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Faculdade de Farmácia

Design synthesis and biological evaluation of new antiplasmodial compounds related to ellagic acid

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Monografia de Mestrado Integrado em Ciências Farmacêuticas apresentada à Universidade de Lisboa através da Faculdade de Farmácia

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Resumo

Actualmente, a malária continua a ser uma das maiores ameaças à vida humana em termos de problemas de saúde pública a nível mundial. Estima-se que cerca de 3,2 biliões de pessoas, em 95 países e territórios, estejam em risco de infecção pelo *Plasmodium* e assim contrair esta doença que, por vezes, se torna fatal. Em 2015, foram registados 212 milhões de casos de malária, verificando-se ainda cerca de 429 000 mortes. A epidemia tem maior impacto no hemisfério sul, principalmente em países em desenvolvimento sendo que mais de 90% das mortes devidas à malária acontecem na zona subsariana de África. As crianças com idade inferior a 5 anos, que representam 70% das mortes registadas em África, e as grávidas constituem os grupos de maior risco no que concerne à infecção, tendo em conta o estado débil dos seus sistemas imunitários. No entanto, têm-se verificado avanços no controlo e tratamento desta doença, com a taxa de incidência a diminuir 41% no período de 2000 a 2010, e 21% entre 2010 e 2015. Este declínio no número de casos e mortes registados deve-se, em grande medida, às melhorias no acesso a cuidados de saúde incluindo o uso de terapias combinadas à base de artemisinina e ainda às grandes campanhas de prevenção e sensibilização organizadas. A infecção pelos parasitas da malária pode levar a um amplo espectro de sintomas, desde a sua ausência ou mesmo sintomas muito leves, tais como náuseas, vómitos, dores de cabeça e febre, até a uma doença com consequências graves e possivelmente fatais, como anemia grave por hemólise ou insuficiência renal aguda.

A malária é, então, uma doença infecciosa transmitida por mosquitos e provocada por parasitas protozoários do género *Plasmodium*, cujo ciclo de vida se verifica extremamente complexo. A transmissão para humanos ocorre pela picada de uma fémea infectada do mosquito *Anopheles*, a qual introduz no sistema circulatório do humano os esporozoítos presentes na sua saliva, que vão posteriormente infectar os hepatócitos, onde maturam desenvolvendo esquizontes. Após a maturação dos esquizontes estas células rompem libertando na circulação merozoítos, que vão infectar os glóbulos vermelhos. Nas hemácias, ocorre a multiplicação assexuada formando um esquizonte sanguíneo cuja ruptura liberta novamente merozoítos com a capacidade de infectar novos eritrócitos. Os parasitas na fase sanguínea são os responsáveis pelas manifestações clínicas da doença, e alguns destes têm a capacidade de se diferenciar em formas eritrocíticas sexuais (gametócitos). Os gametócitos masculinos e femininos, micro e macrogametócitos respectivamente, são

ingeridos pela fémea do mosquito *Anopheles* através de uma picada dando-se início ao ciclo esporogónico com a multiplicação dos parasitas no mosquito. Uma vez no estômago do mosquito, os microgâmetas penetram os macrogâmetas dando origem a zigotos que se tornam alongados e móveis (oocinetes) e invadem a parede do intestino onde se desenvolvem em oocistos. Estes oocistos crescem e após a sua ruptura libertam esporozoítos que através da circulação sanguínea chegam às glândulas salivares do mosquito. Quando ocorre nova picada do mosquito *Anopheles* num outro ser humano, os esporozoítos são inoculados conjuntamente com a saliva do mosquito e começa, então, outra infecção humana.

Quanto à prevenção da malária, os seus principais objectivos são reduzir consideravelmente a transmissão do parasita, limitar a propagação da doença e, em última instância, diminuir a morbidade e a mortalidade. Existem várias estratégias de prevenção para alcançar os objectivos supramencionados, como a quimioprevenção, através de fármacos que suprimem as infecções; por controlo do vector, evitando as picadas de mosquitos em seres humanos e, eventualmente, no futuro, pela vacinação. A utilização destes métodos é essencial para evitar o aparecimento e desenvolvimento de resistências aos tratamentos já existentes.

Durante o século XX ocorreram vários progressos científicos e tecnológicos no desenvolvimento de novos fármacos, onde a cloroquina e a artemisinina figuram como as descobertas mais relevantes. Contudo, o *Plasmodium falciparum* possui a capacidade de desenvolver resistência a estas moléculas e, assim, comprometer a eficácia continuada dos tratamentos. Este facto desperta então uma grande necessidade de novas abordagens terapêuticas, bem como o desenvolvimento de novos fármacos, de preferência com diferentes mecanismos de acção. Em resposta à propagação da resistência adquirida pelo *Plasmodium* à cloroquina e outros antimaláricos como a sulfadoxina e pirimetamina, as Terapias de Combinação à base de Artemisinina (TCAs) estão actualmente recomendadas para o tratamento da malária não severa, e o seu uso tem sido um factor determinante no notável decréscimo do número de casos desta doença infecciosa a nível mundial.

Apesar de todos os esforços desenvolvidos nas últimas décadas, ainda não existe nenhuma vacina disponível. RTS,S/AS01, uma vacina anti-esporozoíta é, neste momento, o candidato em ensaios clínicos mais promissor. Actualmente já passou, com sucesso, os testes clínicos de fase III em mais de 15.000 crianças africanas. Durante um ano de seguimento, a vacinação com uma série de três doses, diminuiu os casos clínicos de malária em 18% em lactentes e 28% em crianças. Esta vacina, que

representa grande esperança para o controlo da malária, está a ser desenvolvida pela GlaxoSmithKline (GSK) em colaboração e parceria com outras entidades.

Neste trabalho o grande foco, na tentativa de encontrar melhorias para a saúde global no combate à malária, esteve num composto natural polifenólico presente em inúmeros frutos e vegetais como morangos, framboesas, nozes e romãs: o ácido elágico. A estrutura do mesmo é composta por dois anéis lactónicos e dois anéis fenílicos com dois grupos hidroxilo cada - $C_{14}H_6O_8$. As principais actividades biológicas deste composto incluem a acção antioxidante, anticarcinogénica, antimicrobiana, anti-inflamatória e antimalárica, como reportado por vários estudos realizados. O ácido elágico possui um valor de IC_{50} entre 100 e 300 nM, contra várias estirpes de *Plasmodium falciparum*, seja qual for o seu nível de resistência contra a cloroquina e a mefloquina, e não mostra toxicidade em células humanas a 100 µM. Demostra também propriedades profilácticas, bem como uma clara sinergia com a cloroquina, mefloquina, artesunato e atovaquona, actuando na parte final da fase eritrocítica do ciclo de vida do parasita. No entanto o seu uso como fármaco *in vivo* encontra-se comprometido devido à sua reduzida biodisponibilidade oral. Esta desvantagem deve-se ao facto do ácido elágico apresentar uma baixa solubilidade em água bem como em outros solventes orgânicos. Esta característica pode ser explicada pela estrutura planar da molécula favorecendo a aglomeração da mesma e tornando bastante difícil a sua separaração. Vários alvos moleculares foram propostos para explicar a actividade antimalárica do ácido elágico, tais como a regulação da expressão da glutationa S-transferase (GST), a inibição da formação de β-hematina e, em menor grau, a inibição das plasmepsinas, mas o seu mecanismo de acção ainda não se encontra totalmente esclarecido.

O presente trabalho visa, então, desenvolver novos compostos antimaláricos derivados da estrutura do ácido elágico na tentativa de melhorar os parâmetros farmacocinéticos relativamente ao composto original. Através da introdução de cadeias alquilo polares na estrutura do composto espera-se uma melhoria das características físicas e químicas dos derivados quando comparadas com os atributos do ácido elágico. Algumas das alterações estruturais realizadas poderiam ser também responsáveis pela obtenção de compostos com uma clara melhoria da actividade antimalárica relativamente ao ácido elágico. Dessa forma, os principais objectivos poder-se-ão dividir em três partes: 1) síntese de derivados do ácido elágico com o intuito de melhorar os seus parâmetros farmacocinéticos; 2) purificação e identificação dos compostos sintetizados; e 3) avaliação da actividade malárica desses mesmos compostos, usando um ensaio *in vitro* com o *Plasmodium falciparum*.

