concentration of 5mg/ml with a 60% acetonitrile concentration in water (60%ACN:40%mH_2O). The C18 isolute cartridge was pre-moistened with 20 ml of mH_2O and preconditioned with 20 ml of the 60% acetonitrile. A volume of 3 ml of this solution was layered on the top of this cartridge. Samples retained on the sorbent beds were rinsed with 20 ml of mH_2O to elute unretained material. Samples retained on the sorbent beds were eluted under vacuum through a step-wise gradient with 40 ml of ACN:H_2O (20%-100%) at an increasing concentration of 20%. The eluates were collected in a fitted bottle. The vacuum pressure was set to control the flow at the rate of 15 ml/min. At the end of each run, the cartridge was washed with 100 ml acetone to wash out any remaining material. The collected fractions were concentrated under pressure by rotary evaporation at 40ºC and freeze-dried. The freeze-dried samples were placed in vials and stored at -20ºC. The 100% ACN fraction was transferred to pre-weighed vials, dried in the fume hood and stored at -20ºC. The *in vitro* antiplasmodial activities of these fractions were determined using the pLDH method described earlier (3.1.8). The SPE fraction, with promising antiplasmodial activity and good selectivity index (P63 100% acetonitrile), was further purified using high pressure liquid chromatography (HPLC), and structurally elucidated using NMR and GC-MS spectroscopic methods, as described below.

3.5.2 High Pressure Liquid Chromatography (HPLC)

The SPE fraction selected for further purification was fractionated on a Shimadzu LC 10AS high pressure gradient system. This was equipped with a desktop PC which runs Shimadzu control software via a Shimadzu CBM10A communication bus module. Other components of the HPLC instrument included an automatic sample injector, two solvent delivery systems (LC10AS pumps) and a diode array detector (Shimadzu SPDM10A). Compounds were detected by UV spectra at 210 nm, 240 nm and 260 nm as acquired by the diode array detector. The solvents used include methanol (Scharlau) and acetonitrile (Scharlau), each of analytical grade. Purified deionized water (Millipore, milli-Qwater system) was also used. The conditions are stated section 3.5.3.

3.5.3 Semi-preparative HPLC conditions

A semi-preparative HPLC C18 column (Discovery®, 25cm x 10mm, 5µm, 56924-U Supelco) was used. Samples were chosen based on their *in vitro* antiplasmodial activity, as well as their cytotoxicity values. Samples were centrifuged in a micro-centrifuge (Abbott, Germany) at 1000 rpm in 5 minutes. The injection volume was 50 µl with a flow rate of 2 ml/min over a 30 min run time and a solvent gradient of 20%-100% acetonitrile in water. The elution time of the peaks was observed, noted, and set up for further collections following multiple injections. The fractions collected were concentrated using the rotary evaporator and freeze-dried. Dried samples were tested *in vitro* against *Plasmodium* parasites, and the active peaks were run on analytical HPLC. The concentrated and dried samples of the two peaks were tested for purity using the analytical HPLC system as specified in section 3.5.4.

3.5.4 Analytical HPLC conditions

The purity of the compounds was monitored by an analytical HPLC using an octadecyl Silica column (Agilent Eclipse, XDB-C18, 4.6x150mm, 5µm, USA). Separations were accomplished at 29.7ºC with a solvent gradient of 20%-100% acetonitrile in water for 30 minutes at a flow rate of 1 ml/min. Pure compounds were identified and structurally elucidated using GC-MS and NMR spectroscopic methods.

3.5.5 Nuclear Magnetic Resonance (NMR)

The NMR spectra were performed on a Varian Unity Inova 600 MHz system available at the University of Stellenbosch, Stellenbosch). The compounds were dissolved in methanol at a concentration of 3 mg/ml. NMR spectroscopy made use of 1-D NMR and 2-D NMR techniques. The 1D NMR; ^{1}H and ^{13}C were used and the 2-D NMR techniques used in this study included HSQC, HMQC, and COSY.

3.6 GC-MS conditions

The HPLC samples were analyzed on a Waters GCT premier spectrometer, model HP5. The column specification was 30 m, 0.25 mm ID, 0.25 µm film thickness. The carrier gas was helium, with a constant flow of 1 ml/min. The injection split was 1:5, the injector temperature and the transfer temperature were 280ºC. The EI ionization energy was 70eV, the scanning mass range was *m/z* 40 to 400 with a solvent hold of 6 minutes. The peaks of the two compounds were identified by comparison with the retention times of reference standards.

3.7 *In vivo* Bioavailability and Activity methods

3.7.1 Animals and diet

This study was approved by the Animal Research Ethics Committee of the University of Cape Town, South Africa in 2007. All animal procedures were carried out in accordance with the suggested ethical guidelines for care of laboratory animals by the Animal Care and Use Committee of the University of Cape Town, as adapted from the recommendations of the Medical Research Council of South Africa (MRC 2004). The test animals were wild strains of C57 BL6 mice. Mice were obtained from the animal unit of the University of Cape Town (South Africa) at the ages between 7-10 weeks old. The animals were transferred to the animal facility of the Division of Pharmacology, University of Cape Town. As soon as they arrived at the facility they were housed in standard cages in groups of five and placed on a pelleted custom diet. They were maintained under conventional conditions with controlled temperature (22±4ºC) and illumination (12h; 6:00 am to 6:00 pm) and had free access to standard diet and water *ad libitum*.

3.7.1.1 Plasma Preparation for bioavailability study

Whole blood samples (O^+) were obtained from the Western Province Blood Transfusion Service, Groote Schuur Hospital, Cape Town, South Africa. Blood was centrifuged at 1300 rpm for 5 minutes. Plasma (supernatant) was stored at -20ºC. This was used in the preparation of standards for method development and evaluation.

3.7.1.2 Sample formulation and treatment of animals

The average weight of each group of mice in a particular cage was calculated and 100 mg/kg of compound was given to groups A, B and C. Group A was dosed orally, group B and group C received subcutaneous and intravenous dosage, respectively. Fatty acid methyl ester (FAME) micelles were prepared

according to the method of Kumaratilake *et al*., (1992). This method was used in preparing all of the fatty acids and their FAME used in both the *in vitro* and *in vivo* experiments. Solutions were freshly prepared before treatment. The unsaturated fatty acids and their esters are insoluble in water. DL-α-Dipalmitoyl phosphatidylcholine (DPPC) (Sigma) was served as a vehicle to these compounds due to their lipophilic nature. Fatty acids were dissolved in chloroform (10-50 mg/5 ml). Mixed fatty acids and DPPC micelles were prepared by adding DPPC at four-fold the amount of the fatty acid. Solvents were evaporated under nitrogen and 1 ml of complete medium (RPMI 1640) was added to the mixture. Thereafter, the mixture was sonicated for 2 minutes and used for the *in vivo* experiments. A volume of 200µl of the resultant solution was given to each mouse in the three routes of administration using a calibrated syringe. The same dose was given to the 3 groups for a better comparison. The subcutaneous injection was administered just behind the ears in between the two fore arms. For the intravenous dosage (i.v), mice were anesthetized with ketamine 15 minutes before dosing. All groups were given free access to food and water immediately after dosing.

3.7.1.3 Blood collection and sample preparation for analysis

Venous blood (50µl) was collected through the base of the eye using heparinized tubes (Lasec 200 capillary tubes; 75mm, 1.40-1.60mm, 80µl; for micro-hematocrit determination) from the Laboratory and Scientific Equipment Company (Lasec), Cape Town, South Africa. Blood samples were taken from experimental animals at four time points (t1 to t4) after administration. Blood samples were immediately transferred to pre-labeled (group A) Eppendorfs in an ice pack and centrifuged at 1300 rpm for 5 minutes. A volume of 20 µl of the plasma was transferred into pre-labeled Eppendorfs in group B. The group B Eppendorfs, now with 20 µl plasma, were kept in a -20ºC freezer for subsequent extraction.

3.7.1.4 Preparation of Standards

An initial stock solution (SS1) of 1 mg/ml concentration of compound 1 was prepared in methanol. Seven standards (STD 1-7; stock solution of standards) from the initial stock solution (SS1) of compound 1 were prepared by two-fold serial dilutions in plasma with a starting concentration of 20 µg/ml to 0.3125 µg/ml. The seven standards STD 1 to STD7 were labeled in decreasing order of concentration such that the highest is STD1=20µg/ml while the least concentrated STD7=0.3125µg/ml. Blank plasma was also included to serve as a control. Samples were stored at -20ºC for short term storage and at -80ºC for longer durations. However, before analyzing the samples, the LC/MS/MS spectrometer and the chromatographic conditions were developed and optimized.

3.7.1.5 Extraction from plasma

The test samples and the standards (STDI-STD7) were extracted. These standards and fatty acid methyl ester were extracted using a modified method of Aleryani *et al*. (2005). A volume of 100 µl of acetone was added into 20µl of spiked plasma (20µg/ml) in labeled Eppendorfs (Group B). Acetone was added to precipitate protein. The solution was vortexed for a minute and placed in ice for ten minutes. It was then centrifuged at 1120 rcf for 5 minutes. The resultant supernatant was transferred to labeled Eppendorfs (Group C). Hexane (150 µl) was added to group C Eppendorfs, vortexed for a minute, placed in ice for ten minutes and centrifuged at 200 rcf for 5 minutes. The hexane layer was transferred to group D Eppendorfs and dried under nitrogen. After drying was completed, 100 µl of mobile phase (acetonitrile: 0.1% formic acid; 50:50) was added to these Eppendorfs. The mixture was vortexed for 30 seconds and transferred into LC/MS/MS vials for injection and bioavailability assessment.

3.7.1.6 LC/MS/MS Analytical and Chromatographic conditions

The LC-system (Agilent 1200 series HPLC) equipped with an API 3200 quadrupole mass spectrometer detector (Applied Biosystems) was used in the experiment. This was carried out by direct injection of 2 µl at a flow rate of 0.5 ml/min using a Phenomenex C18 (5 cm x2 mm i.d., 5 µm) column. The mobile phase (MP) was acetonitrile: 0.1% formic acid (50:50) which was run isocratically for 1.9 minutes. The stability of the eluent system was efficient for identifying the retention times of compounds. Further *in vivo* study was carried out as shown in section 3.7.2.

3.7.2 *In vivo* antiplasmodial experiments

3.7.2.1 Test animals and diet

The test animals and their diet were the same as outlined earlier (3.7.1).

3.7.2.2 *In vivo* toxicity study of crude extracts

The *in vivo* toxicity testing of extracts was investigated at a dose of 800 mg/kg for 4 days. The extracts dissolved completely in 200 µl ethanol, 20µl Tween 80 and 780 µl phosphate buffered saline (PBS). The dosage was administered orally. The test animals were monitored for 4 weeks to ascertain if there were any adverse events. The same formulation was used for the *in vivo* antiplasmodial study of all of the extracts in this work.

3.7.2.3 Parasite strain

The parasites used for this experiment were of the cryopreserved *P. berghei* (ANKA) strain. This parasite strain was donated by the Swiss Tropical Institute, Basel, Switzerland. Parasite stock was preserved in liquid nitrogen at -80ºC in the Division of Clinical Pharmacology, University of Cape Town. Parasite stock was sustained by serial passage of blood from infected mice to uninfected mice. Parasitemia was monitored regularly. At a desired parasitemia, the mice were bled and euthanized. Blood samples collected were frozen in cryotubes and stored in liquid nitrogen at -80ºC.

3.7.2.4 Infection of experimental animals

The parasite stock which was frozen in cryotubes was thawed. The thawed parasites were diluted in phosphate buffered saline. The inoculum was injected intra-peritoneally into two mice which received 200 µl each. After a period of 6 days, the parasitemia was determined. An incision was made in the tail vein and a drop of blood was placed on the slide. Thin smears were made and fixed in methanol and stained in 10% giemsa stain for 25 minutes. This is ten minutes longer than the duration of staining slides of *in vitro* experiments. After the staining, a drop of immersion oil was placed on the slide and viewed under a light microscope. The parasitemia was determined. A volume of 200 µl of $1x10^6$ parasitized cells/ml was used to infect each mouse. The number of cells/ml was calculated using a haemocytometer and was diluted to a suspension of $5x10^6$ using phosphate buffered saline (PBS). All animals used in the *in vivo* antiplasmodial study were each infected with 200 µl of $1x10^6$ parasitized cells/ml. The percentage growth inhibition was determined according to Tona *et al*. (2001) as follows;

$$\% \text{ growth inhibition} = \frac{\text{Parasitemia of negative control} - \text{Parasitemia of test sample}}{\text{Parasitemia of negative control}} \times 100$$

3.7.2.5 Treatment of experimental animals

The *in vivo* testing of crude extracts and compounds against *Plasmodium berghei* was done using the 4-day suppressive test described by Peters *et al*. (1993). Each experiment had a positive control group and a negative control group. The positive control group received 200 µl of CQ (reference drug) at a dose of 10 mg/kg in Millipore water. The negative control group received 200 µl Millipore water only. Solvent control test fot the formulation (200 µl ethanol, 20µl Tween 80, and 780µl phosphate buffered saline) used in dissolving the extracts showed no antiplasmodial activity. Test groups and control groups were grouped as five animals in separate cages. Mice and cages were distinctively marked and labeled, respectively with permanent marker. The test animals were uniformly treated with different doses of extracts, singly or in combination. The extracts dissolved completely in 200 µl ethanol, 20µl Tween 80, and 780µl phosphate buffered saline. The extract was dissolved first in ethanol and Tween 80 before adding PBS. Extracts were administered orally. Each mouse in the test group received 200µl of drug formulation 24 hours post infection. The mice were monitored on a daily basis and physical appearance and weights were noted. Parasitemia was determined 3 days post-infection and this was regularly monitored. The length of time prior to the mice being sacrificed was subject to the parasitemia, mouse disposition and general well-being of animal, as recommended by the animal care and use committee.

3.7.2.6 4-day suppression test

The four-day suppression test used in all *in vivo* antiplasmodial experiments was as described by Peters *et al*. (1993). Animals were infected on day 0, (D_0) while treatment started 24 hours after infection (D_1). The parasitemia of each group was determined and the standard deviation values taken as the mean value for the group. Mice were sacrificed day 9 post infection, except on occasions where mice were very sick before day 9, or still showing no parasitemia on day 9. Mice with no parasitemia on day 9 were left for 28-days

post infection in accordance to WHO, 2006 specifications for determining cure of infected mice. During this period, in order to establish cure, mice were monitored daily and parasitemia determined two to three times a week.

3.7.2.7 *In vivo* data analysis

Parasitemia of all groups was monitored, and growth inhibition calculated, as shown earlier (3.7.2.4). The standard deviation values of parasitemia and weight were determined using the Microsoft Excel® 2002. The percentage parasitemia relative to the number of days post infection was evaluated using the Graph Pad Prism 4 version.

CHAPTER FOUR

In vitro activity of extracts and structural elucidation of compounds isolated from *C. papaya*

4.1 Introduction

Seven plants were collected in this study based on their traditional use to treat malaria in Nigeria. They were sequentially extracted using solvents of different polarities (Petroleum ether, Dichloromethane, Ethyl acetate, Methanol and Millipore water). Fractions from each plant were screened for in vitro antiplasmodial activity singly. Selected fractions with IC_{50} ≤10 µg/ml were further tested in combination. The figure 4.1.1 schematically illustrates the procedures whose results are explained in this chapter.

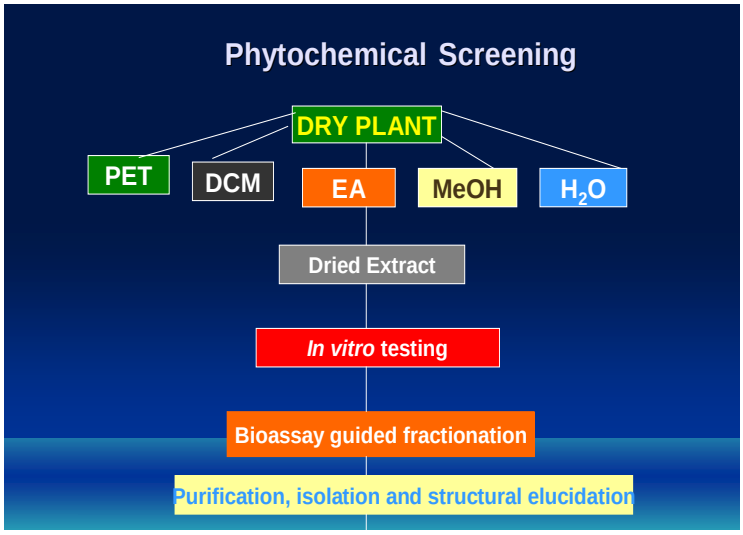


Fig 4.1.1: Schematic representation of phytochemical screening to isolate antiplasmodial compounds. Extraction solvents include; Petroleum ether (PET), Dichloromethane (DCM), Ethyl acetate (EA) Methanol (MeOH) and Millipore water (H_2O).

4.2 Plant collection, extraction and yield

4.2.1 Study Site

Nigeria is a West African country located at latitude 10º00´ north of the equator and longitude 8º00´ east of Greenwich. It is a tropical country with characteristic hot and wet conditions. These conditions are due to the two major seasonal variations, the rainy season and the dry season. However, the south eastern part of Nigeria, where the plants were collected, has an additional shorter version of each season coming between the two major seasons. This is not the case in the northern part of Nigeria where the two major seasons prevail. The two major seasons with their shorter versions at the collection site are: the long rainy season, the short dry season, the short rainy season, and the long dry season. The plants were collected during the long rainy season. The long rainy season starts in March and continues to the end of July. The peak of this season is in June. Malaria transmission reaches its peak during the rainy season (Falade *et al*., 2007). The long rainy season is followed by what is commonly known as August break which is the short dry season. As the name suggests, it is indeed a short, dry period experienced in August for about 2-3 weeks. A short rainy season commences after the August break. It starts in early September and runs to mid-October, with its peak usually at the end of September. The rains in the short rainy season are usually not as heavy as those experienced during the long rainy season. Last, but not the least, is the long dry season which spans through October and early March. The peak of this period is between early December and late February. This is also commonly known as the harmattan period and is usually dry with dusty winds. The moisture content of the leaves is reduced during this period.

4.2.2 Collection of Plants

A total of seven plants, each from a different plant family, were collected from Okigwe, Imo state in Nigeria. These plants were collected during the long rainy seasons in Nigeria. The choice of these plants for investigation was made due to their popularity in Nigerian folk medicine (Igoli *et al*., 2005). These plants were commonly used by traditional healers in the diverse ethnic regions of the country for the treatment of febrile illnesses and related ailments (Igoli *et al*. 2005). The plants collected for this study are available all through the year and there is little or no difficulty in their propagation. The plants used for this study were collected and identified by the Plant Science and Biotechnology Department, Abia State University, Uturu, Nigeria. These plants were air dried at room temperature and voucher specimens (Table 4.2.2.1), were deposited in the herbarium of Abia state University, Uturu, Nigeria. The air dried plant materials were packaged and couried to the University of Cape Town in South Africa. Collection of these plants in Nigeria was met with no difficulty or restraint, since the plants are used as food, and are found growing in homes. This study established collaboration between University of Cape Town in South Africa and the Abia state University in Nigeria. The Division of Pharmacology in the University of Cape Town requested for the importation of these plants from Nigeria. The Nigerian custom services granted the demand of the Abia State University to export these materials to the University of Cape Town South Africa for research purposes.

Table 4.2.2.1 Voucher specimen numbers of collected plants

Botanical Names	Voucher specimen numbers
Mangifera indica	PM/ABSU/06-20
Citrus limon	PM/ABSU/06-32
Musa sapentium	PM/ABSU/06-40
Psidium guajava	PM/ABSU/06-52
Carica papaya	PM/ABSU/06-63
Cymbopogon citratus	PM/ABSU/06-72
Vernonia amygdalina	PM/ABSU/06-82

Table 4.2.2.2: Names and dry weight (g) of plants collected

	Botanical Name	Common name	Local names	Part used	Dry weight of plants (g)
1	*Mangifera indica*	Mango	Mango	Leaves	115.6
2	*Citrus limon*	Lemon	Oroma nkrisi	Leaves	48.3
3	*Musa sapentium*	Plantain	Plantain	Leaves	48.4
4	*Psidium guajava*	Guava	Ugofa/ uguava	Leaves	84.4
5	*Carica papaya*	Paw paw	Pawpaw/ okwurukwa	Leaves	38.6
6	*Cymbopogon citratus*	Lemon grass	Achara	Leaves	43.7
7	*Vernonia amygdalina*	Bitter leaf	Olugbu/ olugbiri	Leaves	122.5

4.2.3 Solvent extraction of plant material

The dry plants were reduced into smaller pieces using a plant blender (Waring, Connecticut, USA). They were sequentially extracted using various solvents of different polarities. This sequential extraction started with petroleum ether, which helps in reducing the chlorophyll pigment in these green leaves. This was followed by dichloromethane extraction, thereafter ethyl acetate, methanol and water in that order. Each solvent was repeatedly used to extract each plant for 4-5 times. Plants were extracted for 24 hours, and during the process the plant material and the solvent were continuously shaken for adequate mixing on a horizontal orbit shaker (Labcon, California, USA). The resultant mixture was filtered and the filtrate concentrated under pressure in a Büchi Rotavapor R-205 (Büchi Labortechnik AG Switzerland), at 24ºC. The concentrated extracts were transferred to pre-weighed vials, dried in the hood at room temperature and stored at -20 ºC. The water extracts were concentrated by freeze drying using a DURA-DRY II instrument (FTS Systems, NY, USA) under a reduced pressure at -82ºC. The freeze-dried extracts were stored at -20ºC. This procedure was carried out for the seven plants collected. The yield from each solvent is as shown (Table 4.2.3.1).

Table 4.2.3.1: Yield of extracts from plant leaves from each solvent (first batch)

Botanical Name	Petroleum Ether (mg)	Dichloro-methane (mg)	Ethyl Acetate (mg)	Methanol (mg)	Millipore Water (mg)	Total extract-ive (%)
Mangifera indica	1260.4	868.9	631.3	8633.6	121.3	10.0
Citrus limon	1162.9	366.9	91.2	2894.8	1223.5	11.9
Musa sapentium	1699.8	106.7	42.5	1634.4	650.7	8.5
Psidium guajava	1617.2	791.6	536.6	7965.8	91.7	13.0
Carica papaya	1432.7	839.4	187.8	6563.1	7.0	23.4
Cymbopogon citratus	1060.5	506.7	84.0	4790.7	1307.9	17.8
Vernonia amygdalina	1864.8	3430.7	637.9	5419.3	864.1	10.0

4.3 *In vitro* activity testing

4.3.1 *In vitro* antiplasmodial activity of the plants collected

Each extract was screened for antiplasmodial activity against the chloroquine sensitive (CQS D10) strain of *P. falciparum*. The method used to measure parasite viability was the parasite lactate dehydrogenase activity (pLDH) method of Makler *et al*. (1993). This is a colorimetric enzymatic method which distinguishes parasite LDH (pLDH) from host LDH activity. Lactate is converted to pyruvate by pLDH and APAD is reduced to APADH in the presence of living parasites. The percentage of living parasites was determined by measuring the

conversion of NBT by *P. falciparum*. Details of this method are described in section 3.1.8.

4.3.2 Screening criteria

Of the seven plants collected, two plants, *M. indica* and *M. sapientum*, showed little antimalarial activity (Table 4.3.2.1), and work with these two was not taken further. In this study, plants with *in vitro* activities of ≤10 µg/ml were chosen. Chloroquine used during this screening showed an IC_{50} of 8.55±2.81ng/ml in the CQS D10 strain The five plants selected (Table 4.3.2.2) all contained fractions with *in vitro* activity ≤10 µg/ml in the chloroquine sensitive (CQS D10) and chloroquine resistant (CQR DD2) strains of the parasite (Table 4.3.3.1). The CQ used in the DD2 strain showed IC_{50} value of 98.5±26.1ng/ml

Tables 4.3.2.1: The *in vitro* antiplasmodial activity of the seven plants extracted with the various solvents using the CQS D10 strain.

Plant Botanical name	PET IC_{50} µg/ml	DCM IC_{50} µg/ml	EA IC_{50} µg/ml	MEOH IC_{50} µg/ml	WATER IC_{50} µg/ml
M. indica	17.5	49.8	39.3	14.6	>50.0
M. sapientum	27.0	52.2	>50.0	>50.0	>50.0
P. guajava	15.5	6.0	21.6	>50.0	>50.0
C. limon	37.2	5.0	>50.0	12.0	>50.0
C. papaya	16.4	12.8	2.6	10.8	>50.0
C. citratus	9.1	7.6	12.1	15.9	>50.0
V. amygdalina	14.2	4.1	10.7	>50.0	>50.0

4.3.2.1 Results and Discussion

Water extracts showed no activity with all IC_{50} values >50µg/ml. Irungu *et al*. (2007) recorded similar results in work with 14 plants. Bhat and Surolia (2001) recorded no activity of the water extracts of *C. papaya.* The petroleum ether extracts of the rind and pulp of the unripe fruit of *C. papaya* demonstrated antiplasmodial activities with IC_{50} values of 15.19 µg/ml and 18.09 µg/ml respectively (Bhat and Surolia, 2001). Their observations using FCK 2 (a local strain of *P. falciparum* from Karnataka state, India) were similar to the IC_{50} value of 16.36 µg/ml from the petroleum ether extracts of the leaves of *C. papaya* investigated in this study usin the D10 of *P. falciparum* (Table 4.3.2.1). Stronger antiplasmodial activities with IC_{50}s of <10 µg/ml were seen mostly in the dichloromethane extracts, except *C. papaya* which showed the highest activity in the ethyl acetate fraction. The IC_{50} values of the five selected extracts which had IC_{50} <10µg/ml compare favourably with those reported for extracts of *Artemisia annua* (3.9 µg/ml) and *Azadirachta indica* (≤10 µg/ml), against *P. falciparum* (Maria do Ceu de *et al*., 2002). Methanol, as well as petroleum ether, extracts recorded activities of >10 µg/ml in most of the plants. The high activity recorded in the dichloromethane (DCM) extracts over extracts from other solvents, like water and methanol, was also reported by Koch *et al*. (2005). This may be traceable to the absence of tannins, polysaccharides and other water- soluble molecules which do not have antiplasmodial properties.

A second batch of the 5 selected plants was further collected and identified by the Botany department of the Abia State University Uturu, Nigeria. The procedures for the second batch were carried out as was done in the first batch. The selected plants in the second batch were also sequentially extracted, as was carried out in the first batch, and the yields from the selected solvents were noted. The HPLC profile, and the *in vitro* antiplasmodial activities, of the selected plants in the second batch, compares favourably with the corresponding plants in the first batch. Samples from the two batches were

pooled together. The total yields from the first and second batch, as well as the total yield of the pooled samples are shown (Table 4.3.2.3).

Table 4.3.2.2: Names and parts of selected plants after screening (second batch)

	Botanical Name	**Common name**	**Local names**	**Part used**
1	*Citrus limon*	Lemon	Oroma nkrisi	Leaves
2	*Psidium guajava*	Guava	Ugofa/ uguava	Leaves
3	*Carica papaya*	Paw paw	Pawpaw/ okwurukwa/	Leaves
4	*Cymbopogon citratus*	Lemon grass	Achara	Leaves
5	*Vernonia amygdalina*	Bitter leaf	Olugbu/ olugbiri	leaves

Table 4.3.2.3: The total weights and yields of the two batches and the pooled samples of each extract.

Botanical name of selected plants	Solvent selected for extra-ction	Weight of Plant (first Batch) (g)	Yield (mg)	Weight of Plant (2nd Batch) (g)	Yield (mg)	Total Weight (first and 2nd batch (g)	Total Yield (g)	% Yield
Citrus limon	DCM	48.3	366.9	746.0	5665.3	794.3	6.0	0.8
Psidium guajava	DCM	84.4	791.6	1264.6	11860.8	1349.0	12.7	0.9
Carica papaya	EA	38.6	187.8	1950.6	9492.3	1989.2	9.7	0.5
Cymbopogon citratus	DCM	43.7	506.7	726.5	8424.0	770.2	8.9	1.2
Vernonia amygdalina	DCM	122.5	3430.7	201.5	5643.0	324.0	9.1	2.8

Key: DCM= Dichloromethane
EA= Ethyl acetate

The extract of interest from *Carica papaya* leaves was the ethyl acetate. The percentage yield of the ethyl acetate fraction was lower compared to the dichloromethane fractions of the other four plants. The *in vitro* antiplasmodial and cytotoxicity activities of the selected extracts are shown in Table 4.3.3.1 The extracts showed little or no cytotoxicity against the CHO cell line with selectivity indices ranging from 9.35-185.37 (Table 4.3.3.1). The control drug for the *in vitro* antiplasmodial experiment was chloroquine while that for cytotoxicity experiment was emetine (Clarkson *et al*., 2004, Chukwugekwu *et al*., 2009, Lategan *et al*., 2009).

4.3.3. *In vitro* activity of selected plants after screening

Table 4.3.3.1 *In vitro* antimalarial activity of the selected extracts on *Plasmodium falciparum* cultures and toxicity towards the CHO cell line

Crude extract/Drug	Solvent	IC_{50} D10 (µg/ml)	IC_{50} DD2 (µg/ml)	IC_{50} CHO (µg/ml)	(SI) D10	(SI) DD2	RI
C. limon	DCM	5.01±0.32	5.99±0.39	247±2.94	49.30	41.23	1.19
P. guajava	DCM	3.38±1.16	4.60±1.00	85.64±2.14	25.33	18.61	1.36
C.papaya	EA	2.96±0.14	3.98±0.42	737.8±0.28	249.25	185.37	1.34
C.citratus	DCM	6.85±0.56	9.44±1.02	331±0.70	48.32	35.06	1.37
V.amygdalina	DCM	3.98±1.21	4.12±0.39	38.54±0.97	9.68	9.35	1.03

IC50 values are given as the mean of three independent experiments

Key: **DCM**= Dichloromethane **EA**= Ethyl acetate

SI=selectivity index**=**Cytotoxic antiplasmodial ratio (IC50 CHO/IC50 *P.falciparum*

RI= Resistance index=IC_{50} DD2/IC50 D10, **CHO**=Chinese Hamster Ovarian cell line

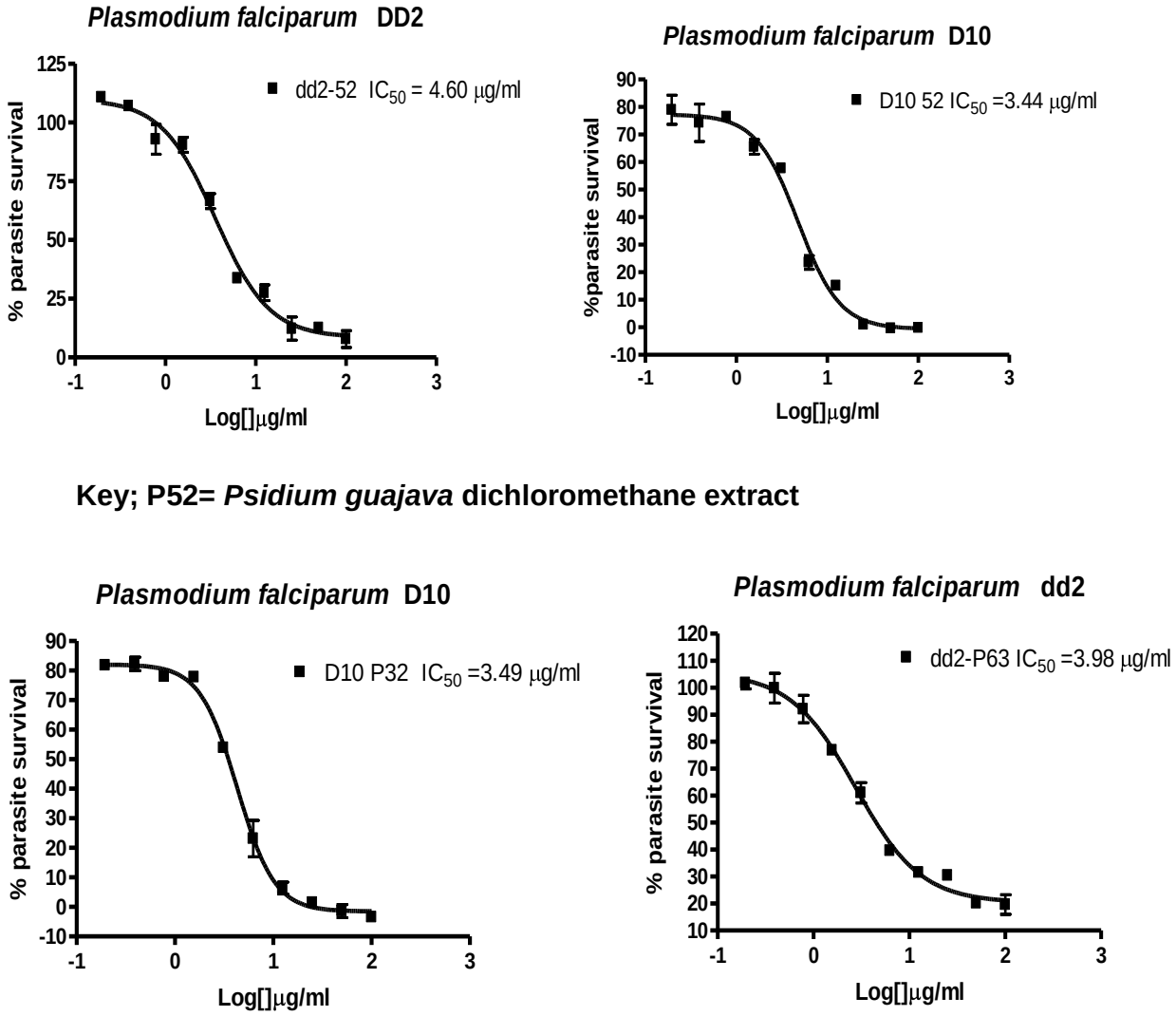
4.3.3.1 Results and Discussion

For the five selected plants after screening the D10 strain used in the experiment was found to be CQ- sensitive with 50% inhibitory concentration (IC_{50}) value of 9.21±3.01ng/ml while the DD2 strain showed IC50 value of 98.5±26.1ng/ml. The representative IC_{50} values of the selected extracts in the cytotoxicity experiments are shown (Fig 4.3.3.3). The activity of the extracts against the chloroquine sensitive (D10) and chloroquine resistant (DD2) strains of *P. falciparum* did not differ significantly. The strongest antiplasmodial activity was observed in the ethyl acetate fraction of *C. papaya. C. papaya* showed a high selectivity for *P. falciparum* with a selectivity index of 249.25 and 185.37 against the D10 and DD2, strains respectively.

In general, an SI ≥10 signifies that biological efficacy is not the result of *in vitro* cytotoxicity (Attioua et. al., 2007, Chukwugekwu *et al*., 2009). Literature reveals

that other *Vernonia* species have been investigated for their antiplasmodial activities on other strains of *P. falciparum*. Irungu *et al*., (2007) reported an antiplasmodial activity of 4.7 µg/ml against the K1 strain for the dichloromethane extract of *Vernonia lasiopus*. This correlates well with the activity of *V. amygdalina* with IC_{50} values of 4.12 µg/ml and 3.98 µg/ml against the DD2 and D10 strains, respectively obtained in this study (Table 4.3.2.4). *V. lasiopus* is one of the plants used to treat malaria in Kenya (Muregi *et al*., 2003). The work of Muregi *et al*., (2003) also recorded similar observations on the antiplasmodial activities of *Vernonia* species investigated. In the work of Pillay *et al*., (2007) the DCM extract of *V. staehelioides* showed an IC_{50} value of 3.0 µg/ml against the D10 strain and is similar to the activity of the DCM extract of *V. amygdalina* in the present study (3.98 µg/ml) against the chloroquine sensitive D10 strain. Extracts from leaves of *V. brasiliana* species using n-hexane fractions also showed antiplasmodial properties (Alves *et al*., 1997). *V. colorata* subspecies *grandis* was active against W2 with an IC_{50} value of 6 µg/ml and 10 µg/ml against human monocytic THPI cells. In this study, *V. amygdalina* showed IC_{50} of 4.12 µg/ml and 38.54 against the DD2 strain and the CHO cell line, respectively. Some Sesquiterpene lactones isolated from *V. amygdalina* such as vernolepin, vernolin, vernolide, vernodalin and hydroxyvernodalin have demonstrated antiplasmodial activity with IC_{50} values of <4 µg/ml (Phillipson *et al*., 1993), however, no cytotoxicity testing was done to establish if the recorded activity was due to the cytotoxic effect of the isolated sesquiterpene lactone. Different species of the genus *Vernonia* have shown promising antiplasmodial activities. In addition, in this work, *V. amygdalina* showed a low RI of 1.03 when compared to other extracts indicating good activity against the CQ resistant strain. Work done by Iwalokun (2008) showed that *V. amygdalina* dose dependently enhanced the efficacy of CQ against *P. berghei*. This suggests that extracts of this species may be good candidates in the reversal of chloroquine resistance. The *in vitro* antiplasmodial activity of *C. citratus* was not significantly different from *C. limon.* Similarly, there was no

significant difference between the *in vitro* antiplasmodial activities of *V. amygdalina* and *P. guajava* (Fig 4.3.3.1 and fig 4.3.3.2).



Key; P52= *Psidium guajava* dichloromethane extract

Fig 4.3.3.1 The *in vitro* antiplasmodial dose response curves of the representatives of the selected extracts.

Key:
P32= ***Citrus limon*** dichloromethane extract
P63= ***Carica papaya*** ethyl acetate crude extract

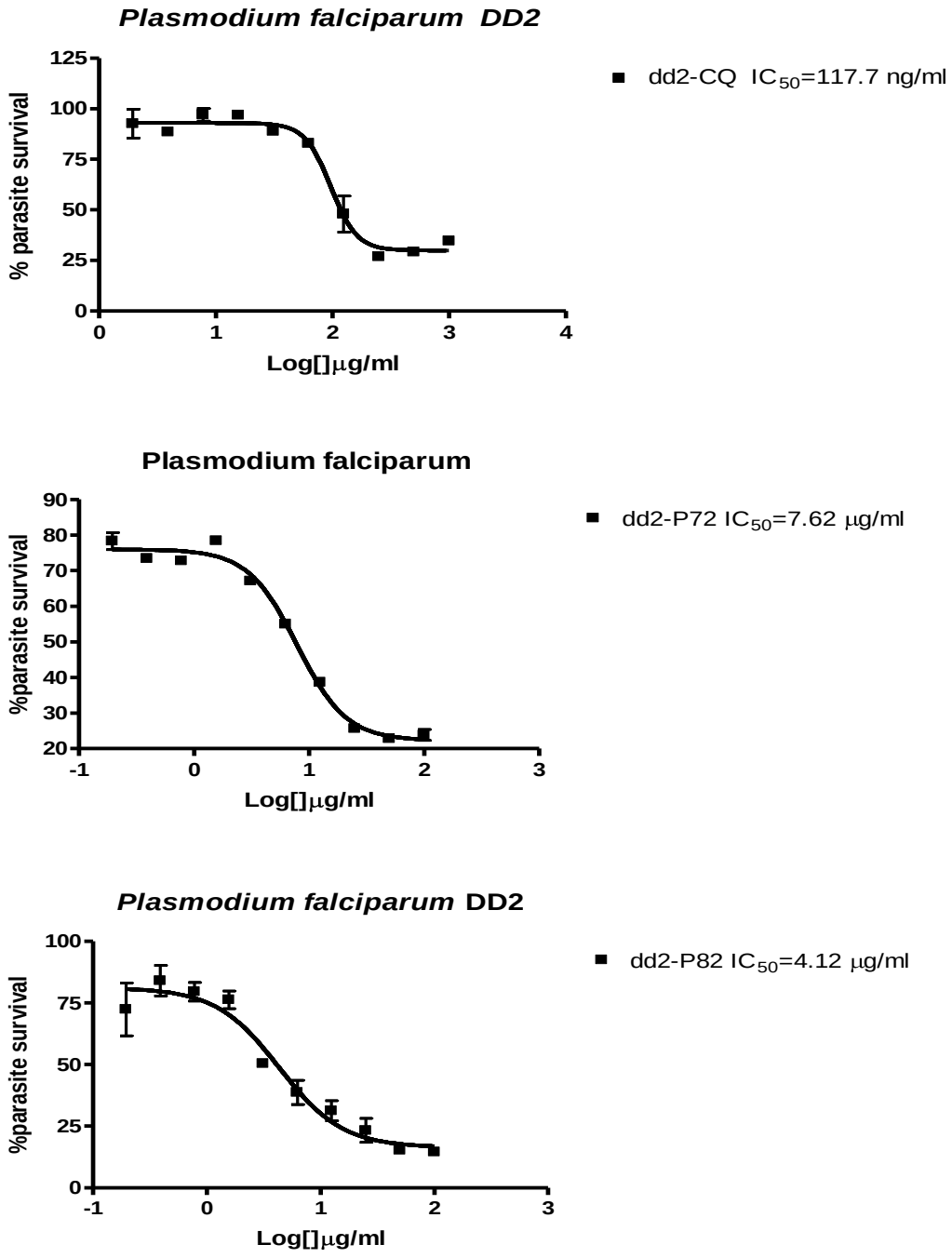


Fig 4.3.3.2 The *in vitro* antiplasmodial dose response curves of the representatives of the selected extracts.

