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IN VITRO THERAPEUTIC VALUE OF PLANT EXTRACTS USED IN BURKINA FASO TO TREAT MALARIA AND PHARMACODYNAMIC STUDIES OF ANTIMALARIAL DRUGS

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ABSTRACT

My PhD project has developed in two main research areas, both related to antimalarial drug discovery. The first part was focused on the study of the antimalarial and anti-inflammatory activity of medicinal plants used in Burkina Faso to treat malaria; the second part was dedicated to the development of new tools to measure in vitro the pharmacodynamic characteristics of current or new drugs to predict early on their clinical behavior. The work was conducted in the Laboratory of Parasitology, Department of Public Health-Microbiology -Virology of the University of Milan and at the Liverpool School of Tropical Medicine, Liverpool, UK.

The antiplasmodial activity, the inhibition of cytokine production, and the antiproliferative activities of three different plants, *Canthium henriquesianum* (K. Schum), *Gardenia sokotensis* (Hutch) and *Vernonia colorata* (Willd) were investigated. Crude and organic plant extracts were tested in vitro for antimalarial activity against chloroquine susceptible (D10) and resistant (W2) strains of *P. falciparum*. Cell cytotoxicity was assessed on Human Dermal Fibroblast (HDF) and melanoma cells by the MTT assay. The selectivity index (SI) was used as the ratio of the activity against the parasites compared to the toxicity of the plant extract against HDF. *C. henriquesianum* aqueous extract had a moderate antimalarial activity ($IC_{50}<50\mu g/ml$) with a good selectivity index ($SI_{(HDF/D10)}>7$). *C. henriquesianum* diisopropyl ether extract was the most potent inhibitor of parasite growth with an IC_{50} 9.5 µg/ml on W2 and 8.8 µg/ml on D10 and limited toxicity ($SI_{(HDF/D10)}<2$). On the contrary, *G. sokotensis* and *V. colorata* aqueous extracts were shown to be weakly active on the plasmodium parasites ($IC_{50}\geq 50\mu g/ml$) with limited toxicity. In addition, when the production of IL-1β and TNFα by human THP1 monocyte was assayed by ELISA, it was found that the extract of *C. henriquesianum* induced a dose-dependent inhibition of IL-1β, but not TNFα production, thus confirming its traditional use as antipyretic. By NMR analysis, we identified the chromone as the compound mostly represented in the diisopropyl ether extract of *C. henriquesianum*. Chromone however was less active as antimalarial than the crude extracts and it did not inhibit cytokine production at not toxic doses, indicating that other molecules in the total extracts contribute to the anti-inflammatory activity. In conclusion, out of the three plants examined, only *C. henriquesianum* seems to possess antimalarial activity in vitro and the capacity to inhibit pyrogenic cytokine production. *Gardenia sokotensis* extract showed moderate cytotoxic activity on melanoma cell line and it could be further investigated for its antitumor activity.

The second part was related to the in vitro study with the objective to develop new tools to measure in vitro the pharmacodynamic features of different antimalarials. Three type of assays were used: dose response curves to measure IC50 and IC90 variables and calculate the slope of the curve; time to kill kinetics using SYBR Green I-based fluorescence (MSF) assay and high content in vitro assays using the Perkin Elmer Operetta High Content Screening microscope to measure the stage specificity of antimalarial action. These tests were first set up with current antimalarials, but the intent is to use them to prioritize the development of new drugs. The results of the dose–response curves confirmed that DHA, atovaquone and CQ are more potent antimalarials compared to pyrimethamine., with a IC50 less than 4 nM for DHA and atovaquone; and 10 nM for CQ. The slope factor, which represents the steepness of the curve, appears to be characteristic of drug class and was >1 for all the drugs suggesting that binding does not follow the law of mass action with a single site and the ligand binds cooperatively to a multivalent receptor. Time to kill studies indicated that DHA has a noticeable effect before 5h as well as CQ and the parasites are irreversibly inhibited after 12hrs of exposure to the drug. The results from the High Content Screening showed that for all the compounds, at the highest dose, the surviving parasites after 48 h are 80-90% young forms and only10-20% mature parasites. No major changes are seen in the distribution of young vs mature forms at doses lower than the IC50. However, at higher doses the percentage of young is increasing and in parallel the percentage of mature forms is decreasing. This indicates that a small number of parasites was able to complete the cycle and re-invade new RBC.The Operetta technique seems to be able to quickly assess the potency as well as the stage specificity of a drug. It should be able to predict the parasite reduction ratios (PRR) without the need to perform the time to kill assays which are more laborious, time consuming and less adaptable to high throughput screenings (HTS). The compounds that inhibit the ring stage would be predicted to have faster time-kill profiles and would be prioritized for further studies in the process of antimalarial drug development.

Keywords: *Canthium henriquesianum, Gardenia sokotensis, Vernonia colorata, m*alaria, antiplasmodial activity, selectivity index, immunomodulatory activity, cytokine human IL-1β, cytokine TNFa, chromone, slope factor, time to kill-curves, High Content Screening.

RIASSUNTO

Il presente progetto di dottorato è stato svolto in due principali aree di ricerca, entrambe relative alla scoperta e sviluppo di farmaci antimalarici.

La prima parte della ricerca ha riguardatolo studio dell'attività antimalarica e anti-infiammatoria di alcune piante medicinali utilizzate in Burkina Faso per trattare la malaria; la seconda parte invece è stata dedicata allo sviluppo di nuove metodologie per misurare in vitro le caratteristiche farmacodinamiche di farmaci nuovi o già in uso, in modo da prevedere già in fase iniziale di sviluppo, il loro comportamento clinico. Il lavoro è stato condotto nel laboratorio di Parassitologia, Dipartimento di Sanità Pubblica- Microbiologia-Virologia dell'Università degli Studi di Milano e nella Liverpool School of Tropical Medicine a Liverpool, UK.

Sono state investigate l'attività antimalarica, l'inibizione della produzione di citochine e l'attività antiproliferativa di tre diverse piante comunemente utilizzate dalla popolazione del Burkina Faso contro la malaria: *Canthium henriquesianum* (K. Schum), *Gardenia sokotensis* (Hutch) e *Vernonia colorata* (Willd).

L'attività antimalarica di estratti crudi ed organici di queste piante è stata saggiata in vitro contro ceppi di *P. falciparum* sensibili (D10) e resistenti (W2) alla clorochina. E' stata testata anche la citotossicità utilizzando due modelli cellulari umani, fibroblasti primari dermici (HDF) e melanoma, attraverso il saggio MTT.

Per ogni estratto è stato calcolato l'indice di selettività (SI), come il rapporto dell'attività antimalarica sui parassiti rispetto alla tossicità su HDF. L'estratto acquoso di *C. Henriquesianum* ha dimostrato una moderata attività antimalarica ($IC_{50} < 50 \mu g/ml$) con un buon SI (HDF/D10 > 7). L'estratto ottenuto con diisopropil etere da *C. henriquesianum* ha dimostrato la migliore attività antimalarica sia su W2 (IC_{50} 9.5 µg/ml) che su D10 (8.8 µg/ml) con una tossicità limitata (SI HDF/D10 < 2).

Gli estratti acquosi di *G. sokotensis* e *V. colorata* hanno mostrato di avere una modesta attività antimalarica ($IC_{50} \geq 50 \mu g/ml$), e limitata tossicità. Inoltre, quando è stata valutata la produzione di IL-1β e di TNFα da una linea di monociti umani (THP-1), gli estratti di *C. henriquesianum* hanno provocato una inibizione dose-dipendente di IL-1β, ma non di TNFα, confermando il suo utilizzo tradizionale come antipiretico. Attraverso l'analisi NMR, abbiamo identificato il cromone come il composto principalmente rappresentato nell'estratto ottenuto con etere di *C. henriquesianum*. Tuttavia il cromone ha mostrato una attività minore in confronto a quella degli estratti crudi e non ha inibito la produzione di citochine a dosi non tossiche, indicando quindi che altre molecole presenti negli estratti totali contribuiscono all' attività antimalarica.

In conclusione, tra le tre piante studiate, *C. henriquesianum* è quella che ha mostrato avere sia attività antimalarica in vitro che la capacità di inibire la produzione di citochine. Gli estratti di *Gardenia sokotensis* hanno mostrato una moderata attività citotossica sulla linea di melanoma, che merita di essere ulteriormente approfondita.

La seconda parte di questa tesi ha avuto come obbiettivo la messa a punto di nuove metodologie per valutare *in vitro* le caratteristiche famacodinamiche di alcuni farmaci antimalarici. Sono stati utilizzati tre diversi tipi di test: curve di crescita dose-risposta per misurare IC50 e IC90 e calcolare la pendenza della curva; studi di cinetica utilizzando una metodologia fluorescente basata sul SYBR Green (MSF) e una metodica che utilizza lo strumento Perkin Elmer Operetta High Content Screening Microscope per valutare lo stadio di crescita del parassita sul quale agisce il farmaco. Queste metodiche sono stati inizialmente messe a punto con antimalarici in uso, ma con l'intenzione di utilizzarle in seguito per lo sviluppo di nuovi farmaci. I risultati delle curve dose-risposta, hanno confermato che DHA, atovaquone e, CQ sono farmaci antimalarici più potenti rispetto alla pirimetamina, con un'IC50 inferiore a 4 nM per atovaquone e DHA; e 10nM per la CQ. La pendenza della curva risulta essere caratteristica per ogni classe di farmaci e risulta essere <1 per tutti i farmaci ad indicare che il legame non avviene ad un singolo sito ma in maniera cooperativa ad un recettore multivalente. Gli studi di cinetica indicano che DHA ha un'attività notevole prima delle 5h, così come CQ e che la crescita dei parassiti è inibita in maniera irreversibile dopo 12h di esposizione al farmaco. I risultati ottenuti con l'High Content Screening hanno dimostrato che i parassiti sopravvissuti alla dose più alta di tutti i farmaci dopo 48h sono per l'80-90% forme giovani e solo per il 10-20% parassiti maturi. Non si sono osservati cambiamenti nella distribuzione di forme giovani e mature a dosi inferiori rispetto all'IC50. Tuttavia alle dosi più alte la percentuale di forme giovani aumenta parallelamente alla diminuzione della percentuale delle forme mature. Questo indica che solo un numero esiguo di parassiti è stato in grado di completare il ciclo e reinvadere nuovi globuli rossi. La metodica "Operetta" sembra essere in grado di stimare velocemente sia la potenza che lo stadio su cui è attivo un farmaco. Sembra essere in grado di predire la riduzione della parasitemia senza la necessità di eseguire test di cinetica che sono molto laboriosi, richiedono tempo e sono meno adattabili ad uno screening ad elevata prestazione (HTS). I composti che inibiscono lo stadio ad anello sembrano avere una rapidità d'azione superiore e quindi questo è un utile criterio di scelta per selezionare un farmaco più promettente.

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PART I: IN VITRO THERAPEUTIC VALUE OF PLANT EXTRACTS USED IN BURKINA FASO TO TREAT MALARIA

1   INTRODUCTION TO THE MALARIA PROBLEM

1.5 Malaria Overview

Infectious agent-viruses, bacteria and parasites, cause the largest burden of disease worldwide, of which the top five are HIV/AIDS, diarrhea, tuberculosis, the childhood clusters (measles, pertussis, tetanus, diphtheria and poliomyelitis) and malaria [*1*]. Malaria (from the Italian expression mal'aria) is the most important eukaryotic parasitic disease, threatening the livelihood of over 2.2 billion people. There are four main species of malaria occurring in humans, *Plasmodium falciparum (P. falciparum), P. malariae, P. ovale* and *P. vivax*. All four species are transmitted by mosquitoes of the genus Anopheles, of which exist over 400 species [2]. A fifth specie, *P. knowlesi* is infact a zoonosis which infects people leaning at the forest border [3]. *P. vivax* which is widespread in Central and South America, Asia and Oceania, rarely leads to a lethal infection. It can exist latent inside hepatocytes, re-emerging after many months to several years. *P. vivax* causes morbidity, is an important cause of low birth weight in pregnancy but is associated with relatively severe complications. Though commonly transmitted, infections due to *P. ovale* are rare as immunity is established early and parasitaemia remains low. It is found principally in Africa causing less than 0.5% of malaria infections [4]. *P. malariae* is found worldwide but with a very patchy distribution. It causes renal complication or chronic nephropathy and if left untreated the patients may remain parasitaemic though asymptomatic for years [5].

P. falciparum is the most virulent species and causes almost all the 1.7-2.5 million deaths worldwide from malaria [6]. The clinical manifestations caused by *P. falciparum* range from a mild asymptomatic parasitaemia to severe and often fatal syndromes such as cerebral malaria, severe anaemia or multi-organ failure. In Africa, *P. falciparum* is responsible for 30-50% of hospital admissions and 50% of outpatient visits [7]. Africa presents the highest endemicity of *P. falciparum*, as shown in figure 1 . The high-risk groups include children below the age of five years, travelers, migrants from non-malarial regions moving into malarial regions, and populations with repressed immune system, including pregnant women (especially first pregnancy) [8].

Over the time, the human immune system reacts to the malaria parasite and adult mortality in endemic areas is fairly low due to acquired immunity.

Beyond the clinical issues, malaria has huge economic impact in developing countries due to the loss of productivity [9]. Other secondary factors that contribute to the burden of malaria include the socio-economic conditions and behavior of individuals [10]. Furthermore, changes in global ecosystems and weather patterns have been implicated in a resurgence of malaria in South America and Africa. The *P. falciparum* disease pattern display enormous geographical variations and as more and more studies are carried out, greater variability is uncovered. Of particular concern are the facts that the number of drug resistant *P. falciparum* field isolates is increasing at a rate that exceeds new drug development. Malaria chemotherapy has been hampered by the emergence of parasites resistance to almost all available antimalarial drugs, including recently the artemisinin derivatives [11] .
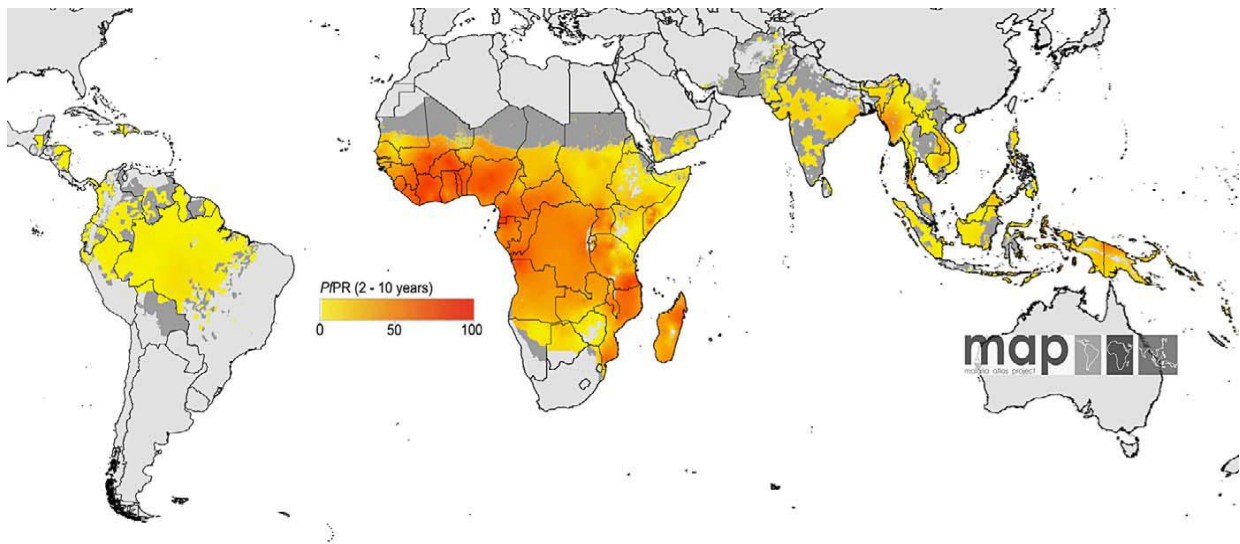
Figure 1. The spatial distribution of *P falciparum* endemicity stratified by parasite rate in people aged from 2 to 10 (PfPR$_{2-10}$) from 2007 adapted by [12]. The stratification is made according the parasite rate in people aged from 2 to 10 (PfPR$_{2-10}$). The yellow colour to red colour corresponds to 0% to 100%. The medium grey areas is defined as land areas with unstable risk and the light grey as no risk.

1.5.1 Malaria parasite and its life cycle

The life cycle of *P. falciparum* is complex (Figure 2), and can be divided into 3 stages: one is sexual (sporogonic cycle) and takes place inside the mosquito while the other two are asexual and occur inside the human host: the erythrocytic cycle (in the red blood cells) and the exoerythrocytic cycle (in the liver). Parasites are transmitted to humans in the form of sporozoites through the bite of an infected female anopheles mosquito. Sporozoites can circulate for up to 45 minutes before entering the parenchyma cells of the liver whereby they undergo development and multiplication forming a large intracellular schizont [13]. After a period ranging from 6 to 15 days, according to the species of *Plasmodium*, thousands of merozoites are released into the bloodstream, where they will adhere to and invade red blood cells initiating the erythrocytic cycle. In *P. vivax* and *P.ovale*, some sporozoites enter into a dormant stage becoming "liver hypnozoites". These forms will remain latent for weeks, some months or even years until reactivated, at which point they will complete the hepatic cycle, giving rise to the relapses characteristics of these two species.

The intraerythrocytic parasites first develop within a parasitophorous vacuole in the form of ring and then into a trophozoite, within which multiple nuclear divisions occur to form a schizont. The mature trophozoites and schizonts stages are not normally seen in the peripheral blood but are sequestered in deep vascular beds where they adhere to endothelial receptors. This cytoadherence phenomenon was first described over a century ago by Bignami and Bastianelli in brain tissue of patients who died of malaria and it is a unique feature of *P. falciparum*, not found in the other human malaria species [14]. Once mature, the schizonts cause the infected red blood cell to burst, setting free other merozoites (8 to 32) that go to invade fresh red blood cells. The destruction of the red blood cells also coincides with the release of pyrogenic substances, which trigger immune mechanisms that ends in fever and other clinical symptoms. The morbidity and mortality associated with malaria derive solely from the erythrocytic stages. The asexual erythrocytic cycle lasts 48 or 72 hours, depending on the species of *Plasmodium,* and continues until brought under control either by the body's immune response or chemotherapy. Some of the invading merozoites develop into sexual forms (gametocytes). If viable gametocytes are taken up by feeding *Anopholes*, gametes from male and female will fuse and form a zygote which then develops into a oocysts containing infective sporozoites. This process takes 10 to 20 days. The sporozoites migrate to the salivary glands ready to be injected into the human host at the next blood meal thus, completing the malaria transmission [15]. Since the female *Anopheles* mosquito requires a blood meal to mature her eggs, it will therefore have several occasions for either acquiring or transmitting malaria parasites.
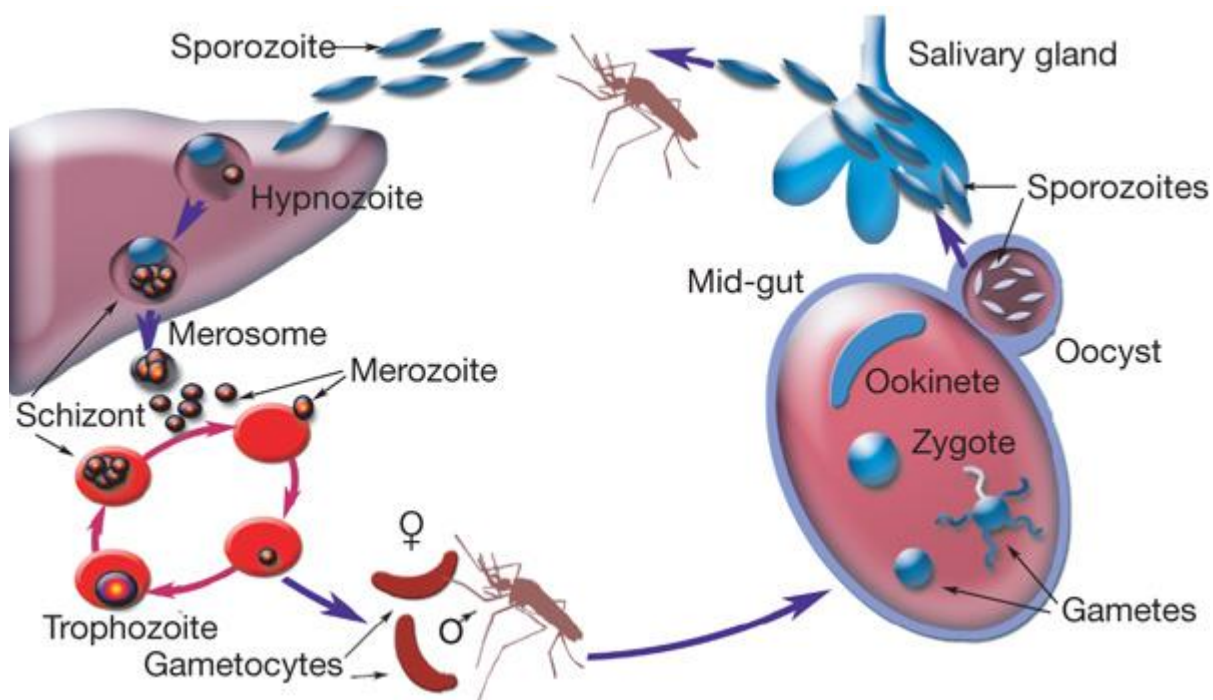
Figure 2. Diagram of the malaria parasite's life cycle. Adapted from Winzeler [16].

