

**Investigating Ethnopharmacology-based Natural Product
leads for Antimalarial Drug Discovery**

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DECLARATION

I certify that this thesis, which I submit to the University of Salford a partial fulfilment of the requirements for a Degree of Doctor of Philosophy, is a presentation of my own research work. Wherever contributions of others are involved, every effort is made to indicate this clearly with due reference to the literature and acknowledgement of collaborative research and discussions. The content of this thesis has not been submitted for a higher degree at this or any other university.

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ABBREVIATIONS

ACT	Artemisinin combination therapy
AMA-1	Apical antigen-1
<i>B. ferruginea</i>	<i>Bridelia ferruginea</i>
<i>B. Coccineus</i>	<i>Bryocarpus coccineus</i>
CAM	Complementary and alternative medicine
CDC	Centre for disease control
CQ	Chloroquine
DDT	Dichlor-diphenyl-trichloroethane
DHFR	Dihydrofolate reductase
DHA	Dihydroartemisinin
DHPRS	Dihydropteroate synthase
EC ₅₀	Half maximal effective concentration
FDA	Food and drug administration (USA)
GNI	Gross national income
HPLC	High performance liquid chromatography
HTS	High thoupout screening
EMEA	European Medicines Agency
IC ₅₀	Half maximal inhibitory concentration
IL ₁₀	Interleukin-10
ITN	Insecticide treated nets
KAHP	Knob-associated histidine rich proteins
MDG	Millenium development goals
MSP	Merozoite surface proteins

NGO	Non governmental organisation
NIH	National institute of health
NIPRD	National Institute for Pharmaceutical Research & Development
NMEs	New molecular entities
TLC	Thin layer chromatography
TNF- α	Tumour necrosis factor
TMP	Traditional medicine practice
WHO	World health organisation

Abstract

A collaborative study between the University of Salford and the National Institute of Pharmaceutical Research and Development (NIPRD), Nigeria, involving *in vitro* tests at the University of Salford and the use of facilities at NIPRD for *in vivo* tests has led to the evaluation of a range of traditional “fever cures” for antimalarial efficacy. The main objective of this study is to identify a suitable antimalarial plant candidate towards the development of a phytopharmaceutical drug (a plant-based medicinal mixture/compound used in preventive or therapeutic medicine) for the treatment of malaria as a cheaper and more accessible alternative, inline with the WHO resolution (WHA 3049) urging nations to use their traditional systems of medicine as part of their healthcare systems.

Traditionally, the mainstay of the antimalarial drug discovery process has been natural products. Their use however diminished over the past few decades due to several advances in molecular targets and technical difficulties encountered in high-throughput screens of natural product leads. Natural products have played a very important role as a source of antimalarials (e.g. quinine and artemisinin derivatives). In this present study, fluorescent-based *in vitro* antimalarial assays including flow cytometry (FCM) and SYBR green microtitre assay (SG) were optimized to screen some aqueous plant extracts which were selected based on their ethnopharmacological usage. Giemsa light microscopy was used to validate the assays. *Plasmodium berghei malaria* animal model was also used to evaluate the anti-plasmodial activity of extracts *in vivo*. The results showed a strong antimalarial activity in all the six extracts. *Bryocarpus coccineus* and *Bridelia ferruginea* were chosen for further investigation due to their efficacy and the collaborative nature of the study. The IC₅₀ values obtained in the *in vitro* antimalarial studies in the region of 70 µg/ml and 15 µg/ml. *Bridelia ferruginea* aqueous and methanolic extract was compared to determine any differences in IC₅₀. In vitro comparison of the aqueous and methanolic extracts of the extract revealed an IC₅₀ value in the region of 25.69 µg/ml for the aqueous extract and 15-16 µg/ml for the methanolic extract. Qualitative phytochemical screening of both extracts revealed the presence of various bioactive compounds including tannins, flavonoids, saponins and cardiac glycosides amongst others in both the aqueous and methanolic extract of *B. ferruginea*. Anthocyanins were found present in the methanolic extract only. Further investigation of the mechanism of action of the methanolic *B. ferruginea* extract showed that the extract inhibited β-haematin formation, indicating the inhibition of haemazoin formation in the parasite. Lastly, the methanolic extract was fractionated using HPLC analysis. Various resolved peaks were obtained and subsequent bioassays of the collected fractions revealed that antimalarial activity was distributed across

the fractions. This may suggest that isolating a single active compound might not be advantageous, making a case for phytopharmaceutical drugs.

CHAPTER 1

INTRODUCTION

1.1 History of malaria and epidemiology

Malaria has been known to man for thousands of years and may have been a human pathogen for the entire history of humanity (Joy *et al.*, 2003). The disease probably originated in Africa along with man. It is believed to be a zoonotic disease affecting primates. Early records dating back to the Xian dynasty in China (2700 B.C) have discussed characteristic periodic fevers and symptoms which we now know to be classical symptoms of the disease (Cox, 2002). The disease has affected the entire continents, except for Antarctica with historic literature mentioning the disease symptoms including during ‘Little Ice Age’. The disease was evident in many parts of Europe and referred to in some of Shakespeare’s plays (Reiter, 2000). In 400 B.C, early Greeks like Hippocrates described the disease manifestations and some scriptures associated the disease to supernatural powers. Recently, evidence of malaria has been found in the bone tissue of two Egyptian mummies dating back to 3500 years (Lalremruata *et al.*, 2013).

During the medieval Italian period (1476), Leonardo Bruno coined the name malaria from ‘mal’ air. During that period, the ancient romans believed that fumes of bad air came from swampy regions and this idea carried on till the 19th century (Opeskin, 2009). The disease continued to be prevalent in different parts of Europe, including the basin of central Switzerland, a breeding ground for mosquitoes (Bruce *et al.*, 1988). Malaria was evident in Munich in the 1940’s and continued to be a global health issue. Countries like Greece have a history of malaria dating back to the 4th century. By the 1950’s, the country was considered one of the worst hit by the disease outside of Sub-Saharan Africa. It took a tremendous improvement in healthcare together with rigorous public health measures, including clearing of swamp to eradicate the disease in 1974. Moving outside of Europe, Russia saw an epidemic that killed nearly 60, 000 people in 1980. It took vigilant public health campaigns and efforts for Europe and the United States to successfully eradicated the disease. These regions however still have the *Anopheles* mosquito species that can transmit the disease, hence, reintroduction of the disease still remains a risk. Recently in 2012, there have been reports that the disease might have found its way back into Europe with some cases of the

disease being recorded in Greece. Austerity induced budget cuts in the country have affected municipal insecticide spraying leaving unsprayed. Only 8 out of 56 municipalities near Athens were sprayed in 2012. This decline in the public health measure in place posed a risk to the region leading to 70 cases recorded. Although the vast majority of cases were imported from abroad, statistics show that up to 10 percent of cases were contracted within the country (Telegraph, UK).

Despite its elimination in Europe and the progress achieved in reducing morbidity and mortality, malaria still remains the leading cause of disease and death in many developing countries. About 3.3 billion people, half the world's population are at risk of malaria transmission in 106 countries and territories. According to the World Health Organization's World Malaria Report 2015, it is estimated that 214 million cases of malaria occurred worldwide leading to 438 000 death. Fifteen countries accounted for 80% of cases, and 78% of death. 89% of the global burden of mortality is borne by some countries in sub-Saharan Africa. The Democratic Republic of the Congo and Nigeria alone account for more than 35% of the estimated global malaria death whilst 10% cases occurred in South East Asia and 2% in the Mediterranean region. In 2015, the millennium development goal (MDG) target to halt and reverse malaria was achieved with a 50-75% reduction in malaria incidence in 18 countries. Death in children under 5 fell from 723, 000 cases in 2000 to 306, 000 in 2015. (WHO Report, 2015). Although there has been an overall decline in the incidence and mortality of the disease, the 2015 report also points out the fact that the rate of decline in cases has been most rapid in countries that reported the smallest amount of cases in 2000. In the same year, countries with a high morbidity and mortality showed a slower decline in incidence rate. Although not all countries have recorded a successful reduction in malaria infections, there has been a successful global decline in cases. Insufficient public health measures for malaria in some regions puts half the world's population at risk. In 2014 alone, 1586 cases were reported in the United Kingdom (UK) due to imported malaria most of which were due to travel to West African endemic areas (Public health England statistics, 2014). Hence reductions in incidence need to be greatly accelerated in those countries lagging behind if sustainable global progress is to be achieved.

The economic burden of malaria cannot be overestimated. Along with direct health costs, there is a significant additional encumbrance of the disease in terms of lost days of work. Malaria mortality rates shows a correlation with gross national income (GNI) per capita. Countries with higher proportions of their population living in poverty (less than US\$ 1.25

per person per day) have higher mortality rates from malaria as a result of low domestic spending per capita on health and malaria control. The ability of malaria endemic countries to strengthen health systems depends on many factors, including a country's physical infrastructure, educational systems, policies surrounding the role of the public sector, and the ability to finance the expansion of the sector. International spending on malaria control is more evenly distributed in relation to malaria burden, but a large proportion of this funding is spent on commodities and does not address fundamental weaknesses in health systems. Hence, innovative ways of providing services may be required to rapidly expand access to malaria interventions, particularly diagnostic testing and treatment. Such innovations will require community based approaches and engagement with the private sector. Malaria continues to pose a serious economic burden on health systems. Since 2001 in sub-Saharan Africa, malaria is estimated to have cost every year, on average, nearly US\$ 300 million annually for case management alone (WHO Report, 2015).

1.2 Malaria distribution and transmission

The parasite is solely transmitted by the mosquitoes of the *Anopheline* species. The disease is transmitted through a bite from an infected mosquito. Parasites enter the blood stream and travel to the liver where the life cycle begins. The highest biting rates occur at night between sunset and sunrise. Rarely, the disease is transmitted congenitally from mother to foetus and through transfusions and organ transplant. Malaria transmission through transfusion was one of the first recorded incidents of transfusion-transmitted infections (Kitchen *et al.*, 2006).

The distribution of the disease depends primarily on different factors including climatic conditions, weak economic development and poor public health measures. Malaria transmission is high in tropical and subtropical regions of the world, which offer suitable breeding conditions for the *Anopheles* mosquito. Certain strains of malaria like *Plasmodium falciparum* (*P. falciparum*) cannot complete its growth cycle in temperatures lower than 20°C, leading to interrupted transmission in cold climatic conditions. *P. vivax*, a different strain of the disease, is more tolerant to lower temperature and hence survives colder conditions. The transmission of the disease is hence seasonal. Warm regions in sub-Saharan Africa and New Guinea have the highest distribution of the disease due to all year round transmission.

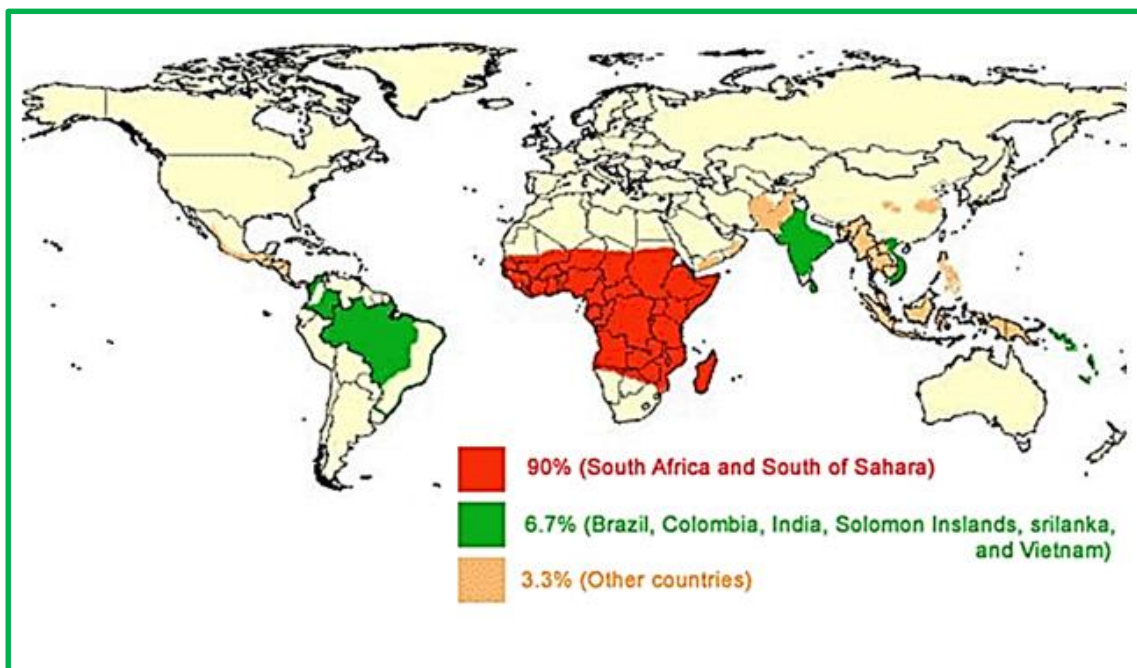


Fig 1.1: Malaria transmission: Showing the global distribution of malaria with 90% cases in Africa. (Source: <http://vcrc.res.in/writereaddata/mapf4.gif>).

1.3 Malaria eradication and control

Malaria was endemic in the United States (U.S) during the military occupation of Cuba in 1906. The workforce and military personnel were greatly affected especially around the Panama canal construction region. At the turn of the 20th century, the American congress approved funds for malaria control with a view to effectively reduce the number of cases. Mosquito breeding sites were reduced by controlling water levels as well as insecticide spraying using DDT (dichloro-diphenyl-trichloroethane), an almost odourless organochloride. In 1942, a centre was established and named the Office for Malaria Control. The primary aim was to reduce the impact of malaria and other vector borne diseases in military zones in the USA and its territories. Several achievements were made, leading to the Centre for Disease Control (CDC) being founded with a major mandate to control, prevent and eliminate the disease both domestically and internationally. By 1947, the national malaria eradication programme began operations. DDT was applied in all affected rural houses, drainages and breeding grounds for mosquitoes. In 1947, there were 15, 000 reported malaria cases and by 1950, these had reduced to 2000 due to the activities of the CDC. The disease was successfully eliminated due to effective control measures and the country was declared malaria free by 1951. The National Malaria Society put forward a

motion that 'Malaria may be assumed to be no longer endemic in an area once an indigenous case has not been recorded for three years'. Europe also successfully eliminated the disease during World War II with the extensive use of DDT amongst other control measures.

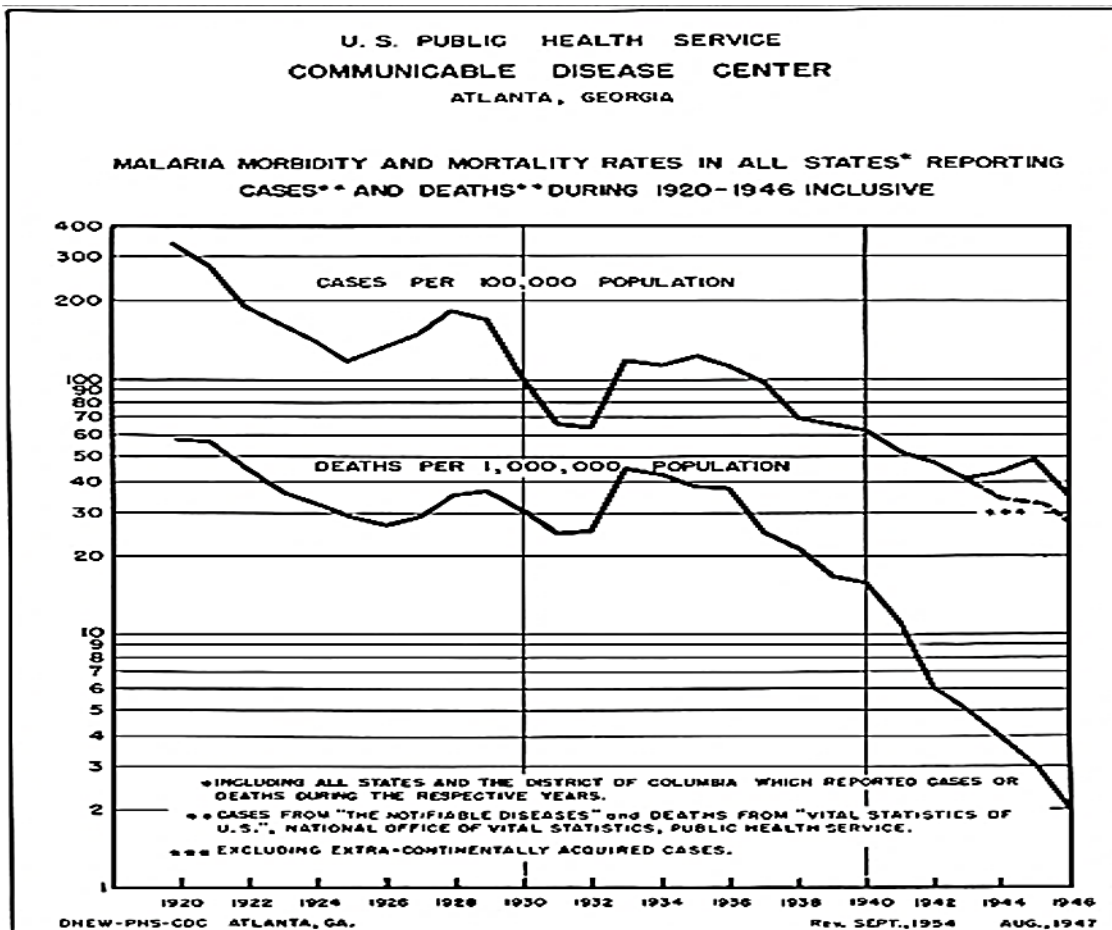


Fig 1.2: Malaria mortality in the US: Showing the mortality and morbidity rates in the United states prior to malaria elimination during World War (Source: http://www.cdc.gov/malaria/images/graph/malaria_us_curves.gif).

Other organisations played a vital role in the fight against malaria. The World Health Organisation (WHO) also commenced regional campaigns to eradicate the disease in the 1940's and by 1955, a global eradication programme was implemented in countries with low to moderate transmission. Eradication efforts included massive mosquito net distribution in malaria endemic regions, swamp clearing, prompt drug treatments, drug and vaccine research and development. DDT was largely used for mosquito control in these regions to reduce transmission. The programme proved successful in eliminating the disease in several countries including Taiwan and Australia whilst dramatically reducing mortality rates in other countries, including Sri Lanka and India (Harison, 1978). The DDT spraying

programme was however not sustained due to several reasons including safety and environmental factors. The significant financial burden required to implement the programme was unsustainable and these critical factors led to the programme being abandoned. The programme was largely successful in countries with high socio-economic status, well organised healthcare systems and relatively low malaria transmission but was never implemented in the sub-Saharan African region where presently, more than 80% of the disease burden occurs. Failure to sustain the programme led to increased parasite resistance to DDT and antimalarial drugs resulting in a resurgence in the disease and reversal of earlier victories previously achieved. By 1992, there was an improved understanding of the social, economic and cultural dimensions of malaria. The improved knowledge and experience derived from post eradication era research fuelled a renewed global focus on malaria. This led to the launch of the Roll-back on Malaria Initiative in 1998 by WHO which led to an Abuja declaration in 2000. The declaration looked into various progressive interventions and control measures designed to eliminate malaria as a public health issue through strengthening of the local healthcare systems. More recently the Bill and Melinda Gates foundation launched the Gates Malaria Forum in 2007 and the WHO defined four phases on the road to malaria elimination (Control, pre-elimination, elimination and prevention of re-introduction). Participating countries use the WHO set goals to achieve these phases with the ultimate goal of reaching the 'prevention of re-introduction phase'. An increasing number of countries are moving towards elimination of the disease. 16 countries have successfully reported zero indigenous cases in 2014 including Sri Lanka, Turkey, Morocco and Uzbekistan (WHO report, 2015). Other countries including Algeria have reached the pre-elimination phase with fewer than 10 indigenous cases. Interestingly, WHO European region, comprising of 53 countries, reported zero indigenous cases for the first time in 2015 in line with the Tashkent Declaration to eliminate the disease in the region by 2015 (WHO Report, 2015). Despite all these efforts and current tools that are available to us, the end is still not near in the fight to end malaria. This can be clearly seen in the map below.

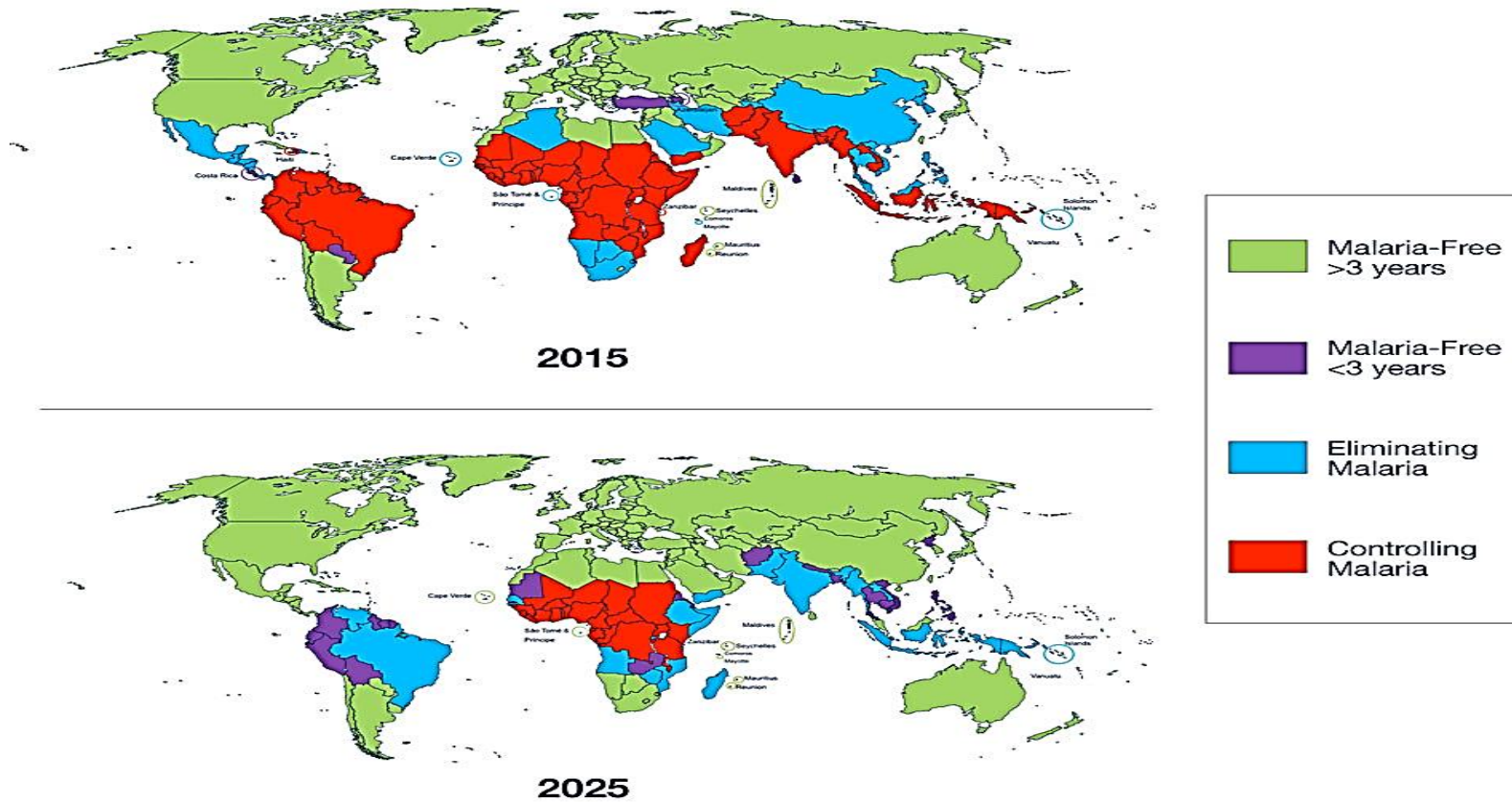


Fig 1.3: Current and forecasted World malaria eradication map: Showing different regions and their stage of the eradication programme. (Source: Malaria elimination initiative).

Although dramatic progress has been recorded in the last decade in the fight against malaria, the disease can only be eliminated worldwide through a strategic vision including strengthening of existing tools to achieve maximum synergy. To implement this holistic approach, several factors need to be considered including improving the economic plight of the nations involved.

Before malaria can be successfully eradicated in any region, the government of that country must make a commitment to end the menace. This must involve pooling all efforts of government bodies, NGOs and international organisations together. Sri Lanka and Egypt are two countries that have demonstrated that developing countries can achieve elimination with government commitment.

1.4 Discovery of the *Plasmodium* from a historical perspective

Centuries ago, events like the discovery of bacteria in 1676 by Antoni Van Leeuwenhoek and the development of the ‘germ theory’ by Louis Pasteur in 1878 steered investigators to intensify the search for the causative agent of malaria. Some prominent microbiologist claimed the disease was caused by a bacterium, *Bacillus malariae* (Cox, 2010). The details were however patchy and it was not until in 1880 that a French army doctor named Charles Louis Alphonse Laveran embarked on a mission to unravel this mystery. He discovered the parasites in the blood cells of his patients whilst working in a military hospital in Algeria. He observed different course of events which we now know to be the different stages of the parasite. Amongst his observations was the bursting of red blood cells which coincided with patient fevers. Laveran also discovered that these crescentic bodies were removed by quinine. He realised that he was not dealing with a bacterium but a protozoan and named it *Oscillaria malariae*. This was a ground-breaking discovery at the time, as it was the first time that a protozoa was identified as a disease causing agent. Unfortunately, his finding was rejected at the time, more particularly by the malariologists and biologists who believed his discovery was nothing other than disintegrating red blood cells. In 1884, Laveran convinced some leading malariologists including Golgi and Bignami of his protozoan theory. His findings were later accepted and he was awarded the Nobel prize for Physiology and Medicine in 1907 (Tan *et al.*, 2008). In 1886, Camillo Golgi further established that there were two presentations of the disease due to the nature of fevers observed. One form of the disease presented tertian periodicity whilst the other a quartan

periodicity. He also further confirmed that these two forms were distinct, as upon maturity, they produced different numbers of merozoites. The rupture of these merozoites coincides with the tertian and quartan fever cycles. He was awarded a Nobel prize in 1906 for his discoveries in neurophysiology.

In 1897, a British army doctor, Ronald Ross demonstrated that malaria could be transmitted from infected patients to the mosquito. He discovered parasites in the midgut of the *Anopheline* mosquito which had been previously fed for four days on a malarious patient, confirming the role of the *Anopheles* mosquito in malaria transmission. Ross continued with his work on malaria but on the avian animal model. After feeding mosquitoes on infected birds, he found the parasite in the salivary glands of the insects allowing them to subsequently infect other birds through a bite. He was awarded a Nobel prize for this breakthrough discovery. His findings became very useful and critical in public health awareness campaigns. In 1899, another team including Bignami and Grassi demonstrated the complete sporogonic cycle by feeding *Anopheles* mosquito on malarious patients and subsequently feeding healthy volunteers establishing an infection.

1.5 *Plasmodium* species and life cycle

1.5.1 Species and symptoms

Although Laveran attributed the disease to a single species in 1890 (*Oscillaria malariae*), the Italian investigators Giovanni Batista Grassi and Raimondo Filetti introduced the names *Plasmodium vivax* and *P. malariae* for two other species that affect humans. Furthermore, William H. Welch, named the malignant tertian malaria parasite, *P. falciparum* in 1897 causing lot of controversy. This name was widely used in literature and accepted. By 1922, John William Watson Stephens described another human malaria parasite, *P. ovale* whilst *P. knowlesi* was described in 1931 by Robert Knowles and Biraj Gupta. Another less severe strain, the *P. vivax* has also been described.

Amongst the species described above, four affect humans including *P. falciparum*, *P. oval*, *P.vivax* and *P. malariae* although a human case of *P. Knowlesi* was first reported in 1965 (Chin *et al.*, 1965). The different species affect different red blood cell types. *P. falciparum*, the most deadly strain infects all red blood cell types. *P. ovale* and *P. vivax* invade young red blood cells or reticulocytes while *P. malariae* prefer senescence red blood cells. The incubation period for the disease varies between 7-18 days depending on the specie due to the differences in the length of cycle between strains.

The presentation of uncomplicated malaria is highly variable depending on the species infecting the human host. Initial symptoms include fever, sweats, headache, vomiting and malaise. *P. vivax* and *P. malariae* tend to bring on mild symptoms in immune individuals and severe symptoms in non-immune persons. The most severe symptoms however occur with *P. falciparum* infections which without prompt treatment could lead to life threatening complications including organ failure and cerebral malaria.

1.5.2 Life cycle

The parasite life cycle was discovered in the late/early 20th century by several scientists including Laveran, Mason, McCallum and Ross. The first stage of the life cycle starts when the anopheles mosquito takes a blood meal from a human during it injects the parasites in the form of sporozoites from its saliva into the host. The parasites travel to the liver via the blood vessels and once there, they invade the liver cells. Over a period of days, the sporozoites grow and multiply through cell mitotic cell division forming haploid cells called the merozoites. Each liver cell is able to produce tens of thousands of these haploid forms. Due to the growing pressure on the liver cells, the cells burst releasing the merozoites back into circulation. Some malaria species are however known to remain in the liver cells in a dormant stage, multiplying at a later date and this is one of the main causes of relapses in treatment.

Once in the blood stream, merozoites invasion of erythrocytes begin very rapidly and specifically through receptor ligand interactions. The mechanism of erythrocyte invasion is very complex and not fully understood both at molecular and cellular levels. The *Plasmodium* is a member of the apicomplexan due to the apical position of certain organelles in the parasite which are involved in parasite-host interactions during host cell invasion. Four distinct processes have been described with regards to parasite-host interaction, which include: initial merozoites binding, erythrocyte re-orientation and deformation, junction formation and parasite entry (Gratzer and Dluzeski, 1993).

The four processes involve a cascade of protein-protein interactions, the exact mechanism of which is only partially understood. In summary, erythrocyte and merozoite interaction is initially assumed to be through a random collision between membrane surface proteins on both the merozoite and erythrocyte membranes. The merozoite reversibly binds the host erythrocyte via merozoite surface proteins (MSP), the best characterized one is called MSP-1 protein, which is abundantly distributed over the merozoite surface. Parasite re-

orientation occurs after merozoite binding with the parasite bringing its apical end into close contact with the erythrocyte membrane. This apical adhesion involves an irreversible interaction between receptors on the merozoite surface and ligands on the erythrocyte cell. Apical antigen-1 (AMA-1) has been implicated in this re-orientation (Mitchell, 2004). Several other events occur including junction formation between the parasite and the host cell, formation of parasitourous vacuole and shedding of merozoite proteins. The main driver for parasite entry is an acto-myosin complex called glideosome.

Once inside the erythrocyte, the *Plasmodium* modifies the red blood cell and makes it more suitable for its habitation. It further undergoes a trophic phase followed by a replicative phase. In the case of *P. falciparum* parasites, cytoadherence of infected erythrocytes to endothelial cells occurs. This results in sequestration of the mature parasites in capillaries and post-capillary venules. This could be responsible for the complications that arise with *P. falciparum* infections. The cytoadherence is an advantage to the parasite as it creates a micro-aerophilic environment which is better suited for the parasite. Another major advantage to the parasite is that through this mechanism, it is able to avoid the spleen, hence subsequent destruction.

P. falciparum infections are also associated with knob formation. This is a structural alteration of the host once invasion occurs induced by the *Plasmodium* (Deitsch and Wellens, 1996). Two main proteins have been identified that are associated to this knob formation. These are the knob-associated histidine rich protein (KAHP) and the erythrocyte membrane protein-2 (PfEMP2) also called MESA. These two proteins are exposed on the outer surface of the infected erythrocyte but localised to the cytoplasmic phase of the host erythrocyte. The exact function of knobs is still unknown but it is thought to be involved in reorganising the submembrane cytoskeleton.

Once completely in the red blood cell, the *Plasmodium* multiplies asexually repeatedly over a period of 1-3 days. The red cells carrying the *Plasmodium* burst once several divisions have occurred hence releasing thousands of new merozoites into the blood stream. These new merozoites re-invade new blood cells within seconds increasing the parasitaemia within the infected person causing symptoms and other complications if left untreated. Some merozoite infected blood cells leave the cycle of asexual replication described and develop into the sexual forms called gametocytes which circulate in the blood stream. The sexual forms are however, not able to mate within the human host so the final stage of the

Plasmodium life cycle only occur when a mosquito takes a blood meal from the infected person.

Once in the mosquito, digestion of the blood cells occurs in its gut leading to the release of gametocytes. The free released sexual forms mate and fertilize forming diploid zygotes which develop into actively moving ookinetes. These burrow into the mosquito midgut wall forming oocysts which grow through mitotic divisions to produce thousands of active haploid forms called sporozoites. After 8-15 days, the oocysts burst releasing sporozoites into the body cavity of the mosquito. The sporozoites travel and invade the salivary gland of the mosquito where they wait to be deposited into the next human victim during the mosquito's next blood meal.

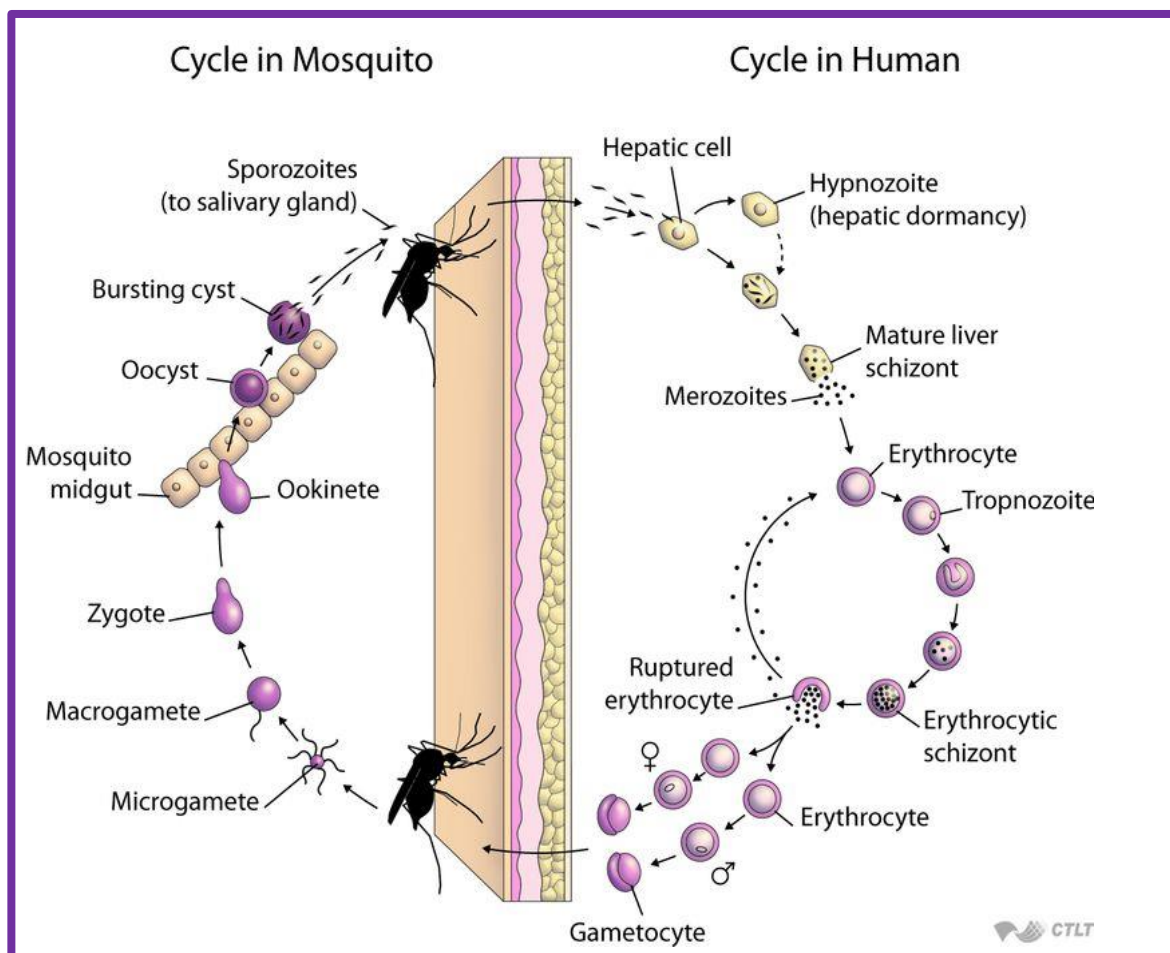


Fig 1.4 Complete life cycle of the Plasmodium: Showing the complete life cycle of the malaria parasite in the human and mosquito hosts.

(Source [http://www.malwest.gr/Portals/0/EID_lec17_slide8-large\[1\].jpg](http://www.malwest.gr/Portals/0/EID_lec17_slide8-large[1].jpg)).

1.5.3 Erythrocytic stages of *P. falciparum*

Within the red blood cell, the *Plasmodium* goes through a trophic period followed by multiplication via fission called schizogony. After red cell invasion, a trophozoite grows in size and this is why it is described as the early trophozoite and late trophozoite stage especially in culture. The trophozoite repeatedly replicates its nucleus and other organelles within the red blood cell forming a schizont which is multinucleated. This further divides via cytokinesis into several identical merozoites. Due to the overwhelming number of merozoites, the red blood cell becomes distorted in shape and eventually bursts releasing several merozoites which reinvade other red blood cells within seconds. This rupture of the erythrocyte causes the release of the parasite waste and cell debris into the circulation which causes the symptoms of the disease.

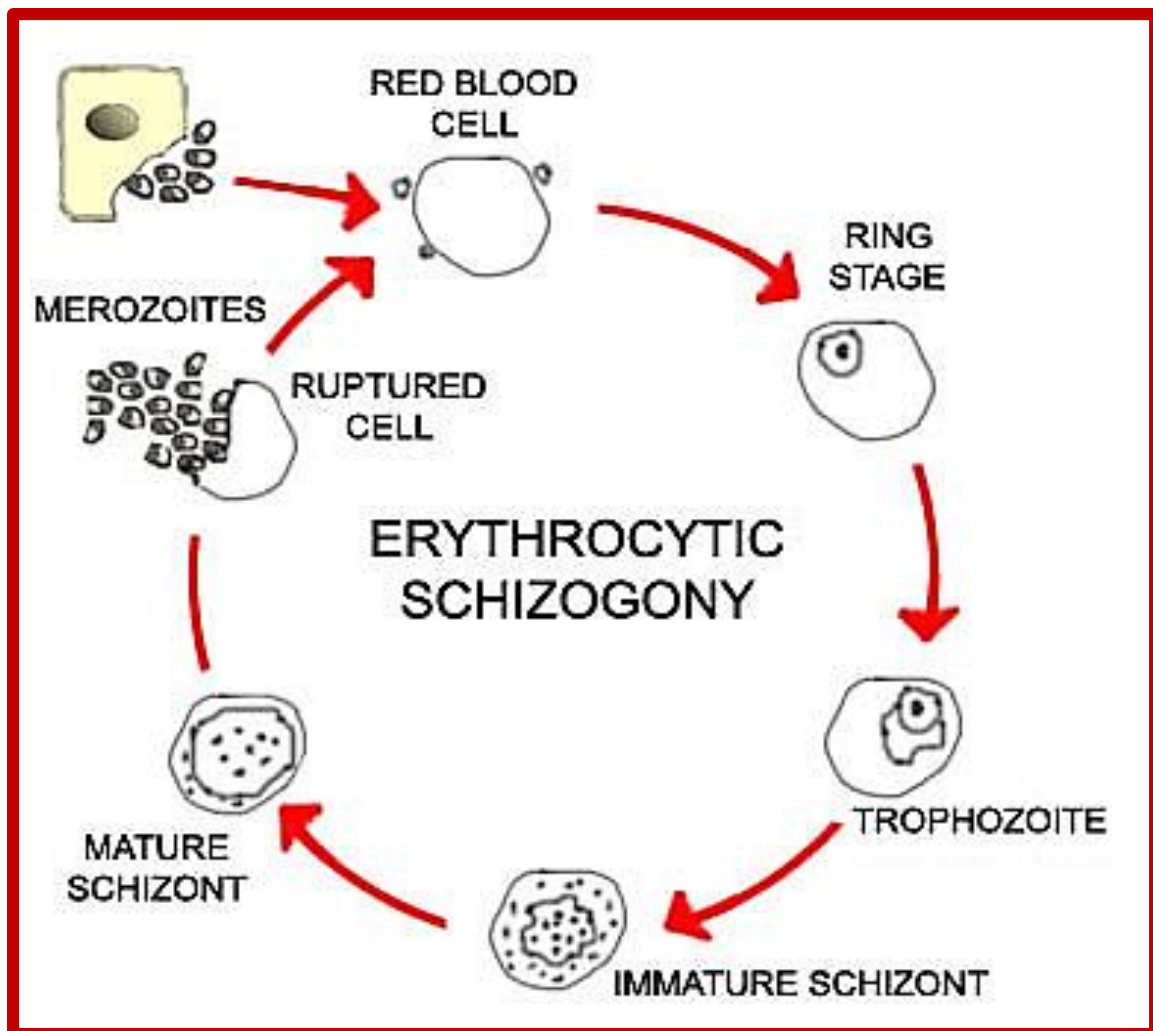


Fig 1.5: Erythrocytic stages of the Plasmodium: Showing a diagrammatic erythrocytic stages (Source http://idshowcase.lshtm.ac.uk/id503/ID503/M1S1/ID503_M1S1_030_020.html).

1.6 Clinical Symptoms

The presentation of uncomplicated malaria is highly variable and can easily be misdiagnosed. The incubation period is between 7-18 days depending on species. Clinical manifestations occur exclusively due to the asexual erythrocytic stage of development of the parasites. Initial symptoms are flu-like and include a high pitch fever, sweats, chills, headache, vomiting and anaemia. Anaemia is one of the major causes of morbidity and mortality in endemic areas. Low reticulocytosis has been observed in infected persons suggesting insufficient erythropoiesis. Studies using *Plasmodium Chabaudi* comparing malaria resistant mutant mice and susceptible mice show that neutralisation of endogenous erythropoietin during infection leads to lethal anaemia, while timely administration of exogenous erythropoietin rescues mice although reticulocytosis is suppressed in proportion to parasitaemia (Change and Stevenson, 2004). Bone marrow suppression and severe anaemia have been shown to be associated with persistent *P. falciparum* infection (Helleberg *et al.*, 2005). Severe anaemia can develop in the aftermath of an infection due to protracted bone marrow suppression possibly due to residual low level parasitaemia. There are several other factors that contribute to anaemia including accelerated removal of erythrocytes, without adequate compensation by the bone marrow, due to hepatosplenomegaly. Bone marrow suppression occurs in all malaria infected patients and also in asymptomatic *P. falciparum* semi-immune individuals (Kurtzhals *et al.*, 1999). Majority of severe anaemia in children residing in malaria endemic regions is due to malaria infection. In some cases, the parasitaemia is undetectable by microscopy and case diagnosis is only possible through antigen detection (Helleberg *et al.*, 2005). In these patients, suppressed erythropoiesis persists until parasites are completely cleared. Data on the duration of bone marrow suppression after malaria infection are conflicting with some data showing hypoproliferative erythropoiesis for weeks following treatment (Camaccho *et al.*, 1998), whilst others show that bone marrow suppression is rapidly reversed post treatment (Abdallah *et al.*, 1980). Malaria infected patients have an increased ratio between the pro-inflammatory cytokine, tumor necrosis factor (TNF- α), and the anti-inflammatory cytokine, interleukin-10 (IL-10). Inflammatory cytokines are proposed to be a causative factor in malaria anaemia (Kurtzhals *et al.*, 1998).

Apart from the debilitating effects of anaemia, cerebral malaria is a more severe presentation. Treatment often involves prompt parenteral administration of an antimalarial (quinine or artemisinins). Mortality rates are lower in adults than in children. The clinical hallmark of the disease is altered state of consciousness, seizures, neurological symptoms with coma being the most severe manifestation. Cerebral malaria is a leading cause of childhood neuro-

disability in affected regions (Idro *et al.*, 2010). The central factor in the pathogenesis of the disease is thought to be sequestration of parasites in cerebral microvasculature resulting in pathophysiological changes in tissue around the sequestered parasites. Sequestration ensues from adherence of parasitized red blood cells (pRBCs) to the endothelial lining (cytoadherence) using parasite derived proteins exposed on erythrocyte surface. A group of parasite antigens including *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP-1) mediate binding to host receptors such as intercellular adhesion molecule-1 (ICAM-1). ICAM expression is upregulated in areas adjacent to sequestered parasites. The sequestered parasite mass is further increased when adherent erythrocytes agglutinate with other pRBCs forming rosettes with non-parasitized erythrocytes or using platelet-mediated clumping to bind to each other. In Addition, the ability of pRBCs to deform and pass through the microvasculature is decreased. Therefore, hypoxia and inadequate tissue perfusion may be major pathophysiological events (Dondorp *et al.*, 2004).

1.7 Prevention of malaria

Prevention and control of the disease can target either barrier methods, vaccination or chemoprophylaxis. The method of control is multifactorial as both the vector and the parasite need to be considered. This has proven difficult over the years as both vector and parasite seem to be highly adaptable, hence multiple strategies need to be employed. For this reason, there is a strong need for governments, Non-governmental organisations (NGOs), international agencies like UNICEF, WHO and healthcare professionals to collaborate and develop a holistic strategy to combat the disease. Early intervention has proven to be an essential key in preventing death and reducing the spread of the disease. Currently, pregnant women in sub-Saharan Africa are given a prophylactic treatment although accessibility might be an issue.

1.7.1 Vector control

WHO recommends several methods for malaria vector control, but also further recommending that the approach should be based on knowledge of the local situation. The most commonly used and successful methods for vector control are insecticide spraying and use of insecticide-treated nets (Raghavendra *et al.*, 2011). Indoor residual spraying is the practice of spraying insecticides on the interior walls of homes in malaria affected areas. Mosquitoes tend to rest on a nearby surface after feeding to digest the blood meal. As previously discussed, the first and most popular insecticide used for IRS is DDT. While it

was initially used exclusively to combat malaria, its use quickly spread to agriculture leading to a large scale usage. The resultant misuse of DDT led to the evolution of resistant mosquitoes in many regions. During the 1960s, awareness of the negative consequences of its indiscriminate use increased, ultimately leading to bans on agricultural applications of DDT in many countries in the 1970s. DDT has never been banned in public health and advocates of its use argue that mosquito resistance stems specifically from its industrial-scale application in agriculture, rather than its use in public health. The WHO currently advises the use of 12 different insecticides in IRS operations. These include DDT and a series of alternative insecticides such as the pyrethroids permethin and deltamethin. Hence, the public health use of small amounts of DDT is permitted under the Stockholm Convention on Persistent Organic Pollutants (POPs), which prohibits the agricultural use of DDT. Many developed countries nevertheless, discourage DDT use, even in small quantities, due to its legacy. One major drawback with all forms of IRS is the likelihood of the development of insecticide resistance via evolution of mosquitos.

Mosquito bednets are also an important barrier method. As some mosquitoes can still pave their way through the net, nets are often treated with an insecticide (often permethin or deltamethin). Insecticide-treated nets (ITN) are estimated to be twice as effective as untreated nets. Although ITN are proven to be very effective against malaria less than 2% of children in urban areas in sub-Saharan Africa are protected by ITNs. ITNs are a very cost effective barrier method. Nets should be re-impregnated with insecticide every six months to maximise effect. This process poses a significant logistical problem in rural areas. New technologies like Olyset or DawaPlus allow for production of long-lasting insecticidal mosquito nets (LLINs), which release insecticide for approximately 5 years.

1.7.2 Vaccine development

Over the years, there have been concerted efforts by several pharmaceutical companies and research organisations to search for a vaccine for malaria. Several vaccine candidates have been tested with limited success. Recently, in 2015, a vaccine candidate (RTS,S) was finally given a positive feedback by the European Medicine Agency for safety and efficacy after taking 30 years to develop at a cost of \$800m. RTS,S was developed by Glaxo SmithKline. It is a recombinant protein engineered using genes and the first vaccine in the World to be licenced for malaria and in fact the first vaccine licenced for any parasitic disease (Walsh and Fergus, 2015). It clinically shows protection against *P. falciparum* and works by triggering the immune system to defend itself against the *Plasmodium* after it enters the blood stream

following a mosquito bite. The vaccine however shows no protection against the liver form, *P. vivax*. It has not yet been cleared for use in children, but is being considered by the WHO due to the ambiguous results obtained from its trials. Furthermore, only 27% protection was observed in children who received up to 4 boosters at 6, 10, 14 and 18 month of age. The groups that did not receive the dose only showed 18% protection (WHO, 2015). Also increased cases of seizures and meningitis were noted in children of older age groups. The fact that repeated boosters are needed for it to be effective will raise questions regarding compliance and cost implications. In 2015, The WHO's Strategic Advisory Group of Experts on Immunization (SAGE) and the Malaria Policy Advisory Committee (MPAC) jointly recommended a pilot implementation of the vaccine in Africa (WHO, 2015 Media Release). In yet another development, another vaccine, PfSPZ developed by Sanaria has been shown in a recent trial to offer up to 1 year protection. It is based on an activated sporozoite. (Nature Medicine Magazine, 2016).

The exact mechanism of action of RTS,S is yet to be elucidated, however the immunologic basis of the protection against malaria offered by the vaccine is via the induction of high levels of antibodies and CD4+ T cells specific for circumsporozoite protein (CSP) (White *et al.*, 2013).

1.7.3 Chemoprophylaxis

Several approaches are involved including disruptive, suppressive and causal prophylaxis. Disruptive prophylaxis is an experimental approach which involves preventing the parasite from binding erythrocytes. This is achieved by blocking calcium signalling between the parasite and the host cell. Suppressive prophylaxis uses drugs that are only effective in killing the erythrocytic parasite stages. Chloroquine, proguanil, mefloquine, and doxycycline are suppressive prophylactics. These drugs have no effect until the liver stage is complete. That is why these prophylactics must continue to be taken for four weeks after leaving the area of risk. Causal prophylactics target not only the blood stages of malaria, but the initial liver stage as well. This means that the user can stop taking the drug seven days after leaving the area of risk. Malarone (a combination drug of atovaquone and proguanil) and primaquine are the only causal prophylactics in current use hence, used as the first line treatment prophylactics for people travelling to malaria endemic regions.

1.8 Chemotherapy in malaria

Chemotherapy remains the most important means of controlling malaria. Treatment usually depends on several factors including severity of diseases, malaria specie and the part of the world infection was acquired. Determination of specie and region is usually useful in order to determine drug susceptibility. Other factors include the clinical situation of the infected person for example adult, child or pregnant female. There are several anti-malarials that are used in practice. Some have previously been used as monotherapy while others are currently used as combination therapy. Combination therapy involves the simultaneous administration of two or more blood schizonticidal agents with different modes of action and different biochemical targets in the parasite. Prior to chemotherapy, prompt parasitological confirmation is required which can be achieved by various methods to be discussed in later section of the report. The gold diagnostic standard is microscopy which is cheap and fast. In *P. falciparum* endemic areas where infection can be fatal in the event of treatment delay, drugs are often given on suspicion of malaria and laboratory confirmation done later. Several antimalarials are used for the treatment of the disease. They are often classified according to their biological activity or according to the structural nature of the compound.

1.8.1 Classification by biological activity

Compounds are classified according to the stage of the parasite life cycle activity resides. Different compounds target the different developmental stages of the parasite both within the host and vectors. According to the Centre for Disease control (CDC), there are five main categories under this classification.

Biological classification	Target
Blood schizonticides	Compounds in this category exert their antimalarial effect in the erythrocytic stage of the life cycle. Examples of drugs in this category include quinine, mepacrine, the 4-aminoquinolines including chloroquine and amodiaquine. The 4-aminoquinolines have a rapid action and are hence used for clinical suppression of symptoms (Bruce-chwatt, 1962).
Gametocidal drugs	Several compounds fall into this class including the 8-aminoquinolines example pamaquine and primaquine. They target the sexual forms of the parasite in blood hence blocking transmission in the mosquito. Quinine, mepracrine, amodiaquine and chloroquine are also active against sexual forms of the parasite in the blood in <i>P. vivax</i> and <i>P. malariae</i> (Kiszewski, 2011).
Sporontocides	These compounds target the mosquito life cycle. They inhibit growth and development of oocysts in the mosquito, hence, preventing transmission. They are also called gametostatic drugs. They include proguanil, chlorproguanil and pyrimethamine (Omar <i>et al.</i> , 1974).
Secondary tissue schizonticides or anti-relapse drugs	These compounds exert their effect on dormozoites of the <i>P. vivax</i> and <i>P. malariae</i> infections of the liver. These dormozoites would ordinarily lead to relapse of symptoms and reoccurrence of disease. Examples of the drugs in this category include pamaquine and primaquine (Alonso <i>et al.</i> , 2011).

Table 1.1 Classification of antimalarials: Showing classification of antimalarials by biological activity.

1.8.2 Classification by structure

Drugs are classified according to similarities in their chemical structure which may sometimes result in similarities in mode of action.

1.8.2.1 4-aminoquinolines

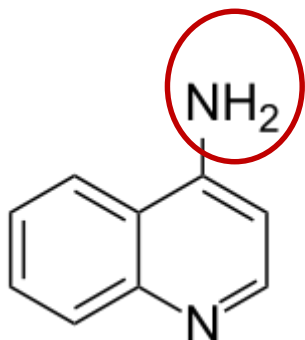


Fig 1.6: Chemical structure of 4-aminoquinoline: Showing the structure of IUPAC name *quinolin-4-amine*

The 4-aminoquinolines are a group of structurally related compounds which all have the quinoline pharmacophore. They have an amino group at position 4 of the quinoline ring as represented in the structure in fig 1.6 above. The 4-aminoquinolines were synthesized from the pharmacophore of quinine. Examples include chloroquine, amodiaquine and hydroxychloroquine.

I) Chloroquine

Chloroquine has the structure in fig 1.7 below with the quinoline ring and an amino substitution at position 4.

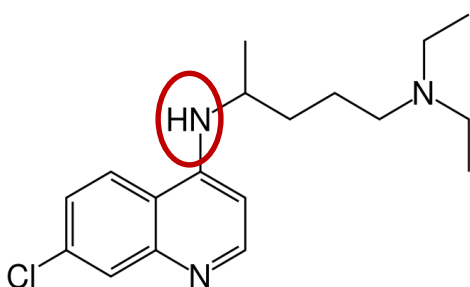


Fig 1.7: Chemical structure of Chloroquine: Showing the structure of CQ, IUPAC name: *RS)-N'-(7-chloroquinolin-4-yl)-N,N-diethyl-pentane-1,4-diamine*

Chloroquine phosphate was the drug of choice for all *Plasmodium* strains except for the chloroquine-resistant strains. *P. malariae* strains tend to be susceptible to chloroquine but

there is a high resistance with *P. falciparum*. The drug can be used as prophylactic treatment for *P. vivax* and *P. ovale*. Chloroquine acts by interacting with DNA, hence, inhibiting certain parasite enzymes. *Plasmodium* resistance to the drug is yet been confirmed in Central America, middle east and the Caribbeans. Chloroquine works by diffusing into red blood cells. Once in the red blood cells, the drug molecule becomes protonated due to its weak basic properties and the acidic nature of the food vacuole preventing it from exiting the vacuole leading to its accumulation in the lysosomes. This is why it is called a lysomotropic agent. The malaria parasite lives in the red blood cell where it acquires amino acids though the degradation of haemoglobin. Once the parasite acquires the amino acids, it uses it to construct its own proteins for energy and metabolism and this digestion occurs in the parasite food vacuole. The end result is the production of the soluble haem which bio-crystallizes to the nontoxic haemazoin, accumulating in the vacuole as crystals. Since chloroquine accumulates in the parasite food vacuole as well, it is thought to prevent the bio-crystallization of haem hence leading to the accumulation of the toxic molecule causing parasite death (Leeds *et al.*, 2002).

ii) *Amodiaquine*

This drug is more effective than chloroquine in treating chloroquine-resistant *P. falciparum* infections. It also offers more protection when used as a prophylactic compared to chloroquine. It differs from chloroquine in structure due to the incorporation of an aromatic structure in the chloroquine side chain. The drug is only susceptible to low chloroquine resistant *P. falciparum* infections. In cases of high resistance, an alternative treatment is sought. The main problem with amodiaquine is hepatotoxicity, which commonly occurs due to biotransformation of the p-aminophenol moiety into a quinoneimine, reducing the therapeutic value of the drug. The drug is no longer in the market in developed countries although the side effect is thought to only occur with long term prophylactic use (Olliaro *et al.*, 1996).

iii) *Hydroxychloroquine*

This has a similar structure to chloroquine and differs by the presence of a hydroxyl group at the end of its side chain hence the name.

