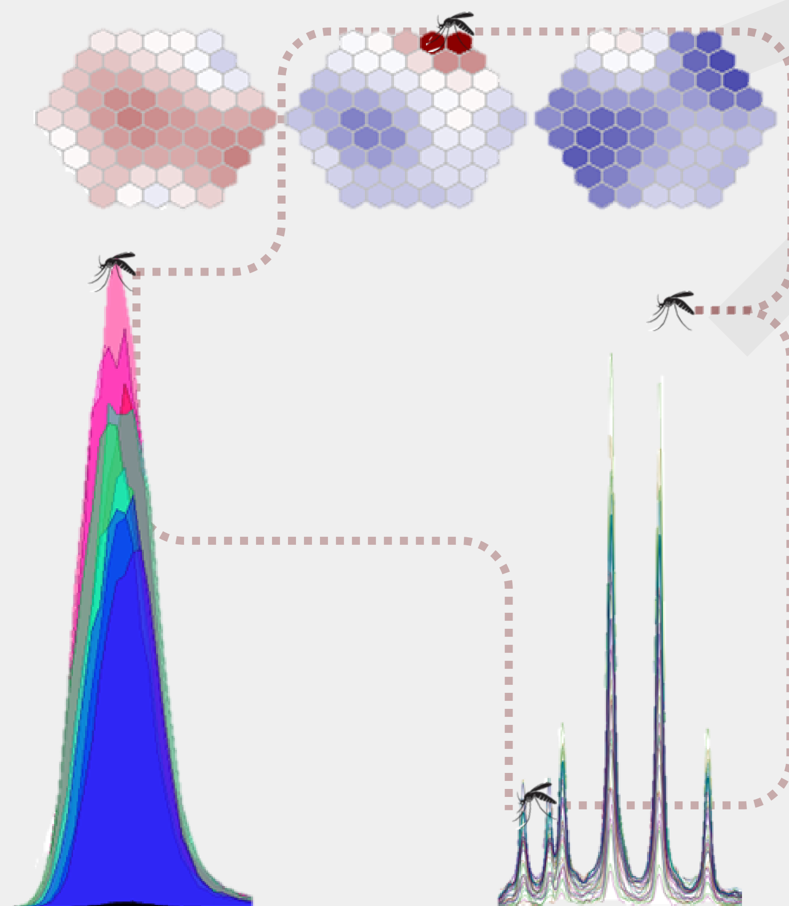

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METABOLOMIC INVESTIGATION OF NATURAL ANTIPLASMODIAL AGENTS

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Abstract

Malaria is one of the deadliest parasitic diseases that still plagues humanity in recent times. Despite being target of several eradication campaigns, the widespread presence of the multiple agents that cause this parasitosis, along with its increased adaptability into developing resistance to treatments, make this old ailment a challenge to modern medicine. Natural products with potential antiplasmodial activity are valuable to discover new therapeutic targets and shed light on new scaffolds that can be optimized into revolutionary treatments. In this thesis, metabolomics, a sensitive and robust approach that can describe the metabolites of the malaria parasite maintained in culture, was used to profile the effects of natural products on the parasite and hypothesize their mode of action. Before this could be done, workflow investigation and protocol optimization were carried out, including a study on metabolic extraction methods with state-of-the-art statistical algorithms. Further exploration revealed that time and complexity are detrimental to repeatability and robustness of the results, so a method with a single washing step followed by methanolic 90% extraction was used and analyzed through ^{1}HNMR and/or LC-MS, depending on sample availability.

Five individual cases were studied in collaboration with the Department of Biochemistry and Molecular Biology and the Huck Center for Malaria Research at Penn State (The Pennsylvania State University), which allowed the use of their inhouse MS system and database for targeted metabolite annotation. First, an exploration of plant extracts and fractions were studied in parallel to the known active antiplasmodial compounds present in those plants to assess how early in plant screening a potential mode of action can be hypothesized. Results show that extract effects can already be distinguished from a control, though fractions with active compounds separate more clearly. The more purified the fraction, the bigger the correlation in mode of action with the isolated natural compounds, possibly due to the lack of matrix effects and interactions, which shows that metabolomics can be introduced early in bioassay-guided fractionation in plant studies. Second, two of the most important plants in traditional medicine against malaria were studied, *Artemisia afra* and *A. annua,* to investigate their phenolic content, presence or absence of artemisinin and correlate the composition with the metabolomic results on a synchronized malaria culture. Results indicate a correlation between activity and artemisinin abundance in these extracts, with *A. annua* presenting a similar parasitic profile to artemisinin whereas *A. afra,* despite trace amounts of this compound, differs significantly. *A. afra* affects various unspecific metabolites but significantly changes myo-inositol distinctly from *A. annua* and artemisinin, which clearly target redox mechanisms. Lastly, three independent studies aimed to investigate three natural scaffolds for their mode of action: alkyl cyclohexenones compounds named poupartones, ellagic acid and derivatives, and a mixture of triterpene esters. Poupartones showed to

interfere with hemoglobin metabolism, DNA and RNA synthesis and redox management systems which correlated to their potential to participate in nucleophilic additions that establish covalent bonds with proteins and generate radical oxygen species, an effective yet not specific type of activity. Our studies on ellagic acid and derivatives support literature data and point to the parasite's digestive vacuole as the site of action of these compounds. Changes in hemoglobin metabolism and redox metabolites suggest possible effects on plasmepsins, enzymes that act early in hemoglobin breakdown, and on glutathione metabolism, essential to maintaining a balanced organelle. Lastly, a mixture of 8 triterpenic esters seems to affect pyrimidine synthesis and amino acid metabolism through N-carbamoyl-L-aspartate, though it is unclear exactly how. Metabolomics is a hypothesis generating approach that gives a snapshot of the effects of innovative natural compounds on the malaria parasite in order to accurately guide antimalarial drug discovery.

List of Abbreviations

ACTs – Artemisinin-based Combination Therapies

ANOVA – Analysis of Variance

AUC – Area Under the Curve

CPMG – Carr-Purcell-Meiboom-Gill pulse sequence

DI-HRMS – Direct-Infusion High-Resolution Mass Spectrometry

DV – Digestive Vacuole

FAS – Fatty Acid Synthesis

FC – Fold Change

FDR – False Discovery Rate

G6PD – Glucose-6-Phosphate Dehydrogenase

GC-MS – Gas Chromatography-Mass Spectrometry

GSH – Reduced Glutathione

GTS – Global Technical Strategy

HILIC – Hydrophilic Interaction Liquid Chromatography

HMDB – Human Metabolome Database

IC_{50} – Half Inhibitory Concentration

iRBC – infected Red Blood Cell

KEGG – Kyoto University Encyclopedia of Genes and Genomes

LC-MS – Liquid Chromatography-Mass Spectrometry

LC-HRMS – Liquid Chromatography High-Resolution Mass Spectrometry

m/z – Mass-to-Charge ratio

MIC – Metabolomic Informative Content

MoA – Mode of Action

NMR – Nuclear Magnetic Resonance

NP – Natural Products

NPP – New Permeability Pathways

PCA – Principal Components Analysis

PLS-DA – Partial Least Squares-Discriminant Analysis

PV – Parasitophorus Vacuole

QC – Quality Control

RBCs – Red Blood Cells

RDTs – Rapid Diagnostic Tests

ROS – Reactive Oxygen Species

RPLC – Reverse Phase Liquid Chromatography

RSD – Relative Standard Deviation

RT – Retention Time

SD – Standard Deviation

SI – Selectivity Index

TCA – Tricarboxylic Acid

TSP – Trimethylsilylpropanoic acid

UPLC – Ultra-High-Pressure Liquid Chromatography

VIP – Variable Importance in the Projection criterion

WHO – World Health Organization

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Mamede, L., Ledoux, A., Jansen, O. & Frédérich, M. Natural Phenolic Compounds and Derivatives as Potential Antimalarial Agents. Planta Med. ***86**, 585–618 (2020). (Review)* 31
Mamede, L., Ledoux, A., Tullio, P. De & Quetin-leclercq, J. Recent metabolomic developments for antimalarial drug discovery. Parasitol. Res. *(2022) doi:10.1007/s00436-022-07673-7. (Review)* 85
Comparison of extraction methods in in vitro Plasmodium falciparum: a 1H-NMR and LC-MS joined approach – ***Submitted*** 140
Metabolomic profiling of Artemisia afra and Artemisia annua reveals divergent modes of action against Plasmodium falciparum – ***In preparation*** 203

Context and Objectives

The research in this work was developed in the context of the project "Metabolomic investigation of natural antiparasitic agents", METNATPAR for short. Its objective is to valorize the library of natural antiparasitic compounds identified in the pharmacognosy laboratories of the Universities of Liège and Louvain (ULiège and UCLouvain) by using MS and NMR metabolomics to clarify these molecule's mode of action. Each laboratory specializes in two different parasitic diseases: *Plasmodium falciparum,* the deadliest causing agent of malaria, in Liège, and *Trypanosoma brucei,* causative agent of Human African Trypanosomiasis (also known as sleeping sickness), in Louvain. Both parasites cause deadly diseases with high degrees of resistance for which innovative treatments are highly necessary. By applying metabolomics, the modes of action of the natural compounds can be compared to established drugs and therefore indicate new putative targets that could be investigated further.

More specifically, the research presented here on out focuses on the malaria parasite and the modes of action of natural compounds with demonstrated antiplasmodial activity. Despite this parasite's complex life cycle and intracellular nature, the hypothesis was that through metabolomic profiling, new antiplasmodial targets could be theorized while revealing potential scaffolds from these active natural compounds.

Research began by investigating the workflows present in the literature and evaluating which parameters and steps were the most robust and reproduceable. Once a workflow was set, a series of proof concepts aimed at demonstrating the applicability of the study design, protocols and processing tools to accomplish fine tuning – these procedures are described in Chapter 2. Chapter 3 is dedicated to each individual case study in collaboration with the Department of Biochemistry and Molecular Biology and the Huck Center for Malaria Research at Penn State (The Pennsylvania State University), which allowed the use of their inhouse MS system and database. An exploration of extracts and fractions in comparison with isolated natural products investigated how early there could be an indication of a mode of action. A study on the different modes of action between *Artemisia afra* and *A. annua* addressed the controversial comparison of the efficacy of both plants in the context of malaria. Regarding potential molecular scaffolds, three examples were tested: alkyl cyclohexenones compounds named poupartones, ellagic acid and derivatives, and a mixture of triterpene esters. A discussion on the modes of action, putative targets and potential applicability as future antimalarials is presented.

INTRODUCTION

Chapter 1

1. Malaria

1.1. The present of an historical disease

Humankind has always struggled with diseases, but some have proven to be more resistant than others, lingering around for millennia. This is the case of a vector-borne parasitic disease that occurs at a global scale called malaria, originally in medieval Italian *"mal air"* which translates to "bad air".[1] Historically, traces of this disease were detected in Egyptian mummies from as early as 3200 BC and literary evidence left by Chinese documents from around 2700 BC or by Hippocrates describing fluctuating fevers suggests its presence many centuries before ancient Greece.[2–4]

This illness carried on to modern times and prompted a global effort led by the WHO with the purpose of eradicating it.[5] This occurred after, at the turn of the 21st century, key political institutions received substantial evidence of the obstacle this disease represented in terms of public health and socioeconomic development and growth.[5] This effort, defined by the GTS for Malaria 2016–2030, is still ongoing and means to reduce malaria incidence and mortality by at least 90% by 2030.[5] Because of this push, global disease burden has been alleviated and eradication was successfully achieved in some countries – as depicted in Figure 1.[6] However, despite these positive signs, recent events and overall indicators still evidence a long way ahead.

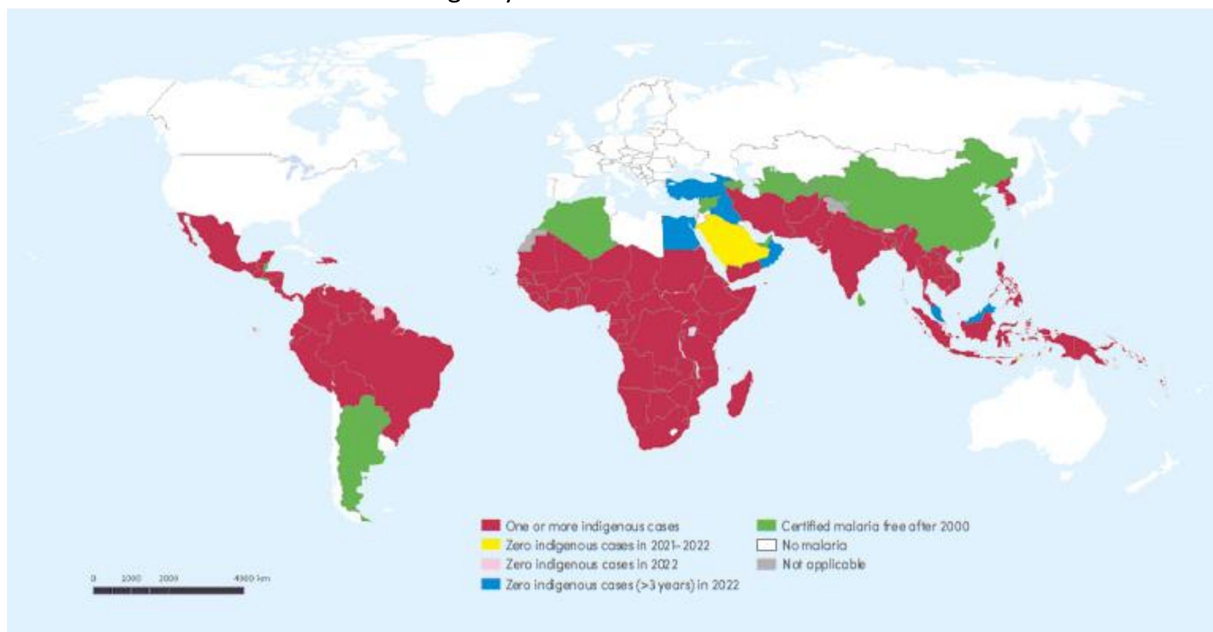

Figure 1 - Countries with indigenous cases in 2000 and their status by 2022. Countries with zero indigenous cases for at least 3 consecutive years are considered to have eliminated malaria.[6]

The latest epidemiological data indicate that there were 249 million estimated malaria cases in 2022, of which 94% originated in Sub-Saharan Africa.[6] In the same year, there were 608 000 deaths, a significant increase from 2019, which accounted for 576 000 deaths. Importantly, 76% represent children, an invaluable decline from 87% in 2000 but that has stayed stagnant since 2015.[6] Indeed,

global malaria incidence and mortality diminished since 2000, but at a relative slower rate since 2015, which is concerning. Not only are the children losses undesirable, but the disease's burden in pregnancy is also significant – 12.7 million pregnancies were estimated to have been exposed to malaria infection in the African region in 2022.[6] This undeniably resulted in increased deaths during delivery due to malaria-related complications, but also in low birthweight, newborn early death or lifelong disability. The surviving children face multiple episodes of malaria and chronic anemia during their life, and if plagued with severe deviations of the disease such as cerebral malaria, they might also suffer from seizures and cognitive impairment for life.[5,7]

Additional hurdles that threat the eradication goals stem from the malaria pathogen itself or world events. As it is recognized, changes in the parasite's genome, *i.e.*, its assortment of genetic information cause further complications. Deletions in the *pfhrp2* and *3* are known causes for detection failure with RDTs in the field and are of particular relevance because if they were to become prevalent, they would incapacitate the most generalized and preferred method of malaria diagnostics. This deletion has now become widespread and is being monitored by the WHO with the defined threshold of 5% prevalence before changes in the RDTs need to be implemented.[8,9] Parallelly, mutations in the *PfKelch13* are being used to monitor the evolution of artemisinin partial resistance (defined as delayed clearance) that is directly linked with treatment failure associated with ACT (further described in section 1.2), which have led to some local first-line therapy changes.[8,9] Simultaneously, widespread resistance to pyrethroids, the insecticides used in insecticide-treated mosquito nets, seriously compromises one of the biggest prevention tools available.[8] To mitigate this situation, strategies are being implemented to fully restore susceptibility in malaria endemic countries. Additionally, the COVID-19 pandemic worsened the situation by disrupting health services and, due to conflation in the fever symptoms, adequate treatment was less likely to be sought and administered.[8] Delays in diagnostic, misdiagnosis (worsened by the possibility of co-infection) and overburdening of laboratories hampered patient treatment and increased the number of severe cases.[9] Between 2020 and 2021, additional 13.4 million cases and 63 000 deaths globally were attributed to disruptions to essential malaria services during the pandemic.[8] Additionally, the number of imported cases of malaria into Europe increased in 2021 compared to 2020 due to easing of travel-restrictions following the pandemic.

As a positive side note, it is important to note that on October 6th 2021 the first malaria vaccine (and parasite vaccine) RTS,S/AS01 was approved by WHO for administration to children in selected endemic areas with moderate to high transmission.[8,10] This pre-erythrocytic recombinant protein vaccine was the fruit of 60 years of vaccine research and is expected to lower disease burden and mortality by inducing antibodies against sporozoites so that they don't infect the liver, the first step into a malaria infection after a mosquito bite. Recently, another vaccine of the same type, R21/MM, has been

recently approved for use in Ghana.[11] Despite RTS,S/AS01 vaccine efficacy of 36%, administration in combination with other interventions is expected to alleviate the burden in the most affected areas.[9,10,12]

Despite significant challenges, the innovations, surveillance, and management by WHO and the local regulatory and health care institutions have never been as impactful and successful. Unfortunately, the current trends lead WHO to predict a failure in meeting the GTS 2025 targets globally, so in order for the strategy to be successful until 2030, a lot of care and effort still needs to be implemented in managing this disease.[8]

1.2. The human as the host

Malaria is caused by an obligate intracellular protozoan with a complex life cycle that alternates between an invertebrate and a vertebrate host – as shown in Figure 2. The *Plasmodium* spp. is host specific and five recognized species infect mankind: *Plasmodium falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi*, a zoonotic parasite that has more recently crossed the species barrier.[13] In humans, after an infected mosquito takes a blood meal, the parasite's sporozoites are injected and seek the liver to develop in a pre-erythrocytic cycle (Figure 2-b) before reaching the RBCs and replicating asexually in what is known as the intra-erythrocytic or blood stage cycle (Figure 2-c). There,

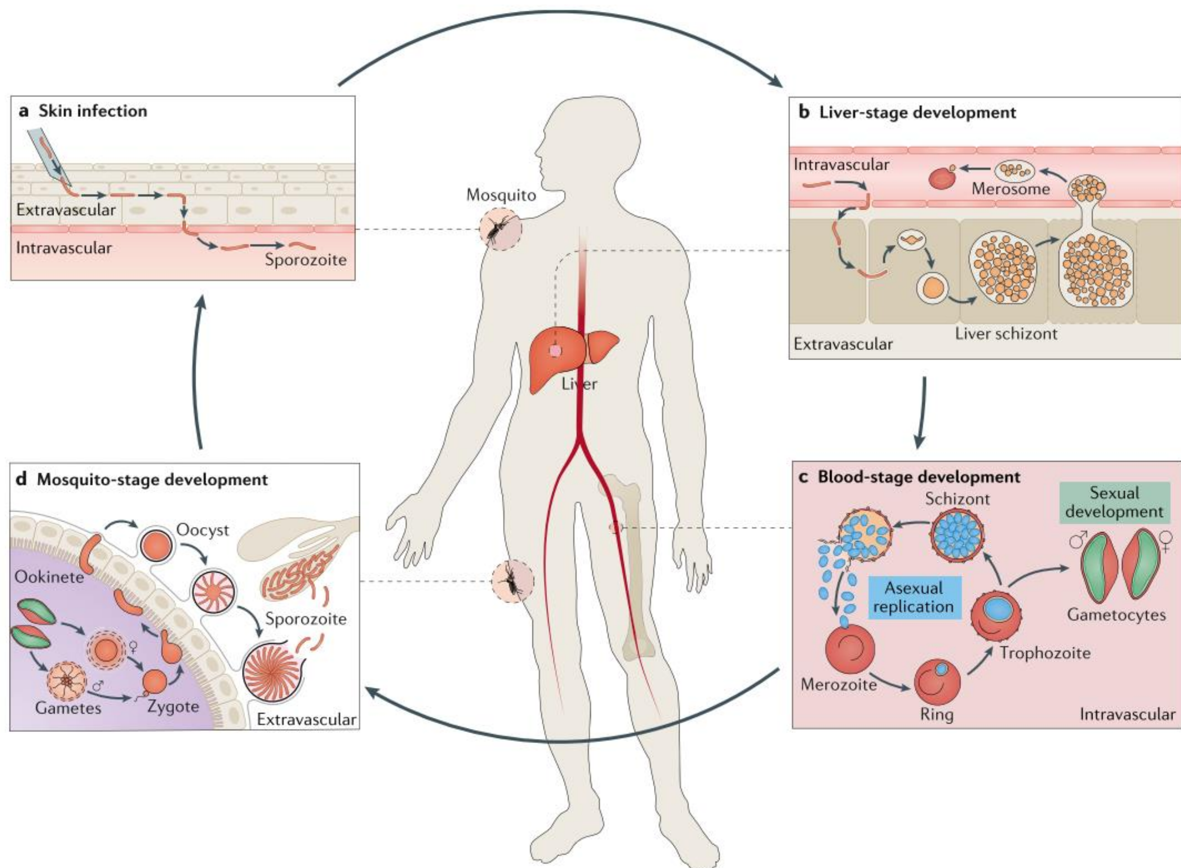

Figure 2 - Life cycle of P. falciparum in humans and mosquitoes.[14]

throughout one lifecycle that ranges from 48h to 72h depending on the species, the parasite evolves morphologically inside the iRBC until it egresses, destroying the host cell, and releasing new merozoites into the bloodstream.[14,15] This is the stage that elicits the disease in variating degrees of severity as this process does not occur in a 1:1 ratio: one schizont can release up to 32 merozoites per lifecycle, depending on the species, which justifies why the parasitic biomass increases rapidly.[15] This in turn increases the glucose consumption and RBC destruction, which leads to the disease's symptomatology: metabolic acidosis, anemia and a great inflammatory and immune response.

During this stage, alternatively, merozoites can differentiate into gametocytes, the forms that can continue the lifecycle in the female *Anopheles* sp. mosquito (Figure2-d).[15] These forms' sole purpose is the sexual replication in the mosquito midgut, thus causing a new infection upon the mosquito's next blood meal. Despite not causing any harm to humans, gametocytes are responsible for transmission and thus recognized as an important target for malaria management and eradication.[10]

Uncomplicated malaria is characterized by a trademark feature – synchronized fevers that correlate to parasite egression from iRBC.[14] The typical febrile malaria paroxysm evolves in three stages: the cold stage, with rigors, the hot stage, with fevers that can reach 41°C accompanied by nausea, vomiting, headaches, and possible seizures, particularly in the pediatric population, and finally the sweating stage where the fever and symptoms subside. Physical examination is generally nonspecific and blood analysis, either through RDTs or microscopy thick or thin smears remains the most effective tool for malaria diagnosis.[14]

The pathogenesis of malaria is, however, more intricate, particularly for the *P. falciparum* species, responsible for 90% of cases and the lethality to infants, making it effectively the most deadly and problematic pathogen.[8,14] When a merozoite invades a RBC, it induces changes to this host cell to make it viable for infection. One of such changes is the introduction of ligands on its surface that will interact with endothelial cells, leading to sequestration in capillaries and postcapillary venules in multiple organs.[13,15,16] This is thought to occur in several species, however, the rate and multiorgan adherence is a key factor in *P. falciparum* infection and confers the virulence and capability to cause severe malaria. This binding of iRBC induces endothelial dysfunction and altered permeability, which occurs notably in cerebral malaria but can also affect the placenta, kidneys, and/or lungs. Additionally, this interaction promotes decreased local blood flow and can cause clogs, tissue hypoxia or ischemia, leading to further complications. This sequestration allows splenic removal evasion and enables the *P. falciparum* to establish an infection.[5,8] With each parasitic blood lifecycle, hemolysis occurs synchronously, releasing inflammatory molecules that induce fever episodes and activate the immune system. During this process, the high production of inflammatory cytokines may, to some extent,

suppress erythropoiesis so no new RBC are being created.[16] Continuous glucose consumption leads to metabolic acidosis, which ensues respiratory distress in children, reaching acute respiratory distress in adults.[16] These and other life-threatening complications evolve rapidly during infection, making diagnosis and symptom management crucial.[14]

Once diagnosis of malaria is confirmed, adequate treatment should be introduced as soon as possible – Table 1 portrays a simplification of the WHO recommended guidelines.[17] Hospital admission is recommended and rapid deterioration should be expected with *P. falciparum* and *P. knowlesi* infections, as both can induce severe malaria.[14] Depending on the plasmodium species, geography, pregnancy status and severity, different guidelines are available for treatment.[14,17,18] Briefly, uncomplicated cases (excluding pregnant women in their first trimester) should be treated orally if tolerated with ACTs such as artemether + lumefantrine or artesunate + amodiaquine, when the causing agent is *P. falciparum. P. malariae* and *P. knowlesi* malaria are still treatable with chloroquine as antimalarial drug resistance is not well characterized, meanwhile, for *P. vivax* and *P. ovale* malaria, it depends on the region.[14,17] As such, for these four species, treatment depends on chloroquine susceptibility: if the infection is susceptible, treatment with chloroquine or ACTs, if resistant, with ACTs.[17] In the field, however, it is unpractical to perform susceptibility tests for every patient, and as such, chloroquine is routinely used. To prevent relapses and the formation of reservoirs of *P. vivax* and *P. ovale* malaria, primaquine must be added to any regiment to destroy lingering liver hypnozoites with the caveat of a supplementary test needed to detect G6PD deficiency, as primaquine is known to cause severe hemolytic anemia if this is the case.

Table 1 - Summary of first-line WHO-recommended therapies for malaria.[16]

	MALARIA INFECTING SPECIES				
	P. falciparum	*P. malariae*[2]	*P. knowlesi*	*P. vivax*	*P. ovale*[2]
UNCOMPLICATED	ACTs	Chloroquine or ATCs	Chloroquine or ATCs	Chloroquine[1] or ATCs + Primaquine	
SEVERE	IV Artesunate				

[1] - If no resistance in the area
[2] - Not indicated to cause severe malaria[17]

Severe malaria is defined by WHO by the presence of one or more syndromes or complications occurring without an identified alternative cause.[17] As mentioned previously, these include but are not limited to: acidosis and respiratory distress, renal impairment, bleeding, shock, hyperparasitaemia, impaired consciousness, and convulsions. The only difference in the definition of severe malaria between species relates to the parasite density, as *P. falciparum* elicits exponentially higher counts. Regardless of the presence of one or many complications, due to the fatality rate of severe malaria

(particularly cerebral) nearing 100%, any patient considered to be at risk of severe malaria should be treated immediately with intravenous artesunate, followed by ACTs as soon as it can be tolerated.[17] As the most affected populations are pediatric and women in their second and third trimesters of pregnancy or immediately after delivery, reaching 50% mortality with fetal death and premature labor as common occurrences, treatment should be administered without delay.[17] Intermittent preventive treatment of malaria in pregnancy should start as early as possible in the second trimester with sulfadoxine-pyrimethamine to improve key pregnancy outcomes.[17] Parasite density and clinical response should be monitored closely until clearance and recovery. Follow-up depends on the severity and side-effects of the treatment and can extend up to 4 weeks after discharge.[14]

The overall pathological process variates according to many factors, such as species involved and the host age or immune status. For instance, cerebral malaria, which translates to an infection by *P. falciparum* with sequestration in brain vessels that lead to loss of function of the blood brain barrier, usually resulting in convulsions, coma or death, is a syndrome more predominant to children aged under five, whereas kidney and lung complications are relatively rare in children and become more frequent with age.[14,16] Regardless of the species, chronic or repeated acute infections induce relevant morbidity and mortality which makes this deadly disease an important public health issue as explored previously.

2. Natural compounds

2.1. Overview of natural antimalarial compounds

Traditional medicine has a history as long as mankind and is often underestimated concerning its role in health care. WHO defines it as the sum of knowledge, skill and practices indigenous to different cultures used in the maintenance, prevention, diagnosis or treatment of an illness.[19] Whether it is the backbone of health care, as 80% of the global population rely on it, or used as a complement to modern medicine, it is present in effectively every country in the world and has served as a guideline to researchers, governments, health care practitioners and populations alike in the management of many public health threats.[19,20] This is particularly the case for malaria, as one of the oldest and deadliest of diseases.

The need for a cure to this burdensome pathology led doctors to explore traditionally used remedies, among which the bark of the *Cinchona* tree used traditionally in South America. In 1820, 60 years before the malaria parasite was discovered, quinine was purified from this bark extract, becoming the first compound to be used to treat malaria.[21] Another example is lapachol, a hydroxynaphthoquinone isolated from the bark of the lapacho tree (*Handroanthus impetiginosus*) also used traditionally in

South America. This compound originated the naphthoquinone drug chemical class, leading to the development of atovaquone.[21] Despite the evolution of drug discovery approaches, antimalarial drug discovery still relies mostly on screenings, most of which include NP or derivatives. Such is the case of how artemisinin, whose derivatives are the current first-line treatment for *P. falciparum* malaria, was discovered. A Chinese project focusing on the screening of traditionally used plants revealed the activity of *Artemisia annua* and in 1971, professor and Nobel-laureate Youyou Tu isolated artemisinin, which reinvigorated the arsenal of no longer effective antimalarial drugs, such as chloroquine and quinine.[21,22] This was the case due to inadequate use that led to resistance emergence and spread, which also became the case for artemisinin derivatives soon after implementation and led WHO to recommend ACTs.[5,22] In short, ACTs comprise two different complementary drugs: an artemisinin derivative, fast acting, usually in therapeutic concentrations for 6h, and a second antimalarial, slower-acting, up to weeks, and with a MoA admittedly different than the first.[22] The strategy is that after the first molecule exerts its effects, the second molecule will remain in the host's system effectively eliminating the surviving parasites. Consequently, the chances of resistance are reduced by eliciting an impossible effort of the parasite to maintain fitness in the presence of two distinct toxic mechanisms. This treatment if effective, but faces some obstacles: it requires multiple doses, usually every 24h, which compromises patient compliance, and carries a heavier financial cost.[23] Additionally, due to resistance to artemisinin derivatives and their short half-life, effective exposure of the vulnerable ring-form of the parasite to this molecule is diminished and the average number of times of exposure also varies. As Khoury *et al.*[23] propose, this means that variations in the parasite's cycle or overall sensitivity to drugs increases the likelihood of treatment failure, even in combination therapies.

Different solutions have been proposed to overcome this and invigorate the eradication plan, from triple combination therapies to the development of new drugs. However, the requirements for the development of new antimalarials are quite steep: (1) new MoA with no cross-resistance to presently used therapies, (2) a single well tolerated cure, (3) active against asexual (accounts for symptoms), sexual (transmission) and hypnozoites (recrudescence) life stages and, ideally, (4) inexpensive.[22] It is unlikely that one single compound would possess all these traits, but it is recognized that at least the (1) is a must. Nowadays, antimalarial drug discovery leans on three pillars: high-throughput screenings that identify new chemotypes, be it natural compounds, semi or synthetized molecules; exploration of combinations of antimalarials, which aid in overcoming resistance and might even improve drug delivery or be synergistic; and repurposing of other drugs, like antibacterials.[22] Combination therapies remain effective but have an elevated cost, and the repurpose of other drugs carries the risk of undesirable side effects and eliciting other problems, like bacterial resistance. As such, there is still a

pertinent interest in the discovery or derivatization of NP into potent antimalarials as nature remains the biggest source of innovative compounds.[24]

2.1.1. Foreword

There are many classes of NP that have reported antiplasmodial and antimalarial activity, and one of the most abundant and interesting is phenolic compounds. This class includes varied chemical structures and is found virtually in every plant. A recent review by Degotte *et al.* evidenced that a significant amount of promising antiplasmodial NP are from this class of compounds.[24] Examples like ellagic acid, catechins, α-mangostin, or licochalcone A are some of the most studied. In light of the work carried out with ellagic acid, it was pertinent to have an overview of the literature to situate the potential of phenolic compounds as antiplasmodial compounds. An integral review of promising phenolic compounds based on a published article is presented in the next section, along with an update the section after.

2.1.2. Phenolic compounds – Published article (adapted)

Reviews

Thieme

Natural Phenolic Compounds and Derivatives as Potential Antimalarial Agents

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ABSTRACT

Malaria is a parasitic disease endemic to tropical and subtropical regions responsible for hundreds of millions of clinical cases and hundreds of thousands of deaths yearly. Its agent, the *Plasmodium* sp., has a highly variable antigenicity, which accounts for the emergence and spread of resistance to all available treatments. In light of this rising problem, scientists have turned to naturally occurring compounds obtained from plants recurrently used in traditional medicine in endemic areas. Ethnopharmacological approaches seem to be helpful in selecting the most interesting plants for the search of new antiplasmodial and antimalarial molecules. However, this search for new antimalarials is complex and time-consuming and ultimately leads to a great number of interesting compounds with a lack of discussion of their characteristics. This review aims to examine the most promising antiplasmodial phenolic compounds (phenolic acids, flavonoids, xanthones, coumarins, lignans, among others) and derivatives isolated over the course of the last 28 y (1990–2018) and discuss their structure-activity relationships, mechanisms of action, toxicity, new perspectives they could add to the fight against malaria, and finally, the difficulties of transforming these potential compounds into new antimalarials.

Introduction

Malaria is a parasitic disease endemic to tropical and subtropical regions with a worldwide distribution. Despite global efforts to eradicate it, the most recent report by the World Health Organization demonstrates a halt towards this goal.[25] In fact, 405 000 deaths are estimated to have taken place in 2018, of which 67% were children under the age of 5, one of the most vulnerable groups.[25] This represents a slight improvement, in comparison to 2017, in terms of mortality, however, the incidence rate change has slowed dramatically in the last 4 years, revealing that, globally, the burden of this disease remains an issue towards eradication. The protozoan responsible for this disease, the *Plasmodium* sp., is transmitted through the bite of a female *Anopheles* sp. mosquito. Of the five human infecting species, *Plasmodium falciparum* and *P. vivax* represent the highest burden and the majority of cases.[25–28] *P. falciparum* remains the most prevalent and deadly, particularly in Africa, with increasing resistance to antimalarial therapy and reports of severe malaria cases, while *P. vivax* represents an additional problem of recurrence derived from its ability to remain in the host's liver as an hypnozoite, a dormant parasitic form that can reactivate and cause the disease later on.[25,29]

Overall, the World Health Organization eradication program faces several obstacles. Reports reveal that important factors, incidence and burden for instance, show signs of slow evolution, particularly in Africa and India.[25] Other problems are responsible for this outcome, such as a difficult access to antimalarial therapies and healthcare, inadequate funding and, in some cases, cultural barriers in the use of modern therapies.[25] Undeniably, these complications are intrinsically connected and, ultimately, lead the population to find other solutions.

Traditional medicine is still recurrently used to treat many infectious diseases.[30,31] The availability, low cost and traditional knowledge that conveys trust in its efficiency are factors that sway populations to rely deeply on plants.[32] Ethnopharmacology has allowed the combination of both traditional and modern pharmaceutical approaches, in order to discover novel compounds and screen them for diverse activities.[31,32] Particularly in malaria, a great number of decoctions, teas and other preparations from plants are traditionally used across different endemic countries.[30,31] Their advantage is that acute toxicity is unlikely, since their use was established from centuries of experience of trial and error.[33,34] Additionally, this incommensurable pool of compounds has already contributed with the majority of modern antimalarials.[28,30,31,33–36] Examples are quinine and artemisinin, largely used today.[18,27,33,37–39] These compounds paved the way for synthetic antimalarial derivatives to be designed and now represent the foundation of the antimalarial therapies, both in the form of ACTs and individually.[18,39,40] In the same way, other natural compounds could contribute in a similar fashion. Phenolic compounds and their derivatives are a group of phytochemical substances found in virtually every plant. Despite their wide presence and recognized value for health-purposes, they are one of the least explored

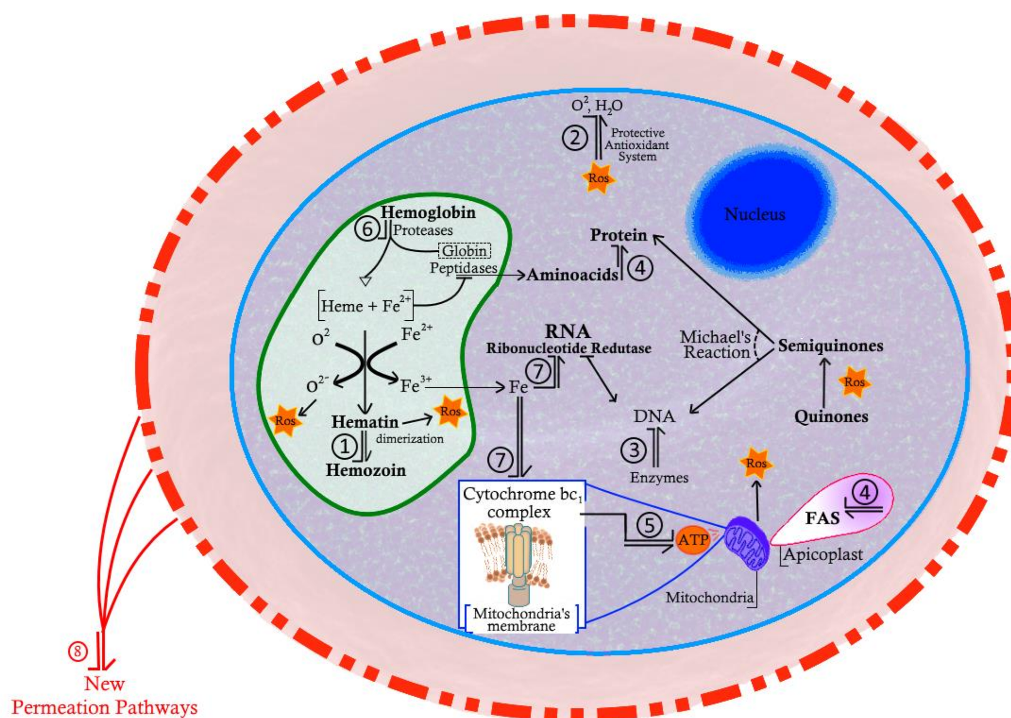


Figure 3 - Representation of an intra-erythrocytic Plasmodium falciparum trophozoite, highlighting key parasite intracellular pathways and the site of action of phenolic compounds. ① - inhibition of the formation of hemozoin; ② - inhibition of the redox homeostasis; ③ - inhibition of the DNA synthesis; ④ - inhibition of the synthesis of proteins or fatty acids (FAS); ⑤ - the inhibition of the cytochrome bc1 complex of the mitochondria's respiratory chain; ⑥ - inhibition of the digestion of hemoglobin; ⑦ - iron chelation; ⑧ - inhibition of new permeation pathways. The interference with any or multiple indicated pathways leads to the parasite's death.

classes, and there is a lack of an integral review on their characteristics and effects on the malaria parasite.

This review aims to integrate and discuss the most promising antiplasmodial phenolic compounds and derivatives isolated from plants over the last 28 years (1990-2018). To gather relevant information, reviews and single publications from that time period were searched on PubMed, Science Direct, SciFinder and similar databases using relevant keywords, such as the classes of phenolic compounds and malaria and/or *Plasmodium sp.*.[26,31,37,38,41–50] Compounds were selected according to their IC_{50} (µM) in *in vitro* assays with *P. falciparum* different strains: chloroquine sensitive (3D7, D6, F32, NF54, T9-96, D10), chloroquine resistant (Dd2, FcB1, FcB2, W2, Fcm29, RKL 303, PFB, BHZ 26/86), multidrug resistant (K1, NHP1337) and/or multidrug sensitive (HB3, FCR-3). The IC_{50} threshold to indicate whether a compound might be interesting for further drug development has shifted over the years from under 11 µM to under 1 µM.[45,51] In this review, the threshold of 2 µM was used because studies would often still describe further molecules with a 2 µM IC_{50} with cytotoxic assays in search for potentially interesting scaffolds.[52] As such, this seemed an acceptable limit predictive of progress into

drug development.[45,52] Attention was given to cytotoxicity tests, to attain the SI, the measurement of differentiated toxicity towards the parasite, and to the plants history as a traditionally used medicine.[33] The purpose of this review is to highlight and compare potential antimalarial hits within the phenolic phytochemical class, to discuss structure-activity, mechanisms of action and new perspectives these compounds could add to the fight against malaria. Figure 3 gives an overview of important parasitic pathways that are known targets of antimalarials. Compounds were selected according to the aforementioned criteria and included in the discussion whenever they impacted research. They are organized by phytochemical classes and displayed in tables with pertinent information, for easy consultation.

Lead phenolic antimalarial compounds from plants

Phenolic compounds are frequent in plants and are distributed within numerous phytochemical classes. They are broadly defined by their molecular structure, with one or more aromatic rings, without a nitrogen, originated from plant's metabolism pathways of the shikimate and/or acetate. The shikimate pathway starts from the same-named acid, which in turn originates aromatic amino-acids that, after deamination, create cinnamic acid and derivatives, like phenylpropanoids. The acetate pathway originates β- polyketide acids that can undergo cyclization steps to create phenolic compounds. From these routes, distinct groups of compounds can be identified, such as coumarins, flavonoids, tannins, stilbenes, lignans, quinones, xanthones, chalcones, among others. It is frequent that a hydroxyl group is associated to the aromatic ring, either free or as a part of other chemical functions, *e.g.*, esters, ethers or glycosides. When this functional structure is repeated, the compound is named a polyphenol.[53,54] Phenolic compounds incur oxidation reactions easily, which makes them strong radical scavengers.

Phenolic acids, phenols and derivatives

Phenols are aromatic compounds that occur very rarely in a free form in a plant, being usually present glycosylated or as polyphenols. Similarly to other phenolic derivatives, when ingested orally, glycosides might be hydrolysed in the digestive tract, which releases the aglycone. These compounds are derivates of the benzoic acid and can be hydroxylated, like in the case of the derivatives of gallic acid and its dimer, ellagic acid.[54] Although simple, this class has prompted interest in some isolates: methyl gallate (1), and compounds with the gallate/galloyl moiety; ellagic acid (3) and curcumin (5), showed in Figure 4.

Methyl gallate (1) exists widely in the plant's kingdom and, more importantly, is reported in plants traditionally used against malaria.[55] With many testified activities, such as antibacterial and antiviral, (1) has been considered a substance of interest since the 1980s.[56] An *in vitro* test with a variation of exposure time with the multi-resistant strain Dd2 was performed to determine the stage-specific

activity of (1). Contrarily to quinine, that significantly affected the parasite growth throughout the entire life cycle, compound (1) had its highest activity on the late stages of the parasite (late trophozoite and schizont).[55] Thus possibly limiting its effectiveness against the disease.[55] In spite of its demonstrated synergy with quinine or additivity with artemether, its highly variable IC_{50} between different strains cautions for limited activity, particularly against resistant strains.[55] Although its mechanism of action has not been elucidated, data suggests the hydroxy groups of the gallate moiety could play a pivotal role as donors in the establishment of bonds where the compound would exercise its activity. Other compounds with the gallate/galloyl moiety have been reported to have antimalarial activity, some of which will be explored in this review. Unfortunately, these compounds have broad

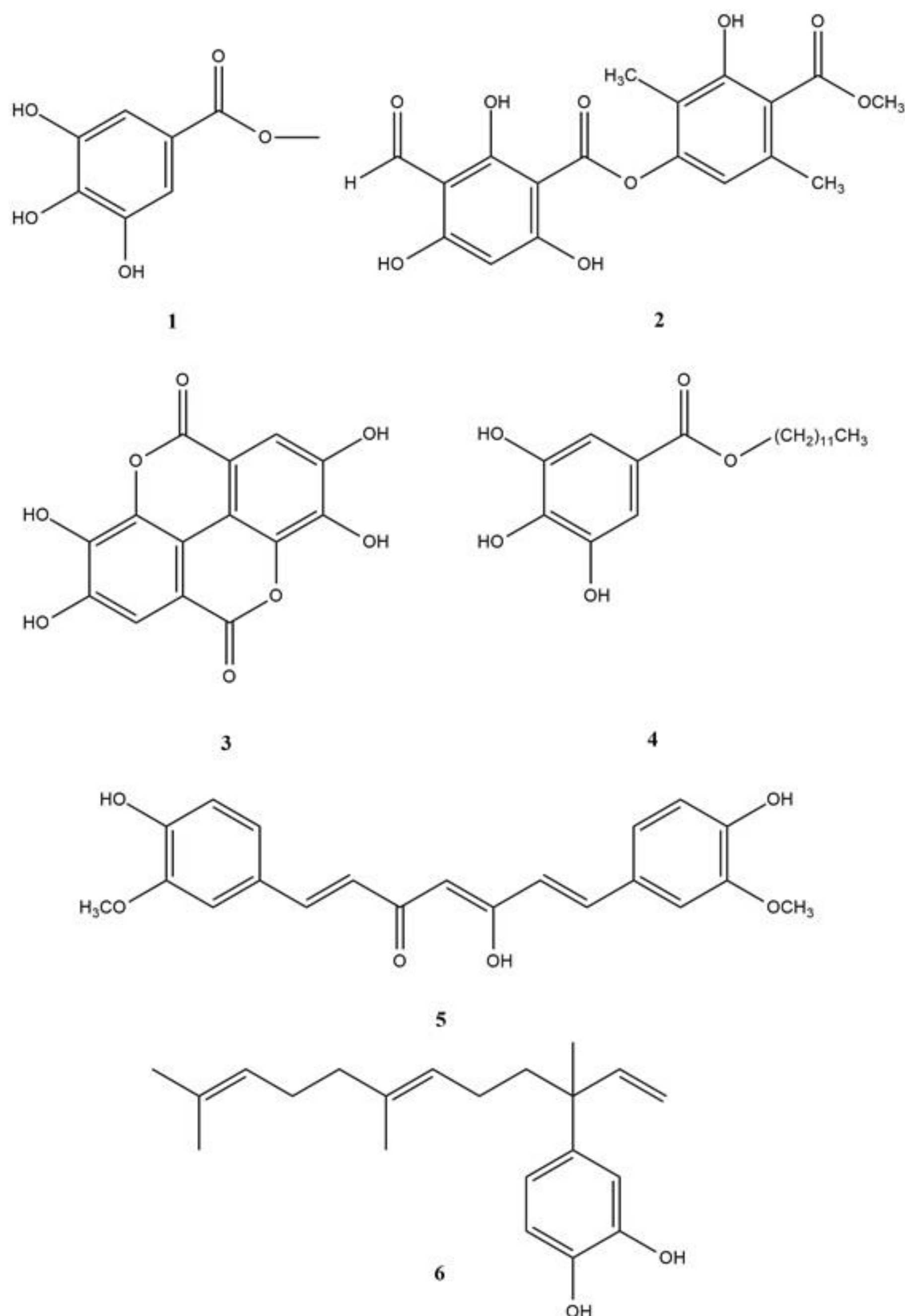


Figure 4 - Molecular structures of phenolic acids, phenols and derivatives.

IC_{50} values and it is not clear if they are selective towards the parasite. It would seem these moieties alone are not sufficient to produce potent antimalarials, but their existence might improve this activity, as it will be discussed ahead.[57,58]

Ellagic acid (3) is a common metabolite found across over 40 species of plants and vegetables, like the pomegranate fruit, a well-known source.[59–61] Present freely or in complex compounds (ellagitannins), it's constituted by a hydrophilic moiety, four hydroxyls, and a lipophilic moiety, two hydrocarbon rings, that render it the capability to act both as an acceptor and donor of electrons. This activity makes (3) act as a preventive and potent antioxidant, with the capability of passing the cellular membranes and entering the parasite. However, it has been reported that this same structure diminishes its oral bioavailability.[54,59–61] In the malaria context, (3) has demonstrated a high degree of activity. Its presence as the active component in several traditionally used plants against malaria has originated many studies and great interest.[57,59,62] Some assays also demonstrate ellagic acid's curative and prophylactic effects *in vivo*. For example, Soh *et al.*[61] demonstrated that in mice infected with *Plasmodium vinckei petteri* treated with (3) at 1, 50 and 100 mg/kg/day intraperitoneally in a 4-day suppressive test, the effective dose was inferior to 1 mg/kg/day and, above this dose, a fully inhibition of parasite growth was attained. Additionally, at 50 and 100 mg/kg/day, no recrudescence was detected over the next 60 days. Likewise, at 10 mg/kg/day intraperitoneally for 4 days before inoculation, a high protective effect was demonstrated, assuring survival by day 9 post-infection. After oral administration, however, the results were in line with the known low bioavailability of this compound.[54,59,60] Even at the highest dose of 1 g/kg/day, no antimalarial activity was reported. Toxicity tests proved its safety in mice, with no toxicity up to 1 g/kg/day. Besides its antiplasmodial activity in the nM range (see Table 2), (3) demonstrated synergy with chloroquine, atovaquone, mefloquine and artesunate in *in vitro* assays.[61] It's important to note that these results occur directly in the erythrocytic stage or when administered intraperitoneally, and do not translate to human effectiveness. It is known that ellagic acid's activity in the erythrocytic cycle occurs on the trophozoite and early schizont forms of the parasite, the most active regarding DNA and protein synthesis.[61,63] When compared to ellagitannins, which have a better oral absorption and can be metabolized into free ellagic acid, the IC_{50} is over ten times the needed concentration for (3) for the same *in vitro* activity.[59] The MoA of (3) is also a matter of discussion. One of the mechanisms might be similar to quinolines, preventing the formation of hemozoin. Its flat molecular structure endows it a dispersed electronic density that allows the formation of a π-π complex.[64] Another mechanism might be the inhibition of enzymes responsible for the digestion of haemoglobin, like plasmepsins, although this occurs in concentrations too high in comparison to those presented in Table 2 – see Figure 3 for context.[64] Finally, in theory, it might act through its chelating properties, with the formation of coordination bonds with the iron present in

heme.[64] Ultimately, (3) demonstrates a broad range of mechanisms and a high selectivity towards the parasite, but its low oral absorption has to be circumvented and its activity in a small part of the parasite's cycle makes it an unlikely antimalarial candidate without further optimization.[61]

Curcumin (5) is a phenylpropane, a derivative of caffeic acid, isolated from the roots of *Curcuma longa*, a food plant reportedly used for over five thousand years.[65–68] The interest on this compound originated from its common dietary use, lack of toxicity and varied pharmacological activities.[66] Studies have demonstrated its immunomodulating, antiparasitic, antioxidant, anti-inflammatory, antiviral, antibacterial and anticancer properties.[65–69] According to the literature, curcumin's antiplasmodial activity in vitro ranges from 5-30 µM, in both sensitive and resistant-chloroquine strains, well above the active threshold recognized for natural compounds.[45,48,67–71] In spite of this, the aforementioned interesting features of (5) led researchers to study its MoA to reach a conclusion on what (5) could add to the fight against malaria. There are at least five proposed MoA for curcumin, that allowed the validation of several potential targets in the parasite. First, the inhibition of Ca^{2+}-ATPase of the sarcoplasmic-endoplasmic reticulum, leading to metabolism arrest.[67,68,70,71] Second, enhancement of cytosolic Ca^{2+} activity and ceramide formation, that lead to the phagocytosis of the red blood cell.[71] Third, in certain conditions, like a range of concentration of 20-100 µM and the presence of transitional metal ions, (5) behaves like a pro-oxidant, possibly through its *o*-methoxyphenol group and methylenic hydrogen, and increases ROS (Reactive Oxygen Species) production, originating damaged DNA and proteins.[67,69,71,72] Forth, (5) inhibits histone acetyltransferases, required for chromatin arrangement and transcriptional activation.[67,69–71] Fifth, more recently it has been demonstrated that (5) disrupts the parasite's microtubule structure, interfering with processes such as merozoite formation, invasion of erythrocytes and nuclear division, although this was only effective at the second cycle of the same culture.[70] Evidently, (5) remains an interesting compound to study in face of the ever-changing parasite. However, being hydrophobic, curcumin has poor oral availability and is rapidly metabolized after administration. These characteristics represent a liability in the pursue of turning this compound into a successful antimalarial. Synthetic analogues have been reported to improve on these characteristics and have better IC_{50} values, as low as 400 nM.[67,71]

Compound (6) is a catechol derivative known as the main secondary metabolite of the *Pothomorphe* genus, a group of Brazilian plants used traditionally for malaria.[44,73] It has a good activity against the multidrug resistant strain K1 (with an IC_{50} of 0.67µM), and it presents low toxicity to mouse 3T3 fibroblast cells, with an acute oral LD_{50} (median lethal dose) in female Swiss mice of > 2 g/kg.[73] This compound demonstrated haemolytic properties in human erythrocytes in a wide range of concentrations, which would caution against administration to malaria patients.[73] *In vivo* toxicity studies demonstrated no death at an oral dose of 2 g/kg, but changes in mice behaviour, such as

Table 2 - Phenolic acids, phenols and derivates.

Number	Compound	Botanical Origin	Family	Antiplasmodial IC_{50} (Strain)	Cytotoxicity IC_{50} (Cell line)	Selectivity Index (SI)	Reference number
1	Methyl gallate	*Dacryoedes edulis* (G Don)	Burseraceae	2.01 µM (3D7) 2.99 µM (Dd2)	> 543 µM (LLC-MK2)	> 182	55
		Swintonia foxworthyi Griffith	Anacardiaceae	10.86 µM (W2) 19.00 µM (D6)	> 109 µM (BC1; Lu1; Col2; KB-V1; LNCaP) 41.27 µM (KB)	> 2.17	56
		Koelreuteria paniculata Laxm.	Sapindaceae	6.95 µM (D6) 4.18 µM (W2)	ND	-	74
2	Atranorin	*Kigelia africana* (Lam.) Benth	Bignoniaceae	4.41±0.35 µM (W2) 2.81±1.07 µM (CAM10)+ 2.78±0.29 µM (SHF4)+	27.72 µM (LLC-MK2)	> 1.7	75
3	Ellagic acid	*Terminalia avicennioides* Guill. et Perr	Combretaceae	0.2 µM (K1)	30.4 µM (HepG2)	152	63
		Terminalia mollis M.A. Lawson	Combretaceae	0.56 µM (3D7) 0.40 µM (F32)	ND	-	76
		Alchornea cordifolia L.	Euphorbiaceae	0.86 µM (Fcm29) 1.52 µM (Nigerian strain)	68.17 µM (Hela)	> 45	62
		Punica granatum L.	Lythraceae	> 39.4 µM (D6 and W2)	> 103 µM (HL-60)	> 2.6	59
		Tristaniopsis calobuxus, Brongniart & Gris	Myrtaceae	0.5 µM (D6) 0.3 µM (W2)	>100 µM (Hep G2)	> 200	58
		Acros Organics (Belgium)*	-	0.33 ±0.027 µM (F32) 0.11 ±0.027 µM (Dd2) 0.30 ±0.017 µM (FcB1) 0.33±0.024 µM (W2) 0.18 ±0.020 µM (FcM29)	>100 µM Vero, KB, and MRC5 cells	> 495	61,77
4	Lauryl gallate	*Cylicodiscus gabunensis* Harms	Leguminosae	2.2 ±0.2 µM (Dd2)	30.0 ±2.0 µM (HepG2)	13.6	57
6	4-Nerolidylcatechol	*Pothomorphe peltata (*L.) Miq.	Piperaceae	0.67 µM (K1)	507 µM (3T3 cells)	756	44,73

* purchased
+ field isolates (CAM10 and SHF4) from Bolifamba, Cameroon, chloroquine resistant
ND – not determined

agitation, reduced water and food consumption and diarrhea.[73] This could be associated with haemolysis or other types of toxicity that make this compound, and its derivatives, unlikely antimalarials. Regarding the antiplasmodial activity, catechols are thought to act by their anti-oxidant activity, possibly through the iron chelating properties or the inhibition of the DNA synthesis, although it is not known with certainty.[64,73]

Coumarins and derivates

Coumarin, 1,2-benzopyrone, is the basic molecular structure that characterises this class of compounds. Further modifications may ensue, like O- or C-prenylation, cyclization and other transformations, that may lead to other derivatives, the case of furanocoumarins or pyranocoumarins.[54] These compounds have been the basis for the discovery of some drugs, like warfarin, but are generally known to have limited pharmacological activity.[78] In the case of malaria, the isolated molecules corresponding to the criteria are presented in Table 3 and Figure 5.

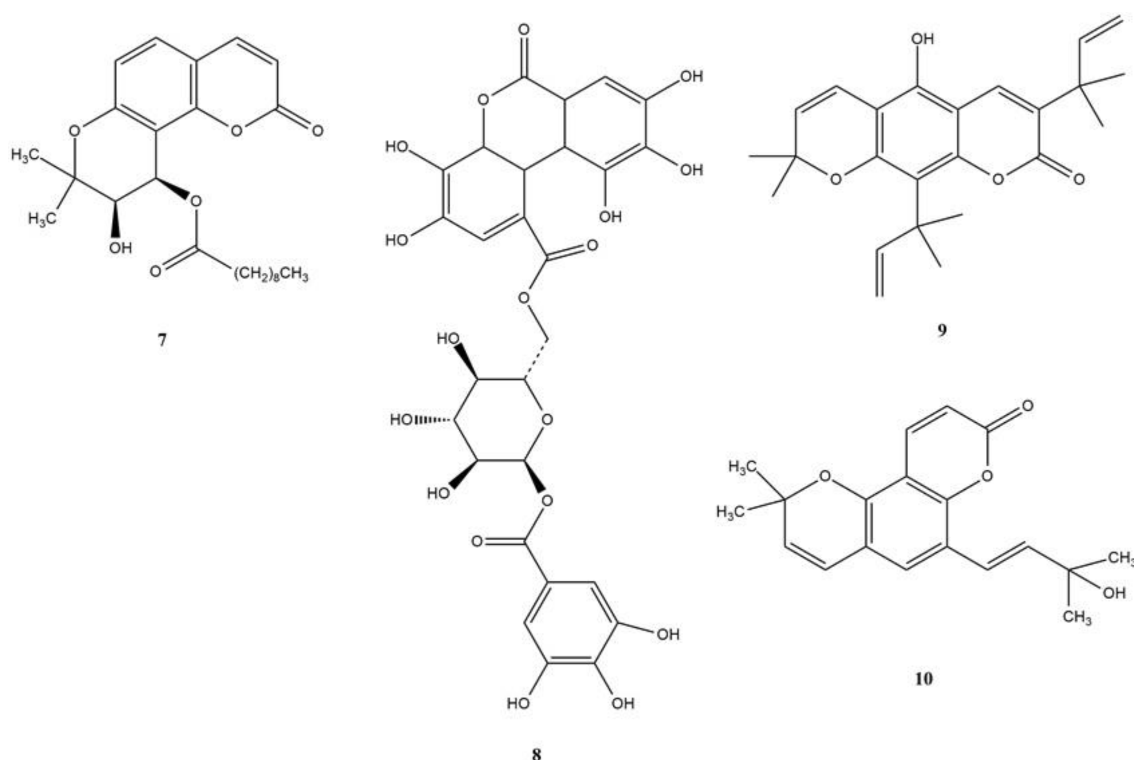


Figure 5 - Molecular structures of coumarins and derivatives.

It is difficult to find highly active antiplasmodial compounds within this class, and there is evidence of a great distinction between sensitive and resistant strains, as evidenced in Table 3. However, coumarins have been identified as the active components in some plants used traditionally against malaria.[32,79]

Compound (7) was identified in the rhizome parts of *Angelica purpuraefolia* Chung, a Korean plant used in folk medicine, after extract tests revealed antiplasmodial activity.[80] Two compounds were

isolated and deemed responsible for the extracts activity. Structurally, the difference between (7) and the other compound ((+)-3'-Decanoyl-cis-khellactone), is the position of the lipophilic chain, from carbon 4' to 3'. This sole change represents an increase in antiplasmodial activity (IC_{50} of 2.4 µM ± 0.2) and, consequently, in SI (> 62.5).[80] It could prove useful to study further the structural implications of both compounds in order to establish structure-activity relations.

Table 3 - Coumarins and derivatives.

Number	Compound	Botanical Origin	Family	Antiplasmodial IC_{50} (Strain)	Cytotoxicity IC_{50} (Cell line)	Selectivity Index (SI)	Reference number
7	(+)-4'-decanoyl-*cis*-khellactone	*Angelica purpuraefolia* T.H. Chung	Apiaceae	1.5 µM (D10)	> 150 µM (SK-OV-3)	> 100	80
8	1-O-galloyl-6-O-luteoyl-R-D-glucose	*Phyllanthus niruri* L.	Euphorbiaceae	2.0-2.4 µM (FCR-3)	ND	-	79
9	Clausarin	*Clausena harmandiana* Pierre	Rutaceae	0.26-1.84 µM (K1)	ND	-	81
10	*trans*-avicennol	*Zanthoxylum chiloperone* var. *angustifolium* Engl.	Rutaceae	7.8 µM (K1) 1.5 µM(F32) 3.5 µM (PFB) 6.4 µM (FcB1)	12.85 µM (MCR5)	> 1.6	32

ND – not determined

Compound (8) was isolated and identified from *Phyllanthus niruri* L., a plant widely distributed in Indonesia with traditional application in malaria.[79] *In vitro* in the FCR-3 multidrug sensitive strain, the activity was barely within the interesting range in discussion (< 2 µM), and unfortunately, no toxicity tests were performed. Microscopical observations detected a stagnation in the ring stage accompanied by morphology changes in the parasite, however no debate ensued.[79] It is possible to infer that (8) exerts antiplasmodial activity in the early stages, affecting possibly the haemoglobin digestion or other metabolomics pathways.

Clausarin (9) was isolated and later identified as one of the antiplasmodial constituents of *Clausena harmandiana* Pierre, a plant from Thailand.[81] Although its antiplasmodial activity is high (0.26-1.84 µM), only one strain was tested and no discussion on toxicity was made.[81] In parallel, another coumarin was also isolated in the same assay, dentatin. The structural difference between the two is an additional prenyl group and an hydroxyl group, instead of a methoxy group.[78,81] This alone represents a 20-fold difference between activities, revealing the importance that the solubility, electromagnetic distribution and proton availability have in the antiplasmodial activity.

Trans-avicennol (10) was isolated from *Zanthoxylum chiloperone* var. *angustifolium* Engl., a tree endemic to central and southern South America, traditionally used to treat malaria.[32] *In vitro* assays with multiple strains revealed various levels of activity, with the highest effect on the chloroquine-sensitive F32 strain (IC_{50} of 1.46 µM).[32] The compound's toxicity was tested on MCR5 cells and

erythrocytes. Results revealed no haemolytic properties and an IC_{50} of 12.85 µM on the MCR5 cells, which does not award the compound a great selectivity towards the parasite.[32]

The MoA of this class of compounds is unknown. There is the possibility of formation of adducts with heme, the case of *trans*-avicennol (10).[32] Whatever the MoA, the presence of hydroxylic groups and carbon chains appears to influence greatly the level of activity. The compound (8), with a gallate moiety, further evidences this structural importance. In any case, it is possible to derive synthetically compounds of interest from this class.[80]

Flavonoids

Flavonoids are one of the most abundant and frequently occurring class of natural compounds. They are broadly present through the entire plant kingdom and, consequently, are consumed daily in the human diet.[28,54,78,82,83] The skeleton is based on a ring framework (C6-C3-C6), in which the A and B rings are aromatic and the C ring interconnects both. The connection between the ring C and B differentiates structurally the classes of compounds, likewise with the saturation at C-2-C-3, or it's substitutes.[28,54,83] This structure is strongly correlated to the classes' pharmacological activities as anti-inflammatory and anti-oxidants. Similarly to other phenolic compounds, flavonoids do not usually occur freely, but as biflavonoids or glycosides.[28,78,83]

As one of the most plentiful secondary metabolites, when plants are analysed for the possible presence of antiplasmodial compounds, flavonoids are frequently referenced.[28] Following this research, the flavonoids relevant for this review are enumerated in Table 4 and 5 and showed in Figure 6. Although this phytochemical class proved to be highly active, at first this would be expected, considering its structural diversity. However, a concept of variety of chemical structures applied to malaria is quickly refuted upon examining the tables. Of the thirty-four compounds exposed in Table 4, twenty-five are flavones and biflavones, seven are biflavanones, and only one other flavanone and one flavanolol are present. Table 5 presents an additional group of seven catechins with antiplasmodial activities. This demonstrates a preference for the benzo-γ-pyrone structure and for the connection of the ring B at the C-2.

Ten active flavones were isolated from the *Artocarpus sp.* (Moraceae), a plant traditionally used against malaria in the tropical and subtropical regions of South- east Asia.[84] The ten were found to be prenylated, but notably only with isoprene groups.[84–87] Compounds (25 and 26) both have an isoprene, at C-3 and C-8 respectively. The B ring differs in one oxygen methylation and a cyclization. Despite this, both activities are very close, with an IC_{50} of 0.12 and 0.18 µM in the 3D7 strain, respectively.[84] Artonin A (29), B (34) and F (35) have the same flavone skeleton and three structural differences regarding

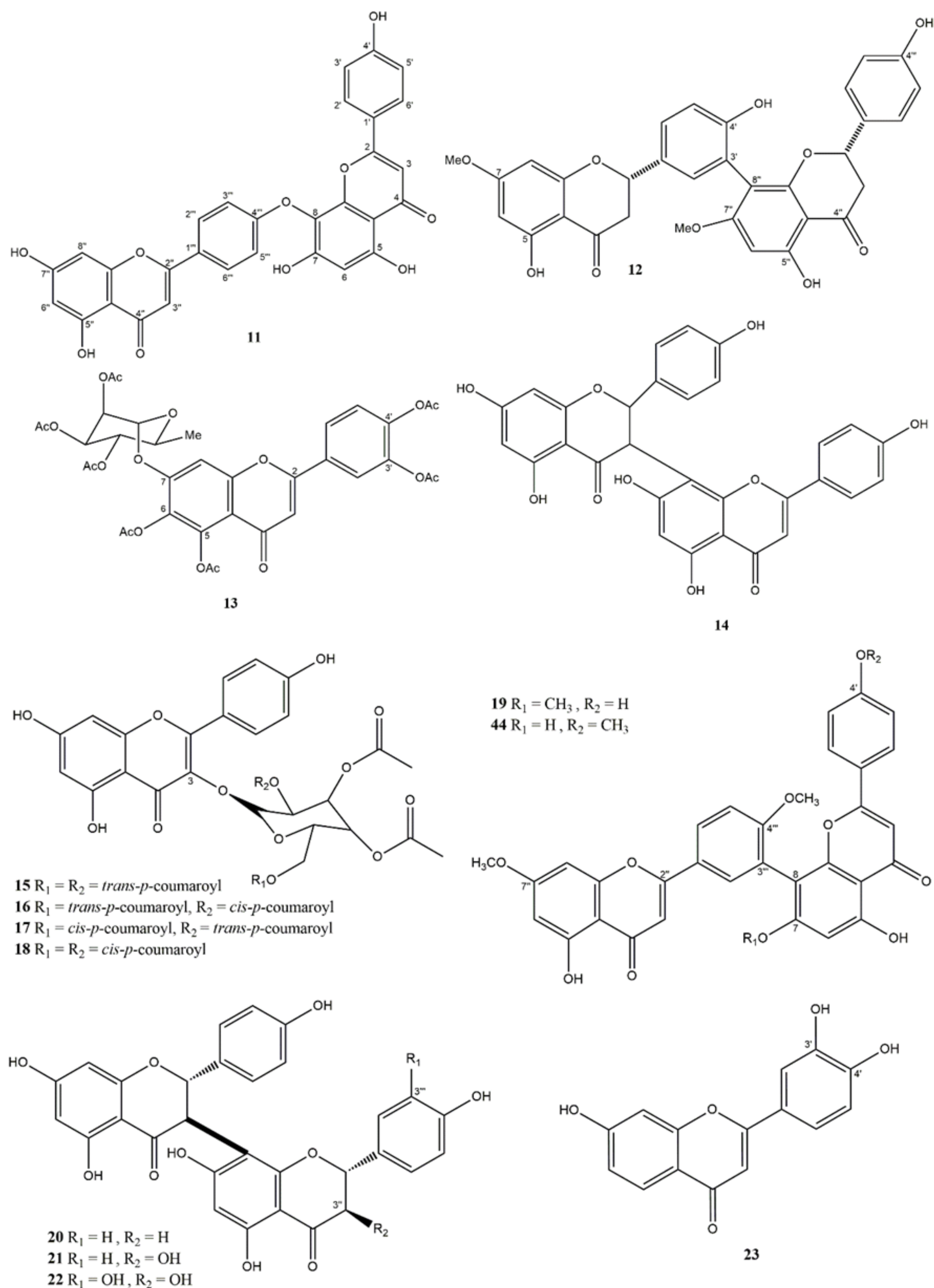


Figure 6 – Molecular structures of flavonoids.

cyclization and isoprene groups.[86] When comparing activities in the same test, the most active compound is (34), with an IC_{50} of 1.56 µM.[86] Considering the sole molecular difference between (34) and (29), with a representative IC_{50} of 4.9 µM in the same assay, it would appear the free hydroxylic group at C-5' is meaningful for activity. Compound's (34) low SI (5.5), however, puts it at a disadvantage

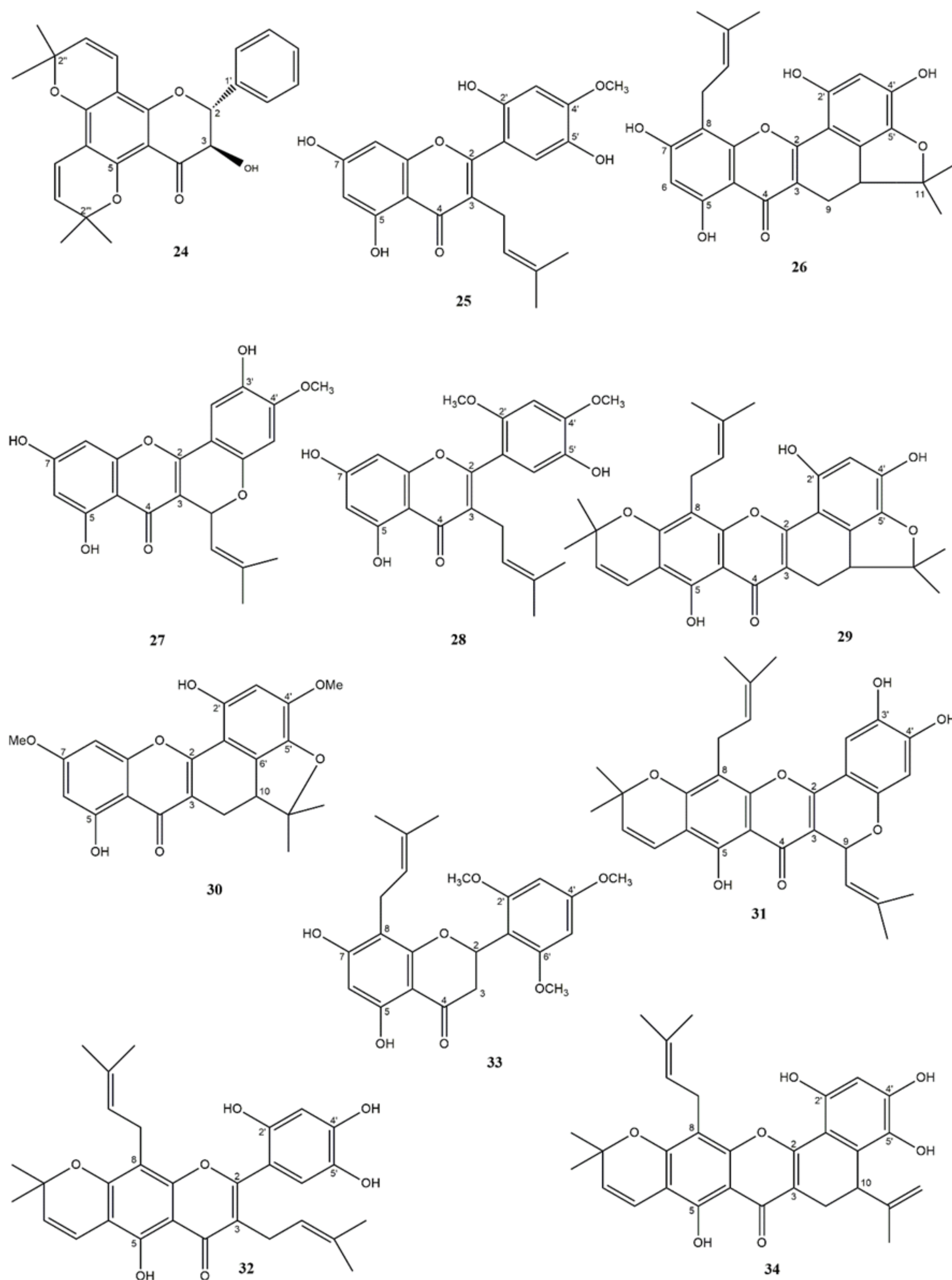


Figure 6 (continued)

in relation to (35), that is slightly less active, with an IC_{50} of 2.2 µM, but considerably more selective (33.0).[86] This would lead to the conclusion that the position of the isoprene and the additional ring are important for the selectivity of these compounds. Compounds (27 and 28) further impose the importance of the isoprene group. Compound (28), with an isoprene at C-3, is significantly more active, with an IC_{50} of 0.66 µM, than (27), with an IC_{50} of 1.31 µM, that includes a ring in that group, leaving

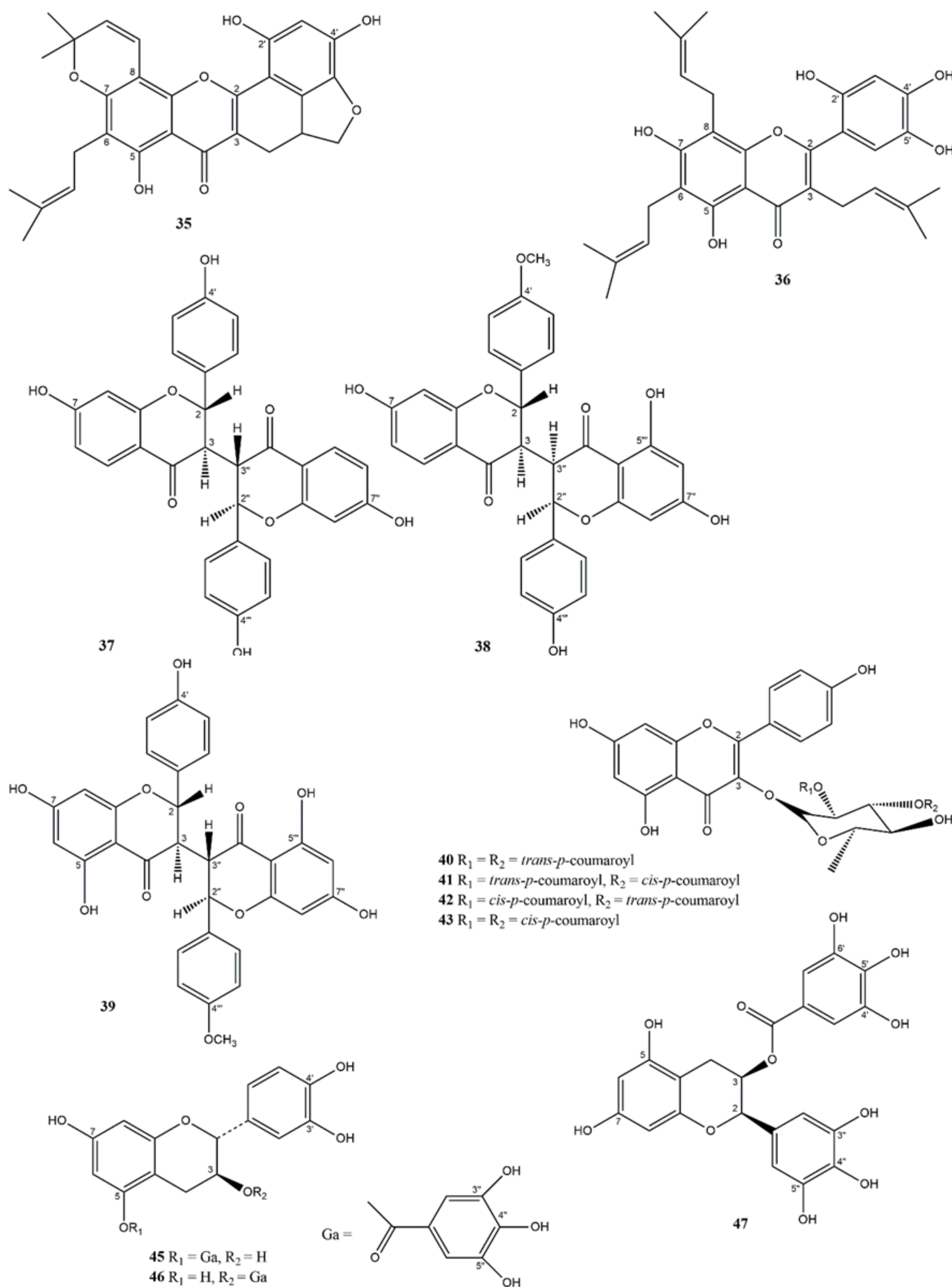


Figure 6 (continued)

free a 2-methylbut-2-ene.[85] Other differences are present in the B ring, but overall it would seem the length of the hydrophobic chain is important. The same occurs between compounds (31 and 32). In this case, the compound with the cyclization at C-3 (31), with an IC_{50} of 0.02 µM, is significantly more active than (32), with an IC_{50} of 1.04 µM, that has two isoprene groups.[85] Lastly, (36) has three isoprene groups at C-3, C-6 and C-8, and has an IC_{50} of 1.12 ± 0.08 µM.[86] The number of isoprene groups should

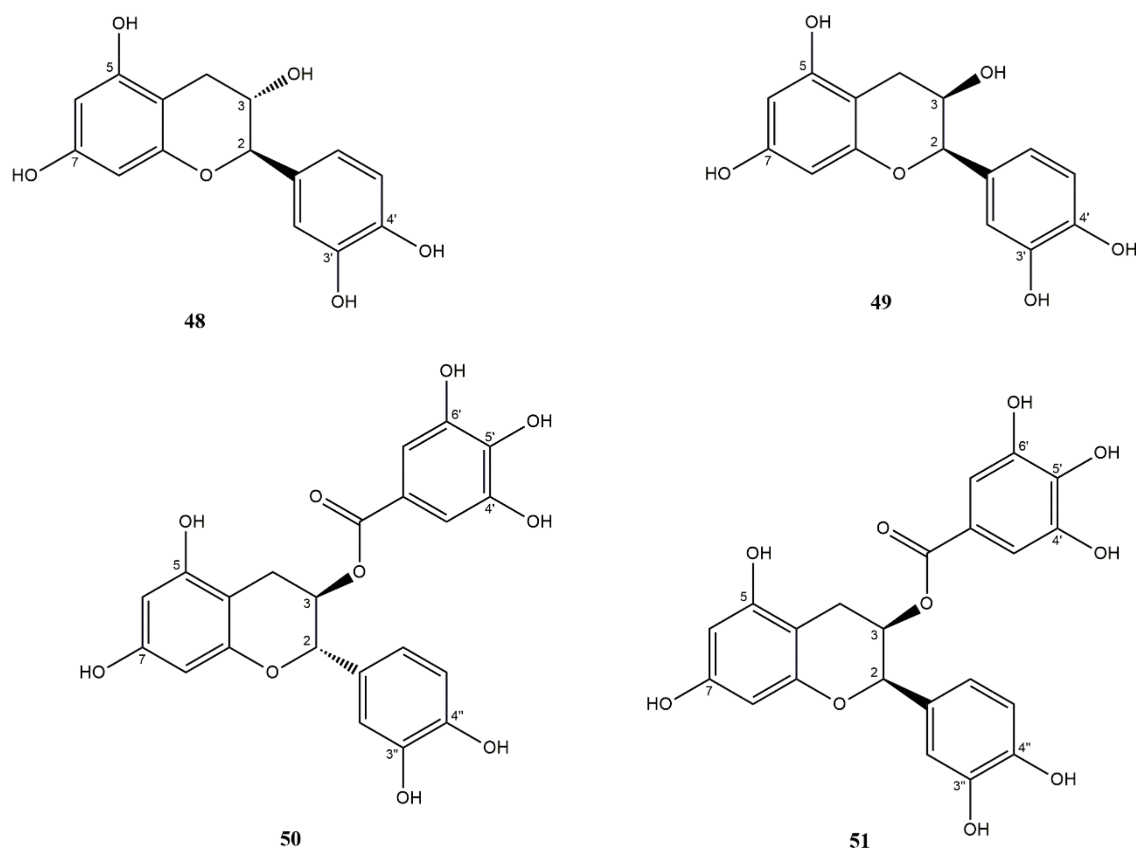


Figure 6 (continued)

increase this compounds lipophilia, even with five hydroxylic groups available. It would appear that the isoprene group at C-8 is important to increase the lipophilia of the compounds so that they can bypass the cytoplasmatic parasitic membrane and exert additional effects on the parasite.[87,88] The most cytotoxic compounds in this group appear to have a isoprene at C-3, which means cyclization at this position might at least decrease toxicity.[86] Moreover, the ten compounds prove to be strongly antiplasmodial, although not confirmed as selective towards the parasite, questioning the safety of the use of the *Artocarpus sp.*[84]

Artopeden A (30) is a simple flavone with a C-3 cyclization and with two methylations. It may be that the methylations increase its lipophilia enough to have a similar activity to prenylated flavones, since their degree of activity is comparable (IC_{50} of 0.114 µM in the 3D7 strain).[85,88] Unfortunately, no further studies are available to discuss this compound's activity. 3',4',7-trihydroxyflavone (23) was isolated from the bark of the *Albizia zygia* (DC.) J.F. Macbr. tree, traditionally used in Sudan as an antimalarial.[89] Although in the same assay other one flavone and one flavanone were isolated, sadly no further information regarding *in vitro* antiplasmodial or cytotoxicity is reported.[89] While with a fairly simple chemical structure, with only hydroxyl substitutions at C-7/3'/4', both good antiplasmodial and cytotoxic results were achieved, which warranted a SI of 5. The lack of specificity of activity might be

connected with the oxidant potential of this compound, which could be a good starting point to further development.

Biflavones are two flavone units, similar or not, connected covalently either through C-C or O-C bonds, that occur rarely.[78,90] Four biflavones were found according to the criteria: compounds (11), (14), (19) and (44). Compound (11) has a C-4'''–O–C-8 bond connecting two monomeric similar flavones. Both its activity (IC_{50} of 0.48 µM) and SI (159) are impressive and might be correlated to the bond between the two units. In the same study, compounds with a hydroxyl group at C-4''' showed no activity.[90] Compound (14) is an example of a biflavone with two different monomeric units, an imposition by the place of the bond between the two molecules (at C-3). Similarly, in the same study, it was demonstrated that, if instead of another unit at C-3, the compound had a simple hydroxylic group, the antiplasmodial activity would drop significantly.[91] Although its activity is not as impressive as (11), it demonstrates the possible advantage of biflavones. Compounds (19 and 44) are structurally similar, with the exception of a methylation at the oxygen at C-7 or C-4'. This single difference in their structure is enough to improve the activity, of 2.0 and 1.4 µM, respectively, and, more importantly, the selectivity of (44) over ten times.[90] The availability of the methoxy group in the C4' position might represent an influence in bonding to specific sites at the human or parasites' cellular structures, enough to prevent a toxic interaction in the hosts cells.[90] Overall, the significant SI of biflavones is interesting towards asserting structural key-features.

Solely one flavanone is relevant in the context of this review: heteroflavanone C (33). Compound (33) is the most active flavonoid, with an IC_{50} of 1 nM.[84] It has key features that distinguish it from the other compounds discussed thus far: first, it lacks the double bond between C-2-C-3; second, it has only two hydroxyl groups; third, the B ring has three oxygens that are all methylated. The availability of the C-3 might be one of the crucial points in the establishment of a bond. Likewise, the methylation of all oxygens on the B ring might improve its lipophilia and allow the compound to enter the parasite more easily. Regrettably, no SI is given, which would complement this compounds' profile.

Seven biflavanones are presented: compounds (12), (20), (21), (22), (37), (38) and (39). Compound (12) has improved activity towards the chloroquine resistant strain.[92] Compounds (20-22) were extracted from *Garcinia kola* sun-dried nuts, which are used traditionally as a chemo preventive of malaria in Central Africa.[93] *In vitro* tests demonstrated the pertinence of this use: compounds (20-22) exhibited high antiplasmodial activity, IC_{50} between 0.16-0.65 µM, and significant selectivity (above 77).[93] *In vivo* studies in mice infected with *P. berghei* (ANKA strain) were performed with compound (21) following a four-day suppressive test.[93] Concentrations ranged from 25 to 200 mg/kg/day and were orally administered. The mean effective dose (ED_{50}) was calculated at approximately 100 mg/kg. The test

demonstrated improved mice survivability, no observable toxicity up to 200 mg/kg/day and a dose-dependent parasite inhibition.[93] Most importantly, these results were accomplished with an oral administration, what proves to be a great advantage towards developing and studying these compounds further. Compound (37) does not have methylated oxygens and has the bond between the C rings – C-3-C-3''. Compounds (38 and 39) have the same bond as (37), but a methylated oxygen at C-4' or C-4''', respectively. Between (38 and 39) there's the additional difference in isomerism of the protons at C-3''. In the same tests, compound (37) was the most active, with an IC_{50} of 0.15 µM (K1) and 0.47 µM (FCR-3), and selective (>116) of the three.[94] This demonstrates that the occupation of the C-3 of the flavanone by another unit is not a disadvantage towards activity or selectivity.