Abstract

Malaria remains a major public health problem, especially in subtropical regions. This endemic disease is caused by parasitic protozoans that belong to the *Plasmodium* type and people who get malaria are typically very sick with high fevers, shaking chills, and flu-like symptoms. Approximately 3.2 billion people worldwide are at risk of being infected and develop this disease. The highest risk is in the sub-Saharan Africa region where approximately 80 % of cases and 90 % of deaths occurs, mostly in children under five years and in pregnant women. The rise and spread of the resistance of *Plasmodium falciparum* malaria to chloroquine, sulfadoxine-pyrimethamine and artemisinin as well as the resistance of *P. falciparum* malaria vectors to pyrethroids (insecticides used to prevent malaria in endemic regions), presents a serious challenge, especially in developing countries. Therefore it is imperative and crucial the design of new antimalarial drugs. Medicinal plants constitute a promising source of new drugs and there is now a real worldwide interest in antiplasmodial plants. Ellagic acid is a polyphenol found in various plant products, whose main biological activities include antioxidant, anticarcinogenic, antimicrobial, anti-inflammatory and antiplasmodial effect. This compound has an *in vitro* IC_{50} between 100 and 300 nM against several *Plasmodium falciparum* strains, whatever the level of resistance against chloroquine and mefloquine, and it shows no toxicity on human cells at 100 µM. It has also prophylactic properties throughout *in vivo* studies, synergistic activities with chloroquine, mefloquine, artesunate and atovaquone, and it acts at the late stage of the erythrocytic life cycle. This natural compound has also *in vivo* activity against *Plasmodium*, thanks to parenteral administration, but modification of the compound could lead to improved pharmacological properties, principally for the oral route because this molecule suffers of a poor oral bioavailability. In this research work, the main objective was to develop new antiplasmodial compounds derived from the structure of ellagic acid and showing improved pharmacokinetic parameters compared to the parent compound. These new compounds should constitute a set of powerful drug candidates for the treatment of malaria, when its antimalarial activity *in vitro* should be evaluated.

Key-words: Ellagic acid, Malaria, Antiplasmodial, Ellagic acid derivatives.

Aknowledgements

List of Abbreviations

ACTs	Artemisinin-based combination therapies
AQ	Amodiaquine
BCS	Biopharmaceutical Classification System
BMGF	Bill & Melinda Gates Foundation
bs	Broad singlet
CDCl3	Deuterated chloroform
CEA	Coruleoellagic acid
d	Doublet
DCM	Dichloromethane
DMAP	4-Dimethylaminopyridine
DMF	Dimethylformamide
EA	Ellagic acid
EtOH	Ethanol
FEA	Flavellagic acid
GNF	Genomics Institute of the Novartis Research Foundation
GSK	GlaxoSmithKline
GST	Glutathione S-transferase
hept	heptet
HHDP	Hexahydroxydiphenoyl
hrs	Hours
IPTi	Intermittent preventive treatment of malaria in infants
IPTp	Intermittent preventive treatment of malaria in pregnancy
IRS	Indoor residual spraying
ITN	Insecticide-treated mosquito net
K13	Gene Pfk13
m	Multiplet
MEM	2-methoxyethoxymethyl
MEMCl	2-methoxyethoxymethyl chloride
min	Minutes
MMV	Medicines for Malaria Venture
MVI	PATH-Malaria Vaccine Initiative
NITD	Novartis Institute for Tropical Diseases

NMR	Nuclear Magnetic Resonance
PE	Pre-erythrocytic
PfATP4	P-type Na^+-ATPase
PfGST	Glutathione S-transferase of the malarial parasite *Plasmodium falciparum*
pLDH	Parasite lactate dehydrogenase
q	Quadruplet
RT	Room temperature
s	Singlet
SEA	Southeast Asia
SP	Sulfadoxine-pyrimethamine
Swiss TPH	Swiss Tropical and Public Health Institute
t	Triplet
TBAF	Tetra-n-butylammonium fluoride
TCAMS	TresCantos antimalarial set
TIPS	Triisopropylsilyl
TIPSCl	Triisopropylsilyl chloride
TLC	Analytical Thin Layer Chromatography
TMS	Tetramethylsilane
UV	Ultraviolet
WHO	World Health Organization
WRAIR	Walter Reed Army Institute of Research, US

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1. Introduction

1.1. The Malaria

Appearing as one of the most significant infectious disease in the world, malaria is a mosquito-borne illness which affects human beings and other animals, and caused by parasitic protozoans that belong to the *Plasmodium* genus. The transmission to humans occurs through the bite of an infected female *Anopheles* mosquito.[1]

Malaria continues to be a disease of worldwide health importance with 3,2 billion people at risk of being infected and develop disease, distributed in 95 countries and territories. In 2015, WHO estimate the number of cases of malaria at 212 million, leading to around 429 000 deaths. This epidemic is generally located in the south countries, as shown in figure 1, with more than 90% of the deaths caused by malaria occurring in the African region, mostly in children (around 70% of deaths in children under 5 years old). The incidence rate decreased by 41% between 2000 and 2015, and by 21% between 2010 and 2015. The decline in the number of infections and deaths was largely facilitated by better access to care including the use of ACTs (artemisinin based combination therapies), and by large prevention campaigns.[2]
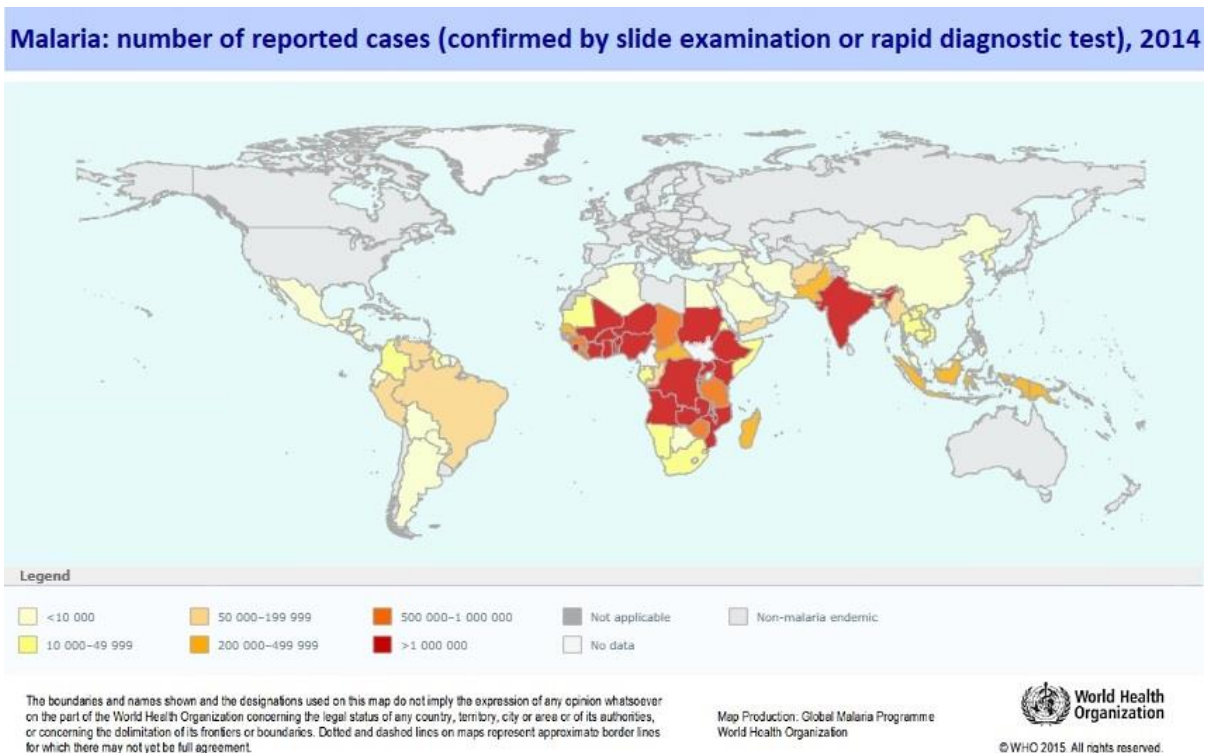

Figure 1 - Malaria: number of reported cases in 2014 [3]

Although the majority of deaths are due to *P. falciparum,* in 2015 the infection caused by *Plasmodium vivax* led to 3100 deaths with 86% of these cases happening outside the African region.[2,4]

The infection with malaria parasites may result in a wide variety of symptoms, ranging from absent or very mild symptoms such as nauseas, vomits, headaches and fever; to severe disease with severe anaemia due to hemolysis, acute kidney failure and even death.[4]

1.1.1. The malaria parasite life cycle

Plasmodium spp. are global pathogens with a complex life cycle (Figure 2) which involving two types of hosts: humans and female *Anopheles* mosquitoes.(4) Throughout a blood meal, a malaria-infected mosquito inoculates sporozoites to the human host that infect hepatocytes and develop into schizont, which rupture and consequently release merozoites.[5]