1.8.2.2 Arylamino-alcohols

These group of compounds consist of alkaloids extracted from the bark of *Cinchona* tree and their synthetic derivatives. Quinine, a naturally occurring alkaloid was the first antimalarial discovered since the 18th century and remained the antimalarial of choice up until the 1940's. Structurally, it consists of a conjugated aromatic quinoline ring fused to a bicycle quinuclidine ring. Other synthetic derivatives were developed by the US army during World War I due to the scarcity of quinine from Germany. Mefloquine and halofantrine are the main synthetic derivatives. Although more antimalarials have been developed and are more widely used, quinine still remains the drug of choice for the treatment of complicated and severe chloroquine resistant *P. falciparum* malaria (Gilles, 1991). The exact mechanism of action is yet to be understood. They differ from the 4-aminoquinolines but also seem to interfere with haem digestion (Famin *et al.*, 2002).

1.8.2.3 8-Aminoquinolines

These classes of aminoquinolines have an amine at position 8 of the quinoline rings. Three main antimalarials belong to this group. These include pamaquine, primaquine and tafenoquine. They are closely related in structure to the 4-aminoquinolines.

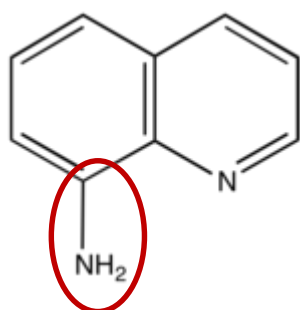


Fig 1.8: Chemical structure of 8-aminoquinoline: Showing the structure of 8-aminoquinoline

The 8-aminoquinolines are the first group of compounds to be synthesized specifically for antimalarial activity. By the 1940s, there was a need for new more potent and less toxic antimalarials. The USA initiated a programme which led to the development of several compounds in this group of which pamaquine and primaquine seemed the most effective (Cooper, 1953). Pamaquine is no longer used anywhere in the world but primaquine is still in use, although it is still not licenced for use in the UK for travellers. Primaquine is effective

against *P. vivax* and *P. ovale* and was also found to be effective as a prophylactic treatment for *P. ovale* as it eradicates malaria hypnozoites from the liver (Jones *et al.*, 1948).

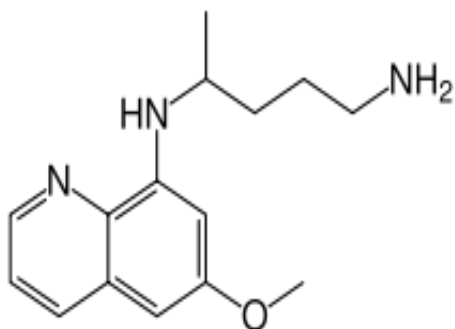


Fig 1.9: Chemical structure of primaquine: Showing the structure of primaquine

1.8.2.4 Artemisinin and related compounds

Artemisinin and its derivatives are a group of compounds with a rapid parasite clearance rate that are active against *P. falciparum* malaria. Artemisinin was discovered from the bark extract of *Artemisia annua*, traditionally used in Chinese traditional medicine. It is a complex sesquiterpene lactone with a peroxide bridge structure as shown in fig 1.10 below.

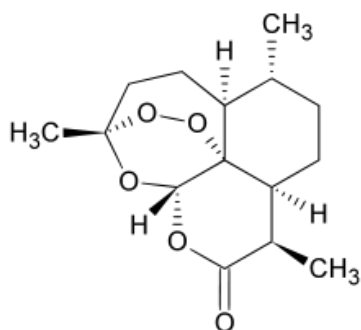


Fig 1.10: Chemical structure of artemisinin: Showing the complex structure of artemisinin.

The complex structure of artemisinin has proved difficult to synthesise. Recently the drug precursor has been produced from chemically engineered yeast. The precursor, artemisinic acid, is then transported out, purified and chemically converted to artemisinin (Ro *et al.*, 2006). This route is still not commercially viable. Various artemisinin derivatives have been produced by modifying its original structure as represented in fig 1.10, forming a very important class of antimalarial drugs, currently the most widely used for the disease. These include artesunate and artemether which are prodrugs. The biologically active metabolite is dihydroartemisinin. Artemisinins work throughout the erythrocytic asexual parasite cycle and kill parasites in all stages including young gametocytes (Olliaro, 2001). The exact mechanism of

action is still unknown although there are various proposed theories for its mechanism of action. Artemisinin derivatives are hypothesized to interact with Fe-II species in the parasite's food vacuole, and early ring stage parasites combat this by slowing down hemoglobin digestion. Artemisinins are fast acting and very potent against blood-stage parasites and show activity against early sexual stages of the parasite, which is important for blocking transmission. . Structure activity relationship studies show that the peroxidic oxygen is important for its activity (Avery *et al.*, 1993). The most widely accepted hypothesis is that ferrohaem ferrous-protoporphyrin IX (Fe(II)PPIX) cleaves the intact peroxide in a reductive reaction generating radicals which in turn alkylate biomolecules leading to parasite death (Olliaro, 2001). Their major limitation is a short half-life, which is why they are partnered with longer lasting drugs.

1.8.3 Antibiotics

These include doxycycline, clindamycin and tetracycline.

ANTIBIOTIC	USE
Tetracycline	Tetracyclines are used as an alternative to clindamycin in children.
Doxycycline	Doxycycline is a tetracycline derivative from oxytetracycline. It is a cheap drug and it is bacteriostatic. It is primarily used in combination with quinine for the treatment of complicated <i>P. falciparum</i> infections or as a prophylactic agent.
Clindamycin	Clindamycin is a blood schizonticide and is used in combination with quinine for the treatment of acute <i>P. falciparum</i> malaria. It is however contraindicated in children due to toxicity

Table 1.2: Antibiotics used for malaria: Showing different antibiotics and their usage as antimalarials

1.8.4 Antifolates

Antimalarial antifolates have been central for prophylaxis and treatment of malaria for decades. Since their discovery in the 1940's during the Second World War, no new

antimalarial antifolates have been developed that have reached Phase I/II stages. Limited work has been carried out to exploit the inhibition of the malaria folate pathway as a target for new drugs. The understanding of the role of folate in man led to the identification and development of current antifolates as therapeutic drugs. One of the major drawbacks of antifolate compounds is their implication in anaemia complications. Much of the work on antifolates was done before the World War II. By the 1930s, evidence showed that some forms of anaemia, mainly megaloblastic anaemia, could be reversed by the addition of yeast or liver extract, leading to the hypothesis that a specific co-factor may be associated with anaemia. In 1941, the co-factor was identified in spinach leaves and named folic acid (Mitchell *et al.*, 1941). The name was derived the word 'folium' which is latin for leaf. Folic acid was subsequently found to be effective in the treatment of anaemia. Folic acid is composed of a pteridine ring, para-aminobenzoic acid (pABA) and glutamate; the compound is also called pteroyl glutamic acid (PGA). Soon after its synthesis, several reports demonstrated that it is not a naturally occurring folate. Its primary function in the body is as a co-factor to various methyltransferases involved in serine, methionine, thymidine and purine biosynthesis.

Antifolates drugs work by antagonising the actions of folic acid (vitamin B9). Folate co-factors are essential for the synthesis and metabolism of amino acids, consequently antifolates inhibit cell division, DNA/RNA synthesis and repair and protein synthesis (Hyde, 2005; Hyde 2007). Some such as proguanil, pyrimethamine and trimethoprim selectively inhibit folate's actions in microbial organisms such as bacteria, protozoa and fungi (Nzila *et al.*, 2005)

Antifolate agents used in the treatment of malarial infection are sub-divided into two classes: inhibitors of dihydropteroate synthase (DHPS), known as class I antifolates and inhibitors of dihydrofolate reductase (DHFR), the class II antifolates. DHFR catalyses the reduction of dihydrofolate to tetrahydrofolate, a very important pathway in most living cells. DHFR is a very interesting target for antifolates from a pharmacological point of view due to its selectivity for the parasite enzyme, binding with a greater affinity than for human enzyme. Although both class I and class II antifolates target the folate pathway, they have been used in combination for malaria therapy. The combination has shown a synergistic activity in antimalarial therapy (Gregson and Plowe, 2005).

1.8.4.1 Inhibitors of DHFR

These mediate their antimalarial activity by inhibiting the enzyme DHFR. They have a higher affinity for binding the *P. falciparum* DHFR than human type. Differences in their binding affinity account for their good therapeutic index. However, a report indicated that the malaria parasite and its host differ fundamentally in the way that translation of DHFR-TS is regulated, such that the parasite enzyme is less readily replenished when targeted by anti-DHFR inhibitors (Zgang *et al.*, 2002). Studies by Nirmalan *et al.*, 2002 however did not support these findings. Their study found that malaria parasites treated with DHFR or thymidylate synthetase (TS) inhibitors resulted in a seven fold increase DHFR-TS levels. The results are not as expected based on the results of previous RNA-protein binding experiments have shown enzyme levels to remain constant in drug treated parasites. In addition, the increase in enzyme levels could not be attributed to an increase in mRNA as the levels remained constant before and after treatment (Nirmalan *et al.*, 2002; Nirmalan *et al.*, 2004).

i) Proguanil

Proguanil was discovered during the Second World War as a result of a British research programme on antimalarials. The drug was found to be more active than quinine against the avian specie of the disease and further research prompted its use in humans. Studies have demonstrated that proguanil is a prodrug and metabolizes to its triazine form chlorcycloguanil, an inhibitor of the parasite DHFR (Crowther *et al.*, 1953). This drug has been used alone as a prophylactic agent against malaria or in combination with chloroquine although its effectivity was not established in regions such as New Papua Guinea (Henderson *et al.*, 1986). Proguanil is also used in combination with atovaquone, an inhibitor of the electron-transport system, targeting a cytochrome P450 enzyme isoform. The combination is synergistic (Indresh *et al.*, 1999). Though proguanil can be converted *in vivo* into cycloguanil, an inhibitor of DHFR, proguanil and atovaquone are the active agents and the mechanism of synergy between these drugs is not well understood. The potency of proguanil led to the search for its analogues. Studies on the analogues demonstrated that chlorination of the phenyl ring and increasing the link between the phenyl ring and the diaminopyrimidine ring, increase the potency of these class of antifolates. These studies led to the discovery of chlorproguanil, clociguanil amongst others (Alexis, 2006).

ii) Pyrimethamine

Pyrimethamine belongs to the 2,4-diaminopyrimidine derivative family. They were synthesized in the 1940s as folic acid analogues for the treatment of tumours. Falco *et al.*, 1951 observed the similarity in their structures with proguanil. This sparked an interest in them as antimalarials. The screening of their antimalarial activity led to the identification of pyrimethamine. This has been the most widely used antimalarial antifolate agent so far. It is used in combination with sulfadoxine or sulfalene, and to a lesser extent it has been used in monotherapy under the trade name, Daraprim®. This drug is widely used as an antimalarial prophylactic agent in sub-Saharan Africa.

1.8.4.2 DHPS inhibitors

DHPS inhibitors are sulfadruugs which block the *de novo* folate synthesis pathway of the malaria parasite. These sulfadruugs belong to two families: sulphonamide and sulphone. Attempts to use sulfadruugs as monotherapy for malaria have not been very successful. The low efficacy and high toxicity of these drugs have discouraged their use. Interest in DHPS inhibitors resurfaced when they were discovered to act synergistically with DHFR inhibitors which triggered their use in combination therapy. Among the available DHPS inhibitors for malaria, dapson is the most potent. Dapson was used in combination with pyrimethamine as Maloprim®, now widely used in combination with chlorproguanil for the treatment of malaria. It is commercially available as lapdap.

1.8.4.3 Pyrimethamine and sulfadruug combinations

Pyrimethamine is a long acting drug and has been combined with several sulfadruugs including sulfadiazine, sulfametopyrazine, sulfamethoxine, sulfadimethoxine, sulfaphenazole and sulfasoxazole (McGregor *et al.*, 1963; Hurly *et al.*, 1959). None of these combinations have been developed further as antimalarial agents. Instead, pyrimethamine was combined with sulfadoxine (Fansidar®), and combined with sulfalene (Metakelfin®). These two sulfadruugs have a long elimination profile, like pyrimethamine, with half-lives of over 80 h (Watkins *et al.*, 1993). The combination of pyrimethamine with the short actiong dapson (Maloprim®) has shown a low effcicacy. The half-life of dapson is around 24h (Winstanley *et al.*, 1997) .

1.8.4.4 Trimethoprim and sulfadruugs

Trimethoprim is a DHFR inhibitor and although a known antibacterial, it was combined with sulfadruugs for the treatment of malaria. Trimethoprim was combined with sulfamethoxazole (this is the antibacterial combination known as Septrim® or Bactrim®, co-trimoxazole), sulfalene and sulfametopyrazine (Peters *et al.*, 1987). The use of trimethoprim did not present any advantage over pyrimethamine, so the drug combination was abandoned as an antimalarial. Clinical studies have demonstrated that this drug remains efficacious against *P. falciparum* infections in areas where resistance to the combination pyrimethamine/sulfadoxine is still low (Thera *et al.*, 2005).

In general antifolate combinations drugs are used both for treatment and prophylaxis of uncomplicated malaria as artemisinin combination therapy is the first line treatment for the disease.

1.8.5 New drugs

Several drugs fall into this classification, the most important of which is atovaquone. Atovaquone is used both for prophylaxis and treatment of malaria infections and also used in combination with proguanil. The exact mechanism of action is unknown but it is thought to interfere with parasite mitochondrial function. Resistance develops rapidly when used as a monotherapy but it is highly efficient when used as a combination. The main draw back for atovaquone is its high cost, hence not accessible to the poor countries where malaria is most endemic.

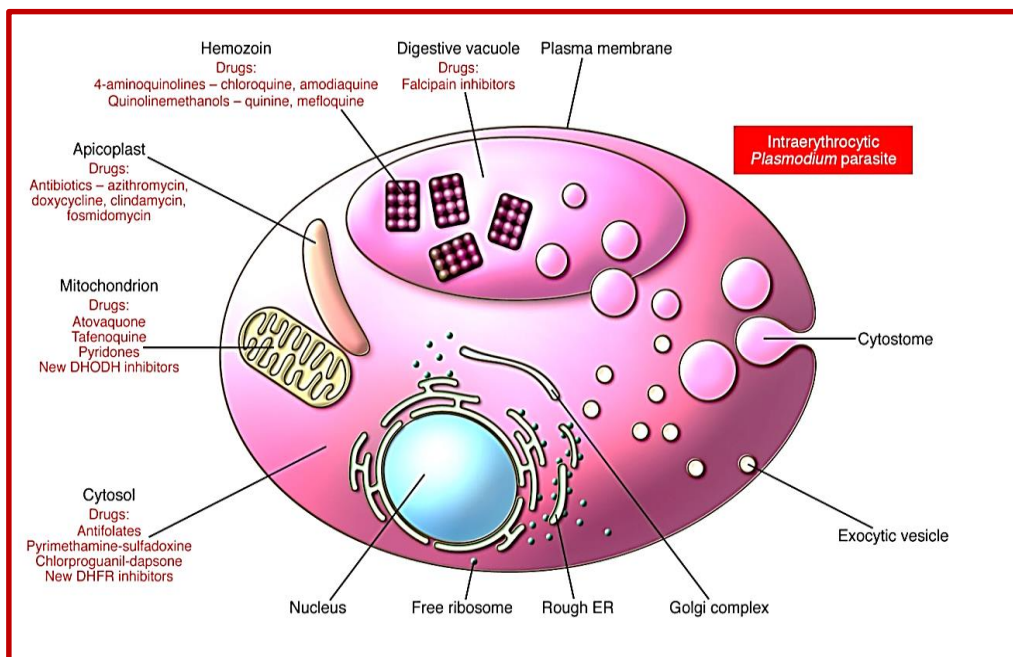


Fig 1.11: Antimalarial targets: Showing the various drug targets for antimalarials. Source: Greenwood et al., 2008.

1.9 Antimalarial drug resistance

Antimalarial drug resistance is often referred to when an antimalarial drug shows reduced or no efficacy in patients or during tests. This often leads to an increase in the half maximal effective concentration of the drug (EC_{50}). This is the concentration of the drug required to produce half of the maximal effect of the drug. The result is a shift in the dose response curve as depicted in fig 1.12 below.

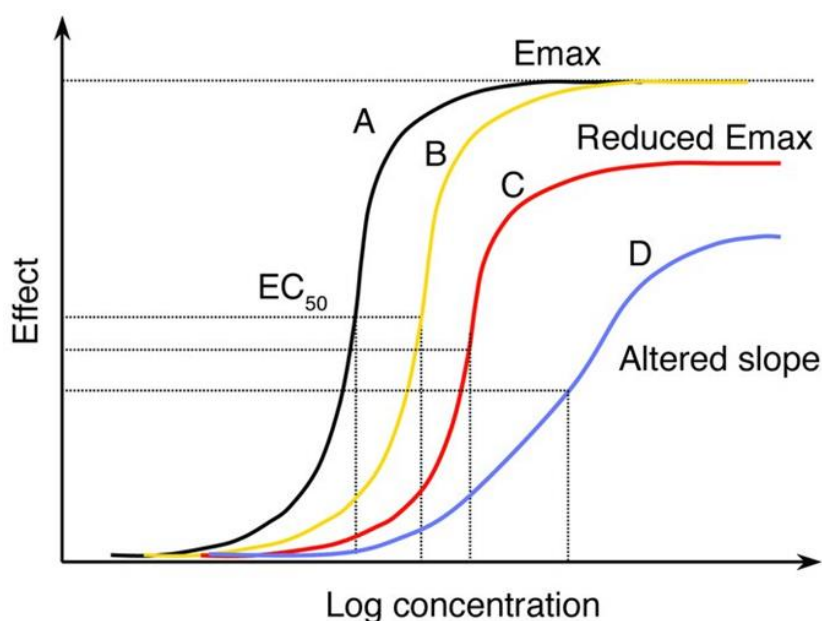


Fig 1.12: Effect of drug resistance on a dose-response curve: Showing the effect of drug resistance on a typical dose-response. Increasing resistance leads to a rightward shift. Hence a higher dose is required to achieve the desired EC_{50} . (Source: White, 2004).

Several methods can be used to investigate drug resistance, including *in vivo* and *in vitro* tests; animal model studies and molecular characterization. In many cases, these tests are not used and presumptive treatment is the rule. Detection of treatment failure is also presumptive and this can be misleading as many symptoms of malaria are non-specific and can be experienced in other diseases. Re-occurrence of symptoms are often thought to be

due to malaria treatment failure although other factors could be attributed to it (Marsh *et al.*, 1998).

1.9.1 Background to antimalarial resistance

The wide spread use and misuse of chloroquine and other antimalarials have stemmed the emergence of parasite strains that are resistant to drug treatment. Parasites have currently shown resistance to almost all drugs in use. This is a colossal public health concern as all efforts to fight the disease are crippled (Marsh, 1998). Furthermore, resistance to other frontline antimalarials is on the increase, which compromises the effort for the fight against the deadly disease (Von seidlein and Greenwood, 2003). Geographical locations may impact on the development of resistance. Drug resistance has also been implicated in the spread of the disease to other regions and re-emergence of the parasites in areas where the disease has previously been eradicated. *P. falciparum* resistance to chloroquine first emerged from South East Asia in the 1950's and the resistance spread to other parts of Asia and eventually Africa within a span of thirty years (Hastings *et al.*, 2000). Other important antimalarials that were discovered after chloroquine such as fansidar (pyrimethamine-sulfadoxine) showed an even worse trend with resistance spreading even more rapidly from the South East Asian region to Africa (Black *et al.*, 1981). Mefloquine, another antimalarial that was introduced showed resistance very quickly within five years of its introduction and this resistance first emerged in Thailand, Cambodia and Vietnam (SE Asia) probably owing to their wide usage. Due to the high resistance of these drugs especially for *P. falciparum* malaria, they are no longer used for the treatment of complicated *P. falciparum* malaria (Wernsdorfer *et al.*, 1994).

Chloroquine is still the drug of choice for *P. vivax* although its efficacy is declining in endemic regions with few resistant strains recorded 30 years after chloroquine resistance to *P. falciparum* emerged (Rieckman *et al.*, 1989). Recent reports indicate resistance to the current frontline treatment, artemisinin. Two confirmed foci for the resistance are the Thai-Cambodian and the Thai-Myanmar borders. Studies have shown changes in parasite clearance half lives in patients over a span of 10 years. Patients have shown an increased parasite clearance half –life. In 2001, reports showed a clearance half- life of 2.6 h whilst in 2010, a clearance half-life of 3.7 h was recorded. The studies also showed an increased proportion of parasites with slow clearance (Phyto *et al.*, 2010). A supportive study by Cheeseman *et al.*, 2012 also demonstrated this by mapping single-letter DNA differences

among 91 parasites. The team found evidence of recent strong selection most likely due to evolutionary pressure to evolve drug resistance in 33 regions of the *P. falciparum* genome. The researchers further looked for associations between each of these genomic regions and clearance rates, using an archive of blood samples taken from Thai malaria patients between 2001 and 2010. Less than 5% of the parasites from 2001 showed slow clearance rates but by 2010, more than 50% did. The study further showed that variations at two adjacent areas on chromosome 13 of the parasite genome were strongly associated with drug resistance. The researchers estimate that this region accounts for at least one-third of the heritable variation in clearance rates. The exact mechanism is however yet to be understood.

The issue of resistance is a great public health concern and hinders the global efforts for the fight against the disease that poses a huge threat to the lives of many. There are no reports of its spread to Africa yet, where the huge burden of the disease lies but it will only be a matter of time if it is not controlled.

Drug	Introduction	Year resistance reported	Difference (years)
Quinine	1632	1910	278
Chloroquine	1945	1957	12
Proguanil	1948	1949	1
Sulfadoxine-pyrimethamine	1967	1967	<1
Mefloquine	1977	1982	5
Atovaquone	1996	1996	<1
Artemisinin	1971	2006?	35

Table 1.3: Drug resistance development: Showing the development of resistance for *P. falciparum* over the decades. (Source: Wongsrichanalai et al., 2002).

1.9.2 Mechanisms of resistance

Antimalarial drug resistance may develop via different mechanisms. Biochemical mechanisms of resistance have been well defined for chloroquine, antifolates and atovaquone. Molecular markers for *P. falciparum* resistance for these drugs are available.

- I. *Chloroquine*: This drug normally exerts its antimalarial activity by accumulating in the parasite food vacuole inhibiting the bio-crystallization of the toxic haem to haemazoin. Resistance occurs when the parasite develops an increased capacity to expel chloroquine via the efflux pump at a rate that is 40 to 50 times faster in resistant strains than in normal strains. This effect will hence not allow the drug to reach therapeutic levels required to cause inhibition (Folay *et al.*, 1997). Proton pump inhibitors such as verapamil have been shown to reverse chloroquine resistance (Martin *et al.*, 1987).
- II. *Antifolates*: Resistance arises due to a mutation in the parasite genome leading to a reduced sensitivity to the given drug. For some drugs, it only takes a single mutation for the parasite to develop resistance to the drug whilst others require multiple mutations. In this circumstance, drug pressure will kill the susceptible population leaving the parasites with the advantageous mutated gene, in this case the resistant ones. The resistant parasites will multiply leading to a new generation of resistant strains. Antifolate drug resistance is mediated *via* this mechanism. Gene mutations encoding for resistance to both dihydrofolate reductase and dihydropteroate reductase, (the two enzymes that are inhibited by antifolates) occurs. Several combinations of mutations may occur in these enzymes leading to varying degrees of resistance to antifolate drugs (Plowe *et al.*, 1998).
- III. *Atovaquone*: Atovaquone normally exerts its antimalarial activity through the electron transport chain. Resistance arises due to a single point mutation in the cytochrome b gene which is involved in the electron transport chain. Atovaquone resistance occurs very rapidly when the drug is used as monotherapy (Korsinczky *et al.*, 2000). Resistance can be slowed by using the drug in combination with proguanil (malarone) or in combination with tetracycline.
- IV. *Sulphadoxine/pyrimethamine resistance*: Specific gene mutations encoding for the DHPS and DHFR enzymes have been identified. The enzymes from resistant strains bind to pyrimethamine with less affinity (400-800 fold) than to enzymes of drug sensitive strains. DHFR mutations appear to be more important in causing treatment failure than DHPS mutations (Gatton *et al.*, 2004)

Some malaria strains are resistant to multiple drugs. Multi drug resistance refers to strains resistant to chloroquine, mefloquine and pyrimethamine sulfadoxine combination. This has been documented worldwide and is attributed to mutations in a specific gene loci (Reed *et al.*, 2000). In Thailand, *P. falciparum* is highly resistant to chloroquine, pyrimethamine + sulfadoxine combination as well as quinine. In Sudan, a high percentage of different chloroquine resistant isolates of *P. falciparum* parasites have been shown to be resistant to more than one drug (Elbashir *et al.*, 2008).

1.9.3 Factors contributing to resistance

Several factors have contributed to the development of resistance. These include parasite biology, treatment failure and pharmacokinetics.

I. Treatment failure: One important point to note is that treatment failure does not necessarily mean resistance but could be a contributing factor to resistance. Treatment failure could arise due to several factors including incorrect dosing, drug interactions, misdiagnosis, patient compliance, poor drug adsorption.

II. Parasite biology: This is an outcome of natural selection. Increased drug pressure leads to an expected Darwinian biological phenomenon of ‘survival of the fittest’. In any large population, a few strains will have the ability to overcome potential noxious stimuli, in this case the ability to overcome antimalarial drug treatment. Antimalarial drugs hence kill the susceptible strains leaving those with the advantageous traits. Susceptible strains can also acquire resistance through mutations, accepting resistant genes from nearby organisms leading to cross resistance. Furthermore, effective antimalarial treatment is thought to clear majority of parasites in the blood stream leaving a few residuum parasites. These residuum parasites get cleared by the immune system. In an immune person, the clearance is in a specific way while in the non-immune person, it is in a non-specific manner. Similarly, in the immunocompromised person or children, residuum parasite clearance also occurs in a non-specific manner as the immune system is weak. This non-specific clearance of residuum parasites is an important factor in the development of resistance. Because the non-specific clearance of the parasites by the immune system is not as effective as the specific clearance of the parasites, immunocompromised patients and children might have some of these residuum parasites uncleared. Overtime, the remaining parasites multiply and grow and because they have previously been exposed to drug treatment, they might have conferred the advantageous resistant gene leading to resistance (Wernsdorfer *et al.*, 1991).

III. Pharmacokinetics: Different pharmacokinetic issues may be a contributing factor in the conferment of resistance. Normally in antimalarial chemotherapy, drugs with long half-life are often preferred over those with a short half-life. This allows for a simpler dose regimen thereby increasing patient compliance. Although this may seem an advantage on one hand, it is also a disadvantage on the other. A drug with a long half-life has a longer elimination period and this could increase the chances of the development of resistance. Drug combinations are often designed based on mechanism of action profiles of individual drugs. The main flaw with these strategies is pharmacokinetic profiles are not often taken into consideration in combination therapy. In the case of the combination of pyrimethamine + sulfadoxine, pyrimethamine has a half-life of 80-100 h while sulfadoxine has a half-life of 100 to 200 h. This means that pyrimethamine clears long before sulfadoxine, leaving the sulfadoxine acting alone without the synergistic effect that occurs with the drug combination (Watkins *et al.*, 1993). In Thailand, combinations of mefloquine with pyrimethamine+sulfadoxine are sometimes used. This situation is even worse than the example previously described as mefloquine on its own has a half-life of 336-432 h. This means that in that combination regimen, mefloquine is left to act alone for over 200 h after pyrimethamine + sulfadoxine have cleared (Palmet *et al.*, 1993).

IV. Drug administration: Dose regimens are based on age rather than weight hence another important pharmacokinetic factor that contributes to emergence of resistance. Children in particular, often have different weights and the assumption that children fall into certain weight ranges according to age groups is not always the case. This might lead to a situation of over dosing or under dosing.

V. Drug pressure: This might be one of the biggest contributing factors for the development of resistance. Several programs involve mass drug administration and these have a great impact on parasite resistance (Wernsdorfer, 1994). Resistance has been shown to be higher in regions with higher accessibility to antimalarials such as urban areas than in regions with less or no accessibility to antimalarials such as rural areas (Ettling *et al.*, 1995).

VI. Counterfeit drugs: Substandard and counterfeit drugs contribute enormously to drug resistance. WHO estimates that 60% of counterfeit drugs do not have any active ingredients, 17% contain too much or too little and the remaining 16% contain the wrong active ingredients ((WHO, 2003). The WHO further estimated that nearly half of the global pharmaceutical market is occupied by counterfeit drugs (WHO, 2006). As parasites are exposed to subminimal doses, this will lead to resistance.

1.10 Combination therapy

The WHO treatment guidelines (2015, report) are based on updated treatment recommendations based on new evidence as well treatment regimens that can be used to prevent malaria in high risk groups. The rational use of antimalarials is very important and highly recommended. It is advocated that treatment should be given to confirmed cases and drugs should be used in combination to prevent or delay the development of resistance. The guidelines also recommend appropriate weight based dosing to improve the chances of curing the disease in affected individuals. Various treatment regimens are currently available. Uncomplicated malaria treatment regimen includes a combination of two or more unrelated drugs with one preferably being an artemisinin derivative. The artemisinins are highly efficacious and act rapidly. They have been shown to act on all parasite developmental stages including reducing gametocytogenesis (Price *et al.*, 1996). Combinations currently prescribed can be categorized into three:

- i) WHO recommended artemisinin based combinations
- ii) Non artemisinin and quinine based combinations
- iii) Other combinations

Examples of combination regimens include Artemether-lumefantrine (Coartem) and amodiaquine-artesunate (Coarsucam). These are the most widely used combinations, whereas dihydroartemisinin-piperaquine (Euartesim) and artesunate-pyronaridine (Pyramax) are the most recently approved combinations (Anthony *et al.*, 2012).

Several re-formulations with doses specific for children and pregnant women are in clinical trials. Chloroquine, a 4-aminoquinoline, is still the recommended treatment for *P. vivax* infections because resistance has not fully developed in contrast to *P. falciparum*. In areas where chloroquine resistant *P. vivax* is endemic, artemisinin combination therapies are recommended as first-line treatment (except artesunate and sulfadoxinepyrimethamine, which is ineffective owing to resistance), in particular those with partner drugs with long half-lives (e.g. dihydroartemisinin and piperaquine and artesunate and amodiaquine). In all cases, a 15-day course of primaquine is required to prevent relapse and provide a radical cure.

For travelers visiting areas with endemic malaria transmission, the CDC recommends atovaquone-proguanil (Malarone), chloroquine, doxycycline or mefloquine, with specific recommendations depending on individual and regional factors.

ARTEMISININ-BASED		NON-ARTEMISININ BASED	OTHERS
Artemether-Lumefantrine		Sulfadoxine-pyrimethamine (SP)	Piperaquine-dihydroartemisinin-trimethoprim
Artesunate Amodiaquine	+	SP + Chloroquine	Pyronardine + Artesunate
Artesunate Mefloquine	+	SP + Amodiaquine	Chlorproguanil-Dapsone + Artesunate.
Artesunate sulfadoxine-pyrimethamine	+	SP + Mefloquine	
Dihydroartemisinin + Piperaquine		Tetracycline or doxycycline + Quinine	

Table 1.4: Antimalarial drug combinations: Showing WHO recommended antimalarial drug combinations. The combinations are classified into three: artemisinin based antimalarial combinations; the non artemisinin based combinations and other combinations.

1.11 Drug discovery process

Developing a new drug from original idea to the launch of a finished product is a complex process which can take 12-15 years and may cost in excess of \$1 billion. The high cost associated with drug discovery process is attributed to several factors, one of which is the fact that for every 5000 to 10000 compounds that enters the research and development (R & D) process, only one gets to the market. This high attrition rate makes the process very time consuming and expensive. The idea for a target usually comes from different institutions including academic, commercial or clinical research sectors.

There is an urgent need to develop new antimalarial drugs that can target the blood stages of the disease to alleviate symptoms; the liver stages to prevent relapses and vaccines to prevent the disease. Currently, there is a robust drug discovery strategy for the blood stages but efforts towards the liver and transmission stages are still in their infancy. Targeting these stages could be instrumental in eradicating the disease. New drugs must meet several stringent criteria to meet the current requirements for new antimalarials. They must be fast acting in order to prevent the disease from progressing quickly, safe for children and pregnant women and ideally a single dose regimen to improve compliance. The high cost associated to R & D of new drugs is perhaps the reason why pharmaceutical companies have not found it economically viable to heavily invests in diseases such as malaria, which mainly

affect the poor. Recent interest in the development of new antimalarials has been revived however, by NGO's such as the Bill and Melinda Gates Foundation.

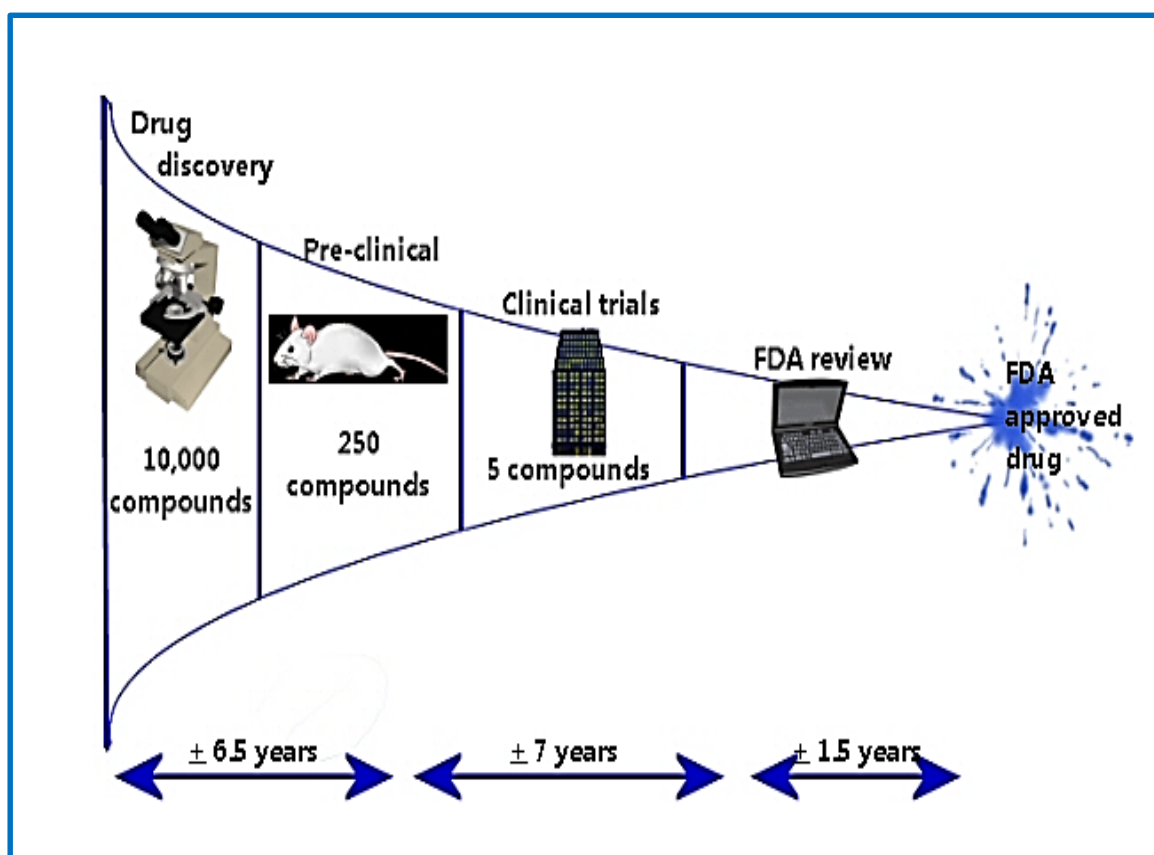


Fig 1.13: Research and development process: Schematic representation of the R & D process with time span, cost and the different stages involved to develop a new drug. (Source: Trends in drug discovery and pharmaceutical research, 2002).

1.12 Approach to drug discovery

The main stakeholders in drug development are pharmaceutical companies and academic institutes. Different approaches are used for the development of new drugs which include rational drug design and random screening.

The recent availability of the genome for humans, *P. falciparum* and *Anopheles gambiae* has steered in an exciting new era for malaria research. Furthermore, fundamental knowledge and bioinformatics has strengthened the basic tools required for functional genomics studies to be developed further. We are now at an era where there is a huge variety of options and different research areas for malariologist all of which are important and

provide a knowledge which can all be streamlined towards a final goal of achieving malaria eradication.

1.12.1 Random screening of compounds

This involves the screening of a large library of compounds against a certain disease using different assays. High throughput screening (HTS) is often used with this approach. HTS is often used as a starting point in the drug discovery process to screen large libraries of compounds against a disease using automated equipment and software. Most drugs that are in use today have been identified from random screening of large libraries. Compounds in the library can be either chemically synthesized products or natural product-based compounds. Historically, the mainstay for lead identification has been natural products which will be discussed next.

1.12.2: Rational drug design

The main goal of this approach is drug target identification. This usually arises from thorough understanding of the pathology and mechanisms of disease. The sequencing of the malaria parasite genome has allowed for systematic analysis of different target proteins. More recently in the past decade or so, the main focus of drug design has been target oriented. 'Druggable genes' identified in parasites have been aided in the identification of various target proteins within the parasite. Compounds have been designed using different computer aided drug design systems to fit the identified targets, which are subsequently tested. The knowledge of the metabolic pathways involved in the disease aids in the design of these highly specific molecules. These usually involve proteins or genes. The main method for protein structure determination is X-ray crystallography. X-ray crystallography determines the structure of a protein by passing a beam of X-rays to the crystal molecule of the protein which causes diffraction of the beams in different directions. The 3D structure is determined based on the angles and directions of the generated beams. The protein is designed to fit the identified target. Although a very novel approach, it is time consuming, costly and some proteins simply fail to crystallize. As a result, the main hitch with this model so far, is a very high attrition rate. Although several targets have been identified through organisations like the Medicine for Malaria Venture (MMV). They have a portfolio of compounds designed to fit targets. Some of these molecules have not been able to go into clinical trials due to toxicity issues (Aguero *et al.*, 2008) that were not addressed at the point

of molecule design. So far, most of the identified hits have proved inefficient as drugs, having little or no effect on actual parasites.

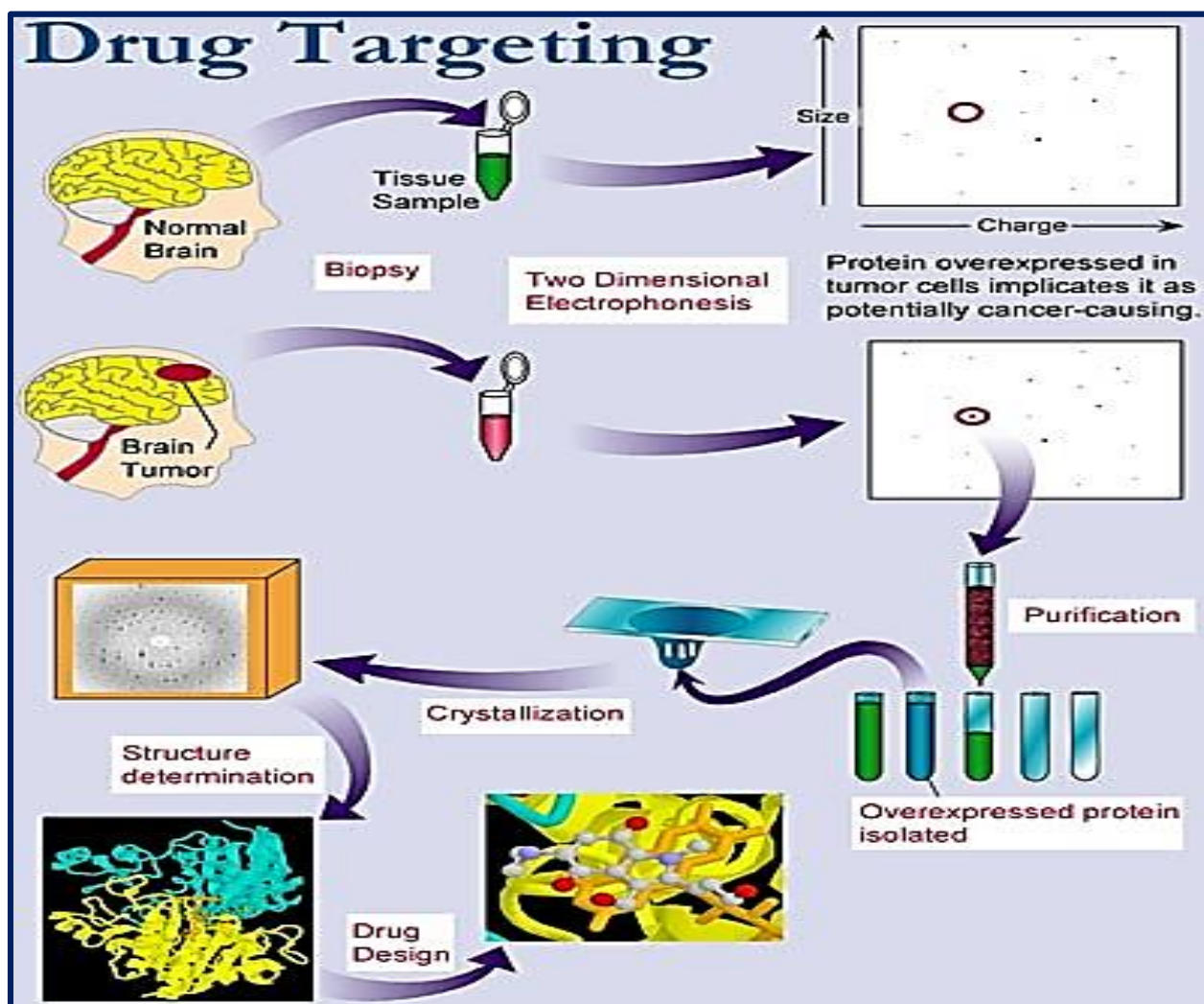


Fig 1.14: Rational drug design: Showing the rational route to drug design. New molecules are designed based on knowledge of biological drug target. (Source: <http://www.wiley.com/college/pratt/0471393878/instructor/activities/proteomics/index.html>)

1.13 Natural products as a route to drug discovery

Primitive man observed and appreciated the great diversity of plants available to him. Man's observation of wild animals through his own trial and error led to much of the development of medicinal plant usage today. Before the era of writing, the knowledge of plant medicine was passed on from generation to generation by word of mouth. Documentary evidence for the use of plant medicine can be traced back to about 4000 years where some writings were

found in a clay tablet in the Sumerian culture. The Egyptians have records of several remedies for different ailments which date back to 3000 years. In India, herbal medicine has played an important role in the health care system. This dates back to the collection of Hindu sacred verses. This practice is called Ayurveda which involves a complete lifestyle including medicine, philosophy, health, medicine, exercise regimens and diet. Currently, it may seem that the mainstay provider is modern medicine but the WHO estimates that up to 4 billion people which is about 80% of the world's population, use herbal or alternative medicine at some point in their life. WHO records also show that 74% of natural product derived medicines used in modern medicine are actually used for the same reasons traditional medicine healers used them for. Furthermore, industrialised nations are increasing the use of natural products example in naturopathy. The main downside is that most of the herbs that are being used have not been scientifically validated nor evaluated in clinical trials. With about 75% of the world's population dependent on medicinal plants and no access to modern healthcare system, it is becoming increasingly important to evaluate natural products. Modern medicines are not affordable or available for the vast majority of the world's population with 75% of the population in third world countries living under less than \$2 a day and over 270million children having no access to healthcare (Global monitoring report, 2015). The pharmaceutical industry today is facing huge challenges in terms of cost and it is proving inefficient due to the amount of time it takes for one drug to be available in the market. The average cost and time of discovering and developing a new drug into the market is consistently increasing and there is no corresponding increase in safer and better drugs. Furthermore, the number of new molecular/chemical entities produced per company have declined and so has the number of approvals for new drugs. In 1996, there were 53 approvals and only 23 in 2010 (FDA). There is a huge challenge today for pharmaceutical companies to grow and sustainability is becoming an issue which has given rise to several mergers and acquisitions within the industry. The cost of R & D has risen from \$2 billion USD in 1980 to \$40 billion USD in 2007 with no correlation between the rising cost and new drugs in the market. Also below are some details of how much cost has risen per approved new drug.

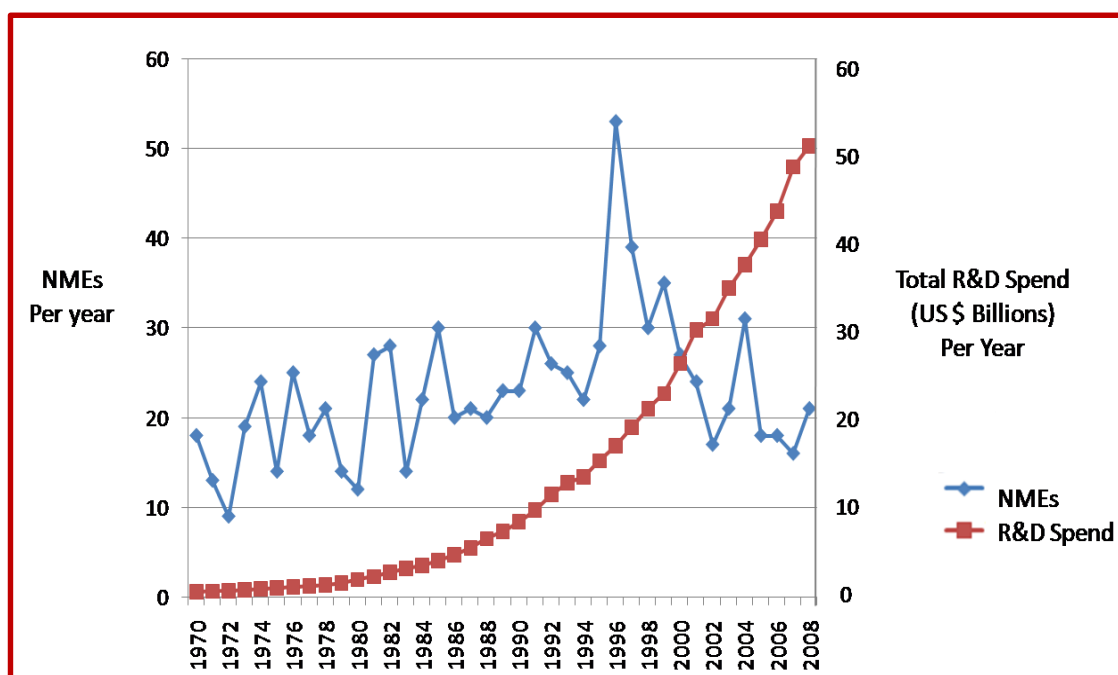


Fig 1.15 New molecular entities (NME) in research and development: Showing NME's per year versus total R&D spend in Billions of Dollars. The number of NMEs per year was trending upward to 1996 but now in recent years, the number appears to be going down. Increased R&D spending has not helped. The R&D spend appears to be out of control. (Source: <http://www.discoverymanagementsolutions.com/wp-content/uploads/2011/09/Figure-1-for-NME-doc.png>).

It is evident that the cost of R & D are exploding hence it is only strategic for other models to be adopted. Approaches based on natural product-based drug discovery such as ethnopharmacology and traditional and complementary medicine are re-emerging as attractive options. Drug discovery need not be confined to molecular entities. Rationally designed, carefully standardized, synergistic traditional herbal formulations and botanical drug products with robust scientific evidence can offer more informed and hopefully safer alternatives in the form of phytopharmaceutical drugs, for example. Due to this concern, WHO initiated the Traditional Medicine Programme (TMP) in some member states. The programme evaluates the traditional medicine systems and practices of different regions to ascertain and establish the basis for the use of different plants separating superstitions and harmful practice from useful real science, hence establishing a dosing system for good prescription services. Moreover, the WHO commission on intellectual property and innovation in public health also duly recognised the promise and role of traditional medicine in drug development for affordable healthcare solutions. TMP has been defined as sum total

of knowledge, skills and practices based on theories, beliefs and experiences indigenous to different cultures used in the maintenance of health as well as in the diagnosis, prevention and treatment of physical and mental health (WHO Traditional Medicine report, 2005).

The Republic of China has successfully achieved incorporating traditional medicine in their healthcare system. Traditional practices including acupuncture and herbs together with modern diagnostic techniques form the basis of their health care system further boosting the economy. Several herbal plantations for the purpose of medicinal plant cultivation have provided various job opportunities.

In India and Africa, traditional medicines although very common, have remained separate from the healthcare system offered by the government despite ayurvedic medicine commonly practised in India. Herbal medicine is becoming increasingly common in Europe and it is dispensed in different forms from essential oils, capsules to tinctures. In the United States, more than 1/3rd of the adult population use complementary and alternative medicine (Complementary and Alternative Medicine press release, 2004). In another survey 18.9% of adults were shown to use some form of alternative medicine (National centre for Complementary Medicine). Several trials have been funded by the National Institute of Health (NIH) to investigate the effectiveness of some herbal medicines.

The World market for herbal Medicine is about \$40 billion USD. In Brazil alone, it is estimated to be around \$1billion USD. Data from WHO shows that more than half the world population makes use of some form of medicinal herb in searching for relief from various ailments. About 30% of these medicines are provided by medical prescription (WHO, 1978).

1.13.1 Historical importance of natural product as antimalarials

Medicinal plants have played an important role in the fight against malaria. Based on the historic high success rate of natural products as a source of medicine with several examples of antimalarials that have been derived from natural products, there is a need to revert to use of natural products as a source for lead identification in malaria drug discovery. Hence by choosing natural products as lead compounds, the time used to understand disease, identify targets, design leads to fit the targets and test the leads in 'rational drug design' can be cut by simply testing the natural product compound in different biological assays. Furthermore,

the lead identification process can be guided by the ethnopharmacological usage of the plant which is more likely to generate a hit than random selection.

The first effective antimalarial known to man was a natural product from the bark of '*Chinchona*' plant, which is what we now know today as quinine.

1.13.2 Discovery of quinine

The tree grows on the slopes of the Andes in Peru (South America). It was mainly used by the natives as a tincture. It has been referred to as 'Jesuits bark', 'cardinal's bark', and 'sacred bark'. The name sprang of its usage by the missionaries in the Andes in the 1630's. A legend suggests earlier use by the native population. According to the legend, an Indian with a high fever was lost in an Andean jungle. When he drank from a pool of stagnant (standing) water, he found it tasted bitter. Realizing it had been contaminated by the surrounding quina-quina trees he thought he was poisoned. But his fever abated, and thereafter his village used extracts made from quina-quina bark to treat fevers. Another legend of quinine is that of the countess of Cinchon who visited Peru and fell ill and her fever was cured by this tree bark. The use of the plant as a fever cure was rapidly accepted (Kaufman and Ruveda, 2005). The tree was named '*Cinchona*' in 1742 by a Swedish botanist Carol Linnaeus (1707-1778) in honour of the Countess of Chinchon. In 1820, *Cinchona* and quinine were isolated as alkaloids from the powdered fever tree bark by Pierre Joseph Pelletier (Kyle *et al.*, 1974). By 1834, a German Physician, Carl Warburg, had used a concoction of different herbs to make an antipyretic tincture called 'Warburg's tincture'. The tincture contained quinine, amongst other herbs. This tincture gained a huge international recognition at the time. The British government supplied this tincture to its troops in India and other colonies (Maclean, 1875).

In the early part of the 20th century, Java (current Indonesia) was the main producer of the naturally produced quinine. The events of World War I, led Germany to have no supply of quinine and hence they developed a synthetic substitute, Atabrine. During World War II when America joined, the quinine plantations in Java were controlled by Japan. American Soldiers fighting in North Africa were badly affected by malaria and pills obtained from captured Italian soldiers were sent to the United states and discovered to be chloroquine also manufactured by Germany. The United States produced its own supply by the end of the war. By 1934, Hans Andersag and co-workers synthesized chloroquine which was named resochin. This compound did not gain popularity due to its toxicity. The drug however, went

into clinical trials for malaria in World War II and was considered as an official antimalarial drug in 1946 naming it chloroquine. The correct chemical structure was not confirmed until 1944 by the American chemists, Robert Burns Woodward and William Von Eggers Doering. Woodward won a Nobel prize. Quinine was used successfully until resistance developed after its synthesis (Kyle *et al.*, 1974). Several other natural product-derived drugs are in use today as antimalarials, one of the most recent examples being the development of artemisinin from the plant *Artemisia annua*.

1.13.3 *Artemisia annua*.



Fig 1.16 *Artemisia annua* plant

The plant has a long history of use in Chinese traditional medicine for haemorrhoids since 168BCE. By the 4th century, Ge Hong recommended its use for intermittent fevers (Wright *et al.*, 2010). Although quinine has been established as an antimalarial earlier, artemisinins have a longer history of being used as ‘fever cures’. Artemisinins were discovered as antimalarials in 1971 when the Chinese Army embarked on a project to screen several plants against malaria. Over 2000 plants were screened and *qinghao* was found to be active against *Plasmodium berghei* in mice by a Chinese pharmacologist Tu Youyou. She later isolated artemisinin from the extract (Qinghaosu and C-ORGO, 1979). The compound went through further research and clinical trials and the findings of this pharmacologist were presented at the United Nations meeting in 1981 which led to the development of artemisinin.

1.13.4 Other natural product derived antimalarials

Tetracycline, a natural product-derived compound was discovered in the 1940's. In 1970, the Walter Reed research foundation discovered mefloquine and halofantrine as result of a random screen of over 300,000 compounds.

1.13.5 Classes of natural product compounds with antimalarial activity

In the previous years, there has been an immense research on natural products for antimalarial activity which has led to the identification of several classes of compounds that are active against malaria. These include alkaloids, terpenes, flavonoids, quinones, xanthenes and coumarins, peptides, phenols and lignans. Furthermore, several compounds have been isolated from various natural products with their structures determined. Fig 1.17 below shows some natural product scaffolds that form the basis of different antimalarial drugs.

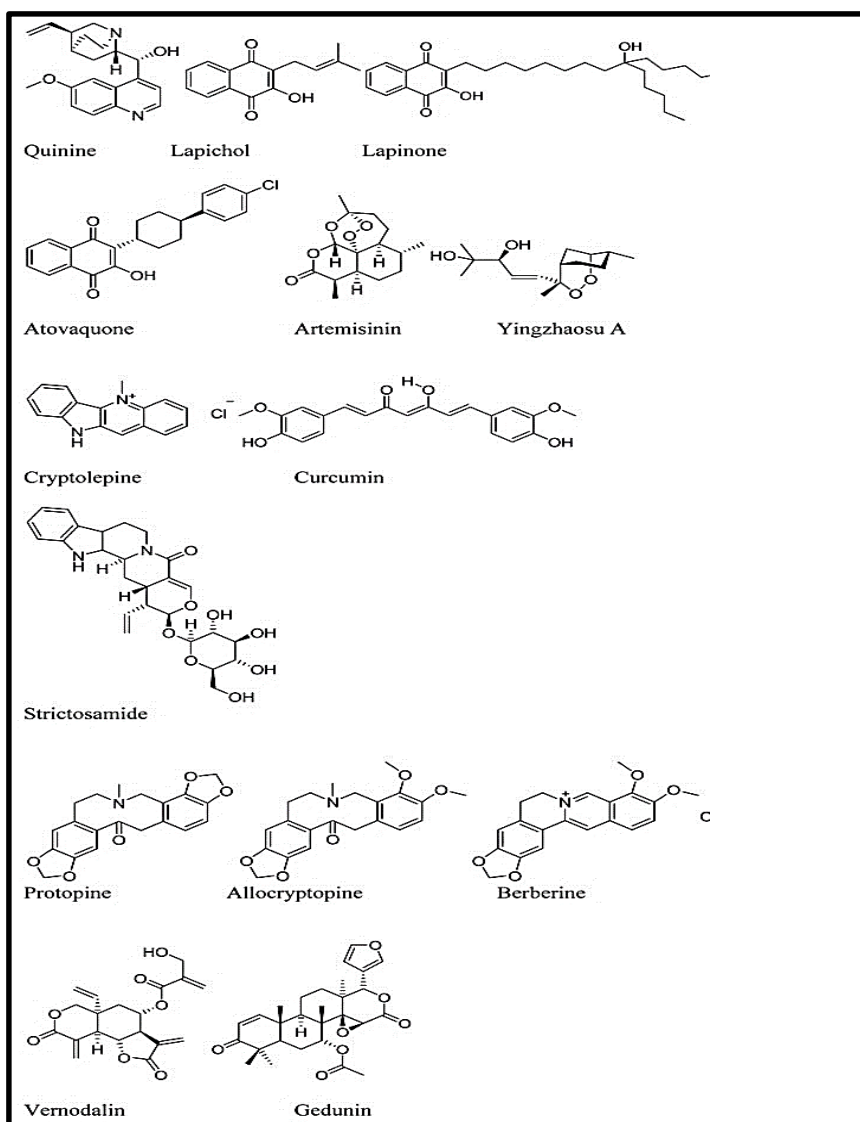


Fig 1.17: Natural product antimalarial scaffold: Structures of molecules from herbal medicinal products with confirmed activities in humans. Adapted from Wells et al., 2011.

