THE EFFECT OF PYROGALLOL ON THE pH OF *Plasmodium falciparum* DIGESTIVE VACUOLE

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THE EFFECT OF PYROGALLOL ON THE pH OF *Plasmodium falciparum* DIGESTIVE VACUOLE

by

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Dissertation submitted in partial fulfilment of the requirements of the degree of

Master of Science (Biomedicine) Mixed Mode

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CERTIFICATE

This is to certify that the dissertation entitled "THE EFFECT OF PYROGALLOL ON THE pH OF Plasmodium falciparum DIGESTIVE VACUOLE" is the bona fide record of research work done by Mr. Alfaqih Hussain Omar during the period from 1st December 2019 to 30th September 2020 under my supervision. I have read this dissertation and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation to be submitted in partial fulfilment for the degree of Master of Science (Biomedicine).

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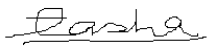

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DECLARATION

I hereby declare that this dissertation is the result of my own investigations, except where otherwise stated and duly acknowledged. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at Universiti Sains Malaysia or other institutions. I grant Universiti Sains Malaysia the right to use the dissertation for teaching, research and promotional purposes.

Alfaqih Hussain Omar

Date: 29.9.2020

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMNS

~	approximately
%	percent
°C	degree Celsius
Ɛ	molar absorptivity
±	plus minus
<	less than
≤	less than or equal to
>	more than
≥	more than or equal to
µM	micromolar
nM	nanomolar
mM	millimolar
µl	microliter
mL	mililiter
mg	milligram
g	gram
cm	centimetre
nm	nanometre

× g	gravitational force
ART	artemisinin
ACTs	artemisinin-based combination therapies
ADP	adenosine diphosphate
AMDP	aminomethylenediphosphonate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BFMP	blood film for malaria parasite
BCECF	5'(and 6')-carboxy-10- dimethylamino-3-hydroxy spiro[7H-benzo[c] xanthene-7,1'(3H) isobenzofuran]-3'-one
CCM	complete culture medium
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CDC	Centres for Disease Control and Prevention
df	dilution factor
DNA	deoxyribonucleic acid
DMSO	dimethyl sulfoxide
DV	digestive vacuole
ECM	endocytosis medium
e.g.	for example

E-64	a specific inhibitor of cysteine protease
EPM	erythrocyte plasma membrane
ES	extracellular saline
ETC	electron transport chain
FACS	fluorescence-activated cell sorting
FCS	Flow Cytometry Standard
Fe	iron
Fe(II)	ferrous iron
Fe(III)	ferric iron
FPFe(II)	ferroprotoporphyrin IX
FPFe(III)	ferriprotoporphyrin IX
FITC	fluorescein isothiocyanate
FSC	forward scatter
FITC-Dextran	fluorescein isothiocyanate–dextran
GSH	(2S)-2-Amino-4-{[(1R)-1 [(carboxymethyl)carbamoyl]-2 sulfanylethyl]carbamoyl}butanoic acid
Hb	haemoglobin
HEPES	hydroxyethyl piperazineethanesulfonic acid
HRPII	histidine-rich protein II

H_2O_2	hydrogen peroxide
H+	hydrogen ion
$IC_{50\text{-}4\ hours}$	4-hour pulse inhibition concentration 50%
ICCM	incomplete culture medium
i.e.	that is
I_g	fluorescence intensity collected at green channel
I_y	fluorescence intensity collected at yellow channel
IDP	imidodiphosphate
IL-1β	interleukin-1β
K^+	potassium ion
KCl	potassium chloride
L	lethal concentration
MACS	magnetic-activated cell sorting
MES	2-[N-morpholino] ethane sulfonic acid
MSF	malaria SYBR Green I-based fluorescence
Mg^{2+}	magnesium ion
MgCl	magnesium chloride
NaCl	sodium chloride
NaF	sodium fluoride
NaH2PO4	sodium phosphate