Key: P72= ***Cymbopogon citratus*** (DCM extract); **P82**= ***Vernonia amygdalina*** (DCM extract)

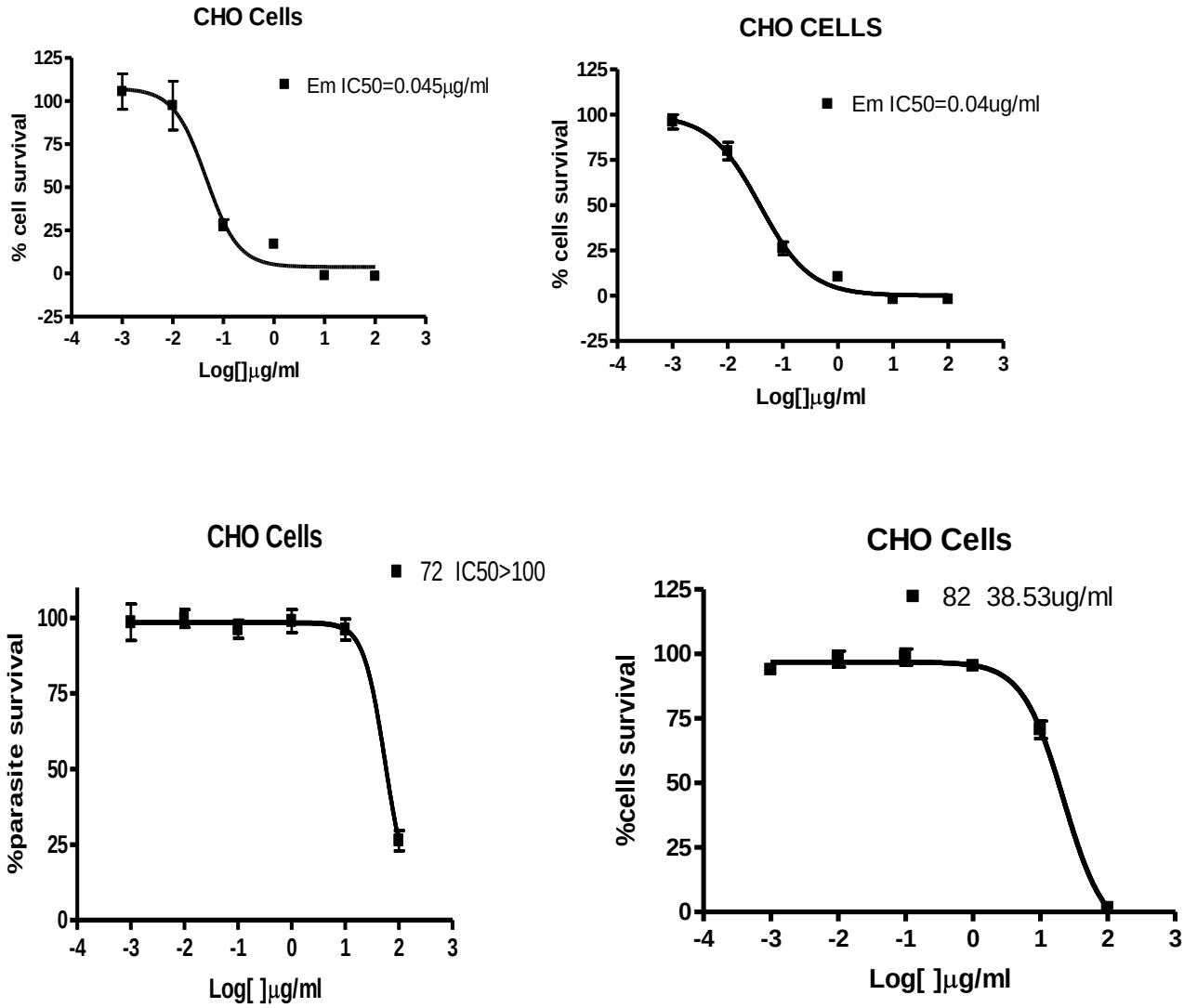


Fig 4.3.3.3 The *in vitro* dose response curves of representatives of the cytotoxicity experiments of selected extracts

Key:
Em= Emetine
P72= ***Cymbopogon citratus*** dichloromethane crude extract
P82= ***Vernonia amygdalina*** dichloromethane crude extract

Most of the extracts showed equipotent activity against the sensitive and resistant strains of the parasite. However, it was apparent that there were slight increases in parasite survival in the DD2 IC_{50} as compared to the D10 IC_{50}. This increase was significant between *C. citratus* and *P. guajava*. Representatives of the *in vitro* experiments with the extracts used in this study are further shown (figs A1-A3). The traditional healers in Nigeria use these plants in combination in the treatment of malaria (Igoli *et al*., 2005). It was therefore decided to test the active extracts in combination for antiplasmodial activity to establish if they act synergistically.

4.3.4 *In vitro* antiplasmodial activity of extracts in combination

Combination therapy, which has been a strategy approved for other multidrug resistance infections such as HIV and tuberculosis, is widely recommended for malaria treatment (White, 1998; WHO, 2001). Over the past decades combination therapy has gradually replaced single drug treatment due to the rapid spread of drug resistance by *Plasmodium* parasites globally (Martinelli *et al*., 2008). Recently, artemisinin combination therapy (ACT) has been the main therapeutic treatment for malaria, but has been met with treatment failures in some regions. This emphasizes the urgent need for the development of new drugs and combination treatments. The antiplasmodial properties of the extracts from five plants were investigated in combination with each other (Table 4.3.4.1). The combination study was confined to combinations of two extracts. The checkerboard method used in the combination studies in this work was adapted from Berenbaum *et al*., (1978), as described in section 3.4.1.1. In this method the concentration of one extract is kept constant while in the other it is varied. The *in vitro* dose response curves of representative extracts in combination using the D10 chloroquine sensitive strain are shown in fig 4.3.4.1 and figs A4 -A9.

Table 4.3.4.1: IC_{50} values (µg/ml) of two extracts in combination (a+b) when extract a is kept constant while extract b is the variable component using CQS D10 strain of *P. falciparium*

Combination a+b	50µg/ml a + 50µg/ml b	50µg/ml a + 25µg/ml b	50µg/ml a + 5µg/ml b
32+52	3.39	3.91	4.17
52+32	3.09	4.89	6.76
32+63	2.73	4.47	4.4
63+32	0.83	1.65	2.28
32+72	4.22	5.62	6.02
72+32	6.6	8.1	11.4
32+82	24.26	25.17	30.83
82+32	17.03	13.5	19.01
52+63	2.72	39.90	48.30
63+52	2.31	2.59	6.32
52+72	9.68	10.02	10.23
72+52	6.08	10.30	13.89
52+82	>50	>50	>50
82+52	43.35	44.05	>50
63+72	3.01	3.8	3.98
72+63	4.07	4.51	4.46
63+82	8.87	15.84	16.03
82+63	4.42	8.74	23.01
72+82	7.23	6.82	10.37
82+72	9.1	9.5	11.2

Key: **P32**= ***Citrus limon*** dichloromethane extract
P52= ***Psidium guajava*** dichloromethane extract
P63= ***Carica papaya*** ethyl acetate crude extract
P72= ***Cymbopogon citratus*** dichloromethane crude extract
P82= ***Vernonia amygdalina*** dichloromethane crude extract

4.3.4.1 Results and discussion

Results are shown in Table 4.3.4.1. Some combinations such as that in 63+72 and 32+52 do not show significant differences in their activity when either extract was kept constant and the other varied at different concentrations. This suggests that their activities were not dose dependent. Combinations of 52+82 showed no enhancement of the activity of either component in combination. However significant enhancement of activity was observed between the extracts 63+32. This suggests that some of the components in this combination may have acted synergistically (Berenbaum, 1978). Combinations with activities ≤10µg/ml were chosen and further tested against CQS and CQR strain (DD2). The results given are the mean of three independent experiments (Table 4.3.4.2). A cytotoxicity experiment was carried out on selected combinations using the CHO cell lines. This was carried out to investigate if the activity of the extracts in combination at different concentrations is cytotoxic to the CHO cell lines or selective to *Plasmodium falciparum*. The combinations were not cytotoxic at the concentrations tested (Table 4.3.4.2). The cytotoxicity values of these combined extracts showed higher selectivity for *P. falciparum* when compared with the cytotoxicity values of extracts singly (Table 4.3.3.1).

Table 4.3.4.2: *In vitro* activity of selected combination of crude extracts on *Plasmodium falciparum* D10 and DD2 strains and toxicity towards Chinese Hamster Ovarian (CHO) cell lines

Extract a+b Each at 50µg/ml	D10 IC_{50} (µg/ml)	DD2 IC_{50}(µg/ml)	CHO IC_{50} (µg/ml)	SI
32+52	3.39±1.85	3.78±0.95	82.60	24.32
63+32	0.83±0.56	0.86±0.62	>100	ND
63+52	3.71±0.22	4.02±1.12	>100	ND
63+72	3.01±0.55	2.95±0.78	>100	ND
32+72	5.01± 1.21	4.98±1.86	>100	ND
72+52	5.01±0.74	5.28±1.03	>100	ND
72+82	4.43±0.21	3.82±0.72	>100	ND

Key: **P32**= ***Citrus limon*** dichloromethane extract
P52= ***Psidium guajava*** dichloromethane extract
P63= ***Carica papaya*** ethyl acetate crude extract
P72= ***Cymbopogon citratus*** dichloromethane crude extract
P82= ***Vernonia amygdalina*** dichloromethane crude extract

a+b=extract a combined with extract b, IC_{50} values are given as the mean of 3 independent experiments. ND= Not determined

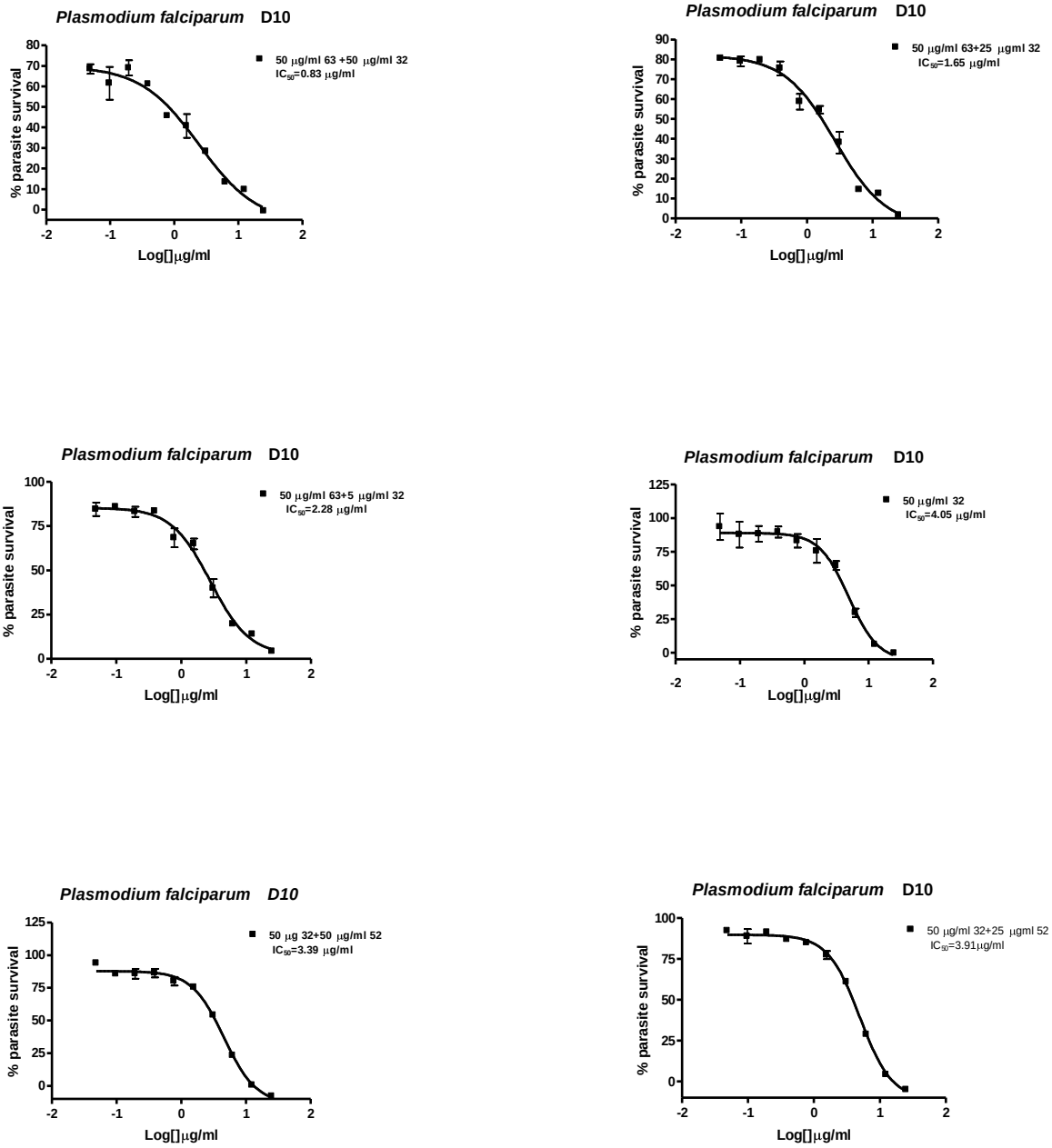


Fig 4.3.4.1 Dose response curves: The dose response curves of some representatives of the extracts in combination

Key: P32= ***Citrus limon*** dichloromethane extract, **P52**= ***Psidium guajava*** dichloromethane extract, **P63**= ***Carica papaya*** ethyl acetate crude extract

4.3.4.2 Determination of interactions between selected combinations

In order to ascertain if the more active combinations were acting synergistically, their fractional inhibitory concentrations (FIC) were determined as described by Berenbaum (1978). This method has been used by other researchers including Canfield *et al*., (1995) and Fivelman *et al*., (2004)). CQ IC50= 9.21±3.01ng/ml.

Table 4.3.4.2.1: The selected combinations and their effect when extract a is kept constant using the D10 chloroquine sensitive strain

Combination a+b	IC_{50} of [a] in combination	IC_{50} of [b] in combination	FIC value
32+52	3.39	4.97	1.99
63+32	0.83	2.43	0.76
63+52	3.71	3.82	2.39
63+72	3.01	3.87	1.56
32+72	5.01	5.63	1.85
72+52	5.01	6.26	2.58
72+82	4.43	9.61	3.09

Table 4.3.4.2.2: The IC_{50} values of the individual extract using the CQS D10 strain values from table 4.3.3.1

Extract	IC_{50} of extracts
32	5.01±0.32
52	3.38±1.16
63	2.96±0.14
72	6.85±0.56
82	3.98±1.12

Key: **P32**= ***Citrus limon*** dichloromethane extract
P52= ***Psidium guajava*** dichloromethane extract
P63= ***Carica papaya*** ethyl acetate crude extract
P72= ***Cymbopogon citratus*** dichloromethane crude extract
P82= ***Vernonia amygdalina*** dichloromethane crude extract

The formula used in calculating the sum of the fractional inhibitory concentrations (FIC) is given below;

$$\frac{IC_{50}\ (\mu g/ml) \text{ of extract (a) in combination}}{IC_{50}\ (\mu g/ml) \text{ of extract (a) alone}} + \frac{IC_{50}\ (\mu g/ml) \text{ of extract (b) in combination}}{IC_{50}\ (\mu g/ml) \text{ of extract (b) alone}}$$

When the sum of the FIC values is less than 0.5 this indicates a clear case of synergism while values between 1 and 0.5 indicate a low grade synergism (Gupta *et al*., 2002). Values between 0.5 and 2 are generally regarded as indeterminate, while those above 2 indicate antagonism (Gupta *et al*., 2002).. FIC values for the five most active combinations are shown in Table 4.3.4.2.1. One combination (63+32) is tending towards synergism while the others show either an antagonistic or indifferent effect.

Five extracts from different plants were investigated in this study. Several combinations of these plants were tested. Each combination was tested with one component being kept constant while the other is varied and vice versa. The combinations with antiplasmodial activity ≤10 µg/ml were shown (Table 4.3.4.2). The activities of these combinations showed no significant difference in the D10 and DD2 strains of *P. falciparum*. The extracts whose activity was significantly enhanced in the combination experiment showed an FIC value of 0.76 (Table 4.3.4.2.1). This was recorded in the combination between the ethyl acetate extract of *C. papaya* and the dichloromethane extract of *C. limon*. This combination was not cytotoxic to the CHO cell lines and had a high selectivity index (Table 4.3.4.2). An enhancement of activity was also noted whenever *C. papaya* ethyl acetate extract was one of the components used in combination. This was noticed whenever the *C. papaya* ethyl acetate (P63) was kept

constant and the other component varied (Table 4.3.4.2.1, Table 4.3.4.1). Due to the good *in vitro* antiplasmodial activity of *C. papaya* against both chloroquine-sensitive and resistant strains of the parasite, the indication that it interacts synergistically with *C. limon* and its low cytotoxicity, it was selected for further investigation. This was carried out through a bioactivity-guided fractionation with the aim of tracing the activity of this extract and isolating the compounds responsible for the recorded activity.

4.4 Bioactivity-guided fractionation

4.4.1 Introduction

The ethyl acetate extract was investigated through a bioactivity guided fractionation and characterization of isolated compounds using the procedures shown in the pyramid chart (Fig 4.4.1.1).

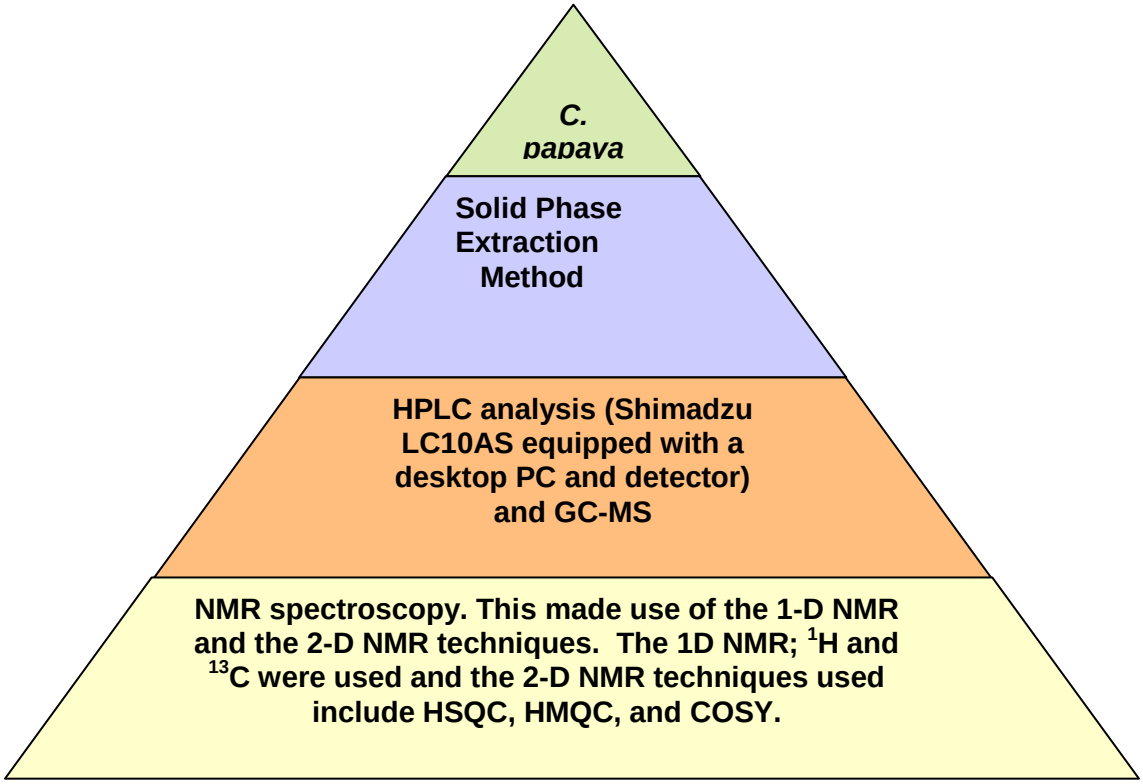


Fig 4.4.1.1 Fractionation procedure of *C. papaya* ethyl acetate crude extract and identification of isolated compounds.

4.4.2 Solid phase extraction (SPE)

Solid phase extraction was used to fractionate the ethyl acetate extract of *C. papaya* (3.5.1). Briefly, SPE involved sequential elution of the extract from an isolute C18 cartridge. The extract (10 mg/ml) was dissolved in methanol and layered into a pre-wetted and preconditioned cartridge. The unretained materials were washed off under vacuum with Millipore water. Different concentrations of acetonitrile in millipore water were used in 20% increments to elute the substances that were absorbed in the sorbent bed. The fractions were collected, dried and tested for antiplasmodial activity against the D10 chloroquine sensitive strain of *P falciparum*. Chloroquine showed an IC_{50} of 12.13ng/ml. The 100% acetonitrile concentration showed an *in vitro* activity with an IC_{50} of 2.24µg/ml. Results are shown in Table 4.4.1.1.

Table 4.4.1.1 Activities of *C. papaya* SPE fractions against the CQS D10 strain

Percentage ACN Fraction	Weight of Fractions (mg)	IC_{50} µg/ml
20%	142.80	>50
40%	295.60	16.55
60%	329.90	2.52
80%	482.51	2.69
100%	369.50	2.24

The 100% ACN fraction was further fractionated using HPLC analytical system and revealed the chromatogram shown in Fig 4.4.2.1.

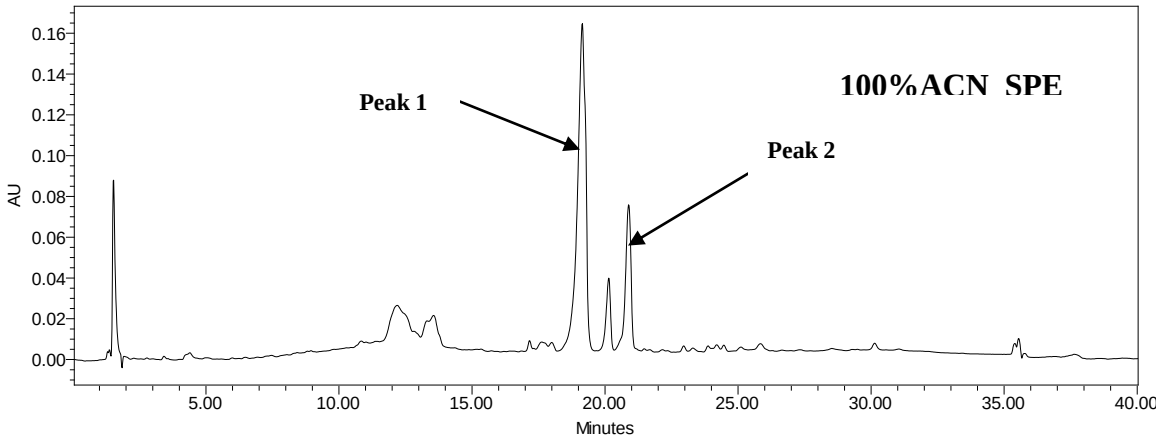


Fig 4.4.2.1 HPLC profiles of SPE fraction using C18 column: Agilent XDB C18 RP analytical: 4.6 x 150mm; 5µ particle size. (λ = 236.8nm)

HPLC conditions: Mobile phase ACN: H_2O using a gradient of 20-100% ACN (30 minutes) 100% ACN hold (3 minutes), 100-20%ACN (2 minutes) 20% ACN hold (5 minutes)
Injection volume: 30 µl of 1 mg/ml
Column temp: 30°C
Flow rate: 1 ml/min

4.4.3 HPLC Results

The HPLC profile of the *C. papaya* ethyl acetate SPE fraction (100% ACN) showed two major peaks. These were isolated using semi-preparative and analytical columns. The CQS D10 and CQR DD2 used in this assay showed IC_{50} of 10.83ng/ml and 118.6ng/ml respectively. Peak1 had IC_{50}s of 3.58 µg/ml and 4.40 µg/ml against the CQS D10 and CQR DD2 of *P. falciparum*, respectively, while peak 2 recorded IC_{50} values of 6.88 µg/ml and 6.80 µg/ml against the CQS and CQR strains of *P. falciparum*, respectively. These two peaks were less active than the SPE fraction (2.2 µg/ml) as well as the ethyl acetate extracts which had an IC_{50} of 2.96 µg/ml against the CQS strain and

3.98 µg/ml against the CQR strain (Table 4.3.3.1). The *in vitro* activities of these peaks are shown in the dose response curves in Fig 4.4.3.1. Neither peak showed significant cytotoxicity (Table 4.4.3.1).

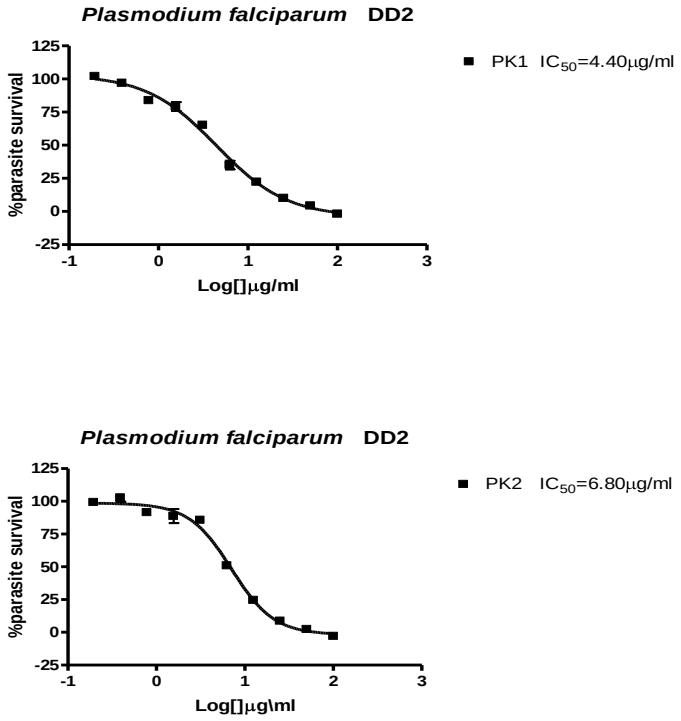


Fig 4.4.3.1 Dose response curves of peaks 1 and 2

Table 4.4.3.1 *in vitro* activity of peaks 1 and 2

Sample name	D10 IC_{50} µg/ml	DD2 IC_{50} µg/ml	CHO IC_{50} µg/ml	SI-D10 CHO	SI-DD2 CHO	RI
Peak 1	3.58	4.40	54.70	15.27	12.43	0.81
Peak 2	6.88	6.80	51.16	7.43	7.52	1.01

4.4.4 Nuclear Magnetic Resonance (NMR)

4.4.4.1 Introduction

An attempt was made to characterize and elucidate the structures of compounds 1 and 2 using the 1D and 2D NMR spectrometric methods. The ^{1}H and ^{13}C spectra used in this study are the most widely used 1-D NMR techniques. ^{1}H –NMR spectra can identify the protons in molecules. The number of ^{13}C signals identified compounds 1 and 2 as unsaturated aliphatic fatty acids. Generally, 1D NMR helps in identification of aliphatic systems, determination of the degree of unsaturation, as well as the identification of functional groups. Further characterization of compounds 1 and 2 using 2-D NMR techniques, which included HSQC, HMQC, and gCOSY met with difficulties due to the similarity in chemical shift of most of the methylene groups and of the olefinic double bonds. In this study the representative 2D NMR data of compounds 1 and 2 using the conventional NMR spectroscopy are shown (Figs 4.4.4.1.1 to 4.4.4.1.6 and figs A18-A23).

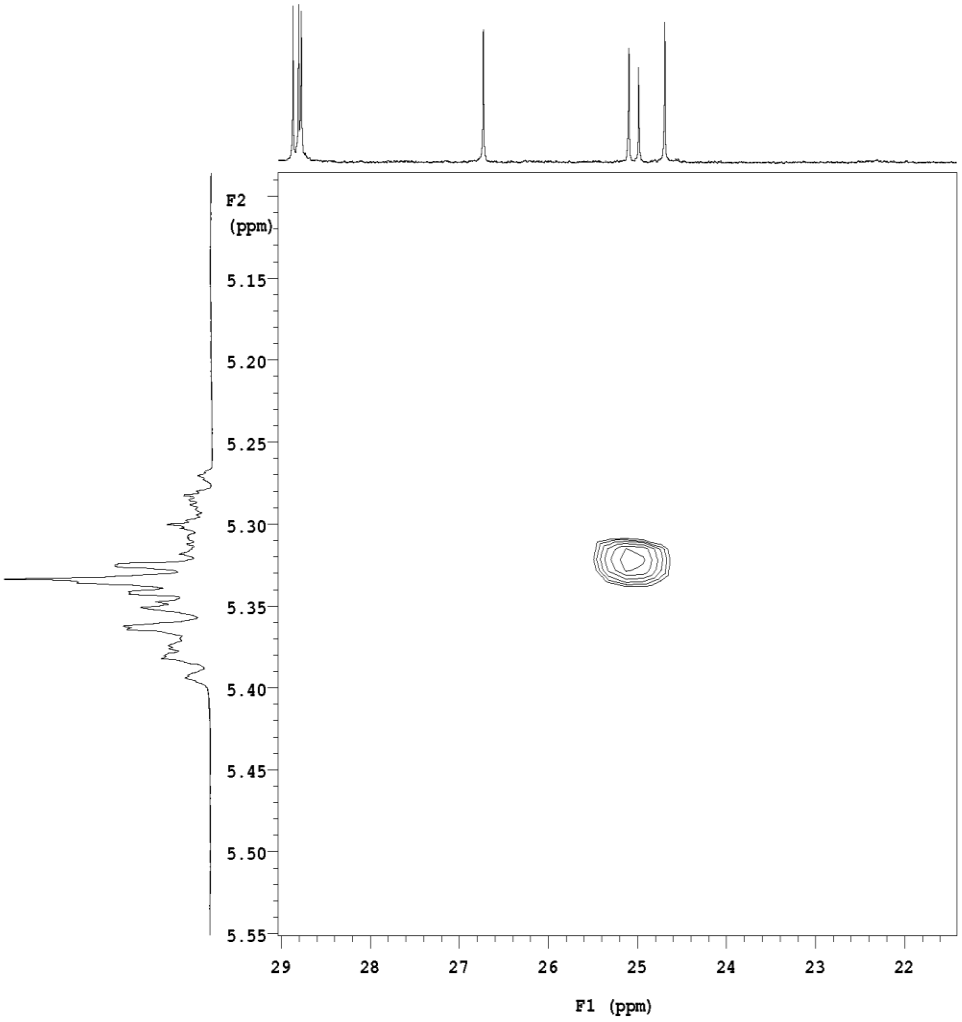


Fig 4.4.4.1.1: A representative of compound 1 gHMQC plot.

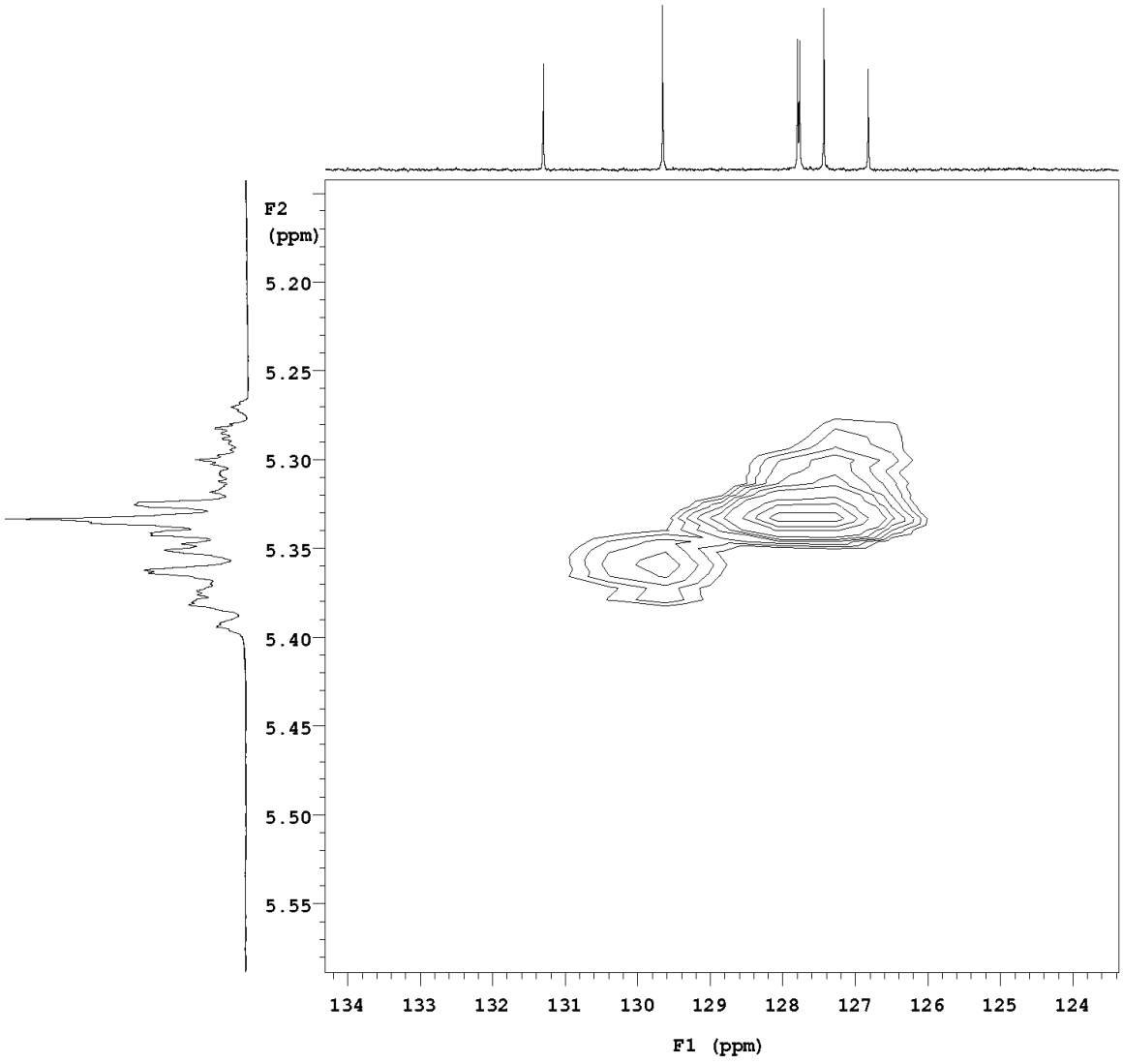


Fig 4.4.4.1.2: A representative of compound 1 gHSQC plot.