1.1.2 Malaria diagnosis

Currently malaria control is heavily dependent on chemotherapy, to which resistance is quickly evolving in endemic regions [17]. One of the main interventions of the global malaria control strategy for effective disease management is prompt and accurate diagnosis. In the tropics, a malaria diagnosis is carried out mainly by microscopic methods while rapid diagnostic techniques (RDTs) are available in non-endemic settings. However, the performance of these diagnostic techniques is subjected to many factors: the different forms of malaria species, the inter-relationship between parasite densities, immunity and symptoms under different levels of transmission and the use of chemoprophylaxis [18].

The phenomenon of sequestration of late stages makes detection of parasites in peripheral blood smears difficult, and this is an important limiting factor of microscopic diagnosis. Although malaria is a common cause of fever in endemic areas, it is not the only cause. Determining which fever can be attributed to malaria is greatly simplified when a parasitological diagnosis can be made. However, even when a parasitological diagnosis of malaria infection has been made in a symptomatic individual, the attribution of those symptoms to the malaria infection is not straightforward. Much depends on transmission dynamics, underlying immunity and local patterns of drug use and susceptibility [19]. A single negative blood film in a child suspected of having a malarial illness is not enough to exclude a positive diagnosis. Some parasitaemias can still cause symptoms and, the majority of parasites may be sequestered in deep tissues for 18-24 hours of the 48-hour life cycle. For this reason, several sequential blood films, repeated at 6- to 12-hourly intervals, are recommended to exclude the diagnosis of a malarial illness [20]. Furthermore a quantification of their cytoadherence or schizogony using parasite density or biochemical markers released from sequestered parasites could resolve the problems in estimating the total parasite burden in patients [21].

1.1.3 Malaria pathology

The clinical outcome of malaria infection involves many factors that include parasite factors (specie and strain of infecting parasite, parasite load, multiplication rate, cytoadherence or sequestration), the host factors (state of immunity, age, genetic constitution, general health and nutritional status) and the geographical origin of the infection and social aspects [22], [23]. Due to changes in one or several of these factors, patients will move from having an asymptomatic infection (presence of *P. falciparum* and absence of symptoms) to developing fever (symptomatic) which can advance to a severe form of the disease, and eventually death. In malaria endemic areas asymptomatic infections are a common occurrence dependent

on transmission intensity [19] and age of host which determines development of partial-immunity and protects against clinical disease [24]. In naïve individuals and, in some cases, in partially-immune hosts asymptomatic infections lead to increase in parasitaemia and develop to symptomatic infections. The characteristic symptoms of uncomplicated malaria are fever, headache, cough, rapid breathing, vomiting and diarrhea while severe malaria comprises several different overlapping syndromes, altered consciousness, respiratory distress, repeated convulsions, hypoglycaemia, acidosis, prostration and severe anemia [20]. Sequestration and cytoadherence are important elements of the pathology of all these different manifestations of malaria disease. Furthermore the malaria symptoms depend on the type of *Plasmodium* which caused the infection. *P. falciparum* takes 7 to 14 days to show any symptoms of malaria while *P. ovale* and *P. vivax* take 8 to 14 days, *P. malariae* takes 7 to 30 days. The general symptoms include headache, nausea, fever, vomiting and flu-like symptoms (figure 3).
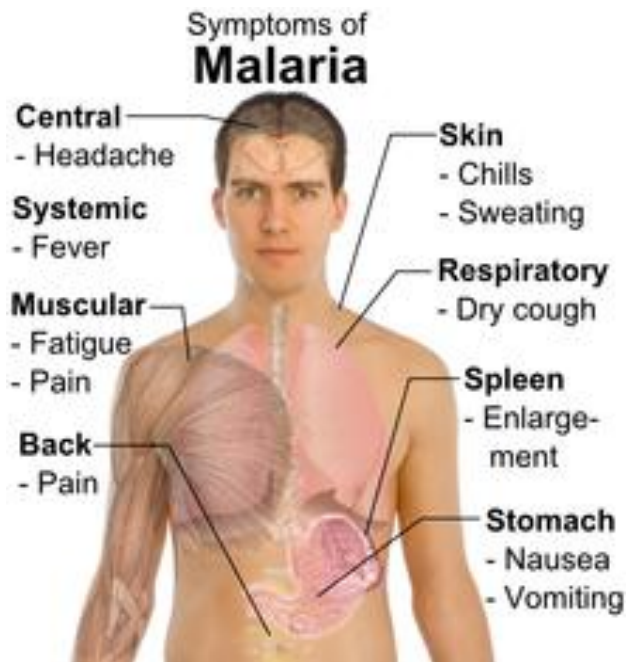
Figure 3. General symptoms of malaria, adapted from Tayyaba [25].

http://www.healthgiants.com/2010/04/26/causes-and-symptoms-of-malaria/

1.1.3.1 Fever and uncomplicated malaria

Malaria fever coincides with the rupture of schizonts which lead to the release of parasite products that are considered to be pyrogens. These products trigger an acute phase response comprising up-regulation of many host proteins including tumor necrosis factor-alfa (TNF α) [26](Clark 1987), chemokines and adhesion moelcules on endothelial cells . In maternal malaria, TNF α is associated with low birth weight and IL-8 is up-regulated in the placenta [27], [28]. It has been demonstrated that six genes (PFD1140w, PFB0115w, PFI1785w, MAL13P1-470, PFL0050c and PFF0435) are preferentially transcribed by the circulating and sequestered forms of *P. falciparum* parasites that infect pregnant women [29]. In endemic areas, many children with malaria parasitaemias are asymptomatic. These parasitaemic patients with fever are defined having uncomplicated malaria and respond to oral antimalarial chemotherapy and recover. But a proportion of them will, for reasons that are not clear, develop complicated (severe) malaria [20].

1.1.3.2 Cerebral malaria

One of the most serious complications of *P. falciparum* infection is cerebral malaria (CM), defined as an acute, diffuse, symmetric encephalopathy [30]. CM is a frequent feature of severe malaria in all ages. The pathogenic mechanism underlying CM and why a small percentage of patients develop CM are not fully understood, but the accumulation of large number of parasites in specific sites such as the cerebral vessels

or placenta associated with adverse clinical outcomes, suggests that organ-specific accumulation of parasites is important [18]. The majority of infected red blood cells found in brain sections are late stage trophozoites, tightly packed within vessels [30]. Blockage of cerebral vessels by parasitized cells, deposition of immune complexes in brain capillaries, reduced humoral or cell-mediated immune responses, action of TNF α all play roles in development of CM. Children with a clinical case definition of CM have many sequestered parasites and intra and perivascular pathology which includes hemorrhages, accumulation of pigmented white blood cells and thrombi [31]; [32]. A very high peripheral parasitaemia (>1000000/µl) is significantly associated with fatal outcome [33].

1.1.3.3 Anemia

Merozoites are released upon schizonts rupture, thus causing destruction of red blood cells. This phenomenon results in a small degree of anemia of which further progresses leading to severe anemia. Severe malarial anemia is arbitrarily defined as a hemoglobin of less than 5g/dL in association with malaria parasites [34]. Apart from destruction of red blood cells by parasites, uninfected erythrocytes are further destroyed by immune sensitization as well as damage by oxygen free radicals [35]. It is thought that suppression of chronic release of cytokine like TNF α in malaria could lead to dyserythropoiesis [36]. Furthermore, it has been demonstrated *in vitro* that elimination of uninfected red blood cells also may contribute to severe anemia [37]. Infact, it has been shown that *P. falciparum* accelerates aging of both infected and uninfected red blood cells, causing a significant shift in the cell population toward the old fraction. In addition, sequestered infected red blood cells are metabolically active and release high amounts of lactic acid as well as hypoxic tissues, leading to hypoglycaemia and lactic acidosis [38] [39]. Severe acidosis has emerged as a major feature of severe malaria [40].

1.1.4 Immunology of malaria

Malaria immunity may be defined as the state of resistance to the infection brought about by all those processes which are involved in destroying the plasmodia or by limiting their multiplication [41]. Malaria is an important cause of morbidity, but not everyone infected with the malaria parasite becomes seriously ill or dies. In stable endemic areas, repeated exposure to the parasite leads to the acquisition of specific immunity, causing mild febrile illness in older subjects, while restricting serious problems to young children. However, individuals with no previous experience of malaria usually become ill on their first exposure to the parasite. They develop a febrile illness which may become severe and in a proportion of cases may lead to death [42]. Immunity to malaria is provided partially by innate mechanisms and subsequently by the development of acquired immunity.

1.1.4.1 Innate resistance to malaria

Natural (innate) immunity to malaria is an inherent property of the host, a refractory state or an immediate inhibitory response to the introduction of the parasite, not dependent on any previous infection with it [41]. The malaria parasite faces a succession of challenge within the host. It has to attach to, enter and thrive in, first, hepatocytes and then erythrocytes. Having overcome these hurdles, it has to leave the host to carry on the next part of its cycle in the mosquito. Along its way, the parasite is susceptible to a whole range of potential interruptions, including simple physical barriers, non-specific protective response, alterations in the supply of essential nutrients and the operation of specific immune mechanisms [43].

The principal innate immunity response to protozoa is phagocytosis through cell mediated mechanisms that have been developed against foreign non-self-cells. Merozoites or parasitized erythrocytes (PE) are phagocytized by neutrophils, monocytes or macrophages, which can also secrete molecules that modulate cellular immune responses or that act against the parasites, such as cytokines and nitric oxide [44]. Though , many of these parasites may overcome cellular immune responses or scape to phagocytic killing. Malaria parasites have evolved resistance to innate immunity and successfully invade and replicate hepatocytes and in the red blood cells. So, the defense against malaria parasites requires the more powerful and specialized mechanisms of adaptive immunity [45].

1.1.4.2 Acquired immunity

Acquired immunity may be either active or passive. Active (acquired) immunity is an enhancement of the defense mechanism of the host as a result of a previous encounter with the pathogen. Passive (acquired) immunity is conferred by the prenatal or postnatal transfer of protective substances from mother to child or by the injection of such substances [41].

Malaria parasites have evolved to maintain a well-balanced relationship with their human hosts. This implies that they can partially escape from protective effector mechanisms of their hosts, but also that hosts can develop partial immunity to the parasite. This immunity requires repeated infections, takes years to develop and is usually of short duration. However, protective immunity to clinical malaria rather than infection may be of long duration. This natural acquired immunity is called premonition since a low parasitemia often persists in the presence of circulating antibodies to the various stages and in the absence of clinical disease. In children who do not have circulating antibodies to the parasite, premonition is probably caused by antitoxic immunity. This could be also the reason why children under the age of 5 are mostly susceptible to severe disease. These poor and slowly developing immune responses to malaria are partly due to immune evasion strategies of the parasite caused by antigenic polymorphism, shedding of parts of parasite proteins, cross-reactive epitopes of antigens of different developmental stages, prolonged exposure to endemic malaria and widespread restricted immunogenicity to defined antigens. Premonition relies on the cooperation between the parasite and human antibodies, leading to the induction of antibody dependent cellular inhibition (ADCI) of the intraerythrocytic growth of the parasite. In stable endemic areas, a heavy burden of morbidity and mortality falls on young children, but malaria is a relatively mild condition in adults [46]. This is due to the acquisition of specific immunity. Children born to immune mothers appear themselves to be relatively immune to malaria for a period [28]. The acquired immunity is mediated by specific antibodies to several conserved and polymorphic proteins, and to the highly variable protein PfEMP1 expressed by the parasite at the trophozoites and schizont stages; and then exported on the surface of infected erythrocytes where it mediates adhesion to the vascular endothelium [47].

1.1.5 Malaria control

Though malaria is a dangerous disease, death is preventable if appropriate treatment is taken in time and correctly. Malarial control is oriented to the disease elimination and eradication. Elimination entails reducing to zero the incidence of locally acquired malaria infection in a specific geographic area as a result of deliberate efforts, with continued measures in place to prevent re-establishment of transmission. Eradication or reducing the global incidence of malaria to zero will be achieved through progressive elimination in countries where feasible [48].

The internationally recognized Global Malaria Action Plan (GMAP) promoted by the Roll Back Malaria Initiative (RBM) – an institution launched by the World Health Organization (WHO), the United Nations Children's Fund (UNICEF), the United Nations Development Program (UNDP) and the World Bank – and adopted by many countries, consists of three elements on the figure 4.

Figure 4. Three components of the global strategy

Elimination is a worthwhile goal in many countries today. It is epidemiologically feasible in more settings than previously thought, specifically in areas of unstable transmission, where over 1 billion people are at risk. Several countries have achieved success in their pre-elimination and elimination campaigns. In 2005, Morocco and Syria reported zero locally acquired malaria cases (figure 5). Furthermore, every successful national elimination effort requires significant politics in order to focus on malaria elimination over the long term, as well as significant financial commitment [49].
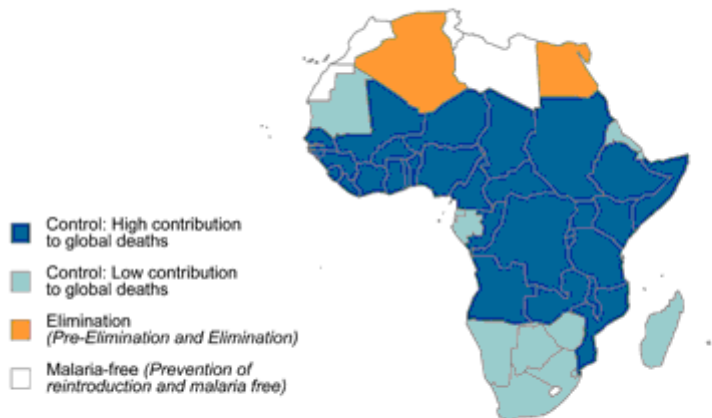
Figure 5. Country categorization in Africa [49]

1.1.5.1 Chemoprophylaxis and Chemotherapy

In spite of the international efforts, a vaccine for malaria has not been discovered, yet. In endemic countries natural host immunity protects adults at least against the severe form of the disease and it would be a satisfactory result to mimic this protection in children with a vaccine. However, only recently the results of phase III clinical trials of RTS, S/AS02, the most promising malaria vaccine candidate have been released. The RTS,S vaccine provided protection against both clinical and severe malaria in African children of about 55% against first malaria attack and 47% against severe disease [50]. Although protection is far to be

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complete, these results are very encouraging. However, while waiting for a vaccine, the available tools for fighting malaria are drugs and prevention. Prevention needs education: ignorance about the causes of malaria or low perception of the gravity of the disease are diffused. The use of treated bed-nets to prevent mosquito bites during the night is one of the possible tools to prevent transmission [51] .

Up to now drug treatment is the only efficacious weapon against malaria. The part on current antimalarial drugs is well detailed in the part II of this thesis.

1.1.5.2 Impact of Malaria on African countries economy

The most affected continent is sub-Saharan Africa, which accounts for 91% of malaria-related deaths. Due to increasing business and tourism travel, malaria cases are also on the rise in Europe and North America. Yet, malaria does not only affect the health of people, but equally represents an enormous social and economic burden for governments and households in developing countries: people cannot work or go to school when they fall ill with malaria; and the disease consumes substantial resources of a country's health system [52]. The direct and indirect economic burden of malaria in Africa is estimated to be US $2 billion annually. To the poor sub-Saharan countries of Africa, the WHO Roll Back Malaria has highlighted that the cost of malaria control and treatment drains African economies. Endemic countries have to use scarce hard currency on drugs, nets and insecticides: Africa's gross domestic product (GDP) today would be up to 32% greater if malaria had been eliminated 35 years ago, according to estimates from a Harvard study. Malaria endemic countries are among the world's most impoverished [53].

According to WHO (2010), in some heavy-burden countries, the disease accounts for:

- up to 40% of public health expenditures;
- 30% to 50% of inpatient hospital admissions;
- up to 60% of outpatient health clinic visits.

A malaria-stricken family spends an average of over one quarter of its income on malaria treatment, as well as paying prevention costs and suffering loss of income. Workers suffering an attack of malaria can be incapacitated for at least 5 days. Malaria-afflicted families on average can only harvest 40% of the crops harvested by healthy families. Malaria disproportionately affects poor people who cannot afford treatment or have limited access to health care, trapping families and communities in a downward spiral of poverty [9].

1.2 African Traditional Medicine

Traditional medicine (TM) is the sum total of knowledge, skills and practices based on the theories, beliefs and experiences indigenous to different cultures that are used to maintain health, as well as to prevent, diagnose or treat physical and mental illnesses. Herbal medicine includes herbs, herbal materials, herbal preparations, and finished herbal products that contain parts of plants or other plant material as active ingredients. Herbal treatments are the most popular from traditional medicine [54]. In some Asian, African and Latino America countries, 80% of the population depends on traditional medicine for primary health care. For many people in these countries, particularly those living in rural areas, this is the only available, accessible and affordable source of health care [9]. TM has always maintained its popularity worldwide [55]. In Africa south of the Sahara and probably in many parts of the tropical world, populations use and rely on traditional medicines more than on modern medicine: this is because traditional medicines and traditional health care are easily accessible to the majority of the populations whether urban or rural. In addition, because traditional healers live within and are part of the community, they have a higher distribution and a lower patient-healer ratio in rural areas than modern medical practitioners. They spent more time with their clients, and knowing the background information of the clients puts them in a better position to satisfy the patient psychologically. Modern medicine is a comparatively foreign culture and is only well known to the scholars and doctors who practice it.

1.2.1 Policy of traditional Medicine

In Burkina Faso, a national policy, laws and regulations and a national program on traditional medicine/Complementary and alternative medicine (TM/CAM) has been developed. A national office on

TM/CAM exists; the Service de medicine et pharmacopée traditionnelles (Traditional Medicine and Pharmacopoeia Service) was established in 1987 and is administrated by the Ministry of Health in Ouagadougou. Burkina Faso has a research institute on herbal medicine founded in 1983 and called the Institut de recherche en sciences de la santé (Institute for Health Sciences Research). The national regulation on herbal medicine is part of the public health code, No.23/94/ADP, established in 1994. Burkina Faso is using the Senegalese pharmacopoeia. Furthermore, Burkina Faso uses the national monographs from the African pharmacopoeia established in 1985 which contains 261 monographs. Regarding the regulatory requirements for manufacturing, the rules for Good Manufacturing Practices used for conventional pharmaceuticals also apply to herbal medicines. A post marketing surveillance system exists, but no system yet exists for monitoring adverse effects of herbal medicines. In Burkina Faso, herbal medicines are sold in pharmacies as over the counter medicines, in special outlets and by licensed practitioners. Burkina Faso reported annual market sales figures for 19 982 000 CFA estimated by the National Traders Network; and in 2000 sales were 3 billion CFA francs [55].

According to the Burkina Faso Government, traditional medicine will always remain an important source of health care for the majority of the population since traditional medicine is part of African sociocultural foundations. The following declarations have been adopted by the Ministry of Health to promote the universal integration of TM in the scientific community:

Declaration of Alma-Ata in 1978 on primary health care, which recognizes traditional medicine and traditional practitioners as essential partners to reach the health for all goals; The Natural Substances Research Institute and a Health Ministry service were created in 1978 to promote traditional medicine and pharmacopoeia.
In 1979, traditional medicine practitioners were officially recognized in Burkina Faso. Title IV of the Public Health Code of 28 December 1970 pertains to traditional medicine. Section 49 state
In 1983, the Government encouraged the formation of associations of traditional medicine practitioners as well as pharmacopoeia units within decentralized sanitary structures of the health system.

The Practice and Organization of Traditional Medicine, Chapter IV of Law 23/94/ADP of 19 May 1994, promulgates the Public Health Code. In July 1996, the Government approved the National Pharmaceutical Policy.
In 1997, the National Pharmaceutical Directive Plan was adopted to define the global objectives of the National Pharmaceutical Policy in concrete terms. One of the aims, as designated by the Ministry of Health, was the development and promotion of traditional medicine and traditional pharmacopoeia within the official Burkina Faso health care system in order to improve the health care delivered to the population. The Plan will be taken into consideration in the development of the National Sanitary Policies, which will cover the years 2001–2010 [56].
Brazzaville Declaration adopted in 2007 on traditional medicine which affirms the commitment of African states to strengthen links between research on traditional medicine, health systems and health policy decision taking.

1.2.2. Safety, effectiveness and quality

The safety and efficacy of traditional medicine as well as quality control have become important concerns for both health authorities and the public. Scientific evidence from tests done to evaluate the safety and effectiveness of traditional medicine products and practices is limited. While evidence shows that acupuncture, some herbal medicines and some manual therapies (e.g. massage) are effective for specific conditions, further study of products and practices is needed. Requirements and methods for research and evaluation are complex. For example, it can be difficult to assess the quality of finished herbal products. The safety, effectiveness and quality of finished herbal medicine products depend on the quality of their source materials (which can include hundreds of natural constituents), and how elements are handled through production processes [54].

1.2.3. Role of Medicinal plants in Malaria treatment

When dealing with traditional medicine, it is important to bear in mind that healers basically treat the symptoms of the disease, especially those that are apparent to them. As malaria can produce a wide variety of symptoms, over 1200 plant species are used to treat this disease. Medicinal plants considered effective in the treatment of malaria are therefore those observed by healers to alleviate or prevent one or more recognized symptoms of malaria. As malaria can occur concurrently with other infectious diseases, accurate

diagnosis can be difficult to achieve, and this makes malaria symptoms somewhat complex. Ethnomedical beliefs of populations also play a role in the choice of plants for the treatment of malaria. Based on these considerations, antimalarial plants can be roughly divided into three categories:

Plants with a direct effect on the parasite, either at the erythrocytic stage (antiplasmodial drugs) or at the hepatic stage (preventive drugs). Some plants with effects on host-parasite relationship (immunostimulants, antipyretics…). The other one with no clear effects on malaria, but with probable psychosomatic action, the uses which originates from ethnomedical beliefs. Currently, most of the new antimalarials are beyond the affordability of much of the sub-Saharan populations living in malaria endemic zones. If these same populations have been passed from generation to generation, this indicates that there must be some empirical evidence for their safety and efficacy.