1.13.6 Natural product drug discovery process for malaria

Traditionally, the drug discovery process for natural products involves the screening of a large natural product library for a disease in order to identify some hits, with the ultimate goal of isolating and synthesizing an active compound. Alternatively, a natural product might be selected for drug discovery based on its ethno-pharmacological usage. The screening process involves the use of various *in vitro* and *in vivo* assays to screen against the disease. The natural product extract is subsequently purified and the active ingredient isolated with the ultimate goal of chemically synthesizing an analogue. Fig 1.18 is a schematic representation of the process.

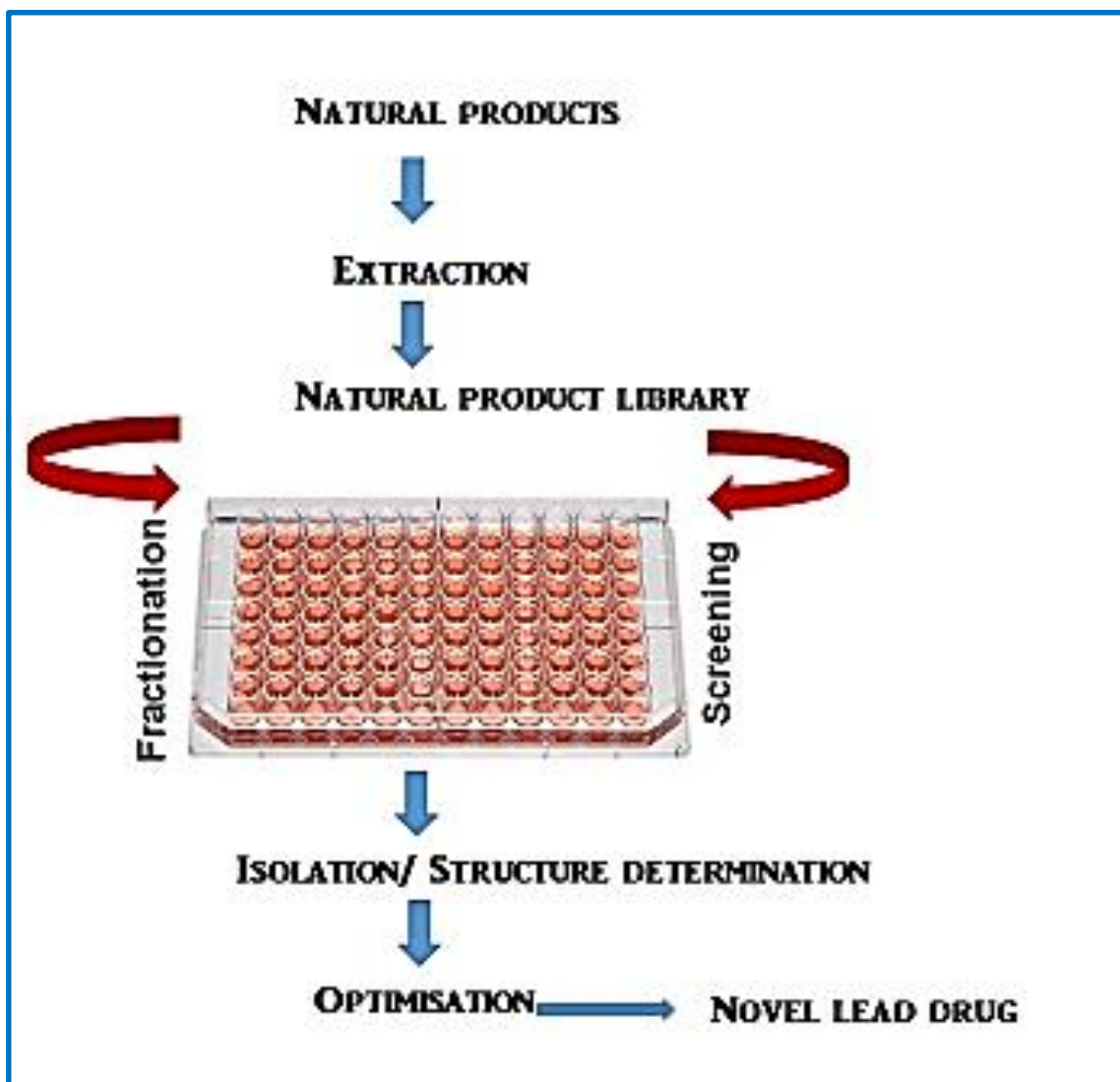


Fig 1.18: Natural product drug development: Schematic representation of the current route to natural product development.

1.13.7 Current techniques in natural product drug discovery

Historically, natural product screening involves using a number of techniques to isolate and purify the active ingredient(s) found within a crude extract. Natural products can be purified and isolated using a wide range of techniques, depending on the constituents to be isolated. Hence, different separation techniques are often used in conjunction with spectroscopic methods in order to achieve a successful purification and isolation of a compound. Natural product scaffolds have been isolated using various techniques including chromatography. Fig 1.19 below is a schematic representation of a typical approach for the isolation of an active compound from a complex natural product mixture.

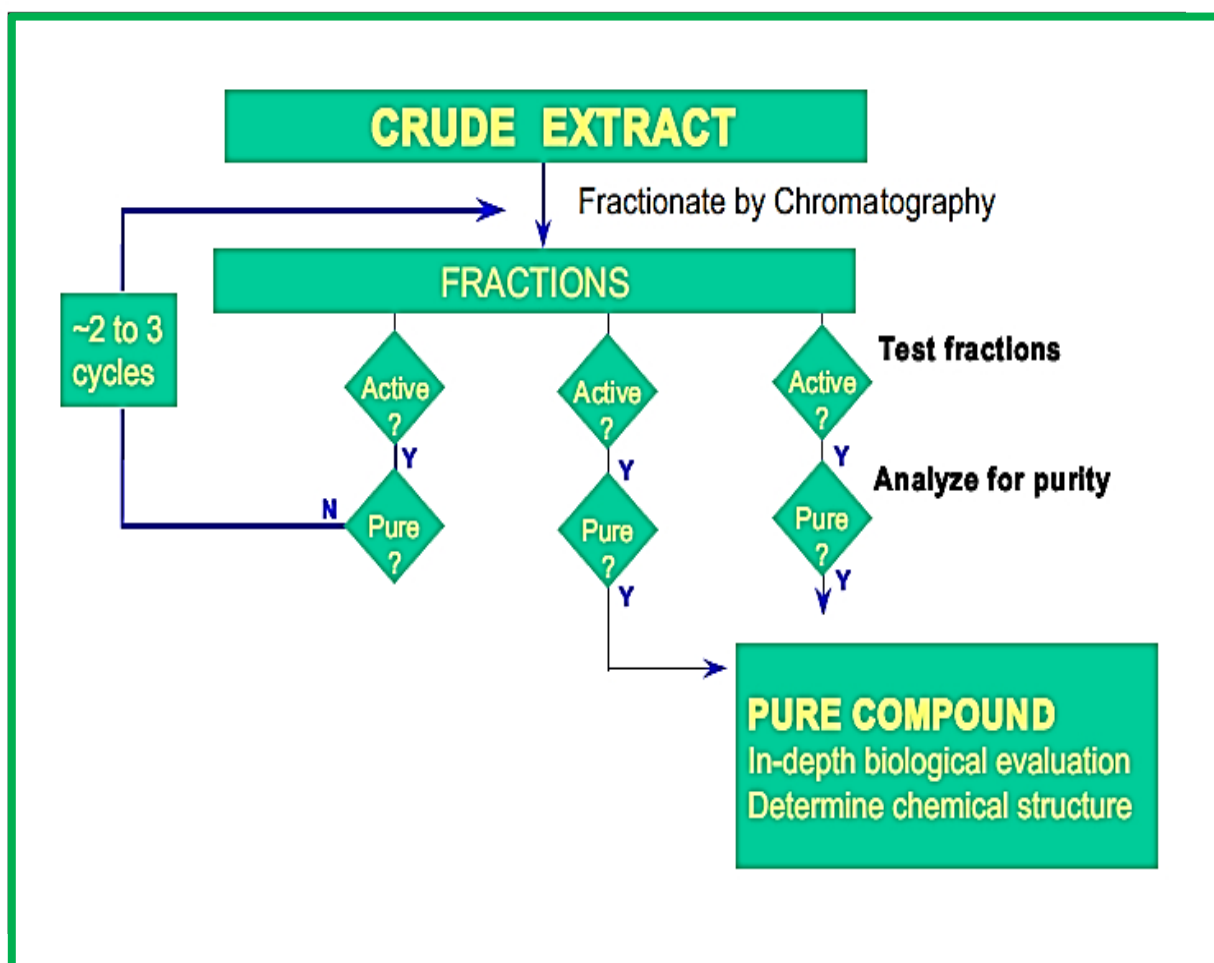


Fig 1.19: Bioassay guided fractionation: Showing a generic schematic flow chart of a typical bioassay guided fractionation process.

The first step in natural product isolation is to have an idea of what components might be present in the extract, which is often researched through existing literature. In some cases, it might be difficult to have an idea, in which case various qualitative tests can be done to reveal the phytochemistry of the extract to confirm the presence of compounds such as phenols, flavanoids, tannins and other phytochemicals. This knowledge can be used to design a protocol for isolation of a compound. Certain information such as knowledge of the solubility, acid base properties of the compound can be critical in the isolation process. In recent years, the most common technique that is used for natural product separation and purification is chromatography. High performance liquid chromatography (HPLC) is the most commonly used technique. There are different types of HPLC including normal phase and reversed phase chromatography. Reversed phase chromatography is commonly used for

isolation of methanolic extracts that have highly polar compounds. Fig 1.20 shows a schematic representation of a HPLC system.

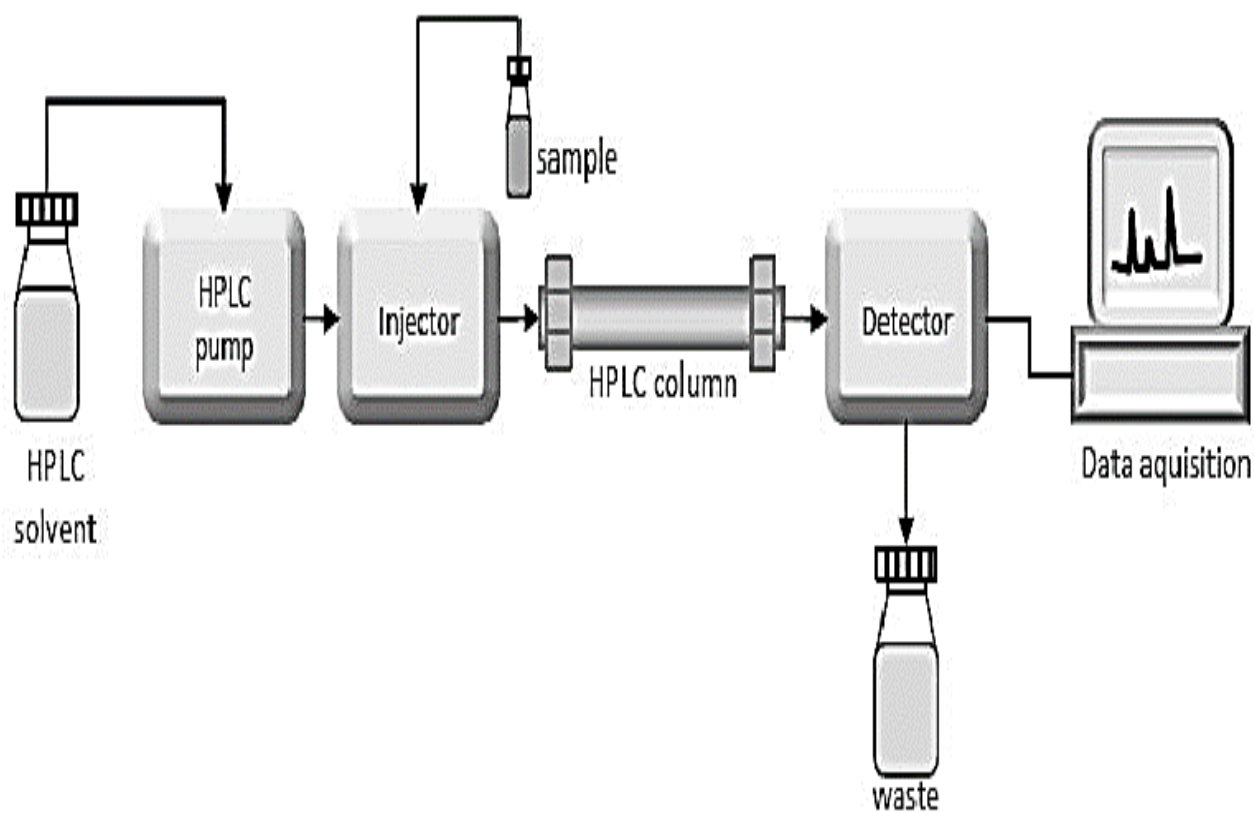


Fig 1.20: HPLC system: Showing the main components of a HPLC system with a data acquisition system at the end which normally produces the chromatogram. (Source: <http://www.laboratoryinfo.com>).

The main draw back with current methods for antimalarial drug discovery, is that so far, the focus has been on purification and isolation of a single active compound from an extract. Although a vast potential of success hit rates have been recorded, many of these have not been translated into drugs. Pharmaceutical companies, government regulatory bodies and investors have not shown enough interest and a lot of effort today is focused on target oriented drug design. The arguments put forward against natural product drug development range from issues related to reliability, accessibility and supply; seasonal or environmental variations in the composition of living organisms and loss of source though extinction or legislation. More practical concerns include complexity of natural product mixtures after

fractionation, the isolation of very small quantities of bioactive substance and challenging physicochemical properties such as solubility and stability. Most anti-malarial compounds isolated from natural sources are usually only moderately active, or possess challenging physicochemical and biological properties, and as such represent ‘hits’ rather than actual lead drug candidates (Vederas *et al.*, 2009). This is probably due to the fact that natural products usually exist as a complex mixture of several biologically active phytochemical compounds. The reason why isolated compounds might only show moderate activity is because they often act in synergy and in some way nature has balanced the toxic effects of some compounds by the presence of other compounds which might neutralise that toxicity, hence achieving an overall effect (Ulrich-Merzenich *et al.*, 2010). The process of purification might therefore lead to the loss of several active components and the synergistic and additive interactions between fractions might be lost too.

There are several solutions today to the main bottlenecks in natural product drug discovery. Recent technological advances in biotechnology, for example have provided us with in-depth understanding of the genomics of natural product biosynthesis. This may enable the efficient production of the same bioactive molecules from plant cell cultures and genetically engineered microorganisms hence combating the problems of supply issues. This has been demonstrated recently with the large-scale microbial production of the artemisinin precursors, amorpha-4,11-diene and artemisinic acid, from genetically modified *Saccharomyces cerevisiae* and *Escherichia coli* (Zeng *et al.*, 2008). Communities could be massively engaged in farming for these products providing economic empowerment.

1.13.8 Research and development pathways for medicinal plants

The WHO has in recent times promoted the development of ethnopharmacological drug formulations, allowing researchers to follow different approaches. One approach that has been of considerable interest over the past two decades is reverse pharmacology. It is the science of integrating documented clinical/experiential hits into leads by transdisciplinary exploratory studies which further develops it into drug candidates. Though reverse pharmacology, complementary and alternative medicine (CAM) can be strengthened in the healthcare system. Botanical formulations with distinct pharmacological properties can be substitutes for the management of several human disorders. Fig 1.21 below shows a summary of the different routes currently in traditional/herbal medicine research available

including the standard route of purifying and isolating the active compound from the natural product.

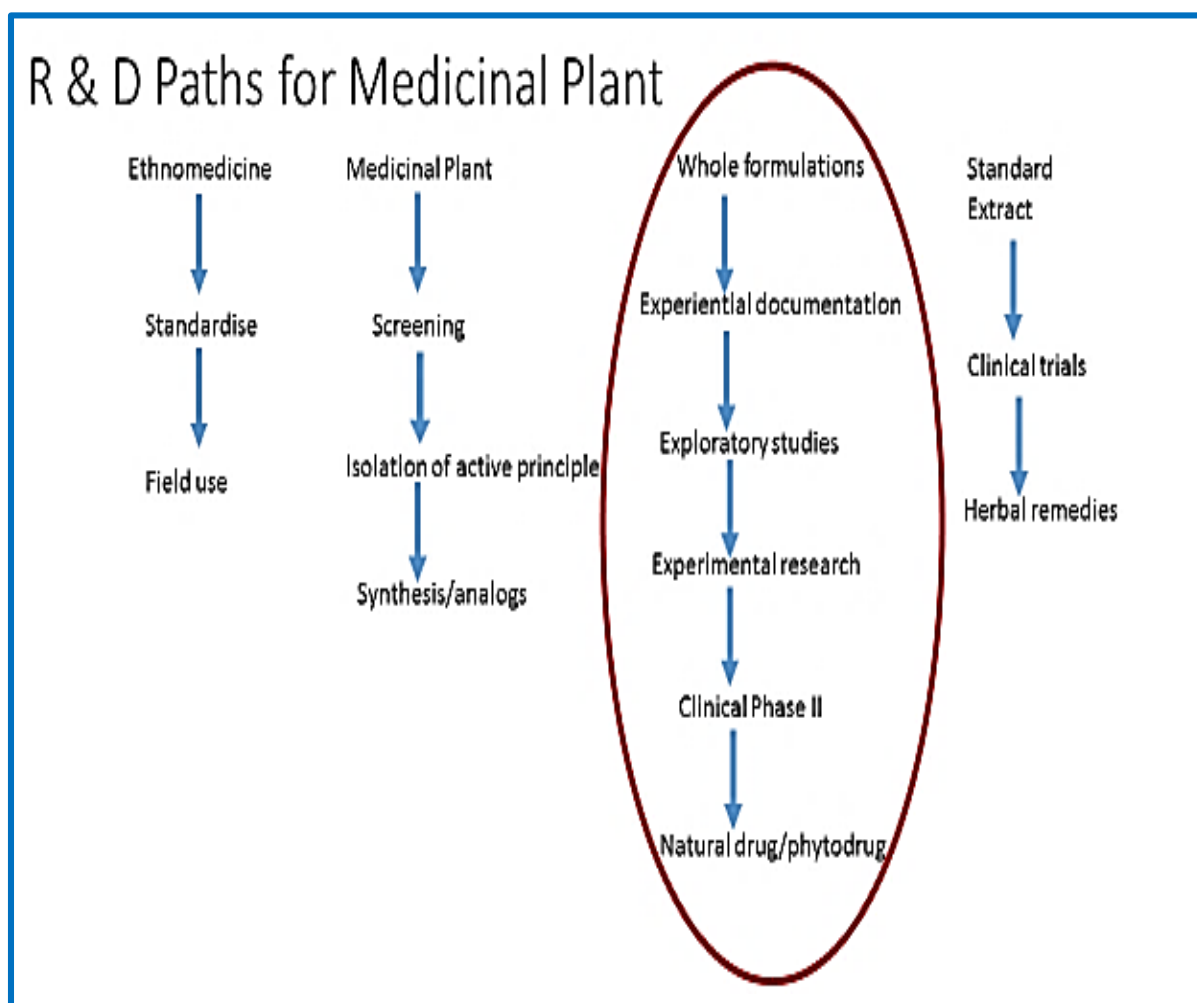


Fig 1.21: Routes to medicinal plant research: Showing different routes for medicinal plant research highlighting the reverse pharmacology approach.

Although the reverse pharmacology approach is very controversial, it has proved to be a much faster alternative and has so far proved very beneficial within communities. There have been a number of case studies that shall be discussed in due course. One of the main benefits/goal of this approach is to develop a phytopharmaceutical drugs. Phytopharmaceutical drugs have a number of benefits. They are considerably faster and cheaper to develop. They can be more sustainable in certain parts of the world as communities tend to feel it is a safer alternative. The fact that it involves agriculture also means it can be incorporated in poverty alleviation programs in developing nations. In the

case of malaria for example, using phytopharmaceutical drugs might ease pressure on standard drug usage hence reducing resistance. The reduction in R & D cost by this approach comes with the fact that proven use of the natural product in question by a certain community for often centuries means it can be accepted as some form of safety. The main advantage of a reverse pharmacology approach is that the starting point is a compound that is accepted to be safe and there is a track record of human usage. With a conventional approach on the other hand, research often starts with finding hits which can be time consuming and costly. After hit identification, most studies move on to efficacy studies after which safety studies are done. On several occasions, hits that have been identified and validated in terms of efficacy are often dropped at a later stage of the research during *in vivo* studies due to safety/toxicity issues leading to a high cost implication.

The concept of reverse pharmacology was first developed in India in Ayurvedic medicine and also by Chinese government with the main aim of developing phytopharmaceutical drug products. Later other governments like Brazil and some African countries adopted the model.

1.13.9 Herbal medicine vs phytopharmaceutical drugs

Herbal medicines are plant-based products that are used as a source of cure/prevention for ailments. Herbalism is considered as a form of alternative medicine. The practice is often based on historical use of plants by communities for certain ailments. The practice is often not evidence-based but rather based on historical beliefs and practices. In recent times, some herbal medicines have been researched and considered an important part of alternative medicine. There is however still major concerns with regards to the use of herbal medicine. The practice is often not scientifically validated, there is poor regulation in several countries and the drugs are often not registered nor regulated by health authorities (GAO, 2009). The safety and efficacy of several herbal products remain a concern due to the lack of scientific validation of the medicinal claims of the products. Several organisations are working in partnership with governments, research institutions and academia to address this issue. The WHO, for example is working with member countries to strengthen the traditional medicine systems. The latest strategy employed by the WHO focused on promoting safety, efficacy and quality of the traditional medicine system through the expansion of knowledge base and assisting in the provision of guidance on quality assurance, regulatory systems and standardisation (WHO 2014-2023 TM strategy, Geneva, 2013).

In recent times, the concept of phytopharmaceuticals have sprang up to address the issues encountered with herbalism. The concept of reverse pharmacology has been often used to develop phytopharmaceuticals which are ‘plant-based medicines’ that are standardised and scientifically researched and validated. Quality assurance and regulatory bodies are often involved in the development of phytopharmaceuticals which is often not the case with herbal medicines.

1.14 The concept of reverse pharmacology

Reverse pharmacology is defined as the science of integrating documented clinical or experiential hits into leads by transdisciplinary exploratory studies and developing these further into a drug by clinical research. This concept is inspired by traditional medicinal plant knowledge and involves reversing the usual ‘laboratory to clinic’ concept in normal drug discovery process to ‘clinic to laboratory’ as represented in Fig 1.22 below.

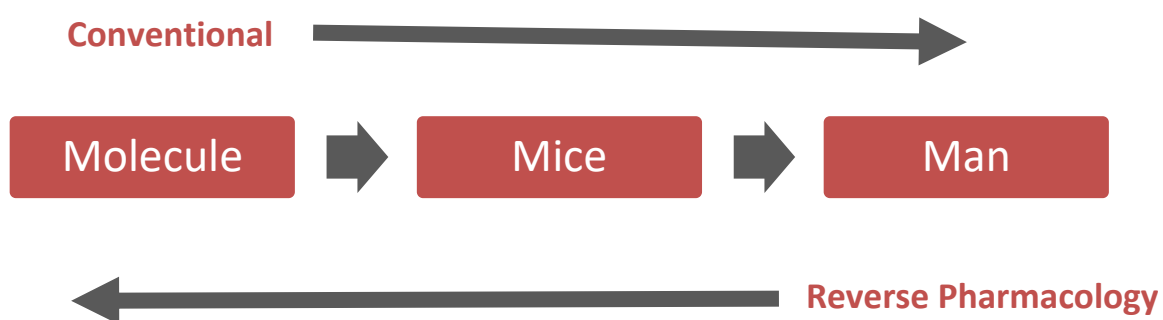


Fig 1.22: Comparison of reverse pharmacology and conventional approach: A diagram comparing the reverse pharmacology vs conventional approach routes in the drug development process.

The scope of reverse pharmacology is to understand the mechanism of action of natural product formulations and optimise their efficacy, safety and acceptability based on scientific evidence. Hence reverse pharmacology is a combined drug discovery approach that merges together both conventional medicine and traditional medicine, the overall effect of which is a faster, cheaper and robust alternative.

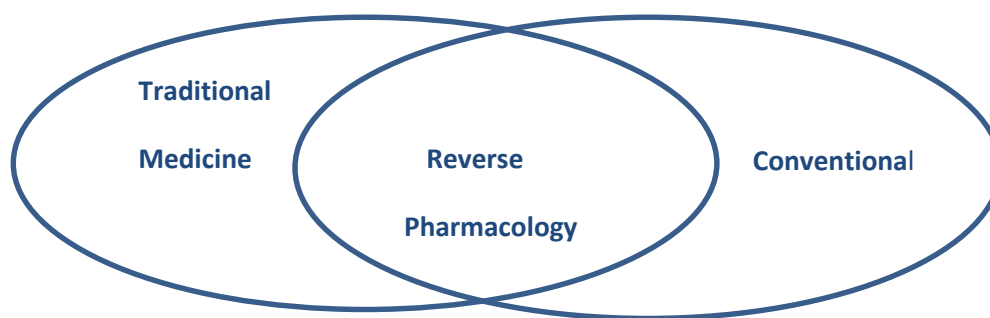


Fig 1.23: Convergence of the two disciplines: Showing how reverse pharmacology is a convergence between traditional medicine and conventional approach.

Reverse pharmacology is a transdiscipline medicine that could involve several phases as detailed below:

1. The first step is the experiential phase which primarily involves a documentation process of clinical observations of the herbal formulation investigated
2. Exploratory studies involves further studies to establish the tolerance/toxicity of the formulation, any possible interactions and establishing exact therapeutic dose ranges both in clinic and also through *in vitro* and *in vivo* studies to evaluate the activity.
3. Experimental studies involve both *in vitro* and clinical studies in various models to validate the safety and efficacy. This is often where most of the laboratory work comes in.

These phases are the main phases that are used in ayurvedic formulation development. In conventional drug development, clinical experiences and observations take place after laboratory validation of a therapeutic claim. These phases are represented methodologically below:

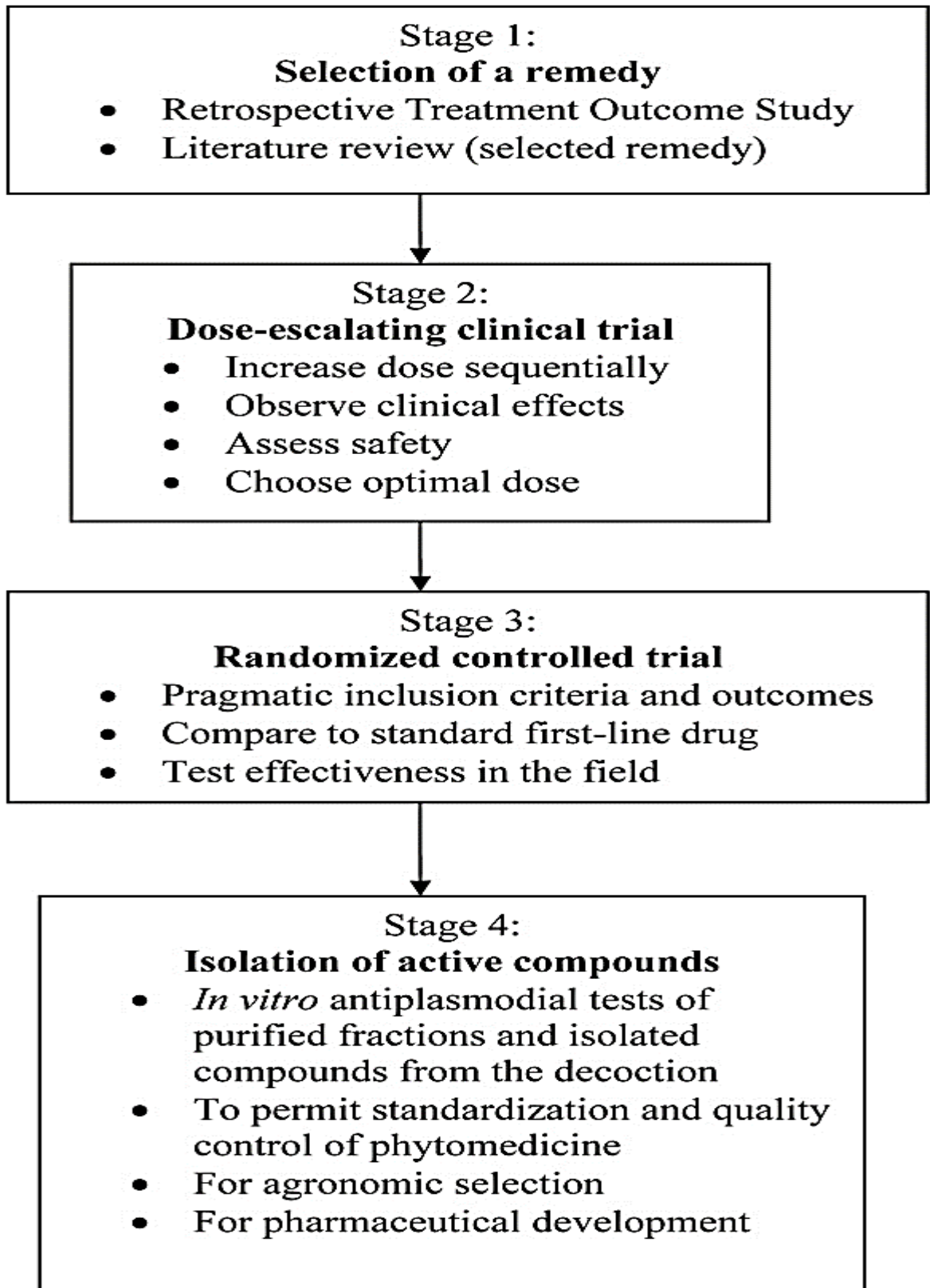


Fig 1.24 Chronological order of a reverse pharmacology: Showing a reverse pharmacology approach (Source: Wilcox et al., 2011).

1.14.1 Case studies of reverse pharmacology/phytopharmaceutical drug development

Different governments across the globe have adopted the reverse pharmacology approach as part of their traditional medicine drug development scheme. Some case studies are discussed in this section.

1.14.1.1 India as a case study for reverse pharmacology approach

Sir Ram Nath Chopra and Gananath Sen are said to be the forefathers of reverse pharmacology. They successfully stimulated the interest of chemists in medicinal plant. Sir Ram, documented the effects of several ayurvedic medicines and used this to lay the foundations of reverse pharmacology. In 1931, Ganannath Sen and Bose reported on the use of an alkaloid extract from *Rauwolfia Serpentina* plant for hypertension and mania. These findings were ignored until 1945 when Nathan Kline, an American scientist, did some studies of reserpine, a derivative of the plant and found that it was more effective than placebo treatment in a trial of about 400 inpatients with psychiatric symptoms (Sen & Bose., 1931; Kline 1954).

Reverse pharmacology in India offers a major paradigm shift in drug discovery, especially with the recent amendment of its drug act to include a category of phytopharmaceuticals. This class of drugs will be developed as medicinal plants with evidence of safety and quality and will be distinct from traditional medicine (TM) usage of herbs and ayurvedic medicine.

The Indian council on medical research (ICMR) recently established an advance centre for reverse pharmacology with initial focus on malaria, sarcopenia and cognitive decline. India can clearly be considered the World's leading country in reverse pharmacology. Recently, an Indian based laboratory did some extensive studies on a plant for psoriasis. The company, Lupin laboratories, successfully filed a new drug application for the drug and phase I trials began in 2004 and was successfully completed. The drug was developed conforming to FDA guidelines for botanicals and is estimated to be considerably cheaper than its Western counterpart. Another success story is in the field of Parkinson's disease. Ancient Ayurvedic physicians have long used *Mucuna pruriens* seeds for the treatment of the disease. The dose used is considerably lower compared to L-dopa, the first line treatment for Parkinson's. These observations led some scientist in India to collaborate with a pharmaceutical company and further investigate the observations. The research led to the development and approval of a national drug for the treatment of the disease. A new drug application was filed with

the FDA, as well as a patent cooperation treaty for the extraction process by the Indian company which led to the development of the drug, Zandopa which is now available. The drug is successfully used as a standardised, safe and economical natural product replacing synthetic L-Dopa preparations in India. Clinical studies with *Mucuna* seed powder suggests that this natural source of L-dopa might possess advantages over conventional L-dopa preparations in the long term management of Parkinson's disease. (Katzenschlager *et al.*, 2004).

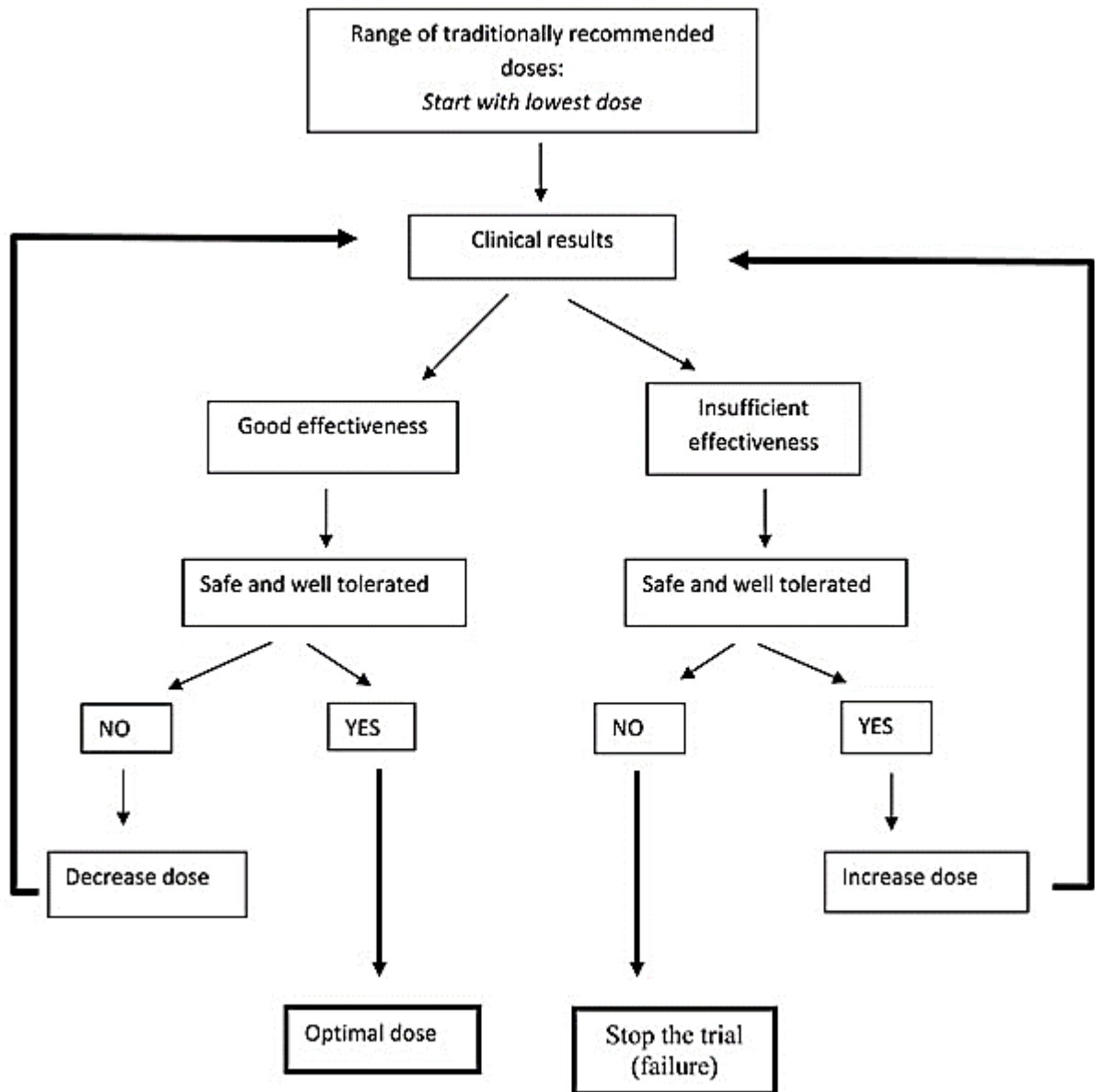
1.14.1.2 Brazil as a case study

In Brazil, about 80% of the population lack access to essential medicine (Toledo *et al.*, 2003). One of the major contributing factor is high cost of medication. For this reason, in 2006, the Brazilian government decided to include phytotherapy as one of its medicinal options through its National policy on Integrative and Complimentary Practices (Brazil, 2006). Since then documentary processes have followed and standardisation procedures of medicinal plants began. Several guidelines have been put in place through relevant government agencies to establish a list of regional plants to represent the popular phytotherapy. The policies adopted advocate for the inclusion of medicinal plant in primary healthcare system. Safety and efficacy standards have been called for by these agencies and it is argued that the same strict standards should be applied to manufacturing of medicinal drugs as it is with conventional drug manufacturing (Barbosa and Pinto 2005). For a drug to be classed as a phytopharmaceutical drugs, it must contain exclusively plant material as active ingredient. Addition of isolated compounds eliminates it from being classed as a phytopharmaceutical drugs. Hence the law allows for a product classed as a natural product to be marketed as long as the relevant authorities are notified and the product is cultivated by those who follow good agronomical practice (Brazil, 2010). Despite the current law and regulations that allow for use of medicinal plants, it seems the use of phytopharmaceutical drugs in public health is still limited.

1.14.1.3 Africa as a case study

A reverse pharmacology approach was adopted for the design and development of an antimalarial phytomedicine in Mali. The drug was successfully developed and standardised in 6 years only. The study involved the selection of a remedy based on ethnopharmacological usage. A clinical dose escalating trial followed to establish a dose response. Based on the results, the safest and most efficacious dose was selected and

another randomised controlled trial followed comparing the phytopharmaceutical drugs with a standard first line treatment. The active compounds within the medicinal plants were identified for standardization and quality control purpose (Wilcox *et al.*, 2011).



*Fig 1.25: Dose optimisation study for an antimalarial phytopharmaceutical drugs: Showing a schematic representation of a dose optimisation study for an antimalarial phytopharmaceutical drugs in Mali. (Source: Wilcox *et al.*, 2011).*

An additional example of a phytopharmaceutical drugs developed in West Africa is called NIPRISAN. The drug is an anti sickling phytopharmaceutical drugs developed by the National institute of pharmaceutical research and development, Nigeria (NIPRD). It was developed from the plant *Vernonia amygdalina*, commonly known as the bitter leaf, due to its bitter/sweet taste. The main clinical trials and laboratory bench work was performed at the NIPRD institute, Abuja. Following the R & D process, NIPRD collaborated with an Indian phytopharmaceutical drugs manufacturer, Xechem in order to commercialise the product. It was commercialised and introduced to the primary healthcare system for the treatment of sickle cell anaemia (Perampaladas *et al.*, 2010).

BF001 is another herbal formulation produced from *Bridelia ferruginia* (*B. ferruginia*) in Ghana at the centre for scientific research and plant medicine (CSRPLM). The extract was developed in collaboration with WHO and is being used as an oral hypoglycaemic agent in the primary healthcare system.

1.15 Methods used for natural product discovery for malaria

Generally, the same screening methods are used for both synthesised compounds and natural products. The main difference is in the approach that is followed. As discussed earlier, some natural product screens begin by screening large database of natural products to identify leads whilst others involve choosing compounds based on ethnopharmacological anecdotal evidences and might begin with human studies (reverse pharmacology). Which ever approach is followed, there are some drawbacks to the current methods used currently in natural product research. In malaria research for example, there is a high successful hit rate, with several compounds showing low IC_{50} values (Cerventes *et al.*, 2012). The main drawback is there is no standard for antimalarial screening of natural product remedies and there is no bench mark IC_{50} value that malariologist could refer to when investigating new compounds.

Several researchers have attempted to address these issues. Wells *et al.*, 2011 suggests that natural product research should be replaced by a patient data led approach which should involve standardised clinical observational system of ethnopharmacological compound used in patients. This approach is in support of previous suggestions by Chen Guofu in 1952. He suggested that the starting point for investigation of natural products should be by checking the activity in humans. This view faced various objections at the time, especially by clinicians and the international community whose major concerns were using unpurified

products in humans. Over the years, several factors have led to an acceptance of Guofu's suggestion. The biggest milestone is the acceptance by authorities such as the European medicines agency (EMA), the United States Food and Drug Agency (US-FDA) and WHO. The main reason for the acceptance of the view was that since patients and communities take these medicinal herbal products, it is a public health concern/responsibility to validate their efficacy. Below is a flow chat of Wells suggestions.

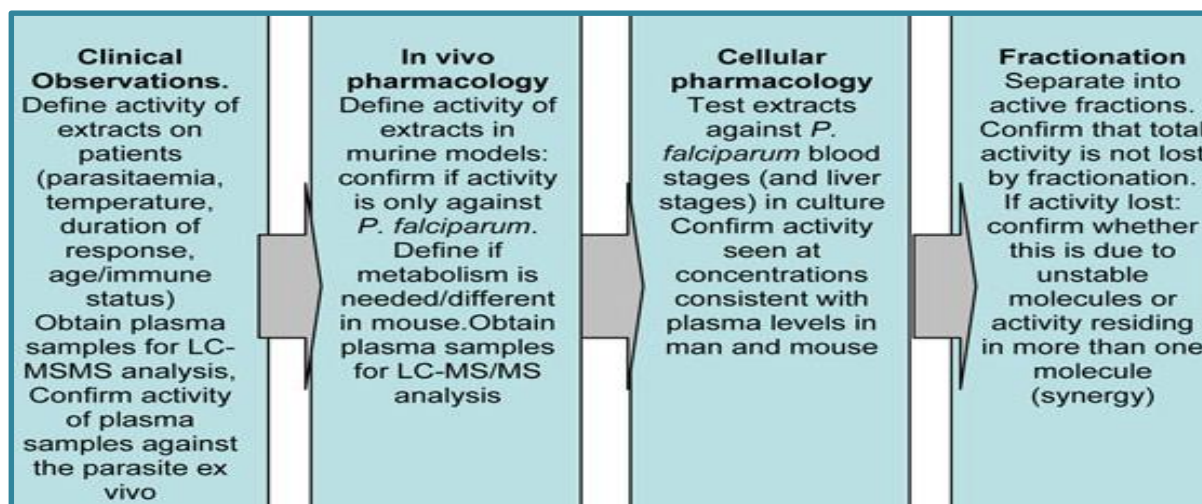


Fig 1.26: Natural product screening: Showing the developmental stages for screening a natural product suggested by Wells, 2011 using a reverse pharmacology approach. The natural product is chosen based on the ethnopharmacological usage and clinical observation begins.

With regard to natural product research, researchers tend to focus on purification and isolation of the active compound in an extract. Natural products usually exist as complex mixtures of various phytochemical compounds. These compounds sometimes act in synergy and the combination gives the total overall effect seen in an extract (Ulrich-Merzenich *et al.*, 2010). Hence by purifying the extract, it is likely that some important compounds are lost along the way. In the case of malaria, the parasite has developed resistance to all new antimalarials that have been developed including artemisinin. Although a number of antimalarials are natural product derived compounds, all have actually gone through the same process of isolating the active compound. The parasite is probably able to develop this resistance due to the simplistic nature of the compounds it is exposed. This theory can be substantiated by looking back at the history of quinine use. Quinine was used in its natural form for centuries with no issue of resistance. Chloroquine was successfully isolated in the

1950's and it only took 10 years for resistance to develop. As mentioned earlier, fansidar, a combination drug of pyrimethamine and sulfadoxine, also developed resistance less than 6 years after its introduction. Another example is mefloquine and the list goes on. Most of these antimalarials are natural product derived. Hence these examples substantiate the argument that the *Plasmodium* is too complicated for simple isolated compounds and might have the ability to evolve quickly and develop resistance. This point can also be emphasised using the artemisinins as a case study. Like quinine, artemisinins have a very long history of use. There has never been a record of resistance. After successful development of artemisinins for malaria, (in a semi-synthetic form) they have remained the last hope over decades. Recent reports have however shown that resistance has developed in these drugs. Fig 1.27 shows is a timeline representation of the development of resistance after isolation of both quinine and artemisinin.

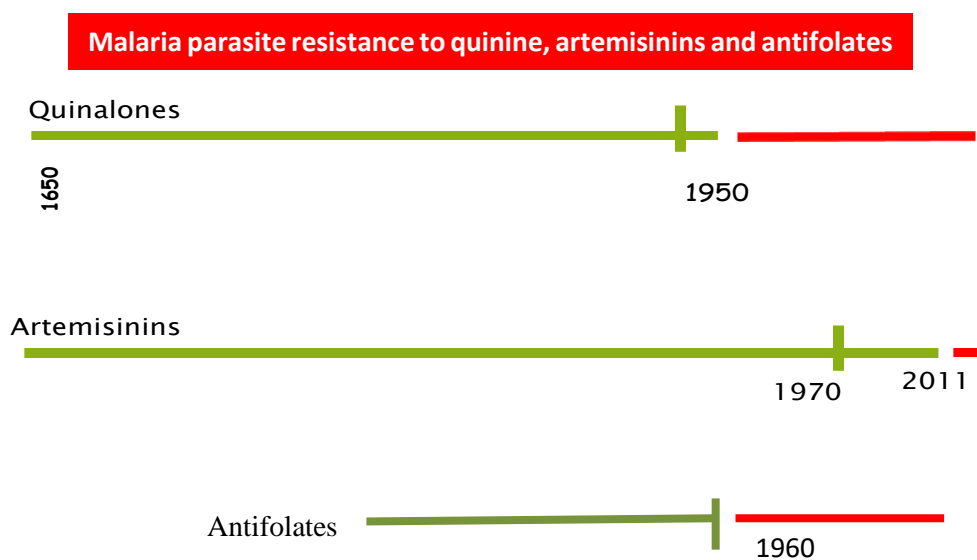


Fig 1.27: A Schematic representation of resistance drug development timeline for malaria: Showing the timeline for the development of resistance of antimalarials after active compound isolation of some antimalarial natural products. The traditional usage of the drug is represented by the green horizontal line whilst point of drug isolation is represented by the vertical green line. Resistance development is represented by the red horizontal line. Malaria parasite resistance developed within 10 years after chloroquine was isolated from the quinolones. The development of resistance took longer with the artemisinins probably owing to their use as semisynthetic drugs and as part of combination therapy regimes.

Antifolate drug resistance developed in less than 5 years after it was isolated as a pure compound.

So far, there has been no successful competitor to the artemisinin. In holding on to this last hope, the WHO has mandated its use as a combination therapy (earlier discussed). The artemisinin started to be used in a 'semisynthetic' form around the 1970s. These drugs are till the cornerstone on antimalarial therapy, thirty years on. In 2006 the first clinical case of resistance was reported in the Thai-Cambodian border (WHO report, 2015). It is thought that resistance might have began as early as 2001 when the drug was used as a monotherapy. The question is why have the artemisinin been successfully used for much longer the quinine? The answer could well lie in the fact that it has been used in a semisynthetic form. Artemisinin has a very complex structure which has kept several chemist on their toes. It has not been successfully synthesised to a commercially viable form and for this reason, it has been used in a semisynthetic form. This could be one of the possible reasons for the delayed resistance seen with these class of drugs. Fig 1.28 shows some structures or artemisinin related compounds.

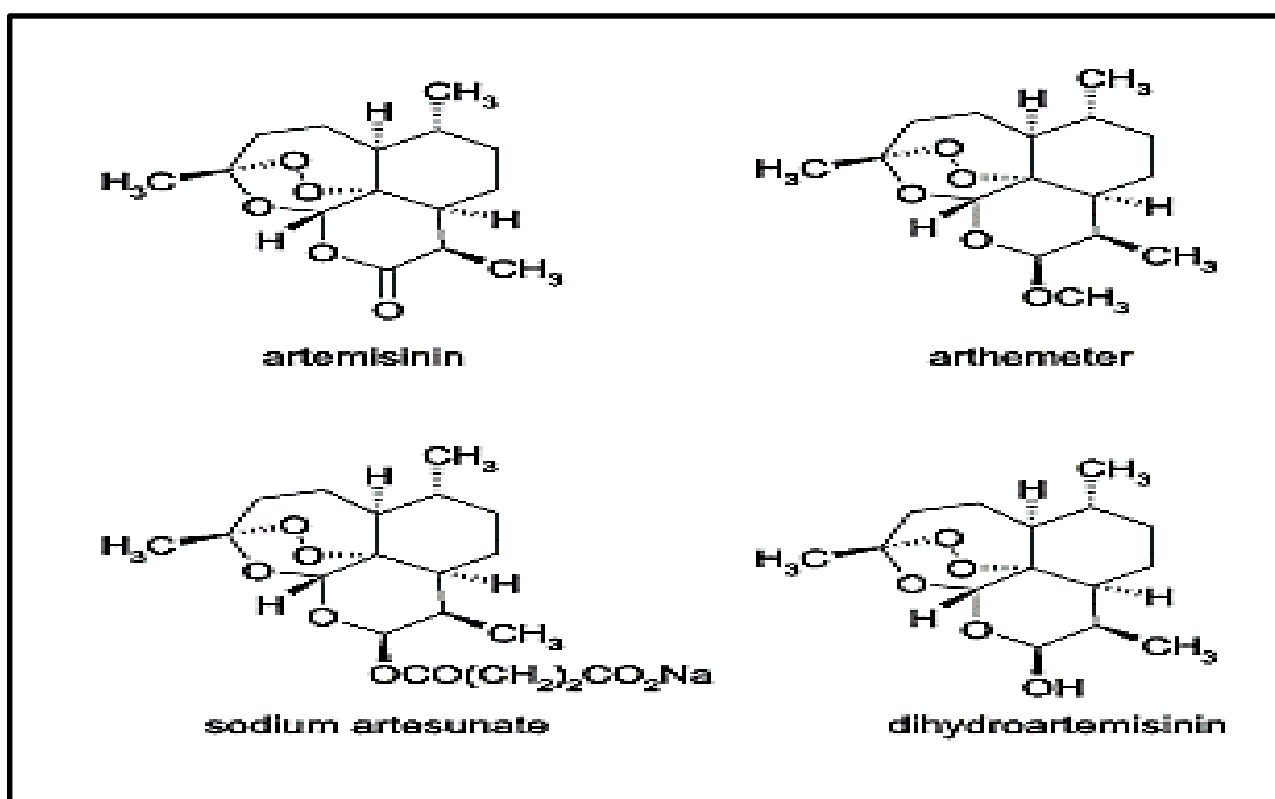


Fig 1.28: Artemisinin related compounds: Showing the complex structures of artemisinin related products. (Source: <http://www.scielo.br/scielo>)

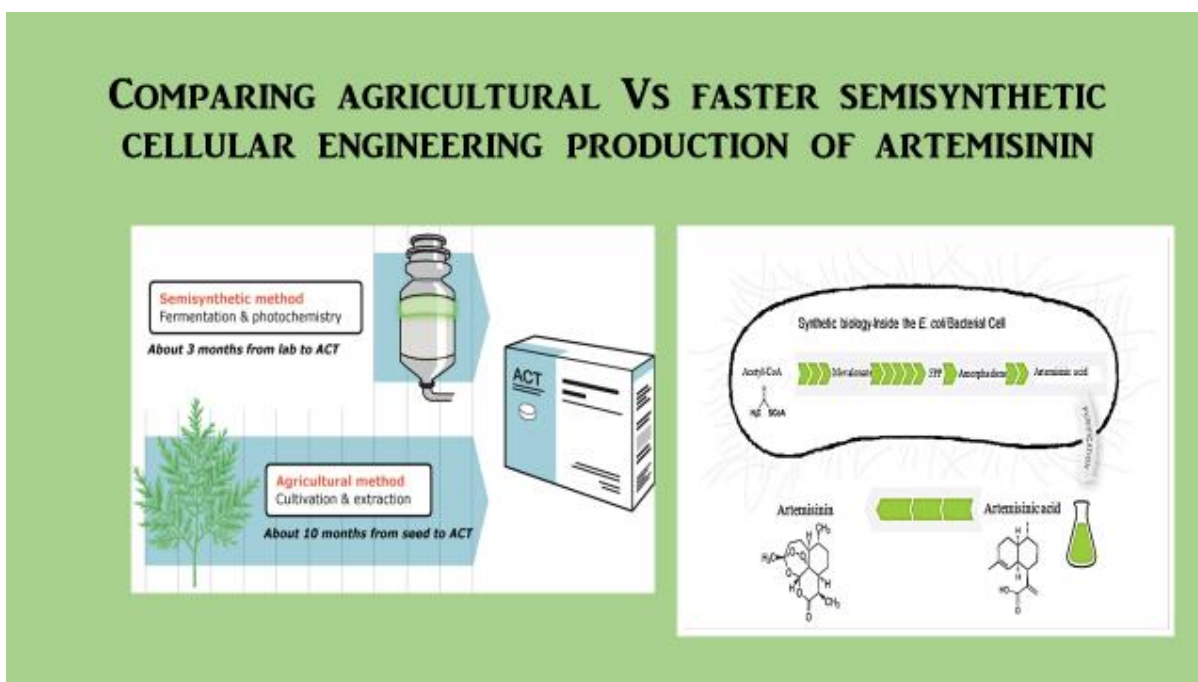


Fig 1.29: Semi-synthetic production of artemisinin: Showing the production of a semi-synthetic form of artemisinin through cellular engineering, allowing for a faster production of the medicine. (Source: <http://www.jsccimedcentral.com/Cell/cell-1-1002.php>).

1.16 Phenotypic screening for malaria

The methods used are mainly either *in vivo* or *in vitro*. *In vivo* methods mainly involve use of animal models for example *P. berhei* (rodent malaria) or *P. relictum* (avian malaria).

Most *in vitro* assays for malaria focus on the erythrocytic asexual stages of the *P. falciparum* and *P. vivax* species. Assays include routine drug assays, rosetting assays, antibody detection, immunoglobulin and serum binding techniques as well as transmission electron microscopy and molecular biology techniques.

