BIOASSAY-GUIDED PROFILING OF *Quercus infectoria* GALL EXTRACTS USING HPLC AND THEIR ANTIMALARIAL ACTIVITY

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by

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LIST OF SYMBOLS

%	percent
^{o}C	degree Celsius
=	equal
±	plus minus
<	less than
≤	less than or equal to
>	more than
≥	more than or equal to

LIST OF ABBREVIATIONS

× g	gravitational force
µM	micromolar
µg/mL	microgram per milliliter
µL	microliter
cm	centimeter
dH_2O	distilled water
e.g.	for example
g	gram
i.e.	that is
mA	milliampere
mg/kg	milligram per kilogram
mg/L	milligram per liter
mL	milliliter
mM	millimolar
n	number of subjects
nm	nanometer
nM	nanomolar
ppm	part per million
pH	potential of hydrogen
pK_a	acid dissociation constant
pLDH	parasite lactate dehydrogenase
v/v	volume per volume
w/v	weight per volume
ACTs	artemisinin-based combination therapies
ANOVA	analysis of variance

CO_2	carbon dioxide
CCM	complete cell culture medium
Dfd	degree of freedom denominator
DFn	degree of freedom numerator
DMSO	dimethyl sulfoxide
HCl	hydrogen chloride/ hydrochloric acid
HEPES	hydroxyethyl piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
HR-LCMS	high resolution liquid chromatography mass spectrometry
IC_{50}	half-maximal inhibitory concentration
ICCM	incomplete cell culture medium
ICH	International Conference on Harmonization
INFORMM	Institute for Research in Molecular Medicine
IIUM	International Islamic University Malaysia
ISO	International Standard of Organisation
ITNs	insecticide-treated bed nets
KCl	potassium chloride
KOH	potassium hydroxide
NaCI	sodium chloride
NMPC	Natural Medicinal and Product Centre
MSF	malarial SYBR Green 1 fluorescence-based
NaH_2PO_4	sodium dihydrogen phosphate monohydrate
prep-HPLC	preparative high-performance liquid chromatography
QI	*Quercus infectoria*
QIA	*Quercus infectoria* acetone extract
QIM	*Quercus infectoria* methanol extract
R^2	correlation coefficient

RPMI	Rosewell Park Memorial Institute
USM	Universiti Sains Malaysia
WHO	World Health Organization

LIST OF APPENDICES

PEMPROFILAN BIOASAI TERARAH EKSTRAK BIJI *Quercus infectoria* MENGGUNAKAN HPLC DAN AKTIVITI ANTIMALARIA

ABSTRAK

Malaria merupakan isu kesihatan awam yang membimbangkan kerana kemunculan parasit malaria yang rintang terhadap ubat menyebabkan morbiditi dan mortaliti yang signifikan setiap tahun. Penemuan ubat antimalaria daripada tumbuhan ubatan dipercayai merupakan strategi penting untuk menangani krisis rintangan ubat antimalaria. Oleh itu, sifat-sifat antimalaria dalam ekstrak-ekstrak mentah daripada biji *Quercus infectoria* (QI) telah dikaji menggunakan fraksinasi berpandukan bioasai. Ekstrak mentah aseton (QIA) dan metanol (QIM) telah dilaporkan mempunyai aktiviti antimalaria terhadap *Plasmodium falciparum* (strain 3D7) dengan nilai nilai IC_{50} 5.85 ± 1.64 µg/mL and 10.31 ± 1.90 µg/mL. Ekstrak-ekstrak ini melalui fraksinasi menggunakan persediaan automatik kromatografi cecair berprestasi tinggi (prep-HPLC) untuk mengenal pasti fraksi yang paling aktif. Sembilan fraksi telah diasingkan daripada setiap ekstrak di mana fraksi QIA6 dan QIM6 masing-masing menunjukkan aktiviti antimalaria dengan nilai IC_{50} 17.65 ± 1.82 µg / mL dan 24.21 ± 1.88 µg / mL. Dibandingkan dengan ubat antimalaria standard, artemisinin mempunyai nilai IC_{50} = 0.004 ± 0.001 µg / mL. Semua fraksi biji *Quercus infectoria* menunjukkan aktiviti antimalaria yang boleh dikaitkan dengan kehadiran pelbagai metabolit sekunder, terutamanya sebatian fenolik seperti asid ellagik. Kaedah analisis HPLC telah digunakan bagi kuantifikasi asid ellagik sebagai sebatian penanda dalam ekstrak mentah daripada biji *Quercus infectoria*. Semua parameter termasuk spesifikasi, ketepatan, akurasi, lineariti, had pengesanan (LOD) dan had kuantiti (LOQ), telah

menepati kriteria yang ditetapkan oleh garis panduan Majlis Pengharmonian Antarabangsa (ICH). Analisis sebatian fenolik yang disasarkan daripada fraksi yang paling aktif dilakukan dengan kromatografi cecair resolusi tinggi digabungkan dengan spektrometri jisim (HR-LCMS). Analisis HR-LCMS dijalankan bagi fraksi yang paling aktif, QIA6 dan QIM6, dan mendapati bahawa kaempferol-3-O-glukosida, kuersetin-3-glukosida dan asid siringik merupakan antara sebatian fenolik dengan jumlah tertinggi yang diperolehi dalam fraksi QIA6 manakala asid siringik dan 3,4-dihidroksibenzoik dalam fraksi QIM6. Korelasi antara aktiviti antimalaria dan sebatian fenolik dalam fraksi QIA6 dan QIM6 membawa kepada pengiraan kuantitatif empat sebatian fenolik yang disasarkan. Oleh itu, kajian ini menunjukkan aktiviti antimalaria biji *Quercus infectoria* (QI) yang memberangsangkan apabila fraksinasi dilakukan, dimana ia boleh digunakan sebagai garis panduan untuk penyelidikan masa hadapan mengenai mekanisme molekul yang mendasarkan tindakan antimalaria dan seterusnya mencerminkan kepentingan penyelidikan antimalaria yang lebih mendalam.