According to [57] [58] and [59] the proposed thresholds for in vitro antiplasmodial activity of antimalarial extracts are (IC50 ug/ml):

    0.1µg/ml very good
    0.1-1.0 good
    1.1-10 good to moderate
    11-25 weak
    26-50 very weak
    100 inactive

1.3 Rationale

The promotion of traditional medicine was a major concern for many countries. Indeed, this remains Burkina Faso authority's willingness to work in this area. An institutionalization of traditional medicine was made through the international agreements and engagements such as the: Strategy of the WHO for the promotion of the role of the traditional medicine in national systems of health. There is also the declaration of Abuja (2001) for the research on the traditional medicine, a priority in Africa. Finally, there is the declaration of Maputo (2003) on the protection and valorization of the African inventions in matter of drugs [60].

At Burkina Faso, the national policy (2004) laid down several objectives such as: to ensure a good exercise of MT, to improve the local production of drugs and to increase the financing of the traditional medicine. It also pledged to regulate the exploitation and the export of the medicinal plants and to reinforce the capacity of the practitioner. Finally, it will reinforce the research on the traditional medicine and improve collaboration Modern Medicine/Traditional Medicine. Thus, International agencies such as the World Health Organization, World Bank and others accompany Burkina Faso in this perspective.

Like the national policy, this study is justified primarily by a desire to promote medicinal plants. It is also justified by the need to develop and facilitate access to improved traditional medicines at an affordable price. Then, it is justified by the interest that the natural molecules with antimalarial activity raise up as the discovery of quinine and artemisinin. Finally, by the fact that very few cases of resistance were observed with natural molecules compared to synthetic molecules.

1.4 Aims

This PhD project has developed in two main research areas, both related to antimalarial drug discovery. In the first period, the intent was to study the antimalarial and anti-inflammatory activity of medicinal plants used in Burkina Faso to treat malaria: *Canthium henriquesianum* (K. Schum), *Gardenia sokotensis* (Hutch) and *Vernonia colorata* (Willd). The work was conducted in the laboratory of Parasitology, Department of Public Health-Virology-Microbiology of the University of Milan and the results are reported in the first part of the thesis. Subsequently, during the stage at the Liverpool School of Tropical Medicine, Liverpool, UK, I was involved in the set up of a methodology to evaluate in vitro the pharmacodynamic properties of current and new antimalarials. Once standardized, this method could help in the selection and prioritization of new compounds to be developed. The results are presented in the second part of the thesis.

The first part of our study is a prospect for the valorization and development of research on the medicinal plants in order to be able to improve the health of the people favoring the access to cheap, easily available natural products. This study will enable us to validate the utilization of *Canthium henriquesianum, Gardenia*

sokotensis and *Vernonia colorata* in the traditional medicine of Burkina Faso. It will also allow us to propose improved methodology for the manufacture of the traditional remedies for the wellbeing of the population and to enrich the data base on these medicinal plants.

In particular, we will present data on

● Efficacy: measured as antiplasmodial activity and anti-inflammatory activity

● Safety: assessed as in vitro cytotoxicity against normal cells.

● Chemistry: Initial identification of the active principle(s)

In the second part of this thesis the pharmacodynamic features of some current antimalarial drugs will be presented . The pharmacokinetic/pharmacodynamics (PK/PD) features are an important predictor of clinical outcome for antimalarial drugs and have recently being used to guide the selection of potential drug candidates and to provide a scientific rationale for selecting dosing regimens that increase the efficacy and reduce the probability for the emergence of drug resistance [61]. These PK/PD approaches need to be applied on the field of malaria since new drugs are urgently needed to solve the problem of drug resistance. PD study allows to reduce time and cost of antimalarial drug discovery. The main objective of the second part of this thesis was to develop new tools to measure in vitro the pharmacodynamic features of different compounds especially the time to kill of current and novel antimalarial drugs. To reach this objective *in vitro* assays were planned to calculate the slope factor for each compounds from dose-response curve and the IC50 and IC90 variables. The slope factor or Hill factor is an indicator of drug efficacy since some drugs can have the same IC50 values but not the same slope. Higher slope achieves much greater inhibition at relevant clinical concentration [62]. For a same drug, according to the time of the drug pressure it is possible to know the time limit of drug efficacy monitoring the variation of the slope from 48h, 72h and 96h of incubation. Furthermore, high content in vitro analysis using the Perkin Elmer Operetta High Content Screening microscope will be done to measure the stage specificity of the activity of known antimalarials namely chloroquine (CQ), dihydroartemisinin (DHA), pyrimethamine and atovaquone. This new tool is a 96-384 well spinning disk confocal microscopy system which can perform many types of assay followed by automated image analysis [63]. It should be able to replace the time to kill assays which cannot be easily automatized and are time consuming.

2. ANTIPLASMODIAL, ANTI-INFLAMMATORY AND CYTOTOXIC ACTIVITIES OF *CANTHIUM HENRIQUESIANUM* (K. SCHUM) G., *GARDENIA SOKOTENSIS* (HUTCH), AND *VERNONIA COLORATA* (WILLD)

2.1 Introduction

Malaria is caused by the single called protozoan parasites called Plasmodium and transmitted to man through the anopheles mosquitos. It currently ranks high among the most important infectious disease around the world. According to the last World Malaria Report there are 225 million malaria cases in 2009. The number of deaths is estimated to have decreased from 985 000 in 2000 to 781 000 in 2009. The majority of deaths from malaria occur in Sub Saharian African regions particularly in children under five years of age. In spite of considerable efforts in many countries there has been little improvement in the control of malaria infections which are causing both economic and human loses. The situation is worsened by the growing problem of parasite resistance to the majority of available antimalarial drugs. The WHO promoted since 2000 the use of Artemisinin based combination therapy (ACT) as the reference treatment for uncomplicated falciparum malaria in order to reduce the resistance risk [9]. However, resistance to artemisinin derivatives has been recently described in Southeast-Asia [64], [65], [66].

The increasing prevalence of strains of *Plasmodium falciparum* resistant to cheap standard treatments has led to search for new antimalarial compounds [67]. The use of plants for malaria treatment extends over at least three continents, including several countries in Africa, in the Americas, and in Asia [68]. Traditional treatments have been investigated and a significant success was achieved with quinine present in the Chincona barks and more recently with Quinghaosu, isolated from the Chinese herbal medicine *Artemisia annua [69].* In Burkina Faso, a registry of more than 72 species used to cure malaria by traditional healers, was compiled by Jansen et al. in 2008 [70]. Only few of them have been systematically and scientifically analyzed, yet. We reasoned that a better knowledge of plants from traditional pharmacopoeias and local valorization of validated traditional remedies could lead to the identification of effective and affordable therapies for the management of malaria by local populations. We selected and collected in the field, 3 of these species belonging to 2 families : *Canthium henriquesianum (Rubiaceae), Gardenia sokotensis (Rubiaceae), and Vernonia colorata (Asteraceae).* They are widely used by the traditional healers but they have not been evaluated, yet, for antiplasmodial activity or only partially studied. All these plants are common in tropical Africa [70].

This investigation on C. *henriquesianum* was initiated because of the absence of published phytochemical reports on this specie and the possibility of the presence of antiplasmodial/anti-inflammatory-antipyretic compounds. But the other *Canthium species* such as *Canthium multiflorum* I was shown to have a good antiplasmodial activity with the dichloromethane extract from the roots (IC_{50}=7µg/ml). The active fraction contains some 3 methoxy-coumarins and 2 pentacyclic triterpenes [71]. This genus is known for its folkloric applications against a number of ailments such as cough, diabete and hypertension. Because these three plants are used in traditional medicine in Burkina Faso to heal malaria and to reduce fever, the aim the present work was to test in vitro the antiplasmodial and/or antipyretic activity of the plant extracts and to identify the active principle. Aqueous and organic extracts were prepared from each of the selected plant and they were evaluated for 1. Antiplasmodial activity in vitro against two *P. falciparum* strains with different resistance phenotype; 2. Effect on the production of anti-inflammatory cytokines (pyrogens) by a human leukaemia monocytic cell line; 3. Cytotoxicity on human mammalian cells.

2.2 Material and Method

2.2.1 Plant material

The classification and the characteristics of the plants *Canthium henriquesianum (K.* Schum), *Gardenia sokotensis Hutch* and *Vernonia colorata Willd* selected for this study are described in Table 1. All these plants are common in tropical Africa. These plants have been respectively collected from their natural habitat in Burkina Faso) in June 2008. The samples were authenticated by prof Adjima Thiombiano, botanist at the Laboratory of *Botany,* Ecology and Plant *Biology* (Botany Department Staff of the "UFR/SVT University of Ouagadougou" in Burkina Faso). A voucher herbarium specimen has been deposited in the laboratory of Ecology at the University of Ouagadougou. The samples, mainly leaves and twigs were cleaned, dried in a ventilated room and then powdered.

Table 1. Selected plants and collected samples

Species /Plant part	Collection place	Voucher number	Traditional use associated with malaria and related symptoms	Previous report of antiplasmodial activity
C.henriquesianum Leave-twigs	Badara	ID12791 No 01. 08/06/2008	Aerial part decoction for Fevers-malaria	No report
G. sokotensis Leave-twigs	Nagreongo	ID12792 No02. 08/06/2008	Aerial part decoction, often in combination with other plants for fevers-malaria associated with gastrointestinal disorder	In vivo activity of the dichoromethane/ methanolic extract on *P. berghei*. $ED_{50}=116mg/kg$ [72]
V. colorata Leave-twigs	Ouagadou-gou	ID12793 No03. 08/06/2008	Leaf decoction or water infusion, often in combination with other plants for Malaria with gastrointestinal disorders	11β, 13-dihydrovernodalin Vernodalol exhibited an antiplasmodial activity in vitro 1.1-4.8µg/ml [73]

2.2.1.1 *Canthium henriquesianum (K.* Schum) G *Syn Psydrax acutifolia (Hiern)*

Local Name: Laagui Fofana

Canthium henriquesianum (annexe 1) called in local language as "Laagui Fofana" is a shrub beside upland streams with shiny and coriaceous leaves and yellow-green flowers. The aerial part (leaves and twigs) are used in the treatment of malaria. The inflorescence is composed of 15-50 flowers under the leaves. Fruits are globular or ovoid, often persisting on the branch. The forest gallery and the shores of rivers are its habitat. It is a plant whose roots are tonic.

2.2.1.2 *Gardenia sokotensis (Hutch)*

Moore Name: Tang-ra-kweenga.

*Gardenia sokotensis (*annexe 2) is a shrub more or less bushy, 1-2.5 m of high. The bark is smooth and yellowish. The branch is short. The leaves are opposite. The flowering occurs early at the rainy seasons. The savannahs Sahelo-Sudanian to Sudano Guinean, usually the dry soils, rocks, and gravel constitute its habitat [74]. The twigs and leaves contain tannins, saponins, triterpenoids, sterols, iridoids and alkaloids [75].

Leaves and stems are fortified and are used in the treatment of malaria with gastro-intestinal pain, diarrhea, vomiting, fever, colic, asthenia, headache, and the prevention of uterine bleeding during pregnancy. The combination of the bark, of roots and leaves would be effective against hypertension [75], [74].

2.2.1. 3 *Vernonia colorata (Willd)*

Moore Name: Koa-safandé

Vernonia colorota (annexe 3) is a shrub of 2-4m tall. The branches are beige, hairless. The leaves are alternate, whole, oval or elliptical. The stalk is more or less pubescent. The inflorescences are the terminal panicles. The flowers are white. The fruits are some achenes, hairless, 3mm of long. The flowering is rather at the end of the rainy season. The forests and savannahs Sudano Guinea and Guinea, the riverside, and on drained land are its habitat. It is in tropical Africa from Senegal to Cameroon [74]. *Vernonia colorata* , known by the name "Kosafune`" in the Dioula language in Burkina Faso, has long been used in traditional medicine by the indigenous people for the treatment of cough, fever, hepatitis, gastritis, stomach pain, gastrointestinal disorders, venereal diseases, malaria and skin eruptions. Commonly, the leaves very bitter, seen as a substitute for quinine are used as water infusions or decoctions and employed to cure ear ache, and as a febrifuge. The extract of fresh leaves, softened by heating, is applied to wounds for cicatrisation [75]. The stems, the leaves and the roots have principles bitter. Through conventional chromatographic techniques

and bioassay-guided fractionation, the following sesquiterpene lactones were isolated and identified by spectroscopic data; vernolide (1), 11beta, 13-dihydrovernolide (2) and vernodalin (3). The leaves have a great antimicrobial activity through the sesquiterpenes lactones such as vernolide, 11beta, 13-dihydrovernolide and vernodalin. Sesquiterpene 11beta and 13-dihydrovernodalin have an antiplasmodiale activity [73]. Plant extract has also a hypoglycemic and antidiabetic effects [76] and anti-Toxoplasmosis effects [77]. The areal parts of plants are prepared before being sold to people at the local market (annexe 4).

2.2.2 Plant extractions and phytochemical screening

Air-dried plants were finely powdered. In traditional medicine, the population drinks the decoction of bark and stem for one to two weeks to treat malaria. The aqueous extracts of *C. henriquesianum, G.sokotensis* and *V. colorata* were prepared as described above following the methods used in Burkina Faso by traditional healers. For the aqueous crude extracts we proceeded by a decoction of 5g of dried plant powder in 100 ml of distilled water. The preparation was filtered and freeze dried (lyophilized) and tested in vitro against different strains of *P. falciparum* and for cytotoxicity on mammalian cells. *C. henrequesianum* aqueous and the diisopropyl extracts were screened for the chemical compositon. The qualitative analysis of the aqueous extract was done for checking the presence of tannins, saponins, flavonoids, coumarins, and the alkaloids (Dragendorff test). We used the n(BuOH) and HCL5% solution to detect the presence of condensed tannins and the ferric chloride solution test for the hydrolysable tannins. The 25 ml of water with NH_3 10% was used to concentrate the alkaloids from the plant powder. The presence of alkaloids was assessed by using the Dragendorff test. The Thin Layer chromatography (TLC) was used to separate the different chemical components of the extracts. The flavonoids were detected with Ethylacetate/Formic acid/Acetic acid/water: 100/11/11/27) and revealed by the polyethylene glycol (PEG) and the Diphenylboryloxy ethylamine (DNP). Saponins were detected by chloroform/methanol/water: 65/50/10 and revealed with the two revelators ethanol-sulfuric acid and ethanol-vanillia; and coumarins detected by Toluene/ether with the methanolic extract: 1/1.

The air-dried and powdered leaves of *C. henriquesianum* (62.3g) were soxhlet-extracted (figure 6) with different organic solvents (300ml, 3 h) according to their polarity with hexane, diisopropyl ether, dichloromethane, ethyl acetate and methanol/water. Then, each preparation was evaporated under reduced pressure obtaining 7.33g (hexan), 3.03 g (diisopropyl ether fraction), 5.26 g (dichloromethane fraction), 4.068 g (ethyl acetate fraction) and 8.64 g (methanol/water fraction), respectively. Each fraction was then tested for antimalarial activity.
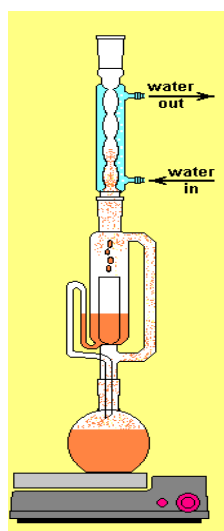
Figure 6. Shoxlet extraction

The chlorophyll from the diisopropyl ether fraction was removed using activated charcoal and the residue (500mg) was chromatographed on silica gel eluting with ethyl acetate-hexan 1:1. The subfractions (5 mL each) obtained were screened by TLC. The organic layers were combined, dried over $MgSO_4$, filtered and the solvent was removed in vacuum to give a residue (15mg) that was analyzed by NMR spectroscopy. The NMR spectra were recorded on a Brucker Avance 400 (1H, 400 MHz; 13C, 100 MHz) spectrometer. The

Carbon Nuclear Magnetic Resonance (^{13}C-NMR) Spectroscopy and the Proton Nuclear Magnetic Resonance (^{1}H NMR) Spectroscopy allow to elucidate the structure of the compound. The chemical shift, the integration and the multiplicity are the characteristics of a NMR spectrum. They are represented in figure 7. The chemical shift defined as the nuclear *shielding / applied magnetic field*. Chemical shift is a function of the nucleus and its environment. It is measured relative to a reference compound. The multiplicity determine the number of neighboring protons. The multiplicity of a multiplet is given by the number of equivalent protons in neighbouring atoms plus one. The integration only gives information on the relative number of different hydrogens [78].

^{1}H-NMR (400MHz, CDCL3)

6.32 (d,1H,H-3, J=6.04Hz), 7.36-7.44(m,2H,H-6,H-8),7.65 (dt,1H,H-7),7.84(d, 1H,H-2, J=6.04Hz),8.17 (dd,1H,H-5)

^{13}C –NMR (400MHz, CDCL3) 112.98 (C3),118.19 (C8),124.83 (C4a), 125.25(C6),125.77 (C5), 133.77 (C7), 155.34 (C2), 156.54(C8a), 177.66(C4) Melting point 55°C ; Elemental analysis calc. C 73.97% H 4.14%; trov.C 74.01% H 4.10% [79] [80].
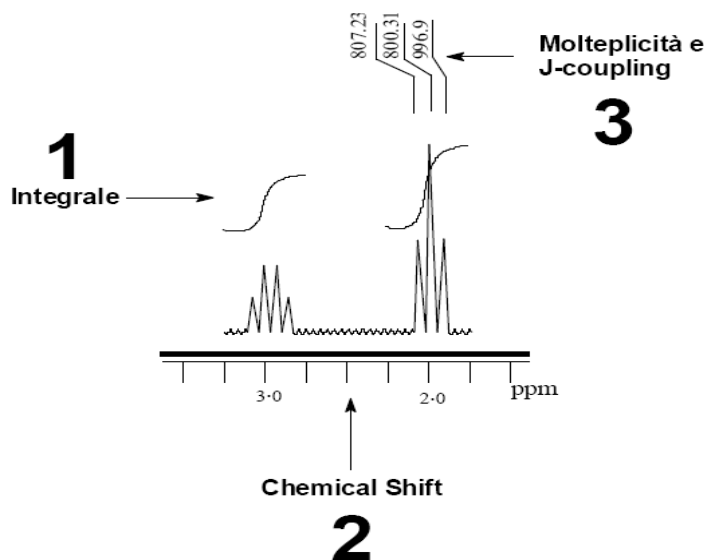

Figure 7. shows the 3 components of a spectrum

2.2.3 In vitro drugs susceptibility assay on Plasmodium falciparum

P. falciparum were cultured according to Trager and Jensen with slight modifications [81]. The CQ-sensitive (D10) and the CQ-resistant (W2) strains were maintained at 5% hematocrit (human type A-positive red blood cells) in RPMI 1640 (EuroClone, Celbio) medium with the addition of 1% AlbuMaxII (lipid-rich bovine serum albumin) (Invitrogen), 0.01% hypoxantine (Sigma), 20mM Hepes (EuroClone), 2 mM glutamine (EuroClone). All the cultures were maintained at 37°C in a standard gas mixture consisting of 1% O_2, 5% CO_2, 94% N_2. Each aqueous, organic extracts and referenced drug (CQ) were dissolved in the complete medium or DMSO to a concentration of 10mg/ml. Drugs were placed in 96 wells flat-bottom microplates (COSTAR) and serial dilutions made (final concentration ranging from 0.125 to 500 µg/ml for crude extracts and final DMSO concentration ≤1%). Asynchronous cultures with parasitaemia of 1–1.5% and 1% final hematocrit were aliquoted into the plates and incubated for 72 h at 37°C. The parasites growth was determined spectrophotometrically (OD_{650}) by measuring the activity of the parasite lactate dehydrogenase (pLDH), according to a modified version of Makler's method in control and drug-treated cultures [82]. Antimalarial activity was determined as concentration of drugs inducing 50% of growth inhibition (IC_{50}).

2.2.4 Cell cytotoxicity assay and cytokines production by the leukaemia monocytic cell line THP-1

The human cell line THP-1 (ATCC NUMBER: TIB-202, American type Culture collection, Manassas, VA, USA) is derived from the peripheral blood of one year old patient, with acute monocytic leukaemia. These cells were maintained in RPMI-1640 medium (EuroClone) containing 10% foetal calf serum, 100 units/ml penicillin/streptomycin, 2mM L-glutamine, HEPES (10mM), Pyruvate sodium (0.01mM) and Beta-Mercaptoethanol (0.05mM) (Invitrogen, s.r.l., Milan, Italy).

For the cytokines production, cells were seeded in 96 well round bottom tissue culture clusters (Costar, NY, USA) at 10^5 cells/well and incubated with different concentrations of the compounds under test (20-500 µg/ml) and LPS as stimulus (10 or 100 ng/ml). After 24 h of culture, the supernatants (150 µl) of THP-1 cells were collected and the levels of human cytokines IL-1β and TNFα were evaluatedTHP1 viability was determined, by the MTT assay, as described above. The levels of human cytokines IL-1β and TNFα were quantified using ELISA kit from R&D Systems according to manufacturer's protocols. All experiments were performed in triplicate and repeated at least three times. Results were expressed as pg of cytokine/ml.

2.2.5 Cell cytotoxicity assay on Human Dermal Fibroblast (HDF) and melanoma cells

Cytotoxicity was evaluated on HDF from skin biopsies. HDF were maintained in DMEM (Dulbecco's modified Eagle's medium) containing 10% foetal calf serum (Euroclone), 100 units/ml penicillin/streptomycin, and 2 mM L-glutamine. Melanoma cells were maintained in RPMI containing 100 units/ml penicillin/streptomycin, 10% foetal calf serum (Euroclone), 2 mM L-glutamine and HEPES (10mM).