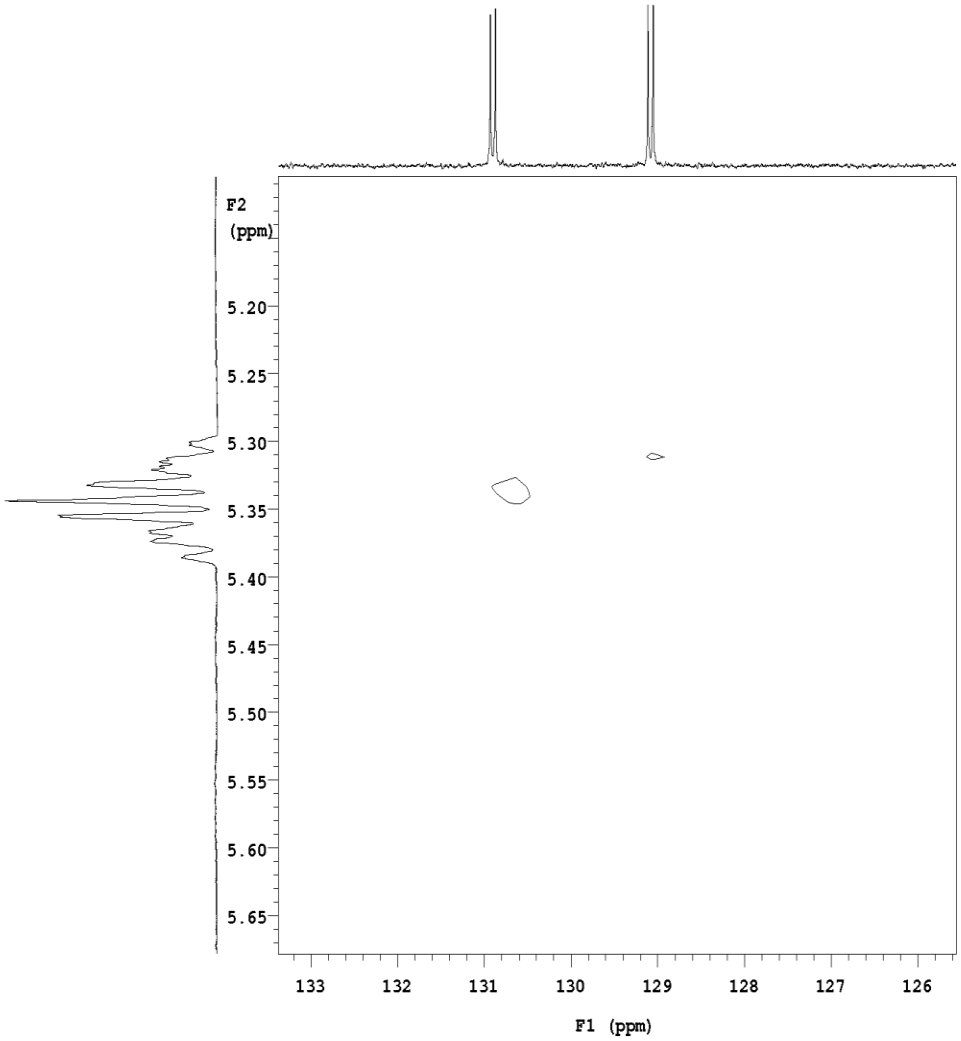


Fig 4.4.4.1.3: A representative of compound 2 gHSQC plot

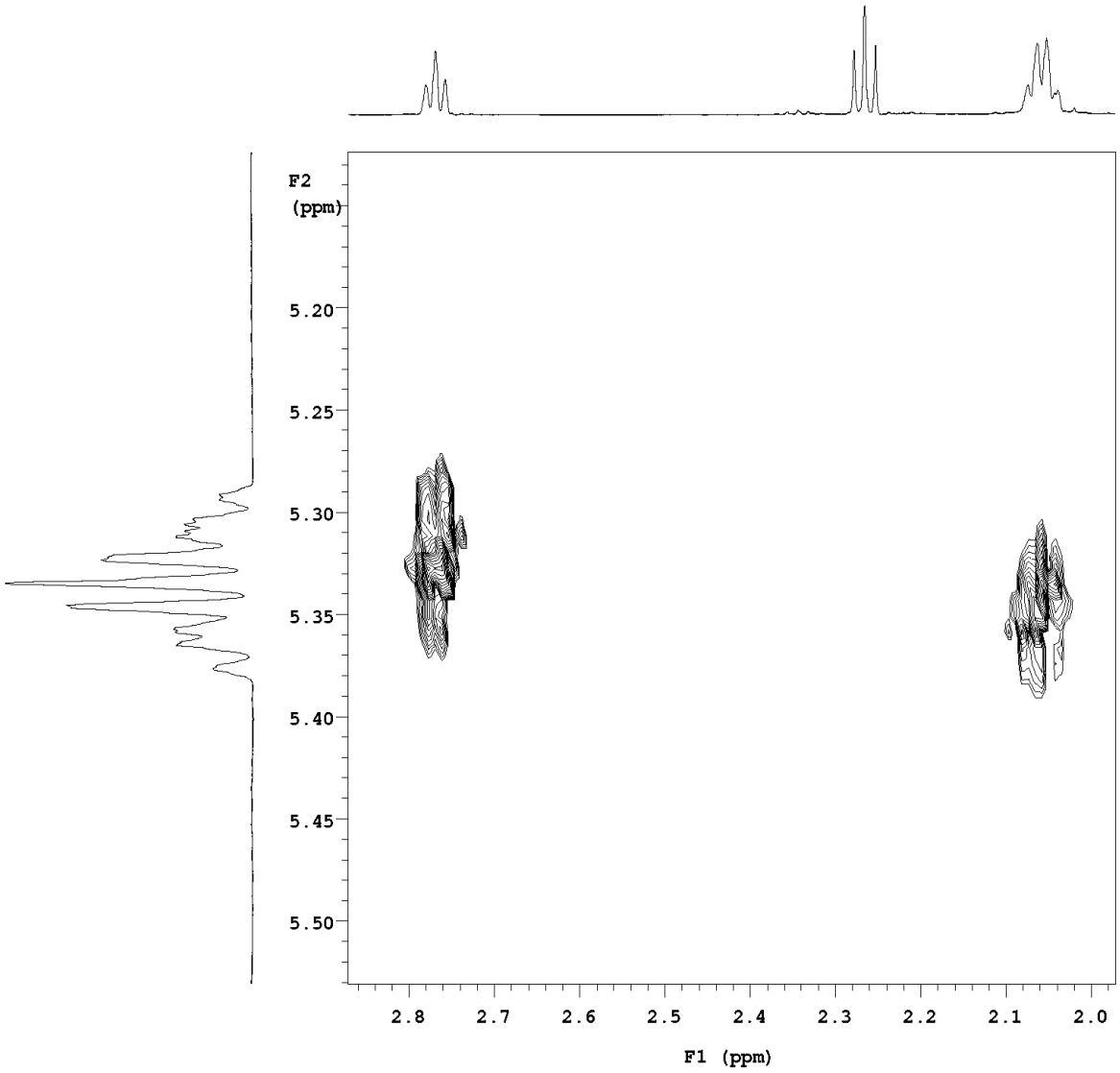


Fig 4.4.4.1.4: A representative of compound 2 gCOSY plot

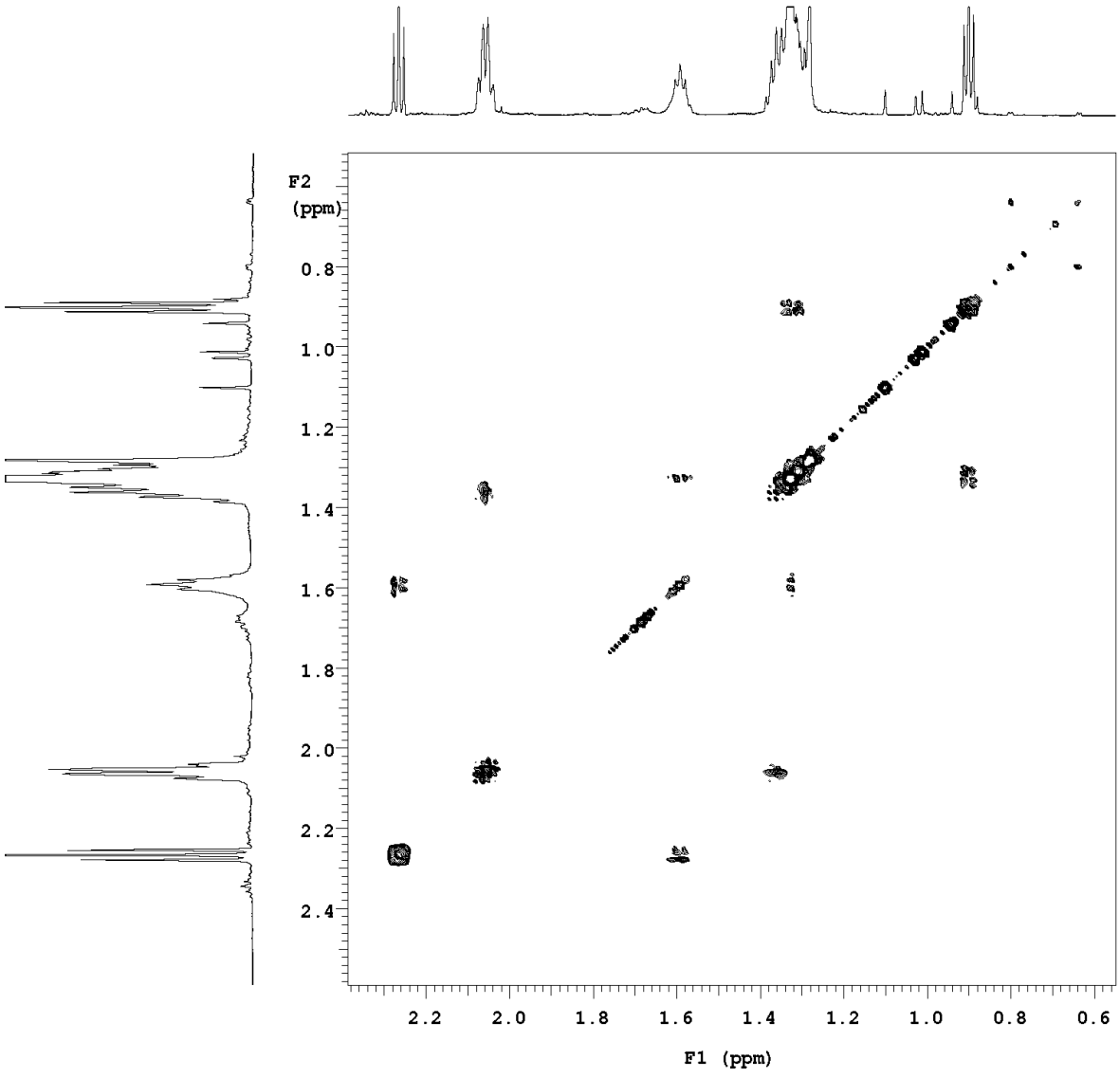


Fig 4.4.4.1.5: A representative of compound 2 gCOSY plot

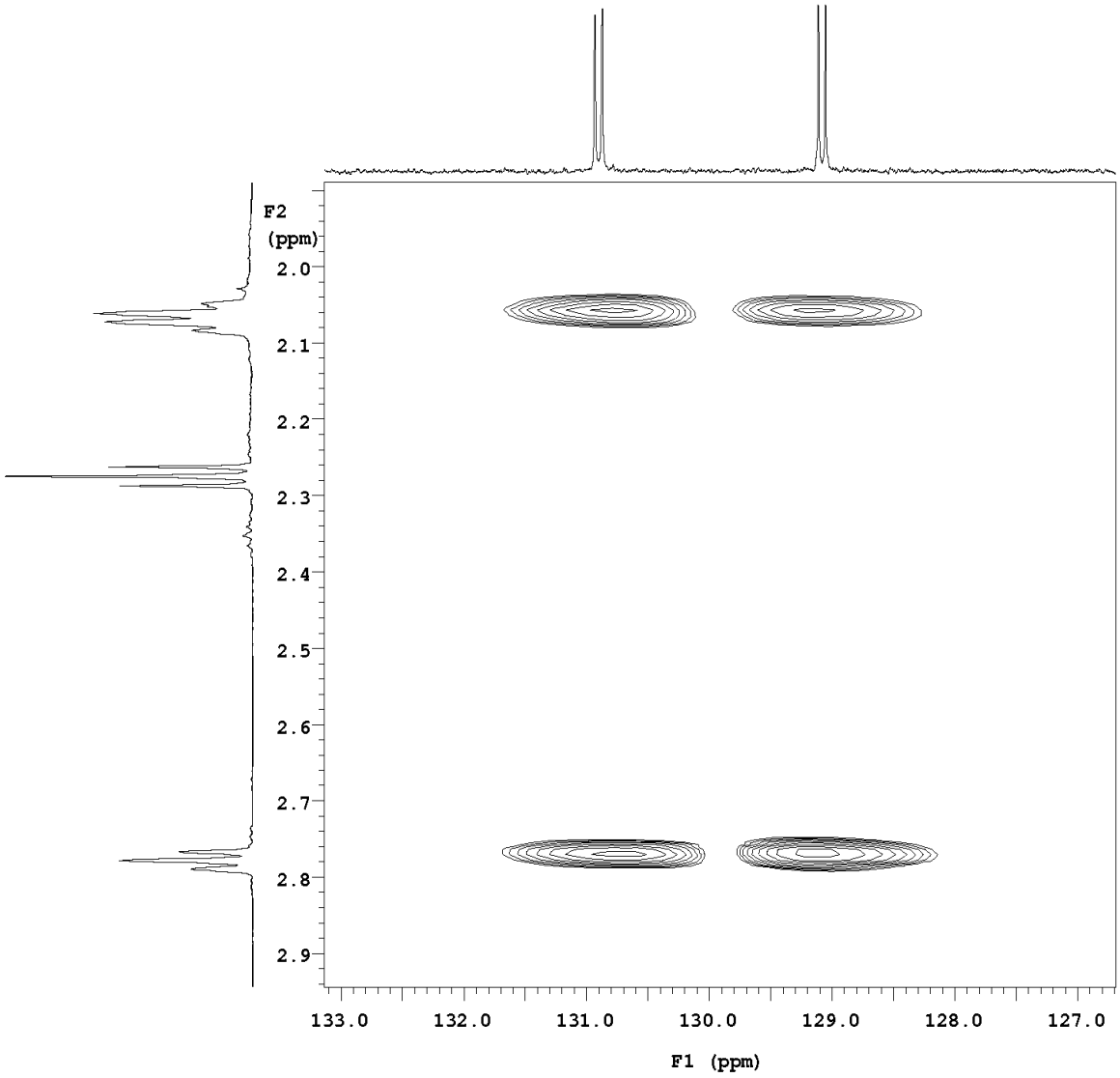


Fig 4.4.4.1.6: A representative of compounds 2 gHMQC

4.4.4.2 The structural characterization of compounds 1 and 2

Two groups could be unequivocally identified. The CH_2 at position 2 gave a triplet at δ2.30, with a coupling constant of 7.50Hz. Similarly the CH_3 at position 18 gave a triplet at δ0.90 with a coupling constant 0f 3.6Hz. The multiple peaks for compound 1, which were at ^{1}H 5.27 - 5.38 (9, 12, 15 H) were connected to carbon signals at ^{13}C 131.0-127.0 in the HSQC spectrum (Fig 4.4.4.1.2), while in compound 2 the multiple peaks at ^{1}H peaks which were at δH 5.30 - 5.39 (9, 12 H) were linked to the carbon signals at ^{13}C 129.0-130.6 in the HSQC spectrum (Fig 4.4.4.1.3). Due to the complexity of the resonances for the olefinic protons we do not believe that it is possible to assign the stereo-configuration of the double bonds using the conventional NMR spectroscopy. The work done by Vatele *et al.,* (1998); Viron *et al*., (2000); Cao *et al*., (2006; 2007), emphasized the difficulties encountered in characterizing this class of compounds. Cao *et al*., (2007), made a significant input in giving a more detailed characterization of the unsaturated fatty acids he studied (ß-eleostearic, α-eleostearic and punicic acids) by using a derivative which converted the acid into a nitrogen containing derivative. Vatele *et al.,* (1998), reported the use of a new 2D NMR technique: SAPHIR-HSQC, in the complete assignment of ^{13} C and elucidation of n-3 polyunsaturated fatty acids. Cao *et al*., (2006), documented the use of silver ion impregnated high-performance liquid chromatography (Ag+-HPLC) in the separation of CLnA methyl ester (CLnAMe) mixture while Ying *et al*., (2007) reported the identification and characterization of conjugated linolenic acid isomers by Ag^{+} -HPLC and NMR**.** Gas chromatography is routinely used to analyze fatty acids due to its high resolution, speed and sensitivity (Horning *et al*.,1964). It was therefore necessary to attempt to structurally identify these aliphatic compounds by GC-mass spectrometry.

4.4.5 GC-MS analysis

The purity of these peaks was further confirmed using the GC-MS spectrometry facility available at the University of Stellenbosch GC-MS laboratory. The HPLC samples were analyzed by a Waters GCT premier spectrometer, model HP5 (3.6). The column specification was 30 m, 0.25 mm ID, 0.25 µm film thickness. The carrier gas was helium with a constant flow of 1 ml/min. The injection split was 1:5, the temperature of the injector and the transfer temperature were 280ºC. The EI ionization energy was 70eV, the scanning mass range was *m/z* 40 to 400 (perfluoro-tri-*N*-butylamine as mass reference), with a solvent hold of 6 minutes. Peaks 1 and 2 were identified as essential fatty acids 9,12,15-octadecatrienoic acid (Compound 1: Linolenic acid) and 9,12-octadecadienoic acid (compound 2: Linoleic acid). These essential fatty acids belonging to the C_{18} fatty acid differ structurally in the position and degree of unsaturation. Compound 1 also known as Linolenic acid has three double bonds, while compound two also known as Linoleic acid has two double bonds. The GC-MS spectra and structure of Compounds 1 and 2 are shown (Figs 4.4.5.1- 4.4.5.2 and figs A12-A17). The spectrum of compound 1 (Fig 4.4.5.1) shows the molecular ion at *m/z* 278. In compound 1 two losses of CH_2 groups were evident (*m/z* 135 - m/z 121; *m/z* 93 – 79), while in compound 2 (Fig 4.4.5.2) the molecular ion was shown at *m/z* 280. Two losses of CH_2 groups were also evident (*m/z* 96 - m/z 82; *m/z* 82 – 67) in the spectrum of compound 2.

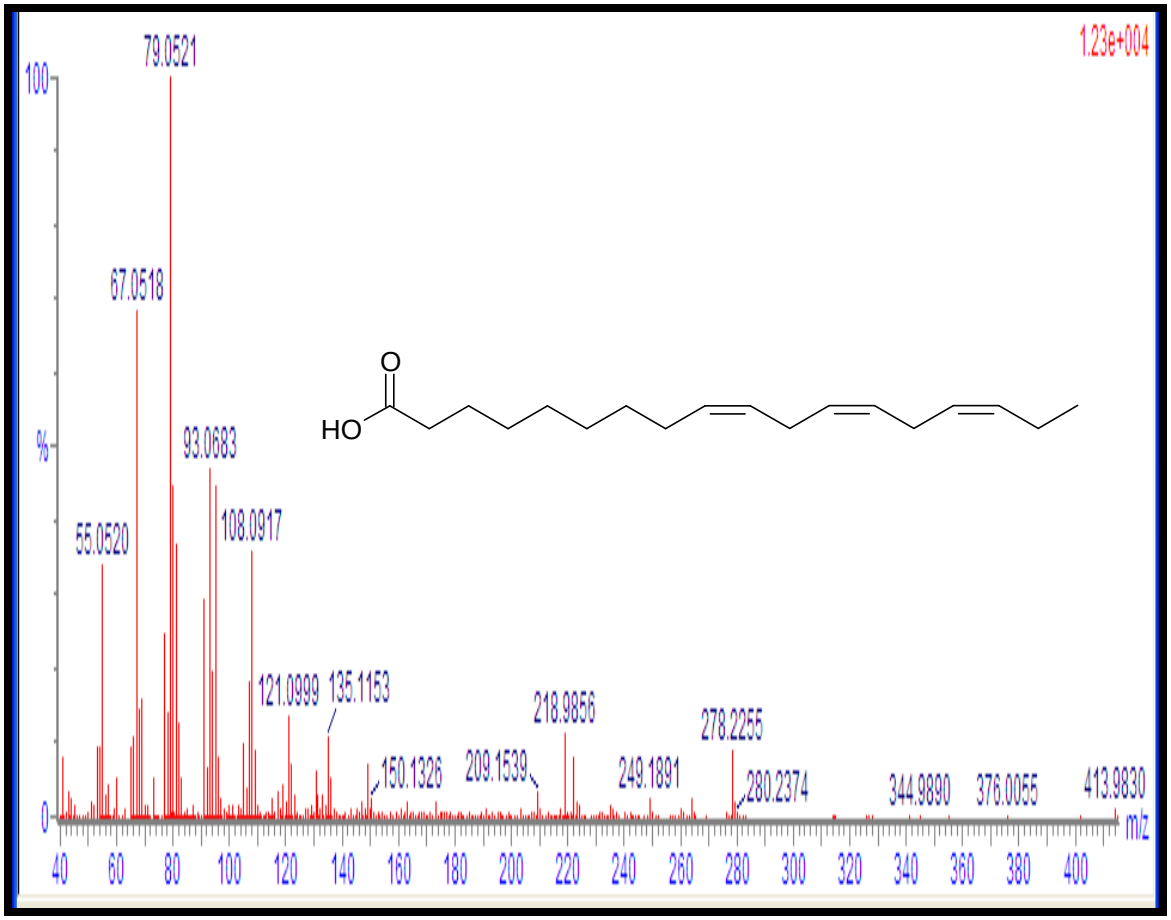


Fig 4.4.5.1: GC-MS spectrum showing the structure of compound 1 (9, 12, 15, Octadecatrienoic acid).

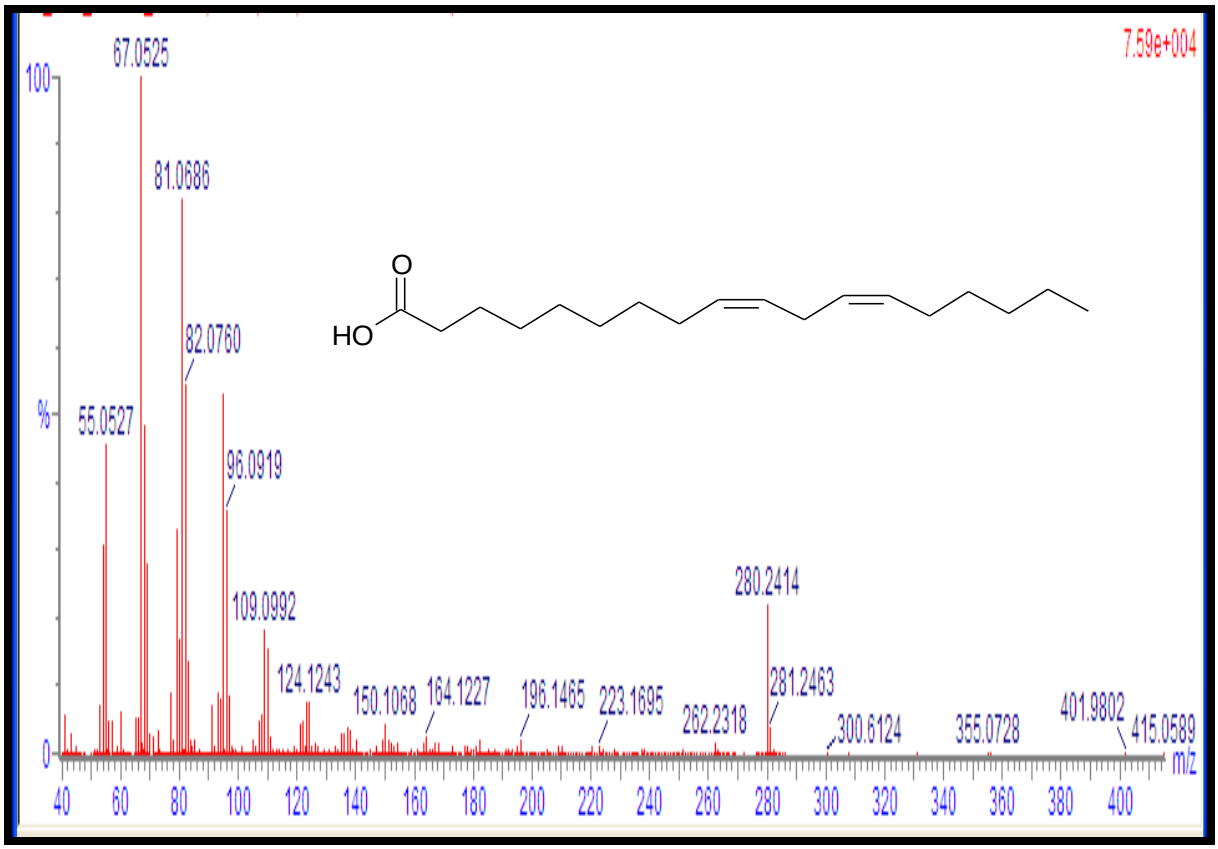


Fig 4.4.5.2: GC-MS spectrum showing the structure of compound 2 (9, 12, Octadecadienoic acid).

4.4.6 Result and discussion

Linolenic and linoleic acids belong to the C_{18} fatty acids. Linolenic acid (compound 1) which has three double bonds, showed a higher antiplasmodial activity with a higher selectivity index when compared to linoleic acid (compound 2) with two double bonds (Table 5.2.1). Previous work on a C18 fatty acid (scleropyric acid) isolated from the twigs of *Scleropyrum wallichianum* Arn. of the family Santalaceae (Suksamrarn *et al*., 2005) reported antiplasmodial activity with an IC_{50} value of 7.2 µg/ml against K1 (CQR) strain of *P. falciparum*, similar

to the antiplasmodial activity of linoleic acid with an IC_{50} of 6.80 µg/ml against the DD2 (CQR) strain in this study. Further study documented the antiplasmodial activities with IC_{50}<5µg/ml showed by fatty acids isolated from *Croton lobatus* against *Plasmodium falciparum* K1 (CQR) strain (Attioua *et al*., 2007). The fatty acids they isolated included (*Z*.,*Z*.,*Z*.)-9,12,15-octadecatrienoic acid methyl ester, 8,11,17,21-tetramethyl-(*E*.,*E*.,*E*.,*E*.)-8,10,17,21-tetraentetracosanoic acid, (*E*.)-3-(4-methoxy-phenyl)-2-phenyl-acrylic acid and betulinic acid (Attioua *et al*., 2007).

The antiplasmodial activity of the unsaturated fatty acids has been reported to increase as the degree of unsaturation increases (Kumaratilake *et al*., 1992, Kumaratilake *et al*., 1997). These researchers reported the marked *in vitro* growth inhibition of *P.falciparum* by Docosahexaenoic acid ($C_{22\text{-}6,n\text{-}3}$), docosahexaenoic acid methyl ester ($C_{22\text{-}6,n\text{-}3}$ methyl ester), eicosapentaenoic acid ($C_{20\text{-}5,n\text{-}3}$), arachidoic acid ($C_{20\text{-}4,n\text{-}6}$), linoleic acid ($C_{18\text{-}2,n\text{-}6}$). They reported that oleic acid ($C_{18\text{-}1,n\text{-}9}$) and docosanoic acid ($C_{22\text{-}0}$) had very little effect on parasite growth inhibition (Kumaratilake *et al*., 1992). In their work, the unsaturated fatty acids $C_{22:6,\ n\text{-}3}$, and $C20{:}4,\ _{n\text{-}6}$ resulted in the death of *P.falciparum* parasites by >90% and 80%, respectively. However, they stated that the effect of C22:0 on parasites was comparable to the parasites which received no fatty acid. The introduction of a single double bond into the mono-unsaturated fatty acid greatly enhanced the antiplasmodial effects of the molecules (Kumaratilake *et al*., 1992). It was demonstrated in their study that oxidized fatty acids exhibit a stronger antiplasmodial effect than the non-oxidized fatty acids. This observation further confirms their previous finding since the saturated and mono-unsaturated fatty acids which are resistant to oxidation are not as active as the polyunsaturated hydrocarbons with ≥2 double bonds. This may help explain the relative increase in the *in vitro* activity of linolenic acid (3 double bonds) with IC_{50} value of 4.40 µg/ml compared to linoleic acid (2 double bonds) with an IC_{50} of 6.80 µg/ml against the DD2 strain in this study. Further study reported that the neutrophil-mediated killing of the

asexual blood forms of *Plasmodium falciparum* could be enhanced by fatty acids (Kumaratilake *et al*., 1997). Previous studies demonstrated other factors that could enhance neutrophil-mediated killing of *Plasmodium falciparum*, such as tumor necrosis factor (Kumaratilake *et al*., 1990) and Lymphotoxin and interferon gamma (Kumaratilake *et al*., 1991)

CHAPTER FIVE

In vivo antimalarial activity of linolenic and linoleic acids and their methyl esters

5.1 Introduction

Polyunsaturated fatty acids especially the essential fatty acids, have medicinal properties. Polyunsaturated fatty acids have been shown to have antiplasmodial properties which are traceable to their degree of unsaturation (Kumaratilake et. al., 1992). Compounds 1 and 2 commonly, known as linolenic and linoleic acids, respectively are polyunsaturated hydrocarbons that belong to the omega 3 and omega 6 fatty acids respectively. Linolenic and linoleic acids are essential fatty acids which belong to the omega 3 and omega 6 fatty acids. These essential fatty acids have recently drawn attention and are recently marketed as health supplements due to the health benefits associated with them (Larsen *et al*., 2003, Gaullier *et al*., 2005). The conjugated forms of these essential fatty acids have also shown promising biological activities. They are conjugated fatty acids due to the geometric position and configuration of their double bonds. Conjugated linoleic acids (CLA) are octadecadienoic acids with two conjugated double bonds. CLA have been shown to suppress human tumor (Tsuzuki *et al*., 2004) and were also recorded to reduce metastasis of cancers to lung tissue (Cesano *et al*., 1998). Other health potentials of CLA are antiathergenic, antidiabetogenic and immune modulating properties (Belury, 2002; McLeod *et al*., 2004). They are the only conjugated fatty acids that can be prepared in large quantities from natural sources (Tsuzuki, *et al*., 2006). Conjugated linolenic acids (CLnA) are octadecatrienoic acids with three conjugated double bonds. Linolenic and linoleic acids, isolated from *C. papaya* ethyl acetate extract in this study, showed *in vitro* antiplasmodial activities of <10 µg/ml. They were further investigated *in vivo* using a mouse model infected with *P. berghei*.

5.2 *In vivo* activity of linolenic and linoleic acids

Due to the poor yield of linolenic and linoleic acids from the ethyl acetate fraction of *C. papaya*, more of these compounds were purchased for further work (Sigma). The very lipophilic nature of these compounds meant that they

were not readily soluble in an aqueous environment. When part of a crude extract, lipophilic compounds such as artemisinin often demonstrate an increased solubility compared to the purified compounds (Mueller *et al*., 2000). This suggests that other components of plant extracts could help make compounds lipophilic in aqueous solution. Due to the lipophilic nature of the purified fatty acids, DL-α- Dipalmitoylphosphatidylcholine (DPPC) has been used as a vehicle to deliver these unsaturated hydrocarbons in *in vitro* and *in vivo* antimalarial assay (Kumaratilake *et al*., 1992). DPPC alone showed no activity against plasmodium parasites (Kumaratilake et. al., 1992). It was decided to adopt this approach. Linolenic and linoleic acids were formulated in DPPC micelles according to Kumaratilake *et al*., (1992). Details have been given in section 3.7.1.2. Briefly, lipids were dissolved in chloroform (10-50 mg/5 ml). A mixture of fatty acids and DPPC micelles were prepared by adding DPPC at four-fold the amount of the fatty acid. Solvents were evaporated under nitrogen. A volume of 1 ml complete medium (RPMI 1640) was added to the mixture. The mixture was sonicated for 2 minutes and thereafter used for the assay. The activity of the purchased compounds (99% pure) were tested *in vitro* and correlated well with the activity of the isolated compounds (Table 5.2.1).

Table 5.2.1: *In vitro* activity of linolenic and linoleic acids isolated and purchased against the CQR DD2 strain of *P. falciparum*

Compound	IC50 DD2 (µg/ml)
Linolenic acid (Isolated)	4.40
Linolenic acid (Sigma)	5.04
Linoleic acid (Isolated)	6.80
Linoleic acid (Sigma)	7.56

Mice infected with *P. berghei* were dosed orally with linolenic and linoleic acids singly and in combination. In the single treatment each compound was administered at a dose of 100 mg/kg using the 4-day suppressive test of Peters

et al. (1993). Linolenic acid significantly inhibited the growth of parasites compared to linoleic acid. The suppressive effect of the free acids when combined together (at a dose of 50 mg/kg each) proved to be more potent than the individual compounds alone (100 mg/kg) (Fig 5.2.1).

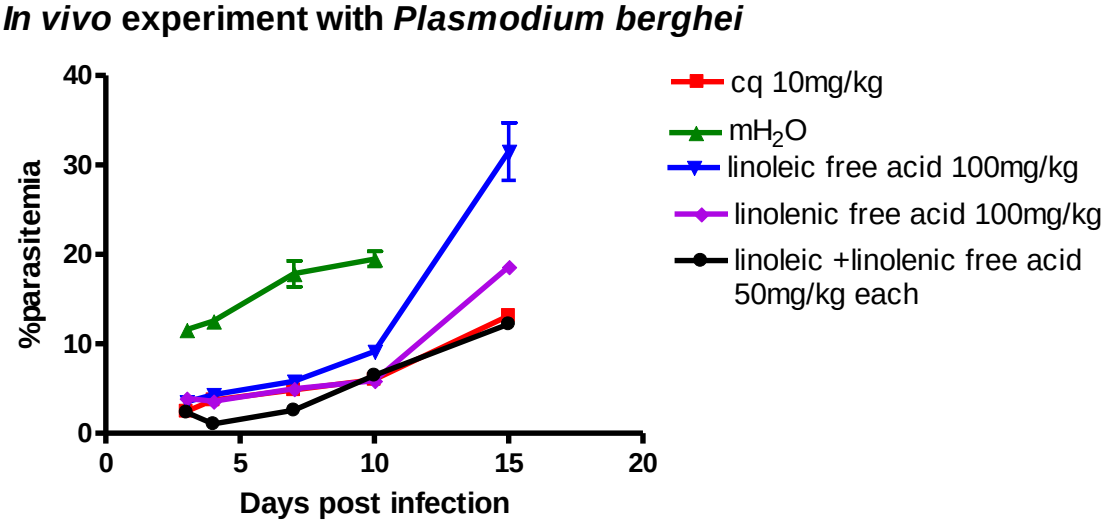


Fig 5.2.1 *In vivo* activity of linolenic and linoleic acids in combination compared with the compounds alone in a 4-day suppressive test using oral administration.

Key: cq = Chloroquine (10 mg/kg), mH_2O = Millipore water

The percentage growth inhibition of the parasites by the free acids, singly and in combination is shown (Table 5.2.2). The untreated control group, which is necessary to determine parasite growth inhibition, could not survive longer, thus day four post treatments was therefore chosen since it gives a good indication, of the rate of parasite suppression, induced by drug in a 4-day suppressive test.

Table 5.2.2: Percentage growth inhibition of compounds 1 and 2 individually and in combination on day 4 of treatment

Compound	%parasitemia	% growth inhibited
Millipore water	12%	Untreated negative control
Chloroquine	3.7%	69%
Linolenic free acid	3.6%	70%
Linoleic free acid	4.3%	64%
Linolenic and linoleic acids combined	0.5%	96%

Parasite growth inhibition was calculated using the formula below:

$$\% \text{ growth inhibition} = \frac{\text{Parasitemia of negative control} - \text{Parasitemia of test sample}}{\text{Parasitemia of negative control}} \times 100$$

5.2.1 Results and discussion

The *in vivo* result from this study demonstrated that linolenic acid was more potent than linoleic acid (Fig 5.2.1). This correlates well with the observations of Kumaratilake *et al*. (1992) which showed that fatty acids vary in their ability to inhibit the growth of parasites. Their work demonstrated that this variation in fatty acid antiplasmodial activity is partly dependent on the degree of unsaturation. The polyunsaturated compounds they investigated: $C_{22:6,n=3}$, $C_{20:5,n-3}$, $C_{20:4,n-6}$ and $C_{18:2,n-6}$ markedly inhibited the growth of the parasites, while the mono-unsaturated fatty acid C18:1,n-9 and the saturated fatty acid C22:0 showed little effect. Kumaratilake *et al.* (1992) further reported that the growth inhibition of parasites by the 22-C fatty acid significantly increased several-fold by increasing the degree of unsaturation of the molecule by six. Similarly, when they introduced a single double bond to the mono-unsaturated

fatty acid, the antiplasmodial effect of the molecule was more than tripled. These researchers concluded that the polyunsaturated fatty acids were more effective against *P. berghei* in mice when compared to the saturated fatty acids which had no effect. Fatty acids were reported to cause degeneration in the intra erythrocytic stages of *P. falciparum in vitro* (Kumaratilake *et al*., 1992). In the present work, compounds linolenic and linoleic acids inhibited the growth of parasites by 70% and 64%, respectively on day 4 of treatment. There was marked inhibition of parasite growth by a 1:1 combination of linolenic and linoleic acids. The growth inhibition of compounds linolenic and linoleic acids in combination was 96% on day 4 of treatment. This suggests a possible synergistic effect. The figures are slides from the untreated control group (Fig 5.2.2) and the group treated with a combination of the two compounds (Fig.5.2.3).

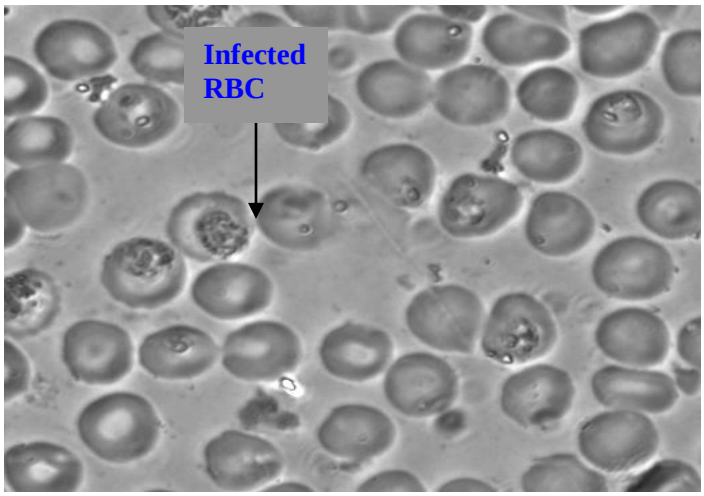


Fig 5.2.2: Morphology of *P. beghei* infected RBC in an untreated mouse

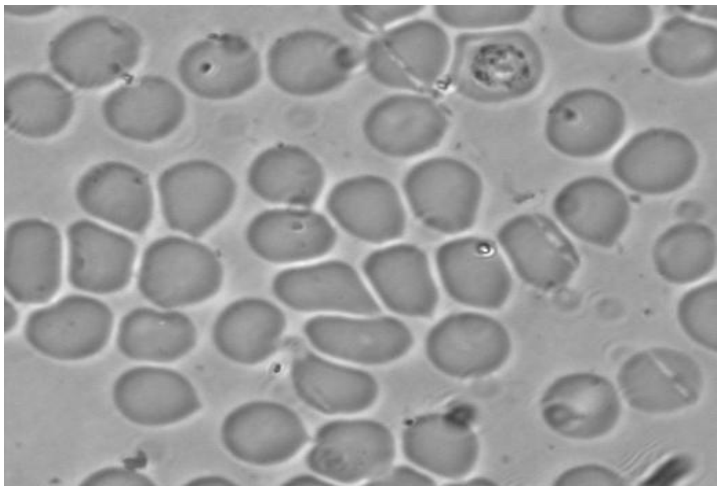


Fig 5.2.3: Combined effect of linolenic and linoleic acids on *P. berqhei* on day 4 of treatment using the 4-day suppressive treatment

After the final dose on day 4, parasite growth remained suppressed until day 10 but by day 15 significant recrudescence was observed for the compounds administered singly, while the result for the mixture suggested that the two compounds were acting synergistically, and were as potent as the chloroquine control by day 15 (Fig 5.2.1). In order to ascertain the reason for the observed recrudescence post treatment using the compounds singly, it was decided to investigate the bioavailability of the more active linolenic acid, so as to establish its absorption and elimination rate from plasma, and the possible reasons for the recorded recrudescence.

5. 3 Bioavailability study of linolenic acid methyl ester

A bioavailability study of linolenic acid requires its extraction from plasma. The bioavailability study was carried out using an Agilent 1200 series HPLC coupled to an API 3200 Applied Biosystems Mass Spectrometer (LC-MS/MS). The standards of linolenic acid were prepared at various concentrations necessary for method development and optimization using the LC-MS/MS spectrometer (3.7.1.4). However, linolenic acid which is a free acid, was not efficiently detected by the mass spectrometer. Previous studies have shown that fatty

acid methyl esters are more stable and show good chromatographic properties compared to the free acids (Viron *et al*., 2000) and would be expected to be more readily detected by mass spectrometry. The methyl esters of linolenic and linoleic acids were therefore purchased from Sigma (purity ≥ 99%). To determine if their antiplasmodial activity was the same as the free acids, the linolenic and linoleic acid methyl esters were tested for antimalarial activity *in vitro* and *in vivo*. The *in vitro* activity was similar for the free acids and their methyl esters. Similarly, no significant difference was shown for the *in vivo* antiplasmodial activity of the two free acids and their methyl esters using the oral administration (Figs. 5.3.1-5.3.2 and fig A11). These findings were similar to those of Kumaratilake *et al*., (1992) who reported similar antiplasmodial activity of a range of free acids and their methyl esters. The dose response curves of the *in vivo* schizontocidal activity of the free acids and their methyl esters are shown below.

Both the *in vivo* and *in vitro* antimalarial activities of linolenic and linoleic acids compares well with their methyl esters. Representative mass spectra of linolenic and linoleic acids are shown in figs A24-A29. The methyl ester of linolenic acid which showed the greatest activity was therefore used for the bioavailability study.

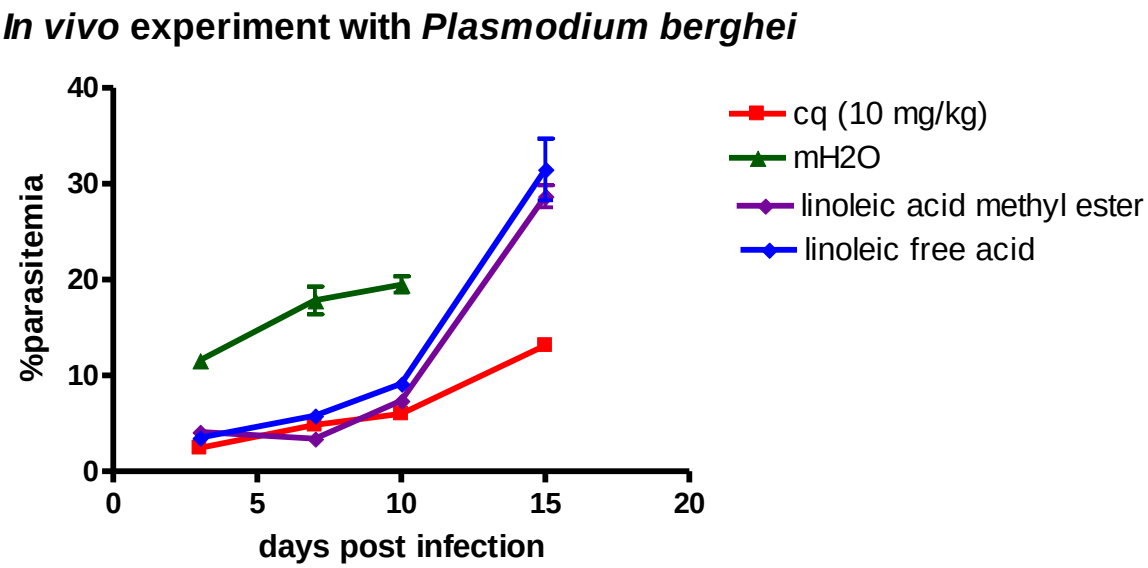


Fig 5.3.1: The *in vivo* activity of linoleic acid and the its methyl ester in a 4-day suppressive test using the oral route of administration

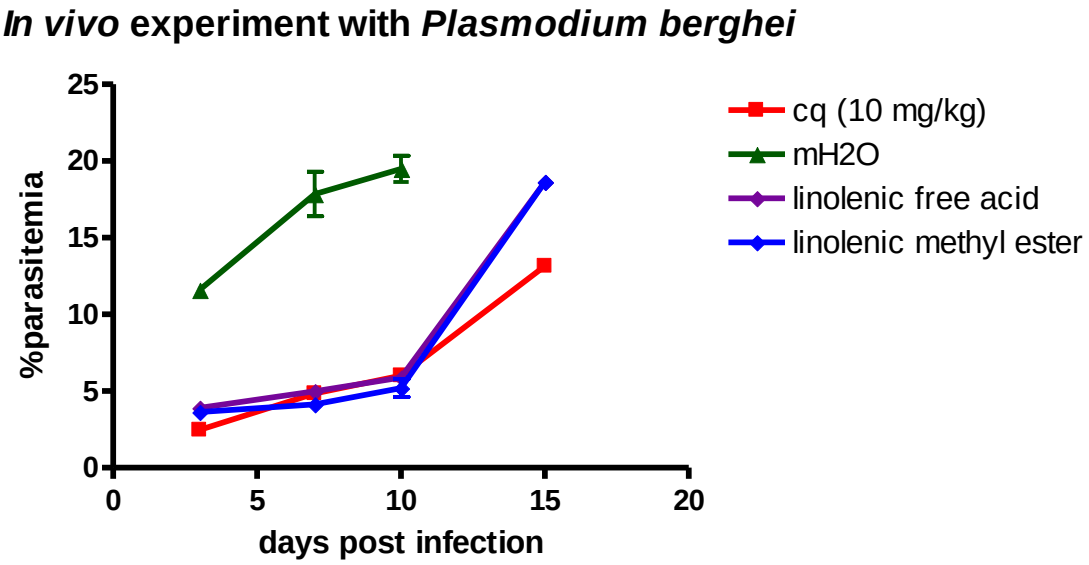


Fig 5.3.2: The *in vivo* activity of linolenic acid and its methyl ester in a 4-day suppressive test using the oral route of administration

5.4 Bioavailability study of linolenic acid methyl ester in plasma

The bioavailability study of linolenic acid methyl ester was carried out in a mouse model using different routes of administration (oral, intravenous and subcutaneous routes). A sensitive and selective method was developed for compound analysis using the LC-MS/MS.

5.4.1 Mass spectrometry method development

Prior to the bioavailability study, the detection, stability and ionization of the compound was established using the LC-MS/MS tandem mass spectrometer.

5.4.2 Optimisation of mass spectrometry conditions

The LC-system (Agilent 1200 series HPLC) was connected with an API 3200 quadrupole mass spectrometer (Applied Biosystems) in the ESI positive ionization mode and multi-reaction monitoring (MRM) was used for this analysis. The optimized mass spectrometry conditions are summarized in Tables 5.4.2.1 and 5.4.2.2.

Table 5.4.2.1 ESI settings

Curtain gas (psi)	**20**
Collision gas (psi)	**5**
Ionspray voltage (V)	**5500**
Source temperature (˚C)	**500**
Gas 1 (psi)	**20**
Gas 2 (psi)	**60**

Table 5.4.2.2 MS/MS settings

Specification	linolenic methyl ester
Linolenic acid methyl ester mass [M+H]+ (mass filter 1)	293.2
Q3 mass(mass filter 2)	67.1
Dwell time (ms)	150
Declustering potential (V)	31
Entrance potential (V)	5
Collision cell entrance potential (V)	20
Collision energy (v)	47
Collision cell exit potential (V)	4
Scan type	MRM
Polarity	Positive
Pause time (ms)	5

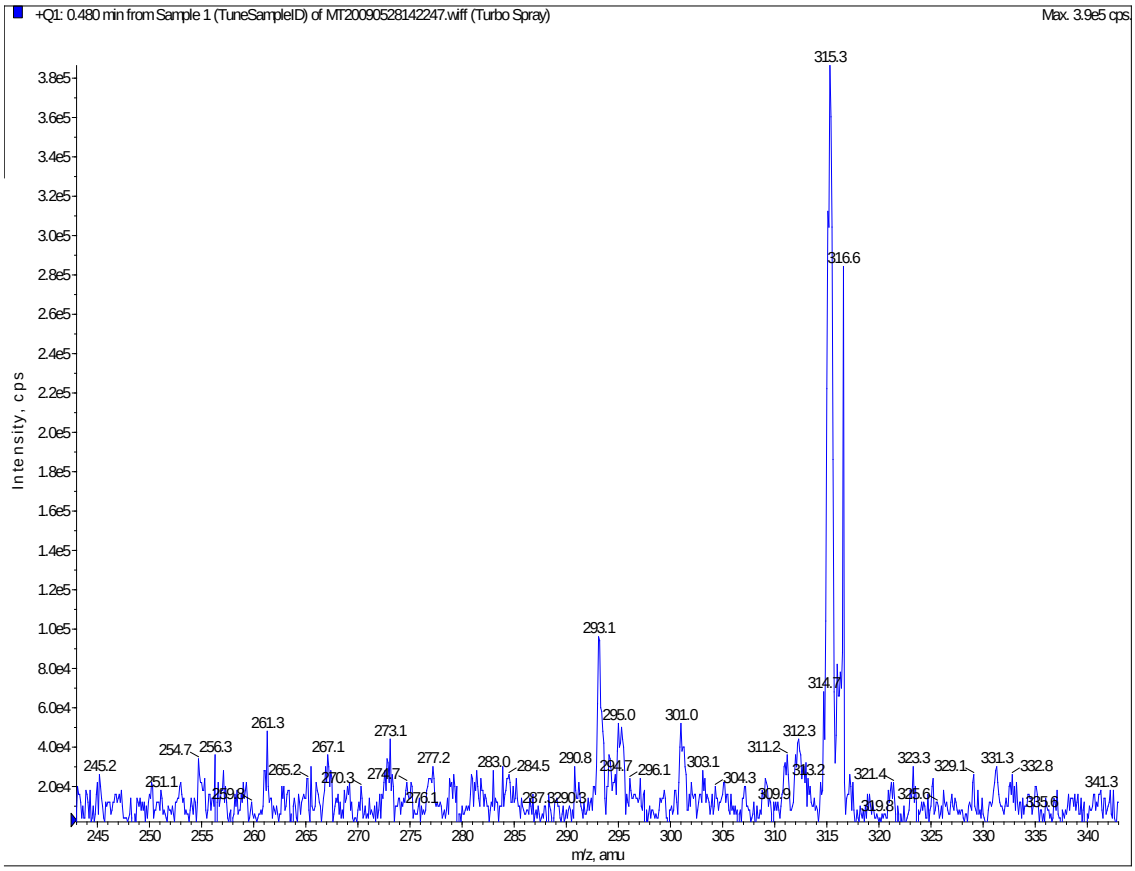


Fig 5.4.2.1 Shows the mass spectrum of lionlenic acid methyl ester with molecular ion of 293.1 $[M+H]^+$

The molecular ion $[M+H]^+$ 293.1 amu is shown in the MS/MS spectrum of linolenic acid methyl ester (Fig. 5.4.2.1). The molecular ion, which was selected in the first mass filter, was subsequently fragmented in the collision cell to give rise to product ions. The mass of linolenic acid methyl ester and the sodium adduct, which usually comes from glass, was shown at *m/z* 315.3 (Fig 5.4.2.1). The most abundant product ion was at *m/z* 67.2, while the protonated molecular ion was *m/z* 293.2 (Fig 5.4.2.2). The product ions at *m/z* 123, *m/z* 94.8, *m/z* 81.2, and *m/z* 67.2 all arise from sequential loss of CH_2 groups (Fig 5.4.2.2). In this experiment the protonated molecular ion *m/z* 293.2 and not the sodium adduct at *m/z* 315.3 has been used to form product ions.