BIOASSSAY-GUIDED PROFILING OF *Quercus infectoria* GALL EXTRACTS USING HPLC AND THEIR ANTIMALARIAL ACTIVITY

ABSTRACT

Malaria is a public health concern as the emergence of drug-resistant malaria parasites causes significant morbidity and mortality annually. The discovery of potent antimalarial drugs derived from medicinal plants is believed to be a crucial strategy for addressing the antimalarial drug resistance crisis. Therefore, the antimalarial properties of crude extracts from *Quercus infectoria* (QI) galls were investigated through bioassay-guided fractionation. Acetone (QIA) and methanol (QIM) crude extracts have been reported to exhibit promising antimalarial activity against *Plasmodium falciparum* (3D7 strain) with IC_{50} values of 5.85 ± 1.64 µg/mL and 10.31 ± 1.90 µg/mL, respectively. These extracts were subjected to fractionation using automated preparative high-performance liquid chromatography (prep-HPLC) to identify the most active fractions. Nine fractions were separated from each extract, of which the fractions QIA6 and QIM6 showed potent antimalarial activity, with IC_{50} values of 17.65 ± 1.82 µg/mL and 24.21 ± 1.88 µg/mL, respectively. In comparison, the standard antimalarial drug artemisinin had an IC_{50} value of 0.004 ± 0.001 µg/mL. The fractions of the *Quercus infectoria* galls exhibited antimalarial activity, which could be attributed to the presence of various secondary metabolites, particularly phenolic compounds such as ellagic acid. The high-performance liquid chromatography (HPLC) analytical method was established for the quantification of ellagic acid as a marker in the *Quercus infectoria* gall crude extract. All parameters including specificity, precision, accuracy, linearity, limit of detection (LOD) and limit of quantitation (LOQ), were found to be in the acceptable criteria of the ICH guideline.

Targeted phenolic compound analysis of the most active fraction was performed by high-resolution liquid chromatography coupled with mass spectrometry (HR-LCMS). HR-LCMS analysis was conducted on the active fractions, QIA6 and QIM6, and revealed that kaempferol-3-*O*-glucoside, quercetin-3-glucoside, and syringic acid were among the major compounds identified in QIA6, while syringic acid and 3,4-dihydroxybenzoic acid were predominant in QIM6. The correlation between antimalarial activity and phenolic compounds in fractions QIA6 and QIM6 led to the quantitation of four targeted phenolic compounds. Thus, this study showed promising antimalarial activity of *Quercus infectoria* (QI) galls when fractionation was performed, which can be used as a guideline for future investigations on the molecular mechanism underlying the antimalarial action and further reflect the importance of an in-depth antimalarial investigation.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Human malaria parasites caused an estimated 241 million cases of malaria in 2020 as compared with 227 million cases in 2019 with most of the cases reported from countries in the World Health Organization (WHO) African Region (WHO, 2021). Approximately half a million people died from the disease since year 2000 until 2019 that was predominantly caused by *Plasmodium falciparum* and mainly affected children under 5 years old (WHO, 2021). The standard treatments for malaria including artemisinin and its derivatives are often administered together with partner drugs in artemisinin-based combination therapies (ACTs) (World Malaria Report 2018, 2018; He *et al*., 2019). Although the ACTs are still working, recent findings have confirmed that *P. falciparum* in Southeast Asia and Africa has developed partial resistance to the key family of antimalarial drugs (Slater *et al.*, 2021). This necessitates a search for new antimalarial drugs from other sources particularly from medicinal plants.

Quercus infectoria Olivier (Fagaceae) is a small-sized shrub primarily distributed in Greece, Asia, Syria and Iran (Morales, 2021) The tree produces galls that arise on its branches due to the attack by a gall wasp, *Cypnis gallae-tincotoriae* (Samuelson *et al*., 2017). For centuries, the galls derived from *Q. infectoria* (QI) have been employed in traditional medicine for their properties as astringent, local anaesthetic, antidiabetic, antitremorine, and anti-inflammatory agents (Galla, 1911; Chopra *et al*., 1956; Dar *et al.*, 1976; Dar & Ikram, 1979; Hwang *et l.*, 2000). Besides

having antibacterial, antiviral, and antifungal properties (Hussein *et al.*, 2000; Baharuddin *et al.*, 2015; Mustafa *et al.*, 2018), the galls have been demonstrated to possess antiparasitic activities against *Entamoeba histolytica*, *Blastocystis hominis*, *Leishmania major* and *P. falciparum* (Sawangjaroen *et al.*, 2004; Sawangjaroen & Sawangjaroen, 2005; Ozbilgin *et al.*, 2013; Kheirandish *et al.*, 2016; Nik Mat Zin *et al.*, 2021).

Phenolic compounds and their derivatives are a group of phytochemical compounds discovered in virtually every plant including QI (Rahman *et al.*, 2015). The phytochemical studies have reported the presence of tannic acid, gallic acid, syringic acid, ellagic acid, amentoflavone hexamethyl ether, methyl oleonate, hexagalloyl-glucose and polygalloyl-glucose in QI gall extracts (Ikram *et al*., 1977; Hwang *et al.*, 2000; Shrestha *et al*., 2014). Despite their wide presence and recognised value for health purposes, there is insufficient research to assess the effects of phenolic compounds isolated from *Q. infectoria* gall extracts on malaria parasites (Tajuddeen *et al*., 2019; Mamede *et al.*, 2020).