Flavanonol MS-II (24) is a flavanolol with two (2,2-dimethylchromene) rings. In spite of having been solely tested on sensitive strains, it demonstrates a relevant variety in activity depending on the strain (see Table 4).[95] Its selectivity towards the parasite is noteworthy, proposing that resistant strains should be tested in order to assess the compound's potential.

Lastly, catechins are a particular sub-group of flavonoids, distinguishable by the absence of the carbonyl group on the C-ring.[54] They are abundantly found in green tea leaves (*Camellia sinensis*) as acid esters and are known for their antioxidant properties.[28,96] Hence, these compounds are frequently isolated with their gallate moieties, as evidenced in [Table 5]. Compounds (45 and 46) are two catechins active against a chloroquine-resistance strain, with an IC_{50} of 1.2 and 1.0 µM, respectively, and with a remarkable SI (> 75). They differ solely on the position of the gallate moiety, either at C-5 or C-3, respectively. However, this does not seem to interfere with neither their activity or selectivity, on the contrary, the existence of the gallate esters seems essential towards their inhibitory activity, independently of the positioning.[97] Compounds (48-49 and 50-51) are structurally related, differing only on the isomerism at C-2 and C-3. Studies specifically targeting the FAS have demonstrated the structural requirements for the parasiticidal effect through FAS inhibition. Catechins with a gallate moiety at C-3 are all potent inhibitors, while a free single hydroxyl group in the same position seems to diminish greatly this activity.[83] Consequently, catechins (50 and 51) may act through this mechanism. The gallate substitution in these compounds appears crucial for their inhibitory activity, especially considering that gallic acid alone is inactive.[57,83,98,99]

Table 4 - Flavonoids and derivatives.

Number	Compound	Botanical Origin	Family	Antiplasmodial IC_{50} (Strain)	Cytotoxicity IC_{50} (Cell line)	Selectivity Index (SI)	Reference number
11	Lanaroflavone	*Campnosperma panamense* Standl. *Lanaria lanata* L. *Ouratea semiserrata* Mart.	Anacardiaceae Lanariaceae Ochnaceae	0.48 µM (K1)	70 µM (L-6)	159	[90,100]
12	(2S,2"S)- 7,7"-di-*O*-methyltetrahydroamentoflavone	*Rhus retinorrhoea*	Anacardiaceae	1.72 µM (W2) 4.92 µM (D6)	NT	-	[92]
13	Peracetyl-6-Hydroxyluteolin-7-*O*-(1"-α-rhamnoside)	*Vriesea sanguinolenta* Cogn. and Marchal	Bromeliaceae	0.52 µM (K1) 0.63 µM (NF54)	ND	-	[101]
14	Volkensiflavone	*Allanblackia floribunda*	Clusiaceae	2.25 µM (F32) 1.81 µM (FcM29)	ND	-	[91]
15	kaempferol-3-O-(3′′,4′′-diacetyl-2′′,6′′-di-*E*-*p*-coumaroyl)-glucoside	*Quercus laceyi* Small	Fagaceae	0.6±0.1 µM (HB3) 2.1±0.6 µM (NHP1337)	< 3 µM (HeLa)	> 5 (HB3) > 3.3 (NHP1337)	[37]
16	kaempferol 3-O-(2′′-cis-p-coumaroyl-3′′,4′′-diacetyl-6′′-*trans*-*p*-coumaroyl)-β-D-glucopyranoside	*Quercus laceyi* Small	Fagaceae	0.9±0.2 µM (HB3) 5±1 µM (NHP1337)	< 3 µM (HeLa)	> 3.3 (HB3) > 0.6 (NHP1337)	[37]
17	kaempferol-3-O-(2′′-trans-p-coumaroyl-3′′,4′′-diacetyl-6′′-cis-p-coumaroyl)-β-D-glucopyranoside	*Quercus laceyi* Small	Fagaceae	0.8±0.1 µM (HB3) 4±1 µM (NHP1337)	< 3 µM (HeLa)	>3.8 (HB3) > 0.8 (NHP1337)	[37]
18	kaempferol-3-O-(3′′,4′′-diacetyl-2′′,6′′-di-Z-p-coumaroyl)glucoside	*Quercus laceyi* Small	Fagaceae	2.1±0.9 µM (HB3) 3.8±0.6 µM (NHP1337)	< 3 µM (HeLa)	> 1.4 (HB3) > 0.8 (NHP1337)	[37]
19	Ginkgetin	*Gingko biloba* L.	Ginkgoaceae	2.0 µM (K1)	9 µM (L-6)	4.1	[90]
20	GB-1a	*Garcinia kola* Heckel	Guttiferae	0.65 µM (FCR-3)	50 µM (KB 3-1)	77	[93]
21	GB-1	*Garcinia kola* Heckel	Guttiferae	0.16 µM (FCR-3)	> 150 µM (KB 3-1)	> 900	[93]
22	GB-2	*Garcinia kola* Heckel	Guttiferae	0.21 µM (FCR-3)	> 150 µM (KB 3-1)	> 700	[93]
23	3',4',7-trihydroxyflavone	*Albizia zygia* (DC.) J.F. Macbr.	Leguminosae	0.29 µM (K1)	1.50 µM (L-6)	5.17	[89]
24	Flavanonol MS-II	*Tephrosia subtriflora* Baker	Leguminosae	4.6±1.1 µM (D6) 1.7±0.1 µM (3D7) 1.5±0.1 µM (KSM009*) 1.4 ±0.3 µM (F32-teM**)	> 247.5 µM (Vero) 247.5 µM (Hep2)	>58	[95]
25	Artocarpone A	*Artocarpus champeden* Spreng.	Moraceae	0.12 µM (3D7)	ND	-	[84]

26	Artocarpone B	*Artocarpus champeden* Spreng.	Moraceae	0.18 µM (3D7)	ND	-	[84]
27	Artoindonesianin A-2	*Artocarpus champeden* Spreng.	Moraceae	1.31 µM (3D7)	ND	-	[84]
28	Artoindonesianin R	*Artocarpus champeden* Spreng.	Moraceae	0.66 µM (3D7)	ND	-	[84]
29	Artonin A	*Artocarpus champeden* Spreng. *A. styracifolius*	Moraceae	0.55 µM (3D7) 4.9 ±1.2 µM (FcB1)	ND	-	[84,86]
30	Artopeden A	*Artocarpus champeden* Spreng.	Moraceae	0.11 µM (3D7)	ND	-	[85]
31	Cycloheterophyllin	*Artocarpus champeden* Spreng.	Moraceae	0.02 µM (3D7)	ND	-	[84]
32	Heterophyllin	*Artocarpus champeden* Spreng.	Moraceae	1.04 µM (3D7)	ND	-	[84]
		Artocarpus styracifolius	Moraceae	1.2 ± 0.004 µM (FcB1)	5.4 µM (KB) 3.8 µM (MRC-5)	3.2	[86]
33	Heteroflavanone C	*Artocarpus champeden* Spreng.	Moraceae	1.0 nM (3D7)	ND	-	[84]
34	Artonin B	*Artocarpus styracifolius*	Moraceae	1.56 ± 0.08 µM (FcB1)	11.3 µM (KB) 8.2 µM (MRC-5)	5.5	[86]
35	Artonin F	*Artocarpus styracifolius*	Moraceae	2.2 ± 0.5 µM (FcB1)	73.5 µM (KB) 72.2 µM (MRC-5)	33	[86]
36	Styracifolin B	*Artocarpus styracifolius*	Moraceae	1.12 ± 0.08 µM (FcB1)	5.6 µM (KB) 4.7 µM (MRC-5)	4.3	[86]
37	*meso* 3,3''-di(7,4'-dihydroxyflavanone-3-yl)	*Ochna integerrima* Merr.	Ochnaceae	0.15 µM (K1) 0.47 µM (FCR3)	54.6 µM (MRC-5)	> 116	[94]
38	Sikokianin B	*Ochna integerrima* Merr.	Ochnaceae	0.98 µM (K1) 0.47 µM (FCR3)	40.9 µM (MRC-5)	> 42	[94]
		Wikstroemia indica (Linne) C. A. Meyer	Thymelaeaceae	0.98 µM (K1) 0.98 µM (FCR3)	41.0 µM (MRC-5)	42	[102]
39	Sikokianin C	*Ochna integerrima* Merr.	Ochnaceae	1.02 µM (K1) 0.62 µM (FCR3)	20.4 µM (MRC-5)	20	[94]
		Wikstroemia indica (Linne) C. A. Meyer	Thymelaeaceae	0.98 µM (K1) 0.32 µM (FCR3)	20.4 µM (MRC-5)	> 21	[102]
40	kaempferol 3-O-α-L-(2'',3''-di-E-*p*-coumaroyl) rhamnoside	*Platanus occidentalis* L.	Platanaceae	0.6±0.2 µM (HB3) 7±1 µMartopeden (NHP1337)	20.6 ±0.5 µM (HeLa)	34 (HB3) 2.9 (NHP1337)	[37]

41	kaempferol 3-O-α-L-(2′′-E-*p*-coumaroyl-3′′-Z-p-coumaroyl) rhamnoside	*Platanus occidentalis* L.	Platanaceae	2.0±0.6 μM (HB3) 4±1 μM (NHP1337)	11.9 ±0.7 μM (HeLa)	6 (HB3) 3 (NHP1337)	[37]
42	kaempferol 3-O-α-L-(2′′-Z-p-coumaroyl-3′′-*E*-*p*-coumaroyl)-rhamnoside	*Platanus occidentalis* L.	Platanaceae	0.50±0.03 μM (HB3) 4.1±0.5 μM (NHP1337)	9.3 ±0.2 μM (HeLa)	19 (HB3) 2.3 (NHP1337)	[37]
43	kaempferol-O-α-L-(2′′,3′′-di-*Z*-*p*-coumaroyl) rhamnoside	*Platanus occidentalis* L.	Platanaceae	1.8±0.4 μM (HB3) 7±1 μM (NHP1337)	16 ±1 μM (HeLa)	8.9 (HB3) 2.3 (NHP1337)	[37]
44	Sciadopitysin	*Sciadopitys verticillate* Thunb.	Sciadopityaceae	1.4 μM (K1)	68 μM (L-6)	49	[90]

NT – not toxic; ND – not determined
* Field isolate
** Artemisinin sensitive

Table 5 - Catechins.

Number	Compound	Botanical Origin	Family	Antiplasmodial IC_{50} (Strain)	Cytotoxicity IC_{50} (Cell line)	Selectivity Index (SI)	Reference number
45	(+)-catechin 5-gallate	*Piptadenia pervillei* Vatke	Leguminosae	1.2 μM (FcB1)	>75 μM (MRC-5)	>60	[97]
46	(+)-catechin 3-gallate	*Piptadenia pervillei* Vakte	Leguminosae	1.0 μM (FcB1)	(>75 μM (MRC-5)	>60	[97]
47	(–) Epigallocatechin gallate	Biopurify Phytochemicals Ltd, Hepes (Sigma- Aldrich) *	-	5.63 μM (3D7)	-	-	[98]
48	Catechin	Biopurify Phytochemicals Ltd, Hepes (Sigma- Aldrich) *	-	0.73 μM (3D7)	-	-	[98]
49	Epicatechin	Biopurify Phytochemicals Ltd, Hepes (Sigma- Aldrich) *	-	0.46 μM (3D7)	-	-	[98]
50	Catechin-Gallate	Biopurify Phytochemicals Ltd, Hepes (Sigma- Aldrich) *	-	0.37 μM (3D7)	-	-	[98]
51	Epicatechin gallate	Biopurify Phytochemicals Ltd, Hepes (Sigma- Aldrich) *	-	2.05 μM (3D7)	-	-	[98]

*purchased, ND – not determined

Compound (47) has other properties that might award it an additional edge against malaria. The epigallocatechin gallate (47), a catechin with both a galloyl and a gallate moiety, is the most studied, with reported activity against tumour cells and as a cell cycle regulator.[96,99] It binds to adhesion molecules, preventing the grip of *P. falciparum*-infected erythrocytes to the endothelium of blood vessels.[96] Additionally, it inhibits the gliding motility of sporozoites, preventing invasion and interrupting the parasite's cycle.[96] It is possible that its eight free hydroxylic groups bind to adhesion molecules on the surface of the sporozoites, inactivating them and rendering the parasite immotile.[96] However, in spite of these demonstrated advantages *in vitro*, *in vivo* data reveals otherwise. In a 4-day suppressive test with *P. berghei* (ANKA strain) infected mice, concentrations between 0-100 mg/kg/day of (47) equivalent in the form of green tea extract, were administered by gavage.[103] Instead of inhibiting, the administration of the extract augmented the parasite's growth over the control's own growth.[103] Thipubon *et al.* discuss that the compound's (47) highly antioxidant activity might prevent the oxidative damage by the mice's immune system, consequently endorsing the parasite's growth. However, there are various studies that support either the suppression or promotion of *P. berghei* by compound (47), which prevents the deduction of conclusions.[83,98,103]

Several studies have been performed in order to assess the MoA of flavonoids. There are three main ways they interfere with the malaria parasite: first, it is thought they inhibit the transport of substances necessary for the parasite's growth; second, they might affect heme detoxification; third, as mentioned for catechins, flavonoids inhibit lipid peroxidation.[28,78,82,104–106] The entry of L-glutamine and myoinositol into infected red blood cells is impaired in the presence of flavonoids.[28,107] The way flavonoids might interfere with heme is not entirely clarified, since their acidic nature, although weak, might prevent their entry in the digestive vacuole, where the heme is.[28] Flavonoids are demonstrated radical scavengers that bind easily with metals, specially iron, and are able to form stable complexes with Fe(II), therefore preventing it from partaking in other reactions, such as DNA synthesis and energy-enzymatic pathways, possibly starving the parasite.[28,82,104,106,108] Theoretically, and similarly to synthetic iron chelators that have been studied towards *P. falciparum*, flavonoids might also interfere with mosquito transmission.[108] Since iron is a key-component in the function of the gametocytes' mitochondrial electron transport chain, chelation is thought to inhibit this system, thus inactivating the gametocyte.[108] Transmission tests with flavonoids would have to be performed to confirm this. Structure-activity studies demonstrated that an *o*-hydroxyl on the B ring of a flavonoid, the number of free hydroxyl groups, the double bond at C-2-C-3 or a C-3 hydroxyl group all contribute to these activities.[28,82,104] All presented compounds possess one or more of this characteristics, imposing the possibility of antiplasmodial activity through multiple mechanisms. Moreover, this flexibility regarding structure is in accordance with the evidence that many structurally diverse flavonoids have

antiplasmodial activity in a wide range of concentrations.[78] In addition, the FAS of the parasites, and to some level, of the host, are inhibited by these compounds.[83,107] The di and tri-hydroxylation of the B and A ring and the double bond at C-2-C-3 (flavanols, isoflavanols, flavones, isoflavones) are common features of the most potent inhibitors.[78,83,99] This evidences the importance of both the planarity of the C-ring and the availability of hydroxylic groups at the B-ring towards antiplasmodial activity.[83] Compounds such as (15-18,31,32,34,36,40-43) fit these characteristics. Catechins might have other characteristics, like the gallate moiety, to favour their inhibition of this biosynthetic pathway, as discussed above. However, it is noteworthy that the most active compounds presented are not the ones with a di- or trihydroxylation of the B ring, but with prenylations of this ring. This ultimately leads to the conclusion that the compound's lipophilia is crucial for activity, even if only to facilitate the entrance into the parasite.

Flavonoids are known for their low toxicity to humans, with interesting half-lives that can go up to 28h.[83] Additionally, their solubility and thermostability convey favourable pharmacokinetic characteristics relevant for a possible antimalarial drug, especially considering their dietary presence.[28,83] Further studies concerning the SI and with differentiated strains might help elucidate the potential of these compounds as future antimalarial drugs.

Quinones, anthrones and derivatives

Quinones are products of the oxidation of phenolic compounds and they occur widely in plants.[54] The quinone unit is a six-carbon diene cycle with two keto groups, *o*-quinone or *p*-quinone. These units can group and are distinguished according to their structure as anthraquinones, benzoquinones, furanoquinones, naphthoquinones, among other derivatives.[54,78] It is noteworthy to mention that quinones undergo reduction to the hydroquinone form, an important property in the transferal of electrons in a biological system.[54] Natural quinones, and naphthoquinones in particular, are a phytochemical class with known parasiticidal activity.[27] In malaria's case, a good example is atovaquone, a synthetic derivative of lapachol, from the *Tabebuia* sp., used in combination with proguanil in Malarone™, recommended for travellers' prophylaxis.[18,27,37] Thirty compounds were found on the literature according to the defined criteria across all subgroups of quinones. They are presented in Table 6 and Figure 7.

Benzoquinones are defined by the presence of the quinone unit, with a single ring. In total, four compounds have this structure. Compound (60) is an active quinone, with an IC_{50} of 0.8 µM, with low selectivity (>1) that can undergo a redox reaction and convert to a hydroquinone, compound (61). This transformation does not prove useful towards *P. falciparum*, diminishing its activity to 1.2 µM, and maintaining its low selectiveness (>5).[109] Compound (79) is a hydroquinone with a long carbon chain.

It is believed its free hydroxyl groups and benzene ring grant its activity, although the chain's lipophilia surely contributes to the entry in the parasite.[110] Thymoquinone (77) is a benzoquinone that was isolated from multiple plant species with antimalarial traditional backgrounds.[111,112] In a first report, its antiplasmodial activity against a multidrug sensitive strain (FCR-3) was relevant (IC_{50} of 1.22 µM).[111] Since, further studies have focused on the anticancer and antitumor potential of the compound.[113] One study reported on the synthesis of analogues towards both ovarian cancer and malaria *in vitro*.[112] Structure-activity studies revealed that the position of the substitution groups in the quinone moiety proved essential, as did the hydrophobicity of the compound, with the chain substitution diminishing the antiplasmodial activity. Overall, in this study, compound (77) displayed a disparate inhibitory concentration of 25 µM, and the optimization of the molecule's structure improved this concentration only to 4.2 µM, which is still far from ideal.[112]

Naphthoquinones, related to natural naphthalene, have a quinone unit coupled with an aromatic ring that may have substitutions, in a total of two rings. Compounds (57 and 78) both fit this description. Compound (57) has a hydroxyl substitution in its aromatic ring and the particularity of an endoperoxide bridge. It shows an improved activity towards a multidrug resistant-strain, with an IC_{50} of 0.76 µM (K1), rather than the sensitive one, with an IC_{50} of 1.55 µM, with an average selectivity (>10).[114] Compound (78), isolated from a traditionally used plant, has an interesting antiplasmodial activity across different strains, but unfortunately, no selectivity.[115] *In vivo* studies with pure (78) orally administered were performed in a *P. berghei* model (ANKA strain) in a 4-day suppressive test.[116] Interestingly, acute toxicity assays were also done, with single oral doses of 500, 200 and 100 mg/kg body weight, and subacute assays, with oral doses of 100, 50 and 25 mg/kg/day for 14 days. Low toxicity was achieved at 100 mg/kg in a single dose and 25 mg/kg/day for 14 days. In spite of no histological damage, behavioural changes, such as anxiety and agitation, were evident, and mice died with a single dose of 200 mg/kg or higher, or cumulative doses of 100 mg/kg/day or higher.[116] These results are indicative of the unideal SI. As for the antimalarial test, a concentration of 25 mg/kg/day resulted in a 41% parasitemia suppression at day 4 with an average survival of 10 days.[116] These results are far from the control Chloroquine, with 100% suppression and over 15 days survival at 10 mg/kg/day. The low oral bioavailability of (78) produces only 39% systemic availability, while mostly being retained in the liver and spleen.[116] Therefore, arguably, its bioavailability could change the *in vivo* outcome, both in toxicity and efficiency.

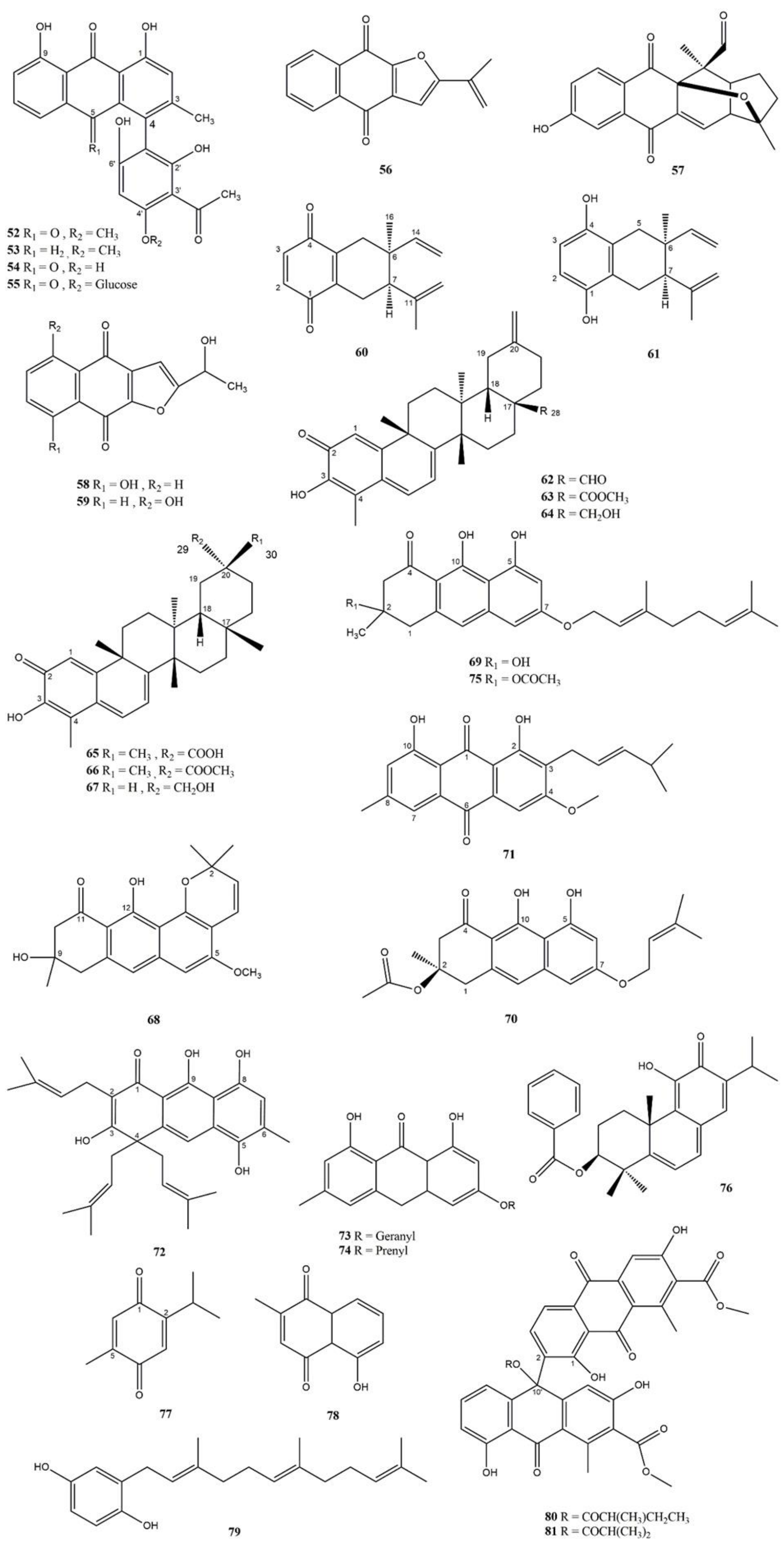


Figure 7 - Molecular structures of quinones.

Table 6 - Quinones and derivatives.

Number	Compound	Botanical Origin	Family	Antiplasmodial IC_{50} (Strain)	Cytotoxicity IC_{50} (Cell line)	Selectivity Index (SI)	Reference number
52	Knipholone	*Bulbine capitata* Poelln. *Kniphofia foliosa* Hochst.	Asphodelaceae	1.06 µM (K1) 1.70 µM (NF54)	76 µM (L-6)	> 45	117
		Bulbine frutescens (L.) Wild	Asphodelaceae	1.59 µM (K1) 2.15 µM (NF54) 4.9 ± 0.6 µM (HB3) 5 ± 2 µM (NHP1337)	78.12 µM (L-6)	> 34	37,118
53	Knipholone anthrone	*Bulbine capitata* Poelln.	Asphodelaceae	0.38 µM (K1) 0.42 µM (NF54)	8.8 µM (L-6)	> 21	117
54	4'-O-demethylknipholon	*Bulbine capitata* Poelln.	Asphodelaceae	1.80 µM (K1) 1.55 µM (NF54)	71 µM (L-6)	> 40	117
55	4′-O-demethylknipholone-4′-O-β-D-glucopyranoside	*Bulbine frutescens* (L.) Wild	Asphodelaceae	0.72 µM (K1) 0.75 µM (NF54)	157.75 µM (L-6)	> 210	118
56	2-(1-hydroxyethyl)naphtho[2,3-b]furan-4,9-dione	*Kigelia pinnata* **DC.**	Bignoniaceae	0.627 µM (K1) 0.718 µM (T_{9-96})	3.9 µM (ED 50 KB)	> 5	114
57	Isopinnatal	*Kigelia pinnata* DC.	Bignoniaceae	0.763 µM (K1) 1.552 µM (T_{9-96})	14.8 µM (ED 50 KB)	> 10	114
58|59	5- and 8-hydroxy-2-(1'-hydroxy)ethylnaphtho[2,3-b]- furan-4,9-dione	*Tabebuia ochracea ssp. neochrysantha* A. Gentry	Bignoniaceae	0.167 µM (*P. berghei*) 0.677 µM (FcB2)	ND	ND	119
60	Cordiachrome C	*Cordia globifera* W.W.S.	Boraginaceae	0.8 µM (K1)	5.6 µM (Vero) 6.0 µM (KB) 7.2 µM (BC-1) 0.8 µM (NCI-H187)	> 1	109
61	Cordiaquinol C	*Cordia globifera* W.W.S.	Boraginaceae	1.2 µM (K1)	6.4 µM (Vero) 27.6 µM (KB) 12.8 µM (BC-1) 7.6 µM (NCI-H187)	> 5	109
62	28-nor-Isoiguesterin-17-carbaldehyde	*Salacia kraussii* Harv.	Celastraceae	0.22 µM (K1) 0.19 µM (NF54)	ND	-	120
63	17-Methoxycarbonyl-28-nor-isoiguesterin	*Salacia kraussii* Harv.	Celastraceae	0.06 µM (K1) 0.08 µM (NF54)	5.30 (HT-29)	> 66	120
64	28-Hydroxyisoiguesterin	*Salacia kraussii* Harv.	Celastraceae	0.27 µM (K1) 0.33 µM (NF54)	14.4(HT-29)	> 44	120
65	Celastrol	*Salacia kraussii* Harv.	Celastraceae	0.40 µM (K1) 0.56 µM (NF54)	2.89(HT-29)	> 5	120

66	Pristimerin	*Salacia kraussii* Harv. *Celastrus paniculatus* Willd.	Celastraceae	0.41 µM (K1) 0.58 µM (NF54) 0.55 µM (K3)*	9.90(HT-29)	> 17	120,121
67	Isoiguesterol	*Salacia kraussii* Harv.	Celastraceae	0.05 µM (K1) 0.13 µM (NF54)	5.75(HT-29)	> 44	120
68	Vismione B	*Cratoxylum cochinchinense* Blume	Clusiaceae	1.86 µM (K1)	3.36 µM (NCI-H187)	1.80	122
69	Vismione D	*Psorospermum febrifugum* Spach	Clusiaceae	0.23 µM (K1)	ND	-	30
70	Vismione H	*Vismia guineensis* (L.) Choisy	Clusiaceae	0.23 µM (NF54)	ND	ND	123
71	Vismiaquinone A	*Vismia laurentii* De Wild.	Clusiaceae	1.42 µM (W2)	NT	-	124
72	Bazouanthrone	*Harungana madagascariensis* Lam.	Hypericaceae	1.80 µM (W2)	> 20000 µM (E)	11111	125
73	3-Geranyloxyemodin anthrone	*Psorospermum glaberrimum* Hochr.	Hypericaceae	1.68 µM (W2)	> 20000 µM (E)	11905	126
74	3-Prenyloxyemodin anthrone	*Psorospermum glaberrimum* Hochr.	Hypericaceae	1.98 µM (W2)	> 20000 µM (E)	10101	126
75	Acetylvismione D	*Psorospermum glaberrimum* Hochr.	Hypericaceae	0.12 µM (W2) 0.85 µM (K1)	> 20000 µM (E)	16667	30,126
76	3-O-benzoylhosloppone	*Hoslundia opposite* Vahl.	Lamiaceae	0.96 µM (K1) 0.53 µM (NF54)	ND	-	127
77	Thymoquinone	*Monarda fistulosa L.*	Lamiaceae	1.22 µM (FCR-3)	ND	-	111
		Sigma Aldrich**	-	25.0 µM (Dd2)	ND	-	112
78	Plumbagin	*Nepenthes thorelii* (Rt.)	Nepenthaceae	270 nM ($T_{9/94}$)	0.06 µM (MCF-7) 1.14 µM (A549)	>0.22	115,128
		Apin chemicals Co. Ltd. (Oxford, UK)**	-	580 nM (3D7) 370 nM (K1)	-	-	116
79	2'E,6'E 2-farnesyl hydroquinone	*Piper tricuspe* C.DC.	Piperaceae	1.37 µM (FcB1) 0.93 µM (Thai)* 1.14 µM (F32) 0.55 µM (PFB) 0.58 µM (K1)	1.075 µM (L-6)	> 0.78	110
80	Scutianthraquinone A	*Scutia myrtina* Kurz	Rhamnaceae	1.23 µM (Dd2) 1.20 µM (FCM29)	7.6 µM (A2780)	6	129
81	Scutianthraquinone B	*Scutia myrtina* Kurz	Rhamnaceae	1.14 µM (Dd2) 5.4 µM (FCM29)	5.8 µM (A2780)	> 1	129

E – Erythrocytes
* Thai field isolate
** purchased
NT – not toxic; ND – not determined

Structure-activity studies with both compounds (57 and 78) undeniably confirmed the quinone structure was crucial for antiplasmodial activity, while substitutions at C-3 were detrimental.[42,114,128] In spite of the low SI of naphthoquinones, authors defend the selectivity towards cancerous cells and the parasites' mitochondria, and not normal hosts' cells.[114] Additionally, (78) is a ubiquinone analogue, and has demonstrated its inhibitory activity against malarial enzymes.[116] Regardless of these arguments, naphthoquinones represent a lead in antimalarial research because they are easily synthetized, which enables the improvement of their bioavailability and facilitates the search of new drugs.[78]

Furanoquinones are a diverse group of compounds where a quinone unit and a furan ring are present. Compound (56) is a furanonaphthoquinone, because in addition to the other structures, it possesses an aromatic ring, characteristic of a naphthoquinone. It demonstrates no significant difference in activity between chloroquine sensitive and resistant strains and, similarly to naphthoquinones, has a low SI (3.9).[114] Likewise, compounds (58 and 59) are also furanonaphthoquinones, this time inseparable compounds extracted from the stem bark of *Tabebuia ochracea* ssp. A. Gentry, traditionally used against malaria.[119] *In vitro* assays demonstrated a preferentiality in activity towards the chloroquine-resistant strain. The alternate hydroxylation at C-5 or C-8 proves more advantageous than the hydroxylation in both positions.[119] Regrettably, the general toxicity of these compounds was not evaluated. In an *in vitro* test with *P. berghei* infected cells, these compounds had an IC_{50} of 0.167 µM, allowing the prediction of good activity *in vivo*.[119] These three compounds demonstrate the benefit of associating the furan ring to the naphthoquinone structure, possibly instigating a new class of antimalarials.

Anthraquinones are frequent metabolites with a three-ring skeleton, namely one quinone unit and two aromatic rings. They can be oxidized or reduced into derivatives in reversible reactions, and the ratio between these structures in a plant depends on several factors, for example, the time of the year.[54] Because they react easily, the method of extraction is important to control the ratio and kind of compounds extracted.[54] This phytochemical class has presented the biggest amount of antiplasmodial compounds, revealing its importance to traditional medicine.

Knipholone (52) is an anthraquinone pigment, found in at least thirty plants, with several known derivatives.[117] Compound (53) is the anthrone derivative of (52), *i.e.*, through reductions, it loses the ketone at C-5. This change is enough to double the antiplasmodial activity, from over 1 µM to 0.4 µM, while its selectivity worsens significantly, from over 34 to 21.[117] Compound (54) is similar to (52), but instead of an O-methyl at C-4', it has a hydroxyl. This difference does not change the selectivity (>40) or activity towards the chloroquine-sensitive strain (of 1.55 µM), but diminishes the activity against the multi-drug resistant strain (IC_{50} of 1.80 µM), indicating the necessity of a hydrophobic chain at that

position against a specific resistance mechanism.[117] Compound (55) is another knipholone derivative, again with a sole difference at C-4', with a glucopyranoside. Contrarily to (53), compound (55) improves both its antiplasmodial activity (IC_{50} of 0.7 µM) and selectivity (>210) with this change, showing the relevance of the substitution at C-4'.[118] Knipholone's antiplasmodial properties seem to be rooted in the special phenyl-anthraquinone skeleton, since neither structure individually has any significant activity.[117,118]

Vismiones are lipophilic anthrones originally isolated from the *Vismia* sp., traditionally used against malaria.[30,123] Other derivatives have been isolated in different species since and revealed the exceptional antiplasmodial activity associated with these compounds' structure. Vismione B (68), D (69), H (70) and Acetylvismione D (75) are all molecularly related, with changes at C-2/9 and C-7. Additionally, (68) has a dimethyl chromene moiety. Although in separate tests, compound (69, 68 and 75) were all tested *in vitro* on the K1 strain. Compound (69) is more active than (75), both better than (68) on this strain, with IC_{50} of 0.23 µM, 0.85 µM and 1.86 µM, respectively.[30,122,126] The fact that (70) remains highly potent on a different strain (IC_{50} of 0.23 µM in the NF54 strain), allows the conclusion that either a prenyl or a geranyl group at C-7 are equally essential for antiplasmodial activity.[123,125,126] Regarding selectivity, however, compound (75) is the only selective and safe compound.[30,126] Inclusively, *Psorospermum febrifugum* Spach, with compound (69), has been ill advised for consumption following reports of toxicity in mice.[30]

Vismiaquinone A (71) and compound (72) are anthrones similar to vismiones, with minor differences in their substitutions. Overall, the number of hydroxyl groups is higher. Unfortunately, the strains studied are not the same, which does not allow a comparison, but other compounds in the same study permit further structure analysis. It would appear that the prenyl group at C-2 and the hydroxyl at C-5 are enhancers of activity, although it is uncertain if they function additively or synergistically.[125] On the same study, compound (72) was the most potent antiplasmodial, possibly because it is a tri-prenylated anthrone.[125] Additional studies would have to be performed to analyse how these characteristics influence the activity of vismiones and anthrones. Regarding (71), because in the same study other related compounds were not tested, it is not possible to infer structure-activity links. However, from this family of compounds, it would seem safe to conclude that the quinone substitution interferes with activity and that the substitution with increasingly lipophilic chains might also improve antiplasmodial activity.[124]

Compounds (73 and 74) are anthrones isolated from the root bark extract of *Psorospermum glaberrimum* Hochr., used traditionally on severe cases of malaria.[126] Their sole structural difference is an O-geranyl or prenyl at C-4, which does not seem to interfere with neither the activity or selectivity.

It would appear, though, that anthrones with their chemical unit on an extremity are preferred activity-wise, when comparing these two compounds with vismiones.[126]

Scutianthraquinone A (80) and B (81) are anthraquinones with the particularity of having a connection C-2-C-10' to an anthrone, which is a rare structure. This precise characteristic conveys better activity than a simple hydroxyl substitution.[129] Unfortunately, the selectivity is not optimal and the complexity and rarity of this chemical structure hinders further studies with these compounds.[129]

Finally, natural quinone methides triterpenes exist in nature as quinone derivatives. Compound (76) was isolated from *Hoslundia opposita* Vahl., a traditionally used plant against malaria in Tanzania.[127] It is a hydroquinone with a sesquiterpenoid sidechain, with its α,β-unsaturated carbonyl moiety possibly responsible for a Michael reaction with the parasites' structures.[127] This is, however, unlikely, as this reaction has been shown to occur more intensively with simple benzoquinones (1,4-benzoquinone) or naphthoquinones.[130] Compounds (62-67) were isolated from *Salacia kraussii* (Harv.) Harv. and were found to have interesting activities. Compounds (62-64) have only a different substitution at C-28, sufficient to distinguish (63) has the most active, with an IC_{50} of 0.06 µM in the K1 strain and 0.08 µM in the NF5684.[120] Likewise, compounds (65-67) have two different substitutions at C-29 and C-30, enough to ascertain (67) as the most active and selective of the trio, but not of the entire group. Compound (63), the most active and selective *in vitro*, was evaluated *in vivo* and revealed both toxicity and inactivity against murine malaria, after oral and parenteral administration. The low SI might justify the high toxicity *in vivo,* and the low bioavailability explains the lack of activity. In fact, quinone methides triterpenoids such as compounds (65 and 66) have been studied for their anti-tumoral activities.[131,132] It was found their quinone methide moiety was responsible for this effect, namely through the induction of apoptotic pathways, be it with mitochondrial targeting, formation of Michael adducts with chaperones, among other interactions.[131–134] Consequently, the *in vitro* and *in vivo* lack of specificity becomes logical. These characteristics make the development of these compounds as antimalarials difficult.[120,135]

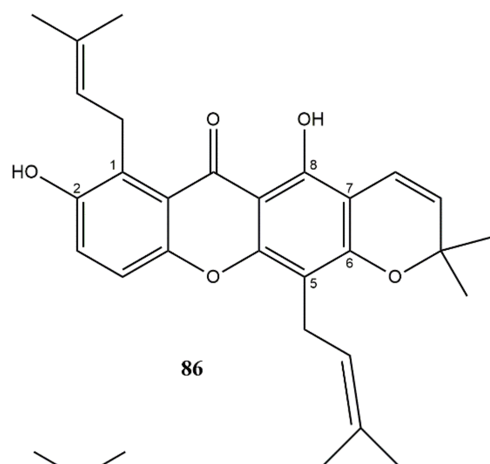
There are two accepted mechanisms of action for quinones: the oxidative stress induction and the inhibition of the mitochondria's electron transport and respiratory chain. Quinones are easily reduced into derivatives, which in turn can be further reduced.[54] The capability of generating ROS or of a nucleophilic addition reaction on the parasite's proteins depends on the quinone, being more frequent on benzoquinones than anthraquinones or naphthoquinones, possibly because of the decreased electron flow on these structures.[28,130,136] Although claimed by many authors, it is unlikely that quinones and derivatives undergo Michael's reactions directly with the parasite's DNA, since in physiological conditions, they are known to interact with proteins that possess thiol groups. This is not

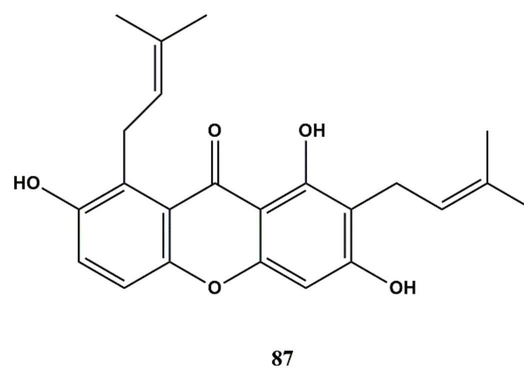
the case of the parasite's DNA, but of other proteins, *e.g.* chaperones.[72,130,131,137] Therefore, for the more complex and fully conjugated structures, it is accepted that they act in a similar fashion to atovaquone.[48] In the long term, the blockage of the parasite's mitochondrial electron transport chain by targeting the cytochrome *bc₁* complex interferes with the redox homeostasis and with the pyrimidine biosynthetic pathway, crucial for the parasite's subsistence.[27] In any case, the high antiplasmodial activity of this class of compounds is balanced with the frequently low selectivity and bioavailability, has evidenced thus far. As with lapachol and atovaquone, synthetic derivatives might be designed and optimized, however, the existing resistance to atovaquone invokes the question on whether there is a necessity for new compounds from this chemical class that will perform through the same MoA.[18,27,114]

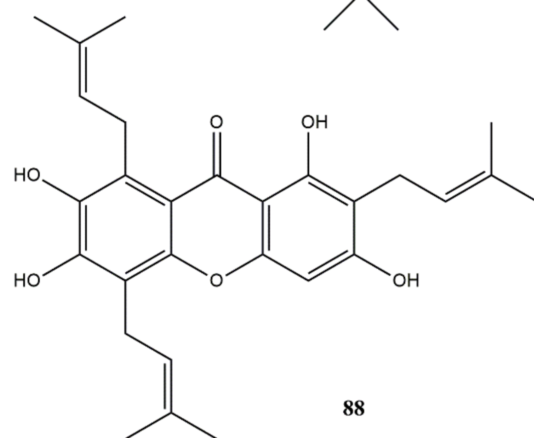
Xanthones

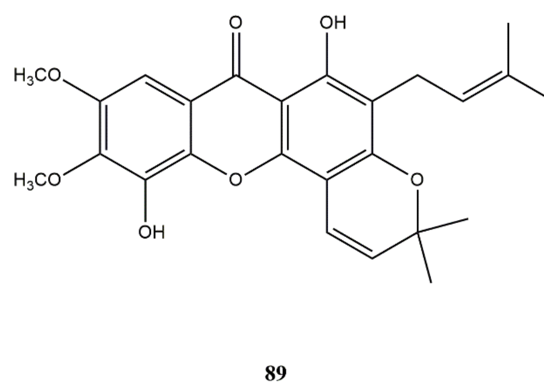
Xanthones are dibenzo-c-pyrone compounds formed by cyclization of a benzophenone.[78,138] The great interest on these compounds is comparable to quinones, because of their high *in vitro* parasiticidal activity and the facility of synthesis.[78,138] The antiplasmodial activity of these compounds against both sensitive and resistant strains, in a similar fashion to the quinoline class of antimalarials, has made it one of the most studied phenolic phytochemical classes.[138] In spite of this, only eight compounds corresponded to the criteria and are presented in Table 7 and Figure 8.

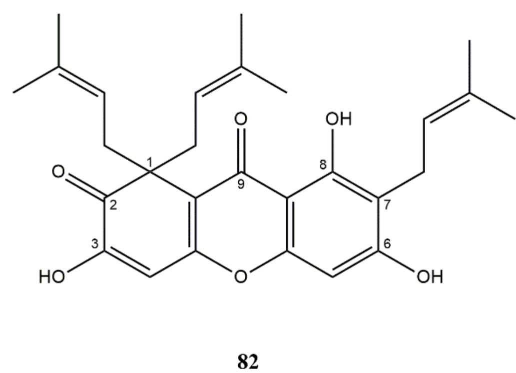
There are two characteristics that stand out across the compounds presented: the prenylation and cyclization of the xanthone structure. α-Mangostin (84) is one of the most studied xanthones because of its several activities: antimicrobial, antioxidant, anti-inflammatory, anti-viral and antimalarial.[139] Compounds (82, 87 and 88) are all structurally related to α-mangostin (84), as Figure 8 suggests. These compounds, besides the xanthone skeleton, have in common one or two prenyl groups at C-1 and C-7, and a hydroxyl at C-6. These characteristics appear to be important for activity.[139–142] Although this group has been tested on different strains and assays, it is possible to point that compound (88) is the most active of the four, with an IC_{50} of 0.41 µM on the W2 strain, while (84) retains activity mostly against the chloroquine-resistant strains.[143] *In vivo* studies with (84) demonstrated a low antimalarial activity when orally administered at 100 mg/kg/day in a 7-day test, when compared to intraperitoneal administration of the same dose twice per day – from 27% to 81% chemo suppression, respectively.[143] This might be due to low bioavailability that hinders absorption at the digestive level.[143] In spite of positive haemolysis results (at 69.7 µM), the SI was found to be high enough, as demonstrated *in vivo* by the reduction of symptoms malaria-related and overall absence of toxicity at all levels tested – hepatic, renal and histological.[143]

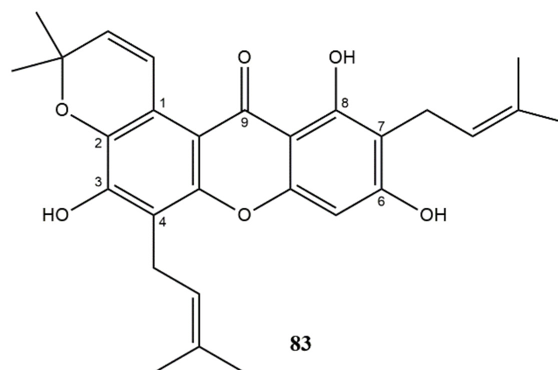


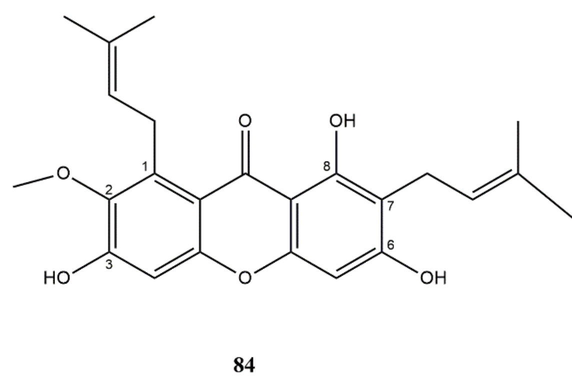












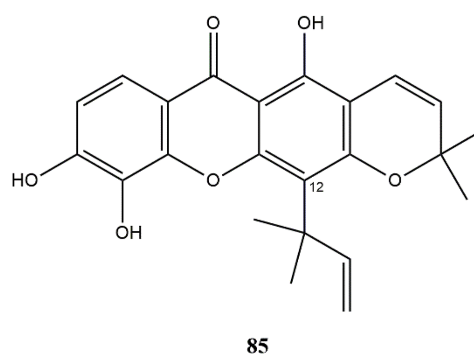


Figure 8 - Molecular structures of xanthones.

Compound (88) structurally differs from the others with an additional prenyl at C-4, which might indicate that lipophilia might play an important role on these compounds' MoA. Further studies to evaluate the structure-activity relation of these compounds revealed that the prenyl group at C-1 and the hydroxyl at C-3 are fundamental characteristics for antiplasmodial activity.[78] On this regard, it would be interesting to study (82 and 88) on different strains in order to ascertain if the double prenyl group at C-1 or the carbonyl at C-2 are important towards a certain type of strain. The fact compounds (84 and 87) were isolated from traditionally used plants further demonstrates their activity.[142]

Compounds (83, 86 and 89) have incurred both prenylations and cyclization. Considering the aforementioned characteristics for active xanthones, compound (83), apparently the most active of the trio, fits the criteria and demonstrates that a cyclization at C-1-C-2 does not interfere with activity.[140]

Finally, compound (85) has a 12-(2-methylbut-3-en-2-yl) group and a dihydropyrano ring. This structure proves to be preferential towards activity in comparison to other isolated structures in the same study, particularly to the F32 and FcM29 strains.[141] Likewise, with the other xanthones thus far, it stands out the difference in activity between chloroquine-resistant and sensitive strains. Most compounds presented have higher activities on resistant strains, rather than the chloroquine-sensitive ones. This is odd, considering the MoA of xanthones is defined as a complexation with heme, preventing the detoxification of the digestion of haemoglobin that, this way, kills the parasite – see Figure 3.[48,78,138,144] This MoA is similar to the quinoline class of antimalarials. Resistance to chloroquine, in particular, originates in the modification of a transporter in a digestive vacuole of the parasite, consequently altering its accumulation and preventing its activity.[145,146] The fact that xanthones are active against chloroquine-resistant strains evidences a bypass through this mechanism of resistance, representing a lead to discover the structural requirements to this ability. However, none of the xanthones have a great selectivity index, which might indicate a lack of structural selectivity that impeaches their utilisation as antimalarials.

Table 7 - Xanthones.

Number	Compound	Botanical Origin	Family	Antiplasmodial IC_{50} (Strain)	Cytotoxicity IC_{50} (Cell line)	Selectivity Index (SI)	Reference number
82	Allanxanthone C	*Allanblackia monticola* Staner L.C.	Clusiaceae	1.3 µM (FcM29) 7.1 µM (F32)	184.5 µM (A375)	> 26	[140,147]
83	Tovophyllin A	*Allanblackia monticola* Staner L.C.	Clusiaceae	0.7 µM (FcM29) 20.3 µM (F32)	83.8 µM (A375)	> 17	[140]
84	α-Mangostin	*Allanblackia monticola* Staner L.C. *Garcinia mangostana* Linn. *Pentadesma butyracea* Sabine	Clusiaceae	2.0 µM (FcM29) 7.7 µM (F32) 2.77 µM (W2) 36.10 µM (3D7) 0.20 µM (FCR3)	79.2 µM (A375) 130.6 µM (U-937)	> 15	[140,142,143]
85	Macluraxanthone	*Allanblackia floribunda*	Clusiaceae	0.92 µM (F32) 0.69 µM (FcM29) 3.78-5.21 µM (FcB1) 3.42 µM (K1)	8.67	> 1.66	[91,122,141]
86	Demethylcalaaxanthone	*Calophyllum caledonicum*	Clusiaceae	1.63-2.55 µM (FcB1)	ND	-	[141]
87	6-deoxy-γ-mangostin	*Calophyllum caledonicum*	Clusiaceae	1.84-2.37 µM (FcB1)	ND	-	[141]
88	Garcinone E	*Pentadesma butyracea* Sabine	Clusiaceae	0.41 µM (W2)	ND	-	[142]
89	Symphonin	*Symphonia globulifera* L.f.	Clusiaceae	1.3 µM (W2)	ND	-	[144]

ND – not determined

Lignans

Lignans are dimeric phenylpropanoids connected by C-C bonds between the β-carbon of the propanoid chains.[78] When the C-C bond is established differently, the compounds are termed neolignans. These compounds, shown in Table 8 and Figure 9, are known for their toxicity and general activity, including antiprotozoal.[148]

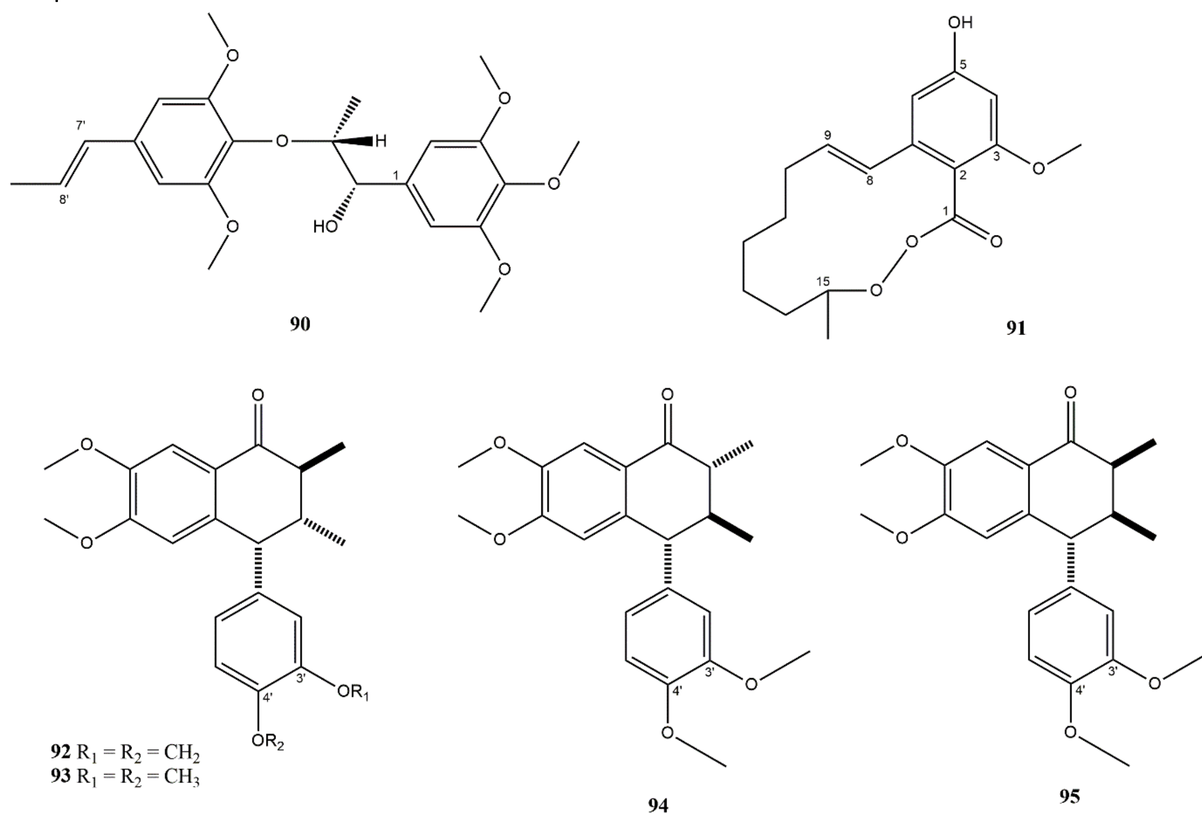


Figure 9 - Molecular structures of lignans.

Compounds (90 and 91) are considered neolignans, because of their unusual linkage between monomeric units. Compound (90) is active equally against resistant and sensitive strains. In a study, three compounds, including (90), were isolated and tested. Compound (90) was the most active of the three, with an average IC_{50} of 0.9 µM between sensitive and resistant strains, and, structurally, that demonstrated that the double bond on C-7'-C-8' was preferential for activity, possibly due to the easiness of the conjugated double bond to generate ROS.[149] Compound (91) has an unconventional molecular structure, with a peroxide ester that might function similarly to the endoperoxide bridge of the artemisinin class of antimalarials.[149] Its activity, however, is not comparable to that class of drugs, with an IC_{50} of over 1.4 µM, which points to a different mechanism of action.[149] Neither compounds have good SI, making them non-ideal for further studies.

Compounds (92-95) were all isolated from the same Brazilian plant.[148] The *in vivo* activity of the hexane extract, with a parasite reduction of 67% and 48%, for the root and leaves extracts, respectively, prompted the isolation and *in vitro* testing of these compounds.[148] All four lignans proved to be very

active against the chloroquine-resistant isolate (BHz 26/86), with IC_{50} between 0.20 and 0.63 µM.[148] It is thus possible to infer the structural requirements for activity: a *trans* orientation in the B-ring and a veratryl A-ring, with no particular difference between an O-methyl or a methoxy at C-3' and C-4'. Contrarily to the previous lignans and to the general idea of toxicity, these four compounds were found non-toxic on Hep G2 A16 hepatic cells.[148] This could mean that traditional lignans present advantages both activity and toxicity-wise. The MoA of these natural compounds is currently unknown, although, as discussed previously, it might be linked to the generation of ROS.

Table 8 - Lignans.

Number	Compound	Botanical Origin	Family	Antiplasmodial IC_{50} (Strain)	Cytotoxicity IC_{50} (Cell line)	Selectivity Index (SI)	Reference number
90	Polysyphorin	*Rhaphidophora decursiva* Schott	Araceae	0.97 µM (D6) 0.88 µM (W2)	4.78 µM (KB)	> 4.93	149
91	Rhaphidecurperoxin	*Rhaphidophora decursiva* Schott	Araceae	1.83 µM (D6) 1.43 µM (W2)	13.6 µM (KB)	> 7.43	149
92	(7'*R*,8*R*,8'*R*)-4,5-dimethoxy-3',4'-methylenodioxy-2,7'-cyclolignan-7-one	*Holostylis reniformis* Duch.	Aristolochiaceae	0.26 µM (BHz26/86)	NT (Hep G2)	-	148
93	(7'*R*,8S,8'*R*)-3',4,4',5-tetramethoxy-2,7'-cyclolignan-7-one	*Holostylis reniformis* Duch.	Aristolochiaceae	0.32 µM (BHz26/86)	NT (Hep G2)	-	148
94	(7'*R*,8*R*,8'*S*)-3',4,4',5-tetramethoxy-2,7'-cyclolignan-7-one	*Holostylis reniformis* Duch.	Aristolochiaceae	0.20 µM (BHz26/86)	NT (Hep G2)	-	148
95	(7'*R*,8*S*,8'*S*)-3',4,4',5-tetramethoxy-2,7'-cyclolignan-7-one	*Holostylis reniformis* Duch.	Aristolochiaceae	0.63 µM (BHz26/86)	NT (Hep G2)	-	148

NT – not toxic

Other derivatives

Other subgroups of phenolic compounds with relevant antiplasmodial activity are displayed in Table 9 and Figure 10. Their individuality demonstrates, coupled with the compounds discussed thus far, the variability of phenolic structures with antiplasmodial activity that may prove to be important for the discovery of new antimalarials.

Licochalcone A (96) is a chalcone, an aromatic ketone, isolated from the Chinese licorice roots.[150] It is not frequent to find chalcones in plants, as they are precursors of flavonoids and, therefore, are often transformed into these compounds and derivatives.[49,50,78,151] Compound (96) was the first chalcone to reveal the potential of the phytochemical class as antimalarials, besides as antibacterial, antiviral and antileishmanials.[49,152] Its activity was demonstrated *in vitro* against all erythrocytic stages of the parasite, proving the effectiveness in important metabolic pathways throughout the parasite's life cycle.[153] *In vivo*, in *P. yoelii* YM infected mice, the administration of 15 mg/kg four times daily intraperitoneally, for a total of 60 mg/kg, for three consecutive days, accounted for parasite clearance (93%) without toxicity.[153] In the same test, (96) was administered orally at doses of 450, 150, and 50

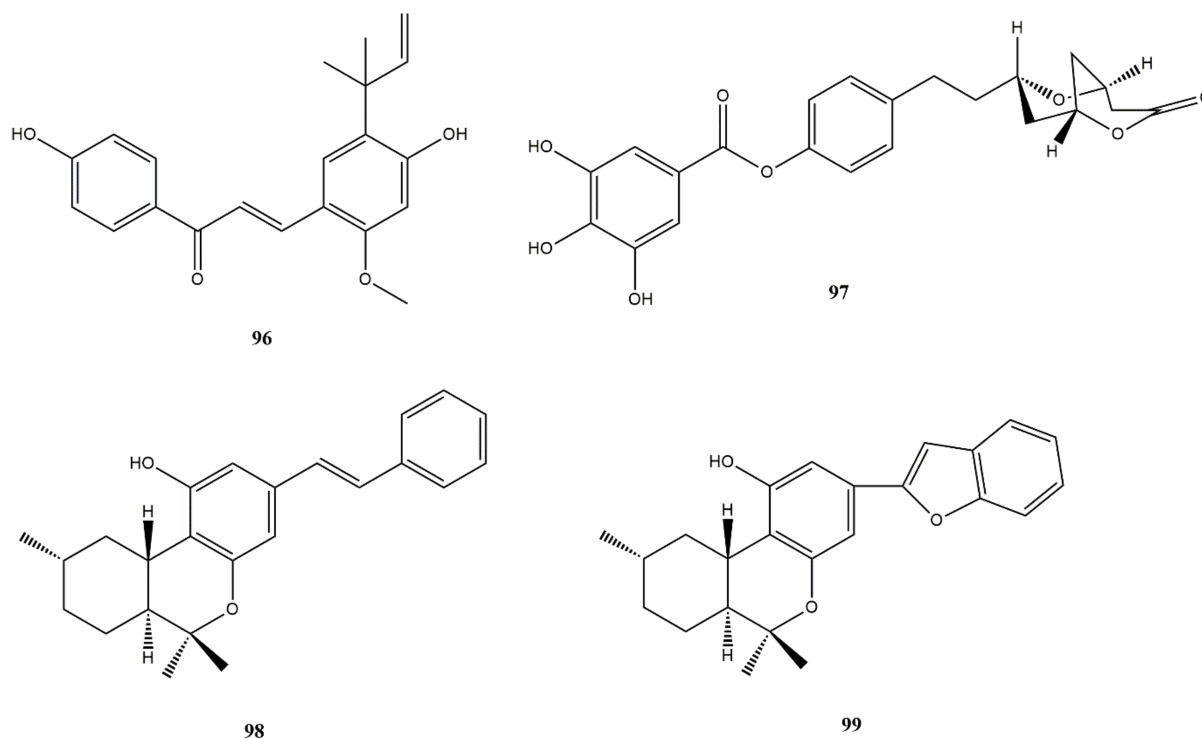


Figure 10 - Molecular structures of other phenolic derivatives.

mg/kg/day and it demonstrated to nearly clear the parasitemia at all doses, with no mortality by the end of the study (21 days).[153] In one hand, the bioavailability of (96) allows for an ideal oral antimalarial, on the other hand, it is unclear whether the administration several times a day and for a long period of time, at least over 3 days, would be practical. This compound's MoA has been thoroughly studied: it is thought the major mechanism is the inhibition of the mitochondria's cytochrome *bc1* complex, similarly to atovaquone.[151] However, chalcones have other MoA, different from the activity reported for compound (96): hematin degradation inhibition, preventing detoxification; hemoglobinase inhibition, preventing digestion of haemoglobin; and new permeation pathways inhibition, leading to the parasite's death – see Figure 1.[78,151] Structural studies revealed that the properties of the B-ring proved important for antiplasmodial activity, as did the number of methoxy substitutions and the hindrance caused by them, thus impeaching the connection to the enzymes responsible for the previous steps.[152,154] Overall, the potential of compound (96) and the facility of creating synthetic derivatives has made chalcones a potential group for derivation of innovative antimalarial structures.[152]

Compound (97) is the only lactone to fill the pre-set requirements. It is a valerolactone, a five-carbon ester cycle, isolated from *Phragmanthera capitate*, an African parasitic plant used to treat fever and parasitic diseases.[155] In the same study it was isolated, a second lactone was also identified with a single structural difference: the phenolic ring substitution of a hydroxyl, instead of a galloyl group. Although compound (97) does not have a promising antiplasmodial activity of its own, it is interesting

to note that the difference between the two compounds is over one-hundred-fold.[155] This demonstrates undoubtably, as mentioned previously, the advantage activity-wise that the galloyl moiety awards, comparing to a single hydroxyl group. It is possible compound (97) acts in a similar way to phenols.

Compounds (98 and 99) are hexahydrodibenzopyrans with a monoterpene moiety isolated from an Amazonian plant.[156] The structural difference between the two is a cyclization at C-1' with an oxygen in a 1',2'-dihydrofuran. This divergence is enough to accentuate the antiplasmodial activity of (99) over two-fold, for the chloroquine-resistant strain, and over five-fold, for the sensitive strain, in relation to compound (98).[156] It is possible the dihydrofuran ring might contribute for the conformational rigidity of the compound or for its electron flow, augmenting its potential antiprotozoal activity. In any case, without the SI, general toxicity cannot be overlooked, making these compounds unlikely future antimalarials.

Table 9 - Other phenolic compounds.

Number	Name compound	Botanical Origin	Family	Antiplasmodial IC_{50} (Strain)	Cytotoxicity IC_{50} (Cell line)	Selectivity Index (SI)	Reference number
96	Licochalcone A	*Glycyrrhiza uralensis, G. inflata, G. glabra*	Leguminosae	1.43 µM (3D7) 4.17 ± 0.16 µM (3D7) 4.23 µM (3D7) 1.88 µM (Dd2) 5.33 ± 0.24 µM (RKL303) 5.38 µM (RKL303)	NT	-	150–153
97	4-{2-[rel-(1R,3R,5S)-7-oxo-2,6-dioxabicyclo [3.3.1] non-3-yl] e- thyl} phenyl 3,4,5-trihydroxybenzoate	*Phragmanthera capitata* (Spreng.) Balle	Loranthaceae	2.4±0.5 µM (NF54) 4.9±3.3 µM (3D7)	ND	-	155
98	Machaeriol A	*Machaerium multiflorum* Spruce	Leguminosae	1.74 µM (W2) 4.36 µM (D6)	ND	-	156
99	Machaeriol B	*Machaerium multiflorum* Spruce	Leguminosae	0.33 µM (W2) 1.99 µM (D6)	ND	-	156

NT – not toxic; ND – not determined

Discussion

Phenolic compounds and their derivatives are found in virtually every plant, as part of their metabolic pathways, and, subsequently, are a major component in both the diet and medicine of the populace. The complexity of plant extracts deters the pharmacokinetic studies and the prediction of clinical outcomes.[36] Therefore, it is logical to focus on the plants' isolated compounds. However, individual molecules, or extracts, might not prove to have a comparable efficacy in *in vitro/vivo* tests. Still, clinical value is not directly defined solely by these assays. The ability to better fevers or anaemia is highly valued by the patients, who often do not have the capability of reaching out to professional help or to pay for the recommended treatments. Future traditional medicine applications will require

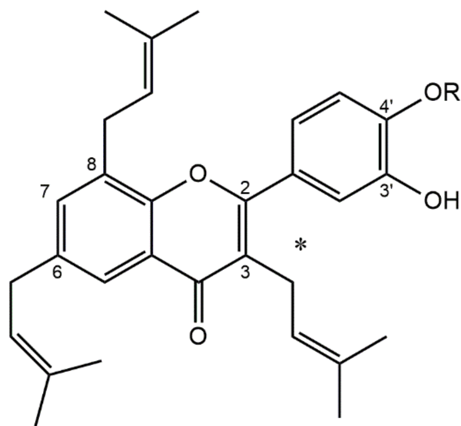
effectiveness validation and standardization into improved traditional medicines, as is recognized by the World Health Organization.[19] As a result, although the cost may rise, these might still prove a preferential option to purified or synthesized drugs, with the added advantage that the process could be developed locally, which would improve the accessibility of these medicines.[19,34,36,157] In order to verify the safety of consumption and enable future medical applications, but also to enlarge the data pool of interesting antiplasmodial compounds, it is important to continue to screen and study traditional medicinal plants.

To target malaria, the six most interesting sub-groups of phenolic compounds were condensed and will now be analysed. For an integral view of the mechanisms of action on the parasite, see Figure 3.

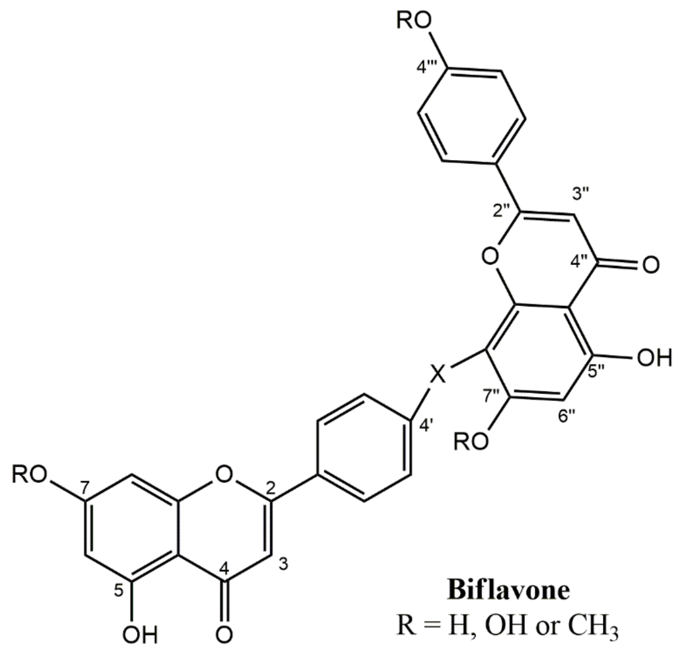
Phenolic acids, phenols and their derivatives conserve simple phenolic moieties and are, therefore, related to several mechanisms of action. They are capable of inhibiting the haemoglobin digestion and the hemozoin formation, disturb the redox homeostasis and, possibly, of iron chelating.[64,73] However broad their reach, no selected compound from this class excels on differentiating themselves as antiplasmodials. In fact, the low bioavailability, rapid metabolization and non-consistent selectivity and activity towards all types of strains represent important characteristics that prevent interest in this class of compounds.[61,67,71] It is noteworthy to mention that in other classes of compounds, the galloyl or gallate moiety substitution has shown to improve the compounds activity, as evidenced throughout this review. Still, these structures show no relevant potential on their own.[57,58]

In a similar fashion, no coumarin stands within the selected compounds. Overall, all present a wide-range of concentrations, unreliable concerning the necessary characteristics of an antimalarial.[45,52] The lack of studies regarding their mechanism of action and selectivity also leave an information void. Further studies would be necessary to confirm the contribution this class could give, especially considering that there are coumarins identified as the active compounds of traditionally used plants.[32,79]

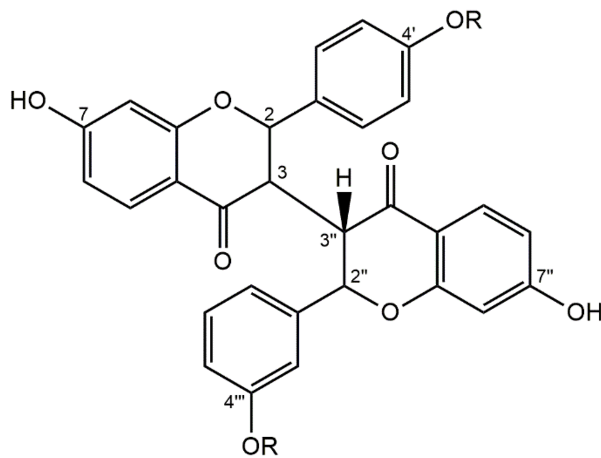
The flavonoid phytochemical class is one of the most extensive and varied classes approached in this review. Its structural variation is great, and, as mentioned previously, the criteria of this review allowed the narrowing of the ideal characteristics of the active structures: flavone, biflavone and biflavanone – see Figure 11. Not only there are multiple molecules with considerable structure-activity co-relation, but the known mechanisms of action by which they act are multiple.[28,83]



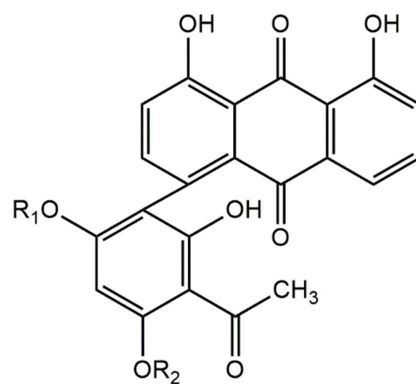
Flavone
R = H, Me or Ac
* or cyclization



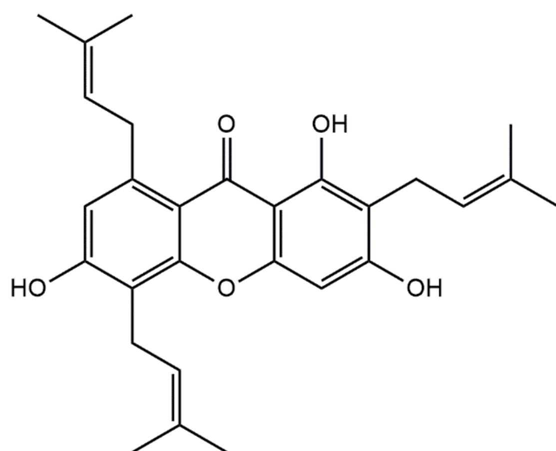
Biflavone
R = H, OH or CH_3
X = O or null



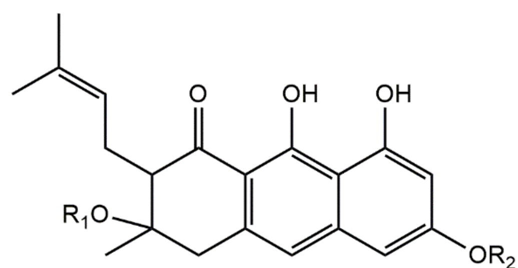
Biflavanone
R = H or CH_3



Anthraquinone
R_1 = H or CH_3
R_2 = CH_3 or glucopyranoside



Xanthone



Anthrone
R_1 = H or Ac
R_2 = Prenyl or Geranyl

Figure 11 – Projected ideal molecular structures per phytochemical class. Based on the discussed information of relation structure-antiplasmodial activity.

Of the thirty-four flavonoids presented, three are of interest: compound (11, 21 and 37). All are dimeric structures, a biflavone and biflavanones. They demonstrate a good antiplasmodial activity against both multidrug resistant and sensitive strains with a great SI.[90,93,94,100] Compound (11) appears to have the ideal structure for FAS inhibition: a planar structure (double bond C-2-C-3) and hydroxy groups at C-5 and C-7.[90,100] Although its B-ring does not possess hydroxy groups, it could be that the other characteristics are enough to represent antiplasmodial activity. Compounds (21 and 37) fall far from these characteristics, possibly justifying its activity through the number and position of the hydroxy groups.[93,94] The most active catechins are the ones with a galloyl moiety at C-3, of which compound (50) is the most active of the presented.[83,99] Unfortunately, the range of concentrations needed for (50) to act as antiplasmodial could coincide with the therapeutic window of other activities, imposing care in its utilisation.[99] In this regard, flavonoids appear safer and more active, and therefore, more promising to study as antimalarials, than catechins.

In the symptomatic phase of malaria, that is understood as the erythrocytic stage of the parasite's life cycle, the parasite has to maintain a relatively sensitive homeostasis. Particularly its redox homeostasis, because not only does its digestion of haemoglobin and the mitochondrial activity originate oxidative stress, but also the host's immune system will attack it with oxygen and nitrogen reactive species.[158,159] The host's erythrocyte is also receiving oxidative insults, which means the parasite must maintain both systems redox homeostasis if it wants to survive.[67,158,159] In the fight to prevent lipid peroxidation, inactivation of enzymes and ion channels, protein oxidation and inhibition of mitochondrial respiration, the parasite is equipped with an antioxidant system that includes glutathione proteins and superoxide dismutase.[67] When antimalarials are able to saturate this system and disturb the already fragile redox balance, the cascade of oxidative damage ensues the parasite's death.[159] Flavonoids are recognized powerful antioxidants that, in certain conditions of concentration and the presence of metal ions, as occurs within the erythrocytic stage of the parasite, act as pro-oxidants. Therefore, disturbing this vital balance and leading to the parasite's death. Particularly the B-ring of flavonoids may be converted to a phenoxy radical anion (as semiquinones) under extreme stress.[28,69]

Flavonoids, and particularly green tea's catechins, remain the main group of phenolic compounds with evidenced anti-FAS activity.[83,90,99] Both mammal cells and the *Plasmodium* sp. use the FAS system to perform the elongation cycle of fatty acids. The structural differences between the type I (eukaryote) and the type II (among others, protozoa), allow this essential system to be targeted by antimalarials.[99] Both the FAS system, and a part of the protein synthesis, occur in the apicoplast, where flavonoids interact with the pertinent enzymes and inhibit this pathway.[83,99]

An ingenious MoA that was ultimately never implemented is iron chelating. Iron is essential for protozoa, among other infectious species, to thrive and is the main concern when invading a host.[39] Iron chelators induce death by iron-starvation, possibly preventing the use of iron on the mitochondria's respiratory chain or for DNA synthesis.[39] Overall, flavonoids represent an alternative to conventional iron chelators that have unreasonable requirements of therapy time and dangerous secondary effects.[108] Flavonoids have the property to starve the parasite of iron, while not being as aggressive to the host as the known synthetic iron-chelators.[82,105]

Additionally, and finally, an innovative, although relatively unexplored, antiplasmodial target: the inhibition of new permeation pathways. After six hours post-invasion, the parasite alters the constitution of the host's cell membrane with its own proteins in order to make it permeable to more compounds.[160] Anions, but not cations, and small compounds, like monosaccharides, but not disaccharides, become available to the parasite.[151,160,161] The homeostasis of the whole structure is again threatened, since the osmotic status and erythrocyte membrane potential might be altered. Flavonoids have the capability of inhibiting these permeations pathways, altering the metabolism of the parasite, leading to its death.[151] Haemolysis and immune recognition might be part of the killing process.[161]

The versatility and multiple approach capacity make flavonoids the most interesting class in this paper. They are highly active against various strains of *P. falciparum*, with a SI of, overall, at least higher than 4. This represents their safety, also expressed by the exclusive and varied pathways through which they target the parasite alone. These compounds have prolonged half-lives and have an history of dietary importance, making them at least apparently safe for children and pregnant women.[28,90,98] Their solubility is enough to quench the long-sought requirement that most identified antiplasmodials cannot meet, not to mention its wide distribution, that should make these compounds low-costly. Their presence in traditional plants used against malaria in endemic places not only validates their use to a certain extent, but evidences their viability as chosen promising compounds.[28,90,98] Lastly, although it depends on the chosen flavonoid, there is no reason to believe the compound could not be used in ACTs, proven activity tests are made to distinguish the ACT's MoA from the flavonoid's.

The quinones and their derivatives presented and discussed have various structures – the most promising appear to be anthraquinones and anthrones. The most interesting anthraquinone is compound (55), a knipholone derivative with a glucopyranoside that awards it an additional edge in both activity and selectivity.[118] Likewise, compound (75) is the most interesting anthrone, a vismione with a geranyl group at C-7 and an acetyl group at C-2, attaining the same advantage.[126] It is noteworthy that, overall, the presented quinones all have high antiplasmodial potentials. It is their SI

that represents a liability in transforming these compounds into antimalarials. Both compounds (55 and 75) may present structure-activity starting points towards a solution to this problem. Figure 11 shows the ideal structures to be considered for further studies. Activity-wise, quinone structures can be oxidized to semiquinones that function as Michael acceptors in nucleophilic additions that establish covalent bonds with proteins, damaging significantly the parasite.[73,130] However, this might not happen exclusively in the parasite, which is why the SI is not always optimal. Naphthoquinones have high affinities to the cytochrome bc_1 complex within the parasite's mitochondria respiratory chain, a specific parasitic structure, justifying the SI of atovaquone and similar acting compounds.[114] However, as formerly discussed, parasites with resistance to atovaquone exist and have limited its use. This represents an obstacle on the interest of developing this class further, unless these new phenolic compounds could prove to bypass this resistance.

Xanthones, as discussed previously, appear to be a particularly interesting class of antiplasmodials. They display somewhat a preference in activity towards chloroquine or multidrug resistant strains. How this happens may be unknown, but it certainly demonstrates an advantage against the resistant parasite. It is noteworthy that all the isolated xanthones originated in the same family, Clusiaceae, broadly distributed in tropical regions, similarly to malaria.[122] The most impactful characteristic towards activity and selectivity seems to be the prenylation at key carbons with 2-methylpent-2-ene groups. A substitution with another group, as in compound (85), demonstrates great activity, but low selectivity, indicative of general toxicity.[91,122,141] As mentioned in the related section, not only do hydroxyl groups at positions C-3, C-6 and C-8 improve xanthones' activity, but also prenylations at C-1, C-4 and C-7 (see Figure 11). A good example of a promising xanthone would be compound (83), with a 20-fold preferential activity towards the chloroquine resistant strain and a good SI.[140] Its cyclization at C-1-C-2 could be the responsible factor, acting in a similar fashion, or better, to the carbon chain at C-1. It is possible the improved lipophilia of the carbon chains facilitates the entrance and accumulation of these compounds inside the digestive vacuole, easing their antiplasmodial activity as inhibitors of the hemozoin formation.[139,162] Also, because the electrostatic potential of xanthones is directly related to their antiplasmodial activity, an additional ring may complement an electrostatic profile that allows the interactions with hematin, preventing its transformation.[138]

Lignans, according to the present findings, are compounds with high antiplasmodial potential and varied SI. Compound (94) is a very interesting lignan with an outstanding activity against a chloroquine resistant strain and no toxicity against Hep G2 A16 cells.[148] However, it is uncertain if lignans could be an innovative antimalarial structure, since most screened lignans are recognised for their general toxicity, unwarranted in malaria's case.[148] Additionally, their mechanism of action is uncertain and would require further studies to ascertain the interest in this phytochemical class.

Table 10 - The antioxidant activity of some phenolic compounds.

Compound	Name	Type of compound	Antioxidant Activity	Reference
3	Ellagic acid	Phenolic acid	1.1 μM[1] 8.24 μM[2] *35.71± 2.5 μM[3]* 13.63 μM[4]	59,63
6	4-Nerolidylcatechol	Catechol	75 % [2]	73
49	Epicatechin	Catechins	1.41 μM[2]	98
50	Catechin-gallate	Catechins	1.27 μM[2]	98
51	Epicatechin-gallate	Catechins	1.18 μM[2]	98
79	2′*E*,6′*E* 2-farnesyl hydroquinone	Quinone	10.8± 0.09 μM[2] *8.4± 0.11 μM[3]**	110
89	Symphonin	Xanthone	23 % ± 0.4[2]	144
96	Licochalcone A	Chalcone	Equal to Vitamin E[5]	163

[1] DCFH-DA (2',7'-dichlorodihydrofluorescein diacetate) method - cell-permeable fluorogenic probe that, after deacetylation inside a cell, can be oxidized by ROS into a fluorescent form. (M)

[2] DPPH (1,1-diphenyl-2-picryl-hydrazyl) radical scavenging assay – DPPH is a free radical that, in the presence of an antioxidant, is reduced to DPPH-H and diminishes the absorbance from the solution, in relation to a DPPH blank, that correlates to the scavenging potential and proton donating ability. The potential might me given in percentage (%) or converted to the concentration required for 50 % inhibition (M)

[3] Ferric reducing antioxidant potential (FRAP) assay – at low pH, colourless ferric complex (Fe^{3+}-tripyridyltriazine) may be reduced to a blue ferrous complex by electron-donation antioxidants. The absorbance of (Fe^{2+}-tripyridyltriazine) are measured and the potential is expressed as Fe^{2+}/g of sample.

[4] $ABTS^+$ radical scavenging activity – $ABTS^+$ is turned into a radical that, in the presence of an antioxidant, decreases the absorbance, that is measured and correlated to a scavenging potential.

[5] Peroxide Value Method (POV) – quantitation of the peroxide formed in the presence of active oxygen through time.

* Values are equivalent to g of ascorbic acid per 100 g of antioxidant compound. The faster the peroxide value increases exponentially, the weaker the antioxidant.

It is pertinent to discuss, considering the topic of phenolic compounds, their anti-oxidant properties in the context of the malaria disease. Phenolic compounds are considered powerful antioxidants with the capacity to turn into powerful oxidants when certain conditions are met, for example, their concentration. The easier the oxidation of the phenolic compound, *i.e.,* the highest the phenoxyl radical/phenol redox ratio, the higher the antiplasmodial activity.[28] Studies have shown that the highest the oxidation potential of phenols, the higher the selectivity towards the parasite.[136] Which means phenolic compounds could be selected for their oxidation potential in order to improve their selectivity and, consequently, antiplasmodial activity. However, considering that this potential depends on certain conditions, if the antioxidant potential overthrows the oxidant, the antiplasmodial activity might become compromised, begging the question if this type of activity is reliable in the context of malaria.[159] Table 10 gives an overview on the antioxidant potential of some of the discussed antiplasmodial phenolic compounds. Most compounds in this table are proton donors, as represented by their high antioxidant values. Compounds (79) is an exception, an electron donor that can act as a nucleophile in Michael reactions.[110] Compound (89) is also a noteworthy exception, the weakest antioxidant of its study, but the most active antiplasmodial, evidencing how xanthones have others MoA.[144] If antioxidants are ever to be included in antimalarial therapy, all factors need to be taken into consideration.

The path that connects the discovery to the development of a new antimalarial is both expensive and time-consuming. A group of accepted criteria exists to help distinguish, as a pre-set, if a compound is promising enough to go on this journey. First, the compound should be a potent antiplasmodial against both resistant and sensitive strains of *P. falciparum in vitro*. This presupposes that the compound targets an essential component to the parasite's survival, an unavoidable route or molecule.[36,41] Second, the compound must be selectively toxic to the parasite, *i.e.*, it must use a unique target, divergent from the host.[36,41] This is represented by a high SI. Some authors argue above 4, others above 1000, while accepting that the SI depends on the strains and types of cells tested and it is only an indicative figure.[36,41] Third, it has been highly suggestive thus far the ability to eradicate the murine malaria in *in vivo* models without any signs of toxicity.[36,41] Fourth, because the ideal antimalarial is perceived as oral, it is advisable to select the compound with its bioavailability in mind.[36,41] Other criteria might also be pointed, such as a long half-life, no recrudescence, active on gametocytes and hypnozoites, no adverse effects, appropriate for pregnant women and children, inexpensive, and a therapy no longer than three days, optimally a compound that could be included in ACTs. These are a set of complicated, at times impossible, guidelines to follow for one single compound, which makes the development of antimalarials harder than it would appear.[27,29,164,165]

In order to obtain information to be able to distinguish promising from not-promising compounds, standard *in vitro* and *in vivo* assays are developed, but possess limitations. The *in vitro* screening tests account only for the erythrocytic stage, responsible for the disease as it is perceived, namely the fevers and its complications. Therefore, the test cannot detect compounds that act in other stages of the *Plasmodium* sp. life cycle that would be equally important to target.[31] Even with the SI information, the compounds may still affect different human cells and organs, leading to unwanted effects. In spite of the restriction on interpretation of these tests, they remain the major mechanism through which relevant data may be attained ethically. Unfortunately, the cost of the required apparatus and tests prevents most studies of plants from having a complete profile. In order to define if a compound could be a promising antimalarial, it is necessary to have a minimum battery of tests that give basic information, such as activity and toxicity, to indicate if *in vivo* tests, for example, would be worth doing.