For the cytotoxicity assay, cells were seeded in 96 well flat bottom tissue culture clusters (Costar, NY, USA) at 10^4 cells/well for HDF, at 10^5 cells/ml for Melanoma cells and incubated overnight. Adherent cells were then treated with serial dilutions of test compounds and cell proliferation was evaluated by the MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (Sigma) test [83] after 72 h at 37°C in 5% CO_2. At the end of the treatment, 20 µL of a 5 mg/mL solution MTT in PBS were added to the cells for an additional 3 hours at 37°C. The plates were then centrifuged, the supernatants discarded and the dark blue formazan crystals dissolved using 100 µL of lysing buffer consisting of 20% (w/v) of a solution of SDS (Sigma), 40% of N,N-dimethylformamide (Merck) in H_2O, at pH 4.7 adjusted with 80% acetic acid. The plates were then read on a microplate reader (Molecular Devices Co., Menlo Park, CA, USA) at a test wavelength of 550 nm and a reference wavelength of 650 nm. The results are expressed as IC_{50} which is the dose of compound necessary to inhibit cell growth by 50%. All the tests were performed in triplicate at least three times.

2.2.6 Data analysis

Data are presented as mean ± standard deviation. Student's t test has been used for the statistical analysis. A probability value of P≤0.05 was considered as statistically significant (fig 12A).

2.3 Results

2.3.1 In vitro antimalarial screening and the selectivity index of aqueous extract against P. falciparum strains

The vegetal samples obtained from the 3 selected plants traditionally used to treat malaria in Burkina Faso were prepared as used by the traditional healers and patients. The antiplasmodial activity of the acqueous extracts were tested against a chloroquine resistant (W2) and a chloroquine sensitive (D10) strains of P. falciparum. The results are presented in table 2.

C. henriquesianum was the most active aqueous extract displaying a moderate activity (<100ug/ml) against both CQ-R and CQ-S P. falciparum strains. On the contrary, G. sokotensis and V. colorata demonstrated a weak antiplasmodial activity only against W-2, CQ-R strain, whereas they were less active against the D10, CQ-S strain. In the same experiments, the IC_{50} for chloroquine (CQ), used as reference drug, was 0.009±0.002 µg/ml and 0.171±0.016 µg/ml against D10 and W2, respectively. C. henriquesianum aqueous extract was also the less toxic when tested for cytotoxicity against human dermal fibroblast with a selectivity index of about 10 (Table.2). G. sokotensis and V. colorata were more toxic with a $SI_{(HDF/D10)}$<1 and <3, respectively. Based on these results, moderate activity and low toxicity, C. henriquesianum was selected for further studies.

Table 2 . *In vitro* antiplasmodial activity and the selectivity index of *Canthium henriquesianum*, *Gardenia sokotensis* and *Vernonia colorata* aqueous extracts against CQ-R (W2) and CQ-S (D10) strains of *P. falciparum*

Plant extracts	*Plasmodiun falciparum*		HDF [a] IC_{50} (µg /ml)	Selectivity index HDF/D10 [b]	Selectivity index HDF/W2 [c]
	D10 IC_{50} (µg /ml) [*]	W2 IC_{50} (µg /ml)			
Canthium henriquesianum	80.0± 26.8	66.7±21.5	615.38 ± 28.9	7.7	9.2
Gardenia sokotensis	268.2 ± 123.8	159.6±80.8	222.03 ± 53.5	0.8	1.4
Vernonia colorata	220.2± 158.9	78.8±10.6	562.2 ± 22.0	2.5	7.1
Chloroquine (CQ),	0.009 ±0.002	0.2 ± 0.02			

[a] HDF: Human Dermal Fibroblast proliferation tested after 72h with MTT
[b] Selectivity index (SI) = IC_{50} of HDF/ IC_{50} of *P. falciparum (D10)*
[c] Selectivity index = IC_{50} of HDF/ IC_{50} of *P. falciparum* W2
[*] IC_{50} is the concentration of drugs inducing 50% of growth inhibition expressed on µg/ml
Values are the means ± SD of results from three different experiments, each performed in triplicate

2.3.2 Cytotoxic activity of organic extracts from Canthium henriquesianum

To identify the chemical class associated with the observed antimalarial activity, different organic extracts were then prepared from *C. henriquesianum* using solvents with different polarity. Each extract was then tested against W2 and D10 *P. falciparum* strains. As shown in Table 3, the highest antiplasmodial activity was associated to the diisopropyl ether extract followed by the hexan and then the dichloromethane extract. When the chlorophyll was removed from the diisopropyl ether extract, the activity against the parasites increased as shown by the change in IC_{50} from 17.6 to 9.5 µg/ml and 21.1 to 8.8 µg/ml on W2 and D10, respectively. These experiments have been repeated on the same strains of parasites with two different preparations of *C. henriquesianum* and the results were comparable.

In order to verify the selectivity of these preparation on the parasite and their safety, the toxicity of the extracts was evaluated on human dermal fibroblast (HDF) and the monocytic cell line (THP1). When compared with the results obtained with the aqueous extract *C. henriquesianum* diisopropyl ether extract showed higher cytotoxicity and a selectivity index >1. However, *C. henriquesianum* diisopropyl ether (without chlorophyll) extracts showed reduced cytotoxicity with a SI>3 (table 3). The MTT results showed also that the high dose of *C. henriquesianum* aqueous extract of was not toxic for THP-1 cells after 24h of incubation (table 4). Indeed similar OD values were found for the control and the higher dose of *C. henriquesianum* aqueous extract.

Table 3. Cytotoxicity of *Canthium henriquesianum* fractions on the monocytic cell line (THP-1) and on Human dermal fibroblasts (HDF) after 72h.

C. henriquesianum extracts	IC_{50} (µg/ml) THP-	SI THP-1/W2	SI THP-1/D10	IC_{50} (µg/ml) HDF	SI HDF/W2	SI HDF/D10
Diisopropyl ether (with chlorophyll)	18.53±3.5	1.05	0.87	28.48±0.02	1.62	1.35
Diisopropyl ether (without chlorophyll)	31.94±11.8	3.34	3.62	-	-	

SI=selectivity index
Values are the means ± SD of results from three different experiments, each performed in triplicate

Table 4. Cytotoxicity of *Canthium henriquesianum* aqueous extract on the monocytic cell line (THP-1) after 24h.

OD $_{650}$	Control	C500ug/ml	C100ug/ml	C20ug/ml
Without LPS	0.75 ± 0.07	0.81 ± 0.03	0.70 ± 0.06	0.74 ± 0.03
LPS 100ng/ml	0.59 ± 0.01	0.60 ± 0.01	0.57 ± 0.01	0.70 ± 0.10

Values are the means ± SD of results from three different experiments, each performed in triplicate

2.3.3 Immunomodulatory activity of aqueous extracts on human monocytic leukemia cell line (THP-1)

To investigate whether the aqueous extract of the three plants used in this study possess an antinflammatory activity to justify their use in traditional medicine, we measured the production of the pyrogenic cytokines IL-1β and TNFα by THP1 cells treated with the extracts and stimulated by LPS for 24 hours. Preliminary experiments were performed to choose non-toxic doses to use on THP-1 cells. The aqueous extract of *C. henriquesianum* reduced the production of IL-1 in a dose-dependent manner, whereas the production of TNFα was not affected (figures 8 A, B). Only the highest dose of *C. henriquesianum* extract used was able to induce a significant inhibition of IL1β production. But statically the difference between the control and highest dose was only significative at p=0.097. The diisopropyl ether fraction of *C. henriquesianum* at doses >100 µg/ml was toxic on THP-1 cells.
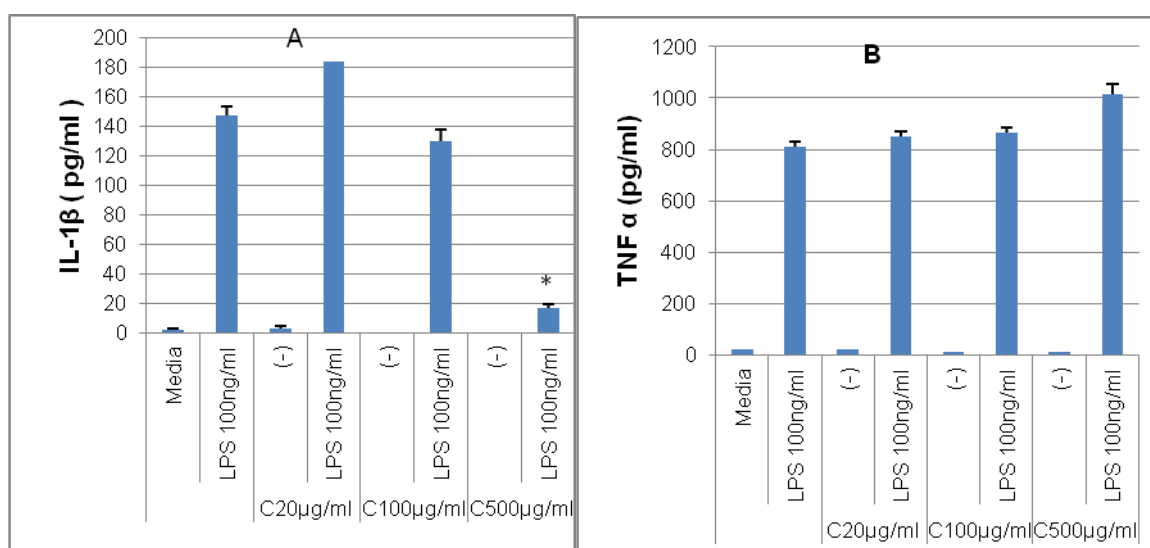
*P=.0097

Figure 8: Dose-dependent inhibition of Il-1β and TNF α production by Human Monocytic leucemia cell line (THP-1) stimulated with LPS in the presence of *C. henriquesianum* aqueous extract.
A: Dose-dependent inhibition of IL-1 production by THP-1
B: TNFα production by THP-1

2.3.4 Anti-tumor effect of plant aqueous extracts on melanoma cells

From the data shown in Table 2, it appeared that the aqueous extract of *G sokotensis* was toxic against human normal fibroblast. This observation prompted us to investigate the potential antitumor activity of the three extracts against a human melanoma cell line kindly provided by Dr ML Sensi of the National Cancer Institute in Milan. The results shown in Table 5 indicate that only the extract of *G sokotensis* shows moderate cytotoxicity against melanoma cells in a 72 h assay. The camptothecin was used as positive control.
The activity of the extract is, however, > 100 fold lower than that of camptothecin used as positive control.

Table 5: IC$_{50}$ values of plant extracts on melanoma cells

Aqueous extract	MELANOMA IC$_{50}$ (µg/ml)[a]
Canthium henriquesianum	767 ± 113
Gardenia sokotensis	66.3 ± 5
Vernonia colorata	572.5 ± 57
Camptothecin	0.0019 ± 0.001

[a] Melanoma cell growth was quantified with the MTT assay as described in M&M

2.3.5 Qualitative analysis of Canthium henriquesianum aqueous extract

As shown in Table 6 the aqueous extract of *C. henriquesianum* contains a mixture of hydrolysable tannins, flavonoids, and saponins. No condensed tannins, coumarins and alkaloids were found.

Table 6. *C. henriquesianum* chemical composition by TLC as described in M&M.

Chemical groups	Presence or not
Alkaloids	-
Coumarins	-
Flavonoids	+
Saponins	+
Condensed tannins	-
Hydrolysable tannins	+

To have a preliminary analysis of the composition of the extract and in the attempt to identify the active principle, canthium diisopropyl ether extract was analysed by NMR. The NMR spectra of the carbon and the hydrogen (figures 9 and 10) revealed that the most representative compound isolated from the canthium diisopropyl ether extract was chromone or 1-4 benzopyrone (figure 11). The spectra indicated that the chromone has 9 atoms of carbon and 7 atoms of hydrogen. Chromone was indeed tested on D10 and W2 to verify its antiplasmodial activity The IC50 was 18.1 and 43.4 µg/ml on D10 and W2, respectively, indicating that the pure compound was less active than the extract. The chromone was toxic on THP-1 cells only at doses >100 µg/ml. However, at not toxic doses (<100 µg/ml) the chromone did not affect IL-1β production by LPS stimulated THP-1 (data not shown).
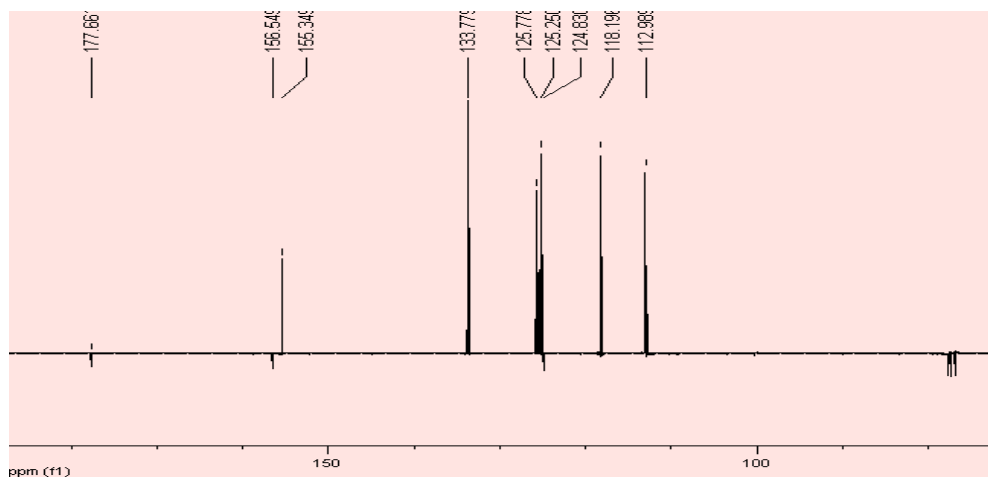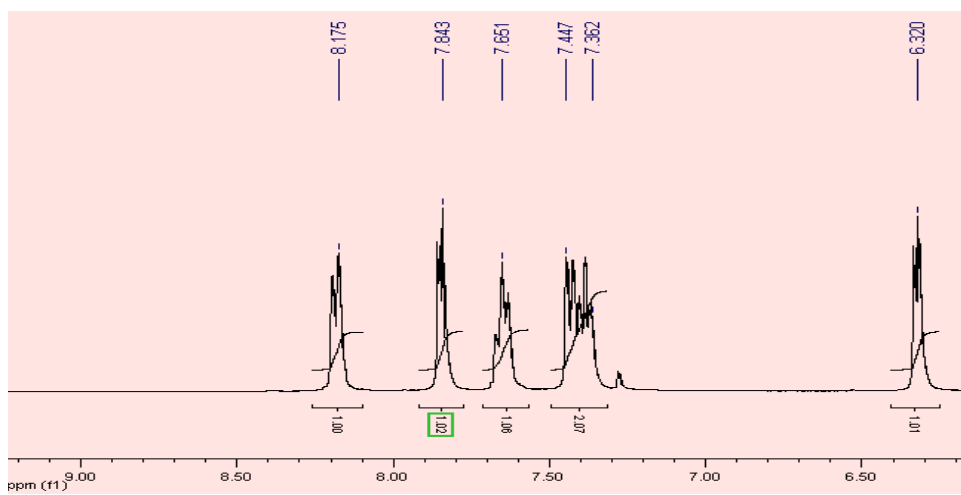
Figure 9. NMR spectra of the carbon (^{13}C NMR)

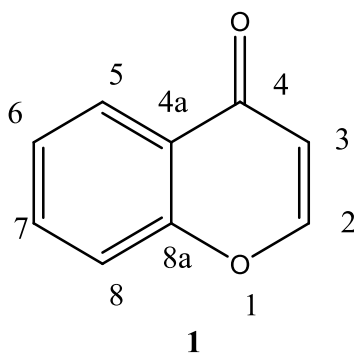**Figure *10.*** NMR spectra of the hydrogen (^{1}H NMR)

1

Figure 11: Chromone or 1-4 benzopyrone

2.4 Discussion

In line with previous study [58], the antiplasmodial activity of natural extracts can be classified as follows: $IC_{50} \leq 15\mu g/ml$: promising activity; IC_{50} between 15-50 µg/ml: moderate activity; IC50>50 µg/ml: weak activity; IC50>100µg/ml: inactivity. Among the 3 selected plants used for traditional malaria healing in Burkina Faso, *C. henriquesianum* aqueous extract exhibited a moderate activity against *P. falciparum in vitro* while *C. henriquesianum* diisopropyl ether fraction displayed a promising antiplasmodial activity, although this fraction (with chlorophyll) was also more toxic against normal cells ($SI_{(HDF/D10)}$=1,35 vs $SI_{(HDF/D10)}$>7 of the aqueous extract). On the contrary, *G. sokotensis* and *V. colorata* aqueous extracts both demonstrated a weak antiplasmodial activity. These findings provide some evidence underlying the traditional use of these plants as antimalarials.

The areal part of *C. henriquesianum* is used in traditional remedies to treat malaria and the related symptoms. This is the first report showing antiplasmodial activity of *C. henriquesianum* although there are few data on the other species of the genus *Canthium*. Indeed, Traoré et al. reported the isolation of the first of 28- hydroxylated ursanoic acid endowed with weak antiplasmodial activity [84]. A new peptide alkaloid named anorldianine has been also isolated from *Canthium anorldanum* . Many plants such as *Sida acuta, Pavetta crassipes* or *Mytragina inermis* were found to exert antimalarial activity related to their alkaloids contents [85], [86]. However, we have to be aware that *C. henriquesianum* aqueous extract is still significantly less, less potent than CQ. This may be due to the fact that the extract is still crude and needs to be purified, and that the active principle is present at a very low concentration .

We have also shown that the aqueous extract of *C. henriquesianum* induced a dose- dependent inhibition of the production of IL-1β, one of the most potent pro inflammatory cytokine responsible for the fever accompanying acute diseases. Inflammation is a complex mechanism developed as an immunological response to viral, bacterial or parasitic infection [58] (Jonville et al., 2010). According to the phytochemical screening performed in our samples, the extract of *C. henriquesianum* contains some flavonoids, 3. hydrolysable tanins and saponins. We can conclude that the aqueous extract of *C. henriquesianum* has an anti-inflammatory and anti pyretic activity which is probably due to the presence of flavonoids. Indeed, Aquila showed that flavonoids have an antinflammatory activity . The antipyretic activity of *C. henriquesianum* justifies the use of this plant in the treatment of the symptoms of malaria in Burkina Faso [87]. The flavonoids have also an analgesic activity [88]. The finding that it inhibits the IL-1β production is of particular interest from a immunologic point of view. The inhibition of IL-1 production seems to be specific since TNF a production was not affected, this implies only a partial reduction of the symptoms and inflammation associated with a malaria attack. This is also the indication that the extracts may not be useful in the cases of severe malaria when it is known that elevated levels of TNF contribute largely to the pathology.

The compound mostly represented in the diisopropyl extract of *C. henriquesianum* has been identified as chromone, which is a flavonoid. The 1H- and 13C-NMR spectra of the chromone were in agreement with those reported in the literature [79], [80]. The authors [79] showed that the ring system of the chromone is a part of the flavonoid nucleus. Part of the antiplasmodial activity was due to chromone, but it appears that some other compounds present in the extract contribute to it., since the IC50 of chromone was higher than that of the extract. Chromone, however, at non-toxic doses (<100µg/ml) did not inhibited IL-1β production by LPS stimulated THP-1, suggesting that it does not possess anti-inflammatory activity.

The results revealed that the *G. sokotensis* leaves/twigs aqueous extract had a weak activity against *P. falciparum* parasites but exhibited significant cytotoxic activity against normal human fibroblasts ($SI_{(HDF/D10)}$<1). The extract from *G. sokotensis* does not modulate the production of IL-1β, either. Few data are available in the literature on *G. sokotensis*. Weak antiplasmodial activity of the aqueous extract has been found *in vivo* by Traoré et al. using the model of *Plasmodium berghei* in Balb/c mice [72]. The same study showed that the dichloromethane and the methanolic extracts of *G. sokotensis* leaves exhibited a promising and moderate antiplasmodial activity, respectively.

It was confirmed by the same authors that the dichloromethane extract exhibited a high cytotoxic activity ($SI_{(WI38/3D7)}$ = 0.9), in agreement with our results. The leaves of this bush tree are used in traditional remedies in Burkina Faso, often in combination with others plants to treat malaria and related symptoms such as fevers, weakness, icterus and gastrointestinal disorders [70] (Jansen et al., 2010). The work *has* also reported the presence of flavonoids, triterpens and carotenoids in the extracts of *G. sokotensis*, but the active compounds were not identified [72]. In addition, the aqueous extract of *G. sokotensis* when tested against human melanoma cells showed a moderate antitumor activity (IC_{50} on the melanoma cells =66.32±5.01 versus 222.03µg/ml±53.52 on HDF). Although the cytotoxicity in vitro is significantly lower than the reference antitumor agent, camptothecin, *G sokotensis* could be further investigated because the extract may contain novel anti tumor molecules yet to be ideintified.

The aqueous decoction from the leaves of *V. colorata* is used in traditional medicine for various purposes particularly for gastro intestinal disorders and malaria [75]. In our study the water extract has been shown to have a weak antiplasmodial activity with a $SI_{(HDF/D10)} < 3$ and does not have an effect on the human pro inflammatory cytokine IL-1b production. Because, usually this plant is used in combination with others plants in the case of malaria attack, the lack of the in vitro antiplasmodial activity may be due to the absence of a synergistic action with other plant mixtures. These antiplasmodial results contrast with those from the Zimbabwe, where the authors found good activity of the lipophilic extract of the leaves from *V. colorata*. Four sesquiterpenes 13-dihydrovernodalin, vernodalol, 13-dihydrovernolide and 13,17,18-tetrahydrovernolide, were identified , the first two being the most active against *P. falciparum* parasites (IC50 values: 1.1 – 4.8 μg/mL) [73]. Furthermore, the twigs from *V. colarata* exhibited a good antiplasmodial activity (14.1 μg/ml) [89]. At present, we cannot explain the difference in the results obtained by ourselves and others. However, several factors may contribute, including the P falciparum strains used and the type of assay. Most important, though, is the fact that we have used an aqueous and not a lipophilic extract. In addition, differences in soil nature or period of harvesting (humid or dry period) may induce changes in the metabolism of the plant and the relative abundance of active metabolites. Other extracts from *Vernonia* species are reported *in vitro* to possess antimalarial activity. Indeed, a good antiplasmodial activity of the isoamyl alcohol fraction from *V. amygdalina* (IC50<3μg/l) has been shown by [90]. A moderate activity was reported for *V. ambigua* (31.62 μg mL^{-1}<IC50<50 μg mL^1) [91].