NLRP3	NOD-like receptor containing pyrin domain 3
NMCP	National Malaria Control Programs
n.d	no drug
PCR	polymerase chain reaction
PE	phycoerythrin
*Pf*ATP6	*Plasmodium falciparum* sacrco/endoplasmic reticulum Ca2+-ATPase
*Pf*PM4	P. falciparum digestive vacuole's aspartic protease, plasmepsin IV
pLDH	*Plasmodium* lactate dehydrogenase
pK_a	acid dissociation constant
PP_i	pyrophosphate
PPM	parasite plasma membrane
PV	parasitophorous vacuole
PVM	parasitophorous vacuolar membrane
Q. infectoria	*Quercus infectoria* plant
RDT	rapid diagnostic test
RNA	ribonucleic acid
ROS	reactive oxygen species
RPMI	Rosewell Park Memorial Institute

RPMI 1640	a growth medium used in cell culture.
R_{gy}	fluorescence ratio
SNARF	5'(and 6')-carboxy-10- dimethylamino-3-hydroxy spiro[7H-benzo[c] xanthene-7,1'(3H) isobenzofuran]-3'-one
SSC	side scatter
SERCA	sarco/endoplasmic reticulum Ca2+-ATPase
SEM	standard error of mean
S.L	sub-lethal
TCTP	translationally controlled tumor protein
TLR9	Toll-like receptor 9
TRIS	tris (hydroxymethyl) aminomethane
V-type H^+-ATPase	vacuolar-type proton-pumping ATPase
V-type H^+-PPi	vacuolar-type proton-pumping pyrophosphatase
WHO	World Health Organization
w/v	weight per volume
-ve	negative control

KESAN PYROGALLOL TERHADAP pH VAKUOL PENCERNAAN

Plasmodium falciparum

ABSTRAK

Quercus infectoria, sejenis ramuan perubatan yang sangat ampuh telah digunakan secara meluas dalam budaya orang melayu bagi pelbagai kegunaan. Kajian terdahulu telah membuktikan aktiviti antimalaria oleh *Q.infectoria*, namun secara tepatnya, unsur fitokimia yang menyebabkan aktiviti antimalaria dalam tumbuhan ini masih belum ditemui walaupun bertahun-tahun usaha penyelidikan telah giat dilakukan. Laporan telah mendapati bahawa pyrogallol telah dijumpai pada *Q.infectoria.* Pyrogallol, sejenis sebatian organik, mempunyai keupayaan untuk menghasilkan radikal bebas sebagaimana ubat antimalaria lain seperti artemisinin. Ini menjadikan artemisinin sebagai ubat yang boleh digunakan sebagai mekanisme untuk meramalkan tindakan pyrogallol terhadap *P.falciparum* di dalam kajian ini. Laporan telah menunjukkan bahawa *"reactive oxygen species" (ROS)* yang dihasilkan oleh artemisinin dalam vakuol pencernaan parasit melalui pengaktifan "*endoperoxide bridge"* mungkin mengakibatkan kematian parasit. Kajian lain telah melaporkan bahawa artemisinin memperlihatkan pencegahan secara langsung terhadap H--ATPase jenis-V, pam proton yang terletak di dalam membran vakuol pencernaan di mana ketidakaktifan boleh menyebabkan perubahan pH pada organel ini. Oleh itu, ujian berdasarkan aliran sitometri telah dilakukan untuk mengukur pH vakuol pencernaan selepas rawatan dengan pyrogallol dan artemisinin. Ujian malaria SYBR Green 1 (MSF) berasaskan pendarfluor telah digunakan untuk menentukan aktiviti antimalaria oleh pyrogallol terhadap *chloroquine-sensitive strain (3D7)* pada *P. falciparum*