In this context, it is important to discuss the emergence of artefacts during assays. It has been discussed that some classes of compounds may appear to be active in various screening tests, only to be discarded when, upon further studies, it is discovered that their activity is not specific.[166] These compounds – called Pan Assay INterference compoundS (PAINS) – are in fact reactive chemicals, and not selective promising drugs, *i.e.*, they interact generally with multiple proteins or enzymes, sometimes interfering inclusively with the reading of the test.[166,167] This represents a problem in drug discovery, since active components should be selective and discriminant in what structures they

interact with. Since most drugs derive from natural compounds, it is only expected some PAINS classes have structures that appear in nature – the case of quinones and catechols.[166–168] In both cases, the issue is in their propensity to establish redox reactions, chelate metals and become nucleophiles that interfere with multiple targets in a cell.[167–169] As discussed previously, these classes have uncertain MoA on the malaria parasite, thus their activity might be justified with these general processes. Tests with curcumin (5), epigallocatechin gallate (47) and quinones have revealed multiple ways in which they interfere with cells, such as connecting easily to proteins through thiol addition, chaperone inactivation and alteration of intramembrane protein function through partition into the (eukaryote) cell membrane.[130,137,170] In the antimalarial discovery context, however, this issue becomes particular. The Plasmodium parasite is intracellular, which means all antimalarials must enter and, to a certain degree, interact with the host cell, the red blood cell. *In vitro* tests, thus, include both cell systems. Although results are usually revealed through a method that correlates specifically to the parasite, it is undoubtably important to also perform other tests, lysis tests for example, to rule out direct toxicity to the red blood cells. Once more the selectivity tests gain importance, allowing for the comparison of IC_{50} on normal cells, which reveals the general toxicity compounds might have. This is the case with compounds (6 and 78), a catechol and a quinone respectively, that reveal good activity, but also low SI, indicative precisely of no selectivity and, thus, no future applications in this respect. Lastly, it is important to note that, although researchers should be conscious that PAINS exist and might adulterate results, the presence of the molecular structure should not be enough to abandon all interest in a compound.[166,169,171] As mentioned previously, atovaquone is a synthetic derivative from lapachol, a quinone, with a described MoA and selective towards the *Plasmodium* sp., that is currently used in the drug Malarone™. This demonstrates how the presence of the quinone moiety was successfully optimized to an approved drug and, likewise, other compounds might follow. In the same order of ideas, ellagic acid (3), exhibiting a catechol moiety, presents high *in vitro* activity on multiple strains, high SI and also high *in vivo* activity in a *P. vinckei petteri* model, which is not coherent with a nonspecific mode of action.[61] Hence, it represents a good example of how future optimization could lead to important findings.

Concerning phenolic compounds, flavonoids and, maybe, xanthones, prove to be the most promising classes. Xanthones are interesting for their capability of bypassing the chloroquine resistance mechanisms, which *per se* might help to work around this issue. Whether it is its lipophilicity, electronic distribution or skeleton conformity and if the structure could be applied to the quinoline antimalarial class would be an interesting subject to explore.

As a side note, attention should be given to the contradiction between the requirement of oral absorption and the evidence that increased lipophilia is a major factor towards antiplasmodial activity.

Thus far, for most phenolic classes, lipophilia successfully improved the oxidant activity, the entry in the parasite and in its the digestive vacuole, also facilitating the compounds accumulation in the parasite's structures. Upon observing Figure 11, it becomes clear most ideal structures include additional rings or hydrocarbon chains, which in turn diminishes the hydrophilia. This presents the importance of maintaining these characteristics in favour of greater antimalarial activities, and, in turn, consider other strategies to develop new drugs, *e.g.*, different administration routes, different pharmaceutical formulations or a pro-drug, such as artemisinin, that transforms in dihydroartemisinin, the metabolite that exerts antimalarial effect.[172]

The emergence of future antimalarials might be linked to the study of natural compounds, such as phenolic compounds. These compounds present novel mechanisms to kill the malarial parasite and even possible molecular keys to fight established resistances. However, a long way still lays ahead to take full advantage of these benefits. Unfortunately, some phytochemical classes remain undescribed or understudied regarding their selectivity and mechanism of action, leaving an incomplete profile to work with. Such is the case of coumarins and lignans, as evidenced in this paper. Furthermore, although the three most interesting classes – flavonoids, quinones and xanthones – have available information on them, allowing inclusively the eligibility of interesting compounds from these groups – compounds 11, 21, 37, 55, 75 and 83 –, it is evident that the incomplete data on some compounds may shift the understanding of which ones are the most pertinent to pursue studies. It is essential to have a complete set of data, both the antiplasmodial activity, ideally on multiple strains, and the selectivity index, to be able to discuss compounds as closely as possible. Additionally, for the chosen compounds, *in vivo* testing remains essential to confirm their potential and should be performed, as part of a complete profile, whenever previous factors support them. Finally, the present revision allowed, for the most interesting phytochemical compounds, to preview an ideal molecular structure in order to optimize the antiplasmodial activity. It would be important to synthetize these compounds, test them and assess further their structure-activity relation. Overall, this review demonstrates the variety of possible future phenolic antimalarials and the potential that natural compounds hold in the fight against malaria.

2.1.3. Update

As pointed out in the previous section, the phenolic class of compounds is an interesting and varied group of metabolites that can yet prove to be important in drug discovery, particularly for antimalarials. Furthermore, it was suggested in the previous section that a few compounds could circumvent known resistance factors in particular strains. This could be helpful in the pursuit of designing innovative compounds that retain activity in these strains.

A similar literature research was conducted choosing the publication date between 2020 and December 2023 to include in this dissertation the latest phenolic compounds with potential antiplasmodial activity. The criteria were chosen as mentioned previously – phenolic compounds of natural origin tested *in vitro* with *P. falciparum* different strains with an IC_{50} inferior to 2 µM.

Unfortunately, no compounds fit the established criteria. Despite the extensive research done in the natural product subject with antiplasmodial applications, many studies identified the presence of phenolic compounds through qualitative phytochemical screening, quantification of total phenolic content or flavonoid or tannin content, but seldomly identifying the NP responsible for the antiplasmodial activities.[173–182] *In vivo* tests were mainly conducted in these studies with the crude extracts or fractions without isolating or identifying the NP, possibly in order to retain the interactions that justify these plants uses traditionally.[175]

Still, two studies are worth mentioning despite not fulfilling the criteria mentioned above. α-Viniferin is an oligostilbene recently isolated from *Dipterocarpus littoralis* in what constituted the first study to describe chemically this species used in traditional medicine in Indonesia.[183] Initial antiplasmodial screenings demonstrated a relevant activity of the ethyl acetate crude extract and fractionation revealed α-viniferin as the active compound in this fraction (IC_{50} of 4.06 µM in a 3D7 strain).[183] This compound has been reported in other species and has been the target of numerous studies that demonstrated its anti-inflammatory, antidiabetic, anticancer and antioxidant effects.[184] A bioavailability assay *in vivo* revealed an oral bioavailability of 4.2% for this compound, in line with other phenolic compounds that are also orally malabsorbed.[184] In light of the important antiplasmodial activity revealed in this initial screening, additional tests should be conducted to investigate the potential for this molecule to reveal an interesting antiplasmodial scaffold.

In the second study, *Dacryodes edulis* was investigated for its antiplasmodial and cytotoxic effects as it is a species used traditionally in Cameroon to cure fever.[185] Screening with leaf and stem bark extracts and fractions yielded interesting IC_{50} *in vitro* which led researchers to isolate and identify 16 compounds and also test them in the same conditions (*P. falciparum* 3D7 and Dd2). Of these compounds, the most active were two ellagic acid derivatives, 3,3'-di-O-methylellargic acid and 3,3''-di-O-methylellagic acid 4-O-(3''-galloyl)-β-D-xylopyranoside with IC_{50} of 3.42 µM and 2.97 µM, respectively for 3D7, and 3.98 µM and 2.81 µM, respectively for Dd2, isolated from the ethyl acetate and dichloromethane extracts of the stem bark. Another active compound is ethyl gallate with an IC_{50} of 5.80 µM for 3D7 and 14.43 µM for Dd2, isolated from the ethyl acetate extract of the leaves.[185] Although bioavailability can be predicted to also be an obstacle, these findings demonstrate that the

hydroxy groups of the gallate moiety can play important roles in the activity of these compounds, further establishing this moiety as a potential scaffold worth developing.[186]

3. Metabolomics

A deeper understanding of the parasite is required to increasingly improve the way its biology and resistance are perceived, which in turn can help develop better treatments and eradicate this disease. This has been made possible in the last decade with the emerging systems biology discipline that comprehensively studies biological systems through various lenses, including *in silico* models and analytical technologies, broadly named omics.[187] These omics sciences began with genome sequencing, which for the *Plasmodium* started with the publishing of *P. falciparum*'s genome in 2002, and saw their exponential growth with the optimization of analytical tools and statistic models.[187,188] But omics don't include exclusively the study of the genome (genomics), but also of the transcriptome (transcriptomics), proteome (proteomics), and metabolome (metabolomics), which includes particular fields that study the lipidome (lipidomics) or the metabolic flux (fluxomics).[187,188] These omics allow the in-depth study of the *Plasmodium* as a unicellular organism, but also as an infection that can be characterized through parasite-host interactions, which has allowed the understanding of malaria to reach an all-time high. Indeed, this omics cascade occurs naturally in cells and can be translated into a phenotype that is characteristic of the cellular, or parasitic, status. However, each one provides different information. For example, the activation or inactivation of a gene might be relevant, but this doesn't always translate to a phenotype or insight into biological pathways due to regulation and other factors that occur downstream.[187,189] This is why the interest in metabolomics has increased over the last decade.

Metabolomics is a broad subject and has been used extensively to optimize crops, study the nutrient composition of food, characterize microbial environments, and aid in bioremediation, without forgetting its contributions to pathway mapping and bioengineering optimization.[189,190] These later methodologies were successfully adapted to the medical field by elucidating biomarkers and mechanisms of disease or aiding in the rational development of drugs. Particularly, the field of cancer and microbial infections have benefitted from the implementation of this omics that is now considered an essential tool.

Specifically, the metabolome comprises metabolites, small molecules (<1500 Da) that reflect accurately and rapidly the activity of enzymes, proteins and pathways, leading to a faithful snapshot of the parasite's status.[187,188] Some examples include amino acids, vitamins, cofactors, nucleotides, fatty acids, among others; all compounds that provide energy, signaling or building blocks essential for parasitic survival. Despite the metabolome's heterogeneity, metabolomics aims to detect or quantify

all metabolites simultaneously at a given moment, thus establishing a metabolic profile under those particular conditions.[189] Additionally, the metabolism is dynamic and is swiftly affected by the surrounding environment of the cell, or parasite, meaning it can reflect the reaction or system's adaptability to an external factor, such as a drug.[190–192] Notwithstanding, with the downstream of events, the increasing specificity sheds light on upstream functions and helps to annotate genes, characterize pathways and protein function, making metabolomics a versatile and clarifying discipline.[187–189]

3.1. The analytical tools

Metabolomics as a science is possible due to sensitive and selective analytical tools. The extracted and sampled metabolome remains an intricate matrix composed of metabolites with variating chemical structures, properties, and concentrations.[193,194] Analytical platforms must detect these chemically different compounds with reproducibility in order to achieve identifiable metabolites with biologically interpretable functions. Additionally, there are molecules with the same nominal mass but different chemical configurations, along with stereoisomers and isomers which also need to be differentiated.[195] Therefore, only robust and powerful analytical technologies with a degree of automatization and structure elucidation capabilities are standardly employed to perform metabolomics studies, namely LC-MS, GC-MS, and NMR.[196] As the purpose of this project was to routinely implement LC-MS and NMR analysis, these techniques are briefly expanded upon to elucidate how data is acquired, processed, and analyzed depending on the tool.

NMR was first described in 1945 and it has evolved into a reliable and cost-efficient tool to analyze samples of different kinds and origins.[197,198] The basis for the applicability of this technology in metabolomics relies on the nearly universal abundance (99%) of ^{1}H, making this method virtually sensitive to almost every molecule in the metabolome.[196] NMR assays expose samples to a magnetic field, making the nuclei, protons in the afore-mentioned case, resonate in a characteristic frequency according to their position, environment, and number in a molecule, thus distinguishing the metabolites, including isomers and diastereoisomers. This resonance is inherent to a molecule and was originally used in the identification and characterization of individual molecules before being transposed to omics sciences due to its elevated reproducibility and unbiased nature, independent from the complexity of the matrix.[199,200] A metabolite's polarity, volatility, or charge does not influence directly this technique's efficiency, so it is always possible to obtain information.

NMR is non-destructive and requires far less sample processing than other methodologies. The important aspects to consider when preparing an NMR sample are the necessity of: a deuterated solvent, a buffer, because pH shifts cause chemical shifts, and a reference standard, to lock the spectra.

This standard, trimethylsilylpropanoic acid (TSP) or sodium trimethylsilylpropanesulfonate (DSS) can also be used to quantify the molecules in the sample, as NMR is inherently not only qualitative, but also quantitative.[197,200] The intensity of a peak is directly proportional to the presence of the molecule in the sample and its concentration, making this a simple process in the right experimental conditions.[201] ^{1}H NMR is the most frequent in metabolomics, but other nuclei can also be detected, such as ^{13}C, ^{15}N, and ^{31}P, either individually or simultaneously. Proton NMR has a smaller chemical shift window, which translates in extensive overlapping, particularly in the 1-4 ppm range.[196] This happens because of the presence of multiple metabolites of the same class that have the same molecular groups and it limits the metabolites that are detectable. The use of other nuclei augments the chemical shift dispersion from around 10 ppm in ^{1}H NMR, to over 200 ppm in ^{13}C, for example, thus increasing the resolution and the information attained.[196] However, other nuclei have other constraints, be it their abundancy (around 1% and 0.37%, for ^{13}C and ^{15}N, respectively) or their seldom presence in metabolites, as is the case for ^{31}P, whose abundance is comparable to protons, but not all metabolites have phosphor. Another way to deconvolute overlapping signals and to obtain more information on the samples is to use multidimensional NMR. In fact, the same sample can be re-analyzed at different timepoints and with 1D or 2D NMR, *e.g.,* homonuclear (^{1}H-^{1}H) or heteronuclear (^{1}H-^{13}C) 2D NMR or ^{13}C-heteronuclear single or multiple quantum correlation spectroscopy experiments.[196] Because of higher acquiring times and the additional complexity of 2D spectra, multidimensional NMR is still not frequently implemented in metabolomics studies. Instead, in metabolomics, the use of stronger magnets and stronger magnetic fields is the strategy to resolve overlapping signals, because the resolution of 1D proton spectroscopy scales linearly with the magnetic field strength, while also augmenting its sensitivity. As metabolomics deals mostly with aqueous samples, NMR pulse sequence techniques to suppress the water signal have evolved greatly in order to reduce its influence on neighbouring metabolite peaks.[202] The most frequent sequence used to achieve this in metabolomics is the nuclear overhauser effect spectroscopy (NOESY) with pre-saturation.[196,201–203] Additionally, the presence of macromolecules can also affect the background and influence the baseline, whose quality is a requirement for statistical analysis.[202,203] Sequences such as the CPMG are commonly used in metabolomics to reduce the signal of proteins by using the molecular-weight difference between metabolites and macromolecules to apply a detection filter.[203] This and other methods should, however, be used carefully, since incorrect suppression will impact the acquired data and influence neighboring resonances. Sampling can also include evaporation of solvents or lyophilization to reduce the necessity of using suppressing techniques by reconstituting the sample directly in D_2O, for aqueous samples.[200] One metabolomics spectra usually allows the identification and/or quantitation of between 50 and 200 metabolites.[196] However, NMR fails to detect metabolites at the sub micromole

level, and as such MS becomes the alternative choice.[190] Still, its high-throughput capability, versatility, universality and fairly fast and simple sampling make this technique ideal in clinical metabolomics.[196]

Another way of analyzing a metabolomics sample involves separative techniques before other analysis can be performed. Chromatography is a separative method that relies on the molecule's interaction or affinity to a mobile and stationary phase. This degree of affinity is directly correlated to the molecule's characteristics, like the polarity or charge, and can be translated in a retention time or indices that are used as identifiers. As most metabolites are naturally soluble in liquid solvents, samples don't require additional processing aside from metabolite extraction. The development of chromatographic columns lead to the development of UPLC, which allows to work with decreased particles sizes (sub 2 µm) and pressures around 800 bar with an increased mobile phase flow, thus rendering the run times significantly lower with improved sensitivity and resolution.[199,204] Moreover, it is frequent to use the same solvents for both metabolite extraction and mobile phase, and although not mandatory, it simplifies the workflow. The chromatographic stationary phase choice is of utmost importance, since it will influence the metabolome coverage and reproducibility.[194] In terms of the type of stationary phase, two methods are most commonly used according to Table 11: RPLC and HILIC. HILIC can be considered complementary of RPLC, because the elution order is inverted.[199] Therefore, one sample can be analyzed twice, with each chromatographic column, or using a column-switching technique, so that the most complete coverage of the metabolome can be assured.[205]

Chromatography is most frequently coupled with MS in metabolomics studies because it allies the high sensitivity of MS with the separation (retention time) of compounds, permitting a solid characterization of metabolites and the building of databases. Other valuable advantages from this association when compared to direct MS analysis are that: it reduces ion suppression, may separate isomers, prevents artifacts and distinguishes real metabolites from fake signals that arise from fragmentation and that would have overlapping peaks.[190] Samples could be immediately detected by MS without separation, but sensitivity would be compromised by matrix effects that happen in complex samples such as the ones from *Plasmodium* related assays.[199] The mass analyzer measures the mass to charge ratio of ions, *m/z,* after the metabolite's ionization, which can be done through multiple techniques, *e.g.,* electron-impact ionization or electrospray ionization.[195,205] The intensity of the peak can be linked to the relative concentration of the metabolite in the sample, thought to make this correlation, an internal standard must be used. This process is not straightforward and usually requires parallel runs with internal standard curves or spiking.[206] Internal or external standards are necessary irrespectively if the assay is quantitative or not because they are used to calibrate the analyzer, normalize the peaks, measure the matrix effect and to evaluate reproducibility of the workflow, injection and ionization.[199] After ionization, ions can be positively or negatively charged,

which can be induced according to the pH and/or the ionizable functions of the molecule. This choice influences the number and type of metabolites detected: positive mode is the most common in metabolomics, but negative mode can also be complementary by detecting organic acids.[205] The most frequent mass analyzers in *Plasmodium in vitro* metabolomics, as according to Table 11, are time-of-flight (TOF), orbitrap and quadrupole (Q). The resulting information, the accurate mass (except for the quadrupole, which has poor mass accuracy), intensity of the fragments and pattern of fragmentation, are the metabolite's characteristics that can be used to compare to databases or pure standards, or to serve as basis for *de novo* identification.[195,205,207] Finally, it's important to note that different analyzers, ionization methodology and acquisition mode variate the detectable metabolites, their *m/z* and intensity, and fragmentation pattern, making it hard to compare data to databases and identify metabolites unless the exact same conditions and systems are used.[199,205]

Individually, both MS and NMR can still be improved. Chromatography columns and systems, both in size, kind of particles and interactions are ever changing. MS tandem methodologies to improve reproducibility and software to help analyze the massive bulk of data will help standardize metabolomics studies. And finally, stronger magnets, personalized sequence pulses and multidimension NMR will allow the in-depth characterization of samples in a highly throughput and reproducible manner. Still, it would be shortsighted to perceive the future of analytical methodologies as standalones, as it is recognized that one single system cannot succeed in detecting robustly and identifying all metabolites in complex matrixes.[196,203,206] Therefore, the successful realization of metabolomics' premise of characterizing the entirety of the metabolome can only be fulfilled by combining multiple platforms. That way, one single sample would benefit from the complementary of multiple analytical methodologies, namely MS' sensitivity and NMR's selectivity.[208] NMR's powerful structure elucidation aids in distinguishing isomers and molecular bonds, aiding MS characterization of features and fragments.[195,196,204,205,209] As such, both techniques were sought to be implemented in each assay of this thesis.

4. Metabolomics Developments in Antimalarial Discovery

Antimalarial drug discovery became possible when in 1976 methods for the continuous *in vitro* cultivation of asexual blood stages of *P. falciparum* were published.[21,51,210] This *in vitro* methodology was key to streamlining antimalarial drug assays and screening libraries of compounds relatively quickly and at a low cost. These high-throughput or medium-throughput screens can include synthetic compounds, NP or known drugs and by determining their IC_{50} against drug-sensitive or resistant strains, paved the way to identify chemical classes of potential interest. By having this preliminary

phenotypic readout, studies were only continued with promising classes or compounds, allowing for a superior rationale and ethical research.

This typical path for antimalarial drug discovery is still implemented today and is invaluable in preliminarily selecting compounds that might be promising.[21,51,211] However, these *in vitro* screenings do not reveal the MoA of these potential compounds, and as such hinder their development into drugs. The industry and regulatory institutions are more likely to invest, develop and approve drugs with clear known targets as this allows for the optimization of the strategy of their utilization, and in the case of malaria, to delay as much as possible the emergence of resistance.[211]

More recently, other tools have been successfully integrated in the antimalarial pipeline discovery, such as screenings to identify essential genes or omics technologies.[21,211] Most techniques focus on how to determine the targets of promising compounds, either indirectly in the case of *in vitro* resistance selection and whole genome sequencing, or directly, e.g. affinity-based probe chemoproteomics or protein stability target identification. While these techniques identify binding sites or compound-protein interactions, they are costly, time consuming, non-compatible with high or medium throughput screenings and can lead to false positives or non-specific results. Omics approaches, and in particular metabolomics, offer hypothesis generating results not with a single target identification, but with a phenotype that allows quick characterization of MoA by comparison with established metabolic signatures of known antimalarials – also called fingerprinting. This process is quicker and can be optimized for screenings, which maintains its time/cost relation at an efficient level. The potency or speed of action of a potential active compound might not be seen in a single timepoint test, but multiple timepoints or simultaneous omics can make up for this limitation.[211] Additionally, phenotypic screening of this type, similarly with IC_{50} determination, does not require prior knowledge of the MoA in order to give an accurate phenotype that can reshape the direction of drug development at an early stage.[212] As most antimalarials are known to interfere with parasitic metabolism, metabolomics has emerged as an unavoidable tool in the pipeline in order not only to triage effectively promising antiplasmodial compounds, but to also establish an early-on image on the compounds potential development. Therefore, metabolic profiling, or fingerprinting, can be repurposed for the discovery and description of compound's MoA, thus aiding immensely in the expansion of the antimalarial pipeline.[22,212] This can be done regardless of the nature of the compounds, making the implementation of this approach in NP studies all the more valuable.

There are three principal approaches to a metabolomics assay: targeted, semi-targeted or untargeted. The untargeted approach, that can also be referred to as metabolic profiling or fingerprinting, aims to universally detect and identify the metabolome. Through this method, there's no necessity of

beforehand knowledge and metabolic interactions can be perceived as a whole, hence creating a holistic global view of the cellular status. Moreover, this strategy elucidates MoA that affect multiple targets and pathways, which happen when a drug is pleiotropic. This strategy, however, has downsides, such as facing the heterogeneity of the metabolites that sometimes cannot be identified or quantified, thus leading to putative annotations and descriptions of interactions. A metabolite does not reflect necessarily a specific pathway, as multiple reactions share the same “building blocks”, and may reflect perturbations up or downstream. Additionally, metabolite annotation is challenging, depending on the analytical tool used, metabolite characteristics and *à priori* knowledge.[195] Semi or targeted approaches, on the other hand, lose the universal sight, but succeed in zooming-in on particular pathways. The sampling and extraction method may be adapted to the metabolites and reactions to be detected and quantified, thus making it a selective and highly accurate approach. Consequently, it is frequent that this strategy is used to confirm hypothesis, instead of generating them.[195] The choice of strategy revolves around the assay’s objective. Particularly for antimalarial drug discovery, it depends on the *à priori* knowledge about the drug. If the MoA is suspected and other information would add noise to the study, a targeted or semi-targeted approach would offer more accuracy and sensibility to the type of metabolites involved. On the contrary, if there are no indications of the affected pathway, a complete overview is more adequate to understand globally the drug’s effect on the parasite.[189,200] Optimally, multiple strategies can be used in sequence and the use of other omics could also prove invaluable.

Lastly, it is noteworthy to comment on the particularity of *Plasmodium* sp. as an intracellular parasite that can adapt to multiple kinds of cells and settings, which testifies to its flexibility.[13] The RBC provides protection during development and replication, but it implies an adaptation in order to have access to nutrition.[213] Upon invasion of the RBC, its permeability is changed with NPP that allow influx of ions and low molecular weight nutrients, such as some amino acids and purines.[213,214] Parasites also seek to incorporate glucose and other carbon sources from the host serum and the RBC, which *Plasmodium* relies on deeply for energy through glycolysis.[215] As the parasite starts developing, and increasingly so during its intraerythrocytic development until schizogony begins, it imports hemoglobin into its DV where it is catabolized to amino acids, with the exception of isoleucine.[213] In fact, the *Plasmodium* is auxotrophic for many essential substances: purines, such as hypoxanthine, vitamins, such as B_5 and B_7, and isoleucine.[213,214,216,217] Finally, through the rest of the lifecycle, other cells, with different characteristics from the RBC, host the parasite, representing a necessary step of adaptability in order to obtain essential components from the surrounding milieu.[213] Metabolomics is, therefore, the most pertinent tool to describe these adaptations and detect what pathways are vital and can be targeted by new antimalarials.

4.1. Foreword

The human being coexisted with malaria parasites for millennia, and it has done so by adjusting to maintain fitness in the presence of infection. Unfortunately, as the most complex species between the two, the flexibility at a genetic or metabolic level is lower, and so is the adaptation rate.[190] Despite developing antibodies due to recurrent infection, this immunity is only partial and quickly dissipates if exposure ceases. Fortunately, mankind has had other ways of dealing with this disease. Presently, as mentioned previously, malaria control methods include insecticide-treated mosquito nets, a vaccine, prophylactic drugs and campaigns to ease the access to proper differential diagnosis and adequate treatment.[5] Antimalarial drugs, thus, play a pivotal role in disease management and in the eradication plan.

In order to better understand the multiple parasitic pathways that can be affected by antimalarials, their mode of action needs to be examined. This integral view of important metabolic pathways and the description of pleiotropic signatures and less specific parasitic targets are crucial to interpret metabolomics assays that intend to hypothesize innovative modes of action. Additionally, as fingerprinting assays rely on known antimalarial drugs effects, their description is critical for biological interpretation. As such, the known mechanism of action of antimalarial drugs and the recent discoveries in this field with resource to metabolomics techniques are resumed in the following review and updates in the next section.

4.2. Mechanisms of Action – Published article (adapted)

Parasitology Research
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REVIEW

Recent metabolomic developments for antimalarial drug discovery

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Abstract
Malaria is a parasitic disease that remains a global health issue, responsible for a significant death and morbidity toll. Various factors have impacted the use and delayed the development of antimalarial therapies, such as the associated financial cost and parasitic resistance. In order to discover new drugs and validate parasitic targets, a powerful omics tool, metabolomics, emerged as a reliable approach. However, as a fairly recent method in malaria, new findings are timely and original practices emerge frequently. This review aims to discuss recent research towards the development of new metabolomic methods in the context of uncovering antiplasmodial mechanisms of action in vitro and to point out innovative metabolic pathways that can revitalize the antimalarial pipeline.

Keywords Metabolomics · Malaria · *Plasmodium* sp. · Mass spectrometry · Nuclear magnetic resonance · Mechanism of action

Malaria: understanding the Plasmodium spp.

Malaria is a vector-borne parasitic disease that remains a global health issue.[218] Clinically speaking, malaria is caused by an obligate intracellular protozoan with a complex life cycle that alternates between an invertebrate and a vertebrate host – see Figure 12.[13] The *Plasmodium* spp. are host specific and five recognized species infect mankind: *Plasmodium falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi*, this last one being a zoonotic parasite that has more recently crossed the species barrier.[13] The latest data indicate that there were 241 million estimated malaria cases in 2020, of which 95% originated in Sub-Sharan Africa.[218] The death toll was 627 000 deaths in the same year, with 77% representing children, an important decline from 87% in 2000.[218] Indeed, global malaria incidence and mortality diminished since 2000, but at a relative slower rate since 2015, which jeopardizes the WHO' malaria eradication plan.[5]

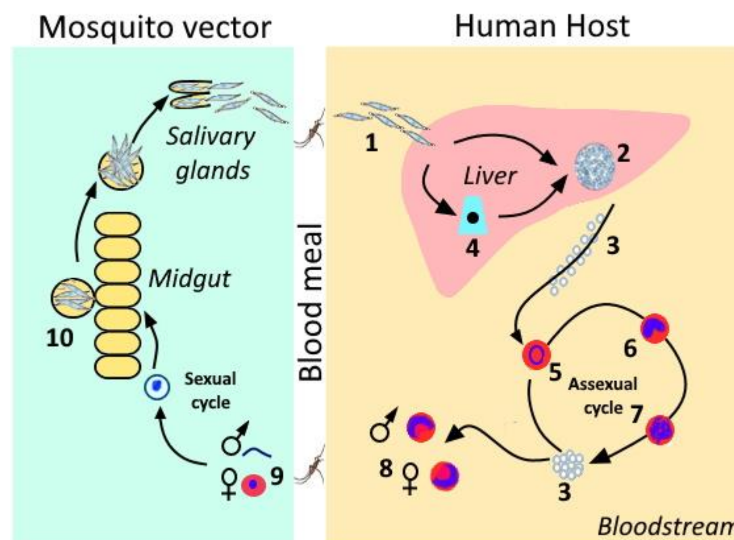

Figure 12 –Human-infecting Plasmodium spp. lifecycle. Upon a bite from the infected Anopheles sp. mosquito, the invertebrate host, sporozoites (1) migrate to the liver of the vertebrate host where a schizont (2) will develop in the hepatocytes, releasing merozoites (3) into the bloodstream. Alternatively, in P. vivax, the hypnozoite (4) form might also develop in the liver, staying in stasis until eventual re-activation. Merozoites invade reticulocytes or erythrocytes, depending on the species, and develop into early trophozoites, also known as the ring stage (5). Over the course of one asexual cycle, the ring stage evolves to a more characteristic trophozoite (6) until it begins schizogony. The schizont (7) releases its merozoites into the bloodstream, recommencing the cycle. Alternatively, merozoites, upon invasion, may develop into male and female gametocytes (8) (stage I-V) that, when taken in a bloodmeal by the invertebrate host, develop into gametes (9) and undergo the sexual part of the cycle in its midgut. Oocysts (10) release sporozoites that migrate to the salivary glands where they readily infect a new vertebrate host upon a new feeding.[14]

Presently, malaria control methods include insecticide-treated mosquito nets, prophylactic drugs and the WHO recommendation of the RTS,S/AS01 malaria vaccine since October 2021.[218] Antimalarial drugs, thus, play a pivotal role in disease management and in the eradication plan. All antimalarial drugs have been impacted by the emergence of resistance and, to tackle this issue, treatment evolved in the sense of Artemisinin-based Combination Therapies.[5,22,219] Although this strategy has been successful in delaying the spread of resistance and diminishing treatment failure, the necessity for

antimalarial drugs with innovative MoA is irrefutable. Antimalarial drug discovery is the key for malaria eradication and tools to aid and optimize it are needed.

Malaria pathological mechanisms presuppose a tight connection between the host cell and the parasite that can be used against the latest. In fact, the presence of the parasite inside the red blood cell (RBC) serves the function of protection, but implies an adaptation cost in order for it to also be a nutritional source.[213] The *Plasmodium* is auxotrophic for many essential substances: purines, such as hypoxanthine, vitamins, such as B_5 and B_7, and isoleucine.[213,214,216,217] This implies that the parasite is partially reliant on external nutritional sources that are relevant to characterize it and, most importantly, that their metabolic pathways can become innovative antimalarials targets.

In order to characterize this new venue for antimalarial development, metabolomics became a stepping stone. This omics discipline that studies the metabolome in depth, including the lipidome (lipidomics) and the metabolic flux (fluxomics), is an invaluable tool in elucidating many aspects of the malaria disease.[187,188,220] The metabolome comprises metabolites, small molecules (<1500 Da) that reflect accurately and rapidly the activity of enzymes, proteins and pathways, leading to a faithful snapshot of the parasite's status.[187,188] Some examples include amino acids, vitamins, cofactors, nucleotides, fatty acids, among others; all compounds that provide energy, signaling or building blocks essential for parasitic survival.

This snapshot can reveal the dynamics of a complex organism like the *Plasmodium sp.* when exposed to a drug, thus establishing a metabolic profile under those particular conditions and portraying the drugs' effects.[189–192] This type of study has become, in the last decade, a significant tool in antimalarial drug discovery.[189,221] In fact, antimalarials are recognized to act by disturbing metabolic pathways that are essential to the *Plasmodium*, but remain segregated from the host, such as folate synthesis or hemoglobin proteolysis.[222] Even if the MoA is not known, the metabolome is capable of capturing the senescence cascade by reflecting how other systems are affected, *e.g.*, the antioxidant system. Knowledge on essentiality of pathways or variating metabolites is crucial to find new targets and for rational drug development.[187,192,217,223] Likewise, the metabolome can aid in describing the pathways linked with parasite resistance in order to help design treatments that can prevent this occurrence. Therefore, metabolic profiling, or fingerprinting, can be repurposed for the discovery and description of drug MoA, thus aiding in the expansion of the antimalarial arsenal.[22,212]

In the recent decade, many important studies regarding the metabolic overview of the *Plasmodium* emerged, particularly in the field of target validation and drug discovery [188,212,224,225]. These advancements have been reviewed before with variant focuses.[187,188,191,199,212,219,225] This review aims to discuss recent and groundbreaking research on the characterization of antiplasmodial targets and

MoA, with a focus on metabolomic *in vitro* studies. To do so, we shed light on recent metabolomic analytical method developments in the field of malaria, and point to pertinent new directions in antimalarial drug discovery.

Metabolomics applied to the Plasmodium spp. parasite

Innovation in metabolomics methodologies

Metabolomics is a broad discipline that has been used extensively in medical field to elucidate biomarkers, mechanisms of disease or aid in the rational development of drugs.[189,190] Despite its huge potential, metabolomics is not without challenges. The metabolome is a very complex assortment of molecules, so it is difficult to detect, characterize or quantify them all simultaneously, as this omics proposes.[189,199] These and other hurdles exist in metabolomic studies on the *P. falciparum* parasite as discussed ahead.

A metabolomic experiment is defined by a sequence of necessary steps, also called the workflow – see Figure 13.[200] Key steps in malaria *in vitro* studies include the sampling and isolation of the parasitic cell from the environment, cessation of all metabolism (quenching) and successful extraction according to the coveted coverage.[190,200] These points are of extreme importance, since they can alter the results, and thus, their interpretation. A discussion on each of these decision steps is given ahead.

Study Design

Metabolomic studies are key to elucidating drug's MoA due to their unbiased nature, global overview and sensitivity.[188] Most study designs of this type in antiplasmodial drug discovery do so through treatment experiments *in vitro,* in which the parasite is exposed to a compound of interest and the modified metabolome is compared to a control. This allows the identification of changes caused by the drug, which are then studied through statistical models and metabolite identification in search for a biological meaning.[192,221,226] This is the basis of metabolic fingerprinting, but the extracellular medium can also be collected and analyzed, thus providing the so-called footprint. Ideally, both the fingerprint and footprint should be used for the most complete dataset possible.[200]

The key aspect of the design is that the collected sample reflects the most directly possible the desired metabolome.[190,200] Metabolomic experiments can also reveal other aspects of parasite biology, such as merozoite invasion and egression, gametocytogenesis, schizogony, mosquito transmission, among others. For each case, the appropriate sampling choice must be considered, likewise the number of samples or parasites *per* sample and their species, stage or synchronicity.[200]

The workflows applied to *in vitro P. falciparum* studies regarding the evaluation of drug's MoA published in the literature since 2015 are resumed in Table 11. These were simplified by the key steps,

from the parasitic stage to data processing and analysis tools, and will be detailed more in-depth in the following sections.

Sample choice and sampling

Metabolomics can reveal fundamental aspects of the metabolism, irrespective of the parasitic stage or species, provided they're not quiescent. The *in vitro* life stages, be it the intraerythrocytic asexual or sexual, characterize the human infection and transmission, respectively. These timepoints of interest for drug development are therefore easily available and information on activity and transmission-blocking capabilities can be readily attained.[227]

Across the intraerythrocytic asexual life stages, most studies focus on the trophozoite and schizont phases, as they are easily magnetically concentrated and are considered metabolically active.[224,228–230] This selection occurs due to the presence of paramagnetic hemozoin, which is retained when in presence of a magnetic field, thus allowing non-invasive synchronization and concentration of late trophozoite and schizont stages.[231] A metabolomic study by Beri *et al*. aimed to discover metabolites as potential malaria biomarkers.[232] It determined that, of the intraerythrocytic asexual life stages, the

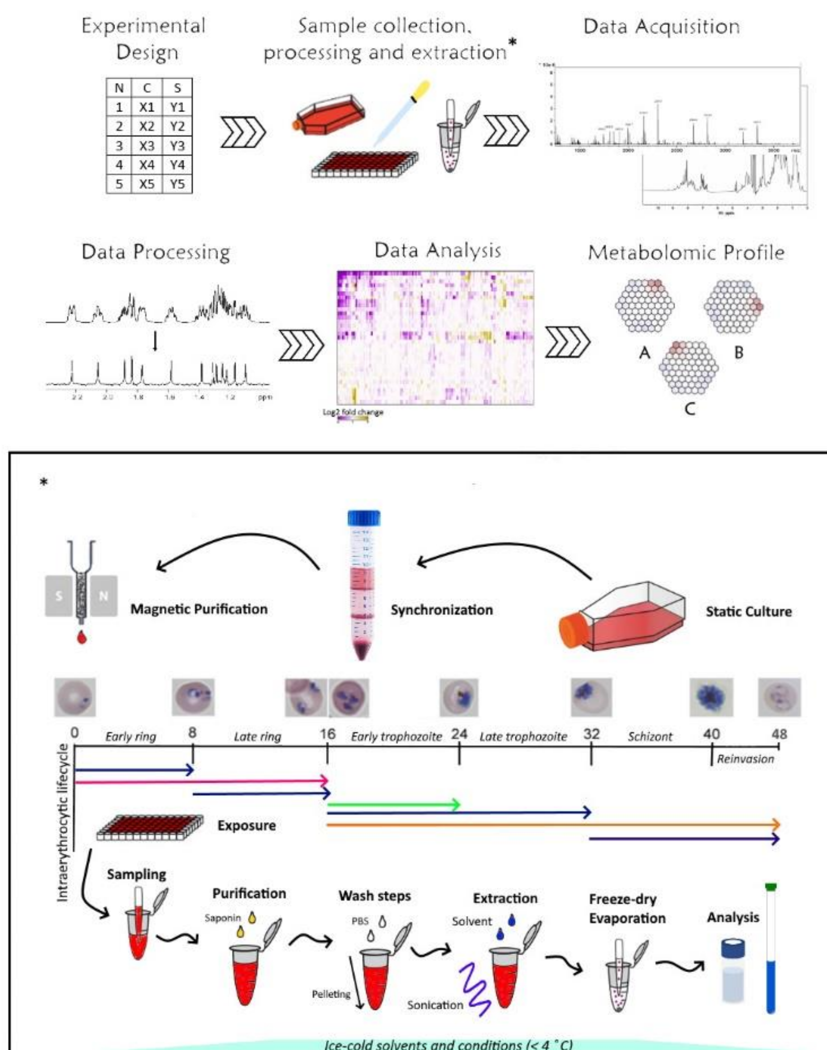

Figure 13 - Workflow of an *in vitro P. falciparum* metabolomic test. Following the preset of the experimental design steps, an *in vitro* assay can yield individual and representative samples that, through processing and extraction*, are analyzed through one or more robust metabolomics techniques. *Depiction of critical steps, including synchronization techniques, magnetic purification, study design (e.g. of an intraerythrocytic time-resolved exposure scheme), purification, washing, extraction and storage.

ring stage was the least metabolically active. Of the 141 metabolites identified, only 10 were common between all stages, reflecting the adaptability and swift changing needs of the parasite over the course of one lifecycle. Additionally, 34% were assigned to lipid metabolism, that proved essential throughout the lifecycle, and 24% were attributed to amino acid biosynthesis, that was upregulated at the trophozoite (24h) and schizont (40h) stage. Interestingly, the assay was stopped at the 40h-mark in order to prevent contamination with the RBC metabolome resulting from lysis and merozoite egression (before reaching 48h), which most studies in the literature don't account for.[232]

The ring form is harder to study than other stages, as it is more difficult to isolate in adequate quantities.[231,232] Still, by isolating schizonts, rings can be studied as well after a few hours.[231,239,258] Whenever possible, metabolomic fingerprinting studies try to distinguish the candidate drug profile across the asexual lifecycle, starting at the ring-stage. Dihydroartemisinin is frequently used as a standard to confirm or compare profiles, since it is recognized that its peak activity happens during this stage.[239] Consequently, and due to the emergence of artemisinin resistance that leads to an unusual prolongation of this form from around 8h to up to 14h, this stage is one of the most interesting.[23] A drug metabolomic fingerprint screening performed by Murithi *et al.* revealed the different profiles and peak activity of 36 compounds, including standard antimalarials.[239] Remarkably, by comparing the fingerprints between chloroquine and other compounds that were theorized to affect hemoglobin catabolism, unique peak activity was found in early ring-stages, indicating that hemoglobin digestion might begin as early as under 8h, before the digestive vacuole (DV) is formed. Multiple drugs with differing clustering profiles seemed to have peak activity at the ring-stage, opening the landscape to the possibility of multiple druggable targets at this stage. Similarly, another study that investigated ozonides' MoA revealed that hemoglobin-derived peptides were impacted as early as 6-12h, further attesting the hemoglobin digestion theory.[259] More studies analyzing the hemoglobin catabolism and proteosome in this early stage would undoubtably advance the understanding of the beginning of the parasitic lifecycle and how it could be targeted in alternative ways to dihydroartemisinin.

Other lifecycle stages should also be considered noteworthy, since they represent timepoints in which interference can prevent transmission or resistance spread.[226,228,241,260–262] This is the example of gametocytes. Only 1%-2% of parasites differentiate in these sexual intraerythrocytic forms, however they are solely responsible for perpetuating the sexual cycle to the invertebrate host.[227,260] Gametocytogenesis is universal in the human-infecting *Plasmodia*, but it changes depending on the species, namely in the number of stages and the cycle duration, for example five stages (gametocytes I-V) in approximately 10-12 days, in the case of *P. falciparum*.[260,261,263] Despite not causing symptoms, a drug with effective gametocidal activity would prove invaluable in blocking transmission. Unfortunately, gametocyte metabolomic studies are not easy, since it is difficult to generate enough

Table 11 - Methods used in *Plasmodium* spp. metabolic studies published since 2015.

Parasitic Stage	Number of cells/sample	Purification	Quenching	Metabolite Extraction Method	Storage	Data Acquisition Method	Data Analysis	Reference
Intraerythrocytic Stages	1 × 10^8	-	Ice-cold PBS	Acetonitrile:Water (4:1,v/v)	-	LC-MS (SeQuant ZIC-pHILIC, Q-TOF)	MAVEN, *R*, MetaboAnalyst 3.0, BioCyc, XCMS	[192,233–237]
Intraerythrocytic Stages	-	-	Dry ice-ethanol bath	Methanol	-80°C	RP UPLC-MS/MS and HILIC UPLC–MS/MS (Q-Exactive, Orbitrap)	MATLAB, MetaboAnalyst, HMDB, SMPDB, KEGG	[221]
Intraerythrocytic Stages - Trophozoite	5 × 10^7	-	-	Methanol	−20°C/ -80°C	LC-MS (SeQuant ZIC-pHILIC, Q Exactive)	IDEOM, XCMS, TraceFinder, MetaboAnalyst	[224,230,238]
Late-stage gametocytes	-	-	-	Direct media was tested	-	^{1}HNMR	ProMetab, MATLAB, HMDB, KEGG	[226]
Intraerythrocytic Stages, Mid to late stage gametocytes (III-IV)	1.0 x 10^5 to 1 × 10^8	-	-	90% Methanol	-80°C	UHPLC-MS (Phenomenex Hydro-RP C18, Orbitrap)	MAVEN, *R*	[228,239–242]
Intraerythrocytic Stages	5x10^7	0.1% saponin	Liquid nitrogen	Chloroform and Methanol/Water (3:1)(GC-MS) 80% Acetonitrile(LC-MS)	-80°C	GC-MS (BD5 capillary column, EI-quadrupole MS) LC-MS (SeQuant ZIC-pHILIC column, Q-TOF)	ChemStation, MAVEN, METLIN	[229]
Intraerythrocytic Stages - Trophozoite	-	-	Ice-cold PBS	Methanol	-80°C	GC-MS (DB5 capillary column)	IDEOM, XCMS, mzMatch, *R*	[230]
Intraerythrocytic Stages - early rings	-	0.15% saponin	Liquid nitrogen	Methanol	-80°C	UPLC-MS/MS (HILIC, Q Exactive)	*R*	[231]
Intraerythrocytic Stages	1 x 10^5	0.07% saponin	-	Series of organic and aqueous extractions 10 v/v 0.1% formic acid in methanol	-80°C	GC-MS (Trace DSQ fast scanning single-quadrupole) HPLC-MS/MS (Zorbax Eclipse Plus C18, QQQ)	GraphPad Prism 5.0, *R*	[232]
Intraerythrocytic, gametocytes and ookinetes	1 x 10^6	-	Dry ice-ethanol bath	Chloroform/methanol/water (1:3:1, v/v), sonication	-80°C	GC-MS (VF5-MS column)	Agilent ChemStation	[243]
Intraerythrocytic Stages	8x10^7	-	Dry ice-ethanol bath	Methanol, Methanol/water (4:1), sonication	-80°C	uHPLC-MS (Hypersil-GOLD C18, QQQ)	-	[206,244]
Intraerythrocytic Stages	1 × 10^8	-	-	Chloroform/methanol/water (1:3:1 v/v)	-	GC-MS (DB5 capillary column)	Mass Hunter, IDEOM, MAVEN	[234]
Intraerythrocytic Stages - Trophozoite	1.2 × 10^{10}	-	-	Acetonitrile:Water (4:1 v/v)	-	LC-ESI-MS/MS (Acquity UPLC BEH C18, Reaction Monitoring	-	[245]

and late-stage schizont						(SRM) Method, Quattro Premier XE MS)		
Intraerythrocytic Stages	-	-	-	Acetonitrile (2:1, v/v)	-	LTQ-FTMS (C18)	Metlin Mass Spectrometry Database, apLCMS, KEGG	[246]
Intraerythrocytic Stages – early ring	-	-	-	90% Methanol	-80°C	Reversed-Phase Ion-Pairing LC-MS, Exactive Mass Spectrometer	MAVEN, *R*	[247]
Intraerythrocytic Stages	-	0.02% saponin	-	Sonication, 1.8 mM perchloric acid and pH adjusted with 5.4M KOH	-20°C	^{1}HNMR	Mestrec software, MATLAB, HMDB, Metaboanalyst	[248,249]
Intraerythrocytic Stages	-	-	-	Methanol: acetonitrile: water 5:3:2	-80°C	uHPLC-MS (Hypersil-GOLD C18, Orbitrap Elite)	GraphPad Prism v 7.04, *R,* HMDB, Metlin, and KEGG	[250]
Intraerythrocytic Stages	1×10^{9}	0.1% saponin	-	Chloroform/methanol/acetonitrile (2:1:1, v/v/v), 2 vols Water	-80°C	LC-MS/MS (Ion-pair reverse-phase chromatography, 4000 QTRAP)	-	[251,252]
Intraerythrocytic Stages and Gametocytes	-	0.1% saponin	-	Isopropanol: hexane (1:2) and KCl (2 M): MeOH (4:1)	-	LC-MS (Zorbax Rx-Sil, QQQ)	MATLAB	[253]
Intraerythrocytic Stage - Late-stage Schizonts	-	Hypotonic buffer (5 mM KH_2PO_4, pH 7,4)	-	Methanol	-	HPLC-MS (ReprosilPur Basic C18, LTQ-Orbitrap Discovery MS) HPLC-MS/MS (HSS T3, TSQ triple quadrupole MS)	XCALIBUR, XCMS, HMDB and METLIN	[254]
Intraerythrocytic Stages	5×10^{7}	-	-	N-ethylmaleimide in 80% methanol with 10 mM ammonium formate, and Acetonitrile	-80°C	HPLC-MS (HILIC-Z column, Q-Exactive Orbitrap)	QuanBrowser 4.2, GraphPad Prism 8.0.2	[255]
Intraerythrocytic Stages	3.5×10^{8} iRBC	0.15% saponin	-	Ethanol/0.01 M phosphate buffer (85:15, v/v), sonication	-80°C	UPLC-MS/MS (ACQUITY, Xevo TQ-S)	MetIDQ, *R*	[256]
Intraerythrocytic Stages – extracellular vesicules	-	Ultrafiltration and size-exclusion chromatography	-	Chloroform:methanol (2:1)	4°C	MALDI-TOF	-	[257]

biological material *in vitro*. Additionally, the late stage, responsible for transmission, is quiescent, awakening only in the conditions of the mosquito's midgut, thus limiting its metabolic studies and hindering drug targetability.[260,261,264] Presently, methods that account for up to 4% gametocytemia in *in vitro* culture exist.[261] Notably, accumulation of homocysteine and redox metabolites in culture were associated with differentiation into sexual stages.[265] Despite being intraerythrocytic parasitic forms, the metabolism between sexual and asexual stages is sufficiently different to allow future development of targeted gametocidal drugs. Specifically, glycolysis and mitochondrial processes were found to be activated distinctly in asexual and sexual life stages, respectively.[243,266] Exclusively during gametocytogenesis, the mitochondria develop tubular cristae and expand in size.[266] It is theorized that these modifications are correlated to an increased function of the Tricarboxylic Acid (TCA) cycle during gametocyte maturation in *P. falciparum* and *P. berghei*, despite these two species different metabolic needs.[243] Additional studies are needed to ascertain and screen compounds MoA against this stage, so that a transmission blocking treatment can be developed.

The mosquito parasite stages represent additional complications, since they require supplementary laboratory apparatus and produce small biological samples, especially in comparison to *in vitro* intraerythrocytic cultures.[241,260] One naturally infected mosquito carries between 2-5 oocysts, while feeding assays can reach a saturation of around 400 oocysts, in comparison to tens of millions of parasites in the human intraerythrocytic stages.[260] An ambitious study by Antonova-Koch *et al.* screened half a million compounds for their capability of inhibiting hepatic invasion, hence possessing chemoprophylactic properties, by dissecting more than one million infected mosquitoes over a 2-year period.[241] This demonstrates the difficulties in generating enough mosquito infective forms to study these and downstream stages. This study found, through phenotypic and metabolic profiling approaches, 58 potential antimalarial drugs, indicated as mitochondrial inhibitors, that would benefit from being explored further.[241] Additionally, parasitic stages in the mosquito host are not stationary and differ between female and male. In a metabolomic and genomic study, upon ingestion by mosquitos, *P. berghei* female and male gametocytes were shown to develop into gametes through different processes.[243] Females conserve the mitochondria, while it degenerates in the male counterpart. The latter is thought to be more vulnerable to drugs because of its higher necessity of glucose for both mobility and the fast-multiple rounds of nuclear replication.[243] Alternatively, female gametes can use glutamine and the TCA cycle to fuel ATP synthesis, making them less dependent on energy storage and more prone to interchanging the carbon source pathway. Other changes related to glucose availability, like the midgut pH, have also been shown to metabolically induce *P. berghei* sexual development.[267] Furthermore, ookinetes remain metabolically active and dependent on both glucose and the TCA cycle. CoA synthesis seems to also play an important role, both in carbon

metabolism and lipid synthesis, thus affecting ookinete to oocyst conversion and oocyst sporogony. Hence, compounds that could disrupt these pathways could prove important transmission blocking properties.[243,260].

Lastly, hepatic schizonts and hypnozoites are also important, permitting intervention before the disease manifests and the depletion of *P. vivax* and *P. ovale* reservoirs [260,261,268]. However, hypnozoites are unsusceptible to most antimalarials as they remain quiescent, which also hampers their metabolic evaluation. Additionally, it is difficult to culture the hepatic stages, as hepatocytes are harder to maintain and have smaller life spawns than RBC. Hence, robust *in vitro* hepatic stage models are an area of interest for malaria research, that would facilitate metabolomic testing. Most models still have issues relative to deficient surface receptors, abnormal cell regulation and loss of hepatic function, responsible for poor sporozoite invasion rates and difficulties in maintaining and in-depth studying this stage [268,269]. Recent developments of culture systems for primary human hepatocytes and immortalized hepatocyte-like cell line models represent important advancements that will enable studying these forms in the future.[268–270]

Finally, a few key obstacles need to be considered when choosing the sample and sampling for metabolomic studies. As metabolites or pathways are sometimes overlapping between host and parasite, care is important when treating data to account for the metabolome that does not originate from the parasite. Specifically, in the case of *in vitro* assays, cultures usually have parasitemia up to 10%, meaning there are significantly more RBC than parasites.[206,231] Some experimental steps can be performed to selectively extract the parasitic metabolome, for example, through saponin treatment. Saponin at a given concentration was demonstrated to lyse RBC cellular membranes, while maintaining the parasitic membrane intact.[271] Additionally, special culturing protocols have allowed to, through this process, have parasites directly in culture without the RBC.[272] Even if for a limited time, this so-called functional isolation enabled different approaches on the study of uptake of certain metabolites and drugs. The saponin isolation method is used recurrently in the methods described in Table 11. Care is still important even when using this technique, considering that RBC remains may stay attached to the parasite and influence analysis.[231] Another method to induce lysis is incubation with streptolysin, yet it is less used because it is recognized that it does not disintegrate the RBC's membranes, leading instead to pores and increased sample contamination.[199,273] Other lysis options exist, based on solutions with NH_4Cl, $KHCO_3$ and EDTA, however incubation times of 15 minutes prevent them from being used in metabolomic workflows, despite their advantage of allowing membrane purification and study, which is important for lipidomics.[273] The lysis step, when performed, should last from a few seconds to a couple of minutes and usually bellow 4°C.

Additionally, studies typically don't distinguish or note the age of RBC used in culture, thus not accounting for a factor that, as mentioned above, can influence parasitic growth and antiplasmodial activity.[274] In culture maintenance, some protocols specify that the conservation of RBC should not surpass one month, ideally two weeks.[210,275,276] This assures that the parasites have fresh cells to infect and ascertains the quality of these cells. Since *P. falciparum*, the most recurrent species in culture, can infect RBC of any age, age or type of cells are not usually a problem and, hence, go unnoticed. Even if type or age are not noted, RBC of the same donor should be used to account for the least variance possible. This was demonstrated to be an important factor in a recent metabolomic study by Tewari *et al.*[276] This team verified if there were significant batch differences or metabolic biases that could inadvertently come from the RBC *in vitro.* As such, this assay maintained uninfected RBC in the same conditions as originally intended for infected Red Blood Cell (iRBC) and tested the metabolome through high-resolution metabolomics. Despite inter-study consistency, individual donors were responsible for metabolic variations, pointing to the necessity of more batch controls in metabolic studies.[276] Parallelly, blood storage was proven to be an important factor, as peptide modifications, due to degradation of methaemoglobin, alongside with variations in glycolysis and in synthesis of glutathione were detected.[276] These variations are often overlooked in metabolomic profiling in *Plasmodium* studies, but could prove to adulterate results and should be carefully analyzed to eliminate biases.[276]

When performing metabolomic studies, care should be given to possible contaminations from the RBC's metabolome or the extracellular medium. This can be done by incorporating washing steps, by sampling RBC standard metabolomes for comparison, and through fluxomics. Via enrichment techniques it is possible to follow some metabolites and confirm branching or extension of pathways. Indeed, studies using metabolically labeled ^{13}C-glucose have successfully distinguished pathway activation between parasite and RBC. Notably, in a study by MacRae *et al.*, TCA cycle intermediates were found in ring stage-iRBC at five times the concentration of uninfected RBCs, with significant labeling of these compounds in the infected sample.[277] The difference in labeling between uninfected and iRBC successfully discerned the extensiveness and rate of metabolic fluxes of the TCA-cycle.[277] More recently, Cobbold *et al.* performed an untargeted LC-MS metabolomic assay with unsupervised isotopologue grouping to describe the metabolic capacity of the *P. falciparum* trophozoite infected RBC and RBC.[237] The basis for the study was that significant protein and metabolic-encoding *P. falciparum* genes are still unannotated, thus failing to shed light on enzyme promiscuity and ambiguous metabolites.[237] By characterizing the metabolites and successfully distinguishing them between infected and non-infected RBCs, this research hoped to identify unexpected parasitic metabolic pathways, complement gene annotation and successfully remove RBC biases. This study was able to describe over 70% of parasite gene-encoded metabolic reactions, detecting 911 and 577

metabolites for the iRBC and RBC, respectively.[237] It was found that the largest group of undescribed metabolites were related to parasitic damage-repair systems, which still need further investigation.[237]

In order to explore the impact of RBC metabolome and media contamination, Carey *et al*. sought to define parameters that could optimize critical and specific *Plasmodium* factors.[231] They demonstrated in an untargeted metabolomic study of intraerythrocytic *P. falciparum* that, despite varying multiple experimental conditions, both the RBC and the culture medium contaminated the samples, influencing the final results. Particularly, over 68% of parasites remained attached to "ghost" RBC, namely cell membranes, that influenced the metabolite counts, predominantly the lipidic component.[231] Similarly, normalization was found to be the second most influential parameter and the method choice skewed greatly the results interpretability. Normalization by DNA content or parasite number still failed to eliminate the contamination effect from the datasets. Additionally, this study showed how the manual elimination of media and RBC contaminants, such as HEPES or phenol red, from the data sets are erroneous and should, instead, be considered for analysis, both for quality control and to evaluate the extension and type of contamination.[231]

When possible, other enzymatic, membrane potential or omics methodologies should be employed alongside metabolomics to confirm or obtain further insight into parasitic specific mechanisms. The presence of HEPES or other culture media exclusive components, as well as RBC specific metabolites should be incorporated as markers to give information on media contamination of samples or overall quality of the washing steps of the workflow. This would prevent result bias and improve overall assay quality.

Quenching

Metabolism shutdown is a crucial step in a metabolomics workflow. This key point, known as quenching, ensures enzymatic arrest as quickly as possible and, thus, that the sample reliably correlates to the experimental conditions [192,200,230]. However, despite its essentiality, this step is sometimes overlooked in workflows. In fact, quenching is not easy to implement and its methodology needs to be adapted to the study's design.

Quenching methodologies are usually based on variating sample temperature and/or adding solvents, although there is no universal method.[190,200] For cultures, cells are frequently placed in contact with cold organic solvents (4° C, usually lower), either after media aspiration, for adherent cells, or filtration, for non-adherent. The objective is to slow the enzymatic activity by diminishing the temperature, and to assure enzyme denaturation, which happens permanently with organic solvents.[190] Despite being non-adherent cells, *in vitro Plasmodium* metabolomic assays do not commonly include filtration steps

in their workflow. However, fast-filtering could present advantages in the speed of segregating the cells from the media in comparison with current centrifugation cycles present in some workflows.[190,200]

Any steps that may alter the metabolism or induce stress are usually advised against. These include cell pelleting and wash steps prior to quenching. However, in the workflows presented in Table 11, it is noticeable that most studies perform cell pelleting. It is a frequent antiplasmodial metabolomic workflow to incubate the sample with saponin below 4 °C, followed by pelleting and washing with cold PBS before extraction. Despite uniformity in the quenching methods in the literature and in Table 11, metabolomics in the malaria field still needs research and viable alternatives in order to optimize this step of the workflow. [192,230,243].

Metabolite Extraction

After quenching, and in this case separation between RBC and parasite, the next step is to obtain the latter's metabolites. The extraction is a key mandatory step in sample processing, allowing the isolation of the metabolome. Organic solvents play multiple roles at this point of the workflow: breaking-down membranes, retaining the metabolome and enzymatic denaturation.[190] An additional role of the extraction step with organic solvents is sample deproteinization, which cleans the sample of interferents for posterior analysis.[206,278] This is important, since *Plasmodium in vitro* cultures contain plasma, and hence proteins and lipoproteins, not to mention the presence of hemoglobin and other parasitic proteins. The solvent choice is crucial, because it influences the range of attained metabolites, based on their physico-chemical characteristics.[189,192] Additionally, the extension of metabolite extraction depends on the time, type and possible mixture of solvents chosen. Hence, these factors need to be chosen carefully.

As shown in Table 11, the most common extraction solvents in *P. falciparum in vitro* metabolomic studies are acetonitrile, methanol, chloroform and water, in various gradients, with few exceptions, such as hexane.[231,233,234] Hence, by variating the gradients and mixtures, the extraction of the most differentiated molecules possible is assured, which increments the likelihood of attaining significant biological data.[189] The time of contact also variates from a few minutes to 30 minutes, which reveals the compromise between long experimental times and the extensiveness of metabolic extraction. Multiple cycles of extraction with a fresh volume of solvent are also frequent to try to obtain a most complete metabolome as possible. Finally, solvents are usually removed through evaporation under nitrogen stream or freeze-drying.[200,206]

Data acquisition

After a sample has been collected and processed appropriately through the methodologies presented previously, it needs to be analyzed. As such, the objective of data acquisition is to detect accurately, and potentially quantify, as many metabolites as possible.[190]

Robust and powerful analytical technologies are needed to perform metabolomic studies.[193] Platforms based on chromatography, such as Liquid Chromatography (LC) and Gas Chromatography (GC), coupled with mass spectrometry (MS), or Nuclear Magnetic Resonance (NMR) made metabolomics possible because of their sensitivity, selectivity and reproducibility.[205,278] These analytical methodologies are versatile in characterizing samples by their chemical composition, be it the affinity for the stationary phase of a column, the ionization profile or chemical shift, among other factors. Their features allow them to be used in diverse circumstances, making them universal in metabolomic studies, including in malaria studies.[189] However, different platforms have unique advantages and disadvantages and may require care during the sample treatment stages. These analytical techniques have been reviewed extensively elsewhere.[202,279,280]

In the *Plasmodium in vitro* context, NMR is not utilized as frequently as MS. This can be because of the lack of access to different platforms or the relatively lower sensitivity of this technique. This characteristic does imply that NMR requires more parasites per sample than MS, above 10^8 parasites. Interestingly, some studies have pointed out the benefits of using NMR instead of MS. For example, glycerol was left undetected in the *Plasmodium* until an NMR assay successfully detected it.[281] Glycerol is a glucose catabolism end product that had been identified in other human parasitic protozoa, but not in the *P. falciparum.* This because glycerol has no chromophore and ionizes poorly through ESI, thus UV detection or MS failed to detect it.[281] Extensive derivatization might have been relevant to make this metabolite detectable, but in global metabolomics there is no *à priori* knowledge of the necessity to implement such techniques, as the content of the metabolome is unknown or putative. ^{13}C NMR successfully detected and identified glycerol in *P. falciparum* samples, suggesting that the parasite maintains carbon and redox equilibrium through the ramification of the use of glucose.[281] This process is significantly different in RBC, that use glucose exclusively for glycolysis. This discovery paved the way for more studies that focused on the carbon metabolism of this parasite.

Data analysis and interpretation

Due to the metabolome's complexity, the interpretation of the obtained chromatograms and spectra is challenging. This is inherent to omics data because one sample originates dozens to thousands of features that are not readily interpretable. These data needs to be simplified and analyzed per sample, frequently without any preconception of the metabolites to be found.[200] Malaria-derived data is

treated similarly to other metabolomics sets, using the same processing software and statistical models.

Only the databases represent a significant difference. Databases are used to facilitate metabolite annotation through the concentration of information from multiple studies that are interchangeable between laboratories.[189,196,282] Their construction is specific and time and labor-consuming, but they allow a fast exploration of metabolic data. Some databases have the option for *P. falciparum* metabolites or are more general, containing biological metabolites from many organisms.[283] Plasmodia-oriented databases are the PlasmoDB (https://plasmodb.org/) or Malaria Parasite Metabolic Pathways (https://mpmp.huji.ac.il/).[193,212,242,266] These don't contain exclusively metabolic reactions, but also the genome and other parasite related data, and their principal objective is to link genes to other structures in order to annotate their functional meaning. Examples of freely accessible databases with information on both MS and NMR analysis are the Human Metabolome Database (HMDB - https://hmdb.ca/) and the Kyoto University Encyclopedia of Genes and Genomes (KEGG - https://www.genome.jp).[189,196,282] Another example that works exclusively with NMR spectra is Chenomx NMRSuite.[196,200] The matrix tables resulting from data analysis can be uploaded to platforms that annotate and map metabolic pathways such as Metaboanalyst or Workflow4Metabolomics, which have a *P. falciparum* option.[190,283] This allows to putatively identify unknown metabolites but also to interpret their biological function.[189] Generally, multiple databases are used in plasmodial metabolomic studies.

The final step of metabolomic data treatment is statistical analysis, although other data mining and analysis methods can be performed.[282] This analysis can be performed before annotation, in a chemometric approach, or after, in quantitative metabolomics. In *Plasmodium* in culture assays, it is more frequent to encounter chemometrics due to its unbiased nature and potential for automatization.[278]

Finally, it is worth mentioning that metabolomics data can also be used to confirm computational models' predictions or provide experimental data to build said models. These *in silico* assays can mirror the metabolic network of the *Plasmodium* through algorithms and thus predict outcomes, essentiality and exclusivity by virtually evaluating the impact of several factors.[284–286] Despite limitations, these models are complementary to experimental studies and are sure to simplify malaria drug discovery in the future.[287,288]

Troubleshooting

As with any troubleshooting, after identifying the weaknesses and short-comings of the workflow processes, it is possible and worthwhile to find workarounds and solutions. Various indicators towards

optimization were pointed out so far, depending on the workflow step. In terms of study design, time or concentration-resolved experiments and fluxomics can be implemented to increase the screen's dimensionality and distinguish unspecific metabolic variations and help to establish causality. In the experimental procedures, in order to reduce or attenuate the host cell or the media impacts some steps can be foreseen. One is parasite purification, by isolation or enrichment, by increasing the parasite:RBC ratio. Another is tracing analytes not metabolized or synthesized by the parasite, such as HEPES or phenol red, that are externally added through the culture media. Finally, samples collected from said media, RBC metabolome or the different washing steps can provide insight on contamination. Microscopy can also be helpful in confirming the integrity of parasitic membranes and the presence or absence of RBC membrane residues still attached, before continuing the metabolomic assay. Isotope marking can also be used to improve data quality, as marked species can be used to confidently remove background or degeneracy features, which can represent over 90% of all detected features in LC-MS.[237] Non-singular acquisition methodologies should be universally implemented in order to attain and explore as further as possible individual metabolites. This would enable identification and interpretability with high degrees of confidence, and thus the building of plasmodial databases that could be exchanged between laboratories. And lastly, the data processing and normalization procedures should be validated and standardized, in order to improve analytical reproducibility across plasmodial metabolomic studies.[192,224,231,282]

Metabolomics in the Discovery of Mechanisms of Action of Antimalarials

The aforementioned workflow warranted the discovery and validation of metabolic targets of antimalarials and other potential compounds in the research pipeline.[224,228,230,234,239] Generally, compounds can have one specific target or be pleiotropic, consequently posing difficulties in defining the MoA through traditional means.[192,234,289] A metabolomic profile is able to disclose a compound's distinguishable fingerprint and evidencing downstream events that better correlate to parasite death. This means that the MoA would not relate to a single target, but to a series of interactions that relay to how the parasite reacted to the drug, hence also shedding light on possible resistance mechanisms. When essential parasitic pathways are targeted, their exclusiveness can increase the molecule's antiparasitic efficacy and downplay side effects. Through metabolomics, traditional antimalarials and promising compounds can be studied, both to optimize current therapies, and to guide drug development to a treatment that will lead to the eradication of malaria.

With this purpose, multiple established and potential antimalarials were metabolomically studied throughout the last decade.[228,230,234] Table 12 summarizes the data collected in this context with a focus on the discoveries since 2015. Overall, seven metabolomic profiles were identified according to the pathway affected, as simplified in Figure 14: hemoglobin metabolism and protein degradation,

Table 12 - Profiles of antiplasmodial compounds found or confirmed through metabolomics.

Compound	**Stage**	**Metabolic Pathway = Profile**	**Putative target/mode of action**	**References**
3361	Ring	Glycolysis	Plasma membrane hexose transporter	[230,234]
2-deoxyglucose	Trophozoite	Homeostasis	Glycolysis	[228]
Artefenomel (ozonides)	Trophozoite	Redox homeostasis	Inductor of oxidative stress	[255]
Atovaquone	Late trophozoite Gametocyte[1]	Pyrimidine synthesis and mitochondrial potential	Mitochondrial electron transport chain, *Bc1* complex	[224,228,230,234,239,261]
C7 (MMV665915), E4 (MMV666600) and P2_A3 (MMV000634)	Trophozoite	Lipid metabolism	Alpha-linolenic acid (ALA) pathway	[230]
Chloroquine	Ring, Trophozoite, Early gametocyte	Hemoglobin metabolism	Hemoglobin catabolism Heme detoxification	[228,230,239,261]
Cycloguanil	Ring	Folate biosynthesis	Dihydrofolate reductase-thymidylate synthase (DHFR-TS)	[234]
Dihydroartemisinin[2]	Ring, Trophozoite	Hemoglobin metabolism Redox homeostasis Pyrimidine synthesis	Multiple hemoglobin-digesting proteases Inductor of oxidative stress	[224,230,239]
DSM265	Late trophozoite	Pyrimidine synthesis and mitochondrial potential	Dihydroorotate dehydrogenase (DHODH)	[228,239]
Ferroquine	Ring	Hemoglobin metabolism Redox homeostasis	Heme detoxification Fenton reaction	[239]
Fosmidomycin	Trophozoite	Isoprenoid biosynthesis	2-C-methyl-D-erythritol 4-phosphate/1-deoxy-D-xylulose 5-phosphate (MEP/DOXP) pathway	[229,234,239,290]
Indolmycin	Trophozoite	Isoprenoid biosynthesis	Apicoplast tryptophanyl-tRNA synthetase (TrpRSapi)	[235]
JPC-3210 (MMV 892646)	Trophozoite	Hemoglobin metabolism	β-hematin polymerisation	[224]
KAE609 (cipargamin), (+)-SJ733, and KAF246	Trophozoite	Homeostasis	*Pf*ATP4	[228,291,292]
KAI407	Schizonts	Hemoglobin metabolism	Phosphatidylinositol 4-kinase (PI4K)	[239]
Lumefantrine	Ring, Trophozoite	Hemoglobin metabolism	Hemoglobin uptake	[239]
Mefloquine	Trophozoite	Hemoglobin metabolism	Heme detoxification	[224,239]
Methylene blue	Ring, Trophozoite Gametocytes	Hemoglobin metabolism Redox homeostasis	Heme detoxification Glutathione reductase Inductive of oxidative stress	[226,230,234,239]
MMV007571 and MMV020439	Trophozoite	New permeability pathways Pyrimidine synthesis and mitochondrial potential	Mitochondrial electron transport chain, *Bc1* complex DHODH	[233,293]
MMV007839 and MMV000972	Trophozoite	Homeostasis	Formate nitrite H^+-transporter (PfFNT)	[236]
MMV08138	Trophozoite	Isoprenoid biosynthesis	2-C-methyl-D-erythritol 4-phosphate	[294]

			cytidyltransferase (MEP/IspD)	
MMV667487 and P218	Trophozoite	Folate Biosynthesis	DHFR-TS	[228]
Naphtoquine	Ring	Hemoglobin metabolism	Heme detoxification	[239]
Piperaquine	Ring, Trophozoite	Hemoglobin metabolism	Heme detoxification	[230,239]
Primaquine[2,3]	Trophozoite, Gametocyte, Hypnozoite	Homeostasis	Inductive of oxidative stress	[261][Review Marcsisin 2016]
Proguanil[3]	Ring	Protein degradation	Arginase enzyme or transporters	[234]
Pyrimethamine	Trophozoite	Folate Biosynthesis	DHFR-TS	[228,254]
Sal A	Trophozoite, Schizont	Lipid metabolism	Serine hydrolases	[289]
Tafenoquine[2,3]	Trophozoite, Gametocyte	Homeostasis	Inductive of oxidative stress	[224,261,264]
Torin 2	Ring, Gametocyte, Hypnozoite	Hemoglobin metabolism	Hemoglobin catabolism	[234]

[1] Atovaquone has limited gametocidal activity. Metabolic fingerprint resulted from 1 µM exposure for 2.5h to stage III-IV gametocytes.
[2] Exact mechanism of action is unknown.
[3] Is a pro-drug.

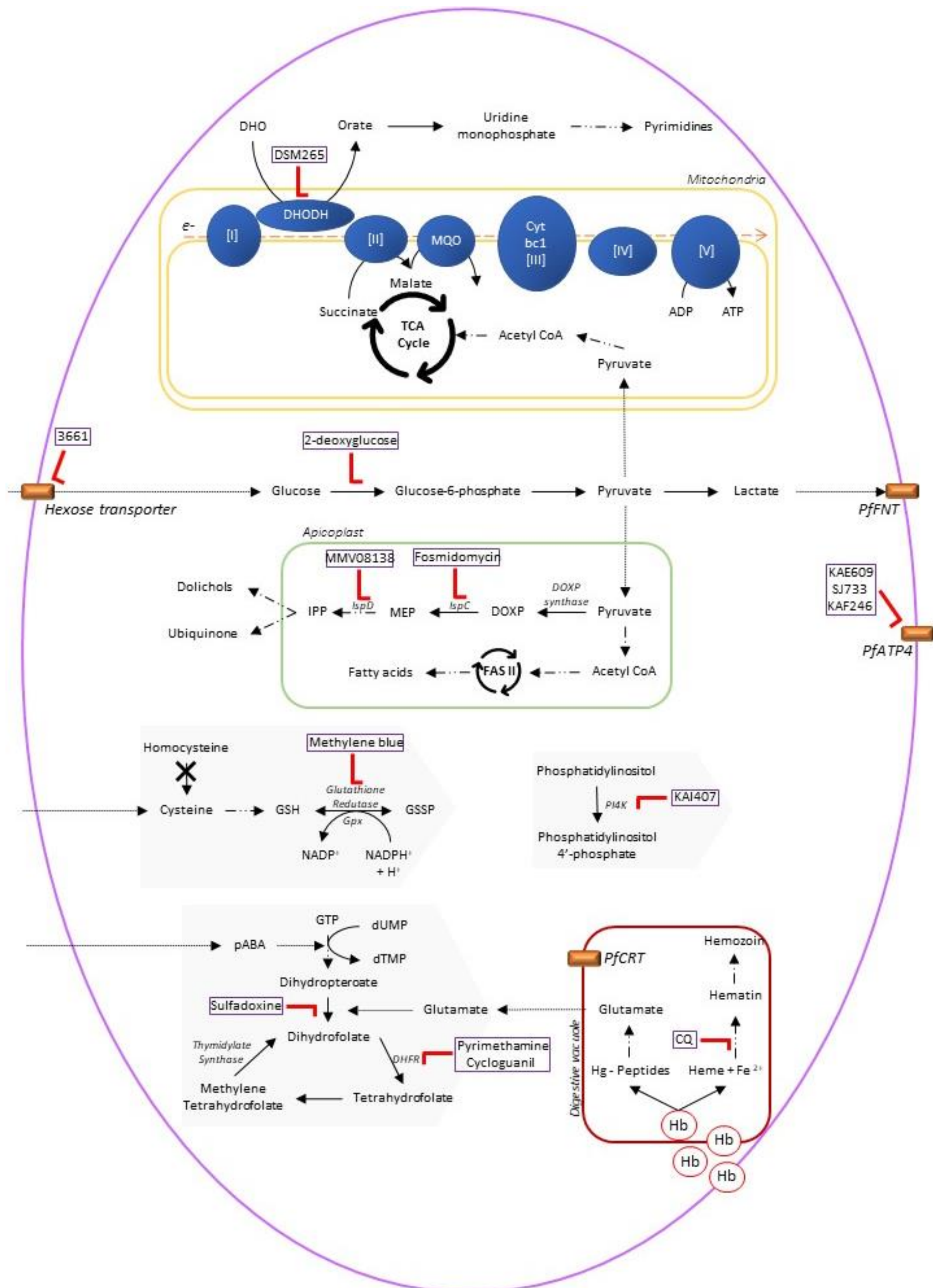

Figure 14 - Schematic view of primary and innovative metabolic targets in the asexual life stages of the human-infecting *Plasmodium* spp. Abbreviatures: CQ – Chloroquine, Cyt bc1 – cytochrome bc1, DHFR – Dihydrofolate reductase, DHO – Dihydroorotate, DHODH – Dihydroorotate dehydrogenase, DOXP – 1-deoxy-D-xylulose 5-phosphate, FAS II – fatty acid synthesis, Gpx – Glutathione peroxidase, GSH – Reduced Glutathione, GSSP – Oxidized Glutathione, Hb – Hemoglobin, Hg-Peptides – Hemoglobin-derived peptides, MQO – L-malate:quinone oxidoreductase, PfCRT – *P. falciparum* chloroquine resistance transporter, PfFNT – *P. falciparum* formate nitrite transporter, PI4K – Phosphatidylinositol 4'-kinases.

pyrimidine biosynthesis and the mitochondria Electron Transport Chain (mitETC), isoprenoid biosynthesis, fatty acid metabolism, folate biosynthesis, homeostasis and unknown.[228,230,234] These profiles are described in the next paragraphs.

Hemoglobin metabolism and protein degradation

Protein catabolism is recognized as one of the most important plasmodial metabolic pathways to target by antimalarial therapies. This stems from the fact that this genus has rudimentary amino acid biosynthesis and has adapted to obtain them from other sources.[295] The *Plasmodium* scavenges amino acids in order to fulfill its protein requirements, both in the invertebrate and vertebrate host. In the intraerythrocytic cycle, the most common sources are the RBC hemoglobin and the importation of free amino acids from the host.[224,239] These metabolites are in turn used by the parasite for various purposes, like signaling, replication and survival.

This pathway can be disturbed at multiple points: during hemoglobin uptake, catabolism or detoxification; amino acid transport; DV homeostasis or its enzymes.[239] When antimalarials interfere with these processes, their metabolomic profile shows variations of hemoglobin-derived peptides, such as prolyl-glutamate, prolyl-aspartate, prolyl-glutamyl-glutamate and aspartyl-leucyl-histidine, and amino acids.[234] One example is leucine, whose efflux out of the iRBC is well described and is considered an indirect measurement of hemoglobin uptake and catabolism. This is mostly because uninfected RBC amino acid efflux is negligible and hemoglobin amino acid composition directly correlates to amino acid relative efflux rates.[296] Further omics are able to detect modifications regarding hemoglobin, heme, hemozoin and intermediary peptides.[224,228,230,234,239]

Because these essential processes are non-concomitant with the host metabolism, they represent an effective target against the malaria parasite. Indeed, many antimalarial therapies are based on drugs whose main MoA is the interference with this metabolic pathway, as displayed in Table 12.[228,230,239,261] One of the most known is the 4-aminoquinoline class, such as chloroquine, that is a recognized inhibitor of the heme biomineralization pathway. Chloroquine is a diprotic weak base that is accumulated in the DV, whose pH differential with the parasitic cytoplasm assures drug retention.[219] Resistance to chloroquine is well described and occurs through the *P. falciparum* chloroquine resistance transporter (PfCRT).[247] PfCRT is a membrane pump responsible for multiple activities, such as the regulation of hemoglobin metabolism and ionic balance of the DV.[219,227] In resistant parasites, it is also responsible for drug efflux, effectively reducing its amount inside the DV, preventing its activity.[247,296] Many metabolomic assays explored the profile of chloroquine, either as a reference or as a focus. For example, a ^{1}HNMR spectroscopy assay by Elmi *et al.* evaluated the antiparasitic effect of a nanocomposite, a dendrimer chloroquine-curcumin conjugate, on the metabolism of *P. falciparum*

asexual ring stage.[249] This study found that the nanocomposite improved chloroquine and curcumin's individual activity, possibly due to synergy, and that metabolites from the glyoxylate and dicarboxylate metabolism were the most affected in parasites treated with the nanoparticle.[249] This was proven to be significantly different from the metabolism changes induced by chloroquine alone, or the control group, evidencing venues of novel targets.[249] Another example is the study by Na *et al.* that sought to characterize the mechanisms underlaying chloroquine resistance at a metabolic level by comparing the signatures of chloroquine-resistant and sensitive strains after chloroquine treatment.[250] Phenotypes between strains pointed to multiple factors of chloroquine resistance. Reduced levels of glutathione and its precursors pointed to oxidative stress in resistant strains.[250] Additionally, depletion of fructose-1,6-bisphosphate and alanine could indicate induction of glycolysis to compensate the increased consumption of inosine monophosphate, necessary for replication, in resistant strains.[250] In addition to changes in phenylalanine metabolism, these variations from the chloroquine-sensitive strain profile could be indicative of metabolic changes that assure fitness under resistance conditions.