After this, merozoites infect red blood cells where asexual multiplication occurs forming a blood schizont, which rupture results in the release of more merozoites that can infect further erythrocytes.[6] The blood stage is responsible for the clinical manifestations of the disease[4], and some merozoites can differentiate into sexual erythrocytic forms (gametocytes).[7]

The male and female gametocytes, micro and macrogametocytes respectively, are ingested by an *Anopheles* mosquito during another blood meal and start the sporogonic cycle with the multiplication of parasites in the mosquito. Once in the mosquito's stomach, the microgametes penetrate the macrogametes generating zygotes, which become motile and elongated (ookinetes) and invade the midgut wall of where they develop into oocysts. The oocysts grow, rupture, and release sporozoites, which make their way to the mosquito's salivary glands.[4,7] When the *Anopheles* mosquito takes a blood meal on another human, the sporozoites are injected with the mosquito's saliva and start another human infection.[4]
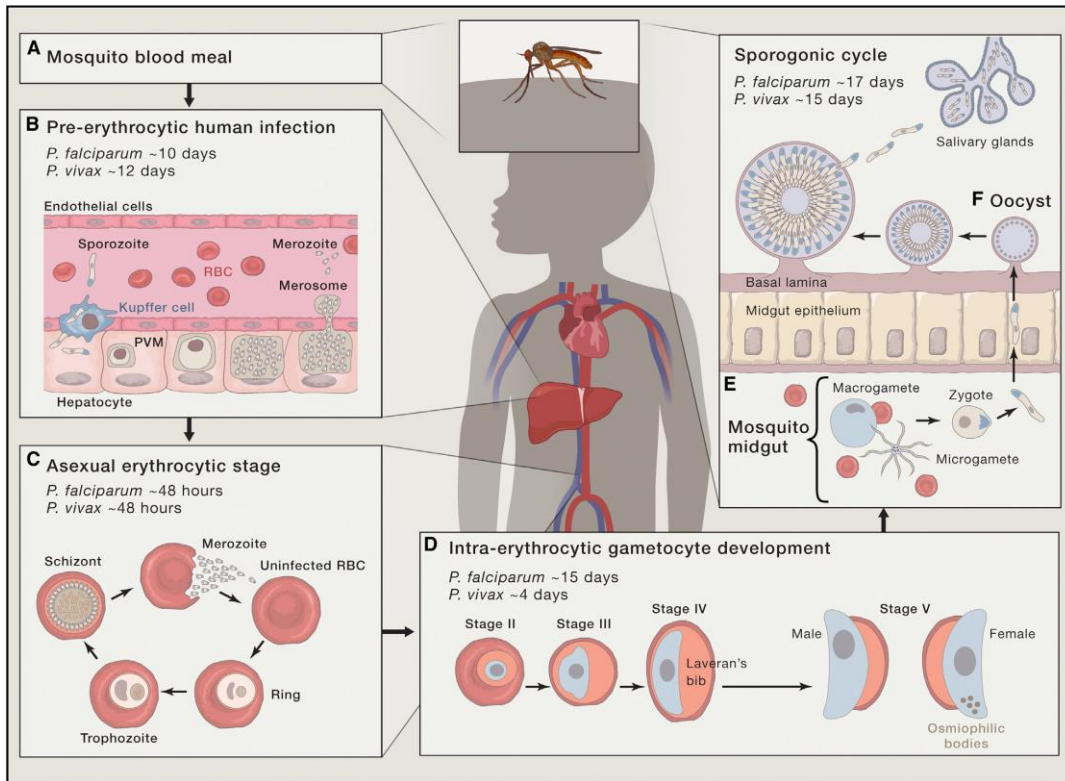
Figure 2 - Life Cycle of *Plasmodium falciparum* [7]

1.1.2. Prevention

The major goals of malaria's prevention are to reduce considerably the transmission of the parasite, to limit the propagation of the malaria and ultimately to decrease both morbidity and mortality.[2,4] There are several prevention strategies in order to achieve the supramentionated objectives, such as the chemoprevention, through drugs that supress the infections; by vector control, avoiding the mosquitoes bites in humans and, eventually in the future, by vaccination. The use of these methods is essential to avoid the appearance and the development of resistances from the existent treatments linked either to natural resistances or to post-infection therapeutic misuse.[2]

a. Chemoprevention:

The intermittent preventive treatment of malaria in pregnancy (IPTp) and in infants (IPTi) with sulfadoxine-pyrimethamine (SP) has been shown to reduce some problems arising from this disease as the anaemia.[2,4] Amodiaquine combined with SP (AQ + SP) has been used in the chemoprevention of seasonal malaria, showing to

decrease the occurrence of clinical attacks and severe malaria when used in children aged 3-59 months. In 2016, 10 African countries had already implemented this therapy.[2]

b. Vector Control:

The main contribution to the decrease in malaria burden since 2000 was made by two core vector-control interventions – use of ITNs (insecticide-treated mosquito net) and IRS (indoor residual spraying).[4] In some specific conditions and situations, ITNs and IRS can be complemented by larval management or other environmental alterations that decrease the suitability of environments as mosquito habitats or that on other hand can limit bites of humans.[2]

c. Vaccination:

At the moment, some research projects about malaria vaccines are ongoing. Only one have completed Phase 3 testing, RTS,S/AS01 (trade name Mosquirix™), which has shown to reduce the clinical incidence by 39% and severe malaria by 31,5% among children aged 5-17 months who completed four doses of this vaccine.[2,7,8] WHO recommended the implementation of RTS,S/AS01 on a pilot scale in some sub-Saharan African countries to provide information on feasibility, safety and mortality impact, and to guide recommendations on the potential wider scale use of this vaccine.[2]

1.1.3. Treatment

a. Antimalarials:

The first use of quinine from the cinchona tree marks the beginning of the treatment against malaria and since there antimalarial drugs have been the main tool of control and prophylaxis. During the 20th century occurred some scientific progresses in drug development, wherein chloroquine and artemisinin were the most important discoveries.[7] However, *P. falciparum* has the ability to develop resistance to these drugs what compromises the continuing efficacy of the treatment. This raised the need of novel therapeutic approaches, as well as the development of new drugs preferentially with different mechanisms of action.[9] In answer to the spread of acquired resistance to chloroquine and other molecules such as sulfadoxine and pyrimethamine, artemisinin-based combination therapies (ACTs) are currently recommended for

treatment of uncomplicated malaria, and their use has been a key factor of the notable decrease in malaria worldwide.[7,10]

b. Mechanism of Artemisinin Action and Resistance

Although, the mechanism of action of artemisinin against malaria parasite is not fully understood, it is already identified the importance of heme-mediated opening of the endoperoxide bridge (arising from the degradation of hemoglobin) for its activity.[7] But how does the endoperoxide bridge break open to form free radicals? In 1991, Meshnick and collaborators presented that artemisinin interacted with intraparasitic heme, and suggested that heme or iron might function to activate artemisinin inside the parasite into toxic free radicals. The malaria parasite is rich in heme-iron, derived from the proteolysis of host cell haemoglobin. This could explain why artemisinin is selectively toxic to parasites. Once formed, the artemisinin-derived free radicals appear to damage specific intracellular targets, possibly via alkylation. [11–13]

For decades, Southeast Asia (SEA) has been fertile ground for the appearance of drug-resistant malaria parasites.[14] After resistance to numerous antimalarials (such as chloroquine, sulfadoxine, and mefloquine) resistance to artemisinin was detected for the first time in 2006 in Cambodia.[15] Two key mechanisms are responsible for *Plasmodium* resistance to practically all antiplasmodial drugs: (i) reduced drug availability at its site of action, essentially due to mutations in transporter genes; and, (ii) modification of the drug target by mutations in corresponding genes.[16] However, artemisinin resistance results from another cellular process, the quiescence. *Plasmodium* resistance to artemisinin occurs due to the enhanced number of young ring forms to enter into a quiescence state upon exposure to artemisinin, which quickly resume the growth after the artemisinin discontinuation. This ability is conferred by mutations of a gene called *Pfk13* (K13).[7,16,17] This gene is currently monitored to follow artemisinin resistance spread according to WHO recommendations.[15,16]