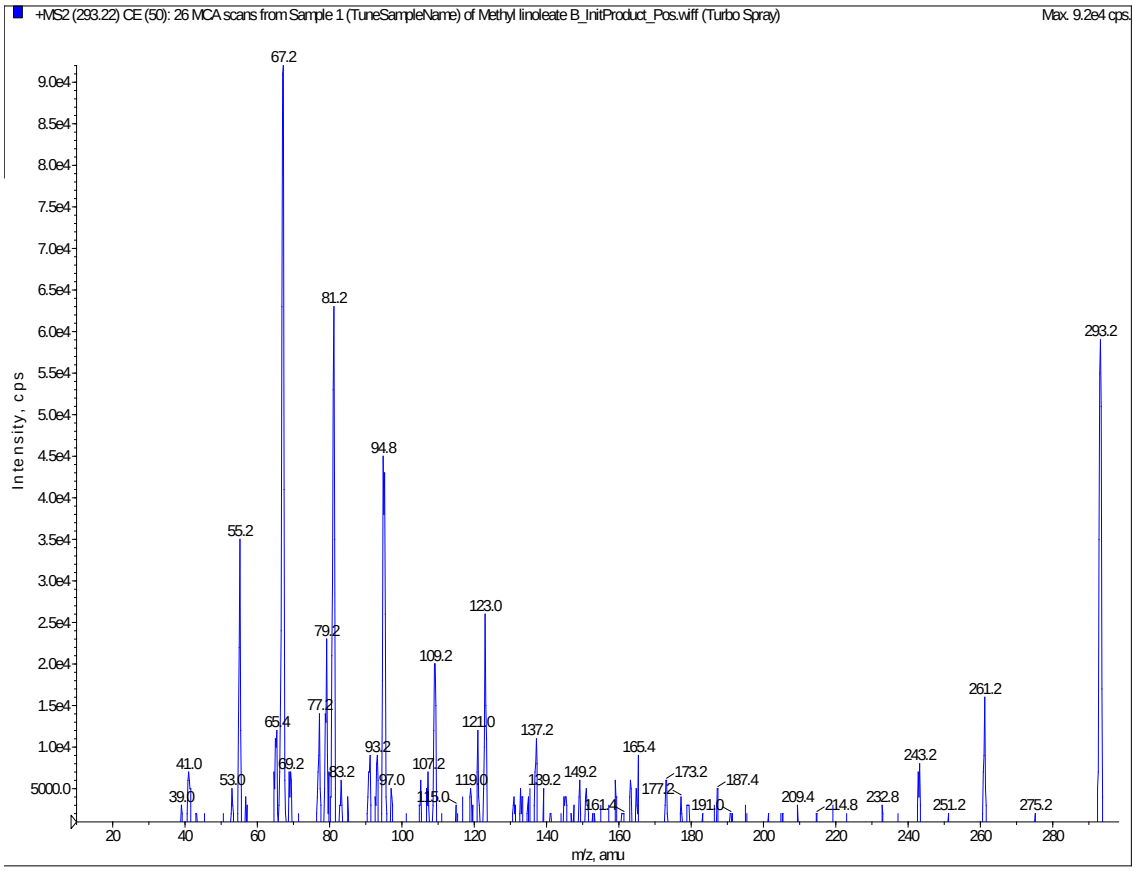


Fig 5.4.2.2 Mass spectrum of linolenic acid methyl ester showing the most abundant product ion at *m/z* 67.2 and the protonated molecular ion at *m/z* 293.2

5.5 Chromatography

The chromatography was determined using the LC-system (Agilent 1200 series HPLC) equipped with an API 3200 quadrupole mass spectrometer detector (Applied Biosystems) coupled with an ESI positive ionization and multi-reaction monitoring (MRM). This was carried out by direct injection of 2µl at a flow rate of 0.5 ml/min. The system is equipped with a flush port wash station which automatically rinses the injection needle with the mobile phase before each injection. The mobile phase (MP) was acetonitrile: 0.1% formic acid (50:50) which was ran isocratically for 1.9 minutes. The stability of the eluent system

was efficient for identifying the retention times of compounds. A representative chromatogram of linolenic acid methyl ester at 1 µg/ml is shown in Fig 5.5.1.1.

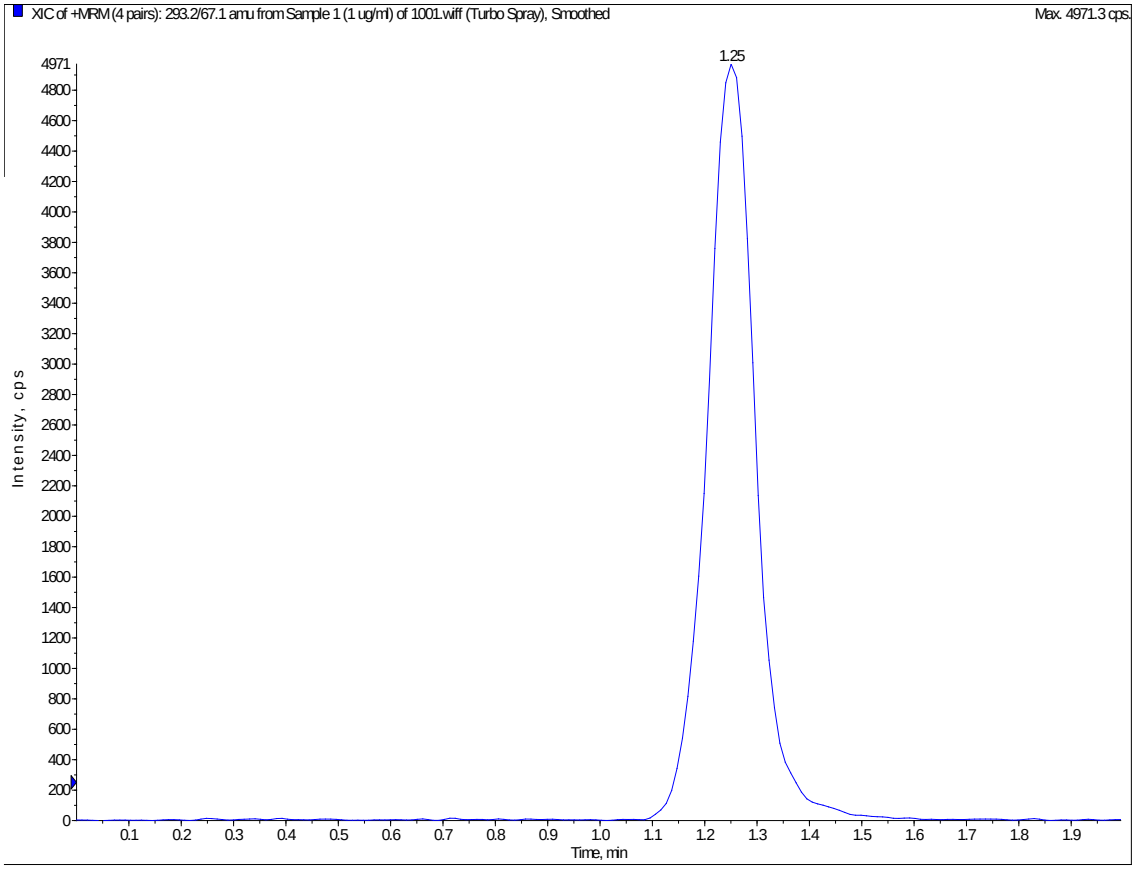


Fig 5.5.1.1 A representative chromatogram of linolenic acid methyl ester at a concentration of 1 µg/ml in the mobile phase using the LC/MS/MS. MRM:293.2-67.1 amu.

5.6 Extraction of linolenic acid methyl ester from plasma

The extraction process was performed on the test samples and the standards (STDI-STD7) which served as controls. Stock solutions of seven standards with known concentrations were prepared in plasma (20 µg/ml-0.3125 µg/ml). Initially, the extraction of linolenic acid methyl ester from red blood cells in pH buffers ranging from pH 2-12 (prepared with Britton Robinson Universal buffers) did not prove very sensitive. Extraction was therefore attempted using the modified method of Aleryani *et al*., (2005) as described in section 3.7.1.5.

Briefly, a volume of 100 µl of acetone was added into 20µl of spiked plasma (20 µg/ml) in labeled Eppendorfs (Group B). Acetone was added to precipitate protein. The solution was vortexed for a minute and placed in ice for ten minutes. It was thereafter centrifuged at 1200 rcf for 5 minutes. The resultant supernatant was transferred to labeled Eppendorfs (Group C). Hexane (150 µl) was added to group C Eppendorfs, vortexed for a minute, placed in ice for ten minutes, and centrifuged at 200 rcf for 5 minutes. The hexane layer was transferred to group D eppendorfs and dried under nitrogen. After drying was completed, 100µl of mobile phase (ACN: 0.1% formic acid; 50:50) was added to these Eppendorfs. The mixture was vortexed for 30 seconds and transferred into LC/MS/MS vials for injection. This method proved sensitive and effective, and was used in all of the extraction procedures in this work.

5.7 Mass spectrometry method validation

A pilot study was carried out using the mass spectrometer to evaluate the concentrations of the linolenic acid methyl ester in plasma, as well as the stability and sensitivity of the different concentrations in plasma. These concentrations were prepared as different standards (STD1-STD 9). The initial and highest concentration (STD 1 = 25 µg/ml) was diluted in plasma to 0.097 µg/ml (STD 9). The intensity of the chromatogram of the lowest standard (STD 9), and the low concentrations observed in mouse plasma prompted another evaluation. This time a starting concentration STD 1 of 6.25 µg/ml and a final concentration STD 7 of 0.098 µg/ml was used (Fig 5.7.1.1).

5.7.1 Preparation of calibration standards

An initial stock solution (SS1) of 1 mg/ml concentration of linolenic acid methyl ester was prepared in methanol. Seven standards (STD 1-7) from the initial stock solution (SS1) of linolenic acid methyl ester were prepared by two-fold serial dilution in plasma with a starting concentration of 6.25 µg/ml to 0.098 µg/ml. Standard 1 had the highest concentration (STD 1 =6.25 µg/ml) while

standard 7 showed the lowest concentration (STD 7 = 0.098 μg/ml). Blank plasma was also included to serve as an internal check. Samples were vortexed briefly and stored at -20ºC for short term storage and at -80ºC for longer durations. A representative calibration curve of linolenic acid methyl ester is shown in Fig. 5.7.1.1.

5.7.1.1 Calibration curve

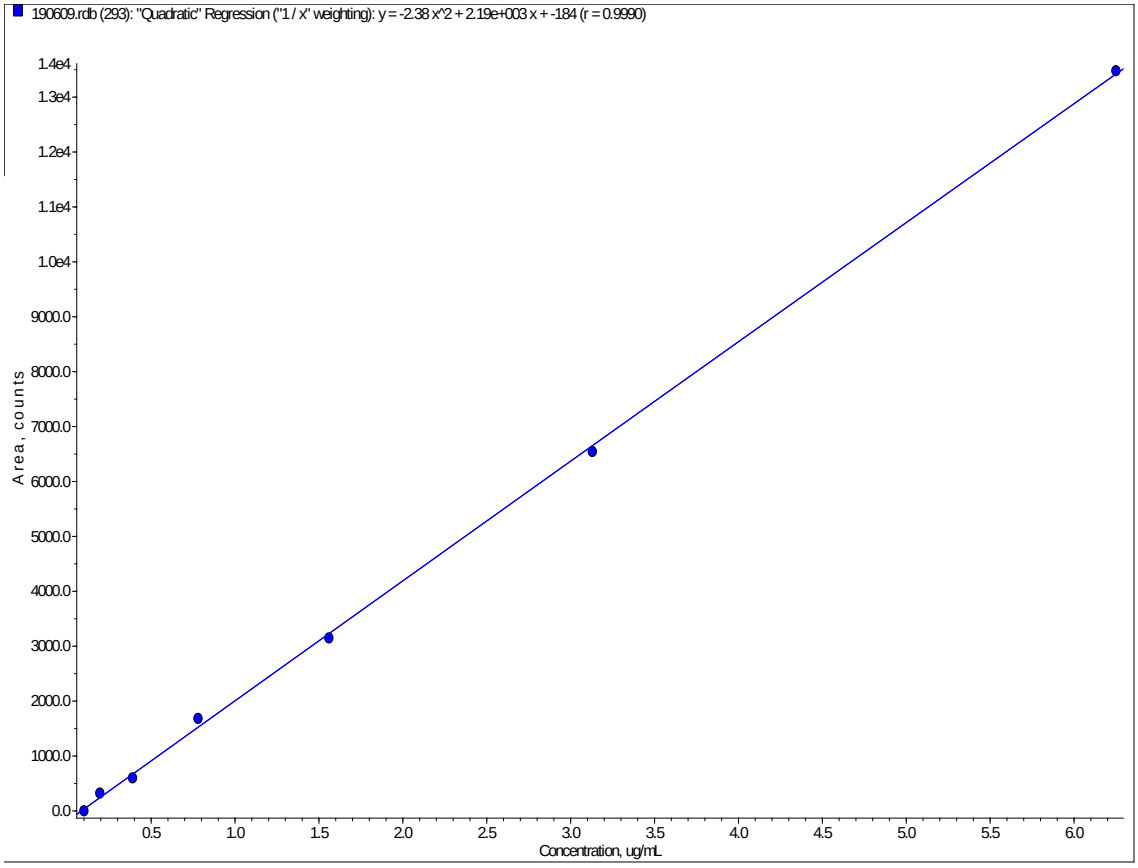


Fig 5.7.1.1 Standard curve of linolenic acid methyl ester using the LC-MS/MS

5.8 Effect of route of administration on the bioavailability of linolenic acid methyl ester

The bioavailability of compounds and their activity *in vivo* are partly influenced by parameters such as the route of administration (Peters *et al*., 2002), as well as the elimination rate of the compound from plasma. Three routes of drug administration, including the oral, subcutaneous and the intravenous routes were analyzed to evaluate the bioavailability of linolenic acid methyl ester in a mouse model. Groups of five male mice were used for each route of administration, as the bioavailability of linolenic acid methyl ester in each mouse is likely to differ, due to different physiological changes in different animals. Samples were taken from each mouse at four time points (T1 - T4) which were the same for all the mice in the three routes of drug administration. The standards shown were run before and after the time points of a particular mouse (T1 - T4). The lower limit of quantification (LLOQ) in this experiment was at a concentration of 0.195 µg/ml. Further use of STD 7 (0.098 µg/ml) was discontinued since it was below the limit of quantification (BLQ). A tabular representation of STD1- STD 6 is shown (Table 5.8.1).

Table 5.8.1: Representative standards showing their percentage accuracy

Sample name	Expected Concentration (µg/ml)	Mean Concentration	Accuracy (%)
Standard 6	0.195	0.2	102
Standard 5	0.39	0.289	74
Standard 4	0.78	0.775	99.4
Standard 3	1.56	1.49	95.3
Standard 2	3.13	3.61	115
Standard 1	6.25	5.88	94.1

Table 5.8.2: Plasma concentrations of linolenic acid methyl ester extractions in the oral route of administration

Sample Name	Analyte Peak Area (counts)	Calculated Concentration (µg/mL)
Mouse 1 T1	358	0.082
Mouse 1 T2	350	0.0813
Mouse 1 T3	313	0.0777
Mouse 1 T4	0	No Peak
Mouse 2 T1	211	0.068
Mouse 2 T2	70	0.0546
Mouse 2 T3	105	0.058
Mouse 2 T4	259	0.0726
Mouse 3 T1	0	No Peak
Mouse 3 T2	0	No Peak
Mouse 3 T3	0	No Peak
Mouse 3 T4	186	0.0657
Mouse 4 T1	101	0.0576
Mouse 4 T2	192	0.0663
Mouse 4 T3	54.4	0.0531
Mouse 4 T4	0	No Peak
Mouse 5 T1	0	No Peak
Mouse 5 T2	0	No Peak
Mouse 5 T3	246	0.0714
Mouse 5 T4	0	No Peak

The first time point T1 was 30 minutes post treatment, the second (T2), third (T3) and fourth (T4) time points were 2, 4 and 6 hours post treatment, respectively. The results of the oral and subcutaneous administrations are shown in Tables 5.8.2 and 5.8.3 respectively. The concentrations of linolenic acid methyl ester in the oral and subcutaneous route were below the limit of quantification (BLQ) for all the time points tested in all mice. However, the intravenous route of administration showed good concentrations of linolenic acid methyl ester (Table 5.8.4).

Table 5.8.3 Plasma concentrations of linolenic acid methyl ester extractions in the subcutaneous route of administration

Sample Name	Analyte Peak Area (counts)	Calculated Concentration (µg/mL)
Mouse 1 T1	72.6	0.0646
Mouse 1 T2	138	0.0706
Mouse 1 T3	210	0.0772
Mouse 1 T4	0	No Peak
Mouse 2 T1	0	No Peak
Mouse 2 T2	77.4	0.0651
Mouse 2 T3	41.4	0.0618
Mouse 2 T4	0	No Peak
Mouse 3 T1	68.2	0.0642
Mouse 3 T2	0	No Peak
Mouse 3 T3	80.5	0.0654
Mouse 3 T4	161	0.0727
Mouse 4 T1	0	No Peak
Mouse 4 T2	0	No Peak
Mouse 4 T3	54	0.0629
Mouse 4 T4	0	No Peak
Mouse 5 T1	253	0.0811
Mouse 5 T2	161	0.0727
Mouse 5 T3	0	No Peak
Mouse 5 T4	0	No Peak

Table 5.8.4 Plasma concentrations of linolenic acid methyl ester extractions in the intravenous route of administration

Sample Name	Analyte Peak Area (counts)	Calculated Concentration (µg/mL)
Mouse 1 T1	112000	14.2
Mouse 1 T2	16100	2.64
Mouse 1 T3	8720	1.42
Mouse 1 T4	1040	0.0527
Mouse 2 T1	126000	15.6
Mouse 2 T2	27700	4.4
Mouse 2 T3	9760	1.6
Mouse 2 T4	2380	0.3
Mouse 3 T1	216000	23.1
Mouse 3 T2	102000	13.3
Mouse 3 T3	1800	0.194
Mouse 3 T4	81.9	< 0
Mouse 4 T1	34900	5.42
Mouse 4 T2	4520	0.687
Mouse 4 T3	751	< 0
Mouse 4 T4	180	< 0
Mouse 5 T1	171000	19.5
Mouse 5 T2	6630	1.06
Mouse 5 T3	1020	0.0503
Mouse 5 T4	574	< 0

5.9 Results and Discussion

The method achieved a lower limit of quantification (LLOQ) of 0.195 µg/ml.

The fact that no plasma concentrations above the LLOQ were seen in mice receiving oral and subcutaneous doses suggests that the compound was either very poorly absorbed or may have been metabolized. Previous work reported the low levels of 18:3n-3 fatty acids, and further pointed out the differences between their metabolism and that of 18:2n-6 which is usually more abundant (Pawlosky *et al*., 1996). Oxidation of the methyl ester to its active metabolites could explain the *in vivo* activity recorded when this compound was

administered orally. Fatty acid methyl esters are metabolized as would be other dietary fats. Higher molecular weight aliphatic esters are readily hydrolyzed to the corresponding alcohol and acid. These are subsequently oxidized to carbon dioxide and water through an established mechanism of metabolic breakdown into two-carbon fragments. These fragments are utilized by the body for energy and serves as building blocks for synthesis. During digestion they are hydrolyzed to the free fatty acids for absorption from the intestine into the blood stream aided by lipase enzymes and bile salts. Once formed, the free acid is metabolized by known oxidative processes or are reconstituted into glyceride esters and stored in the fat depots in the body. Further work by Kumaratilake *et al*. (1997) confirms the oxidation of this class of compounds. This suggests that the *in vivo* activity recorded when this compound was administered orally could be traceable to the active metabolites. Rapid metabolic conversion of linolenic acid to linoleic acid isomers in the oral administration, has been reported by other researchers (Tsuzuki *et al*., 2004). The relatively high levels of the linolenic acid methyl ester, achieved after the intravenous injection, allowed determination of the elimination rate of the linolenic acid methyl ester from plasma. The result showed the highest concentration at T1 (30 minutes post dosing) and the lowest at T4 (6 hours post dosing) (Figs 5.9.1, 5.9.2, and 5.9.3). This decrease in concentration as the time after dosing increases, coupled with the very low concentration at 6 hours post-dosing, shows a fast elimination rate from plasma within 6 hours of administration.

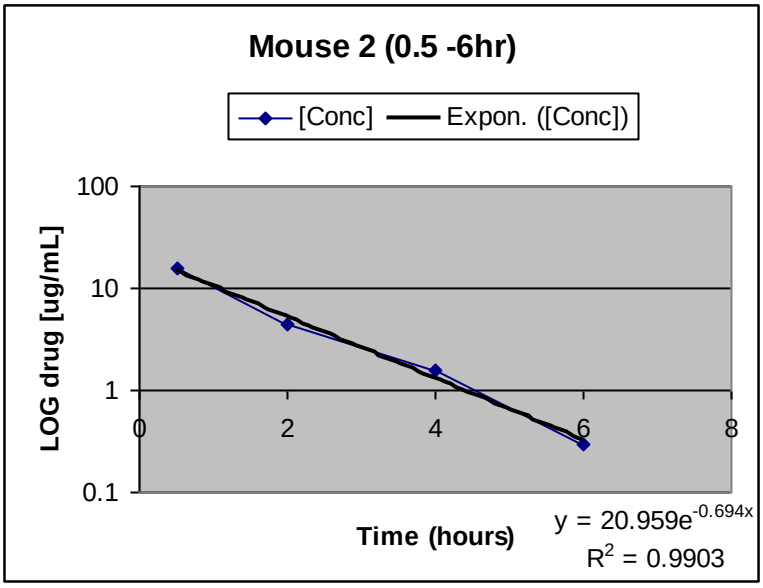


Fig 5.9.1 Concentrations of linolenic acid methyl ester between 0.5-6 hr in mouse 2 following i.v. dosing

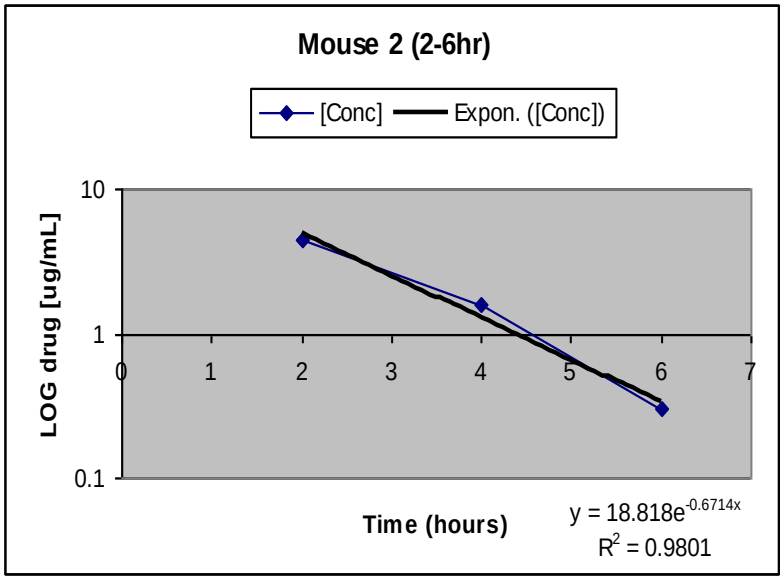


Fig 5.9.2 Concentrations of linolenic acid methyl ester between 2-6 hr in mouse 2 following i.v. dosing

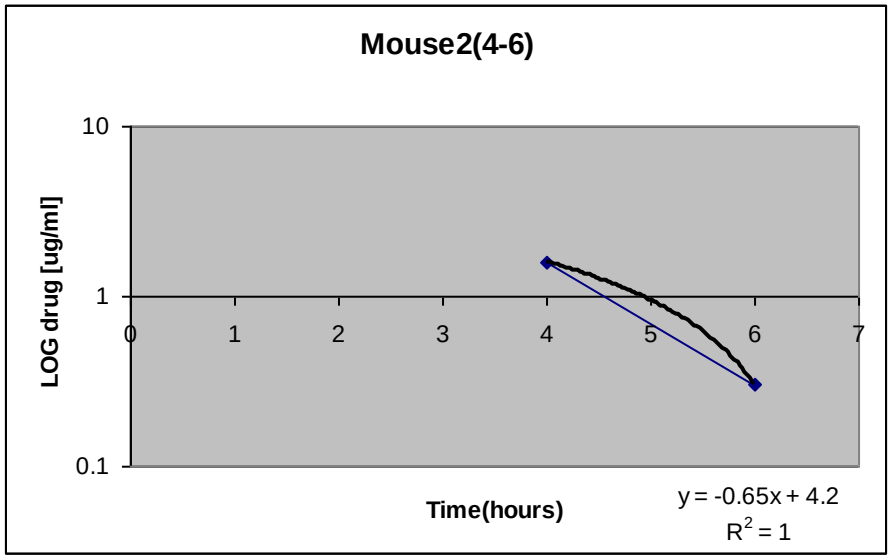


Fig 5.9.3 Concentrations of linolenic acid methyl ester between 4-6 hr in mouse 2 following i.v. dosing

The formula below was used to calculate the half life (t1/2) and to evaluate the elimination rate constant (K) for linolenic acid methyl ester after the intravenous administration.

$t_{1/2}$ =Ln2 / K, $t_{½}$ =0.693/ K

Mouse two was chosen for this evaluation since it recorded no value below the limit of quantification (BLQ). The other mice all had some data values below the lower limit of quantification of the assay which did not allow data to be used for half life determination. The first group in this evaluation is 0.5-6 hrs using the formula above =0.693/0.694 equals -0.99856 hr. The second and the third groups (2-6 hrs) and (4-6 hrs) had K values of -1.032217 hr and -1.066156 hr, respectively. The standard deviation of the elimination rate constant was calculated as -0.6718 and this gave rise to the mean K =0.693/-0.6718 = -1.03156 hrs. This value represents the estimated half life of linolenic acid methyl ester in plasma between 0.5-6 hrs. It correlates well to the calculated value in mouse 1 between 0.5-6 hrs (Fig 5.9.4), suggesting that the longer range 0.5-6 hrs could be the best fit for this evaluation. The bioavailability evaluations of linolenic acid methyl ester in mouse are shown in Figs 5.9.4 and 5.9.5.

The formula used in evaluating the half life of linolenic acid methyl ester in mouse 1 is stated below;

$t_{1/2}$ =Ln2

$t_{1/2}$ =0.693/K

$t_{1/2}$ =0.693/-1.1225

$t_{1/2}$ =-0.617

K=0.693/-0.617

K (elimination rate constant) = -1.123 hours

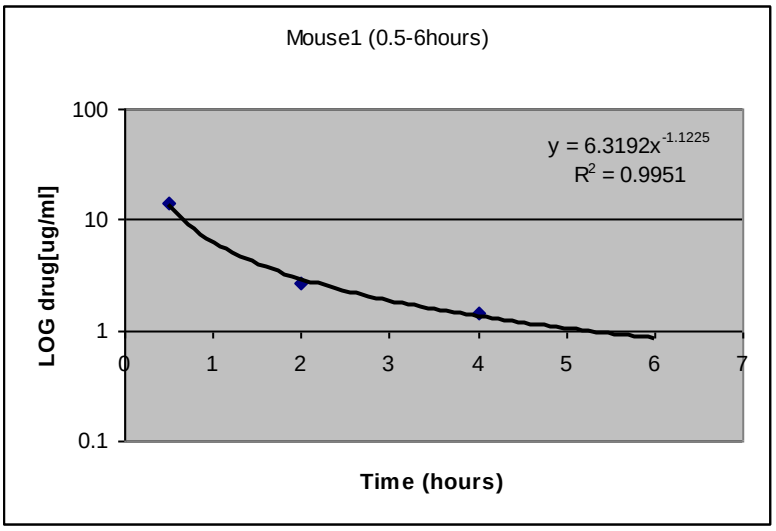


Fig 5.9.4 Concentrations of linolenic acid methyl ester between 0-6 hr in mouse 1

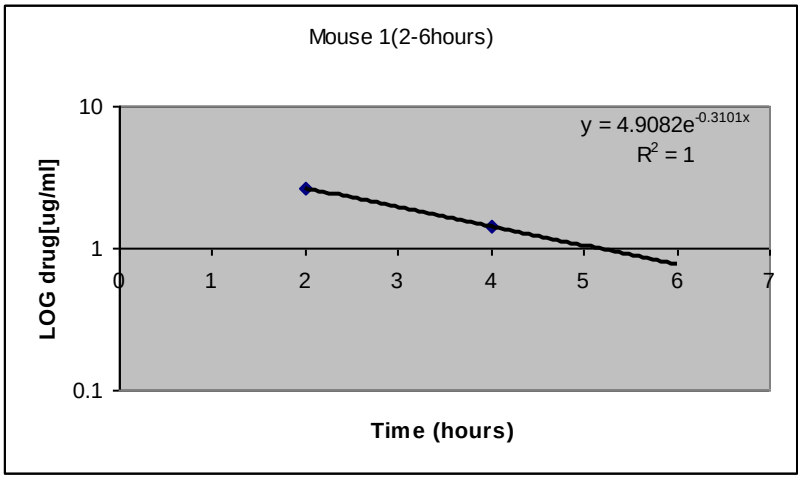


Fig 5.9.5 Concentrations of linolenic acid methyl ester between 2-6 hr in mouse 1

5.9.1 Mean elimination rate

The mean value of the elimination rate constant (K) in mouse 1 was calculated to be -1.123 hours. This value did not differ significantly with the value calculated for mouse 2 with a mean value of K= -1.0322 hours. Two mice were used to evaluate and compare the elimination rate of linolenic acid methyl ester from plasma, since there may be slight variations due to physiological differences. The remaining two mice showed values that were below the limit of quantification (BLQ) which may be due to technical errors and thus were not used in this evaluation. The highest concentration recorded at 0.5 hrs (30 minutes) and the significant decrease in concentration recorded 2 hours post treatment could be traceable to its half-life, which showed a fast elimination from plasma after 1 hour of treatment.

5.10 *In vivo* schizontocidal experiment using the intravenous route of administration

As linolenic acid methyl ester showed relatively good concentrations in the intravenous route of administration, as compared to the oral and subcutaneous routes, it was decided to check the schizontocidal effect of the compound after intravenous administration. A group of mice were infected with *P. berghei* and

treated intravenously with linolenic acid methyl ester (100 mg/kg) once a day for four days. The positive control compound is Cq at a dose of 10mg/kg as used in the previous *in vivo* experiments in this study. Results are shown in fig 5.10.1.

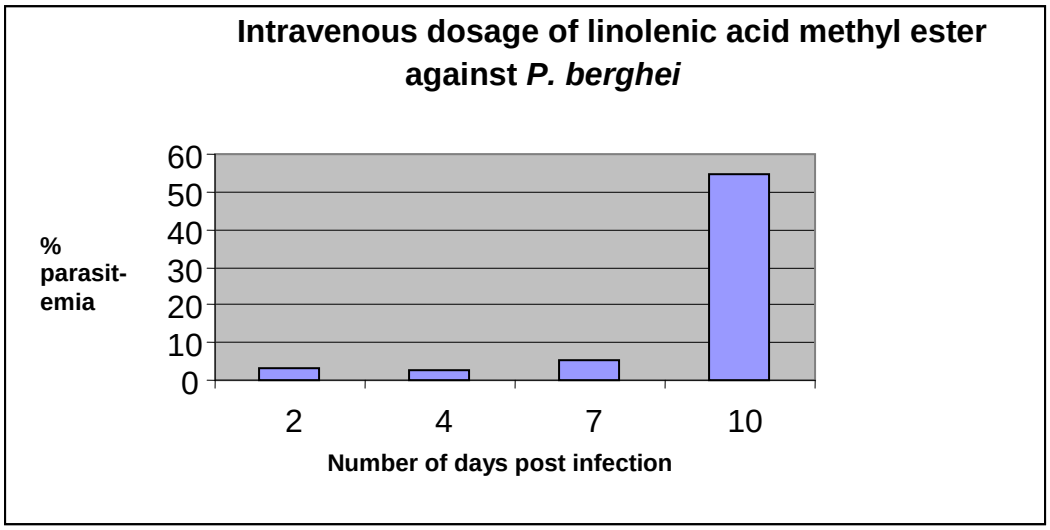


Fig 5.10.1 Percentage parastemia post-infection using a 4-day suppressive treatment against *P. berghei*

5.11 Results and Discussion

Parasites were markedly suppressed during the 4-day treatment, but a rapid recrudescence with parasitemia of >50% was recorded on day 10 post treatment (Fig 5.10.1). The fast recrudescence observed during the *in vivo* antiplasmodial study of this compound intravenously could be attributed to its short half-life. Chemical modification of this compound to increase its solubility could help it to be a better pharmacokinetic match with other compounds having similar half-life, like the artemisinin derivatives since this class of compounds seem to have a different mechanism of action. Previous studies reported that the biosynthesis of type II fatty acid synthase (FAS II) which takes place in the apicoplast of *P*. *falciparum* could be altered by fatty acids

(Carballeira, 2008). The type II FAS II system is vital to these apicomplexan parasites and is also found in bacteria and algae. The fatty acid biosynthesis of parasites differs from that of humans or higher eukaryotes and this makes it possible for the parasite to be destroyed without harming the host (Carballeira, 2008). . The type normally found in higher eukaryotes is the type I fatty acid synthase (FASI) system (Carballeira, 2008). .

Linolenic and linoleic acids isolated from the ethyl acetate fraction showed synergistic properties when combined. It is possible that these compounds and other numerous components in the crude extract may have added to the relatively increased activity observed in the ethyl acetate extract. Researchers have shown that plants contain complex mixtures of chemical agents, and the possibility of synergism existing between the various compounds may not be ruled out (Kirby, 1996). Such complex interactions may help fight recrudescence but may also help delay or prevent the onset of resistance. It was therefore decided to investigate the *in vivo* activity of the crude extracts from the five selected plants. The objective here is to investigate the activity of each crude extract as an entity alone, or in combination with another extract, using the 4-day suppressive test. These experiments are detailed in Chapter six.

CHAPTER SIX

In vivo antiplasmodial activity of crude extracts singly and in combination

6.1 Antiplasmodial activity

6.1.1 Introduction

The five plants investigated in this study are used to treat malaria by traditional medical practitioners in Nigeria. These plants include *Citrus limon, Psidium guajava, Carica papaya, Cymbopogon citratus,* and *Vernonia amygdalina.* Herbal remedies from these plants are prepared singly and in combination as decoctions, infusions or macerations. An attempt has been made in this study to scientifically evaluate the *in vitro* antiplasmodial efficacy of these plants singly and in combination. A bioassay-guided fractionation of the most active plant extract (*C. papaya* ethyl acetate fraction) in this study yielded two compounds with *in vitro* activity less than that of the crude extract (IC_{50}=2.96 µg/ml). The activity of *C. papaya* ethyl acetate fraction could be due to the synergistic activity of mixtures of several compounds present in the extract. The extracts of *Citrus limon* (DCM)*, Psidium guajava* (DCM)*, Carica papaya* (EA)*, Cymbopogon citratus* (DCM)*,* and *Vernonia amygdalina* (DCM) investigated in this study showed good to moderate *in vitro* antiplasmodial activity (IC_{50} values between 2-10 µg/ml) and were not cytotoxic to the Chinese Hamster Ovarian (CHO) cell line at the concentrations tested. However, since *in vitro* results are not necessarily predictive of *in vivo* activity, this chapter describes the *in vivo* schizontocidal activity of these extracts singly or in combination using the 4-day suppressive test.

6.2 *In vivo* schizontocidal activity of extracts singly

The crude extracts of these plants were tested in mice infected intraperitoneally with *Plasmodium berghei*, using the 4-day suppressive test of Peters *et al*. (1993). The extracts were dissolved in SMEDDS formulation (solution of 200 µl ethanol, 20 µl Tween 80 and 780 µl PBS), and showed no *in vivo* toxicity when tested at doses >1000 mg/kg. Each extract was administered orally at a dose

of 800 mg/kg. The SMEDDS formulation when tested alone had no effect on parasitemia. The extracts dissolved well in the SMEDDS formulation (solution of 200 µl ethanol, 20 µl Tween 80 and 780 µl PBS). The composition of the formulation includes a test sample, lipids, surfactants and co-surfactants which when exposed to the aqueous environment in the gastrointestinal tract form a fine emulsion of oil in water (Wu *et al*., 2006). The extracts were dissolved first in ethanol followed by PBS and Tween 80 was added last. This was done to give room for optimal solubility. A volume of 200 µl of each extract formulation was administered orally to the test animals 24 hours post infection. The positive control group received CQ at a dose of 10 mg/kg while the negative control group received millipore water only since the formulation alone had no effect on parasitemia. The number of cells/ml was calculated using a haemocytometer and was diluted to a suspension of 5 x 10^6 cells/mlusing phosphate buffered saline (PBS). There were five test groups for the five extracts, with each group comprising of five mice. The positive and negative control groups had three mice each. The effect of each extract on the parasitemia of each group is shown below.

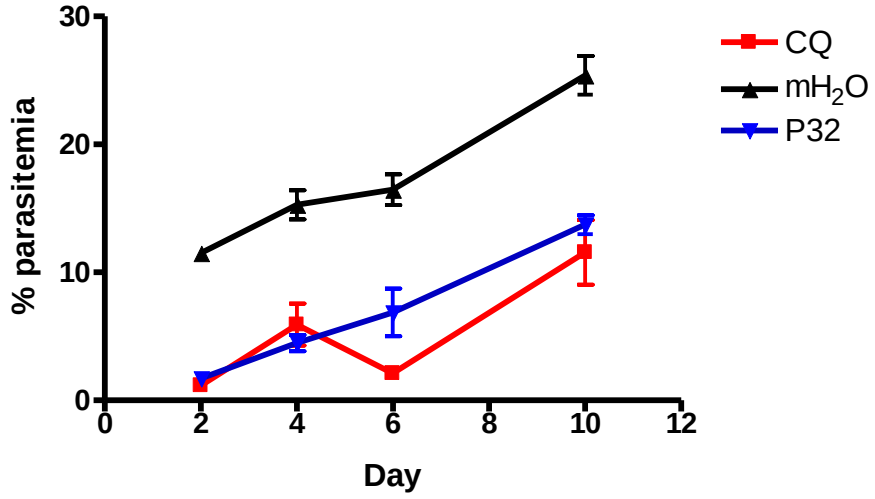


Fig 6.2.1: Percentage parasitemia of *C. limon* DCM extract (P32) during and post treatment.

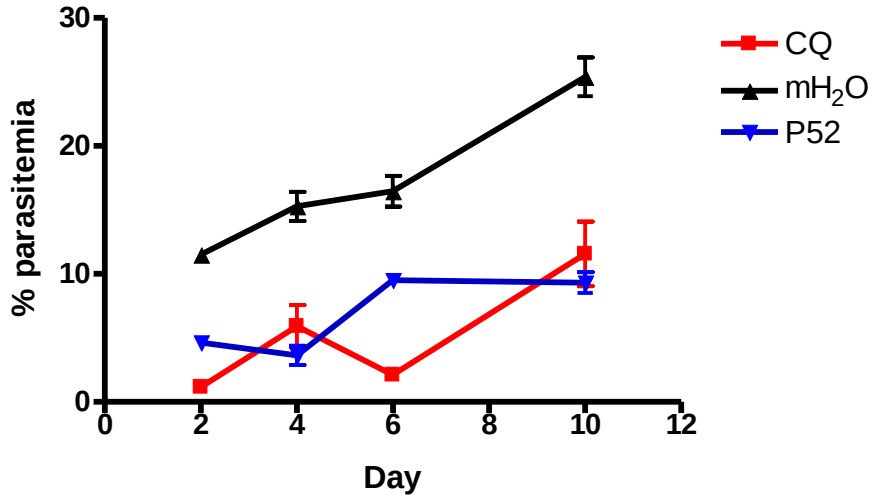


Fig 6.2.2: Percentage parasitemia of *P. guajava* DCM extract (P52) during and post treatment.

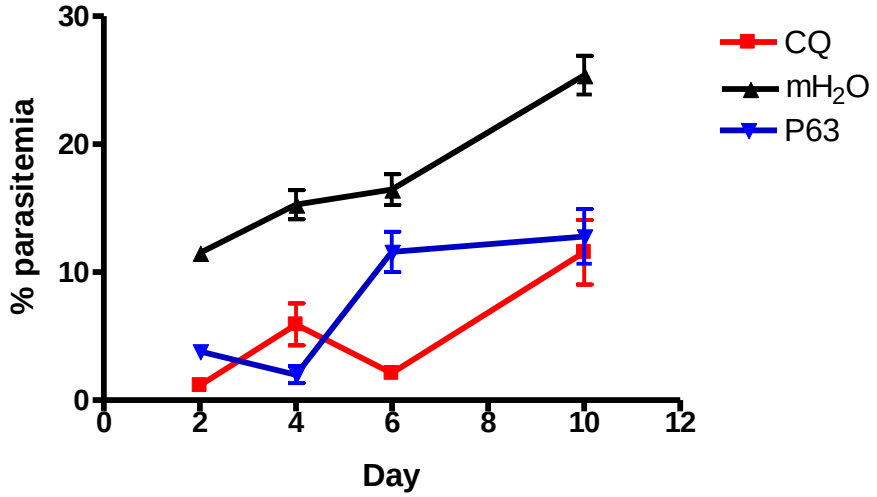


Fig 6.2.3: Percentage parasitemia of *C. papaya* ethyl acetate extract (P63) during and post treatment.

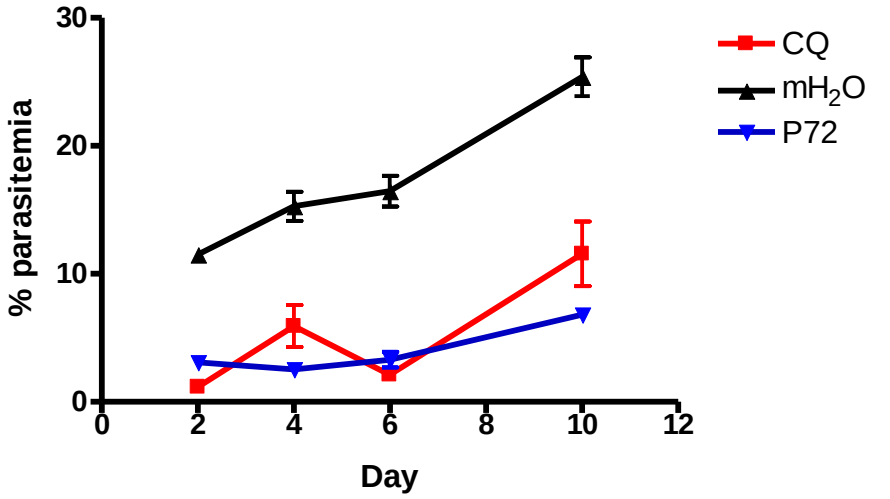


Fig 6.2.4: Percentage parasitemia of *C. citratus* DCM extract (P72) during and post treatment.

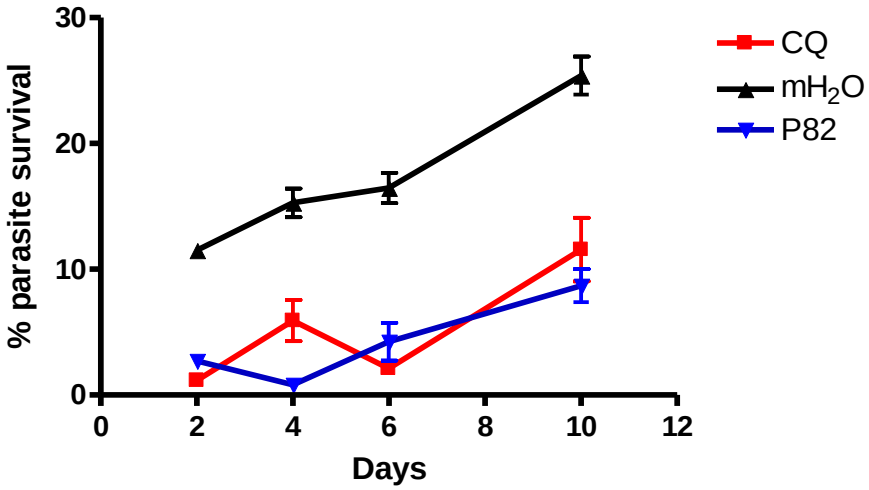


Fig 6.2.5: Percentage parasitemia of *V. amygdalina* DCM extract (P82) during and post treatment.