Ellagic acid is one of the phenolic compounds abundantly in QI galls (Rahman *et al.*, 2015; Abdullah *et al.*, 2018), which might be the major compound responsible for the antimalarial activity of the plant (Aldulaimi *et al.*, 2017; Dell'Agli *et al.*, 2003). Previous studies on the antimalarial activity of ellagic acid showed that this compound could inhibit beta-haematin (haemozoin) formation (Dell'Agli *et al.*, 2003; Simões-Pires *et al.*, 2009; Soh *et al.*, 2009) and alter pH of the acidic digestive vacuole of the mature stage parasites (Nik Mat Zin *et al.*, 2021; Muchtar *et al.*, 2022).

1.2 Problem statement

The problem of antimalarial drug resistance is not only obscured the treatment of malaria, but also has caused difficulties for the global effort to eliminate the disease (Antony *et al.*, 2016; WHO, 2021). Although the quest for the development of malaria vaccines continues, none of them is readily available and licensed to be employed to treat single drug-resistant and multidrug-resistant malaria parasites (WHO, 2021). In addition, most populations affected by malaria are in poor and developing countries that have a little access to modern medicine and vaccine, and thus turn towards the use of medicinal plants for maintaining their health. (WHO, 2021).

The discovery of potent antimalarial drugs from medicinal plants such as QI is believed as a major approach to tackle the crisis of antimalarial drug resistance. Therefore, this is the first study that searches for the potential phenolic compounds from the acetone and methanol extracts of QI galls that exhibit a promising antimalarial activity against *P. falciparum*. The existence of phenolic compounds such as ellagic acid in the acetone and methanol extracts of QI galls might offer an alternative and more effective treatment against drug-sensitive and drug-resistant strains of the malaria parasites. The outcomes could provide possible explanations on the antimalarial effect of the gall extracts, which can be used as the guideline for future investigation on the molecular mechanism underlying the antimalarial action and further reflect the importance of the in-depth antimalarial investigation.

1.3 Objectives of the study

1.3.1 General objective

The study aimed to determine the antimalarial constituents from the acetone and methanol extracts of QI galls.

1.3.2 Specific objectives

i. To determine the phenolic profiles of the acetone and methanol extracts of QI galls by using high performance liquid chromatography (HPLC).

ii. To fractionate the acetone and methanol extracts of QI galls by using preparative high performance liquid chromatography (Prep-HPLC).

iii. To determine the antimalarial activity of the fractions by using malarial SYBR Green 1 fluorescence-based (MSF) assay.

iv. To identify the potential phenolic compounds from the most potent fractions by using high resolution liquid chromatography mass spectrometry (HR-LCMS).

1.4 Experimental design

Figure 1.1 summarises the overall flow of the study starting with the extraction of the QI galls by using two solvents (i.e. acetone and methanol)