To date, more than 200 non-synonymous mutations in the K13 gene have been reported in South-East Asia, but these mutations are still rare and highly diverse in Africa countries.[15] To avoid the spread of this drug resistance, some factors should be controlled which include mobile populations and migrants, uncontrolled use of ACT's, artemisinin monotherapy, the use of subtherapeutic levels of artemisinin, substandard and counterfeit drugs and co-use of artemisinin derivatives as prophylactic agents.[18]

c. Development of Novel Antimalarials

Malaria caused by *P. vivax* and *P. falciparum* has effective therapy to treat and control it. But *Plasmodium* acquired resistance against antimalarials which rises serious worries about the long-term usage of the existing therapies.[7] WHO defined numerous objectives regarding this disease for 2030, but the current situation doesn't seem promising because the available therapies are scarce to achieve these purposes.[2,7] Concerns about artemisinin resistance have led universities and research institutes, funding agencies, governments, non-governmental organizations, the military and public-private partnerships to work together to find possible replacement medicines.[19] To reach the malaria elimination it has become crucial to discover new drugs that feat broadly to cure the asexual blood stage to mitigate symptoms, clean the liver stage which is responsible for relapses for *P. vivax* and *P. ovale* malaria, and stop transmission.[7]

There are different strategies to reach the development of new antimalarial drugs. Though efforts have been made in existing molecules (for example by modifying a scaffold to work against parasites that have acquired resistance to the parent scaffold), most novel classes of antiplasmodial compounds have come from high-throughput screens. In this approach, a huge compound library is screened to identify compounds that are active against the parasite in what is called a "phenotypic" or "whole-cell" assay.[19] This has been increased by the availability of the TresCantos antimalarial set (TCAMS) of 13,000 compounds that have antimalarial activity.[7,20] From this, Medicines for Malaria Venture (MMV) assembled 400 unique compounds with bloodstage antimalarial activity that has been called the Malaria Box.[7,21] MMV, in collaboration with many researchers, have built a research and development portfolio from lead compounds to novel antimalarials currently in clinical trials.[22]

Whole-cell screens of asexual blood stages of P. falciparum identified the spiroindolone class of compounds, leading to the candidate KAE609 (cipargamin) currently in clinical trials.[7] KAE609 is a novel synthetic spiroindolone developed by the Novartis Institute for Tropical Diseases (NITD) in Singapore in collaboration with the Swiss Tropical and Public Health Institute (Swiss TPH), the Dutch Biomedical Primate Research Center, and the Genomics Institute of the Novartis Research Foundation (GNF) in San Diego.[18] Cipargamin and related compounds bind the P-type Na^+-ATPase (PfATP4) expressed on the parasite plasma membrane. This disrupts sodium homeostasis, thus blocking asexual blood-stage development and transmission to the mosquito.[7,23]

d. Vaccine Development

Despite all the efforts during the last 30 years, there is no vaccine available yet. The attempts have targeted pre-erythrocytic (PE), blood and sexual stages. A fully effective PE vaccine would prevent malaria by stopping establishment of blood-stage infection. Vaccines impacting the asexual blood stages replication have been considered to control morbidity and mortality. Vaccines against the sexual stages would interrupt the transmission cycle but not have a direct effect on an established infection. Ideally the malaria vaccine would be based on preserved targets that cause, for all lifetime, sterile protection with as less doses as possible but until now this has not been achieved.[7]

The development of RTS,S/AS01 malaria vaccine is being conducted by GlaxoSmithKline (GSK) in collaboration and partnership: the early stage development was undertaken in collaboration with WRAIR (Walter Reed Army Institute of Research, US) and the paediatric clinical development through an innovative Public Private Partnership with PATH-Malaria Vaccine Initiative (MVI), funded by the Bill & Melinda Gates Foundation (BMGF). Clinical trials are carried out with African Research Institutes and their Northern hemisphere partners.[24]

RTS,S/AS01, an antisporozoite vaccine, is the most promising clinical candidate up to date and has proceed through phase III clinical trials with over 15,000 African children. During a year of follow-up, vaccination with a three-dose series decreased clinical malaria cases by 18% in infants and 28% in young children. After these trials, it was possible to conclude that the period of protection was very short and efforts to understand the reason of this are urgently needed. A comprehensive study of interaction between malaria parasites and the human immune system and how they co-evolve is another area that will help in designing better vaccine strategies.[7,24,25]

1.2. Ellagic Acid
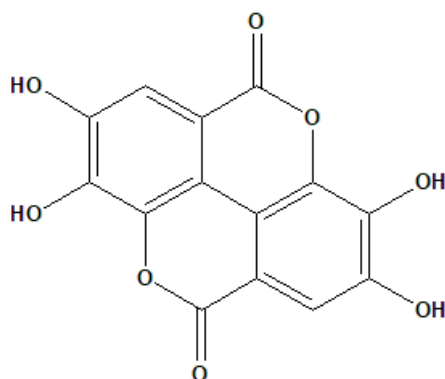
(1)

Ellagic acid, 1, is a natural polyphenol compound found in numerous fruits and vegetables such as raspberries, strawberries, walnuts and pomegranates.[26,27] This compound is present as structure element of hydrolysable and highly concentrated tannins.[28] In plants and fruits the tannins are called the ellagitannins and are composed by moieties of hexahydroxydiphenoyl (HHDP) esterified to a sugar, usually the glucose. Once hydrolysed these tannins (HHDP) allows the production of ellagic acid.[29] Clinical studies reported that the main biological activities of ellagic acid include antioxidant, anticarcinogenic, antimicrobial, anti-inflammatory and antiplasmodial effects.[26,27]

The structure of ellagic acid is composed of two lactonic rings and two benzene rings with two hydroxyl groups each.[30] Its molecular formula and weight are, respectively, $C_{14}H_6O_8$ and 302,194 g/mol.[31] According to the Biopharmaceutical Classification System (BCS), ellagic acid is classified as a class IV molecule.[32] Thus, ellagic acid has very low solubility in water, polar solvents as well as in organic solvents, which makes it very difficult to use.[31,33] Therefore, its oral bioavailability does not exceed 1%, and this feature doesn't allow its use *in vivo* as a drug.[34] The very low solubility is explained by the flatness of the molecule enabling the creation of flat stack of molecule very difficult to separate.

Ellagic acid has a promising effect *in vitro*, shows high curative antiplasmodial activity and has prophylactic activity without any toxicity *in vivo* after intraperitoneal injection. Moreover this compound shows a synergy of action with chloroquine,

atovaquone, mefloquine and artesunate but in the presence of artemisinin, it will exhibit a slight antagonistic effect.[35]

According to Soh et al. (2009) mice infected with *Plasmodium vinckei petteri* were treated with 1, 50, and 100 mg/kg/day of ellagic acid by the oral and intraperitoneal routes. The results showed that with the 4-day suppressive test, the ED50 of ellagic acid administered by the intraperitoneal route was inferior to 1 mg/kg/day, and at doses of 50 and 100 mg/kg/day, a 100% inhibition of parasite growth was obtained. On the other hand, mice treated orally showed very little inhibition of parasite growth. In prophylactic-curative tests, mice treated with ellagic acid by the intraperitoneal route before parasite inoculation had a high-level reduction (between 79 and 93%) of parasitemia compared with the controls at day 6, suggesting a prophylactic effect of ellagic acid.[35]

According to the same study, ellagic acid acts on late stages of the erythrocytic *Plasmodium* life cycle. During the erythrocytic life cycle, the period of activity of ellagic acid at pharmacological doses was between the 24th and the 40th hours. This period corresponds to the trophozoite and early schizont forms of the parasites; which is the most metabolically active phase, where protein, RNA, and DNA synthesis taking place.[35]

Several molecular targets have been proposed to explain the antiplasmodial activity of ellagic acid, such as regulation of glutathione S-transferase (GST) expression, inhibition of β-haematin formation and, to a lesser extent, inhibition of plasmepsins, but its mechanism of action remains not yet fully understood.[36,37]

So *P. falciparum* possesses only one GST isoenzyme (PfGST), which differs structurally from all other known GST classes. Functional studies have shown that the inhibition of PfGST's system may lead to:

— A decrease in the general detoxification capacity;
— A reduction in the antioxidant capacity of the parasites;
— An increase in the free heme concentration in the vacuoles of the parasite.

All these considerations make PfGST's system a potential target for antimalarial drug development.[26] For this reason, Sturm et al.[38] decided to investigate the inhibitory effect of ellagic acid on the PfGST's system and discovered that these compounds - EA, flavellagic acid (FEA) and coruleoellagic acid (CEA) - are very good inhibitors of PfGST's system.[26,38]

2. Objectives

The present project aims to develop new antiplasmodial compounds derived from the structure of ellagic acid and showing improved pharmacokinetic parameters compared to the parent compound. Ellagic acid was found to be highly active *in vitro* an *in vivo* against malaria parasites with interesting selectivity and therapeutic indices after intraperitoneal administration. Nevertheless, its low oral bioavailability, linked to poor water solubility, precludes its oral use. This project will thus focus on the improvement of ellagic acid's critical physiochemical (i.e. hydrosolubility) and pharmacokinetic parameters through the design of prodrugs and original derivatives bearing polar alkyl chains, starting from the structures of ellagic acid and its metabolites. Some of the pharmacomodulations envisaged in this work could also be responsible for an improvement of the antimalarial activity. These new compounds should constitute a set of powerful drug candidates for the treatment of malaria.