The antiplasmodial activities of the five extracts and the control groups on day 4 of treatment against *P. berghei* are shown in Fig 6.2.6, and Table 6.2.1. Percentage parasitemia of representative extracts in the *in vivo* experiments are futher shown in fig A10.

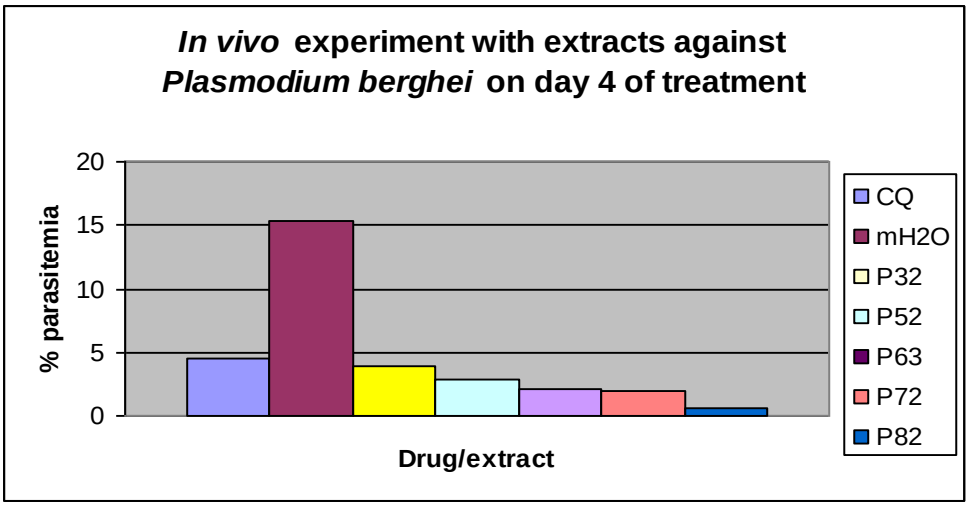


Fig 6.2.6 The mean parasitemia of each group on day 4 of treatment

Key: P32= ***Citrus limon*** dichlromethane extract
P52= ***Psidium guajava*** dichloromethane extract
P63= ***Carica papaya*** ethyl acetate crude extract
P72= ***Cymbopogon citratus*** dichloromethane crude extract
P82= ***Vernonia amygdalina*** dichloromethane crude extract

Table 6.2.1: Percentage growth inhibition against *P. berghei* on day 4 of treatment using the 4-day suppressive test.

Extract/Drug	Drug /extract codes	Average parastemia on day 4 of treatment (%)	Percentage growth inhibition on day 4 of treatment
Chloroquine(+control)	CQ	4.50±2.00	70.5%
Milliporewater(-ve control)	mH_2O	15.27±1.96	
***C. limon* (DCM)**	P32	3.87±1.16	74.7%
***P. guajava* (DCM)**	P52	2.92±1.45	80.9%
***C. papaya* (EA)**	P63	2.14±0.83	86.0%
***C. citratus* (DCM)**	P72	1.96±0.71	87.2%
***V.amygdalina* (DCM)**	P82	0.64±0.39	95.8%

The percentage growth inhibition of each extracts and control on day 4 of the treatment was calculated using the formula below:

$$\% \text{ growth inhibition} = \frac{\text{Parasitemia of negative control} - \text{Parasitemia of test sample}}{\text{Parasitemia of negative control}} \times 100$$

6.2.1 Results and Discussion

Treatment lasted for four days and thereafter mice were monitored and parasitemia checked regularly. Percentage parasite suppressions of 74.7%, 80.9%, 86.0%, 87.2% and 95.8% were recorded by *C. limon, P. guajava, C. papaya, C. citrates,* and *V. amygdalina* respectively on day 4 of treatment. The extracts of four plants (*P. guajava* (DCM), *C. papaya* (EA) *C. citratus* (DCM) and *V. amygdalina* (DCM) significantly inhibited the growth of the parasites. The percentage inhibition of of *C.limon* extracts compares well with that of chloroquine in this study (Table 6.2.1). These extracts showed no indication of toxicity in rats. There was a marked suppression of parasites by the dichloromethane extracts of *C. citratus* and *V. amygdalina* with growth inhibition of 87.2% and 95.8%, respectively (Table 6.2.1). Previous work with the essential oils of *C. citratus* in mouse models showed a dose dependent activity with 86.6% parasite suppression at a dose of 500 mg/kg (Tchoumbougnang *et al*. (2005). This is similar to the growth inhibition of *C. citratus* DCM extract (87.2%) at a dose of 800 mg/kg demonstrated in this work. Abosi and Raseroka (2003) demonstrated a dose-dependent *in vivo* antimalarial activity of ethanol extracts of *V. amygdalina* leaf with chemo suppression of 41.5% and 67% at doses of 250 mg/kg and 500 mg/kg, respectively using *P. berghei.* A previous study reported that extracts from the leaves of *Vernonia* species have shown good hepatoprotective effects in mice models at doses of 500 mg/kg (Roy *et al*., 2006). In the present work, however, there was an increase in parasitemia post treatment due to recrudescence. The rate at which

recrudescence occurred could be shown by the increase in the parasitemia of each group on day 7 post-treatment (Fig 6.2.7).

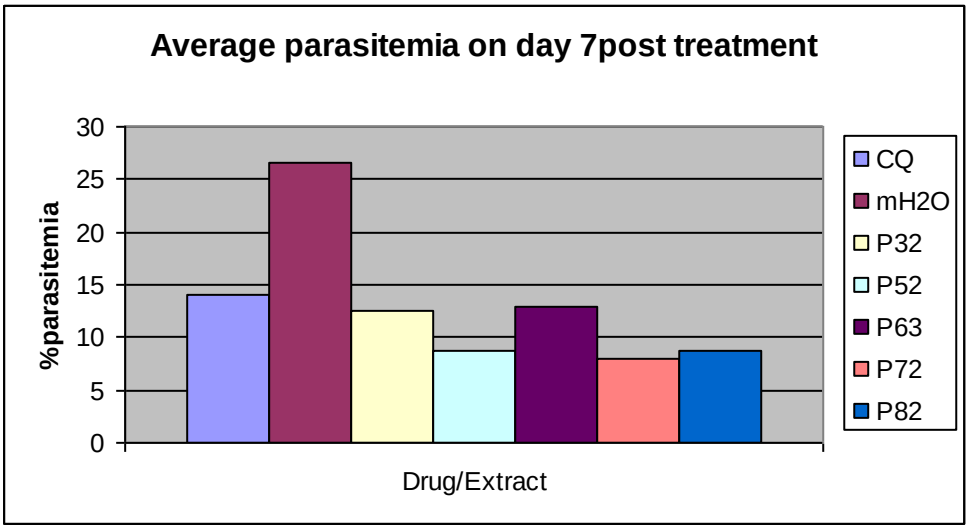


Fig 6.2.7: The mean parasitemia of each group on day 7 post-treatment.

Key: P32= ***Citrus limon*** dichlromethane extract
P52= ***Psidium guajava*** dichloromethane extract
P63= ***Carica papaya*** ethyl acetate crude extract
P72= ***Cymbopogon citratus*** dichloromethane crude extract
P82= ***Vernonia amygdalina*** dichloromethane crude extract

The parasitemia of the groups treated with the dichloromethane extracts of *C. citratus* or *V. amygdalina* on day 7 post-treatment was lower compared to *C. limon* (DCM) and *C. papaya* (EA) extracts (Fig 6.2.7). *C. limon* demonstrated an increase in parasitemia comparable to that of *C. papaya* at 7 days post treatment. Similarly, growth inhibition of *P. guajava* (DCM*)* extract compares well to that *C. citratus* and *V. amygdalina* (DCM) extract. The growth of the surviving parasites treated with *C. citratus* and *V. amygdalina* was markedly restricted and most of them appeared as “dots” during the course of treatment

(Figs 6.2.8 and 6.2.9). The untreated control and CQ treated mouse are shown in figs 6.2.10 and 6.2.11 respectively.

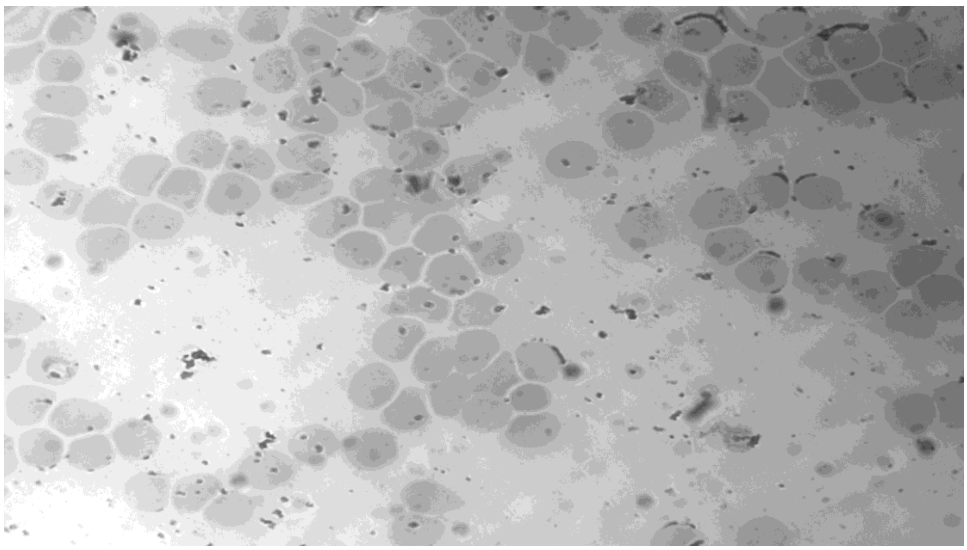


Fig 6.2.8: Thin blood smear of an infected mouse treated with *V. amygdalina in vivo*

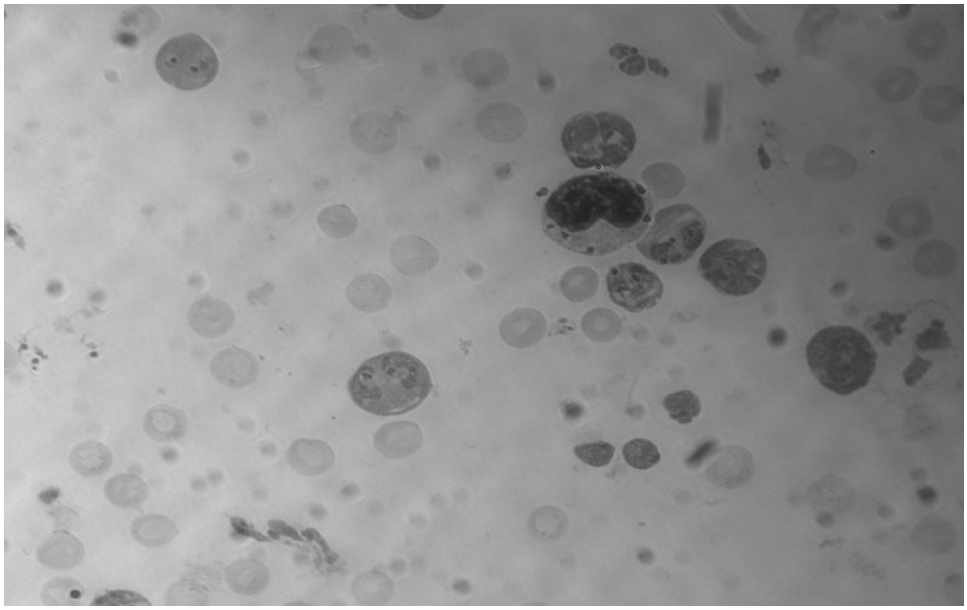


Fig 6.2.9: Thin blood smear of an infected mouse treated with *C. citratus in vivo*

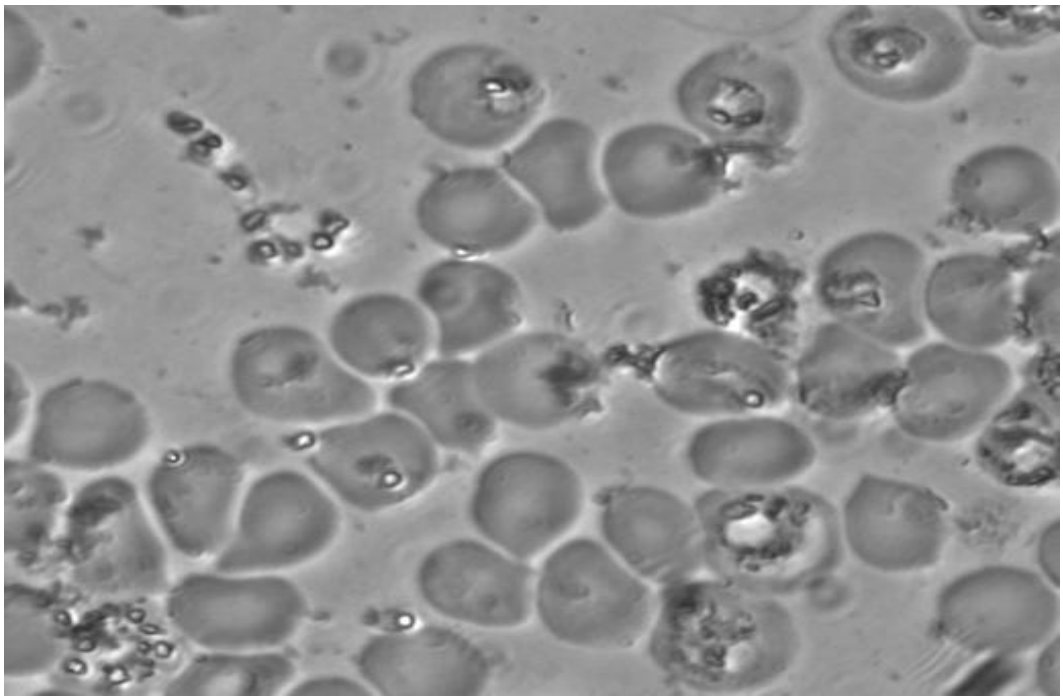


Fig 6.2.10: Thin blood smear of an untreated mouse

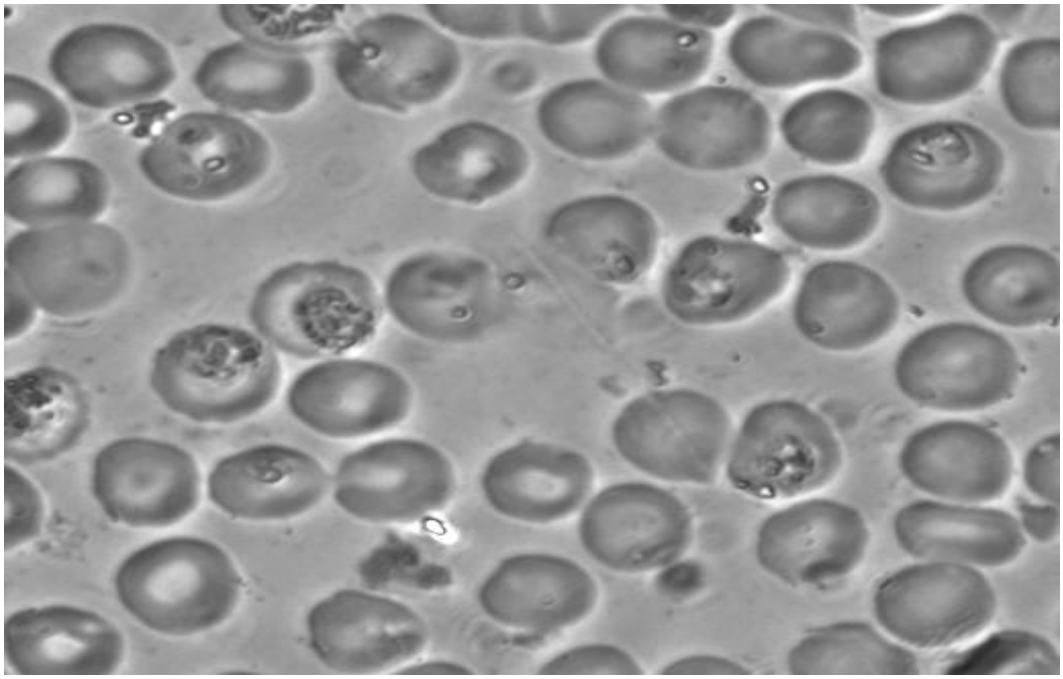


Fig 6.2.11: Thin blood smear of an infected mouse treated with chloroquine

The weight of the mice during the experiment was monitored and the values are shown in Table 6.2.2.

Table 6.2.2: Average weight (g) of mice using the 4-day suppressive test against CQ sensitive *Plasmodium berghei* (±SD).

Day post infec-tion	Cq control	mH2O	P32	P52	P63	P72	P82
0	19.84±2.06	18.97±0.39	17.76±1.24	19.29±1.19	18.07±1.68	18.03±0.76	18.68±1.62
2	19.82±2.59	18.27±0.84	17.81±1.59	19.20±2.01	17.57±1.74	17.38±1.14	18.91±1.88
4	19.68±2.92	18.60±0.65	17.71±1.61	18.90±1.32	17.62±1.81	17.88±1.21	18.52±2.26
6	20.11±3.1	17.75±0.22	17.40±1.62	18.68±2.30	16.97±1.50	17.02±1.21	18.13±1.52
8	20.99±3.14	16.72±0.74	17.88±1.69	17.85±3.09	17.06±1.44	17.24±1.19	18.08±1.63
10	20.13±2.52	14.70±0.02	17.50±1.56	18.68±1.42	16.57±1.40	17.09±1.15	17.03±2.39
14	_____	----------	17.02±1.14	18.10±1.28	16.35±1.10	16.71±0.58	16.47±3.34

The weights of the mice treated with *C. limon* appeared to be stable, however, parasitemia was increasing post-treatment. This may help explain that stability in weight does not rule out an increase in parasitemia. The mean parasitemia of the groups treated with either *C. papaya* or *C. limon* was higher compared to the groups treated with either *P. guajava*, *C. citratus* or *V. amygdalina*. The groups treated with extracts of *C. citratus* or *V. amygdalina* appeared healthier than the others. In India, the leaves of *Vernonia* species are traditionally used to treat cough. Traditionally, *V. amygdalina* is used alone in the form of decoctions, however, other plants may be added to reduce the side effect of nausea which often occurs due to the characteristic bitter taste of this species (Ohigashi *et al*., 1991). A further investigation was carried out to demonstrate the schizontocidal activities of *C. citratus* and *V. amygdalina* in combination using the 4-day suppressive test by Peters *et al*. (1993). The details are reported below.

6.3 *In vivo* antiplasmodial activities of extracts in combination

This study investigated the antiplasmodial activities of combinations of plant extracts such as *C. papaya* and *C. limon*, *C. citratus* and *Vernonia amygdalina* using *P. berghei*. These plants were chosen for this combination experiment due to their observed activity when used singly in this study. Having investigated the toxicity of these plants in mice model at higher doses >800mg/kg, the extracts from these plants were tested in combinations, at two different doses (600 mg/kg and 400 mg/kg). The first combination comprised 600 mg/kg of each extract and the second combination was dosed at 400 mg/kg of each extract. The first group of extracts was the two extracts that gave the best *in vitro* activity (*C. papaya* (EA) + *C. limon* (DCM)), while the second group of extracts (*C. citratus* (DCM) + *V. amygdalina* (DCM)) was the two extracts that gave the best *in vivo* activity in this study. These two groups were tested in combination at two different doses (600 mg/kg and 400 mg/kg), which were administered to the mice orally. This was done to maintain the same route of administration used for the extracts singly, for a good comparison. The drug formulation and volume given to the test group remained the same as in the previous experiment with crude extracts administered singly. In these combinations, the group which received 600 mg/kg of each extract inhibited the growth of parasites more than the group which received 400mg/kg showing a dose dependent activity (Fig 6.3.1). However, recrudescence occurred rapidly post treatment.

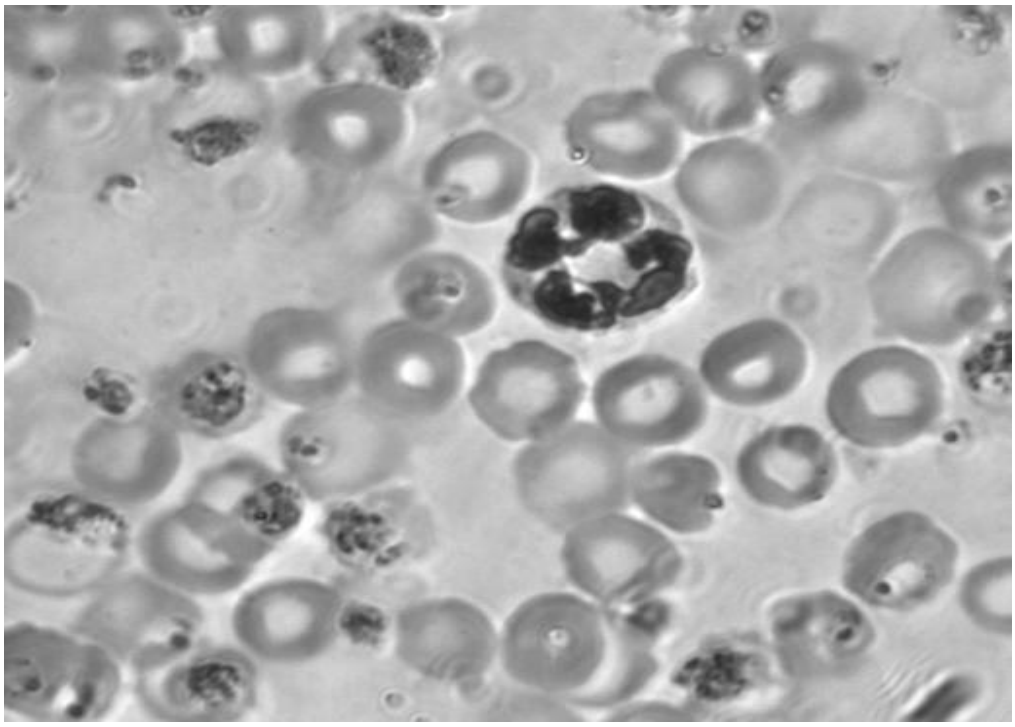


Fig 6.3.1 *In vivo* slide of an infected mouse treated with *C. papaya* (EA) + *C. limon* (DCM) (each 600 mg/kg) on day 1 post treatment

6.3.1 Results and Discussion

The first group of extracts was a combination of the two extracts that gave the best *in vitro* activity (*C. papaya* (EA) + *C. limon* (DCM)). The group which was dosed with 400mg/kg inhibited the growth of parasites by 59% and the group which received 600mg/kg inhibited parasite growth by 76% on day three of treatment. The second group of extracts (*C. citratus* (DCM) + *V. amygdalina* (DCM)) was the two extracts that gave the best *in vivo* activity. These extracts were combined at two different doses (600 mg/kg and 400 mg/kg). The group which received 400 mg/kg showed growth inhibition of 85% on day 3 of treatment, while the group treated with 600 mg/kg inhibited the growth of the parasite by 95% (Table 6.3.1). In the group treated with 400 mg/kg of the mixed extracts, it was observed that one mouse which received 300 µl instead of 200 µl of the dosage recorded a lower parasitemia than all others in the group. This suggests that the activity of the extracts is dose dependent. The

dose dependent antimalarial activity of *C. citratus* essential oil was shown in a previous study (Tchoumbougnang *et al*. (2005). The combinations which were tested at a dose of 600 mg/kg of each extract showed good *in vivo* antiplasmodial activity. The mice treated with this combination showed no parasites after the 4 days of treatment. On day 1 post treatment there was a 100% growth inhibition of parasites in the group treated with *C. citratus* and *V. amygdalina* (600 mg/kg each), while the group that received 400 mg/kg recorded 87% growth inhibition (Table 6.3.1). This shows a dose-dependent activity of the extracts. The significant difference recorded by this group called for a repeat experiment using the same dosage and experimental conditions. The repeat experiment recorded 100% parasite inhibition day 1 post treatment as was observed in the first experiment. Mice were monitored for >30 days. No recrudescence was seen in any of the mice in that group (Fig 6.3.2). According to WHO guidelines mice are considered cured if no parasites are seen ≥ 28 days of treatment (WHO, 2006).

Table 6.3.1: Percentage parasite inhibition of the combinations *in vivo*

Extract a+b (dose mg/kg)	Parasite growth inhibition on Day 3 of treatment (%)	Parasite growth inhibition on day 1 post treatment (%)
Chloroquine(10 mg/kg)	**71**	**70**
63+32 (400 mg/kg)	**59**	**69**
63+32 (600 mg/kg)	**79**	**76**
72+82 (400 mg/kg)	**85**	**87**
72+82 (600 mg/kg)	**95**	**100**

Key: 63+32 = *C. papaya* (EA) combined with *C. limon* (DCM)
72+82 = *C. citratus* (DCM) combined with *V. amygdalina* (DCM)

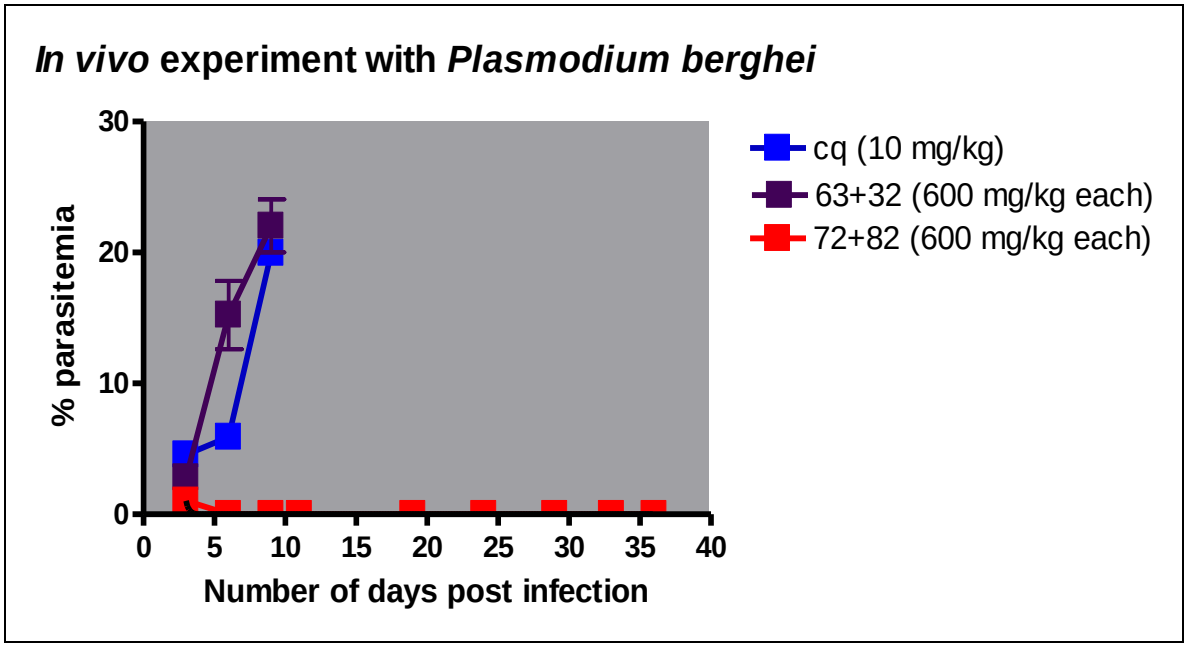


Fig 6.3.2: The schizontocidal activity of *C. papaya* + *C. limon* and that between *C. citratus* and *V. amygdalina* in combination.
Key: 63+32=*C. papaya* (EA) combined with *C. limon* (DCM)
72+82=*C. citratus* (DCM) combined with *V. amygdalina* (DCM)

Figs 6.3.3 and 6.3.4 show the slides of mouse treated with a combination of *C. citratus* and *V. amygdalina* at a dose of 600 mg/kg each and a control group.

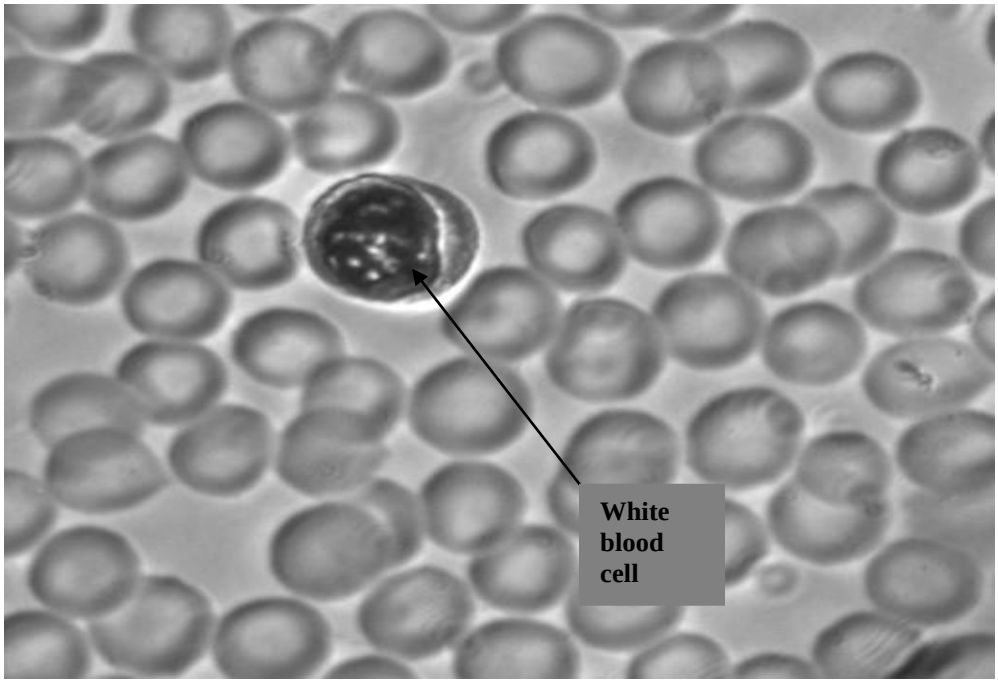


Fig 6.3.3: Thin blood smear from an infected mouse treated with P72+82 (600mg/kg) showing no parasites >30 days post treatment.

Key: P72+82 = *C. citratus* + *V. amygdalina* dichloromethane extracts (at 600 mg/kg each)

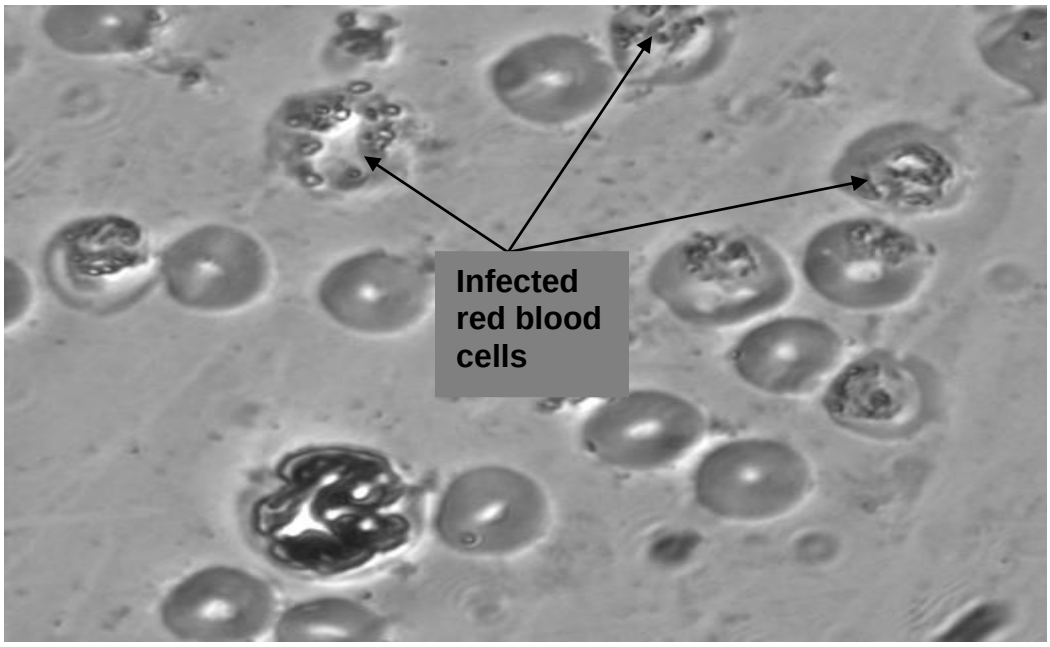


Fig 6.3.4: Thin blood smear of untreated control mouse at 7 days post infection

The mice treated with a combination of *C. citratus* (DCM) and *V. amydalina* (DCM) at a dose of 600 mg/kg increased in weight by about 13% compared to their original weight before infection. The weights of the group that was cured were monitored for 37 days post-infection to establish cure of infected mice according to WHO guidelines (WHO, 2006). The rest of the other groups died due to increased parasitemia, including the CQ group which recorded a high recrudescence post treatment (Fig 6.3.2). The only surviving group after eleven days post-treatment was the group treated with a combination of *C. citratus* (DCM) and *V. amydalina* (DCM) at a dose of 600 mg/kg. The weight details are shown in Fig 6.3.2.

Table 6.3.2: Weights of the groups during the combination experiments

days post infection	CQ	mH2O	63+32 (600 mg//kg)	63+32 (400 mg/kg)	72+82 (600 mg/kg)	72+82 (400 mg/kg)
0	22.01±1.71	23.50±1.31	25.79±0.22	24.49±1.41	23.65±1.81	24.15±1.87
1	21.98±1.24	22.39±0.73	26.06±0.68	25.23±0.96	24.60±1.73	24.94±1.68
2	21.88±0.73	21.91±1.26	25.77±0.83	25.05±1.01	24.47±1.55	24.64±1.79
3	20.87±0.50	21.87±0.84	25.98±0.82	24.88±0.95	24.28±1.54	24.64±1.65
4	21.03±1.30	22.12±0.86	24.04±0.63	24.63±0.66	22.28±0.91	23.77±1.33
5	20.93±1.80	21.23±0.59	22.38±0.75	23.12±1.09	21.6±1.46	22.92±1.40
8	18.47±1.18	19.7±0.87	19.28±0.91	19.28±1.06	20.95±2.14	19.36±2.41
10	18.35±0.64	---	18.75±0.72	18.02±1.44	22.9±1.63	18.73±2.35
11	17.8	---	18.6±0.78	17.73±1.60	23.23±1.61	18.3±2.61
14	---	---	---	---	22.89±1.11	---
16	---	---	---	---	23.59±0.99	---
18	---	---	---	---	23.01±0.81	---
23	---	---	---	---	24.11±0.86	---
28	---	---	---	---	25.17±0.69	---
32	---	---	---	---	25.91±0.56	---
35	---	---	---	---	25.86±0.82	---
38	---	---	---	---	25.87±0.48	---
37	---	---	---	---	26.62±0.55	---

Key: 63+32=*C. papaya* (EA)combined with *C. limon* (DCM)
72+82=*C. citratus* (DCM) combined with *V. amygdalina* (DCM)

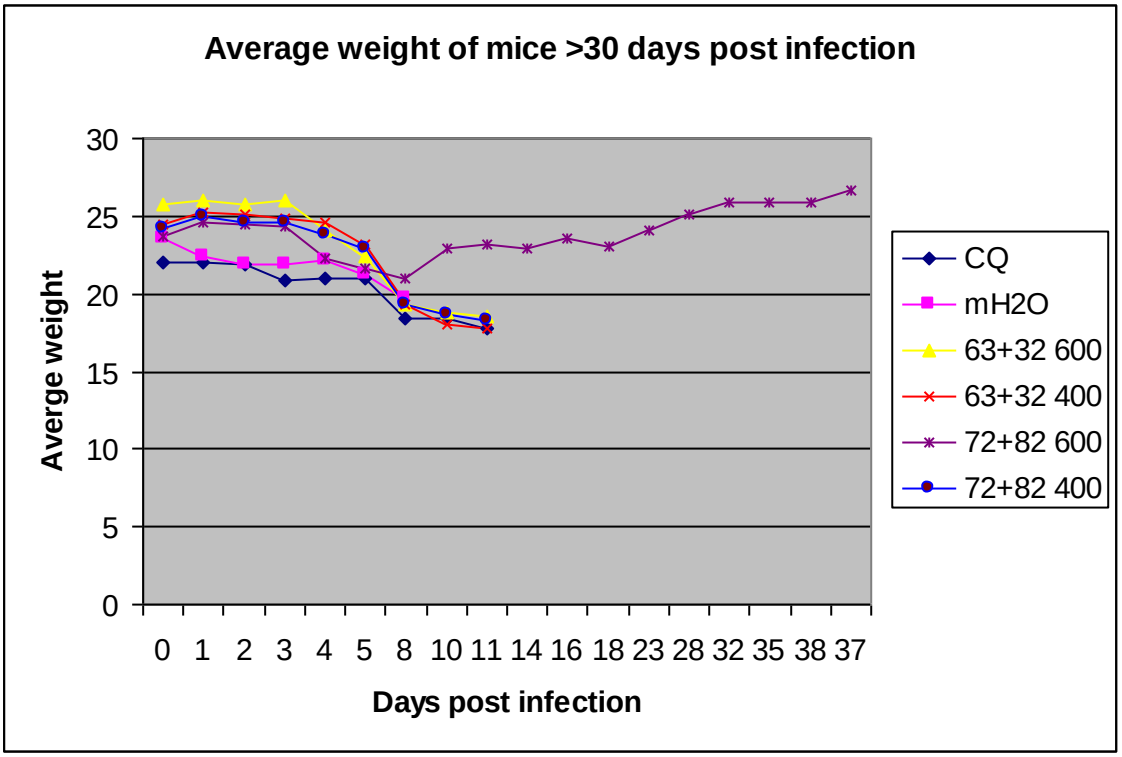


Fig 6.3.5 Trend in weight of the various groups of mice post infection

Key: 63+32=*C. papaya* (EA) combined with *C. limon* (DCM)
72+82=*C. citratus* (DCM) combined with *V. amygdalina* (DCM)

CHAPTER SEVEN

Discussion, Recommendations and Conclusions

7.1 Background

The traditional medicinal use of the seven plants *M. sapientum*, *M. indica*, *C. papaya, C. limon*, *P. guajava, C. citratus* and *V*. *amygdalina* in the treatment of malaria and febrile illnesses in Nigeria initiated this scientific investigation. In Nigerian folk medicine, the traditional healers prepare aqueous decoctions, infusions, or macerations using these plants singly or in combination. These plants were investigated singly in an *in vitro* system to determine their antiplasmodial activities. Antiplasmodial activities of ≤10 µg/ml were regarded as active in this study. Gessler *et al*. (1994) recommended that very good extracts should display IC_{50}s of ≤10 µg/ml. Extracts of *C. papaya, C. limon*, *P. guajava, C. citratus* and *V*. *amygdalina* showed activities in this range and were selected for further investigation. These plants were investigated singly and in combination using *in vitro* and *in vivo* systems to establish their potential therapeutic effectiveness.

7.1.1 *In vitro* antiplasmodial activity

The five extracts selected after screening were chosen out of the seven plants collected because they showed promising *in vitro* antiplasmodial activities with IC50 ≤ 10 µg/ml, against the chloroquine sensitive (D10) and chloroquine resistant strains (DD2) of *Plasmodium falciparum*. There was no significant difference in the activity of each extract against the CQS (D10) and CQR (DD2) of *P. falciparum*. Of the seven plants investigated, *Musa sapientum* and *Mangifera indica* were not selected for further work because of their weak *in vitro* activity. These two plants, however, have gained popularity and wide application in the traditional treatment of malaria and febrile illnesses in Nigeria. The absence of good *in vitro* activity could possibly mean that these plants may be acting as antipyretics or immune stimulants which perform better in relieving the symptoms rather than a direct parasiticidal activity against *P. falciparum* (Phillipson *et al*., 1993). Some plants which have shown no *in vitro* activity could be active *in vivo*. Some chemical compounds in plants may require being

modified *in vivo* before activity is exhibited. Several other environmental factors, as well as harvesting and storage conditions, geographical location of plant species could also influence the secondary metabolites, thus affecting the bioactivity of the plants. Leaves of *Carica papaya* are used in the traditional treatment of febrile illnesses in Nigeria. This may help explain the traditional use of the fruits in the treatment of anaemia which could also be caused by malaria. However, there is scanty data regarding the antiplasmodial properties of plants in this family.

The investigation of these extracts singly revealed that the ethyl acetate fraction of *C. papaya* was the most active extract *in vitro*. This extract recorded an IC_{50} of 2.96 µg/ml and 3.98 µg/ml against the CQS (D10) and CQR (DD2) of *P. falciparum*. *C. papaya* crude extract showed a high selectivity index of 249.25 and 185.37 against the D10 and DD2 strains respectively, which indicates good specificity against *P. falciparum*. It also shows that the recorded activity of the *C. papaya* extract was not due to cytotoxicity to the CHO cell lines. The *in vitro* antiplasmodial activity of the petroleum ether extract of *Carica papaya* pulp and rind have been reported to show IC_{50} values of 18.09 µg/ml and 15.19 µg/ml, respectively (Bhat and Surolia, 2001) against FCK 2 strain of *Plasmodium falciparum*. Interestingly, this correlates well with the IC_{50} value of petroleum ether extract of the leaves (16.36 µg/ml) demonstrated in the present study against the *Plasmodium falciparum* D10 strain (Table 4.3.2.1). The methanol and water extracts showed activities >100 µg/ml (Bhat and Surolia, 2001) which was similar to that observed in this study. This is due to the inability of these solvents to effectively extract the active lipophilic constituents from these plants.

The sequential extraction carried out in all the extractions in the present study enabled the identification of greater antiplasmodial activity in the ethyl acetate fraction with IC_{50} value of 2.63 µg/ml when compared to other solvents. I am yet to come across literature data on the antiplasmodial properties of *C.limon*.

Vernonia amygdalina in this study showed IC_{50} values of 3.98 µg/ml and 4.12 µg/ml against the D10 and DD2 strains, respectively in the dichloromethane (DCM) extracts. This correlates well with the activity of *V. colorata* Drake, *V. myriantha* Hook.f. and *V. oligocephala* Gardner naturalized in South Africa which showed IC_{50} values of 4.7 µg/ml, 3.0 µg/ml and 3.5 µg/ml, respectively against D10 (Clarkson *et al*., 2004). *Vernonia amygdalina* leaves have previously been reported to show *in vitro* antiplasmodial activity (Masaba *et al*., 2000; Alawa *et al*., 2003; Tona *et al*., 2004). Several classes of compounds have been isolated from these species; flavonoids, sesquiterpenes, triterpenoids, alkaloids, and steroids, and the triterpenes have been implicated in the antiplasmodial activity of this plant (Alves *et al*., 1997). *Vernonia colorata* DCM root extract showed an IC_{50} of 6 µg/ml against the W2 strain of *P. falciparum*. Vernolide and vernoladin isolated from the leaf extract of *Vernonia colorata* were shown to be responsible for the recorded activity in the acetone fraction (Chukwujekwu *et al*., 2009). *Vernonia* species have shown antipyretic, analgesic, and anti-inflammatory properties (Valverde *et al*., 2001; Iwalewa *et al*., 2003; Mazumder *et al*., 2003; Cioffi *et al*., 2004). They have shown antitumoral (Jisaka *et al*., 1993), antibacterial (Reid *et al*., 2001; Rabe *et al*., 2002; Erasto *et al*., 2006), anti-inflammatory (Cioffi *et al*., 2004) and antioxidant activities (Erasto *et al*., 2007). *In vitro* growth inhibition of malaria parasites with a value of 57.9% was recorded for the chloroform/ethanol extract (Bidla *et al*., 2004). These reports further emphasize the antimalarial potential of *Vernonia* species.