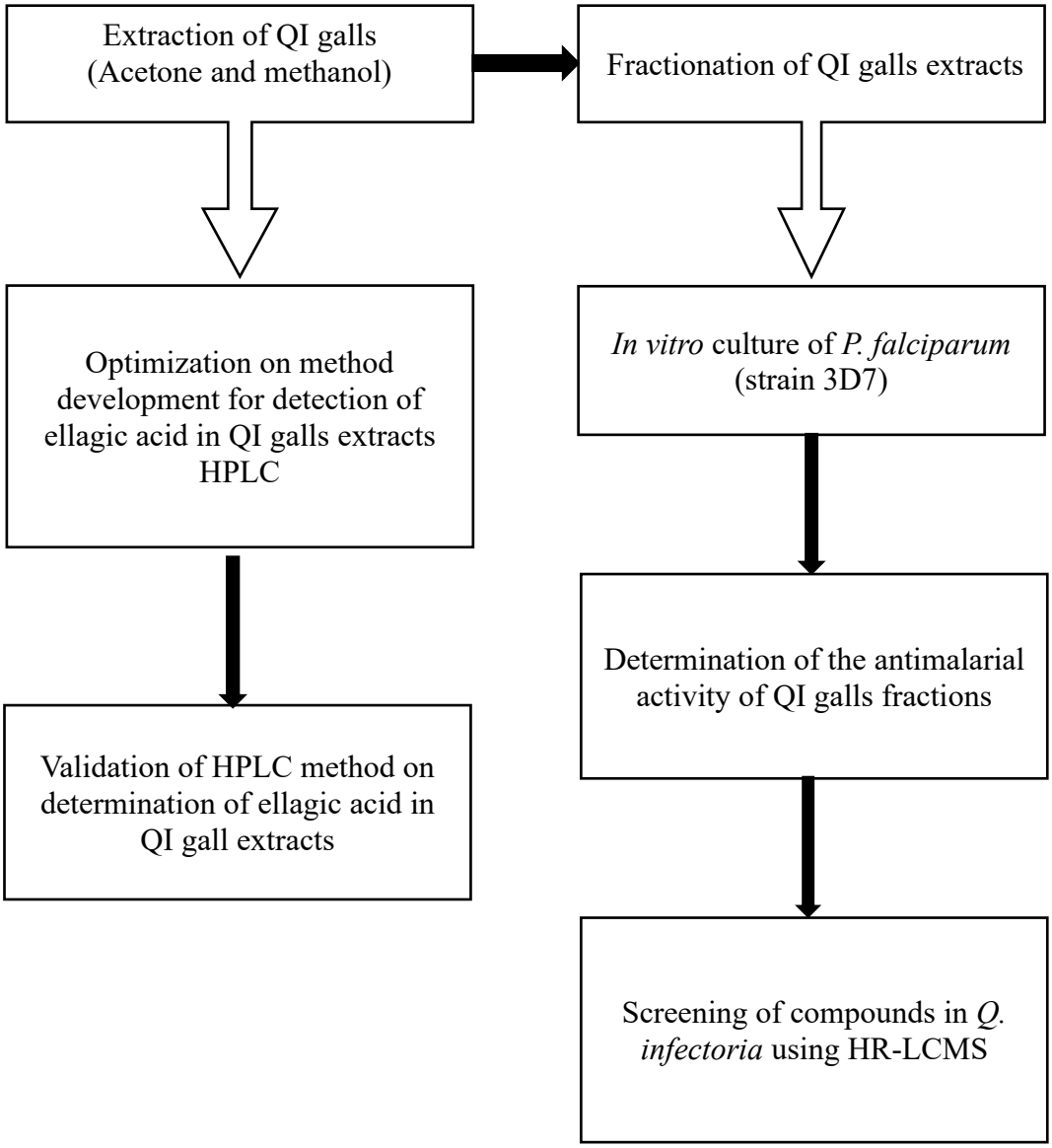


Figure 1.1: Flowchart of the experiments carried out through all the study

with different polarities to produce two different crude extracts. The extracts were analysed by using HPLC to determine their phenolic profiles. Chromatographic separation was performed by using a gradient elution on a C18 (250 mm × 4.6 mm, 5 µm) analytical column and maintained at 30°C. Injection volume was 5 µL where the flow rate was set at 0.8 mL/min. Mobile phase consisting of 0.1% trifluoracetic acid, methanol and 0.1% trifluoracetic acid: acetonitrile was applied gradient. Ellagic acid was used as the standard to compare with the QI extracts and fractions.

The QI gall extracts were further fractionated by using Prep-HPLC. The extracts were dissolved by using methanol and acetone, respectively, and filtered by membrane filter (0.22 µm) before 100 µL of the extracts were injected into preparative-HPLC system. Separation was conducted by using reverse phase C18 preparative column (10 mm × 250 mm, 5 µm particle size) and solvent A (100 % acetonitrile) and solvent B (0.1 % trifluoroacetic acid in deionised water) as mobile phase. The isolation was conducted with flow rate of 3.8 mL/min and detection wavelength was monitored at 254 nm. The fractions eluted by preparative HPLC were collected and dried under vacuum below 45°C.

The antimalarial activities of the QI gall fractions were determined by using malarial SYBR Green I-based fluorescence (MSF) assay. Artemisinin and ellagic acid were used as control drugs of the assay. In this standard 48-hour drug sensitivity assay, 96-well plates containing triplicate of two-fold serial dilutions of tested samples in complete culture medium were prepared before being added into plates containing synchronised cultures of ring stage parasites (2% haematocrit, 2% parasitaemia). The parasites were incubated at 37°C for 48 hours. Samples were

collected for assessment of parasite growth and parasitaemia by adding 20 µL of 20× SYBR Green I solution. After 60 minutes incubation, the fluorescence signal was measured by using excitation and emission wavelengths of 490 and 530 nm, respectively by a microplate reader. The inhibitory concentration of the gall fractions that kills 50% of the parasite population (IC_{50}) was analysed.

The high-resolution liquid chromatography coupled with mass spectrometry (HR-LCMS) was used for analysis of compounds in the QI gall fractions. The column used was HSS XSelect Waters C18 (4.6 × 250 mm, 5µm). The gradient was eluted with (A) consist of acetonitrile:water:formic acid in ratio of 5:95:3 (v/v/v) and acetonitrile (B) buffered with 0.1% formic acid, starting at 5% B and equilibrated for 5 minutes. The gradient was increased to 95% in 45 minutes and held for 5 minutes. Gradient was equilibrated for 5 minutes prior to next injection. The flow rate was 0.8 mL/min and the column temperature was maintained at 35°C. Sample injection was 3 µL. MS detection was performed in positive and negative atmospheric pressure ionization-electrospray source mode. The drying gas temperature was 250°C with gas flow of 11 L/min. Nebulizer pressure monitored at 110 psig, while nebulizer assistant gas temperature at 350°C. Capillary voltage sustained at 400V, with collision energy of 30 to 50 eV.

Identified compounds were elucidated by using Thermo Fisher Mass Frontier and the Compound Discoverer 3.1. This software incorporates the mzCloud search feature, offer an online fragmentation library comprising meticulously curated MS/MS and MSn spectra generated from various collision types and energies. Additionally, it includes tools such as predicted compositions derived from high-

resolution full mass data and ChemSpider search to aid in the partial identification of compounds. To further validate hits obtained from ChemSpider against MS2 data, the built-in FISh scoring system was employed.

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of malaria

Malaria is a parasitic disease that has a negative impact on global health (Ferguson, 2018). The disease causes major complications leading to high morbidity and mortality in many developing countries (Nigussie & Wale, 2022). Five *Plasmodium* species responsible for human malaria are *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* (Nigussie & Wale, 2022; Nosten *et al.*, 2022). *P. falciparum* is the most threatening species in terms of morbidity and mortality (Berg *et al.*, 2021).

2.1.1 History of malaria

Malaria outbreak was documented to have occurred since the dawn of civilisation (Moss *et al.,* 2008). It was even reported to be the cause of major military defeats as well as the collapse of some nations (Lehrer & Pavličić, 1981). Although malaria and its symptoms were common to the ancient people, the fever that stroke patients was believed to be attributed to supernatural forces and angry divinities (Dugački, 2005). In the fourth century BC, Hippocrates linked malaria with evaporation from swamps that caused the disease when inhaled. The belief was held until 1880 before Charles Louis Alphonse Laveran, a French military surgeon discovered the cause of the disease (Talapko *et al.,* 2019).