To conclude, the objectives of this work could be divided in three main goals:

I.   Synthetize the ellagic acid's derivatives, to improve the pharmacokinetic parameters of this antimalarial compound;
II. Purify and identify the compounds previously synthesized;
III. Evaluate the antiplasmodial activity of those compounds, using an *in vitro* assay with *Plasmodium falciparum*.

3. Experimental

3.1. General Chemistry Experimental

Melting points were determined on a Büchi Tottoli capillary apparatus and are uncorrected.

The ^{1}H NMR spectra were recorded on a Bruker Avance (500 MHz) instrument using deuterated chloroform ($CDCl_3$) as the solvent and tetramethylsilane (TMS) as an internal standard; chemical shifts are reported in δ values (ppm) relative to that of internal TMS. Coupling constants were determined using MestreNova and the values are quoted in Hz. The abbreviations s = singlet, d = doublet, t = triplet, q = quadruplet, hept = heptet, m = multiplet and bs = broad singlet are used throughout.

Analytical Thin Layer Chromatography (TLC) was performed using normal phase Merck silica gel 60 F_{254} aluminium-supported thin layer chromatography sheets. The sheets were visualised using UV light absortion ($λ_{max}$ 254 or 365 nm) or thermal development after staining with nihydrin or potassium permanganate.

Chemicals were purchased from Sigma Aldrich, ABCR, Acros, or Fluorochem and were used without further purification unless otherwise stated.

3.2. Synthesis of Ellagic Acid Derivatives

The present project was focused on the preparation of antimalarial derivatives with an improved hydrosolubility, oral bioavailability and/or antiplasmodial activity. Based on the structure of ellagic acid, some approaches to introduce various (substituted or not) alkyl chains on selected phenolic functions (as R_1 and R_2 shown in structure below) were made. With these modifications, we expect to modulate the pharmacokinetic parameters and to obtain compounds with a safety profile close to that of ellagic acid. The Table 1 schematize the attempts achieved in this work and the numbers of compounds represented will be used in the rest of the report, where compound 1 represents the ellagic acid.
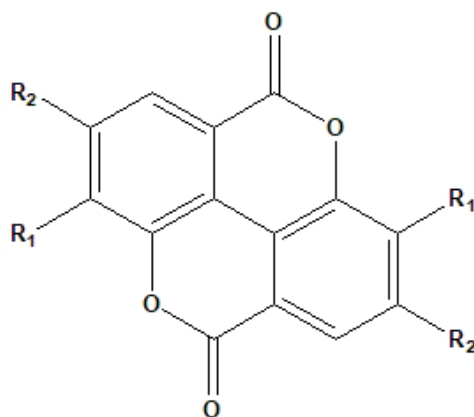
Table 1 - **Resume of the derivatives of ellagic acid tried to synthetize**

Compound	R1	R2
1	OH	OH
2	$OCOCH_3$	$OCOCH_3$
3	$OCOCH_3$	OH
4	$OCOCH_2CH3$	$OCOCH_2CH3$
5	$O(CH_2)_2OH$	$O(CH_2)_2OH$
6	OCH_2CH_3	OCH_2CH_3
7	O-TIPS	O-TIPS
8	O-TIPS	O-MEM
9	O-TIPS	$O(CH_2)_2I$

3.2.1. Synthesis of 3, 3', 4, 4' – tetra – O – acetylellagic acid (2)

(1)   **(2)**
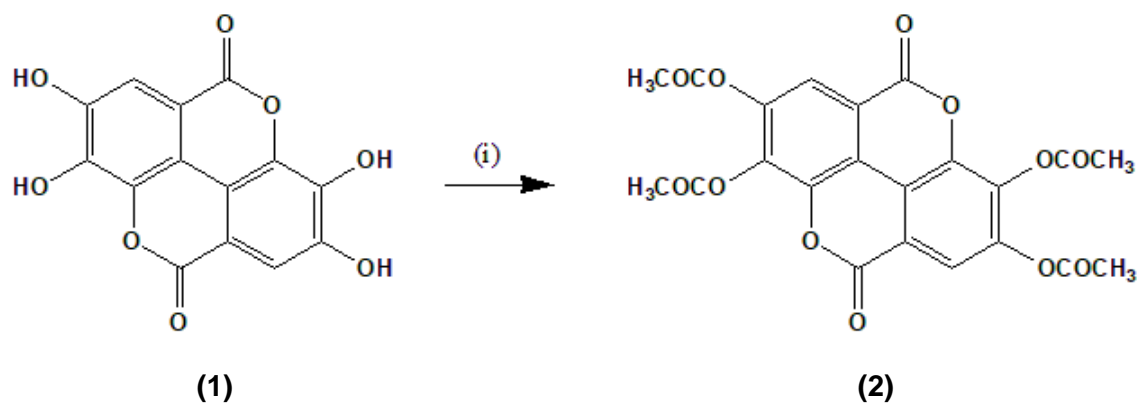
Scheme 1:

Reagents and conditions: (i) CH_3COONa, $(CH_3CO)_2O$, 110 ºC, 2 hrs.

Compound **2** was synthesized after adaptation of the method found in Heur et al. (1992).[39] A suspension of ellagic acid (1,0 g; 3.31 mmol), sodium acetate (0,2 g; 2,4 mmol) in 10 ml of acetic anhydride was refluxed at a temperature of 110 ° C during 2 hours. The reaction was followed by TLC. At the end of the chemical reaction, 20 mL of CH_2Cl_2 and 60 mL of EtOH were added to promote precipitation and the precipitate was recovered by filtration and placed in the oven at 30 °C to remove the last traces of solvents. The identification of the product was made by ^1H NMR.

3.2.2. Synthesis of 4, 4' – di – O – acetylellagic acid (3)

(2)   **(3)**
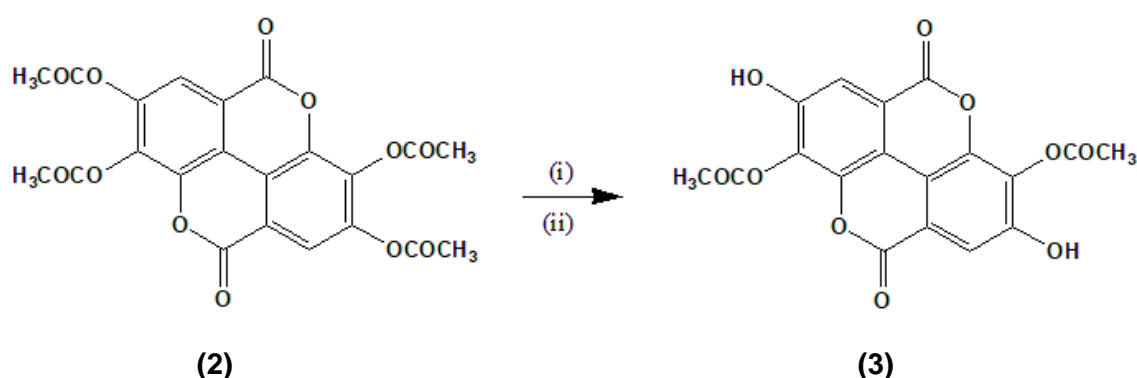
Scheme 2:

Reagents and conditions: (i) Pyridine, heat to boil; (ii) H_2O, steam bath, 3 min.

Compound **3** was synthesized according to the method of Jurd (1959).[40] A suspension of compound **2** (2,0 g) in pyridine (10,0 ml) was heated to boiling. Water (5,0 ml) was added, the mixture was heated briefly to boiling and then on a steam-bath for 3 minutes. The compound **2** dissolved to give a yellow solution. Almost immediately yellow needles began to separate. Water (30 ml) was added, the mixture was cooled and the crystalline product was collected. After washing with water, methanol and acetone the product was recrystallized from N,N-dimethylformamide. Compound **3** separated in almost colorless needles, m. p. 325-327 ºC wich did not give a color with methanolic ferric chloride. The identification of the product was confirmed by ^{1}H NMR.