At present, and using only the present *in vitro* data, it is not possible to draw definitive conclusions to suggest or not the use of these plants in traditional medicine, particularly in the field of malaria, a disease which involves many different symptoms and which has a complex physiopathology [92]. Indeed, many plants that are frequently reported to be used as antimalarial in traditional medicine in different countries do not show necessarily high activity in *in vitro* screening. This in part is due to the fact that extracts from only one species or isolated compound behave differently in vitro than in the mixture that constitutes the decoction used by the patients. Additive or even synergistic affects are lost.

In our study, the detected antiplasmodial and anti-inflammatory activity in the extracts of *C. henriquesianum* clearly help to explain its traditional use for the relief of malaria symptoms, such as fever. For the less active plants as *G. sokotensis* and *V. colorata*, we cannot exclude completely their advantages in traditional malaria healing. In fact several elements might explain the absence or the weak activity of the plants extracts. Firstly, the *in vitro* test model reproduces only the erythrocytic stage of the parasite, but some plants could also be active against the liver stage of parasite development [93]. This activity against hepatic schizonts could not be detected with the *in vitro* model, but such plants could be advantageous for prophylaxis as well as for the treatment of the dormant hypnozoites which can relapses sometimes several months after the first infection (*Plasmodium vivax, Plasmodium ovale*). Moreover, traditional remedies often consist of a combination of several different plants mixed together in the decoctions (*G. sokotensis* and *V. colorata*) and several therapeutic effects could be present at the same time. In addition to the anti parasitic effects, some plants could have antipyretic, analgesic, hepatoprotective, anti-inflammatory or immunomodulatory effects. The different constituents of the mixture can exert an additive or synergistic action against several symptoms of malaria, and this synergy may have an impact on the patient's recovery. Finally, plants could be more effective in vivo than in vitro, since they may contain prodrugs [70]. A prodrug is not active by itself, but it needs to be metabolized in order to become an active drug; it cannot therefore be detected in an *in vitro* test model.

2. 5 Conclusion and perspectives

Among the selected plants from Burkina Faso, the preliminary results show that *Canthium henriquesianum* was the most active extract against *P. falciparum*, although the chromone, which is the component mostly represented, is not potent as the total extract, suggesting that a mixture of compounds contribute to the antimalarial activity observed in vitro. The therapeutic value of *C. henriquesianum* used in traditional medicina has been confirmed by the inhibition of human Il-1β production, indicating that it may possess anti - inflammatory activity, as well. Further studies may help elucidate which are the components that contribute to the immunomodulatory activity compared to the antimalarial activity. The results indicated also that the extracts from *G. sokotensis* and *V.colorata* have a weak antiplasmodial activity and are slightly toxic to normal human fibroblasts. *G. sokotensis* extract had a moderate antitumor activity when tested against melanoma cells, with IC_{50} lower than that on HDF. In conclusion, *G. sokotensis* does not express antiplasmodial activity but could be further investigated for its antitumor activity.

PART II: PHARMACODYNAMIC STUDIES OF ANTIMALARIAL DRUGS: GROWTH INHIBITORY ASSAYS, SLOPE FACTOR, TIME TO KILL AND STAGE-SPECIFITY ASSAY

1 INTRODUCTION

The elimination of malaria is now considered a realistic goal because of good surveillance and high intervention coverage between 2000 and 2007 which resulted in the reduction of malaria cases and deaths by 50% or more in some countries and regions of Africa [7]. An early treatment with effective anti-malarial drugs is the main life-saving intervention but the spreading of *P. falciparum* strains resistant to drugs that were once effective is posing a tremendous challenge to malaria control. Recognition of this problem by the international community and the engagement of the pharmaceutical industry in Public Private Partnerships has catalyzed the concerted search for novel targets and new antimalarial drugs [94]; [95]. Increased effort in antimalarial drug discovery are needed to achieve the objectives of the Global Malaria Action Plan (GMAP) namely the reduction in the burden of malaria in the mid-term and the elimination followed by the global eradication by 2050 [96]. New tools are absolutely needed for such ambitious goals.

However, drug discovery has main concerns. Due to the continuous increase in time and cost of drug development and the considerable amount of resources required by the traditional approach, companies can no longer afford to continue to late phase 3 with drugs which are unlikely to be therapeutically effective. The challenge for the pharmaceutical industry is to slash its research and development costs by achieving a significant cut in the attrition rate for drugs entering preclinical and clinical development to reduce the development time and to increase the probability of success in later clinical trials by streamlining the development processes [97]. At present. the scientific community has already generated thousands of antimalarial hits [98]. However, a rigorous selection process for picking candidate compounds out of the pool and a quick kill process for the candidate is mandatory to avoid wasting time, energy and money.

Antimalarial drug development is constrained by the same factors as any drug development program in that new agents must demonstrate efficacy, be safe and in the case of malaria, they have to be cheap, since there is a major need for widespread treatment of malaria in developing countries. To be widely useful, antimalarial drugs must be very inexpensive (<$1) and thus available to populations in need. However, since the markets for antimalarials are primarily in poor countries, marketing opportunities and returns have generally been considered to be limited, and so investment in antimalarial drug discovery and development has been small [99]. Thus, drug discovery directed against malaria is particularly reliant upon shortcuts that may obviate excess cost [100].

Pharmacokinetic/pharmacodynamic (PK/PD) knowledge has been critical for the drug discovery since it gives preliminary information on the efficacy, the safety and the dosing of a new compound. PK describes the time course of plasma concentration of a drug resulting from administration of a dosage regimen and accounts for its absorption, distribution, metabolism and excretion in the body. PD describes the intensity of drug effect in relation to its concentration. It is the study of the biochemical and physiological effects of drugs on the body or on microorganisms or parasites within or on the body [61]. PD studies look at mechanisms of drug action and the relationship between drug concentration and drug effect.

To avoid wasting time, and money some PK/PD studies in antimalarial drug discovery need to be taken into account as for the discovery programmes of other antimicrobials [61]. Indeed, in recent years, integrated PK/PD approaches have been developed as a more sophisticated methodology to assess the efficacy of antimicrobials based on dynamic kill curves. These PK/PD-based approaches are being used to guide the selection of potential drug candidates and to provide a scientific rationale for selecting dosing regimens that increase the efficacy and reduce the probability for the emergence of drug resistance [101].

The knowledge of the antimalarial PK/PD properties is fundamental to optimizing the therapeutic regimens [102]. Tin-Chao indicated that PK per se determines neither efficacy nor toxicity whereas PD plays much more critical role [103] since it allows an adequate prediction of the magnitude and rate of the drug's subsequent biological effects [104]. Pharmacodynamic data can help define the clinical potential of a new drug and identify the strengths and weaknesses. Furthermore. pharmacodynamic experiments can help focus the clinical phases of drug development by providing key information on the pharmacodynamic parameters that influence efficacy and the pharmacodynamic targets that should be achieved to optimize clinical success. Characterization of these pharmacodynamic properties for a new drug in development can help direct the design of the best dose and dosing strategy for clinical trials [105].

The preclinical development of a drug is that part of drug development occurring before the drug enters human trials. The elements associated with preclinical development include in vitro and in vivo studies of efficacy, safety, pharmacokinetics, and pharmacodynamics in order to produce pharmaceutical grade

materials and to make effective submission for investigational new drug status with the US Food and Drug Administration (FDA) and other regulatory authorities [106]. Any new therapeutic agent that has shown activity in *in vitro* and *in vivo* study still needs to be thoroughly investigated in order for it to move successfully from the laboratory to the clinic. For example. the pharmacodynamics properties of antimalarial drugs are relevant to the prevention of resistance [107].

The PD features of a drug are therefore an important predictor of clinical outcome. However, at present. which pharmacodynamic features are best at predicting clinical outcome for an antimalarial at this stage are not known. In addition, the in vitro assays that best measure some of these pharmacodynamic features (e.g. time to kill) are also hitherto not known. The time to kill curves are the profiles that follow the antimalarial killing and growth as a function of both time as well as antimalarial concentration. The use of the in vitro model is crucial for the early drug discovery and development processes. This technique is generally applied during the earliest stages to identify new targets (target discovery) and lead compounds (drug discovery) as well as for subsequent drug development. This *in vitro* model is also used to resolve equivocal findings from the in vivo studies in laboratory animals, to guide selection of the most appropriate preclinical in vivo models and help to define the mechanistic details of drug activity and toxicity [108]. Indeed, in vitro PK/PD models have been shown to be some useful tools to evaluate the efficacy of new anti-TB drugs under development: they come with benefits of reduced cost, flexibility and adaptability. Therefore, they are an important guide for the early drug development process [61]. Two in vitro PK/PD models have previously been defined these are the static models and the dynamic models. The static models used in the study evaluate how a culture responds to a constant environment with a fixed antibiotic exposure. Since there is no exchange of medium in these systems and the culture utilizes the same medium during the course of the experiment, its growth is limited by nutrition, space, aeration and toxic metabolites in addition to the effect of the drug. However, the dynamic models involve a periodic administration of the drug with varying concentrations. The ability to prioritize inhibitors based on their pharmacodynamic features will become more essential in order to reduce the cost and time of drug development.

Currently, most antimalarial drugs target the asexual erythrocytic stage of the parasite. as this leads to the clinical symptoms of disease. The major classes of antimalarials include: (i) quinolines (e.g. chloroquine and amodiaquine) which target the parasite's haemoglobin digestion. (ii) antifolates. that target pyrimidine biosynthesis through the inhibition of the key enzymes dihydrofolate reductase (DHFR, by inhibitors such as pyrimethamine and proguanil) and dihydropteroate synthase (DHPS, by inhibitors such as sulfadoxine and dapsone); (iii) quinolones. e.g. atovaquone targeting the mitochondrial bc1 complex; and (iv) endoperoxides. e.g. artemisinin and its semi-synthetic derivatives e.g. artemether, arteether, artesunate.Using the above antimalarials which have distinct modes of action, the objective of this study was to develop new tools to measure in vitro the pharmacodynamic features of different compounds. In order to do so we compared a variety of existing and novel in vitro assays including growth inhibitory assays (with measured IC50 and IC90 variables), time to kill kinetics and high content in vitro assays able to measure the stage specificity of antimalarial action.

1.1 Chemoprophylaxis and Chemotherapy

Malaria control requires an integrated approach that ideally should include vector control and measure treatments with effective, possibly transmission blocking drugs, such as ACT and chemoprophylaxis. The latter is particularly important to protect high risk groups from the disease and from its severe evolution to cerebral malaria and death. Chemoprophylaxis through intermittent preventive treatment (IPT) is recommended by WHO for women during pregnancy. Sulfadoxinepyrimethamine (SP) is preferentially used (three tablets of SP in a single dose) and given during prenatal visits to pregnant women with more than 16 weeks of gestation, in two doses, separated by one month to healthy women and three doses to HIV-positive subjects. In children. IPT with SP is administered during the first year of life during Expanded Program on Immunization campaigns. Recent trials on IPTi (IPT in infants) with sulfadoxine-pyrimethamine have demonstrated considerable protective efficacy reducing clinical malaria by 30%. all-cause hospital admissions by 24%. malaria-related hospital admissions by 37% and anemia by 15% [109].

In addition, in some studies conducted by Garmer and Gulmezoglu, IPT with SP was found to reduce antenatal parasite prevalence and placental malaria when given to women whatever the parity status. It also was shown to have positive effects on birth weight and possibly on perinatal death in low-parity women. Due to financial constraints National Malaria Programs promote and implement chemoprophylaxis only for young children and pregnant women. However, there is an urgent need to extend the prevention measure also to other high risk groups such as immigrants, Refugees, individuals with sickle cell anemia or HIV/AIDS and visitors from malaria free countries or from low transmission areas [110]. Whatever the epidemiological

characteristics of a given setting, access to effective treatment remains the most important factor to reduce malaria mortality. Drugs derived from *Artemisia* plants hold enormous potential for combating the global malaria epidemic. Following the recommendations of WHO, Burkina Faso and other sub-Saharan Africa countries have adopted artemisinin-based combination therapy (ACT). as first line treatment for simple cases of malaria whereas severe malaria cases must always be treated with quinine. Although tools to reduce substantially the global burden of malaria are available in remote areas, accessibility and the cost of these tools remains a big obstacle for their implementation.

Effective malaria control interventions such as the use of insecticide-treated nets (ITNs), artemisinin-based combination therapies (ACTs) and intermittent preventive therapy (ITP) still do not reach all who would are in need; much remains to be done to achieve the aim of 80% ACT and ITN coverage as set by WHO for 2010. However, the recognition and rational integration of complementary and alternative medicine into the national health systems may contribute to reach this aim. Given the fact that currently 80% of the world population makes use of traditional medicine for malaria management.

The current antimalarial drugs for malaria treatment recommended by WHO include artemisinin based combination therapy (ACT), chloroquine, amodiaquine, pyrimethamine and sulfadoxine and mefloquine. Furtheremore. the available antimalarial fall into five(5) broad groups such as the quinolinene-related compounds, the antifolates the artemisinin compounds, the tetracyclines and the napthioquinone and target different stages of the malaria cycle albeit the majority of them act on the intra-erythrocytic phases of development of the malaria parasite [111].

Several antibacterial drugs including sulphonamides and sulphones , the tetracyclines and macrolides, also have antiplasmodial activity. although in general their action is slow and they are used in combination with other antimalarial drugs. The tetracyclines are particularly useful in that they retain activity against multidrug resistant parasites [112].

1.1.1 Quinoline antimalarials

Cinchona alkaloids

The first quinoline antimalarial drugs were alkaloids extracted from the cinchona tree. which was introduced from South America into Europe by the Jesuits. Quinine has remarkable antimalarial properties. is still one of the most important drugs in treating of uncomplicated malaria particularly when combined with other drugs [113] and often the first therapeutic choice for the treatment of severe malaria as it can be administrated intravenously. intramuscularly and rectally [114].

The mode of action of quinine is not fully elucidated. Quinine is however. known to accumulate in the acid food vacuoles of malarial parasites and may inhibit the parasite haem polymerization process [111]; [115]. Structure-activity studies suggest that the conformation around the atoms C-8 and C-9 of the cinchona alkaloid. particularly the direction of aliphatic N-H and (9C) O-H bonds relative to each other. are crucial to antimalarial activity [116]. Quinine has relatively low potency and a narrow therapeutic range [117].

8-aminoquinolines

The 8-aminoquinolines were the first drugs to be synthetized for use against malaria. Currently. primaquine introduced in 1950 as an antimalarial. is the only available drug for the treatment of exoerythrocytic stages of *P. vivax* and *P. ovale* while acts as a gametocytocidal drug in *P. falciparum* infections [118].

4-aminoquinolines

Chloroquine (CQ) (figure 1) has been one of the widely used antimalarials due to its high efficacy. low toxicity. low production cost and high availability. Unfortunately. massive administration of this drug in single drug regimens in malaria eradication campaigns during the 1950s and 1960s led to the emergence of chloroquine-resistant parasites that spread in all areas where malaria was endemic. The 4-aminoquinolines antimalarials were mainly schizontocidal are weak bases diprotonated and hydrophilic at neutral pH. whereas the 8-aminoquinoliones antimalarials are gametocidal. weaker bases and lipid soluble at neutral pH. More likely to reduce malaria transmission the 8-aminoquinoliones antimalarials became a more promising approach for malaria treatment and control [119]. They were found to be effective against asexual parasites of *P. vivax* in the liver and blood as well as sexual blood forms but lacked activity against asexual stages of *P. falciparum [120]*. Despite years of use. the mechanism of action of the resistance to the quinoline drugs is far from being resolved. Various mechanisms have been proposed for the action of chloroquine and related

compounds. either extra- or intravascuolar [121]. A newer generation of quinoline related antimalarials that emerged as a response to the increasing problem of drug resistance include the development of mefloquine (quinoline methanol) lumefantrine and halofantrine (phenanthrene methanol).
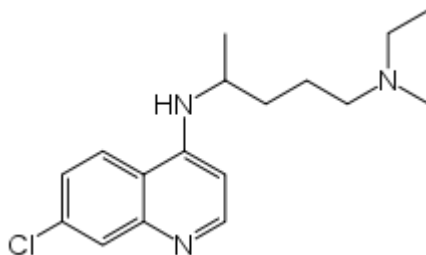

Figure 1. Chemical structure of chloroquine

Synthetic quinoline methanols

The quinoline methanols, structural analogs of quinine, were the most promising group of compounds. Mefloquine has efficacy against the late trophozoite stage of all species of human malaria. The mode of action of mefloquine is unknown, but may be similar to quinine due to the structural similarity [122]. Mefloquine has a very long plasma half-life (15-33 days and a mean of 21.4 days) and this makes it a practicable drug for chemoprophylaxis [123]. Unfortunately, mefloquine is a non-competitive inhibitor of acetylcholinesterase and butyrylcholinesterase, which is thought to account for some of the gastrointestinal (e.g nausea and vomiting) and central nervous system toxicities (e.g hallucinations and disorientation) seen at relatively high drug concentrations [124]. Mefloquine-artesunate is effective for the treatment of *P. falciparum* malaria and also prevent more new infections [125].

Phenanthrene methanols

This is another important class of compounds in which the quinoline portion of the 4-quinoline methanols is replaced by a different aromatic ring system to form the aryl amino alcohol e.g. halofantrine and novel lumefantrine. Halofantrine is effective against the erythrocytic stages of all four human Plasmodial species. Both *in-vitro* and *in-vivo* studies suggest that it has activity against chloroquine-resistant parasites and has similar antimalarial potency to mefloquine [126].

Proposed mode of action of quinolines and related compounds

Quinine (QN), extracted from the bark of the Cinchona tree, takes its name from the Inca word for this substance (quina-quina; bark of barks). Its structure is built upon the quinoline ring system, as are those major synthetic derivatives chloroquine (CQ), amodiaquine (ADQ), Primaquine, piperaquine (PIP) and mefloquine (MQ). Two other important antimalarial, more loosely related to QN structurally are halofantrine (HF) and lumefantrine (LMF) based on phenanthrene and fluorine ring systems, respectively [127]. All of these compounds, with the exception of primaquine (used primarily to combat *P. vivax* infections), are thought to share a common target: the formation of the parasite-specific substance hemozoin., and thus the detoxification of haemoglobin –derived heme, although additional targets may also be involved in some cases.

The 4-aminoquinoline CQ became clinically available in 1947 as a less toxic and more readily produced substitute for quinine. CQ acts by interfering with the sequestration of toxic heme (ferriprotoporphyrin IX) moieties produced when hemoglobin is digested the intra-erythrocytic parasite to obtain a major portion of its amino acid requirements. The heme, in the form of hematin dimers, is normally complexed by a process of biomineralization into hemozoin. which is deposited in a crystalline form in the lysosome like acidic food (or digestive) vacuole (pH≤5.5), into which the hemoglobin is initially imported by endocytosis [128]. CQ and related drugs bind to heme, preventing the detoxification process of dimerization and crystallization. and producing complexes that are detrimental to both membranes and enzymes [129]. These are ultimately lethal to the parasite [130].

Pharmacokinetic/Pharmacodynamic of quinolines and related compounds

Quinine, a Cinchona alkaloid with rapid antimalarial activity and relatively short half life (10–18 h), is mainly used at present for the treatment of severe malaria or as a last line chemotherapy for cases resistant to treatment with chloroquine and sulfadoxine/ pyrimethamine [131].

2.1.1   Antifolate antimalarial

Inhibition of enzymes of the folate pathway results in decreased pyrimidine synthesis, hence, reduced DNA, serine and methionine formation. Activity is exerted at all growing stages of the asexual erythrocytic cycle and on young gametocytes. Traditionally, antifolates are classified into two classes:

(1) Type-1 antifolates (sulfonamides and sulfones) mimic p-aminobenzoi acid (PABA). This kind of antifolates prevent the formation of dihydropteroate from hydroxymethyldihydropterin, catalized by dihydropteroate synthase (DHPS). (2) Type-2 antifolates (pyrimethamine, biguanides and triazine metabolites, quinazolines) inhibit parasite dihydrofolate reductase (DHFR), also a bifunctional enzyme in plasmodial coupled with thymidylate synthase, thus preventing the NADPH dependent reduction of H2folate (DHF) to H4folate (THF) by DHFR. THF is the necessary cofactor for the biosynthesis of thymidylate purine nucleotides and certain amino acids [111].

The biguanides, proguanil and chloroproguanil are metabolized *in vivo* to the triazine compounds cycloguanil and chlorcycloguanil, Respectively, which are structurally similar to the diamino-pyrimidine pyrimethamine. Proguanil and chloroproguanil can inhibit all malaria parasite stages thus making them useful to be used as causal prophylactics and effective in preventing the growth of sporogonic stages in the mosquito. Proguanil is synergistic in combination with atovaquone (Malarone™), a drug that inhibits parasite mitochondrial electron transport. Pyrimethamine (figure 2) is available only as formulations with either a sulphonamide (sulfadoxine,sulfalene) or for chemoprophylaxis, with dapsone. Pyrimethamine in combination with dapsone (as Maloprim or Deltaprim) has been used for the prophylaxis of mildly chloroquine-resistant falciparum malaria. Pyrimethamine with sulfadoxine (as Fansidar) or with sulfalene (as Metakelfin) is used for the treatment of uncomplicated chloroquine-resistant falciparum malaria. Fansidar is also used in combination with short-course quinine in the treatment of uncomplicated multiresistant *falciparum* malaria and in two doses during pregnancy for the intermittent presumptive treatment of chloroquine-resistant falciparum malaria in Africa [132].
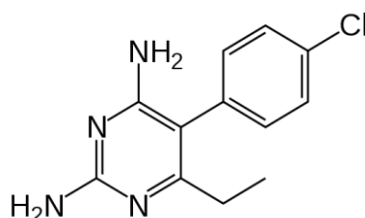

Figure 2. Chemical structure of Pyrimethamine

Pharmacokinetic/Pharmacodynamic of antifolates

Sulfadoxine-pyrimethamine is a fixed combination of a long-acting sulfonamide and the antifolate pyrimethamine. These are synergistic against sensitive parasites. Minor adverse effects are unusual. Serious sulfonamide toxicity is unusual with a single-dose treatment of malaria. The anti-folate properties of pyrimethamine rarely produce toxicity [133].