2.1.2 Statistics of malaria

According to the World Health Organization (WHO), around 247 million malaria instances were documented worldwide in 2021, in contrast to 245 million cases in 2020.(WHO, 2022). This resulted in 619 000 deaths of which the WHO African Region accounted for the majority of malaria cases (95%) and deaths (96%), and children under five years of age (80%) were mainly affected by the disease (Figure 2.1). In contrast to human malaria, the spread of zoonotic malaria infections caused by *P. knowlesi* in Malaysia has frequently been reported (Solhi, 2022). Malaysia recorded zero human malaria infections for four consecutive years between 2018 and 2021.

Figure 2.1: Countries with indigenous cases in 2000 and their status by 2021

Countries that remain free of indigenous malaria cases for a minimum of three consecutive years are classified as malaria-free. In 2021, the Islamic Republic of Iran and Malaysia recorded zero indigenous cases for the fourth consecutive year, whereas Belize and Cabo Verde reported zero cases for the third consecutive year. China and El

Salvador attained malaria-free status in 2021, following four years without any reported cases. Modified from WHO , World Malaria Report (2022).

2.2 Life cycle of the malaria parasite

The asexual and sexual cycle of the malaria parasite is completed in humans and female *Anopheles* mosquitoes, respectively (Ibraheem *et al.*, 2023) (Figure 2.2).

2.2.1 Sexual cycle of the malaria parasite

A female *Anopheles* mosquito ingests gametocytes, the sexual stages of the malaria parasite at the time of human blood feeding (Figure 2.2A) (Sinden, 1983). In the midgut lumen of the mosquito, a male microgametocyte bursts from its host erythrocyte, transforming into eight flagella-like structures called microgametes through a process known as exflagellation. (Bousema & Drakeley, 2011; Leplingard *et al.*, 2003). A macrogametocyte (female) sheds the erythrocyte membrane and forms a

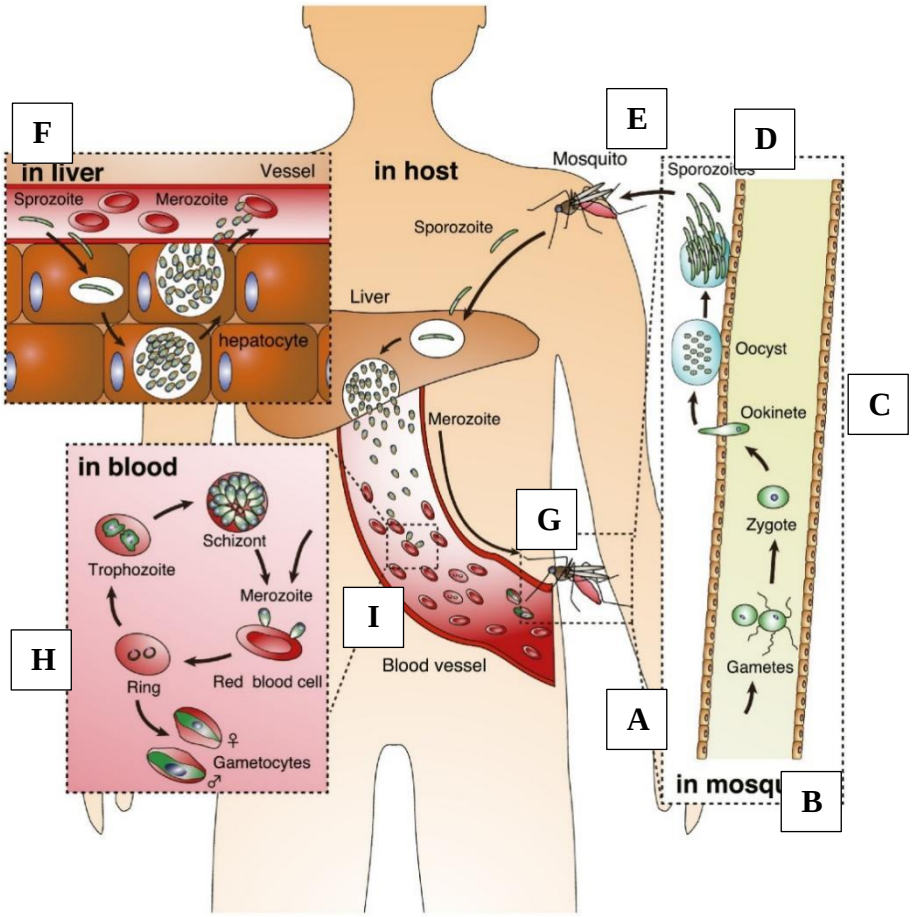


Figure 2.2: Life cycle of *P. falciparum*

(A) Female Anopheles mosquitoes ingest male and female gametocytes during blood feeding and undergo gametogenesis to generate gametes. (B) Zygotes grow into ookinetes from male and female gametes. (C) The midgut's epithelial cells are traversed by the ookinetes as they grow into oocysts. (D) Sporozoites exit mature oocysts and go to the salivary gland, where they await injection into a new human host. (E-F) After a mosquito bite, sporozoites enter the bloodstream and swiftly infiltrate hepatocytes, producing thousands of merozoites. (G-H) Infected hepatocytes produce merozoites, which infiltrate erythrocytes and grow into rings, trophozoites, and schizonts. (I) Schizont rupture releases merozoites, which enter new erythrocytes. Modified from Ibraheem *et al.* (2023).

macrogamete. The microgamete fertilises the macrogamete, forming a zygote (Figure 2.2B). The zygote transforms into a mobile ookinete with a tapering end. The ookinete passes through the midgut epithelial cell membrane and forms an oocyst (Figure 2.2C). During sporogony, the parasite transitions to a haploid state and goes through multiple rounds of mitosis. This process leads to the oocyst containing a significant number of active sporozoites. After the oocyst wall ruptures, the sporozoites are liberated and proceed to travel towards the salivary glands, where they penetrate the secretory cells. Later on, the sporozoites make their way to the salivary ducts, ready to infect another human host through a mosquito bite (Figure 2.2D).