dengan menentukan 50% pencegahan kepekatan (IC_{50}). Keluk penentukuran pH telah dibina dengan menggunakan *fluorescein isothiocyanate (FITC) -dextran*, penunjuk ratiometrik pH yang telah dimasukkan ke dalam vakuol pencernaan parasit trophozoite tahap terpencil yang diletakkan ke dalam penyangga pelbagai nilai pH dengan kehadiran *ionophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP).* Parasit bebas CCCP telah memperlihatkan keseimbangan pH vakuol pencernaan pada 5,42 ± 0,11, oleh itu mengesahkan potensi penggunaan lekuk penentukuran pH yang telah dihasilkan. Seterusnya, kepekatan artemisinin "*$IC_{50\text{-}4\ hours}$ (60 nM) and near $IC_{50\text{-}4\ hours}$ (15 and 30 nM)"* telah dipilih dari kajian sebelumnya untuk memastikan perubahan pH vakuol pencernaan yang telah diperhatikan dalam eksperimen berikutnya bukan disebabkan oleh kematian parasit. Ubat uji nadi selama 4 jam dengan artemisinin (15, 30 dan 60 nM) telah dilakukan dengan menggunakan parasit trophozoite tahap pertengahan untuk menentukan perubahan terhadap pH vakuol pencernaan. Kepekatan artemisinin yang telah dipilih telah meningkatkan pH vakuol pencernaan sebanyak 1 (15 nM, pH = 6,6 ± 0,1), 1,48 (30 nM, pH = 7,1 ± 0,08) dan 1,6 unit pH (60 nM, pH = 7,3 ± 0,1), masing-masing berbanding dengan vakuol pencernaan yang tidak dirawat (pH = 5.6 ± 0.1). Hasil yang sama dari perubahan pH vakuol pencernaan yang telah dicetuskan oleh satu piawai perencat pam proton, concanamycin A telah diperhatikan. Hasilnya menunjukkan bahawa artemisinin dapat menghalang H--ATPase jenis-V, menyebabkan perubahan pH pada vakuol pencernaan. Kesimpulannya, kajian ini menyumbang kepada pemahaman yang lebih tepat mengenai mekanisme tindakan pyrogallol berdasarkan hasil yang telah diperolehi dengan menggunakan artemisinin sebagai ubat untuk meramalkan aktiviti sebatian tersebut.

THE EFFECT OF PYROGALLOL ON THE pH OF *Plasmodium falciparum* DIGESTIVE VACUOLE

ABSTRACT

Quercus infectoria, a highly potent medicinal herb is widely used in a Malay culture in several applications. Previous studies proved the antimalarial activity of *Q. infectoria*, however the exact phytochemical constituents leading to the antimalarial activity of this plant remains searchable despite years of intensive research efforts. It has been reported that pyrogallol is found among the phytoconstituents of *Q. infectoria*. Pyrogallol, an organic compound, has a capability of generating free radicals like other antimalarial drugs such as artemisinin. This has made artemisinin an acceptable drug to predict the mechanism of action of pyrogallol against *P. falciparum* in this study. It has been reported that reactive oxygen species (ROS) generated by artemisinin in the parasite's digestive vacuole through endoperoxide bridge activation may cause parasite death. Another study reported that artemisinin displays direct inhibition of V-type H^+-ATPase, a proton pump located on the digestive vacuole's membrane in which the inactivation might cause pH alteration of this organelle. Hence, the flow cytometry-based assay was performed to measure the digestive vacuole pH after treatment with pyrogallol and artemisinin. The malarial SYBR Green 1 fluorescence-based (MSF) assay was used to determine the antimalarial activity of pyrogallol against the chloroquine-sensitive strain (3D7) of *P. falciparum* by determining the 50% inhibitory concentration (IC_{50}). A pH calibration curve was constructed by using fluorescein isothiocyanate (FITC)-dextran, a ratiometric pH indicator incorporated into the digestive vacuole of isolated trophozoite