In vitro assays are mainly used in malaria research to monitor the amount of parasites in the blood (parasitaemia). The gold standard is microscopy, a cheap and highly reliable technique allowing malaria species and stages to be differentiated microscopically. The main drawback is it can be tedious for multiple samples and it also requires a highly skilled person to correctly estimate parasitaemia. Furthermore, it is subjective, as count variations do occur from one microbiologist to another and it is also labour intensive. Several other tests have been developed over the years to increase the accuracy of parasitaemia estimation

and allow for automatic reading of parasitaemia. Some of these assays are briefly discussed below:

- I. *Isotopic assays*: The most popular of this is the incorporating of radiolabelled hypoxanthine assay. This involves the use of high cost equipment such as scintillation counters and radioactive material which is expensive and requires expensive disposal of the materials. New guidelines only allows for use of isotopes when there is no other alternative hence the need for new methods.
- II. *Parasite lactate dehydrogenase assay (pLDH)*: This assay involves the use of pLDH, a terminal enzyme in the glycolytic pathway as a measure of cell viability in vitro. The parasite pLDH is different from that of the hosts and the level of the enzyme is used as an indication of parasite density. Decrease in the level of pLDH is an indication of lower parasite density and this is used as a marker in drug sensitivity assays.
- III. *Histidine rich protein II (HP2)*: This assay measures the level of histidine and alanine rich proteins produced by the parasite during multiplication. HP2 levels are closely related to parasite density and multiplication. Drug sensitivity assay tests are designed to monitor the levels of these proteins. The protein levels are measured using a simple commercially available enzyme linked immunosorbent assay (ELISA).
- IV. *Fluorescent based methods*: Several fluorescent based methods are used for antimalarial drug susceptibility testing. These methods take advantage of the fact that mature red blood cells lack nucleic acids that is deoxynucleic acid (DNA) and ribonucleic acid (RNA). The dyes bind to parasite DNA hence an increased fluorescence corresponds to increased parasitaemia. Dyes that have been previously used include SYBR green I, picoGreen and YOYO-1 (Weismann *et al.*, 2006; Smilkstein *et al.*, 2004; Quastine *et al.*, 2006). SYBR Green is the most sensitive amongst the available DNA stains. It works by intercalating and binding to double stranded DNA which once bound, becomes fluorescent (Vossen *et al.*, 2010). Hence any fluorescence is due to binding of parasite DNA as erythrocytes have no DNA. The dye also has a lower affinity for RNA making it selective. Hence during a drug screen, high fluorescent reading means a high presence of parasite and a lower reading depicts less parasite. Thus control samples will show higher fluorescence whilst treated samples will show lower fluorescence if a drug has antimalarial activity, indicating parasite killing. The SYBR Green assay has several advantages including the fact that it is simple, quick and a relatively cheap method (Rason *et al.*, 2008). The only technical equipment

required is a fluorescent plate reader and the dye is cheap to buy. The method is highly adaptable involves few steps and incubation period is only 1h (Smilkstein *et al.*, 2004). The main disadvantage of this method is the high background that might often be seen. For this reason, some studies involve an additional erythrocyte lysis step to combat this (Johnson *et al.*, 2006). Another drawback is that the dye is also non specific hence it will bind any available double stranded DNA hence any sample contamination example white blood cells will cause interference in the reading or cause a high background. The dye is also capable of binding to single stranded DNA and RNA but at a much lower affinity than double stranded DNA, again contributing to background interference (Smilkstein *et al.*, 2004). SYBR green can also be used in flow cytometry to detect malaria parasite. The flow cytometer uses the same ideology as the plate reader. The only DNA is that of the parasite since erythrocytes lack DNA (Grimberg *et al.*, 2011). The FCM was used to measure nuclear division of the parasites within the red blood cell using a nucleic acid stain. Like the SYBR green plate reader assay, it also takes advantage of the fact that red blood cells do not contain DNA hence any nuclear material is that of the parasite. Apart from giving information about the nuclear division within the parasite, the technique also gives an accurate percentage parasitaemia. With the flow cytometer, a whole range of nucleic acid dyes are available as alternatives but for this project, the SYBR green is used due to its high specificity for DNA. Fluorescent based techniques are very attractive due to their ease of use, relatively cheap and less complicated compared to other techniques like radioactive isotope or ELISA based techniques. For this reason SYBR green plate reader and flow cytometry along side light microscopy are the chosen techniques for the scope of this project¹⁷

1.17 Conclusion

In view of the issues discussed in this current chapter, the main aim of this study is to screen some plants based on ethnopharmacological usage towards the development of a phytopharmaceutical drugs for malaria using *in vivo and in vitro* methods. Some novel extracts were screened and used to design a suitable workflow. Methods were optimised with the aim to validate the ethnopharmacological claim of the plants' use for malaria.. The natural product crude extracts will be screened for antiplasmodial activity in a cell based assay approach. Once activity is established, a dose response curve will be generated using different doses and the IC₅₀ value will be determined. The crude extract will be fractionated using high throughput liquid chromatography (HPLC) separation method to obtain broad fractions for testing as evidence that different components within the plant act possibly in

both additive and synergistic way to give an overall antimalarial activity. The main objective of this project is mainly to validate the ethnopharmacological claim of the plants using a model that will screen the crude plant for malaria. It is argued that previous plant extracts that were used for the treatment of malaria developed resistance few years after being isolated to a single active compound, as is the case with chloroquine and other antifolate drugs. Hence in this project, the plant material will be maintained in a wholesome form, as the complexity of the compound mixture might delay/prevent resistance. With these results, the ultimate aim of this project is to work through a collaboration with National institute for pharmaceutical research and development, Nigeria (NIPRD) to develop a phytopharmaceutical drugs that will be beneficial and cost effective to malaria endemic regions in Africa, as well as tackling the issue of resistance. The development of the phytopharmaceutical drugs is however beyond the scope of this project.

1.18 Project objectives

1. To screen ethnopharmacological-based plants against the multi-drug resistant malaria strain, K1.
2. To optimise and use three different methods to ascertain the antimalarial efficacy of the selected plant extracts.
3. To establish dose-response curves of the selected plants.
4. To choose a plant based on the best IC_{50} and easy accessibility in West Africa for further characterisation. The accessibility of the plant is based on its biodiversity, distribution and ease of cultivation in West Africa and more specifically Nigeria.
5. To investigate the possible mechanism of action and the onset of antimalarial action.
6. To explore the *in vivo* pharmacological actions of the selected plants including antimalarial, antipyretic and analgesic activity.
7. To use HPLC analysis to identify active fractions in the plant extract.
8. Illustrate the suitability of the extract for development into a phytopharmaceutical medicine and identify standardization pathways for the extract.

CHAPTER 2

Materials and Methods

2.1 Establishing *P. falciparum* cultures and maintainance

Routine cultures were established and maintained according to methods previously described by Read and Hyde (1993). Standard operating procedures (SOPs) were followed with regards to use of Class II pathogens and biological safety rules. Experiments were carried out in the University of Salford pathogen laboratory sterile hood (ESCO class II Biological safety cabinet). All equipments were pre-sterilised using aseptic techniques. A 2% virkon solution (Antec International, UK) was used to disinfect waste material before autoclaving and disposal.

2.1.1 Complete media: RPMI Medium 1640 (+)25 mM HEPES and (+) L-glutamine (Gibco life technologies, UK) was made complete by adding 50 mg/mL gentamicin (Sigma, UK), 2.5 ml of a 1mg/ml hypoxanthine in PBS (Fischer chemical, UK), 2.5 ml 40% glucose (Dextrose anhydrous, Fisher chemicals, UK), 2.5 g Bovine serum albumin (Sigma, UK). The above additives were dissolved in 15-20 ml of pre-sterilised RPMI media taken from a 500 ml bottle. Once dissolved, it was filter sterilized back into the bottle using a 0.22 µm filter. Solutions were kept sterile by performing all procedures in the sterile hood. The complete media (CM) was stored at 4°C until use for up to two weeks.

2.1.2 Blood washing: O⁺ human blood was obtained from the NH Blood Bank at Manchester Royal Infirmary. 200 ml of the blood was transferred into sterile falcon tubes and the remaining blood transferred into sterile culture flasks and kept in the fridge for future use. The blood in the falcon tubes were centrifuged at 3400 rpm for 5 mins. The supernatant, containing plasma and leukocytes, was removed and discarded. Normal RMPI media [Wash media, (WM)] was added to each tube and the centrifugation process repeated. The supernatant was discarded and the process was repeated three times ensuring all leucocytes were depleted. Then CM was used to perform a final wash. Once the supernatant was discarded, an equal volume of CM was added to the 100% hematocit blood left in the tubes making it a 50% haematocrit blood ready for use in culture. The washed blood was labelled and stored in the fridge at 4°C until use.

2.1.3 Retrieving new cultures from frozen: A cryovial containing 0.5 ml of a 3D7 or K1 *P. falciparum* frozen culture was retrieved from a nitrogen tank and thawed in an incubator set at 37°C. The contents were transferred to an eppendorf tube and centrifuged at 13000 g for 90 seconds and then transferred to a sterile hood where the supernatant was discarded. The pellet, consisting of the parasitized blood was gradually re-suspended in 1 ml of 10% sorbitol solution in PBS, with continuous mixing. The process was repeated twice and a third wash was performed using 5% sorbitol in PBS. A final wash in CM was performed and the pellet was re-suspended in 0.5 ml of CM. The re-suspended culture was added to a culture flask containing 10 ml complete media and 0.5 ml non parasitized blood giving a total of 1 ml blood in a flask containing 10 ml CM achieving a 5% haematocrit. The culture flask was gassed with 5% CO₂, 5% O₂ and 90% N₂ gas (Boc Limited, UK) and then incubated at 37°C for 72 h in an incubator (Leec culture safe touch 190 CO₂, Leec Limited, UK).

2.1.4 Light microscopy (Giemsa staining): Briefly, light microscopy was carried out by performing a thin blood smear on a microscopic slide. The slide was air dried and fixed with 100% methanol. The slide was immersed in a 1:10 diluted Giemsa stain in a buffered thiazine-eosinate solution (BDH/WVR, UK). Oil immersion microscopy was used at X 100 magnification using a Leica DM 500 compound microscope to view the parasites. Infected red blood cells were counted in 3-5 different fields of view and parasitaemia was estimated by the average percentage of infected cells counted against the non-infected cells. An estimated 1000 cells were counted in order to establish parasitaemia. Based on the parasitaemia determined, subculturing was carried out at 0.5-1% parasitaemia. All experiments were initiated at parasitaemia levels between 0.5-1%.

2.1.5 Maintenance of routine P. falciparum cultures: Routine cultures were performed as previously described by Read and Hyde, 1993. Cultures were carried out in either 25 cm³ flasks (10 ml culture volume) or 75 cm³ flasks (30 ml volume). The experimental set up was however usually in smaller 12.5 cm³ flasks (5 ml volume) at 5% hematocrit. Briefly, the procedure was performed by removing cultures from the incubator and carefully placing them in a sterile culture hood carefully in order to avoid dislodging the settled parasitized blood layer. The clear spent media at the top was removed by slightly tilting the flask to an angle. Once all spent media was removed, the settled parasitized blood was mixed and a drop taken out to prepare a thin blood smear as described in section 2.1.4. Once parasitaemia was established, the culture was diluted down to between 0.5 to 1% parasitaemia. Typically

a 3% parasitaemia culture was diluted by adding 2 parts new blood to 1 part parasitized blood making it 1% parasitaemia. Cultures that were going to be left for 72 h were diluted down to 0.5% parasitaemia to prevent culture overgrowth and maintain healthy cultures. The cultures were gased as previously described and placed in the incubator at 37°C.

2.1.6 Sorbitol synchronisation: Newly set up cultures do not need synchronisation as only the ring form survives the cryopreservation process. After several cycles however, the cultures lose synchrony. For best experimental results, sorbitol was used to maintain this synchrony in established cultures. The method previously described by Trager and Jensen, 1976 was used. Briefly, cultures with a high parasitaemia (about 8% in our lab) of predominantly ring stage were centrifuged. The spent media was discarded leaving the parasitized pellet. A 5% w/v sorbitol (in distilled water) was filtered through a 0.22 µm filter for sterilisation. 9 ml of the sorbitol solution was added to 1 ml of culture pellet and incubated for 5 min at room temperature. The culture was centrifuged at 3400 rpm for 5 min and the supernatant discarded. The pellet was washed three times in complete media leaving a parasite pellet. The parasitaemia of this suspension was estimated using light microscopy and subculturing performed as previously described in section 2.1.5. The resultant culture was a synchronised ring stage culture. Once cultures lost synchrony, this synchronisation process was repeated to maintain it.

2.2 Drug susceptibility assays

Based on careful consideration of the various assays that are available for the estimation of parasitaemia, three simple, reliable and efficient techniques were chosen in the present study, to investigate the effect of drugs on parasitaemia. The three methods were compared based on the half maximal inhibitory concentration (IC₅₀). The three methods include Giemsa light microscopy, SYBR green microplate assay (SG-Microplate) and SYBR green flow cytometry (SG-FCM). The Giemsa assay is the gold standard in malaria parasite detection.

2.2.1 Optimisation of the SG-Microplate assay: The SG-Microplate assay was performed as previously described by Rason *et al.*, 2008. Briefly 3D7 *P. falciparum* culture was grown in 10 ml culture volume as previously described. Once the parasitaemia reached 2.5% or more, 100 µl of the culture was put into different wells in a 96 well, black bottom culture plate (Nunc, Denmark). 100 µl of a 0.2 µl/ml SYBR green 1 solution (10, 000X Sigma, UK) diluted to 5 X in PBS was added to each well. Plates were covered and incubated in the dark

for an hour. Fluorescent intensity was measured with a micro plate reader (Genius Tecan) at an excitation of 485 nm and emission of 535 nm. The gain of the instrument was set to a maximum of 80 and both top read and bottom read options were compared. The assay was optimised to investigate the effect of several factors on background fluorescence. The first experiment involved a two-fold serial dilution of a high parasitaemia culture using RPMI media in order to determine the correlation between fluorescent intensity and parasite density. The results obtained necessitated further optimisation. The effect of several factors including haematocrit level, amount of CM present in the wells were investigated in subsequent experiments.

2.2.2 Effect of haematocrit level and media on background fluorescence: The effect of several factors were investigated using a 96 well plate in a SYBR green assay as described above. 100 µl of samples were loaded in wells and the medium and amount of haematocrit varied in different wells. The samples were loaded as below and serially diluted from a high parasitaemia to a low parasitaemia.

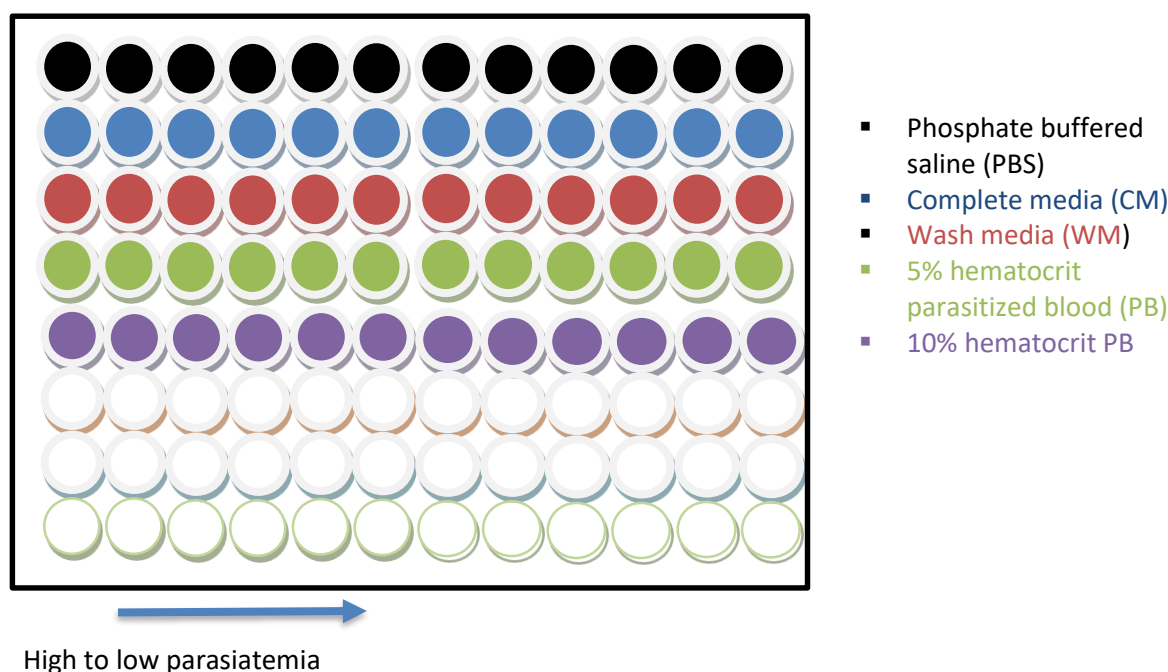


Fig 2.1: *Serial dilution format of the SG-Microplate assay: Showing the 96 well plate format of the samples. Arrow indicates direction of serial dilution.*

2.2.3 Effect of complete media on the SG-microplate assay

The results of the experiment in section 2.2.2 necessitated further investigation on the effect of complete medium due to the high background detected. Briefly wells were loaded with

200 µl of complete medium, 150 µl of complete medium, 100 µl of complete media and 50 µl of complete media. Each sample well was made up to 200 µl volume with PBS and the SG-Microplate protocol carried out

2.2.4 The adopted optimised SG-Microplate protocol

As mentioned previously in section 2.2.1, method described by Rason *et al.*, 2008 was originally followed. Based on the optimisation assay results, the method was slightly modified by omitting the erythrocyte lysis step. 100 ul of cultures were placed in 96 well Nunc plate and 10,000 X SYBR green diluted to 5 X in PBS was added. The haematocrit level was maintained at 2.5% and PBS was used as CM caused a high background. All other parameters were as described in section 2.2.1.

2.2.5 Optimisation of the SG-FCM method

The method used has previously been described by Karl *et al.*, 2009 although minor modifications were made to optimise and adapt the method for our laboratory. Cultures were challenged either at ring stage or trophozoite stage at 0.5-1 % parasitaemia and incubated for 48 h or 72 h respectively. After the incubation period, samples were obtained from control and drug challenged culture flasks. 50 µl was taken from culture flasks incubated with varying doses of extract as well as the control flasks which had no treatment, each placed in different labelled eppendorf tubes. The samples were washed with 1 ml PBS by centrifuging for 1 minute at 14000 g and the supernatant discarded. 950 µl PBS was added to each of the samples as well as 50 ul of a 1:100 SYBR green solution. Samples were incubated for 1 h after which the samples were washed to remove the SYBR green. The samples were subsequently fixed by adding a 250 µl solution of a 1:15 dilution of formaldehyde. Samples were refrigerated at 4°C for 15 minutes and the fixative was removed by washing the sample three times in PBS. The samples were ready to be read in a BD biosciences FACs flow cytometer machine. Fifty thousand events were acquired from each sample. The BDS biosciences software was used to generate a density plot as well as a forward scatter plot versus fluorescent intensity. Statistical data of events was obtained from the software. Gating was performed using standard gating procedures as previously described by Karl *et al.*, 2009. The control sample was used to perform the initial gating and the challenged samples were run under the same gating procedure allowing comparison to be made between non-challenged and extract challenged cultures. The SG-FCM also allowed for gating of two different cell populations, hence unsynchronised cultures were

gated as mononuclear and multinuclear cell populations depicting ring stage/trophozoite as one sub-population and shizont as another sub-population. This method has been previously described by Karl *et al.*, 2009. The BD FACS verse software was also used to accurately determine parasitaemia by recording the percentage of infected cells relative to the total number of events recorded, in this case the erythrocyte count.

2.2.6 Comparison of the Giemsa microscopic test, SG-MicroPlate and SG-FCM

As the different assays were used to evaluate antimalarial activity in this project, the validity and reliability of these assays was evaluated by determining the IC₅₀ values of dihydroartemisinin (DHA) and chloroquine (CQ) against the K1 strain of *P. falciparum* and comparing the results to literature values. Briefly, high parasitaemia culture that had previously been synchronised was used at ring stage. The culture was microscopically examined and diluted to a 1 % parasitaemia (5 % hematocrit) and divided into flasks of 5 ml final volume in a 12.5 cm³ flask. Two fold doubling concentrations ranging from 1.25 nM to 40 nM were added to the flasks in duplicate for DHA. A flask of untreated culture was made to serve as drug-negative control. All flasks were incubated at 37°C. The cultures were incubated for a 72 h time period. At the end of the 72 h drug exposure, the three assays were performed simultaneously as described in sections 2.1.4, 2.2.4 and 2.2.5. Briefly, cultures were removed from the incubator at the end of incubation period and carefully placed in a sterile hood without dislodging the settled cultures. 4 ml of the spent media was removed and thin blood smears were prepared for each sample for Giemsa light microscopy. The haematocrit was restored to 5% with 4 ml of warm PBS (37°C). 100 µl of each sample was taken in triplicate and placed in a 96 well Nunc plate for the SG-Microplate assay while 50 µl of each sample was pipette into labelled eppendorf tubes for subsequent SG-FCM analysis. Each experiment was repeated three times for statistical validation. The percentage parasite inhibition compared to infected control samples was used to analyse data. IC₅₀ values were determined using normalised dose-response analysis (Graphpad Prism).

2.3 Optimisation assay results

The results obtained during the optimisation assays were plotted on a graph using Graphpad Prism 6. The results are detailed below:

2.3.1 Effect of haematocrit and CM on SG-Microplate assay

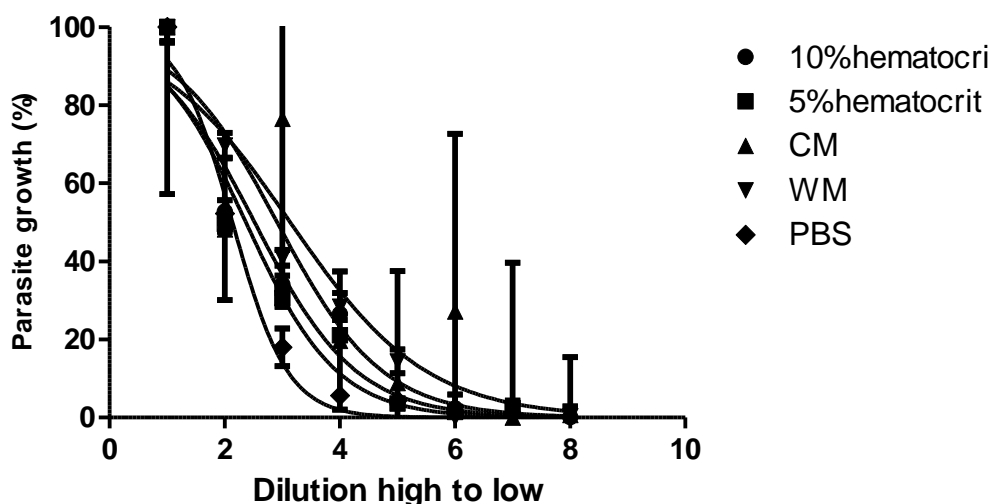


Fig 2.2: SG-Microplate assay for using different conditions: The results of the assay showing the influence of various factors on the SG-Microplate assay. Different conditions were varied including percentage haematocrit, changing media conditions (alternating CM, WM or PBS). The effect of these factors on background fluorescence can be seen in the graph above. The data is based on three independent experiments.

The results of this assay represented in fig 2.2 showed that high haematocrit had a high background fluorescence on the assay. Reducing the haematocrit from 10% to 5% lowered the background fluorescence but both readings are likely to interfere with the results of the assay. CM contributed to the high background influence and it was observed that using WM or PBS as an alternative reduced the background fluorescence to almost nil. Based on these results, further optimisation experiments were carried out to determine the best assay conditions for the SG-Microplate assay.

2.3.2 Effect of varying the amount of CM on the SG-Microplate assay

Media caused a high background influence in the previous assay hence necessitating this assay to establish if reducing the amount of CM will reduce background fluorescence. The results of the assay showed that CM caused an increased background fluorescence. The higher the CM present in the wells, the higher the background fluorescence.

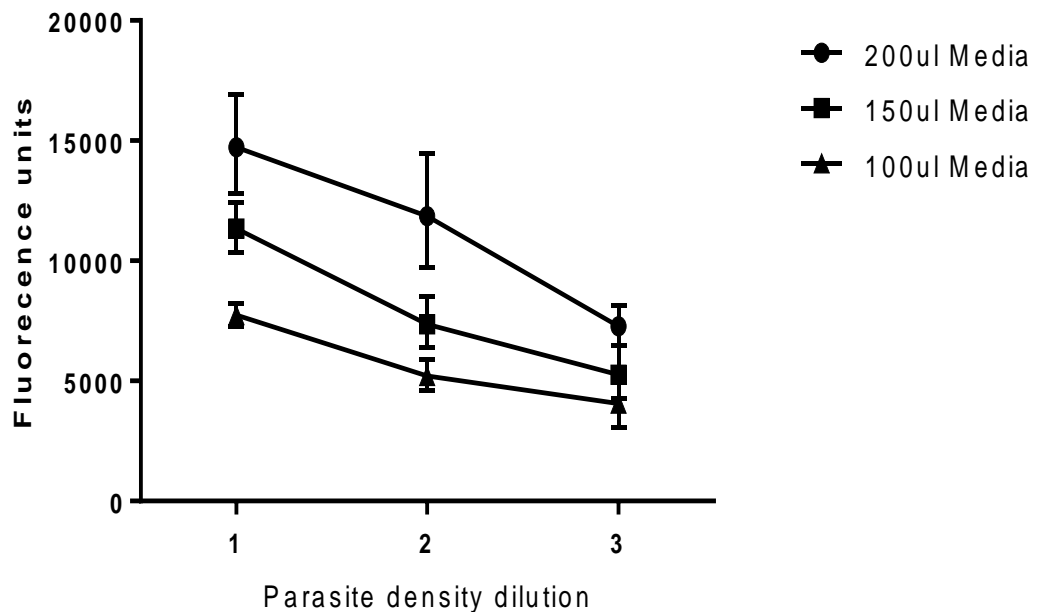


Fig 2.3: Effect of complete media on the SG-Microplate assay: Showing the effect of CM on background fluorescence. CM was replaced with PBS in some wells. For example well with 100 μ l CM was made up to a volume of 200 μ l by adding 100 μ l PBS. The results show that CM contributes to high background fluorescence.

Though a series of experiments, some data not shown, it was established that several factors could cause background interference in fluorescence reading. CM and a high haematocrit caused a high background fluorescence interfering with experimental readings. Changing from CM to WM or PBS got rid of the high background. Another experiment that specifically investigated the effect of CM, in order to establish how much CM is acceptable showed that replacing CM with PBS caused a great reduction in high background. The higher the volume of CM removed from the wells, the lower the background interference. These experiments were necessary as the parasites are cultured in CM. Hence based on these results, the optimised protocol to be taken forward for this project will use a low haematocrit

of 2.5% and also replacing 150 μ l of CM with PBS in wells prior to SYBR green addition in order to reduce background fluorescence.

2.3.3 SG- FCM optimisation

The a sample of the of the optimised SG-FCM assay data is shown in fig 2.4 below. The results show a population of red blood cells in orange and F1 and F2 boxes showing two population subsets of the *Plasmodium* representing mononucleated cells and multinucleated cells respectively.

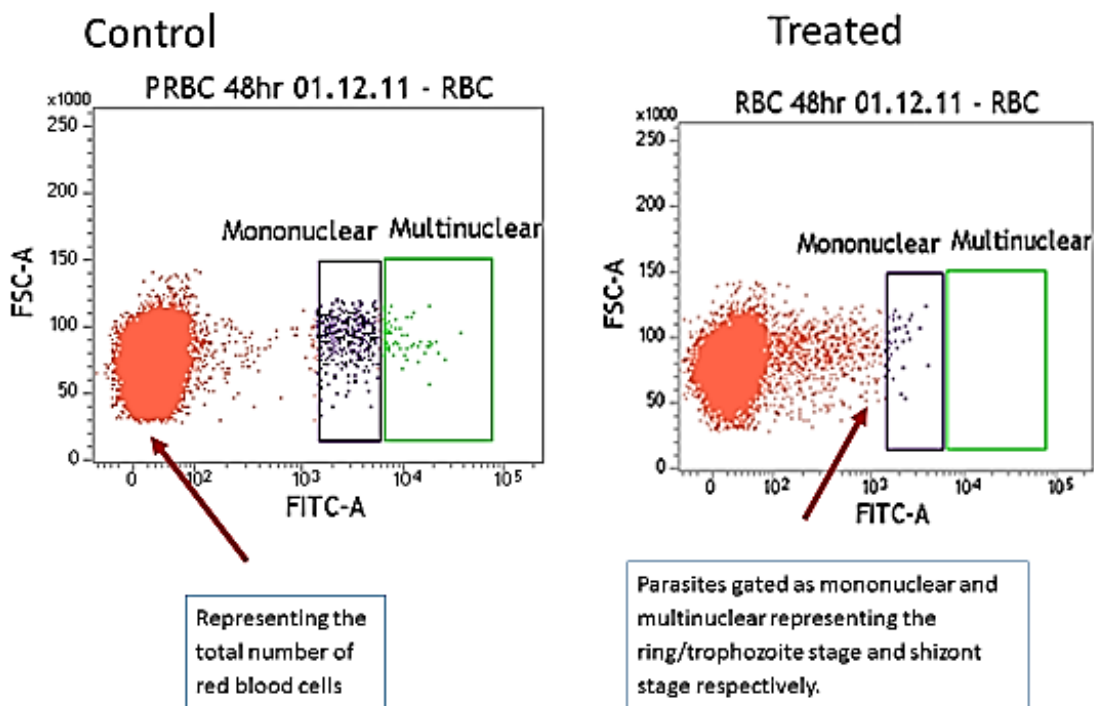


Fig 2.4 FCM image showing the Plasmodium at different stages: Showing SG-FCM analysis of control and DHA treated cultures with a near 100% parasite growth inhibition at a dose of 10 nM.

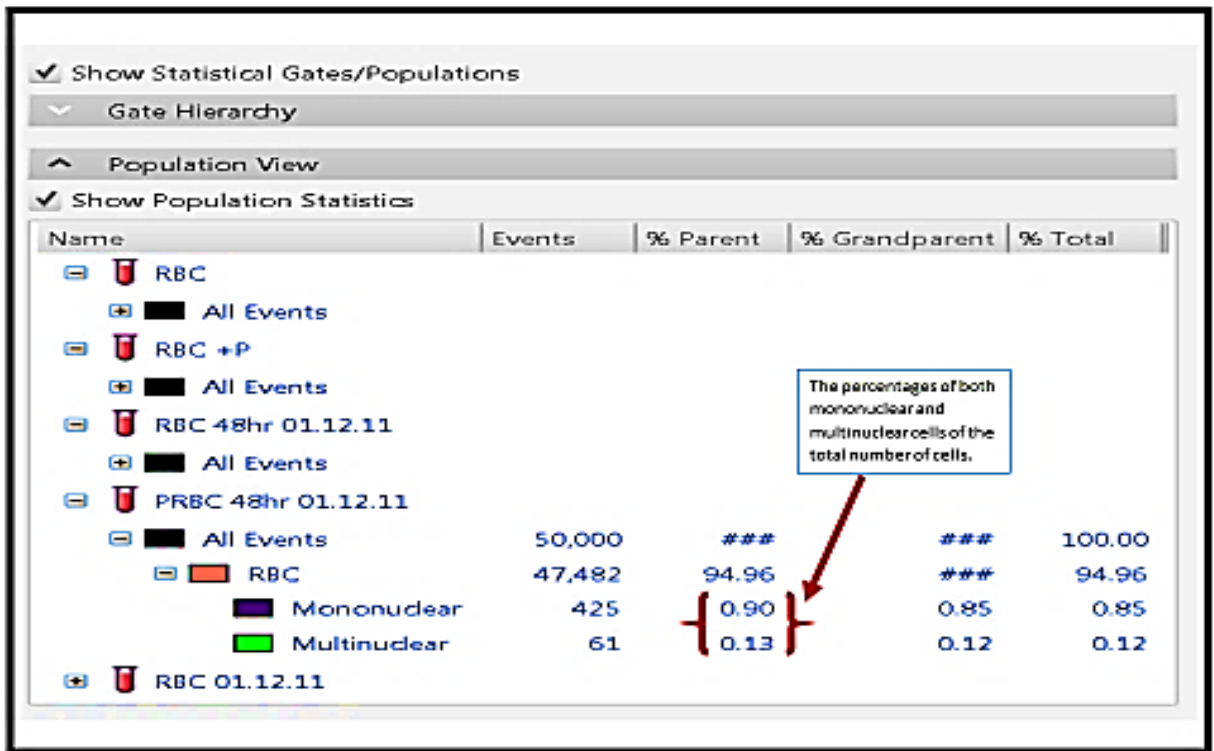


Fig 2.5 Statistical hierarchy for the FCM analysis: Showing the statistical hierarchy generated by the BDS system in the SG- FCM analysis.

The results from fig 2.5 above show the normal red blood cell indicated as control represented by a plot of forward scatter (FSC) against fluorescent intensity (FITC). From the gating procedure, it can be seen that the cells in the orange region are normal red blood cells with no parasites hence they do not fluorescence. Cells in the first gate, in blue show a high fluorescence due to DNA content of the parasitized erythrocytes and the statistical data generated in fig 2.5 shows the percentage of these cells in the mononuclear region which is 0.90%. The cells in the second gate, in green, are indicative of the multinucleated cells due to shizont formation. Hence the mononuclear gate indicates rings and trophozoite stage parasites while the multinuclear gated cells indicate shizonts.

2.3.4 Comparing the Giemsa light microscopy, SG-Microplate and SG-FCM assays.

A *P. falciparum* culture was treated with varying doses of DHA in order to determine the IC₅₀ using all three assays.

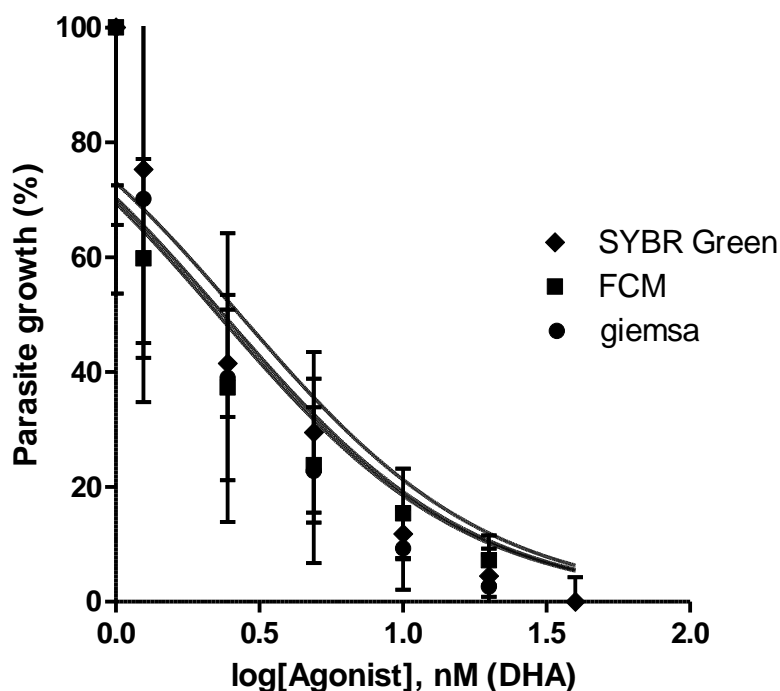


Fig 2.6 Comparison of the three antimalarial assays: Comparing the selected three assays by determining the IC_{50} value of DHA. Parasites were incubated at trophozoite stage for 48 h with doses ranging from 0.625-20 nM. A dose-response curve generated using the normalised dose-response plotted against the log drug concentration. The IC_{50} value was determined for each assay using non linear regression analysis on Graphpad Prism 6.0. Error bars represent standard error of the mean (SEM) of a triplicate data.

Light microscopy IC_{50}	SYBR Green IC_{50} (nM)	SG-FCM IC_{50} (nM)
2.369 (1.343-4.179)	2.700 (1.456-3.542)	2.271 (1.557-4.681)

Table 2.1: IC_{50} values obtained from the three assays: Showing the actual IC_{50} values obtained from the three different assays in the experiment represented in fig 2.5 above. The IC_{50} values in all three assays are comparable ranging from 2.271 to 2.700 nM with an average of 2.430 nM. Literature IC_{50} of DHA as determined by Fivelman et al., 2004 and Zongo et al., 2011 are 1.67 ± 0.43 nM and 3.15 nM which ± 1 respectively. These compare favourably with the average value of 2.43 nM obtained in the three assays in this current study.

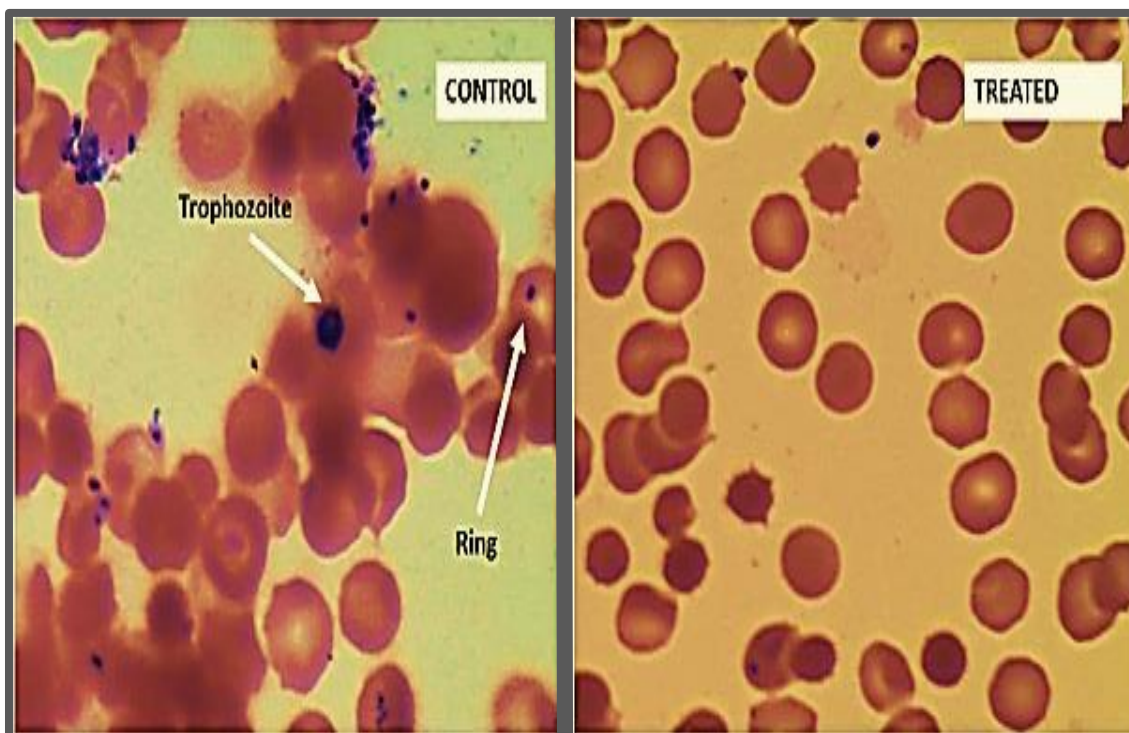


Fig 2.7 Light microscopy image of control and treated parasites after Giemsa staining: Showing pictures obtained from the Giemsa light microscopy representing infected control and DHA treated cultures.

2.4 Aqueous or methanolic extraction of plant material

100 g of dried plant material was air dried in shade and pounded into a coarse powder using a pestle and mortar. The plant material was subjected to cold maceration in water (aqueous) or 70% methanol (methanolic extraction) for 24 h on a shaker. The plant was filtered using whatmann paper and the resultant filtrate was concentrated using a rotary evaporator maintained at 70°C until a dried powder was obtained.

2.5 Drug/extract stock preparation.

Dihydroartemisinin (DHA) was prepared by making a 5 mM stock solution. This was prepared by considering its molecular weight (MW) of 284.35 and using it for standard molar calculations. Briefly, 1.4 mg of the powder stock was dissolved in 1 ml of DMSO to obtain a primary stock concentration of 5 mM. The stock was aliquoted into small eppendorfs of 5 µl volume and stored at -20°C until use. Extract stocks on the other hand were made as 20 mg/ml stock by weighing 0.02 g and dissolving in 1ml DMSO. The dissolved extracts were aliquoted into eppendorfs at 50 µl volume and stored at -20°C for future use.

2.6 Phytochemical test methods

The aqueous and methanolic bark extracts were subjected to phytochemical screening for the presence of terpenoids, saponins, flavonoids, sterols, glycosides, alkaloids, and anthaquinones according to standard procedures previously reported by Trease and Evans, 1989.

- i) Flavonoids: A few drops of 1% ammonia solution was added to the extract sample in a test tube. The presence of flavanoid was confirmed by a yellow coloration.
- ii) Saponins: 0.5 g of test extract was dissolved in boiling water, allowed to cool, and shaken vigorously forming a froth. The froth was mixed with 3 drops of olive oil, and the formation of an emulsion indicated the presence of saponins.
- iii) Terpenoids: 0.5 g extract sample (aqueous or methanolic) was mixed with 2 ml of chloroform in a test tube. 3 ml of concentrated sulphuric acid (H_2SO_4) was carefully added to the mixture to form a layer. Presence of terpenoids was confirmed by the formation of an interface with a reddish brown coloration.
- iv) Glycosides: A 10 ml quantity of 50% concentrated H_2SO_4 was added to 0.5 g of the test extract in a test tube. The mixture was heated in boiling water bath for 5 min. 10 ml of Fehling's solutions (5 ml of solutions A and B each) was added, and the mixture was boiled. The presence of glycosides was indicated by the formation of a brick red precipitate.
- v) Tannins: 0.5 g of the test extract was boiled in 10 ml of water in a test tube. A few drops of 0.1% ferric chloride was added. Presence of tannins was indicated by the emergence of a brownish green or bluish black coloration.
- vi) Phenolic compounds: A few drops of 10% aqueous ferric chloride solution was to a 1ml alcoholic solution of the test extract in a test tube. The presence of phenolics was confirmed by the emergence of blue or green color.
- vii) Alkaloids: 1 ml of 1% hydrochloric acid was added to 3 ml of the extract in a test tube. A few drops of Dragendorff's reagent was added to the mixture. The presence of alkaloids was confirmed by the formation of a reddish brown precipitate.
- viii) Sterols: 0.5 g of the extract was extracted in 10 ml chloroform. 2 ml of acetic anhydride was added followed by 2 ml of concentrated H_2SO_4 . A color change from violet to blue or green confirmed the presence of sterols.
- ix) Anthaquinones: 0.5 g of the extract was boiled with 10 ml of concentrated H_2SO_4 . The mixture was filtered and the filtrate was shaken with 5 ml of chloroform. The

chloroform layer was removed, and 1 ml of 10% aqueous ammonia was added. The appearance of a pink, violet, or red tint in the solution confirmed the presence of anthraquinones.

2.7 Chapter Conclusion.

In this chapter, two SG fluorescent-based assay methods were explored for the investigation of malarial parasite growth in cultures. Giemsa light microscopy, the standard gold malaria detection method, was used as a positive control to confirm the effectiveness of the assays. Percentage parasitaemia of cultures was calculated in all the three methods. The results of this chapter showed a relatively comparable results in all three assays: namely the SG-microplate assay, the Giemsa light microscopy and the SG-FCM. The SG-FCM method was very useful and proved to be the most desirable method due to its accuracy in giving the exact parasitaemia and further information about the exact stages of the parasite. Giemsa light microscopy also had the advantage of giving information about parasite stages, via viewing the morphological changes in parasites. The main disadvantage of this method is its subjective nature. In the case of the SG-Microplate assay, the method showed a correlation between the level of fluorescence units with parasitaemia. The higher the parasitaemia, the higher the level of fluorescence units. Hence, it is a useful method in drug assays as treated samples can be compared with control (non treated) samples in terms of fluorescence levels. The limiting factor with the SG-Microplate assay is parasitaemia can not be established and parasite stage cannot be detected. Hence data from this assay can only be interpreted in terms of percentage dose inhibition which can be used to establish the IC_{50} value. Hence the results of this chapter has shown that SG fluorescent-based methods can be a fast and reliable method of detecting parasitaemia and compare favourably to the standard detection method, light microscopy. The SG-FCM assay was shown to be the most specific and accurate method in our laboratory, as it gives an exact parasitaemia with detailed stages of the parasite. The main drawback with this assay is although the reagents are cheap, it requires expensive laboratory equipment and it is more time consuming. The Giemsa light microscopy is the cheapest and fastest method, but it requires expertise and can be subjective. It can also be used to determine the exact stages of the parasites.

CHAPTER 3

Ethnopharmacological validation of selected plants from Nigeria.

3.1 Introduction

The 21st century has witnessed a paradigm shift from man's reliance on traditional medicine (TM) to over reliance on modern medicine for the treatment of many ailments. In most developing nations, governments have adopted and only recognise modern day medicine in their healthcare system. This approach has however led to a disconnect between the common practices of the people and what is available within various healthcare systems. This is reflected in the fact that the mainstay of healthcare for over 80% of the world population in these regions is based on traditional medicine. The main reasons for this include poverty, lack of accessibility to healthcare systems and traditional beliefs such as lack of trust in modern medicine. In developed countries however, a new concept of herbal technology is gaining attention internationally with a vast amount of literature available, thereby creating more awareness. In recognition of this, the WHO has passed a number of resolutions in response to this resurgence on the study and use of TM in healthcare systems. The WHO has urged nations to utilize their traditional medicine systems and develop a comprehensive approach to the subject of medicinal plant usage (Mashelkar *et al.*, 2001). In view of this, this chapter aims to validate the ethnopharmacological claims of the antimalarial properties of some selected plants from Nigeria.

3.2 Validating the ethnopharmacological claims of some plants used for malaria.

Ethnopharmacology is the scientific study of plants that are used by a group of people within the same ethnicity or sharing the same cultural practices. It is usually centred around folk medicine and is typically linked to plant use, ethnobotany. The ethnopharmacological usage of plants for different diseases have been extensively studied. In malaria alone, over 1200 plant species from 160 families have been documented for the treatment of malaria or fever (Willcox and Bodeker, 2004). An ethnobotanical survey is the first step in the identification, selection and development of the therapeutic agents from medicinal plants, however a detailed survey is beyond the scope of this study. In ethnobotany and natural products

chemistry, the mode of preparation and administration of herbal preparations are often crucial variables in determining efficacy in pharmacological evaluation (Levine, 1981; Lewis *et al.*, 1998; Albers–Schonberg *et al.*, 1997). Traditionally, plants are generally prepared by hot water extraction, alcoholic extraction or soaking plant material in fermented corn water. The remedies are usually taken based on a strong beliefs and trust in the herbalist, whose knowledge is usually passed down generations. Most people do not consider the possibility of toxic effects that could arise from these herbs. The herbalist usually gives recommendations by giving a certain amount of plant material and asking individuals to boil in a specific amount of water for daily oral consumption. Some plants are used in bath, steam inhalations or as incense. In this study, plants were selected based on recommendations by herbalists in Kano and Abuja region of Nigeria. The objective was to validate the ethanopharmacological claims for malaria of the selected plants and ascertain the safety profile using both *in vitro* assays and *in vivo* tests.

Local name	Botanical name	Collection point
Tazargade (whole plant)	<i>Artemisia maciverae</i>	Kano
Kizni (bark)	<i>Bridelia ferruginea</i>	Suleja
Kimbar mahalbar/halillua/tsamiyar kurmi (bark)	<i>Byrsocarpus Coccineus</i>	Kano
Bagaruwa	<i>Bombax buonopozense</i>	Kano
Hannu	<i>Boswellia dalzielii</i>	Kano
Coded NIPRD extract	<i>Coded</i>	Coded

Table 3.1 Selected plants for antimalarial studies: Showing the plants selected for this study and their botanical and local names.

3.3 Background on selected plants

3.3.1 *Artemisia maciverae*:

Artemisia maciverae belongs to the plant genus *Artemisia*. This is a large, diverse genus of plants with between 200 and 400 species belonging to the daisy family *Asteraceae*. Most species have strong aromas and bitter tastes, most likely due to the presence of terpenoids and sesquiterpene lactones. *Artemisia annua* from which artemisinin was derived from belongs to this large group of family. Plants in this family grow as herbaceous shrubs. *Artemisia maciverae* commonly grows in the wild in northern Nigeria. It is locally known as ‘**tazargade**’ and commonly used for various ailments. Locals believe it cures up to 99 ailments. For this reason, this plant is found in various herbaceous mixtures and is the master ingredient in the concoction of several herbalist.

3.3.2 *Bridelia ferruginea*:

It is a shrub belonging to the family *Euphorbiaceae*. Sometimes it reaches the size of a tree under suitable conditions. It is the most common savannah *Bridelia*. The tree is 6-15 m high; the bark is grey and often scaly. Geographically, it spreads through West Africa all the way to Zaire and Angola. It has various local names in different languages, **kizni** (Hausa), **iralodan** (Yoroba), **mareni** (Fulani) and **ola** (Igbo). The leaves, bark and fruits are used for the treatments of various diseases (Dalziel *et al.*, 1937). It is commonly used for various ailments including as a purgative and vermifuge (Imanga *et al.*, 1999). The bark is used for the coagulation of milk and as a traditional mouth gargle (Orafidiya *et al.*, 1990). It is commonly used as a chewing stick and the bark has also been reported to have molluscicidal activity (Iwu, 1984). The root and bark are also used for intestinal disorders and certain skin conditions (De-Bruyne *et al.*, 1997). It has also been reported to have anti-inflammatory, antipyretic and analgesic properties (Olajide *et al.* 2000). It is used in various paediatric preparations the most common called ‘**agbo**’ by the Yoroba people of Nigeria. It is also commonly used for the treatment of malaria infections in both children and adults (Iwu, 1993). The plant has several other uses as well as having trypanocidal activity. Studies by Atawodi *et al.*, 2005 and Ekanem, 2008 compared different solvent extracts of the plant against *Trypanosoma* species and found the methanolic extract to be the most active. Pasternek *et al.*, 1979 have also shown the plant to increase platelet counts in rats. The bark is also used in rural communities for waste water treatment. Studies by Kolawole and co-workers showed that the extract caused a significant clarification and sedimentation of total

suspended solids in a comparative manner to alum and ferric chloride. Interestingly, another study by Owoseni *et al* 2010 and Tall *et al.*, 2002 have shown the plant to possess antimicrobial activity against different microbes. This is an added advantage in its use for waste water treatment since studies by James (2000) have shown presence of human excreta in domestic waste water as major public health concern.



Fig 3.1: *Bridelia ferruginea*

3.3.3 *Byrsocarpus coccineus*:

Byrsocarpus coccineus (*B. coccineus*) is a scrambling shrub or small tree with leaves folding downward. It belongs to the family *Connaraceae* which grows in various parts of West Africa including Nigeria, Ghana and Burkina Faso. It has different local names in different communities for example ‘**kimbar maharba, halillua or tsamiyar kurmi**’ in Hausa; ‘**oke abolo**’ in Igbo and ‘**awuje wewe**’ in Yoruba. It is used as a traditional medicine in various concoctions. Different parts of the plant are used for different ailments. The plant is most commonly used for the treatment of haemorrhoids (northern Nigeria and Ghana), venereal diseases and dysentery (southern Nigeria), swellings, tumours and malaria (northern Nigeria). Several studies have evaluated the ethnopharmacological properties of this plant. Studies by Akindele *et al.*, 2010 investigated the hepatoprotective properties of the plant against carbon-tetra chloride induced liver damage in rats. The study further evaluated the

antioxidant properties of the plant, with the outcome suggestion that the plant has significant hepatoprotective properties. In the same study, the acute toxicity of the plant was expressed by the median lethal dose value (LD₅₀). The extract was found to be safe up to a high dose of 158.4 mg/kg in the acute toxicity tests. Subchronic tests in rodents revealed that the plant causes no significant changes in both biochemical and haematological parameters at that dose. Chronic tests however showed that the extract significantly increased white blood cell count, reduced liver enzymes, total cholesterol, triglycerides and total protein. The weight of certain organs including the heart and lungs was found to increase after prolonged exposure. This implies that the plant might be safe to take for short term use but some adverse effects could occur in long term use (Akindele *et al.*, 2010). Other pharmacological properties reported include pain relief and diarrhoea (Akindele *et al.*, 2006) and pyrexia (Akindele *et al.*, 2007).



Fig 3.2: *Brysocarpus coccineus*

3.3.4 *Bombax buonopozense*:

Bombax buonopozense is commonly known as the ‘gold coast bombax’ or red-flowered silk cotton tree. It belongs to the family *Malvaceae* formerly *Bombacaceae*. It has a good geographical distribution across West Africa where it is primarily native, spreading right

from Sierra Leone to Uganda, Nigeria, Ghana and Gabon. It grows typically at elevations of 900 to 1200 metres and can grow up to 40 metres (130 feet) height with buttress roots up to 6 metres (20 feet) in diameter. The tree is leafless and its bark is often covered in spines with deep pink-to-red flowers emerging from it. It is called '**bagaruwa**' amongst the hausa people. The plant material has several uses. It's cotton like seeds are used as a source of clothing and pillow fibre. Different parts of the plant are used as part of herbal preparations by herbalist, especially the bark. The bark is burnt to produce a smoke that is believed to drive away evil spirits called in certain cultures, it is used in treating wounds and swellings and also the gum is used as an incense preparation called '**turaren wuta**' which is a very common tradition within the hausa and kanuri ethnic groups. The wood is also commonly used in building canoes due to its light nature. The bark is rich in tannins making it find applications in the traditional tanning industry.



Fig 3.3 Bombax buonopozense: Showing a Bombax buonopozense stem bark, its geographical distribution and its flower.

3.3.5 *Boswellia dalzielii*:

Boswellia dalzielii belongs to the plant genus ***Boswellia*** found in Africa. *Boswellia dalzielii* belongs to the family, *Burseraceae*. It is a tree of the Savanna forest recognizable by its papery bark peeling off in a ragged manner. The bark yields a whitish gum resin, which dries readily and is friable. The bark of the tree is popular among the locals in north-western Nigeria as a potent source of ethnomedicine. The Hausa speaking people refer to it as **hannu, harrabi or ararrabi**. The leaf extract is used for the treatment of diarrhoea in poultry. The root decoction of the plant is used for wound healing (Etuk *et al.*, 2006). The fresh bark is eaten to induce vomiting and relieve symptoms of giddiness and palpitations. The root decoction of the plant boiled along with *Hibiscus sabdariffa* is used for the treatment of syphilis. The fragrant gum resin from the plant is used locally for fumigation of houses and as a deodorant (Etuk *et al.*, 2006). Oil from the leaves of *B. dalzielii* was found to exhibit significant activity against *Staphylococcus aureus*, *Bacillus subtilis* and *Candida albicans* (Nwinyi *et al.*, 2004). The aqueous stem bark extract of the plant was reported to show anti-ulcer activity and reduced gastrointestinal motility (Nwinyi *et al.*, 2004) and to possess anti-diarrhoeal effects, which may be related to anticholinergic mechanisms (Etuk *et al.*, 2006). Crude extracts of the stem bark of this plant have been found to show antibacterial activity against both gram-positive and gram-negative bacteria (Olukemi *et al.*, 2005). Despite the widespread uses of this plant in treating a plethora of human and animal diseases in this environment, little work has been done on its phytochemistry and its effects on some major organs in the body. The plant products (such as the gum resin) and different parts of the plant are widely employed in traditional medicine. The gum resin is used along with other medicines as gastrointestinal ailments and for the treatment of venereal diseases.



Fig 3.4: *Boswellia dalzielii*: Showing stem bark of *Boswellia dalzielii* and the whole tree in a typical sahara in North Western Nigeria.

3.3.6 SNO5 extract:

Preliminary *in vitro* work was done our laboratory and the plant is taken forward for *in vivo* studies by colleagues at the NIPRD, Abuja, Nigeria.

3.4 Phase I screening for initial phenotypic antimalarial activity determination

3.4.1 Plant collection and extract preparation

Bombax buonopozense, *Bridelia ferruginea*, *Brysocarpus coccineus*, *Artemisia maciverae*, *Boswellia dalzellii* and the NIPRD coded extract, were all collected from Nigeria and water extraction was performed in NIPRD. The plant materials were shade dried, grinded and and soaked in water for 24 h on a shaker. The plant material was filtered using a Whatmann paper and the filtrate was evaporated o dryness in a water bath. The dried extracts were stored in dried bottles and sent to the University of Salford for use in our laboratory.

3.4.2 Antiplasmodial screening of extracts

Plants were screened for antimalarial activity using the three optimised assays decribed in chapter 2, namely the SG-Microplate assay, SG-FCM and Giemsa light microscopy. The

extracts were screened by initially challenging K1 *P. falciparum* parasites with three randomly selected doses (20, 40 and 80 µg/ml) of the extracts. The parasite culture was incubated for 72 h under standard culture conditions described in section 2.1.5. At the end of 72 h, thin blood smears were prepared for each sample for analysis by Giemsa microscopy as well as following the respective protocols for the SG-Microplate assay and SG-FCM.

3.4.2.1 SG-Microplate screen of plant extracts:

Following the SYBR green assay, the results were collated and dose-response plots were obtained by normalising the percentage parasitaemia values for each group. Experiments were represented as mean value of triplicate data from three independent experiments, on Graphpad Prism. Fig 3.5 below shows the dose-response analysis

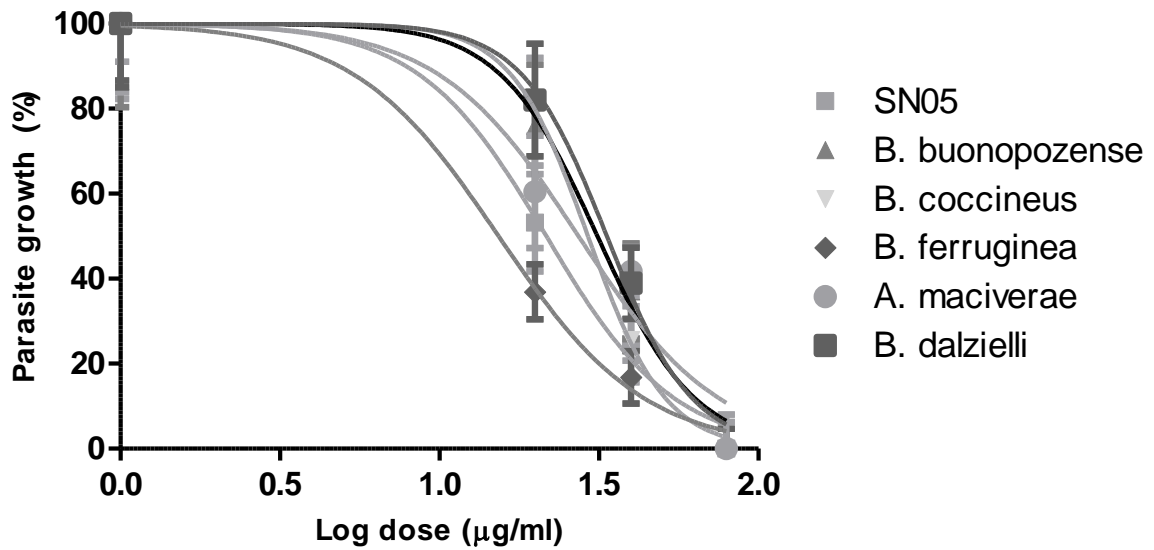


Fig 3.5 Preliminary phase I screen using SG-Microplate assay: Showing the preliminary phase I screen of various extracts against K1 cultures using the SG-Microplate. Control and extract- challenged extracts at ring stage for 72 h. Each curve represents independent experiment. Experiments were performed in triplicate and IC_{50} values were generated using graphpad prism for SN05, *B. buonopozense*, *B. coccineus*, *B. ferruginea*, *A. maciverae* and *B. dalzielli* were 21.63, 31.28, 29.23, 15.33, 27.50 and 33.48µg/ml respectively.