2.2.2 Asexual cycle of the malaria parasite

An infected female *Anopheles* mosquito injects sporozoites during the blood feeding (Figure 2.2E) (Shah *et al.*, 2020). The sporozoites infect hepatocytes where they replicate to form hepatic schizonts through the process of exoerythrocytic schizogony (Figure 2.2F). Inside each schizont, thousands of merozoites are generated and released into the blood stream where they circulate and invade erythrocytes (Figure 2.2G). In the process of intraerythrocytic schizogony, the merozoite transforms into a ring stage followed by trophozoite and schizont stages (Figure 2.2H). The schizont ruptures and releases merozoites that infect new erythrocytes (Figure 2.2I). The intraerythrocytic stages of the malaria parasite are responsible for the clinical indications of the disease (Ibraheem *et al.*, 2023).

2.3 Malaria prevention tools and strategies

The implementation of successful vector control, preventative antimalarial medications, and vaccines has significantly lessened the global malaria burden (Alout *et al.*, 2014; Gachelin *et al.*, 2018; Hemingway *et al.*, 2016; Pinto *et al.*, 2021).

2.3.1 Vector control

Insecticide-treated nets (ITNs) and indoor residual spraying (IRS) are two main interventions used in vector control programmes as they are highly effective in preventing infection and reducing disease transmission (N'Guessan *et al.*, 2022). ITNs function by hindering mosquito blood feeding through both physical barriers that prevent human-mosquito contact and chemical mechanisms that deter, irritate, and kill mosquitoes. The physical barrier of ITNs prevents mosquitoes from entering, while the pyrethroid treatment induces mosquito repellence and paralysis, ultimately leading to mosquito mortality (Shah *et al.*, 2020). IRS has successfully eliminated malaria in several countries and significantly reduced disease incidence in others (Choi *et al.*, 2021).

Progress in vector control has however been threatened by emerging resistance to insecticides among the *Anopheles* mosquitoes (Berg *et al.*, 2021). The mosquitoes have been shown to have changed their behaviours, which bite early before people go to bed and rest outdoors, thereby evading exposure to insecticides (Zhang *et al*., 2020). Other threats to vector control include inadequate access and loss of nets due

to the tensions of day-to-day net replacement and lack of affordable, alternative insecticides (Malima *et al.*, 2017).

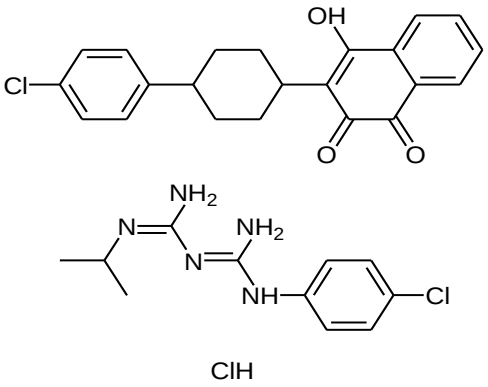
2.3.2 Preventive chemotherapy

Antimalarial drugs such as atovaquone-proguanil, doxycycline and mefloquine (Figure 2.3A-C) have been utilized alone or in combination to prevent malaria among exposed populations of newborns, children under 5 years old and pregnant women during specific periods when malaria risk is at its peak (Al Khaja & Sequeira, 2021; Blanshard & Hine, 2021; Pandey & Shingadia, 2022). This includes perennial and seasonal malaria chemoprevention, intermittent provision of malaria treatment during pregnancy and for school-aged children, post-discharge malaria chemoprevention, and large-scale drug administration (Lalloo *et al.*, 2016; Yeboah *et al.*, 2016; WHO, 2022; WHO, 2023). These strategies are intended to complement ongoing vector control programmes, rapid diagnosis of the disease, and treatment of positive cases with antimalarial drugs.

2.3.3 Vaccine

RTS,S/AS01 is the primary and currently sole malaria vaccine endorsed for utilization by the WHO (WHO, 2023). The vaccine has extended its protective benefits to over a million children in Ghana, Kenya, and Malawi through vaccination (Pandey & Shingadia, 2022). Despite significant advancements in malaria vaccine research and development, significant obstacles still exist in creating highly effective and durable vaccines against *P. falciparum.* (Nadeem & Bilal, 2023). Enhanced comprehension of

A



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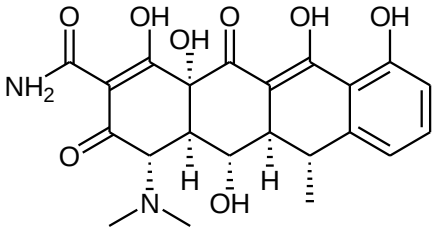

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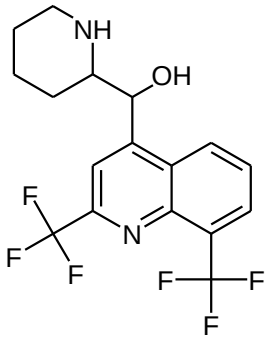


Figure 2.3: Chemical structures of (A) atovaquone-proguanil, (B) doxycycline and (C) mefloquine.

immune mechanisms and vital targets are essential, along with strategies for generating robust, enduring, and functional immunity against multiple antigens. Innovations to ease and enhance delivery of vaccines to malaria-endemic areas are also demanded (Ashley *et al.,* 2018; Olotu *et al.,* 2011; White *et al.*, 2015)

2.4 Malaria treatment with antimalarial drugs

Treatment with antimalarial drugs is given to cure the infection by ensuring quick and complete elimination of the malaria parasites from a patient's blood stream (Menard & Dondorp, 2017) It also prevents the progress of an uncomplicated malaria to severe malaria or death and minimises the transmission of the infection by reducing the parasite reservoir. Before the treatment is commenced, a suspected patient should undergo diagnosis of the disease with either microscopy or rapid diagnostic test (RDT) (Bennett *et al.,* 2017; Hemingway *et al.,* 2016; Slater *et al.,* 2021).