Another drug class example is the aryl amino-alcohol group of antimalarials, which include lumefantrine and mefloquine. These two drugs do not act through the same mechanism. While lumefantrine is thought to interfere with hemoglobin uptake, mefloquine is thought to be pleiotropic and its main MoA is still a topic of discussion.[224,239,296,297] Initially, while trying to elicit resistance to mefloquine through selective pressure *in vitro*, it became evident that its primary resistance factor is the *P. falciparum* multidrug resistance 1 (PfMDR1) that encodes the Pgh-1 protein, a DV membrane transporter that is originally responsible for solute influx.[224,239,297] Subsequently, studies have suggested mefloquine's MoA is similar to chloroquine or lumefantrine, albeit with lesser activity. Metabolomic studies profiled these antimalarials and showed that lumefantrine clustered with GNF-Pf-5660, a known hemoglobin uptake inhibitor that does not affect hemozoin detoxification, while separating greatly from mefloquine's profile.[239] The former dissociated also from chloroquine and other hemoglobin catabolism inhibitors, indicating that its overall MoA is in fact different.[239] Metabolomic profiles also evidence stage peak activity: lumefantrine and chloroquine are active in both ring and trophozoite stages equally, whereas mefloquine peaks in trophozoite only, further distinguishing their targets.[239] It is believed that mefloquine's cytosolic target may be the Pf80S ribosome, where the direct binding inhibits protein synthesis.[297] Despite different MoA, both lumefantrine and mefloquine are affected by the *Pfmdr1*.[298,299] This, however, suggests a level of adaptation that confers indiscriminate protection to multiple drugs, not specific resistance, as amodiaquine and artemisinin derivatives efficacy is also affected by this gene.[239] Curiously, chloroquine resistance is not associated with this transporter, which confirms the distinguished metabolomic profile found in multiple studies between these drugs.[224,239,296,298,299] Dihydroartemisinin,

the active metabolite of artemisinin derivatives, is also thought to affect hemoglobin metabolism as one of its pleiotropic ways.[224,239] This occurs upon activation with heme-bound iron that induces the formation of cytotoxic radicals.[217,219] In untargeted metabolomic studies, however, it has become evident that this is not its main MoA, as its profile diverges greatly from those of chloroquine, lumefantrine or mefloquine.[239]

In spite of the existing widespread resistance to this group of antimalarials, hemoglobin digestion and amino acid and protein biosynthesis are still interesting targets for new drugs. The reason being multiple opportunities are present within this pathway while being exempt of PfCRT or *Pfmdr1* interference. In this context, one MoA that has gained attention is the phosphatidylinositol phosphate pathway. It includes three ubiquitous kinases that phosphorylate lipids at different positions with the purpose of regulating cellular processes, such as survival, proliferation, signaling and membrane trafficking.[300] In the case of the *Plasmodium*, despite initial uncertainty regarding their presence, two have been validated as drug targets through metabolomic studies: phosphatidylinositol 3'-kinases (PI3Ks) and phosphatidylinositol 4'-kinases (PI4Ks).[300] PI4K was validated as the target for imidazopyrazines, such as KAI407, while PI3K was described as the target of Torin 2.[239,301] KAI407 was shown to act selectively during the late-trophozoite to schizont stages, while exposure from early-rings up to 24h showed no loss of parasitic viability.[239] In fact, exposure between 34h to 44h blocked parasitic reinvasion and left an accumulation of multinucleated schizonts.[301] Although this kinase is present throughout the entire intraerythrocytic cycle, the consequences of the depletion of phosphatidylinositol-4-phosphate pools may become only apparent with merozoite formation, justifying why KAI407 peak activity occurs at this time.[239] Interestingly, in the metabolomic study by *Murithi et al.*, KAI407 fingerprint clustered alongside lumefantrine and other hemoglobin catabolism interfering drugs.[239] This evidences the interference at the hemoglobin-derived amino acid level that is perceived in a phenotype of disorganized membrane segmentation, and leads to defective merozoites.[301] Torin 2 was developed as an inhibitor of the mammalian target of rapamycin (mTOR), a protein kinase of the PI3K family.[234] This compound is reported to be active throughout all human parasitic stages, albeit without certainty of its MoA, since the mTOR was never described in the *Plasmodium*. Studies have demonstrated that Torin 2 interacts with various parasitic proteins, one of which is a putative nutrient transporter present in the asexual stage in the DV and parasite plasma membrane.[234] This interaction is pertinent because Torin 2 metabolomic fingerprint showed a rapid decrease of hemoglobin-derived dipeptides and tripeptides as soon as within 1 hour of treatment, maintaining this tendency at every time point over the 6h study.[234] Torin 2 may be pleiotropic, but considering the low fold-change of other metabolites, it is likely that it targets hemoglobin catabolism through this putative transporter. Lastly, JPC-3210 is a 2-aminomethylphenol synthesized from the

nonquinoline chlorophenylphenol WR 194,965.[302] Despite optimization, its MoA was uncertain and a multi-omics study was conducted to determine it. Untargeted metabolomics portrayed significant depletion of hemoglobin-derived peptides within 1h of exposure, clustering the profiles of JPC-3210, mefloquine and dihydroartemisinin together.[224] Both long and short-chained peptides were found, suggesting early digestion interference, like hemoglobin uptake or DV changes, rather than heme detoxification, as evidenced by the differential metabolomic profile in regards to chloroquine and other 4-aminoquinolones. Additionally, JPC-3210 proved active against mefloquine-resistant *P. falciparum* strains, suggesting that its activity is independent from PfMDR1-associaded transporter and cross-resistance is unlikely. This compound's fast activity and promising pharmacokinetics have made it a lead for future preclinical studies. Globally, new antimalarials that target this pathway remain relevant, if proven to bypass resistance mechanisms, be fast-acting and target innovative mechanisms. The PI3K[303] or the PI4K[304,305] are good examples that are currently being purified and studied in order to develop assays to easily evaluate anti-enzymatic activity, with some inhibitors having reached clinical development (further reviewed by *Arendse et al.*[306]).[307]

Pyrimidine biosynthesis and the mitETC

Similarly to the DV, another structure that is absent in the RBC but is crucial to the malaria parasite is the mitochondria. Despite its presence in other human cells, the parasitic mitochondria possess exclusive and essential enzymes and structures that ascertain a tangible difference and, thus, a target opportunity for antimalarial drugs.[232,308] Indeed, the RBC depends exclusively on glycolysis for ATP production, whereas the *Plasmodium* utilizes both the glycolysis and the TCA cycle, to different extents depending on the life stage. Irrespectively of the energetic pathway, the mitochondria has other functions: the mitETC is intimately involved in pyrimidine biosynthesis.[243] It is in the mitochondria inner membrane that five dehydrogenases are present and their re-oxidation is maintained by the ETC, assuring their availability for RNA/DNA synthesis. Thus, the mitochondrial membrane potential is maintained through the TCA cycle in order to produce essential pyrimidines and ATP.[239]

The redox balance and membrane potential are partially sustained by two metabolites: pyruvate and glutamate. Pyruvate is diverted from glycolysis and enters the TCA cycle by, for example, converting to acetyl CoA (see Figure 14).[309] Glutamate enters the TCA cycle as 2-oxoglutarate and it is synthesized from glutamine, obtained from hemoglobin digestion. Alternatively, glutamate can be further transformed to γ-aminobutyric acid (GABA).[243] GABA represents a significant metabolic difference between *Plasmodium* and RBC, as it is only present in iRBC. This is due to the incomplete GABA shunt in the *Plasmodium* that, contrarily to Apicomplexan *Toxoplasma gondii*, lacks the enzymes to reroute GABA back to the TCA cycle, leading to its accumulation.[232,243,277] Its high levels in malaria patients could make it a viable marker of infection, and its effects as an inhibitory neurotransmitter are thought to

be involved in cerebral malaria pathology.[232] In metabolomic studies, GABA and other amino acids can be used to evaluate disturbances in the mitETC and establish drug profiles.[230]

The TCA cycle interacts with the mitETC through two of the five membrane dehydrogenases, succinate:quinone reductase (SQR or complex II) and *L*-malate:quinone oxidoreductase (MQO) – see Figure 14.[310] Of this chain, complex III, MQO and DHODH are known to be essential for intraerythrocytic parasite survival.[310] Interestingly, mitochondrial ATP production is non-essential at this parasitic stage, accentuating the involvement of the mitETC in other key biological processes.[243]

Atovaquone is the hydroxynaphthoquinone component in Malarone® and is a known inhibitor of the complex III used in combination with proguanil.[224,310] Though resistance is well described through single mutations in the *cytB* gene, it is still used in association.[224,239]

As the mitETC is considered a valuable antimalarial target, other molecules are currently being developed, such as DSM265. DSM265 is a triazolopyrimidine-based inhibitor of DHODH, the enzyme of the forth step in the pyrimidine *de novo* biosynthesis, hence blocking DNA and RNA synthesis.[311] Clinical data confirmed patient safety and parasitic clearance with a single-dose regiment, but revealed a subset of *P. falciparum* infection recurrence due to a single-point mutation in the *Pfdhodh* gene.[311] *In-vitro* assays confirmed this mutation protected against DSM265 treatment, further ascertaining, as with atovaquone, the necessity to use combination therapies.[227,311] In addition to this factor, metabolomic studies have shown that atovaquone and DSM265 are active in intraerythrocytic asexual stages only against late trophozoites, further limiting its use in monotherapy.[239,311] This life-stage specificity is predictable with the DNA synthesis peak before proceeding to replication.[239,243]

The metabolomic profile of antimalarials that target the mitETC is characterized by higher levels of pyrimidine precursors, dihydroorotate and N-carbamoyl-L-aspartate most notably, among other precursors of the TCA cycle.[224,228,233,308,310] This is true for both complex III and DHODH inhibitors, as cytochrome bc1 complex inhibition indirectly inhibits the DHODH because of the interference with ubiquinol oxidation.[224,230] Hence, in this case, atovaquone and DSM265 inhibit two different targets of the same chain, but present the same metabolomic profile.[239]

Interestingly, it is recognized that the mitochondria are active differently across different parasitic stages. These differences are aligned with their requirements, as the asexual forms depend mostly on glycolysis for energy, whereas gametocytes, having limited access to glucose, namely in hypoglycemic malaria patients and in the mosquito hemolymph, rely more on mitochondrial ATP production.[243,263,277] Metabolomic assays have demonstrated the differences in the TCA cycle substrate between sexual and asexual stages, revealing that inhibition of this cycle in the asexual stages does not affect viability, whereas in the gametocyte it arrested development and killed the transmissible parasite.[277] *In vitro*,

DSM265 did not show gametocidal activity, contrary to blood and liver-stage activity, which is in line with the drug's effect prior to schizogony.[312] Atovaquone has demonstrated gametocidal effects *in vitro* at 1 µM, while being known for not possessing gametocidal activity *in vivo*.[228,261] Since gametocytes are dependent on TCA cycle ATP production, particularly in early to mid-stage gametocytes, drugs that target this pathway could be promising antimalarials and should be metabolically investigated further.[239,243,263]

Isoprenoid biosynthesis

Another organelle that differentiates the parasite from the host cells is the apicoplast.[235,244,286,290,294] Specific to the Apicomplexan, and hence the filo name, it is responsible for various metabolic functions. The sole essential metabolites to be produced in the apicoplast are the isoprenoid precursor isopentenyl pyrophosphate (IPP) and its isomer dimethyallyl pyrophosphate (DMAPP). These are synthetized through the 7-enzyme Methylerythritol Phosphate (MEP) pathway, essential in the blood-stage malaria infection, as proven by recovery through the presence of IPP.[235,244,258,290] IPP and DMAPP can be further transformed through farnesyl/geranylgeranyl diphosphate synthase to longer prenyl chains, as happens with ubiquinone, cyclized/conjugated by a variety of prenyltransferases, or originate *cis*-polyisoprenoids in the cytosol.[258,290] These metabolites are vital for the posttranslational modification of proteins in the *Plasmodium* and are involved in many cellular tasks, including signaling, replication and in membrane structures.[235,244,251,286,290,294]

Isoprenoid biosynthesis is not limited to Apicomplexan, it also exists in bacteria. As such, repurposed antibiotics with antiplasmodial activity mostly target this process. Examples of antibiotics are doxycycline, clindamycin and fosmidomycin.[234,235,239,244,290,294]. Human cells also synthetize isoprenoids through a different pathway. However, homology exists between certain human and parasitic enzymes of this pathway.[290,294] Consequently, toxicity assays are important to verify cross-activity and it is recognized that broad inhibition of these system's enzymes could lead to toxicity in patients.[290]

Plasmodial isoprenoid biosynthesis can be targeted at multiple points and it has been linked with disturbances at multiple cellular pathways.[290] All enzymes of the MEP pathway can be potential targets, as is the case for the IspC and IspD, targeted by fosmidomycin and MMV08138, respectively. Inhibition of the MEP is characterized by diminished levels of isoprenoid precursors, 1-deoxy-D-xylulose-5-phosphate and 2-C-methyl-erythritol-2,4-cyclodiphosphate and downstream compounds.[235,244] Precisely due to the branching of isoprenoid uses, downstream metabolic effects can also be affected, *e.g.* in variations of hemoglobin-derived peptides and amino acids, nucleotides and lipids.[244]

The antibiotics doxycycline and clindamycin are known to inhibit prokaryotic transcription and translation in bacteria. They also interfere with the plasmodial apicoplast genome, inhibiting protein synthesis, thus being active antimalarials.[294] Because they present the "delayed death" phenotype, they are used in combination therapies as slow acting partner drugs. The "delayed death" signature happens when parasitic death is only achieved by the second lifecycle.[235,286] Fosmidomycin acts by inhibiting the IspC of the MEP, bypassing the "delayed death" profile.[286] Despite immediate onset, with sudden arrest in IPP biosynthesis, it has a short half-life and, consequently, high recrudescence rates when used in monotherapy.[286] Despite acting in the same life stage, the trophozoite, when most prenylation reactions occur, fosmidomycin acts immediately in blocking this process, while delayed-death drugs lead to gradual depletion of IPP and eventual loss of prenylation.[235,290,294] This is thought to lead to the transmission of defective apicoplast to the merozoites during replication, which leads to unviable parasites in subsequent cycles.[235,244,286]

Metabolically speaking, when these drugs are used, there are no significant changes in the first lifecycle because the parasite is able to adapt its metabolism to compensate the isoprenoids deficit and successfully complete its lifecycle.[235,294] A multiple omics study by Tewari *et al.* looked into what compensation mechanisms the *P. falciparum* used to assure survival when treated with 1 µM of fosmidomycin, its half-inhibitory concentration (IC_{50}).[286] The study revealed adjustments through the polyamine metabolism in which enhanced purine-recycling and decreased phosphatidylcholine metabolism let the parasite maintain synthesis of purine nucleotides by suppressing phospholipid synthesis.[286] This had already been hinted by the Swift *et al.* metabolomic study, where variations in lipid and sphingolipid metabolism would suggest a linked compensation mechanism for the loss of the apicoplast.[244]

Resistance to fosmidomycin has been described *in vitro* by metabolomics as an enzymatic amplification that induces the parasitic glycolysis flux and, consequently, the metabolite intermediates available for the isoprenoid biosynthesis pathway. As precursors become increasingly available, the inhibition of IspC by fosmidomycin becomes less important and the MEP isn't totally arrested.[229,290]

Innovative compounds that target isoprenoid biosynthesis are still pertinent, seeing the multitude of target options available that would be parasite-specific. However, compounds would have to present faster killing effectivity and longer half-lives.[290] Kennedy *et al.* used a metabolomic study to describe the molecular mechanism of the "delayed death" profile.[235] To do so, indolmycin, an antibiotic inhibitor of the apicoplast tryptophanyl-tRNA synthetase in *P. falciparum* was used. [235] Similarly to other antibiotics that target translational processes, it induced "delayed death" that can be rescued with IPP *in vitro*.[235] It was found that no metabolic variations occurred during the 1st intraerythrocytic

asexual lifecycle.[235] Afterwards, severe disruption of the morphology of the DV could be observed, along with diminished heamoglobin-derived peptides levels, and it was deduced aberrant haemoglobin uptake could be one of the mechanisms that kills the parasites.[235] Additionally, isoprenoid precursors and downstream species levels decreased, consistent with a defective apicoplast that does not produce isoprenoids.[235] Promising compounds like MMV-08138 have been studied, but showed a similar profile.[294] Omics research that elucidates MoA of promising isoprenoid biosynthesis inhibitors could prove beneficial for the development of innovative antimalarial therapies.[290] The apicoplast is a necessary structure throughout the intraerythrocytic lifecycle, thus it could present important blocking transmission and chemoprophylaxis activity that is worth pursuing.[258,290,294]

Fatty acid metabolism

The *Plasmodium* apicoplast also harbors enzymes necessary for lipid metabolism, namely *de novo* Fatty type II Acid Synthesis (FASII) and elongation.[232,235,243] Despite the similarities in this pathway with the host cells', structural differences between eukaryote and prokaryote enzymes make this pathway noteworthy as a potential target.[289] Fatty acids, phospholipids and lipids are required at multiple *Plasmodium* lifecycle points for proliferation, protein trafficking, hemoglobin degradation, signaling and host interaction.[221,243,253,263,289,313] Phosphoenolpyruvate is a precursor of the TCA cycle, glycolysis and fatty acid synthesis. In the latter case, it is transported into the apicoplast where it is transformed into pyruvate by pyruvate kinase 2 and into acetyl CoA by pyruvate dehydrogenase.[243] Acetyl CoA is the precursor for FASII synthesis that will generate the key building blocks of phosphatidylcholine, phosphoethanolamine, or phosphatidylinositol 4-phosphate, involved in signaling and in membrane structures of the vacuolar system and development of merozoites.[253] Parallelly, the parasite can scavenge fatty acids from the serum to be incorporated into parasitic membranes by modifying them through elongases and desaturases, thus optimally adapting its resources.[253,313] Interestingly, lipidic metabolites have been linked to various host interactions that are known to be relevant players in malaria pathology. Specifically, the metabolomics study by Beri *et al.* demonstrated how the lipid metabolism of the *P. falciparum* is deeply intertwined with the host, going as far as interfering with RBC deformability.[232] Accumulation of membrane precursors/degradation metabolites (choline, ethanolamine and glycerol 3-phosphate) and decrease of choline-containing lysolipids were consistent with RBC membrane rigidity and composition changes caused by the *Plasmodium* infection.[232] Additionally, a more recent metabolomics assay by Tewari *et al.* revealed the increase of eicosanoid-signaling precursors in iRBC, which once released can modulate inflammation, further confirming the role of lipids in host immune-modulation.[221]

This pathway is not essential for all *Plasmodium* species or life stages. Some species, like *P. berghei* or *P. lophurae*, are known to scavenge CoA from the host cells during the asexual stages, while *P. falciparum*, being dependent on mature RBC, lacks external supplies and is instead dependent on *de novo* synthesis in the apicoplast and mitochondria.[243] Debate exists on the extent to which the *P. falciparum* scavenges fatty acids from the host, while parallelly synthesizing them *de novo.* A lipidomic study by Gulati *et al*. analyzed 304 lipids found in *P. falciparum* blood stages and in non-infected and iRBC.[253] The study used GW4869, an inhibitor of parasitic ceramide production, that failed to achieve 100% growth inhibition *in vitro*, even at 170 times its IC_{50}.[253] Since the RBC possesses up to 10 times the parasite's level of ceramide, survival could be due to scavenging it from the host, revealing that even though GW4869 has potent antiplasmodial activity *in vitro* (IC_{50} of 6 nM), the inhibition of the hydrolysis of sphingomyelin is not unsurmountable for the parasite.[253] In the same study, Orlistat, a triacylglycerol lipase inhibitor, revealed a lower IC_{50} (0.9 µM), but a better profile as antimalarial, as it targets the metabolic pathway of a lipid that cannot be scavenged without cannibalizing the RBC.[253] Likewise, in this study, phosphatidylglycerol, acyl phosphatidylglycerol, lysophosphatidylinositol, bis(monoacylglycero)phosphate, monosialodihexosyl-ganglioside, diacylglycerol were found to also be unsalvageable and to require likely *de novo* synthesis.[253] These results show that some pathways might, hence, be promising targets for antimalarial therapies, while others could be bypassed through host metabolite importation. Consequently, it is theorized that blood stages are not as reliant on general fatty acid synthesis and this could prove relevant when developing inhibitors of this pathway.[313] Another way to affect the asexual blood stage could be through preventing merozoite invasion, which is theorized to be possible if the membrane lipid composition is affected.[314]

Concerning the gametocyte stage, the same lipidomics analysis by Gulati *et al*. showed that the lipid environment can induce gametocytogenesis.[253] Supplementation with serine, that increases levels of phospholipids and sphingolipids, and N,N-dimethyl-sphingosine, that decreases ceramide catabolism, were shown to augment gametocytemia.[253] A previous ^{1}HNMR-based lipidomics study by Lamour *et al.* had already hinted at the correlation between depletion of lipid moieties and gametocyte maturation, and the same study by Tewari *et al*. referred above also denoted the high abundance of polyunsaturated fatty acids in the iRBC promoted gametocytogenesis.[221,263] These results indicate that lipids play a part in signaling the gametocytogenesis commitment and development and could, hence, be used to block transmission.

Contrary to the blood stages, mosquito stages are known to be reliant on fatty acid synthesis. A metabolomic and genomic study by Srivastava *et al.* gave insight on this theory by studying both *in vitro* and *in vivo P. berghei* carbon metabolism.[243] Indeed, the carbon metabolism was proven essential in the mosquito stages, that showed high sensitivity to variations in the TCA cycle, CoA synthesis and

glutamine catabolism.[243] Additionally, FASII was shown to be essential for complete sporogony of *P. Falciparum* in the mosquito midgut.[243,315] This points to the important role of inhibitors of the FASII or of the CoA synthesis in blocking transmission.

Although no traditional antimalarials directly affect this metabolism, it is possible it is disturbed indirectly through the disruption of the apicoplast or the glycolysis pathway.[235,243] Promising compounds that target the plasmodium lipid metabolism have been metabolomically investigated. A study by Creek *et al.* aimed to determine the MoA of 100 antimalarial compounds from the Malaria Box collection through untargeted metabolomics in *P. falciparum* iRBC *in vitro*.[230] In this study, three compounds were found to disturb unique aspects of parasite fatty acid metabolism: C7, E4 and P2_A3. C7 showed a 10-fold depletion of traumatic acid, a product of the plant-like α-linolenic acid pathway whose role in the *Plasmodium* parasite remains unknown.[230] Still, C7 (IC_{50} of 448 nM) had a unique signature, with no changes in α-linolenic acid precursors or other end products, proving there might be merit in targeting this metabolic branch.[230,316] E4 also diminished traumatic acid levels, but to a lesser degree, although with a promising IC_{50} (215.5 nM).[230] P2_A3 induced a decrease of linoleic acid specifically and uniquely, with no other changes.[230] Considering linoleic acid is an upstream precursor of the α-linolenic acid metabolism pathway, its MoA could be an indirect effect, *e.g.* in fatty acid uptake, since *P. falciparum* might not synthesize linoleic acid.[230,316] Overall, the three compounds point to the α-linolenic acid pathway as a potential antimalarial target. Another metabolomic assay by Yoo *et al.* sought to study Sal A, a natural compound produced by a *Salinispora* sp. bacterium with a promising IC_{50} (50 nM) against *P. falciparum* and a selectivity index > 1000 over many mammalian cell lines.[289] Accumulation of acylglyceride metabolites, and profile resemblance with Orlistat, revealed that Sal A inhibits α/β serine hydrolases, which lead to incomplete merozoite formation and failure in egression from iRBC.[289] Overall, these three compounds revealed metabolomic profiles specific of fatty acid metabolism disruption, including traumatic acid, linoleate, lysophospholipids, sphingolipids, glyceroplipids, monoacylglycerol and others, as referred thus far. However, it cannot be discarded that compounds targeting this pathway may show changes at other levels, like the TCA cycle, central carbon metabolism, glycolysis and even hemoglobin catabolism.[221,230,232,243].

Folate biosynthesis

Folate biosynthesis is an essential pathway for any replicating biological system, as it is directly responsible for the production of purines. For the *Plasmodium* this need is even higher, as the parasites undergo schizogony every 48h or 72h. In order to meet such demands, this genus adapted to synthetize folate metabolites *de novo*. To do so, two metabolites are key: *p*-aminobenzoic acid (pABA), which can be obtained through the shikimate pathway or by importing it from the host, and glutamate, diverted from hemoglobin catabolism or the host, as described in Figure 14.[217,254,317] Folate

biosynthesis can be described in three major axes: the generation of pteridine metabolites, the building blocks of purine metabolism; the synthesis of dihydrofolate and the pyrimidine synthesis cycle.[254] The first axis is the chain conversion of GTP to hydroxymethyl dihydropteridine pyrophosphate, which relies on multiple enzymes. The second axis depends on pABA and hydroxymethyl dihydropteridine pyrophosphate components to be joined into dihydropteroate, which will then be converted to dihydrofolate, dependent on glutamate. Finally, the third axis involves the cycle that converts dihydrofolate to tetrahydrofolate, to methylene tetrahydrofolate and back to dihydrofolate. All reactions of this pathway are catalyzed by enzymatic complexes passible of interreference by drugs that would effectively block parasite replication.[254]

Several antimalarials act as inhibitors of this pathway. Sulfonamides, as sulfadoxine, are analogs of pABA, effectively blocking the second axis.[219,239,254] Pyrimethamine and cycloguanil are known inhibitors of the dihydrofolate reductase-thymidylate synthase (DHFR-TS).[228,234] DHFR-TS is an important complex of the third axis that mediates the folate-dependent conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP), while using NADPH as a cofactor.[228] Other drugs are known to possess antimalarial activity through this MoA, such as the antibiotic trimethoprim, WR99210 and methotrexate, a structural analog of folate.[219,228,254] Folate antagonists might interfere at other stages, like preventing the formation of dihydropteroate or of dihydrofolate. Unfortunately, resistance to antifolates by the *Plasmodium* is well described in the field after accumulation of point mutations.[219,227,318] Parallelly, it is possible that the parasite upregulates genes that increase the flux of upstream substrates, which make downstream substrates more abundant, thus counteracting enzymatic inhibition. This has been reported for the GTP-cyclohydrolase, the first enzyme of the folate biosynthesis pathway, for example.[219] Still, cycloguanil derivatives have shown that structural modifications are able to bypass antifolate drug transmission on the basis that the parasite is limited to maintain a functional DHFR-TS.[318]

A metabolomics screening by Allman *et al.* successfully demonstrated the clustering of antifolate drugs and reported on their profiles.[228] DHFR-TS inhibitors showed increased dUMP and NADPH as a unique signature, which also clustered compounds P218 and MMV667487.[228] This profile also displayed decreased downstream intermediates, dTMP and deoxythymidine triphosphate.[228,234] In a multi-omics study, genetic deletion of a parasitic membrane transport in *P. falciparum* blood-stage schizont allowed for the identification of resistant factors through untargeted metabolomics. It was described that depletion of folate created parasites resistant to methotrexate, while maintaining sensitivity to pyrimethamine.[254] This assay successfully demonstrated that this metabolic pathway is reliant on more structures besides the DHFR-TS, like specific membrane transporters, for which new antimalarials may be developed. Disruption of this biosynthesis affects multiple intraerythrocytic stages, although the

most active in DNA synthesis are the trophozoite late stages and during schizogony, which is successfully blocked by this class of compounds.[254] Other studies are underway to determine what key targets could make antifolates potential antimalarials, as this pathway still offers multiple specific inhibition targets.

Homeostasis

The *Plasmodium*, as an obligatory intracellular parasite, faces the challenge of maintaining homeostasis.[232,246,265] At one level, the parasite introduces morphological and chemical changes in the iRBC and has to ascertain its integrity, including membrane potential, osmolarity, free radicals and pH. On a second level, the parasite itself goes through multiple changes throughout its lifecycle, imposing other threats to this fragile system.[246] Thus, the parasite needs powerful systems to assure homeostasis. Amongst them is a potent antioxidant system that relies on superoxide dismutase, thioredoxin and glutathione. Additionally, by alternating between scavenging and *de novo* synthesis, while maintaining redundancy of some metabolites and metabolic pathways, the *Plasmodium* ascertains a wide safety net for its development.[230,232,265,319,320] These systems are robust and known to be powerful in countering antimalarial effectivity, alongside point mutations and other resistance factors.

All metabolic pathways are intertwined and co-dependent in a complex network. For example, pyruvate from glycolysis can be fermented into lactate directly, enter the TCA cycle in the mitochondria, or be imported into the apicoplast to serve as the precursor of isoprenoid or fatty acid biosynthesis. Hence, when a metabolic pathway is overwhelmingly stressed, and the parasite cannot compensate it, a cascade that affects multiple systems will ensue.[232,234] For example, a disruption of the apicoplast can lead to changes in lipidic and isoprenoid metabolites, but it can go as far as disturbing hemoglobin uptake and digestion, a process that occurs in an altogether different section of the parasite. In addition to the problems that have been discussed so far, such a disruption would precipitate osmotic difficulties, which speed parasite death.[235,239,247] This death cascade can be perceived as a general metabolic shut down and pleiotropic antimalarials often arise this kind of profile in omic studies. Various metabolomic assays analyzed the profile of antimalarials with this kind of MoA, such as dihydroartemisinin, primaquine and tafenoquine.[228]

Dihydroartemisinin, the active metabolite of artemisinin, is a potent pleiotropic antimalarial, whose unconventional resistance mechanism forced a reform on how antimalarials were used in therapy.[227,234,238,321] It is thought to be activated upon endoperoxide bridge opening, possibly in the DV by hemoglobin-derived Fe^{2+}-heme, therefore releasing free radicals.[242] These will interact mainly with proteins, but its oxidative damage has other ramifications, causing lipid peroxidation and maybe

depolarization of the mitochondrial and plasma membranes, thus altering homeostasis in multiple fronts.[219,227,234,238,321] Artemisinin resistance is a known example of how omics studies successfully uncovered both the resistance mechanism and the MoA that had eluded the scientific community for years. This resistance phenotype differs from others, in which the parasites tolerate high drug concentrations and instead show development deviations.[23] Several characteristics are now known to be associated with this resistance: *Pf*Kelch13 mutations, the dominant determinant of resistance; redox detoxification and early-ring dormancy.[23,242] Omics helped establish the *Pf*Kelch13 gene, and subsequent effects, as the artemisinin-resistance factor and demonstrated how changes in protein folding disable an otherwise potent antimalarial.[238,321] Specifically, untargeted metabolomic studies successfully identified the decrease of the *Pf*Kelch13 protein and hemoglobin-related peptides, and the increase of glutathione and gamma-glutamylcysteine. These variations confirm these metabolites as biomarkers of resistance and dihydroartemisinin's link with heme activation and oxidative damage.[238] A genomic study confirmed the role of a parasite endoplasmic reticulum enzyme, the *Plasmodium* eIF2α Kinase PK4, that begins the cascade of latency and leads to recrudescence post artemisinin treatment.[322] This assay found that inhibition of this enzyme successfully blocks this cascade and assures artemisinin treatment, giving leads to partner drugs to use alongside artemisinin-derivatives.[321,322] Additionally, another study identified PI3Ks as an artemisinin target and found lipidic product phosphatidylinositol-3-phosphate levels to increase in resistant strains.[323] Further omics studies demonstrated that this metabolite is involved in a vesicular system that neutralizes artemisinin interactions with the proteome and ensues modifications that increment resistance and enable recrudescence.[227,324] *In silico* models based on experimental data were used to predict metabolic differences between artemisinin-resistant and sensitive parasites.[325] This study pointed to unique genes differentiating pyrimidine and folate biosynthesis and mitochondrial reactions in resistant parasites, beside other changes mentioned thus far.[325] More recently, a study focused on integrating multiple omic tools to ascertain the intricate mechanisms through which the Plasmodium resists artemisinin treatment. This ambitious assay by Mok *et al.* discovered new functionalities of the *PfKelch13* gene and validated others suggested thus far.[242,326] Notably, the *PfKelch13* gene was shown to interfere with pyruvate and glutamate-linked carbon metabolism, with alterations in glycolysis/gluconeogenesis, the TCA cycle and purine metabolism.[242] Specifically, the low levels of malate and 2-ketoglutarate, TCA intermediates, points to ring-stages that mimic conditions of anoxia and lower the mitETC complex III activity.[242] Additionally, variations in phosphoenolpyruvate, glutamine synthetase and NADP-specific glutamate dehydrogenase reveal resistant parasites that rely more on reverse glutaminolysis.[242] This energy rewiring might be involved in the recovery after treatment, as had been suggested before.[242,327] Parallelly, the *PfKelch13* showed to facilitate this energetic shift while augmenting the proteostatic capacity of the resistant parasite by facilitating the

elimination of damaged proteins.[242] Generally, resistant parasites are predicted to display higher metabolic flexibility, but metabolomic assays should be performed to confirm this.

Primaquine and tafenoquine are prodrugs metabolized by the hepatocytic CYP2D6, which awards them hepatic schizonticide activity.[261,268] Their exact MoA is unknown, but similarly to dihydroartemisinin, they are considered to induce oxidative stress.[261] Because of their metabolization and rapid onset of action, they are effective in preventing relapses, which limits their use, as the hepatic stage is asymptomatic and not all *Plasmodium sp.* create hypnozoites.[219] Primaquine is reported to be toxic in glucose-6-phospate dehydrogenase-deficient individuals and to have low drug tolerance, which further compromises its clinical utilization.[268,269]

Commonly, not only pleiotropic antimalarials can generate a general death cascade profile. This because, as discussed thus far, metabolic pathways are interconnected and if one chain link fails, the others fail too. A metabolomic study by Cobbold *et al*. assessed the MoA of front-line antimalarials, including dihydroartemisinin, atovaquone, Torin 2, and others, in order to compare their metabolomic fingerprints.[234] In it, pleiotropic drugs clearly separated from one-target mechanistic drugs. Compound 3361 is a selective inhibitor of the *P. falciparum* plasma membrane hexose transporter. Incubation with [^{13}C]-U-glucose labeling revealed disturbances in carbon metabolism.[234] Unsurprisingly, 3361 disrupted glycolysis, but not exclusively.[234,236] TCA cycle and purine alterations are probable downstream consequences of the loss of energy production and carbon skeletons.[234] Moreover, hemoglobin-derived peptides were also disturbed, demonstrating how the lack of ATP can interfere with DV acidification or transporters, which would prevent correct hemoglobin catabolism.[234] This profile was significantly different from dihydroartemisinin, that affected hemoglobin digestion within 1h of treatment, consistent with reports that drug activation occurs inside the DV.[234] Compound 3361 ultimately led to nonrecoverable parasites, indicating the likelihood of downstream damage that prevented parasite replication.[234] Similarly to 3361, 2-deoxyglucose is known to competitively inhibit the conversion from glucose to glucose-6-phosphate in glycolysis.[228,243,308] This compound has been studied extensively in multiple assays, including metabolomics, as is the case by *Allman et al.* and *Srivastava et al*. The first study pointed to a widespread metabolic collapse profile in blood-stage *P. falciparum*.[228] The second focused on finding how dependent gametes are on glycolysis, finding that *P. berghei* male gametogenesis was completely reliant on this pathway, pointing towards transmission blocking.[243] Regrettably, RBC and other cells, likewise to the parasite, rely heavily on the glycolysis pathway for energy. Hence secondary effects are expected, unless specific parasitic targets can be focused, as is the case of compound 3361, that does not inhibit glucose import to the RBC.[234,243]

Other targets can also ensue homeostasis disruption. Compounds that target the parasite formate nitrite H^+-transporter (*Pf*FNT), responsible for lactate efflux and pH maintenance, also demonstrate a profile of metabolic collapse. This inhibition leads to hemoglobin metabolism and pyrimidine biosynthesis disruption, besides arising cytosolic alkalinisation.[236] It's the MoA of two compounds, MMV007839 and MMV000972, found in the metabolomics study by Hapuarachchi *et al*.[236]

Additionally, the previous study mentioned by Allman *et al.* (2016) also included in the homeostasis cluster KAE609, (+)-SJ733 and KAF246, compounds that target the *Pf*ATP4, a Na^+/H^+-ATPase in the parasite plasma membrane that is restricted to apicomplexan parasites.[228,328] *Pf*ATP4 inhibitors prevented Na^+ efflux, that would occur in exchange of H^+ intake, disturbing the parasite's ionic gradient, pH and osmolarity.[228,328] In the same study, 2-deoxyglucose displayed a different metabolomic profile from *Pf*ATP4 inhibitors, evidencing that compounds targeting glycolysis develop a unique print.[228,243,308] Creek *et al.* goes further in this analysis, and clusters dihydroartemisinin with *Pf*ATP4 inhibitors, suggesting that this metabolomic profile derives from potent and fast-acting antimalarials that disrupt multiple metabolomic pathways simultaneously, rather than a specific target.[230] Further studies with KAE609 and others with the same MoA showed that the Na^+ uptake provokes swelling of both parasite and iRBC, increasing these cell's osmotic fragility and killing them through this mechanism.[328] KAE609 underwent one phase 2 clinical trial that ended in 2019 to adjust the dose in regards to reported hepatotoxicity, and is undergoing another to evaluate its intravenous efficacy and safety in severe malaria patients, set to end in 2024 (registered with ClinicalTrials.gov, NCT03334747 and NCT04675931, respectively).[291,329]

Lastly, peroxide antimalarials like ozonides, are known to target homeostasis systems by disrupting redox processes. A recent proteomics and targeted LC-MS-based thiol metabolomics assay by Siddiqui *et al*. sought to describe ozonides MoA.[255] To do so, the protein and thiol changes were assessed after dihydroartemisinin and artefenomel treatment, an ozonide clinical candidate.[255] Artefenomel provoked disproportionate alkylation of proteins involved in redox homeostasis, confirming that this group of antimalarials might act similarly to dihydroartemisinin.[255]

Homeostasis is a fragile balance to maintain for the blood-stages, which opens the way for pleiotropic drugs like dihydroartemisinin or *Pf*ATP4 inhibitors like KAE609 to be fast and effective antiparasitic agents. Generally, the metabolomic profile associated with homeostasis disturbance displays variances in thioredoxin reductase, glutathione precursor cysteine, oxidized glutathione and cysteine-glutathione disulfide, deoxyribonucleotides, ribonucleotides, carbon metabolites, such as glucose-6-phosphate and other glycolytic intermediates, and hemoglobin-derived peptides, demonstrating the extent of this perturbance.[228,232,234,319] Overall, the parasitic homeostasis is a fragile balance that

requires enormous amounts of energy and multiple structures to maintain it, which makes it a valuable antimalarial route. However, careful selective targeting of these systems is necessary to avoid toxicity.

Unknown and others

Lastly, the attained metabolomic profile might not have statistical sturdiness to cluster with other classes and, in that case, the MoA remains uncertain.[228,230] In antimalarial drug discovery, compounds usually start by being screened *in vitro* against *P. falciparum*, in which the IC_{50} is used to rank activity. Thus, when compounds are further explored through metabolomics and their profile is ambiguous, it shouldn't be linked to a lack of activity, but to other factors. The compound might target metabolic pathways inactive or non-essential in the tested stage, have a slow time of action, require metabolomic activation from the host or higher concentrations.[228,230] Lastly, the altered metabolites might not be detected or reproducibly quantified, which would leave gaps in the attained results.[228,230]

Alternative profiles have emerged in metabolomic studies that correlate to innovative targets that do not yet have validated clinical efficiency. Such is the case of MMV007571 and MMV020439 that were shown through untargeted metabolomics to inhibit New Permeation Pathways (NPP), along with pyrimidine synthesis and the mitETC.[233,293] Since NPP may be redundant in importing and exporting nutrients and waste products, it is difficult to link a metabolomic fingerprint to this particular pathway, more so since the compounds also interfered with other metabolomic pathways. However, NPP are recognized potential antimalarial targets, as they are induced by the parasite and important for its viability.[233,293] Another alternative profile that has emerged is associated with the acetyl CoA anabolism. This pathway has been successfully targeted by pantothenamide bioisosteres, not only in the asexual stages, but also in sexual phases, consequently blocking transmission.[262] Pantothenate is the precursor of CoA and its phosphorylation by the *P. falciparum* pantothenate kinase is the first step in CoA biosynthesis.[330] The inhibitors profile was established by detection of converted panthothenamides to CoA-panthothenamides, along with a reduction in the levels of acetyl CoA and other downstream metabolites, consistent with findings that these inhibitors are likely activated by pantothenate kinase, whose products will inhibit other downstream enzymes or reactions or behave as antimetabolites.[262,330] Preclinical characterization of the antimalarial pantothenamide MMV693183 was recently described, further ascertaining this promising antimalarial target.[331]

Many other antimalarial targets are still expected to be discovered, especially through the implementation of omics or multiomics studies in compound screenings. Although metabolomic profiling is not always easy, metabolic fluxes and interactions are reliable indicators of parasitic death mechanisms and will never fail to give a glimpse on drug's MoA.

Conclusion

Metabolomics is a comprehensive omics field that transformed and continues to innovate the way diseases and therapies are studied. In fact, its application in the malaria field has enabled the characterization of both established and potential antimalarials, thus contributing to a rational drug design. By providing an in-depth view of the metabolic reactions of the asexual parasite when exposed to antimalarials, accurate and interesting phenotypic profiles have been discovered that will contribute to the development of innovative antimalarials. More precisely, extraction with a proportion of methanol:water analyzed through LC-MS seems to be most recurring method towards antimalarial drug discovery, as revealed by pioneer studies. [224,235,236,239,262] However, metabolomics inherent complexity exposes the lengthy path ahead before it can become a routine technique. The *Plasmodium* poses obstacles in the variety of host cells it parasitizes across multiple life stages, and the broad metabolic interactions and modifications intrinsic to each of them. It would be interesting if omic sciences could be broadly implemented to study all life stages and other species, such as *P. vivax.* This would certainly shed light on stage and species specificity, thus predicting differential antimalarial activity and guiding drug development successfully.

Despite the increasing number of assays in the field, standardization of experimental procedures and data processing and analysis are lacking. This, in turn, compromises study's reproducibility and interpretation, leading to an uncertain link of causality between the original design question and the attained results. Particularly, techniques such as HILIC-based stationary phase chromatography, although pertinent, do not have the capability to thoroughly analyze the lipidome. This represents a significant mishap, since the lipidome is a promising source of information in the malaria context that appears to have been neglected in most metabolomic studies. The coupling of analytical platforms such as LC-MS with GC-MS or NMR, though intricate, has been shown to complement the information attained and seems to be an important strategy towards new target discovery. Additionally, isotope tracing and fluxomics should be explored and be more vulgarly used, as the obtained information would add dimension to malarial discovery. Methodologies still need to evolve in order to allow a true holistic view and complete metabolic fingerprint. These data can then be used to build metabolomic databases based on concrete characteristics, like the retention time, mass or NMR spectra, that will ease interpretability of further studies and improve the predictability power of *in silico* models. Additionally, the integration of multiple omics, as demonstrated by the cited studies, presents invaluable advantages in result interpretation and should be encouraged. Software and statistical tools that can easily analyze such complex datasets are also readily needed, as they would facilitate interpretation and make this research field more accessible.

Lastly, it would appear there is a delimited number of essential pathways vulnerable to pharmacological intervention that can be explored in the context of the malaria disease, however new metabolomic studies are portraying innovative targets. This would seem to indicate that chemotherapy can now be expanded through the help of powerful and robust omic technologies that will shed light on innovative antiparasitic MoA. Additionally, an in-depth characterization of plasmodium's biological functions would prove invaluable towards the disclosure of resistance before implementation of new therapies. As metabolomics could close the gap between clinical studies and experimental research, proper chemotherapy design could be achieved faster and to a greater result in eradicating malaria. Overall, metabolomics proves to be a promising field for the exploration and comprehension of pathology intricacies.

4.3. Update

Metabolomics applied to antimalarial drug discovery is a complex topic that can evolve in multiple directions based on the versatility and extensive information provided by this technique. As such, new research is published every day and a few key studies are worth mentioning to compliment the previous section with the latest research.

Metabolomics can be used to improve, validate or complement experimental methods and models. The Streptolysin O-Percoll (SLOPE) concentration method is a recent example.[256] Streptolysin is a pore-forming toxin that was demonstrated to selectively lyse uninfected RBC in detriment of parasitized ones which can make it a reliable tool in enrichment protocols. However, the presence of excess ghost erythrocytes was an obstacle and the technique required optimization. Metabolomics aided in confirming that iRBC were successfully selected by detecting increased levels of glycerophospholipids and sphingolipids, likely from the parasite's membranes and structures, amino acids, from hemoglobin catabolism, and metabolites from the polyamine synthesis pathway.[256] Additionally, the differentiating factor in selectivity was found to be the cholesterol levels on the host cell's membrane.[256] In fact, the parasite is incapable of synthetizing *de novo* cholesterol and is, thus, forced to extract it from the RBC's membrane, altering its rigidity, integrality and cholesterol content.[256] Hence, metabolomics confirmed that the lipid levels and, specifically, the cholesterol levels, greatly interfered with the SLOPE method and contributed to its optimization. Yu *et al.* recently conducted a study to investigate the metabolic mechanisms associated with artemisinin resistance and used a modified SLOPE method to obtain ring-stage parasites.[332] This study confirmed through metabolomics the clear distinct metabolic profiles obtained with this method versus traditional enrichment techniques and showed that metabolic remodeling plays a significant role in growth arrest and artemisinin tolerance.[332] Additionally, pH assays were validated by metabolomics as satisfactory primary screens of drugs that target *Pf*FNT or

*Pf*ATP4, known parasitic proton pumps whose inhibition represents an effective antimalarial mechanism, as discussed previously.[236]

New research also came to light regarding previously discussed or new potential antimalarial targets. Mutations on the *Pf*ATP4 were shown to elicit resistance to multiple classes of compounds and to describe how the parasite adapts to this mutation, a metabolomics and transcriptomics study was carried out by Tewari *at al.*[287] This study demonstrated an immediate survival phenotype reliant on carbonic anhydrase and aquaglyceroporin to increase pH and transport water across the parasitic membrane, respectively, and an adjusted carbohydrate metabolism with increased myo-inositol and phospholipids levels. Myo-inositol is a precursor for phosphatidylinositol biosynthesis and its accumulation was linked to a decreased rate of RNA synthesis and increased oxidative stress. However, this might be the cost to adjust to the aggression of drugs targeting the *Pf*ATP4 through the osmoregulatory function of this precursor.[287] A second study by the same research group demonstrated a strong correlation between Na^+ homeostasis disruption and an increased production of lipids, which highlights a regulatory pathway that could be targeted to prevent resistance emergence.[333]

A new potential target in an already established important pathway is polyprenyl synthase. This enzyme of the MEP pathway proved to be essential to synthesize longer-chain isoprenoids, which are critical to keep apicoplast membrane fluidity and assure apicoplast inheritance.[334] Other potential targets in fatty acid metabolism have been elucidated through metabolomics: PfGDPD and LCAT. *De novo* phosphatidylcholine biosynthesis is essential for parasite survival and it depends on choline scavenging from the host. In a study by Ramaprasad *et al.*, PfGDPD was the esterase found to release choline from exogenous lysophosphatidylcholine, a reaction essential for phospholipid production and parasitic survival.[335] The same research team investigated the activity of phospholipase LCAT, known to participate in merozoite egression from hepatic schizonts, and demonstrated with lipidomics the role of this enzyme in the membrane lysis to allow egression in the asexual blood stage. Interestingly, the study was inconclusive about how this happens: either through direct lysis of membrane phosphatidylcholine or by producing lysophosphatidylcholine, which itself can be membranolytic.[336] Lastly, due to the differences between host and *Plasmodium* mitochondria, this structure and associated reactions remain a point of interest. *P. falciparum* has a very small mitochondrial genome so this organelle's function depends on the import of proteins and their regulation. Recently, a study using multiple omics demonstrated the essential role of regulating the mitoribosome of two RAP proteins encoded by the nuclear genome but localized to the parasite mitochondria.[337] The decrease of metabolites involved in pyrimidine biosynthesis in knocked down parasites further established this vital function and a promising innovative antimalarial target. In parallel, genomics and metabolomics

were coupled to demonstrate that the only TCA enzymes fumarate hydratase and malate–quinone oxidoreductase thought to be essential for parasite survival in blood stages are not.[338] Oxaloacetate production was still present when these enzymes were knocked out, proving this metabolite to be essential and possibly obtained through an alternative pathway in case the TCA is disturbed.

The discovery of potential antimalarials is also an ever-evolving subject and a recent study is of note. Azithromycin is a macrolide that can inhibit translation by the bacteria-like ribosomes of the *Plasmodium* apicoplast, eliciting a 'delayed death' phenotype. Despite its reported activity *in vitro* against gametocytes and *in vivo* on the liver stage, this antibiotic is still not recommended as an antimalarial.[339] Metabolomics profiling was part of a study on the potential MoA of azithromycin analogues and revealed differential build-ups of hemoglobin-derived peptides that point to the DV as the target of these compounds, as opposed to azithromycin that seems to retain its activity in the apicoplast.

Therefore, metabolomics is shown further as a promising tool in the optimization and drug design of future antimalarials. Interestingly, a new class of antiparasitic drugs is on the rise whose predominant MoA is the interaction with enzymes, proteins or membranes with the purpose of damaging metabolic pathways. Peptide-based drugs have shown parasiticidal effect, however none of these studies have made use of metabolomics' invaluable angle in evaluating the antimalarial phenotype, which is critical in the exploration of this class of compounds.[340] This is one example that shows the necessity of a streamlined implementation of metabolomics assays in antimalarial drug discovery so that molecules with potential develop faster into drugs.

4.4. Mode of Action – Summary

Many antimalarials exist that target multiple pathways in the *Plasmodium* spp. as can be seen in Table 12. In order to simplify consultation regarding the modes of action of drugs used in the experimental section, these MoA are resumed in Table 13.

Table 13 – Profiles of antimalarial drugs used in the metabolomics studies (synthesized from Table 12).

Compound	Stage	Metabolic Pathway = Profile	Putative target/mode of action	References
Atovaquone	Late trophozoite Gametocyte[1]	Pyrimidine synthesis and mitochondrial potential	Mitochondrial electron transport chain, *Bc1* complex	224,228,230,234,239,261
Chloroquine	Ring, Trophozoite, Early gametocyte	Hemoglobin metabolism	Hemoglobin catabolism Heme detoxification	228,230,239,261
Dihydroartemisinin[2]	Ring, Trophozoite	Hemoglobin metabolism Redox homeostasis Pyrimidine synthesis	Multiple hemoglobin-digesting proteases Inductor of oxidative stress	224,230,239
Quinine	Trophozoite, Schizont	Hemoglobin metabolism	Hemoglobin catabolism Heme detoxification	22,219,224

[1] Atovaquone has limited gametocidal activity. Metabolic fingerprint resulted from 1 µM exposure for 2.5h to stage III-IV gametocytes.
[2] Exact mechanism of action is unknown.

The selection of these antimalarials related to the possibility of eliciting a signature *in vitro*, notably for atovaquone used frequently as a standard in this context. Additionally, quinine's use was pertinent due to the experimental work in Chapter 3. Finally, artemisinin and chloroquine, the most common and relevant antimalarial drugs used in antimalarial therapies nowadays, were included to immediately compare potential MoA to these establishes drugs.[9,17,228]

5. Metabolomics in the search of natural antimalarials

The antimalarial research focus on NP is a wide topic that could benefit from a metabolomics approach. This would not only complement the known information on these molecules but hasten their possible development, which would expand the arsenal of antimalarial drugs, as is sorely needed. However, the literature shows a distinct direction in the use of metabolomics and plants or NP with potential antiplasmodial activity.

There are recent studies that profile plant extracts through metabolomics in order to identify NP that could be responsible for their antiplasmodial activity, some in conjunction with bioassay-guided fractionation.[341–344] Another study by Alhadrami *et al*. took a different approach and profiled fungal endophytes associated with *Artemisia annua* in the hopes of finding additional potential antiplasmodial compounds and detected 8 active fungal metabolites that were subsequently studied in growth inhibition assays.[345] Thissera *et al*. applied metabolomics profiling to optimize the media growth conditions of *Pantoea agglomerans* and identify new hits that later revealed antiplasmodial activities in growth inhibition assays possibly as hemozoin inhibitors as suggested by computational investigation.[346] Molecular networking of metabolomics data has also been used to identify bacterial natural analogues of falcitidin, an inhibitor of falcipain-2, an essential enzyme in the early stages of *Plasmodium* hemoglobin digestion.[347] These results guided synthesis and studies on protease inhibitions that confirmed these analogues as potential antiplasmodials.[347] A common theme in all these studies is the use of metabolomics to describe or orient other assays, like bioassay-guided fractionation, computational investigations, or protease activity tests, which is in line with metabolomics hypothesis generating ability. This tactic, however, does not follow the effects on the pathogen, but rather the natural source extract or compound showing the activity. This approach is undoubtedly interesting to concentrate the attention of researchers on the plants and compounds that have potential, but shows a lack of routinely used metabolomics assays to uncover the potential MoA before specifically engaging in target tests, like hemozoin or protease inhibition. Additionally, none of these assays coupled NMR and LC-MS data, which could be a venue worth exploring.

Only two studies in the literature used a metabolomics approach to describe the activity of a NP or plant extract as we propose in this project. Yoo *et al*. synthesized an analog of the antimalarial natural

product Sal A and used LC-MS lipidomics to confirm the enzymes being targeted by this compound in *P. falciparum.*[289] The second study was by Parvazi *et al.* who aimed to characterize the effect of the aqueous extract of cinnamon on the metabolome of *P. falciparum* by ^{1}HNMR.[248] Several pathways were affected significantly by this extract and the lack of specificity could be attributed to the complexity of the crude extract. Moreover, the lack of other control groups illustrates the need to have a comparison base to help guide the profiling and data interpretation.

The complexity and high cost of the purchase and maintenance of metabolomics tools might justify its lack of routine implementation. Still, the argument could be made for the advantages that this approach offers in facilitating NP research and elucidating MoA in the *P. falciparum* towards accelerating antimalarial drug discovery.

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METABOLOMICS WORKFLOW

Chapter 2

1. Foreword

As described previously, metabolomics as an approach is complex and its workflow has to be adapted to the type of biological system under study. Many workflows exist in the context of *in vitro Plasmodium* spp. studies, as seen in Chapter 1 Section 4.1[1], but one needed to be chosen in order to establish a routine benchtop methodology to supplement NP screening and studies. To do so, a part of this work was dedicated to exploring the stages of the workflow and optimizing each one with the tools available.

2. Extraction Methodology

2.1. A vague rulebook

After reading the section **"Metabolomics applied to the *Plasmodium* spp. parasite"** of the previous chapter, two things become clear: multiple parasite stages can be studied through metabolomics, and there is no consensus on the workflow to be implemented.[1]

In order for the antimalarial drug pipeline to expand, it's not only important to have effective drugs, but also to tackled multiple parts of the lifecycle of the parasite. As explored previously, targeting different stages would block the parasite at different levels which would help manage the disease from other angles than just the symptomatology itself. However, in light of multidrug resistance and malaria-associated morbidity and mortality, it is undeniable that the asexual cycle remains one of the most important to target and control. As such, the intraerythrocytic lifecycle is the most interesting. In the context of investigating compounds with traditional use against fevers, plants have been selected and recorded based on the perceived symptomatology and disease evolution or cure. Thus, while studying other forms might shed light on new venues, the most accurate tests to explore these reports are linked with the blood asexual stages. Of these stages, one is mostly studied: the early or the late trophozoite. The ring, or early stage, is the most difficult to study and the least metabolically active, but interesting in the context of artemisinin and similar drugs that act at the start of the cycle. The late trophozoite is relatively easier to concentrate and hence study to have informative phenotypes.

Once the life stage has been chosen, however, there is no set of rules for how to conduct a study, only a general guideline. As is noticeable from Table 11, many different workflows have been reported in the literature. However, many of them fail to assess the quality in extracting reproducibly parasite metabolites. As mentioned previously, a compromise inevitably happens at an experimental level: the longer the experiment, the most effective the extraction but at the risk of metabolite stability, leading to enzymatic reactions or degradation that elicits a less accurate snapshot of the metabolome. This is

more so the case if the studied life stage is the ring trophozoite, because they are difficult to concentrate in adequate amounts without retaining the RBC or ghost membranes, which can pollute the results. Additionally, most methodologies have been developed with LC-MS data acquisition in mind and were never evaluated for their performance through another method, like NMR. It is recognized that a more complete metabolomics assay will have multiple complementary approaches, but often costs, accessibility and time are hurdles in making this idea come to fruition.[2,3] Although hyphenated techniques are a growing field in metabolomics, it is still in its infancy and seldom used in plasmodial assays.[2]

It was with these thoughts in mind that a study was carried out to explore current extraction methods in the literature that focus on the *in vitro P. falciparum* asexual stages. The purpose was to evaluate their performance and comment on what factors should be kept in mind when choosing an extraction method that aims at working with early trophozoites. Due to time and cost constraints, three methods were picked from Table 11 and analyzed through both LC-MS and ^{1}HNMR, and the results can be found in the publication bellow. These methods were chosen based on their relevance and frequency in the literature, with method A developed by Vo Duy *et al*.[4] validated for LC-MS quantification of an assortment of metabolites, including lipids, which seemed to be a complete and robust method that would allow for quantification if needed down the road. Method C was adapted from method A in another publication, as were multiple workflows presented in Table 11, and was pertinent due to the acetonitrile solvent of extraction being the same as the mobile phase of the LC-MS method used at the MASSMET platform of UCLouvain at the time.[5] Lastly, Method B was published by Teng *et al.*[6] and was the first research paper in the literature to compare *Plasmodium* sp. *in vitro* metabolomics extraction methods and analyze them by ^{1}H-NMR, hence assuring that the signal was repeatable enough for accurate characterization through NMR. None of these methods A through C had ever been characterized by both analytical tools (^{1}HNMR and LC-MS), hence an additional level of interest existed in demonstrating the applicability and robustness of these methods with a joined acquisition approach. By comparing these metabolome extraction methods, not only were the protocol steps and other data analysis parameters investigated, but also the methods' variability across analytical tools and the possibility of biological interpretability, which is the ultimate goal of this thesis. The results obtained are presented in the submited article in the next section.

2.2. Ring-stage Extraction Method

2.2.1. Methodology comparison – Submitted

(Submitted to Biochemical and Biophysical Research Communications)

Comparison of extraction methods in *in vitro Plasmodium falciparum:* a ^{1}H-NMR and LC-MS joined approach

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Keywords: Metabolomics, Malaria, Plasmodium sp., Mass Spectrometry, Nuclear Magnetic Resonance, Methodology

Abstract

Malaria is a parasitic disease that remains a global concern and the subject of many studies. Metabolomics has emerged as an approach to better comprehend complex pathogens and discover possible drug targets, thus giving new insights that can aid in the development of antimalarial therapies. However, there is no standardized method to extract metabolites from *in vitro Plasmodium falciparum* intraerythrocytic parasites, the stage that causes malaria. Additionally, most methods are developed with either LC-MS or NMR analysis in mind, and have rarely been evaluated with both tools. In this work, three extraction methods frequently found in the literature were reproduced and samples were analyzed through both LC-MS and ^{1}H-NMR, and evaluated in order to reveal which is the most repeatable and consistent through an array of different tools, including chemometrics, peak detection and annotation. The most reliable method in this study proved to be a double extraction with methanol and methanol/water (80:20, v/v). Metabolomics studies in the field should move towards standardization of methodologies and the use of both LC-MS and ^{1}H-NMR in order to make

data more comparable between studies and facilitate the achievement of biologically interpretable information.

Introduction

Malaria is a vector-borne parasitic disease that remains a global health issue[7]. The latest data indicate that there were 249 million estimated malaria cases in 2022, with a death toll of 608 000 of which 76% are children[7]. These rates of incidence and mortality are still relevant and make eradication all the more important. The *Plasmodium sp.* is a protozoon that affects hundreds of species and when it infects the human host, its cyclic invasion of red blood cells leads to fevers in a specific diagnosis characteristic of malaria. This implies that the parasitic metabolism has uniquely adapted to this environment, making antimalarial drug discovery a challenging field[8].

The study of the metabolism was made possible with the emerging discipline that comprehensively studies a biological system through various lenses, including *in silica* models and analytical technologies, also broadly named omics.[9] These omics sciences began with genome sequencing, which for the *Plasmodium* started with the publishing of *P. falciparum*'s genome, and saw their exponential growth with the optimization of analytical tools and statistic models.[8,9] Specifically in the case of metabolomics, the metabolome is analyzed, which comprises metabolites, small molecules (<1500 Da) that reflect accurately and rapidly the activity of enzymes, proteins and pathways, leading to a faithful snapshot of the parasite's status.[8,9] Some examples include amino acids, vitamins, cofactors, nucleotides, fatty acids, among others; all compounds that provide energy, signaling or building blocks essential for parasitic survival. Powerful and robust techniques such as Liquid Chromatography (LC) coupled with mass spectrometry (MS), or Nuclear Magnetic Resonance (NMR) make metabolomics reliable because of their sensitivity, selectivity and reproducibility.[10,11]

Extensive research published on the *Plasmodium* metabolome makes use of metabolomics analysis unrivaled advantages.[8] It has the capability of reflecting the adaptation of the parasite to exposure to a drug, revealing both the senescence cascade or the resistance mechanisms, crucial to find new targets and for rational drug development.[9,12–14] Notwithstanding the many studies on the *Plasmodium* metabolome, there is no consensus on a standardized method for metabolite extraction, despite it being the key departure point for metabolomics studies.[1] In the literature, dozens of methods exist that variate in the use of saponin for red blood cell (RBC) lysis, quenching, extraction solvents, among other factors. The disparity in methodology might mean a different range of metabolites is extracted with each study, consequently changing the biological deductions that can be inferred. Moreover, most literature methods were developed for LC-MS analysis, raising the question on whether they can be used for ^{1}H-NMR as well. Ideally, a metabolite extraction method should be

repeatable, reproducible and extract a metabolome as representative as possible.[1] Additionally, most methods have been developed with either LC-MS or NMR in mind, and have rarely been evaluated with both tools. In this work, three methods frequently used in the studies of the *P. falciparum* metabolome *in vitro* were reproduced, analyzed through ^{1}H-NMR and verified once through LC-MS, and evaluated to reveal which parameters award the most reproducibility in order to achieve methodology optimization. These methods were chosen based on their relevance in the literature, with the addition of one method developed with ^{1}H-NMR analysis in mind.[4–6]

Materials & Methods

Reagents

All pipettes, bottles and sterile materials to handle the culture were acquired from Greiner Bio-One. Sorbitol was obtained from Sigma. Saponin (Alfa Aesar), and PBS were obtained from Thermo Fisher Scientific. Methanol of HPLC grade was obtained from Merck, chloroform (Merck), acetonitrile LC-MS-grade was obtained from VWR. Milli-Q water was obtained with a milli-Q reference A+ system® from Merck. Trimethylsilyl-3-propionide acid-d4 (TMSP) and deuterium oxide (D_2O, 99.96% D) were purchased from CortecNet (France).

Parasite culture and maintenance

Blood-stage *P. falciparum* 3D7 parasites obtained from the Malaria Research and Reference Reagent Resource 453 Center (MR4) were cultured in human erythrocytes at 3% hematocrit in various volumes in complete media.[15] This media consisted of RPMI 1640 (Gibco, Fisher Scientific, Loughborough, U.K.) containing $NaHCO_3$ (32 mM), HEPES (25 mM), and L-glutamine, supplemented with 1.76 g/L of glucose (Sigma-Aldrich, Machelen, Belgium), 44 mg/mL of hypoxanthine (Sigma-Aldrich, Machelen, Belgium), 100 mg/L of gentamycin (Gibco, Fisher Scientific, Loughborough, U.K.), and 10% human pooled serum (A+). Cultures were microscopically verified for stage and parasitemia and were kept gassed (90% N_2, 5% O_2, 5% CO_2) and incubated at 37°C. Parasites were kept synchronously by weekly 5% sorbitol (w/v) treatment and additionally treated 24h before each assay.

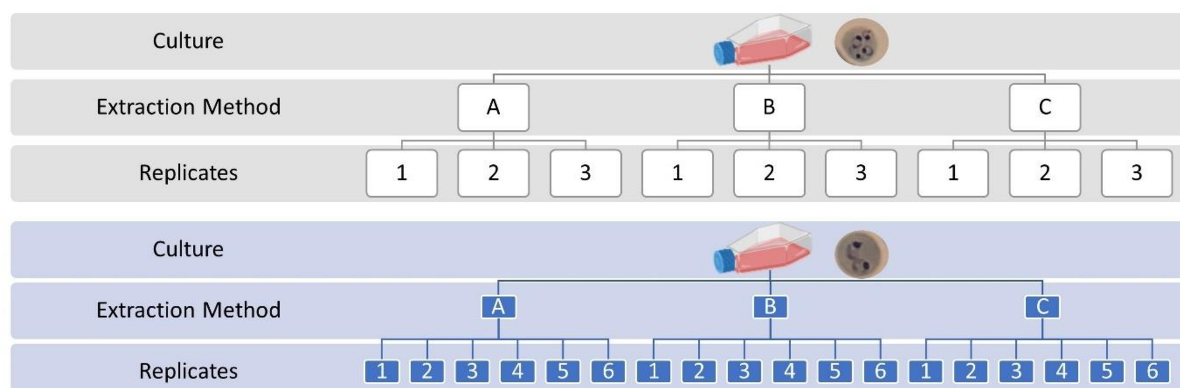

Figure 15 – Experimental design, in gray the N=3 analyzed by ^{1}H-NMR, and in blue the assay (N=1) analyzed by LC-MS. All cultures were synchronized with sorbitol and microscopically verified to start the extraction no later than 8h post-infection.

Metabolomics extraction

In each assay, asexual intraerythrocytic ring-stage cultures not older than 8h as observed microscopically (> 90%) were aliquoted equally to a minimum of 10^8 parasites/sample and extracted by either method according to the design shown in Figure 15. One extra assay was performed in the same conditions, this time in six replicates per method, and analyzed through LC-MS. Samples were placed in an ice bath before extraction. Three methods from the literature were performed with slight adaptations as shown in Figure 16.

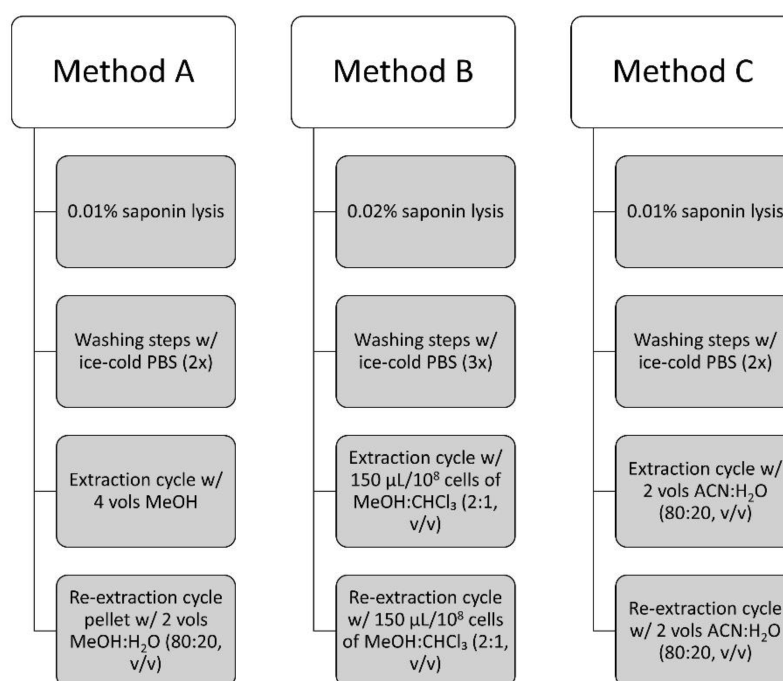

Figure 16 – Schematic workflow of each extraction method under study.

Method A was published by Vo Duy *et al.*[4] involved RBC lysis with 0.01% saponin (w/v) for 3 min, followed by centrifugation (3000 rpm for 8 min) and a PBS wash. The parasite pellet is then extracted in two cycles: first, incubated 15 mins at -20°C with cold methanol (1:4, v/v), then centrifuged at 11,000 rpm for 10 min to keep the supernatant; second, resuspended with 2 pellet volumes of a mixture of cold methanol/water (80:20, v/v) followed by an incubation of 15 mins at -20°C, followed by centrifugation. Both supernatants were pooled, evaporated, lyophilized and kept at -20°C until resuspension for analysis.

Method B was published by Teng *et al.*[6] and it comprised saponin lysis (0.02%, w/v) for 90s, followed by centrifugation at 3000 rpm for 8 min and two subsequent PBS washes before the pellet was centrifuged 7700 rpm for 2 min before being stored at -20°C. The frozen pellet was extracted at first with a mixture of methanol/chloroform (2:1, v/v) at a proportion of 150 µL/10^8 cells and vortexed until a suspension is formed. Ice-cold water was then added at a proportion of 20 µL/10^8 cells and incubated

for 15 min in an ice bath, followed by a thawing at 4°C for 3 minutes. A second volume of ice-cold water (150 µL/10^8 cells) was added, vortexed and centrifuged at 11,000 rpm for 10 min. After supernatant collection, the pellet was re-extracted with methanol/water (2:1, v/v) at a proportion of 150 µL/10^8 cells and incubated 5 minutes on ice. After centrifugation at 11,000 rpm for 10 min, the supernatant was collected, pooled, evaporated, lyophilized and kept at -20°C until resuspension for analysis.

Method C was published by Dickerman *et al.*[5] and it encompassed lysis with 0.01% saponin (w/v), vortexing, pelleting by centrifugation at 3000 rpm for 8 min followed by a PBS wash. The pellet was extracted with acetonitrile/water (80:20, v/v), followed by centrifugation at 11,000 rpm for 10 min before the supernatant was collected and this process repeated. Supernatants were pooled, evaporated, lyophilized and kept at -20°C until resuspension for analysis.

Samples to be analyzed by NMR were dissolved in 400 µL of buffered D_20 at pH 7.4 with TMSP as internal reference, and transferred into 3 mm NMR tubes (Bruker) for analysis. For LC-MS analysis, mixture of 100 µL of formate/acetonitrile (20:80 v/v) was used for resuspension and transferred in an LC-HRMS vial.

Instrumentation

NMR spectra were acquired on a Bruker NEO Ultrashield Plus 700 MHz equipped with a helium cold probe (cryoprobe). ^{1}H-NMR experiments were performed with a CPMG sequence with 128 scans collected over a spectral width of 20 ppm. All spectra were phased and baseline-corrected manually using TopSpin v4. Spectra were stacked, aligned and integrated between δ0.5-9.5 ppm using MestReNova v14. Spectra were divided into buckets of 0.04 ppm, integrated to the sum of intensities and normalized to the number of parasites per sample.

Other samples were analyzed using a LC-HRMS system consisting in a Thermo Accela pump, autosampler, photodiode array detector and Thermo Scientific LTQ orbitrap XL mass spectrometer at the MASSMET platform of UCLouvain. Samples were injected (10 µL) into a hydrophilic interaction liquid chromatography (HILIC) column with a Phenomenex Luna 3 mm x 150 mm, 200 A HILIC (Louvain, Belgium). The mobile phase consisted of A: 10 mM pH 3.8 ammonium formate, and B: acetonitrile and the gradient elution started with 5% solvent A until 3 min, then increased to reach 95% at 25 min and maintained for 5 more min, then back to 5% and equilibrated for 10 min. Flow rate was 0.3 mL/min; oven temperature was 40°C and total run time was 40 min.

Metabolomics Data analysis

The bin tables generated by the NMR spectra were analyzed using MetaboAnalyst v5.0 and *R* (packages MBXUCL, PepsNMR and limpca).[16–18] The NMR spectra were annotated using Chenomx NMR Suite 9.0 database and the Human Metabolome Database (HMDB), as according to literature.[4,6]

Data acquisition was done in positive mode and raw LC-HRMS data profiles were converted into mzXML format with msConvert (ProteoWizard) using the Filter "Peak Picking". MzXML format files were then processed using XCMS package to Worklow4Metabolomics 3.3 (W4M). The CentWave algorithm was used for automatic peak detection. Statistical analysis was performed with MetaboAnalyst v5.0.

Results

Chemometric Visualization and Description

Metabolomics is a discipline that generates complex datasets with multidimensional data. Generally, the first step in data analysis is data visualization through a chemometric tool, such as Principal Component Analysis (PCA), which can then be followed by more telling models, such as ASCA+ (ANOVA-Simultaneous Component Analysis) and APCA+ (ANOVA-Principal Component Analysis).[17,19] These enhanced versions of the original statistical methods use general linear models instead of ANOVA to correct the bias of unbalanced experimental designs to generate a tool that incorporates multivariate analysis of variance with PCA for eased data visualization in a reduced space.[17] This tool was used in this context to analyze the assay effect and remove its interference from the outcome.

Figure 17A shows the PCA score plot of the original ^{1}H-NMR data for each extraction method (of which one representative spectrum can be found in Figure S1 in the Supplementary Data. It reveals two clear outliers, one for method A (sample A32) and one for method B (B32), which were removed for subsequent analysis. A new PCA scores plot was generated and is shown in Figure 17B in which it is clear that each group of samples separated by assay and extraction method group together. However, it also becomes clear that samples tend to gather in either quadrant of the PCA in regards to the assay, which shows the influence of this factor that was then removed.

Because assays were conducted at three independent times, it was important to decompose the outcomes matrix in the effect matrices: Method + Assay + Residuals. This was done using ASCA+ with the package limpca and the results are shown in Figure 18A and 17B. Figure 18A shows the percentage of variance explained by the effects and it is perceivable how the effect 'assay' represents the biggest variance. Because this effect is not interesting for the purpose of this study, it was removed by centering the data for the assay variability and a new PCA score plot was generated – Figure 18B. Method A has less intragroup variability, as seen by the distribution across the PC1 (56.5%), which

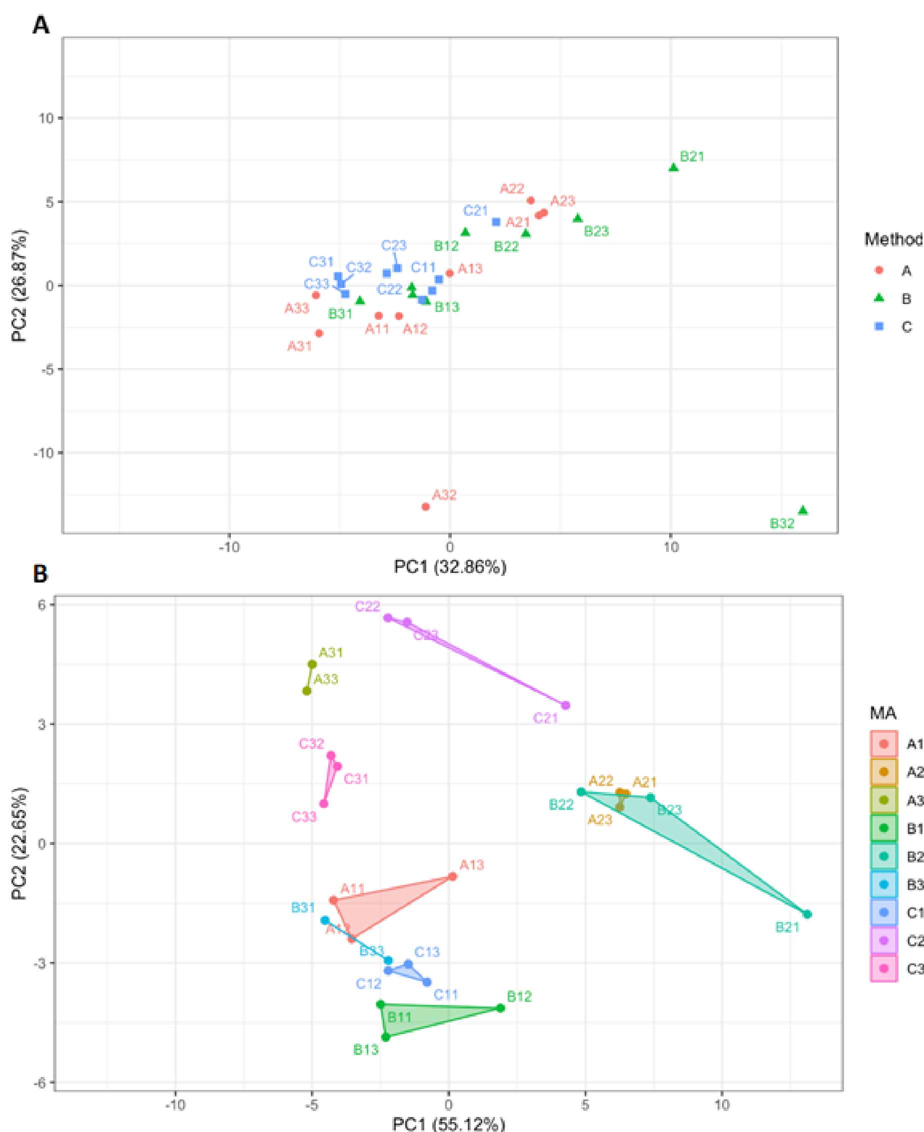

Figure 17 – (A) PCA score plot of the original 1H-NMR bin data organized by method where the two outliers are clear. (B) PCA score plot of the original 1H-NMR bin data without outliers organized by method and assay, points represent triplicates. (MA – Method-Assay)

reveals this method as the most homogenic. Method C has some variability associated with two samples from the second assay and Method B is the most variable across both PC1 and PC2. PC2 (18.29%) has the least variability for method C, which would indicate that this PC is related to a source of variability that is more impactful on the other two methods.

Figure 19 shows the PCA scores plot of the LC-MS-analyzed assay, where groups correspond to each individual method A, B or C. All methods are clearly separated from each other with their respective samples clustering closely. The variation explained by components 1 and 2 accounts for a big part of the variation of the data (70.5 %). Method B would appear the most variable, as perceivable by its variance across the PC1, followed by method A and C.

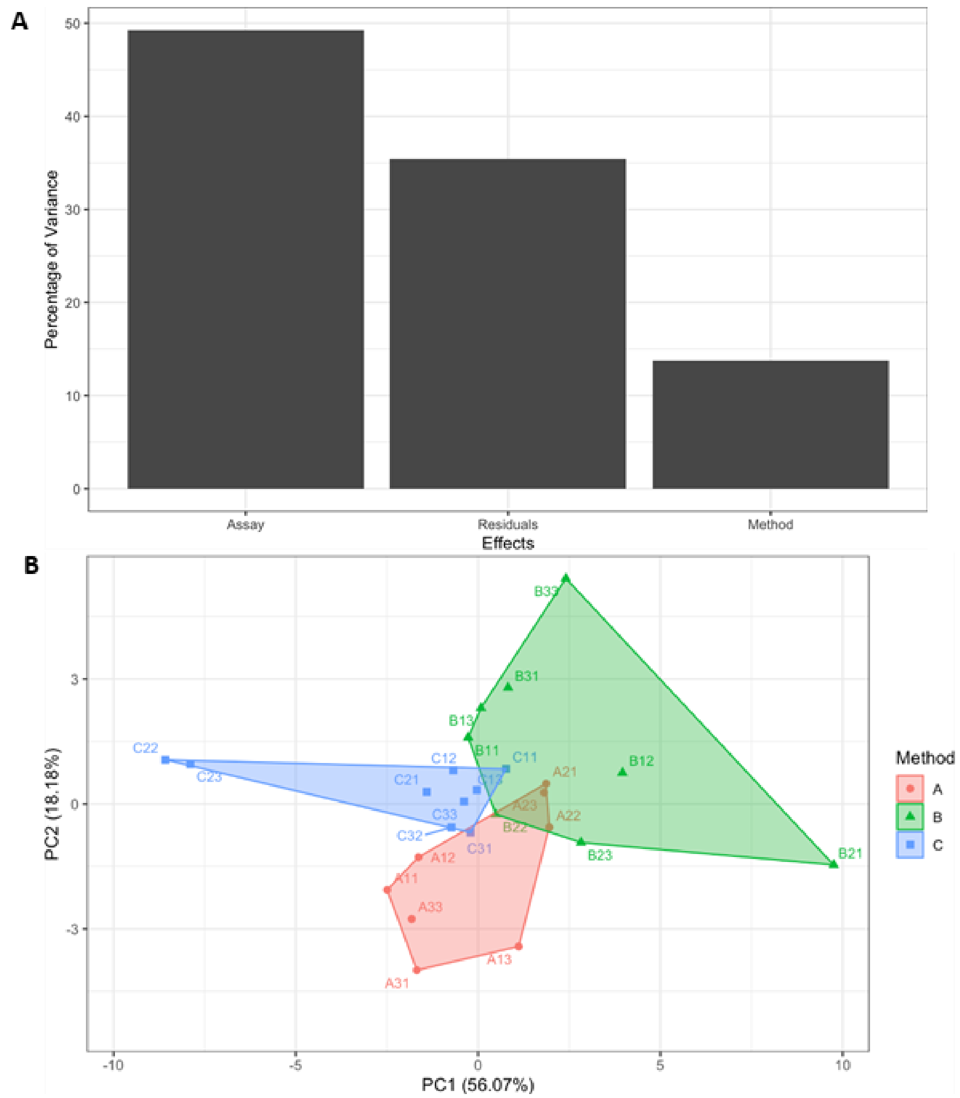

Figure 18: (A) Percentage of variance explained by the decomposition of effect matrices of the 1H-NMR data, (B) PCA score plot obtained by centering the data to the 'assay' effect after rebuilding the data matrix with the 'limpca' package.

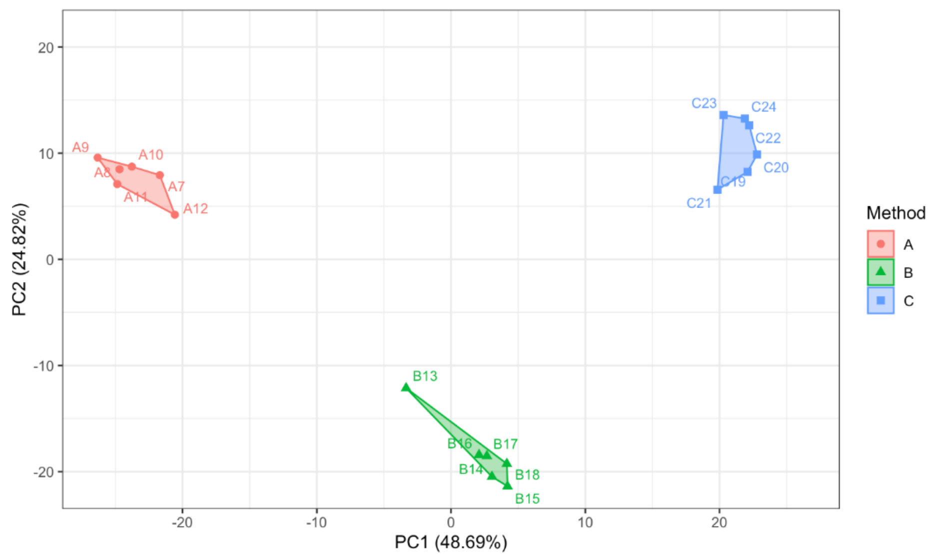

Figure 19: PCA scores plot of the LC-MS data organized by method.

Inertias

One technique developed specifically for the evaluation of the Metabolomics Informative Content (MIC) in NMR metabolomics studies was also used to analyze this dataset.[20] Contrary to using just repeatability in spectrometry, the MIC uses a clustering approach that evaluates the amount of captured information (*i.e.* signal) compared to noise that would unintentionally come from other factors. The method considers that a signal is responsible for group clustering, thus dependent on the group's characteristics and non-identical between groups, whereas noise is independent and identically distributed, as it is likely to come from other factors such as the experimental design, the operator, analytical apparatus, etc. To differentiate them, the model decomposes the total variance into two parts: between groups, *i.e.* intergroup, and within the groups of observations, *i.e.* intragroup.[21] Ideally, the variance, or inertia, within groups should be small, as it would mean that observations are the most similar. Likewise, if the inertia between groups is big, then it is indicative of the quantity of captured signal that has informative value.

Table 14 resumes the inertia of the three methods as analyzed by ^{1}H-NMR. Method A has the least intra group variability demonstrated by the lowest measure of inertia within assays of the three methods (13.2%). Method B and C have similar inertias, 25.41% and 25.65%, respectively, which indicates similar levels of variability. A high inertia between assays and consequently small inertia within groups translates into a better signal over noise ratio with more metabolomic informative content, which reveals repeatability and robustness across assays.

Table 14 – Measurement of the Metabolomic Informative Content (MIC) through inertia per method of the original ^{1}H-NMR data (without outliers).

	Inertia Between Assays	Inertia Within Assays
Method A	86.8%	13.2%
Method B	74.59%	25.41%
Method C	74.36%	25.65%

Because the MIC measures inertia between repetitions, it could not be applied to the LC-MS assay, which was conducted only once. However, a measure of inertia can be performed by calculating the volume of the PCA ellipses for all PCs for each method. This calculation can be found in Table 15 and it translates an abstract measure of variability that allows to compare the inertia per method between analytical tools. Similarly with the MIC, the smaller the value, the smaller the variability associated with a method analyzed with that tool. Interestingly, the methods that have the least inertia are not the same for both analytical tools – method A is the least variable when analyzed by NMR, followed

closely by method C, whereas through LC-MS method C is clearly less variable. Unsurprisingly, method B is the most variable with both analytical tools.

Table 15 - Inertias calculated by method through both the NMR (assay centered data) and LC-MS data.

	NMR	**LC-MS**
Method A	0. 06860552	1.308199
Method B	0.1092255	1.578083
Method C	0.07758823	0.9162912

Through exploration models, a general overview of the datasets is possible. PCA of the original data exposed two outliers for methods A and B, respectively, in ^{1}H-NMR analysis. ASCA+ and APCA+ analysis, in which the 'assay' effect was removed, confirmed the variability of method B regardless of this effect. Through MIC analysis, method A displayed the minimal within assays inertia and maximal between assays inertia, which correlates with the least intragroup variance and the highest metabolomic informative content. Lastly, LC-MS analysis generally demonstrated a good separation between samples extracted by method A, B or C. A second calculation of inertias shown in Table 15 to compare the variability across analytical tools showed that the methods perform differently between platforms – method A shows less variability for NMR and method C for LC-MS. Chemometrics analysis points to a close variability and repeatability between methods A and C while confirming that method B is not reliable.

Metabolite Detection

Metabolite detection can be broadly assumed as the number of signals detected in the analysis of a metabolic extract. As one single molecule may have multiple protons, which will elicit multiple ^{1}H-NMR peaks for one sole metabolite, the number of peaks cannot be translated to number of metabolites. Still, this number can be correlated with the success of the metabolic extraction, as if there are no metabolites there will be no signals.

For this reason, automatic peak picking was performed with MestReNova v14, which uses a Global Spectral Deconvolution algorithm to automatically pick only positive peaks, in this case. The average number of peaks variated between assays, though it remained similar across methods: 281 ± 34 peaks for method A, 289 ± 65 peaks for method B and 295 ± 37 peaks for method C. Through the standard deviation (SD) it is evident that the method B has the biggest variation, whereas the other two methods are fairly similar.

In LC-MS, features are detected, i.e., a two-dimension signal of retention time per *m/z*. Though the whole chromatographic run is analyzed, missing values are a common occurrence in which zero

intensity is detected. These can have biological or technical reasons, and the way of dealing with this issue is a topic of discussion in the metabolomics field.[22] Regardless, missing values may also be used as a metric of the quality of a metabolic extraction method, as it can play a role in the detection by MS. Table 16 summarizes the results obtained. The mean peak intensities for each extraction method along with reproducibility were calculated, which in this context is evaluated as the same metabolite being detected in at least 5 out of 6 replicates. After the application of filters to remove peaks present in the blanks, a total of 187 peaks were detected. When evaluating reproducibility, 144 peaks are detected in at least 5/6 of the replicas with method A, followed by method C, with 139 peaks and method B, with 117 peaks. Method A displayed a higher number of detected features with higher average intensities and fewer missing values.

Table 16 - LC-MS analysis: missing values, mean of peak intensities with and without MV, reproducibility and intragroup SD for each extraction method.

	Method A	Method B	Method C
Missing Values (MV)	192	345	254
Mean with MV	5.03	4.05	4.61
Mean without MV	6.07	5.85	5.96
Peaks in at least 5/6 samples	144	117	139
Within SD	0.35	0.42	0.35

Detection of peaks through both ^{1}H-NMR and LC-MS hence points method B as the least consistent, with method C showing the biggest average peak number through NMR and method A through LC-MS. Method A appeared more consistent through LC-MS with the least missing values and highest number of features detected in at least five out of six samples.

Annotation

Annotation of metabolites is a crucial step in processing metabolomics data. It is effectively the step that allows for biological inference and result interpretation. However, it is also the most complex and time consuming stage, as the degree of certainty of annotation can play a large role in the confidence of the results.[19] Previous metabolomic studies with *P. falciparum* successfully detected and annotated parasitic metabolites, thus annotation can be used as a measurement of quality for an extraction method in this context.

As such, the table of detected metabolites published by Teng *et al.*[6], where method B was published, was used as a frame of reference for the annotation of ^{1}H-NMR data. Chenomx and HMDB databases were used to identify specific chemical shifts attributed to each metabolite. The full list of metabolites can be found in the Supplementary Data, and it consists broadly of amino acids, membrane precursors, nucleotides, carboxylates and contaminants (ethanol, methanol, and for this study, acetonitrile which

was added as the extraction solvent of method C). In total, 53 metabolites and contaminants were searched in the spectra, 43 through Chenomx and 10 manually with HMDB's spectra references.

For all methods the same range of metabolites was annotated, with emphasis on the lowest annotated spectra in the case of method B (13 metabolites). Method B's variability didn't make possible to annotate metabolites reported to be extracted through this method, such as γ-aminobutyric acid, putrescine or spermidine.[6]Between method A and C, the range was similar, between 18-32 and 17-34 metabolites, respectively. It was not possible to annotate every metabolite as reported in the reference table, however, this might be a consequence of the lower amount of parasites used in these assays (~ 10^8), in regards to the amount used by Teng *et al.* (~1-4 x 10^8).[6]

Visibly, all methods were in the same range of metabolites annotated, but there were differences in the metabolites, as shown in Table 17. A total of 13 metabolites from diverse classes were identified differently across extraction methods. Method C accounts for more consistency as all the 13 metabolites were identified in most samples. Method B displays the highest variation regarding detection of these metabolites, with neither being found in all samples. For method A, only $NADP^+$ was not found in any sample, possibly because of the quicker experimental time or another technical factor, as Vo Duy *et al.* reported annotation of this cofactor through this extraction method.[4]

Table 17 - Differences in the annotated metabolites per method, according to the class. Annotation performed with Chenomx.