Statistical analysis of the preliminary data represented in fig 3.5 using a one- way ANOVA analysis on Graphpad prism showed a significant difference between control and extract-treated cultures with a $P < 0.0001$ in all six extracts. A Bonferri post test analysis was performed and there was no difference between extracts $P > 0.05$ in all six extracts.

The results of the preliminary screen for the six extracts using SG-Microplate assay shows that all the extracts exert antimalarial activity on cultures in a dose-dependent manner.

3.4.2.2 Giemsa light microscopy screen for plant extracts.

Slides prepared for Giemsa light microscopy were visualised under light microscopy and parasitaemia counted using oil immersion at X 100. The results were plotted using grouped data analysis on Graphpad Prism.

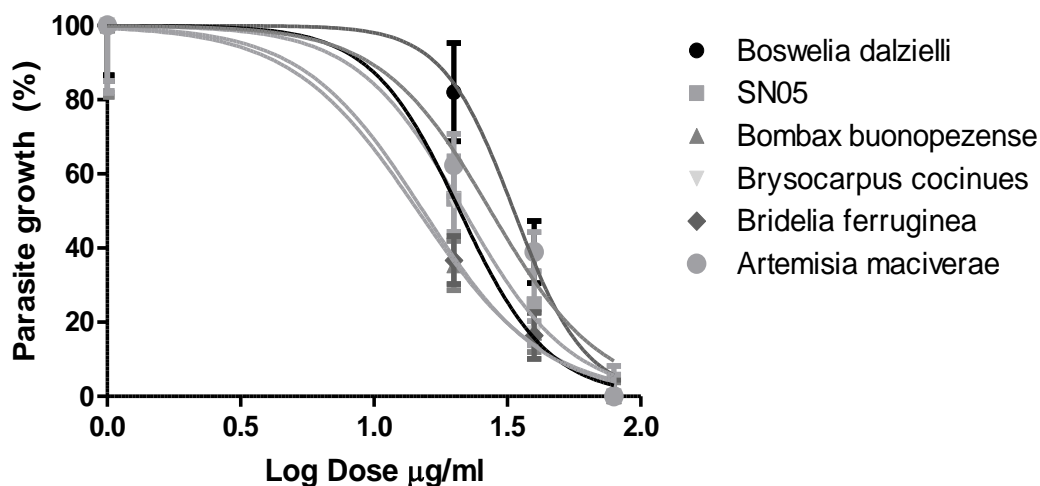


Fig 3.6 Preliminary phase I screen using Giemsa light microscopy: Showing the preliminary phase I screen of various extracts against K1 cultures using the Giemsa light microscopy. Control and extract- challenged extracts at ring stage for 72 h. Each curve represents independent experiment of triplicate data. IC_{50} values generated using graphpad prism for SN05, *B. buonopozense*, *B. coccineus*, *B. ferruginea*, *A. maciverae* and *B. dalzielli* were 21.70, 14.64,, 20.95, 15.29, 27.38 and 33.48 μ g/ml respectively.

Statistical analysis of the preliminary data represented in fig 3.6 above using a one- way ANOVA analysis on Graphpad prism showed a significant difference between control and extract-treated cultures with a $P < 0.0001$ in all six extracts. A Bonferri post test analysis was performed and there was no difference between extracts $P > 0.05$ in all six extracts.

The results of this assay compares with that of the SG-Microplate test. There was a significant difference observed between control and all extract-treated groups at all dose levels.

3.4.2.3 SG-FCM Screen for plant extracts

Results for control and extract treated cultures that were analysed using the SG-FCM analysis as described in chapter two were analysed using Graphpad Prism 6. Grouped data method analysis was used. The graphical and statistical results are presented below:

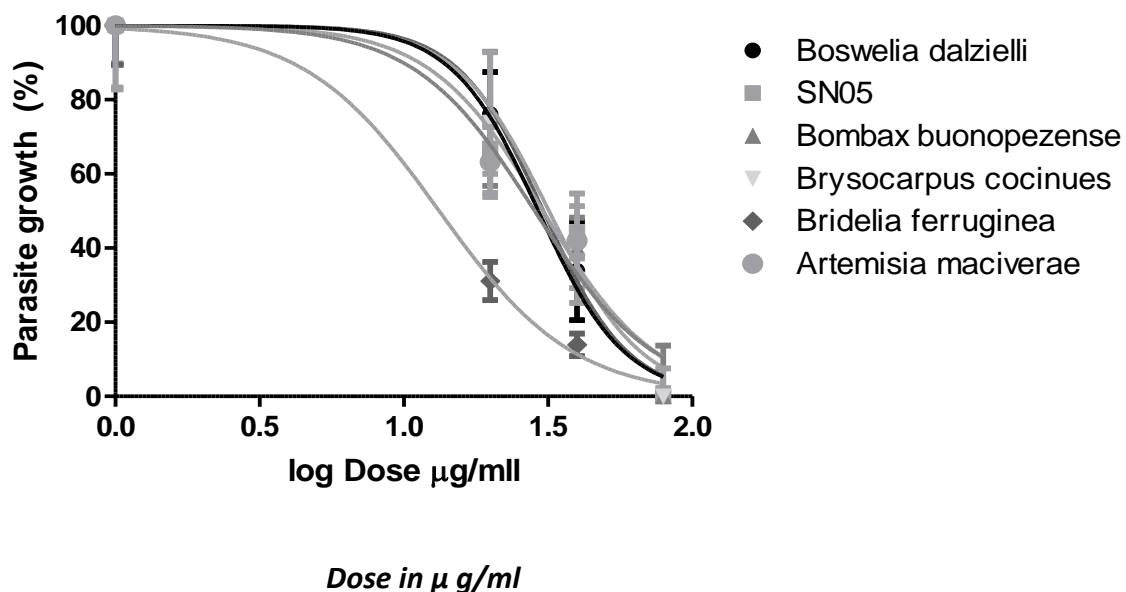


Fig 3.7 Preliminary phase I screen using SG-FCM assay: Showing the preliminary phase I screen of various extracts against K1 cultures using the SG-FCM. Control and extract-challenged extracts at ring stage for 72 h. Each curve represents independent experiment of triplicate data. IC_{50} values generated using graphpad prism for SN05, *B. buonopozense*, *B. coccineus*, *B. ferruginea*, *A. maciverae* and *B. dalzielli* were 30.42, 31.87, 29.27, 13.26, 28.44 and 30.47 μ g/ml respectively.

Statistical analysis of the data for the SG-FCM analysis using one-way ANOVA revealed a significant difference between control and all six extract treated cultures with P values < 0.001. A bonferri post test was used to investigate differences between the various extracts and there was no difference with each showing a P value > 0.05.

Hence preliminary tests have revealed that the extracts show strong antimalarial activity in all the three assays used with a high level of significance and low IC_{50} values < 50 μ g/ml in all six extracts.

3.4.2.5 SG-FCM, SG-Microplate and Giemsa assay of chloroquine and dihydroartemisinin (DHA) as positive controls

Chloroquine was used as a positive control in order to validate the assays and compare the IC_{50} with reference values.

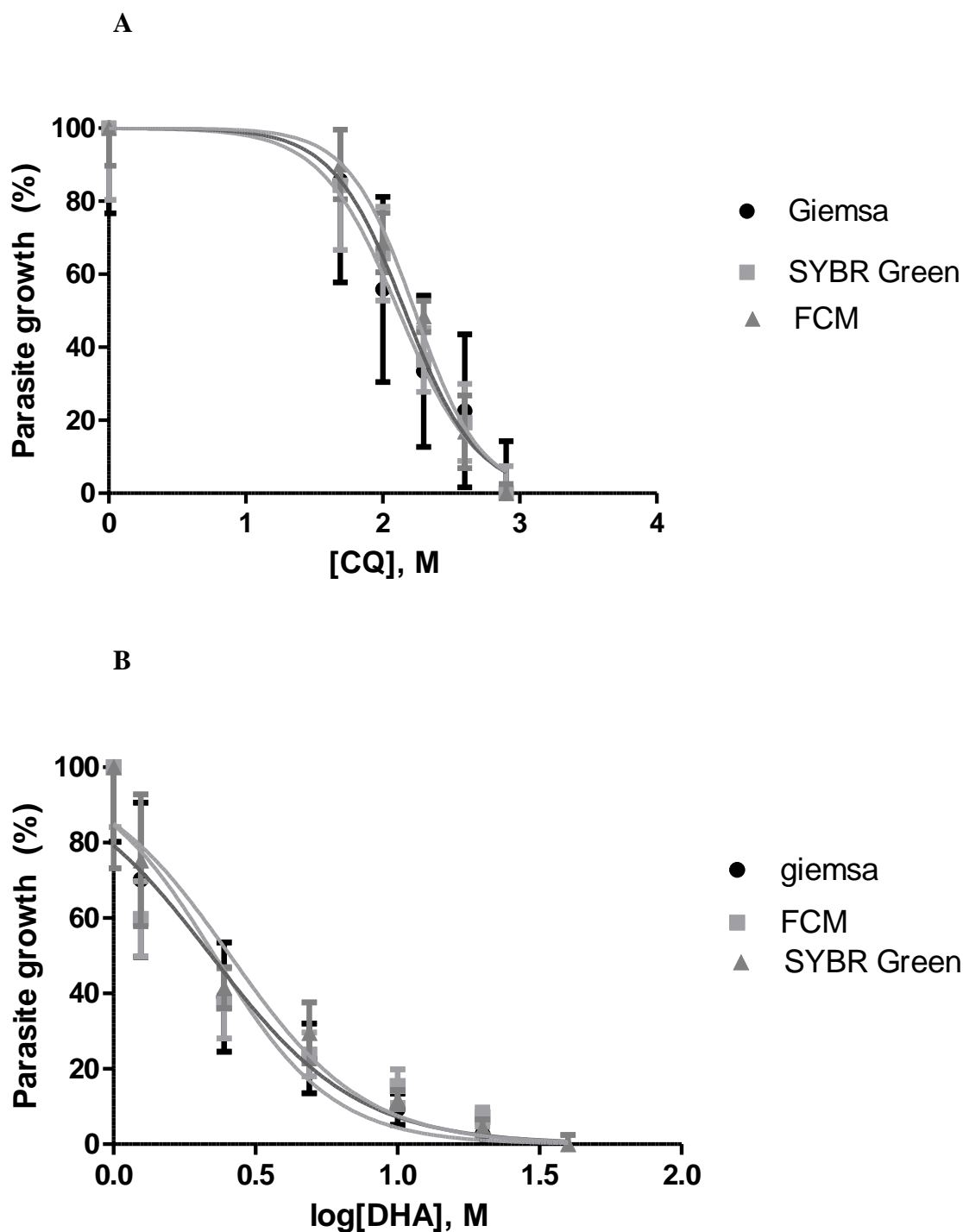


Fig 3.8 IC₅₀ determination for chloroquine (CQ) and DHA using SG-Microplate, SG-FCM and Giemsa light microscopy: Fig 3.8A IC₅₀ values of 130.4, 145.2, 171.2nM were obtained for Giemsa, SG-Microplate and SG-FCM respectively for CQ using normalised plots with Graphpad prism software. IC₅₀ values of 2.27, 2.21 and 2.58 nM were determined for DHA from the Giemsa light microscopy, SG-FCM and SG-Microplate assay respectively.

3.5 Phase II Screen of plant extracts

Preliminary Phase I screens showed significant and promising antimalarial activity on the multi-drug resistant K1 *P. falciparum* strain of the parasite. The collaborative nature of the project with NIPRD meant that phase II screening responsibilities were shared between the two institutions. *Bryoscarpus coccineus* and *Bridellia ferruginea* were selected to be taken further for second phase screening at the University of Salford due to their effectivity and wide biodiversity, hence availability, in West Africa. Furthermore, *B. ferruginea* has already been developed as a phytopharmaceutical drug for the treatment of diabetes mellitus, hence will have a defined toxicity profile data which could be accessed in order to go into clinical trials for malaria at the NIPRD clinical trial unit.

3.5.1 *Bridellia ferruginea* collection

The stem bark of *B. ferruginea* was collected from Odenigbo Nkalagu Obukpa district in Nsukka, Enugu State, Nigeria. The plant was identified by its leaves in the department of medicinal plant research at the National Institute for Pharmaceutical Research and Development (NIPRD), Nigeria. Specimen was deposited at the institute's herbarium with the voucher number (NIPRD/H/6414). The international index label is *Euphorbiaceae Bridellia ferruginea* Niger FL. [W.J.Hooker].5111.1849[NOV-DEC 1849](IK). The plant bark was cleaned and cut into pieces and air dried at room temperature for a week. The bark was grounded to a powder form from which aqueous and methanolic extracts were prepared. The grounded plant material was soaked in water and 70% methanol respectively and left on a shaker for 24 h. The dissolved extracts were filtered the next day and the filtrate was evaporated to dryness in a water bath equipment.

3.5.2 Dose response analysis for *Bryoscarpus coccineus* using different assays

The three assays (SG-Microplate, SG-FCM, *Giemsa* light microscopy) were used to obtain a dose-response analysis of *B. coccineus* in order to estimate the median inhibitory concentration (IC₅₀). This is the dose required to kill 50% of the parasites. In order to calculate the percentage parasite growth inhibition for each dose level, the following formula was used:

Percentage parasitaemia = (Control-Treated/Control) *100.

The values were plotted on a graph Prism software and the three assays were compared.

All three assays were comparable and the IC₅₀ value was in the region of 72 µg/l.

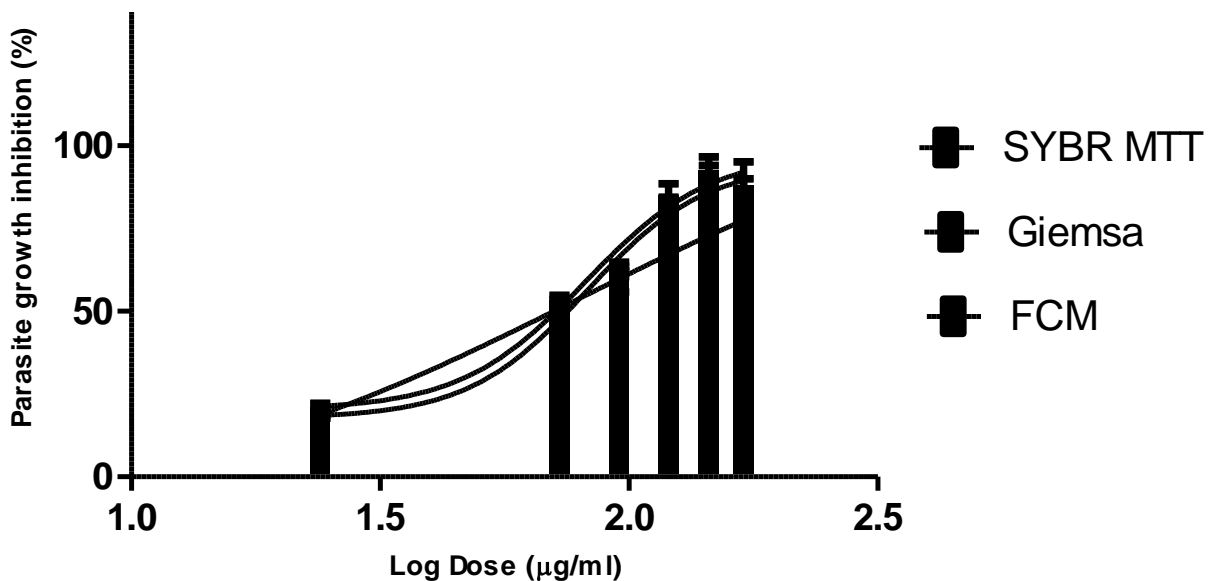


Fig 3.9 *Antiplasmodial activity of B. coccineus using different assays: Showing the antiplasmodial activity of B. coccineus against Kl cultures using three different assays (SG-Microplate, Giemsa light microscopy and SG-FCM). The IC₅₀ value was in the region of 72.83, 78.98 and 81.37 µg/ml for SG-Microplate assay, Giemsa light microscopy and SG-FCM. This was obtained through dose-response simulation using Graphpad prism software.*

One-way ANOVA analysis was also performed on the data represented in Fig 3.9 above to determine if there was any significant difference between the three methods of analysis. The results showed that in all three assays, there was a significant difference between control values and treated values with a P value < 0.01 in all treatment groups across all the assays. Further analysis also revealed that there was no significant difference between the SG-Microplate assay Vs Giemsa light microscopy with a P value of 0.0823; SG-Microplate assay Vs SG-FCM with P < 0.05; SG-FCM Vs Giemsa light microscopy with P < 0.05.

The results of this study shows a dose dependent parasite growth inhibition of the extract. The IC₅₀ value in all three assays was in the region of 72 µg/ml and analysis by Graphpad Prism (see table 3.5 above) show that these findings are significant. The treated culture is significantly different to control cultures at all the dose levels tested.

3.5.3 Dose response analysis for aqueous *B. ferruginea* using different assays

The dose-response activity of aqueous *B. ferruginea* was evaluated by incubating ring stage parasites for 72h. On the final day of incubation, the challenged parasites were prepared for SG-microplate assay, Giemsa microscopy and SG-FCM using methods previously described. The results of these assays are presented in the graph in fig 3.9

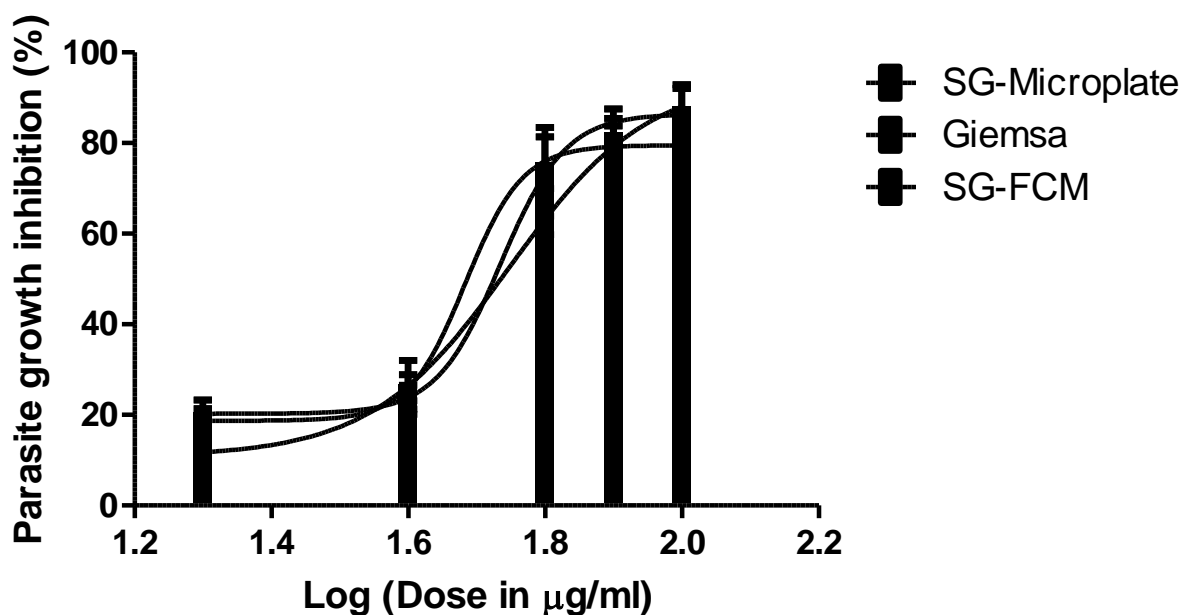


Fig 3.10 Antiplasmodial activity of aqueous *B. ferruginea* against K1 *P. falciparum* cultures using different assays: Showing the assay results are the same for the three assays with a comparable IC_{50} value in the region of 50 µg/ml for all the three assays as indicated in the graph.

The IC_{50} values determined using the Graphpad prism software for the data represented in Fig 3.10 above were 48.25, 55.95 and 54.32 µg/ml for SG-Microplate, Giemsa microscopy and SG-FCM respectively. There was no significant difference between the three assays with $P > 0.05$ in all cases.

3.6 Comparing aqueous and methanolic *B. ferruginea*

For the scope of this study, *B. ferruginea* was chosen as the candidate to proceed with. The main reason for this decision is, it already exist as a phytopharmaceutical drugs for diabetes hence commencing clinical trials for malaria will be likely. The extract has also shown an

IC₅₀ dose range within an acceptable value for plant extracts (up to 100 µg/ml) and also lower than that of *B.coccineus*. Furthermore, the plant has a proven track record of extensive use within various communities and it grows abundantly in the wild. In view of these factors the methanolic extract was explored to see if it is more potent due to the fact that methanolic extracts tend to be rich in flavonoids and terpenoids which have been previously implicated in malaria (Ntie-Kang *et al.*, 2014).

3.6.1 Comparing the aqueous and methanolic extract of *B. ferruginea* using the SG-Microplate assay

Both the aqueous and methanolic extract of *B. ferruginea* were incubated in trophozoite stage cultures for 48 h and subsequently analysed using SG-Microplate assay. The results are represented in fig 3.10 below:

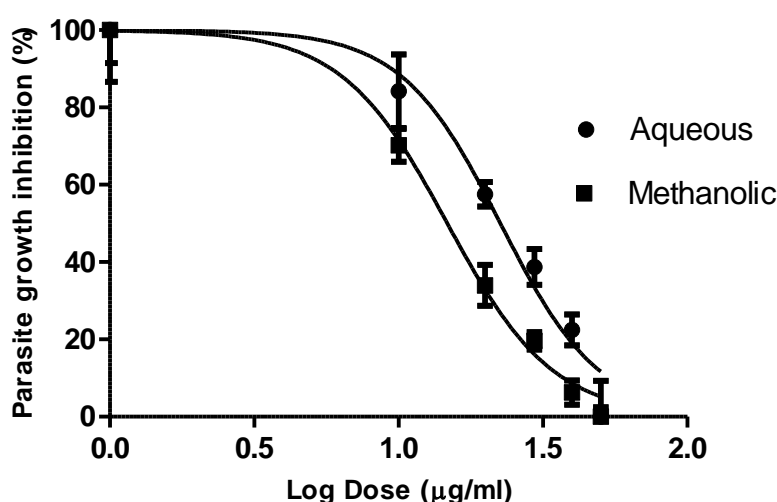


Fig 3.10 Comparing the activity of aqueous and methanolic *B.ferruginea* against *K1* cultures using SG-Microplate assay: The results show that the methanolic extract of *B. feeruginea* showed a higher inhibition of parasitaemia at all dose levels compared with the aqueous extract. The IC₅₀ for the aqueous extract was 22.52 whilst that for the methanolic extract was 14.75 µg/ml. was generated using the average of three independent experiments on graph pad prism. There is a left-ward shift in the dose-inhibition curve.

The results were further analysed using one-way ANOVA in Graphpad Prism . The statistical analysis showed that the aqueous and methanolic *B. ferruginea* was significantly different with P value of 0.013.

3.6.2 Comparing the aqueous and methanolic extract of *B. ferruginea* using Giemsa light microscopy

Aqueous and methanolic extracts of *B. ferruginea* were incubated in K1 cultures at trophozoite stage for 48 h and the cultures were analysed using the Giemsa light microscopy described in section 2.1.4. The data was analysed using Graphpad Prism and presented in fig 3.11 below.

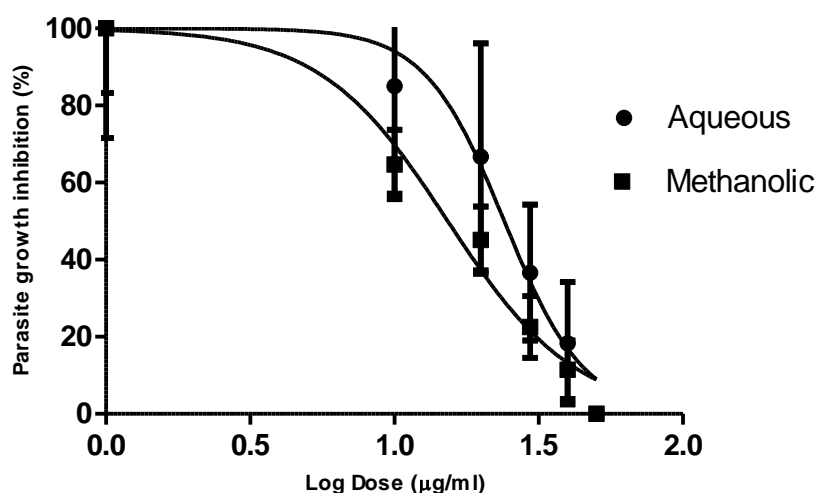


Fig 3.11 Comparing the activity of the aqueous and methanolic extracts of *B. ferruginea* against K1 cultures using Giemsa light microscopy analysis: The results showed that the methanolic extract exerted a higher parasite killing compared with the aqueous extract. The average IC_{50} for the aqueous extracts was 24.05 whilst that for the methanolic extract was 15.31 as generated using graphpad prism.

Results analysed using one-way ANOVA in Graphpad prism showed that the methanolic *B. ferruginea* was significantly more potent than the aqueous extract with a P value of 0.002.

3.6.3 Comparing the aqueous and methanolic extracts of *B. ferruginea* using SG-FCM assay

K1 *P. falciparum* cultures at trophozoite stage were challenged and incubated for 48 h. After the incubation period, the cultures were prepared for SG-FCM analysis as previously described in chapter 2.

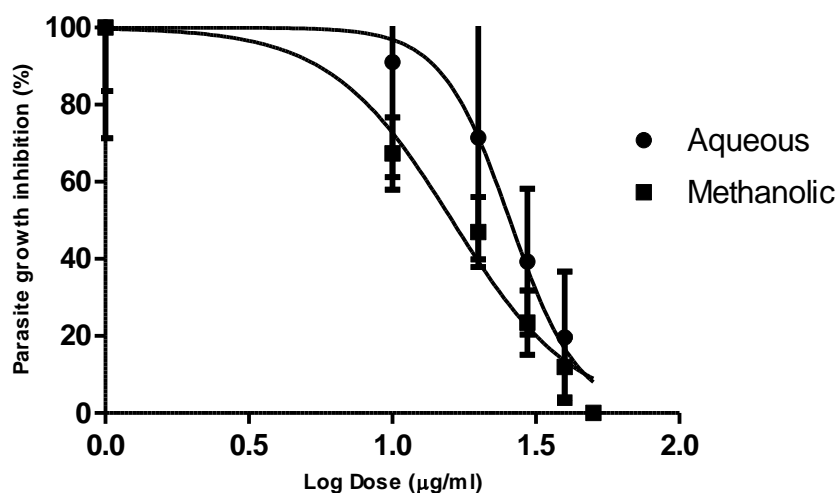


Fig 3.12 Comparing antiplasmodial activity of aqueous and methanolic *B. ferruginea* on K1 *P. falciparum* cultures using SG-FCM analysis: The results show that the methanolic extract is more efficacious with a lower IC_{50} value of 16.14 whilst the aqueous extract had an IC_{50} value of 25.69.

Column Statistical analysis of the data represented in Fig 3.12 above using One-way ANOVA showed that the difference observed between doses are significantly different $P < 0.001$ whereas there is no significant difference between the three assays $P > 0.01$

3.7 Time point assay for methanolic *B. ferruginea*

As the methanolic extract seemed more potent, with an IC_{50} near 15 µg/ml, a time point assay investigation was performed using SG-FCM assay, as it is the most specific and accurate amongst the three assays. Cultures were split into 5 ml culture volumes in 25 cm³ flasks for each timepoint. Moreover, each timepoint had a control and treated flask with the control being the unchallenged culture whilst the treated culture was challenged with 20 µg/ml of the methanolic extract. The experiment was initiated at ring stage. The parasitaemia of the culture was checked at the commencement of the experiment and at various intervals i.e 0 h, 2 h, 4 h, 6 h, 24 h, 48 h and 72 h. The experiment was repeated three times for statistical analysis. The data was analysed using Graphpad Prism and the graphical results are represented in fig 3.13. The experimental data was represented after calculating the percentage inhibition of the challenged extracts relative to control cultures.

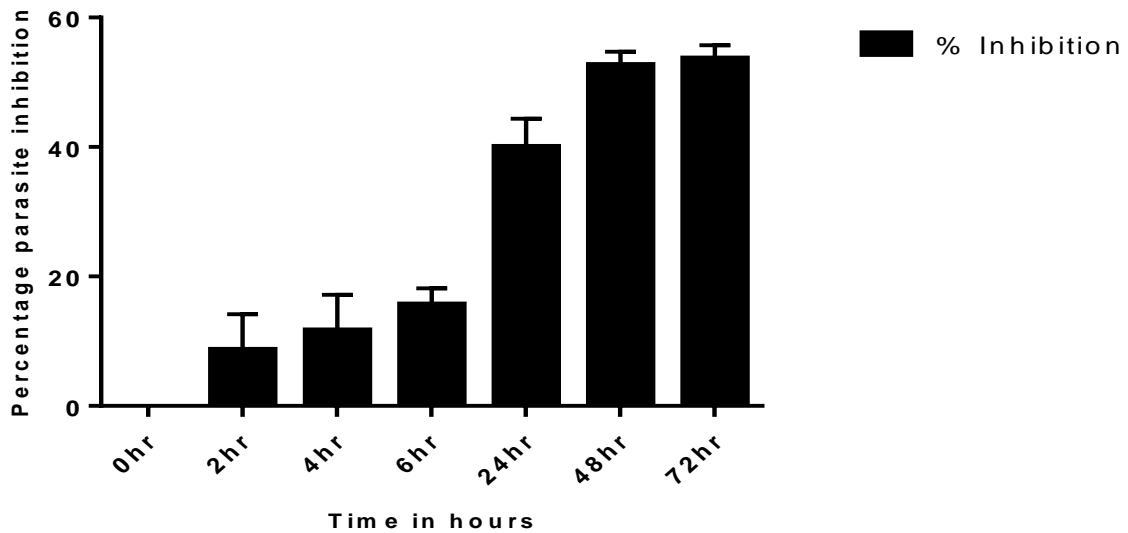


Fig 3.13 Timepoint assay for methanolic *B. ferruginea*: Graph representing the percentage inhibition of parasite growth during the time point assay after challenging cultures with 20 µg/ml methanolic *B. ferruginea* at ring stage.. The extract showed an early onset of action as early as 2 h post challenge.

The results of this experiment showed a time dependent parasite killing. At 2 h, 4 h and 6 h, percentage parasite inhibition gradually increased to about 20% and about 58% inhibition was observed at 48 h and 72 h. One-way ANOVA shows that at 2 h, 4 h and 6 h, although a difference was observed, the results were not statistically significant with a P value of 0.18, 0.15 and 0.12 respectively. At 24 h, 48 h and 72 h, the statistical analysis shows that there is a significant difference with control with a P value < 0.005.

3.8 Effect of *B. ferruginea* on erythrocytic stages

Different drugs act on different stages of the malaria parasite via different mechanisms. The main aim of this experiment was to investigate the effect of the extract incubated at different erythrocytic stages of the parasite life cycle. Synchronised cultures at either ring or trophozoite stages were challenged with 20 µg/ml of the extract and harvested at 24 h, 48 h and 72 h. Different flasks were set up for different timepoints and each timepoint had its control flask in parallel with the treated culture. The results of this assay are graphically represented below.

3.8.1 Effect of *B. ferruginea* extract on ring stage cultures

Cultures were synchronised using method previously described in section 2.1.6. Briefly, the synchronised cultures were challenged at ring stage with 20 µg/ml. The effect of the extract was evaluated at four time points (0 h, 24 h, 48 h and 72 h). Each timepoint was set up with three 12.5 cm³ flasks (5 ml culture volume), challenged with 20 µg/ml *B. ferruginea*, a positive control flask challenged with 5 nM DHA and a negative control unchallenged flask. Hence a total of 12 flask per experiment. The experiment was repeated with the same conditions in triplicate. The results are graphically presented in fig 3.14 below:

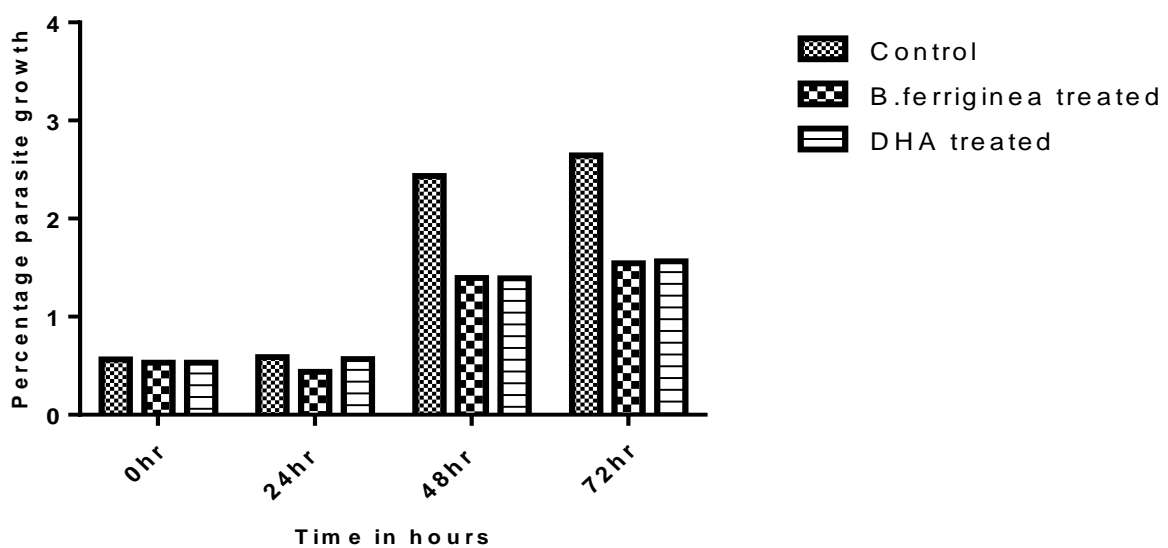


Fig 3.14 Effect of *B. ferruginea* and DHA on *Plasmodium* ring stages: Shows that the extract is effective on ring stage parasite stages with the highest activity observed at 48 h and 72 h for both *B. ferruginea* and DHA. DHA was used as a positive control as it is known to act on all parasite stages. One-way ANOVA analysis of the data using Graphpad Prism 6 also showed that there was a significant difference between control and treated samples at 48 h and 72 h. $p < 0.001$.

3.8.2 Effect of *B. ferruginea* extract on trophozoite stage cultures

Synchronised cultures at trophozoite stage were challenged with 20 µg/ml of methanolic *B. ferruginea* and also 5 nM DHA as described in section 3.7.1. At 0 h, 24 h, 48 h and 72 h, the cultures were harvested for SG-FCM analysis and the results are graphically represented below.

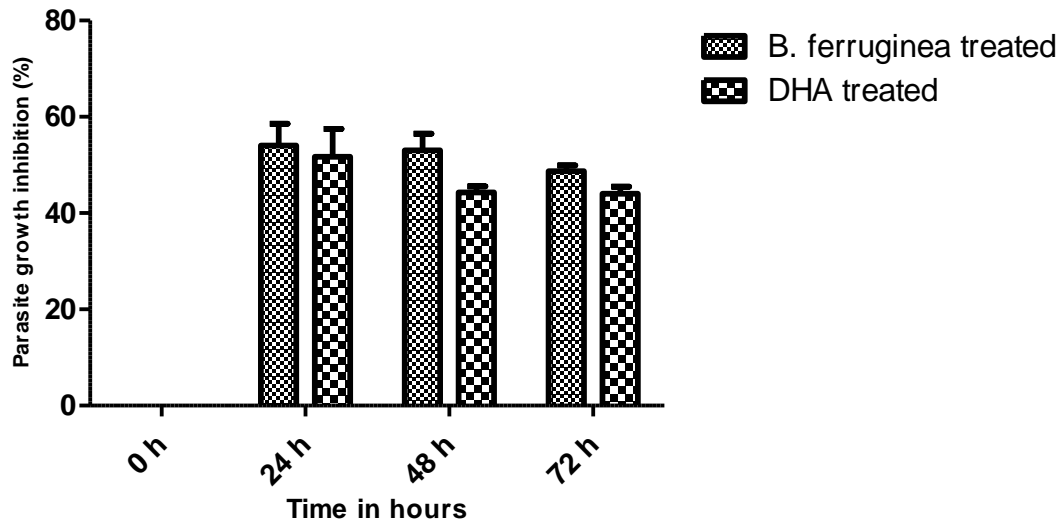


Fig 3.15 Effect of methanolic *B. ferruginea* and DHA on Plasmodium trophozoite stages: Showing the effect of methanolic *B. ferruginea* and DHA on the trophozoite stage parasites with a higher inhibition compared to the ring stage experiment. One-way ANOVA analysis using Graphpad Prism showed the difference between control and its parallel treated culture was significant, $P < 0.001$.

3.8.3 Comparing ring and trophozoite stage challenged cultures

The graph below shows a comparison of cultures challenged at ring and trophozoite stage with *B. ferruginea* methanolic extract

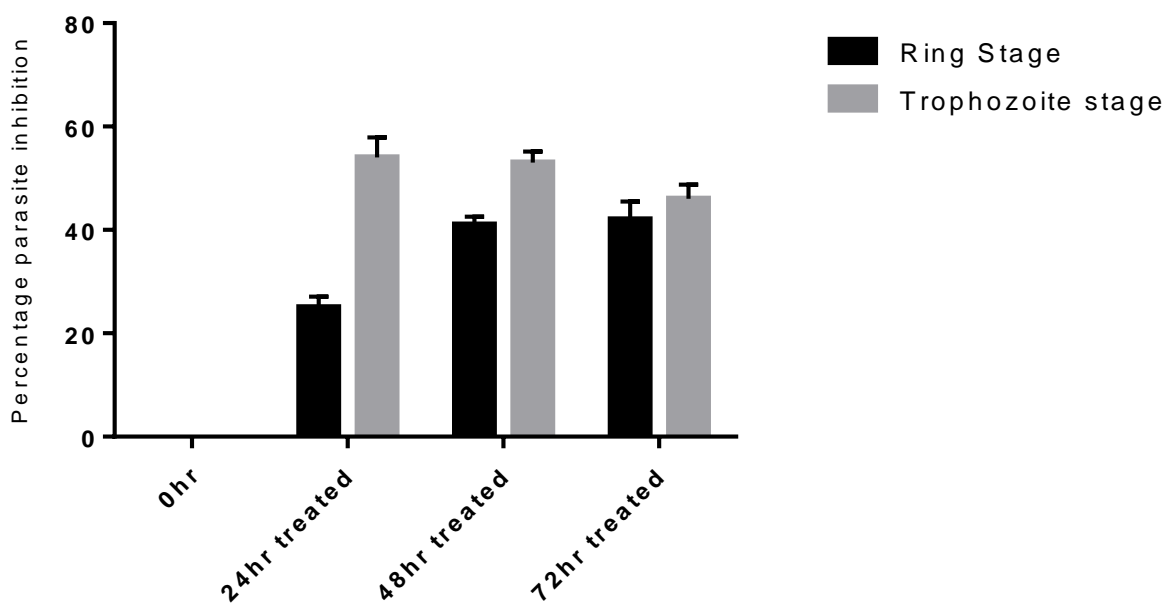


Fig 3.16 Comparison of the effect of *B. ferruginea* on ring and trophozoite stages: Showing the the percentage inhibition of parasitaemia by methanolic *B. ferruginea* on parasite erythrocytic stages.

One-way ANOVA analysis using Graphpad Prism 6 showed there was a significant difference between inhibition in ring and trophozoite stage cultures at 24 h for *B. ferruginea* treated cultures, $P < 0.01$ and at 48 h $P < 0.05$. The difference between the two stages was not significant at 72 h ($P > 0.05$). The inhibition was noted to be less at 72 h at both parasite stages possibly because the parasites have gone into the next cycle.

3.9 Phytochemical screening of *B. ferruginea*

Qualitative phytochemical analysis were performed for alkaloids, saponins, flavonoids, terpenes, tannins, sterols, glycosides and phenols using standard procedures. The results could be used to establish the possible active secondary metabolites in both the aqueous and methanolic extract of *B. ferruginea*. Aqueous extraction methods are known to extract anthocyanins, starch, tannins, saponins, terpenoids, polypeptides and lectins. Methanolic extraction method on the other hand is known to extract anthocyanins, terpenoids, saponins, tannins, flavones, phenols, polyphenols and lactones (Tiwari *et al.*, 2011).

Phytochemical test results

Test	Aqueous	Methanolic
	<i>B. ferruginea</i> Bark	<i>B. ferruginea</i> Bark
Alkaloids	Present	Present
Flavanoids	Present	Present
Tannins	Present	Present
Terpenes	Present	Present
Cardiac Glycosides	Present	Present
Saponins	Present	Present
Phlobatannins	Present	Present
Anthraquinone	Absent	Present
Sterols	Present	Present
Cyanogenic glycosides	Absent	Absent
Resins	Present	Present

Table 3.2 Phytochemicals in aqueous and methanolic *B. ferruginea* stem bark: Showing the presence of different phytochemicals in *B. ferruginea* stem bark. Anthraquinone was found to be absent in the aqueous bark extract but present in the methanolic extract.

The results of the phytochemical test have confirmed the presence of various bioactive compounds including alkaloids, saponins, tannins, flavanoids and terpenes amongst other constituents.

3.10 Conclusion

The five plants screened in this study have several ethnopharmacological usage, including: tumour management, malaria, fever, diarrhoea amongst other disorders. The bio-evaluation of the pharmacological actions of the plant extracts in the current chapter shows that they have a strong antimalarial activity. Based on the results, *B. ferruginea* was chosen as a candidate for this study due to its wide biodiversity and also the fact that it is already used as a phytopharmaceutical drug for diabetes in Ghana. The extract showed a dose-dependent inhibition of K1 *P. falciparum* malaria culture, supporting the ethnopharmacological claim of its activity against malaria. A very interesting finding is the fact that the extract showed an early onset of action as early as 2 h, 4 h and 6 h post incubation. The results were however statistically different to control, with a P value > 0.05, but they were consistent in all triplicate experiments. There was a statistical difference between the control group and treated groups at 24 h, 48 h and 72 h post challenge with a P value < 0.005. The results in this chapter have shown that the methanolic *B. ferruginea* extract has anti-malarial activity and is active against all both the ring and trophozoite stage of the erythrocytic parasite cycle. The results also show that the extract has an early onset of action, hence will be potentially relevant in the clinical application for malaria.

The results of the phytochemical analysis in this chapter revealed the presence of several secondary metabolic compounds (phytochemicals). Phytochemicals have shown to be a useful source of human medicine (John *et al.*, 2013). The results of this study can be used in crude plant quality control and elucidation of the therapeutic mechanism of action of the plant extract.

CHAPTER 4

Investigating the mechanism of action of methanolic *Bridelia ferruginea*

4.1 Introduction

Over the years, several antimalarial targets have been discovered some of which have already been discussed in Chapter 1. Quinine, for example, has been implicated in targeting the haem detoxification pathway whilst other drugs like atovaquone have been shown to target the mitochondria.

The malaria parasite consumes host cellular resources to grow and multiply as explained in the parasite life cycle (the erythrocytic stage). During this multiplication stage within the host red blood cell, the parasite consumes up to 80% of its host cell haemoglobin. This digested haemoglobin is used by the *Plasmodium* as a source of amino acid as well as for maintaining osmotic balance. The entire process occurs in the parasite food vacuole which is acidic in nature. Once this digestion occurs, **haem** is released (a porphyrin) as a by-product. Haem is potentially toxic to cells as it catalyses the products of certain reactive oxygen species (ROS). Haem degradation is very critical to the *Plasmodium* as concentrations as low as 20 μM have been shown to lyse the parasite within a span of 10 minutes. The *Plasmodium* has a mechanism of detoxifying this toxic product to a safe insoluble crystalline product called **haemozoin**. Haem has been shown to disrupt cell membranes, damage cell structures and cause cell lysis (Fitch *et al.*, 1983). The haem detoxification pathway hence is an attractive target for antimalarial drug candidate. For the scope of this study, the haem detoxification pathway was explored to determine if *B. ferruginea* acts through this pathway.

4.2 Haem detoxification

The mechanism of haem detoxification is highly efficient. The malaria parasite consumes and digests about 70% of the host haemoglobin releasing free amino acids. About 16% of these amino acids are used for protein biosynthesis. As a small amount of the released amino acids are used up, the excess are discharged out of the infected erythrocytes to the surrounding plasma (Rudzinska *et al.*, 1965; Krughak *et al.*, 1986; Zarchin *et al.*, 1968; Kutner *et al.*, 1985; Trager, 1994). The exact reason why parasites expend so much energy

in ingesting and digesting excess haemoglobin is yet to be understood. The mechanisms of the haem detoxification pathway is also not fully understood, although several controversial pathways have been suggested. One pathway that is believed to be the most important and predominant is **sequestration of haem into haemozoin**, also known as malaria pigment. The process is a biocrystallization pathway converting the haem from a soluble monomeric form to an insoluble dimeric form, haemozoin (Hempelmann *et al.*, 2007). Each haemozoin crystal consists of 80,000 haem molecules. Haemozoin accumulates in the parasite food vacuole which is acidic in pH. Several proteases including cysteine, aspartic and metalloproteases have been characterized from the food vacuole of *P. falciparum*. (Rosenthal, 2000). A multi-step mechanism has been proposed, suggesting more than one protease is involved in the degradation process, with the proteases cleaving the protein molecule at different sites (Sijwaoli and Rosenthal 2004). These proteases digest the haemoglobin into small peptides and subsequently amino acids in a multi-step process involving cleavage of different proteases. Once the amino acids are released, a free ferrous protoporphyrin IX (Fe (II) PPIX) is released and rapidly converted to Fe (III) PPIX (haem) by oxidation. When this multiple degradation process successfully releases haem, the free haem becomes a toxic by-product as mentioned earlier. This non-sequestered haem once accumulated, could lead to red cell lysis amongst other biochemical consequences.

Other pathways that have been suggested for haem degradation include the involvement of enzymes. In mammals, haem oxygenase breaks down excess haem into three components (biliverdin, carbon monoxide and iron). Kituchi *et al.*, 2005 have however shown that the *Plasmodium* lacks this enzyme hence it is a pathway unique to mammals. This difference is clearly exploitable for antimalarial drug design

More recently, Huy *et al.*, 2013 have proposed a phospholipid membrane-mediated pathway for haemozoin formation. Other groups have previously reported the involvement of histidine rich proteins (Sullivan *et al.*, 1996). It is likely that a combination of these mechanisms occur as different pathways have been speculated. In support of this, Pandey *et al.*, 2003 showed the involvement of both lipids and histidine rich proteins in haemozoin formation although another pathway has been proposed outside of the parasite food vacuole. This is the glutathione-mediated and enzymatic degradation pathway. There is however no evidence of how the haem molecule translocates into the food vacuole (Egan *et al.*, 2002).

4.3 Structure of haemozoin

Haemozoin was initially thought to be identical to haematin but this has been proved otherwise due to their differential solubility characteristics in weak bases such as bicarbonate buffer and DMSO (Slater *et al.*, 1991). Haematin solubilises in both solvents whilst haemozoin doesn't.

Haemozoin extracted from parasite lysate that accumulated in the liver and spleen have shown similar spectroscopic characteristics and solubility. Infrared (IR) data of haemozoin shows some peaks which are characteristics of the Fe-carboxylate bond at 1664 and 1210cm^{-1} . Haem is a porphyrin molecule found in haemoglobin which has an extensive aromatic structure with a rich phytochemistry consisting of several π - π transitions appearing in the visible part of the electromagnetic spectrum. The UV absorption spectra of haem consists of an intense Soret band around the region of $\lambda=400$ nm and also medium bands appearing at $\lambda=450$ - 700 nm. The figure below shows a descriptive spectra of haem.

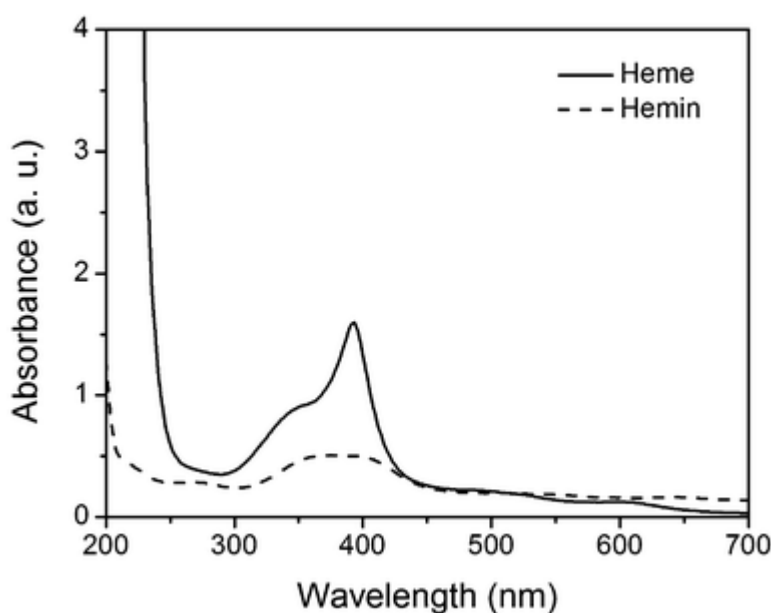


Fig 4.1 UV spectra of haem and haemin: Showing the Ultraviolet (UV) spectra of haem and haemin at 405 nm.

(Source: <http://pubs.rsc.org/en/content/articlelanding/2013/an/c3an00310h#!divAbstract>).

The infrared spectrum of haemozoin shows that it is significantly different from haematin or haem but very similar to β -haematin. β -haematin exist in a dimeric form consisting of two

haematin molecules that are joined by H-bonding, just like the haemozoin molecule. Each haematin molecule consist of a central iron molecule which forms an iron-oxygen coordinate bond, linking the central iron of one molecule to the carboxylate side chain of the next haematin molecule, forming a dimer. These corresponding iron-oxygen bonds are unique to β -haematin and have not been observed in any other porphyrin structure. The molecule can exists in both linear and cyclic forms but never in a polymeric form (Hempelmann *et al.*, 1994).

4.4 Haem aggregation

Haem aggregates as a result of mutual attractions between several haem molecules that are held by hydrogen bonding. These aggregates that form may vary slightly, but all have a similar absorbtion spectra ranging from 395 nm to 450 nm. This is why during *in vitro* studies for haemozoin assay, non uniform aggregates form, leading to excitation at different wavelenth within the stated range. The formation of side products can be minimised by several measures including using haemin rather than haematin as a starting material in the assay.

4.5 Role of haemozoin in malaria pathogenesis

Free haemozoin is released into circulation during the re-infection stage (i.e once the shizonts rupture). The haemozoin is phagocytosed into host cells leading to several biochemical changes including inhibition of erythropoiesis and red cell lysis. This is the main reason for anaemia in malaria patients (Skorokhod *et al.*, 2010). Livers and spleens of patients with malaria appear dark and black upon necropsy after death, hence it is not known where and for how long haemozoin is deposited in the body (Hanschield & Egan 2007).

4.6 Haemozoin as a target for antimalarials

Haemozoin seems a very interesting target for antimalarials due to several reasons. Haemozoin formation is critical to parasite survival as the build up of haem is toxic to the parasite. Hence blocking the pathway will lead to the accumulation of haem in the parasite food vacuole causing parasite death. Another crucial factor that supports targeting haemozoin as a drug pathway is the mechanism of haemozoin formation. Biocrystallisation seems a unique pathway to parasites. As mentioned earlier, mammalian cells rely on an enzymatic pathway for haem formation, this enzyme is absent in the parasite. Hence by targeting the biocrystallisation pathway, the drug will be selective for the parasite and safe

to the host cell. Several blood schizonticidal antimalarials including quinolone drugs such as chloroquine and mefloquine act via the haem biocrystallisation pathway by inducing disruption of haem (Tripathi *et al.*, 2001). Some 8-aminoquinolones have also been shown to inhibit this process (Vennerstrom *et al.*, 1996). The drugs bind to both haem and haemozoin crystals, forming a non-covalent complex with haem, hence preventing the addition of new haem units to the crystals (Sullivan *et al.*, 1996).

Haemozoin formation inhibition has been demonstrated in several *in vivo* and *in vitro* experiments. *In vitro*, the quinolones block the polymerisation of micromolar haem into haemozoin which is mediated by crude trophozoite lysates. Quinolones generally act by incorporating a drug-haem complex into the growing polymer of haemozoin. Polymer elongation seems important for significant drug-haem interaction. Without polymer elongation, the interaction with haemozoin is weak. This mechanism of haem-quinolone complex incorporation into the growing polymer might be a possible explanation to the dramatic accumulation of drug in the parasite food vacuole. Chloroquine for example, has been shown to hyperconcentrate to near millimolar concentration in the acidic parasite food vacuole of *P. falciparum* from the nanomolar concentration in the plasma. The rate at which this drug accumulation occurs in the food vacuole could lead to a 1 mM concentration (Sullivan *et al.*, 1996). Once the drug accumulates, it disrupts haem biocrystallisation either from drug-haem interaction with sequestration of the monomeric substrate or by direct interaction of chloroquine with a protein that is involved in haem aggregation (Yayon *et al.*, 1985).

Studies of the chemical reaction of haemoglobin with artemisinin have shown that the protein bound haem in haemoglobin reacts with artemisinin much faster than free haem does. It seems that the uptake of haemoglobin and the accumulation of artemisinin into the parasite food vacuole together with the preferential reactivity of artemisinin with haem in haemoglobin, may make the haemoglobin the primary target of artemisinins as antimalarials. Two haem artemisinin derivatives have been isolated, which include the monoalkylated and the dialkylated haem derivatives. These have been shown to inhibit haemozoin formation by binding to PfHP II, ultimately killing the *Plasmodium* (Kannan *et al.*, 2005).

4.7 *In vitro* methods used for haemozoin detection and inhibition

Studies using X-ray crystallography, nuclear magnetic resonance technology (NMR) and spectroscopic techniques have shown haemozoin and β -haematin to be identical in structure and solubility profile (Slater *et al.*, 1991). Hence β -haematin formation has been extensively studied *in vitro* to aid in the understanding of haemozoin formation and aggregation. Monomeric haem aggregates in acidic conditions, pH < 4.5. These aggregates are soluble in sodium bicarbonate buffers and DMSO. Interestingly, β -haematin is a highly insoluble substance and can only be dissolved in sodium hydroxide solution. This differential solubility has been invaluable for the separation of formed β -haematin in a reaction from unreacted haem aggregates. Haem can be converted to β -haematin, although certain conditions have to be met for this chemical synthesis to occur. Simply incubating haematin in glacial acetic acid at high temperatures of 70-80°C for 18 h leads to β -haematin formation, with a reaction yield of about 40-50% (Bohle *et al.*, 1993). Several different reactions have been used by different teams for chemical synthesis of β -haematin. Egan *et al.*, 1994 proposed that the reaction is spontaneous in the malaria parasite. He suggested that for the reaction to be spontaneous, there are certain pre-requisites namely pH (acidic), temperature (60-65°C) and certain buffer conditions, in most cases, acetate buffers ranging from 0.1- 4.5 M. These conditions are certainly not physiological. Although β -haematin is identical to haemozoin, its formation cannot be achieved under the same physiological conditions as with haemozoin. This has led much research in the field. Further work has shown that β -haematin formation *in vitro* can be achieved in aqueous acidic conditions under physiological temperature only in the presence of certain biological factors. These factors include the parasite lysate, histidine rich proteins, preformed haemozoin or β -haematin and certain unsaturated lipids (Fitch, 2004; Tripathi & Tekwani, 1999; Dorn *et al.*, 1998; Fitch *et al.*, 1999).

4.8 Method optimisation for the inhibition of β -haematin formation for extract screening

Several methods were explored to achieve the optimised protocol used for this study. The three methods that were used are described in this section.

4.8.1 Protocol 1

This method was designed based on the method previously described by Egan *et al.*, 2001.

4.8.1.1 Materials:

Haemin-Cl (Sigma, UK) freshly made in DMSO (Sigma Aldrich); **0.5 M acetate buffer** made with acetic acid (Fisher scientific) pH 4.8, 100 mM Tris HCl buffer (Fischer scientific) made in 5% SDS (Sigma, UK) pH adjusted to 7.4 with HCl (Sigma, UK); Sodium bicarbonate (Fisher scientific) buffer adjusted to pH 8.8 with NaOH (Fisher scientific); 2N NaOH; Drugs included chloroquine (Sigma, UK) and *B. ferruginea* (Nigeria).