2.4.1 Artemisinin-based combination therapies

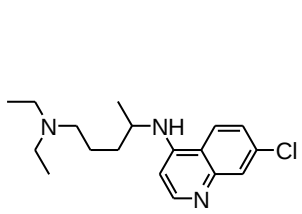
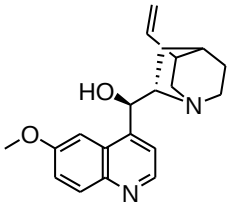
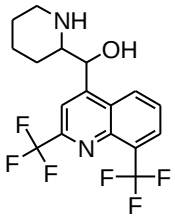
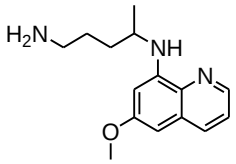
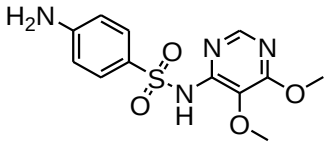
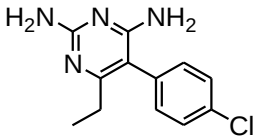
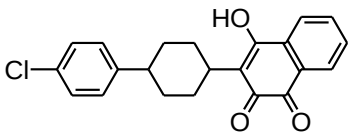
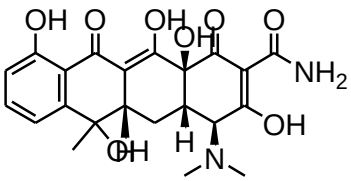
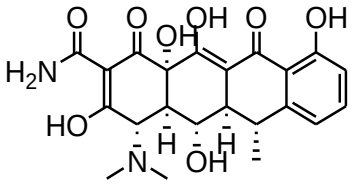
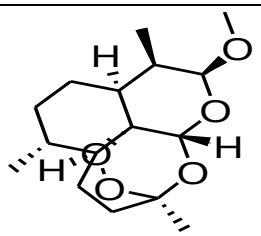
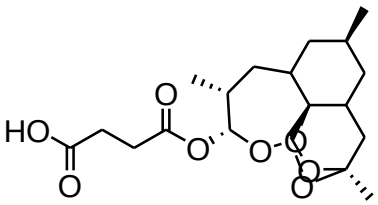
Due to the global spread of chloroquine-resistant strains of *P. falciparum*, ACTs have been recommended as the primary therapy of uncomplicated and complicated malaria (Arya *et al.*, 2021). Like other drug combination treatments, ACTs have been developed to inhibit or delay the development of drug resistance in the human population (Ashley *et al.,* 2018). Quinoline derivatives (i.e. chloroquine, quinine, mefloquine and primaquine), antifolates (i.e. sulfadoxine-pyrimethamine and atovaquone), antibiotics (i.e. tetracycline and doxycycline) and artemisinin derivatives (i.e. artemether and artesunate) have been used in ACTs (Table 2.4)

(Hanboonkunupakarn & White, 2016; Menard & Dondorp, 2017; Parhizgar, 2017). In ACTs, an artemisinin derivative rapidly reduces parasitaemia and a partner drug eliminates remaining parasites over a longer period of time.

2.4.2 Challenges in artemisinin-based combination therapies

Following the emergence of parasites resistant to chloroquine, sulfadoxine-pyrimethamine, quinine, and mefloquine, artemisinin and its derivatives-resistant parasites have surfaced in Southeast Asia (Hanboonkunupakarn & White, 2016; Woodrow & White, 2017). A prolonged P. falciparum clearance time has been observed in patients located in Thailand, Cambodia, and the neighboring countries for a significant period of time. This has raised concerns regarding the efficacy of artemisinin and its derivatives, whether used alone or in combination with other drugs (Phyo *et al.,* 2016). Artemisinin resistance may be present in *P. falciparum* mutant K13 due to its inability to bind to a transcription factor believed to be translocated to the nucleus and responsible for activating genes associated with the antioxidant response (Chhibber-Goel & Sharma, 2019). The ability of the parasites to endure the oxidative stress induced by activated artemisinin appears to be through the restoration or repair of proteins that have been damaged by oxidants (He *et al.,* 2019). In accordance with the evidence, mutant K13 binds to P13K, thereby increasing P13P levels and activity. Artemisinin induces elevated concentrations of P13P, which enables the survival of parasites (Mok *et al*., 2021).

Table 2.1: **Chemical structures of compounds used in artemisinin-based combination therapies (ACT's).**

Chemical structures
 Chloroquine  Quinine  Mefloquine  Primaquine   Quinoline derivatives
  Sulfadoxine-pyrimethamine  Atovaquone   Antifolates
 Tetracycline  Doxycycline   Antibiotics
 Artemether  Artesunate   Artemisinin derivatives

2.5 New antimalarial drug discovery from plants

Plants are an excellent source of novel natural products. For many years, medicinal plants have been used as a source of medicinal treatments and demonstrated beneficial uses in a variety of applications (Yuan *et al.*, 2016). Despite intense competition from synthetic compounds, numerous bioactive substances found in plants have significantly been important in advancing human health (Tahir *et al.*, 2022). Between 1981 and 2014, the Food and Drug Administration (FDA) approved 1 562 drugs in the United States of which 4% were unaltered natural products, 9% were botanical drugs, 21% were natural derivatives and 4% were synthetic drugs containing natural pharmacophores (Newman & Cragg, 2020). This has increased the interest in herbal medicine among people (Che & Zhang, 2019).