3.2.3. Synthesis of 3, 3', 4, 4' – tetra – O – propionylellagic acid (4)

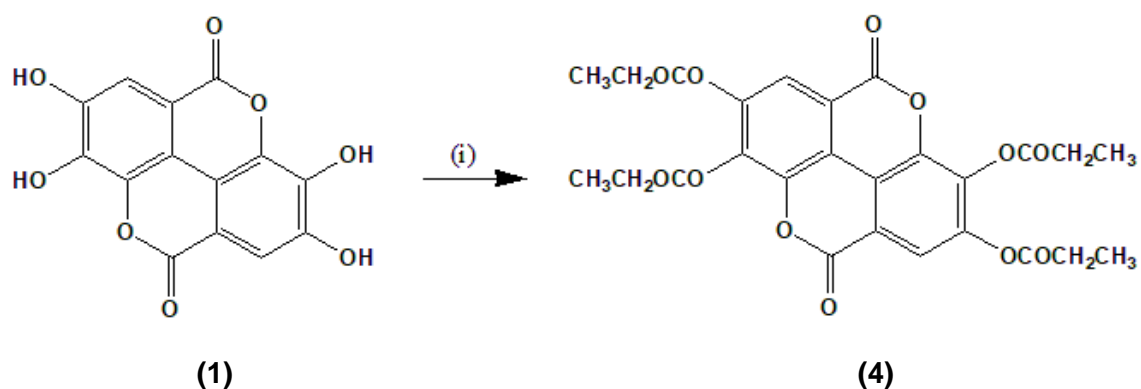**(1)**   **(4)**

Scheme 3:

Reagents and conditions: (i) CH_3COONa, $(CH3CH_2CO)_2O$, 110 ºC, 2 hrs.

Compound **4** was synthesized after adaptation of the method found in Heur et al. (1992).[39] A suspension of ellagic acid (1,0 g; 3.31 mmol), sodium acetate (0,2 g; 2,4 mmol) in 10 ml of propionic anhydride was refluxed at a temperature of 110 ° C during 2 hours. The end of the reaction was verified by TLC, and after that 20 mL of CH_2Cl_2 and 60 mL of EtOH were added to promote precipitation and the precipitate was recovered by filtration and placed in the oven at 30 °C to remove the last traces of solvents. The compound was confirmed by ^{1}H NMR.

3.2.4. Synthesis of 3, 3', 4, 4' – tetra – O – hydroxyethylellagic acid (5)

(1)   **(5)**
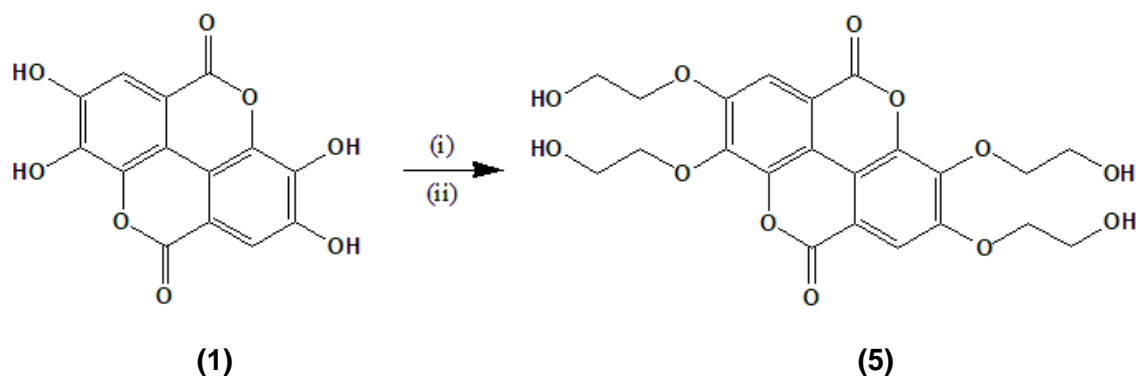
Scheme 4:

Reagents and conditions: (i) Cs_2CO_3, DMF, RT, 30 min; (ii) 2-bromoethanol, 110 °C, 6 hrs.

Compound **5** was synthesized after adaptation of the method of White.[41] A suspension of ellagic acid (0,2 g; 0,662 mmol), and cesium carbonate (1,73 g; 5,3 mmol) in 4 dimethylformamide (4 ml) was stirred at room temperature. After 30 minutes of stirring, 2-bromoethanol (0,4 ml; 5,3 mmol) was added and the mixture was heated at 110 °C during 6 hours. Lithium chloride (10 ml) was added and the mixture was extracted 3 times with chloroform. Brine wash of organic phase was realized. A Dry Column Vacuum Chromatography was realized to separate the products, and the final product was confirmed by ^{1}H NMR.

3.2.5. Synthesis of 3, 3', 4, 4' – tetra – O – ethylellagic acid (6)

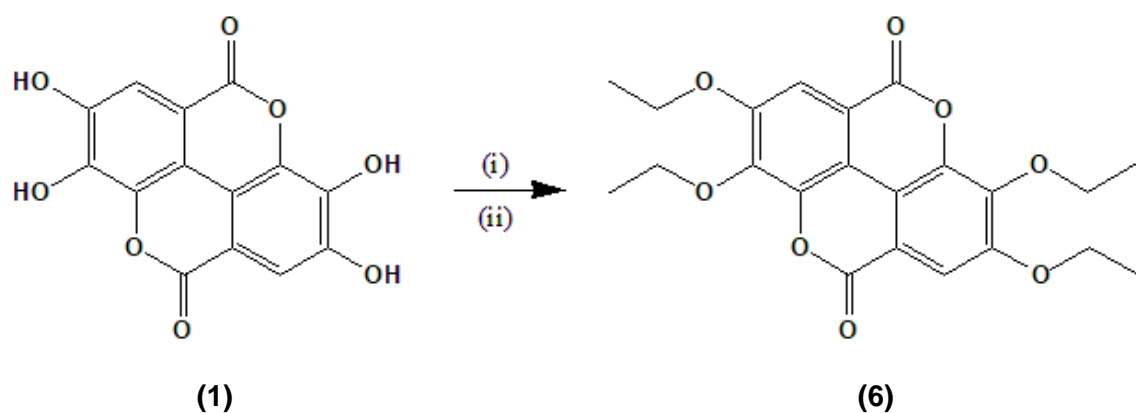**(1)**   **(6)**

Scheme 5:

Reagents and conditions: (i) Cs_2CO_3, DMF, RT, 30 min; (ii) diethylsulfate, 110 °C, 6 hrs.

Compound **6** was synthesized after adaptation of the method of White.[41] A suspension of ellagic acid (0,2 g; 0,662 mmol), and cesium carbonate (1,73 g; 5,3 mmol) in dimethylformamide (4 ml) was stirred at room temperature. After 30 minutes of stirring, diethylsulfate (0,7 ml; 5,3 mmol) was added and the mixture was heated at 110 ºC during 6 hours. Litium chloride (10 ml) was added and the mixture was extracted 3 times with chloroform. Brine and magnesium sulfate were added to wash and dry the mixture, respectively, and the filtration and evaporation in vacuum were realized. The recrystallization with ether and hexane was tried, unsuccessfully. So the product was collected by filtration and identified by ^{1}H NMR.

3.2.6. Synthesis of 3, 3', 4, 4' – Tetrakis – O – triisopropylsilylellagic acid (7)

(1) **(7)**
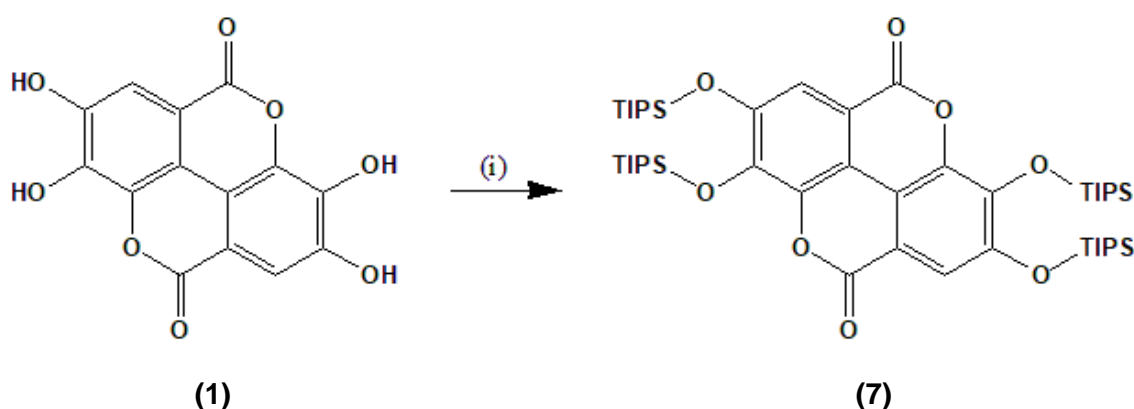
Scheme 6:

Reagents and conditions: (i) imidazole, DMAP, DMF, DCM, TIPSCl, 50 ºC, 12 hrs.