4.8.1.2 Method:

Assay mixture: Haemin-Cl was prepared at 5 mg/ml in DMSO. 0.5 M acetate buffer was made with acetic acid to achieve a final pH of 4.8, Tris HCl buffer was made to 0.1 M in 5% SDS and pH was adjusted to 7.4 with HCl. 0.1 M Sodium bicarbonate buffer was made to pH 8.8 by adjusting with NaOH. Chloroquine and *B. ferruginea* stocks were prepared such that 1000 µl was added in reaction mixture to achieve 300 nM and 30 µg/ml respectively. Eppendorf tubes were labelled as: control, chloroquine and *B. ferruginea*. The reaction mixture was prepared in each eppendorf by adding 1000 µl of the 5mg/ml haemin-Cl, 2000 µl of sodium acetate buffer and 1000 µl of test compound (sterile water for control tube). The reagents were added in the order mentioned and the reaction mixture was mixed gently and an average pH of 4.9 was recorded. The reaction mixture was incubated at 37°C for 24 h.

Washing step: After 24 h incubation, the eppendorfs were removed from the incubator. The mixture was centrifuged at 13000 g for 5 mins. The absorbance of the mixture was taken at two different wavelength (405 nm and 750 nm) using a UV/Vis spectrophotometer.

4.8.1.3 Result:

There was no separation in the reaction mixture after 24 h incubation. A pellet was not formed when the mixture was centrifuged in both treated and control mixtures hence haematin did not form under these conditions.

Treatment	Abs @ 405nm	Abs @750nm
Control	0.018	0.002
300 nM Chloroquine	0.017	0.008
30 µg/ml <i>B. ferruginea</i>	0.018	0.009

Table 4.1 Absorbance values for β -haematin formation inhibition: Showing the average triplicate data for the experiments. There is no difference between control and treated and the absorbance readings are negligible, hence no haematin formation.

The results of this assay were not as expected. After 24 h incubation, there was no separation of the reaction mixture into two phases indicating that β -haematin did not form. The washing step should have produced a pellet (haematin) which could have been washed with the Tris buffer for 5 mins twice. A final wash in sodium bicarbonate buffer would have been carried out. The expected β -haematin pellet could have been dissolved in NaOH. NaOH breaks down the hydrogen bonds and converts the formed dimeric haematin aggregate back to the monomeric form allowing for high absorbance reading at 405 nM. In this reaction, the two last steps could not be performed as there was no pellet. The reaction did not form β -haematin as expected.

4.8.2 Protocol 2: Optimisation assay of β -haematin formation

The method used in section 4.8.1 was repeated with minor modifications.

4.8.2.1 Materials:

Sodium acetate, NaOH, haemin-Cl, Chloroquine, *B. ferruginea*, DMSO, double distilled water.

4.8.2.2 *Method:* 3 M sodium acetate was prepared (**modification: not the buffer in this case**), 0.1 M NaOH, 6.5 mM Haemin-Cl in 40% DMSO, 5 mM chloroquine working stock solution, 40 mg/ml working stock for *B. Ferruginea* and finally, glacial acetic acid which is 17.4 M.

The reaction mixture consisted of the mixture below in the same order mentioned:

- 100 µl of the haemin-Cl
- 50 µl of either drug or water in the case of control

- 200 μ l of 3 M sodium acetate
- 50 μ l of 17.4 M acetic acid (glacial)

The reaction mixture was mixed gently for 5 mins and the pH reading taken.

The mixture was incubated at 37°C for 24 h after which the eppendorfs were removed from the incubator. The eppendorfs were centrifuged at 13000 g for 15 min. The supernatant was collected in another eppendorf and labelled fraction I. The pellet left was washed in DMSO for 15 min @ 13000 g and supernatant labelled Fraction II. The pellet was re-suspended in DMSO and washed for the third time to obtain fraction III. The final pellet obtained which should be the formed β -haematin was re-suspended in 600 μ l of 0.1 M NaOH and this was labelled fraction IV. An aliquot (100 μ l) of fraction IV was further diluted into 3 mls of 0.1 M NaOH. Absorbance was read at 405 nm.

4.8.2.3 *Results:* The average pH for the reaction mixture was 4.92 (triplicate data). The table below shows the absorbance recorded at 405nm which represents monomeric haemin. The pellet formed in this assay is the dimeric form, β -haematin. But β -haematin is only soluble in NaOH. In order to read the absorbance to obtain a semi quantitative value, the β -haematin was dissolved in NaOH which converted it back to the monomeric form, allowing for readings to be taken at 405 nm. Fractions I, II and III represent the unreacted monomeric haemin whilst fraction IV represents the dimeric haemin that was converted back to the monomeric form. The results are represented in the table below:

Fraction I	Fraction II	Fraction III	Fraction IV
1.872	1.376	0.323	1.967
1.733	1.238	0.285	1.899
1.897	1.031	0.216	1.888

Table 4.2 Absorbance of supernatant fractions: showing average triplicate data set for the supernatant fractions I-III (unreacted haemin-Cl and the dissolved pellet fraction IV (formed β -haematin)).

Table 4.2 above shows monomeric haemin readings at 405 nm of the different fractions obtained from the reaction with fraction I, II and II. Fractions I and II show high readings which represents the unreacted haemin that solubilised in the DMSO. Fraction III has a much lower absorbance because much of the unreacted haemin had been removed after three washes. The β -haematin pellet left was insoluble in DMSO. In order to obtain the absorbance value of the β -haematin, it was dissolved in NaOH. The absorbance was recorded as fraction IV which gave a high reading indicating a high β -haematin formation in the reaction. The results of this protocol has demonstrated that a good optimisation was achieved. The incubation time for the reaction was 24 h hence, protocol 3 was designed to see if the reaction rate could be increased.

4.8.3 Protocol 3 The effect of varying temperature on β -haematin formation.

The reaction was performed under different temperature conditions to investigate its effect on reaction rate.

4.8.3.1 Method:

The reaction was carried as previously described in section 4.8.2 with slight modifications. In this case, the reaction was performed in three parallel sets. The experiments were set up on the same day, using the same stock solutions and conditions except for the temperature variations applied. The reaction was carried out by adding the reagents to form the reaction mixture just as described previously.

The reaction mixture was mixed gently for 5 mins and the pH was checked. The eppendorf tubes were split into three sets with each set consisting of control (water) and treated samples. The sets were incubated as follows:

Set 1: Incubated at 37°C for 24 h

Set 2: Incubated at 60°C for 1 h

Set 3: Incubated at 70°C for 1 h.

After each incubation period, the eppendorfs were washed 4 times in DMSO, removing the supernatant with each wash, until all the unreacted haemin was removed as indicated by a clear supernatant. The final pellet was solubilized in 0.1 M NaOH solution and the converted β -haematin was read at 405 nm.

4.8.3.2 Results:

The average pH reading for the triplicate experiments was 4.88. The results obtained from the absorbance reading at 405m in all triplicate experiments was plotted in a graph using Graphpad Prism 6.

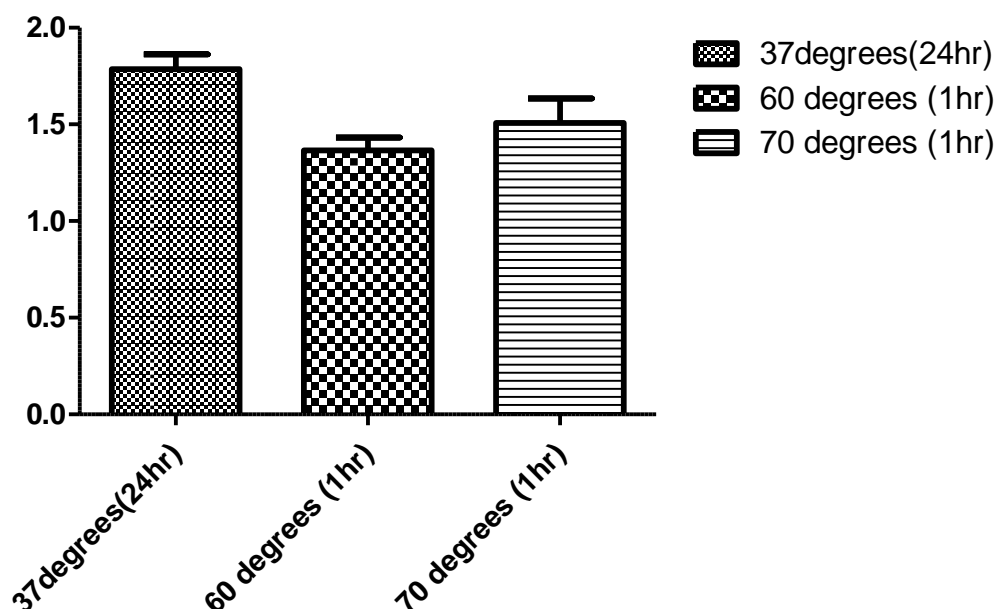


Fig 4.2: The effect of temperature on β -haematin conversion: The highest conversion occurred with a 24 h incubation, although a reasonable conversion was obtained with a 1 h incubation period at 60°C and 70°C. One-way ANOVA test on Graphpad Prism showed that the means between the three groups were significantly different with a value of $P < 0.05$, when compared to the 37°C in 24 h incubation period. Bonferri post test was performed to investigate if there was any significant difference between the two groups incubated for 1 h (60 and 70°C). The results of this tests shows that there is no significant difference between these two groups $P > 0.05$.

The data in this experiment shows that the incubation time for β -haematin conversion can be decreased with increasing temperature. High absorbance readings were obtained at 60°C and 70°C. Increasing the temperature from 60°C to 70°C increased the absorbance reading hence more β -haematin had been converted. There was no conversion at 37°C within 1 h incubation.

4.9 Preliminary investigation of the effect of *B. ferruginea* and chloroquine on β -haematin formation.

In this experiment, the effect of the methanolic *B. ferruginea* extract on β -haematin conversion was investigated using protocol 3.

4.9.1 The effect of chloroquine and B. ferruginea on β -haematin formation

Several studies have shown that quinoline compounds are classified as inhibitors of haem detoxification (Egan *et al.*, 2000; Egan & Ncokazi, 2005; Sandlin *et al.*, 2014). Furthermore, structure activity studies of 4-aminoquinoline antimalarials have shown that the 4-aminoquinolone nucleus is implicated in haem binding and the chloroquine atom at position 7 of the quinolone ring is vital for optimal inhibition of haemozoin formation (Kaschula & Egan, 2002). The effect of chloroquine on β -haematin formation is well documented and several studies have shown that chloroquine inhibits β haematin formation. This was the basis of its choice as a positive control in this study.

4.9.1.1 Method: Preliminary experiments were performed using protocol 3, to investigate the effect of β -haematin formation on 300 nM chloroquine (the IC_{50} in literature) and 20 μ g/ml *B. ferruginea* (IC_{50} value) was used.

4.9.1.2 Results: Absorbance readings were recorded from the experiment after incubating with chloroquine and water (control). The data collected were plotted in a graph using Graphpad Prism as shown in fig 4.3.

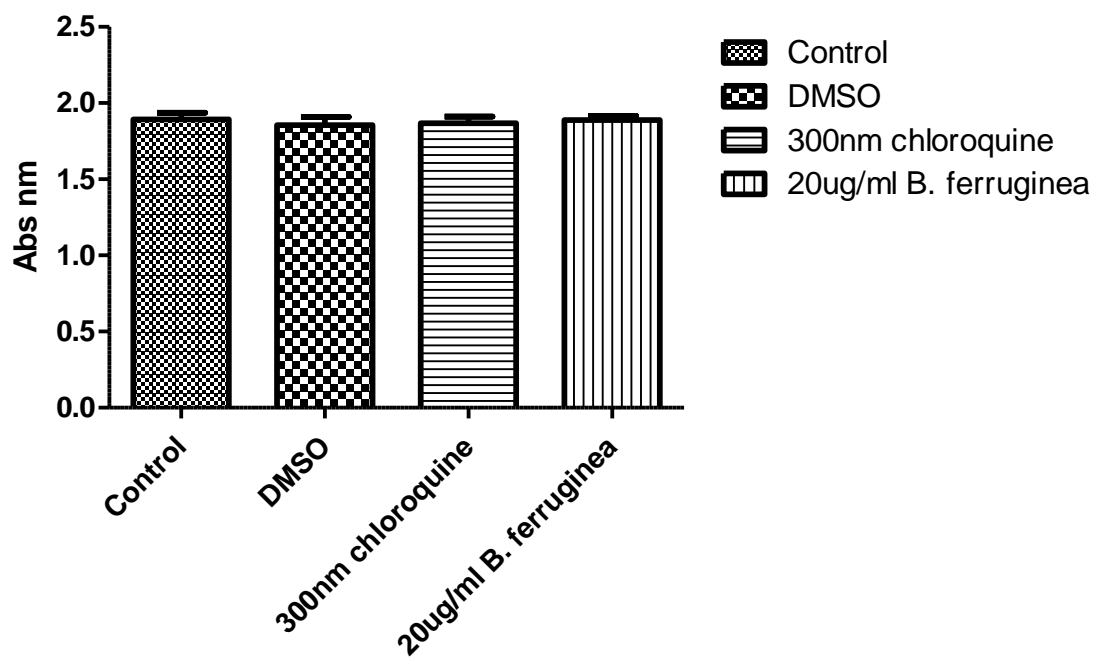


Fig 4.3 Preliminary investigation of the effect of chloroquine and *B. ferruginea* on β -haematin formation: β -haematin formation was not inhibited using the IC_{50} values of methanolic *Bridelia* and CQ. There was no inhibition of β -haematin formation at these dose levels.

4.9.2 Dose-response inhibition of chloroquine on β -haematin formation

A series of experiments were performed to obtain the working doses for the experiment. The initial experiments used IC_{50} values of chloroquine (300nM). As β -hematin formation was not inhibited by this dose, a 10-fold dose escalation was performed until inhibition was achieved. As no β -haematin formation occurred, the dose was increased to millimolar concentrations. The results are graphically presented in fig 4.4 below.

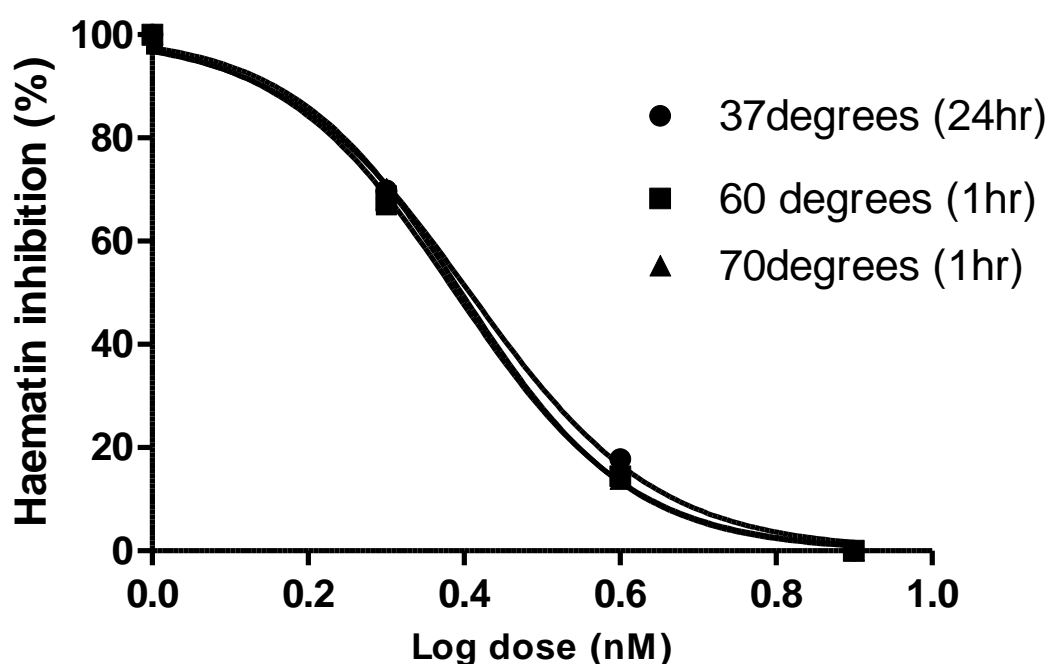


Fig 4.4: Effect of temperature on β -haematin formation inhibition by chloroquine: A dose-response inhibition of β -haematin formation was achieved with CQ, with a 100% inhibition achieved at a dose of 8 mM. Statistical analysis using Two Way ANOVA in Graphpad Prism 6 shows that there is no difference between control and 300 nM treated group; there is a significant difference between control and samples treated with 2 mM, 4 mM and 8 mM samples with $P < 0.001$ and $P < 0.0001$ respectively for last two doses. The calculated IC50 using Graphpad prism for the beta haematin inhibition was 2.55, 2.245 and 2.49 at 37, 60 and 70°C respectively.

The results show that chloroquine inhibits β -haematin formation at very high doses. Near 100% inhibition was achieved at doses higher than 4 mM. The data for the 10 fold-escalation is not shown as there was no inhibition. Hence though these experiments, it is evident that chloroquine inhibits β -haematin formation. As previously suggested in other studies (Egan *et al.*, 2000; Egan & Ncokazi, 2005; Sandlin *et al.*, 2014).

4.9.3 Dose-response inhibition of *B. ferruginea* on β -haematin formation

In an attempt to investigate the possible mechanism of action of *B. ferruginea*, which has shown strong anti-malarial activity *in vitro*, inhibition of β -haematin formation was explored.

4.9.3.1 Methods:

Methods used are as stated in section 4.8.3 (Protocol 3). The IC₅₀ of *B. ferruginea* is 20 µg/ml as determined by the fluorescence-based methods and Giemsa light microscopy described in Chapter 2. This dose was not chosen for the experiment based on the fact that with chloroquine, near IC₅₀ values did not inhibit β-haematin formation. Dose ranges from 200 µg/ml were tested until inhibition was noted at high dose levels, in milligrams/millilitre. Based on the series of preliminary experiments performed (data not shown), a starting dose of 1mg/ml was selected for the experiments.

4.9.3.2 Results:

After the incubation period described previously in protocol 3, the absorbance reading obtained were recorded for each experimental set and the results were plotted in a graph using Graphpad Prism as represented in fig 4.4 below:

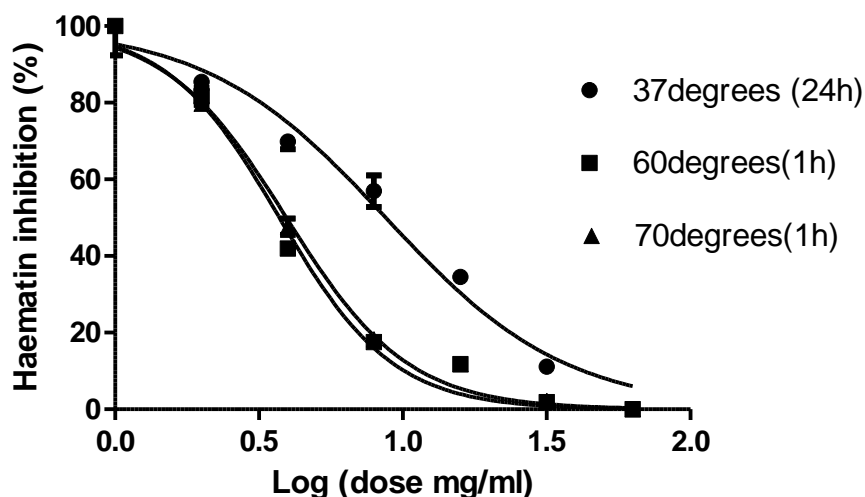


Fig 4.5: Effect of temperature on β-haematin formation inhibition by methanolic *B. ferruginea*. The result of this experiment shows that methanolic *B. ferruginea* inhibits β-haematin formation in a dose-dependent manner. At high doses of 32 mg/ml and over, near 100% inhibition was achieved. Lower doses ranging from 20 µg/ml were tested prior to the

high doses seen in this graph and there was no difference compared to control (data not shown). The results of this experiment showed a lower IC_{50} with increasing reaction mixture. The IC_{50} calculated using Graphpad prism were 8.73, 3.73 and 3.95 mg/ml at 37, 60 and 70°C respectively.

4.9.4 Pyrimethamine and sulfadoxine controls for the β -haematin formation assay

Pyrimethamine and sulfadoxine are drugs that are known not to work on the hemozin pathway. Their primary mode of action is by inhibiting the folate pathway enzymes, dihydrofolate reductase and dihydropteroate synthase. Hence in this study, they were used to further confirm that the inhibition of β -haematin formation was not due to any artefacts. Both drugs were used to investigate β -haematin formation inhibition using three doses (2 mM, 4 mM and 6 mM). For all doses tested, no inhibition was noted. Control and treated values remained the same as represented in fig 4.5.

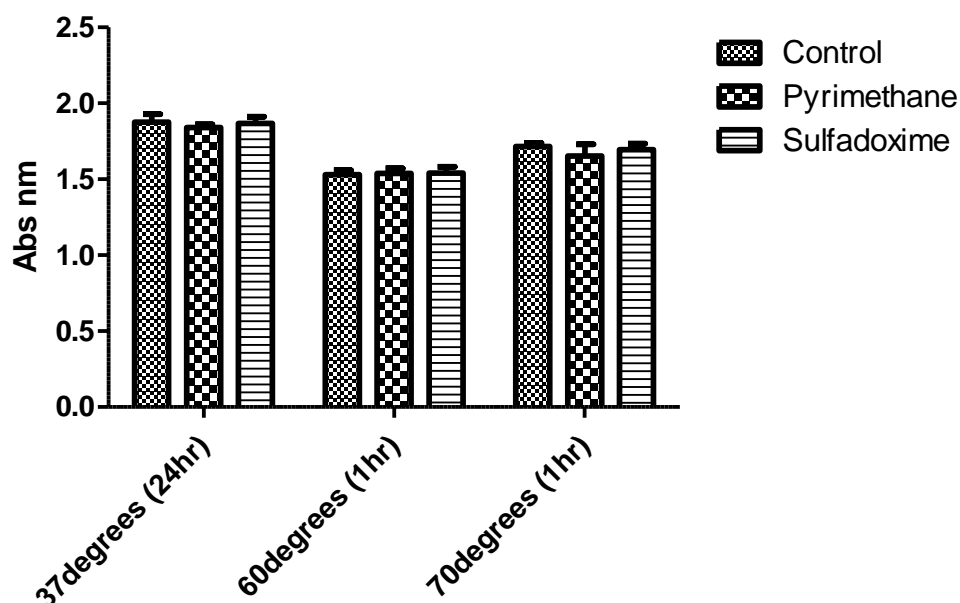


Fig 4.6: Effect of temperature on β -haematin formation inhibition by pyrimethamine and sulfadoxine: Pyrimethamine and Sulfadoxime were used as further controls for the β -haematin assay. The two drugs did not inhibit β -haematin formation. Control and treated values were the same.

4.10 Chapter conclusion

The objective of this chapter was to investigate the effect of *B. ferruginea* on the haemozoin pathway, in order to gain some insight into its possible mechanism of action. The haemozoin biocrystallization pathway is an attractive target for many efficacious antimalarials due to its specificity to the *Plasmodium*. Drugs that have been shown to act through this pathway include quinine, chloroquine, mefloquine, lumefantrine, piperaquine and halofantrine (Hempelmann, 2007 and Kumar *et al.*, 2007). Pyrimethane and sulfadoxine do not target this pathway hence were used as positive controls in this chapter. The haemozoin pathway appears a stable target for antimalarials and resistance has only been shown to occur due to removal of the antimalarial compound from the site of action, the parasite food vacuole, hence reducing drug accumulation (drug resistance). The inhibition of haemozoin conversion seems to only occur at very high drug concentrations, beyond physiological doses. In this study, it was demonstrated that for chloroquine, inhibition was only achieved at doses above 1 mM whilst for *B. ferruginea* at doses from 2 mg/ml. These doses are no doubt lethal to the human body. The only explanation to these dose levels is that physiologically, the drugs enter the parasite food vacuole and accumulate to a high dose. Hence *in vitro*, the assay could only be achieved with very high doses. Chloroquine has been demonstrated in several studies to enter the parasite food vacuole through simple diffusion. Once it enters, the drug is protonated due to the acidic pH (4.7) and this prevents it from diffusing out. It may be recalled that in this study, the experiment only worked at pH ranging from 4.6-4.9 which models what happens physiologically. This build up of chloroquine in the food vacuole relative to the rest of the parasite prevents further biocrystallisation of haem leading to a haem build up which is toxic to the parasite. Chloroquine resistance has been demonstrated to be due to less accumulation of the drug in the parasite vacuole due to a mutation in the chloroquine resistant transporter (PfCRT), hence reducing its concentration to non lethal levels in the food vacuole (Ehlgen *et al.*, 2012). Hence the results of this study has shown that *B. ferruginea* inhibits β -haematin formation and up to 97% inhibition was achieved at high doses which is probably what may be happening physiologically as is the case with chloroquine. It may therefore be right to suggest that the extract possibly mediates its effect by accumulating in the parasite food vacuole and possibly binding to haem, hence preventing the biocrystallisation process, just as it has been demonstrated with chloroquine in previous studies.

CHAPTER 5

***In vivo* animal studies in collaboration with National Institute for pharmaceutical research and development (NIPRD), Nigeria.**

All animal studies done in this study were performed at the National Institute for Pharmaceutical Research and Development following the ethical guidelines and SOPs of the department of pharmacology approved by Professor Uche Osunkwo. The extracts used identified at the NIPRD herbarium by Dr Jemilat Ibrahim and samples deposited at the herbarium.

5.1 Introduction

Animal studies are very useful as they limit the use of human volunteers, addressing some ethical concerns. The use of animal models is also more financially viable and practical than humans, where applicable. Although humans and animals may look different, at a physiological and anatomical level they are extraordinarily similar. Organs and organ systems of animals, from mice to monkeys, function in almost the same way. Nearly 90% of veterinary medicines are the same or very similar to medicines used in human patients. Humans share 99% of their DNA with mice hence the similarities far outweigh the minor differences that exists. These similarities have meant that over the years, animal disease models have remained a critical tool in *in vivo* testing in pre-clinical drug screening. Testing usually considers different factors including animal species, age, route of drug delivery, treatment regimen amongst other factors. Another very interesting way animals models have been used in research is ‘knockout mice’. These mice are genetically modified by inactivating, or "knocking out", an existing gene by replacing it with an artificial piece of DNA. These animal models play an important role in studying genes, which have been sequenced but whose functions have not been determined. By ‘switching off’ a specific gene in the mouse, differences between normal mice and the knockout mice can be observed. The result can be used to design better treatments for diseases.

In malaria research specifically, animal models have proved very useful over the years due to the limitations on the nature of the interpretations of *in vitro* studies, particularly with regards to host immune cells (Michelle *et al.*, 2009). Several animal models exist for malaria including the avian, simian and morine models. For the scope of this study, the main focus

will be on the mouse model. There are four different species that exist for the mouse model which include *P. berhei*, *P. vinkei*, *P. chabaudi* and *P. yoelli*. Each species has different strains which have proved useful in different areas of malaria research for example, *P. berghei* infects mature erythrocytes and reticulocytes hence used in C57BL/6 and CBA/T6 mice to investigate pathogenesis of malaria and immune mechanisms of protection (Shofield *et al.*, 2005). Certain strains of *P. Yoelli* are lethal to mice, hence have been successfully used in vaccine research whilst *P. chabaudi* has commonly been used in the study of immune mechanisms as it only infects mature erythrocytes. Due to the several advantages offered by animal models, including; the ease for investigating disease progress, practicality of using organs for research and finally the ease with which behavioural changes can be observed, *in vivo* activity of *B. ferruginea* is explored. The study objectives of this chapter are to investigate toxicity as well as antimalarial, analgesic and antipyretic activity of the *B. ferruginea*.

5.1.1 Animal models used and ethical consideration

Plasmodium Berghei infected mice were used as an animal model in this study. Other animal models used were the acetic acid induced writhing model and the tail flick model for analgesia; and the yeast induced pyrexia model as well. The welfare of the animals used were considered carefully and international guidelines were adhered to in accordance with NIH guide for care and use of laboratory animals was strictly adhered to (NIH publication 23-83) revised (NIH 1985). The three Rs were also considered to ensure humane use of animals in the study which are namely replacement (in which case *in vitro* studies was considered and performed for preliminary data), replacement (in *in vitro* models were replaced for the mechanistic studies) and refinement (only 5 animals were used per group, just enough to statistically validate the studies).

5.2 Acute Toxicity:

The acute toxicity of methanolic *B. ferruginea* was investigated using the Locke Method, 1983 with slight modifications. This method has been shown to be very effective in rapid assessment of chemical/extract toxicity. It uses only 5 rats for each dose level. In this study, three dose levels were investigated hence the extract was assessed for toxicity using 20 rats. Acute toxicity in this method is represented as the geometrical mean of the dose that resulted in 100 percent lethality and that which caused no lethality at all.

5.2.1 Housing and diet

Animals were housed in metal cages using saw dust as bedding. Standard laboratory food pellets were given *ad libitum* with free access to water from water bottles. NIPRD standard operating procedures (SOPs) and ethical guidelines were followed. The experimental design was approved by the NIPRD ethical committee in accordance to Nigerian guidelines prior to commencement of experiments. NIH guide for care and use of laboratory animals was strictly adhered to (NIH publication 23-83) revised (NIH 1985). Normal 12 h day and 12 h dark light pattern was followed.

5.2.2 Experimental animals

Wistar rats of average weight between 250-300 g of both male and female sexes were used. Animals were randomly grouped into 4 groups of 5 animals each. Animals were marked with a yellow marker on their fur for identification and observation purpose only. Different parts of the body were marked differently for each animal including the head, leg, back and arms. Below is a table with the weight and ID of each animal in the groups

Control I (saline)		Grp II (1250mg/kg)		Grp III (2500mg/kg)		Grp IV (5000mg/kg)	
<i>Label</i>	<i>Weight</i>	<i>Label</i>	<i>Weight</i>	<i>Label</i>	<i>Weight</i>	<i>Label</i>	<i>Weight</i>
LSRS	255	HD	280	LA	261	HDRL	292
LARA	276	TL	254	LS	264	BK	300
LLHD	281	LL	293	RS	275	HDBK	255
TLLS	261	RL	271	HDTL	284	RLLL	251
TLBK	298	RA	256	HDRA	259	TLLL	259

Table 5.1 Random allocation of experimental animals: Showing the random allocation of animals into groups, their weight and the labelling system used to identify each animal. (HD Head; TL tail; LL left leg; RL right leg; RA right arm; LA left arm; RS right side; LS left side; BK back, HDRA head right arm; HDTL head tail; RLLL right leg left leg; TLLL tail left leg).

5.2.3 Extract preparation

B. ferruginea bark was obtained and shade dried. The dried bark was grounded and soaked in 70% methanol for 24 h on a shaker. The extract was filtered and the filtrate was

evaporated to dryness in a water bath. The dried aqueous extract was obtained and stored in an airtight bottle until use.

5.2.4 Mode of Administration

Animals were fasted 6 h prior to dose administration with free access to water. Animals were dosed with a single oral dose using a rat oral cannula. Rats in Groups I, II, III and IV received saline, 1250 mg/kg, 2500 mg/kg and 5000 mg/kg of the extract respectively. Food was given 2 h post drug administration.

5.2.5 Observation Period

Animals were critically observed for behavioural changes periodically every 30 mins for the first 6 h and three times daily post 24 h for 14 days. Several parameters were looked out for, including hyperactivity, excessive sleep/coma, changes in grooming behaviour, loss of appetite, tremors and death.

5.2.6 Results

Following observation of the animals for a two week period, the following observations in table 5.2 were made.

Dose	Observations
1250mg/kg	No different observations compared with control group, no death
2500mg/kg	No different observations compared with control group, no death
5000mg/kg	Abdominal writhing and signs of distress including weakness and reduced activity were observed from 72 h but no death recorded throughout the observation period.

Table 5.2 Acute toxicity in rats: Showing observations of the acute toxicity tests using Locke method. There was no difference between control and treated groups up to a dose of 2500 mg/ml. At a very high dose of 5000 mg/ml, there was signs of distress but no death was recorded.

5.3 Antimalarial activity of methanolic *B. ferruginea*

5.3.1 *Innocula*

Plasmodium berghei parasitized erythrocytes was obtained from a donor mouse from the NIPRD animal house. Tail blood of the donor mouse was smeared on a slide and normal Giemsa staining was performed for microscopic analysis to establish parasitaemia. Some blood was collected. Depending on the level of parasitaemia observed, the blood was diluted in normal saline prior to inoculating the new mice for the present study, The mice were intra-peritoneally inoculated with 0.2 ml of infected blood containing 10^7 parasitized erythrocytes.

5.3.2 4-Day Peter's suppressive tests

25 swiss albino mice of weight ranging from 23-30 g were divided into 5 groups of 5 mice each. They were labelled as below:

Animal grouping and labelling table

Grp I (saline)	Grp II (60mg/kg)	Grp III (120mg/kg)	Grp IV (240mg/kg)	Grp V CQ(10mg/kg)
HD	RA	TLHD	BKLL	HDRL
TL	LA	TLLL	BKRL	HDLL
LL	RALA	TLRL	RABK	HDLA
RL	LLRL	BKHD	RLBK	TLSR
BK	BKTL	BKLA	HDRA	TLLS

Table 5.3 Random allocation of mice: Showing the random allocation of animals into groups, their weight and the labelling system used to identify each animal. (HD Head; TL tail; LL left leg; RL right leg; RA right arm; LA left arm; RS right side; LS left side; BK back, HDRA head right arm; HDTL head tail; RLLL right leg left leg; TLLL tail left leg; BDY body).

5.3.2.1 Experimental method

All animals in the above groups were inoculated as previously described in section 5.3.1 on day 0 (D0). Treatment was administered orally 3 h post infection. Grp 1 received saline solution whilst group II, III, IV received 40, 80 and 160 mg/kg aqueous *B. ferruginea* respectively and group V received chloroquine (CQ) 10 mg/kg to serve as positive control.

Mice in group I, II, III, IV and V were orally administered again with saline, 60 mg/kg, 120 mg/kg, 240 mg/kg and 10 mg/kg CQ respectively on days 1, 2, 3 and 4 post infection. On day 5, tail blood of the mice was collected and smeared on slides. The slides were prepared for Giemsa staining and microscopic analysis was performed using oil immersion light microscopy. Percentage parasitaemia suppression was calculated by comparing parasitaemia of infected control (Grp I) and mice from the different treatment groups

Percentage suppression = $100 - (\text{mean parasitaemia treated} / \text{mean parasitaemia control}) * 100$.

5.3.2.2 Results

The parasitaemia of each mouse was estimated using standard Giemsa staining and light microscopy. The raw data collected is represented in the table 5.4 below. The percentage suppression was calculated relative to the mean value obtained in the control group.

Group	Percentage Parasitaemia	Mean parasitaemia	% Suppression
Grp I Saline	4,3,5,5,0,5,0,4,3	4.3	-
Grp II 40 mg/kg	2,0,2,5,2,0,1,33,1,28	2.0	53.35
Grp III 80 mg/kg	1,5,1,0,1,7,0,9,1,3	1.28	70
Grp IV 160 mg/kg	0,5,0,7,0,6,1,1,0,6	0.7	83.72
V CQ 10mg/kg	0,0,0,0,0	0	100

Table 5.4 Antimalarial 4-day Peters' suppressive test: Showing the effect of methanolic *B. ferruginea* on malaria suppressive cell. The data was obtained on day 5 of the experiment.

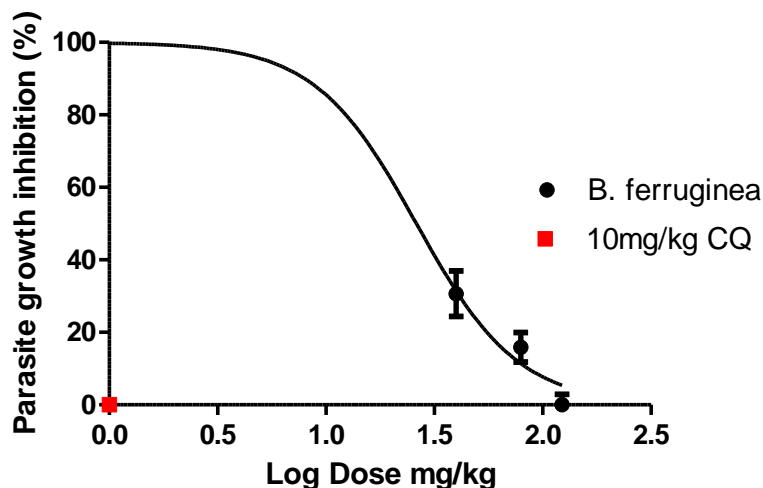


Fig 5.1 Percentage inhibition of *P. berghei* in the 4-day suppressive test: The figure shows the percentage suppression of *P. Berghei* growth in the suppressive tests where the extract was given on the same day as inoculation and daily dose of the extract 4 days post infection. Tail blood was taken on day 5. The IC_{50} value generated using Graphpad prism revealed a dose of 26.14 mg/kg.

The results of the suppressive tests shows that the methanolic *B. ferruginea* possesses good antimalarial properties. This was demonstrated by the chemosuppression of *P.berghei* infection in a dose dependent manner during the 4-day suppressive tests. The 4-day suppressive tests have been as previously described by Knight and Peters 1980. In this study, parasite growth was suppressed all dose levels used, with the highest suppression of 84% noted at 160 mg/kg dose. Chloroquine suppressive tests at 10 mg/kg was used as a positive control and there was 100 percent suppression in the test. These results show that the extract might be very useful in ethnopharmacological usage for a suspected malaria contact or as a prophylactic. The results could be used towards the validation of its usage in ethnopharmacological medicine for malaria.

5.3.3 Curative tests (Rane test)

The curative test otherwise known as the rane test is a test that is performed on mice during an established malaria infection. This tests was previously described by Rhley and Peters. 1970.

5.3.3.1 Method:

Briefly, 25 mice of average weight 22-30 g were used. The animals were grouped into 5 groups as in the suppressive tests with the same labelling codes and patterns. Mice were grouped 5 per cage and labelled grp 1 (control), Grp II (60 mg/kg), Grp III (120 mg/kg), GpIV (240 mg/kg) and finally grp V 10 mg/kg CQ. Infected erythrocytes from a donor mouse was taken on Day 0. All mice were inoculated i.p with 10^7 *P. berghei*. Prior to inoculation, tail blood was obtained from the donor mouse and slide count was roughly 5×10^7 , which was diluted to 0.1×10^7 with saline solution. 0.2 ml of the diluted infected blood was used to inoculate the uninfected mice in all groups on Day 0. 72 hours later i.e Day 3 animals in groups I, II, III, IV and V were treated orally with saline, 60 mg/kg extract, 120 mg/kg and 240 mg/kg extract respectively. Group V received CQ 10 mg/kg. Treatment was continued daily on days 4, 5, and 6. On day 7, tail blood was collected from all animals and smeared on a microscopic slide for *Giemsa* staining and subsequent light microscopy. Mice were returned back to their respective cages and fed *ad libitum* with free access to water. The animals were observed for 30 days and the mean survival noted.

5.3.3.2 Results

The raw data obtained from the study are represented in table 5.5 below.

Treatment Grp	Pre treatment Day 3	Post treatment Day 7
Grp I Saline	22, 29, 26, 25, 28	35, 29, 36, 31, 29.
Grp II 60 mg/kg	29, 26, 20, 21, 26	13, 11, 12, 10, 12
Grp II 120 mg/kg	26, 24, 24, 18, 29	10, 9, 11, 8, 11
Grp III 240 mg/kg	24, 23, 29, 25, 22	8, 7, 6, 7, 16
Grp IV 10 mg/kg CQ	23, 28, 25, 23, 22.	2, 7, 5, 2, 3

Table 5.5 Rane test results: Showing the raw data obtained from the rane tests for the percentage parasitaemia for the mice both on day 3 (pre-treated) and day 7 (post-treated).

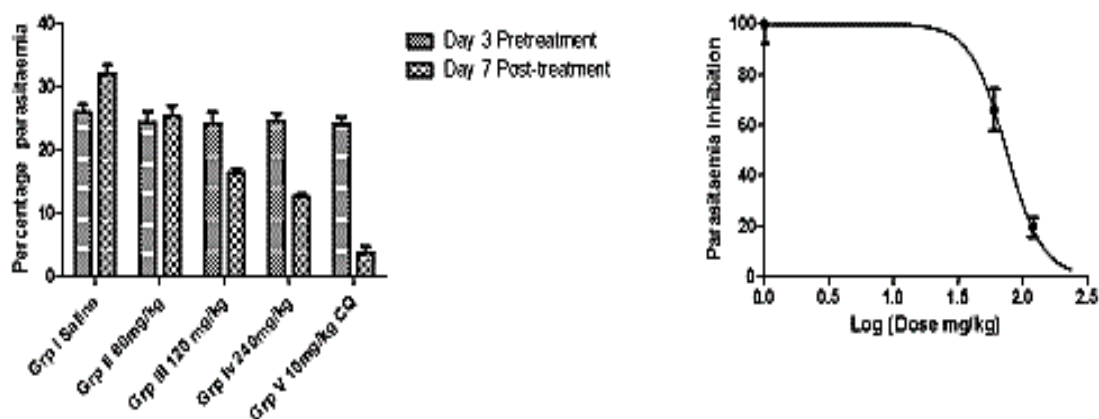


Fig 5.2 The effect of *B. ferruginea* on the rane test. The results have shown antimalarial activity on established plasmodial infections. Repeated measures ANOVA test on Graph Pad prism showed that there was a dose-dependent parasite killing on day 7 post treatment compared to day 3 pre-treatment ($P < 0.01$). Upon comparing the parasitaemia between control, saline treated group and the treated groups at all dose levels (Day 7 data), there was a significant difference $P < 0.001$. The IC_{50} determined using Graphpad prism was 74.91mg/kg.

This study results demonstrates the curative properties of *B. ferruginea* with an IC_{50} of 74.91 mg/kg. The animals were inoculated and left untreated for 72 h allowing the parasite to go through a complete cycle. The results on Day 3, pretreatment show the parasitaemia of the animals before treatment commenced. After the commencement of treatment which was daily oral dose for 4 days, i.e on Day 7 of the experiment, the parasitaemia of animals in each treatment group are represented in the graph. It can be clearly noted that the extract produced a dose dependent inhibition of parasitaemia as compared with the untreated group (saline). Animals in group IV showed the highest inhibition with the extract treated groups and there was about 78% inhibition. Animals in group V were treated with CQ as positive control and a 90% inhibition was obtained with this dose. Survival of the animals was also monitored up to 30 days post treatment. Two animals in the saline treated control group died on day 4 and by day 10, all were dead. There was 2 death recorded in the 60 mg/kg treated group and no death in the other extract treated groups. There was no death in the CQ treated group.

The results of the *in vivo* antimalarial activity of methanolic *B. ferruginea* extract shows that the extract is effective both in suppressive and curative chemotherapy of malaria with IC₅₀ doses. This validates the ethnopharmacological usage of the plant for malaria treatment by the locals. Furthermore, the extract has potentials of being used in malaria prophylaxis due to the activity shown in the suppressive tests. It can also be used to treat established cases of malaria infection as demonstrated in the curative tests.

5.4 Analgesic study

Fever control is a common measure used in malaria infection. Although the use of antipyretics in malaria treatment control is highly controversial, the practice is very common (Brandts *et al.*, 1997). In this study, *in vivo* analgesic properties of the extract was investigated as pain is known to be one of the critical symptoms of malaria. An extract with both antiplasmodial and analgesic properties will be highly beneficial in terms of treatment and will eliminate the need to use an analgesic, which is controversial. Two animal pain models were used to investigate the analgesic effect of the methanolic *B. ferruginea* extract. These include the acetic acid induced writhing method and the tail immersion test, both in mice. Each pain model reveals a different information about the mechanism of pain which will be discussed later.

5.4.1 Effect of B. ferruginea on acetic acid induced writhing method

5.4.1.1 Method:

Method has previously been described by Koster *et al.*, 1959. Briefly, 25 swiss albino mice were randomized into 5 groups of 5 mice each. Three dose levels were investigated. The weight range of the animals were 23-28 g both male and female. As the dose was i.p, lower doses were explored as compared to the antimalarial study which was an oral dose. In this study, animals in grp I received saline, grp II received 15 mg/kg extract, grp III and IV received 30 and 60 mg/kg extract respectively whilst animals in grp V received 150 mg/kg acetyl salicylic acid, aspirin (ASA). This was the positive control group. Animals were pretreated with the extract i.p 1 h prior to giving 10 ml/kg of a 0.7% acetic acid solution, which was given i.p. The animals were observed and number of writhmic abdominal contractions were observed and noted for 10mins. Treatment groups were compared to control group and percentage inhibition was calculated based on the formula below:

Percentage inhibition = (control mean-test mean) / control mean * 100

5.4.1.2 Results

The results obtained from the acetic acid induced writhes investigation are presented in the table below.

	No of writhes		Percentage inhibition
		Mean	
Control (saline)	13,14,16,18,12	14.6	-
15mg/kg extract	10,11,11,12,13	11.4	20.28
30mg/kg extract	6,7,8,7,9	7.4	49.3
60mg/kg extract	2,3,5,4,3	3.4	76.22
ASA 150mg/kg	2,1,1,2,0	1.2	91.78

Table 5.6 Effect of methanolic *B. ferruginea* on acetic acid induced writhe: Showing a good dose dependent inhibition of acetic acid induced writhe produced by mice after being administered with *B. ferruginea*.

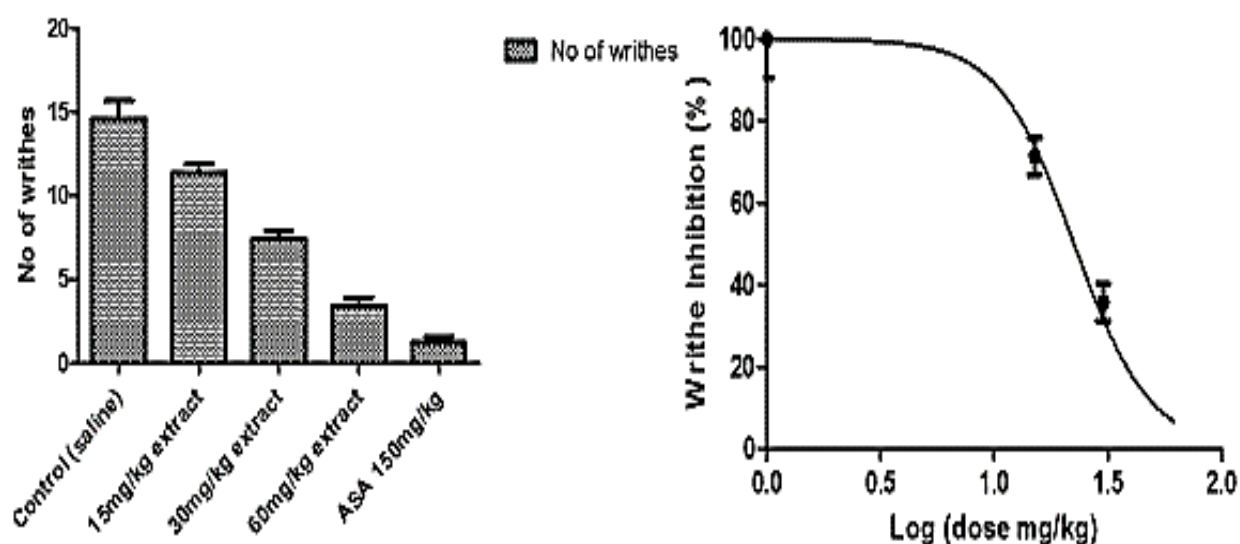


Fig 5.3 Effect of *B. ferruginea* on acetic acid induced writhe: Showing a dose dependent analgesic property of *B. ferruginea*. An IC_{50} dose of 22.49 mg/kg was calculated from the normalised data in Graphpad prism.

Statistical analysis a One way ANOVA tests showed that there was a significant difference between control and extract treated mice at all dose levels ($P > 0.001$). There was also a significant difference between aspirin (ASA) treated mice and control.

The results of this study shows a dose dependent inhibition of acetic acid induced writhes by the extract. Mice that were pre-treated with the extract showed pain inhibition at all dose

levels. The injection of acetic acid irritates the peritoneal cavity of mice causing a classical writhmic contractions due to pain. The positive control group was given ASA and almost 92% inhibition of pain was observed. These results demonstrate that the extract in addition to antimalarial properties possesses analgesic properties. This makes it a very good candidate for malaria therapy development as fever is one of the symptoms of malaria disease. The IC₅₀ dose for analgesia is well below that obtained for curative and suppressive tests which is advantageous. Hence the dose for treating malaria would treat the malaria associated symptom of fever.

5.4.2 Tail immersion tests

As pain has different mechanisms, the tail immersion test was used to investigate the central analgesic of the methanolic *B. ferruginea* extract. The test measures the complex response to non inflammatory acute nociceptor input. Opioid analgesics exert central analgesic properties via different spinal receptors (Nemirovsky *et al.*, 2001), hence block pain sensation in this animal model. Any extract/drug that blocks pain in this model is assumed to have central analgesic properties.

5.4.2.1 Experimental method

This method has been previously described by Jansen and Jagenov, 1959. Minor modifications were applied in this study. Briefly, 25 mice were randomized into 5 groups of 5 mice each and labelled as in table 5.3. Animals in group I were treated with saline, groups II, III, IV and IV received 15, 30 and 60 mg/kg extract while animals in group V received 10 mg/kg morphine. All treatments were given i.p. Mice were restrained in horizontal cylinders (purposely designed for the study) leaving the tail hanging outside freely 30 mins post drug administration. The hanging tail was immersed into a water bath maintained at 50°C ± 2. The time taken for the mouse to flick its tail out of the water was recorded using a timer. The latency was evaluated at 0 min, 30 min, 60 min and 90 min. 0 min was the initial reading prior to drug administration.

5.4.2.2 Results

The raw data obtained from the tail immersion pain study are presented in table 5.7 below:

Treatment	0 min	30 min	60 min	90 min
Control	10,11,11,10,9	10,10,11,12,9	10,11,10,9,8	9,11,12,13,12
15 mg/kg	10,11,9,12,10	10,12,11,12,10	12,11,13,12,11	13,12,12,13,11
30 mg/kg	9,9,11,11,12	14,15,10,13,14	13,12,13,15,12	14,13,15,13,14
60 mg/kg	10,10,10,11,13	11,12,11,13,11	13,14,13,15,12	14,16,15,13,16
Morphine	11,10,10,8,11	14,12,11,14,13	16,16,15,14,17	19,18,19,21,22

Table 5.7 Tail immersion test data: Showing the latency period at different dose levels over a period of 90 mins. 0 min is at the start of the experiment prior to drug administration.

The results of the data from table 5.7 are represented graphically in figure 5.4 below.

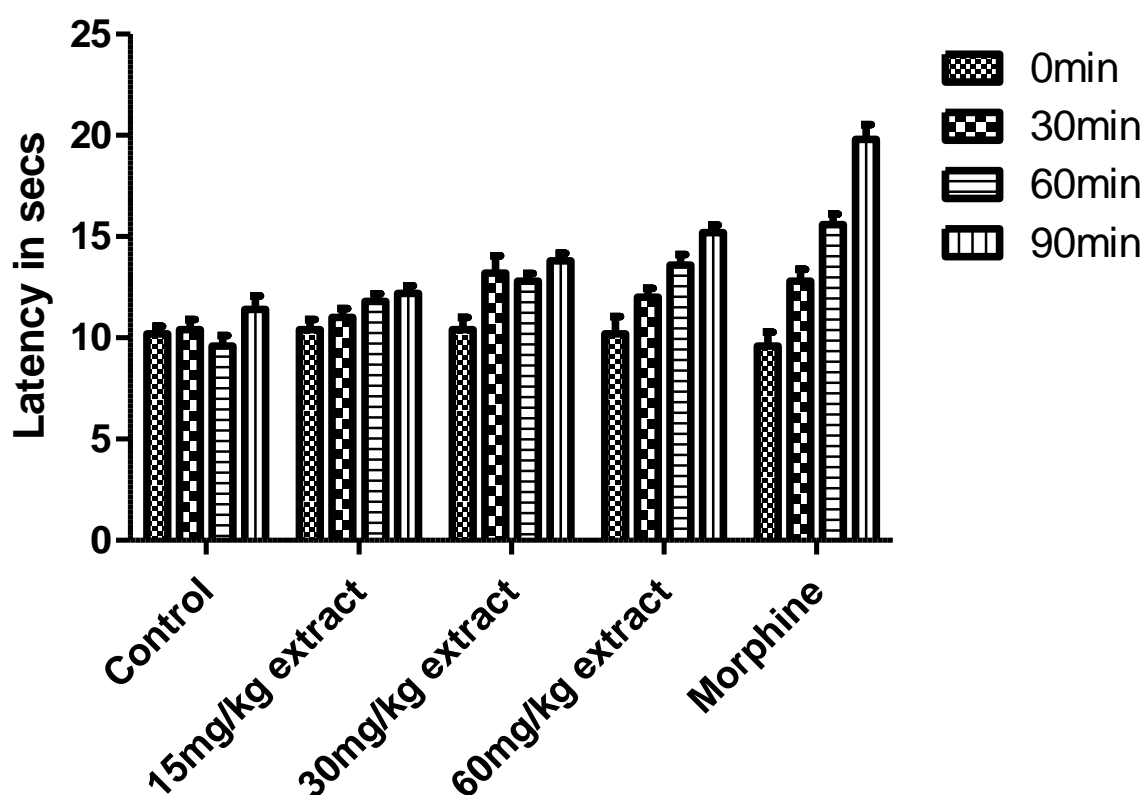


Fig 5.4 Effect of methanolic *B. ferruginea* on tail immersion test: Showing a dose dependent increase in latency period for a mouse to flick its tail out of the warm water. The methanolic *B. ferruginea* extract has demonstrated a dose-dependent analgesic activity as seen with morphine. Statistical analysis using repeated measures Anova in Graphpad Prism 6 showed that there was a significant difference between control and treated mice at all three dose levels. There was also a significant difference between control and morphine treated mice ($P < 0.01$). The data at 90 min was normalised to obtain an EC_{50} value which was 23.76 mg/kg.

The results of this study shows antinociceptive activity of the methanolic *B. ferruginea* extract in both the acetic acid writhe induced method, which mediates peripheral pain, and the tail flick test, which is selective for centrally acting drugs. A dose dependent inhibition of nociception was revealed in both tests. The EC₅₀ value, which is the concentration of the extract that produces half maximal response was determined for the tail immersion tests as against IC₅₀ value as with the other test. This is because the response in this case is not determined as an inhibition.

5.5 Antipyretic study

The aim of this study was to investigate the antipyretic activity of the methanolic *B. ferruginea* extract.

5.5.1 Yeast induced pyrexia

The brewers yeast method previously described by Loux *et al.*, 1972 was used to induce pyrexia in wistar rats of either sex.

5.5.1.1 *Method:* 25 rats weighing 200-250 g were randomized into 5 groups of 5 rats each and marked just as in the previous studies for identification. The body temperature of each rats was taken by inserting a rectal thermometer deep into the rectum. The normal body temperature averaged about 36.5°C for all animals. Pyrexia was induced using the Loux *et al.*, 1972 method. Briefly, a subcutaneous injection of 20 ml/kg a yeast suspension in methyl cellulose was performed on each rat. The animals were returned to their cages and allowed access to food and water. Rectal temperatures were taken 24 h post induction. Extracts/drug were administered at different doses to the animals according to grouping. Animals in group I received saline while those in groups II, III and IV received extract at 15, 30 and 60 mg/kg respectively whilst animals in group V received drugamol , the positive control, at 20 mg/kg.