stage parasites suspended in buffers of various pH values in the presence of an ionophore, carbonyl cyanide m-chlorophenylhydrazone (CCCP). The CCCP-free parasite displayed the steady-state digestive vacuole pH of 5.42 ± 0.11, hence validating the potential application of the generated pH calibration curve. Subsequently, the concentrations of $IC_{50\text{-}4\ hours}$ (60 nM) and near $IC_{50\text{-}4\ hours}$ (15 and 30 nM) of artemisinin were selected from the previous study to ensure the pH change of the digestive vacuole observed in the subsequent experiment was not resulted from parasite death. The assay of 4-hour drug pulse with artemisinin (15, 30 and 60 nM) was carried out by using mid trophozoite stage parasites to determine the pH change of the digestive vacuole. The selected concentrations of artemisinin increased the pH of the digestive vacuole by 1 (15 nM, pH = 6.6 ± 0.1), 1.48 (30 nM, pH = 7.1 ± 0.08) and 1.6 pH unit (60 nM, pH = 7.3 ± 0.1), respectively as compared with the untreated digestive vacuole (pH = 5.6 ± 0.1). The same result of the pH change of the digestive vacuole induced by a standard proton pump inhibitor, concanamycin A was observed. The result indicates that artemisinin might inhibit the V-type H^+-ATPase, causing the pH change of the digestive vacuole. In conclusion, this study contributes to a better understanding on the mechanism of action of pyrogallol based on the results obtained by using artemisinin as a drug to predict the compound's activity.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Malaria is a life-threatening disease caused by *Plasmodium* parasites. The disease is often transmitted to humans via the bites of infected female *Anopheles* mosquitoes (World Health Organization, (WHO) et al., 2019). It can also be transmitted through blood transfusion, shared use of needles contaminated with infected blood, organ transplant and congenitally from a mother to her infant during delivery (Centres for Disease Control and Prevention (CDC), et al., 2019). Common symptoms and signs of malaria infection include fever and flu-like illness including headache, shaking chills, tiredness muscle and aches. Vomiting, diarrhoea and nausea may occur. The disease may result in jaundice and anaemia because of the decrease in erythrocyte production and increase in erythrocyte rupture. Unless immediately treated, the infection can be transformed to a severe form of malaria causing seizures, kidney failure, coma and death.

The WHO estimated that there were 228 million cases of malaria worldwide and the number of malaria deaths was 405 000 in 2018 (WHO et al., 2019). In Asia, the estimated number of malaria deaths per 100 000 population was 0.7 in 2018. Despite a significant decrease in indigenous human malaria cases in Malaysia in 2018 (WHO et al., 2019), 15 cases of malaria were reported by Ministry of Health in 2018. Furthermore, new malaria cases of several species of *Plasmodium* parasites appeared and became a serious issue (Sato et al., 2019). Generally, human malaria is

caused by *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi*. Insecticide-treated bed nets (ITNs), indoor residual spraying and antimalarial drug chemotherapies have a positive impact in reducing morbidity and mortality rate (WHO et al., 2019).

Artemisinin is an antimalarial drug derived from a plant known as *Artemisia annua*. Artemisinin and its derivatives are a class of endoperoxide compounds with a desired antimalarial efficacy against chloroquine-sensitive and chloroquine-resistant strains of *P. falciparum* (Agarwal et al., 2015). The 1,2,4-trioxane ring system, which forms the endoperoxide structural scaffold is the key pharmacophore and plays an important role in increasing the pharmacodynamics potential of endoperoxide-based antimalarial drugs (Rudrapal et al., 2016). Strategies have been implanted for postponing the artemisinin resistance through the use of artemisinin-based combination therapies (ACTs) (Yeung et al., 2004). Artemisinin reduces the number of parasites during the first three days of treatment, while the partner drug eliminates the remaining parasites. Partial resistance towards artemisinin and resistance towards the partner drug have however been reported in the Greater Mekong Subregion, which has been spread from the epicentre in Cambodia to other parts of Vietnam, Laos, Thailand and Myanmar (WHO et al., 2019).

The antimalarial drug resistance problem sparks the screening of traditional medicinal plants with desired antimalarial properties (Karamati et al., 2014). Several traditional medicinal plants have been tested for the treatment of malaria in Malaysia (Karamati et al., 2014). The traditional medicinal plants that were evaluated for antimalarial activity against the malaria parasites include *Eurycoma longifolia, Tinospora crispa. Azadirachta indica, Coscinium fenestratum, Quercus infectoria* and

many more (Mohamed et al., 2015; Ahmad et al., 2016; Bedri et al., 2013; Taher et al., 2019).

Q. infectoria (Olivier) is a small tree of family *Fagaceae* that is mainly distributed in Greece and Syria (Hussein et al., 2016). The galls of this plant or called manjakani grow on young branches of the trees due to the attack of the gall wasps (*Adleria gallae-tinctoria*) (Basri et al., 2014.) In Malaysia, women after childbirth have used the galls in combination with other herbs to keep the uterine wall elastic (Fan et al., 2014). In other Asian countries, the galls have been used for treating inflammatory diseases, toothache and gingivitis (Fan et al., 2014). *Q. infectoria* also has a potency as antimalarial, antidiabetic, anticancer, antimicrobial and anti-inflammatory (Zin et al., 2019; Magbool et al., 2018).