Compound **7** was synthesized according to the method of Kobayashi et al. (2013).[42] Ellagic acid (1 g; 3,31 mmol), imidazole (1,73 g; 5,3 mmol) and 4-Dimethylaminopyridine (0,12 g; 0,094 mmol) were added into a flask. Using syringes, dry dimethylformamide (1,375 ml), dry dichloromethane (10,375 ml) and triisopropylsilyl chloride (3,55 ml; 16,62 mmol) were added, while the mixture was under stirring. The suspension was stirred at 50 ºC during 12 hours. Saturated aqueous ammonium chloride (10 ml) was added and the mixture was extracted 3 times with chloroform. Brine and magnesium sulfate were added to wash and dry the organic layer, respectively, and the filtration and evaporation in vacuum were realized. The product was recrystallized from isopropyl alcohol and the filtrate was collected by filtration. The

identification of the product was made by ^{1}H NMR. ^{1}H-NMR δ_H (500 MHz, CDCl$_3$): 1.13 (36H, d, J = 7.7 Hz, CH_3), 1.14 (36H, d, J = 7.6 Hz, CH_3), 1.40 [6H, hept, J = 7.7 Hz, Si-CH-(CH$_3$)$_2$], 1.56 [6H, hept, J = 7.6 Hz, Si-CH-(CH$_3$)$_2$], 7.61 (2H, s, Ar-H).

3.2.7. Synthesis of 3, 3' – Bis – O – methoxyethoxymethyl – 4, 4' – Bis – O – triisopropylsilylellagic acid (8)

(7)   **(8)**
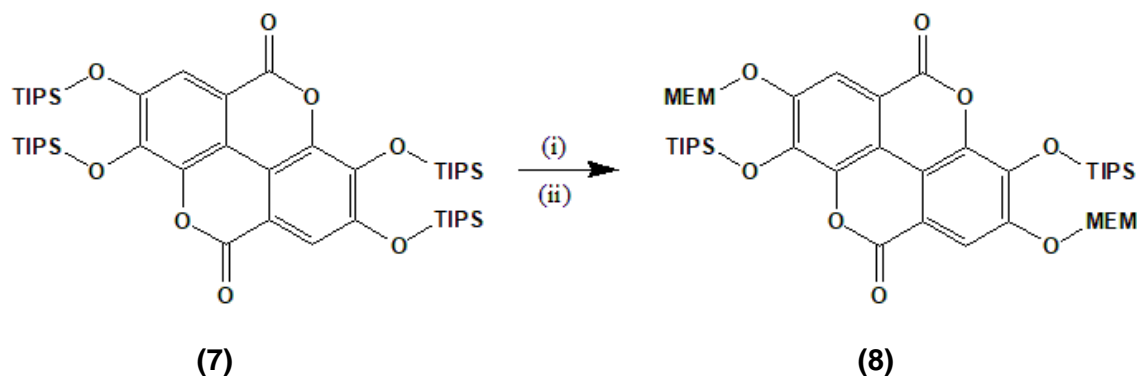
Scheme 7:

Reagents and conditions: (i) Molecular sieves 4A, DCM, TBAF, RT, 10 min; (ii) Cs$_2$CO$_3$, MEMCl, -50 ºC, 4 hrs.

Compound **8** was synthesized according to the method of Kobayashi et al. (2013).[42] Compound **7** (0,4 g; 0,43 mmol), and 800 mg of molecular sieves 4A were added into a flask. Using syringes, dry dichloromethane (8 ml) and tetra-n-butylammonium fluoride (0,85 ml) were added and the mixture was under stirring at room temperature. After 10 minutes, cesium carbonate (0,42 g; 1,3 mmol) and 2-methoxyethoxymethyl chloride (0,154 ml; 1,3 mmol) were added and the mixture was stirred at -50 ºC during 4 hours. Saturated aqueous ammonium chloride (10 ml) was added to adjust the pH for 5-6. The molecular sieves and the insoluble material were removed by filtration and the mixture was extracted 3 times with chloroform. Brine and magnesium sulfate were added to wash and dry the organic layer, respectively, and the filtration and evaporation in vacuum were realized. The product was filtrated and concentrated in vacuum, and the identification was made by ^{1}H NMR.

3.2.8. Synthesis of 3, 3' – Bis – O – 2-iodoethyl – 4, 4' – Bis – O – triisopropylsilylellagic acid (9)

(7)   (9)

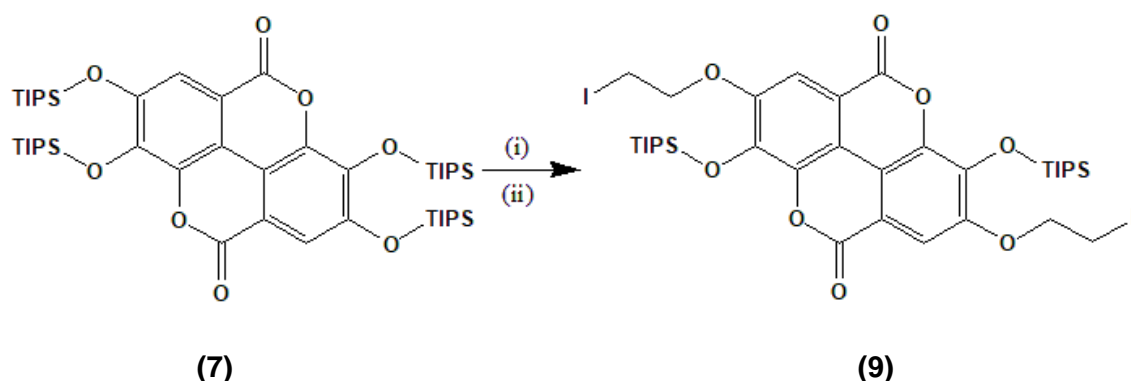**Scheme 8:**

Reagents and conditions: (i) Molecular sieves 4A, DCM, TBAF, RT, 10 min; (ii) Cs_2CO_3, iodoethane, -50 ºC, 4 hrs.

Compound **9** was synthesized according to the method of Kobayashi et al. (2013).[42] Compound **7** (0,4 g; 0,43 mmol), and 800 mg of molecular sieves 4A were added into a flask. Using syringes, dry dichloromethane (8 ml) and tetra-n-butylammonium fluoride (0,85 ml) were added, and the mixture was under stirring at room temperature. After 10 minutes, cesium carbonate (0,42 g; 1,3 mmol) and iodoethane (0,105 ml; 1,3 mmol) were added and the mixture was stirred at -50 ºC during 4 hours. Saturated aqueous ammonium chloride (10 ml) was added to adjust the pH for 5-6. The molecular sieves and the insoluble material were removed by filtration and the mixture was extracted 3 times with chloroform. Brine and magnesium sulfate were added to wash and dry the organic layer, respectively, and the filtration and evaporation in vacuum were realized. The product was filtrated and concentrated in vacuum. The final product was confirmed by ^{1}H NMR.

4. Discussion

Ellagic acid is a molecule highly insoluble in almost all solvents and to improve its bioavailability is imperative to increase its solubility. This very low solubility could be explained through the flatness of the molecule which leads to the creation of a flat stack of molecules with intermolecular bounds very difficult to separate.

During this work, the attempts to synthesize ellagic acid's derivatives with improved pharmacokinetic parameters proved to be a difficult process. Due to several obstacles found in the performed approaches, it became impossible to assemble a robust set of compounds to perform the assessment *in vitro* of the antiplasmodial activity.

The follow-up of the reactions has shown to be truly difficult to achieve, mainly due to two important facts about the ellagic acid and its derivated compounds: i) the low solubility in several solvents; ii) the rapid oxidation of these compounds. Therefore, the mentioned factors were preponderant in the impossibility of following the synthesis by TLC.

Another key issue was the particular difficulty to obtain the final product purified, sometimes due to the impossibility to remove certain solvents such as DMF. On the other hand, there is a possibility that the desired final compound that have been lost during various extraction processes with some solvents such chloroform or methanol.

About the identification by ^{1}H NMR of the synthesised products, there were also some obstacles created by special characteristics of the compounds. Firstly, the low solubility of the compounds observed in water and in other solvents made it difficult to identify them. The ellagic acid and its derivatives have a few number of protons which was also an obstacle to understand the compounds presented in NMR analysis, as well as the possible presence of other compounds and impurities.