2.1.2   Artemisinin based antimalarial

Artemisia annua, the plant sweet wormwood, has been used for over 2.000 years as a Chinese herbal remedy. Artemisinin is a sesquiterpene lactone endoperoxide, first identified as the active anti-malarial constituent of *A. annua* and its derivatives were found to be the most potent of all anti-malarial drugs [134]. Furthermore, artemisinins are *in vitro* extremely effective against chloroquine and sulfadoxine-pyrimethamine resistant *P. falciparum* strain*s* and *in vivo* produce faster parasite clearance and fever resolution time than any other licensed antimalarial and consequently a rapid resolution of symptoms [135]; [136]. The parasite biomass is reduced by 10.000-fold per asexual life cycle, compared to 100- to 1000-fold for other antimalarial. Artemisinin and derivatives have a broader spectrum of activity than other anti-malarial drugs, extending from the young ring stage of parasite development through to the early schizont. They also

decrease gametocyte carriage by 90%. thus reducing transmission of malaria [111]. Artemisinin derivatives are chemically modified semisynthetic forms of artemisinin designed to improve the pharmacological properties of artemisinin: these are artemether arteether, artesunate and dihydroartemisinin (figure 3). Dihydroartemisinin (DHA) is both a drug on its own and the active metabolite of the other artemisinins Artemisinin based combination therapy (ACT) is now recommended as a first-line treatment worldwide and used to treat millions of patients each year [137].

However, artemisinins are limited by their short half-life, cost and safety concerns in pregnant women as they are reported to be embryotoxic in animal models [138], [139]. Indeed, Finaurini et al., using an *in vitro* model which reproduce human erythropoiesis (CD34+ from human peripheral blood) reported that artemisinin inhibits both cell growth and early erythroid differentiation [140] . D'Alessandro et al showed also that the inhibition of microvascular endothelial cell (HMEC-1) growth was induced by dihydroartemisinin (DHA) [141] This work reported that low doses of DHA (required for anti-angiogenic or anti-tumor activity) caused apoptosis of HMEC-1 and G2 cell cycle arrest. As for the antimalarial activity, the peroxide bridge of the artemisinins has been shown to be essential for cellular toxicity and for inhibition of erythroid maturation [141]. The mechanism of action is not completely understood and several hypothesis have been put forward. The prevailing theory is that reductive cleavage of the intact peroxide by ferrous-protoporphyrin IX (Fe(II)PPIX) generates C-centred radicals, which, in turn, alkylate biomolecules, leading to the death of the parasite [111] [142] [143]. However, it has also been shown that the haem iron is not absolutely needed for the antimalarial effect of artemisinin [144]. More recently, Haynes et al suggested that artemisinins interfere with the regeneration of reduced of flavins and NAD/NADPH co-factors leading to parasite death by excess oxidative stress [145]. The synthetic endoperoxides OZ 277 and RKA182 were the new developed antimalarials. The OZ277 the fully synthetic endoperoxide antimalarial was tested against field isolates from Gabon. A comparison of activities of OZ277 with artesunate, mefloquine, and chloroquine showed OZ277 to be highly active against all parasite isolates [146]. RKA 182 a novel 1,2,4,5-tetraoxane synthetized by O'neill et al., has been identified to have a outstanding antimalarial activity, stability, low toxicity [147].

Pharmacokinetic/Pharmacodynamic of artemisinin and derivatives

Artemisinin and its derivatives (e.gartesunate. artemether) belong to the sesquiterpene lactone group and possess a functional endoperoxide bridge. After oral administration the artemisinin derivatives are quickly absorbed and undergo fast metabolic transformation, resulting in short half-lives (0.5-1.4h) [148]. At the recommended dose regimens they are well tolerated and highly effective against multidrug-resistant *P. falciparum* being the fastest acting class of antimalarials available [149].
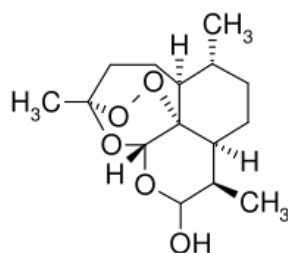

Figure 3. Dihydroartemisinin

2.1.3 Naphthoquinones

Atovaquone (figure 4) is a highly substituted naphthoquinone derivative representing a new class of drugs having a metabolic target different from existing antimalarial drugs. Atovaquone is a structural analogue of coenzyme ubiquinone in the mitochondrial electron transport chain and used therapeutically to treat *P. falciparum* [150]. Atovaquone binds to the ubiquinone binding side of the cytochrome bc1 complex and prevents translocation of an iron-sulfur cluster containing a protein domain that is required for electron transfer to cytochrome bc1 from ubihydroquinone bound to cytochrome b within complex III . Thus inhibition of cytochrome b complex III leads to the inhibition of the parasite's respiratory chain and consequently the blockage of pyrimidine biosynthesis causing ultimately parasite death [151].
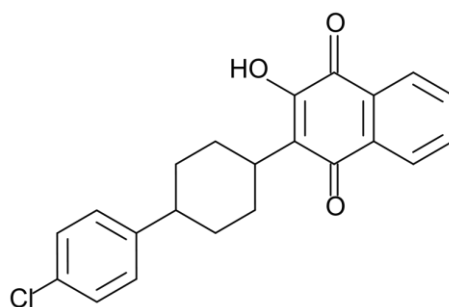
Figure 4. Chemical structure of atovaquone

Pharmacokinetic/Pharmacodynamic of atovaquone

Atovaquone is a hydroxynaphthoquinone is a highly lipophilic compound with low aqueous solubility. The bioavailability of atovaquone shows considerable inter-individual variability. Dietary fat taken with atovaquone increases the rate and extent of absorption. Atovaquone is highly protein bound (>99%) over the concentration range of 1 to 90 mcg/mL. The elimination half-life of atovaquone is about 2 to 3 days. It is a well-tolerated antimalarial drug, active against both chloroquine-sensitive and chloroquine resistant *P. falciparum* [152].

2.1.4   Antibiotic antimalarial

Some antibiotics have shown potent antimalarial activity and have been effective in the treatment of uncomplicated malaria *in vivo*. These are tetracycline, doxycycline, azithromycin, clindamycin and fosmidomycin [153]. Diamidines are good antiprotozoal agents. Pentamidine, the lead compound, is used against Trypanosoma and Leishmnia. It is active against plasmodium, but the advent of better antimalarials made the interest in diamidines decline. Since these antibiotics are well characterized, well tolerated and approved for human use, they provide an attractive alternative for the treatment of uncomplicated or even severe *P. falciparum* malaria when used in combination with a fast-acting antimalarial [112]. The most commonly used combination therapy involving antibiotics is quinine combined with tetracycline or doxycycline, a combination that has been used extensively as a second line therapy in several countries in Southeast Asia [64]. Clindamycin and azithromycin are shown to be highly active and generally very well tolerated antimalarials and they have been used in children and pregnant women [154]. The WHO now recommends the use of oral quinine plus clindamycin in all trimesters of pregnancy and artesunate plus clindamycin during the second and third trimester [49].

1.2 Combination therapy for malaria

The rationale for using a combination of drugs for treating uncomplicated malaria cases is based on the understanding that if the parasite is attacked on several fronts by drugs with independent modes of action then it will be less likely to develop resistance to each of the constituent drugs.

Drug resistance is commonly due to point mutations occurring in the parasite genome, though gene duplications can also contribute to resistance. If at least two drugs with a different mode of action and a different mechanism of resistance are used at the same time. the probability of the parasite surviving the treatment (i.e. having the resistant genetic profile with mutations conferring resistance to both drugs) is much lower than that for each of the drugs separately [107]. This is based on the assumption that there are about 10^{17} parasites worldwide and that the probability of developing *de novo* resistance to a given drug is 1 in 10^{12}. Therefore, a mutant that is simultaneously resistant to two drugs used in combination will arise every 1 in 10^{24} parasites. This is less than the total cumulative number of parasites in one year with the consequence that a resistant parasite will arise once every 10.000 years, assuming the drugs are always used in combination [155]. The investigations involving combinations of antimalarial drugs that will impede the selection of *P. falciparum* drug resistance, is currently focused on the use of a number of the artemisinin family in association with a relatively long-acting blood schizonticide, such as mefloquine. pyronaridine or lumefantrine. Artemisinins achieve a massive reduction of the parasite biomass but have a short half-life, whilst the long acting partner eliminates any survivors [156]. Four ACTs for the treatment of uncomplicated falciparum malaria, namely artemether-lumefantrine (AL). artesunate + amodiaquine (ASAQ), artesunate + mefloquine (ASMQ) and artesunate + sulfadoxine–pyrimethamine (ASSP), are currently recommended [157]. Indeed, Artemether-lumefantrine (coartemether; Coartem) (Novartis Pharma AG. Basel. Switzerland)

is currently the only fixed-combination ACT widely available. Each tablet consists of 20 mg artemether and 120 mg lumefantrine. The rationale for this combination is that artemether rapidly reduces parasite biomass, and the long-acting lumefantrine eliminates residual parasites [158]. More recently a novel fixed combination of antimalarial has been registered by EMEA. It is called Eurartesin, it contains DHA + pyronaridine and has been developed as a public-private partnership between Sigma-Tau and MMV(Nov 2011)

1.3 Potential targets on the developmental stages of the parasite life cycle

As summarized in table 1, the currently used antimalarials act on various developmental stages of the parasite life cycle.

Table 1: Classification of antimalarial drugs according to the stages of the malaria parasites [159].

Developmental Stages	Drugs
Intraerythrocytic schizontocidal	Quinine, chloroquine, amodiaquine, mefloquine, halofantine, pyronaridine, atovaquone, pyrimethamine, sulfadoxine, artemisinins, tetracycline, clindamycine and tafenoquinechloroquine
Tissue schizontocidal drugs	Primaquine, tafenoquine, proguanil, tetracycline and pyremethamine
Gametocytocidal drugs	Primaquine, tafenoquine, atovaquone, quinine, mefloquine, chloroquine, amodiaquine (*P. vivax, P. ovale, P. malariae* only)
Hypnozoitocidal drug	Primaquine
Sporotocidal drugs	Proguanil, chloroguanidine, pyremethamine and atovaquone

The antimalarial drugs target different stages of the malaria life cycle albeit the majority of them act on the intra-erythrocytic forms. The Plasmodium life cycle timing in the erythrocyte with phases targeted by antimalarial drugs is presented below (figure 5)
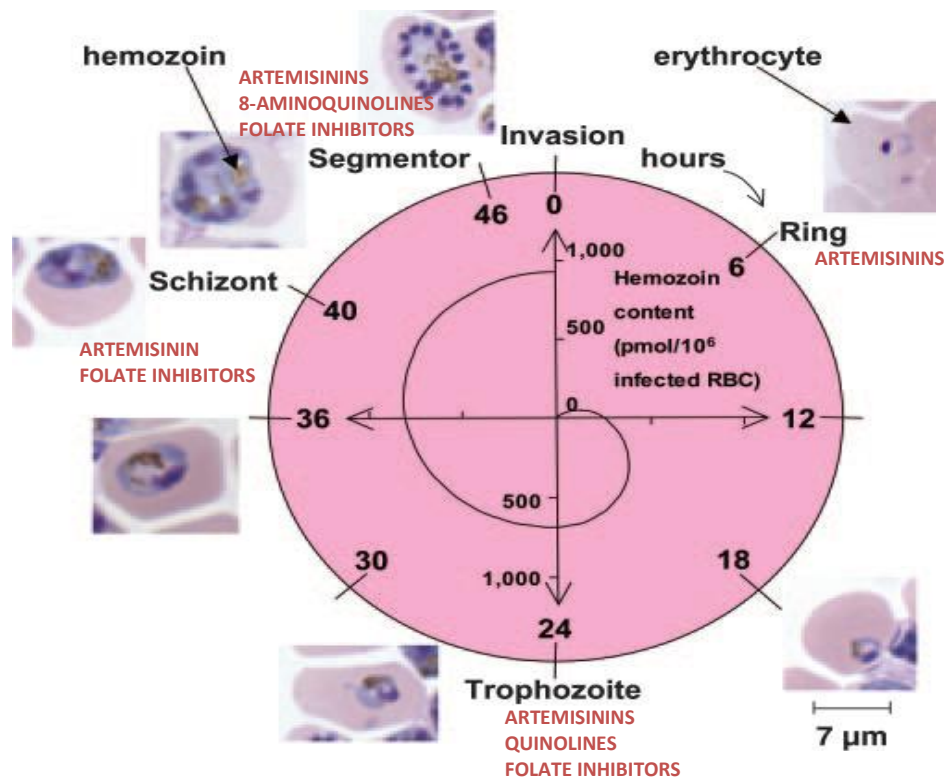
Figure 5. Schematic diagram of *P. falciparum* parasite life cycle timing in the erythrocyte Adapted from [160]

1.4 Resistance to antimalarial drugs

Resistance to antimalarial drug can be defined by the ability of the parasite strain to multiply or survive in the presence of concentrations of a drug that normally destroy parasites of the same species or prevent their multiplication. The control of malaria rests on the use of mosquito vector control and antimalarial chemotherapy. Artemisinin and its derivatives (artemether. arteether. artesunate) are used increasingly in Asia and Africa where multidrug-resistant *P. falciparum* is prevalent. They are rapidly effective and well-tolerated treatments. Combination anti-malarial therapy including artemisinins, has been advocated to improve efficacy and limit the spread of resistance [161]. However, the latter strategy is hampered by the emergence of antimalarial resistance. Currently. there is no antimalarial drug that malaria parasites have not developed resistance against. Worryingly, there is evidence that malaria parasites in Cambodia are becoming resistant to artemisinin derivatives [162] [66] providing a potential threat to artemisinin-based combination therapy (ACT).

This emphasizes the need to reduce drug resistance selection pressure by withdrawing monotherapies to develop new and affordable drug combinations, and to understand the mechanisms that underlie resistance to existing antimalarials [163]. This could lead to the development of simple means of tracking the selection and spread of resistance, and to the design and discovery of new and more potent antimalarials. To study mechanisms of resistance, one needs to obtain well characterized drug-resistant strains. However, such strains are not generally available for most antimalarials. Murine malaria *Plasmodium berghei. Plasmodium chabaudi. Plasmodium yoelii* and *Plasmodium vinckei* have been used as surrogates for *P. falciparum* to study the mechanisms of drug resistance by inducing resistance in vivo. This approach has led to the selection of drug-resistant parasite lines and subsequent studies on mechanisms of drug resistance by inducing resistance *in vivo* [164]. However, for drugs such as chloroquine and probably artemisinin, mechanisms of resistance in murine and *P. falciparum* malaria are different, highlighting the limitations of the murine malaria model [165] [166].

2. MATERIAL AND METHOD

2.1.Method of assessment of antimalarial drug activity

The parasites 3D7 were routinely maintained in continuous culture in RPMI 1640 medium supplemented with 5% albumax II. 1M HEPES. 4mM hypoxanthine in an atmosphere of 3% CO_2 and 4% O_2 with a balance of N_2. The resistant strain K1 was maintained with RPMI supplemented with AB+ human serum. 1M HEPES pH 7.4. Gentamycin (50 mg/ml) was added to avoid contaminations. Cultures were synchronized using sorbitol 5% to obtain mainly ring stage parasites after 48h prior to use for in vitro drug sensitivity assay in 96 well plates

The malaria SYBR Green I-based fluorescence (MSF) assay is used to monitor parasite growth and drug efficacy. SYBR Green is a fluorescent dye which binds to parasite DNA in infected erythrocytes. A linear relationship exists between parasitaemia and fluorescence intensity [167] (Johnson et al. 2007). Triplicate wells of parasitized erythrocytes (ring form or an asynchronous culture with 50% of rings and 50% of trophozoites) were diluted with noninfected erythrocytes at a constant 1% hematocrit and 2% parasitaemia in culture medium and with serial dilutions of the drugs 00ul final volume), After 48h incubation, 100µl of lysis buffer [20mM trizma base (pH 7.5). 5mM EDTA salt. 0.008% (wt/v) saponin, 0.08% (vol/vol) Triton X-100] containing SYBR Green I (1x final concentration) were added directly to the 96 well-plate to lyse the red blood cells to recover SYBR Green. The plates were then incubated for another hour at room temperature in the dark and examined for the fluorescence intensity by using the Varioskan spectral scanning multimode reader at 485/518nm; the Varioskan system is connected to a PC with Skanlt 2.0 software. Finally, the data analysis was performed by MS Excel. The drug concentration that inhibit 50% of parasite growth (IC50) was calculated and the drug concentration that inhibit 10%, 20%.,and 90% of parasite growth (respectively IC10, IC20, IC90) were derived from a non-linear regression analysis using the Grafit computer programme package.

The antimalarial drugs used were from different groups:

The aminoquinolines: chloroquine diphosphate (CQ), isoquine, dehydrofluoro4-amodiaquine (FAQ); the peroxides (dihydroartemisinin (DHA), arthemeter, RKA182, OZ277), the bc1 inhibitor (atovaquone),the folates synthesis inhibitors (pyrimethamine, cycloguanil) and an antibiotic (pentamidine).

2.2 Slope factor determination

For the inhibition of *P. falciparum* growth, IC50 is the drug concentration that produces half-maximal inhibition and SF is the slope factor, a parameter which describes the steepness of the dose-response curve. It is also called the Hill slope [62]. A dose-response curve with a standard slope has a Hill slope of 1.0 (figure 6). A steeper curve has a higher slope factor, and a shallower curve has a lower slope factor. The figure 6 represents the dose-response curve of an agonist (is a drug that causes a response) and the variation in the slope [168]. The slope is also a measure of cooperativity in the binding of multiple ligands to the binding sites.

Some drugs can have the same IC50 values but not the same slope. Higher slope achieves much greater inhibition at relevant clinical concentration [62]. We therefore hypothesized that slope factor might have a substantial effect on antimarial activity. To test this hypothesis, we obtained dose-response curves for antimalarial drugs using the Sybr green fluorescent method. Parasite growth inhibition values and corresponding drug concentrations from at least 3 different experiments were used to plot the curve and to calculate the IC50 and the slope. The slope factor has been determined graphically using the Grafit computer programme package or Kaleidagraph.
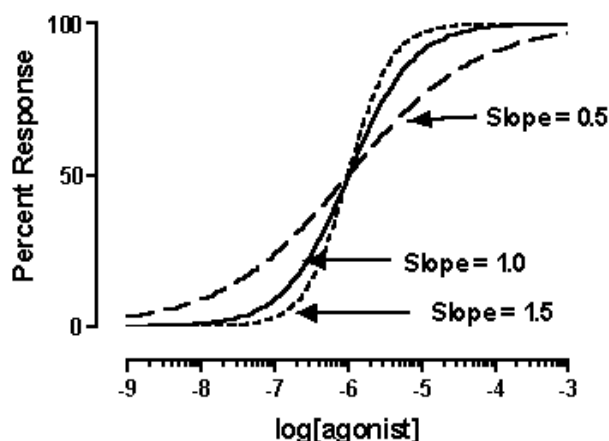
Figure 6. Representing the dose-response curve of an agonist and the slope variation [168]

2.3 Determination of the time-dependency action

An *in vitro* model has been set up to characterize the time required to achieve irreversible drug toxicity against *P falciparum* parasites following CQ, DHA, pyrimethamine and atovaquone treatment. Four flasks were set up for each drug tested and another flask was used for the negative control. Culture of *P. falciparum* 3D7 or K1 strains were used as the target parasite. Synchronous parasites at 1% hematocrit and a 2% parasitaemia were added to 25ml of complete medium in each flask destined to drug pressure and 50ml for the negative control. The four flasks containing the parasites were exposed to a final drug concentration corresponding to their IC10, IC20, IC50 and IC90 as calculated from the dose response curves using the Grafit computer programme package. The fifth flask which is the drug free control was maintained under the same condition. Subsequently, 2ml per flask were carefully removed at time intervals 0, 1, 3, 5, 8, 12, 24, 48 hours post drug exposure. The aliquots were aseptically washed x3 times with 10ml of complete medium at 2500 g for 5min. After the last washing, the medium was completely removed and the parasitized RBC resuspended in 2ml of complete medium in order to maintain the 1% initial hematocrit. Aliquots of 50µl of these samples (in triplicate) were then distributed into 96 micro-well plates and incubated at 37 C for 48 hours. At the end of 48h of incubation, 100µl of the mix lysis buffer-Sybr Green were added and incubated for another hour. The parasites viability at the various drug concentrations was assessed by the Sybr green I-based fluorescence. The time –kill curves were constructed based on the time course of the control growth (drug-free control).