Psidium guajava is commonly used to treat malaria and diarrhea in Nigeria. The aqueous stem bark of this plant showed IC_{50} values of 10-20 µg/ml against the D10 strain of *P. falciparum* (Nundkumar and Ojewole, 2002). In the present study, the activity of this plant in the petroleum ether, dichloromethane and

ethyl acetate extracts were found to be 15.45 µg/ml, 5.95 µg/ml, and 21.62 µg/ml, respectively (Table 4.3.2.1). *Psidium guajava* had a selectivity index (SI) of 25.33 and 18.61 against the D10 and DD2 strains, respectively. The greater activity in the dichloromethane extract suggests the presence of active lipophilic agents. The essential oils from *C. citratus* have demonstrated antioxidant and radical scavenger properties (Menut *et al*., 2000).

7.1.2 *In vitro* study of extracts in combination

Combination therapies are a vital strategy to prevent or delay resistance of parasites and have been approved for other multidrug resistant infections, such as HIV and tuberculosis. Although the combination of compounds for the treatment of malaria has been recommended (White, 1998; WHO, 2001), this study explored the *in vitro* and *in vivo* activity of plant extracts investigated when combined. Several combinations of these plant extracts were tested. The combination that recorded a significant enhancement in its activity *in vitro* was between the ethyl acetate extract of *C. papaya* and the DCM extract of *C. limon* with an IC_{50} of 0.83 µg/ml. This combination was not cytotoxic to the CHO cell line as evidenced by a selectivity index >100. Ngemenye *et al*. (2006) recorded the use of *Carica papaya* leaves in combination with *Turraeanthus africanus* (Welw.) Pellegr. belonging to the family Meliaceae, lime and *Aframomum melegueta* K. Schum. of the family Zingiberaceae in the traditional treatment of malaria in Cameroon. In this study, *C. papaya* extract was demonstrated to enhance the activities of other extracts in combination. The dominant effect of *C. papaya* activity could be traceable to its high selectivity index for the sensitive and the resistant strain of the parasites (Table 4.3.3.1).

7.1.3 Bioactivity guided fractionation of *C. papaya* ethyl acetate

Fractionation of the ethyl acetate fraction of *C. papaya* by solid phase extraction was carried out to isolate and identify the active components. The SPE extraction of the ethyl acetate fraction of *C. papaya* revealed the greatest activity in the 100% ACN concentration with an IC_{50} of 2.2 µg/ml. This activity was very close to the activity of the parent ethyl acetate crude extract of 2.96 µg/ml against *P. falciparum*. Further purification of the 100% ACN fraction by HPLC yielded two peaks. Identification and characterization of these compounds was accomplished using NMR and GC-MS which identified the compounds as two essential fatty acids namely 9,12,15- octadecatrienoic acid (Compound 1) and 9,12- octadecadienoic acid (Compound 2). Compound 1 (linolenic acid) and compound 2 (linoleic acid) with IC_{50} values of 3.58 µg/ml and 6.88 µg/ml, respectively were not as active as the ethyl acetate SPE (100% ACN, IC_{50} 2.2 µg/ml) fraction or its crude extract. This may suggest an enhancement of activity by other chemical constituents present in the extract. These essential fatty acids are very useful in the body, but cannot be synthesized in the body. Essential fatty acids (EFAs) and their polyunsaturated metabolites are major lipid components of all tissues and they play a vital role in modulating the fluidity and function of cell membranes (Minich *et al*., 2000). They can be acquired through diet or dietary supplements, however, the quantity needed for a therapeutic dose may not be obtained in a normal diet due to poor yield, and low concentrations of these lipophilic compounds in an aqueous or similar environment. This class of compounds has various related health benefits and can be prepared in large quantities from natural resources (Tsuzuki *et al*., 2004). They are also commercially available. The activity of linolenic acid, which is a polyunsaturated fatty acid with three double bonds, was higher and more selective for *P. falciparum* compared to linoleic acid which has two double bonds. Previous work by Kumaratilake *et al*., (1992) demonstrated that the antiplasmodial activity of the fatty acids is influenced by the degree of unsaturation. The work done by this group of researchers using

varying degrees of unsaturated fatty acids demonstrated that the polyunsaturated fatty acids significantly inhibited parasite growth when compared to the saturated fatty acids (Kumaratilake *et al*., 1992). The free acids and their methyl esters in the present study were equipotent in their activity against *P. falciparum* and the data compare well with the observations of Kumaratilake *et al*., (1992). Since the antiplasmodial activities of this class of compounds are dependent on the degree of unsaturation; (Kumaratilake *et al*., 1992, Kumaratilake *et al*., 1997), chemical modification of these compounds by the introduction of two or three more double bonds could be of immense help in increasing its activity. Furthermore, long chain fatty acids with >18 carbon atoms, such as arachidonic acid [20:4(n-6)] are vital ingredients in infant nutrition and they serve as precursors for eicosanoids which mediate immune and vascular functions (Decsi and Koletzko, 1994).

Further investigation of the *in vivo* schizontocidal activity of both compounds was carried out to evaluate their efficacy in a mouse model using a 4-day suppressive test. The results of the *in vivo* experiments further demonstrate the antiplasmodial properties of polyunsaturated fatty acids singly and in combination. The *in vivo* antiplasmodial activities of linolenic acid and linoleic acid in combination were significantly higher than those of the individual compounds. This observation suggests a possible synergistic effect. Linolenic acid and its methyl ester significantly suppressed the growth of parasites during the course of treatment with percentage growth inhibition of 70% and 76%, respectively. However, there was a rapid recrudescence post-treatment. The rate at which recrudescence occurred in mice dosed with linolenic acid methyl ester led to an investigation of its pharmacokinetic properties in a mouse model. This study was aimed at evaluating its absorption, elimination rate and half-life in plasma.

7.1.4 Bioavailability study of linolenic acid methyl ester

Linolenic acid methyl ester was preferred to its free acid for the bioavailability study using the LC-MS/MS because it was readily detected by mass spectrometry. Previous studies have shown that the methyl esters of fatty acids have preferential chromatographic qualities compared to the free acids (Viron *et al*., 2000). The molecular ion of linolenic acid methyl ester was recorded as 293.1 $[M+H]^+$. Linolenic acid methyl ester was extracted from plasma using the method adapted from Aleryani *et al*., (2005). The limit of quantification (LOQ) was 0.132 µg/ml. The bioavailability of linolenic acid methyl ester was assessed through three routes of drug administration: oral, subcutaneous and intravenous routes. The compound achieved good plasma concentrations through the intravenous route only. The oral and subcutaneous routes showed concentrations that were below the limits of quantification (BLQ), and as such were not used to determine its elimination rate from plasma. This suggests that the compound could have been metabolized or have been poorly absorbed (Minich *et al*., 1999). Though the traditional use of the plants is by the oral route, the claim of therapeutic efficacy of these plants may be traceable to the activity of the various compounds in the mixture which may have acted synergistically and such interactions between extracts could help keep lipophilic compounds in aqueous solution. However, the recorded lower limits of linolenic acid methyl ester in plasma could be due to malabsorption, oxidation, desaturation and elongation, which are all the end products of increased metabolism. This may further indicate the need to establish the dosage used by the traditional healers, route of administration and possibly the optimal conditions necessary for effective use of these plants. A previous study reported that patients with cholestatic liver disease were noted to have a compromised bile secretion into the intestinal lumen which negatively affects the absorption of adequate amount of their dietary lipids (Minich *et al*., 2000). Kobayashi *et al*. (1983) further pointed out that lipid absorption was reduced by

30% in patients with biliary atresia. Rats with permanent biliary drainage showed decreased percentages in the concentrations of linoleic acid absorbed (Minich *et al*., 1999). Cholestatic liver disease in infants alters the efficiency of hepatic elongation and denaturation (Socha *et al*., 1997; Socha *et al*., 1998).

These observations may help explain that the physiological state and health status of any experimental model could influence the experimental results. The intestines are the first recipients of nutrients, and microbial hydrogenation of these acids takes place in the lumen before they are subsequently delivered to other body tissues (Garton, 1961). By virtue of their strategic position, they may modulate the bioavailability and biological activity of the fatty acids (Tsuzuki and Ikeda, 2007). The oxidized form of these fatty acids showed increased antimalarial activity (Kumaratilake *et al*., 1997). This may help explain that the *in vivo* activity observed after the oral administration of this compound may be traceable to the active oxidation products of these fatty acids investigated in the present study. The presence of vitamin E, which is an anti-oxidant, suppresses the antiparasitic activities of this class of compounds (Kumaratilake *et al*., 1992). This suggests that diets rich in antioxidants such as vitamin E could counteract the antiplasmodial effect of these fatty acids. This may also indicate that using this class of compounds as antimalarials will require that diets of experimental animals should contain no antioxidants. However, since antioxidants are present in most diet, eliminating them is almost impossible hence fatty acids are not likely to be good antimalarials. Kumaratilake *et al*. (1997) further demonstrated the significant structural differences between the fatty acids which induced killing by acting directly on the parasites, and those whose antimalarial activity are products of neutrophil priming. The mechanism of parasite adherence to the neutrophils, and how this can result in the death of parasites, is not known.

When infected mice were treated intravenously with linolenic acid methyl ester, parasite growth was restricted by 97%, with a 69% recrudescence 3 days post-

treatment. A bioavailability study to determine the elimination rate of the compound from plasma revealed that linolenic acid methyl ester is quickly eliminated from plasma, with an estimated half life of 1 hour 18 minutes. In this study, the highest concentrations were recorded at 1 hr post-administration (T1), and this concentration continuously decreased, with very low concentrations at T4, which was at 6 hours post administration. This indicates fast elimination from plasma within the first 6 hours of administration. A previous study demonstrated that the absorption of conjugated linolenic acid (CLnA) is slow in the rat intestine and is rapidly converted to conjugated linoleic acid (Tsuzuki *et al*., 2006). Their report, which was based on the CLA isomers investigated, α-eleostearic acid (9*Z*,11*E*,13*E*-18:3) and punicic acid (9*Z*,11*E*,13*Z*-18:3) showed that after 1 hour of administration, the lymphatic recovery of the conjugated fatty acids with three double bonds was significantly lower compared with the conjugated linoleic acid (CLA) with two double bonds and they recorded that this observation persisted for 8 hours (Tsuzuki et. al., 2006).

Their study suggested that this difference in absorption of CLA and conjugated triene fatty acids could be due to the rapid conversion of conjugated triene fatty acids to CLA. This, they further explained, was due to Δ^{13}-saturation enzyme reaction which the conjugated triene fatty acids undergo as a result of their slow secretion from the intestine. The study recorded a lymphatic recovery ≥ 90% for the most conjugated triene fatty acids investigated 24 hours after administration (Tsuzuki *et al*., 2006). This slow absorption and rapid conversion to CLA could possibly be responsible for the very low concentrations (BLQ) of linolenic acid methyl ester in the oral and subcutaneous routes observed in this study. This may also help explain the high rate of recrudescence observed soon after the intravenous treatment with linolenic acid methyl ester was completed. In other words, the parasites gather more momentum even before good concentrations of the compounds are achieved.

Another study demonstrated that deuterium-labeled ethyl linolenate ($^{2}H_{5}$-17,17,18,18,18-18:3n-3) and deuterium-labeled ethyl linoleate ($^{2}H_{5}$-17,17,18,18,18-18:2n-6) after oral administration in rats reached maximum concentrations in plasma, stomach and spleen at the time points before 8 hours, while in the red blood cells and other internal organs, maximum concentration was reached at 8 hours (Yu Hong and Norman, 2007). Similar observations were reported in a study with linoleic acid and palmitic acid where maximum concentrations were achieved at 6 and 4, hours respectively after bolus administration orally (Minich *et al*., 2000). This may help explain that absorption of CLA by the oral administration is significantly different from the absorption of conjugated triene fatty acids, due to the fast conversion of conjugated triene fatty acids to CLA. It is therefore very likely that linolenic acid methyl ester (linolenic acid methyl ester) investigated in this study may have been rapidly converted to CLA (Minich *et al*., 2000). In the present study with linolenic acid methyl ester, the maximum concentration was reached 1 hour post administration within 6 hours using the intravenous route. The high rate at which recrudescence occurred could be due to the short half life of this compound, and this could be averted by increasing the duration of treatment or by administering them 2 to 3 times a day using the four-day suppressive treatment. During the *in vitro* combination of extracts in this study, *C. papaya* ethyl acetate fraction, from which linolenic and linoleic acids were isolated, acted synergistically and influenced the activity of the components in combination. In the *in vivo* experiments with linolenic and linoleic acids, it was observed that suppression of parasites when the two compounds were combined was greater than that observed with the individual compounds. The *in vitro* antiplasmodial activity of the ethyl acetate (EA) extract may be traceable to the combination effect of linolenic and linoleic acids, which was relatively stronger than the activities of the individual compounds. However, the pharmacokinetic study of linolenic acid methyl ester revealed a short half life, and since its fatty acid appears to be the dominant compound in the EA fraction

its short half life could possibly be responsible for the rapid recrudescence observed during the *in vivo* test with the parent ethyl acetate extract of linolenic acid. It was also evident in this study that linolenic acid methyl ester was poorly absorbed in the oral and subcutaneous route of administration. The recrudescence recorded in the *in vivo* combination study of *C. papaya* and *C. limon* extracts which acted synergistically in the *in vitro* experiment could therefore be attributed to the rapid elimination of linolenic acid methyl ester present in *C. papaya* ethyl acetate extract. Poor absorbtion of this compound in the oral route of administration is another factor that could be responsible for the observed recrudescence in the oral administration of the parent ethyl acetate extract.

7.1.5 *In vivo* antiplasmodial investigation of extracts singly

The extracts of four plants, *P. guajava* (DCM), *C. papaya* (EA), *C. citratus* (DCM) and *V. amygdalina* (DCM), significantly inhibited the growth of the parasites with percentage growth inhibition of 80.9%, 86.0%, 87.2% and 95.8%, respectively in a 4-day suppressive test. The least growth inhibition was recorded by *C. limon* (DCM) 74.7% and this was close to that recorded in the group treated with chloroquine (10 mg/kg) with a growth inhibition of 70.5%. Mice treated with these extracts showed no obvious signs of toxicity at the concentrations tested during the course of the experiment. *V. amygdalina* extracts have been reported to act dose dependently (Anoka *et al*., 2008). In a 4-day suppressive test, the ethanol leaf extracts and root bark of *V. amygdalina* suppressed parasites by 67% and 53.5%, respectively (Abosi and Raseroka, 2003), at a dose of 500 mg/kg and 250 mg/kg. In the present study, there was a marked growth inhibition of parasites with values of 87.2% and 95.8% by the dichloromethane extracts of *C. citratus* and *V. amygdalina*, respectively at a dose of 800 mg/kg. This suggests that these extracts could be acting in a dose-dependent manner. The growth of the parasites treated with *C. citratus* and *V. amygdalina* was highly restricted, and most of them appeared

as “dots” during the course of treatment. The two groups of test animals treated with either of these two extracts outlived all others, including the group which received chloroquine. Recrudescence was delayed in the groups treated with *C. citratus* or *V. amygdalina* when compared to other groups. The *in vivo* activity of *C. citratus* has been reported to show IC_{50}s from 6-9.5 µg/ml with a 20 µg/ml chloroform/ ethanol extract (Tchoumbougnang *et al*., 2005).

7.1.6 *In vivo* study of extracts in combination

7.1.6.1 Combination of *C. papaya* and *C. limon*

The extracts investigated in combination were in two groups. The first group (*C. papaya* + *C. limon*) was selected based on their activity *in vitro* with an IC_{50} of 0.83 µl/ml, and the second group was a combination of the best extracts *in vivo* (*C. citratus* + *V. amygdalina*). Two groups of mice were treated with each combination at two different doses (400 mg/kg and 600 mg/kg). These plant extracts were not cytotoxic at the concentrations tested, rather, they showed a dose-dependent activity. The combination of *C. papaya* + *C. limon* recorded a significant suppression of parasites at a dose of 600 mg/kg each (76%) but was met with recrudescence on day 1 post-treatment. This result may help explain why the *in vitro* activity does not mean that the chemical compound is equally active *in vivo* (Phillipson *et al*., 1993). This is because some physiological factors and immune response that are inevitable in an *in vivo* system are not applicable in the *in vitro* experiment. In the *in vivo* system, the effectiveness of the extracts is partly influenced by factors such as the gastrointestinal uptake, oxidation of active compounds and half-life in plasma (Franssen *et al*., 1997). The *in vivo* activity to a large extent is influenced by pharmacokinetic, as well as pharmacodynamic, properties of the chemical compounds. The aqueous decoctions from these plants used in the traditional treatment of malaria have not been reported to show cytotoxic activities. Aqueous decoctions may contain low concentrations of active constituents which could account for the reduced toxicity (Kirby *et al*., 1996). In this study, these plants showed no

toxicity to mice, however, the evaluation approach used here is likely to demonstrate acute toxicity and not chronic effects of the extracts on vital organs in the body such as the brain, liver, kidney, or even the central nervous system. Assessment of the functional state of these organs, size and shape, as well as full blood count, may offer a better and more reliable assessment of *in vivo* toxicity.

7.1.6.2 Combination of *C. citratus* and *V. amygdalina* extracts

An exciting result was recorded in the combination between *C. citratus* + *V. amygdalina* at a dose of 600 mg/kg each. The mice treated with this combination showed no parasitemia after treatment in the 4-day suppressive test. The animals were monitored for >30 days and no recrudescence was observed in any of the mice. Their weight also increased by 13% of the original weight before infection. A repeat experiment recorded the same result. WHO recommended that treated mice should be considered cured if no parasites are seen after ≥28 days of treatment (WHO, 2006). This result shows that the combination of *C. citratus* + *V. amygdalina* DCM extracts cured mice infected with *P. berghei* using a 4-day suppressive test. *V. amygdalina* has also been shown to enhance the *in vivo* activity of chloroquine (Iwalokun, 2008). The plants investigated in this study are traditionally prepared as aqueous decoctions which involve boiling the plant and drinking the extract. Moreso, the traditional healers make use of these plants in their fresh state. Poor activity of the water extract from plants has been shown in several studies (Francois *et al*., 1996, Clarkson *et al*., 2004). The water extracts of plants investigated in this study showed very weak *in vitro* activities with IC_{50} values >50 µg/ml (Table 4.3.2.1). It could be possible that preparing these plants in their fresh state, following the traditional recipe which involves boiling for several hours may have increased their activities. However there is a possibility of denaturing some active components in the crude extracts since their heat tolerance has not been established. Stronger antiplasmodial activities were mostly displayed

by the dichloromethane extract, with the exception of *C. papaya*, where activity was shown in the ethyl acetate fraction. This may help explain why the aqueous extracts showed no activity since the active lipophilic components from these plants were extracted with the non-polar solvents. This could suggest that the claim of therapeutic efficacy of these plants may be traceable to the activity of the various compounds in the mixture which may have acted synergistically and such interactions between compounds could increase their solubility in an aqueous environment. The ideal animal model that could display precise clinical characteristics of human malaria is not yet available. However, the murine model of PbA in (BALB/C x 57BL/6) has shown several features in common with human cerebral malaria (Hearn *et al*., 2000). Results from the use of these models may not be directly extrapolated to humans. They can only be predictive since major differences exist between the small mammals and humans.

7.2 Recommendations

The primary objective of investigating the chemotherapeutic effectiveness of these extracts in the treatment of malaria was achieved in this study. Out of the seven extracts investigated in an *in vitro* system, *M. sapientum* and *M. indica* were inactive with IC_{50} >50 µg/ml and further work with them was discontinued. The active five extracts tested in the *in vitro* experiment as single entities exhibited antiplasmodial activities with IC_{50} <10 µg/ml against the sensitive (D10) and resistant (DD2) of *P*. *falciparum*. The extracts were not cytotoxic with CHO cell line at the concentrations tested. *C. papaya* ethyl acetate showed good antiplasmodial activity of 2.96 µg/ml and a high selectivity (>100) to *Plasmodium falciparum*. *In vitro* testing of these extracts in combination revealed a combination of the extracts of *C. papaya* and *C. limon* with an IC_{50} of 0.83 µg/ml. The potency of these extracts was enhanced by combining them. It is therefore recommended that researchers should exhaustively explore the *in vitro* properties of extracts in combination before considering them inactive. A follow up investigation of the ethyl acetate extract of *C. papaya* revealed that the long chain polyunsaturated hydrocarbons that were responsible for the recorded activity are the essential fatty acids (linolenic and linoleic acids).

The linolenic acid methyl ester showed an IC_{50} of 3.58 µg/ml and 4.40 µg/ml with a selectivity index greater >10. The activity of this class of compound is partly influenced by the degree of unsaturation (Kumaratilake *et al*., 1992). Chemical modification of this compound by the introduction of additional double bonds could enhance its activity. The oxidized form of these fatty acids showed increased activity (Kumaratilake *et al*., 1997). Therefore chemical oxidation of this class of compounds could also enhance efficacy. Recently, researchers have reported the medicinal properties of this class of compounds (Sala-vila *et al*., 2006; Ren-Wang Jiang *et al*., 2008), therefore a thorough investigation of their biological activities is recommended. Some of these polyunsaturated fatty

acids could be obtained naturally in diets, natural products or food supplements. However, the quantity may be grossly insufficient for therapeutic doses and effort to reduce the lipophilic properties of this class is necessary. The linolenic and linoleic acid and their methyl esters investigated are equipotent and are commercially available. The linolenic acid methyl ester has better chromatographic behaviour than the free acid, and thus was used for the bioavailability study. The bioavailability study of linolenic acid methyl ester showed that it is best administered intravenously and showed good concentrations in plasma. However, due to its short half- life as revealed in the pharmacokinetic study, recrudescence occurred rapidly in mice after a 4-day suppressive treatment. It is recommended that treatment with this compound be given 2 to 3 times a day and for a longer duration, and possibly be evaluated in combination with existing antimalarials. This is necessary because in addition to acting directly on the parasites, these fatty acids and their methyl esters could activate the neutrophils and their effector cells thereby enhancing their antimalarial properties (Poulos *et al*., 1991). Combinations of this nature with a different mechanism of action and similar half-life are vital tools necessary to fight, prevent or delay resistance, as well as to augment cure rates. Artemisinin combination therapies have been highly recommended by WHO in the treatment of malaria (WHO, 2001).

It would be necessary to use animal models to validate the therapeutic efficacy of extracts or chemical agents. In this study, the combination that cured mice infected with *P. berghei* was not the combination which gave the best activity *in vitro*. This research could help to justify that *in vitro* antiplasmodial activity should be confirmed by *in vivo* studies in experimental models. The extrapolation of *in vitro* results to *in vivo* efficacy is not encouraged. This is because the *in vivo* system is more complex than the *in vitro* system which is closely controlled. Traditional remedies which have been the source of antimalarial agents are prepared mostly in aqueous environment. They have played indispensable roles in drug discovery and have been readily accessible

where pharmaceuticals are unavailable, inaccessible, or expensive. However, in this study, these plants were extracted using different solvents with varied polarities. The strong activity of the DCM extract shows that the active components are lipophilic and may not be extracted by water. The hot water treatment of these extracts could extract lower concentrations of these active lipophilic components, but may not be available at therapeutic doses. This result may help explain the increase in parasite survival despite continuous treatment with herbal remedies. The number of existing antimalarials is still inadequate especially in the face of parasite resistance. It is therefore highly recommended that researchers should explore more of the antiplasmodial properties of extracts *in vivo* for more potent antimalarial remedies. Good ideas of plants to explore could be obtained from the traditional healers who use them locally to prepare herbal remedies. It is therefore necessary for the traditional healers and the scientific community to work in collaboration. Since the traditional healers are patronized by the majority of the populace in developing countries, such a relationship will help the traditional healers to know the dosage and duration of treatment that could be toxic to humans, and also the concentrations and the right choice of extracts to administer singly or in combination.

7.3 Conclusions

This study has demonstrated that the extracts from the leaves of these Nigerian plants investigated are potential sources of antimalarial compounds. That the activities of these plants investigated were seen in the leaves is also of interest since it does not endanger the plants species. This work has revealed a combination of two extracts from Nigerian plants that cured mice infected with *P. berghei*. To the best of our knowledge this is the first study which has shown that mice infected with *P. berghei* could be cured by a combination of the two Nigerian plants *C. citratus* (DCM) and *V. amygdalina* (DCM) at a dose of 600 mg/kg using a 4-day suppressive treatment. Nigeria has a rich flora and fauna which are yet to be explored. Exhaustive exploration of the combination

potentials of plant extracts should form part of any drug development program. Such extracts with curative potentials could be further investigated and clinically tried in a controlled study. The results of this study could help encourage more identification and validation of natural products which has shown antiparasitic properties thus facilitating the development of a new generation of antimalarials. Quinine and artemisinin are products based on the documented use of medicinal plants in treating malaria. However, that the disease is still endemic in Nigeria where the majority of the populace patronize traditional healers could suggest the possibility that these healers may not have administered the right dosage (since they could be dose dependent as observed in this study). It also could be that the right combinations were not given. The presence of active lipophilic compounds which may not be extracted in water as practiced traditionally is another hindrance to its antimalarial activity. Very importantly also, the duration of the treatment could play a vital role. Collaboration between the natural product scientists and the traditional healers could be of immense help and that could assist traditional healers to administer the right doses to avoid the risk of toxicity that may result from these herbal remedies. This active combination is a product of this study, and since it has not been proven clinically, the observed efficacy in a mouse model may not be directly extrapolated to humans. They can only be predictive.

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APPENDICES

Appendix I

Dose response curves

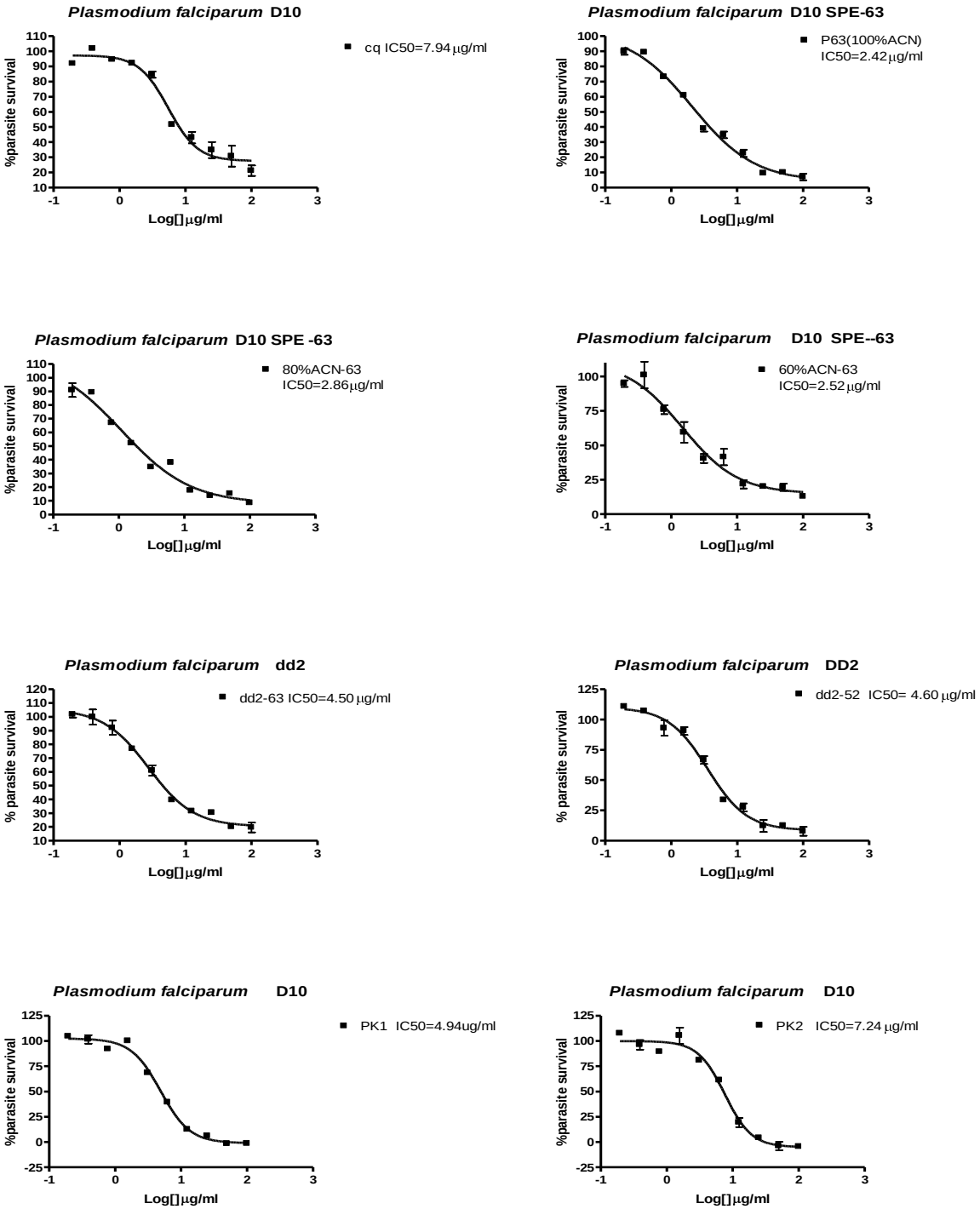


Fig A1 *In vitro* dose response curves of *C.papaya* EA SPE fractions and HPLC peaks

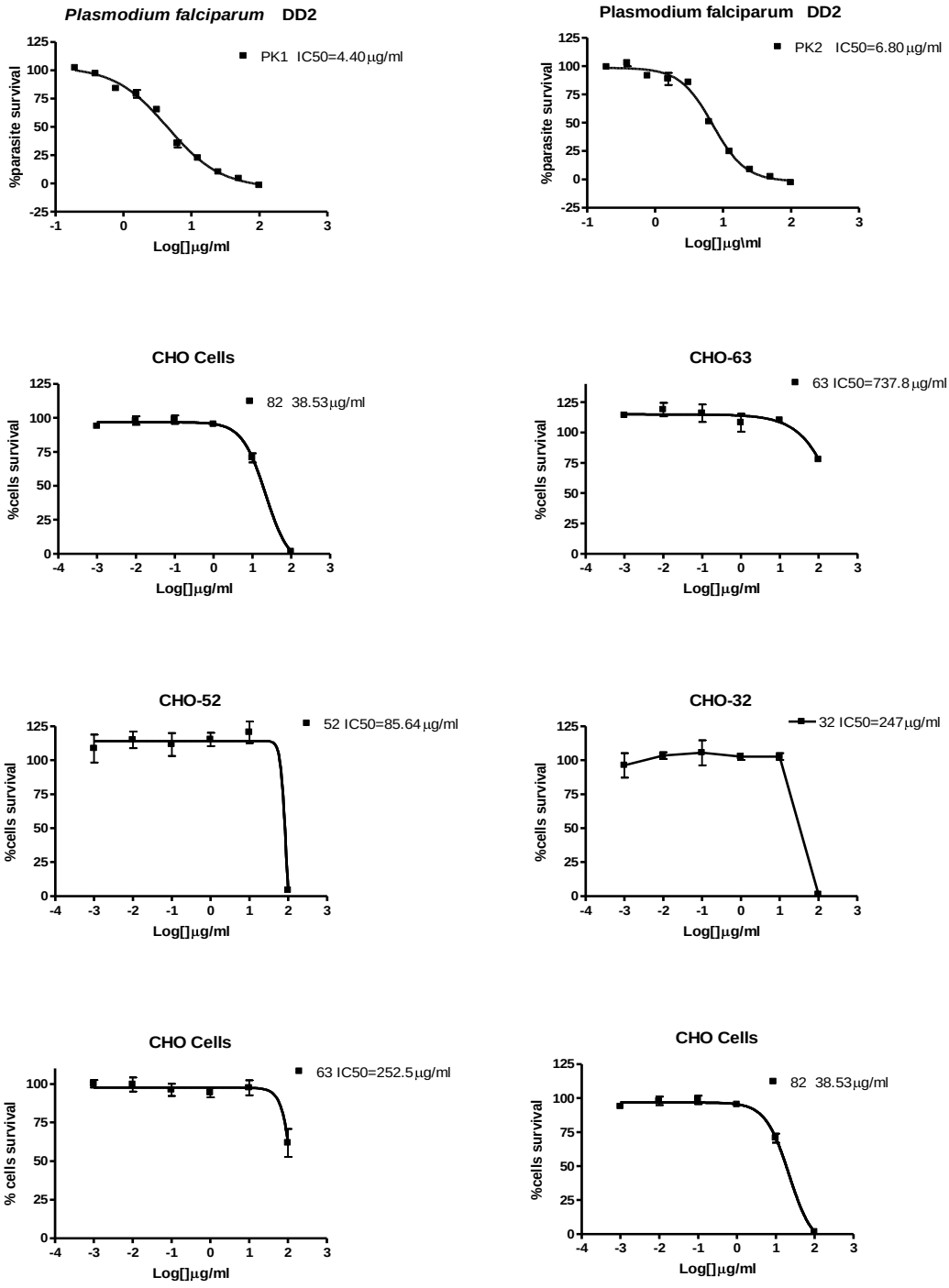


Fig A2 *In vitro* dose response curves of extracts and isolated compounds

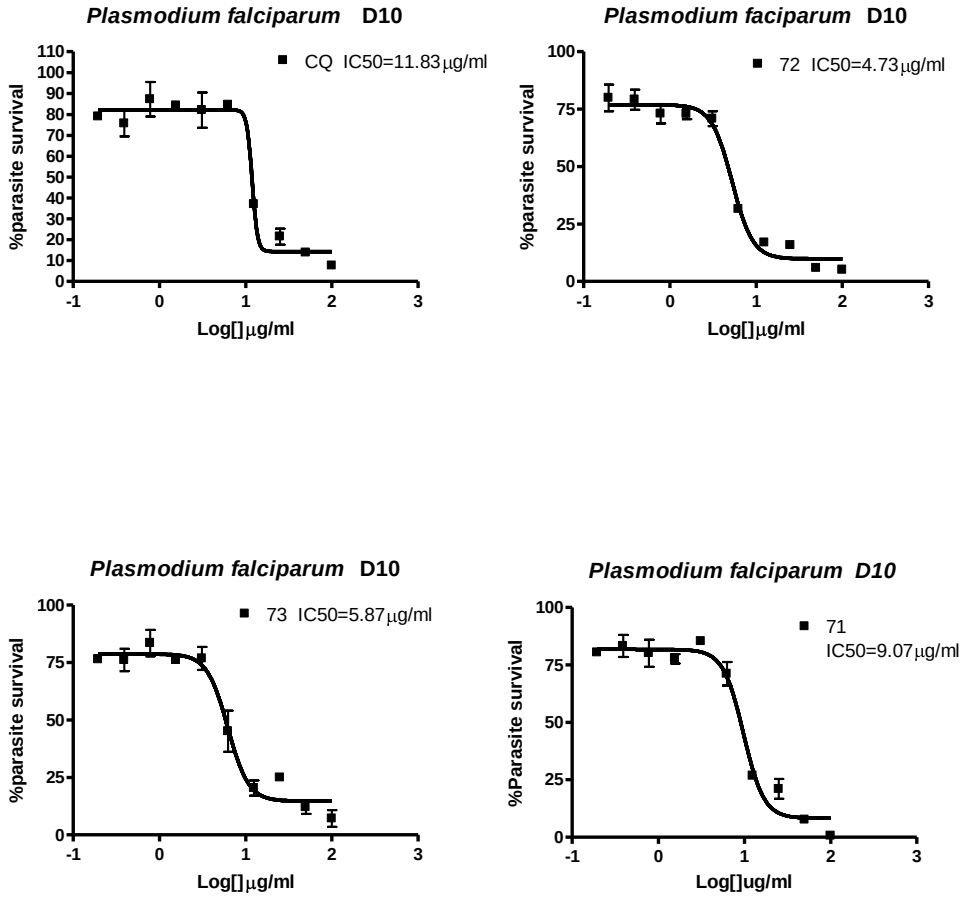


Fig A3 *In vitro* dose response curves of extracts from *C.citratus*

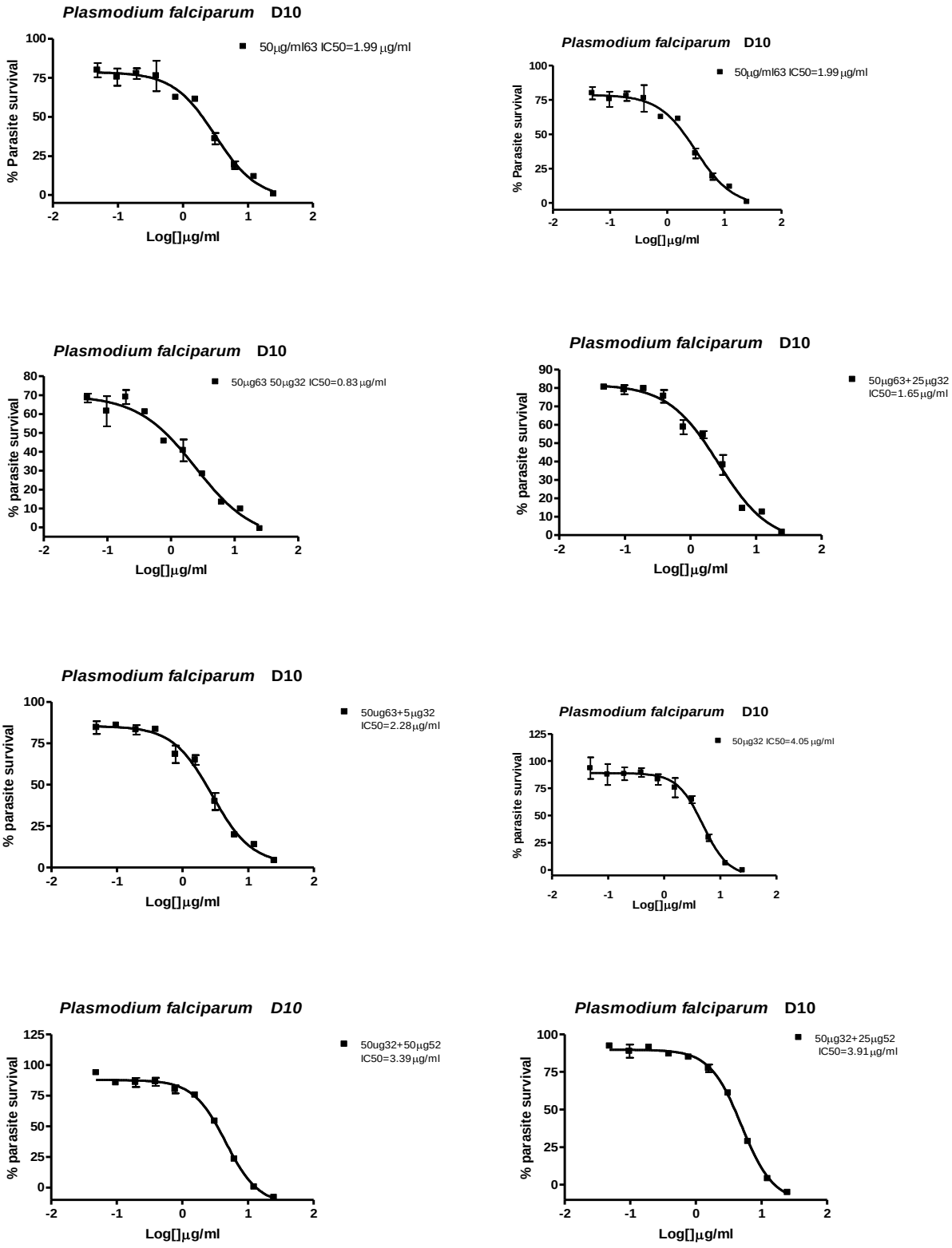


Fig A4 *In vitro* dose response curves of extracts in combination against *P. falciparum*

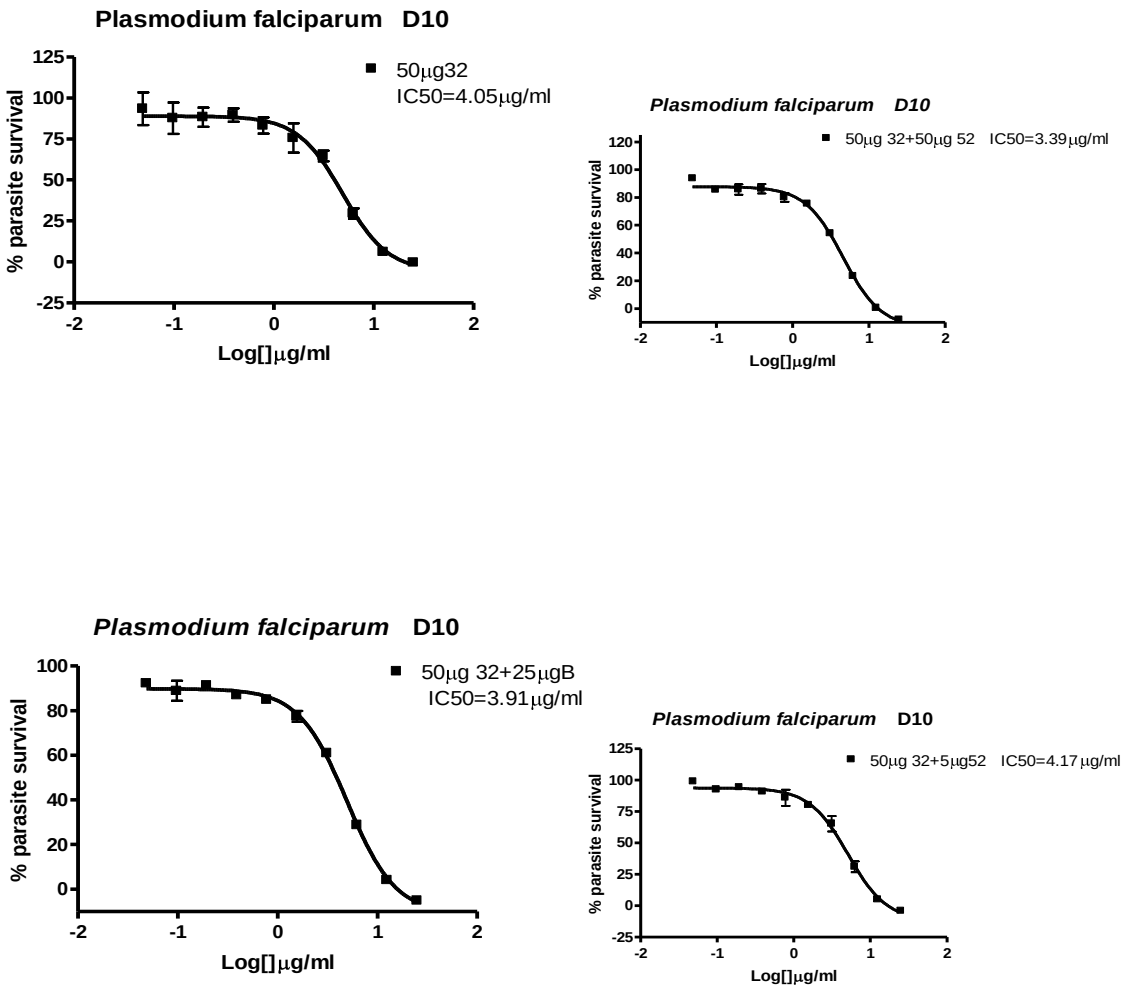


Fig A5 *In vitro* dose response curves of *C. limon* in combination with *P. guajava*