Class	Metabolite	Method A	Method B	Method C
Amino acids	Asparagine	Yes[1]	Yes[1]	Yes[1]
	Glutamate	Yes	Yes[1]	Yes[1]
	Glutamine	Yes[1]	Yes[1]	Yes[1]
	Phenylalanine	Yes[1]	Yes[1]	Yes[1]
	Serine	Yes[1]	Yes[1]	Yes[1]
	Tyrosine	Yes[1]	Yes[1]	Yes[1]
Nucleotides and related compounds	AMP	Yes	Yes[1]	Yes
	Hypoxanthine	Yes[1]	Yes[1]	Yes[1]
	IMP	Yes	Yes[1]	Yes
	$NADP^+$	No	Yes[1]	Yes[1]
Glutathione	Reduced	Yes[1]	Yes[1]	Yes[1]
Carboxylates	Fumarate	Yes	Yes[1]	Yes[1]
Soluble membrane precursors	*myo-Inositol*	Yes	Yes[1]	Yes[1]

[1] – not found in all samples.

Discussion

In this study, three extraction methods from the literature were compared by ^{1}H-NMR and verified once by LC-MS analysis in order to choose the most robust and reliable method towards studying the *Plasmodium falciparum* metabolome. Several methods in this context have been published in the literature, but the evolution of metabolomics technology warrants for further changes in order to develop one or more methods that are reproducible and robust across all analytical platforms. Method A, developed by Vo Duy *et al*.[4], was validated for quantitative LC-MS and it was a starting point to more recent extraction methods in the field, including method C, adapted from by Dickerman *et al.*[5].[1] Both methods have not been, to the authors knowledge, studied and evaluated through ^{1}H-NMR, hence the interest in this study. Method B was published by Teng *et al.*[6] and was the first research paper in the literature to compare *in vitro* metabolomics extraction methods and analyze them by ^{1}H-NMR, hence assuring that the signal was repeatable enough for accurate characterization. Other methods have been published and adapted in the last decade in the field, but for the sake of time and complexity, only these three were studied in detail. Additionally, as most have already been studied or adapted in LC-MS studies, the focus stayed mainly on ^{1}H-NMR analysis with one LC-MS assay confirmation.

The *Plasmodium* spp. complex lifecycle that involves human infection presents multiple opportunities for effective blocking of malaria, be it as a prevention, treatment or transmission blocking. However, as the mortality and mobility associated with this disease remain high and resistance is a growing concern, studies that aid in antimalarial drug research can still focus on the life stages that elicit symptoms and complications, namely the intraerythrocytic stages. Within this cycle, the parasite evolves morphologically and can be distinguished both metabolically and visually from an early trophozoite, also named ring-stage, to late trophozoite and into a schizont that can release multiple merozoites that will reinfect new red blood cells. The ring-stage was chosen in the context of this study to pave the way for assays that could routinely be implemented with resistant strains. Whether to evaluate the effects of promising antiplasmodial compounds or facilitate the discovery of compounds with activity in the early stages, characteristic for being the stage to support artemisinin resistance, this stage seemed the most challenging.[23] Despite reports of the ring-stage being the least metabolically active, our study supports the exploration of this stage with robustness as long as a few parameters are controlled, like synchrony and high enough number of parasites per sample.[24,25] Additionally, none of the extraction methods studied are limited to the ring-stage; purification with other methods like magnetic cell sorting or Percoll are described in the literature and could be implemented prior to these methods.[1,14] This could be an interesting future venue to explore.

Samples were processed as indicated previously and prepared for either analytical platform. As conservation was not a parameter of this study, all samples were freeze-dried to remove solvents and assure stability until analysis. Despite this, traces of water, ethanol and methanol could still be found through ^{1}H-NMR, noting how difficult it is to eliminate solvent traces. These residues were however significantly minimized through this processing and the implementation of a water suppressing sequence during acquisition, which made their impact negligible.

Additionally, it is worth mentioning that extraction methods were performed as indicated in the literature, but new approaches and factors have since been acknowledged as important in the context of these types of metabolomics assays.[24] In culture, even high levels of parasitemia (*e.g.* 10%) remain relatively low in what might represent significant RBC contamination. Other techniques, like culturing the *Plasmodium* sp. without the RBC or through other enrichment techniques, like magnetic cell sorting or the SLOPE method have since become more frequent than methods with saponin, which lyse RBC but has been shown to be associated with the presence of "ghost contamination" from the RBC membranes.[24–26] In this work, all samples were treated with saponin to induce RBC lysis and its effect should be similar across all samples, regardless of the following extractions steps. Still, for the development of future extraction methodologies, this parameter is essential and should be the focus of careful research.

Chemometrics analysis was done through PCA for both analytical platforms as it is the most often used model to graphically give an overview of the variability of the data. Additionally for the NMR data, two other approaches were used: an ASCA+ analysis, to eliminate the assay effect from the analysis, and the MIC algorithm, which consists of a more recently developed model in NMR metabolomics.[19,20] It is important to consider factors external to the method of extraction such as the different assays, which can change the outlook on the results and the choice of method of extraction for further experiments. The assay effect was chosen as a known fixed categorical factor that could be easily evaluated and was relevant in this study. The consensus of the models used for ^{1}H-NMR data analysis is that method A has less intragroup variability and is the most repeatable. Through LC-MS, it is clear that the three methods are distinct from one another, with method A and C clustering in different sides of the PC1 (48.69%), which would indicate relevant differences between the two. The inertia per method for both analytical tools was different and it could be linked to the fact that method C's extraction solvent is also present in the LC-MS mobile phase. Despite lyophilization prior to reconstitution and analysis, the use of acetonitrile at both stages could facilitate solubilization and hence remove a factor that could introduce variability. Of the three methods, method B had the most variability across both ^{1}H-NMR and LC-MS data.

Other chemometrics and statistical models could have been used, but all would have suffered from the same issue: the number of replicates. This study had three triplicates in three independent assays for ^{1}H-NMR, and one set of six replicates for LC-MS. These numbers are considered small in what statistical analysis is concerned, as the number of replicates translates directly into the statistical model's robustness and predictability. This remains a bottleneck in statistical analysis of metabolomics data, as the number of variables is much bigger than the replicates. Indeed, it is still very challenging to obtain high quantities of purified parasite *in vitro* which explains the limitations in the number of replicates. Still, PCA, ASCA+, APCA+ and MIC are not predictable tools and are less affected by this issue than other models, hence their application in this study.

Additionally, metabolite detection was evaluated through the spectral signals. It is important to note that not all signals are metabolites, as background noise or matrix effects can also produce misleading signals. Still, the number of peaks or features is indicative of the number of metabolites in a sample, which can give information on the capability of an extraction method. In this context, method C was found the most repeatable, maintaining a high number of peaks without variating as much as methods A and B between assays for ^{1}H-NMR. It is worth noting that particularly in NMR, superposition plays a role in metabolite detection and peak picking, as metabolites with the same functional groups will have clusters around the same chemical shift window. Fortunately, even in such cases, most metabolites can be distinguished by other characteristic chemical shifts (if there is more than one), allowing annotation even in the presence of hundreds of compounds. Techniques like 2D NMR can be used to tackle this issue, but have further sensitivity issues and are thus less used in metabolomics, especially in malaria *in vitro* studies, where biological material is a limitation.

Through LC-MS, method A was found to be the most repeatable, with the highest number of features detected in at least five out of six samples, and the method with the least missing values. This result could be somewhat predictable, as the method was originally developed for LC-MS, but there might be other factors to consider. As LC-MS is a more sensitive technique, it could be that it can detect more metabolites more consistently across the different methods, hence reporting faithfully a greater consistency for method A. Simultaneously, method A did show the best results through chemometrics and the MIC approaches to ^{1}H-NMR data, so there is a consistency in the results.

Annotation is the analysis step that transforms all the metabolomics data into biologically interpretable results and its success is paramount. As such, a table published by Teng *et al.* was used as a reference to search for *P. falciparum* metabolites and evaluate if the extractions were successful.[6] All three methods succeeded in extracting parasite related metabolites, though to different degrees. All methods allowed the identification of between 13 and 34 metabolites, with method B being the

most variable. Differences in annotation were revealed across diverse metabolic classes, such as amino acids, nucleotides and related compounds, and others. Method C was the most consistent method in regards to annotation, followed by method A. The use of a list of metabolites as reference was important in this context to reliably search for metabolites previously reported in *P. falciparum* and detected through ^{1}H-NMR. However, it should be noted that the use of a list as a framework fails to account for other metabolites that a method might have successfully extracted that were not part of the list, but could prove significant in establishing a method as more thorough than the others.

Conclusion

It should be noted that in the case of a metabolomics extraction method, repeatability and reproducibility should be valued as the most important criteria, as metabolomics suffers from metabolites with very fast turnover and technical problems, like matrix effects, missing values, among others. These factors influence significantly the amount of reliable information that can be deduced from a study's dataset, which is already limited by the number of samples in comparison to the hundreds to thousands of variables to be analyzed. As such, a method that is the most repeatable and robust will diminish, though not eliminate, experimental bias and should be selected as a standard. Accordingly, the most reliable method evaluated in this study proved to be method A, *i.e.,* two cycles of extraction with methanol and methanol/water (80:20, v/v), followed closely by method C, a double extraction with acetonitrile/water (80:20, v/v). Metabolomics studies, whose workflows should be optimized towards reproducibility, are pivotal in the learning of plasmodial pathology and in rational drug design in the path towards malaria eradication.

2.2.2. Playing with the rules – what can be improved?

There are multiple steps in a metabolomics workflow that can be evaluated and optimized in order to reach a more complete, and consequently more telling metabolome. Despite the constraints associated with this experiment, such as time, number of samples, possible loss of parasite material and contamination, a few supplementary tests were carried out to try and enhance a few factors. Figure 20 resumes the steps of each method explored in the previous section with the (*) indicating the moment of sonication, when applicable.

As mentioned previously, pelleting and washing with cold PBS before extraction is a common workflow step in the kind of assay we wish to perform. However, the number of times this is done and the time it takes variates between studies in the literature. If the sample isn't clean enough, the RBC's metabolome or "ghost" membranes might be extracted alongside the culture media, which would represent contaminations to the parasite metabolome. Likewise, if there are too many washing steps, there could be leakage and degradation of metabolites. As such, the number of necessary washes was

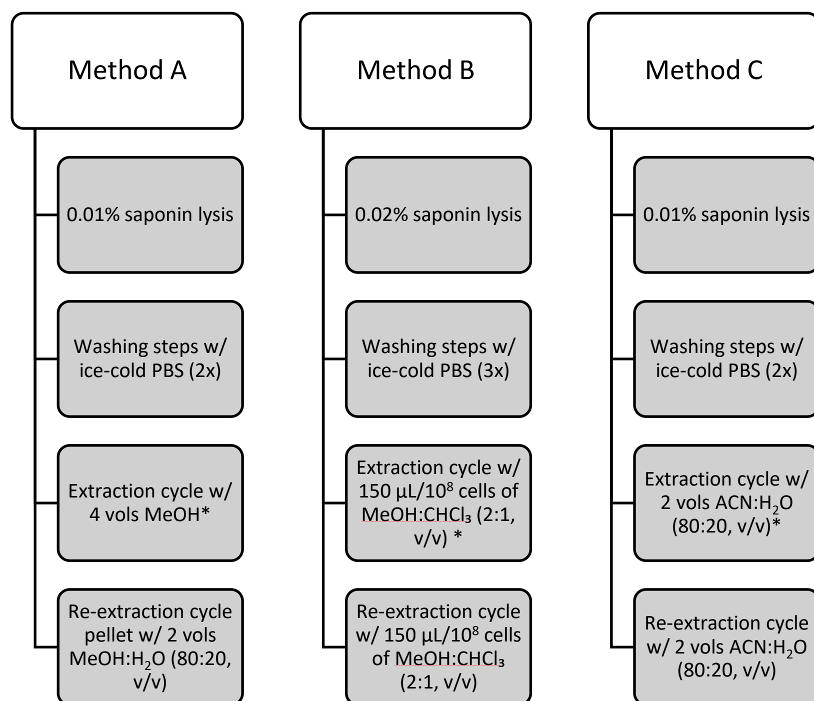

Figure 20 – Resume of each extraction method as explored in the previous section. * indicates the sonication step when applicable.

evaluated by collecting the PBS at each washing step from the previous assay. Washes were done for each sample two or three times (as according to Figure 20), and each wash supernatant was lyophilized and analyzed through ^{1}HNMR in the same conditions as in the previous section. The total spectrum intensity was calculated with MestReNova v14 and the average and standard deviations were calculated with excel and presented in Figure 21.

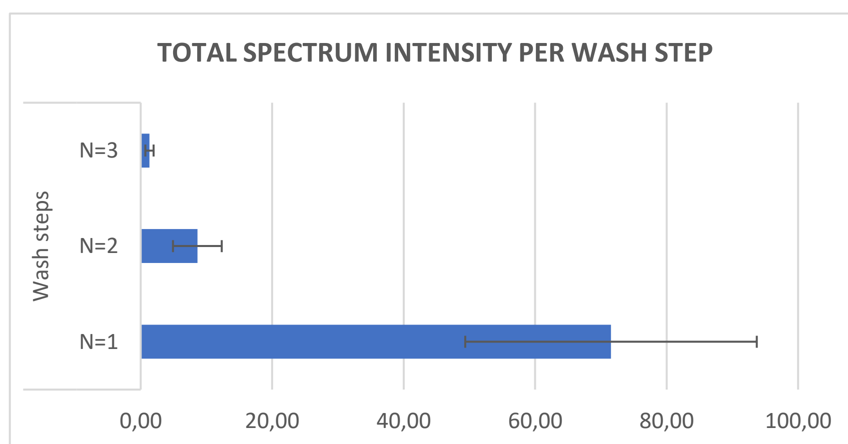

Figure 21 - Average total spectrum intensity per wash step (with the SD).

One washing step is essential in the context of *Plasmodium* blood stage metabolomics studies to remove the culture media as much as possible. Because RBC are in suspension, the culture media can't be aspirated as easily as with adhesive cells. This means that it is easy for each sample to not be perfectly clean after one wash. Additionally, iRBCs might also be collected by mistake, leading to the variability present in the spectral intensity for these samples (N=1 bar in Figure 21). As such, not only

is the intensity bigger for the first wash step, because culture media was retained in this wash supernatant, but the variability of intensity is the biggest.

A second washing step is not always found in the protocols available in the literature, and a third even less because it extends the experimental time and increases the risk of metabolite degradation, even if it is done in cold conditions. Additionally, when predicting the number of washing steps, the use of saponin to lyse RBC is an important factor. Saponin lysis RBC membranes, which releases these cells' metabolome in the supernatant so it can be easily removed in a PBS wash. This lysis usually occurs early in the protocol, either after collection of the cell pellet or after one wash, so the total number of washes can be directly correlated with the contamination of the sample by RBC's metabolites.[26] Saponin was used in this test for all samples right after collection of the pellet, as seen in Figure 20. So not only is the first wash important, but the intensity of the second wash supernatant is still representative and possibly necessary with this protocol. Meanwhile, the third supernatant represents approximatively 1/54 of the spectral intensity of the first washing step, and as such might not be worth it due to the additional experimental time and induced stress to the parasites that can provoke leakages.

After the initial study to compare the three methods – A, B and C – as they are in the literature, the same test was repeated but with a sonication step added to the extraction to evaluate if this technique could improve metabolite extraction – as shown in Figure 20. This was done after the first extraction step by sonicating in 3 cycles of 20s/40s on/off with a vibrating probe (Vibra-Cell, Sonics and Materials Inc., Newtown, USA) in an ice bath. The outcome was analyzed similarly to the previous section: through chemometric visualization and description, metabolite detection and annotation of the ^{1}HNMR.

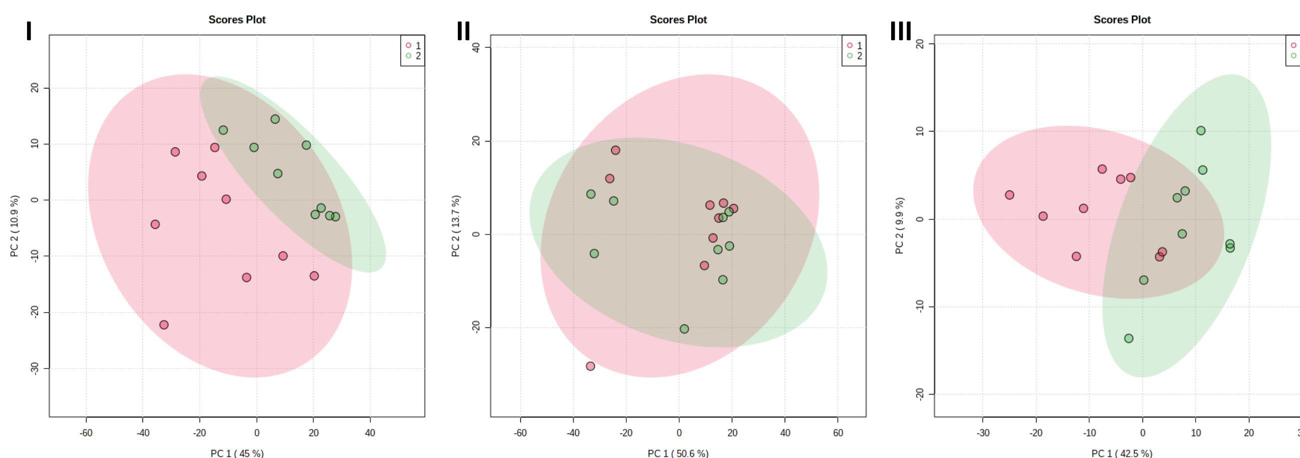

Figure 22 - PCA scores plot with 95% confidence regions of method A (I), method B (II) and method C (III); the two groups correspond to the traditional (red) or with sonication (green) methods, respectively. Each point is a sample.

Visualization of the data through PCA showed in Figure 22 allows to interpret how the variability is distributed between samples extracted with the traditional method A, B or C (in red) or with those methods with a sonication step (in green). Only method A achieves clear separation between groups, though method C isn't far behind with only two samples causing an intersection between the groups. Meanwhile, method B shows overlapping which would mean that there are no significant differences between the traditional method or with a sonication step as analyzed by PCA. For both method A and C, the variability for the traditional method is bigger and spread across PC1 when compared to the sonicated samples. This could mean that sonication uniformized extraction, thus making samples more closely resemble one another. However, further analysis was needed to verify any of these theories.

Table 18 - Measurement of the MIC through inertia per method.

	Inertia Between Groups	*Inertia Within Groups*
Method 1 Traditional	26,62%	73,38%
Method 1 Sonicated	44,76%	55,24%
Method 2 Traditional	58,04%	41,96%
Method 2 Sonicated	66,46%	33,54%
Method 3 Traditional	35,8%	64,2%
Method 3 Sonicated	37,23%	62,77%

The MIC in the form of inertia was also calculated and is presented on Table 18. As explained in the previous section, the smaller the inertia within groups, the more consistency can be found among samples, which translates into a more robust and reproducible method. As is perceivable through Table 18, all tested groups have high within group inertia, even bigger than reported in the previous section. This can be due to the fact that two operators performed these tests, thus introducing an important variability to the samples. Additionally, since samples were processed all at the same time and sonication augments the experimental time, it could be that the traditionally processed samples were impacted by this timing difference, which introduced variability. In general, no method, with sonication or not, seemed to have a good MIC as expressed by the inertia within groups, which could indicate a loss of homogeneity when dozens of samples are processed at the same time.

Metabolite detection was simplified to the average number of peaks as these should represent the metabolic content in the samples, even if not directly proportionally as explained in the previous section. The graphical representation of the average number of peaks per method, either traditional or with sonication, detected through peak picking in the same way as mentioned in the previous section is represented in Figure 23.

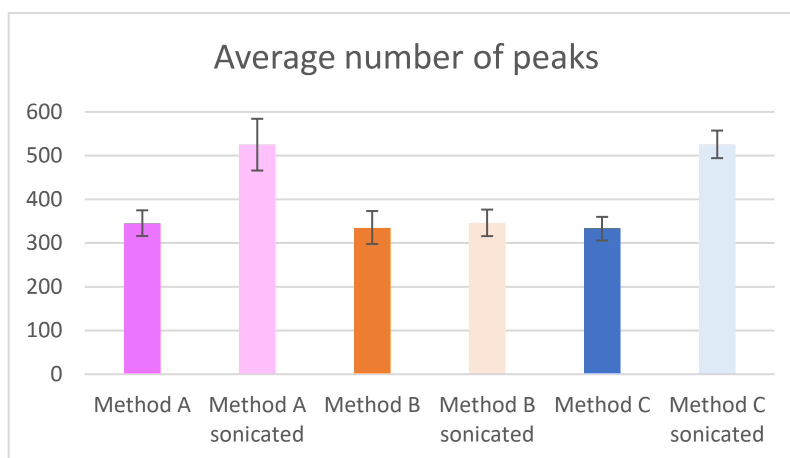

Figure 23 - Average number of peaks between each method, traditional or sonicated (with the SD).

Table 19 – Number of metabolites annotated through either Chenomx or HMDB databases per method, traditional or with sonication, according to the literature reference list (found in Annex IV).[13]

	CHENOMX (43 TOTAL)	**HMDB (10 TOTAL)**	**TOTAL**
Method A	24-33	1-2	26-35
Method A with sonication	29-34	1-2	31-36
Method B	18-34	1-2	19-36
Method B with sonication	26-35	1-2	28-37
Method C	31-33	1	32-34
Method C with sonication	33-35	1-2	34-37

It is clear for methods A and C that sonication increased significantly the number of peaks detected (p-value < 0.001 with a Student's t test), whereas for method B that was not the case. In order to evaluate if the peak number corresponds to a better extraction, spectra were annotated as described in the previous section. The number of metabolites annotated per extraction method is resumed in Table 19 and distributed in a box plot in Figure 24. The intervals in Table 19 indicate the range of annotation per technique, either with Chenomx or HMDB, per samples extracted with each method. In order to best visualize this, Figure 24 shows the distribution of annotation across samples per extraction method.

Across all methods, sonication increased the minimum number of metabolites annotated in a sample but kept the maximum nearly intact. This means that for samples extracted with the addition of the sonication step more metabolites were annotated but that the maximum number annotated didn't change. This changed occurred mostly for annotation with Chenomx, which could be due to a better sensitivity, spectral resolution or processing.

In order to evaluate the impact of this shift, Figure 24 is important to observe the distribution of the total (Chenomx + HMDB) number of annotated metabolites per extraction method. It is visible that for each method the mean of annotated metabolites increases with the addition of the sonication

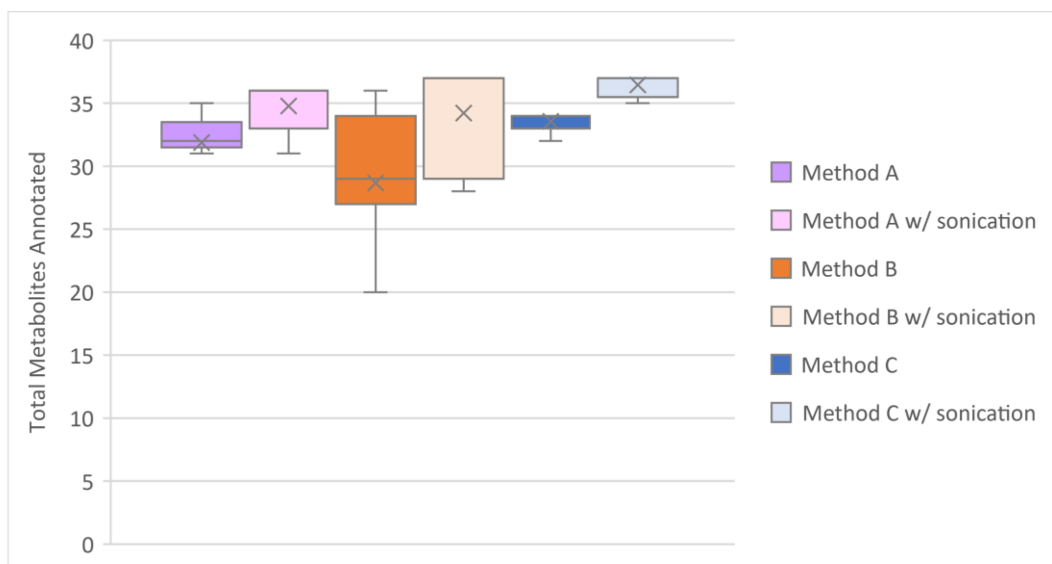

Figure 24 – Box plot of the total number of metabolites annotated per extraction method. X represents the mean.

step. In order to investigate if the groups were statistically significantly different from one another (between traditional and with sonication), a Student's *t*-test was performed for each pair. All methods were statistically significantly different when sonication was performed: method C was the most impacted ($p < 0.001$), followed by method A ($p = 0.018$) and method B ($p = 0.034$). This reveals that annotation was significantly impacted by the addition of the sonication step.

The difference in annotated metabolites varies across different metabolite classes and is not similar across methods, indicating that sonication didn't help extract a particular class or metabolite. Aside from method B, whose experimental workflow is more intricate and introduces more variability, the extraction methods seem to gain in homogenization among samples and uniformity of annotation. Despite the increased experimental time and possibility of metabolite turnover and degradation when samples heat up during the procedure, faster experimental timings could be a mitigating factor. Optimization of the workflow would thus allow to improve homogeneity between samples while incrementing the quality of annotation and thus biological interpretation. As such, initially, this step was thought to be worth implementation.

Despite showing multiple venues for improvement, a workflow should remain fairly simple and streamlined in order to allow the fast collection of the metabolome from dozens of samples in the same assay. To diminish the variability associated with the total experimental length and because not all samples can be processed at the same time due to real-life constraints, for example the number of positions in a centrifuge, the collection, quenching and extraction need to be performed as fast and systematically as reasonably possible. This ensures that samples are collected and treated within a comparable timeframe that makes their phenotype comparable. In this case, for the parameters

explored, two washing steps and sonication were thought to be worthy steps to keep or add, respectively, in the extraction method A.

2.3. Late Trophozoite Extraction Method

The trophozoite stage is the most metabolically active due to the import of metabolites, hemoglobin digestion and preparation for schizogony, with DNA and membrane synthesis. This does not only make this stage an invaluable target for antimalarials, but also a reliable sample for metabolomics and other metabolism-based assays. Because of the extensive hemoglobin digestion at this stage which results in hemozoin accumulation (depicted in Figure 3 and 14)[1,27], parasites can be magnetically purified in the presence of a magnetic field, whereas RBC and other earlier stages can be eliminated or re-cultured. This non-invasive purification technique can be applied prior to many different kinds of assays and has been extensively reported in the literature.[1,14] In *in vitro* metabolomics studies in blood stages published from 2009 to 2023, nearly 42% had samples magnetically purified before the assay began. These studies occasionally included other omics, displaying the usefulness of such a tool. The value of this technique before an experiment is also that not only are the parasites synchronized, but nearly at 100% concentration because non-infected RBCs get sorted out. This decreases significantly the contamination with RBCs, which are generally much more numerous than the parasites in culture, making the obtained metabolome as close as possible to a pure parasitic sample.

Because of the multitude of publications based on this purification step, it seemed interesting to also conduct experiments with this protocol.[14,28] Interestingly, only one study used magnetic cell sorting before a metabolomics assay analyzed through ^{1}HNMR and did not rely on LC-MS analysis.[6] This does not mean this protocol cannot be incorporated for ^{1}HNMR analysis, but it shows that there is a lack of studies in this context making use of the complementarity of both techniques.[3] The studies in the subsequent chapters fill this gap and provide valuable information on not only the joint use of both analytical tools, but on the plants and compounds explored.

A simplification of the methodology applied generally for the metabolomics assays with late trophozoites can be found in Figure 25.[29] $^{13}C_4$,^{15}N-Aspartate and chlorpropamide are used as internal standards to normalize raw ion counts to correct for technical variation introduced during sample handling and ion detection, respectively. It is noteworthy to point out that after magnetic purification, there is one single washing step and one single extraction step before storage at -80°C or immediate drying of the solvent under a stream of nitrogen (N_2). These constitute two main differences with the methods described so far, where multiple washing and extraction steps are carried out, inclusively with different solvents (percentage or composition).

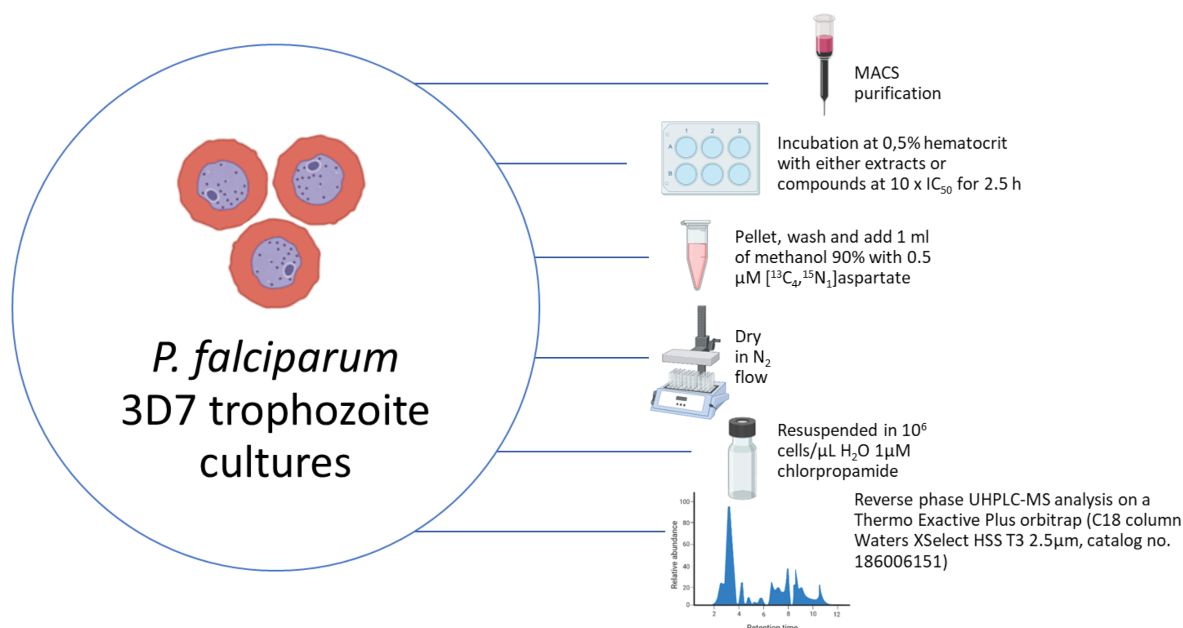

Figure 25 – Metabolomics workflow with extraction method D schematic (Created in BioRender.com).

2.4. Extraction methodology discussion: advantages, drawbacks and troubleshooting

Metabolomics is a broad science that can be applied to a multitude of different samples, but regarding drug discovery, cell culture (*in vitro*) is an uncontested source.[30] Not only does it allow to collect samples in controlled conditions, but its low cost, fairly simple and ethical nature facilitate various types of studies and allow for preliminary data to be drawn out swiftly and robustly. This is also the case for antimalarial drug discovery, although *Plasmodium* sp. cultures require a bit of extra care.

The asexual blood stages, often the most interesting target for antimalarials, have a relatively fast and complex cycle, so the choice of life stage to study is relevant. As referred previously, the ring stage is metabolically less active and thus difficult to study, whereas late trophozoites are more active and easier to study due to non-invasive purification techniques such as magnetic cell sorting. There are other synchronization and purification techniques such as sorbitol and Percoll, but these may introduce contaminants if performed immediately before an assay. Additionally, it is not viable to wait for merozoite invasion post magnetic purification because it relies on available non-infected RBCs that will then also decrease the purity of sample (bellow 100% concentration) and re-introduce RBC-related contaminants.

The sampling is an important factor to obtain accurate and interpretable data. While multiple solvents are reported in the literature, most metabolites in *Plasmodium* samples are hydrophilic, so methanol is the most used for its optimal polarity.[1,31–33] The current trend seems to be to avoid evaporation, and thus metabolite turn over, by choosing an extraction solvent that can be immediately analyzed.[30] This

is a significant point because depending on the analytical technique, this might not be feasible: NMR requires deuterated solvents to reduce solvent background noise and these solvents are fairly more expensive; GC-MS requires volatilization; and LC-MS can variate in mobile phase, column or type of chromatography. The hydrophilic nature of the sample might be an advantage, but water does not extract as extensively as methanol, nor does it rupture or dissolve membranes as easily, meaning that any part of the lipidome would be lost. Evaporation might also allow for samples to be interchangeably used between analytical tools which would allow them to be analyzed from different angles and elicit far more telling results. As demonstrated in the previous sections, despite the lack of metabolomics studies in *Plasmodium in vitro* blood stages analyzed by both LC-MS and NMR, the same sampling can be analyzed by both techniques with no additional steps needed aside from sample reconstitution before analysis, *i.e.,* D_2O for NMR and water with buffers for LC-MS, each with their respective appropriate internal standards. The biggest hurdle in this context proves to be having enough parasites for larger studies, as NMR is less sensitive than LC-MS.

As mentioned thus far, experimental times can alter metabolite turn over or allow enzymatic reactions to happen that change metabolome composition.[30] Additionally, the more steps in the workflow, the harder it becomes to perform on time when dozens of samples need to be processed within a short time frame.[30] A faster and simpler method of extraction like method D might not extract absolutely every metabolite available, but the fact that it does so more reliably allows for the phenotypes to be reproducible and possibly biologically interpreted based on a higher quality dataset.

Finally, no single method can extract the whole of the metabolome due to its wide composition, so choosing the extraction method should depend on the purpose of the study. At this stage, extraction method A with sonication seemed like the best available option. Subsequent assays to define a design were carried out along with a series of "proof of concept" tests. Finally, a comparison between method A with sonication and method D was carried out. These results are described in Section 4.

3. Data acquisition, processing and analysis

3.1. Data acquisition

As mentioned previously, the purpose of this work was to use both NMR and LC-MS data as complementary tools in the elucidation of NP's MoA. This increases the confidence and level of metabolite annotation by matching the two analytical technique's detection, selectivity, and sensitivity.[3] However, as fundamentally different types of data, the merging of the data matrixes obtained by each analytical tool is not straightforward.[3,34] A hardware-based approach, also called hyphenation, was not within our team's reach and is widely considered a cumbersome approach due

to the numerous constraints associated with it.[35–37] Innovative approaches based on cheminformatics or statistics are the most frequent in the latest literature – reviewed recently by Bingol et al.[38,39] These approaches use NMR data to query databases to then calculate the *m/z* of all their possible ions and adducts and characteristic isotope distributions to then match MS experimental data.[34] The opposite approach has also been reported, in which high-resolution MS coupled with liquid chromatography allows the determination of the metabolite's chemical formula. This information is then used to generate all feasible candidate structures which are used to predict MS/MS and NMR spectra. These can be compared to the experimental data and ranked according to the agreement between prediction and experimental results.[40] Other methods relying on incomplete chromatographic separation or none before detection through MS and NMR and based on mathematical data correlation have also been described in the literature.[37] Statistical approaches that correlate NMR and MS data exist and have shown potential to link biological pathway activities with the pathological states being studied.[41] However, none of these tools are routinely used despite their demonstrated applications, potentially due to their limitations. The query of databases and prediction of all possible MS or NMR information creates heavy sets of data that are not easily analyzed. That very same reason impedes most studies of concatenating MS and NMR datasets – the matrix size becomes an issue and amplifies the already underlying problem of the studies having a much larger number of variables than samples or replicates/group. This is inherent to metabolomics assays, as described previously, and significantly diminishes the power of statistical analyses.[3] Additionally, due to differing sensitivities associated with the techniques, not all NMR signals will automatically correspond to an MS ion and vice versa, not all ions will be detected with NMR. This means that the complex datasets still need to be curated in order to tackle this issue and models that try to optimize this process are still under development. As such, most metabolomics studies process and statistically analyze NMR and MS data separately and then integrate the information at the biological interpretation/pathway level.[3,34]

In particular for metabolomics studies with the *Plasmodium* spp., a few restrictions are of note. The cheminformatics approaches described rely mostly on databases for the automatic annotation or correlation of metabolites across datasets and these do not readily exist for the *Plasmodium* spp. And lastly, as an intracellular organism, the number of parasites cultivated *in vitro* for testing is limited. This means that for multiple analytical tools to reliably work at ideal levels, metabolites need to be in high enough concentrations and be accurately detected and annotated with each technique, which is not easily achieved in the context of this work.[41] In many of these approaches, manual assignment and verification also remain necessary, which limits automation.[37]

In light of the tools available, data acquisition was done independently through LC-MS and NMR. After drying under nitrogen flow or lyophilization, as indicated in each experimental section, samples to be

analyzed by LC-MS were resuspended in 10^6 cells/µL in HPLC-grade water with 1µM chlorpropamide for reverse phase UHPLC-MS analysis on a Thermo Exactive Plus Orbitrap. 10 µL were injected on C18 column (Waters XSelect HSS T3 2.5µM, catalog no. 186006151) and ran using a 25 min gradient of 3% aqueous methanol–15 mM acetic acid–10 mM tributylamine-2.5µM medronic acid ion pairing agent (A) and 100% methanol (B). Detection was performed in negative-ion mode, using a scan range of 85 to 1,000 *m/z* and a resolution of 140,000 at *m/z* 200.

Alternatively, samples to be analyzed by ^{1}HNMR were resuspended in buffered D_2O at pH 7.4 with TSP as internal reference and analyzed in a Bruker NEO Ultrashield Plus 700 MHz equipped with a helium cold probe (cryoprobe). ^{1}H-NMR experiments were performed with a CPMG sequence with 128 scans collected over a spectral width of 20 ppm.

3.2. Data processing

In a metabolomics design, multiple conditions are tested simultaneously to allow phenotypes to be compared. For accurate measurements, multiple replicates are necessary per condition, which amplifies the dataset one single study can produce.[42] Additionally, it is frequent that samples from the same conditions have slight variations between them that are not correlated to the assay's conditions but to other factors, such has the analyst, time of the workflow or the calibration of the analytical instrument.[43] Study conditions, such as treatment with antimalarials, induce metabolic changes but these need to be distinguishable from the previous factors. Thus, data treatment is necessary to attenuate unwanted biases and experimental variations as much as possible in order to faithfully extract results from a complex dataset, irrespective of the data acquisition method.[42–44] The focus of the study must not be forgotten: profiling metabolomics studies seek to characterize parasitic pathways, interactions or phenotypes by detecting, identifying and/or quantitating metabolites in a given sample.

As such, raw data or spectra are corrected and treated before any interpretation starts. For LC-MS this means mainly format conversion and data compression followed by peak picking, alignment and filtering in order to centralize all the information about a peak by their *m/z*, retention time and intensity in a way that is comparable across samples.[19,45] [10,31]Other models define regions of interest before applying these filtering algorithms, in which the regions are linked to the presence of metabolites, be it through searching for a threshold of intensity or a minimum number of fragments.[46] Unfortunately, LC-MS datasets are complex and there is no consensus on how exactly the data should be filtered and processed in order to achieve the most faithful matrix.[10,28] From tens of thousands of peaks, often a few hundred to a thousand can be reliably detected and confidently annotated.[19] Indeed, a compound might originate multiple ions by generating multiple adduct species (Na^+,NH_4^+,

and K^+ in positive ion spectra, for example), or common charge-neutral losses ($-H_2O$, $-NH_3$, -HCOOH) upon ionization, but dimers or multimers can also occur.[19] Processing algorithms detect peaks through noise filtering and deconvolution, group and align them by retention time, and deal with missing peaks before originating a feature table that can be further explored or annotated.[19,47]

In this work, a targeted approach was used in the analysis of the LC-MS data: samples were analyzed in the same system and conditions that were used to build a database with over 300 pure standards. This database was curated and built through years of metabolomics data of *in vitro P. falciparum* fingerprinting assays and as such constituted the most reliable tool to annotate the metabolites found in this work's experiments. LC-MS data were acquired as mentioned in the previous section and converted to mzML spectra in MS Convert-Proteo Wizard using a filtering system that performed peak picking through centroiding. Centroiding is a data dimension-reducing algorithm that simplifies peak detection in order to portray clean chromatogram peaks that are more easily processed and analyzed.[19,48] It increases the selectivity and reduces the noise, reducing the number of false positives. Then, data was visualized, corrected and annotated with El-MAVEN. Alignment was performed using the OBI-Warp (also known as 'obiwarp') algorithm with the central QC sample as a reference for retention time correction.[49] The 'Isotope Settings' were set: the isotopic tracer was set to ^{13}C and ^{15}N and the filtering was kept with the default parameters. Peak curation was done automatically by choosing "Compound database search" to match *m/z* and RT values to the inhouse database (with a RT window of ± 2,00 minutes). After the matching was complete, chlorpropamide and aspartate peaks were searched to verify the integrity of the data before each suggested peak annotation was manually marked as "good" or "bad" based on several factors, in order:

1. Peak presence/area in QCs, blanks, water and culture media samples:
    a. Absence in blanks, water (HPLC-grade water with 1µM chlorpropamide) and culture media was privileged,
    b. Presence in QCs and repeatability of area were essential to assure repeatable detection and consistency;
2. Peak alignment – sample peaks should be aligned with QCs and as close as possible to the RT of the standard;
3. C13 labeling – the % labeling of found vs expected should be similar (central QC as the reference sample);
4. Peak area consistency across groups – all samples from the same group should have comparable peak areas;
5. Peak symmetry – chromatogram peaks should be symmetric.

List of confirmed annotated metabolites (marked as "good") were exported as .csv to be processed through Excel.

For NMR, spectrum correction generally starts with the set of the chemical reference, both TSP or DSS have a singlet at 0 ppm. Correction of baseline and phase are important preprocessing steps. Peak alignment across samples is also crucial to reduce chemical shift variations due to matrix effects, as tends to happen in aqueous samples due to pH changes, and improve spectra interpretability.[10,31,50] All these preprocessing steps are critical in reducing sampling and technical variability as much as possible so that they don't tamper with statistical analysis.[3,50] Preprocessing can be achieved through R packages like PepsNMR or in software programs like MestReNova or TopSpin.[3,16] These steps are commonly followed by binning, in which the spectra are separated into bins, or buckets, per a set width interval representing the original spectrum, in ppm for NMR.[51] This technique reduces the data dimension by compressing the data into a matrix representation while also aligning the spectra.[10] The width choice is relevant because too wide bins will include too many peaks and fail to distinguish small peaks from noise, while small bins will divide peaks in smaller parts.[49] This technique originates a data matrix that can be further explored by statistical models.[42]

In this work, ^{1}HNMR spectra were phased and baseline-corrected manually using TopSpin v4. Spectra were stacked and aligned graphically (without derivative use, missing values: linear, reference spectrum active: QC) between δ0.5-9.5 ppm using MestReNova v14. Additionally, solvent regions were removed: residual water region (4.675-5.00 ppm), DMSO – 2.71 ppm (singlet), EtOH – 1.17 ppm (triplet), HEPES buffer – 3.03 ppm (multiplet), MeOH – 3.34 ppm (singlet). Spectra were divided into bins of 0.04 ppm and integrated to the sum of intensities.

Additionally, all data needs to be normalized before factual interpretation is tried.[51] This ensures that the measured intensity observed for every metabolite is equally reliable and on the same scale, thus uniformizing the samples.[43] Normalization is required regardless of the data acquisition tool. There are multiple normalization techniques, including sum normalization, cubic spline normalization, normalizing to a standard peak or the total volume or mass of the sample, or even other factors, such as the number of cells.[19] There isn't a consensus on which method is the best and it remains an area of active research by data analysts.[19,43] In this work, chlorpropamide was used to normalize LC-MS dataset's peak areas per metabolite, which were subsequently corrected with the blank's intensity and filtered based on the RSD (Standard Deviation/Mean) of the QCs. Metabolites were removed from the final dataset when they showed low repeatability (RSD > 25%). Bin tables obtained from NMR processing were normalized to the total spectrum intensity.

Overall, processing tools need to be used carefully in order to assure transposition of accurate spectral data into matrixes. These processing methodologies play a vital role in the accurate result construction that will be explained next.

3.3. Data analysis

After processing, two paths can be taken to obtain results: metabolite annotation or statistical analysis. Annotation, be it before or after statistical analysis, is paramount because only identified metabolites can be studied further or allow biological interpretation.[50,52] The most common kind of annotation is the matching of *m/z* or NMR peaks to databases.[19] However, further exploration of the data should be done to increase confidence in metabolite identification. Ideally, all metabolites should be confirmed by comparison with pure standards, but that is not always possible.

Consequently, four levels of identification exist, as defined by the Metabolomics Standards Initiative, scaling with the certainty of the identification.[19] The highest level of identification, level 1, mandates that two or more properties (*i.e., m/z*, retention time or indices, NMR peaks, chemical shifts and coupling patterns) must match authenticated chemical standards.[19] Software that allows curve-fitting and databases to use the NMR spectra of authentic standards make use of the high ubiquity and reproducibility of NMR to identify metabolites.[10,53] Indeed, despite the same metabolite class having the same chemical groups, it is unlikely that one molecule will have the exact same number of peaks at the defined chemical shifts with the same spin coupling, peak shape and intensity. Thus, redundancy is not a factor and level 1 metabolites can be annotated with NMR. On the other hand, the high variability between analyzers, ionization processes and chromatographic columns make LC-MS level 1 identification far more challenging, since it usually relies solely on the *m/z* and fragmentation patterns.[10] Putative annotation through the probable molecular structure follows as level 2.[19] Similarly, level 3 is also tentative but only the class of metabolite is characterized. Lastly, level 4 is the lowest degree of annotation that happens when the compound is unknown. This system of annotation was designed to implement a standard and ensure reproducible description of metabolites. In the end, putative metabolite annotation should be taken with care, as the confidence on the results and their interpretation may vary.

Databases are an invaluable tool in metabolomics because they allow for the integration of accumulated knowledge regarding metabolite annotation, thus facilitating finding them again in new similar studies.[31] Databases can be built in-house, usually with pure standards, or be freely accessible online through multiple trusted sources – as reported in the review in Chapter 1 section 4.1. A distinction is made between LC-MS and NMR databases, where the variety of LC-MS systems makes exact comparison between different analytical conditions difficult, whereas in NMR spectroscopy, as

long as the magnetic field and solvent are the same, simply matching chemical shifts can be enough to identify a metabolite.[53]

In this work, the LC-MS data was exported as an annotated metabolite table that was analyzed in two ways: log2 fold changes of intensities were displayed in a self-organizing map, or metaprint, for profile comparison; and intensities were loaded directly in MetaboAnalyst v5.0 for statistical analysis.[29,50] Parallelly, the bin table generated by the NMR spectra was analyzed using MetaboAnalyst v5.0 and/or R (packages MBXUCL, PepsNMR and limpca) as stated in each experimental chapter.[16–18] The NMR spectra were annotated using Chenomx NMR Suite 9.0 database and the Human Metabolome Database (HMDB), as according to literature.[4,6]

3.3.1.Statistical analysis

Finally, after spectral data is converted to a matrix, statistical analysis is possible, usually through chemometrics in which an analysis can be performed that doesn't require à priori knowledge of the metabolites present in the sample.[1,19] Its biggest weakness is the necessity of an increased number of samples biologically identical to be able to develop robust and non-overfitting models. Despite metabolomics limited number of samples, between dozens and hundreds, while presenting thousands of variables, the strict workflow allows to diminish experimental factors and link the results to the study's conditions.[10] Chemometrics is, thus, implemented to give sense to the enormous pool of data generated by analyzing spectral patterns or intensities and statistically or mathematically comparing them.[10] This can happen through supervised or unsupervised learning methods, meaning, by taking into consideration the samples classes (*e.g.*, treated vs control) or not, respectively.[31]

The first stage is data exploration that allows the unbiased study of the data's structure, be it trends or clusters.[33] This can be accomplished through unsupervised methods, of which the universal choice is PCA.[10,51] PCA is a clustering algorithm that reduces data dimension by establishing the variable's variance. Thus, a representation of the sample set becomes easily interpretable through a plot where samples group based on their covariance. In metabolomics, important information can be deduced from a PCA: the presence of outliers, if samples variate by aggregating in groups, what variables induce the highest differences between samples and the direction of that variability, *i.e.*, if they are correlated or uncorrelated.[10] Other methods include heatmaps, hierarchical clustering and K-means.[10]

After exploration, hypothesis testing and supervised methods are options to analyse the groups in the study. Hypothesis testing like Student's *t*-test or ANOVA, when there are more than two conditions to be compared, are reliable models that compare the significance or p-value between groups for different hypothesis and are used to distinguish which variables or metabolites are significantly altered between groups.[46,51] FDR correction is important in this context to adjust for the increasingly

small p-values associated with performing these tests with small datasets.[19,46] Volcano plots are commonly relied upon in metabolomics tests as hypothesis-testing plots (through a Student's *t*-test) that simultaneously display the metabolite's or variable fold change in order to indicate biologically pertinent metabolites that differentiate the two groups being compared.[54]

Supervised methods use the given information on sample classification to find patterns, establish models and find variables of interest, and PLS-DA is the most frequent.[51,55] This method looks for the maximum correlation, *i.e.*, the variables (x) that are the most responsible for the study's response (y), thus establishing patterns that can be correlated to sample's class.[33] The variables responsible for the class separation are considered candidate biomarkers and can be ordered by their importance as VIP that can be further interpreted.[55] Its most important shortcoming is the tendency to overfit the dataset, but this can be countered in two ways: by augmenting the dataset, *i.e.*, the number of samples, and by internal cross-validation using the goodness of fit (R^2) and the goodness of prediction (Q^2).[10,33] However, frequently and despite correction, this method leads to a high number of markers that might have biological interest, which increases the hurdle in interpretation.[55,56] Many models have expanded on PLS-DA to try to reduce the number of indicated metabolites with significance to a condensed list of *very* significant and more easily interpretable markers.[21] One such model is sparse PLS-DA and is based on the concept of sparsity in which the coefficients used to appoint variables as significant are recalculated to force the less significant ones to equal zero while reinforcing the most significant biomarkers.[21,55] This penalization leads to a reduced and more selective list of candidate metabolites responsible for the classification, but performance still needs to be evaluated with each test.[56] This can be achieved by doing cross validations that use a subset of data to calculate the classification error rates.[56]

These methods can be used with or without annotation, thus they can reduce significantly the burden of metabolite annotation by guiding the analyst towards the most important peaks and features to be identified or quite simply just by shedding light on what pre-annotated metabolites can be responsible for a biological response.[10]

Finally, after significant variables have been discriminated through statistical models and identified, mapping of metabolic *Plasmodium* networks can be done through databases and platforms, such as Metaboanalyst, KEGG, among others.[10,46,50] In this work, Metaboanalyst's "Pathway Analysis" module was used with the "*Plasmodium falciparum* 3D7" pathway library selected which used KEGG pathway information. KEGG was used further to confirm the results and have a bigger view of the pathways and possible interactions between them. Fitting of metabolites in particular pathways reveals their

function in the parasite and their relevance for survival, infectivity or other factors that help establish phenotypes and elucidate MoA.

4. Proof of concept

Despite the amount of published studies in the literature, there aren't studies that report phenotyping drug or natural compounds effects on the *P. falciparum in vitro* through both LC-MS and NMR. Additionally, despite general conflating workflow steps, like spinning and washing, each laboratory will have access to different instruments, columns and systems, making every protocol unique in its own right. Because of this, parameters other than the extraction method had to be studied in order to develop a reliable workflow that could be performed by different researchers under the same streamlined conditions.

The design of a study is an important factor. It is what allows for comparisons to be made between groups, for example treatments, which is the purpose of this work. The design is comprised of many factors which can become limitations depending on the metabolomics study to be performed. The number of samples is an important parameter because the robustness of statistical analysis is greater the more replicates exist when compared to the number of variables, as explained previously. In *Plasmodium* metabolomics, this correlation is severely skewed, not only because there are thousands of variables for one single sample, but because parasitemia is a limiting factor alongside how many samples can be handled simultaneously. Even if parasites are cultured in hundreds of milliliters, a great number is necessary per sample to elicit reproducible signals depending on the analytical approach, and even more so here since the aim is to perform both LC-MS and ^{1}HNMR. Additionally, losses during

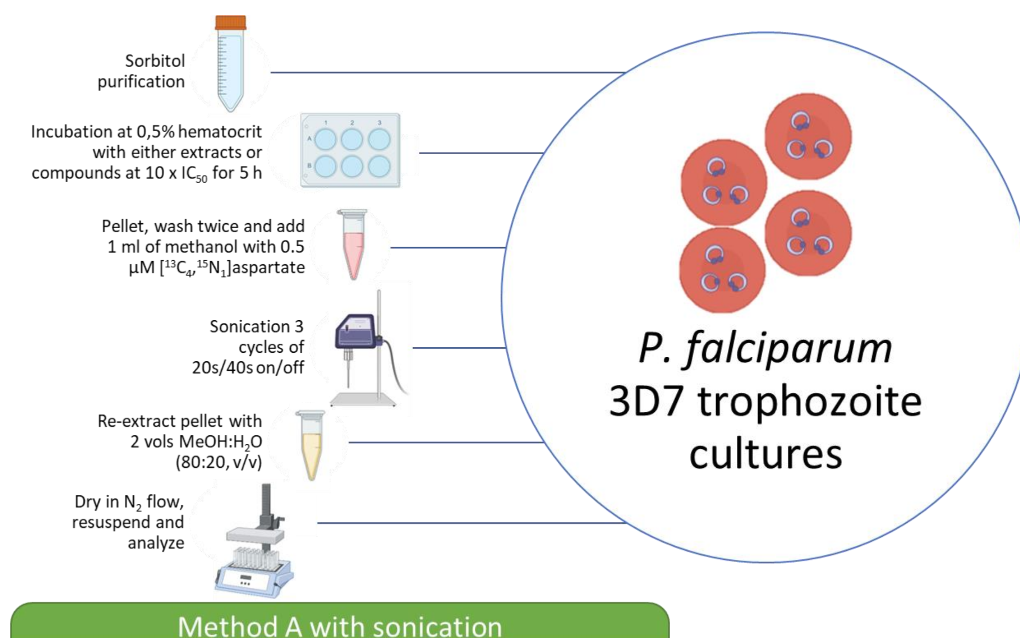

Figure 26 – Metabolomics workflow with extraction method A with sonication schematic (Created in BioRender.com).

the experiment are inevitable, due to purification or just transferring from plate to Eppendorf, meaning the amount of initial parasite counts needs to be high enough to account for all these factors. Furthermore, it would seem ideal to study multiple timepoints because the parasite evolves so greatly within a fairly small timeframe, however, since multiple conditions are studied at a given moment in order to make comparisons possible, the number of samples and necessary parasites also increases. Constraints associated with time, instruments and overall technical differences that introduce biases among replicates are also pertinent factors that might destroy any biological interpretability by making samples far too different from each other within groups for reasons other than treatment.

As such, an initial test was carried out to investigate which time should be used to incubate the parasites with treatments when carrying out an assay so that the differences in phenotype would be maximized between groups. To keep the number of samples relatively low, only three groups were tested: control (DMSO), artemisinin and chloroquine. These two antimalarial drugs were chosen because they reportedly elicit different fingerprints due to their divergent MoA.[25,29] DMSO was added to control to account for the drugs' vehicle (0.1% v/v) and both artemisinin and chloroquine were used at 10xIC_{50} as commonly reported in the literature. At this time, method A with sonication (shown in Figure 26) had been optimized and was considered the best extraction method, given the results. So, this was the method used to extract samples incubated at 0h, 1h, 2h, 4h, 6h, and 24h. Samples were

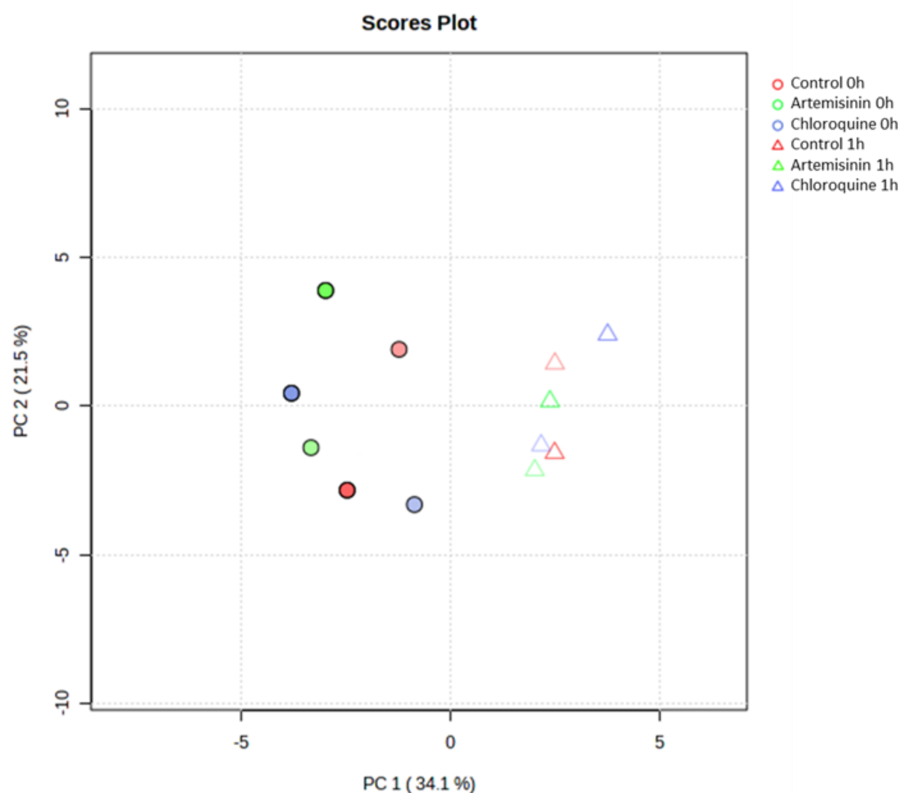

Figure 27 – PCA scores plot of samples incubated 0h or 1h. Colors represent treatments (control, artemisinin and chloroquine), symbols represent incubation time (circles 0h or triangles 1h).

analyzed through ^{1}HNMR and annotated as reported previously. Bin tables were analyzed in MetaboAnalyst v5.0.

When exploring the data, a first comparison between 0h and 1h of drug incubation made it immediately obvious that one hour is enough to show a degree of differential variability through PCA as can be seen in Figure 27. Samples could be separated by time across PC1 rather than by group, with no clear distinction between treated and control samples, which portrays the impact that just time can have. This information is not enough to comment on the speed of action of the drugs or necessarily how one single hour is enough to change the parasite's metabolism, but it is important to identify that long experimental times can have important repercussions on the outcome. This could mean that long workflows (close to 1h) could introduce a higher variability to samples that will mask the treatment's effects. Since the average length of method A with sonication is between 1h to 1h15 per sample, it becomes clear that when dozens of samples are involved that cannot be processed simultaneously, this experimental time becomes a factor that should be taken into consideration during data analysis.

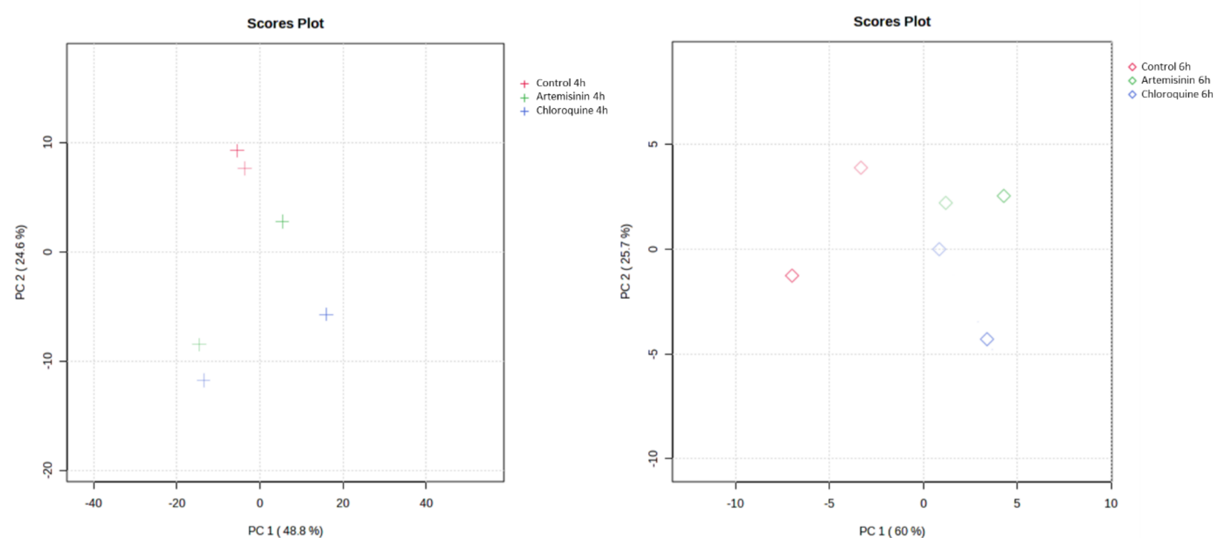

Figure 28 – PCA scores plot of samples incubated 4h or 6h. Colors represent treatments (control, artemisinin and chloroquine), symbols represent incubation time (cross 4h or rhombus 6h).

Upon further exploring the data through chemometrics, it became clear that there was a maximal separation between groups at 4h and 6h as can be observed in Figure 28. At 4h incubation with treatments or DMSO (control), the PC2 separated the groups although with a bit of PC1 influence. Only at 6h can the control be separated from the treatments through the PC1 (60%) and each individual treatment by PC2 (25. 7%). As there were only two replicates per group, this data can only be accounted as preliminary and its analysis as indicative.

As such, an incubation time of 5 or 6h would seem ideal when trying to maximize the output of these metabolomics tests. However, when the samples were observed under the microscope through a

Giemsa stain, counts gave a different perspective. Figure 29 shows the parasitemia per group at each studied time. It is perceivable that control parasitemia peaks around 2h before sinking, showing barely any signs of recovery by 24h.

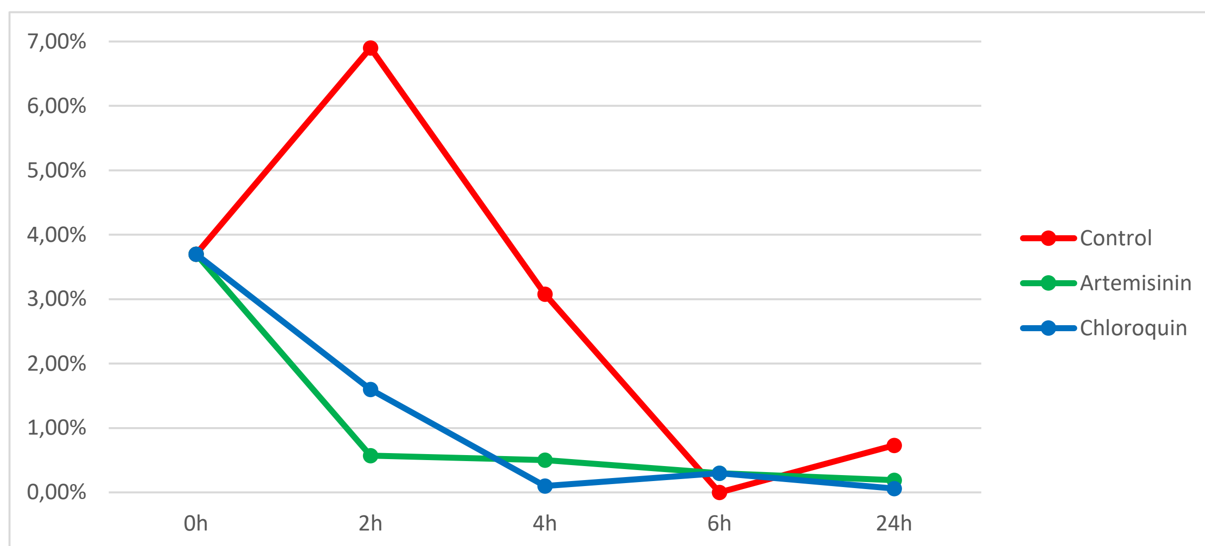

Figure 29 – Parasitemia (%) calculated by Giemsa stain and microscopic observation.

Despite starting from the same culture at the same parasitemia, the evolutions in parasitemia are clear: treated groups loose parasites overtime, consistent with the drugs being effective, however the control shows a peek. This peek was unexpected because as the parasite replicates every 48h and the tests were conducted in ring-stages, the parasitemia was expected to remain the same on average across the 24h. A near duplication in parasitemia by 2h meant new invasion by merozoites, which is possible if the culture is not tightly synchronized. This increase in parasites led to natural death overtime due to overpopulation as it happens naturally when the resources are finite. At this time, cultures were synchronized with sorbitol frequently, but upon these results, the importance of a tighter synchronization before metabolomics assays became clear and the protocol was adjusted.

With these results, the parameters of the metabolomics studies seemed to have been found: with a tightly synchronized ring-stage culture, 5h incubation with the treatment of choice followed by extraction with method A with sonication. Two tests were independently conducted to confirm these conditions and samples were analyzed by ^{1}HNMR. The same treatments in the same concentrations were used.

Chemometrics visualization can be seen in Figure 30. In both tests, PC1 separates control from treatments and describes most of the control group's variability. PC2 separates the treatments in the first test, while not accomplishing this in the second test (Figure 30B). Annotation of the bins responsible for the distribution in Figure 30A revealed lactate to be significantly different among control samples and between this group and the treatments, which could be due to the fact that parasites in the control group are metabolically active and thus producing lactate in higher quantities

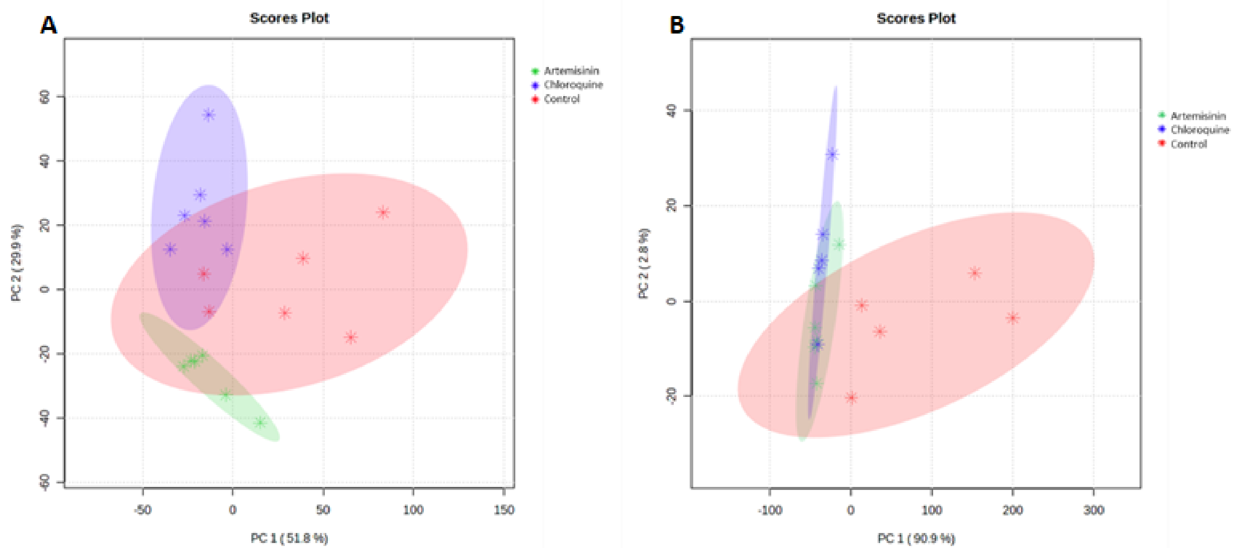

Figure 30 – PCA scores plot with 95% confidence regions of samples incubated 5h, (A) first test, (B) second test. Colors represent treatments (control, artemisinin and chloroquine).

through glycolysis. This can be assumed because the intraerythrocytic form of the *Plasmodium* is known for relying mostly in this pathway for energy.[1] The separation across PC2 seems to occur due to a bin that can have multiple annotations, like aspartate and/or sarcosine, in which the artemisinin treated group has less intensity. These same metabolites are responsible for Figure 30B PC2 (and to some extent PC1) separation, with this test pointing to other amino acids and GSH as metabolites of interest to distinguish treatments from control in the second test.

To further explore these datasets, PLS-DA was also used to investigate if a better separation could be achieved and, if so, what metabolites were responsible. Figure 31 resumes the results for the first (A) and second test (B) with their respective important features or loadings.

For the first test, components 1 and 3 are plotted because they display a better group separation with increased R^2 (0.57 and 0.74) and Q^2 (0.34 and 0.43), whereas component 2 was not able to separate the treatment groups and had a lower Q^2 (0.36). Groups are skewed across both components, revealing that neither axis achieves clear separation by itself. However, seeing the percentage of variation represented by each component, only the top 15 bins responsible for the separation across component 1 (47% variability) were investigated. Annotation of these bins confirms the impact of lactate and the same aspartate and/or sarcosine bin, but brings attention to many other metabolites of different classes. Glucose appears in different bins with an overall similar relative concentration among groups with an opposite tendency from lactate. Notably, lactate is less expressed in control samples while glucose is more, consistent with glycolysis being an active pathway. Contrarily, treated samples show the opposite tendency. Acetate is less expressed in treated groups, which can be in line

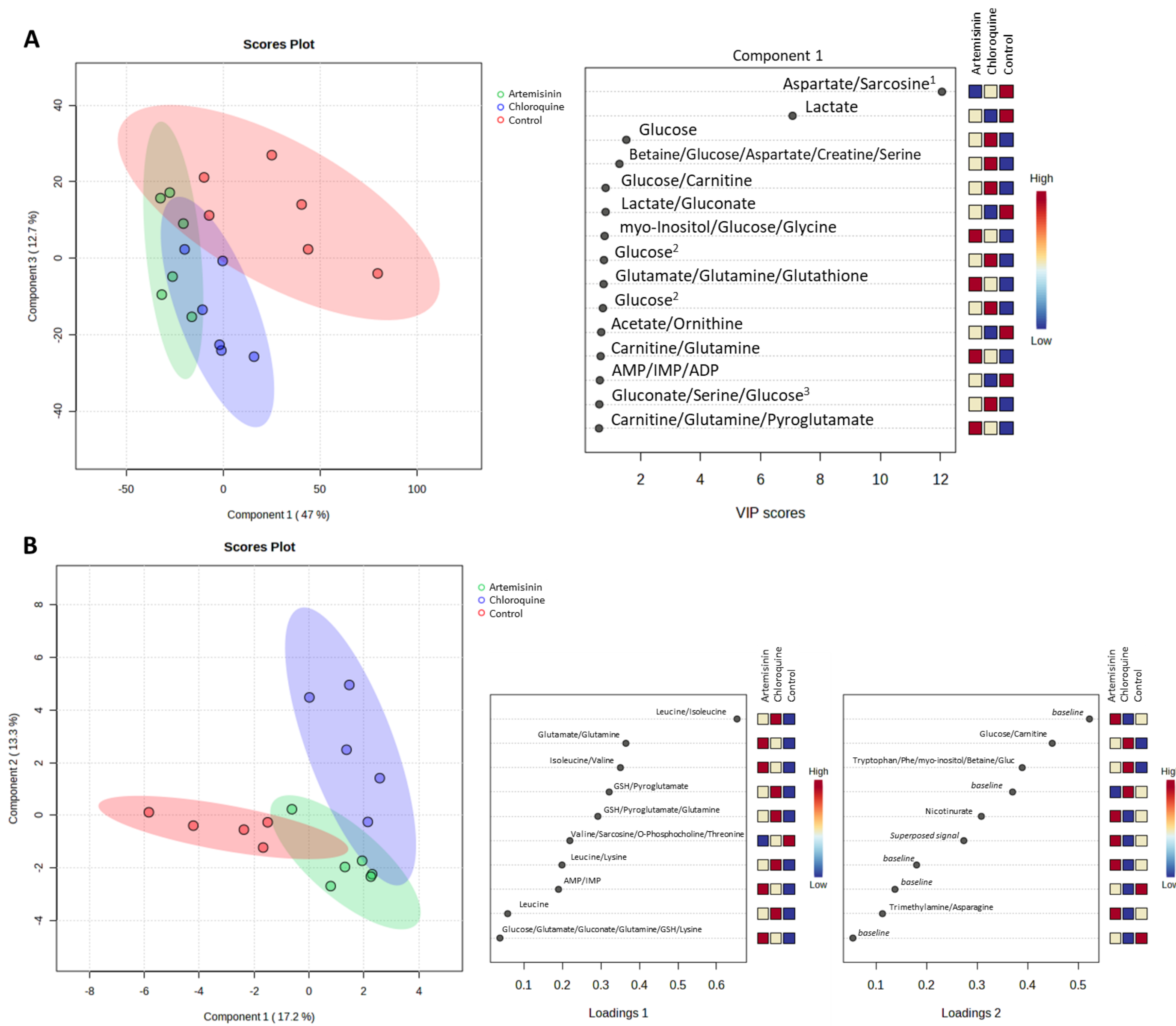

Figure 31 – PLS-DA and sparse PLS-DA scores plots with 95% confidence regions displayed of the proof of concept studies 1 (A) and 2 (B) with the VIP and loadings plots, respectively. The colored boxes to the right of the VIP scores and loadings represent the relative spectral intensity of the corresponding bin in each group under study.

[1] – DMSO peak influence,
[2] – Water peak influence,
[3] – HEPES peak influence.
(A - R^2: PC1 - 0.57, PC3 - 0.74; Q^2: PC1 - 0.34, PC3 - 0.43; B – Error rate: PC1 – 37.5 %; PC2 – 25%)

with an increased consumption to produce acetyl CoA to enter the TCA cycle as a glycolysis substitution. This is supported by the increase of glycine and serine in treated groups, central metabolites to amino acid biosynthesis that participate in carbon metabolism. Additionally, the effects on these two metabolites along with aspartate and ornithine reveal downstream effects on hemoglobin metabolism, as they are mainly obtained from this source.[57] Accumulation of ornithine is toxic for the parasite and thought to be the target of maintenance systems that prevent it, through membrane transporters and/or enzymatic activity, and could be a sign of downstream effects of these antimalarials.[57,58]

Finally, glutamate and glutamine are involved in many major pathways: carbohydrate metabolism, energy, and amino acid metabolism. Glutamate is directly obtained from hemoglobin-derived peptides and their higher relative concentration in treated samples might reflect a rerouting to the TCA cycle.[1] Finally, effects to glycine, glutamate, and glutathione all express higher relative concentrations for the artemisinin-treated group as opposed to others, which shows a clear interference at this level in line with a recognized artemisinin effect on redox systems.[1]

For the second test, PLS-DA didn't improve separation when compared with the PCA, so a Sparse PLS-DA was performed instead and the result is shown in Figure 31B. Pathway analysis of the first component loadings confirms the effects on the hemoglobin metabolism by pointing to the amino acids that are precursors of aminoacyl-tRNA biosynthesis along with amino acid pathways. Again, glutamate and glutamine have relative concentrations between groups that tie these amino acid effects to carbon metabolism, supporting the theories of the first test. Interestingly, lysine and leucine are indicated in this assay instead of ornithine, though they correlate to this amino acid by competing for transporters involved in the transporters of arginine, the precursor of ornithine.[59] This still points to effects on the complex balance of ornithine to prevent toxicity and use this amino acid in other metabolic pathways, consistent with the results of the first test. Analysis of the third component loadings points to other amino acids affected, though it also highlights a limitation of this technique: the solvents' effects. As pointed out in the caption of Figure 31, there are multiple bins affected by the presence of solvents despite their manual removal from the spectra. Additionally, the *"baseline"* description indicates zones of the spectra where a signal could not be distinguished from a possible baseline shift. For the *"superposed signal"* tag, it remarks a zone of the spectra where deconvolution was not possible.

Better resolution could have been achieved with a stronger magnetic field, although 700 MHz is already considered a high-field instrument.[2] Deconvolution would have also benefited from a stronger magnetic field as it increases sensitivity, contrary to the 2D NMR techniques that could have been

implemented.[2] In this case, these techniques would have suffered sensitivity-wise from the limited amount of biological material as described thus far. Finally, acetyl CoA, oxaloacetate, oxoglutarate, and spermidine were not found in the samples possibly due to their low concentrations. However, detection and investigation of their trends would have confirmed the link between the aforementioned metabolic pathways and allowed for a more in-depth comment on the effect taking place per treatment. Techniques like LC-MS would be complementary in this case.

Interestingly, lactate does not get highlighted by PCA loadings, ANOVA or PLS-DA as an important metabolite to distinguish groups in the second study, but glucose does, pointing to the precursor rather than the end product of glycolysis, but showing that this pathway is differently expressed between control and treatment, which is in line with the parasites being metabolically active or not, respectively.

These two datasets were also used to study the impact of preprocessing parameters in ^{1}HNMR and choose the parameters to be used in data analysis henceforth. Namely, normalization is an important step that uniformizes datasets and makes statistics all the more powerful in searching for significance and patterns. There are multiple ways of doing this, such as through the spectral or total intensity sum, probabilistic quotient normalization, quantile, zero-sum regression, number of cells among others, as discussed previously.[42,51] The best technique should be the one that most faithfully makes the samples comparable while not losing data that could be significant towards distinguishing groups and biologically important factors. Additionally, data transformation might be necessary to distribute the data normally and as such allow for many statistical methods to be used. This processing can be done through logarithmic transformation, Pareto, mean centering or auto scaling, among others, and it does not change the samples, only their scale.[51]

Four normalization techniques were tested: total intensity sum, probabilistic quotient normalization, quantile and number of cells. The data were compared by PCA, K-means and one-way ANOVA to verify which processing accounted for the most relevant biological information, including information on outliers. Overall, total intensity sum was the normalization method that pointed to better group separation by PCA, better non-hierarchical clustering outcomes through the K-means method, and more biologically interpretable significant bins through ANOVA that represented biological differences confirmed by spectra observation. A supplementary step of mean centering was then added to center the normalized curve to 0 before statistics is correctly carried out. A summary of the processing of ^{1}HNMR spectrum from acquisition to statistical analysis can be found in Figure 32. These steps had already been described in section 3.

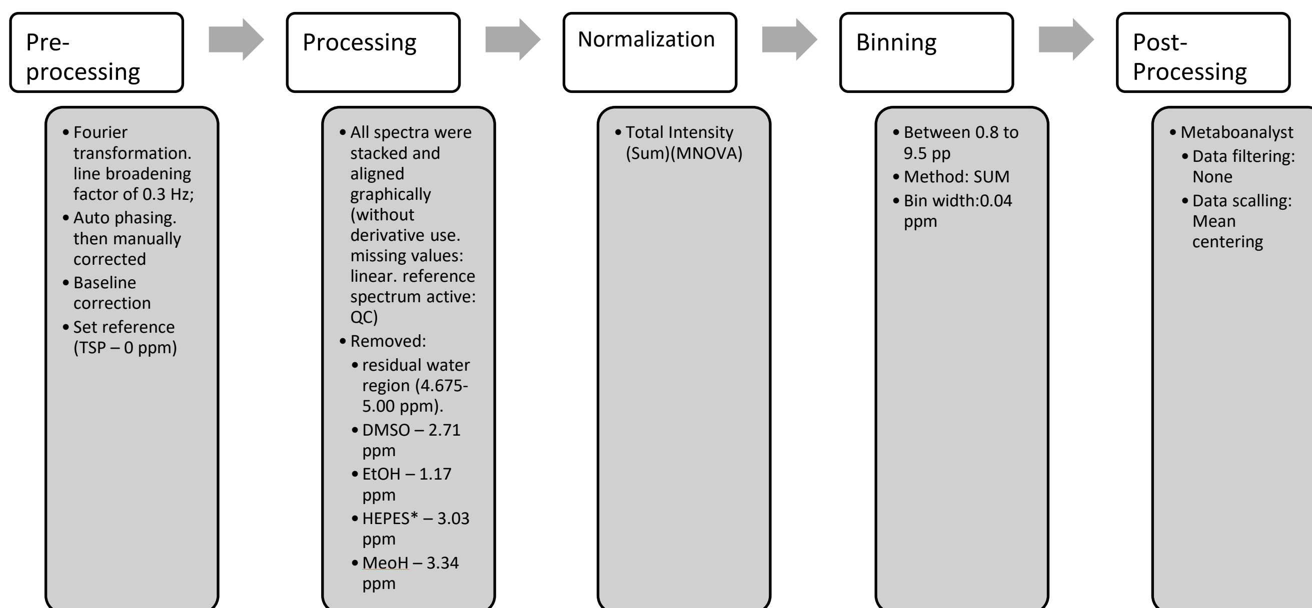

Figure 32 – Summary of the processing workflow of ^{1}HNMR spectrum.

A third assay was carried out under the same conditions but with the addition of a natural compound of interest (poupartone B) to evaluate if the design would be able to still separate the treatments from the control and amongst each other. This compound's MoA was unknown, but being an active compound *in vitro* (IC_{50} = 0.69 ± 0.200 µM) it should elicit a phenotype and serve as a basis to show if the experiment is valid.[60]

Chemometric visualization can be seen in Figure 33. Despite not separating the control group as clearly as in the assays before, the control was separated from treatments. Interestingly, chloroquine and poupartone B treated samples seem to cluster together, which could point to similarities in their metabolic fingerprint. As chloroquine is a known inhibitor of the heme biomineralization pathway in the parasite's DV, poupartone B could share an effect on hemoglobin metabolism or interfere with important processes at the DV.[1] Despite their big intragroup variability across both PC1 and 2, these were exciting results that paved the way forward.

Analysis of the PCA loadings shows that many amino acids are differently expressed between poupartone B and chloroquine versus artemisinin and/or control, namely cadaverine, ornithine, asparagine, histidine, phenylalanine, among others. Both chloroquine and poupartone B might affect amino acid synthesis in multiple different ways, be it through interference in hemoglobin metabolism, amino acid import/export or peptide metabolism and synthesis. Regardless, these tests were positive preliminary results that green-lit further exploration with other plant extracts and natural compounds.

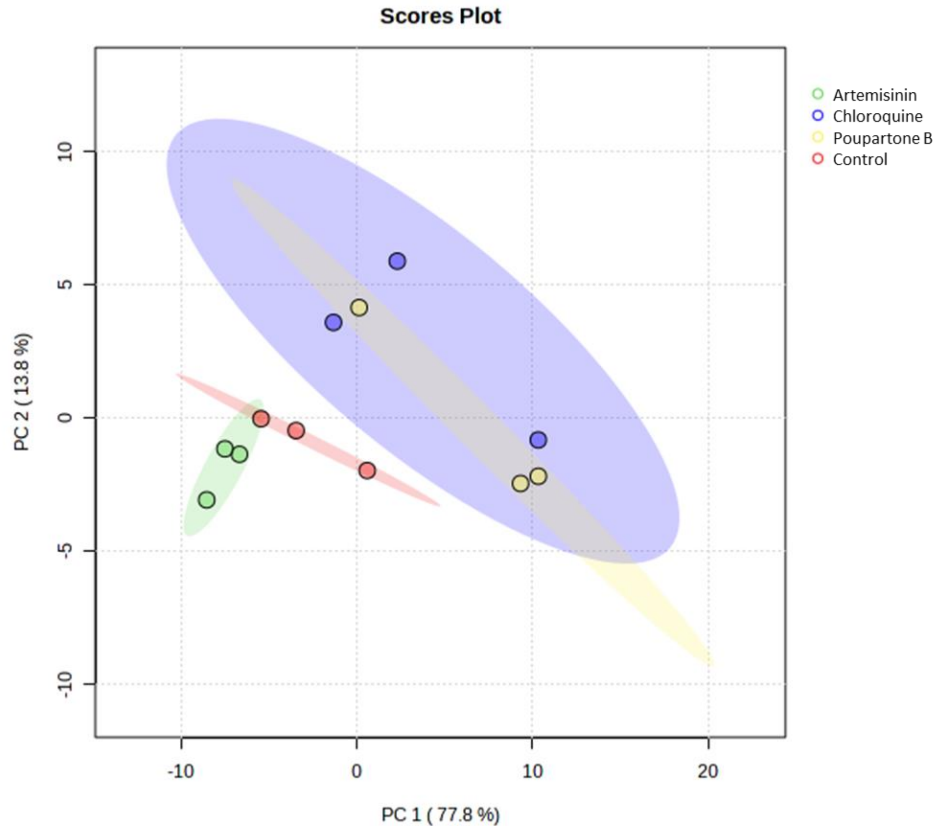

Figure 33 – PCA scores plot with 95% confidence regions of samples incubated 5h with different treatments represented in different colors (control, artemisinin, chloroquine and poupartone B).

4.1. Interchanging methodologies

Despite the work and expertise gained in the efforts of developing an optimal workflow, it became increasingly obvious that the long experimental time would become problematic once more than four conditions were tested. The more antimalarial drugs used to compare metabolic signatures with NP samples the better. Moreover, the robustness of the statistical models to indicate metabolites related and influence by the effects of treatment are directly influenced by the number of replicates, which needed to be at least three.[18,61,62] Additionally, despite the interest in studying the trophozoite ring stage, it became clear that the potential to obtain information was relatively lower to late trophozoite stages, where purification techniques and advanced parasitic metabolism could diminish RBC contamination and expose more clearly different MoA. As such, and upon a stay at the Department of Biochemistry and Molecular Biology and the Huck Center for Malaria Research at Penn State (The Pennsylvania State University) to use Manuel Llinás Laboratory's in-house database of plasmodial metabolites, another method of extraction was suggested. Named method D as shown in Figure 25, this method had been developed in this lab and published in more recent research papers in this context.[23,25,29] The opportunity to learn directly and use the same inhouse methodology and material allowed for a direct comparison on whether a few parameters could be optimized so that the final metabolomics assay will be as robust and informative as possible.