2.4 Stage-specificity assay

2.4.1 Operetta machine

The High Content Screening (HCS) platform integrates fluorescent microscopy, application software and bioinformatics with necessary instrumentation to automate cell analysis. The automated screening and analysis of cells provide resolved information and extracts features at the cellular and subcellular level. Thus. HCS allows target identification. the evaluation of the cytotoxicity and many drugs can be screened on its platform in one time. The image based analysis identifies compounds which inhibit parasite proliferation and the concerned arrested stage. The automated cellular systems allows the rapid visualisation of infected and uninfected red blood cells. The Perkin Elmer Operetta High Content Screening microscope (annexe 1) is a 96-384 well spinning disk confocal microscopy system, that has live cell imaging capacity and an automated quantitative image analysis (Harmony). It can perform many types of assay followed by automated image analysis -including cell cycle, apoptosis, cell shape, G-proteins, cytoskeletal reorganisation, texture, membrane texture, nuclear foci. The image based antimalarial screening assay has proven to be stable, riable and sensitive. This assay is capable of detecting both early and late (mature) antimalarial compounds allowing further biological profiling [63].

2.4.2 Stage specificity activity-Parasite imaging

The Operetta was set up to be able to distinguish between all of the stages of the intra-erythrocytic development, using SYBR green to stain for nuclei and TMRE to stain for the cell membrane.

After incubation 48h, 72h and 96h, the cultures were taken out of the incubator. The lysis buffer-Sybr green has been added to the triplicate test wells, while the last row of the 96 well plate has been used for the imaging of the parasites using the TMRE (tetramethylrodhamine, ethyl ester, perchlorate) staining. The accumulation of TMRE was used as an indicator of parasite viability. Indeed, 100µl of the mix Sybr green (1µl/ml) and the TMRE (400nM) (Invitrogen) was added to each well. Then the culture has been mix well and 50µl from each well has been transferred into 384 well-plate (PerkinElmer 384 well CellCarrier) and proceeded to the imaging. A suitable well layout (number of image fields and image field pattern) has been selected for imaging applications. A measurement using one image field per well at x40 or x100 magnification. The area of the well that is illuminated during image acquisition is slightly larger than the image field. The proportion of young and mature parasites in each well was plotted against drug exposure.

3. RESULTS

3.1 In-vitro sensitivity of different *P. falciparum* isolates to antimalarial drugs

The IC50 represents the concentration of drug at which 50% inhibition of growth is achieved in vitro. The Table 2 shows the sensitivity of CQ-sensitive *P. falciparum* strain (3D7) and CQ-resistant strain (K1) to different antimalarial drugs The assay was conducted for 48 hours measuring SYBR green fluorescence. The results show that atovaquone and the peroxides antimalarials, which include DHA, artemether, RKA 182, and OZ277, are highly potent against both CQ-sensitive and CQ-resistant strains, with mean IC50 values less than 10nM. As expected, the aminoquinolines are more potent on the CQ-sensitive strain with IC50 less than 10nM than on CQ-resistant strain. The cycloguanil and pyrimethamine are effective at nM concentration against both strains. The results also show an inverse relationship between the CQ-sensitivity pattern and that of pentamidine. The CQ-resistant strain shows greater sensitivity than the CQ-sensitive to pentamidine.

Table 2: In vitro antimalarial activities of selected compounds versus CQ-sensitive and CQ-resistant *P. falciparum* strains. Data also shows ± SD from at least 5 independent experiments.

DRUGS	IC50 (nM) 3D7 (CQ-S)	IC50 (nM) K1 (CQ-R)
CQ	7.4 ± 2.6[a]	91.7±25.1
Isoquine	7.3±1.8	22.3±7.7
Amodiaquine	5.2±0.5	41.4±12.6
Dehydro-4-fluoroamodiaquine (FAQ)	6.3±2.6	34.8±13.5
DHA	3.4±1.2	2.7±1.3
Artemether	4.4±1.4	2.2±1.4
RKA 182	1.3±1.1	1.3±1.5
OZ277	6.1±1.3	6.0±2.3
Atovaquone	0.8±0.1	3.2±1.9
Cycloguanil	12.4± 2.6	14.7±6.9
Pyrimethamine	55.0±21.3	ND
Pentamidine	151.2±55.7	90.5±43.6

[a]The assay was performed using the SYBR Green assay for 48 has described in M&M

We then plotted the dose-response curves of CQ, DHA, atovaquone and pyrimethamine against 3D7,CQ-S (Figure 7A), and K1 (Figure 7B), CQ-R strains of *P. falciparum.* The results indicate that atovaquone and DHA are the most potent compounds at all the doses tested against both strains (IC50 less than 5nM). Vice versa, the dose-response curve of CQ against the CQ-R strain, K1 is significantly shifted to the right, IC50>90 nM , compared to that against the 3D7, CQ-S strain
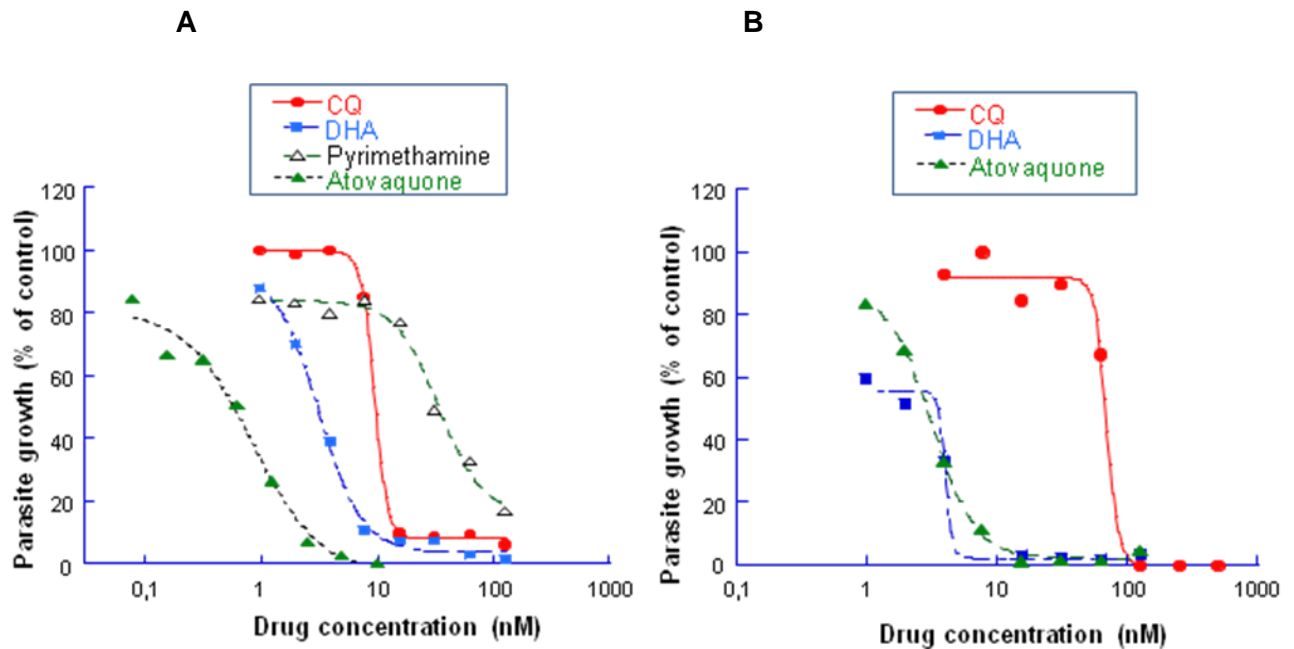

Figure 7. Dose-response curves of CQ- sensitive 3D7 (A) and CQ- resistant K1 (B) strains of *P. falciparum* to CQ, DHA, atovaquone and pyrimethamine assay using the SYBR green test for 48 h.

3.2 Comparison between the IC50 data and the slope factor

From the dose-response curves against CQ-R and CQ-S parasites, we calculated the slope factor of each drug and we compared them with the IC50 values (Table 3). Each drug class had a characteristic slope. The slope of quinoline-related drugs were between 6 and 9. The peroxides had a slope around 3, and the antifolates showed a slope close to 2. The napthoquinolone and the antibacterial had a slope of 1.4 and 1.3, respectively. In our study all the drug classes has a slope >1. All the data used to calculate the slope factor for CQ, DHA, atovaquone and pyrimethamine are shown in Figure 8. The growth curves plotted together indicated that the mean IC50 value for CQ was 7.4 nM with a slope factor of 6.1. For DHA the IC50 value was 3.4 nM and the slope factor was 2.4. The atovaquone and the pyrimethamine had an IC50 value and a slope factor of 0.8 nM and 1.4and 55 nM and the slope factor was 2.1, respectively. Thus antimalarial drugs acting through different mechanisms show distinct slope values. The slope factor might influence the antimalarial activity.

Table 3. The representative IC50 value and slope factor of all antimalarial drugs against CQ-sensitive *P. falciparum* 3D7 isolates

Drugs	IC50 (nM)	Slope Factor (SF)
CQ	7.4 ± 2.6 [a]	6.1 ±3.9
Isoquine	7.3±1.8	7.7 ±1.8
Amodiaquine	5.2±0.5	5.7 ±1.5
Dehydro-4-fluoroamodiaquine (FAQ)	6.3±2.6	7.2 ±1.5
DHA	3.4±1.2	2.4 ±5.5
Artemether	4.4±1.4	3.4 ±0.9
RKA 182	1.3±1.1	2.7 ±1.3
OZ277	6.1±1.3	3.6 ±3.7
Atovaquone	0.8±0.1	1.4 ±1.4
Cycloguanil	12.4± 2.6	1.9 ±0.6
Pyrimethamine	55.0±21.3	2.1 ±0.3
Pentamidine	151.2±55.7	1.3 ±0.02

[a]The assay was performed using the SYBR Green assay for 48 has described in M&M
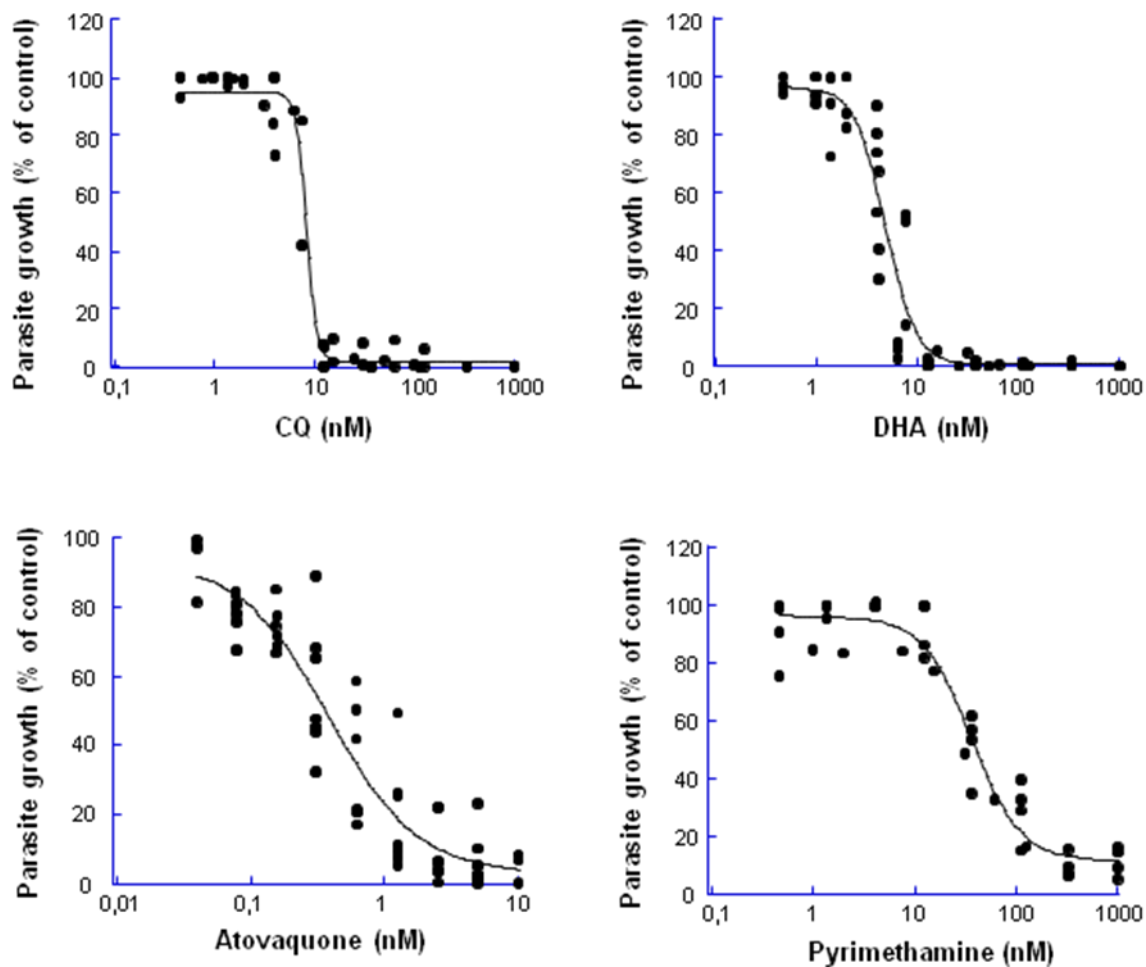
Figure 8. Dose-response curves of the effect of CQ, DHA, Atovaquone and pyrimethamine on the growth of the CQ-sensitive 3D7 strain of *P. falciparum* in vitro. Data from at least 3 independent experiments have been plotted together. Every single experiment has been performed using the SYBR green test for 48h.

The table 4 presents the preliminary data of IC50 (nM) and the slope factor of drugs against an asynchronous CQ-sensitive *P. falciparum* 3D7 strain observed after 3 different time of incubation (48h, 72h and 96h). It can be seen that the IC50 values of CQ are increasing from 48hrs to 96hrs whereas the SF decreases. In terms of DHA, both IC50 and SF do not change significantly during the 72 h assay. For the atovaquone the IC50 is slightly decreasing but the slope factor is increasing slightly from 72 to 96hrs. The slope factor for all drugs in this study is more than 1 suggesting that binding does not follow the law of mass action with a single site and the ligand binds cooperatively to a multivalent receptor.

Table 4: IC50 concentrations (nM) and slope factor data of drugs against an asynchronous CQ-sensitive *P. falciparum* 3D7 strain. The data are from 48h, 72h and 96h of incubation.

DRUGS	48H		72H		96H	
	IC50 **(nM)**	Slope Factor	IC50 **(nM)**	Slope Factor	IC50 **(nM)**	Slope Factor
CQ	7.9	7.2	10.8	4.5	11.3	6.8
DHA	3.9	3.2	3.7	3.6	ND	ND
ATOVAQUONE			0.4	1.1	0.3	1.5

The assay was performed using the SYBR Green assay for 48, 72h and 96h.

3.3 Characterisation of the time required to achieve irreversible drug toxicity against plasmodium parasite following chloroquine. atovaquone and dihydroartemisinin treatments.

The geometric mean IC_{10}, IC_{20}, IC_{50} and IC_{90} of CQ, DHA, atovaquone and pyrimethamine derived from linear regression analysis in 3D7 and K1 parasites used to set up the time to kill assays, are summarized in table 6.

Table 6: The geometric mean IC_{10}, IC_{20}, IC_{50} and IC_{90} of CQ, DHA and atovaquone derived from linear regression analysis in 3D7 and K1 parasites

	IC_{10} (nM)		IC_{20} (nM)		IC_{50} (nM)		IC_{90} (nM)	
Strain	*3D7*	*K1*	*3D7*	*K1*	*3D7*	*K1*	*3D7*	*K1*
CQ	7.9±4.9	57.6±11.1	8.6±4.6	69.7±12.8	10.1±4.7	91.7±25.1	14.3±3.0	149.3±81.8
DHA	1.2±0.2	1.6±0.9	1.6±0.4	1.7±1.2	3.8±0.5	3.2±1.1	10.1±4.5	6.7±0.5
Atovaquone	0.3±0.1	1.1±0.2	0.3±0.1	1.5±0.6	0.7±0.1	3.4±1.6	2.0±0.3	8.2±2.5
Pyrimethamine	12.6±6.4	ND	21.2±11.4	ND	37.8±18.2	ND	81.±15.4	ND

IC_{10}, IC_{20}, IC_{50} or IC90 is the drug concentration giving 10%, 20%, 50% and 90% inhibition of parasite growth.

The time dependent inhibitory effects of the drugs is reported in Figure 9. The untreated cultures were used as control. The results of the treated cultures of 3D7 indicate that DHA is a fast acting antimalarial (effective at less 10nM concentration) with remarkable effects in the first hour of exposure and that after 5hours of exposure, the parasites are irreversibly damaged with drug concentration less than 4 nM (refer to table 6). Furthermore, there is not significant parasite survival at IC10 and IC20 after 12hrs of exposure to DHA.

CQ displays potent inhibitory effects but at a drug concentration more than 7 nM. As DHA, the inhibitory effect of CQ occurs rapidly with noticeable effects seen at less 5hrs and the parasites are irreversibly inhibited after 12hrs of exposure to the drug. There is no significant residual parasite growth at the IC10 and IC20 concentrations. Pyrimethamine is not a fast acting antimalarial drug only effective after 12hrs of exposure when parasites are irreversibly damaged. Atovaquone is a highly potent (effective at less 4nM) and a fast acting antimalarial drug with remarkable effects at less 5hrs of exposure. However, atovaquone is slower acting than DHA and CQ.
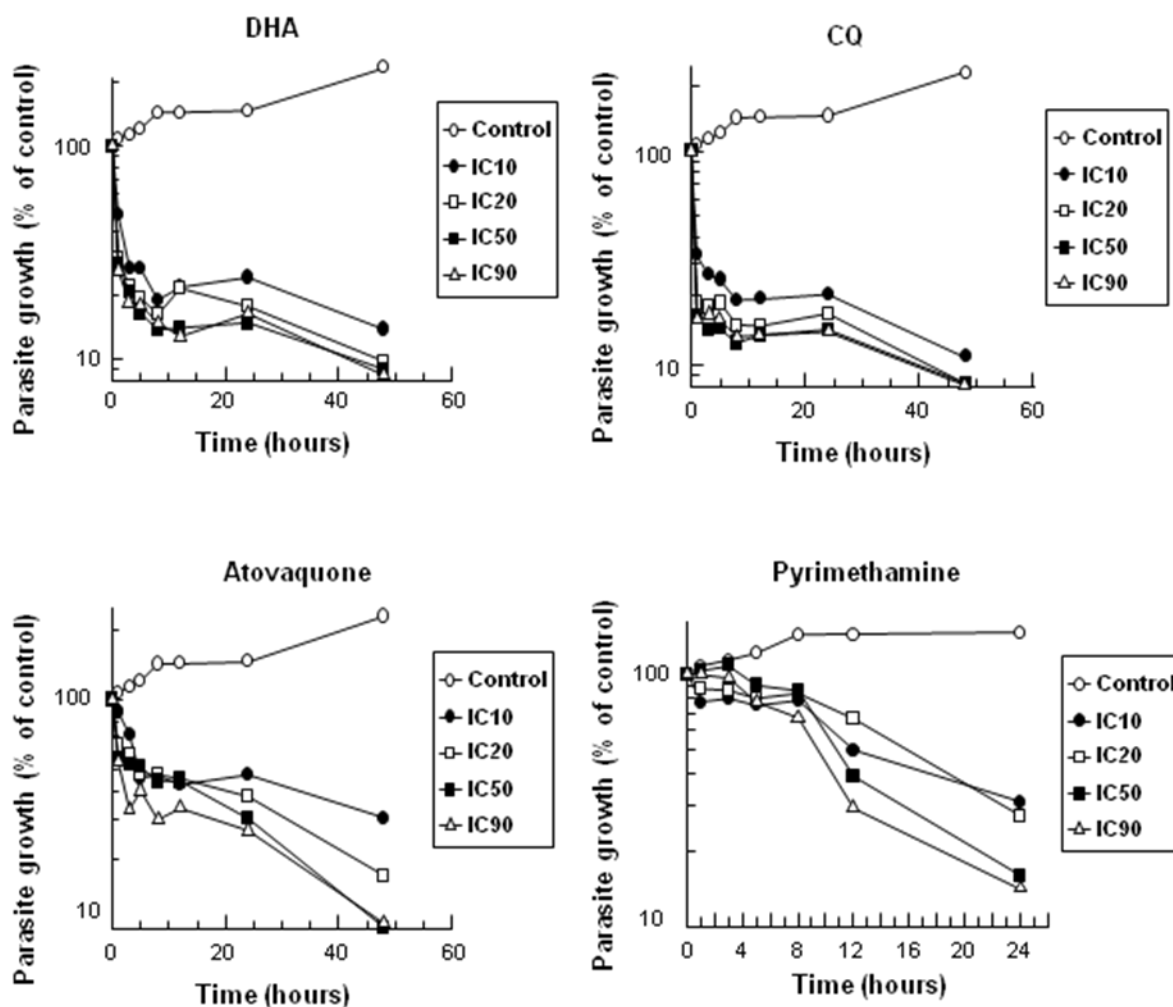
Figure 9. Time dependent effect of CQ, dihydroartemisinin, atovaquone and pyrimethamine, on 3D7 strain. The parasites viability was assessed by the Sybr green I-based fluorescence and the time –kill curves were constructed based on the time course of the control growth (drug-free control).