Modern drug discovery cannot be separated from the study of medicinal plants, which is clinically more cost-effective (Tahir *et al.*, 2022). There are numerous examples of incredibly effective contemporary drugs based on medicinal plants especially in the finding of new drug candidates for the treatment of many infectious diseases such as malaria (Atanasov *et al.*, 2015; Nakalembe *et al*., 2019). Medicinal plants have enormous potential for the effective management of various strains of malaria parasites including those resistant to available antimalarial drugs (Shah *et al.*, 2014).

2.5.1 Plants with antimalarial activity

In 1820, French scientists Pierre-Joseph Pelletier and Joseph-Bienaimé Caventou isolated quinine from the bark of *Cinchona* species (Rubiaceae) and used it as the first antimalarial drug (Dolabela *et al*., 2012). In the 1940s, chloroquine and its derivative, 4-aminoquinoline were discovered and widely used as the antimalarial drugs (Ashley *et al.*, 2018). The potency of these drugs was rapidly dropped since the 1960s, owing to the development of chloroquine-resistant *P. falciparum* strains. Mefloquine is a total synthesis of the 4-quinolinemethanol derivative and was introduced as a new antimalarial drug to treat mild to moderate malaria in 1985 (Fidock *et al.,* 2004). The current antimalarial medication of choice is artemisinin, which was discovered in the 1970s during the Chinese Cultural Revolution from the leaves of *Artemisia annua* L (Klayman, 1985). In 2015, Professor Youyou Tu was awarded the Nobel Prize in Physiology or Medicine for her significant role in the discovery of artemisinin (Su & Miller., 2015). The plant has been used for more than 2 000 years in the Chinese traditional medicine to treat malaria fever (Klayman, 1985). Although artemisinin and its derivatives have been shown to be effective against chloroquine-resistant *P. falciparum* strains, partial resistance to these drugs has been demonstrated (Li & Zhou, 2010; Tu, 2011). This increasing burden caused by artemisinin-resistant *P. falciparum* strains has encouraged efforts to discover new antimalarial drugs mainly from medicinal plants and their drug targets (Siddiqui *et al.*, 2021).

2.6 *Quercus* species

Quercus trees, also known as oaks, are members of among the most important genera in the Northern Hemisphere (Tantray *et al.*, 2018). The *Quercus* genus has undergone several reclassifications, resulting in the recognition of over 500 species spread across the globe (Denk & Grimm, 2009). The timber, bark and acorns for human and animal consumption, and leaves for medicinal and nutritional infusions, have been the most used parts of the *Quercus* trees (Costa *et al.*, 2019; Gomez *et al.*, 2017; Vinha *et al.*, 2016). Some of the oak trees such as *Q. ilex, Q. robur, Q. arizonica* and QI are valued across Europe, Asia and North America for their medicinal properties (Morales, 2021).

2.6.1 *Quercus infectoria*

Q. infectoria (QI), known as the gall oak tree (Figure 2.6A). It is a small shrub with 4-6 feet tall and primarily originated from Greece, Asia Minor and Iran (Askari *et al.*, 2020). The formation of galls of QI (Figure 2.6B) occurs when wasp species of *Adleria gallae-tinctoria* or *Cynips gallae-tinctoria* deposited their eggs on branches of young gall oak trees. Subsequent enzymatic reactions ultimately resulting the formation of hard galls (Wan Nor Amilah *et al.*, 2022). The spherical galls are identified as *majuphal* or *machakai* in India, and as *manjakani* in Indonesia and Malaysia (Samuelsson, 1999; Fatima *et al*, 2001). The galls measure between 0.8 to 2.5 cm in diameter, characterized by their firm texture, rough surface, and greyish-brown to brownish-black in colour (Figure 2.6C) (Imtiyaz *et al*, 2013).

A

B

C

Figure 2.6: **(A) The tree, (B) young galls and (C) dried galls of *Quercus infectoria***

2.6.2 Traditional uses of *Q. infectoria*

Due to its versatility, the galls of QI have become one of the most common parts of the plant used in the Uyghur traditional medicine in Central Asia to treat intestinal dysmotility, dysentery, functional enteritis, alopecia areata, dental caries, periodontitis, halitosis, pharyngolaryngitis and tympanitis (Iminjan *et al.,* 2014). The galls hold significance in Indian traditional medicine as a component of toothpowder or toothpaste to treat gum and oral cavity diseases (Khare, 2007). To effectively treat gingival diseases, the galls have been made into gels, ointments, mouthwashes and powders (Arina & Harisun, 2019). Additionally, gargling an aqueous extract of the galls can treat inflamed tonsils, and applying the galls to the skin can treat swelling or inflammation (Nigussie & Wale, 2022). The use of the gall powder as an ointment has been reported to have cured haemorrhoids caused by inflammation (Kaur *et al*., 2004). In the Malay traditional medicine, the gall extracts have been used to restore postpartum uterine elasticity and stimulate vaginal muscle contraction (Basri *et al*., 2012; Zakaria & Mohd, 2010).

2.6.3 Pharmacological activities of *Q. infectoria*

Traditional uses of QI galls have prompted researchers to explore and validate their biological activities and therapeutic benefits. Extracts, fractions and single compounds of the galls have been shown to have a variety of pharmacological activities including antioxidant, anti-inflammatory, antitumor, antibacterial, antiviral, antifungal and antiparasitic activities (Arina & Harisun, 2019; Basri *et al.*, 2012; Hwang *et al*,