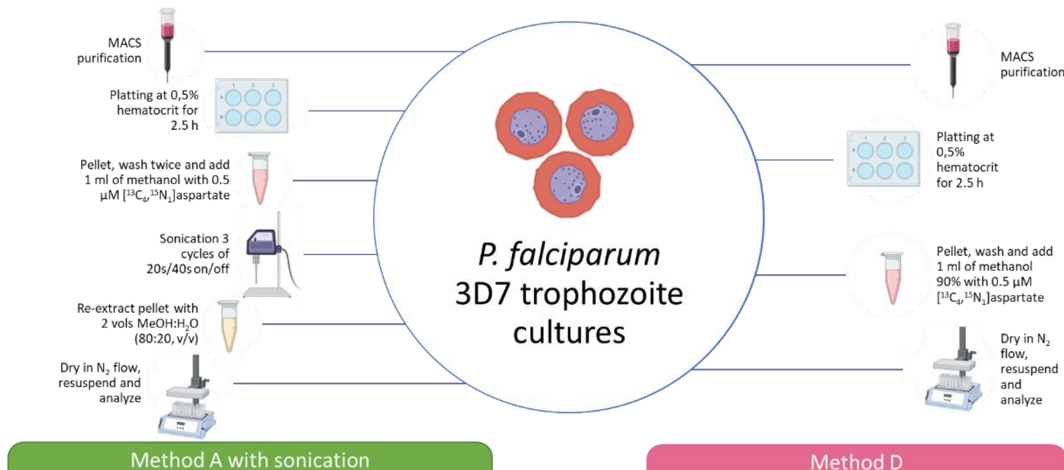

Figure 34 – Metabolomics workflow with the extraction methods under study: method A sonicated on the left, and method D on the right (Created in BioRender.com).

To verify this, a study was carried out at three independent times following the protocol in Figure 34. In this study, purified late trophozoite samples were extracted with either method A with sonication or method D. In this circumstance, parasites were not incubated with a treatment, but simply plated and allowed to stabilize before extraction. Analysis was performed both through LC-MS and ^{1}HNMR as described previously.

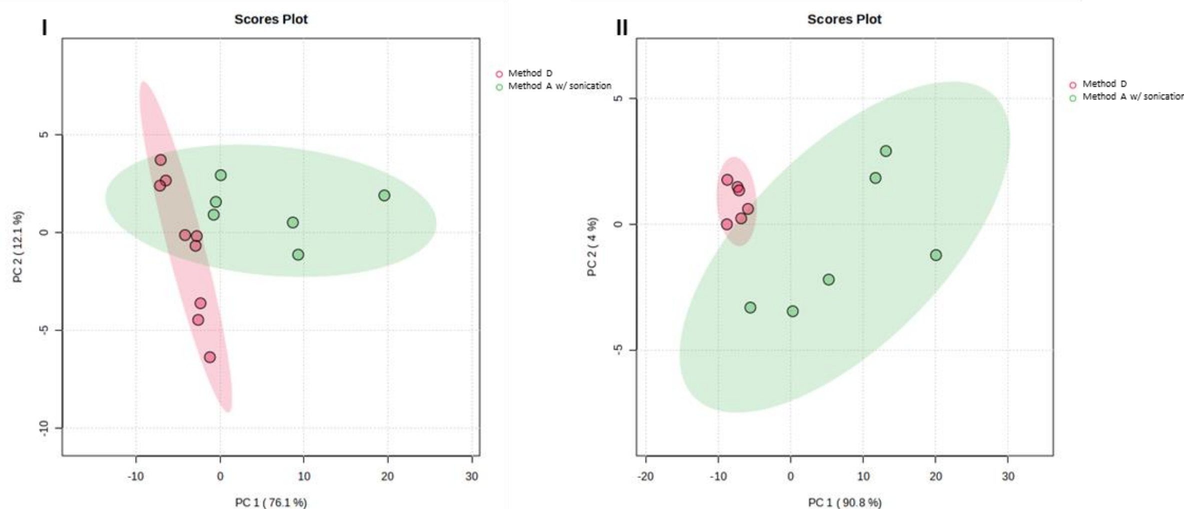

Figure 35 - PCA score plot with 95% confidence regions of samples extracted by method A with sonication and method D analyzed by LC-MS (I) and ^{1}HNMR (II), respectively.

Chemometric visualization of both LC-MS and ^{1}HNMR analysis can be found on Figure 35. It is clear through both analytical approaches that method A with sonication is far more variable, presenting its distribution across both PC1 and 2, for 88.2% and 94.8% variability each tool, whereas method D has its variability described mostly by the PC2 in both techniques, which amounts to 12.1% and 4% variability each, depending on the analytical tool. This overview is enough to present method D as far more robust and reproducible than method A with sonication.

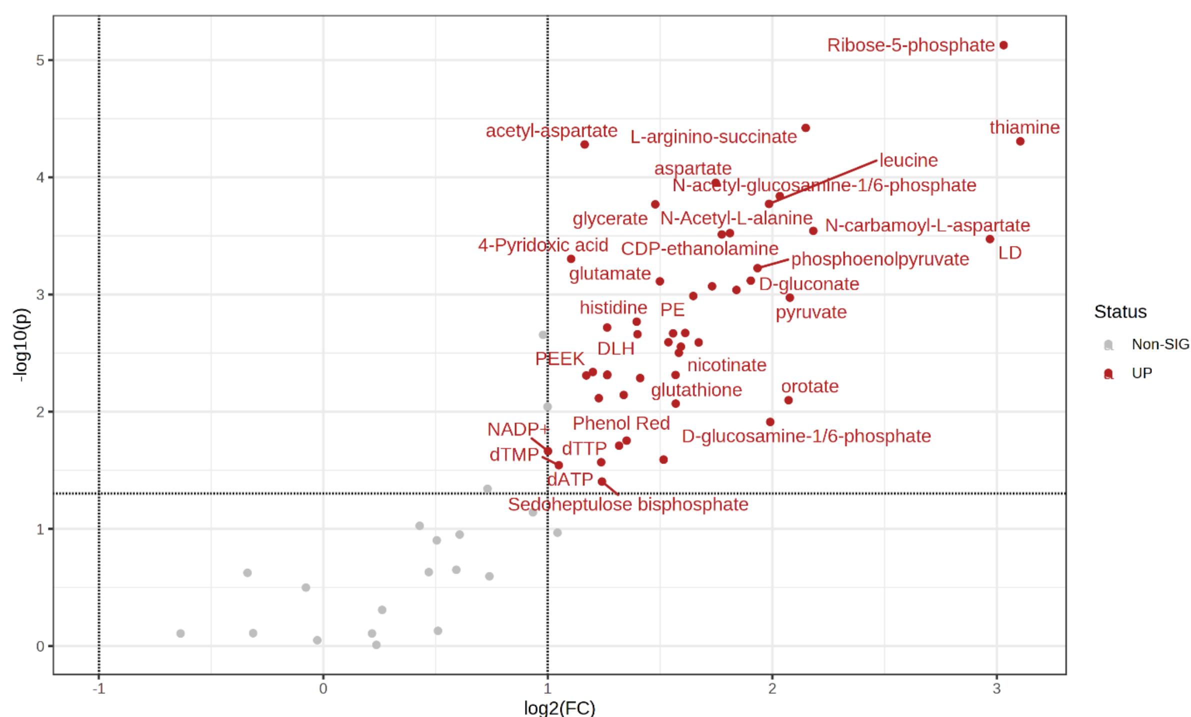

Figure 36 – Volcano plot of LC-MS data – method D/method A with sonication (*p-value: 0.05, FC threshold: 2.0).* Points represent metabolites: in gray (non-significant), red (positive fold), blue (negative fold).

In an effort to understand how these methods were different, the data were explored further. Namely, a volcano plot (p-value: 0.05, FC threshold: 2.0) was performed to quickly see how *t*-test significant metabolites are expressed between methods, and the result can be found on Figure 36. All significant metabolites have a positive fold change in favor of method D, which indicates that they are more present or more reliably extracted and detected through this method. As the solvent of extraction is the same, polarity doesn't seem to be a factor. Alternatively, other factors should be considered.

The quicker and simpler workflow elicited faster sampling which might have reduced metabolite turn over, losses between washes and extractions and the possibility of contamination. This can be correlated with a more consistent and reproducible detection of metabolites. Moreover, a quicker protocol allows for a bigger number of samples per study as the variability associated with experimental times is diminished. Considering the amount of NP and antimalarials to be tested in each study and ideally in triplicate, method D was chosen for subsequent studies in order to have reliable data for biological interpretation. A new proof of concept was not carried out, as the adapted method was the in-house method of the hosting lab and had been previously published in numerous papers.[23,25,29] Therefore, every case study in Chapter 3 followed a workflow with metabolome extraction method D.

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1. EXTRACTS & COMPOUNDS

Chapter 3

Exploration of Modes of Action of Natural Compounds and Extracts

The work presented in this chapter was carried out in collaboration with Pr. Manuel Llinás and Dr. Gabriel W. Rangel at the Huck Center for Malaria Research (CMaR) at Penn State (The Pennsylvania State University) which kindly allowed the use of their inhouse LC-MS database for metabolite annotation.

1.1. Foreword

Traditional medicine often relies on preparation of the plant before consumption, examples being infusions, decoctions, tinctures or even pastes.[1] In research, the first approach is to learn how the plant is traditionally used in order to prepare it the same way before exploring a wide range of solvents or methods of extraction. Routinely in antimalarial drug discovery, *in vitro* screenings with growth inhibition assays on *P. falciparum* of traditionally used plants guide the interest in further exploring these plants or not. If the crude extracts have interesting IC_{50} (< 15 µg/ml, deemed highly active at below 5 µg/ml), they are often further analyzed by bioassay-guided fractionation.[2] This technique relies on *in vitro* results to further fraction an extract or fraction through chromatographic separation techniques until the compound(s) responsible for the perceived activity can be isolated and identified.[3] This (these) compound(s) can then be used in other types of studies in order to evaluate its potential as a new bio-based drug.

Although this technique has been shown to be effective in accomplishing the isolation and identification of new active NP, this funnel-like approach invariably leads to losses along the way. Traditional medicines rely on the use of a full plant part, for example leaf or root, and upon isolating a single compound, important interactions between the constituents of the complex mixture will be lost.[4] Metabolomics can be used in conjunction with bioassay-guided fractionation of a plant extract to make use of the MS sensitivity and molecular databases through molecular networking to detect and annotate metabolites and correlate them to a biological activity.[5,6] Further isolation of NP can lead to identification and/or confirmation, and further exploration through metabolomics on a biological assay can elucidate their MoA. In this work, the focus was on the MoA of previously identified and described NP, but it is noteworthy to mention that this approach exists.

In the context of plant screenings, it becomes relevant to ask whether metabolomics can offer insight regarding the potential MoA of an extract or fraction in early stages of investigation or if it can only be reliably used when a purified active compound has been isolated. To answer this question, a brief study using three plants and respective isolated compounds was performed as described ahead.

Figure 37 - A. annua L.[8]

Artemisia annua L.

The Asteraceae plant family has a long history in traditional medicine, of which the most known *A. annua* (see Figure 37) for its contribution to the antimalarial pipeline.[1,7] This commonly used plant in Chinese Traditional Medicine, also known as

sweet wormwood, sweet annie, sweet sagewort, annual mugwort, annual wormwood, or Qing Hao, has been used for more than 2000 years for multiple ailments.[8] It was first recorded as a cure for malaria in the book **A Handbook of Prescriptions for Emergencies** by Hong Ge (283-363), a physician of the Eastern Jin Dynasty, where it stated "*administer the juice wrung out of fresh A. annua L.*".[8,9] This report and repeated use along the centuries validated the rational of its use, which was later confirmed in a study with the subsequent discovery of artemisinin as explained previously.[8,9] Other studies have since reported on its antiparasitic activity on other parasites, along with antiviral, antifungal and antibacterial activities.[10] Multiple variants of this plant are cultured everywhere in the world and interest remains in trying to categorize or explore further other compounds in this plant that might have antiplasmodial activity. The plant's activity was first described by Youyou Tu in an ethyl acetate extract and later it was revealed that artemisinin is extracted more easily by less polar solvents.[8,11] As such, this extract and compound were part of this preliminary study.

Cinchona officinalis L.

This plant is perhaps one of the biggest and earliest examples of traditional medicine being explored through modern medicine approaches to a remarkable success.[12] *C. officinalis* (Figure 38), also known as Peruvian bark or yellow bark, is a tree of the Rubiaceae family naturally occurring in Central and South America where it was well known for its antipyretic properties.[13–15] Upon investigation of its bark, quinoline alkaloids were isolated, of which quinine was subsequently approved as an antimalarial treatment, as mentioned previously.[16] However, quinine was not the only alkaloid from this class to be isolated and identified, there were also quinidine, cinchonine and cinchonidine, all of which have antiplasmodial activity.[16,17] Dozens of alkaloids have been isolated from different *Cinchona* sp. barks with variating degrees of activity *in vitro*, and interestingly, the combination of alkaloids, such as quinine with quinidine and cinchonine has showed 2-10 times lower IC_{50} against quinine-resistant strains.[17] This is relevant because standardized mixtures of quinine, quinidine and cinchonine have proven to be as effective against malaria but less inducing of side effects such as cinchonism, a syndrome characterized by neurological, cardiovascular and gastrointestinal toxicity. This is a testament to their synergetic

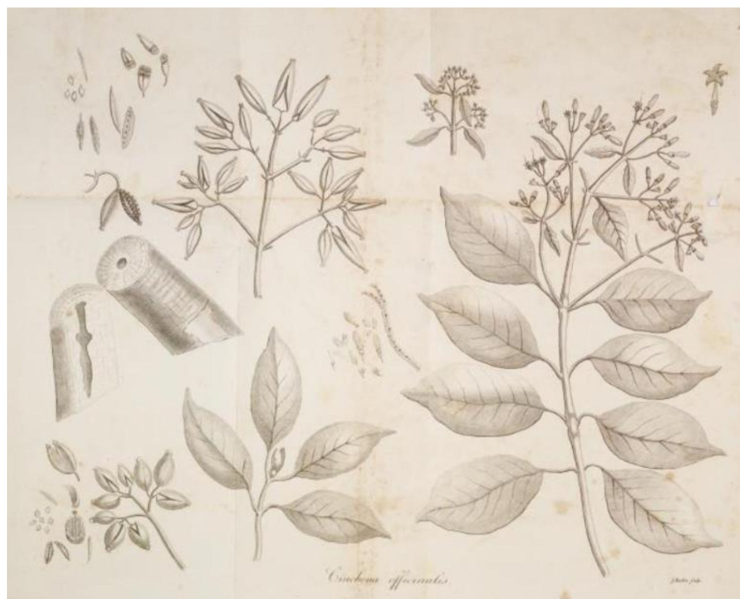

Figure 38 - C. officinalis.[13]

potential that diminishes the concentration required of each compound separately to exert an effect, making administration safer.[17,18] As such, for this study, an alkaloidal extraction of the *Cinchona* bark tree was performed to be analyzed through metabolomics assays in parallel with quinine.[19]

Poupartia borbonica Gmel.

Despite the invaluable contribution of traditional medicine to the populations across the globe, it has long been accepted that much of its knowledge has been lost across the centuries.[13] Only bits and pieces of information constructed on empirical evidence through the millennia have survived to this day, and as such, there is a necessity to study and reassert the properties of many treasure troves across the globe. This basis funded projects such as the European Fund for Regional Development that coordinated efforts and universities to study endemic plants for their potential therapeutic activities. From this study, a plant stood out in antiplasmodial assays in our laboratory – *P. borbonica* (Figure 39). This critically endangered tree is endemic to the Reunion and Maurice islands of the Mascarene Islands and is the type species of this genus belonging to the Anacardiaceae family.[20,21] Locally known as "bois-blanc rouge", "Zevi-marron" and "bois-de-Poupart", it was first reported for medicinal use by Jacob de Cordemoy in 1895 to render women infertile.[20,21] Later, other traditional uses were identified pertaining to kidney, blood or asthma issues through the decoction or infusion of the bark.[21,22]

Figure 39 - P. borbonica (adapted from Flore des Mascareignes 1997)[20]

Since the pharmacological properties of this plant were not yet described, our laboratory focused on exploring this species and its potential. Studies with the ethyl acetate leaf extract showed interesting activity *in vitro* (IC_{50} = 2.43 ± 0.500 µg/ml against 3D7 strain) and bioassay-guided fractionation led to the discovery of new alkyl cyclohexenone derivatives (Figure 40).[23] These three new molecules proved to be responsible for 50% of the extract's antiplasmodial activity and were subsequently studied in regards to their *in vivo* activity and toxicity.[23] Indeed, poupartones' A–C antiplasmodial selectivity index is not great (between 1.51 and 2.86 compared to WI38 human fibroblasts) and further analysis revealed cardiac toxicity, with cytotoxicity assays showing morphological changes and ultimate paraptosis-like cell death on triple-negative breast carcinoma MDA-MB-231 cells.[23,24] Despite its relevant toxicity

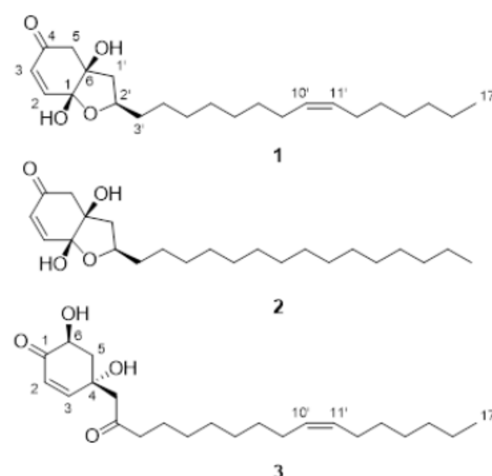

Figure 40 - The three cyclohexenone derivatives. Compound 1 – poupartone A, compound 2 – poupartone B, compound 3 – poupartone C.[23]

that proved to diminish with encapsulation inside liposomes, poupartones have an interesting and unexplored molecular structure that could provide insights on new antiplasmodial targets.[25] As such, poupartone B was studied alongside the fraction obtained from bioassay-guided fractionation that allowed its identification.

The purpose of this study was to obtain metabolic fingerprints of *P. falciparum* after incubation with the three plant extracts and NP to determine if extracts can be used preliminarily to identify MoA. The results were presented in the poster found in Annex III at the WORLD MALARIA DAY SYMPOSIUM: Blood Stage Malaria | Staving Off the Firestorm on April 25th 2023 at the Johns Hopkins Malaria Research Institute, Johns Hopkins University in Baltimore, MD, USA.

1.2. Materials & Methods

Growth inhibition assays of *A. annua* leaf ethyl acetate extract, *C. officinalis* bark alkaloid extract, *P. borbonica* leaf ethyl acetate extract, artemisinin, quinine, and poupartone B were done following the method described by Trager and Jensen.[26]

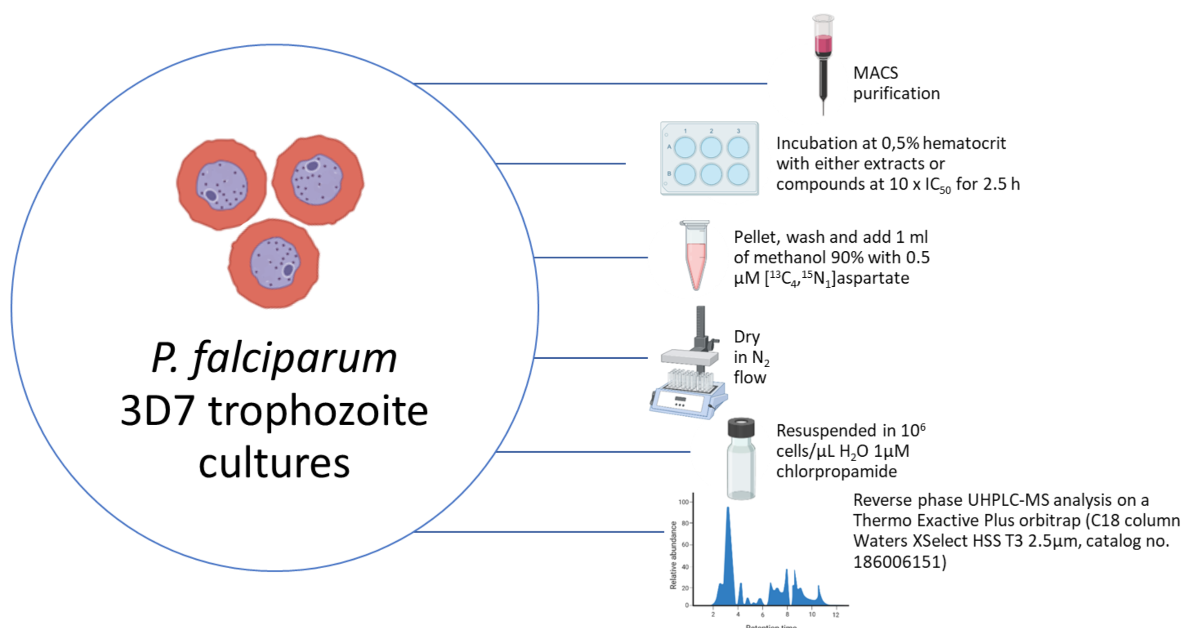

Figure 41 – Resumed methodology of the metabolomics test (equivalent to method D). (Created in BioRender.com)

The metabolomics test was performed with extraction method D and the workflow was simplified in Figure 41. Asexual *P. falciparum* 3D7 cultures were observed microscopically for stage and parasitemia and magnetically purified before being incubated in 6-well plates at 0.5% hematocrit with extracts or compounds at 10 x IC_{50} for 2.5 h. Additionally, a set of triplicates with atovaquone was also performed in each study as a fingerprint control. After pelleting and washing, infected red blood cells were resuspended in 1 ml of methanol 90% with 0.5 µM [$^{13}C_4$,$^{15}N_1$]aspartate. Supernatants were dried under nitrogen flow and stored at -80°C before being resuspended in 10^6 cells/µL in HPLC-grade water with 1µM chlorpropamide for reverse phase UHPLC-MS analysis on a Thermo Fisher Exactive Plus orbitrap.

10 µL were injected on C18 column (Waters XSelect HSS T3 2.5µM, catalog no. 186006151) and ran using a 25 min gradient of 3% aqueous methanol–15 mM acetic acid–10 mM tributylamine-2.5µM medronic acid ion pairing agent (A) and 100% methanol (B). Detection was performed in negative-ion mode, using a scan range of 85 to 1,000 m/z and a resolution of 140,000 at m/z 200. Data was converted to mzML with MS Convert-Proteo Wizard and then visualized, corrected and annotated with El-MAVEN through an in-house database. The internal standard was used to evaluate technical reproducibility and peaks were manually identified based on the proximity of the retention time with the targeted library, mass *m/z* and the signal/ blank ratio. Chlorpropamide was used to normalize the peak areas per metabolite, which were subsequently corrected with blanks and filtered based on the RSD (Standard Deviation/Mean) of the QCs and removed when they showed low repeatability (RSD > 25%). The remaining metabolites were analyzed in two ways: log2 fold changes were displayed in a self-organizing map, or metaprint, for profile comparison; or loaded directly in Metaboanalyst 5.0 for statistical analysis.[27]

1.3. Results & Discussion

Despite the uncontestable antimalarial activity of artemisinin and quinine or the reported activity of the extracts and poupartones, a traditional growth inhibition assay was still carried out first to determine the IC_{50} in these experimental conditions. Results can be found in Table 20.

Table 20 - IC_{50} determined from in vitro growth inhibition assays on 3D7 P. falciparum strain (n=3 with the SD).

Study groups	Extract vs. pure molecule	IC_{50} (ng/ml)
I	*A. annua* extract	67,94 ± 57,70
	Artemisinin	7,33 ± 3,88
II.	*C. officinalis* extract	302,51 ± 65,76
	Quinine	352,61 ± 180,18
III.	*P. borbonica* extract	458,74 ± 170,89
	Poupartone B	1140,86 ± 190,06

All extracts and compounds had IC_{50} in line with those reported in the literature.[23,28–30] Artemisinin exhibited the lowest IC_{50} as was to be expected followed by the *A. annua* extract. The *C. officinalis* extract, *P. borbonica* extract and quinine follow, with poupartone B already in the microgram range. Remarkably, the *P. borbonica* extract is more active than its isolated poupartone B, which is the opposite of what has been reported.[23] Upon re-isolation, poupartone B was confirmed through ^{1}HNMR albeit there was a possibility of it not being 100% pure (see Annex VI). Due to the limitations in obtaining more plant material and time constraints, studies were carried out with this sample. In

addition, there could have been stability issues for samples analyzed a long time after their first preparation, as is the case for the *A. annua* and *P. borbonica* extract. Still, since the IC_{50} are all in the expected range, the metabolomics assay was carried out.

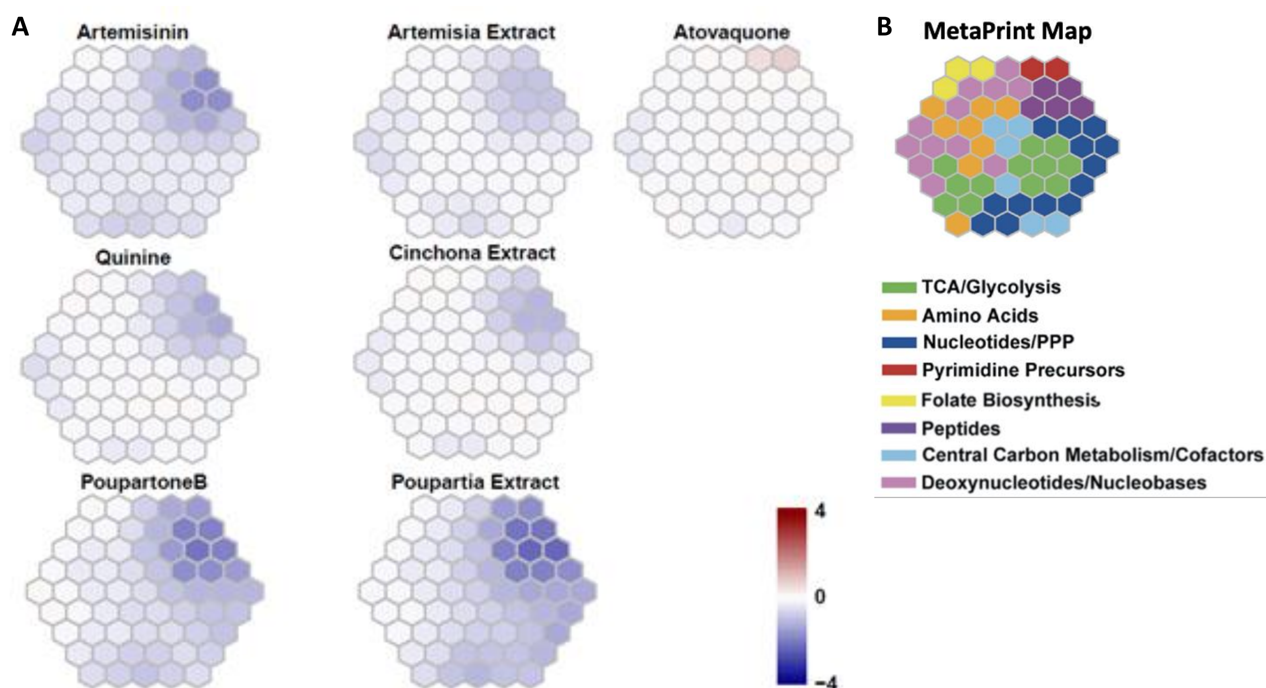

Figure 42 - (A) Metaprints of the study groups. (B) Metaprint map with a color-coded legend adapted from Allman et al. 2016.[27]

LC-MS data was first visualized through a self-organizing metaprint – Figure 42. The position of each group of metabolites is established as according to the map – Figure 42B – and the color is determined based on the fold change of selected metabolites following drug treatment relative to an untreated (DMSO, 0.1% v/v)) control. This means that when key metabolites have higher relative concentration in comparison with the control, they appear as red (hot), and the opposite in blue (cold), similarly to a heatmap. Atovaquone displays a specific signature as a mitochondria bc1 complex inhibitor with an increase in pyrimidine precursors, namely N-carbamoyl aspartate and dihydroorotate. Artemisinin and *A. annua* extract show similar negative fold change in peptides and nucleotides/deoxynucleotides, similar to the other groups. Interestingly, the study group III fold change is far more negative besides also showing central carbon metabolism and TCA/glycolysis with a negative fold change. No fingerprint is particularly distinguished, none is similar to atovaquone, and each plant extract-compound pair seems to have a similar map.

Chemometrics analysis with PCA can be observed in Figure 43. Initially, extracts were plotted with the control group to assess if they could be distinguished – Figure 43A. Despite the separation of the control group from extracts across PC1, this is not the dominant separation force. This seems to be the *P. borbonica* extract that is separated both through PC1 and 2 from all others. Observing the loadings

score plot, nucleotides like dCMP and xanthosine-5-phosphate, and glutathione seem to be the most evident metabolites responsible for this difference.

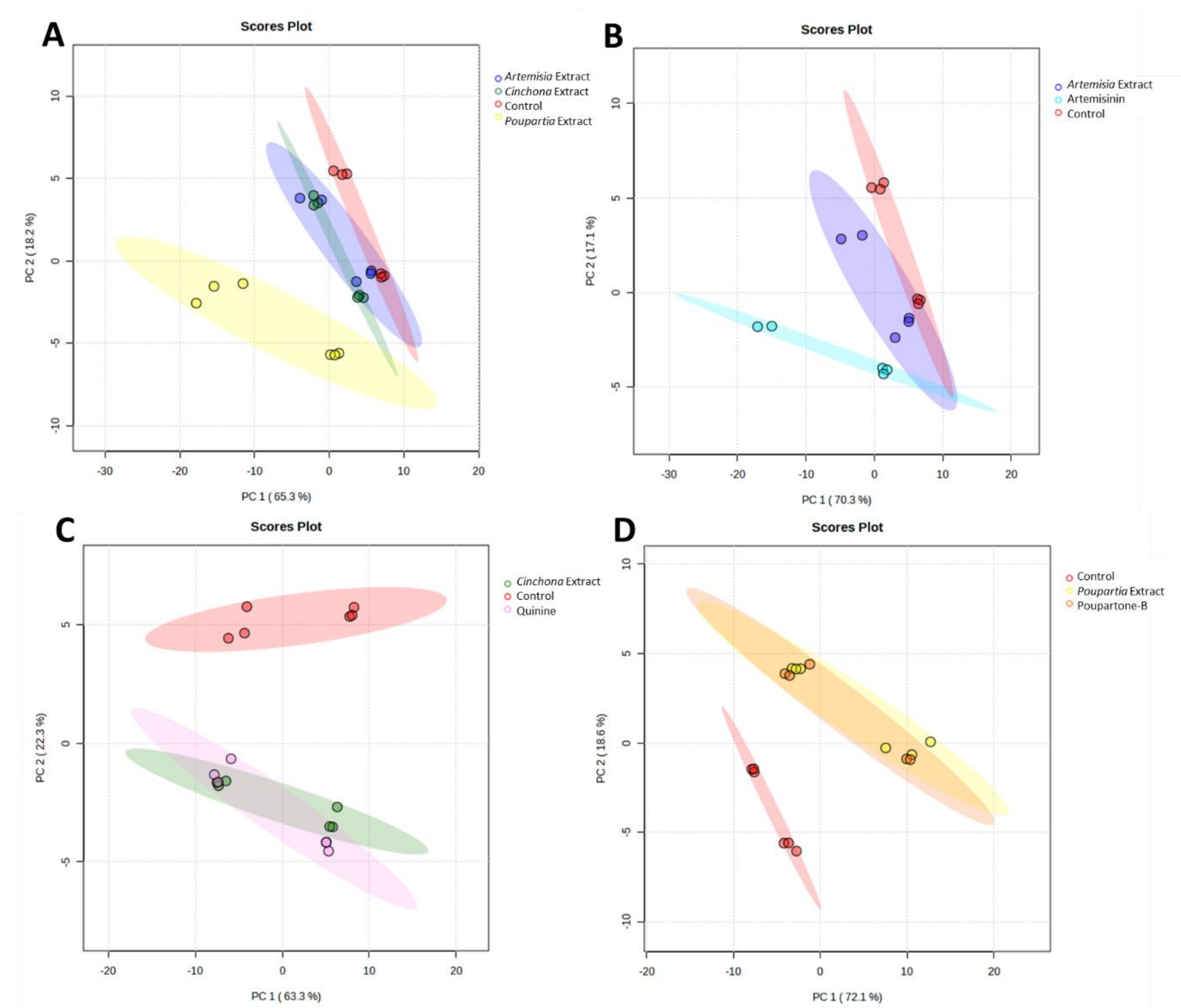

Figure 43 - PCA 2D scores plot with 95% confidence regions displayed. (A) Extracts and Control plot; (B) *A. annua* extract and Artemisinin plot; (C) *C. officinalis* extract and quinine plot; (D) *P. borbonica* extract and poupartone B plot.

Since extracts could be distinguished from one another, the next question was whether they could be distinguished from their NP counterparts. To facilitate this analysis, each pair was separated in study groups I, II and III as according to Table 20. Each PCA scores plot can be observed in Figure 43B, C and D, respectively.

Study group I does not show clustering between *A. annua* extract and artemisinin due to differences in peptides and amino acids. This can be seen by the fold change of the loadings responsible for this tendency: they're similar between the extract and the control but differ from artemisinin treated samples. This difference between profiles is maximized in the PCA, as the model tries to describe the most variability across all samples with the Principal Components, but this variability might not be statistically significant. Indeed, artemisinin is an important and potent antimalarial found in this extract and although its level of activity differs from the extract's (see the IC_{50}s in Table 20), there was an

expectation for convergence in the profiles of parasites treated by both the extract and artemisinin. Parallelly, the extract is complex matrix composed of more than artemisinin alone, hence some of the observed differences might also be associated with the presence of other compounds that influence the effects of the crude extract.

To further verify what metabolites could distinguish the profiles of *A. annua* extract and artemisinin a *t*-test was used and plotted in a volcano plot shown in Figure 44. Only two hemoglobin-derived peptides are statistically significant between the two groups: prolyl-aspartate (PD) and L-prolyl-L-valyl-L-asparaginyl-L-phenylalanine (PVNF). Despite being expressed positively for the extract, their fold change is not very big as seen by the distance of the points to the threshold. This relativizes their impact: these metabolites are statistically significant, but their FDRs are fairly high (0.03, cutoff of 0.05). This could mean that the difference in profile between the extract and artemisinin isn't in fact marked, in line with the metaprint results.

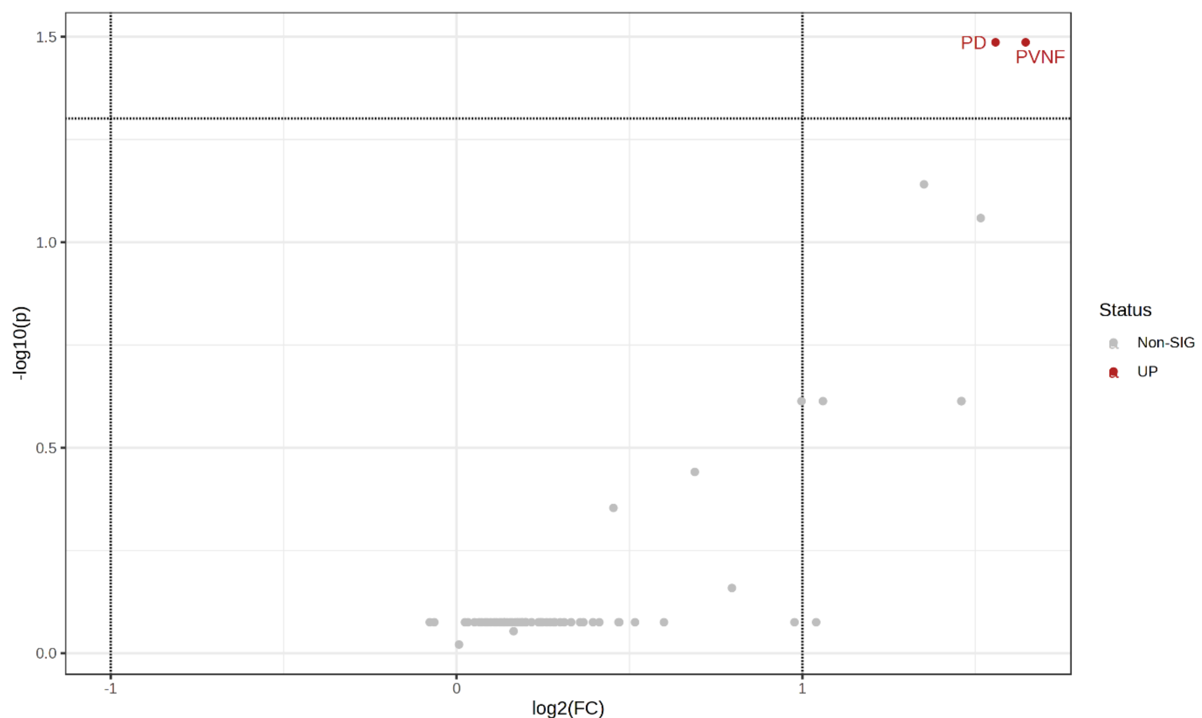

Figure 44 – Volcano plot of *A. annua* extract/artemisinin (*p-value: 0.05 FDR, FC threshold: 2.0). Points represent metabolites: in gray (non-significant), red (positive fold), blue (negative fold).*

The PCA of study case II (Figure 43C) shows the *C. officinalis* extract and quinine clustering together, which is indicative of an identical profile. This is to be expected, as the extract was specific to extract alkaloids. Quinine is not the only alkaloid available in this extract, but the MoA is thought to be shared amongst other alkaloids present in this extract as targeting hemoglobin metabolism.[17,31] Accordingly, the metabolites that separate the control from the extract and quinine can be linked to peptides that are connected to hemoglobin metabolism. A volcano plot of the *C. officinalis* extract with quinine

showed no statistically significant metabolites (p-value: 0.05 FDR, FC threshold: 2.0), further confirming their similarity in profiles.

Likewise, in Figure 43D, study group III clusters together due to their seemingly equal interference with peptides, amino acids and glycolysis related metabolites like acetyl-CoA and aspartate. Despite poupartone B not being the major compound of this extract, the poupartones are selectively present in this fraction, leading to the possibility that they could act similarly. A volcano plot of the *P. borbonica* extract with poupartone B showed no statistically significant metabolites (p-value: 0.05 FDR, FC threshold: 2.0), further confirming their similarity in profiles.

In the light of the preliminary results shown in Chapter 2 Section 4, poupartone B was thought to act by interfering with hemoglobin digestion. Since quinine prevents heme detoxification in the DV, a possible relation between the profiles of quinine and poupartone B was investigated through a volcano plot shown in Figure 45.[32]

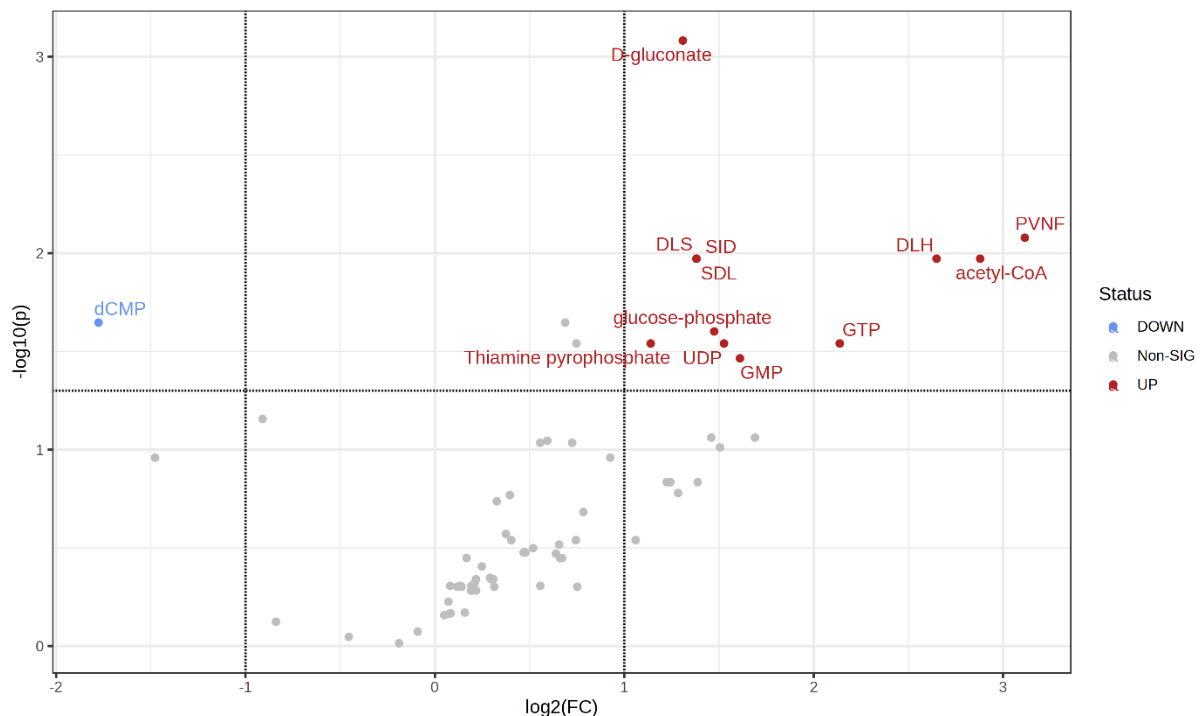

Figure 45 – Volcano plot of quinine/poupartone B (*p-value: 0.05 FDR, FC threshold: 2.0).* DLH – aspartyl-histidyl-leucine; DLS – leucyl-aspartyl-serine; PVNF – L-prolyl-L-valyl-L-asparaginyl-L-phenylalanine; SID – aspartyl-isoleucyl-serine; SDL – seryl-aspartyl-leucine. Points represent metabolites: in gray (non-significant), red (positive fold), blue (negative fold).

Only dCMP was significantly less expressed by quinine in comparison to poupartone B. On the contrary, many metabolites are positively demarked in parasites treated with quinine when compared with a poupartone B treatment. It can be deduced that hemoglobin metabolism is affected because the hemoglobin-derived peptides are significantly positively expressed for quinine along with acetyl-CoA and thiamine pyrophosphate. The latter two connect these effects to glycolysis, pyruvate metabolism, or TCA cycle – in short, carbohydrate metabolism. This distinction in treatment profiles cannot exclude

fully the possibility that poupartone B's MoA is related to hemoglobin metabolism, but it points to a different profile from quinine.

This preliminary assay successfully demonstrated the applicability of metabolomics assays of plants with traditional medicine use, namely in *in vitro P. falciparum* asexual assays. Plant extracts showed a different fingerprint from the control, but only the *P. borbonica* extract stood out from all other groups. Further analysis of the study groups revealed that an already purified bioassay-guided fraction or a specific class of compounds-targeted method of extraction show more similarities with the isolated compounds, with no statistical significance found between extract and NP. Parallelly, a general ethyl acetate extract is a complex mixture that might lead to interactions, competition or instability that ensues a relatively different profile from its active isolated compound, as is the case for artemisinin and *A. annua* extract. As seen through the volcano plot, however, the profiles of the latter two treatments are not that different, which could mean that artemisinin or artemisinin-like pleiotropic effects are also caused by the ethyl acetate extract. This would also be in line with reports that *A. annua* attributes more than 90% of its activity to the artemisinin composition and concentration.[33] Along with the *Plasmodium* pathways affected by the *P. borbonica* and *C. officinalis extracts,* this could mean that crude extracts can be used to have an overview of which pathways are likely to be involved in the perceived antiplasmodial activity.

These findings suggest that metabolomics is a viable tool to profile plant extracts and natural compounds with promising antiplasmodial activity and should be incorporated as a standard screening method in drug discovery. To further demonstrate this, the following chapters focus on the study of *A. annua* and *A. afra* extracts, then on the *P. borbonica* extract and poupartones A and B, followed by the study of ellagic acids and derivatives and a mixture of eight triterpenic esters.

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Chapter 3

Exploration of Modes of Action of Natural Compounds and Extracts

2. ARTEMISIAS STUDY

The work presented in this chapter was carried out in collaboration with Pr. Manuel Llinás and Dr. Gabriel W. Rangel at the Huck Center for Malaria Research (CMaR) at Penn State (The Pennsylvania State University) which kindly allowed the use of their inhouse LC-MS database for metabolite annotation.

2.1. Foreword

As mentioned previously, the Asteraceae plant family has since long contributed to traditionally used medicine due to its survivability across all kinds of habitats which allowed for global distribution.[1] Despite the most known being *A. annua* because of artemisinin's discovery, other *Artemisia* species, of which over 300 species have been recorded, have accounts of use against fevers in different regions, for example, *A. absinthium, A. afra, A. vulgaris* or *A. dracunculus,* among others.[1,2] For all of these, *in vitro* antiplasmodial activity has been described and inclusively the presence of artemisinin has been reported with variating degrees of certainty.[1,2] The presence of artemisinin in species other than *A. annua* remains intriguing due to the theorized method of synthesis in this plant itself – photo-oxidation of dihydroartemisinic acid which originates self-assembling allylic hydroperoxides – which does not seem compatible with other species as they lack dihydroartemisinic acid.[2] This is especially the case for *A. afra,* a plant endemic to eastern and southern Africa used in traditional medicine to placate fevers which has a controversial standing in regards to its artemisinin composition or lack thereof.[3] Additionally, as *A. annua* and *A. afra* can grow in the same areas and look fairly similar, it is speculated that *A. afra's* reported activity is due to contamination or confusion with the *A. annua* counterpart.[2]

Thus, to shed light on this matter, these two species were collected in the same region in the same season by Lahngong Methodius Shinyuy and authenticated by a botanist. One selected extract of each plant based on their *in vitro* preliminary activity was chosen to be analyzed through *P. falciparum in vitro* metabolomics and the MoA of each plant along with their artemisinin content and phenolic profile were described and discussed in the following publication. The metabolomics assay was conducted as described previously using method D (Figure 25).

2.2. Article – In preparation

Metabolomic profiling of *Artemisia afra* and *Artemisia annua* reveals divergent modes of action against *Plasmodium falciparum*

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Keywords: Metabolomics, Malaria, *Plasmodium* spp., *Artemisia annua, Artemisia afra*, Mode of Action

Abstract

The *Artemisia* spp. have been used for millennia in traditional medicine to treat a variety of ailments, including malaria. The discovery of artemisinin from *A. annua* reinvigorated the antimalarial drug roster, but recent resistance emergence threatens the current management strategies. The plant extracts of *A. afra* and *A. annua* may yet prove important tools to counter this tendency, but the mode of action and their potential as standalone treatment remains unknown. In this work, we explored the phenolic profile of *A. afra* and *A. annua* extracts, detected and quantified artemisinin, and performed metabolomic analysis of *in vitro*-cultured *Plasmodium falciparum* trophozoites to investigate the mode of action of these plant extracts. Despite containing trace amounts of artemisinin, *A. afra* elicited a different metabolic response from *A. annua*, which correlated closely with the profile of artemisinin. *A. annua* affected glutathione metabolism in line with artemisinin's known redox activity, while *A. afra* appears to affect lipid precursors. Our study shows that both plants have different antiplasmodial effects and should be further explored as complex matrices for the treatment of malaria.

1. Introduction

Malaria is an infectious disease that still poses a significant threat to global public health. As one of the deadliest parasitic diseases in the world, killing over half a million people annually, and mainly affecting children under 5 years of age, malaria is a top priority for eradication.[4]

Despite concerted efforts to control, eliminate, and eradicate malaria parasites, widespread antimalarial resistance of *Plasmodium falciparum*, the most deadly and prevalent species responsible for malaria, has significantly hindered these efforts. To tackle this obstacle, the World Health Organization recommends the use of Artemisinin-based Combination Therapies (ACTs) for treatment of malaria. ACTs are comprised of at least two mechanistically dissimilar drugs, including one artemisinin derivative, and are the compound combinations are designed to mitigate the development of resistance and recrudescence.[5,6] Despite the reliance on this approach, the emergence of partial artemisinin resistance along with partner drug resistance is a major concern and raises questions about the current strategy for malaria elimination and eradication efforts. Additionally, recent reports of artemisinin resistance in Chad, Eritrea, Kenya, Mali, Uganda, and Rwanda establish that resistance to the first-line malarial treatment has reached Africa, where the burden and mortality are the highest.[7–12] This reveals not only the need for innovative antimalarial targets but also the necessity of improvements to disease management that can diminish transmission and delay resistance emergence to extend the use of new-found therapies, including combinations.

Meanwhile, ethnomedical alternatives are relied upon by 80% of the global human population, including in malaria-endemic countries, where the availability, low cost, and traditional usage of certain plants remains important.[1,13] The Asteraceae plant family has a long history in this context and is well known for the discovery of artemisinin from *Artemisia annua*.[1,2,14–16] Similarly, in Africa, *A. afra* is cultivated for the management of malaria, among other diseases, because of its traditional use.[1,3,15] This, however, remains controversial since artemisinin is thought to be largely absent in this species while being responsible for the antimalarial activity of *A. annua*.[1,2] Multiple *in vitro* growth inhibition assays with *A. afra* extracts have reported a relevant activity against *P. falciparum,* but its mode of action remains elusive.[1] Many non-artemisinin compounds can be responsible for this activity because they represent known antiplasmodial pharmacophores, but an in-depth study is still absent.[2] Recently, previously thought anecdotical transmission blocking activity by *A. afra* was demonstrated *in vitro* along with the isolation of two guaianolide sesquiterpene lactones potentially involved in this effect.[15,17] Moreover, both *A. annua* and *A. afra* infusions were shown to be significantly incapacitating to sporozoites and disruptive to hypnozoites, revealing additional angles from which these traditionally used plants can aid in malaria disease management.[18] This effect was also deemed

artemisinin-independent, which further attests to other compounds, or their interaction, in these plant extracts that could be used to formidable results.

This study aimed to define the antiplasmodial mode of action of extracts from these two plants by several cross-validation techniques including detecting the presence of artemisinin, exploring the plant's phenolic profile and using metabolomics to investigate the relationship between the altered parasitic metabolism and the composition of the extracts.

2. Materials & Methods

2.1 Description and collection of plant material of *A. annua* and *A. afra*

Leaves and twigs of both plants were collected at the flowering stage from the town of Bafia, in the Center region of Cameroon (Coordinates; 4.7546, 11.2240), during the dry season. Voucher specimens were deposited at the Limbe botanical gardens, Cameroon. The samples were authenticated by a botanist and voucher number assigned.

2.2 Preparation of crude extracts.

Acetone crude extracts were prepared by maceration using the protocol described by Mbah *et al.,* 2011.[19] Briefly, each plant material was oven dried at 30 °C and ground to fine powder, and each powder was macerated at room temperature for 72h using acetone. The macerated mixture was filtered through a funnel with Whatman N001 filter paper and the filtrate was concentrated by rotatory evaporation using the R-205 brand Rotavapor. Each crude extract was weighed and stored at 4 °C for subsequent use.

2.3 Antiplasmodial assays

Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* chloroquine-sensitive strain 3D7 were maintained and used in antiplasmodial assays conducted similarly as described elsewhere.[20,21] Briefly, dried acetone extracts were dissolved in dimethylsulfoxide (DMSO) at a concentration of 10 mg/ml and tested three times (n=3) in a 48h *in vitro* colorimetric growth inhibition assay using lactate dehydrogenase activity as a proxy for parasite biomass, according to the method described by Makler *et al*.[22]

2.4 UHPLC-MS Metabolomic Characterization

The IC_{50} values (concentrations inhibiting 50% of parasitic growth) determined by antiplasmodial assays were used to adjust extract concentrations in metabolomic assays, performed as described by Allman *et al.*.[21] Briefly, the *P. falciparum* cultures were microscopically verified for presence of sufficient trophozoite stage synchronicity at the desired parasitemia before they were magnetically purified. The sample of ~95-100% pure infected erythrocytes was distributed in in 6-well plates at ~0.2% hematocrit and incubated for 1 hour for a recovery period. After recovery, the parasites were

exposed to extracts or compounds at 10 x IC_{50} for 2.5 h. Artemisinin (Sigma-Aldrich 361593), atovaquone (Sigma-Aldrich A7986), chloroquine (Sigma C6628) and quinine (Sigma 22620) were used as metabolomic profile controls. After pelleting and washing in ice cold PBS pH 7.4, infected red blood cells were resuspended in 1 ml of 90% methanol with 0.5 µM [$^{13}C_4$,$^{15}N_1$] aspartate as an internal control. Supernatants were dried under nitrogen flow and stored at -80°C before being resuspended to 10^6 cells/µL in HPLC-grade water with 1µM chlorpropamide as an additional internal control for reverse phase UHPLC-MS analysis on a Thermo Exactive Plus orbitrap. 10 µL were injected on C18 column (Waters XSelect HSS T3 2.5µM, catalog no. 186006151) and ran using a 25 min gradient of 3% aqueous methanol–15 mM acetic acid–10 mM tributylamine-2.5µM medronic acid ion pairing agent (A) and 100% methanol (B). Detection was performed in negative-ion mode, using a scan range of 85 to 1,000 *m/z* and a resolution of 140,000 at *m/z* 200.

After data acquisition, .raw files were converted to centroided .mzML files using MSConvert of the ProteoWizard package[23] and then visualized, corrected and annotated with El-MAVEN[24]. The internal standard intensity was used to evaluate technical reproducibility and peaks were manually identified based on the proximity of the retention time with the targeted library, m/z and the signal/ blank ratio. Chlorpropamide was used to correct the peak areas per metabolite for instrument variation during the run. Subsequently, blank signals were subtracted and metabolites were filtered based on the RSD (Standard Deviation/Mean) of the QCs and removed when they showed low repeatability across technical replicates (RSD > 25%). The remaining metabolites were analyzed in two ways: log2 fold changes were displayed in a self-organizing map, or metaprint[21], for profile comparison; or loaded directly in Metaboanalyst 5.0 for statistical analysis.

2.5 ^{1}H-NMR Metabolomic Characterization

NMR spectra were acquired on a Bruker NEO Ultrashield Plus 700 MHz equipped with a helium cold probe (cryoprobe) with samples dissolved in buffered D_2O at pH 7.4 with TSP as internal reference. ^{1}H-NMR experiments were performed with a CPMG sequence with 128 scans collected over a spectral width of 20 ppm. All spectra were phased and baseline-corrected manually using TopSpin v4. Spectra were stacked, aligned and integrated between δ0.5-9.5 ppm using MestReNova v14. Spectra were divided into buckets of 0.04 ppm, integrated to the sum of intensities and normalized to the total spectrum intensity. The bin tables were analyzed using MetaboAnalyst v5.0 and *R* (packages MBXUCL, PepsNMR and limpca).[25–27] The NMR spectra were annotated using Chenomx NMR Suite 9.0 database and the Human Metabolome Database (HMDB), as according to literature.[28,29]

2.6 Plant extract profiling and Artemisinin quantification

Plant extracts were analyzed to obtain a phenolic composition and detect and quantify artemisinin, if present. Briefly, extracts were resuspended in methanol, filtered (0.45 µm) and 10 µL were injected

in an Agilent Technologies 1200 series HPLC to obtain a phenolic profile. A gradient of acetonitrile (A) and formic acid 0.1% (B) was used on a Luna PFP (CQ-039 (250 x 4.6mm, 5µ) for a duration of 87 mins with a flow of 1ml/min. HPLC detection was done through DAD at 330 nm.

Artemisinin detection was done in two instances: by HPLC with the method described by Diawara *et al.*[30], and MS. In the former, derivatization was achieved with NaOH at 50°C for 30 min followed by cooling with EtOH for 10 min before completion with acetic acid (0.2N). Samples were filtered before 20 µL were injected in the HPLC system. The isocratic method consisting of phosphate buffer (pH 6.3) (A) and methanol (B) ran for a total of 25 min with a 1 ml/min flow on a CQ-039 (250 x 4.6mm, 5µ) column. HPLC detection was done through DAD at 260 nm for artemisinin. Quantification was achieved through this method as described elsewhere.[30]

Detection of artemisinin was also done by direct-infusion high-resolution MS (DI-HRMS) of the plant extracts. To do so, 10 mg/ml of AFE and ANE were prepared as well as 1 mg/ml of artemisinin and filtered (0.45 µm) before injection. DI-HRMS was performed on a Thermo Scientific LTQ XL Orbitrap mass spectrometer of the MASSMET Platform (Thermo Scientific, Bremen, Germany). Data were acquired in positive ion mode using full-scan MS with a mass range of 100-1000 m/z. The orbitrap operated at 30000 resolution (FWHM definition). All experimental data were acquired using daily external calibration prior to data acquisition. Appropriate tuning of the electrospray ion source was done. The following electrospray inlet conditions were applied: flow rate, 100 µL min-1; spray voltage, 5 kV; sheath gas (N2) flow rate, 20 a.u.; auxiliary gas (N2) flow rate, 10 a.u.; capillary temperature, 275 °C; capillary voltage, 45 V; tube lens, 80 V.

3. Results

3.1 *A. afra* and *A. annua* have different phenolic compositions

Chromatography techniques were used to describe the extracts at different levels: phenolic composition and artemisinin presence. First, A. afra extract (AFE) and A. annua extract (ANE) were analyzed through HPLC-UV with a reverse phase method to have an overview of their phenolic composition – results are shown in Figure 46.

Since the samples are acetone extracts, it was expected that some phenolic compounds would be extracted, such as phenolic acid, flavonoid and coumarin derivatives, and artemisinin, if present.[31] Four standards were used to verify the presence of these phenolic compounds by matching the UV spectra and retention time. This selection was based on preliminary results on the ANE and AFE compositions and the literature: phenolic acid chlorogenic acid, flavone luteolin and coumarin derivatives scopoletin and esculetin.[1,31] From these, ANE contained chlorogenic acid as confirmed by the retention time and UV spectra despite the small peak, while AFE didn't contain this compound.

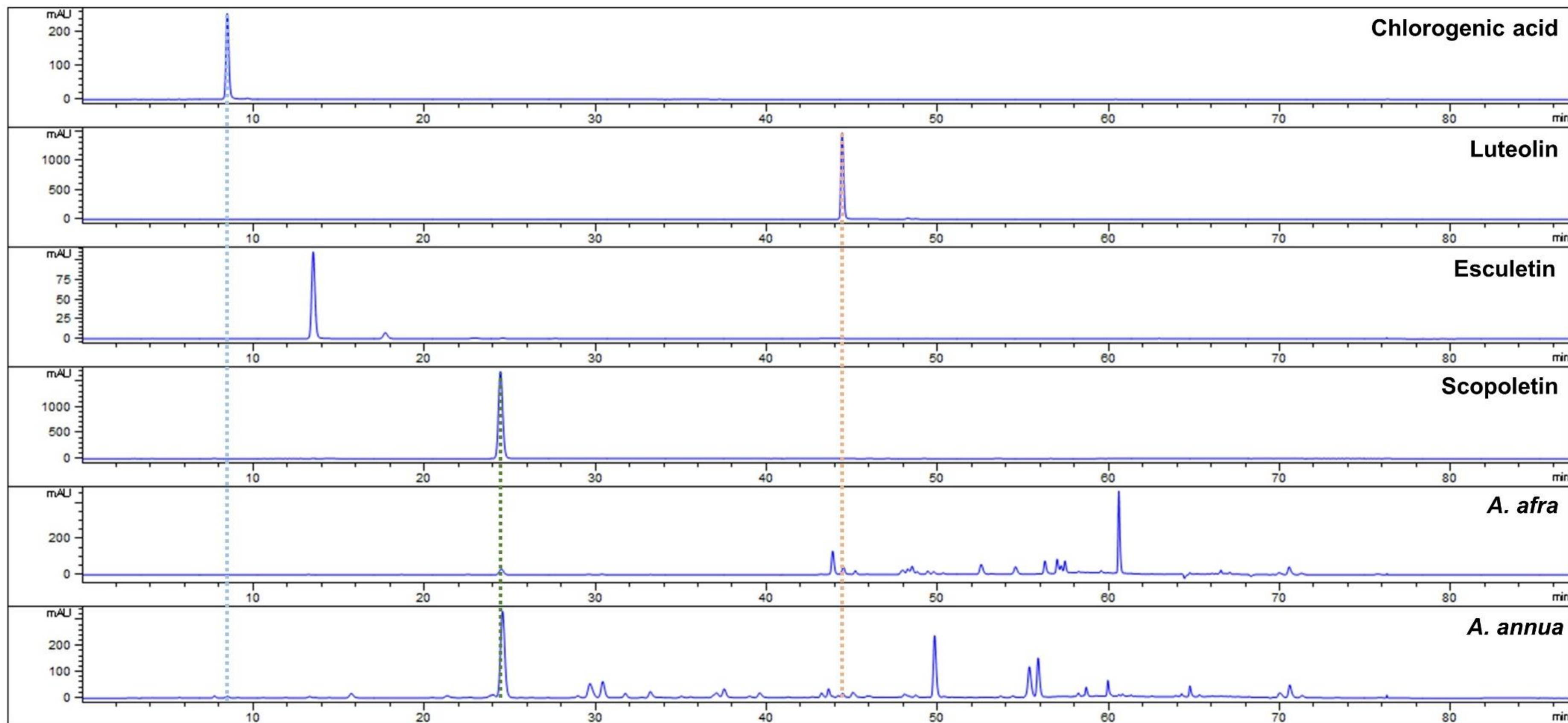

Figure 46 – HPLC-UV chromatogram of standard phenolic compounds and A. afra and A. annua extracts detected at 330 nm.

Both contained luteolin and scopoletin, though with different concentrations. Neither extract had the esculetin peak. Through this analysis, it is clear that there are phenolic compounds with differing abundances between species, which confirms that, despite the time and place of collection being similar, the metabolomes of these two *Artemisia* species are inherently different.

3.2 DI-HRMS confirms the presence of artemisinin in both *A. annua* and *A. afra*

Initially, artemisinin detection through HPLC-UV after derivatization was performed and it showed the presence of this compound in both extracts (Figure 47). Specifically, ANE showed a clear peak that matched the standard (UV and Retention Time) and was within the quantification range, meanwhile AFE had a very small peak at the same retention time, but too small to indicate confidently the presence of this compound. Artemisinin concentration in ANE was calculated to be 0.19 % (m/m) of dry extract, which translates to 1.93 mg artemisinin per gram of extract or 0.019 % (m/m) artemisinin in dry plant. Meanwhile, the peak in AFE fell below the limit of detection (2.73 µg/ml) of this technique, but there could be trace amounts of artemisinin.[30] This difference in artemisinin concentration may be responsible for the differences in activity reported in the literature between these two species.

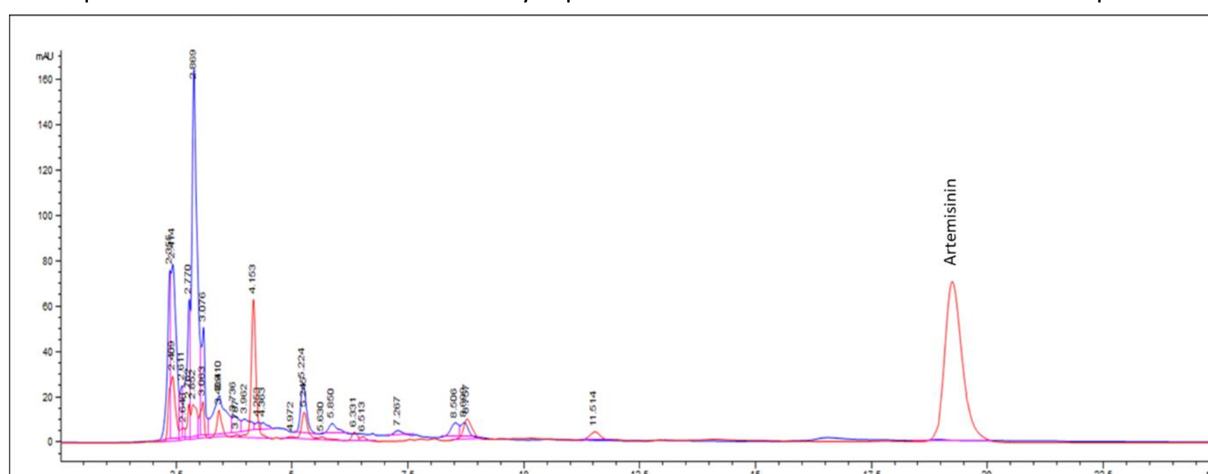

Figure 47 – HPLC-UV chromatogram of ANE (red) and AFE (blue) derivatized and detected at 260 nm.

Since derivatization can induce artefacts, artemisinin detection was further confirmed by DI-HRMS in which the specific ions were found at *m/z* 305.13550 ($[M+Na]^+$) and *m/z* 587.28107 ($[2M+Na]^+$), which was also detected in the spectrum for the artemisinin standard (Figure 48A). In these conditions, a molecular ion at *m/z* 283.15379 $[M+H]^+$ was also detectable. Spectra obtained from ANE and AFE demonstrate the differential composition of these extracts, with ANE clearly displaying *m/z* $[M+Na]^+$ and $[2M+Na]^+$ (Figure 48B, 46C). A manual search for these peaks in AFE revealed that they are also present though at significantly reduced intensities as seen in Figures 48D and E. The detection of these ions demonstrates that artemisinin is present in AFE, although the abundance is much lower, and therefore unlikely to be the active agent in this extract. All other detected analytes are from other

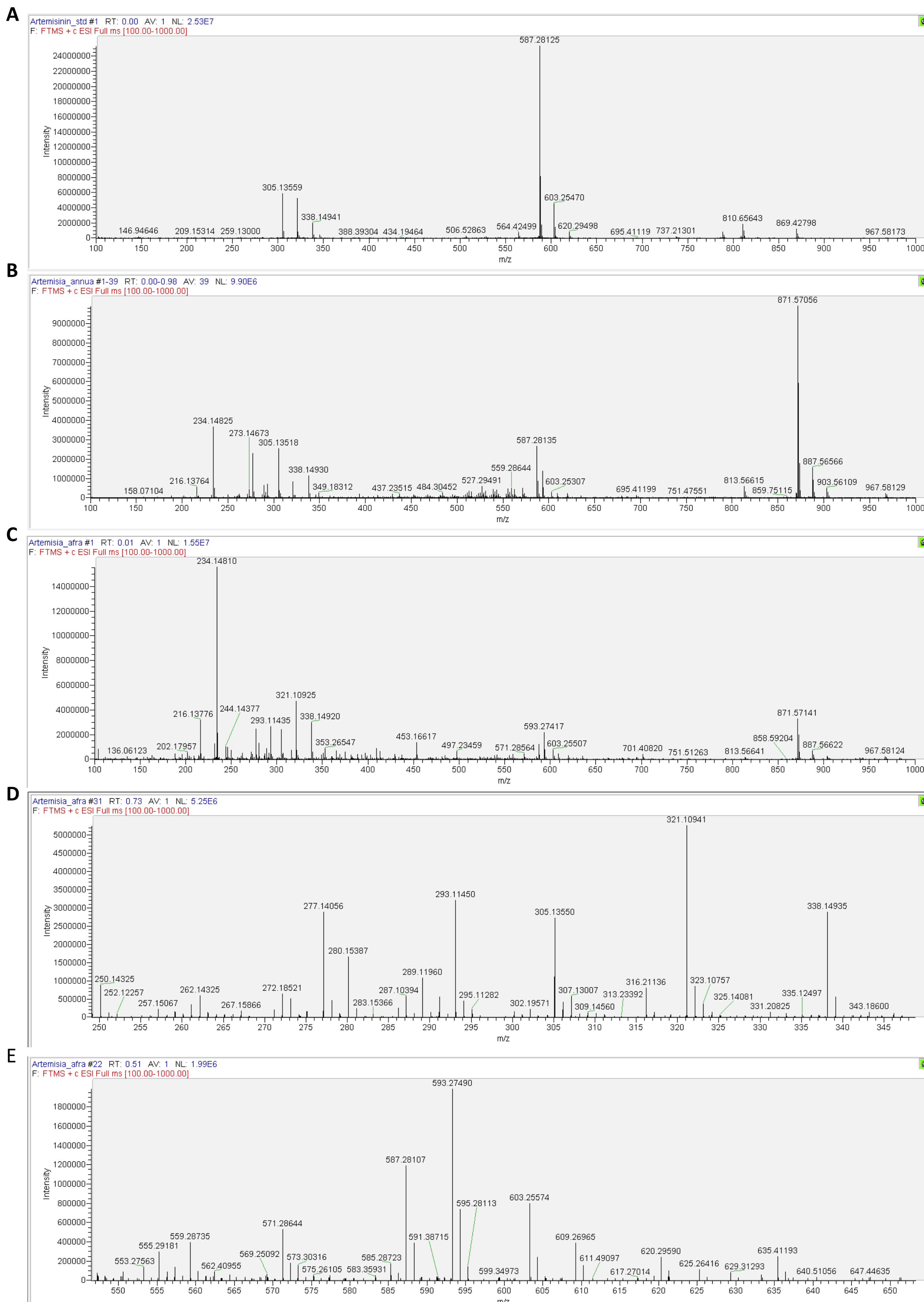

Figure 48 – DI-HRMS spectrum of (A) artemisinin, (B) ANE, (C) AFE, (D) zoomed in AFE spectrum 250 and 350 m/z to visualize artemisinin $[M+Na]^+$, and (E) 550 and 650 m/z to visualize artemisinin $[2M+Na]^+$.

positively ionized molecules present in the extracts, as no separation method was used before ionization as is standard in DI-HRMS.

3.3 *P. falciparum* trophozoites have diferente responses to *A. afra* and *A. annua*

3.3.1 In vitro activity

To determine the antiplasmodial activity of the AFE and ANE extracts, the IC_{50} was calculated using the lactate dehydrogenase colorimetric revelation. The difference in IC_{50} (AFE: 11.73 ± 2.15 µg/ml, ANE: 0.086 ± 0.007 µg/ml) could be expected based on the relative presence of artemisinin as detected by HPLC-UV and DI-HRMS in these extracts (Figure 47 and 48).[2] However, despite the over one hundred-fold contrast, AFE activity would still be considered interesting in an antiplasmodial screening test.[32] This activity could be due to the trace amounts of artemisinin present in AFE or an effect of other compounds in the extract. A metabolomics assay was carried out on *P. falciparum* purified trophozoites to investigate which is the case.

3.3.2 Metabolomic assays

Metabolomic assays were conducted to complement the aforementioned studies and shed light on the additional metabolic perturbations experienced by the parasite upon incubation with the extracts. Late-stage trophozoites were magnetically purified and incubated for 2.5h with either extract, the vehicle DMSO as a negative control, or other established, pure antimalarial drugs as positive controls. Parasite metabolites were extracted with methanol and analyzed through LC-MS and NMR.

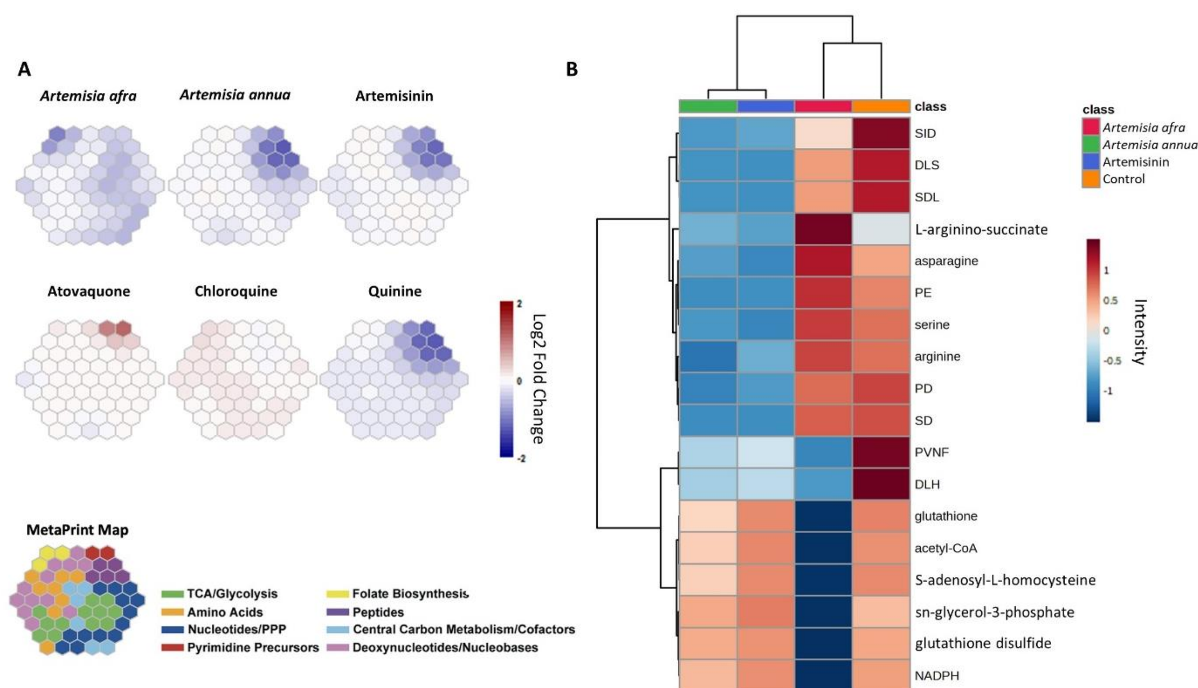

Figure 49 – LC-MS metabolomic analysis of the in vitro antiplasmodial effects of AFE, ANE, artemisinin, atovaquone, and control (DMSO). (A) Metaprint profiles in which metabolite clusters are associated to eight major metabolic pathways as attributed in the MetaPrint Map.[21] (B) Heatmap of the metabolites scored as significant by ANOVA.

The MS data clearly demonstrate that ANE and artemisinin elicit similar metabolic responses, whereas AFE is different (Figure 49A, 47B). In particular, ANE & artemisinin result in a decrease in hemoglobin-derived peptides not seen upon AFE exposure. Furthermore, AFE demonstrates a decrease in folate biosynthesis.

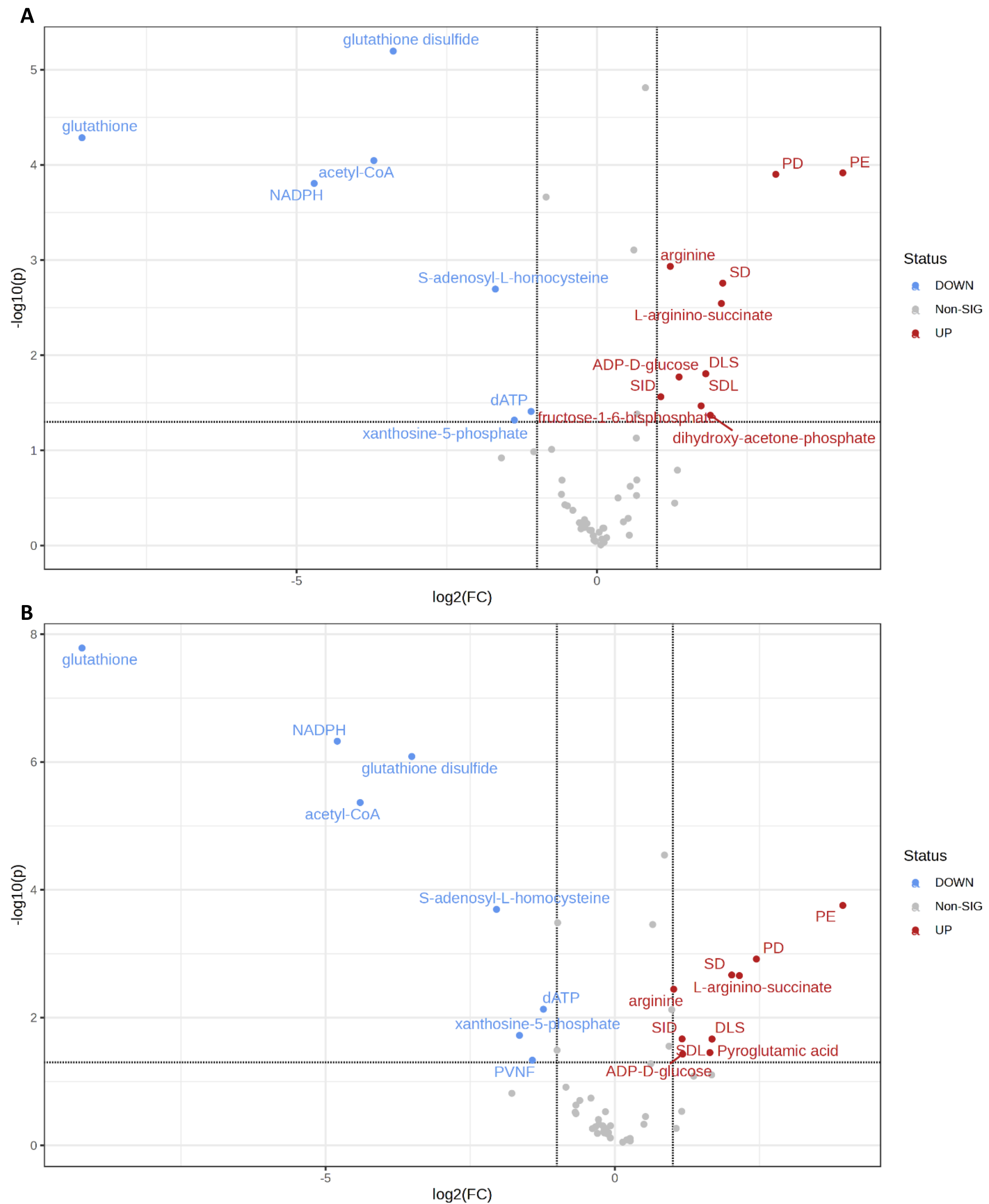

Figure 50 – Volcano plots (fold change threshold: 2.0; p-value threshold: 0.05) of comparisons (A) AFE/ANE and (B) AFE/artemisinin. The comparison ANE/artemisinin had no significant metabolites. Points represent metabolites: in gray (non-significant), red (positive fold), blue (negative fold).

For the metabolites in which AFE and the control resemble one another but differ from the other two groups, 6 are peptides and 4 are amino acids, indicating a disparity with ANE and artemisinin that is in agreement with the metaprints. Alternatively, AFE has a distinct heatmap profile linked with glutathione metabolism and lipid metabolism, *e.g.* acetyl-CoA and sn-glycerol 3-phosphate. Volcano plots were plotted to visualize the metabolic differences between extracts and each extract and artemisinin (Figure 50).

In the case of AFE/artemisinin (Figure 50B), glutathione, glutathione disulfide and NADPH show the biggest changes with the most significant p-values. This points to glutathione metabolism as the most significant pathway affected by artemisinin out of the measured metabolites in this study when compared to AFE elicited response. This is also shown in the volcano plot of AFE/ANE (Figure 50A). Through the same statistical model, ANE extract and artemisinin had no significant differences (p-value < 0.05).

Two statistical methods were implemented to analyze the NMR data to reveal statistically significant bins connected with the different treatments: ASCA+ and ANOVA2.[25,26] ASCA+ was implemented with a general linear model in an enhanced version to correct the bias of unbalanced experimental designs and detect whether the treatment was the most impactful factor in the study.[26] The biggest percentage of variance (45.67%, p-value < 0.01) was attributed to the treatment, while the assay was linked to 10.85% (p-value = 0.04). This percentage is relatively small, but it demonstrates how assays in the same conditions are never identical. The score plot for the treatment effect separates AFE through PC1 (88%) from ANE and artemisinin using multivariate analysis (Figure 51B). This was also preliminarily verified with principal components analysis (PCA) with the original data (PC1 of 44.55%) (Figure 51A).

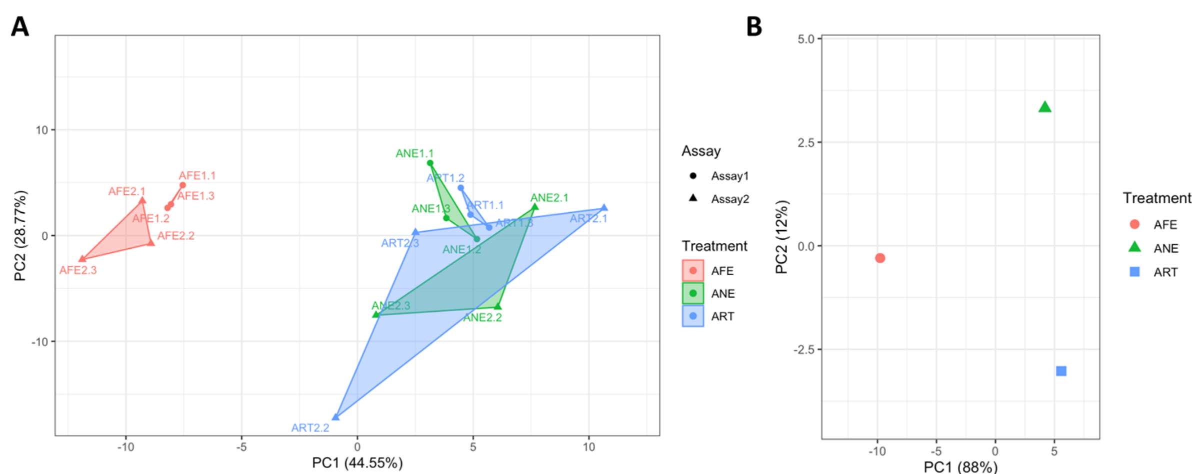

Figure 51 – (A) PCA scores plot of the original (before ASCA+ and ANOVA2) 1HNMR data, (B) Multivariate analysis ASCA+ via limpca R package: score plot for the treatment effect. ART – Artemisinin.

ANOVA2 was implemented and FDR corrected to classify metabolites as significantly different in abundance between different treatments (AFE, ANE and artemisinin). To do so, treatments are paired and subset t-tests are performed. Interestingly, no significant ^{1}H-NMR bin was found for the pair ANE-artemisinin, demonstrating their profile as the closest. However, for the comparisons with AFE, 70 and 72 bins were found to be significantly different with ANE and artemisinin, respectively, and were further annotated (Figure 52). Of these, 4 bins couldn't be annotated, 57 bins were shared between the pairs and 28 bins were different. The common bins between the pairs represent all kinds of classes of metabolites: amino acids, carboxylate, nucleotide, glutathione and assortments of multiple metabolites due to peak superposition. Three amino acids (leucine, valine, histidine), glutathione, NAD^{+}, ADP, O-phosphocholine, myo-inositol, glucose, N-acetylglucosamine and glycine were found to be significantly different in abundance as can be seen in Figure 52. As metabolites tend to have more than one ^{1}H-NMR cluster, metabolites with multiple statistically significant clusters (in this case, bins) were found relevant even when not all possible clusters were indicated by ANOVA2. For the pair AFE-ANE, the only metabolite significantly different from the artemisinin profile is myo-inositol, whose role as an essential membrane precursor is interesting in this context. All other metabolites were similar, showing that ^{1}HNMR also points to a similar profile between ANE and artemisinin, which differ similarly from AFE.

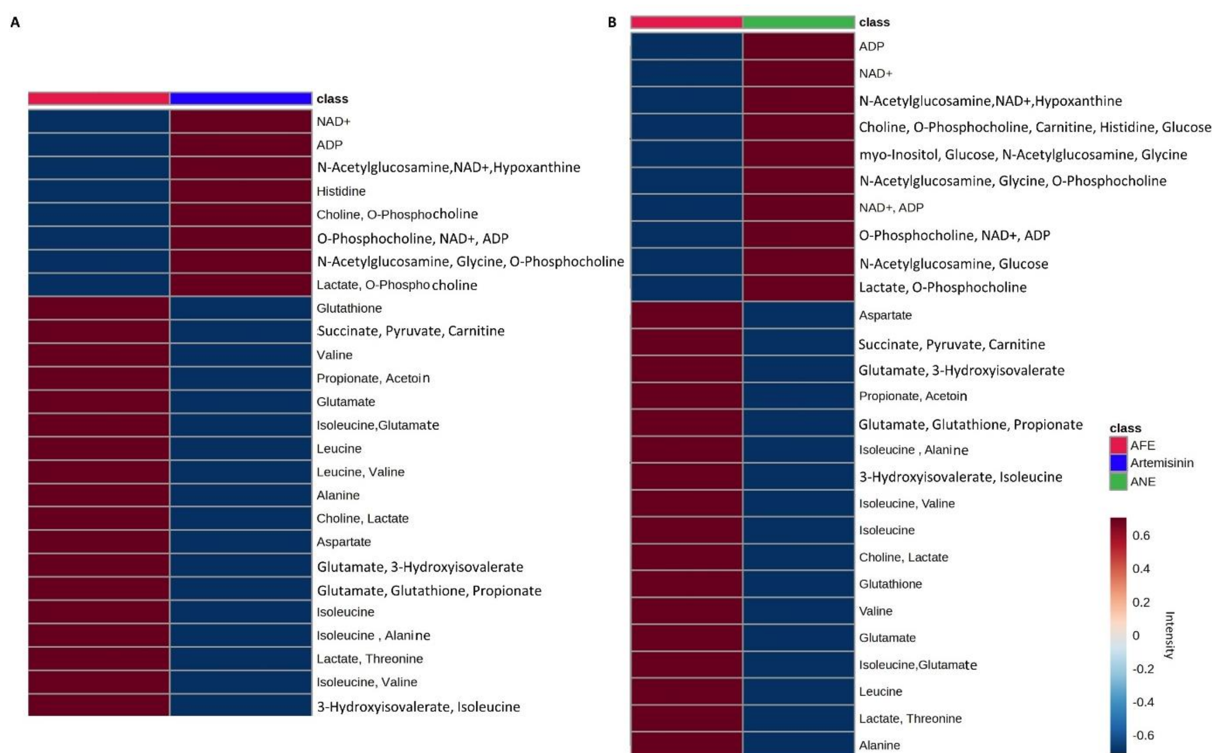

Figure 52 – Heatmap of ANOVA2 significant annotated ^{1}H-NMR bins: (A) AFE-ANE pair, (B) AFE-artemisinin pair.