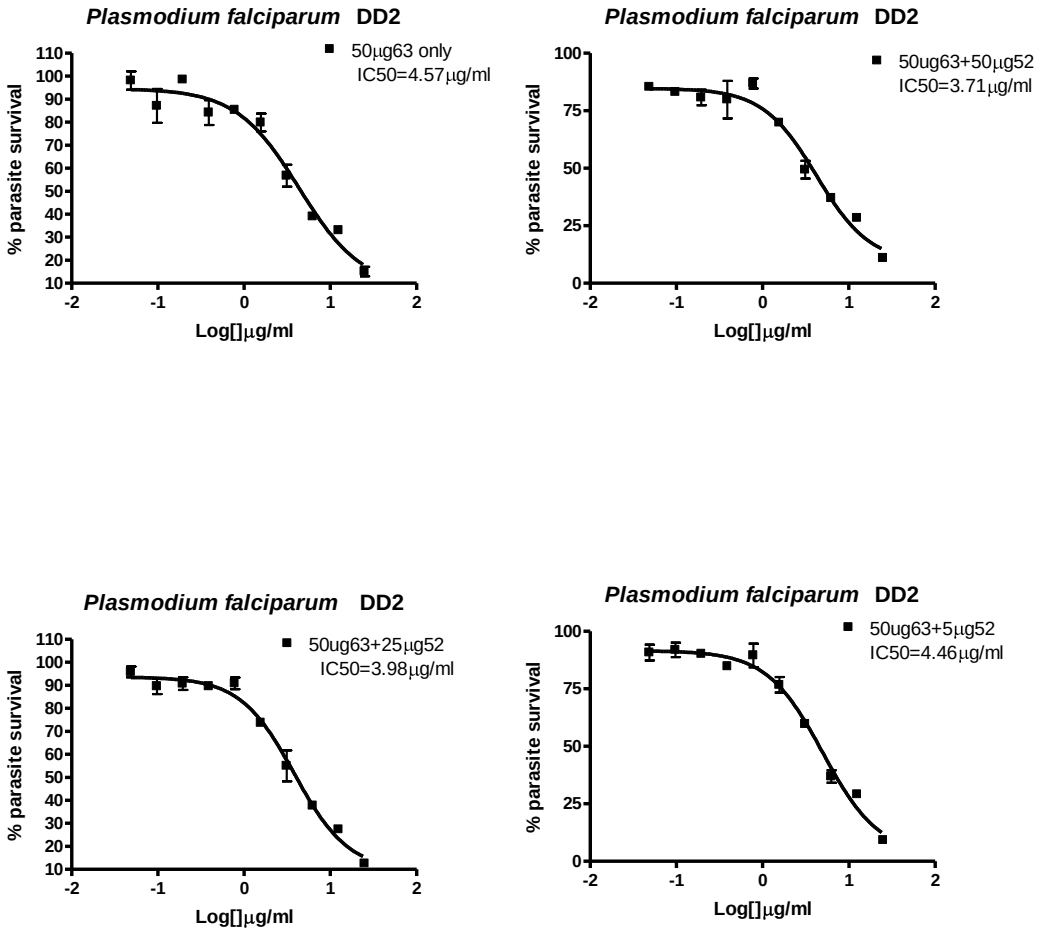


Fig A6 *In vitro* dose response curves of *C.papaya* in combination with *P. guajava*

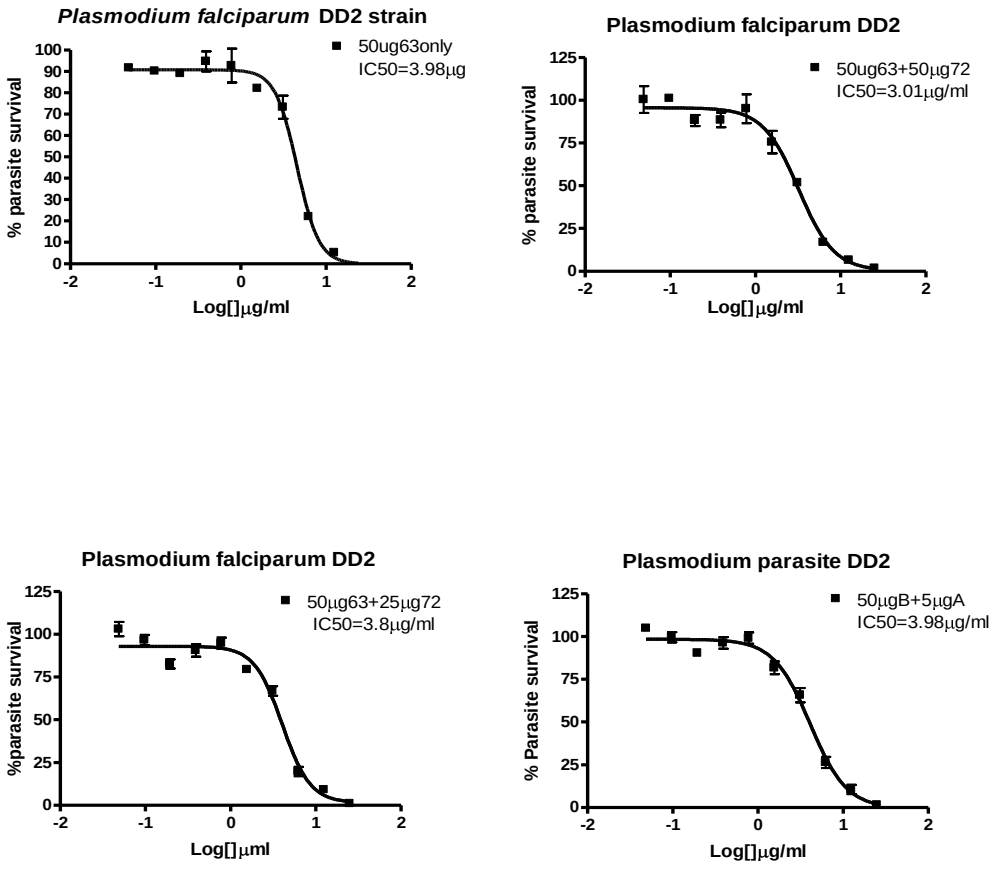


Fig A7 *In vitro* dose response curves of *C. papaya* in combination with *C. citratus*

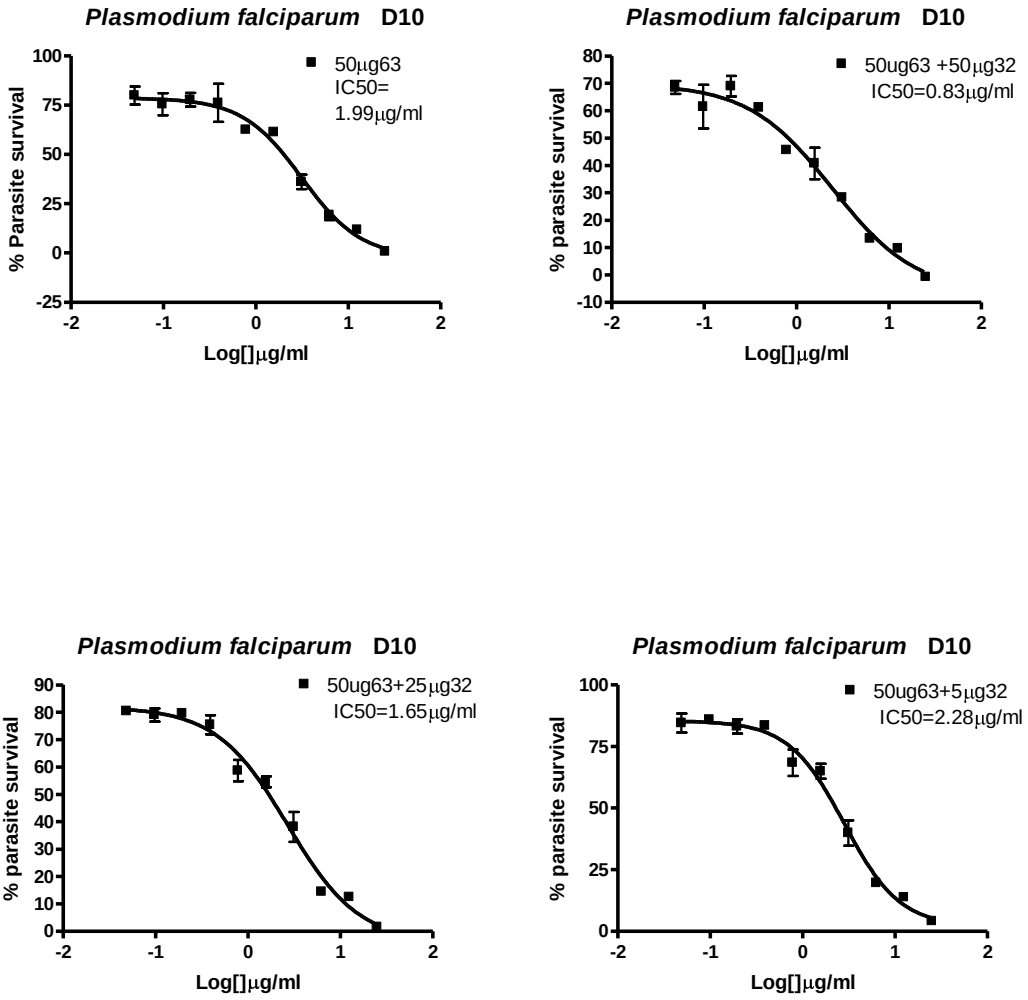


Fig A8 *In vitro* dose response curves of *C. papaya* in combination with *C.limon*

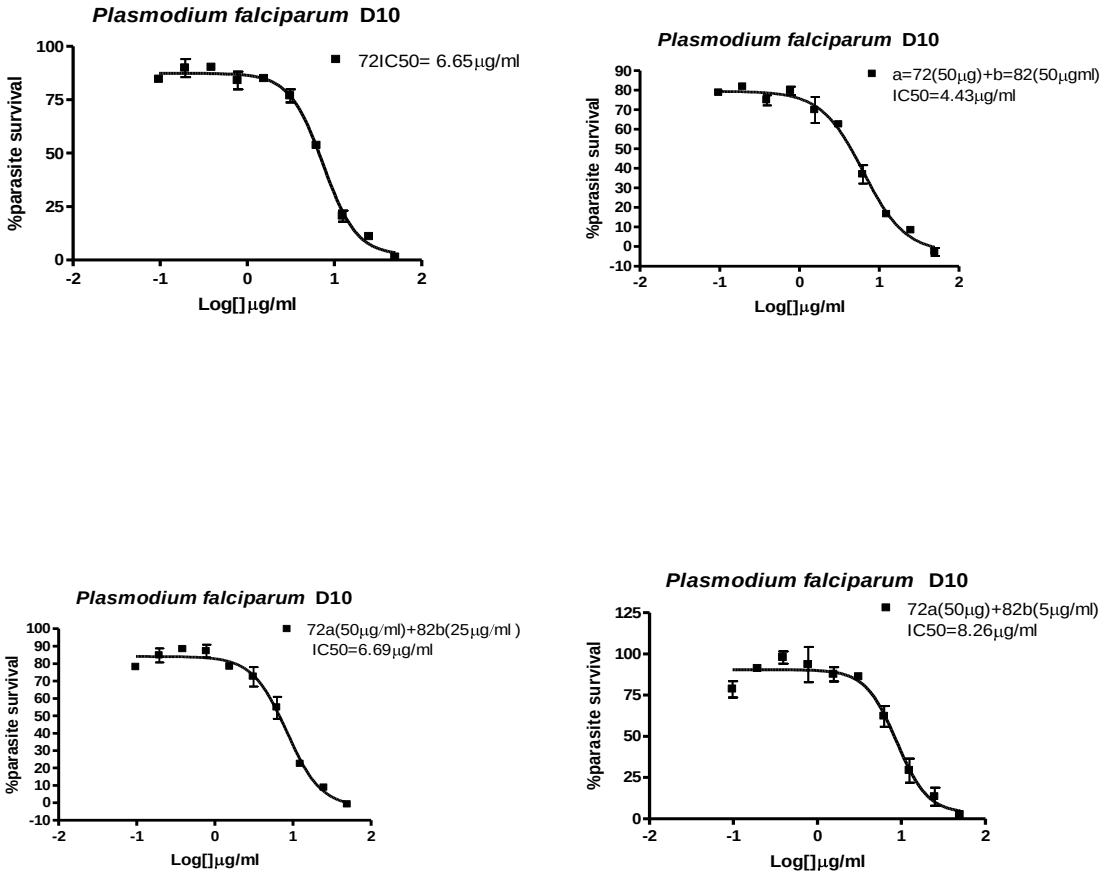


Fig A9 *In vitro* dose response curves of *C. citratus* in combination with *V. amygdalina*

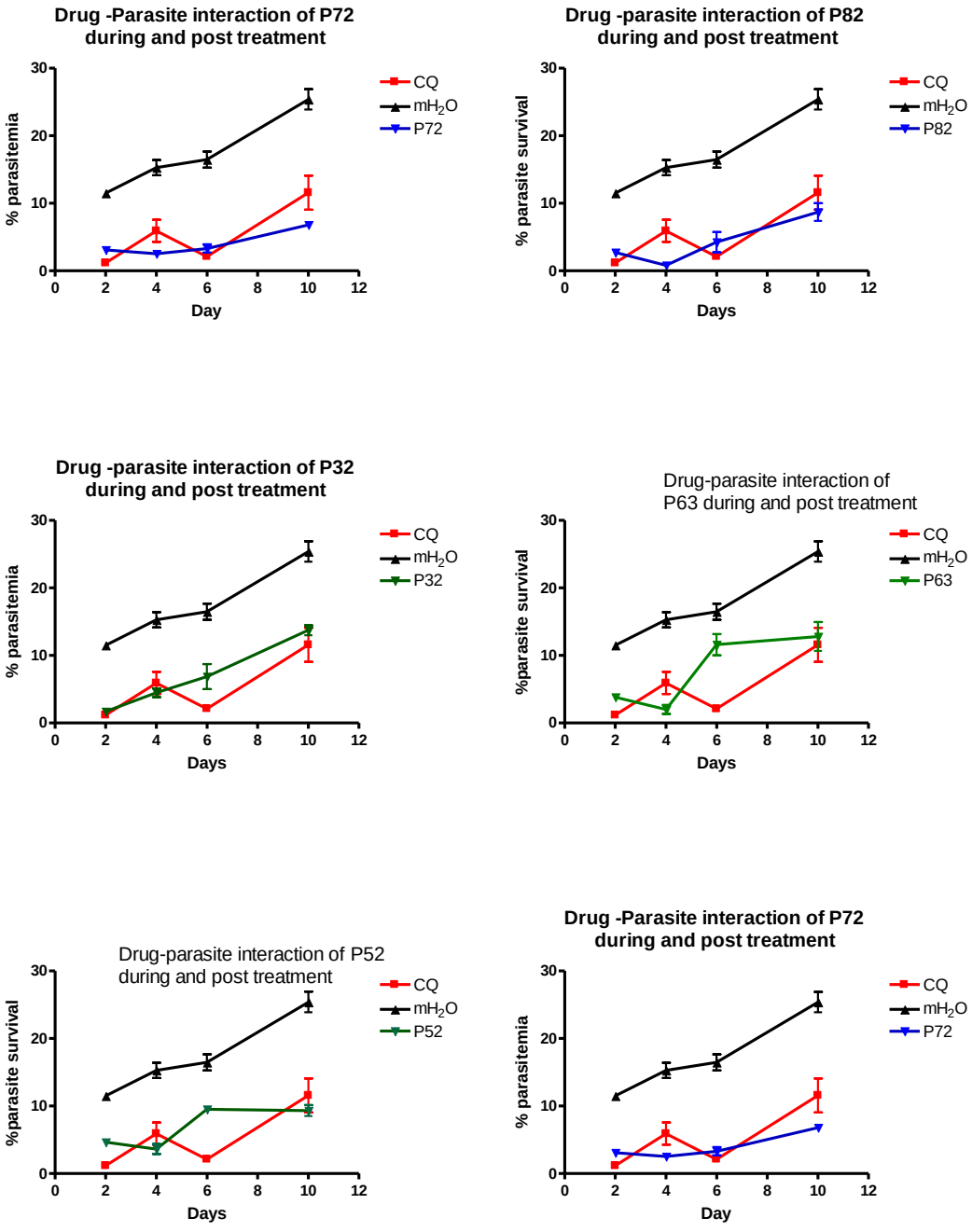
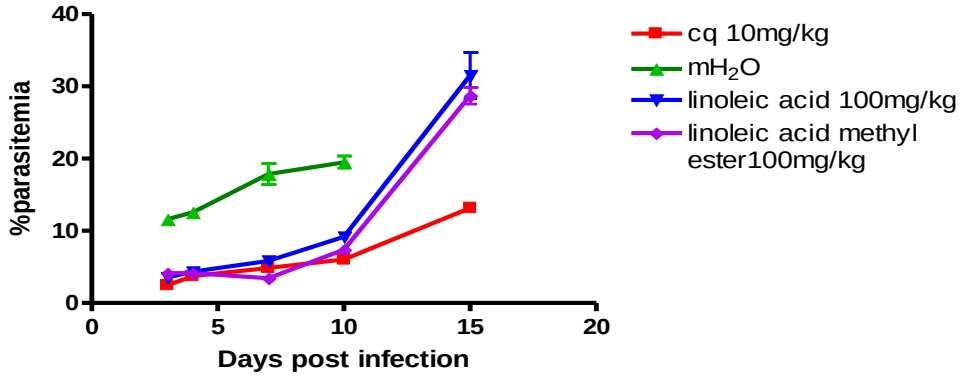
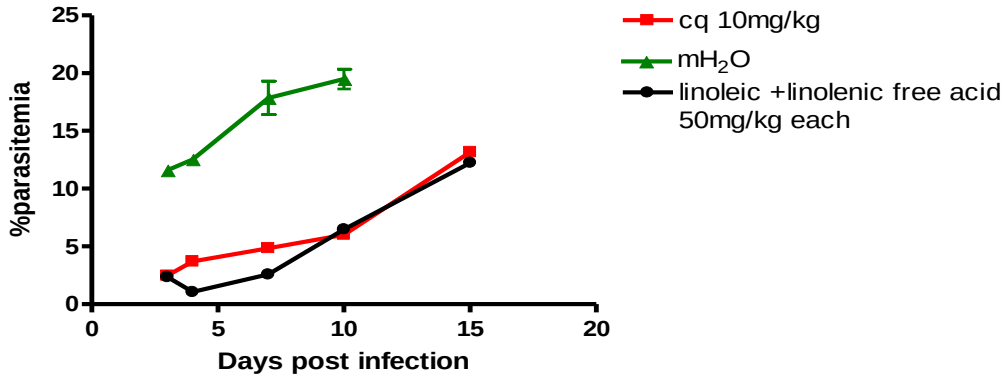


Fig A10 *In vivo* experiments with extracts singly

In vivo* experiment with *Plasmodium berghei



In vivo* experiment with *Plasmodium berghei



In vivo* experiment with *Plasmodium berghei

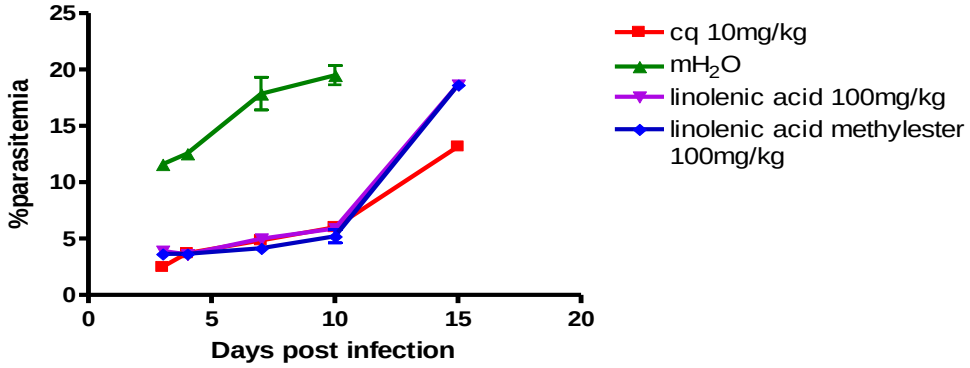


Fig A11 *In vivo* experiments with linolenic and linoleic acids

Appendix II

GC-MS spectrometry data of compounds 1 and 2

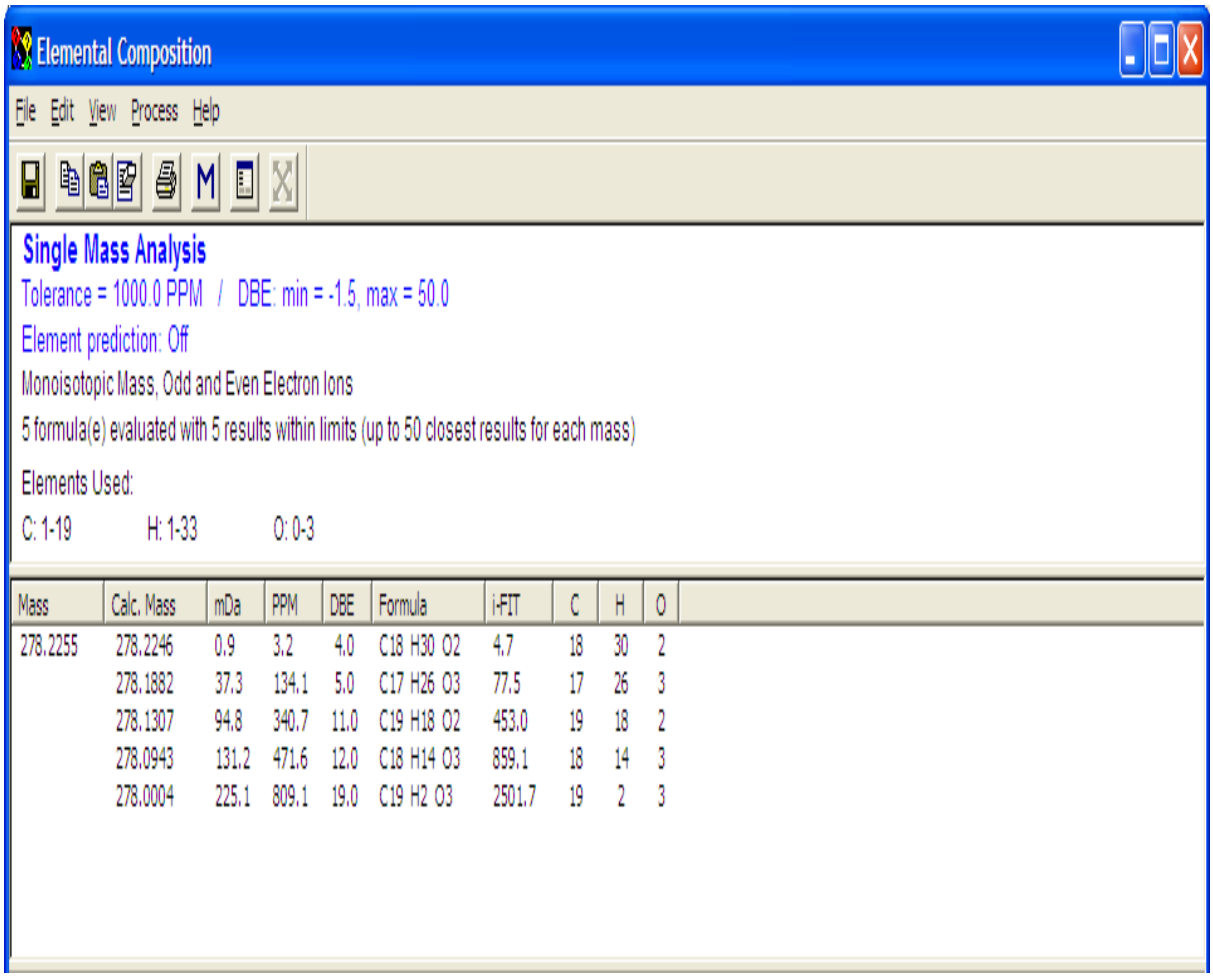


Fig A12 Compound 1 Single mass analysis

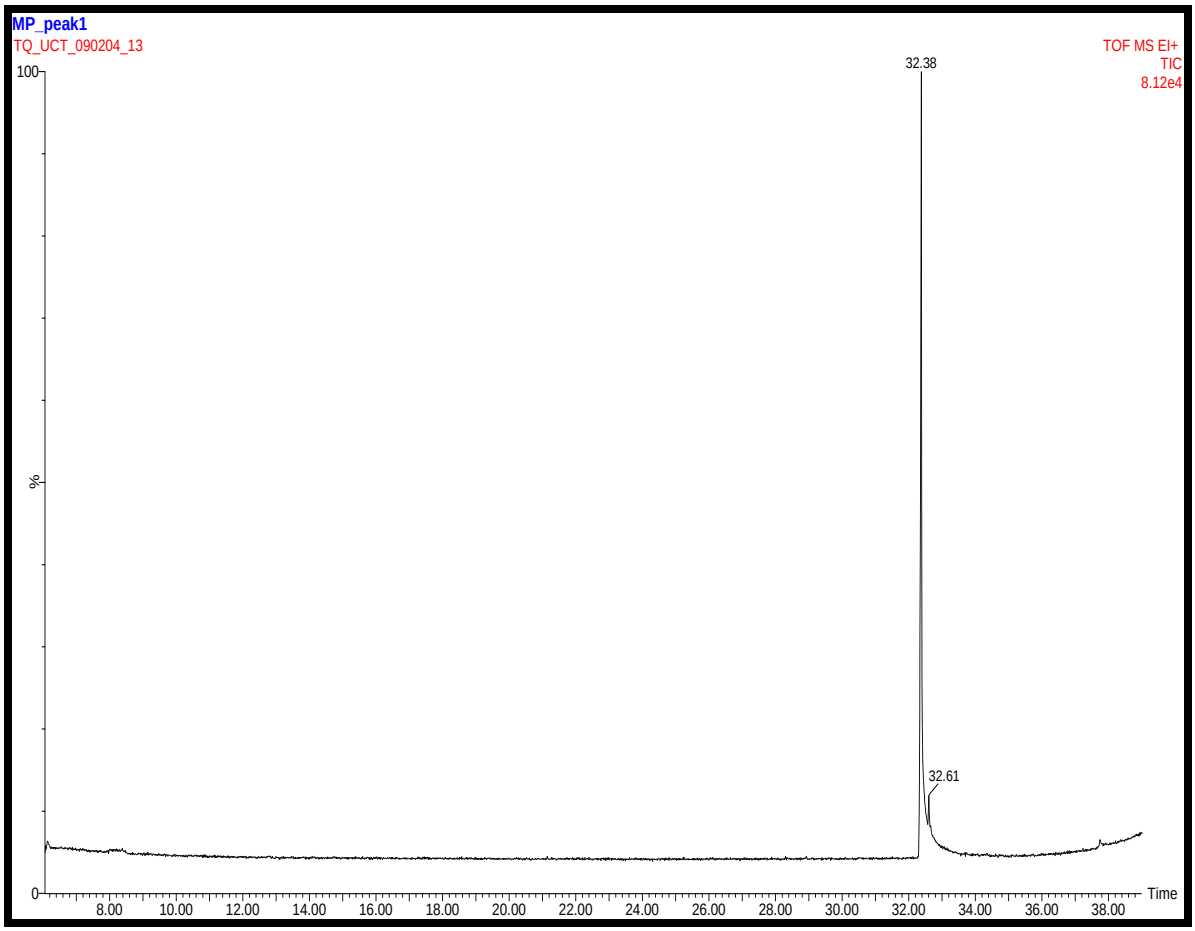


Fig A13 TIC of compound 1

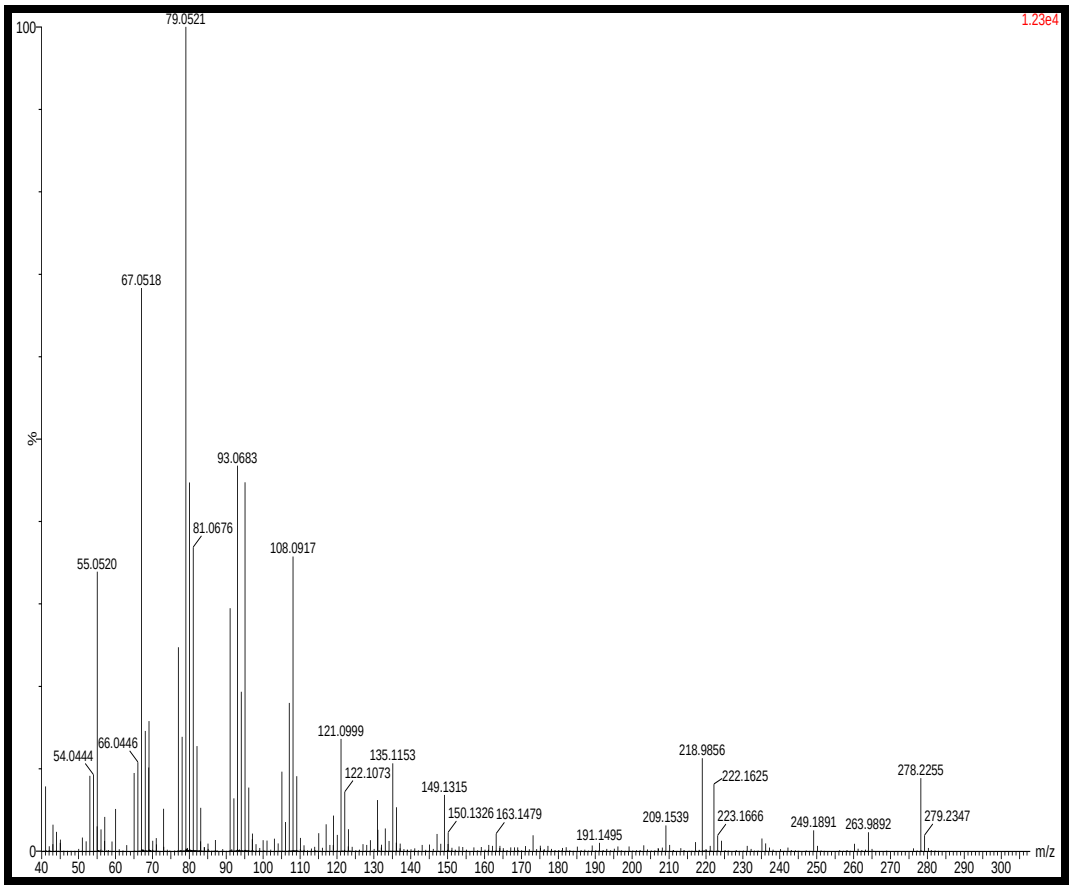


Fig A14 GC-MS spectrum of compound 1

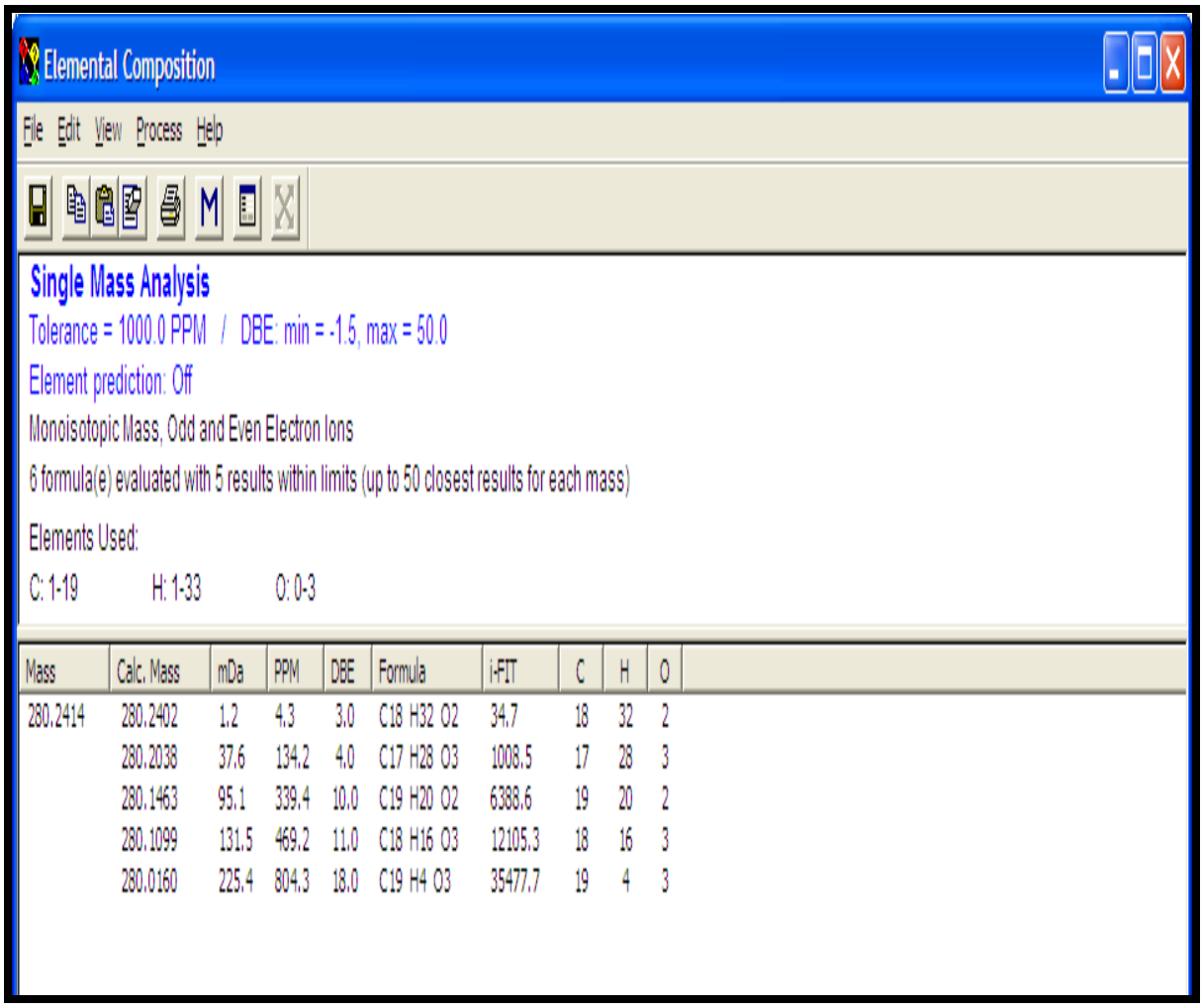


Fig A15 Compound 2 GC-MS single mass analysis

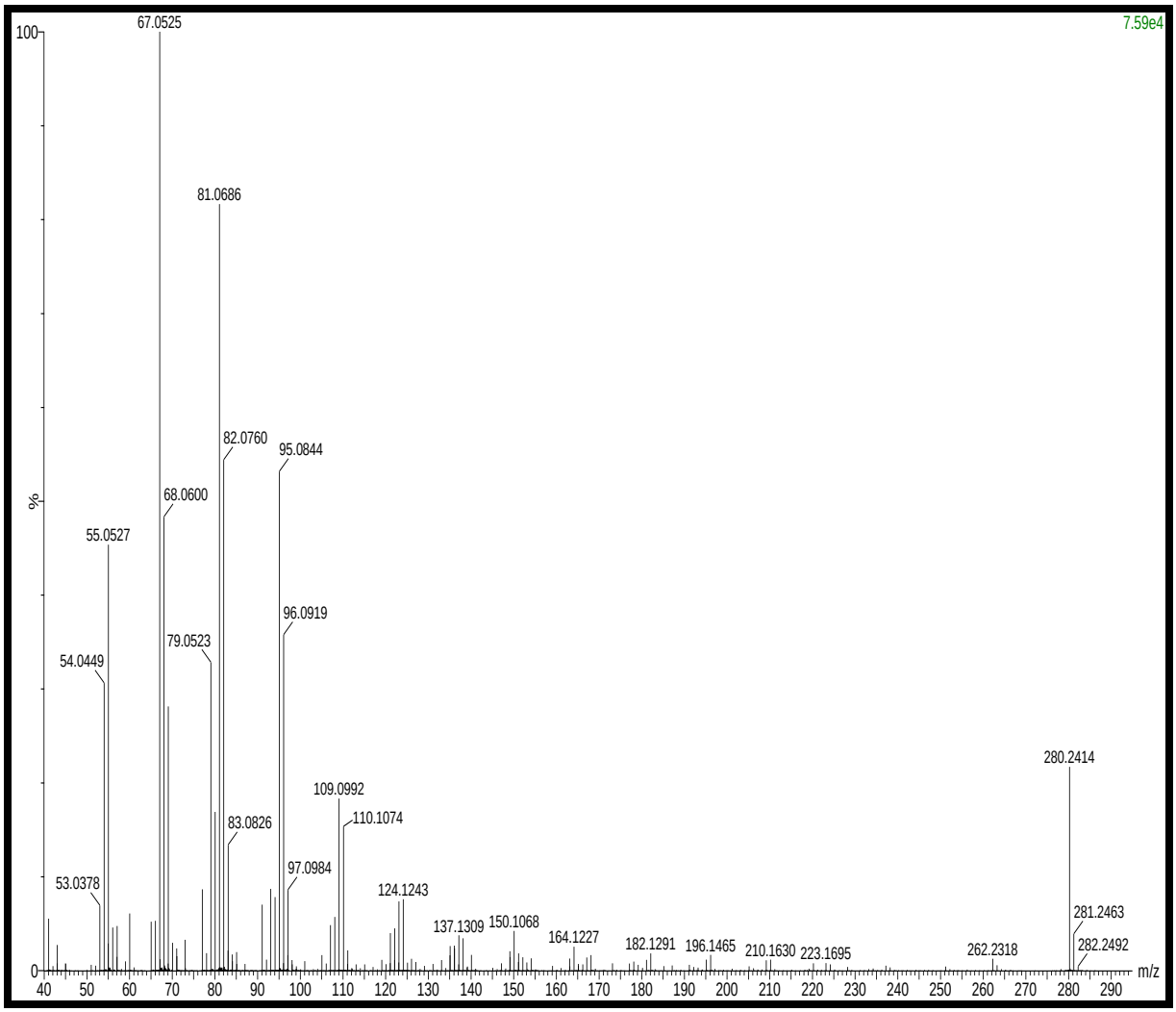


Fig A16 Compound 2 GC-MS product ion spectrum

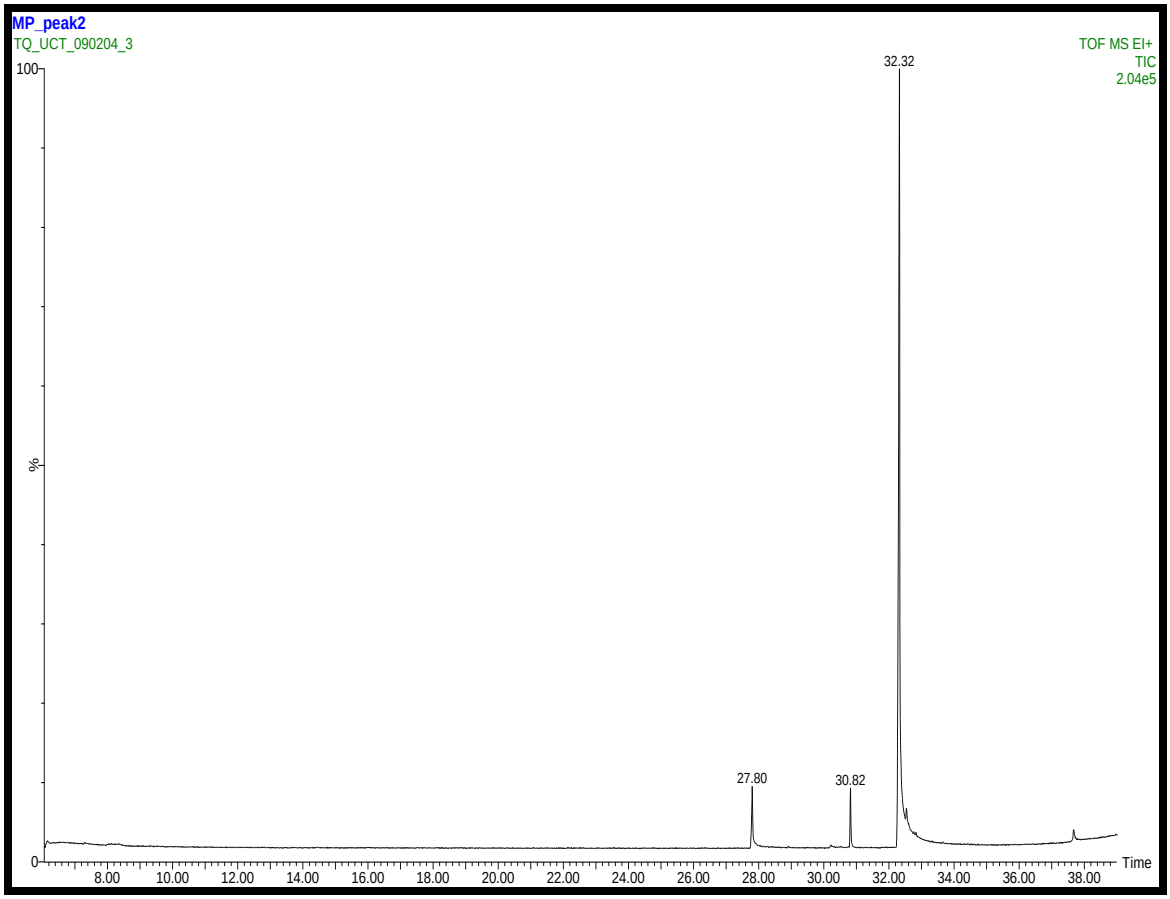


Fig A17 TIC of compound 2

Appendix III

NMR spectra of compounds 1 and 2

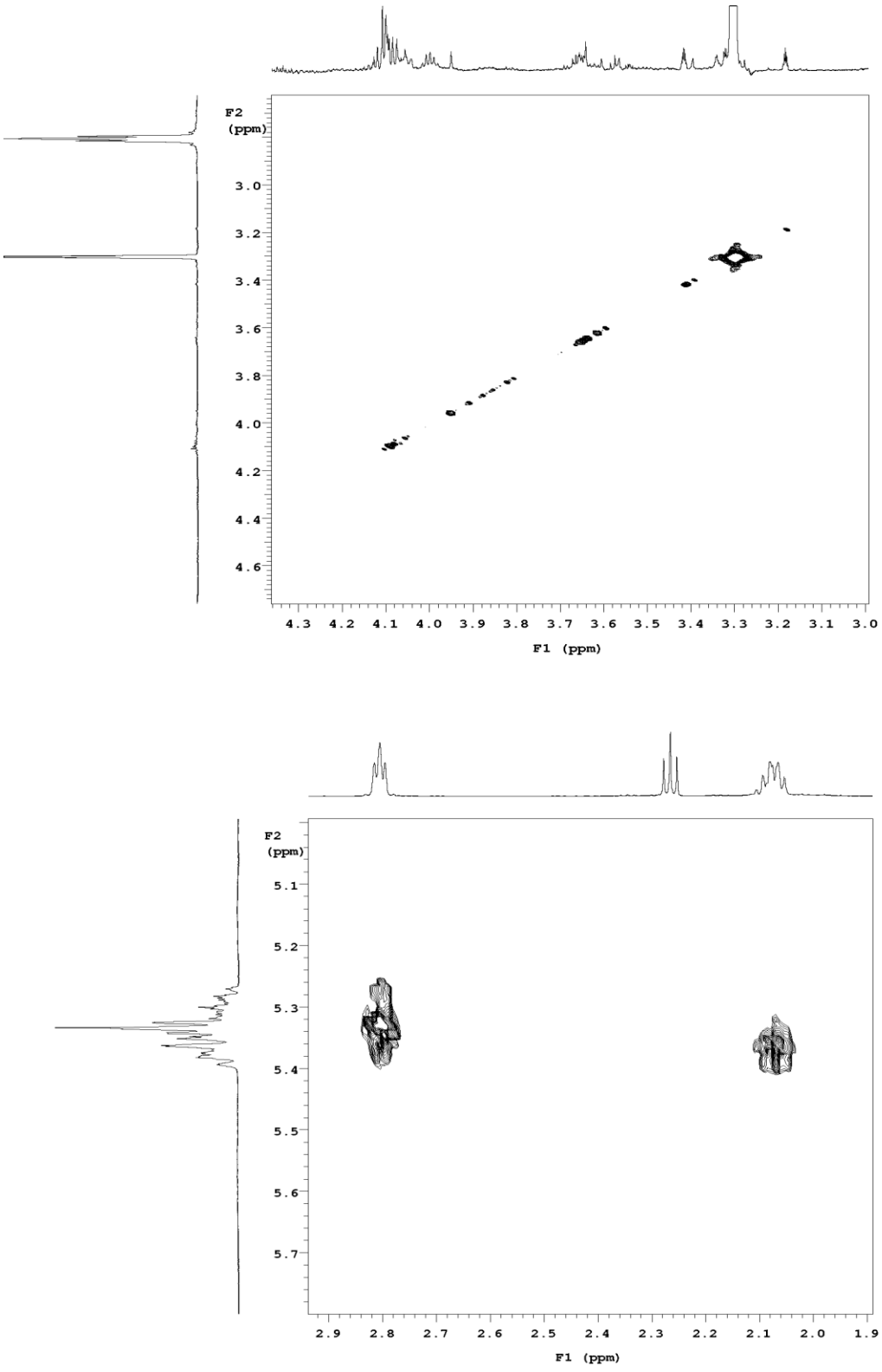
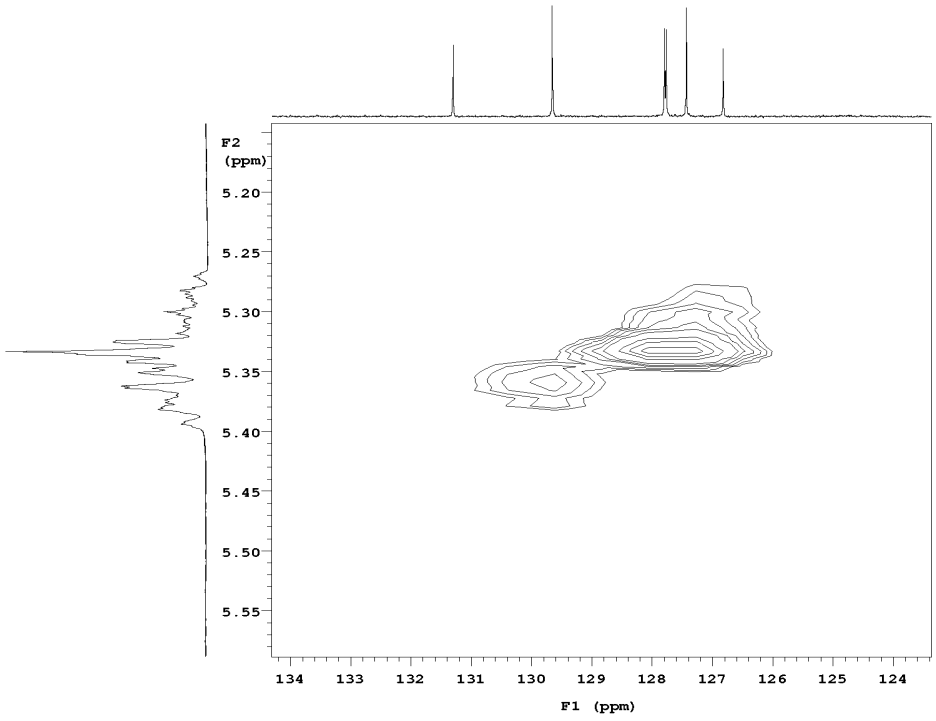