Similar time-dependent inhibitory effects of CQ and DHA, at varied concentrations, were observed using the CQ-R, K1 strain (Figure 10). For the DHA, a noticeable effect can be observed with the IC50 and IC90 before 5h. The parasite growth is strongly inhibited and parasites are irreversibly damaged before 5hours of incubation. However, different results were obtained with CQ which is less active on the CQ-resistant strain. A noticeable effect was only seen using the IC90 and the parasites were irreversibly inhibited after 5 hours of incubation. Significant residual parasite growth was observed for the concentrations corresponding to the IC10, IC20 and IC50.
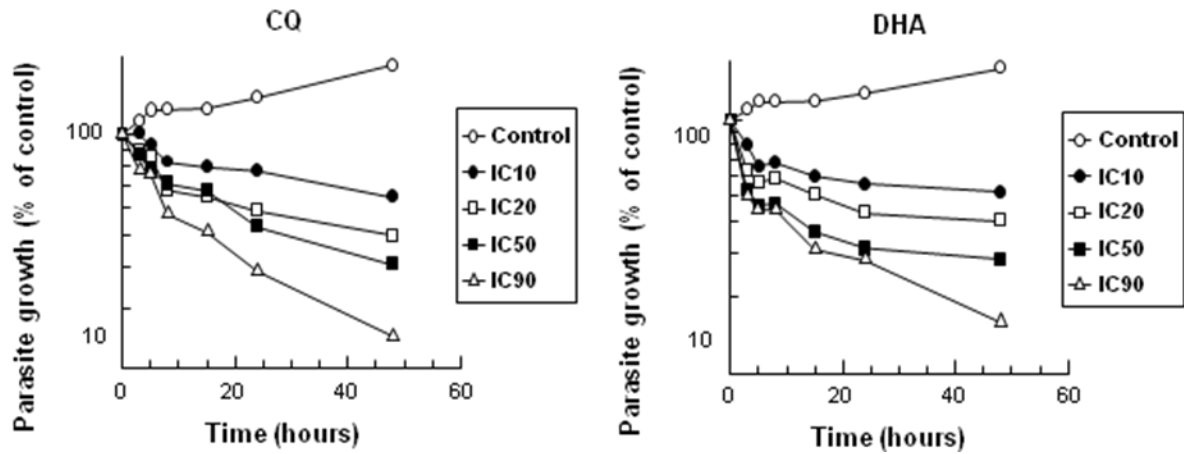
Figure 10. Time dependent effect of CQ and dihydroartemisinin on K1 strain. The Sybr green I-based fluorescence has been used for the assay. The softwares, Excel and Grafit have been used to calculate the percentages and to plot the data, respectively. The time –kill curves were constructed based on the time course of the control growth (drug-free control).

In Figure 11 A and B, the relationship between the time dependent effect of chloroquine. atovaquone and dihydroartemisinin and pyrimethamine on 3D7 strain are directly compared using the IC50 and IC90. The Figure 11 C is the expanded part of the Figure 11 B and allows to see better the trend of the curve before 8h. The graphs show that the time to kill is similar for both concentrations.
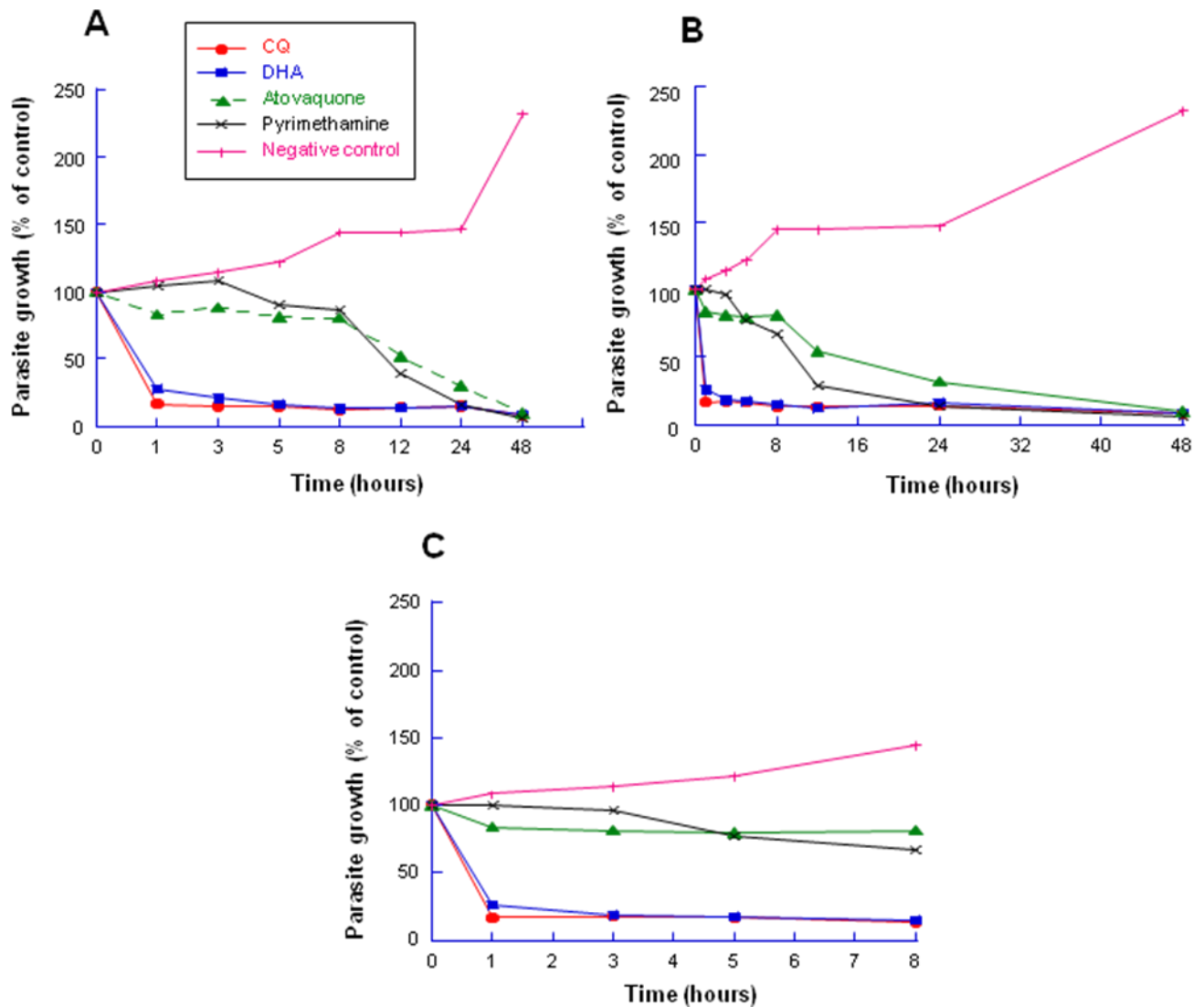
Figure 11 A, B and C: Graph representing the relationship between the time dependent effect of chloroquine. Atovaquone, dihydroartemisinin and pyrimethamine on 3D7 strain using the IC50 (A) and IC90 (B) data obtained from the SYBR Green assay. IC90 data in the 0-8h range are shown at higher resolution (C). The softwares Excel and kaleidagraph have been used to calculate the percentage and to plot the data, respectively.

3.4 Determination of the stage-specificity using high-content screening:

Synchronous cultures of 3D7 parasites were treated with chloroquine, DHA, pyrimethamine and atovaquone and at the end of 48 h they were stained with TMRE and SYBR green lysing buffer. Images of isolated parasites were then collected using Operetta and the percentage of young versus mature parasites is reported in Figure 12. In red is superimposed the dose-response curve of the drugs calculated on the total number of parasites recovered. It can be seen that for all the compounds, at the highest dose, the surviving parasites after 48 h are 80-90% young forms and only10-20% mature parasites. No major changes are seen in the distribution of young vs mature forms at doses lower than the IC50. However, at higher doses the percentage of young is increasing and in parallel the percentage of mature forms is decreasing. This indicates that a small number of parasites was able to complete the cycle and re-invade new RBC.
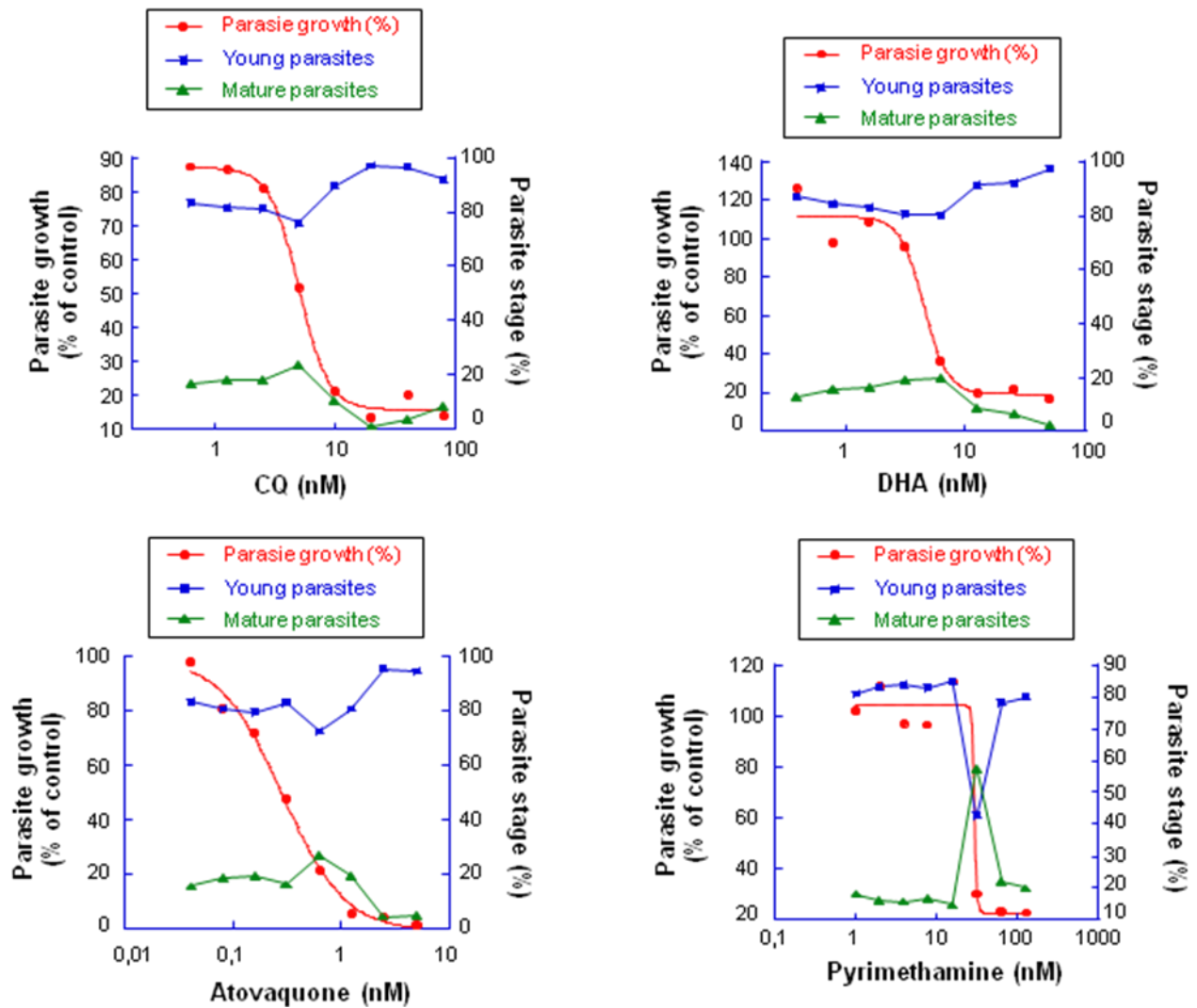
Figure 12. Stage- specificity action of CQ. DHA. Atovaquone and Pyrimethamine on the CQ-sensitive strain of *P falciparum*. The Perkin Elmer Operetta High Content Screening microscope has been used to perform the test and analyse the data. Excel has been used to calculate the percentages. Graphs were plotted with the software kaleidagraph.

It was practicable to perform the imaging studies using the confocal fluorescent microscope. The accumulation of the TMRE was used as an indicator of parasites viability. Using this technique the panel of drugs were assessed for antiplasmodial activity and for stage-specific activity. The results indicated that all potential drugs caused a reduction in the fluorescence signal and a reduction of parasites number indicating an inhibitory effect. The parasites can be seen in the Figure 13.
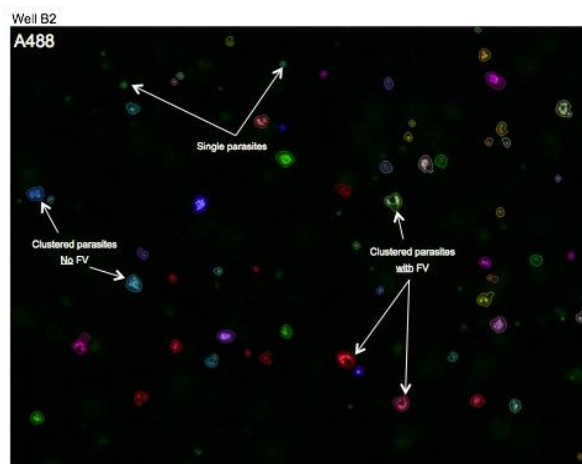
Figure 13. Operetta confocal microscopy images of *P falciparum* parasites. Image obtained from the Perkin Elmer Operetta High Content Screening microscope.

4. DISCUSSION

First, the parasite sensitivity studies clearly show that the endoperoxide and napthaquinolone were the most potent antimalarials. The endoperoxides such as artemether and its active metabolite DHA and the naphthoquinone, such as atovaquone, described in this thesis, have very potent antimalarial activity in vitro against the CQ-S 3D7 and the CQ-R K1 with IC50 values less than 10 nM, as reported by several authors over the last years [169], [158]. DHA appears to be the most potent endoperoxide compound confirming its clinical use in combination therapy. The quinoline-containing drugs and the antifolates were slightly less potent than the endoperoxides and at least for the quinoline family high degree of cross-resistance was seen against the K1 strain. It is worth noticing that the drugs from these two classes are not used any longer as monotherapy against malaria, but only in ACT based therapeutic regimens, exploiting their pharmacokinetic and pharmacodynamic properties in order to reduce the risk of inducing resistance. Over the last years, the malaria treatment policy changed because malaria parasites became increasingly resistant to the combination of chloroquine+sulfadoxine-pyrimethamine (SP). At present, the first line treatment for uncomplicated malaria is artemether-lumefantrine (Coartem) or artesunate-amodiaquine. A new fixed combination of DHA + piperaquine has recently been licensed by the EMEA, but it is not yet largely used in the field. For prophylaxis, the combination of atovaquone-proguanil (Malarone) is presently recommended for travelers to endemic regions [170]. In sum, artemisinin-based combination therapies (ACTs) have been adopted as first line treatment for uncomplicated malaria in most endemic regions [9]. To be used in a combination therapy, any compound should have pharmacokinetic and pharmacodynamic properties compatible with the companion drug. For example, in the case of artemisinin –based combination therapy, the companion drugs should have a different mode of action. It should retain efficacy against existing populations of drug-resistant parasites, and have no adverse pharmacologic interactions or additional toxicity [171]. The assessment of the in-vitro sensitivity of different *P. falciparum* isolates to antimalarial drugs provides the preliminary data for the selection of promising drugs.

In addition to making dose-response curves to determine IC50 for known compounds, we calculated the slope for each class of drugs and we found that the slope is characteristic of drug class A mechanistic explanation can be found by reconsidering the concept of cooperativity in drug action: aslope > 1 classically represents positive cooperativity between ligand binding sites on a multivalent receptor [62]. There may be different mechanistic explanations for high slope values. The variability of the slope indicated that the CQ and the DHA are effective at the first moment of drug assumption (slope decreasing from 48h to 96h) while the atovaquone is effective later (slope increasing from 48h to 96h). This results are in agreement with those found on the time to kill curves. However, we need further analysis of the data to be able to correlate better the influence of the slope on antimalarial drug activity. A new parameter or index must be developed which can provide a more accurate in vitro pharmacodynamics measure of antimalarial activity taking account the slope. For example, Shen et al. in order to show that slope had a noteworth effects on antiviral activity, developed a new index termed instantaneous inhibitory potential (IIP) which equals the log reduction in single-round infectivity at clinical relevant concentration (log [1/(fraction of unaffected viruses by drug)]). Their results demonstrated that IIP provides a more accurate in vitro pharmacodynamic measure of antiviral activity than the traditional measures because it takes into account the slope parameter [62]. A similar study

can be applied to the field of malaria: the stepness of the dose-response curve should be a good parameter for understanding the influence of the slope on the drug activity.

Next, the optimal chemotherapy in malaria entail rapid, sustained clearance of parasites, short duration of treatment and low toxicity. The time to kill of drug effects is an important pharmacodynamic factor. However. relatively little is known about the time to kill of parasites and selective toxicity of antimalarials against the morphologically distinguishable different stages of *P. falciparum*. These pharmacodynamic factors may be important determinants of antimalarial drug efficacy and could provide important clues about the mechanism of action and essential to reduce the cost and time of drug development. However, in recent years on the field of antimicrobials, some integrated PK/PD approaches have been developed as a more sophisticated methodology to assess the efficacy of antimicrobial drugs based on dynamic kills curves [101]. The artemisinins or endoperoxide antimalarials such as DHA has a fast parasitocidal action. This author, using another technique reported that artemether was a fast acting antimalarial drug. In terms of CQ, our time to kill data is similar to DHA. This means that CQ and DHA both might kill all the young parasites. We showed with the figures that CQ and DHA killed more rapidly than pyrimethamine. This result is consistent with other published study which concluded that OZ277 artemether and chloroquine attenuated parasite growth more rapidly than pyrimethamine [172]. Nosten and White indicated that artemisinin and its derivatives have the highest clinical Parasite Reduction Ratios (PRR) among all antimalarials drugs [173]. If we compare the rank order of the time to kill-curves obtained in our study, which is ART=CQ>Pyr>ATOV, we found that it is the same as the rank order of the PRR (ART>CQ>PYR>ATOV) reported by Nosten et al. This means that the pyrimethamine and atovaquone react later on the parasite cycle. Therefore, this time-kill assay could be a good in vitro way to assess the speed in which drugs work in the clinics. This model is suitable for *P. falciparum* parasites since they are fast growing parasites and the parasite growth rate is sufficient to offset any lost during the dilution process and the washing steps.

The *in vitro* PK/PD models can be also used to study the antimalarial effect of single and combination drug compounds and dosing regimens before the in vivo efficacy studies as already done with antimicrobials [61]. The advantages of these models is that the appropriate human/animal pharmacokinetic profiles can easily be simulated and the effect of these changing concentrations on culture growth (i.e via kill curves) and emergence of drug induced tolerance and resistance can be assessed [101]. The advantages of an *in vitro* model is that the influence of the immune system is absent and the drug-culture interactions can be measured precisely. Thus, *in vitro* models offer a safer and more ethical way of assessing the PK/PD relationships of antibiotics compared to animal or human studies [174]; [175]. Nevertheless some drawbacks of the in vitro models exist and can be classified under the host, the pathogen and PK factors. The *in vitro* model lack the ability to mimic the complex host-defense mechanism and therefore approximate more to conditions of an immunodeficient patient where the infection is more bacteremic in nature. The growth rate for most microorganisms is faster *in vitro* compared with growth *in vivo* or in human serum. Since antimalarial efficacy in an *in vitro* model relates to the rate of parasite growth, this can be a potential limitation of *in vitro* models, especially where are significant differences in the growth rates. Drug distribution is technically challenging to simulate in vitro. For example plasma protein binding of the drug is absent in *in vitro* model and needs to be account for [61]. In perspective, the models used in anti-TB, and in general for the bacteria can be applicable to antimalarials. The in vitro PK/PD models have been employed a useful tools to evaluate the efficacy of new anti-TB drugs under development. Since they come with benefits of reduced cost, flexibility and adaptability they can be used to guide the early drug development process and establish efficacy in experimental disease models. Also from predicting the relevant PK/PD indices to studying the effect of novel therapeutics on resistant clinical isolates these in vitro models in combination with preclinical data can help in optimizing dosing regimens that can drive efficacy [101] [61]. In spite of the potential drawbacks associated with in vitro models the wealth of information they provide will be exploited to understand mechanisms of drug resistance and develop new drugs that can target resistant strains. In vitro model will finally likely be more often applied in academic research and clinical settings. Finally in this study we performed preliminary tests of fluorescence-imaging of drug-treated parasites to identify the stage-specificity of antimalarials Maximum drug effects occurred at the late ring and early trophozoite stage, which corresponds to the time at which the most rapid increases in synthetic and glycolytic activities occur [104]. The results from previous study indicated that pyrimethamine is only effective against young schizonts as reported by Maerki et al [172]. The same author propose that CQ is active on mature stages of *P. falciparum* and slightly sensitive on young ring forms. Schizonts multiplied successfully in the presence of relatively high concentrations of chloroquine quinine and artemisinin [104]. DHA is effective against the young stage of parasites directly as it induce more rapid reduction of parasitaemia. There is currently no consensus as to the asexual stage that is most sensitive to artemisinin: it has been suggested that late ring stages and early trophozoites are the most susceptible stages to artemisinin than other stages [104]. Some investigators believe that all asexual parasites stages are susceptible to the antimalarial peroxides [176] . Because the artemisinin compounds had the broadest time window of action they may be particularly suitable for the treatment of severe malaria [104]. The compounds that inhibit the rings would be predicted to have faster

time-kill profiles (and therefore better PRR values) and would be prioritized for mode of action and safety studies. Therefore the Operetta technique which is able to quickly assess the potency as well as the stage specificity of a drug should be able to predict the PRR without the need to perform the time-kill assays which take longer. This will be useful in HTS screens.

5. CONCLUSION AND PERSPECTIVES

The in vitro pharmacodynamics study, are still in their infancy for antimalarial drug development, although they have been already employed for selecting new anti bacterial drugs. The data in this thesis confirm that similar type of parameters (time to kill, dose response curves, slope factor) can also be calculated for new antimalarials and can be related to the clinical behaviour of a drug. The high content screening method using the Operetta machine once standardised will help in defining in a short time and with high reproducibility the stage specificity effects of new compounds. All together these assays should facilitate the in vitro PD studies on new drugs, reduce time and costs for the prioritization and selection of new lead and speed up the development process of new antimalarials, which are absolutely required for achieving malaria eradication by 2050 as stated in the GMAP.

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ANNEXES

Annexe 1: Canthium henriquesianum (K. Schum)

Annexe 2: Gardenia sokotensis (Hutch)

Annexe 3: Vernonia colorata (Willd)

Annexe 4: Traditional preparation of arial parts

Annexe 5: The Perkin Elmer Operetta machine

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