In agreement with the MS metabolomics results, the NMR also points to a similar mode of action between the ANE and artemisinin, with no significantly different metabolites found between the two

of them. Glutathione was also found to be significantly different through NMR, which suggests an impact on redox pathways. Pathway analysis of NMR data points to differences in the aminoacyl-tRNA biosynthesis due to the numerous amino acids, and glyoxylate and dicarboxylate metabolism because of acetate, glycine, and glutamate significant changes (Figure 53). Interestingly, the last two are also part of glutathione metabolism, providing further support that this pathway may contribute to the difference in modes of action between these two plant extracts.

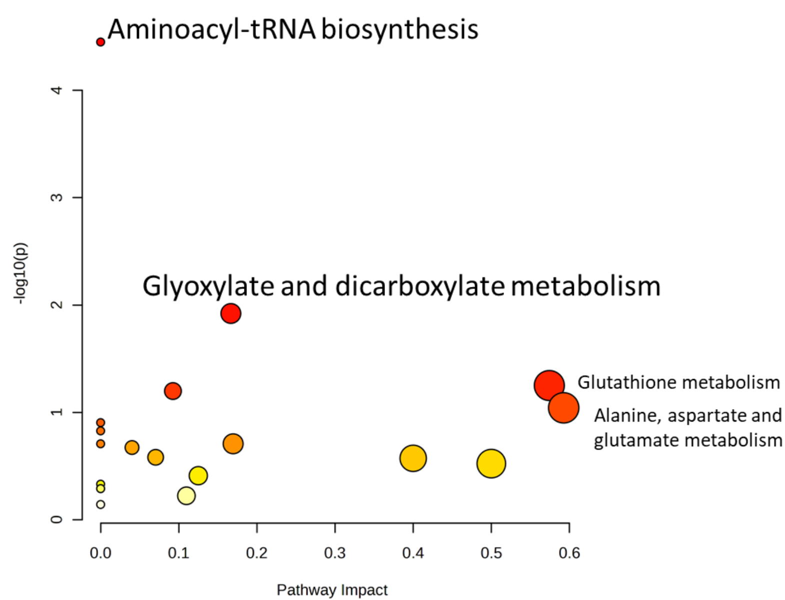

Figure 53 – Pathway analysis performed with the list of significant metabolites indicated by ANOVA2 of ^{1}HNMR data. (MetaboAnalyst v5.0)

4. Discussion

In light of the diverging reports in the literature regarding the antiplasmodial activity of *A. afra* and *A. annua*, this study was conducted to elucidate aspects related to these plants, namely their phenolic composition and artemisinin presence and abundance, and investigate whether they act similarly against asexual *P. falciparum* trophozoites.

It is relevant to mention that these extracts were not prepared as the plants would be traditionally consumed – the leaves and twigs were extracted with acetone as opposed to a traditional infusion.[1,3] These extracts were chosen based on their preliminary antiplasmodial activity *in vitro,* in comparison with other extraction methods or solvents, to test the most potent option and achieve a clearer conclusion in regards to their effects on the malaria parasite.

Initial tests sought to describe the composition of these plant extracts to correlate it with the antiplasmodial activity. This was done with two focuses, phenolic content and artemisinin, because both are considered active antiplasmodial components of these plants.[1,2] Both plants contained phenolic compounds and derivatives that were distinct between them. This is important because

contamination and interchangeability between *A. afra* and *A. annua* is speculated to be a reason why the former is active.[2] Flavonoid luteolin was found in both extracts and is thought to be, among others, partially responsible for the antiplasmodial activity of these plants, though its antimalarial potential (µM IC_{50} range) is not at the level of artemisinin (nM IC_{50} range).[2,33,34] Quercetin was not found in either extract (data not shown), which is in line with a reported cultivar-dependent presence of this flavonoid and its derivatives.[2] Flavonoids and other phenolic compounds have demonstrated synergy with artemisinin, which potentiates their antiplasmodial activity and reportedly expands the multitude of ways the parasite might be affected, from additional kinase inhibitions to apicoplast targeting and artemisinin-enhanced interaction with heme.[2,31,35] Additionally, phenolic compounds have multiple putative antiplasmodial modes of action, i.e., inhibition of heme detoxification (flavonoids), inhibition of fatty acid synthase II (flavonoids and phenolic acids), and/or redox cycling.[2,13] Any of these compounds could participate in these plant's antiplasmodial activity because since they are readily found in extracts.[2,14] The full phytochemical characterization of these extracts is hence of paramount importance and will constitute the objective of another study.

Artemisinin was detected through two different means: HPLC with UV detection after derivatization and DI-HRMS. The first technique revealed a clear disparity in relative artemisinin abundance between AFE and ANE with the latter having a high content of artemisinin, while the former fell beneath the limit of detection, despite the assumed presence of a peak. Because of the possibility of artifacts resultant from derivatization that could lead to an erroneous assumption on the presence of artemisinin, and the limitations of detection associated with the method (2.73 µg/ml), DI-HRMS was used to search for artemisinin in these extracts. Artemisinin was found in very small amounts in AFE after searching for the ions manually, whereas ANE showed a higher relative intensity of artemisinin ions, which confirmed its presence. There is controversy associated with whether these plants share a biosynthetic process to produce artemisinin and related sesquiterpenes or if its presence could be a result of contamination.[2] A shared process to synthesize artemisinin has not been demonstrated between these plants, and, despite the best efforts during collection, contamination can never be fully excluded. Regardless, the evident different abundance of artemisinin between these extracts remains interesting in the interpretation of the metabolomic assays that follow.

Antiplasmodial assays started with the determination of the extract's IC_{50}, not only to confirm their activity, but also to establish the concentration (10 x IC_{50}) to use in the metabolomic tests. Growth inhibition assays revealed over one hundred-fold difference between IC_{50} with ANE as the most active as was to be expected due to the abundance of artemisinin. Antiplasmodial activity associated with *A. annua* extracts has been shown to be almost exclusively (> 90%) attributed to the concentration of artemisinin, hence this activity was to be expected.[2] However, compounds other than artemisinin

might be responsible for the activity of *A. afra* since AFE activity would still be considered promising in the setting of an antiplasmodial screening.[2,32] In this context, the IC_{50} is a unidimensional figure that can give an indication of the level of *in vitro* activity but fails to give information regarding the type of activity, timing, or life stage, as the assay is typically done over 48h (one lifecycle).[32] A metabolomics assay in this context sheds more light on the potential type of activity exerted by these extracts by comparing them to the effect of artemisinin alone and displaying which metabolites are affected. Additionally, since parasites were purified before the assay, results can be directly attributed to the effects on mature trophozoites.[21]

Metabolomics samples were analyzed using two different strategies: first through LC-MS, and then ^{1}H-NMR. The MS results indicate that AFE has a different mechanism of action from artemisinin and ANE, which cluster together through hierarchical cluster analysis (Figure 49). AFE profile differs in regards to glutathione metabolism and precursors acetyl-CoA and sn-glycerol-3-phosphate, which are significantly less expressed in parasites treated with this extract than with ANE or artemisinin. Additionally, a different profile regarding amino acids and peptides is also shown for artemisinin and ANE, which could be due to the endoperoxide bridge opening by hemoglobin-derived Fe^{2+} heme, which releases free radicals and possibly interferes with hemoglobin and protein-related metabolism.[31,36] These results are further confirmed by NMR, where no significant difference between ANE and artemisinin was found and for which amino acids and glutathione metabolism-related metabolites were found as the most significant difference between these treatments and AFE. Oxidative damage has been associated with lipid peroxidation and mitochondrial and plasma membrane changes, so the phenotype reported herein aligns with the reported effects of artemisinin, which can be extended to ANE due to its high artemisinin content.[36] AFE has a similar profile to the control (DMSO) regarding amino acids and peptides (Figure 49), which would indicate that AFE does not particularly affect this pathway, and hence has a different mechanism of action from artemisinin and ANE.

Through the differences in metabolomic profiles of parasites treated with AFE with ANE or artemisinin, a comment can be made on the potential pathways affected by AFE. Pathway enrichment analysis reveals changes to aminoacyl-tRNA biosynthesis linked with amino acid changes and glycine, which is also a precursor for glutathione. Other metabolites are harder to interpret in light of their ubiquitous nature, but two lipid precursors are of note: O-phosphocholine and myo-inositol. Both are expressed significantly differently in parasites treated with AFE when compared to ANE or artemisinin and are involved in several pathways, such as glycerophospholipid metabolism or phosphatidylinositol signaling. Therefore, the potential inhibition of fatty acid synthase II by the flavonoids and phenolic acids present in this extract cannot be overruled as one of the contributing modes of action of AFE.

This enzyme is responsible for fatty acid synthesis in the parasite apicoplast, which has been shown to disrupt the recovery of dormant parasites and be essential in the pre-erythrocytic and sporozoite development in the mosquito stages, though not for intraerythrocytic parasites due to fatty acid scavenging.[13,37,38]

Myo-inositol is a fundamental metabolite for *Plasmodium* asexual forms survival and was found to be significantly different between AFE and ANE in this study.[39] Myo-inositol is scavenged from the host and produced *de novo* by the parasite, and accumulated in trophozoites and schizonts to be incorporated into glycosylphosphatidylinositol glycolipids.[39] Flavonoids are thought to inhibit the entry of myo-inositol into the iRBC, which would hamper the parasite's development by interfering with phospholipid synthesis and osmoregulation.[13,40] Both AFE and ANE present flavonoids in their composition, and studies with an *A. annua* flavonoid, casticin, have shown synergy with artemisinin and inhibition of myo-inositol import to iRBCs, further demonstrating how such kind of interactions could disrupt the parasite.[33] Hence, it is possible that a variety of compounds in AFE exert their activity at different parasitic sites or metabolic pathways, with the possibility that one of them affects myo-inositol import or lipid metabolism in some other way.

Despite affecting peptides and amino acids metabolism, AFE, ANE, and artemisinin do so differently than chloroquine and quinine, known for affecting hemoglobin metabolism.[36] Neither AFE, ANE or artemisinin signatures resembled chloroquine's profile, but the peptide regions of the metaprint were similar to quinine (Figure 49). ANE would seem to be the most similar, but both the presence of artemisinin and flavonoids could suggest multiple ways of interacting with this pathway, from preventing heme detoxification to creating free radicals.[13] Thus, the ANE mode of action seems more similar to artemisinin, with a pleiotropic nature, than to quinine.[36]

Notwithstanding reports of synergy between phenolic compounds and artemisinin, neither plant extract elicits a profile that would seem indicative of this effect. ANE and artemisinin displayed no statistically significant differences in this test's conditions, which would indicate a similar mode of action in this setting, regardless of other compounds. On the other hand, AFE showed statistically significant differences in profile, indicating that this extract's activity is not due to a main effect from artemisinin or its potentiation due to synergism with other compounds. As such, the exploration of this plant's extract could reveal promising compounds with innovative antiplasmodial targets.

These results align with the reported benefits of *A. afra* and *A. annua* when used against malaria. *A. afra*, despite vestigial amounts of artemisinin as detected by DI-HRMS, does not owe its activity to this sesquiterpene and instead seems to rely on the presence of other compounds, like flavonoids, to interfere with the parasite, and lipid precursors to some extent. The findings presented here echo

other studies in the literature that report an *A. afra* activity correlated with compounds other than artemisinin, including other sesquiterpenes that have been found to be gametocidal.[15] Contrarily, *A. annua* seems to have its activity mostly related to its content in artemisinin, with no statistically significant differences in metabolites between the two treatments. Indeed, both the ANE and artemisinin acted by affecting glutathione and lipid metabolism possibly due to oxidative reactions (Figure 49, 50 and 52). Interestingly, despite the long-term usage of this plant traditionally, artemisinin resistance was not discovered or otherwise noticed until the implementation of artemisinin and derivatives as an antimalarial drug.[2] Recent studies have found that the use of *A. annua* whole plant can overcome artemisinin resistance, which attests to the complex matrix as an interesting option for malaria treatment.[41–43] This could mean that although artemisinin plays a central role in the extract's antiplasmodial activity, other components in the extract or perhaps their interaction are able to prevent resistance, retain efficacy, and modulate other aspects of malaria at an inflammatory and immunological level.[1,16]

5. Conclusion/Perspectives

Both *A. afra* and *A. annua* have extensive reports of traditional use against malaria. Whereas the latter led to the discovery of artemisinin, the former retains activity despite seemingly vestigial amounts of this potent compound, according to the literature and our DI-HRMS detection. Our study demonstrates that *A. annua*'s activity is mostly related to artemisinin content and aligns with this compound's effects on glutathione. Meanwhile, *A. afra* may affect lipid precursors and signaling which would relate more to an interaction among extract constituents rather than artemisinin's effects, as these two profiles are distinct. While *A. annua* could prove an important tool to control the emergence and spread of artemisinin resistance, *A. afra* could offer innovative avenues in transmission blocking. Metabolomics assays could profile the gametocidal effects of *A. afra* and help the development of new antimalarials. Both plants act distinctly and should be further explored to accomplish malaria eradication.

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Chapter 3

Exploration of Modes of Action of Natural Compounds and Extracts

3. POUPARTIA & POUPARTONES

The work presented in this chapter was carried out in collaboration with Pr. Manuel Llinás and Dr. Gabriel W. Rangel at the Huck Center for Malaria Research (CMaR) at Penn State (The Pennsylvania State University) which kindly allowed the use of their inhouse LC-MS database for metabolite annotation.

3.1. Foreword

As mentioned previously, poupartone's activity in *in vitro* antiplasmodial assays jumpstarted an exploration regarding the activity of these new compounds. Notably, due to the toxicity found across multiple tests – zebrafish model, *in vivo,* and *in vitro* on multiple cancer lines and human skin fibroblasts – questions regarding a possible structure-activity that could justify these NP's potential are pertinent.[1,2] It is noteworthy to point out that there was no toxicity in hemolysis tests, which means the activity found against multiple strains of *P. falciparum in vitro* and *P. berghei in vivo* is not due to a general toxicity mechanism, but might be targeted. Metabolomics is a hypothesis-generating technique that rarely identifies single targets and thus is not appropriate to study structure-activity relationships as a standalone.[3] However, by comparing the profile of established antimalarial drugs, suggestions can be made concerning the effects of these compounds on the parasite's metabolome and as such, enlighten their MoA. Poupartone A and B were chosen for this study because they are the most active and abundant of the three poupartones (Figure 40).[2]

3.2. Materials & Methods

P. borbonica extract, a purified fraction from crude ethyl acetate extracts rich in poupartones (A, B, and C), poupartone A, poupartone B, artemisinin, atovaquone, chloroquine and quinine were studied in metabolomics assays as described in Chapter 3.1 Section 1.2. Samples were analyzed by LC-MS in the same system and conditions and spectra were processed and analyzed similarly.

3.3. Results & Discussion

The data was first observed through a self-organizing metaprint as described in previous sections, and the result is shown in Figure 54.[4] The extract of *Poupartia,* similarly to the poupatones A and B, seems

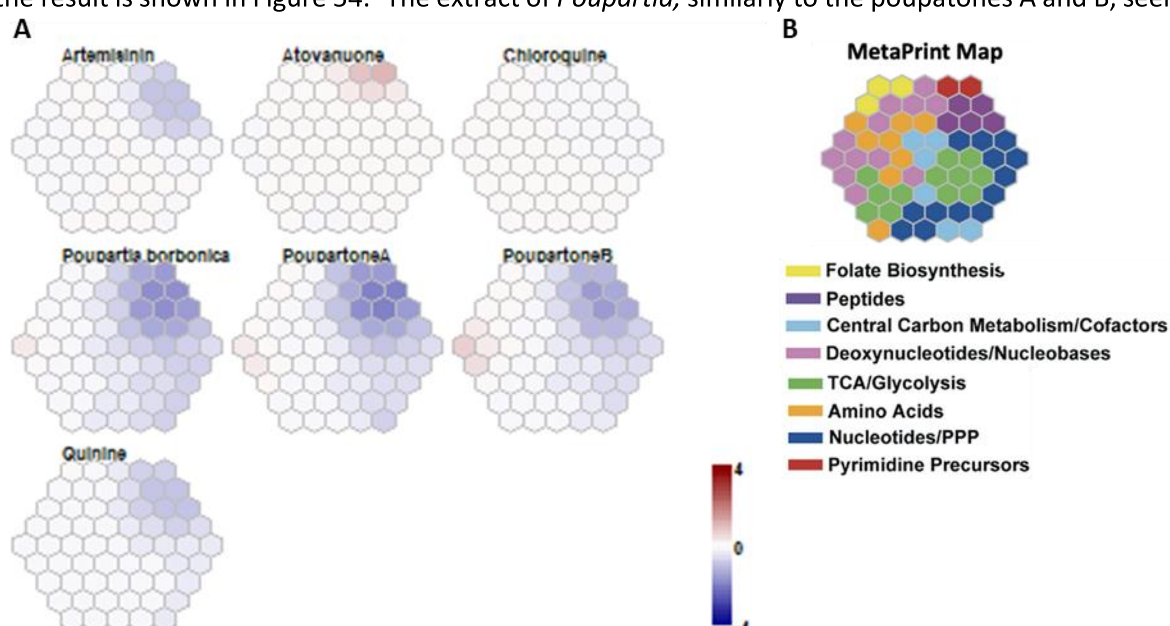

Figure 54 - (A) Metaprints of the study groups. (B) Metaprint map with a color-coded legend adapted from Allman *et al.* 2016.[4]

to have a unique print with negative fold in the pyrimidine precursors, peptides, nucleotides and TCA/glycolysis metabolites areas, and a positive fold in deoxynucleotides/nucleobases area. This profile does not match chloroquine's or atovaquone's, and preliminary PCA observation (data not shown) also showed a clear difference from artemisinin on opposite sides of the PC1 (approximately 50%). Contrarily, quinine clustered on the same side of the PC1, and as such this group was kept in subsequent studies. Through the metaprints, it is visible that the *Poupartia* extract and poupartones share the negative fold region pyrimidine precursors and peptides, which could represent a degree of conflation in their MoA worth exploring.

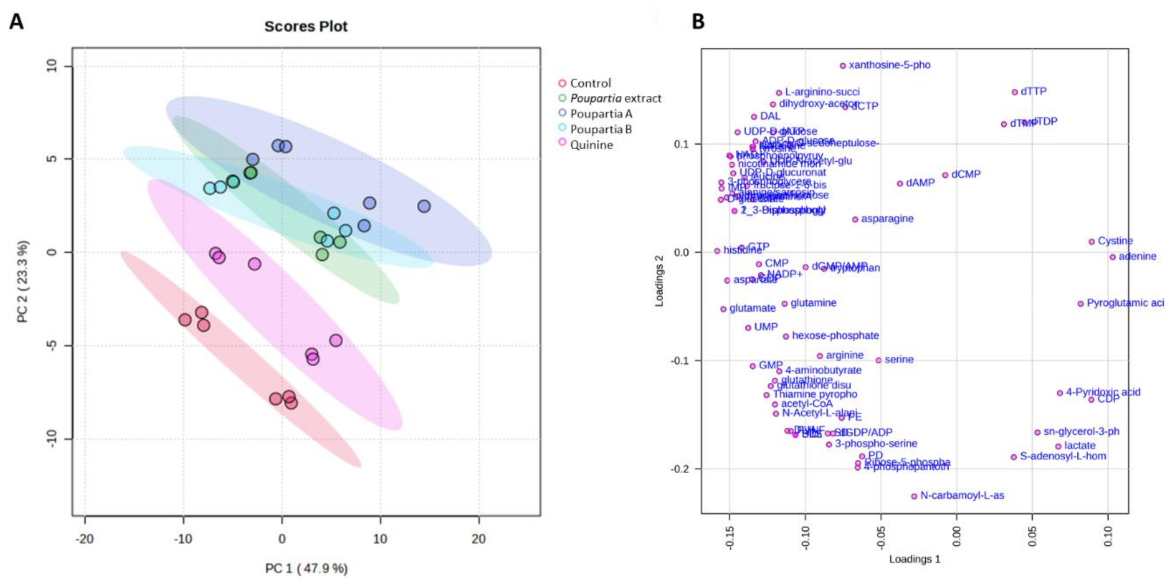

Figure 55 – (A) PCA scores plot with 95% confidence regions displayed with the respective loadings plot (B) of samples treated with poupartones, *Poupartia* extract, quinine and control.

To simplify visualization, PCA scores plot were constructed only with the poupartones, the extract, quinine and control. The result is shown in Figure 55. Visually, it is noticeable that the groups' variability can be described by both PC1 and 2 (71.2 %) by the way the groups are skewed across the plot. Notably, the control group is isolated on one side, while the poupartones and the extract cluster on the opposite end, with quinine in between. The loadings are also distributed across both PCs, making interpretation difficult between group effects or assay effects. Other models were used to further explore the data and try to identify the metabolites responsible for the treatment clustering.

The PLS-DA model was used to search for the metabolites that could identify the groups through a supervised model. The scores plot is shown in Figure 56. Component 1 appears to be responsible for the separation of the control group from the treatments. The cross-validation parameters are not optimal however with Q^2 of 0.03, which indicates an overfit model. Component 3 is portrayed because it separates the quinine-treated group from the other treatments, whereas component 2 related only with the intragroup variation (24.9%, R^2 - 0.62, Q^2 - 0.34). Component's 3 cross-validation parameters

are better than the first component's, but still both top 15 VIP metabolites were investigated due to the information that each component awards individually and are displayed in Figure 57.

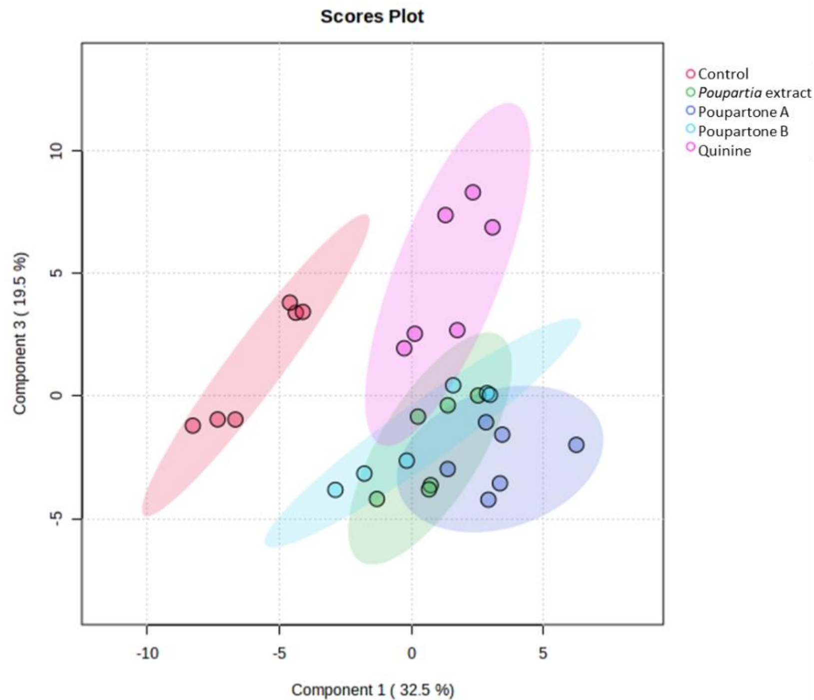

Figure 56 – PLS-DA scores plot with 95% confidence regions of the *Poupartia* study. (R^2: PC1 - 0.33, PC3 - 0.77; Q^2: PC1 - 0.03, PC3 - 0.52)

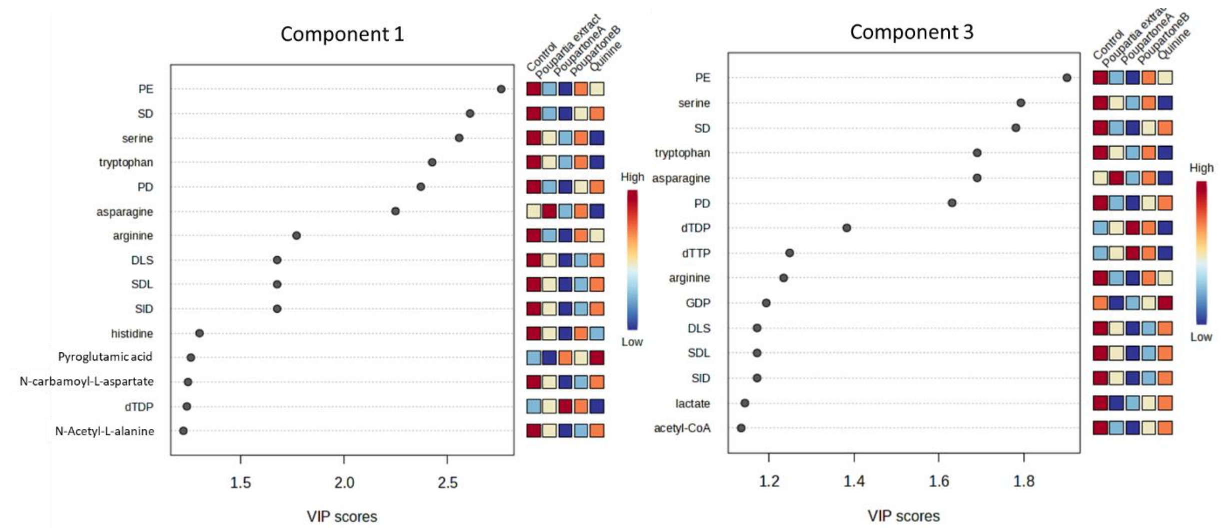

Figure 57 – Top 15 PLS-DA VIP scores of components 1 and 3. The colored boxes to the right of the VIP scores represent the relative intensity of the corresponding metabolite in each group under study. (PE - Prolyl-Glutamate; SD - Serylaspartic acid; PD - Prolyl-Aspartate; DLS - leucyl-aspartyl-serine; SDL - seryl-aspartyl-leucine; SID - aspartyl-isoleucyl-serine)

The VIPs for component 1 are mostly peptides and amino acids whose fold change is higher for the control group than for the treatment groups. This could reflect their availability following hemoglobin breakdown and justify why pathway analysis points to amino acid metabolism and aminoacyl-tRNA biosynthesis being affected distinctly between groups. The latter is crucial for the synthesis of proteins which is essential for parasite replication and survival. For component 3, the fold of a few hemoglobin-

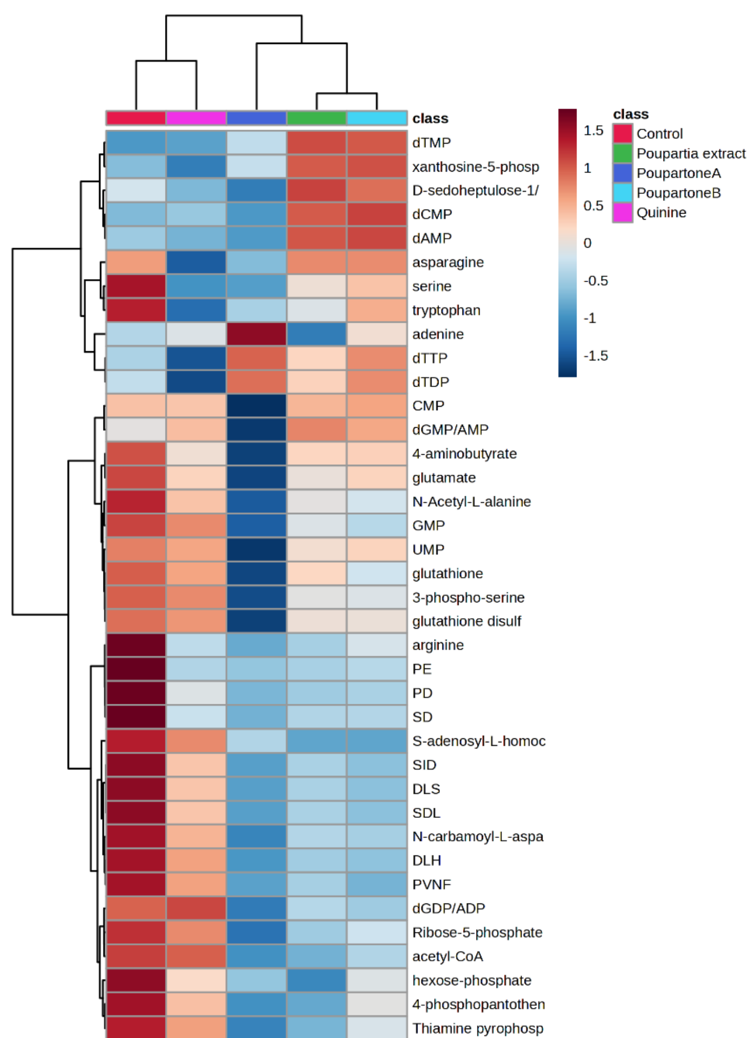

Figure 58 – Heatmap of the metabolites scored as significant by ANOVA. DLH – aspartyl-histidyl-leucine; DLS – leucyl-aspartyl-serine; PE - Prolyl-Glutamate; PD - Prolyl-Aspartate; PVNF – L-prolyl-L-valyl-L-asparaginyl-L-phenylalanine; SD - Serylaspartic acid; SID – aspartyl-isoleucyl-serine; SDL – seryl-aspartyl-leucine.

derived peptides (prolyl-glutamate, serylaspartic acid, prolyl-aspartate, leucyl-aspartyl-serine, seryl-aspartyl-leucine, aspartyl-isoleucyl-serine) in particular is different for quinine, which justifies this separation. Both components indicate dTDP as an important metabolite that has the highest fold for poupartone A, which could indicate a pyrimidine synthesis selective effect by this compound, and an interference at this level for alkyl cyclohexenones.

ANOVA was performed and the significant metabolites were plotted in a heatmap with the clustering shown in the form of a dendrogram (distance measure: Euclidean, clustering algorithm: Ward) in Figure 58. 38 metabolites were classified as significative (p-value < 0.05) and distributed in a heatmap that appears to visually separate in two: top, in which control and quinine have negative fold, and bottom, in which the opposite occurs. Interestingly, poupartone A seems to have a unique profile in between the two clusters: to the right, poupartone B and the extract, to the left, quinine and the control. As the major compound in the extract, it would be logical for the two's profile to align more. The hierarchical clustering confirms the chemometrics visualization that poupartones do not cluster with quinine or the control and shows the fold of the metabolites responsible for this separation. There are three peptides (prolyl-glutamate (PE), prolyl-aspartate (PD), and serylaspartic acid (SD)) with similar fold change between quinine, the poupartones and the extract, but most have a different fold. This suggests that even if hemoglobin metabolism is affected, it might happen in a different way from quinine's effects. These results are consistent with the results in Chapter 3.1 where

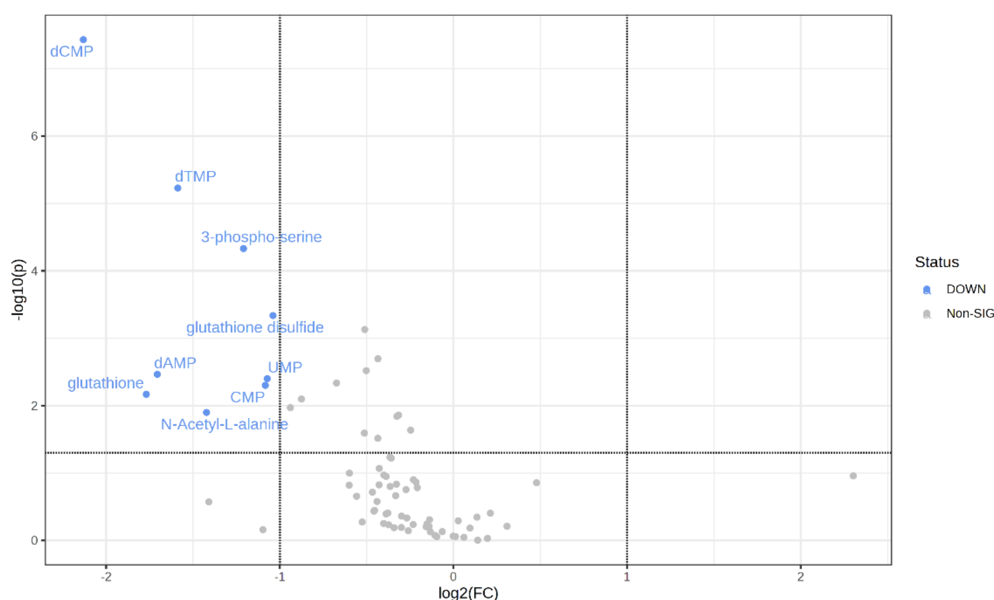

Figure 59 – Volcano plot between poupartone A/poupartone B (p-value: 0.05, FC threshold: 2.0). Points represent metabolites: in gray (non-significant), red (positive fold), blue (negative fold).

the profiles between quinine and poupartone B didn't align fully (Figure 45). Glutathione, 4-aminobutyrate, and nucleotides seem to be differently affected as well, particularly for poupartone A.

Because of this difference, a volcano plot was used to in the same graphic portray both the significance (*t*-test, p-value < 0.05) and fold change (threshold: 2) between poupartone A and B. This plot is shown in Figure 59. Notably, significant metabolites all are negatively expressed, *i.e.,* have a negative fold for poupartone A when compared with poupartone B. These comprise nucleotides, mostly pyrimidine metabolism precursors, and glutathione metabolism metabolites. Poupartone A would seem to cause oxidative damage more severely than poupartone B and distinctly from quinine, that is not known to exert its antimalarial activity this way.[1,5] Artemisinin is linked with oxidative reactions, but the simultaneous interaction with heme and other structures elicits a different profile. This activity by poupartone A could be due to the α-β unsaturated ketone that can act like Michael acceptor, but it wouldn't justify the different intensity between the two poupartones, since their ring is the same (as shown in Figure 40). Toxicity of other alkyl cyclohexenones compounds also seems to be linked with the number of oxygens, but poupartones A and B have the same number.[1] The only structural difference between poupartone A and B is a double bond at C10' and C11', which could mean this link has an impact on the antiplasmodial activity and the difference between profiles, though this needs to be confirmed.

In general, poupartones seem to affect hemoglobin digestion, DNA and RNA synthesis and redox management systems. The latter could be due to an interaction with the mitochondria respiratory chain, but as the profile is different from atovaquone, a inhibition of the cytochrome bc1 complex can be excluded.[5,6] Their α-β unsaturated ketone could participate in nucleophilic additions that establish

covalent bonds with proteins, inactivating them, and generate ROS, as happens with quinones.[6] This would not only disable important peptides and proteins like chaperones, but would tamper with the redox managing systems, leading to a cascade effect that would elicit irrecoverable toxicity. This MoA would be in line with a lack of *in vitro* and *in vivo* specificity found for these compounds and justify how their development into anticancer drugs might be more pertinent.[1]

3.4. Bibliography

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Chapter 3

Exploration of Modes of Action of Natural Compounds and Extracts

4. ELLAGIC ACID & DERIVATIVES

The work presented in this chapter was carried out in collaboration with Pr. Manuel Llinás and Dr. Gabriel W. Rangel at the Huck Center for Malaria Research (CMaR) at Penn State (The Pennsylvania State University) which kindly allowed the use of their inhouse LC-MS database for metabolite annotation.

4.1. Foreword

As mentioned in Section 2 of Chapter 1, phenolic compounds hold great interest in the context of drug discovery from natural sources based on their abundance and potential activities.[1] This is the case of ellagic acid (Figure 60), a dimeric gallic acid derivative, dilactone of hexahydroxydiphenic acid (HHDP), present in high concentrations in fruits, particularly berries, either in free form or in ellagitannins.[2,3]

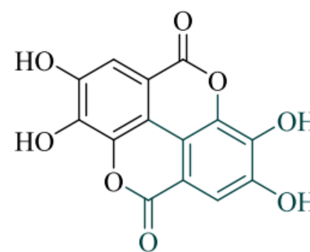

Figure 60 - Ellagic acid. Back and green represent the two gallic acid moieties.

These secondary metabolites are tannins, water soluble phenolic compounds that can release ellagic acid in the human gastrointestinal tract after hydrolysis. However, because ellagic acid has very low water solubility, it is only partly absorbed in the small intestine.[2] This low solubility and consequential low bioavailability is thought to be due to its planar structure, which facilitates intra and intermolecular bonds into a knit-tight structure.[2,4] The capability to both accept and donate electrons is thought to be responsible for the many different activities reported for this molecule, especially *in vitro.*[1,2,4] Its antioxidant, anti-inflammatory and antiparasitic activities are of note as they all interconnect in the context of malaria.[5] Literature findings supporting these activities indicate that the traditional use of plants containing high concentrations of ellagic acid against malaria is because of the inflammation and immunity management more than a direct antiplasmodial activity. This is supported by the numerous studies that demonstrate *in vivo* that there is little to no significant parasitemia decrease when ellagic acid is administered *per os*.[1,4,6] However, new studies of traditionally used plants against malaria continue to isolate and point to ellagic acid or derivatives as their main antiplasmodial constituents.[7–12] Despite the intricacies linked with this particular topic, the versatility of ellagic acid still represents a good opportunity to expand on the antiplasmodial activity and develop it into *in vivo* and future use as an antimalarial.

To accomplish this, a former PhD student at the CIRM took ellagic acid and developed multiple analogues based on the premise that if water solubility was improved, so would bioavailability be and hence a potential hit scaffold could be identified. There were two major multistep synthetic pathways selected for the synthesis of analogues described by Degotte et al.[4] Briefly, one modulated the para-substituents on the 3,4,5-trihydroxybenzoate moieties and created many sub products, of which compound A (Figure 61A), the second pathway parallelly synthesized dimeric gallic acid products with different numbers of phenolic functions with

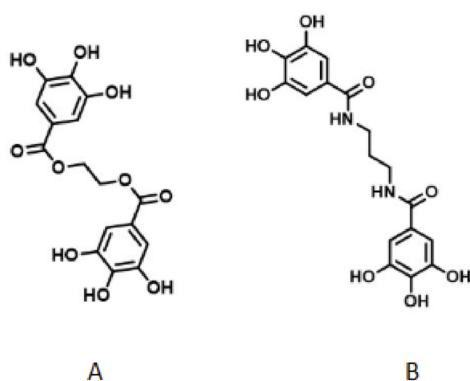

Figure 61 - Chemical structures of the polyphenol analogues studied. (A) Ethane-1,2-diyl bis(3,4,5-trihydroxy)benzoate), (B) N,N'-(propane-1,3-diyl)bis(3,4,5-trihydroxybenzamide).

different linkers, of which compound B (Figure 61B). These analogues were studied extensively and described as the focus of two papers by Degotte et al.[4,5] Table 21 resumes the most recent findings regarding ellagic acid and analogues activity and solubility.

Table 21 - Update on ellagic acid and analogues activity.

	Strain/Assay	Ellagic Acid	Ethane-1,2-diyl bis(3,4,5-trihydroxy)benzoate) (A)	*N,N'*-(propane-1,3-diyl)bis(3,4,5-trihydroxybenzamide) (B)	*References*
Antiplasmodial assays *(P. falciparum)* *(µM)*	3D7 FcB1 W2	2.8 ± 1.9 7.5 ± 2.8 8.9 ± 5.9	3.7 ± 1.1 3.7 ±1.2 6.7 ± 3.2	9.9 ± 3.2 26 ± 6.7 12 ± 1.5	4
Antioxidant	DPPH	5.64 ± 0.47	3.36 ± 0.25	3.70 ± 0.45	5
	ABTS	6.01 ± 0.26	5.77 ± 0.01	5.86 ± 0.18	5
	HRP	ND	2.84 ± 0.00	3.06 ± 0.07	5
Cytotoxicity (µM)	HUVEC	> 165	69 ± 12	130 ± 6.8	4
Solubility (Cmax in mM)		0.018 ± 0.003	0.43 ± 0.25	0.61 ± 0.006	4
cLogP		1.1	1.04	1.6	4

Ellagic acid's IC_{50} more than doubled between chloroquine-sensitive (3D7) and resistant strains (FcB1), tripling with multi-resistant W2 strain. Interestingly, the exact same does not happen for the analogues: compound A has the same IC_{50} independently of chloroquine resistance or not, only augmenting with multi-drug resistance, and compound B shows a lower effect against FcB1 than any other strain. This could suggest that compound B might interact with *P. falciparum* chloroquine resistance transporter, which would suggest an action site at the DV and a possible interference with hemoglobin digestion.[4]

Regarding the antioxidant activity, all three radical scavenging tests demonstrated a great antioxidant potential for ellagic acid, in line with the results reported in Table 10, and analogues, with their IC_{50} being similar or better. Structure-activity data indicate that the presence of free phenol groups seems a requirement for the antioxidant activity of these molecules, whereas other parameters of the analogues didn't seem to impact this activity.[5] As the parasite is heavily dependent on antioxidant systems to maintain homeostasis during the intraerythrocytic cycle, a target at this level cannot be excluded, as seems to be the case with molecules like artemisinin or methylene blue.[5,13]

Both Human Umbilical Vein Endothelial Cells (HUVEC) *in vitro* and zebrafish (*Danio rerio*) were used to account for possible toxicity of ellagic acid and analogues. No significant toxicity was reported in either model, including morphological alterations, with the exception of compound B displaying a negative effect on embryo hatching. Embryos developed correctly, but only 20% were able to hatch when in contact with 30 and 100 µg/ml of compound B, even 5 days post-fertilization.[4] An accumulation of compound B on the chorion could be an explanation, so the experiment was repeated without the

chorion and there were no developmental changes to note with equal survivability across all concentrations (>95%).[4]

Solubility in water was improved for the analogues as it is visible in Table 21, but the lipophilicity (cLogP) stayed approximately the same. This demonstrated that a multitude of molecular factors could play a role in solubility, so their impact on activity was evaluated *in vivo* with compound B in a Peters' 4-days suppressive test.[4] This compound was more effective in reducing parasitemia *per os* (54%) than ellagic acid (0%), despite a big standard deviation (± 43%) that could be due to incomplete solubilization or mice regurgitation. In spite of fast recrudescence at Day 7, compound B demonstrated a significant improvement in activity when administered orally, and as such a potential scaffold for future enhancements.[4]

To supplement this information and try to shed more light on possible MoA, and verify if it is indeed related to hemoglobin digestion, metabolomics assays were conducted in the same conditions as explained previously and the results and discussion follow in the next sections.

4.2. Materials & Method

These experiments were conducted in similar conditions described in Chapter 3.1 Section 1.2 (Figure 41) in three independent moments (n=3). The drugs used for fingerprint comparison were atovaquone, chloroquine and artemisinin, and three products were tested: ellagic acid, compound A and B. LC-MS data was processed similarly and results are presented in the next section.

4.3. Results & Discussion

To have an overview of the fingerprints elicited by each drug and compound, a self-organizing metaprint was generated first (Figure 62A) according to the map on Figure 62B.[14] All three antimalarial

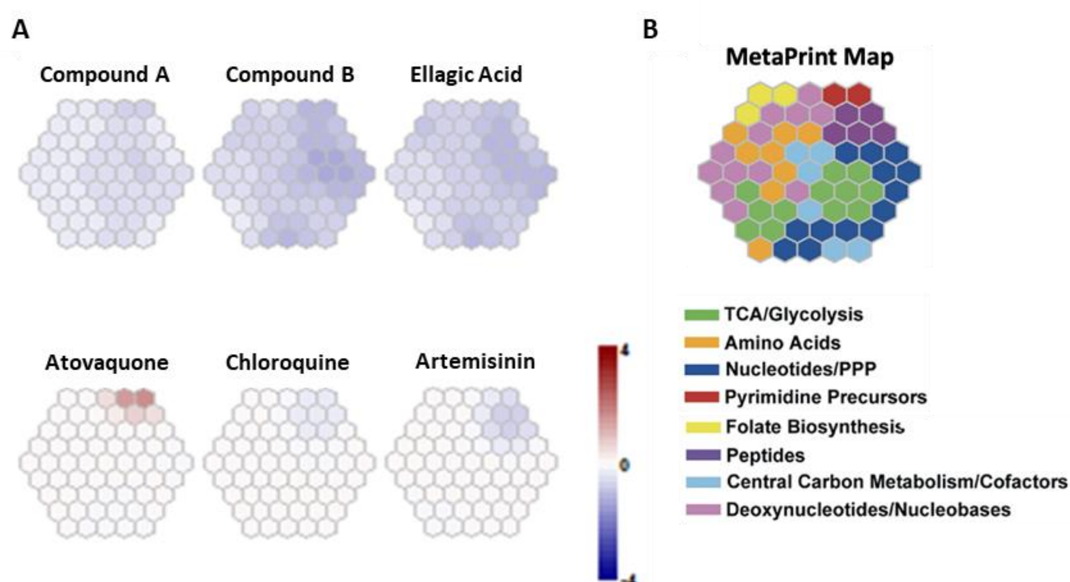

Figure 62 – (A) Metaprints of the study groups. (B) Metaprint map with a color-coded legend adapted from Allman et al. 2016.[14]

drugs elicited a profile similar to previous assays. Perhaps not so surprisingly, ellagic acid and compound A and B displayed fairly similar profiles, with an overall negative fold across the entire map, different from the three drugs tested. Interestingly, compound A had the closest fold to 0 for many hexagons, which could be related to a closeness to the control and a lack of specificity in the MoA, meanwhile compound B and ellagic acid had a lower fold, especially for peptides, nucleotides and nucleobases.

Visualization through PCA was of difficult interpretation due to the many groups in the study. PLS-DA did not achieve separation between groups regardless of the component used, while sparse PLS-DA did accomplish separation but with classification errors above 70% for the first 2 components (the ones that achieved separation).

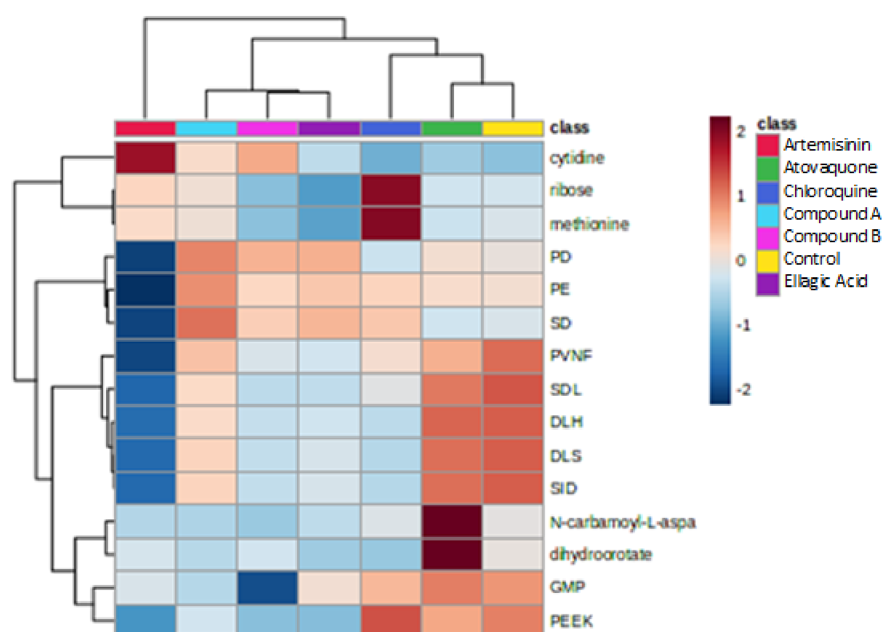

Figure 63 - Heatmap of the metabolites scored as significant by ANOVA. DLH – aspartyl-histidyl-leucine; DLS – leucyl-aspartyl-serine; PE - Prolyl-Glutamate; PD - Prolyl-Aspartate; PEEK – methanone; PVNF – L-prolyl-L-valyl-L-asparaginyl-L-phenylalanine; SD - serylaspartic acid; SID – aspartyl-isoleucyl-serine; SDL – seryl-aspartyl-leucine.

The heatmap for all compounds tested is presented in Figure 63 and was built by performing an ANOVA test and plotting the 15 significant metabolites (p-value < 0.05) and clustering them in the form of a dendrogram (distance measure: Euclidean, clustering algorithm: Ward).

It is noteworthy to mention that the analogues and ellagic acid cluster together based on their close profile, with the exception of compound A that seems to have a higher fold for a few peptides. Likewise, these compounds don't seem to cluster closely with any of the drugs used or the control.

The second exploration of the data was done in relation to ellagic acid alone. An initial PCA with this compound and the antimalarial drugs tested is shown in Figure 64A. PC3 (7.9 %) is displayed to achieve separation between groups in which only artemisinin stands out as different from the rest. The

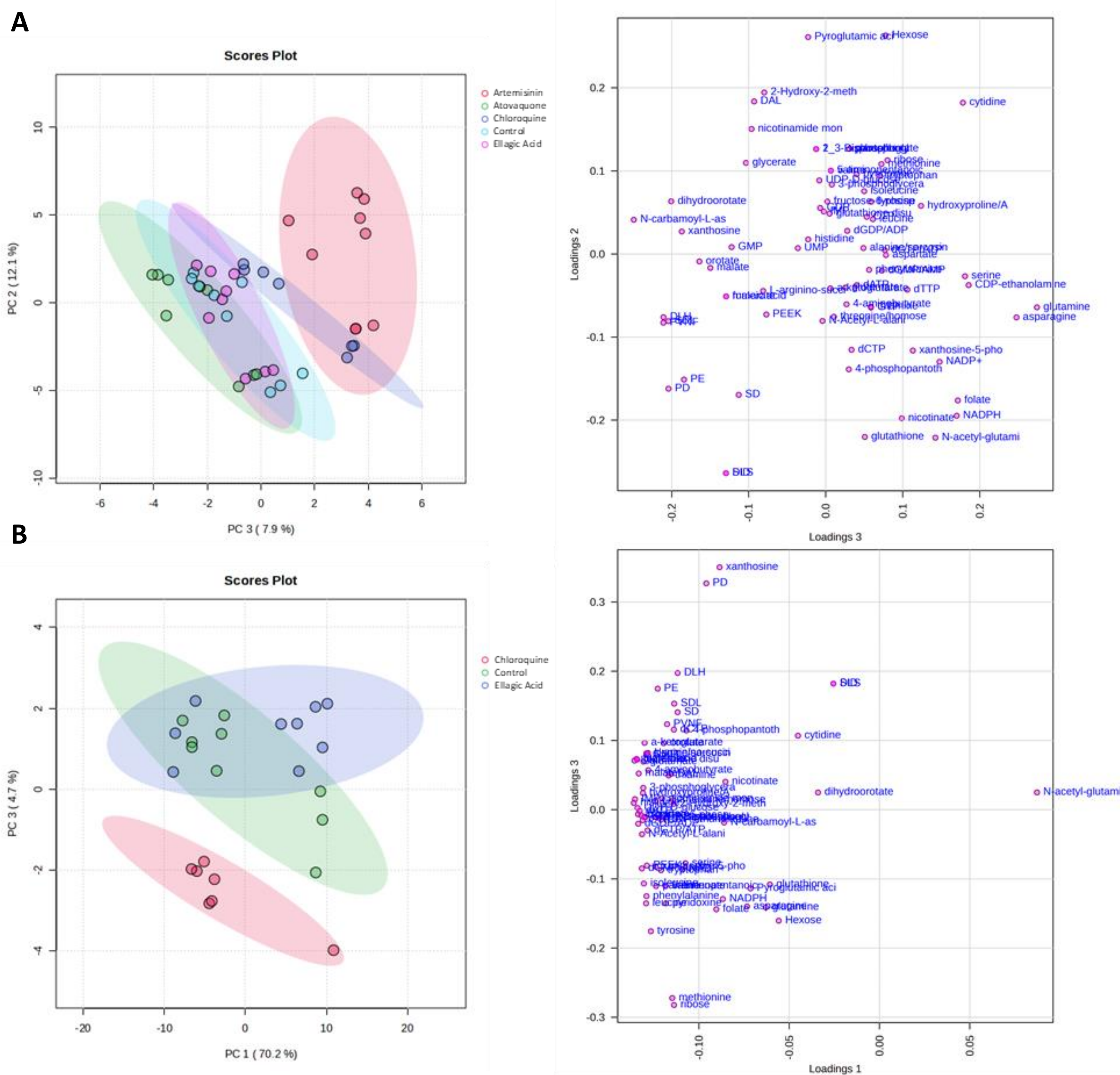

Figure 64 - (A) PCA scores plot with 95% confidence regions of ellagic acid and antimalarial drugs studied, and the corresponding loadings plot. (B) PCA scores plot of ellagic acid, chloroquine and control, and the corresponding loadings plot.

loadings plot doesn't appear conclusive, so an ANOVA test was plotted in the same way as previously and is shown in Figure 65. Clustering groups ellagic acid and chloroquine is based on their similar profile for 9 peptides in the 14 significant metabolites, and as such a second PCA was plotted for just these two compounds and control, and it is shown in Figure 64B. Again, PC3 (4.7 %) was used to separate the groups, which does not account for a big variability difference between them. The loadings plot pointed to PD (prolyl-aspartate), xanthosine, methionine and ribose as the metabolites responsible for this separation, but as building blocks involved in many important metabolic routes, these seem inconclusive to a specific MoA.

Concerning compounds A and B, as mentioned previously, their profiles seem to cluster closely with ellagic acid, which could be in line with the analogues retaining the active scaffolds of the original

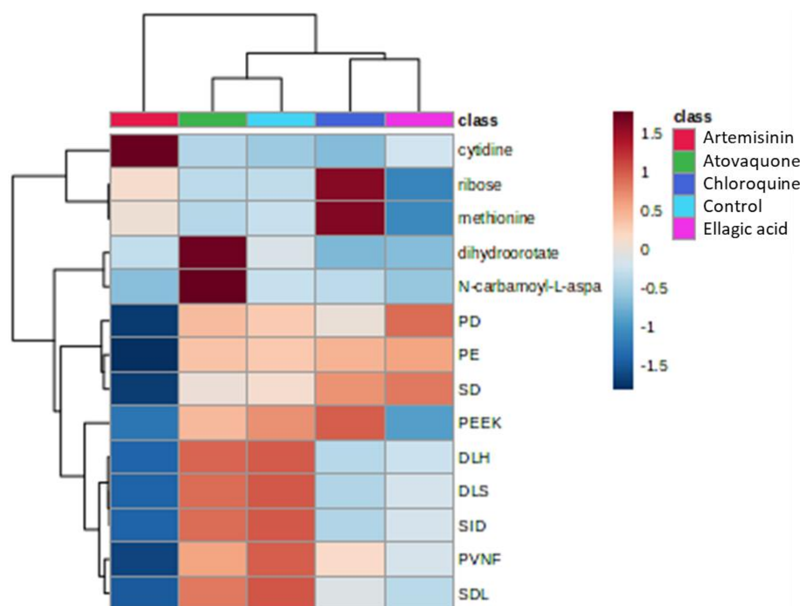

Figure 65 - Heatmap of the metabolites scored as significant by ANOVA – antimalarials and ellagic acid only.

molecule and acting similarly. Statistical exploration of these compounds and ellagic acid showed superposition in PCA (82%) and no ANOVA significant metabolites (p-value < 0.05).

Due to the possible similarities between ellagic acid and chloroquine profiles, individual studies with volcano plots were carried out and are presented in Figure 66. Volcano plots conflate a fold-change algorithm (threshold: 2.0) and *t*-test (p-value < 0.05) in order to create an easily interpretable plot that associates significance with different tendencies. The furthest a point (metabolite) is from the center, the more significant it is in representing a difference between two groups.

It is interesting to note that there aren't many significant metabolites no matter the pairing studied (Figure 66A, B and C). For ellagic acid, a few of the same redundant metabolites as before appear as significative, meaning there seem to be differences between chloroquine's and ellagic acid's MoA, but it is difficult to tell exactly how. Regarding compound A, only three metabolites appear as statistically significant: PD (p-value = 0,0005, intensity variation between groups explained in Figure 67) and xanthosine with positive fold, and glutathione with negative fold. These are common with the plot concerning compound B/chloroquine, which in turn has more nucleotides. Interestingly, if the p-value for the *t*-test is the FDR (corrected p-value) instead, all metabolites fall below the threshold aside for a positive PD when compound A is compared with chloroquine, giving it further pertinence in the discussion of a potential MoA of these compounds.[15]

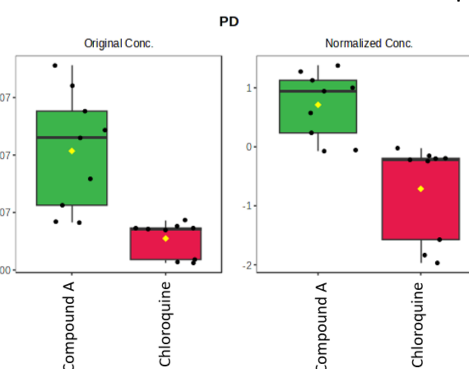

Figure 67 - Box plot with the original and normalized intensity of PD in samples treated with compound A and chloroquine.

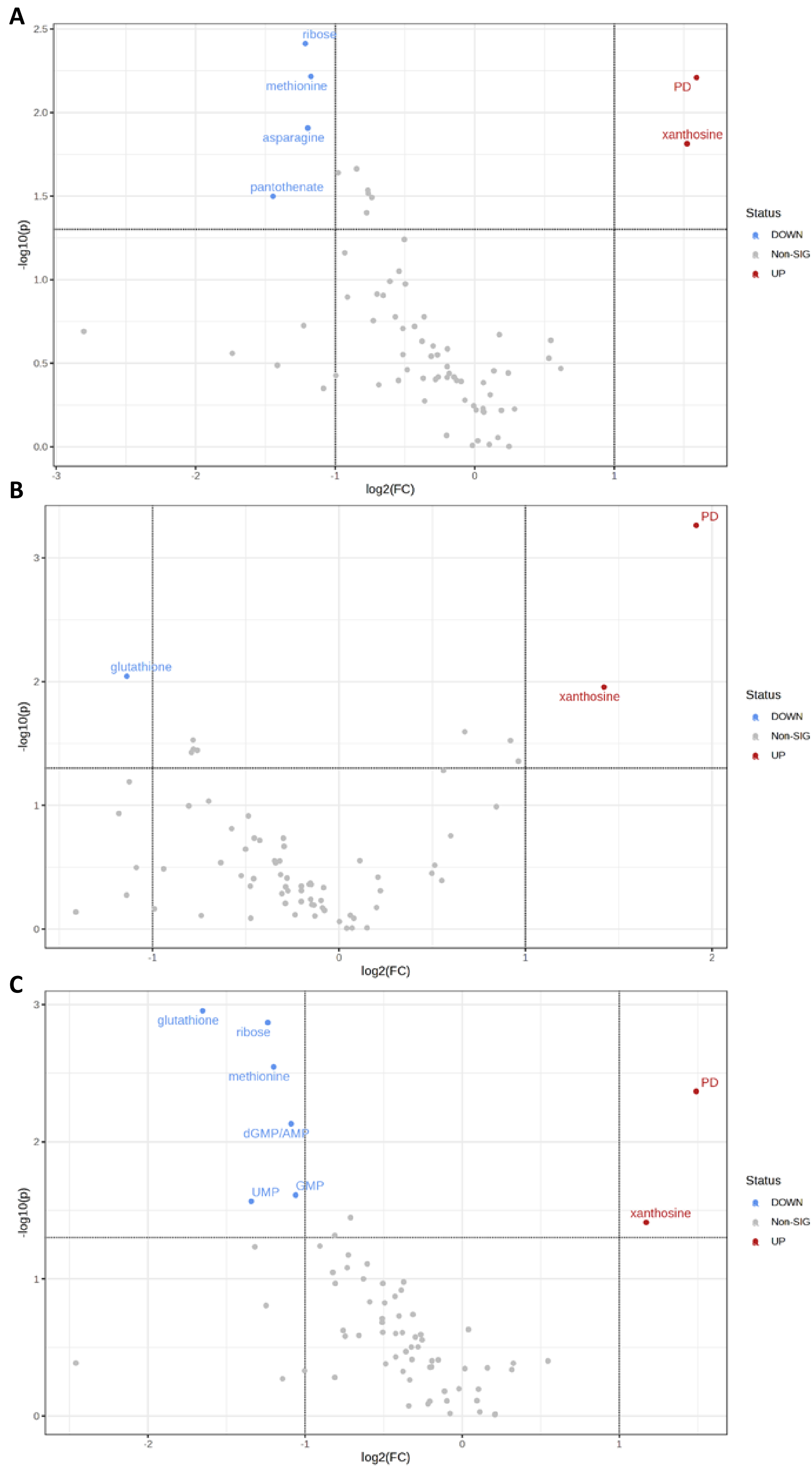

Figure 66 – Volcano plots : (A) Ellagic acid/chloroquine, (B) Compound A/chloroquine, (C) Compound B/chloroquine (p-value: 0.05, FC threshold: 2.0). Points represent metabolites: in gray (non-significant), red (positive fold), blue (negative fold).

As mentioned previously in Section 2 of Chapter 1, ellagic acid has three potential MoA according to studies in the literature: interfering with hemoglobin metabolism, chelating the iron in heme, and redox associated reactions.[1] Some of these are transversal to other phenolic compounds and, if the scaffold is maintained while synthesizing analogues, these MoA could be transposed as well.

According to the metabolomics results presented in this section, ellagic acid and analogues seem to have differing MoA from artemisinin and atovaquone, standing closer to chloroquine. Importantly, this clustering is due to hemoglobin-derived peptides, which is in line with chloroquine's known MoA and further supports the idea that at least one site of action of ellagic acid is in the DV.[4]

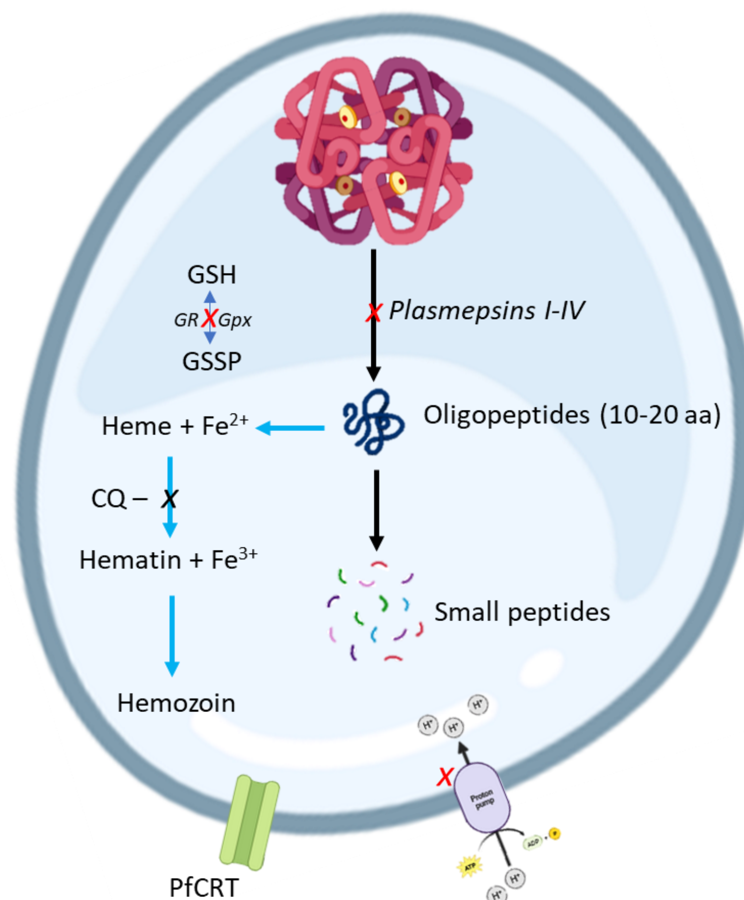

Figure 68 – Plasmodium sp. digestive vacuole. Italic represents enzymes and red crosses possible sites of action of ellagic acid and analogues. Aa – amino acids, CQ – chloroquine, *Gpx* - glutathione peroxidase, *GR* – glutathione reductase, GSH – reduced glutathione, GSSP – oxidized glutathione, PfCRT - *P. falciparum* chloroquine resistance transporter.[1,16,17] (Created in BioRender.com)

However, these mechanisms might not be the same – as depicted in Figure 68.[1,16,17] Chloroquine inhibits heme biomineralization within the DV, which indirectly interferes with hemoglobin metabolism and creates oxidative stress.[13,18] However, the DV is a specialized acidic lysosomal organelle with a specific pH (3.7–6.5), membrane transporters and localized enzymes that can also become as direct antimalarial targets.[18,19] The acidic pH of this compartment is essential for the parasite to obtain most of the amino acids required for its proliferation and deviations from this acidic interval could result in enzyme inactivation and ionic homeostasis difficulties.[18,19] A study by Muchtar

et al. sought to evaluate the effects on the DV pH in ellagic acid-treated parasites *in vitro* and found that it changed significantly in a concentration-dependent way from 6.11 to 6.74 ($p < 0.001$).[20] This result was comparable to a concanamycin A, a standard proton pump inhibitor, and seem to suggest that ellagic acid might change the DV pH by inhibiting proton pumps and preventing its acidification. However, other crucial targets exist within this organelle. Notably, hemoglobin digestion begins by the action of a group of aspartic proteases named plasmepsins (I, II, HAP, IV).[21] Since PD is pointed as a significant metabolite for ellagic acid and analogues, a possible interaction with these enzymes could be theorized. Interestingly, studies in the literature support this interaction. Two studies by Dell'Agli *et al*. aimed to assess the inhibitory activity of the methanolic extract of sun-dried rind of immature fruits of *Punica granatum* L. (pomegranate), its fraction enriched in tannins and ellagic acid against recombinant plasmepsin II in enzymatic tests.[22,23] Ellagic acid inhibited plasmepsin II but needed ten times its antiplasmodial IC_{50} to achieve this effect, hence it is likely not the main MoA. In the metabolomics tests of this chapter, 10 x IC_{50} was also the tested condition, and as such, the same effect on plasmepsin II is possible and could be reflected on the effect in PD. Additionally, only 3 times the IC_{50} was needed to inhibit β-hematin formation in the same study, which would have put the profiles between ellagic acid and chloroquine much closer, which seems to be the case in our study.[22]

However, the lower fold of glutathione as a significant metabolite for the analogues when compared with chloroquine does point to an activity at the redox level greater than chloroquine's, so a potential target at this level cannot be completely excluded. This was further confirmed by a study by Soh *et al*. where ellagic acid's antiplasmodial activity decreased against an *ex vivo P. yoelii* strain that expresses higher levels of glutathione (IC_{50} of 4.867 ± 0.009 µM) compared to the wild-type (IC_{50} of 0.906 ± 0.219 µM).[24] Additionally, tests on the FcM29 strain of *Plasmodium falciparum* (Cameroun chloroquine-resistant) showed a decrease in activity of ellagic acid when associated with antioxidants, and a decrease in 43% reduced glutathione when parasites were treated with ellagic acid.[24] This confirmed that glutathione metabolism is closely linked with ellagic acid's activity, and the same could be extrapolated to its analogues according to the results presented in this section.

It could be argued that ellagic acid is a compound with known antioxidant properties, so these results seem contradicting.[1,2] However, polyphenols are capable of both anti and prooxidant activities depending on the chemical environment and concentration, which inside the acidic DV with the accumulation of heme [Fe(II)] during hemoglobin metabolism could prove beneficial by introducing prooxidant conditions and saturating glutathione-related antioxidant systems.[5] Clinical reports that associated the addition of antioxidants to conventional antimalarial therapies with positive outcomes might be linked with this mechanism, *i.e.* by disturbing redox systems within the parasite's DV, or simply by managing the immunological response and inflammation processes in the host, or both.[2,5]

Mohanty *et al*. recently reported on ellagic acid's neuroprotective role in a cerebral malaria murine model in which pro-inflammatory cytokines were significantly inhibited and the blood brain barrier integrity was restored in a dose dependent manner, further confirming this beneficial capacity.[6]

In humans, ellagitannins are hydrolyzed and release ellagic acid, which is then metabolized into urolithins, which are reportedly not active *in vitro* (IC_{50} > 100 µM) and do not inhibit plasmepsins (IC_{50} > 50 µM).[2,23] Hence metabolism reduces the activity of plant-containing ellagic acid, which together with the low bioavailability does not make this compound the likely active antiplasmodial compound in traditionally used plants as most studies indicate.[23] However, since plasmepsins have low homology with human enzymes, a scaffold targeting these essential enzymes could prove innovating as a new antimalarial drug.[21]

Our results support ellagic acid's and analogues' site of action at the DV and are compatible with the reports of pH changes possibly through proton pumps inhibition that would change heme biomineralization and plasmepsins activity, thus influencing hemoglobin metabolism and the redox homeostasis as shown by PD and glutathione statistically significant changes. In order to confirm and complement this rationale, it would be interesting to test ellagic acid and analogues *in vitro* on strains with known mutations on the genes that encode plasmepsins, as is the case for piperaquine resistance, to verify if activity would change.[25,26] Additionally, other tests with recombinant plasmepsin II or that evaluate the levels of glutathione or DV pH can be performed for the analogues to complement their profile and discuss on whether the molecular scaffolds retain or lose targets in comparison with ellagic acid.

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Chapter 3

Exploration of Modes of Action of Natural Compounds and Extracts

5. TRITERPENE ESTERS

The work presented in this chapter was carried out in collaboration with Pr. Manuel Llinás and Dr. Gabriel W. Rangel at the Huck Center for Malaria Research (CMaR) at Penn State (The Pennsylvania State University) which kindly allowed the use of their inhouse LC-MS database for metabolite annotation.

5.1. Foreword

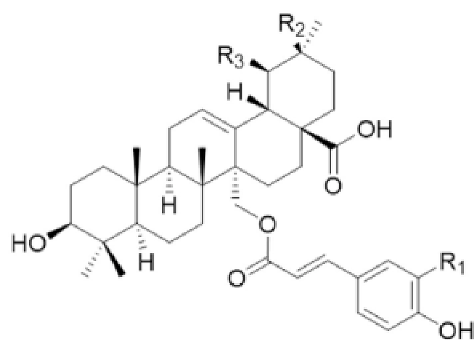
Thus far, as described, traditional medicinal plants have proven to be rich sources of potentially active and innovative molecular structures, making them the focus of research teams worldwide.[1] The UCLouvain-GNOS, a collaborating team of the METNATPAR project, investigated plant species commonly employed in traditional practices in Benin, with *Keetia leucantha* among the subjects of their study.[2] This plant, whose main traditional use in Benin appointed in the literature was for the treatment of malaria in a decoction soup three times a day, showed an interesting antiplasmodial activity with no toxicity (resumed in Table 22).[3] As the selectivity of the dichloromethane twig extract proved to be the most interesting from the conditions studied, further analysis was carried out to elucidate on this activity.

Table 22 – Activity of *Keetia leucantha* extracts and isolated compounds found in the literature.

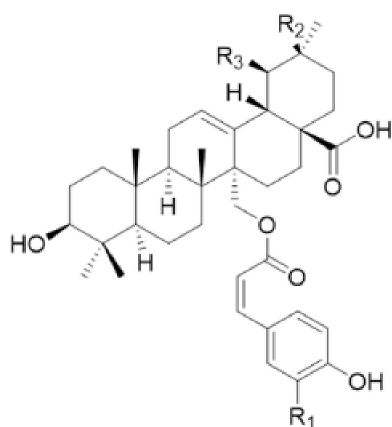
Part Studied	**Extract**	**Antiplasmodial activity (µg/ml)**		**Cytotoxicity (µg/ml)**		**Selectivity index**	**Reference**
		3D7	W2	J774	WI38	WI38/3D7	
Leaf	Dichloromethane	13.8 ± 8.3	26.5 ± 9.5	91.5 ± 3.1	65.6 ± 1.3	4.8	2
	Methanol	> 100	ND	> 100	> 100	1.0	2
	Water	> 100	ND	> 100	> 100	1.0	
Twig	Dichloromethane	11.3 ± 3.8	15.8 ± 2.3	50.5 ± 4.2	> 100	8.8	2
	Methanol	> 100	ND	> 100	> 100	1.0	2
	Water	> 100	ND	> 100	> 100	1.0	
8TTE (6-13)		1.66 ± 0.54	-	-	57.4*	34.5*	5
6-10		2.35 ± 0.44	-	-	ND	-	5
9-13		2.93 ± 1.23	-	-	ND	-	5
Ursolic acid		14.8*	-	-	6.7*	0.45*	5
Oleanolic acid		27.1*	-	-	59.6*	> 2.19	5

J774 – macrophage-like cell line from BALB/c murine reticulum cell sarcoma
WI38 – human normal fibroblast cell line
ND – Not determined
* Tested once

In vivo assays on *P. berghei* NK 173 in a 4-day suppressive test with the dichloromethane extract intraperitoneally and aqueous extract *per os* of *K. leucantha* twigs at the dose of 200 mg/kg/day demonstrated significant parasite inhibition of 56.8% and 53.0% (p-value < 0.0001) on day 7 post-infection.[5] Bioassay-guided fractionation of the dichloromethane twig extract using *P. falciparum* 3D7 *in vitro* led to the identification of antiplasmodial triterpenic acids (ursolic and oleanolic acids) and eight triterpenic esters (8TTE) shown in Figure 69.[5] 8TTE was first obtained as a mixture, which was then characterized by LC-HRMS, HPLC and multiple NMR techniques. Purification of pairs of *E/Z* isomers of each compound showed similar antiplasmodial properties.[3] A later study quantified these compounds in the dichloromethane twig extract and revealed a lower concentration in 8TTE (1.8%) in comparison with ursolic and oleanolic acid (16.9%), with both compounds responsible for the *in vivo* or *in vitro* activity as could be inferred from the results in Table 22.[6] To further verify 8TTE's activity,



1. R_1 = H, R_2 = CH_3, R_3 = H **(27-O-p-(E)-coumaroyloxyoleanolic acid)**
2. R_1 = H, R_2 = H, R_3 = CH_3 **(27-O-p-(E)-coumaroyloxyursolic acid)**
3. R_1 = OCH_3, R_2 = CH_3, R_3 = H **(27-O-p-(E)-feruloyloxyoleanolic acid)**
4. R_1 = OCH_3, R_2 = H, R_3 = CH_3 **(27-O-p-(E)-feruloyloxyursolic acid)**



5. R_1 = H, R_2 = CH_3, R_3 = H **(27-O-p-(Z)-coumaroyloxyoleanolic acid)**
6. R_1 = H, R_2 = H, R_3 = CH_3 **(27-O-p-(Z)-coumaroyloxyursolic acid)**
7. R_1 = OCH_3, R_2 = CH_3, R_3 = H **(27-O-p-(*Z*)-feruloyloxyoleanolic acid)**
8. R_1 = OCH_3, R_2 = H, R_3 = CH_3 **(27-O-p-(Z)-feruloyloxyursolic acid)**

Figure 69 - Molecular structures of 8TTE isolated from *K. leucantha* (from Beaufay et al 2019)[3]

this mixture was tested intraperitoneally at 50 mg/kg/day *in vivo* in a 4-day suppressive test of Peter's with *P. berghei*.[6] Parasitemia was shown to be significantly (p-value <0.01) inhibited (27.8 ± 5.4 %) on day 4 compared to vehicle control. Since pentacyclic terpenes are associated with toxicity, additional tests with extracts and 8TTE demonstrated no hemolysis and no *in vivo* acute toxicity up to a cumulative dose of 150 mg/kg.[6] Hence an argument was made that 8TTE are the interesting components responsible for the activity of this extract, albeit their low relative quantity, due to the selectivity they demonstrate.

Freire *et al*. correlated the metabolic content of *Keetia* sp. twig and leaf extracts, including the one discussed this far (dichloromethane extract of twigs of *K. leucantha*) with their antiplasmodial activity as reported in Table 22.[4] ^{1}HNMR spectroscopy followed by 2D techniques identified statistically relevant signals from chemometrics (PCA) and supervised multivariate data analysis (orthogonal PLS-DA). PCA loadings plot showed that triterpenoids were more present in *Keetia* sp. twigs than in leaves

and were prevalent for *K. leucantha*, from which they were originally isolated, than *K. venosa*.[4] Interestingly, orthogonal PLS-DA analysis between metabolic extract content and antiplasmodial activity revealed a negative correlation with the presence of triterpenoids.[4] Meaning that the higher the triterpenoid content, the lower the IC_{50} against the parasite. This is in line with previous results as it seems to suggest that the more 8TTE are present, the lower the antiplasmodial IC_{50}, portraying a clear link.[4]

Due to these promising results, 8TTE was tested in a metabolomics assay to further explore the potential MoA involved.

5.2. Materials & Methods

Artemisinin, atovaquone, chloroquine and 8TTE were studied in metabolomics assays as described in Chapter 3.1 Section 1.2. Samples were analyzed by LC-MS in the same system and conditions and spectra were processed and analyzed similarly.

5.3. Results & Discussion

A self-organizing metaprint is shown in Figure 70A to allow for a straightforward comparison between the profile of 8TTE and the antimalarials in this study.[7] The antimalarial drugs displayed their expected profiles and 8TTE would seem to resemble more closely artemisinin's metaprint by the negative fold in the upper right region characterized by pyrimidine precursors and peptides.

Statistical analysis began with a PCA visualization which is shown in Figure 71A. PC1 (70.3%) is related to the variability within the 3 experiments, which is fairly significative. PC2 (10%) is the component that distinguishes groups and interestingly, artemisinin and 8TTE both fall on the negative side,

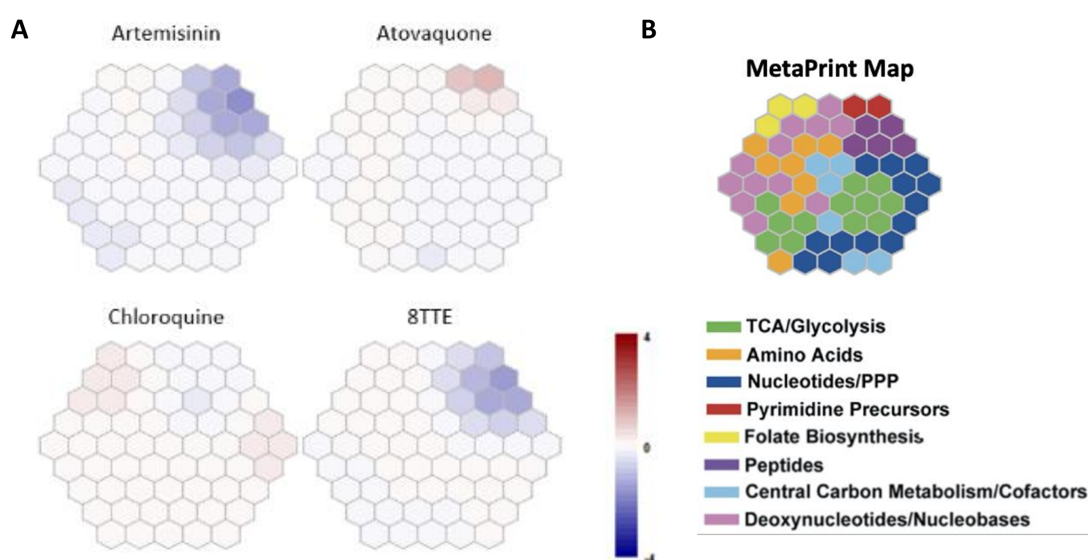


Figure 70 - (A) Metaprints of the study groups. (B) Metaprint map with a color-coded legend adapted from Allman et al. 2016.[7]

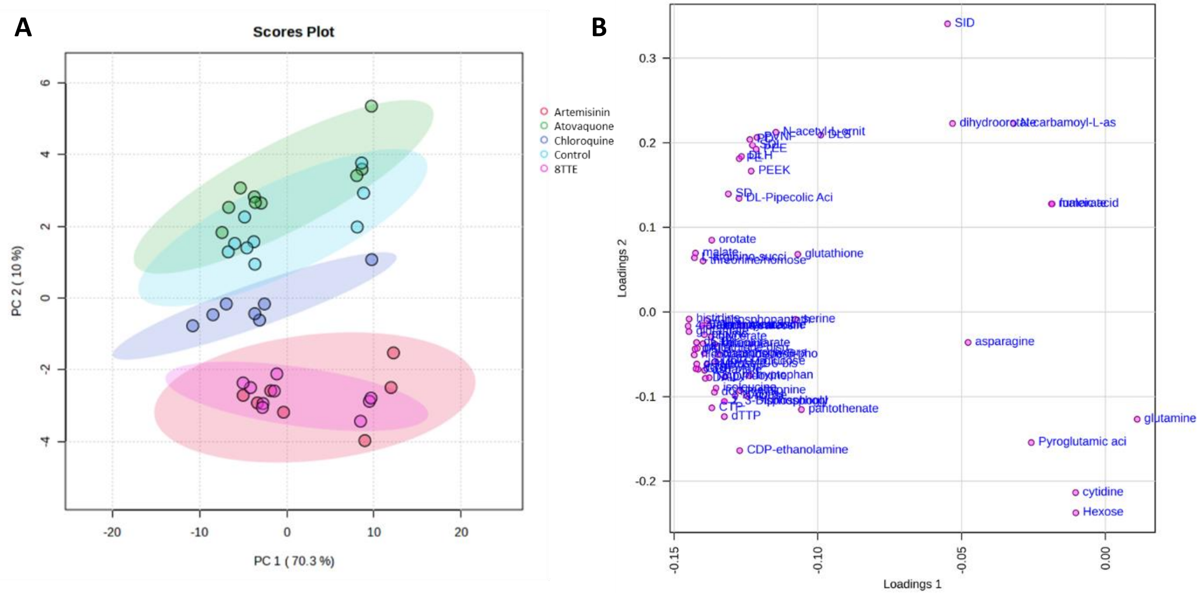


Figure 71 - (A) PCA scores plot with 95% confidence regions displayed with the respective loadings plot (B).

followed by chloroquine fairly across the 0 of the axis. Figure 71B shows which metabolites are responsible for the PCA scores plot separation and a few stand out, like cytidine, glutamine, pyroglutamic acid, CDP-ethanolamine and hexose. Pyrimidine, glutathione and carbon metabolisms might be affected, but it is not specific enough to draw any conclusion.

PLS-DA was used to try to optimize separation through supervision and the result is in Figure 72.

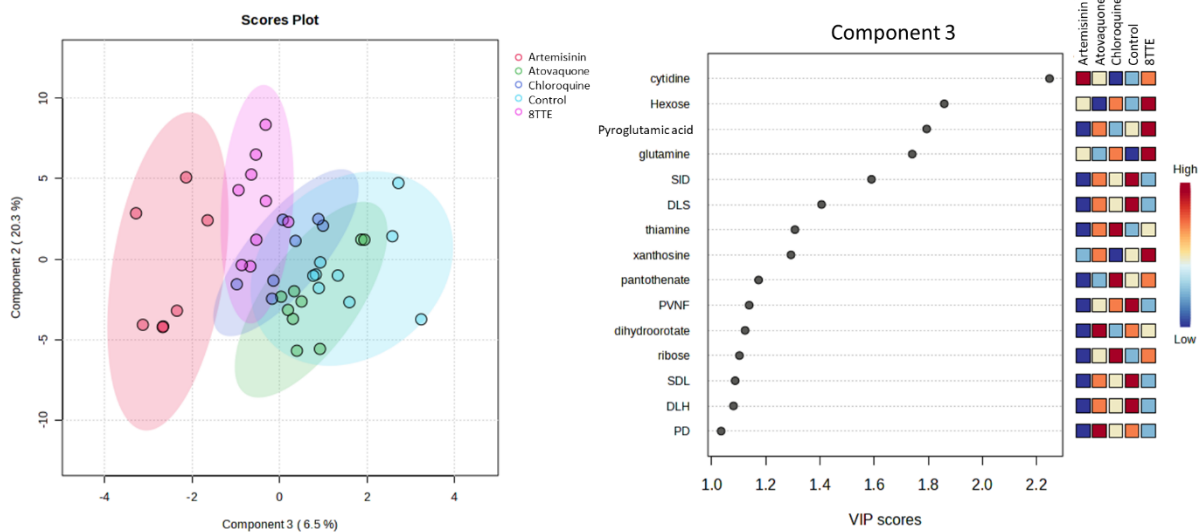


Figure 72 – PLS-DA scores plot of the 8TTE study with 95% confidence regions displayed and the top 15 PLS-DA VIP scores from component 3. The colored boxes to the right of the VIP scores represent the relative intensity of the corresponding metabolite in each group under study. (R^2: PC2 - 0.30, PC3 – 0.46; Q^2: PC2 – 0.02, PC3 - 0.10) (DLH - aspartyl-histidyl-leucine; DLS - leucyl-aspartyl-serine; PD - prolyl-aspartate; PVNF - L-prolyl-L-valyl-L-asparaginyl-L-phenylalanine; SDL - seryl-aspartyl-leucine; SID - aspartyl-isoleucyl-serine)

Component 1 is not shown because it did not separate any groups and had poor cross-validation markers (56.4%, R^2 - 0.09, Q^2 - -0.15), indicating that a representative amount of the data wasn't separated accurately. However, components 3 separated group treatments and was therefore chosen

to investigate markers of activity. This component's percentage is fairly small (6.5%), and does not represent much of the data. This means that the biggest difference among the groups found by a supervised model was not correlated with their classification (treatment). Cross-validation parameters are not ideal but the model is not overfitted, so VIPs were explored to verify what metabolites were responsible for the separation. Unfortunately, no particular pathway seemed affected. Metabolites involved in carbon, amino acid, peptide and nucleotide metabolisms were indicated, which makes interpretation difficult.