In general, the approaches to synthesize and identify these derivatives of ellagic acid, reveals to be a very difficult task. The synthesis and purification to obtain derivatives **(2)**, **(3)**, **(4)**, **(5)**, **(6)**, **(8)** and **(9)** was unsuccessfully performed based on all the features previously mentioned.

The attempts used to produce the compounds **(2)**, **(3)** and **(4)** revealed a problem in the purification of the desired product. It was difficult to follow the reaction by TLC and when the identification by NMR was done it was impossible to detect the final product, probably due to the presence of other components and impurities.

The reactional scheme of the syntheses using DMF as main solvent - attempts to synthesize the compounds **(5)** and **(6)** - showed a huge difficulty to remove it completely and to obtain the final product purified as intended.

The synthesis of the product **(7)** was made successfully, with a yield of 77 %. The processes of purification and identification occurred as expected, and it was possible to identify clearly the desired compound by ^{1}H-NMR as showed in Figure 3.
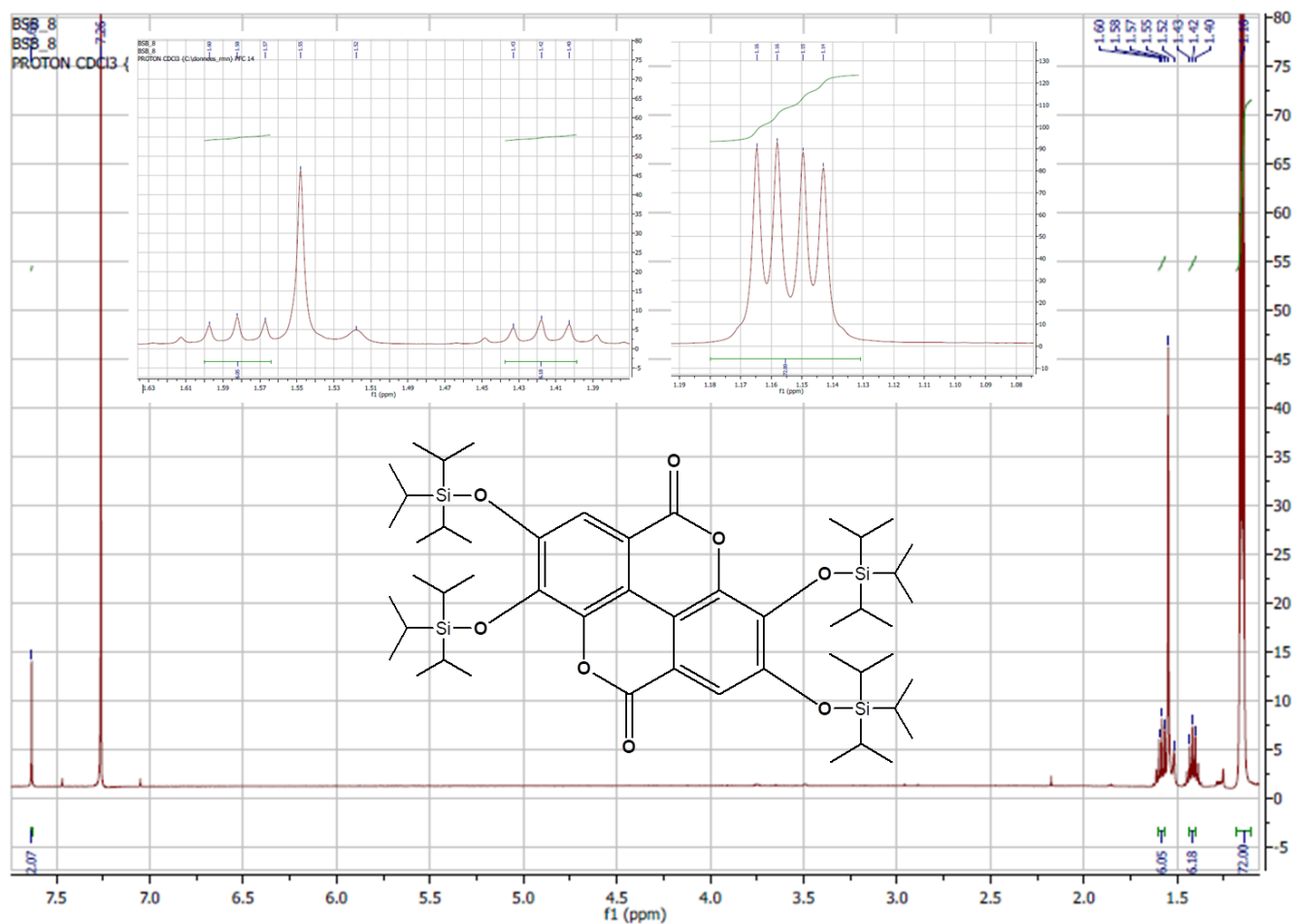
Figure 3 - [1]**H-NMR spectra of 3, 3', 4, 4' – Tetrakis – O – triisopropylsilylellagic acid, compound 7**

Regarding ^{1}H-NMR spectra (Figure 3), aromatic signals from phenyl rings at a downfield shift, corresponding to 2 protons, appeared as a singlet with a δ_H value of 7.61 ppm. The assignment of the chemical shifts on the aliphatic region reveals 72 protons, corresponding to 24 methyl groups that appeared as two doublets (two times 36 protons) with δ_H values of 1.14 ppm and 1.16 ppm. In the same region a signal corresponding to 12 protons, appeared as two heptets (two times 6 protons) with δ_H

values of 1.42 ppm and 1.58 ppm. This signal is due to the CH of the isopropyl group, which is coupled with two methyl groups.

Finally, compound **(7)** was used as intermediate to produce new derivatives substituted with TIPS in positions 4 and 4' and MEM for compound **(8)** and iodoethyl for compound **(9)** in positions 3 and 3'. Again, it was impossible to obtain the desired compounds. It would be interesting to try different approaches using other reactants and conditions in order to obtain these compounds properly purified.

Ideally, the assessment of antimalarial activity was planned according to the method developed by Makler (43). It would consist in the measure of the parasite lactate dehydrogenase (pLDH) activity, in order to determine parasitemia. Chloroquine and artemisinin would be used as positive controls and the medium as a negative control. In presence of lactate dehydrogenase, a tetrazolium salt added to the medium would be reduced to blue formazan, which could be measured through the coloration. Finally, the absorbance would be measured at 630 nm allowing estimation of parasitemia. As previously mentioned, once we could not obtain the desired compounds, it became impossible to perform this assessment.

5. Conclusion

During the 21st century, despite the great scientific and technological advances achieved, there are still some threats to human life and malaria continues to appear as one of the biggest health worldwide current problems. World Health Organization estimates that 3.2 billion people live in areas at risk of malaria transmission in 95 countries and territories, and aims to eradicate this disease by 2030. However, to achieve this milestone, it is strictly necessary to discover new drugs and improve the therapies currently used. It is also important to clarify the mechanism of action of the current antiplasmodial compounds as well as discover the mechanism of resistance to these drugs. For five times, the Nobel Prize in Physiology or Medicine has been awarded for work associated with malaria: to Sir Ronald Ross (1902), Charles Louis Alphonse Laveran (1907), Julius Wagner-Jauregg (1927), Paul Hermann Müller (1948), and Youyou Tu (2015). This curious fact highlights the importance of this serious and potentially fatal disease.

Two important currently used antimalarial drugs are derived from plants whose medicinal values had been noted for centuries: artemisinin from the Qinghaosu plant (*Artemisia annua*, China, 4th century) and quinine from the cinchona tree (*Cinchona spp.*, South America, 17th century). Ellagic acid, a natural polyphenol compound found in numerous fruits and vegetables, is a molecule with great cytotoxic and antitumour potential, as well as immunomodulatory, antimicrobial, antifungal and antiplasmodial effects. To date, it is not possible to use it orally as a result of its almost total insolubility in water and other solvents. This insolubility is translated *in vivo* by a reduced bioavailability.

During the research work, it has been attempted to increase this solubility and therefore the bioavailability of ellagic acid *in vivo*. The main objective of this project was to gather a set of compounds derived from the parent molecule, the ellagic acid, with improved pharmacokinetic parameters and to evaluate their antiplasmodial activity *in vitro*. Unfortunately, due to several reasons, it was impossible to accomplish those objectives. Nevertheless, previous studies proved the efficacy *in vitro* and *in vivo* of the ellagic acid and it is important to continue working with this molecule. Therefore, further studies need to be conducted in order to become possible the oral administration of this natural compound, not only by structural alterations but also through other strategies, such as the incorporation of the molecule in micellar vehicles.

6. References

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