5.5.1.2 Results

Raw data for the antipyretic study are presented in the table 5.9 below

Treatment	0 h Preinductio n	24 h post induction	1 h	2 h	3 h	4 h
Control	36.1,36.8,36 .5 35.8,35.4	37.9,37.6,37.1 37.5,37.4	37.1,38.2,37.4 37.9,37.7	37.5,38.3,37.4 37.6,37.9	37.6,37.4,3 8.2, 37.9,37.8	37.1,37.7,3 8.8 38.4,37.6,3 7.5

15mg/kg	36.4,35.6,35.9 36.7,36.8	37.1,37.9,36.9 37.4,37.3	37.1,37.4,37.5, 37.2,37.1	37.2,37.1,37.3 37.1,36.8	37.1,36.8, 36.7,37.4,37.2	37.1,36.9, 37.2, 37.2, 37.1
30mg/kg	36.8,36.1,36.7 35.4,35.9	37.4,38.1,36.9 37.7,37.4	36.9,36.8,37.1 36.6,36.7	36.4,36.6,36.8 36.3,36.5	36.3,36.5, 36.1, 36.2, 36.0	34.8,36.2 36.5,35.9 ,36.1
60mg/kg	35.3,34.8,36.1 34.3,36.1	37.8,36.9,37.5 37.8,37.6	36.7,36.6,36.3 36.4,36.2	36.5,36.3,36.5 36.4,36.3	36.1,36.3 ,34.8, 36.2,36.5	34.2,36.1 34.5,35.8,36.7
Drugamol 20mg/kg	35.8,36.8,35.6 35.1,36.4	37.7,37.4,37.9 37.6,36.9	36.4,36.3,36.7 36.1,36.3	36.2,36.5,36.1 36.3,36.6	36.2,36.6 ,34.4, 36.5,36.1	35.3,34.7 34.1,35.4,34.7

Table 5.8 Effect of *B. ferruginea* on yeast induced pyrexia: Showing the rectal temperatures recorded at 0 min (pre-induction) and 24 h h post induction of pyrexia. Temperature was recorded at 60 min intervals there after. Statistical analysis using repeated measures ANOVA showed that there was no significant difference between 15 mg/kg extract treated rats and control $P < 0.05$; there was a significant difference between control rats and extract treated rats at 30 mg/kg and 60 mg/kg $P < 0.05$; there is a significant difference between control and drugamol treated rats $P < 0.05$.

The results of the antipyretic study shows a dose dependent inhibition of pyrexia at dose levels of 30 and 60 mg/kg. There was no difference between rats treated with 15 mg/kg extract and control (saline treated) rats.

5.6 Chapter conclusion

The *in vivo* evaluation of the pharmacological properties of the methanolic *B. ferruginea* extract has revealed that the extract has several activities. The results of the antiplasmodial study showed that the extract is active against the *P. berghei* rodent model of malaria, demonstrating a dose dependent inhibition of parasitaemia. Both the suppressive and curative models for malaria were used in this study and antiplasmodial activity was demonstrated in the two models. This validates the traditional usage of the plant as an antimalarial herb by herbalist. The results of this study further supports, and is in line with the results obtained during the *in vitro* analysis of the extract. Moreover, the results also shows that the extract has a potential of being used as a phytopharmaceutical drugs both for prevention and treatment of malaria.

B. ferruginea is often used as part of a concoction of herbs for the treatment of other diseases, according to information obtained by herbalist in Nigeria. For this reason, other

pharmacological properties were explored. Investigation into the antinociceptive activity of the extract revealed that the extract has antinociceptive activity. Two different nociceptive models were used in this study, including the acetic acid induce writhing method and the tail flick test. Extracts that show activity against the acetic acid induced writhing method demonstrate peripheral mechanism of nociception while extracts that show activity against the tail flick tests demonstrate a central mechanism of pain inhibition. In this study, *B. ferruginea* demonstrated antinoceptive activity in both pain models. Hence, this shows that the extract mediates its activity though both the peripheral and the central mechanism. There was a higher inhibition noted in the acetic acid induced writhing method. This means that the extract mediates its antinoceptive activity more though the peripheral mechanism which is discussed in Chapter 7. Interestingly, the extract exerted its effect at IC₅₀ doses below 100 mg/kg in all the *in vivo* studies. Hence the extract could be used to treat malaria and its associated symptoms of pain and fever.

The results of work from this study can be used to develop a *B. ferruginea* phytodrug for malaria in Nigeria, the use of which can spread across West Africa, just like the example of *B. ferruginea* currently used in Ghana for the management of diabetes mellitus in a standardised capsule form.

CHAPTER 6

HPLC optimisation for fractionation of *Bridelia ferruginea* crude extract

6.1 Introduction

Natural products are usually found as a complex mixture of compounds, often acting in synergy to produce a certain effect within a biological system. Natural product compounds exist in different forms and hence have different structures and stability. Isolation of a compound from a crude extract can be complicated. Sometimes compounds isolated do not exert the same response as the crude extract. The crude extract may exert a higher efficacy due to the possible presence of other compounds within the mixture, which might have additive, synergistic or antagonistic interactions (Junio *et al.*, 2011).

Over the decades, several techniques have evolved for the separation and isolation of natural product compounds. Modern techniques like NMR have been invaluable in the area of chemical structure elucidation amongst other techniques. Several research laboratories explore small molecule research and discovery of new chemical entities from natural product scaffolds. Active natural product skeletons have been modified to develop natural product analogue libraries that have been used for the screening of several diseases by different research groups.

Typically, separation/isolation of a compound(s) from a natural product extract requires multiple steps. Some compounds may be easy to separate and isolate while others prove very complex. Sometimes compounds may exist in very small quantities within a mixture requiring specialist techniques to isolate and enrich them. Compounds may also be chromophores or non chromophores. Non chromophoric compounds are very challenging to separate using conventional methods.

A wide range of techniques are used for purification and isolation of compounds from natural products. In most cases of isolation of a compound, there is no prior knowledge of what is to be isolated, making the process a form of a 'hit and miss' situation. Furthermore, the choice of the technique requires some pre-requisite research and information about the possible nature of the compound that may be present. The stability of the compounds present

and their nature also play an important role in deciding the choice or sometimes combination of techniques to use.

A very good example of the importance of how certain factors affect isolation was demonstrated in the isolation of penicillin. After the discovery of penicillin as a useful antibiotic, Alexander Fleming could not isolate it. For this reason, it was not regarded as clinically useful. Fleming was able to successfully isolate the compound in aqueous solution but the compound was unstable once water was removed. This is a clear demonstration of the impact of compound stability on isolation. It was not until the advent of new techniques like freeze drying and chromatography that penicillin was successfully isolated (Clayton *et al.*, 1944).

The mainstay method used for the separation of compounds is chromatography.

6.2 Chromatography

6.2.1 Chromatography

Chromatography is a technique that separates compounds by distributing them between two phases, namely the mobile phase and the stationary phase. The separation occurs due to the differential affinity of the compounds to the two phases, leading to a difference in the distribution constants between the two phases. There are several types of chromatography techniques, the detailed discussion of which is beyond the scope of this project.

6.2.2 Types of chromatography: There are three main categories of chromatography. These include gas chromatography, thin layer chromatography and column chromatography. Each type has different sub-types depending on the nature of stationary and mobile phases used.

6.2.2.1 Thin layer chromatography (TLC): The stationary phase is a thin layer as the name suggests, spread over a glass or plastic sheet. The mobile phase is a liquid (solvent) drawn up plate by capillary action. Different factors will affect solvent choice for example hydrophobicity/hydrophilicity.

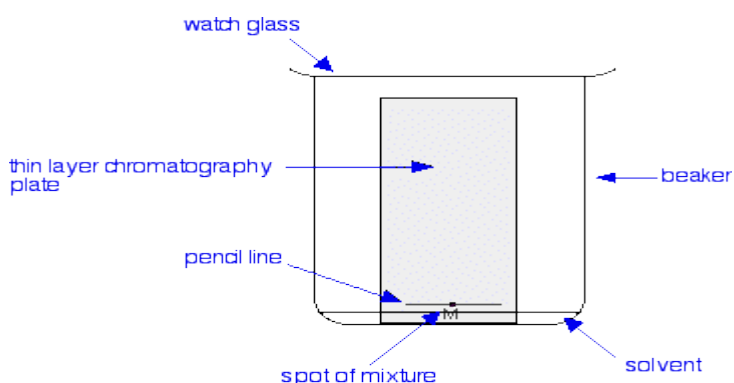


Fig 6.1 Illustration of TLC: system showing the tank with the solvent; the plate and sample spot. (Source from www.chemguide.co.uk)

6.2.2.2 Column chromatography: The stationary phase is contained in a column whilst the mobile phase liquid (solvent) passes through a column. The sample is injected through an injection port and passes through the column. The separated compound, called the eluant, elutes from the column. The speed with which a compound elutes depends on its relative affinity to the stationary and mobile phase. Compounds with a high affinity for the mobile phase will elute first whilst those with a higher affinity for the stationary phase will elute last. The mobile phase is moved down the column by either gravity or pressure. There are different types of column chromatography. High pressure liquid chromatography (HPLC) is one of the most commonly used types of column chromatography techniques.

6.2.2.3 Gas chromatography (GC)

In this case, the stationary phase is contained in a column while the mobile phase is a gas that passes through column (pressure).

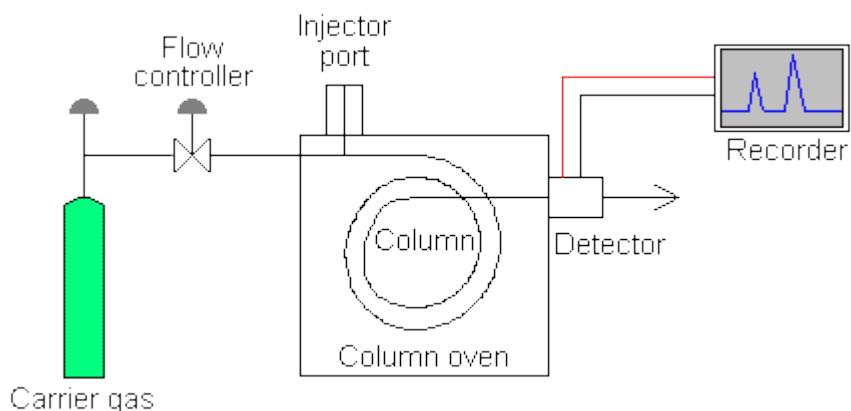


Fig 6.2 Illustration of a GC system: Showing the various components including the detector and recording system. (Source: from www.teachingshu.ac.uk).

6.3 HPLC and natural product separation/isolation

HPLC is one of the main techniques used for the separation of natural products mixtures. This method is highly improved over the traditional column chromatography technique where a liquid is allowed to elute through gravity. In the case of HPLC, the liquid is forced by a very high pressure. This allows for packing of small molecules in the stationary phase which in turn increases the surface area, allowing for more interactions between the stationary phase and the compounds passing through it. HPLC is a highly automated technique. There are several types of HPLC which include normal phase, reversed phase, size exclusion and ion exchange.

6.4 Separation of methanolic *B. ferruginea* using HPLC

6.4.1 Choice of technique

The choice of which technique to use can often be difficult especially if the compound to be isolated is unknown. Certain factors may however give clues as to what technique to choose. In recent times, HPLC has been the mainstay for natural product isolation. There are different types of HPLC techniques and the nature of the compound to be isolated determines which technique to use. In this study, the extracts that were bioassayed were methanolic and aqueous extracts. Both had antimalarial activity with the methanolic one being more potent. The fact that these extracts were extracted with polar solvents is an indication that the nature of the compounds responsible for the activity is polar. This is a valuable tool in the choice of solvents for isolation. The extract was also evaporated to dryness in a water bath which means it is stable to heat. Phytochemical analysis in this study has shown *B. ferruginea* to contain tannins, terpenes, flavanoids and glycosides. Literature suggests that flavanoids play a role in antimalarial activity (Lehane *et al.*, 2008) hence, the main compounds we suspect to be implicated in malaria in this plant extract are the flavanoids. Flavanoids have previously been isolated using reverse-phase chromatography and it is usually the choice for most organic compounds. In this study, we therefore choose reverse-phase chromatography as a separation technique for the crude extract.

6.4.2 Reverse-Phase Chromatography

This refers to any chromatography technique that uses a hydrophobic stationary phase. In normal phase chromatography, the stationary phase is hydrophilic, for example silica or alumina resins. This makes the stationary phase have a strong affinity for hydrophobic compounds in the mobile phase. The hydrophilic compounds will hence elute first. In the case of reverse phase, the stationary phase is hydrophobic example alkyl chains (C18) and the mobile phase has a strong affinity for hydrophilic compounds. Hydrophobic compounds elute first. The polarity of the mobile phase can be decreased in order to reduce the hydrophobic interactions with the stationary phase hence allowing for hydrophobic compounds to elute.

6.4.3 Solvent choice

Choosing a solvent for separation can often be tedious due to the compounds being unknown. In this project, reversed phase chromatography was chosen, hence reversed phase TLC was performed to help choose the solvents to be used for the HPLC. Polarity of different solvents were considered as well as the solvent miscibility chart. This, together with information from literature about solvents used in separation of organic compounds, were used as a guide in solvent choice. Table 6.1 below was used.

Solvent	Polarity	Compound class
Hexane	Lowest polarity	Hydrocarbon
Benzene		Aromatics
Ether		
Chloroform	Modest polarity	Many classes
Ethyl acetate		
Acetone		
Methanol	Highest polarity	Amino acids, carbohydrates
Water		
Acidic water		

Table 6.1 Polarity of solvents: Showing the polarity of the compound depends on the ratio of the polar functional group (hydrophilic part) to the C-H moiety (hydrophobic) part of the molecule.

6.5 Reversed-Phase TLC

This was performed in order to determine the solvents to use for the reverse phase HPLC separation.

6.5.1 Method development

This was performed using a reversed phase glass plate precoated with silica gel. The methanolic extract of *B. ferruginea* was dissolved in water to make a 100 mg/ml stock solution. A 1 cm drop of sample was applied from the edge of the plate as illustrated in figure 6.1.

Several plates were prepared and developed in different solvent systems and ratio combinations. The solvent systems used are described in the table below:

Solvents used	Ratio
Methanol: Water: acetonitrile in the ratio	70/20/10; 60/30/10; 50/50/0
N butanol: water: acetic acid	70/20/10; 60/30/10; 50/50
Ethylacetate:butanol:acetic acid:water	60/30/5/5
Methanol;chloroform:petroluem ether	20/30/5/0
Petroluem ether:ethylacetate	50/50
Methanol/ethylacetate	50/50
Methanol:ethylacetate:water	30/60/10

Table 6.2 Solvent systems for the reversed TLC: Showing the solvent systems chosen for the TLC experimental run. More than one ratio combinations was applied with some solvent systems.

6.5.2 Plate development

The plate was developed by immediately drawing a line to mark the solvent front. Then visible spots were marked with a pencil. The plates that had spots that were not obvious were viewed using an ultraviolet (UV) lamp, allowing spots to be viewed and marked.

6.5.3 Results:

i) Methanol/water/acetonitrile system: This system gave a good separation and there was a spread across the entire plate, indicating the presence of several compounds, hence a complex mixture. The compounds however, travelled all the way to the solvent front with some of the ratio combinations used within this system. Methanol is a highly polar solvent with a polarity index of 4.1, hence a system with a lower polarity was investigated to see if separation will improve.

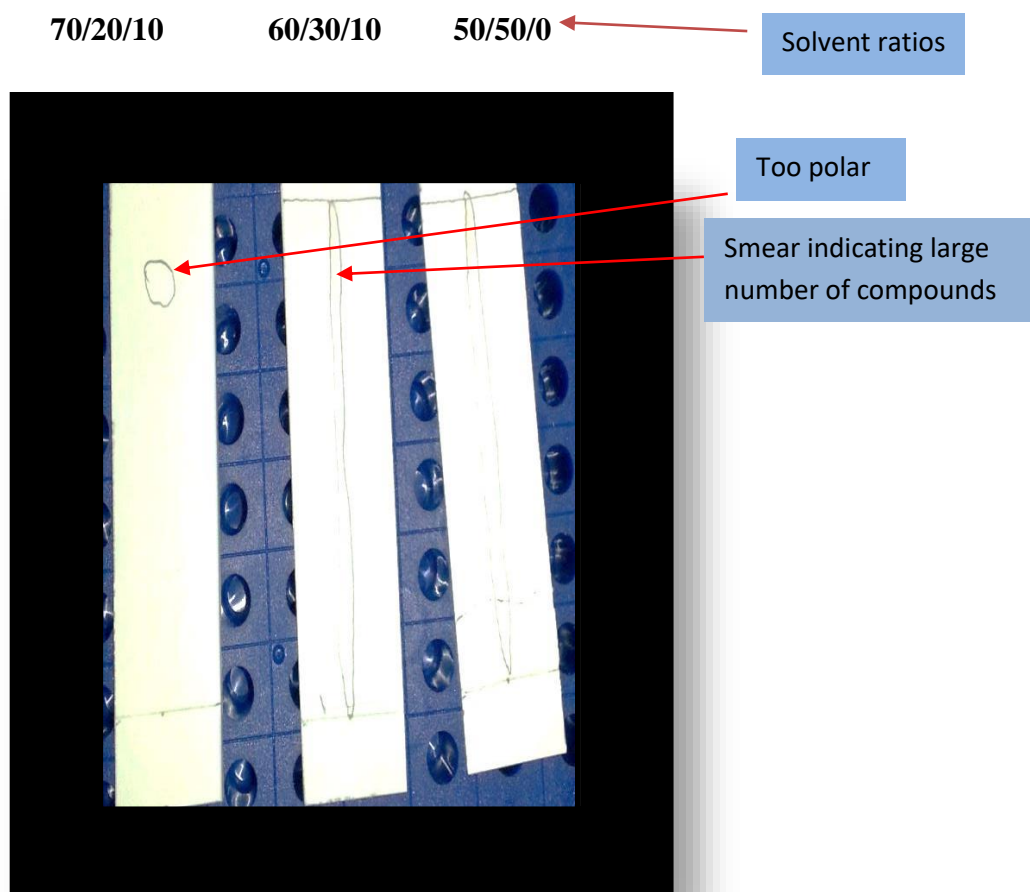


Fig 6.3 Plate development for the methanol/water/acetonitrile solvent system: Showing a separation in some of the solvent ratio combinations. The first system was too polar.

The results from this solvent system indicates that decreasing the polarity improves the separation.

ii) N-butanol/water/acetic acid: This solvent system did not move the analyte from the sample spot, indicating that the polarity of the analyte is greater than that of the solvent. Butanol has a polarity index of 1 while acetic acid has a polarity index of 0 and water 7.2. It will be important to choose a different system with a higher polarity based on this result. Figure 6.5 below shows the image of the TLC plate.

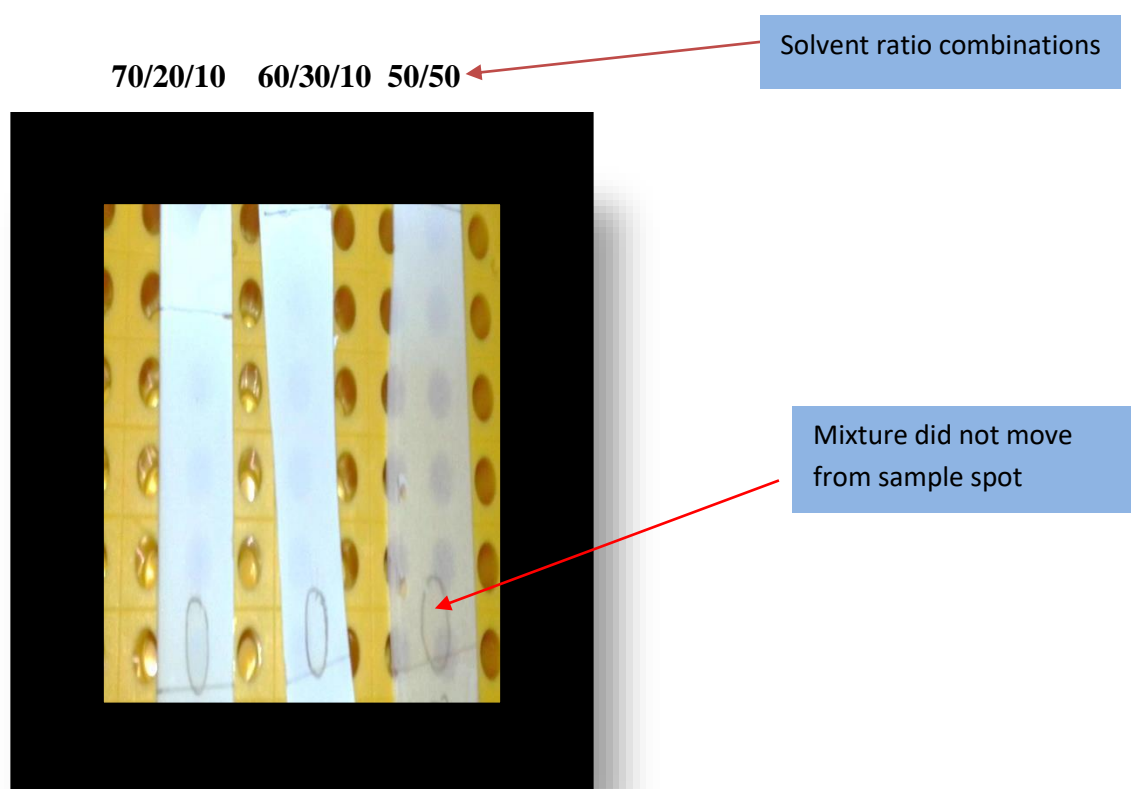


Fig 6.4 *TLC plate of the solvent system, N-butanol/water/acetic acid: There was no separation in this solvent system.*

This solvent system did not move the analytes to the solvent front hence, a better system will have to be explored.

iii) Ethyl acetate/butanol/acetic acid/water.

The polarity of the solvent system was increased by adding ethyl acetate which has a polarity index of 4.4.

60/30/5/5

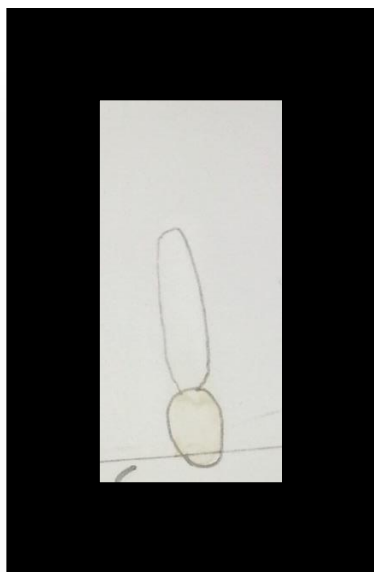


Fig 6.5 TLC plate for the solvent system, ethyl acetate/butanol/acetic acid/water.

A slightly improved chromatogram although the mixture has not separated sufficiently. This is indicated by the fact that the analyte has not travelled all the way to the solvent front. Ethyl acetate has a polarity index of 4.4.

iv) Other solvent systems: In order to get a better separation on the TLC plates, more solvent systems were explored and the results of the plates are represented in Fig below

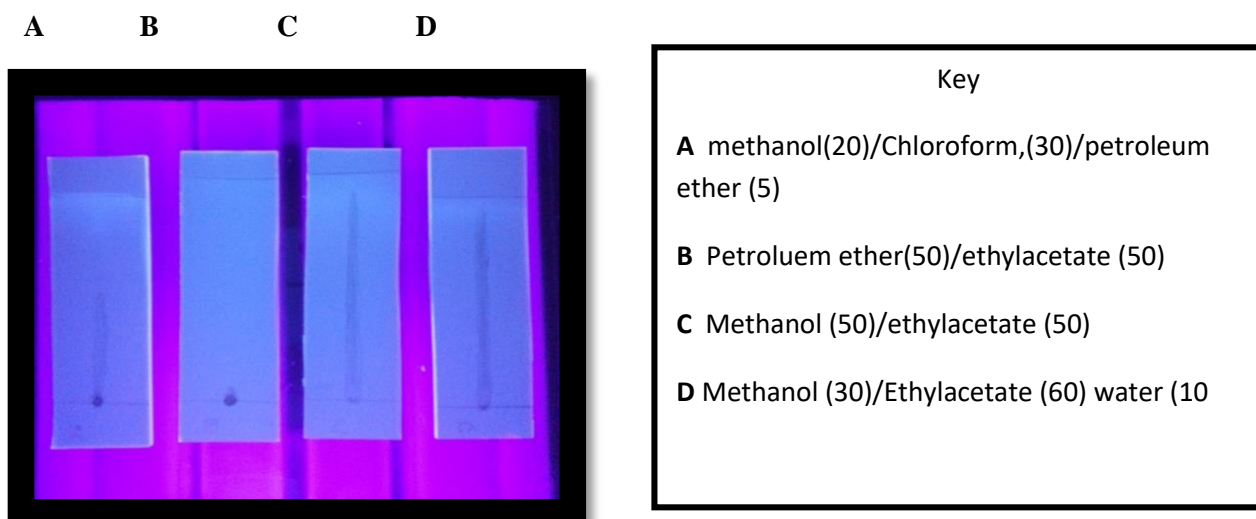


Fig 6.6 TLC plate for other solvent systems: Solvent system A was slightly effective whilst B was in effective. Systems C and D were effective and similar to the methanol/water systems.

In view of the results, acetonitrile/water and methanol/water will be explored as solvent systems for the reversed-phase chromatography.

6.6 Reversed-phase HPLC

6.6.1 Method development analytical HPLC

The HPLC analysis was carried out using a Gilson 307 HPLC pump with Varian Prostar 320 UV detector. The column was a reversed phase Luna ® on a C₁₈ stationary phase with a 5 µm particle size and a 100 Å pore size. The column size was 30 mm length X 4.6mm diameter. A Rheodyne 7725i injection valve was used for the sample injection. The mobile phase was chosen based on the results of the reverse phase TLC. An isocratic mode elution was used with 0.1% trifluoroacetic acid (TFA) and a methanol/water mobile phase. Different solvent ratios were used in the analytical method development this included methanol/water (20/80; 40/60; 60/80; 80/20) and acetonitrile/water solvent system. The separation was performed using an isocratic mode elution. A 50 µl sample was injected into a 50 µl injection loop and the flow rate was set to 1 ml/min. Samples were run for 30 min to 1 h.

6.6.2 Method development for preparative HPLC

The preparative column was a reversed phase Kinetex ® on a C₁₈ stationary phase with a 5µm particle size and a 100 Å pore size. The column size was 50 mm length X 21.2 mm internal diameter. A guard column was used. The guard column was a SecurityGuard preparative mode on a C₁₈ stationary phase with a 21.2 mm internal diameter. An isocratic mode elution was used with 0.1% trifluoroacetic acid (TFA) and a methanol/water mobile phase and an acetonitrile/water mobile phase. The sample was injected into a 500 ml sample injection loop.

6.6.3 Protocol: The HPLC system is automated and uses a computer software system to operate. The pumps can be operated both manually or using buttons on the pump. Initial runs start with getting rid of the storage solvent and replacing it with the solvent system of choice for the run. This was performed by washing the HPLC lines with water and replacing with the mobile phase system. Isocratic elution was used, hence a single pump was used. To wash the column, the purge valve was opened in an anticlockwise direction which transferred the liquid into the waste bottle (Closing the valve directs liquid to the column so this was only done during a sample run or column wash). Once the purge valve was opened,

the PRIME button located on pump A unit was pressed. The maximum flow rate on this unit is 25ml/min. For this study, a flow rate of 1 ml/minute was chosen for the analytical run and 10 ml/min for the preparative run. The output signal was processed using a PicoLog data computer software (Pico technology Ltd, Cambridge, UK). All solvents were HPLC grade and the extract sample was filtered using a 0.45 μm filter (Millipore, Bedford, USA).

6.6.4 Detection: UV absorbance was used for detection in this study. The detector was connected to the HPLC system. Different compounds absorb UV under different wavelength due their characteristic absorbtion coefficient. In most cases, short wavelength of 200 to 300 nm are chosen when monitoring unknowns as most organic compounds fall within this range. Flavanoids absorb at 254 nm, hence this wavelength was chosen for the HPLC analysis due to their implication in malaria. At longer wavelength, compounds tend to be more specific. Wavelength less than 200 cause interference with the mobile phase.

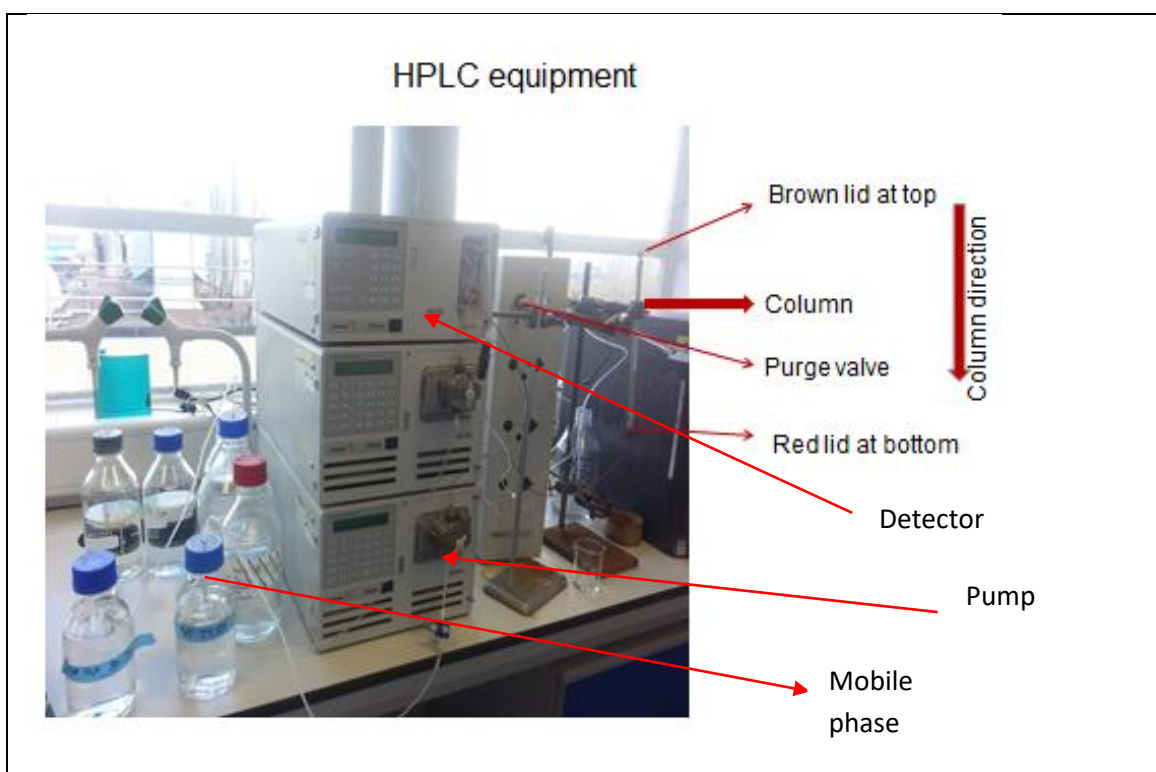


Fig 6.7 A HPLC system: Showing a HPLC system used in our laboratory, indicating the mobile phase, pump, injection port, the column and other components.

6.6.5 Column storage: The column was stored in 70% methanol after running five column volumes of solvent and kept until used.

6.6.6 *Method optimisation:* The robustness of the method was investigated by deliberate alterations in the method parameters. Different mobile phases were ran, sample concentrations was altered. The results of these validated the method. Samples that were ran under the same conditions produced the same chromatogram. Altering the test parameters altered the chromatogram.

6.7 Results

The results of the HPLC analysis was obtained from the software as chromatograms.

6.7.1 Analytical HPLC results for methanolic *Bridelia ferruginea*

i) Acetonitrile/ water as a mobile phase system for separation of *B. ferruginea* components.

30/70 acetonitrile water

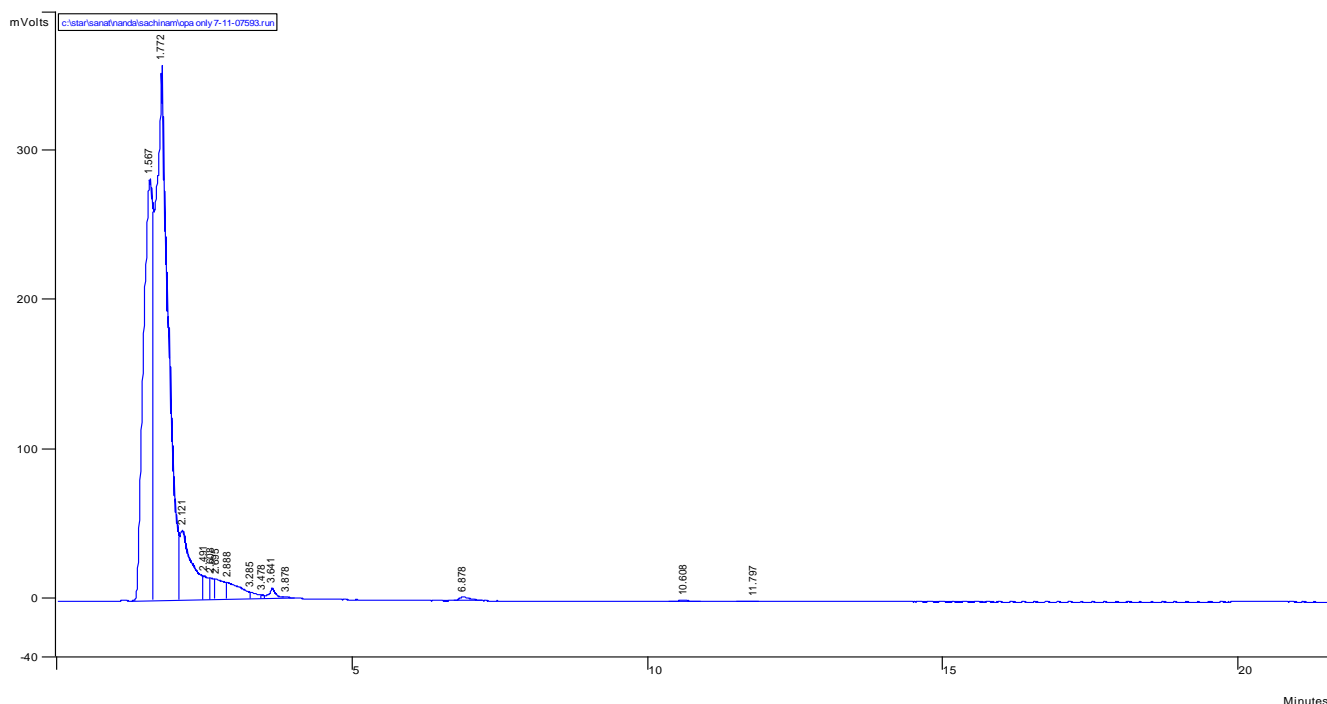
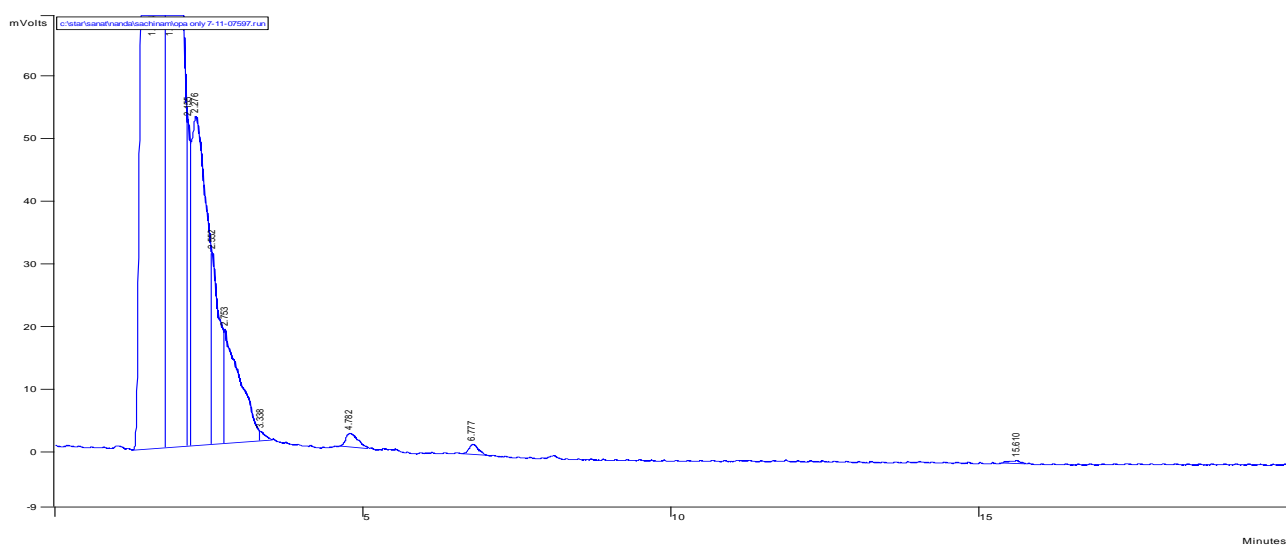


Fig 6.8 A 30/70 acetonitrile/water chromatogram: Showing a chromatogram of the crude extract using 30/70 acetonitrile water solvent system in an analytical column.

The solvent system used in Fig 6.8 above produced several peaks although the peak resolution was poor. Fraction collection will prove difficult due to analytes eluting too quickly. Hence increasing the polarity of the solvent might be useful. A more polar system was tested.

ii) 40/60 acetonitrile/water mobile system



iii) **Fig 6.9 40/60 acetonitrile/water chromatogram:** Showing a chromatogram of the crude extract using 40/60 acetonitrile/water mobile system in an analytical column. The peaks were unresolved

There was a poor peak resolution with this solvent system. Hence further improvements need to be made in order to increase resolution and reduce retention time. Hence 80/20 acetonitrile/water was explored.

iv) 80/20 acetonitrile/water solvent system

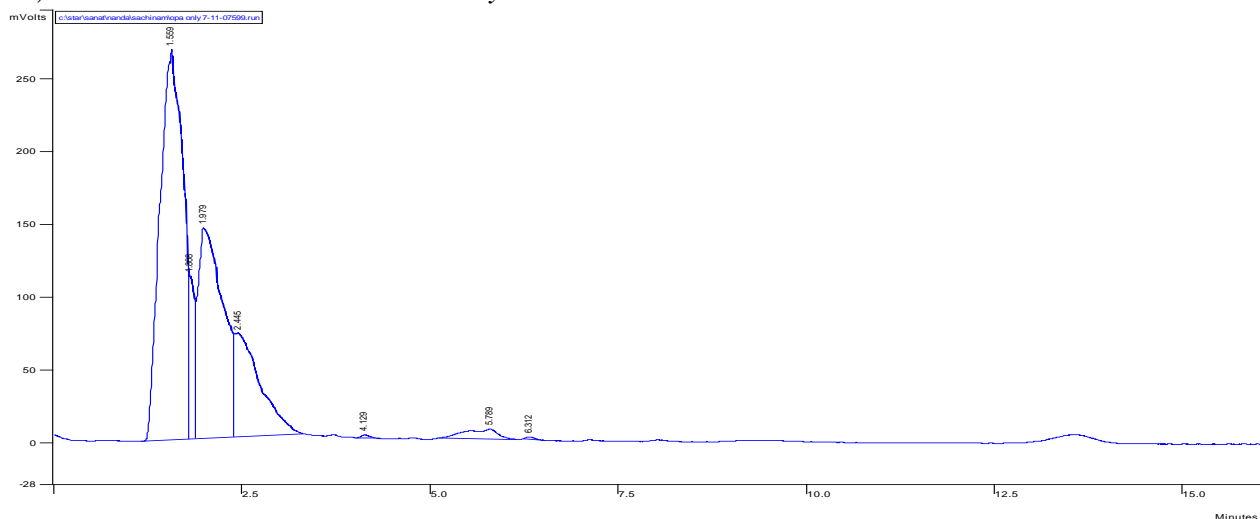


Fig 6.10 80/20 acetonitrile/water system: Showing a chromatogram of the crude extract using 80/20 acetonitrile/water solvent system in an analytical column.

This solvent system has produced a poor peak resolution hence further mobile phase modifications need to be made. Due to this, a different solvent system was explored.

2) Methanol/ water as a mobile phase system for separation of *Bridelia ferruginea* using HPLC

i) Methanol: Water 80/20

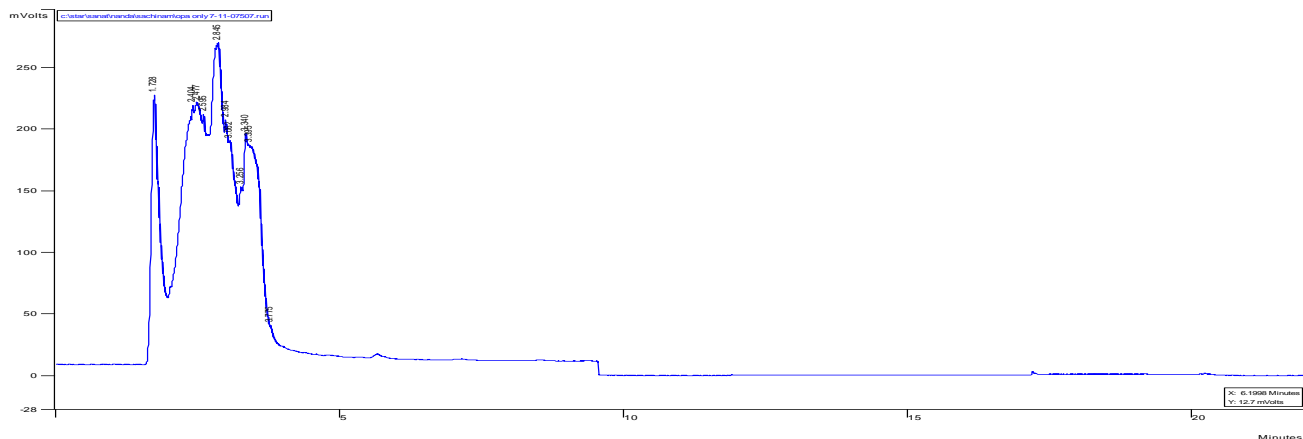
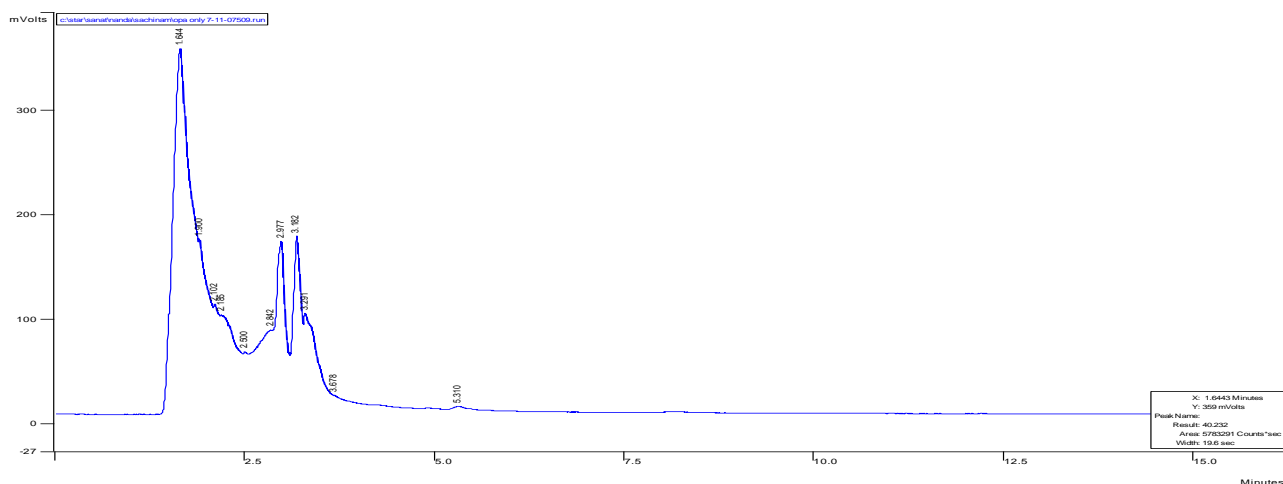


Fig 6.11 80/20 methanol/water chromatogram: Showing a chromatogram of the crude extract using 80/20 methanol/water solvent system in an analytical column.

Several peaks were produced in this run as indicated by the chromatogram above. The analytes have however eluted within the first 5 mins of injecting the sample. There is a slightly better resolution in the first peak but the other peaks are unresolved. The solvent ratio is most likely too polar. Reducing the polarity might slow down the movement of the analytes though the mobile phase to give a better resolution and increase retention time.

ii) Methanol:water 60/40 solvent system



Reducing the polarity of the mobile phase system gave the chromatogram above, which improved the resolution. The retention time was increased.

iii) *Methanol:water 50/50 solvent system*

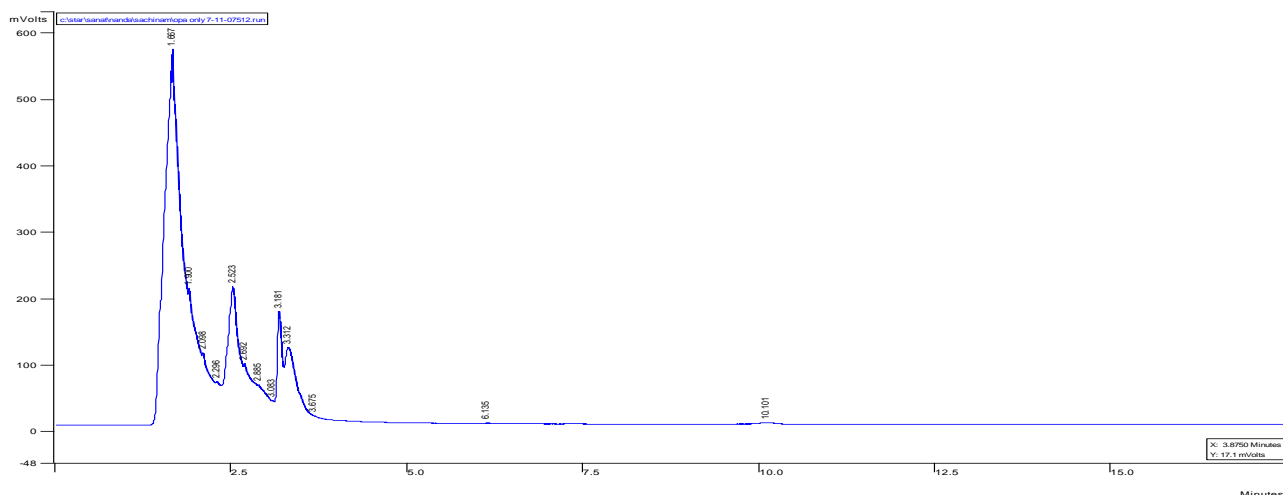


Fig 6.13 50/50 Methanol/water chromatogram: Showing a chromatogram of the crude extract using 50/50 methanol/water solvent system in an analytical column

Changing the solvent system to 50/50 methanol:water has led to an improved resolution as can be seen in the chromatogram above. The peaks are more resolved but the analytes have eluted within a 5minute time span.

iv) *Methanol:water 40/60*

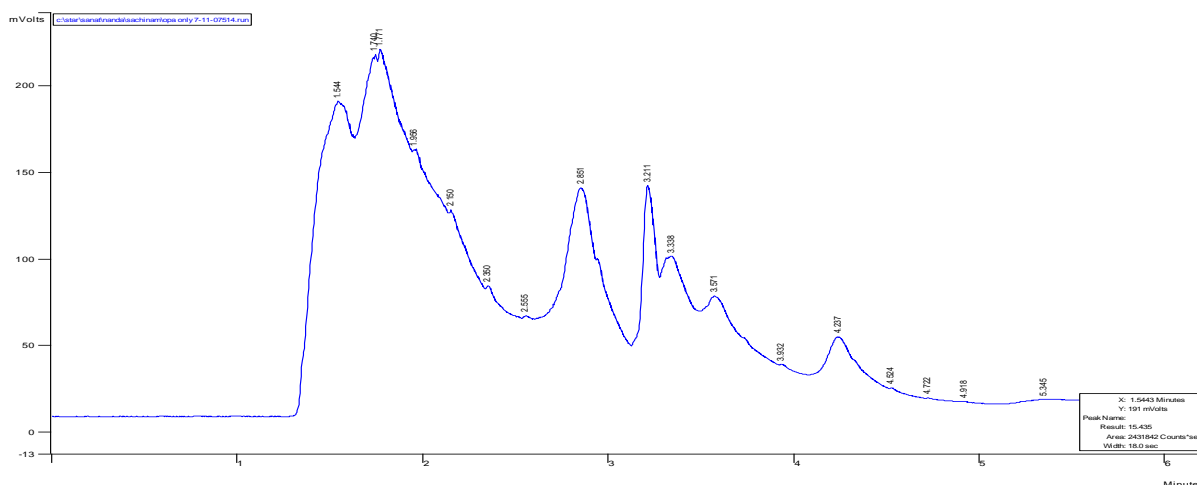


Fig 6.14 40/60 Methanol/water chromatogram: Showing a chromatogram of the crude extract using 40/60 methanol/water solvent system in an analytical column.

This solvent system produced more analytes as indicated by more peaks although the resolution was poor with multiple analytes eluting at the same time.

Methanol:water 30/70

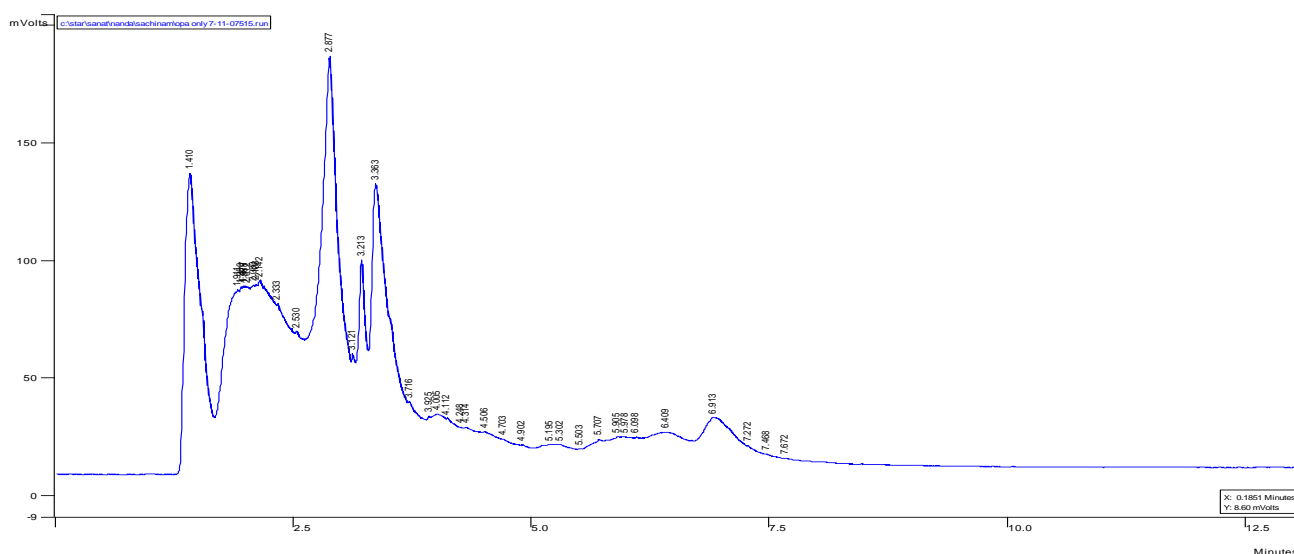


Fig 6.15 30/70 Methanol/water chromatogram: Showing a chromatogram of the crude extract using 30/70 methanol/water solvent system in an analytical column

Overall, methanol/water as a mobile phase seems to give a better separation compared to acetonitrile/ water in the analytical phase although the analytes eluted quicker most likely owing to the polarity of the mobile phase. Overall, increasing water polarity of the mobile phase improved resolution. One of the major disadvantages of the detection method used in this study is it is not quantitative, as with UV detectors. Although several small peaks appeared, they are not an indication of the sample amount, hence the small peaks could contain a reasonable amount of material rather than trace. Collecting fractions according to peak heights could mean reasonable analyte quantities could be lost. Based on this, a timed fraction collection is planned for the preparative HPLC analysis. Based on the results obtained from the above analytical HPLC phase, the methods were scaled up using a preparative column.

6.7.2 Comparative analysis of the aqueous and methanolic extract of *Bridelia ferruginea*

The aqueous and methanolic extract were ran under same HPLC conditions in the analytical HPLC column. The Injection volume was 50 μ l and the concentration was 20 mg/ml stock. Mobile phase was 40/60 methanol water using an analytical column with UV detector set at 254 nm. The chromatogram below was obtained.

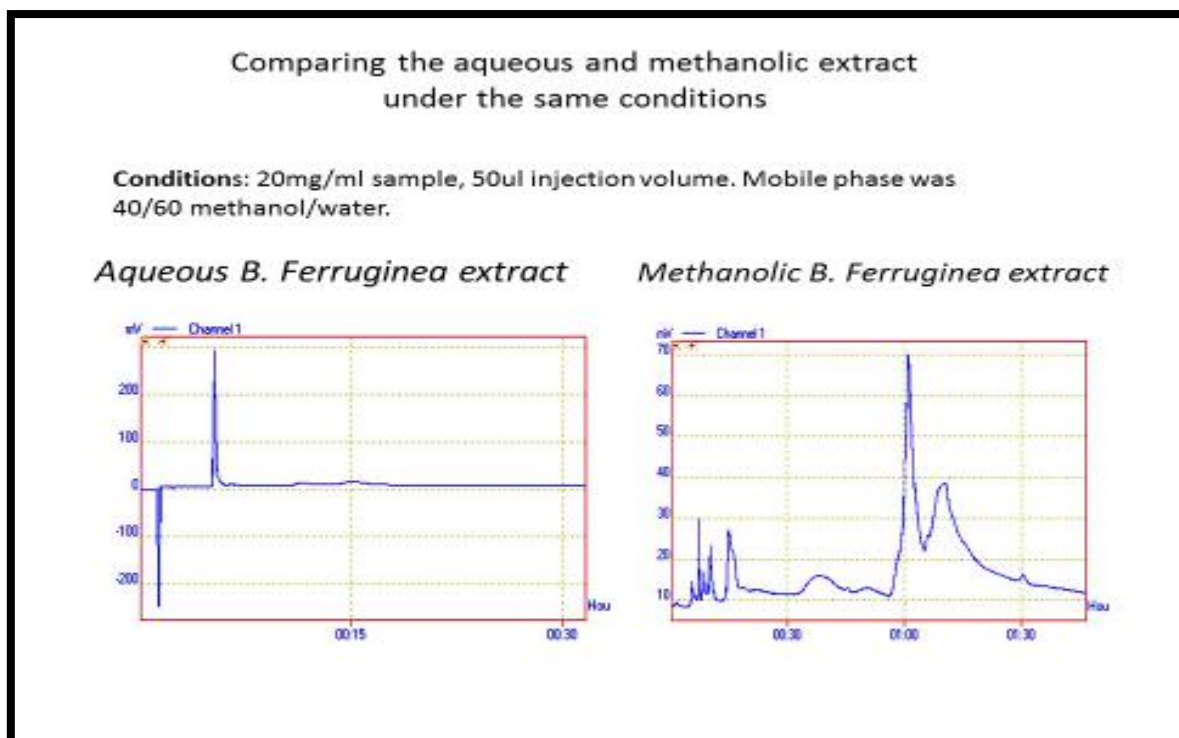


Fig 6.16: *Comparing the aqueous and methanolic extract under the same conditions: Showing the two extracts under same conditions produced multiple peaks in the methanolic extract but just a single peak in the aqueous run. The run was performed for 30 mins*

6.7.3 Preparative HPLC

The preparative HPLC is a scale up from the analytical run using the preparative column. The columns have a high flow rate systems hence allowing for the collection of analytes unlike the analytical HPLC. Furthermore, the preparative HPLC can be used as a quantitative technique. Briefly, the method involved dissolving the extract in water and placing in a 37°C incubator for 1 h to enhance the dissolution. The first series of runs were performed using a 40/60 methanol/water solvent system with injection volume of 1 ml using a 1 ml sample loop at a flow rate of 10 ml/min with the detector set at a wavelength of 254 nm.

6.7.3.1 Methanol/ water system

Methanol/water system at the ratio 40/60.

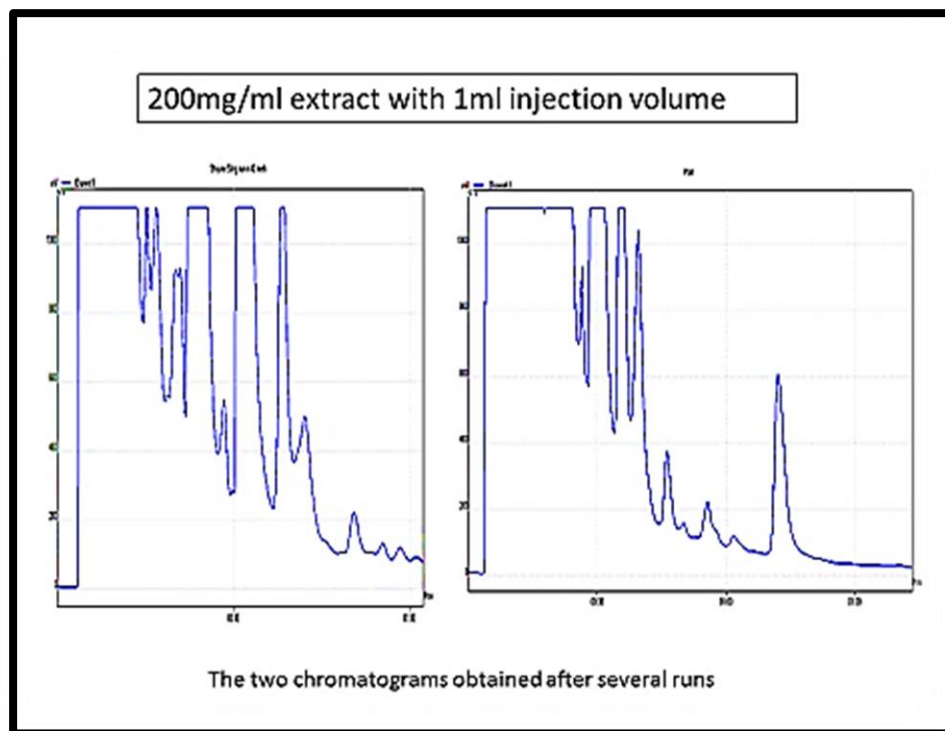


Fig 6.17 Comparing chromatograms between runs: Showing some peaks were lost between runs but most peaks were consistent. The lost peaks were identified based on peak shapes and retention times. Overall, the method was reproducible.