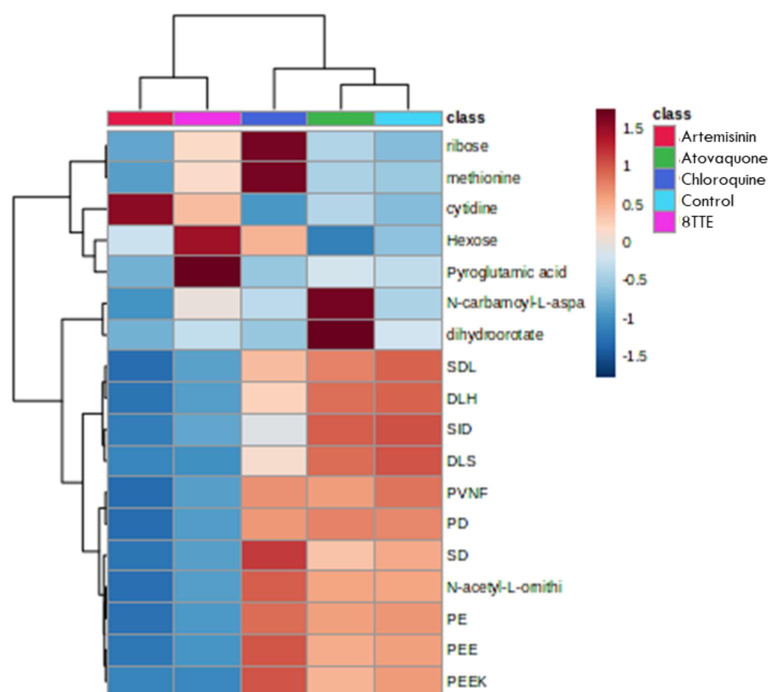


Figure 73 - Heatmap of the metabolites scored as significant by ANOVA. DLH – aspartyl-histidyl-leucine; DLS – leucyl-aspartyl-serine; PE - Prolyl-Glutamate; PD - Prolyl-Aspartate; PEE – proline-proline-glutamate; PEEK – methanone; PVNF – L-prolyl-L-valyl-L-asparaginyl-L-phenylalanine; SD - serylaspartic acid; SID – aspartyl-isoleucyl-serine; SDL – seryl-aspartyl-leucine.

To further explore what metabolites might be responsible for the apparent proximity in profiles between artemisinin and 8TTE, ANOVA was performed and the resulting 18 significative metabolites (p-value < 0.05) were plotted in a heatmap with a clustering dendrogram (distance measure: Euclidean, clustering algorithm: Ward). The result is shown in Figure 73.

Visually, a clear separation in two parts can be made: the bottom part with a negative fold for most peptides for samples treated with artemisinin and 8TTE, and the top where the fold change varies between treatments. The clustering of artemisinin and 8TTE seems to rely on their similar fold change for these peptides, which are positively expressed in the heatmap for the control, atovaquone and chloroquine groups. Interestingly, this peptide profile does not resemble chloroquine's, so it could be that 8TTE MoA does not involve heme biomineralization.

8TTE's heatmap points to a few metabolites with a different fold change from all other groups, namely pyroglutamic acid, hexose, cytidine, methionine and ribose. Methionine and ribose indicate an alteration to amino acid biosynthesis and consequently, to protein homeostasis as well. Ribose is also a precursor of nucleotide metabolism and is involved in the pentose phosphate pathway, which as a central carbohydrate metabolism essential pathway can have multiple ramifications. Cytidine is a central metabolite in pyrimidine metabolism, which is also affected by the amino acid glutamine in the synthesis of N-carbamoyl-L-aspartate. Lastly, pyroglutamic acid, also known as 5-oxoproline, is a marker of oxidative stress by its participation in the synthesis of glutathione.[8–10] This effect is interesting because this metabolite's fold change between 8TTE and artemisinin treated groups is opposite, thus an effect on the glutathione metabolism pathway is significantly different between the two.

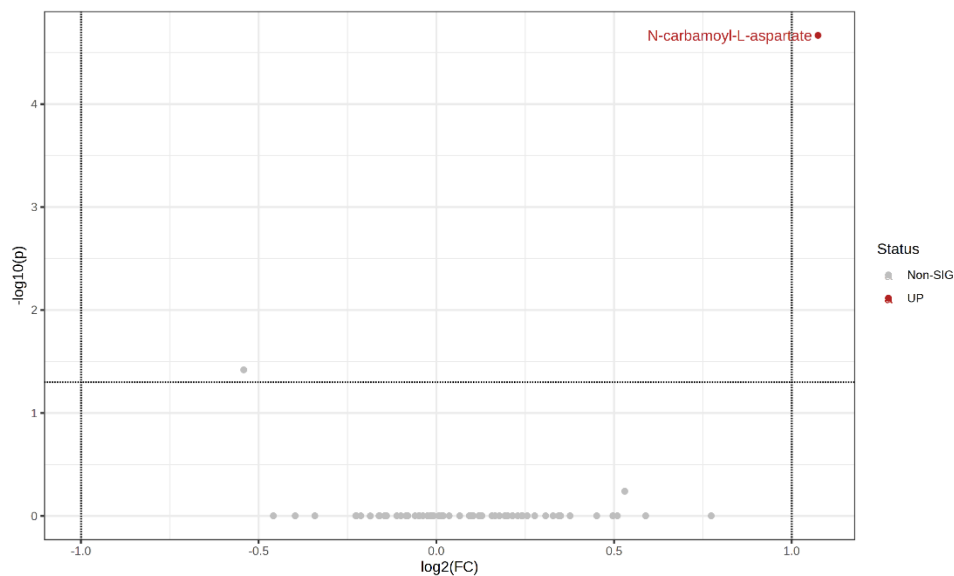


Figure 74 - Volcano plot between 8TTE/artemisinin (p-value: 0.05, FC threshold: 2.0). Points represent metabolites: in gray (non-significant), red (positive fold), blue (negative fold).

To further test the similarity of profiles between 8TTE and artemisinin, a volcano plot was generated and is shown in Figure 74. It is noteworthy that only one metabolite is significant in this plot – N-carbamoyl-L-aspartate (p-value = 0.0000215). This metabolite is involved in amino acid and pyrimidine metabolism and interestingly is a marker of atovaquone as a precursor in pyrimidine metabolism.[11] Through Figure 74 it is evident that the fold change of this metabolite and dihydroorotate are not the same between 8TTE and atovaquone, which was further confirmed by a *t*-test (p-value < 0.001). Hence, the MoA of 8TTE doesn't appear to focus on the mitochondria electron transport chain.

There are reports of artemisinin's ability to alkylate carbamoyl phosphate synthetase and aspartate carbamoyl transferase, enzymes involved in the synthesis of N-carbamoyl-L-aspartate in the parasite's cytosol from glutamine, an amino acid derived from hemoglobin metabolism.[12] This could justify the negative fold of N-carbamoyl-L-aspartate for the artemisinin treated group, as seen in Figure 73.

Contrarily, in this Figure it is visible that the opposite fold occurs for atovaquone due to the accumulation of this precursor. The fold of this metabolite in the group treated with 8TTE is almost neutral in comparison with the others, which could mean that it is not consumed for the synthesis of pyrimidines as with the control, not accumulated due to enzyme inhibition downstream as with atovaquone, or not synthesized due to enzymatic alkylation upstream and/or lack of glutamine from hemoglobin metabolism as with artemisinin. Figure 74 points to a statistically significant higher presence of N-carbamoyl-L-aspartate in samples treated with 8TTE as opposed to artemisinin. This could mean that 8TTE interferes with hemoglobin-derived peptides similarly to artemisinin, as pointed by the similar fold in the heatmap for these metabolites, but does not affect glutamine in the same way, hence the difference in relative abundance of its downstream product. The relative presence of glutamine and dihydroorotate as shown in Figure 72 between artemisinin and 8TTE seems to point to this option. Other assays would be needed to confirm this and what could this target be.

Computational models have predicted a thermodynamic link between the Kennedy pathway, which produces phosphatidyl-ethanolamine, with pyrimidine synthesis through substrate channeling.[13] Although it is not exactly clear how this occurs, lipidomics could be interesting to explore further the potential effect of 8TTE on the Kennedy pathway.

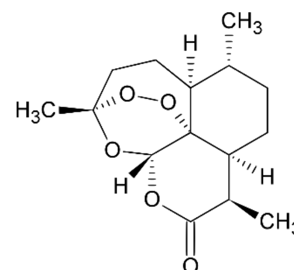


Figure 75 – Artemisinin.

The molecular structure of artemisinin is shown in Figure 75. Artemisinin is a sesquiterpene lactone which does not resemble the structures of the triterpenic esters in Figure 69. The terpene structure is fundamentally different due to the different number of carbons, but more importantly, there's the absence of the endoperoxide bridge in 8TTE and the lactone that are thought to be responsible for the great efficacy of artemisinin against the *Plasmodium* sp.[11] However, the presence of a phenol in the 8TTE compounds allows these compounds to behave as pro or antioxidants depending on the conditions, which might influence their antiplasmodial activity.[1]

But triterpenes are not unknown compounds and many have been tested on antiplasmodial assays. Triterpenes are also known for their anti-inflammatory properties which contribute to the effects of traditionally used plants, despite their poor bioavailability.[6,14] Ursolic acid, also present in *K. leucantha* as mentioned before, has had analogues synthetized and studied for their potential MoA.[5] Initially, interactions of the triterpenic skeleton with heme in dynamic simulations supported a MoA at this level, but experiments demonstrated a weak capacity to inhibit β-hematin formation and an inability to inhibit mitochondrial membrane potential, which is in line with the metabolomics results presented in this section.[14] Triterpenes reportedly can disturb membranes due to their lipophilic nature, though a specific impact at this level is unlikely.[6]

More specifically regarding triterpenic esters, not many are known for their antiplasmodial activity aside from the ones reported in *K. leucantha*.[5] Messagenic acids A and B were isolated from *Gardenia saxatilis* Geddes (Rubiaceae) and displayed IC_{50} of 1.5 and 3.8 mg/ml on a multidrug resistant strain of *P. falciparum* (K1). A mixture of uncarinic acid E and 27-O-p-(E)-coumaroyloxyursolic acid isolated from the same plant also showed antiplasmodial activity on K1 (2.9 mg/ml).[15] Triterpenic esters from the root bark of *Ziziphus cambodiana* Pierre (Rhamnaceae) also elicited a few compounds with interesting IC_{50} (0.9 and 3.7 mg/ml).[16] These IC_{50} are close to those reported in Table 22 for 8TTE despite the strain being different. More importantly, activity was correlated to the coumaric moieties and the esterification on the 27-position.[5] More recently, 27-O-coumaroyl derivatives have been shown to have more antiplasmodial activity (strain 3D7) than 3-O-coumaroyl derivatives, but presence of an ester in C-3 doesn't seem to be the only ruling factor in this decline.[17] Interestingly, antitrypanosomal and antileishmanial effects were reported for the extract containing 8TTE (IC_{50} < 15 mg/ml), but the lack of selectivity across all parasitic species might be a warning sign to the lack of a specific MoA that does not align with the results shown in Table 22 for the 8TTE mixture. Recent preliminary studies with 8TTE *in vitro* have demonstrated an effect on PfA-M17, enzymes that regulate the amino acid pool in *P. falciparum,* which could be in line with the metabolomics results presented here that display changes in peptides and amino acids.[18] Consequently, it could be that the 8TTE have a particular molecular structure that could interfere with the *Plasmodium* sp. at the beginning of pyrimidine synthesis, an exclusive and crucial pathway for this parasite that relies on hemoglobin metabolism to obtain essential amino acids. Further studies to confirm this activity are warranted.

5.1. Bibliography

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Chapter 4

GENERAL DISCUSSION, CONCLUSIONS & PERSPECTIVES

Many diseases affect mankind and are the target of effective management and eradication plans. Despite global efforts, malaria is significantly harder to eliminate due to its virulence and mutating nature. Although the understanding of its complex lifecycle, metabolic necessities and resistance factors has never been better, malaria is still one of the deadliest diseases in the world. The elevated morbidity and mortality in special groups like infants and pregnant women underscores the long-lasting impacts of infection and presses for the necessity of finding a definite and effective treatment plan.[1]

The WHO and governmental institutions that regulate healthcare have recognized the role of medicinal plants in disease management and supported the efforts into categorizing and describing these species further.[2] Most drug scaffolds are or are based upon NP. The long history of use and detailed descriptions of the plants where these compounds can be found in provides invaluable preliminary information.[3] NP research is not without a few hurdles, namely the complexity of a crude extract that may or not have biological activity depending on the solvent and conditions used, the interactions between NP are not always perceivable in a laboratory setting and are often hard to identify, the difficult isolation of single compounds and their identification, especially when they're in small amounts, and lastly, the unknown targets for NP when their activity is identified in phenotypic assays.[4] This last point is also true in malaria research, in which an antiplasmodial screening can reveal the efficacy of a NP, expressed by the IC_{50}, but does not reveal information on a MoA, which is known to be invaluable in drug research and development.[5]

Advances in analytical techniques have opened a new venue for NP research. Dereplication tools, streamlined extraction and fractionation, genome mining, extensive databases and machine learning are a few examples of how NP can now be further explored and utilized in antimalarial drug discovery. To facilitate the inhouse screening and study of these extracts and compounds, metabolomics emerged with the increased sensitivity of acquisition tools and advanced data analysis and statistical models to allow for a more informative rationalized discovery. In order to demonstrate the applicability of these techniques in NP antiplasmodial research, the project METNATPAR committed to study and optimize metabolomic workflows with *in vitro* cultures of *P. falciparum,* and then apply it on the study of MoA of several extracts and NP with interesting antiplasmodial activities that could gain with the additional knowledge of their MoA.

Despite the application of metabolomics in the context of antiplasmodial screenings not being unheard of – the first metabolomics test in this setting reports back to 2009[6,7] –, the wide variety of methodologies found in the literature each had their own setbacks and advantages.[8] Moreover, the

project aimed at using both NMR and LC-MS as acquisition tools, and as such the workflow should reflect an application for both methodologies.

Because the parasite evolves morphologically during the 48h intraerythrocytic cycle *in vitro*, two stages were initially studied: the early trophozoite, also known as ring-stage, and the late trophozoite. To study the ring-stage, three metabolome extraction methods from the literature variating in timing and extraction solvent were compared in parallel and analyzed by both ^{1}HNMR and LC-MS. Chemometrics tools allowed for data visualization, outlier detection and to remove variability associated with the assays in order to have information concerning only the quality of the parasite metabolome extraction methods. Additionally, peak detection and annotation in NMR, missing values and mean of peak intensities for LC-MS, and inertias for both were calculated to evaluate the most repeatable and consistent extraction method for both analytical tools. The most reliable method in this study proved to be a double extraction with methanol and methanol/water (80:20, v/v) (method A) because it accounted for less variability and more repeatability across samples and assays for both ^{1}HNMR and LC-MS.[9] Other parameters were also evaluated, namely the number of washing steps after saponin lysis or a sonication step, and the latter was deemed valuable to improve annotation and reduce variability. Method A was subsequently complemented with this technique in future tests.

The purpose of comparing methods was to choose the one that afforded the most reliable biologically interpretable results while retaining a fairly simple methodology that could be implemented in a day to day basis in NP research. However, there are constraints associated with this logic that are well described in the literature and regrettably posed obstacles in this project as well. There isn't a method that can extract every metabolite, as discussed previously.[8] Additionally, despite improvements to analytical tools, limits of detection, ionization and solvent/matrix effects still represent factors that influence the results. These aspects reduce the quality of the snapshot of the metabolome and limit its interpretation, keeping metabolomics as a hypothesis generating tool rather than also a single-target confirmation technique.[10]

The strategy to associate two analytical techniques would seem to be the key to solving a few of these issues: the sensitivity of LC-MS allied with the selectivity of NMR seem to complement one another and offer a less blurred snapshot. However, the complexity associated with the generated data still poses problems in the merging and interpretation of said data simultaneously. Not many algorithms are available that merge the matrices obtained by both analyses, and for those that do, they often require annotation to be applicable. This would seem logical, as annotation reduces the data dimension by associating a single variable *x*, like the metabolite name, to only a few *y* variables, like MS and/or NMR intensity, as opposed to multiple dimensions (*i.e., m/z,* retention time, ions, chemical

shift and spectral intensity). However, this exercise represents an additional complication because level 1 annotation is often not possible. Confidence in annotation is essential in this case to assure that information from different techniques is being matched correctly. Otherwise, raw data could be tentatively used, but complications arise depending on the platform. ^{1}HNMR has a relatively small chemical shift window, which elicits superposition of peeks and hinders interpretability. Meanwhile, LC-MS frequently detects thousands of features of which only a fraction can be reliably annotated through use of standards or databases generated with standards, as multiple features can stem from the same metabolite while others might represent matrix effects or background noise.[11] Hyphenation is possible, but not widely used because of the many constraints associated with it: solvent compatibility and suppression, sample volume, flow-through probe design, and difficulties in purification that introduce matrix effects and pollute detection.[12,13] As such, interpretation of LC-MS and NMR metabolomic data is most frequently done independently as in this work, in which individual annotation of each dataset from either analytical technique was performed before being matched and complemented post statistical analysis.[14] Recently, algorithms based on the matching of databases of NMR and LC-MS are gaining relevance and open the perspective of one day having an algorithm or program that could reliably suggest annotations and match patterns between the different data.[14] Such a tool would undoubtedly revolutionize the way metabolomics is performed and facilitate the introduction of this approach in drug discovery research, particularly for those who lack NMR or LC-MS expertise.[15]

Since it seemed feasible to reliably extract the parasite's metabolome with method A with sonication (seen in Figure 26), the next step was to verify if by doing so, the MoA of antimalarial drugs could be distinguished. Initial studies sought to distinguish the effects of chloroquine and artemisinin, antimalarial drugs with different MoA, and the control (with the vehicle, DMSO).[8] Because method A with sonication makes use of ring-stages, multiple timepoints were initially studied in an attempt to optimize the separations of the antimalarial profiles. This study revealed that 1h incubation with the antimalarial drugs or DMSO was enough to distinguish these samples (seen in Figure 27) and that separation between samples of different treatments as observed by PCA was possible at 4h and 6h (see Figure 28). A second and third tests were performed with 5h incubation, analyzed through ^{1}HNMR and demonstrated a separation between treatments with a few metabolites responsible for this distinction (seen is Figure 31). These datasets were used to explore normalization and preprocessing techniques vital to NMR metabolomics that were henceforth used in every analysis – a schematic of the workflow can be seen in Figure 32. A third proof of concept introduced a third treatment with a NP (poupartone B) and sought to investigate through ^{1}HNMR whether samples would cluster or if this NP had a different MoA. This preliminary test clustered poupartone B with chloroquine in a PCA while

separating the control and artemisinin groups clearly, demonstrating that the workflow accomplished the goal of distinguishing the profiles elicited by treatment with different compounds, including a NP (seen in Figure 33). These proofs of concept were important because results can variate greatly depending on simple parameters during both the experimental stages and the data processing. Parasite purification proved essential to increase data robustness, but likewise, normalization and preprocessing techniques proved to greatly influence the results. Whereas the experimental design and sampling uniformized samples of the same group (treatment), processing influences the quality of the data and normalization makes samples comparable and minimizes experimental biases, which is crucial to interpret the data.[11,16–18] It is accepted that the bins or features indicated by statistical models as significant are directly correlated to the normalization technique used, and that results can only be reproduced if the same model is applied.[17] In light of this, a normalization method was chosen and kept throughout to maintain consistency in the data analysis. Still, it is important to note that there is no consensus on which normalization technique should be used in metabolomics.[11,17]

A collaboration with the Department of Biochemistry and Molecular Biology and the Huck Center for Malaria Research at Penn State (The Pennsylvania State University) allowed the use of their inhouse workflow – which incorporated extraction method D – and database. A small study was conducted to compare the robustness between extraction methodologies: method A with sonication and method D, as seen in Figure 34. The variability of method A with sonication was bigger than method D (seen in Figure 35) and the detection of most metabolites was significantly increased for method D (seen in Figure 36). While both methods reliably extracted and detected parasite metabolites, method D proved to be more consistent across both LC-MS and ^{1}HNMR. This was found to be due to the uniformization introduced through magnetic purification instead of saponin lysis and the significantly smaller timing of the workflow which might contribute to less metabolite turnover. While no extraction method could be expected to extract the whole metabolome, robustness and repeatability are important factors to assure the confidence in the biological interpretability and reproducibility of the results obtained, which are crucial to implement a workflow in NP antiplasmodial research. Therefore, before proceeding with further experiments, extraction method D was ultimately chosen.

The first exploration of MoA was performed for three extracts with traditional use against fever and their respective isolated NP with antiplasmodial activity: *A. annua* leaf ethyl acetate extract and artemisinin, *C. officinalis* bark alkaloid extract and quinine, and *P. borbonica* leaf ethyl acetate extract and poupartone B. The objective was to determine if the extracts' profile could be used to indicate a MoA before the NP responsible for the activity was isolated, and if so, if the profile aligned with that NP. This could be useful to have a preliminary idea of whether the plant being studied will offer an innovative MoA, but also to verify if the NP is solely responsible for the extract's activity. Extracts have

various NPs that may have individual MoA or act through synergy, and metabolomics could be an approach to detect these interactions early on. This study successfully demonstrated that it was possible to distinguish the profile elicited by the traditional medicinal plants (seen in Figure 43), thus this type of study is an interesting tool to use in early NP research. Furthermore, the profiles aligned with the NPs found to be active in these extracts, further attesting that metabolomics can offer an indication of the antiplasmodial effect even with a crude extract. Notably, the *A. annua* is thought to attribute most of its activity to artemisinin, hence a similarity in profiles was to be expected. A study with a wider variety of plants could prove insightful, especially to early and quickly detect possible innovative targets worth exploring further.

Despite these positive results, it is difficult to extrapolate the exact nature of the interactions that can occur from a complex matrix such as an extract: are the NPs competing for the same receptors or enzymes, or do the compounds affect different targets and cause a chain reaction? A metabolomic workflow in antiplasmodial screenings can give information starting from the extract and fractions on what kind of pathways the NPs will affect, but it can't discern the type of interactions between NPs. Furthermore, it is possible that once the NPs are separated, their effect will change, which would change the profile as well. Metabolomics would remain a valuable approach in quickly following the trend through bioassay-guided fraction and guiding research toward active but also potentially innovative NPs. However, this approach remains a hypothesis-generating tool – other assays are still needed to confirm the potential interactions and targets.

The second study focused on the *A. afra* and *A. annua* plants, both known for their traditional use in the malaria context. The latter is widely known for the artemisinin discovery that revolutionized malaria treatment, while *A. afra* is shrouded in mystery in regards to its activity being linked with a vestigial amount of this compound or not at all.[19] Profiling of the two plants acetone extracts demonstrated multiple differences in phenolic composition and more importantly, the presence of artemisinin in *A. annua* and *A. afra* confirmed by both an HPLC derivatization technique and DI-HRMS. The relative abundance of artemisinin was higher in *A. annua* than in *A. afra,* which is line with the metabolomics MoA results obtained that point to a similarity in activity between *A. annua* and artemisinin, whereas *A. afra* diverges from the two. These results are supported by both LC-MS and ^{1}HNMR, which reveal that artemisinin and *A. annua* interfere with redox systems and amino acids metabolism as direct consequences of the formation of free radicals possibly at the DV, which would interfere with hemoglobin metabolism. Contrarily, *A. afra* has a different profile at the level of amino acids, peptides and membrane precursors, indicating a distinct MoA. Interestingly, myo-inositol was found to be significantly changed with this extract and this could be due to the flavonoids present inhibiting this metabolite uptake, thus interfering with phospholipid synthesis and osmoregulation, which could justify the antiplasmodial activity of *A. afra.*[20,21] This test contributed to debunking a

longstanding doubt regarding the MoA of *A. annua* and *A. afra.* These plants are both active and with distinct MoAs, which does not retract from their traditional use and should instead be the base to expand on what kind of interactions and new potential antimalarials could be extrapolated from them.

Interestingly, the results from this study support the indications from the first assay. The extracts used are not the same – the first is an ethyl acetate extract of *A. annua* collected in Musanze-Rwanda, whereas the second is an acetone extract of *A. annua* collected in Cameroon. Their IC_{50} are 0.086 ± 0.007 µg/ml and 0,068 ± 0,058 µg/ml, respectively, which are comparable. Studies on artemisinin's solubility have demonstrated that it is more soluble in ethyl acetate than acetone, regardless of the temperature, but it is important to note that artemisinin and other metabolites present in *A. annua* depend on many factors, including season and region.[19,22] Despite this, both extracts elicited parasite profiles that were statistically similar to the response generated by artemisinin treatment, which is in line with the reports that correlate over 90% of these plant's activity with the presence of artemisinin.[23]

Next, the *P. borbonica* and the poupartones were studied. This plant was screened inhouse and elicited the isolation of alkyl cyclohexenones compounds, named poupartones. This innovative molecular scaffold could serve as a good molecular basis to develop new antimalarials if the MoA could be elucidated, since the toxicity of these compounds on other cells and models was not negligible.[24] The MoA found in the metabolomic studies *in vitro* on *P. falciparum* is in line with this toxicity. Indeed, poupartones could act like Michael acceptors through their α-β unsaturated ketone, disabling important proteins and producing ROS, which would both justify their lack of specific activity and the metabolomic profile found to affect amino acids, glutathione and nucleotides. In light of the known information about these compounds and the results in this work, alkyl cyclohexenones could become important scaffolds to develop anticancer drugs rather than antiplasmodial compounds.

Ellagic acid is a NP known for multiple biological activities, but with the constraint of low solubility and thus, low bioavailability. In order to improve this, derivatives were synthesized and tested in a wide range of assays to determine not only a possible MoA, but also if the *in vivo* outcomes improved.[25,26] There was no conclusive evidence on the antiplasmodial MoA of these compounds, so metabolomics assays were used to investigate them. Results from the metabolomics tests performed display significant changes for glutathione and a peptide, prolyl-aspartate, particularly for the two derivatives studied. Ellagic acid and derivatives clustered closer with chloroquine based on their peptide's profile, but were still distinct, supporting the DV as the site of action rather than the same MoA of chloroquine. Indeed, ellagic acid can reportedly change the pH of the DV, inhibit plasmepsins and is less effective *in vitro* against strains that overexpress glutathione.[27–29] These results from the literature support the

metabolomics results found for the derivatives and point to an interesting pleiotropic scaffold that is worth investigating further.

Lastly, a mix of triterpene esters was investigated. This mixture was considered as containing the most active molecules that partially explain *K. leucantha* antiplasmodial activity and as such the MoA was profiled through metabolomics. An initial clustering with artemisinin was present, but due to this compound's pleiotropic nature, its profile is not specific. Further statistical analysis revealed one metabolite – N-carbamoyl-L-aspartate – as significantly different between artemisinin and the mixture. Due to the differences in fold change among groups for this metabolite, it stands out that the mixture affects N-carbamoyl-L-aspartate's precursor glutamine, an amino acid obtained by the parasite from hemoglobin catabolism at the DV. This could indicate a target at this level, though other tests would be necessary to evaluate the reliance of the effect on this pathway. Additionally, N-carbamoyl-L-aspartate is thermodynamically involved with other pathways, so lipidomics could be an interesting approach to further describe the potential ramifications or causes of this mixture's effects.

These five case studies demonstrated the applicability of metabolomic assays to complement the antiplasmodial screening of medicinal plants and the investigation of NP with known potential. Hurdles associated with the workflow, like the timing versus the amount of washes, or the normalization method were factors that proved important for the quality and repeatability of the data, but to which ultimately there was no single answer to. Metabolomics could still gain from more comprehensive algorithms to facilitate data integration from different analytical tools, processing and annotation, especially regarding automation, but new tools are published every day in this direction. This is the example of the 'limpca' package used in Chapter 3.2 that removed the variability associated with the different tests to focus the data on the effect of treatment, or the Pathway Analysis tool in Metabonalyst that facilitates the integration of the pathways affected by the treatments that can then be further explored through KEGG or PlasmoDB. This difficulty in data interpretation can also be gapped by matching other omics studies, like gene knockout, to confirm targets and changes in phenotype.[5]

After reading through Chapter 3, it is noticeable that only the article in drafting from Chapter 3.2 about the *A. afra* and *A. annua* study makes use of both LC-MS and NMR analysis. The samples used to compare extraction methods A with sonication and D (presented in Chapter 2 Section 4.1) were also analyzed by both tools. To do this, the samples were analyzed by LC-MS at the CMaR at Penn State and conserved at -80°C before analysis by NMR in Belgium. This procedure was also performed for all samples used in the studies of Chapter 3.4 (Ellagic acid & Derivatives) and 3.5 (Triterpene Esters). However, after NMR analysis, only the samples of the *Artemisia* plants were used due to the quality of

the data. This selection occurred because of the known limitations associated with the parasite number per sample. The parasite numbers were always above those needed for LC-MS analysis, but not always for NMR, which requires more cells.[8] Because the tests were performed at the CMaR, LC-MS analysis occurred almost instantly, meaning that whatever was left of the sample was dried and conserved for NMR analysis. This would invariably mean that samples with lower initial parasite numbers could fall below the advised threshold for optimal detection through NMR. That was seen upon NMR spectra observation – there was significantly less signal and peaks in normally crowded areas. As such, only the samples of the studies that uniformly had good spectra and increased parasite levels at the start of the study were further analyzed and are presented as part of the results. This situation was not ideal, but the study in Chapter 3.2 shows that the combined analysis is possible and beneficial to data interpretation, leading to surer results.

Despite this situation, NMR was an instrumental tool in the establishment of the workflows and experimental designs that would later be used in Chapter 3. Its fast, reproducible, and non-destructive nature allowed for samples to be analyzed quickly and efficiently. Moreover, despite peak superposition that hinders annotation, its reproducibility allows for comparison to databases with confidence, which makes both untargeted and targeted approaches possible with fairly simple data processing workflows. The complementary nature of this technique with LC-MS relies on the straightforward confirmation of the presence of metabolites that correspond to features in MS that can be annotated in multiple ways due to important RT and ionization shifts across different columns, types of chromatography, and MS techniques. This confirmation adds certainty and dimension to LC-MS data, making metabolomics data more readily interpretable. The necessity of additional parasitic material in the context of both analytical tools can be accounted for during culturing and experimental planning, so in the future, both techniques could be frequently and reliably used in tandem with the same protocol. Additionally, the results found in Chapter 3 (except Chapter 3.2) can still be repeated and analysed with NMR in order to confirm the findings or discover new leads.

Another aspect of the NMR analysis is that it easily allows for the use of the footprint to complement information about the fingerprint. However, during the metabolome extraction with method D, a sterilized glass pipette connected to a vacuum pump was used to aspirate the well media as quickly as possible. This was a significant step that allowed for the sampling to occur in 15 minutes and hence, a bigger set of treatments per study. The collection of the media would have taken more time but it would have been interesting to investigate how much the timings would change and if that step would constitute a factor in the quality of the samples and consequently, data interpretation.

Lastly, these essays were conducted only *in* vitro, but NP research is not limited to cellular tests. It is frequent that upon isolation, a NP with promising biological activity, low or no cytotoxicity and good selectivity indexes is tested *in vivo* for their effectivity.[30] *In vivo* benefits from a full macroscopic picture of effects not only correlated with the pathogen under study, the *Plasmodium* sp. in this case, but also with the host. *In vitro* metabolomics studies can help to anticipate innovative effects *in vivo,* but these should still be confirmed.[5] Furthermore, alternative targets and effects can also be identified that could attest further to the potential of the compound or extract under study as preclinical candidates. This type of assay was not within the scope of this project, but could be a future venue for the compounds/extracts that do get tested *in vivo,* as samples like blood or urine can be routinely used without being invasive to the animals.[5,31]

A natural follow up to the work developed in this thesis would be to add more complexity to the tests performed. Other antimalarial drugs with additional MoA exist and were not used in the studies, *e.g.* mefloquine or clindamycin. Both drugs act independently, interfering with hemoglobin metabolism and isoprenoid biosynthesis, respectively, and would have elicited a signature that could also be compared to those of the NP studied.[8,32] Adding more group treatments increases the number of samples needed and parasites per study, but this could be planned ahead of time. Additionally, other strains exist that have different resistances as described in section 2.1.2 of Chapter 1, like chloroquine resistant (for example, Dd2, W2) or multidrug resistant (for example, K1, NHP1337).[20] Studying the effects on these strains could elucidate potential molecular scaffolds that allow to retain antiplasmodial activity, even in the presence of resistance.[20] On another hand, NP could be used to develop resistant lines through pressure *in vitro*, which could then be evaluated through metabolomics to compare their metabolic changes to the original sensitive strains.[33–37] This would not only validate the theorized MoA, but also what kind of resistance mechanism these compounds would provoke, for example whether chloroquine or mefloquine-like resistance with transporters mutations, atovaquone-like with single mutations in the *cytB* gene, or artemisinin-like with delayed parasitic development.[8,32,35,38] Other omics, like genomics, transcriptomics and proteomics could also be parallelly implemented to give additional levels of information on how the parasite might react to the NP. Finally, other *in vitro* stages can be tested with little protocol changes, like with sporozoites or gametocytes at different stages (I through V), which could shed light on potential activities of these compounds other than curative.

Finally, in light of the difficulties associated with streamlining and automating the data processing of omics data, it is worth mentioning that the data generated during this work and with the protocols developed can be used to optimize or train programs, scripts or packages like the ‘limpca’ *R* package

mentioned previously to aid in developing tools that would make this kind of technique possible as routine in antiplasmodial drug discovery.

Main perspectives in a few words

Overall, these studies demonstrated the applicability of metabolomics as a supplemental tool in early NP screenings to the standard IC_{50}. This information completes the profile, along with toxicity screenings and *in vivo* tests, and contributes to the evaluation of the potential of these compounds as antimalarials. Additionally, the use of both ^{1}HNMR and LC-MS proved valuable to cross the identification capability of the former with the sensibility of the latter, increasing the level of annotation and the confidence in the biological interpretability. Of course, metabolomics is a hypothesis-generation tool that does not suffice to give the absolute view of the mode of action. Specific enzymatic tests should be used to confirm these hypotheses and the targets.

In the future, supplementary work including other antimalarial drugs and NP could be carried out while aiming for a double ^{1}HNMR and LC-MS analysis, which could then be used to develop and train innovative software or statistical models. This work would facilitate the automation of the processing and interpretation of metabolomics data applicated to antiplasmodial drug discovery and finally transform this methodology in a benchtop procedure.

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Chapter 5

ANNEXES

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Exploration of untargeted metabolomic extraction methods for in vitro malaria samples by 1H NMR analysis

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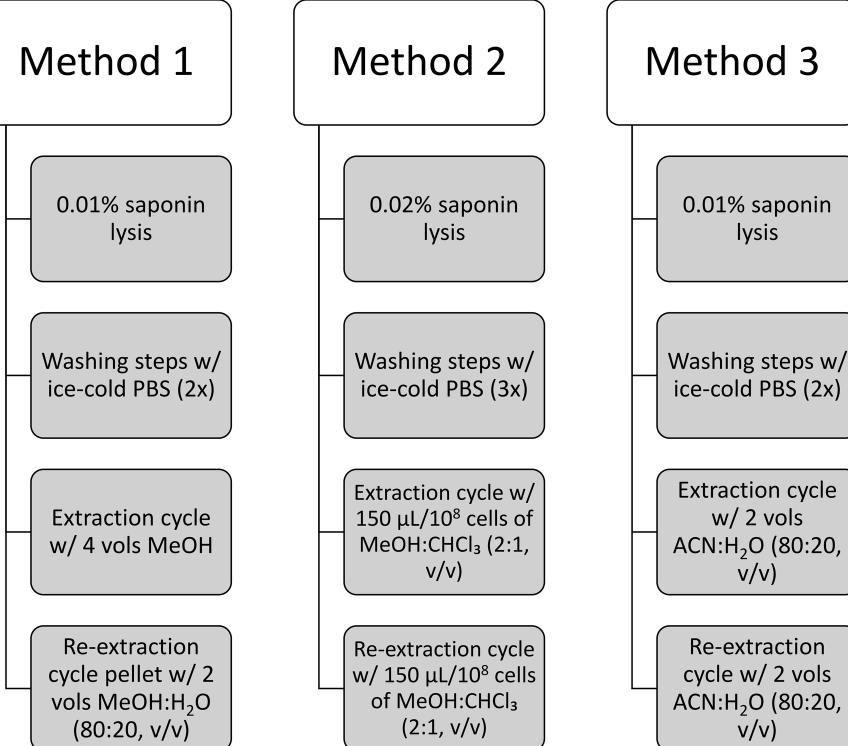
Introduction

- Metabolomics is a reliable omics tool to study the metabolome, the assortment of metabolites that provide energy, signaling or building blocks essential for biological systems survival.
- Malaria is a deadly disease, especially severe when caused by *Plasmodium falciparum*, that still affects over 200 million people yearly.[1]
- The metabolome closely reflects the state of the biological system, and if applied to the *P. falciparum,* it can be used to characterize antimalarial mechanisms of action or study resistance.
- The parasite's intracellular nature, in red blood cells in suspension, introduces significant hurdles to metabolomics extraction methods.

Methods

P. Falciparum 3D7 cultures were magnetically purified and microscopically verified for stage and parasitemia. Ring-stage samples were extracted by either method, as according to the scheme, in triplicate.[2,3] The whole process was performed 3 times, hence noted as groups. Assembled extracts were evaporated and freeze-dried to remove all solvent traces. Samples were dissolved in 400 µL of buffered D_2O at pH 7.4 with TMSP as internal reference, and transferred into 3 mm NMR tubes (Bruker) for analysis. NMR spectra were acquired using TopSpin software on a Bruker Ultrashield Plus 700 MHz equipped with a helium cold probe (cryoprobe). ^{1}HNMR experiments were performed with a CPMG sequence with 128 scans collected over a spectral width of 20 ppm. All spectra were phased and baseline-corrected manually using TopSpin v4. Spectra were stacked, aligned and integrated between δ0.5-9.5 ppm using MestReNova v14. Spectra were divided into buckets of 0.04 ppm, integrated to the sum of intensities and normalized to the number of parasites per sample. The data sets were processed using MetaboAnalyst v5.0 and R. Lastly, the spectra were annotated using Chenomx NMR Suite 9.0 database and the Human Metabolome Database (HMDB), as according to literature.



	Chenomx (43 total)	HMDB (10 total)
Method 1	18-32	1*
Method 2	13-32	1*
Method 3	17-34	1*

Table 2: Number of metabolites annotated through either Chenomx or HMDB databases per method according to the literature. Annotation is variable between samples, mostly due to parasite count variation that interferes with NMR detection.

* - highly tentative annotation of glycerolphospho-ethanolamine.

Class	Metabolite	Method 1	Method 2	Method 3
Amino acids	Asparagine	Yes[1]	Yes[1]	Yes[2]
	Glutamate	Yes	Yes[1]	Yes[2]
	Glutamine	Yes[1]	Yes[1]	Yes[2]
	Phenylalanine	Yes[1]	Yes[3]	Yes[1]
	Serine	Yes[1]	Yes[1]	Yes[2]
	Tyrosine	Yes[1]	Yes[1]	Yes[2]
Nucleotides and related compounds	AMP	Yes	Yes[1]	Yes
	Hypoxanthine	Yes[1]	Yes[1]	Yes[2]
	IMP	Yes	Yes[1]	Yes
	$NADP^+$	No	Yes[3]	Yes[1]
Glutathione	Reduced	Yes[1]	Yes[1]	Yes[2]
Carboxylates	Fumarate	Yes	Yes[1]	Yes[1]
Soluble membrane precursors	*myo-Inositol*	Yes	Yes[1]	Yes[2]

[1] – not found in all samples;
[2] – only not found in outlier;
[3] – only found in 2 samples.

Table 3: 13 metabolites were annotated differently between extraction methods. Method 3 would account for more consistency without the outlier, followed by method 1. Method 2 displays the most variation regarding detection of these metabolites. For method 1, only $NADP^+$ is not found, possibly because of the quicker experimental time. Metabolites were annotated with Chenomx.

Objectives

To discern the most reliable untargeted metabolomic extraction method from 3 literature methods through ^{1}H NMR analysis, using multiple parameters:

- ❑ Chemometrics exploration – Principal Components Analysis (PCA);
- ❑ Number of peaks per spectra;
- ❑ Metabolite annotation;
- ❑ Metabolomic Informative Content (MIC) – Between and within group inertia

Results & Discussion

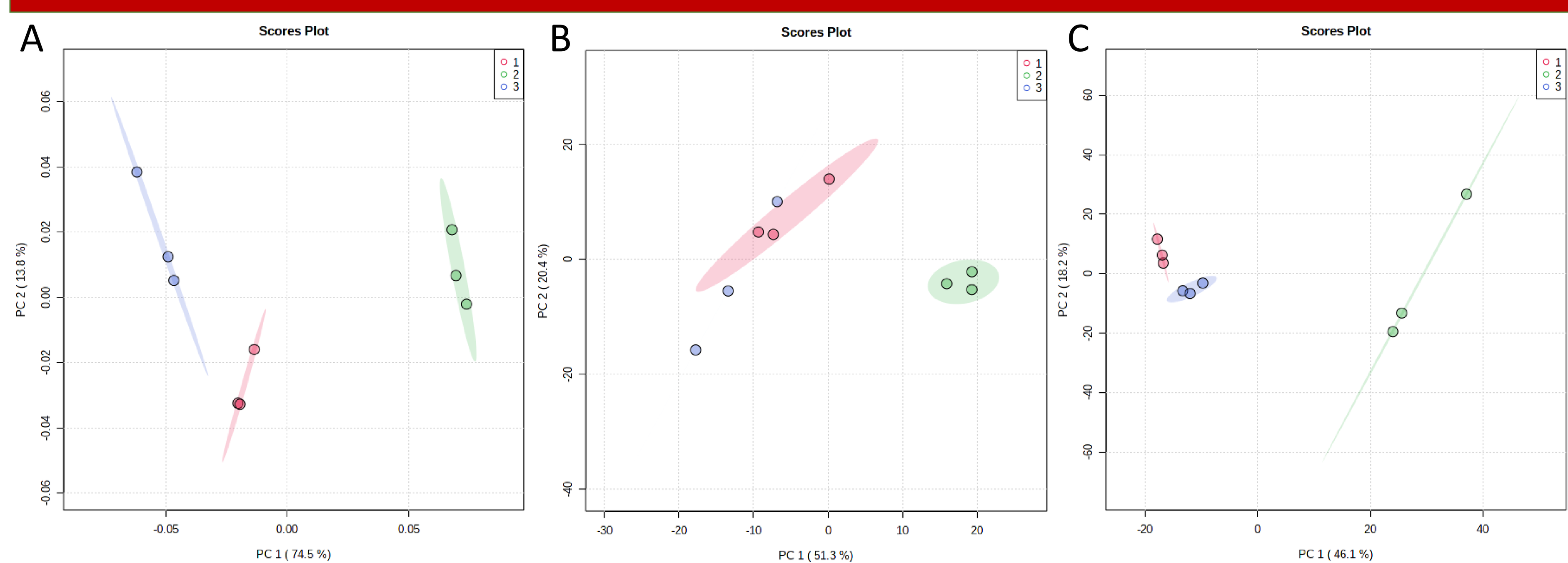


Figure 1: PCA 2D scores plot with 95% confidence regions displayed, of method 1 (A), method 2 (B) and method 3 (C), groups correspond to the three assays. A and C show separation across groups, whereas B does not achieve separation. Method 1 has less intragroup variability, as seen by the distribution across the PC1 (74.5%), which reveals this method as the most repeatable. Additionally, the variation percentage explained by the components 1 and 2 is greater for method 1 than for the others. Both B and C present one outlier each, for group 3 and 2, respectively.

	Group 1	Group 2	Group 3
Method 1	361 ± 60	162 ± 11	370 ± 18
Method 2	304 ± 67	165 ± 30	438 ± 88
Method 3	311 ± 14	224 ± 63	406 ± 13
Parasite count	1.39×10^8	5.22×10^7	2.42×10^8

Table 1: Average number of peaks and parasite count per sample. The 2nd group rendered less peaks across all methods, reflecting the necessity of having high (> over 10^8) parasitic counts for reliable NMR detection.

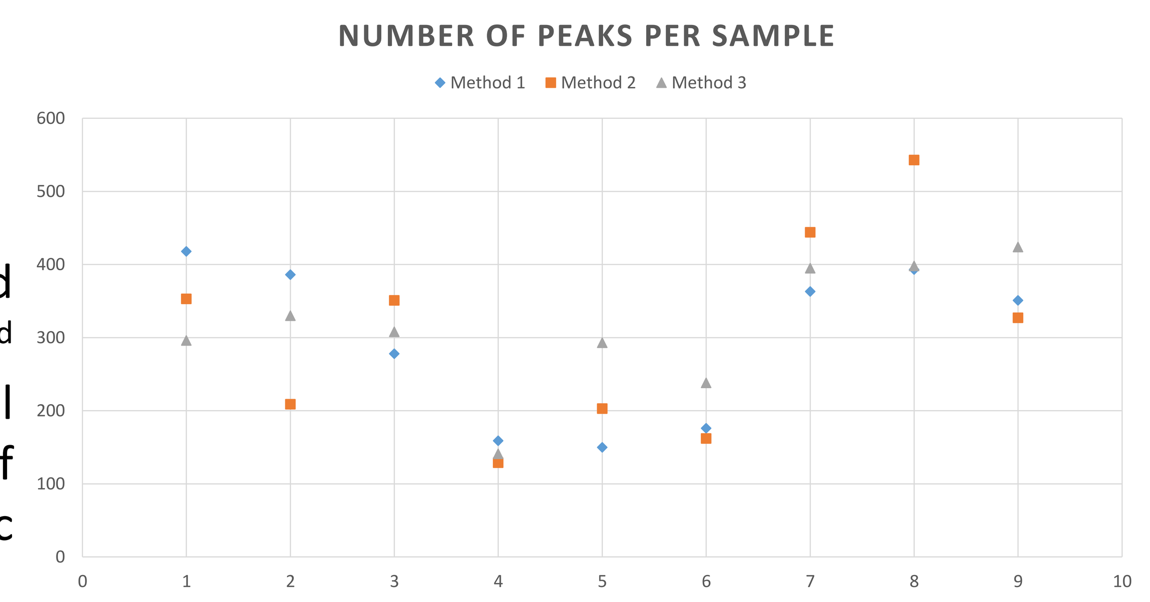


Figure 2: Number of peaks across all samples.

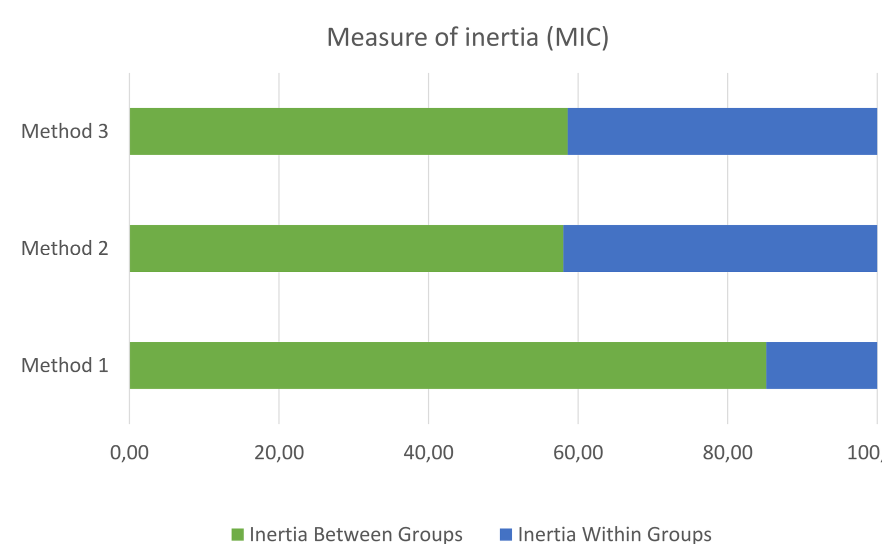


Figure 3: Graphical representation of between and within group inertia. Method 1 has the least intra group variability, demonstrated by the measure of inertia within group, and thus higher separation between groups, which asserts robustness. Method 2 and 3 are similar in terms of inertia.

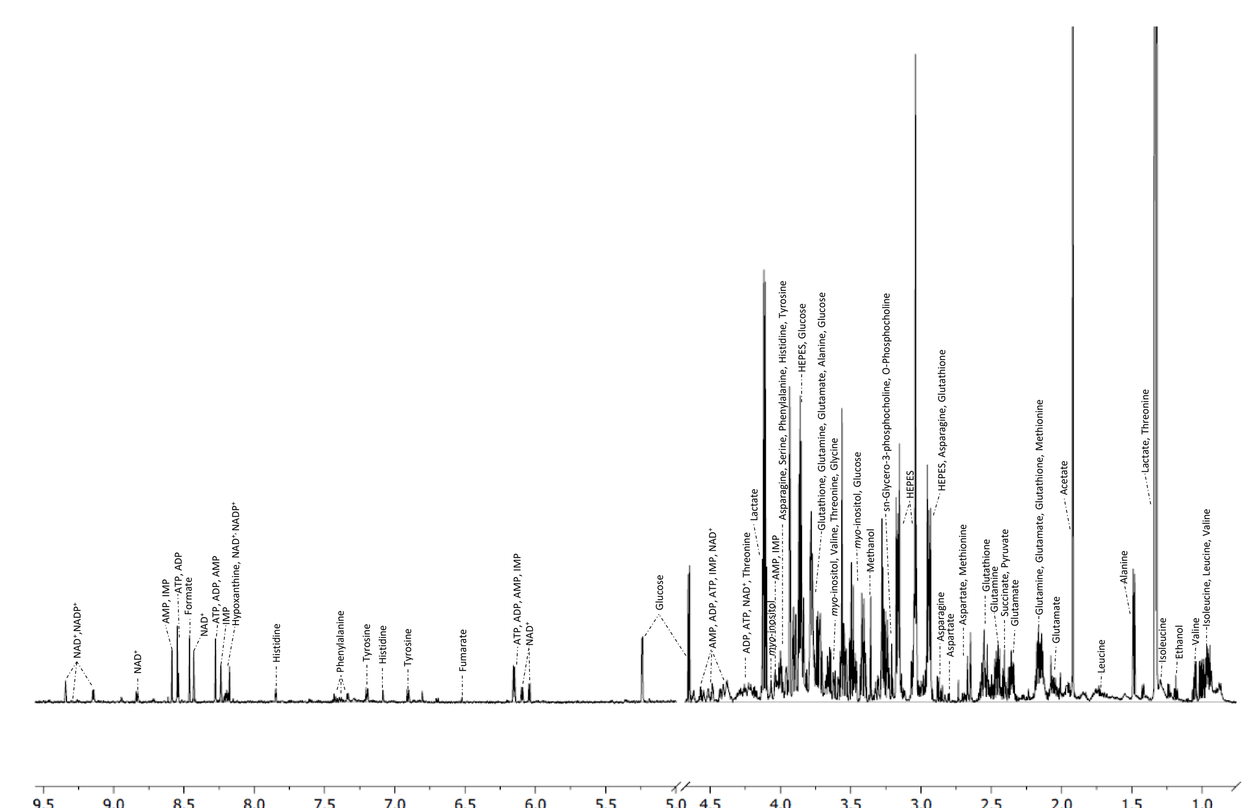


Figure 4: Representative ^{1}HNMR spectra, sample 9 from Method 3.

Conclusion

All methods successfully extracted *Plasmodium* related metabolites, although with different levels of replicability. Method 1 showed more repeatability and robustness across all parameters, followed by Method 3 that accounted for the highest metabolite annotation, and lastly Method 2, that displayed the least promise of the three. Both methods 2 and 3 had one outlier each, which didn't alter the conclusions even if removed. Parasite counts and workflow timings proved to be important factors in extraction and detection through ^{1}HNMR. This preliminary assay allowed for the exploration of *Plasmodium* related metabolites and to choose method 1 for further studies.

Aknowledgements

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CENTER FOR INTERDISCIPLINARY RESEARCH ON MEDICINES

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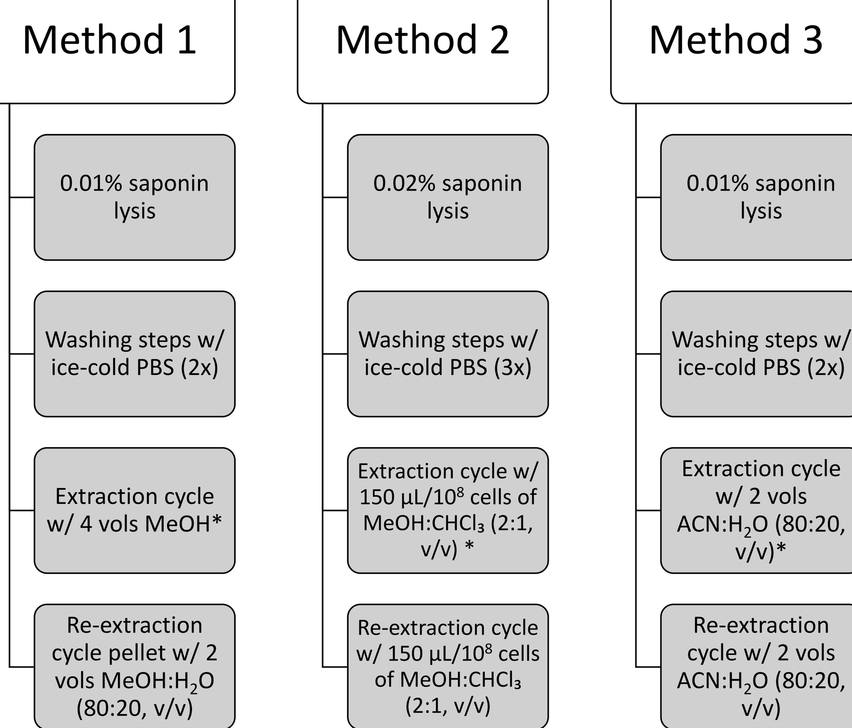
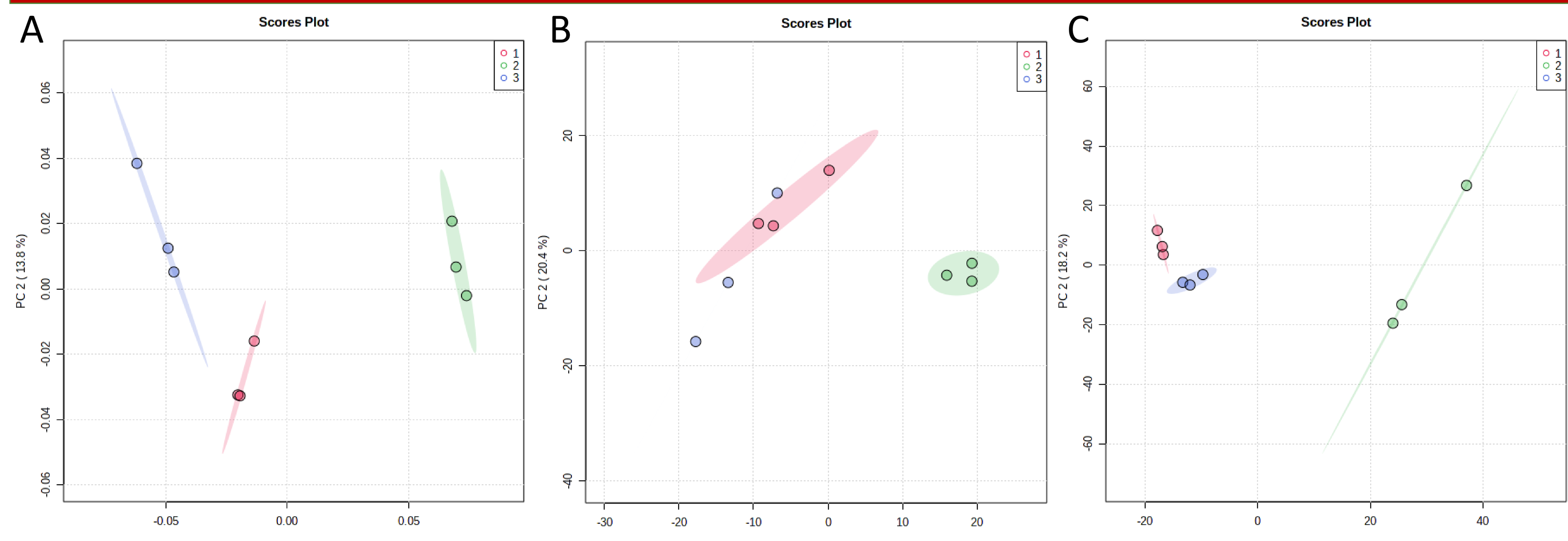


Objectives

- To discern the most reliable untargeted metabolomic extraction method from 3 literature methods through ^{1}H NMR analysis.
- To explore additional methodology parameterization: the number of washes and an optional sonication step to the first solvent extraction.

Results & Discussion



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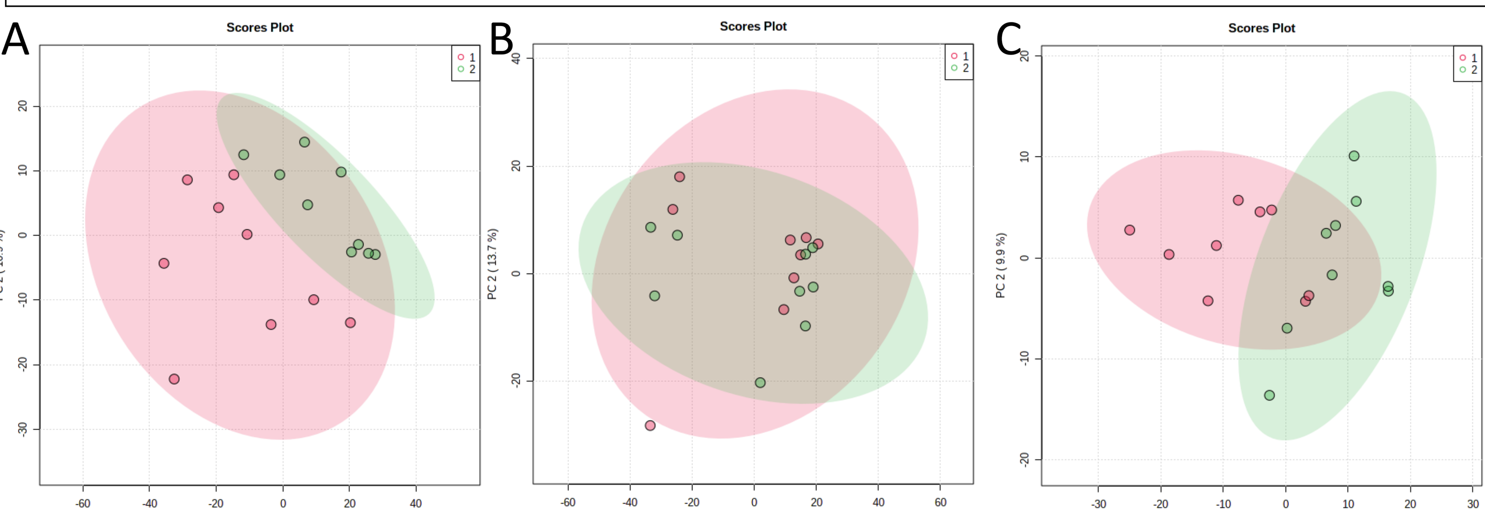

Figure 2: PCA 2D scores plot with 95% confidence regions of method 1 (A), method 2 (B) and method 3 (C); the two groups correspond to the traditional or sonicated methods, respectively. Only A achieves separation, revealing differences between sonicated and traditional samples, which also happens for C. For both A and C the sonicated group shows less intragroup variability. This would suggest sonication increases method 1 and 3 robustness. B has complete superposition, so sonicated samples are not different from the normal ones.

	Chenomx (43 total)	HMDB (10 total)	Total
Method 1	24-33	1-2	26-35
Method 1 sonic	29-34	1-2	31-36
Method 2	18-34	1-2	19-36
Method 2 sonic	26-35	1-2	28-37
Method 3	31-33	1	32-34
Method 3 sonic	33-35	1-2	34-37

Table 4: metabolites annotated through either Chenomx or HMDB databases per method, traditional or sonicated, according to the literature reference list. Annotation becomes consistent with sonication, despite the same maximum annotation.

	Inertia Between Repetitions	Inertia Within Repetitions
Method 1	85,18%	14,82%
Method 2	58,03%	41,97%
Method 3	58,61%	41,39%

Table 2: Inertia table of between and within group inertia for traditional methods. Method 1 has the least intra group variability, demonstrated by the measure of inertia within group, which asserts robustness and repeatability. Method 2 and 3 are similar in terms of inertia.

Class	Metabolite	Method 1	Method 2	Method 3
Amino acids	Asparagine	Yes[1]	Yes[1]	Yes[2]
	Glutamate	Yes	Yes[1]	Yes[2]
	Glutamine	Yes[1]	Yes[1]	Yes[2]
	Phenylalanine	Yes[1]	Yes[3]	Yes[1]
	Serine	Yes[1]	Yes[1]	Yes[2]
	Tyrosine	Yes[1]	Yes[1]	Yes[2]
Nucleotides and related compounds	AMP	Yes	Yes[1]	Yes
	Hypoxanthine	Yes[1]	Yes[1]	Yes[2]
	IMP	Yes	Yes[1]	Yes
	NADP^{+}	No	Yes[3]	Yes[1]
Glutathione	Reduced	Yes[1]	Yes[1]	Yes[2]
Carboxylates	Fumarate	Yes	Yes[1]	Yes[1]
Soluble membrane precursors	*myo-Inositol*	Yes	Yes[1]	Yes[2]

[1] – not found in all samples; [2] – only not found in outlier; [3] – only found in 2 samples; Annotation with Chenomx.

Table 3: 13 metabolites were annotated differently between traditional extraction methods. Method 3 would account for more consistency without the outlier, followed by method 1. Method 2 displays the most variation regarding detection of these metabolites. For method 1, only NADP^{+} is not found, possibly because of the quicker experimental time.

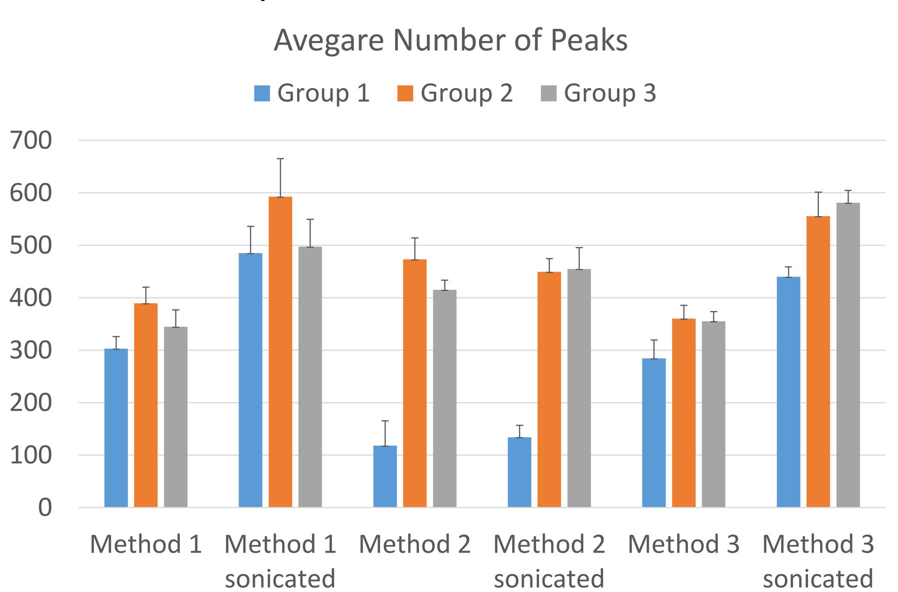

Figure 3: Average number of peaks between each method, traditional or sonicated. For both Method 1 and 3 the number raises significantly with sonication, which could mean an increase in the number of extracted metabolites. Method 2 stays roughly similar.

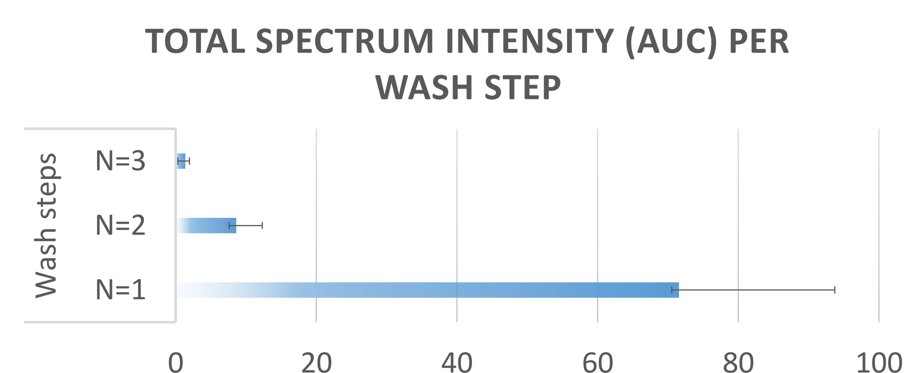

Figure 4: Total spectrum intensity per wash step. First washing step is essential to clean culture media and RBC metabolome, second is still representative, third represents approximatively 1/54 of the spectral intensity of the first washing step. A third washing step might not be worth it, since it entails additional experimental time and parasite leakages cannot be ruled out.

Conclusion

All methods successfully extracted *Plasmodium* related metabolites, although with different levels of replicability. Method 1 showed more repeatability and robustness across all parameters, followed by Method 3 that accounted for the highest metabolite annotation, and lastly Method 2, that displayed the least promise of the three. Exploration of parameters revealed that two washing steps were enough to remove culture media and contaminants and that sonication added real value to methods 1 and 3, with more metabolites detected more robustly. This assay allowed choosing method 1, a two-step extraction with methanol and methanol:water (80:20, v/v), with the addition of a sonication step, for future metabolomic analysis of *P. falciparum in vitro*.

Aknowledgements

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Profiling plant extracts with antimalarial bloodstage activity using metabolomics

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Introduction

- Medicinal plants have contributed significantly to malaria treatment through the use of decoctions, teas, and other preparations traditionally used across endemic countries.[1]
- Studies of these plants led to the discovery of natural products such as quinine and artemisinin that served as models for synthetic antimalarial derivatives.[1]
- Growth inhibition assays are standard to guide natural products isolation methods, which allow for the selection of active plant extracts, fractions and eventually isolated natural compounds with promising IC_{50}. However, IC_{50} are insufficient to determine the mode of action (MoA), an important factor in antimalarial drug discovery due to ongoing general resistance.
- Therefore, metabolomics profiling is a robust approach that can improve drug discovery of antiplasmodial natural compounds as a complement to growth inhibition assays by determining the MoA.

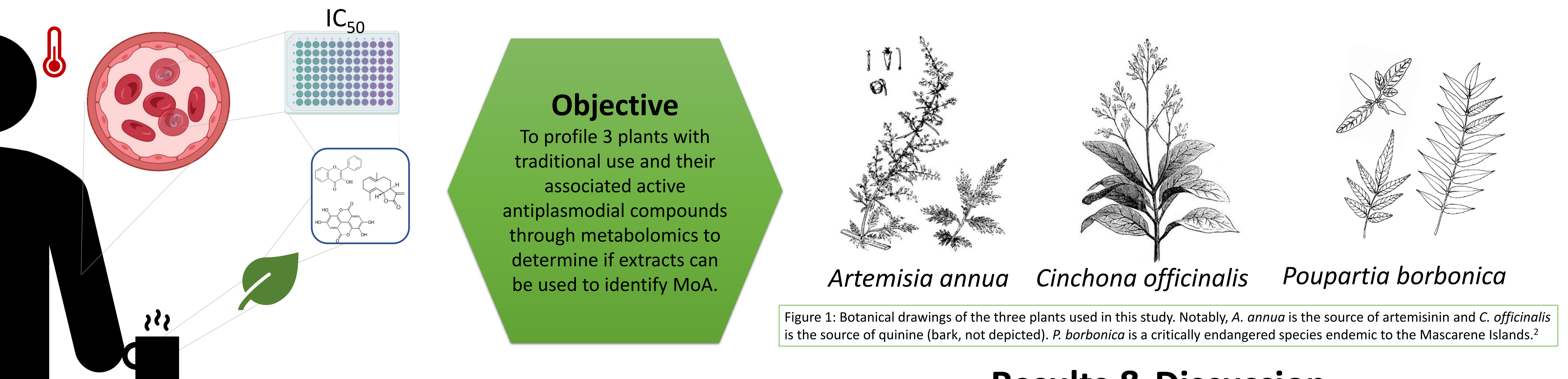
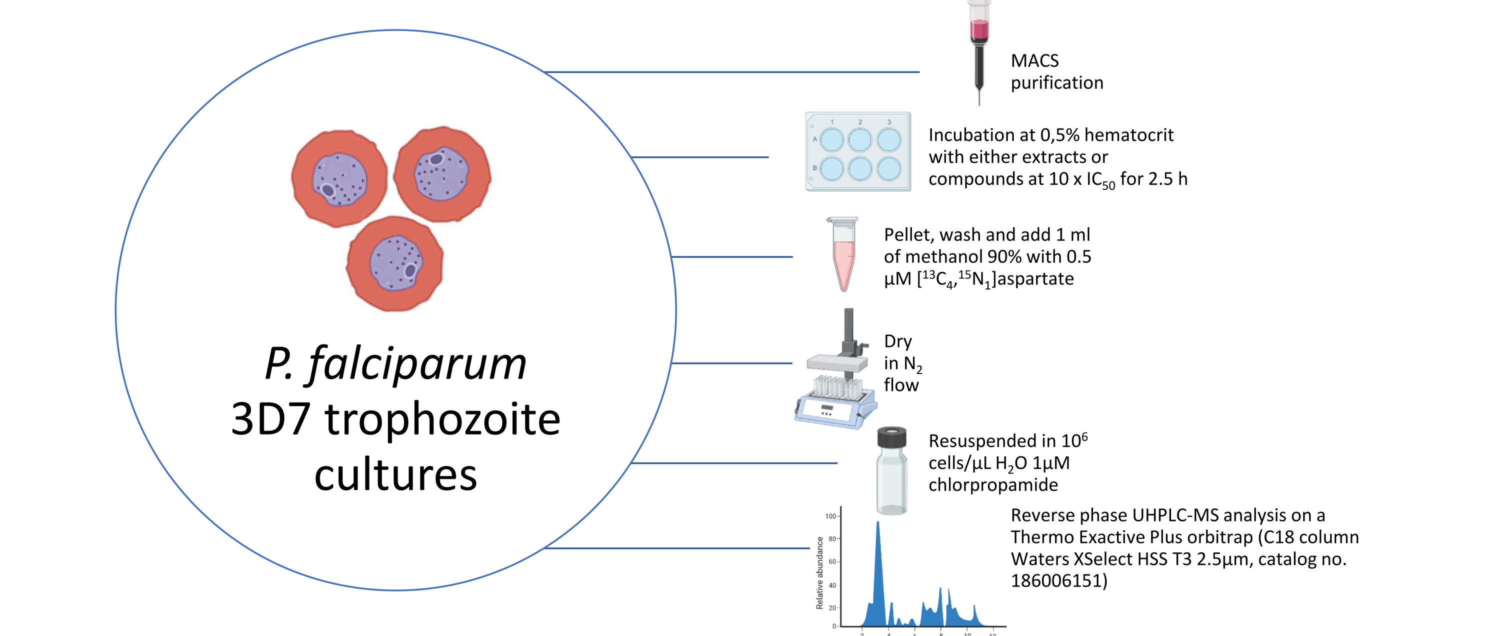


Figure 1: Botanical drawings of the three plants used in this study. Notably, *A. annua* is the source of artemisinin and *C. officinalis* is the source of quinine (bark, not depicted). *P. borbonica* is a critically endangered species endemic to the Mascarene Islands.[2]

Methods[2,3]

Growth inhibition assays of *A. annua* leaf ethyl acetate extract, *C. officinalis* bark alkaloid extract, *P. borbonica* leaf ethyl acetate extract, artemisinin, quinine, and poupartone-B were done as explained elsewhere.



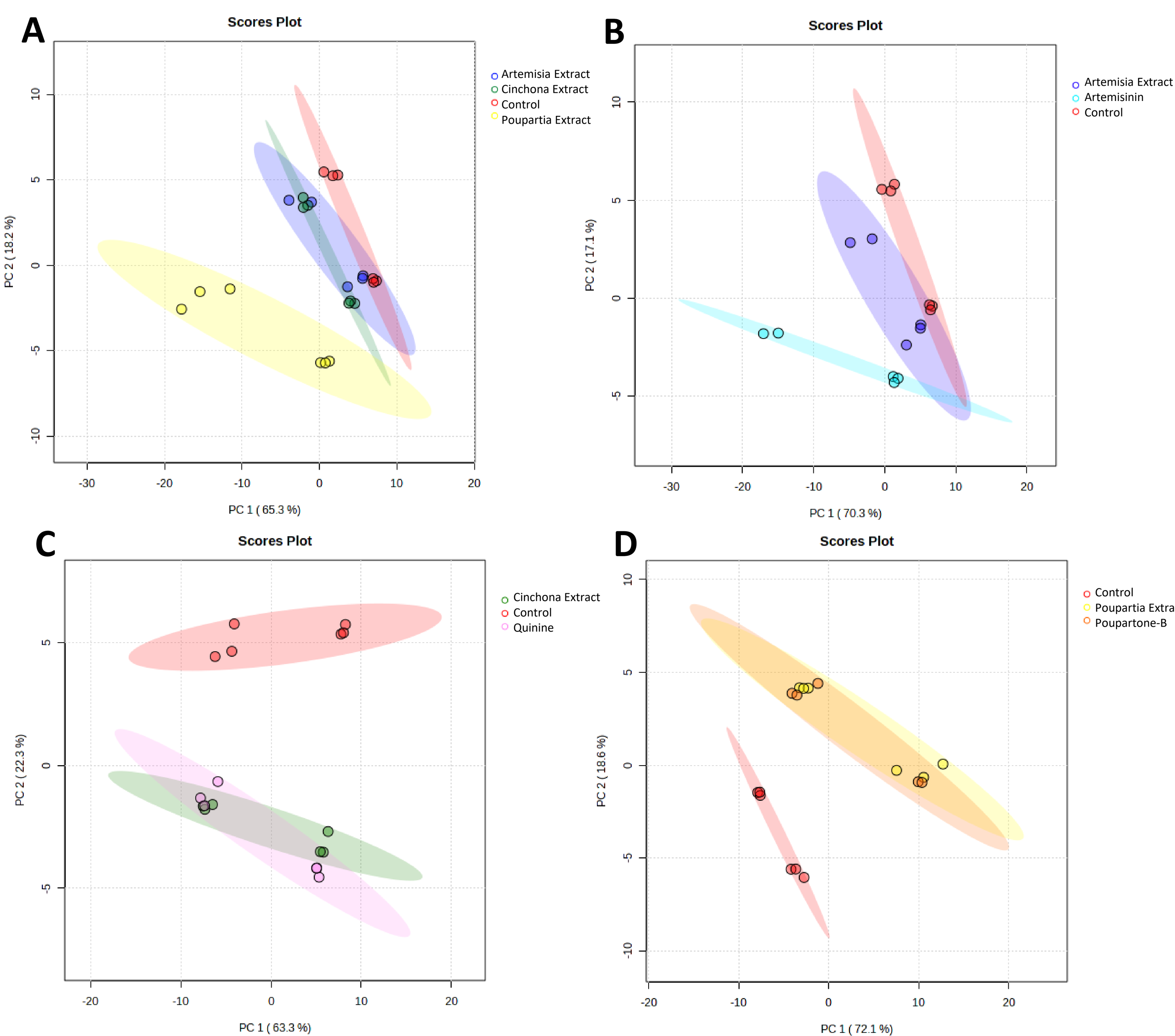


Figure 3: PCA 2D scores plot with 95% confidence regions displayed. A – Extracts and Control plot; B – Artemisia extract and Artemisinin plot; C – Cinchona extract and quinine plot; D – Poupartia extract and Poupartone-B plot. Control represents DMSO only.

Results & Discussion

Study groups	Extract vs. pure molecule	IC_{50} (ng/ml)
I	*A. annua* extract	67,94 ± 57,70
	Artemisinin	7,33 ± 3,88
II.	*C. officinalis* extract	302,51 ± 65,76
	Quinine	352,61 ± 180,18
III.	*P. borbonica* extract	458,74 ± 170,89
	Poupartone-B	1140,86 ± 190,06

Table 1: IC_{50} determined from *in vitro* growth inhibition assays.

I. The Artemisia extract and Artemisinin don't cluster together despite a seemingly similar metaprint due to a different fold change for a few peptides and amino acids, though the exact profile is unclear.

II. The Cinchona extract and Quinine cluster together, which is indicative of an identical profile attributed mostly to peptides linked to hemoglobin metabolism.

III. Despite differing IC_{50}, **the Poupartia extract and Poupartone-B** cluster together and seem to identically interfere with peptides and amino acids. The extract seems to differentiate from the others due to the different fold change of several peptides, nucleotides and translation amino acids.

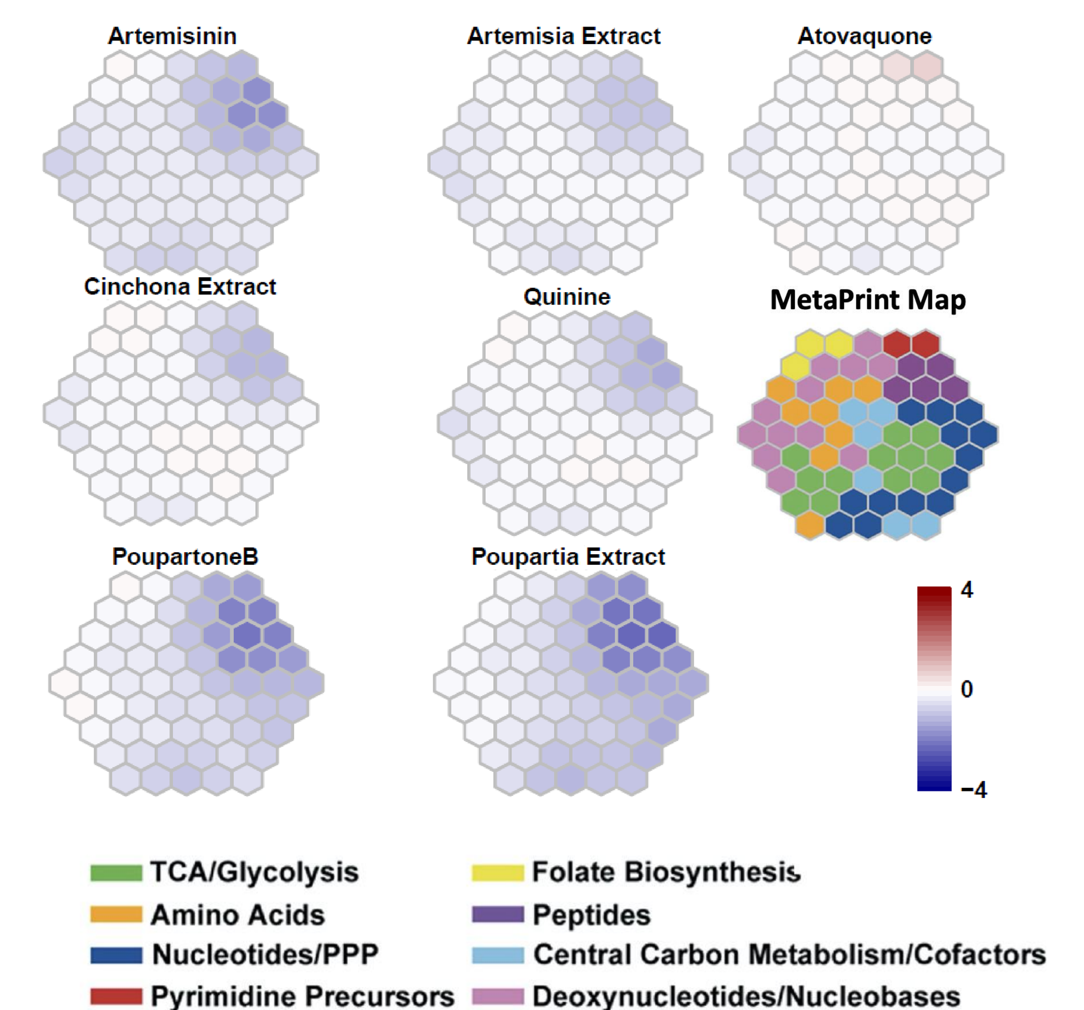


Figure 2: Metabolomic profiles displayed in suprahexagonal metaprints determined from the log2 fold change values of targeted metabolites following drug treatment relative to an untreated (no-drug) control. Atovaquone is displayed as example of a specific signature (inhibitor of bc1 complex in mitochondria)

Conclusions

- Initial assays with the plant extracts point to a general profile, with the exception of the Poupartia Extract, which differentiates further.
- Individually, the extracts have a metabolic profile that is undifferentiated from the isolated natural compounds, except for the Artemisia extract and Artemisinin.
- These findings suggest that metabolomics is a viable tool to profile plant extracts and natural compounds with promising antiplasmodial activity and should be incorporated as a standard screening method in drug discovery.

Acknowledgements This work was supported by the Belgian National Fund for ScientificResearch (FNRS, grant PDR T.0092.20) and the Fondation Léon Fredericq.

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Annex IV

Adapted from: **Supplementary Table: ^{1}H and ^{13}C NMR data for metabolites identified in parasite extracts (pH 7.6)** from **Teng, R. et al. Metabolite profiling of the intraerythrocytic malaria parasite Plasmodium falciparum by 1H NMR spectroscopy. NMR Biomed. 22, 292–302 (2009).**

Compounds
Amino acids
Alanine
4-Aminobutyrate *
Arginine
Asparagine
Aspartate
Glutamate
Glutamine
Glycine
Histidine
Isoleucine
Leucine
Lysine
Methionine**
Phenylalanine
Serine**
Threonine
Tyrosine
Valine
Glutathione
Glutathione (GSH)
GSSG
Soluble membrane precursors
Glycerophosphorylcholine
Glycerophosphorylethanolamine
myo-Inositol
Phosphorylcholine
Phosphorylethanolamine
Nucleotides and related compounds
AMP*
ADP*
ATP*
GMP***
GDP*
GTP***
Hypoxanthine***
IMP***
UMP*
UDP***
UTP***
CMP*
CDP*
CTP*
NAD^+
$NADP^+$ ***
Carboxylates
Acetate
Citrate
Formate***
Fumarate
α-Ketoglutarate
Lactate
Malate
Pyruvate
Succinate

Profiling plants with antimalarial blood stage activity using metabolomics: an added dimension to drug discovery

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Natural products and their derivatives have historically contributed significantly to the treatment of malaria. However, despite their promising potency diversity, it is challenging to determine their mode of action (MoA), which is essential for antimalarial drug discovery. Metabolomics is a robust tool that had been successfully used to profile the MoA of antimalarial compounds, but it has seldom been applied to complex matrices such as plant extracts and natural compounds. In this study, three plants were used: *Poupartia borbonica* Gmel, a dioecious endemic plant from the Mascarene Islands belonging to the Anacardiaceae family, and its alkyl cyclohexenone derivatives with antiplasmodial properties[1]; *Artemisia afra* and *Artemisia annua*, both known for their antimalarial activities[2], collected from the Adamawa Region of the Republic of Cameroon in the same season. Trophozoite-synchronized *Plasmodium falciparum* cultures were treated with extracts and purified compounds derived from these plants and were analysed by LC-MS and ^{1}H NMR metabolomics to estimate their metabolic profile. Results were compared against metabolic responses to established antimalarial drugs with defined MoAs – atovaquone, chloroquine, quinine and artemisinin. Our findings suggest that *P. borbonica* and isolated compounds are active on hemoglobin metabolism related pathways, whereas *A. afra* and *A. annua* have differing profiles, with *A. afra* affecting nucleotide metabolism and *A. annua* impacting glutathione metabolism and associated redox pathways. Therefore, metabolomics is a viable tool to profile plant extracts and natural compounds with promising antiplasmodial activity and should be incorporated as a standard screening method in drug discovery.

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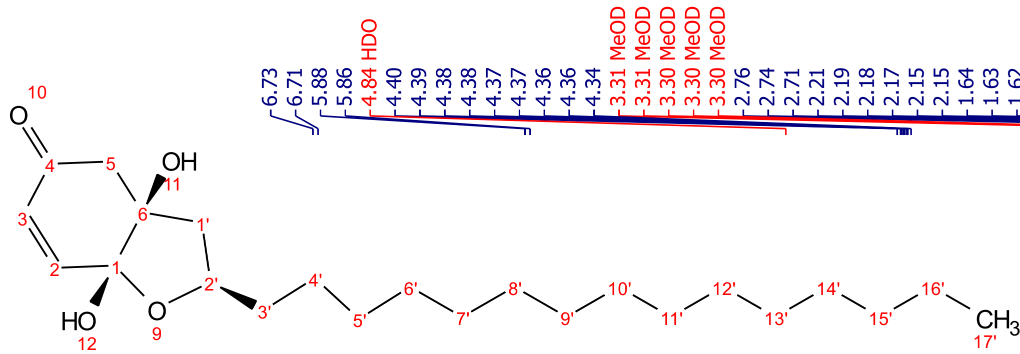

^{1}H NMR (500 MHz, MeOD) δ 6.72 (d, J = 10.2 Hz, 1H), 5.87 (d, J = 10.2 Hz, 1H), 4.37 (dq, J = 8.5, 6.6 Hz, 1H), 2.73 (dd, J = 16.0 Hz, 2H), 2.19 (dd, J = 12.9, 6.6 Hz, 1H), 1.62 (dd, J = 12.9, 8.5 Hz, 1H), 1.57 – 1.50 (m, 1H), 1.46 – 1.22 (m, 35H), 0.90 (t, J = 6.7 Hz, 5H).

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Publications and communications of Lucia Cristina Coelho Cristino Mamede

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