Fig A18 Representatives of gCOSY plots of compound 1



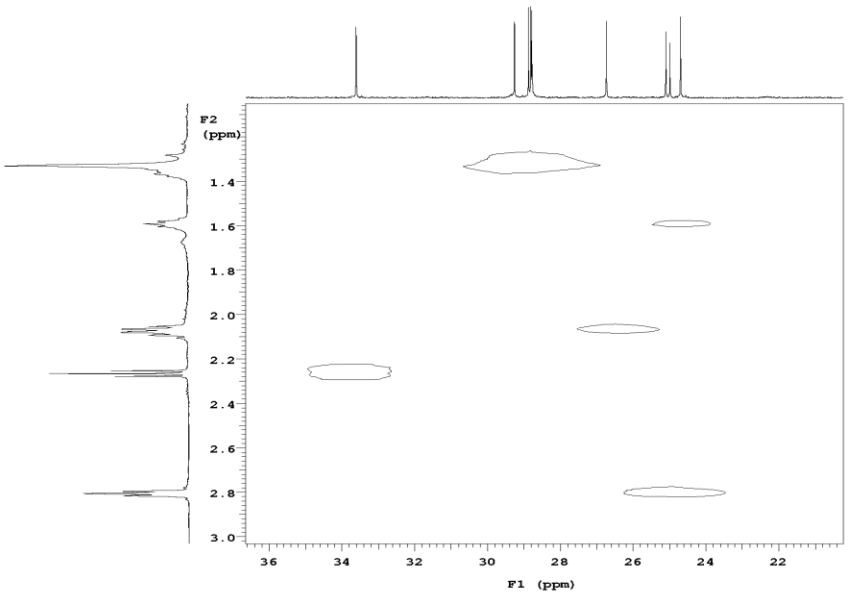
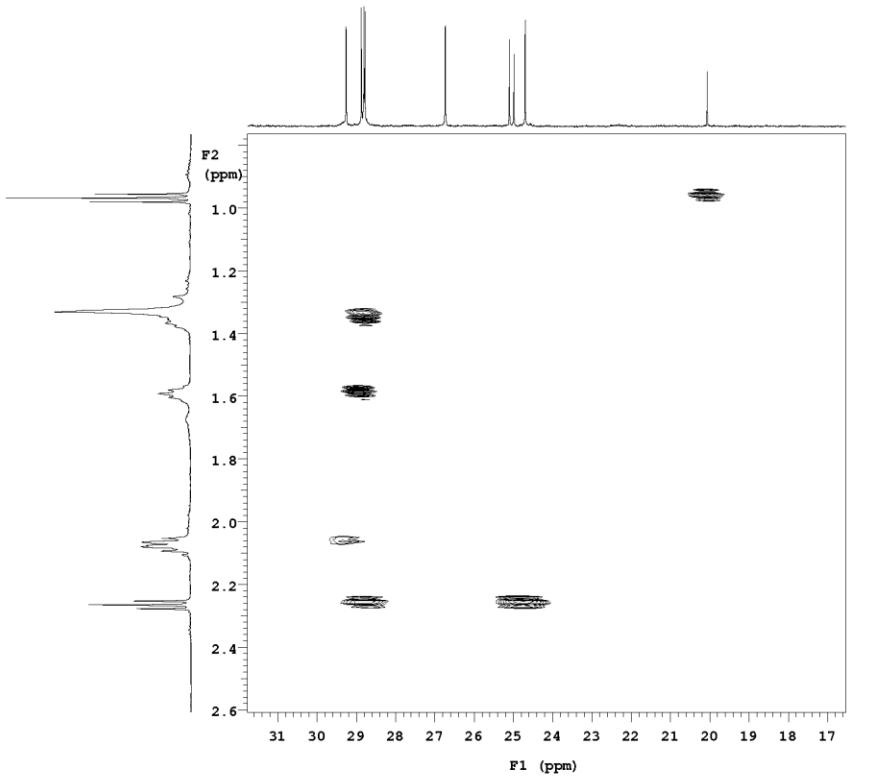


Fig A19 Representatives of gHSQC plots of compound 1



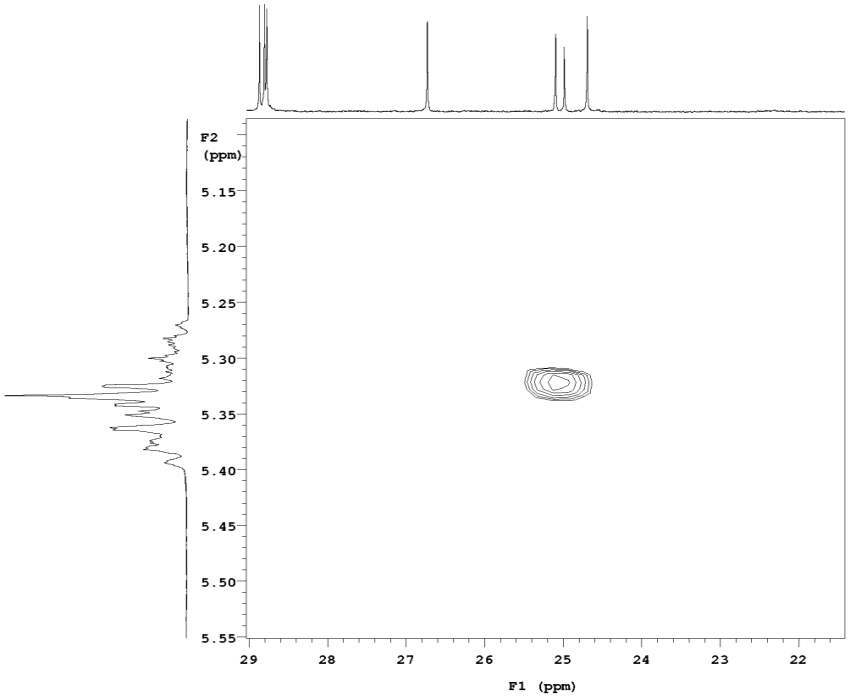
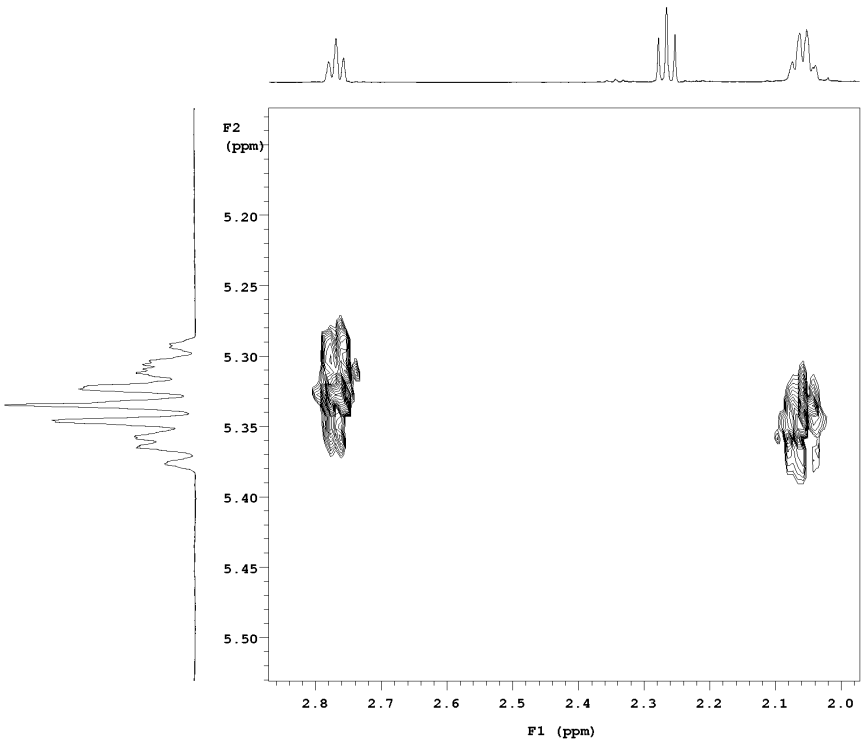


Fig 20 Representatives of gHMBC plots of compound 1



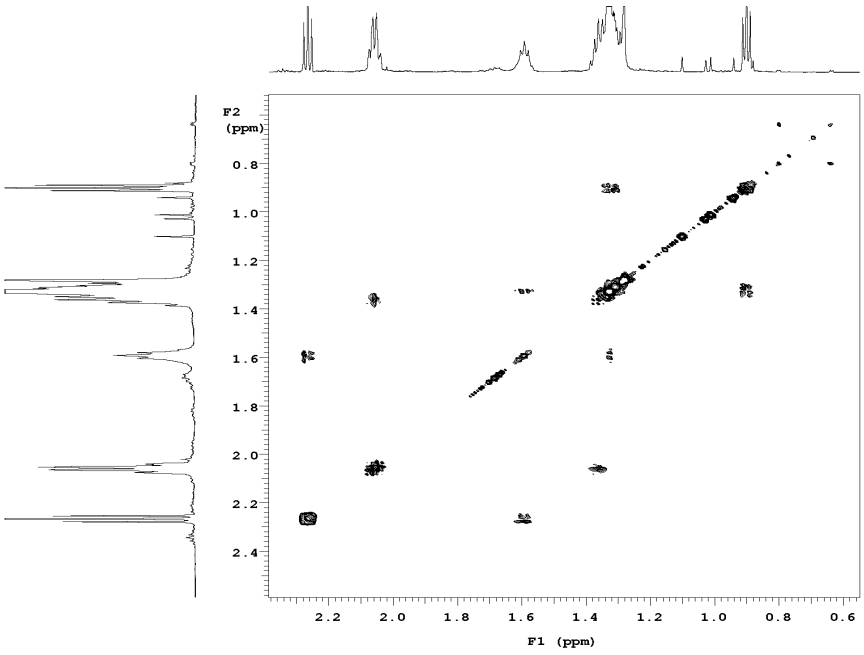
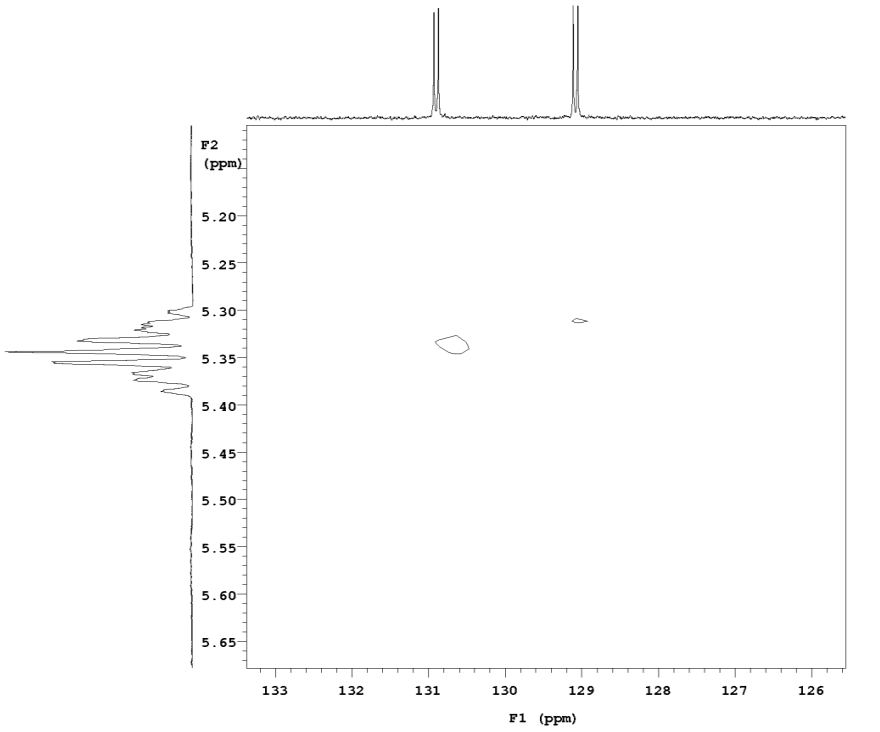


Fig A21 Representatives of gCOSY plots of compound 2



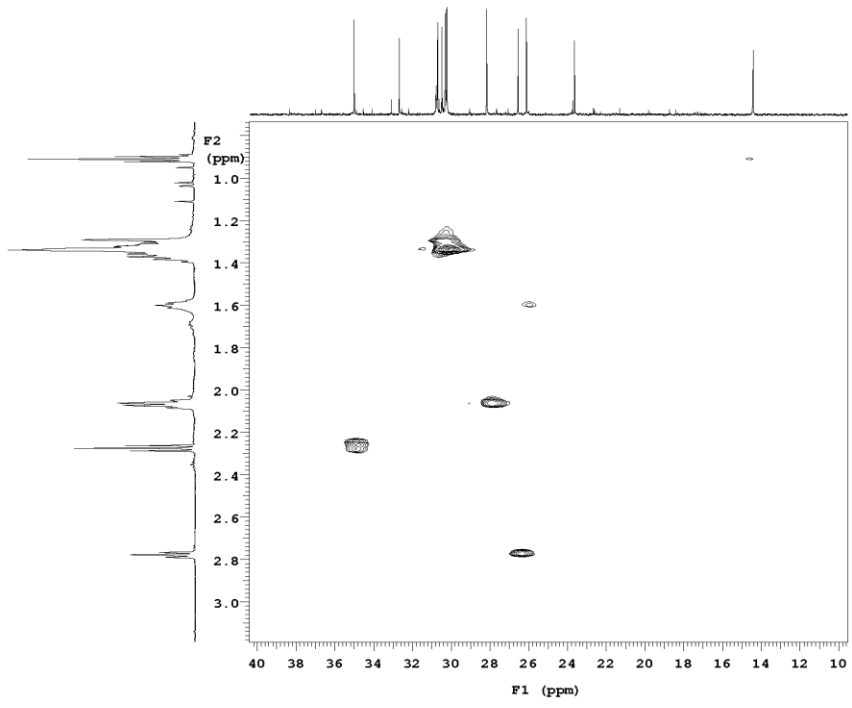
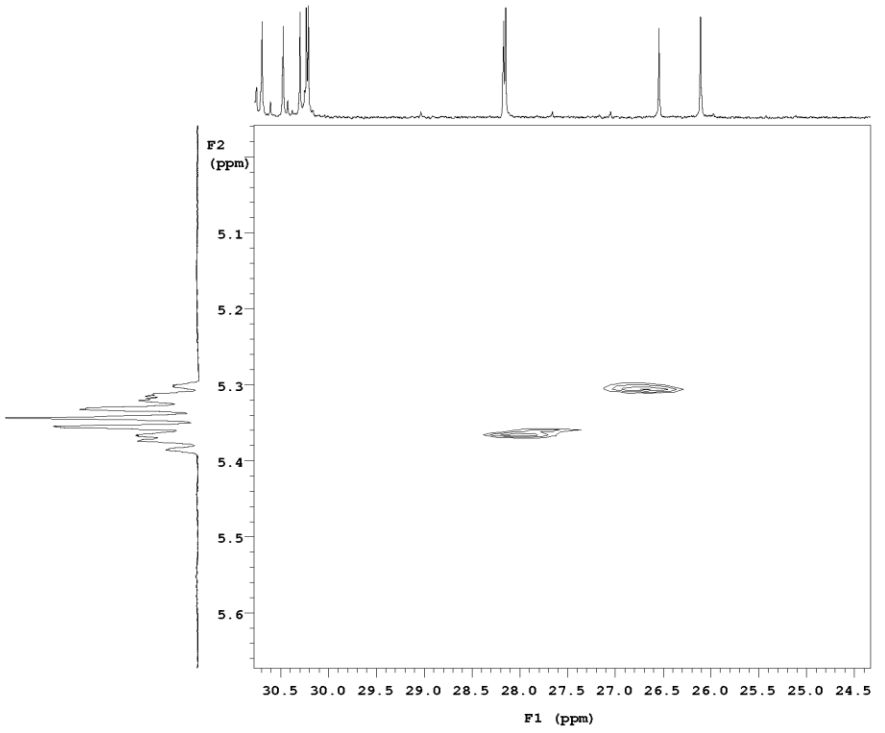


Fig A22 Representatives of gHSQC plots of compound 2



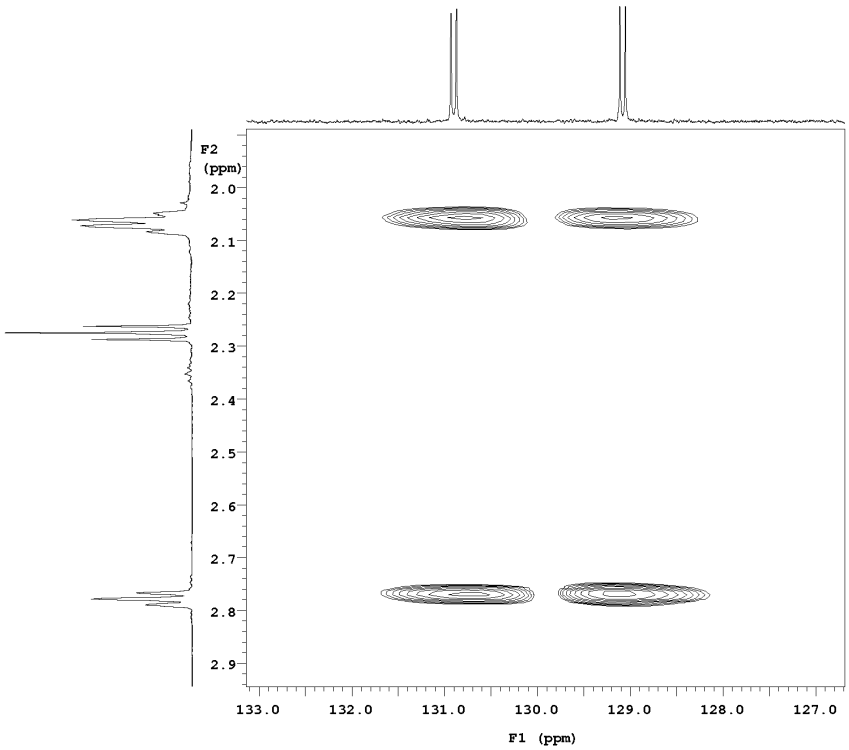


Fig A23 Representatives of gHMQC plots of compounds 2

Appendix IV
Mass spectrometry data of linolenic and linoleic methyl esters

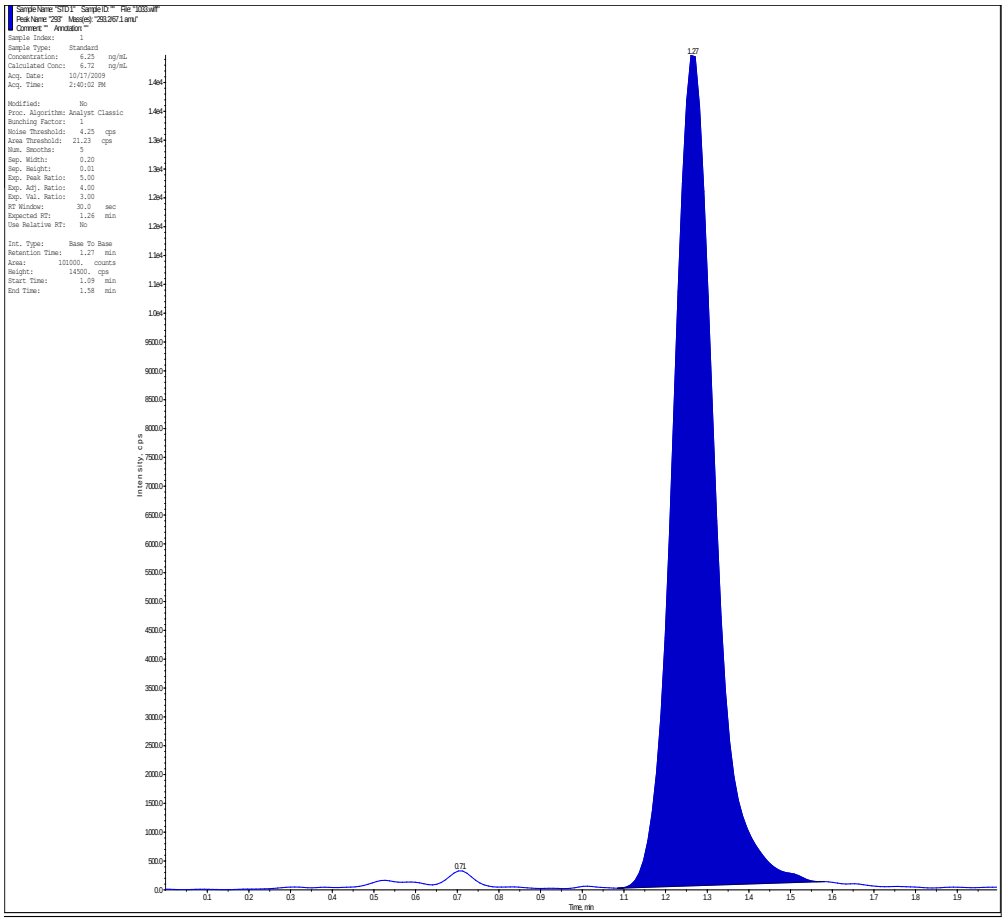


Fig A24 Concentration of standard 1 of linolenic acid methyl ester at 6.25μg/ml

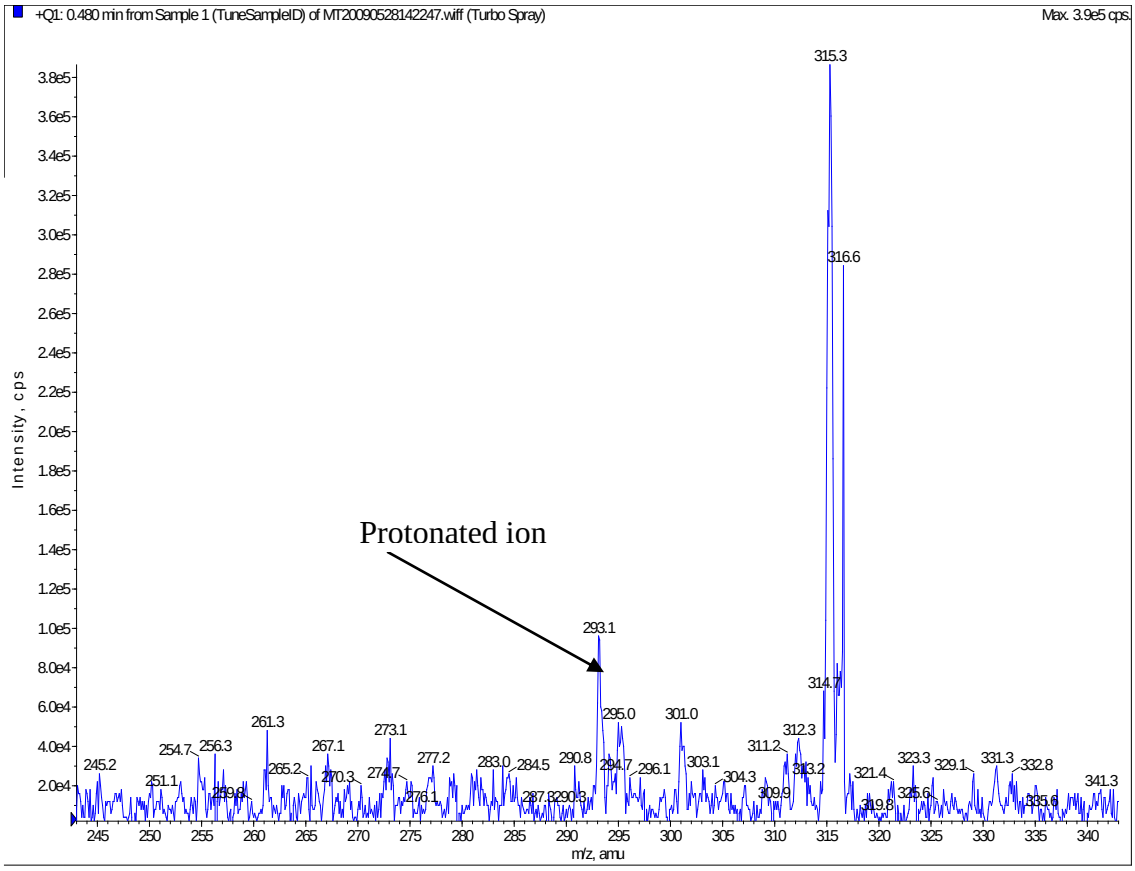


Fig A25 Q1 mass spectrum of linolenic acid methyl ester showing protonated ion

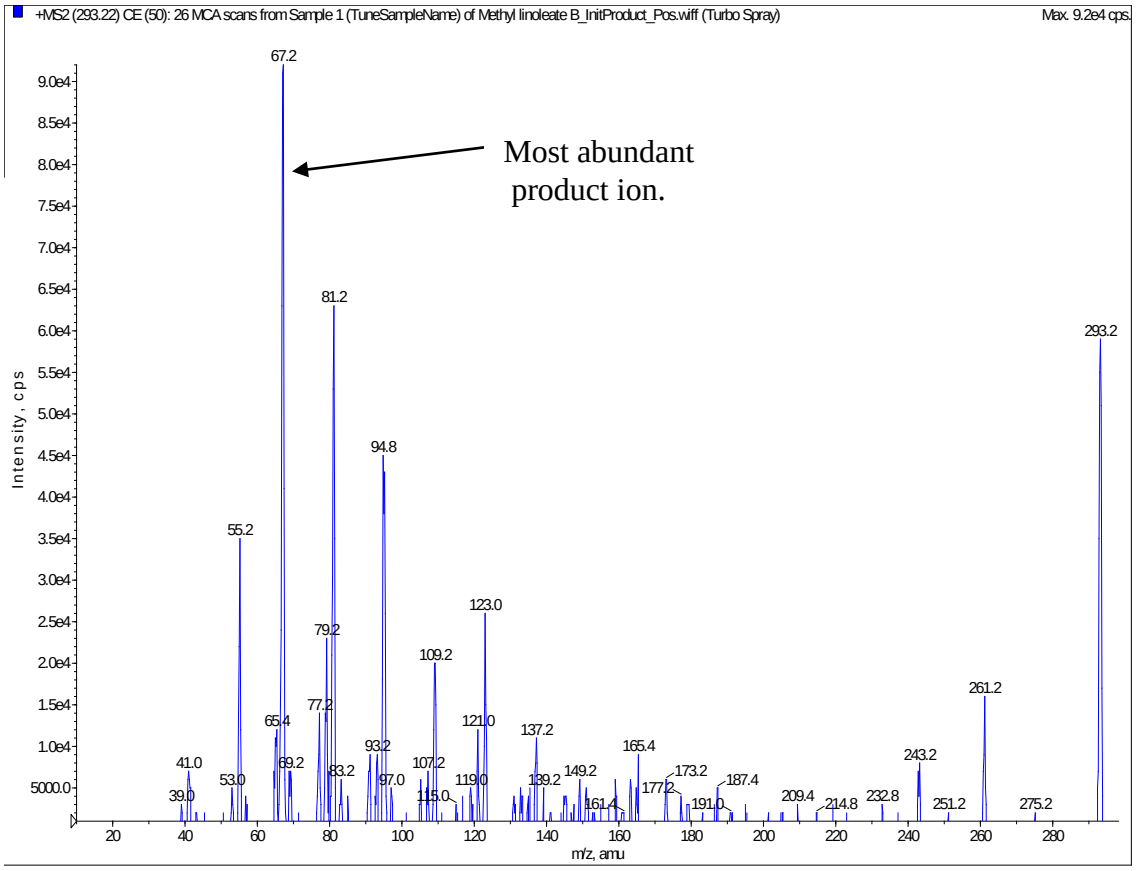


Fig A26: Product ion mass spectrum of linolenic acid methyl ester showing the most abundant product ion

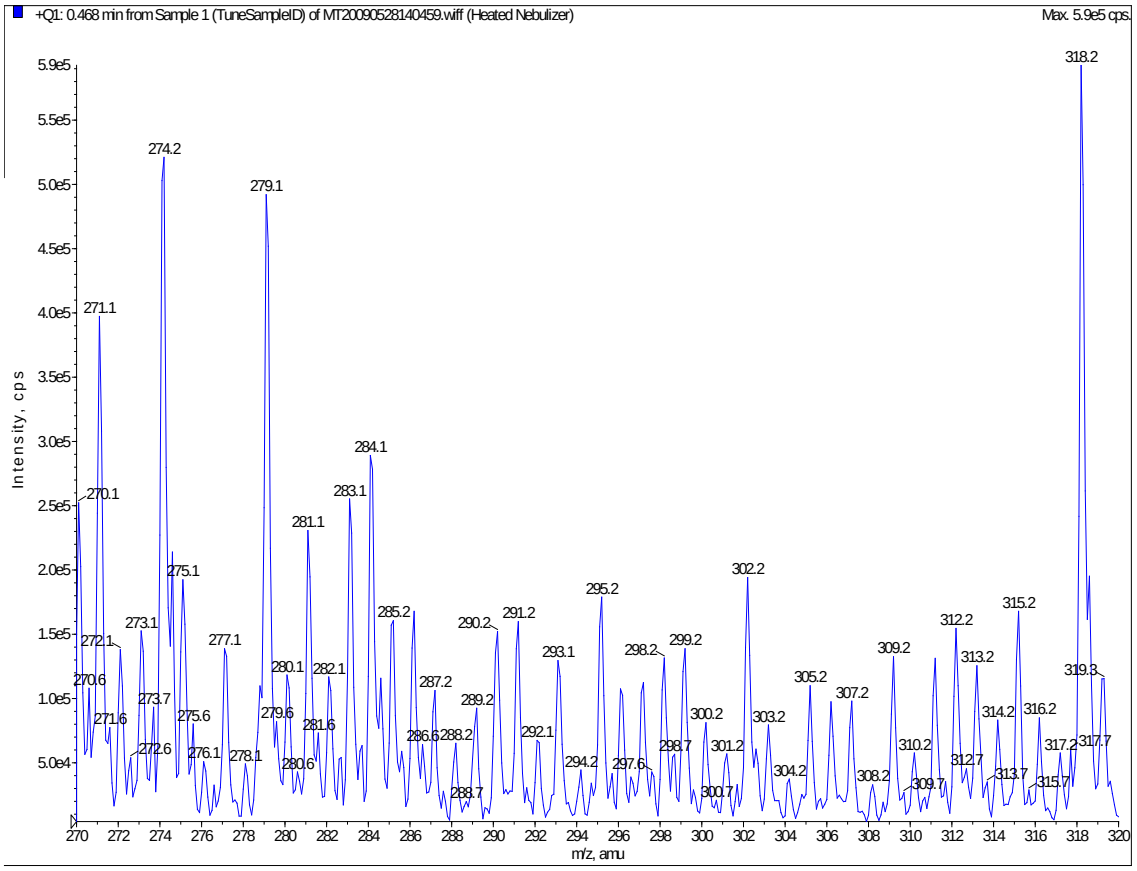


Fig A27 Mass spectrum of linoleic acid methyl ester showing the product ion

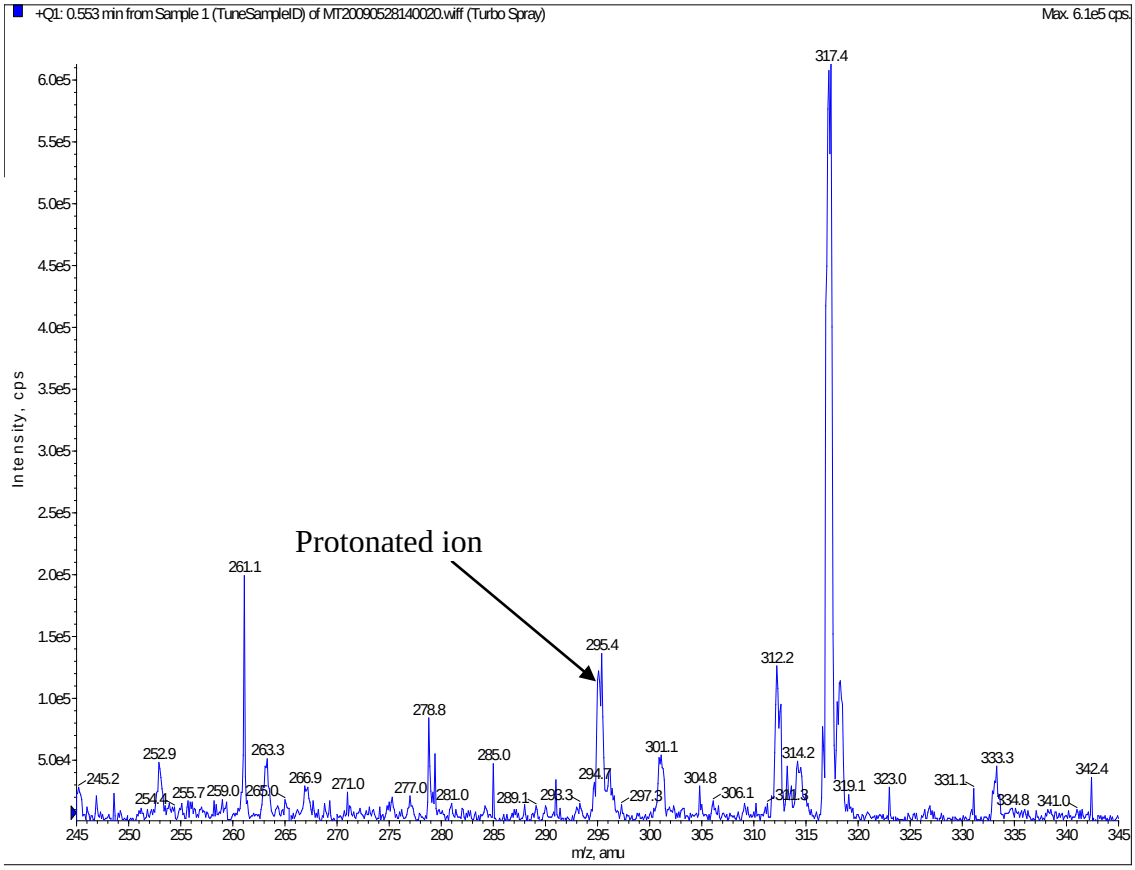


Fig A28 Q1 mass spectrum of linoleic acid methyl ester showing protonated ion at *m/z* 295.4 and the sodium adduct at *m/z* 317

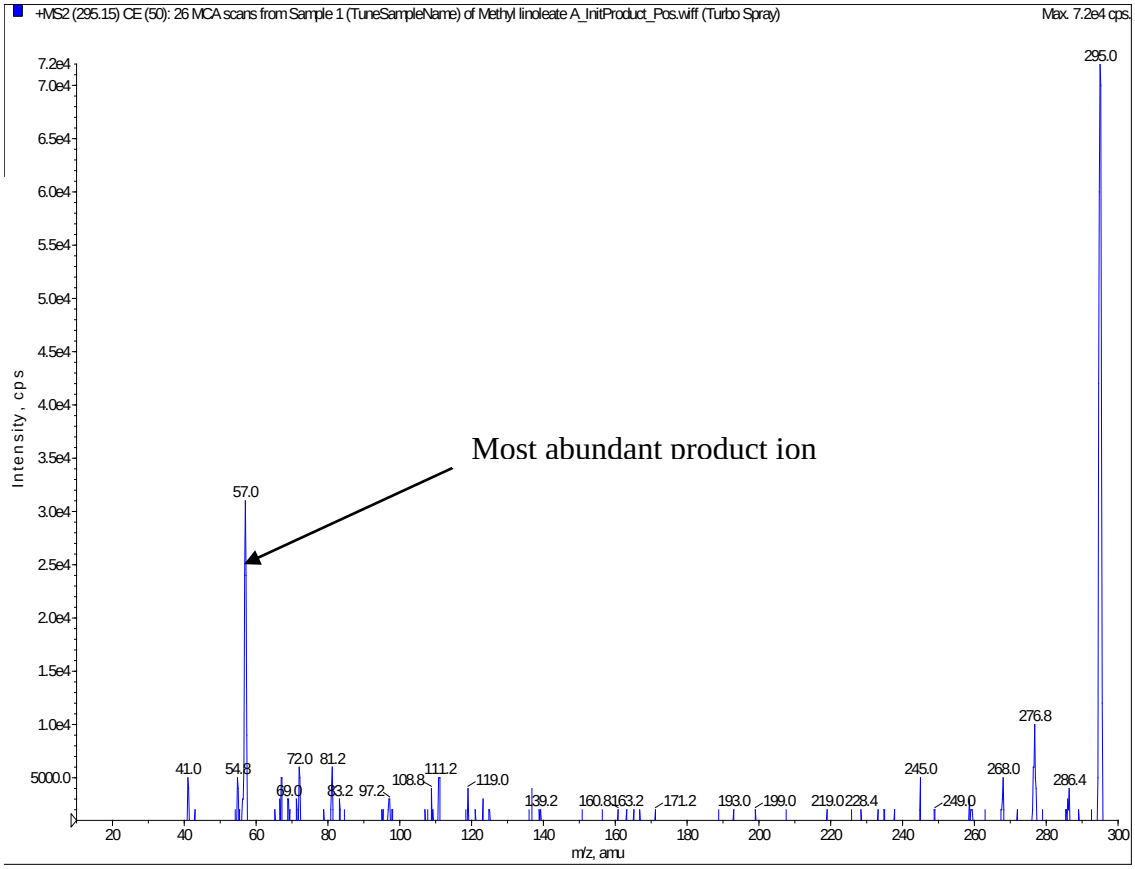


Fig A29: Product ion mass spectrum of linoleic acid methyl ester showing the most abundant product ion at *m/z* 57.0