The unstable peaks were identified in the chromatogram below.

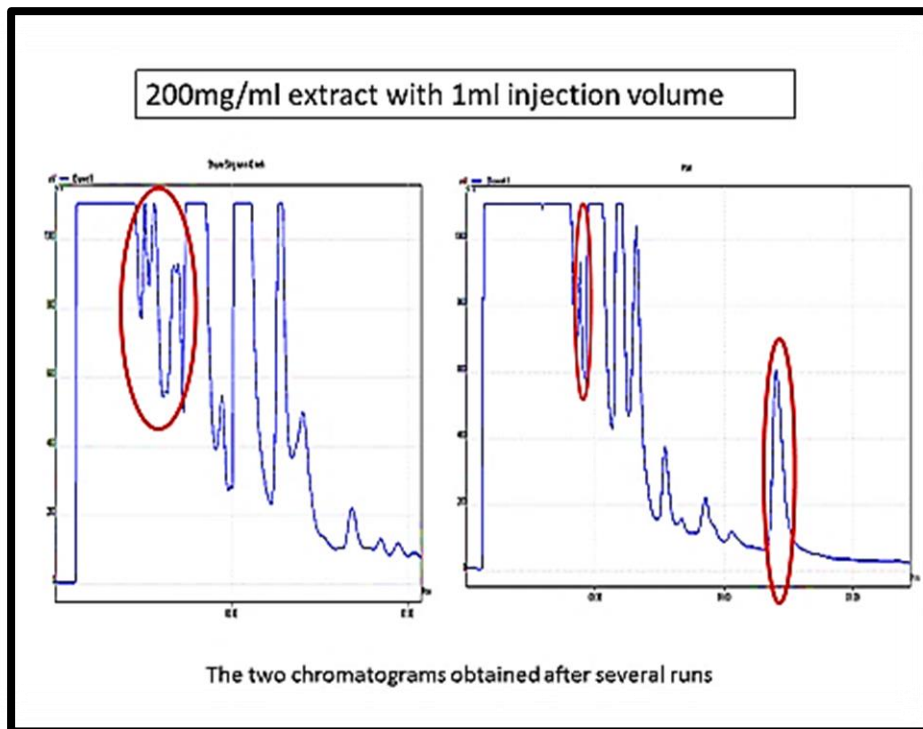


Fig 6.18 Lost peaks in the chromatogram: Showing unstable peaks that were lost in some runs. This was used as a guide in determining what fractions to collect Based on these results, fraction collection was started and the fractions collected are labelled in the diagram below.

200mg/ml extract with 1ml injection volume

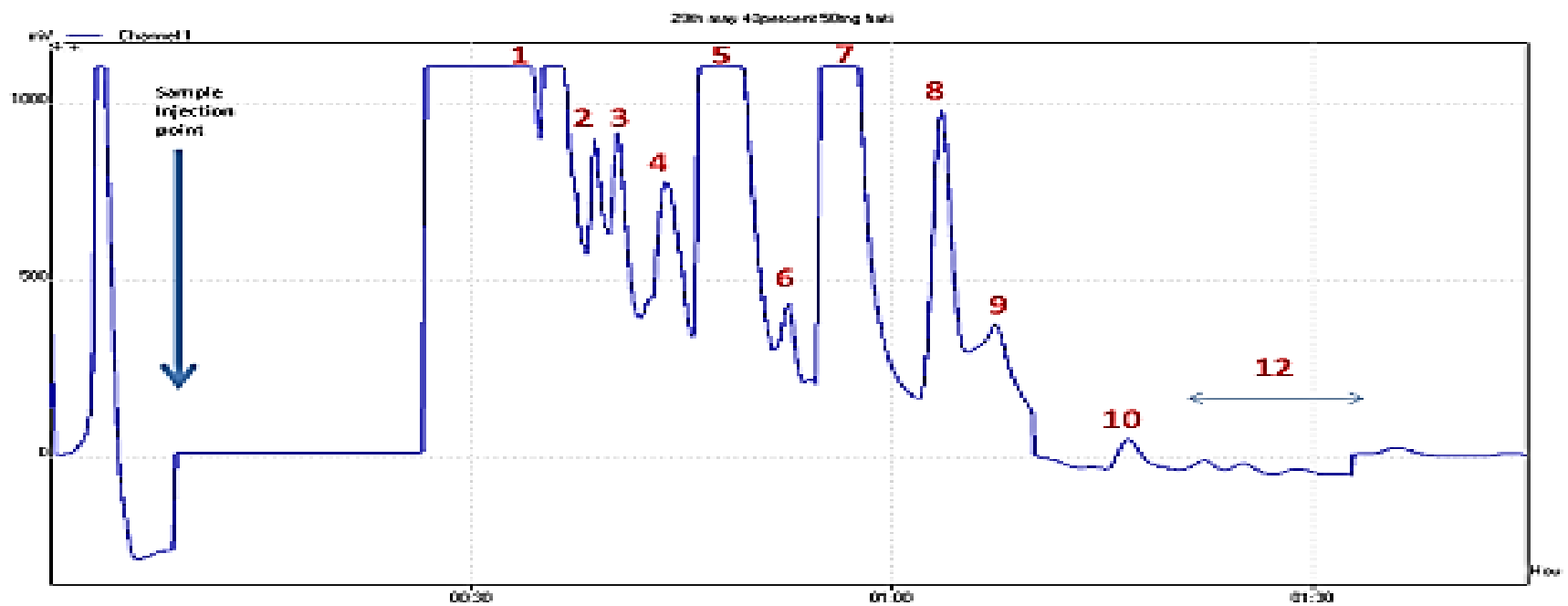


Fig 6.19 Fractions collected: The retention time of each fraction was noted and used as a guide during collection. Fractions that were lost in some runs example peak 2 were collected in runs that they appeared. A total of 12 runs were performed.

6.7.3.2 Evaporation of samples to dryness

The fractions obtained were dried in a rotary evaporator at 70°C under vacuum conditions. Fraction 1 was obtained in reasonable quantity (10 mg). The other fractions were however in negligible quantities hence collection was not possible. The process was performed by weighing a vacuum flask before putting the liquid sample. Once the fraction was dried, the bottle was re-weighed as detailed below.

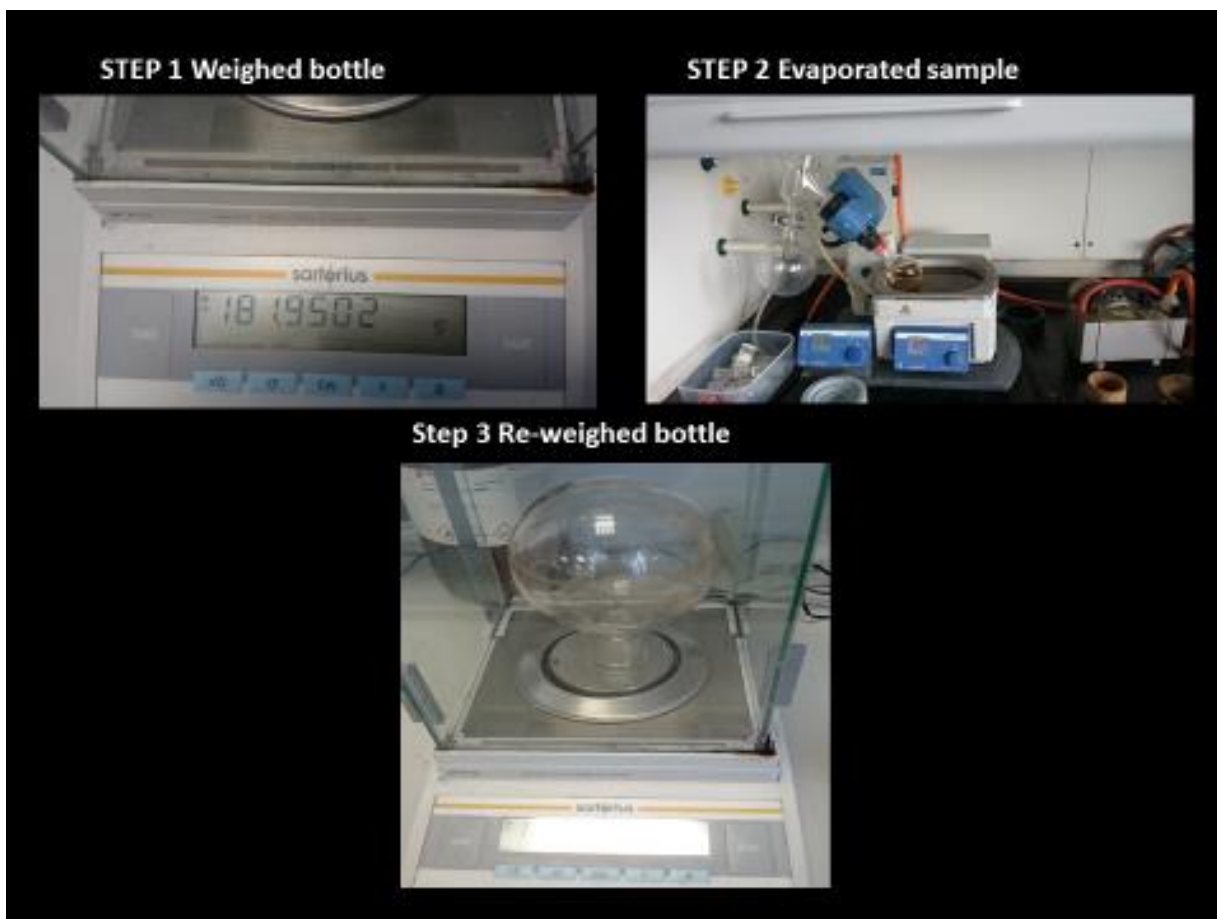


Fig 6.20 Determining fraction quantity: Fractions collected after several runs were weighed in the bottle as in the picture above. It was decided to change the solvent system at this stage.

6.7.3.3 Acetonitrile/ water system

The acetonitrile/water system was explored in the preparative column.

6.7.3.3.1 Run 1 15/85 Acetonitrile/water (50 mg)

20 mg of the extract was injected into the column and ran at a flow rate of 10 ml/min as in previous runs. This system gave a good separation and the time taken for all fractions to be eluted was an hour.

The chromatogram is represented in fig 6.21 below.

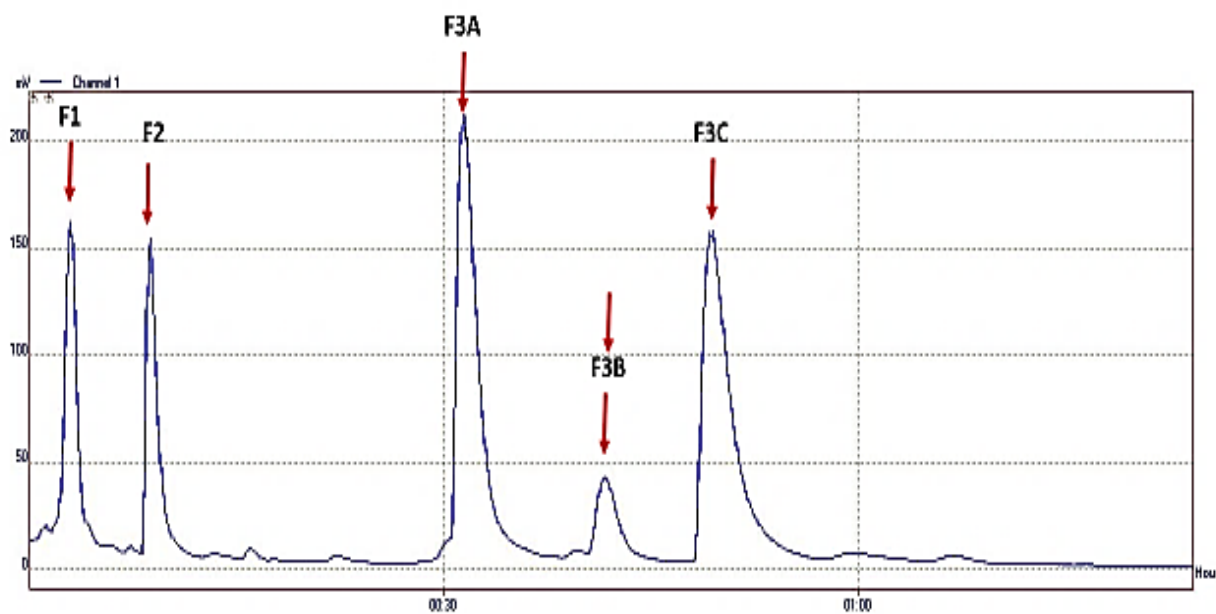


Fig 6.21 15/85 acetonitrile/water preparative chromatogram: Showing a chromatogram obtained during a preparative chromatography run using a 15/85 acetonitrile/water solvent system with fractions eluting within an hour.

6.7.3.3.2 Run 2 20/80 acetonitrile/water (50 mg)

A 20/80 acetonitrile/water system was explored in the preparative HPLC with a 50 mg sample of the extract injected as a 1 ml volume in a 1 ml sample loop. The trace below was obtained and the fractions were collected in different tubes.

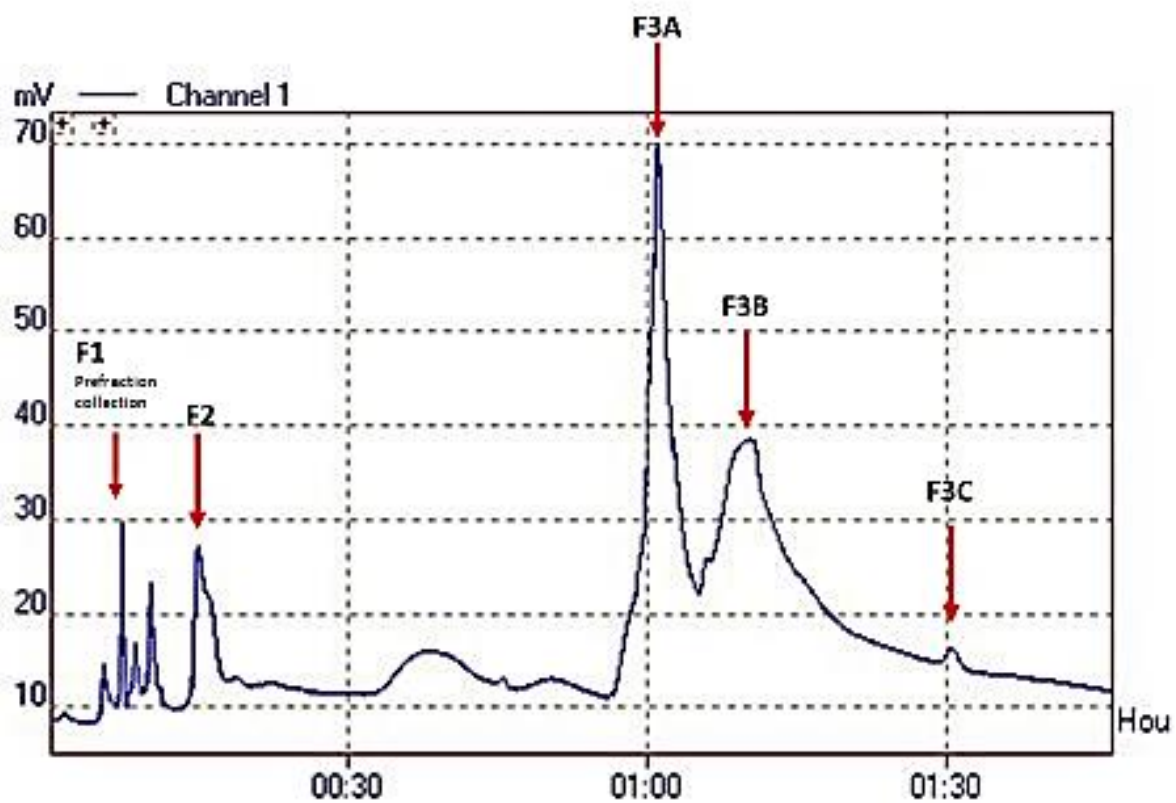


Fig 6.22 20/80 acetonitrile/water preparative chromatogram: Showing the chromatogram obtained for the 20/80 run and the fractions.

6.7.3.3.3 Run 3 30/70 acetonitrile/water (50 mg sample)

In order to speed up the runs, the amount of acetonitrile was slightly increased to 30/70 acetonitrile/water and the amount of extract amount of 50 mg in 1 ml volume.

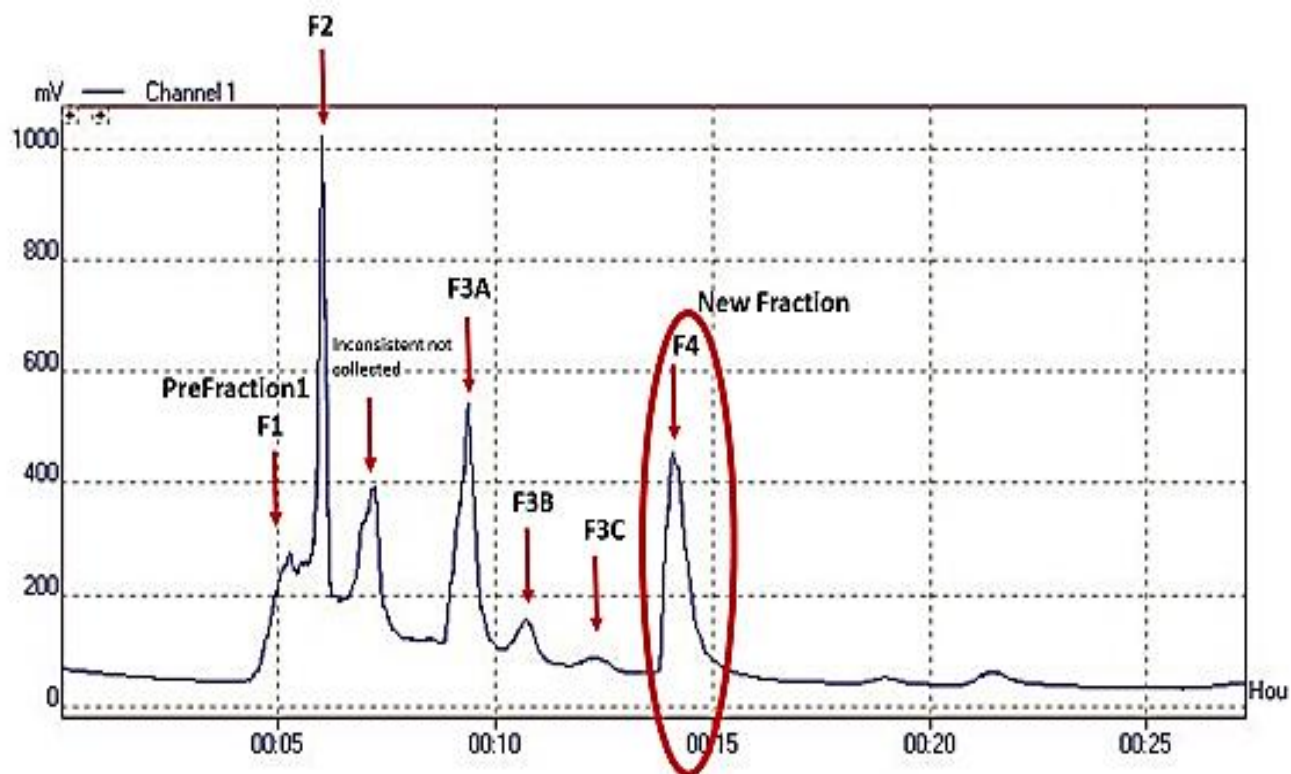


Fig 6.23 30/70 acetonitrile/water preparative chromatogram: Showing a chromatogram obtained from a preparative run with the fractions collected.

Increasing the acetonitrile strength reduced the time taken for the run significantly. The run was shortened to 15 to 20 mins. The runs produced the chromatogram above but some and analytes eluted within 15-20 mins. Peak shapes were consistent in all runs allowing fractions collection. Below is a sample of a chromatogram that ran for over 15 minutes.

Other runs with the same solvent system were consistent.

6.7.4 Troubleshooting for the preparative HPLC column

Troubleshooting was required due to loss of some fractions after several runs. After about 10 runs, fractions were lost. Various trouble shooting procedures were performed to restore fractions. A vigorous column restoration procedure had to be performed. The traces below were obtained.

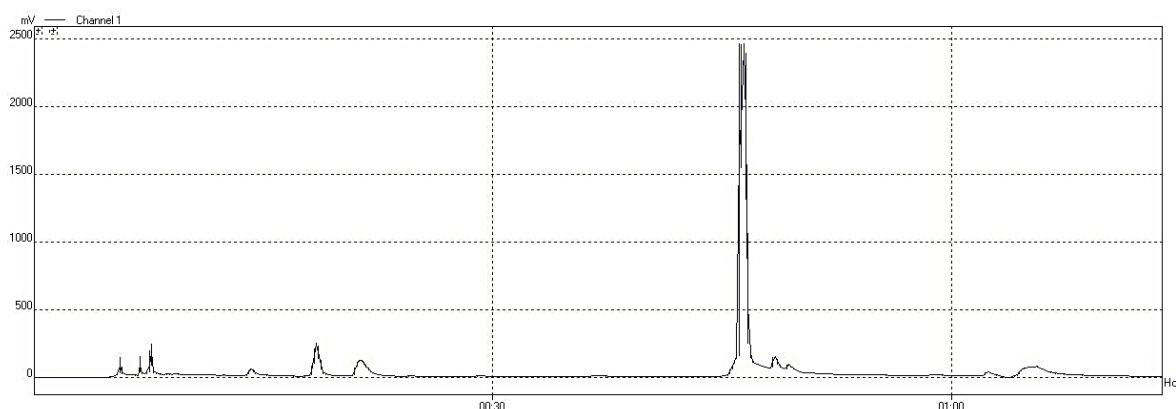


Fig 6.24 Trace for column restoration: Showing the first trace obtained after column restoration All efforts to restore peaks after several washes with 40/60 acetonitrile/water did not restore peaks. Hence an intensive column regeneration procedure was necessitated after contact with column manufacturer.

6.7.4.1 Column regeneration procedure

Step 1

The Column was flushed with pure 100% acetonitrile at a flow rate of 1 ml/min until 5 column volumes were flushed though. The trace below was obtained during the cleaning procedure.

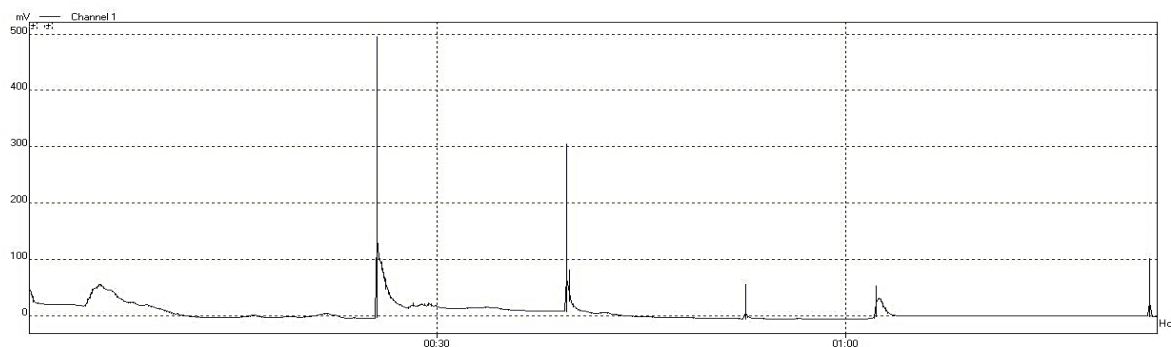


Fig 6.25 Showing the chromatogram obtained during the wash.

In order to test the column. A sample with 50 mg extract was injected at a at flow rate of 10 ml/minute in a 1 m sample loop. The trace in fig 6.27 below was obtained.

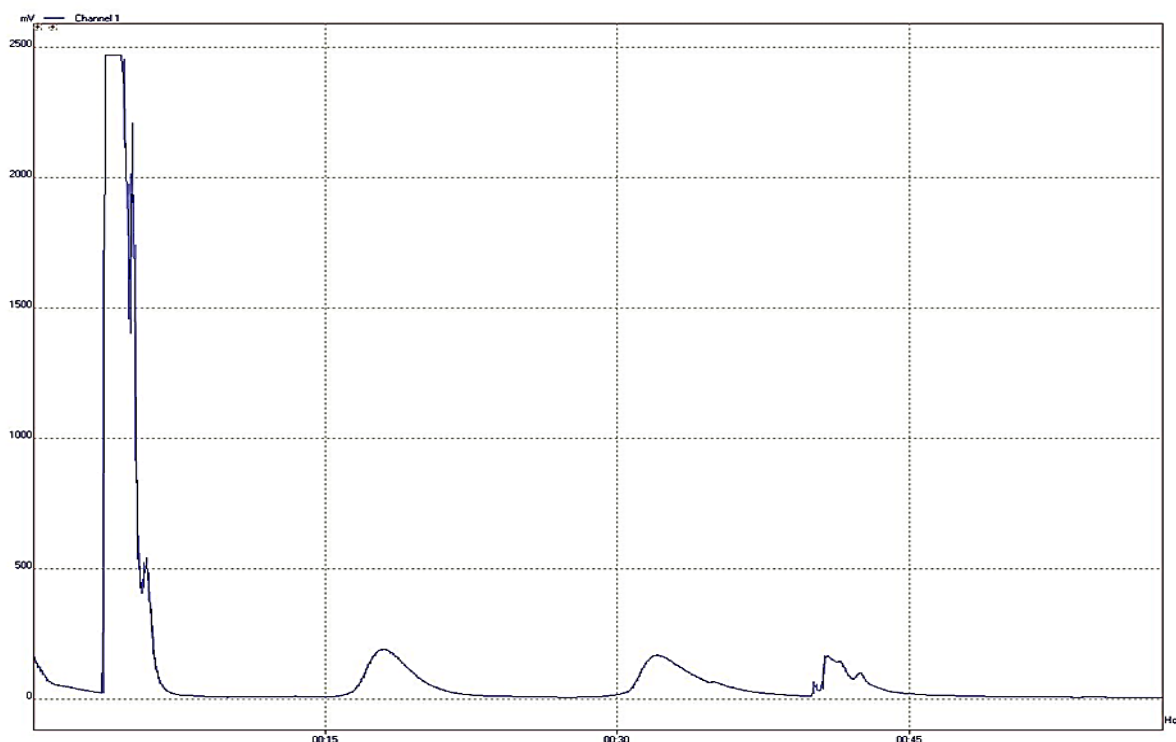


Fig 6.26 30/70 acetonitrile water run after cleaning: Showing a 30/70 acetonitrile/water run after column cleaning.

Based on the chromatogram in fig 6.27 above, it seems the 100% acetonitrile flush did not restore the trace to expectations. Hence, a column regeneration procedure was performed to regenerate the column.

6.7.4.2 Regeneration procedure for reversed-phase columns

The column was disconnected and reconnected to the the pump with the flow in the the reverse direction. The buffers were flushed out using HPLC grade water. 25 mL of water was pumped though the column at 1 mL/min. The column was thereafter flushed with 25 ml of isopropanol, 25 ml of methylene chloride, 25 ml of hexane, 25 ml of methylene chloride again and finally with 25 ml of isopropanol in the order mentioned. The column was reconnected back to the pump in the proper direction. Then it was flushed with the mobile phase without the buffer, and finally the buffer was re-introduced. The column was equilibrated with 50 ml of mobile phase. A sample was injected to perform a normal run

and confirm if column was restored. The column was disconnected from the detector the restoration procedure to protect the detector from contaminants.

The column was restored and collection continued. The trace below was obtained.

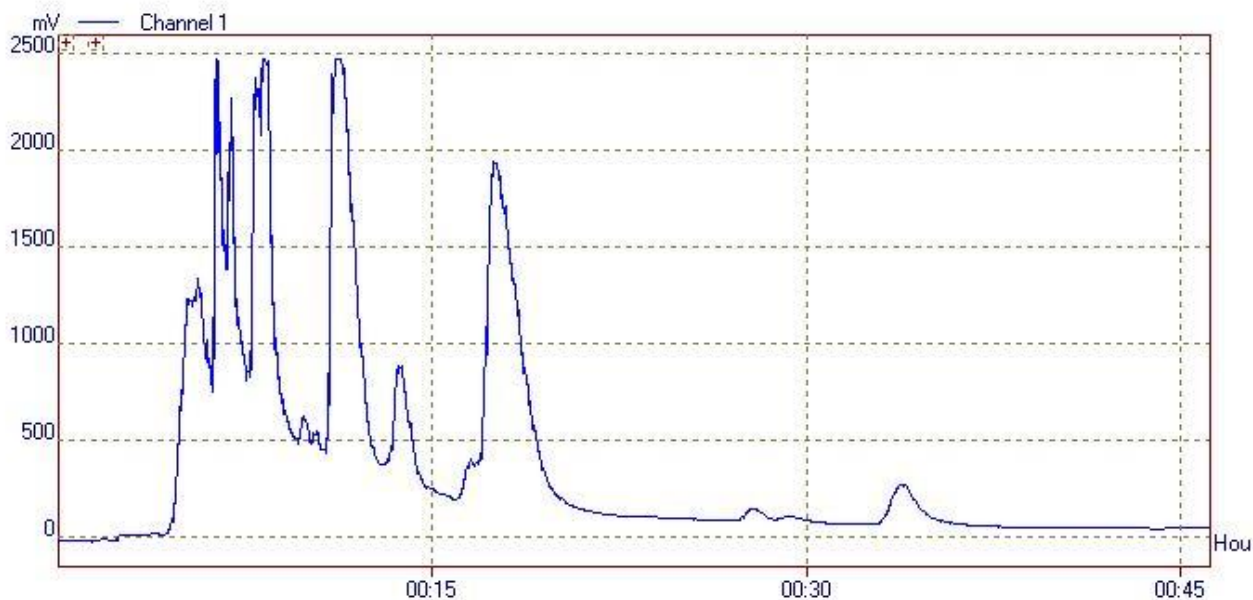


Fig 6.27 Restored chromatogram: Showing chromatogram after column regeneration

Runs were continued until enough was collected to dry in a rotary evaporator as described previously. The dried fractions were weighed and the weights are labelled in the chromatogram below.

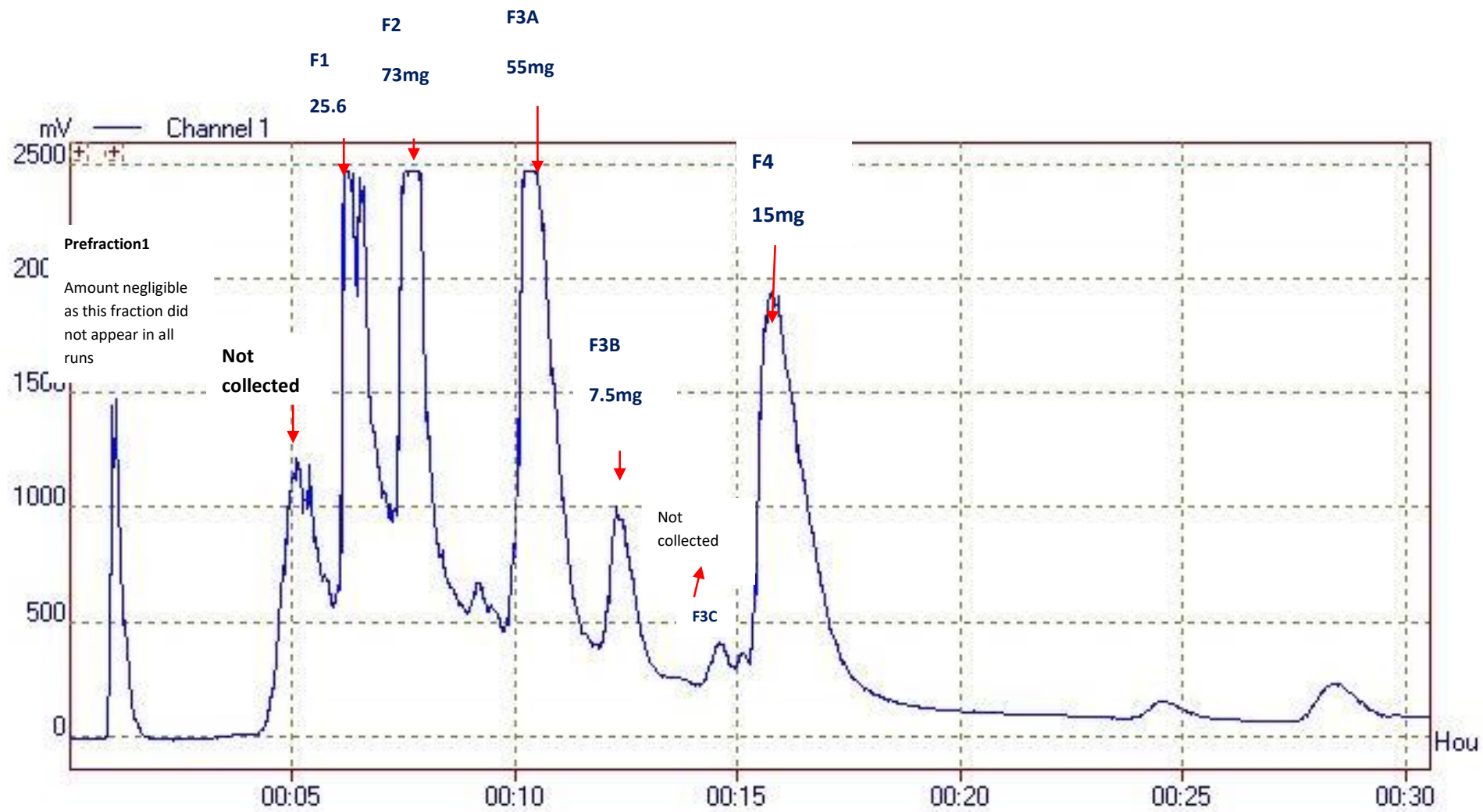


Fig 6.28 Fractions collected: Showing the fractions collected and total amount in milligrams after evaporating to dryness.

6.8 Bioassay of collected fractions:

The collected fractions were made into stock stock solutions of 5 mg/ml. The fractions were prepared for the SG-Microplate assay as previously described in section 2.2.5. Briefly, *P. falciparum* cultures were challenged with 20 µg/ml of each fraction. Control cultures with no drug was also prepared as well as a positive control group of cultures challenged with 20 µg/ml methanolic *B. ferruginea*. The results of this screening procedure are represented in fig 6.29. The results of this assays shows a near 50% inhibition by methanolic *B. ferruginea*. This was performed to validate the assay as well as compare the crude extracts with individual fractions. Based on these results, it can be seen that the antimalarial activity is distributed across all the fractions with the highest inhibition observed in fraction 1 and fraction 3B. Increasing the dose of the methanolic *B. ferruginea* and all the other fractions gave an even higher inhibition as can be seen in fig 6.30 This shows that the fractions exert their antimalarial activity in a dose-dependent manner. The highest inhibition was observed with fractions F1 and 3B in the higher dose levels.

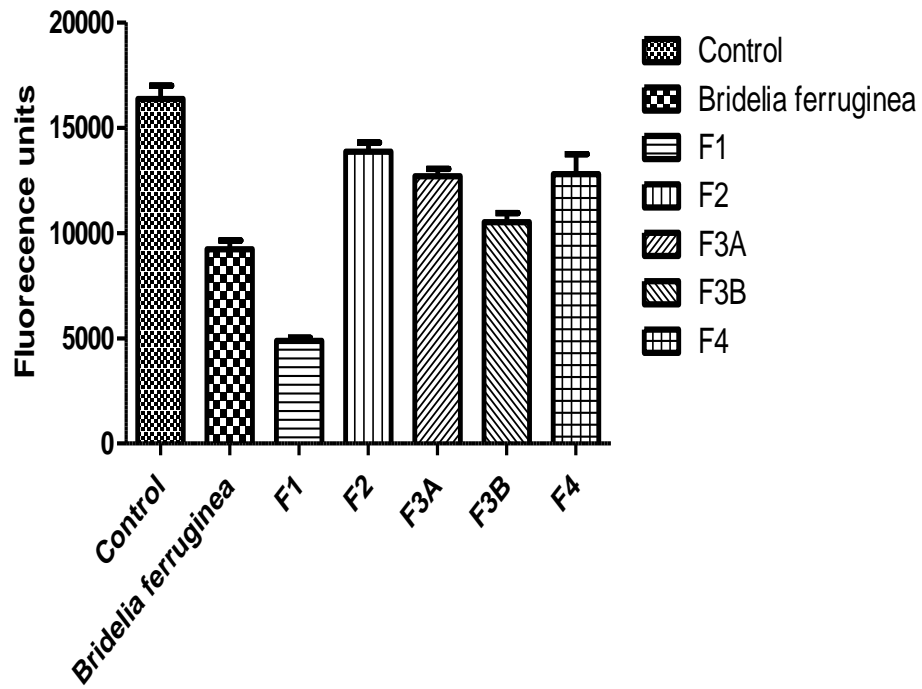


Fig 6.29 Bioassay of fractions 20µg/ml: SG-Microplate assay showing the effect of 20µg/ml fractions or *B. ferruginea* on *P. falciparum* culture.

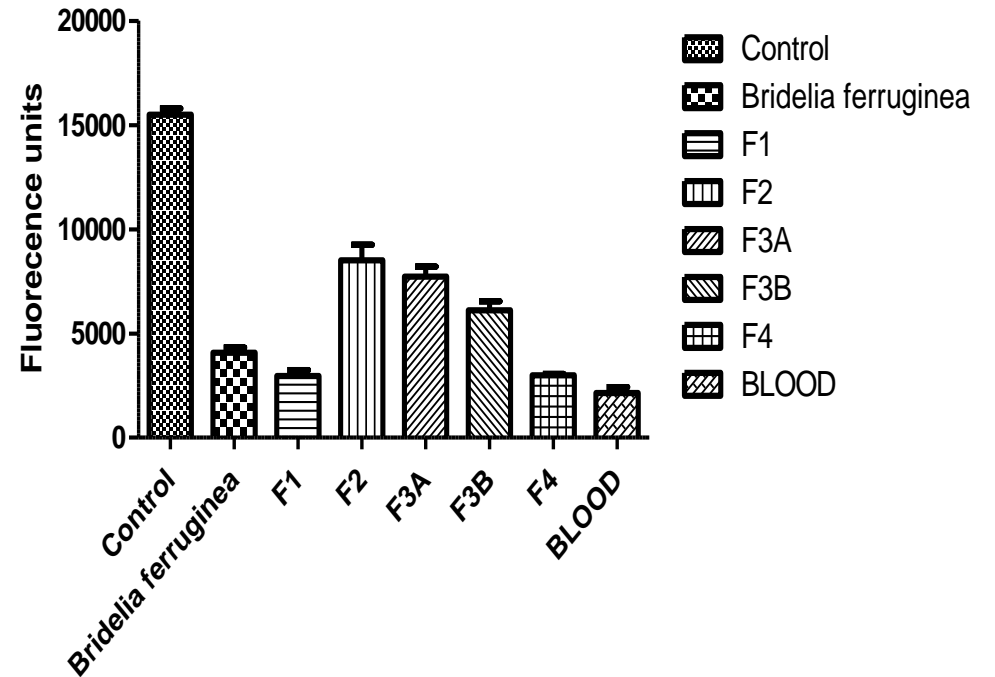


Fig 6.30 Bioassay of fractions 40µg/ml: SG-Microplate assay showing the effect of 40µg/ml fractions or *B. ferruginea* on *P. falciparum* culture.

Tukey's Multiple Comparison Test	Mean Diff.	Significant? P < 0.05?	Summary
Control vs Bridelia ferruginea	11418	Yes	***
Control vs F1	12524	Yes	***
Control vs F2	6976	Yes	***
Control vs F3A	7759	Yes	***
Control vs F3B	9387	Yes	***
Control vs F4	12493	Yes	***
Control vs BLOOD	13342	Yes	***
Bridelia ferruginea vs F1	1106	No	ns
Bridelia ferruginea vs F2	-4442	Yes	***
Bridelia ferruginea vs F3A	-3659	Yes	***
Bridelia ferruginea vs F3B	-2031	Yes	*
Bridelia ferruginea vs F4	1075	No	ns
Bridelia ferruginea vs BLOOD	1924	No	ns
F1 vs F2	-5548	Yes	***
F1 vs F3A	-4765	Yes	***
F1 vs F3B	-3137	Yes	**
F1 vs F4	-30.67	No	ns
F1 vs BLOOD	817.7	No	ns
F2 vs F3A	783	No	ns
F2 vs F3B	2411	Yes	*
F2 vs F4	5517	Yes	***
F2 vs BLOOD	6365	Yes	***
F3A vs F3B	1628	No	ns
F3A vs F4	4734	Yes	***
F3A vs BLOOD	5582	Yes	***
F3B vs F4	3106	Yes	**
F3B vs BLOOD	3955	Yes	***
F4 vs BLOOD	848.3	No	ns

Table 6.3 Statistical analysis for fractions data: Showing statistical analysis of the data represented in Fig 6.30. The results were analysed using One-way analysis of variance and a tukey post test. The results showed that there is a significant difference between control and all *B. ferruginea* fractions. There was however no significant difference between *B. ferruginea* and F1 and F4 with $P > 0.05$. There was a significant difference between *B. ferruginea* and F2, F3A, F3B with $P < 0.05$. There was a significant difference

between fractions for example F1 Vs F2; F1 Vs F3A and F3B. There was no significant difference between some fractions however for example F1 vs F4, F2 Vs F4, F3A vs F3B.

6.9 Chapter conclusion

HPLC separation conditions were optimized in order to achieve separation using different solvent systems. The solvent systems were chosen based on the results of the reverse phase TLC. The methanol/water and acetonitrile water gave the best separation in the TLC plates. These solvent systems were hence chosen to optimize the HPLC separation of the extract using an analytical column. The results obtained from the analytical HPLC were used to scale up the technique using a larger preparative column. After several runs of methanolic/water solvent system fractionation, good resolved peaks were obtained as seen in this chapter. Evaporating the fractions to dryness however produced a negligible amount of fractions except for fraction 1. About 10 mg was obtained from fraction 1 but the other fractions could not be collected. Based on this, acetonitrile/water solvent system was used as an alternative. The results were a very good separation with reasonable quantity of all fractions collected, with up to 73 mg collected in some fractions as can be seen in Fig 6.28. Bioassay of the fractions has produced a good dose-dependent antimalarial activity in *P. falciparum* K1 cultures using the SG-Microplate assay. The antimalarial activity was spread across all fractions, hence isolating a single active compound would have been both tedious and most likely not offered an advantage. The results of this chapter hence make a very good case for *B. ferruginea* to be put forward for further optimization into a phytopharmaceutical drugs for malaria.

CHAPTER 7

7.1 General discussion

As with most developing countries, plant-derived natural products constitute a significant source of medicine for the majority of the population in Nigeria. In this study, we evaluated the *in vitro* and *in vivo* antiplasmodial activity of some commonly used plant extracts for malaria. Different assays were optimised for use in the antiplasmodial screen. Fluorescent-based assays including SG-Microplate and SG-FCM assay were validated against the gold standard Giemsa light microscopy. Based on the results obtained from this study, all the extracts showed activity against the K1 (multidrug-resistant) *P. falciparum* strain.

Recent evidence has shown that malarial parasite viability can be detected using DNA stains like SYBR green I (Karl *et al.*, 2009; Johnson *et al.*, 2007 and Rason *et al.*, 2008). The SG-FCM methodology had the additional advantage of being able to differentiate between the uni and multinucleated stages of the erythrocytic phases of the parasite. The SG-Microplate assay could not detect individual parasitic stages. Another major limitation of the SG-Microplate assay is that the actual parasitaemia of the culture could not be established. The activity of the extract/ drug could only be detected by calculating the percentage inhibition relative to control (untreated) cultures. The optimisation assay further established other issues with regards to the SG-Microplate assay based on background fluorescence. It was discovered that blood and complete media increased background fluorescence hence interfering with the reading. A high background fluorescence meant that drug inhibitions at certain low doses could be masked. Upon comparing complete media with wash media, it was established that high fluorescent background was indeed due to added albumin in the complete media. This was a novel, previously unreported finding (Rason *et al.*, 2008). Moreover, the SG-Microplate assay performed in this study omitted the erythrocyte lysis step previously reported in other studies. It was found that the assay could still be performed without this step hence, simplifying the procedure.

Previous studies have used a 72 h treatment cycle owing to the fact that the parasite life cycle is 72h, spanning from ring, to trophozoite and finally schizont stages. Previous studies by Wein *et al.*, 2010 have demonstrated a 72 h treatment period in order to obtain reliable results with SG assays. In this study, treatment was established using a 48h cycle starting at trophozoite stage. The extract showed activity when treatment was initiated both at ring

stage and at trophozoite stage. It may however still be more preferable to treat for 72 h for drugs with unknown action, starting with the ring stage and analysing at the trophozoite stage. This also covers for drugs that act selectively on specific stages of the erythrocytic cycle as well as slow acting drugs.

7.2 Validation of the ethnopharmacological properties of selected plants in Nigeria.

Five plants namely, *Artemisia maciverae*, *Bridelia ferruginea*, *Bryoscarpa coccineus*, *Bombax buonopozense*, *Bosewellia dalzellii* and a NIPRD coded extract were evaluated for their antimalarial claims. The results obtained in this study supports the traditional usage of these plants for malaria hence validating their ethnopharmacological usage for malaria. *B. ferruginea* and *B. coccineus* were chosen as candidates to take forward for the scope of this study for reasons previously discussed. One of the main concerns associated with the use of unregulated herbal/traditional medicines is the issue of appropriate dose and drug-induced toxicity. A dose response analysis of both extracts gave IC₅₀ values of 15 µg/ml and 72 µg/ml for *B. ferruginea* and *B. coccineus* respectively. Rasoanaivo *et al.*, 2004, suggested that extracts with IC₅₀ greater than 50 µg/ml should be considered inactive, hence *B. coccineus* was excluded from the study after its IC₅₀ had been established.

Phytochemical analysis of the aqueous crude extract of *B. ferruginea* extract revealed the presence of tannins, terpenes, flavonoids, phenols, cardiac glycosides and sterols. Flavonoids have been previously implicated in malaria. Studies by Lehane *et al.*, 2008 revealed 11 dietary flavonoids to be active against certain *P. falciparum* malaria strains including the CQ-sensitive (3D7). Flavonoids are a common constituent in several medicinal plants but their exact molecular mechanism of action have not yet been elucidated. It is believed that inhibition of fatty acid biosynthesis in the parasite is one of the possible mechanism (Freundlich *et al.*, 2005, Elford, 1986). Others groups have shown flavonoids to inhibit the influx of L-glutamine and myoinositol into infected erythrocytes (Yenesew *et al.*, 2003). Flavonoids come in different forms including chalcones, isoflavones and retinoids. These have been previously implicated in malaria (Yeneswe *et al.*, 2003; Midiwo *et al.*, 2007; Yeneswe *et al.*, 2004). Phenolics, which were also present have shown activity against malaria parasites in various *in vitro* studies and been used in combination with other antimalarial compounds including artemether to demonstrate synergy (Pimenta *et al.*, 2014). As methanolic plant extracts tend to be rich in flavonoids, phytochemical analysis of the methanolic extract was performed. Interestingly, the results revealed the

presence of the same compounds including anthraquinones which were absent in the aqueous extract. Anthraquinones extracted from the bark of some medicinal plants have been shown to possess moderate antiplasmodial activity against certain *P. falciparum* strains (Hou *et al.*, 2003). Based on this information, the methanolic bark extract of *B. ferruginea* was investigated for its antiplasmodial activity using the fluorescent-based assays and Giemsa light microscopy. This allowed for the comparative analysis of the aqueous and methanolic extract. The methanolic extract was more efficacious than the aqueous extract with an IC₅₀ value of 20 µg/ml. The higher antiplasmodial activity seen in the methanolic extract could be owing to the presence of anthraquinones, which were absent in the aqueous extract. The methanolic extract was hence used in all further experiments. Moreover, the phytochemical tests can be used as markers for quality control and standardization when a phytopharmaceutical drugs is developed and also for argonomic selection of the best plants. (Patel *et al.*, 2012, Pandey and Tripathi, 2014; Lapornik *et al.*, 2005)

One major drawback associated with using natural products as a source of medicine is the seasonal variability that may occur with phytochemical constituents within the plant. In this study, the plant was collected from two regions in Nigeria. Seasonal variations have been demonstrated in phytochemical properties of certain bulbous plants in South Africa (Ncube *et al.*, 2011). Certain phytochemicals, including phenols were shown to be higher in spring compared to the other seasons. The *B. ferruginea* plant in this study was collected in June and November. There was no variation observed in the IC₅₀ values obtained in the extracts collected between the two seasons. The phytochemical analysis performed in this study supports this finding. The phytochemical analysis in this study was qualitative rather than quantitative. The comparative analysis antiplasmodial study between the aqueous and the methanolic *B. ferruginea* was used to bridge this gap.

7.3 Investigating the mechanism of action of methanolic *B. ferruginea* extract

The malaria haemozoin pathway plays an important role as an antimalarial target. Malaria parasites produces large quantities of inert haemozoin though a highly efficient mechanism the detoxifies lethal haemoglobin degradation products (Roth *et al.*, 1986). Several pathways have been described for the degradation of haem (Tekwani *et al.*, 2005). The most critical pathway and predominant mechanism is the sequestration of haem into haemozoin. This is also called the biocrystallisation pathway in which, haem is incorporated into a crystalline black pigment, accumulated within the parasite food vacuole (Saliba *et al.*, 1986).

The biocrystallisation pathway is unique to the *Plasmodium*. This selectivity makes it an important antimalarial target. Several studies have been performed to gain further insights into the actions of antimalarials on this pathway. Studies on this pathway have shown haemozoin synthesis to be inhibited by chloroquine in both *P. falciparum* and *P. berghei* species of the parasite (Chou *et al.*, 1993; Chou *et al.*, 1992; Orjih and Fitch, 1993). The haemozoin molecule has been studied by several groups over the years. One major finding that has aided the study of this molecule and the mechanisms involved in pigment formation is the similarity between the haemozoin and β -haematin molecules revealed by X-ray crystallography studies (Slater *et al.*, 1991; Pagola *et al.*, 2001). Investigators have previously taken advantage of this similarity between the two molecules to study the effect of extracts/drugs on β -haematin formation. Screening of 178 plants extracts from the pharmacopeia of Bolivia ethnia Tacana using the β -haematin formation assay revealed promising *in vitro* antimalarial activity of several extracts (Baemans *et al.*, 2000). In this current study, we take the advantage of this similarity to investigate the effect of methanolic *B. ferruginea* extract on β -haematin formation. The inhibition of the β -haematin formation assay takes advantage of the differential solubility between haem and β -haematin in NaOH. Different assays were used to optimise β -haematin formation as described in Chapter 4. The results demonstrated that for chloroquine, inhibition was only achieved at doses above 1 mM whilst for *B. ferruginea*, at doses from 2 mg/ml. These doses are no doubt lethal to the human body as explained previously in Chapter 4. Physiologically, the drugs enter the parasite food vacuole and accumulates to a high dose, this has been demonstrated by CQ. CQ enters the parasite food vacuole through simple diffusion. Once it enters, the drug is protonated due to the acidic pH (4.7), which prevents it from diffusing out. It may be recalled that in this study, the experiment only worked at pH ranging from 4.6-4.9, in line with the physiological pH of the food vacuole. This build up of chloroquine in the food vacuole relative to the rest of the parasite prevents further biocrystallisation of haem leading to a haem build up which is toxic to the parasite (Ehlgen *et al.*, 2012). Unfortunately, the assays currently available cannot model the concentration effect in the food vacuole and hence require higher than physiological doses of the drug. They are however, useful surrogates to investigate the parasite haemozoin pathway.

7.4 HPLC analysis of methanolic *B. ferruginea*.

Chromatographic analysis of the extract in this study demonstrated the presence of several compounds as revealed by the presence of multiple, well resolved peaks. This study

optimized HPLC conditions for the extract using a suitable detection technique, the UV detection method. The main objective of the HPLC was to contribute towards standardization procedures in the development of the phytopharmaceutical drugs. The collected fractions were tested and all fractions demonstrated antimalarial activity, albeit some higher than others. Isolating an active compound was not pursued for two reasons; firstly, the antimalarial activity appeared in many fractions with evidence to suggest that a collective advantageous, synergistic trait contributed to the overall effectivity of the whole extracts and secondly the main aim of this study was to work towards developing a wholesome phytopharmaceutical drugs for malaria. For a drug to be classed as a phytomedicine, it must contain purely natural products. Additionally, the work supports the argument that phytomedicines as crude compounds might offer a better alternative for the treatment of malaria due to previous history of resistance that has developed with known antimalarials when they were dispensed as purified active ingredients. In addition to impeding the development of drug resistance, maintaining the extract in its crude form might offer additive and synergistic benefits that might be lost when a single active compound is isolated (Wilcox *et al.*, 2011). Based on the results obtained in this study, it could be argued that this is the case with *B. ferruginea* as antiplasmodial activity was demonstrated in different broad fraction collections obtained from the HPLC analysis. The HPLC could prove invaluable in standardization procedure of the extract for commercialisation.

7.5 *In vivo* activity of methanolic *B. ferruginea*

In vivo antimalarial activity of *B. ferruginea* confirmed a number of its pharmacological properties in mice. The extract showed significant antimalarial, analgesic and antipyretic activity. Compounds sometimes show activity *in vitro* but lose activity when tested *in vivo*, due to factors like lack of absorption and other pharmacokinetic related issues. The fact that this extract demonstrated good antimalarial activity both *in vivo* and *in vitro* makes a very good antimalarial candidate as it is unlikely that pharmacokinetic issues will be encountered in clinical trials.

Hence the results of this studies have successfully demonstrated that *B. ferruginea* as a very good candidate to put forward for clinical trials and it is likely a good phytopharmaceutical drugs can be produced in the near future for malaria from it. Its usage as an antimalarial traditional medicine is hence validated.

7.6 Future directions

The results of this study revealed a strong antimalarial activity of six different extracts that are used for malaria treatment in West Africa. Various *in vitro* and *in vivo* techniques were used to ascertain the antimalarial claim. The *in vitro* work focused on the K1 resistant *P. falciparum* strain. Future studies could investigate the activity of the extract on other malaria strains. The phytochemical studies in this study was qualitative. The results revealed the presence of various phytochemicals in both the aqueous and methanolic extracts of *Bridelia ferruginea*. Future work could further investigate the quantitative phytochemical presence of the various compounds found. This is likely to give a better insight into why the methanolic extract of *B. ferruginea* was significantly more efficacious than the aqueous extract in both *in vitro* and *in vivo* assessments. As stated earlier, flavones and terpenes have been implicated in malaria. These compounds were revealed in both the aqueous and methanolic extract. It has however been shown that methanolic extraction technique tends to extract more flavonoids and terpenoids than aqueous extraction methods (Ncube *et al.*, 2008) Hence it is very likely that the amount of these two compounds are higher in the methanolic extract than the aqueous extract, hence the higher antimalarial activity observed.

B. ferruginea has long term use within communities for various purposes, hence it's safety is established to a certain extent. Moreover, this study evaluated the *in vivo* acute toxicity profile of the methanolic *B. ferruginea* extract and found it safe to use up to a dose of 5000 mg/kg. *In vivo* sub-chronic and chronic toxicity studies were however beyond the scope of this study. Pursuing these investigations will prove invaluable in establishing and documenting an improved toxicity profile for the extracts. Once this is established, clinical trials could commence towards the development of a phytopharmaceutical drugs.

In addition one of the fractions showed strong antimalarial properties. These could be taken further in order to isolate the active compound which could be a new lead for antimalarial drug development.

Finally, the HPLC could be use to determine fingerprints for the extract as a quality control tool in further development of the extract as a phytothraupeutic drug.

CHAPTER 8

References

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