A preliminary study has shown that the gall crude extracts of *Q. infectoria* have an *in vitro* antimalarial activity against a 3D7strain of *P. falciparum* (Zin et al., 2019). The antimalarial effect of these gall extracts might be attributed to the abundant presence of phenolic compounds such as pyrogallol (Baharuddin et al., 2015; Venancio et al., 2016). In the present study, the efficacy of pyrogallol was assessed against a chloroquine-sensitive strain (3D7) of *P. falciparum.*

Pyrogallol has a capability of generating free radicals as other antimalarial drug therapies (e.g. artemisinin) (Upadhyay et al., 2010; Percário et al., 2012; Torii et al., 1994). The free radicals generated by pyrogallol includes hydroxy radicals (OH-), peroxide nitrite or hydrogen peroxide H_2O_2 (Torii et al., 1994). This interesting finding leads to determination of the effect of pyrogallol on pH of the *P. falciparum* digestive

vacuole. Alteration of the digestive vacuole pH is likely to result in detrimental physiological disturbances of its function.

1.2 Rationale of the study

The *P. falciparum* digestive vacuole has always been the focus of the antimalarial drug research and development (Hayward et al., 2006). Pyrogallol are the phenolic compounds majorly detected in *Q. infectoria.* The digestive vacuole could be a validated target for pyrogallol by altering the pH of this organelle following destruction of susceptible proteins such as proton pumps located on the digestive vacuole's membrane. The proton pumps such as V-type H^+-ATPase and V-type H^+-translocating pyrophosphatase located at the *P. falciparum* digestive vacuole's membrane have been known to regulate the acidic pH of this organelle (Saliba et al., 2003). In the present study, artemisinin was utilised as alternative result to predict the effect of pyrogallol on the parasite's digestive vacuole pH. Through using an established flow cytometry method, the change of pH of the malaria parasite' digestive vacuole following treatment of artemisinin was investigated. This event might underlie the basis for toxicity of pyrogallol in *P. falciparum* and could be useful for the development of new antimalarial drugs.

1.3 Objectives of the study

1.3.1 General objective

To determine changes of the digestive vacuole pH following treatments with pyrogallol against the malaria parasite *in vitro*.

1.3.2 Specific objectives

i. To determine the antimalarial activity of pyrogallol against the chloroquine-sensitive strain (3D7) of *P. falciparum*.

ii. To measure the pH of the digestive vacuole of the chloroquine-sensitive strain (3D7) of *P. falciparum* following treatments with artemisinin, an alternative of pyrogallol.

1.4 Experimental design

The study aimed to measure the pH of the digestive vacuole of the chloroquine-sensitive strain (3D7) of *P. falciparum* treated with pyrogallol. The general flow for the study is illustrated in Figure 1.1. The parasites were cultured in T-75 flasks containing washed O-positive type human blood and complete culture media (CCM). CCM consists of RPMI 1640 supplemented with gentamicin in H_2O, 50% glucose in H_2O, hypoxanthine in 1 M NaOH and Albumax II. Blood samples were collected from volunteers recruited at Health Campus, Universiti Sains Malaysia (USM).

The antimalarial activity of pyrogallol was performed to determine the concentration that kills 50% of the parasite population (IC_{50}) by using a malarial SYBR Green I fluorescence-based (MSF) assay. The parasites at ring stages were synchronised with sorbitol to obtain a synchronous population of ring stage parasites. The parasites were treated for 48 hours with different concentrations of pyrogallol. After 48 hours, the SYBR Green I solution (2 × final concentration) was added to the parasite suspensions and the fluorescence signal was measured (excitation and emission wavelengths of 490 nm and 530 nm, respectively). Giemsa-stained thin blood smears were also prepared at 24- and 48-hour post-treatments to observe the morphology of treated parasites.

The effect of artemisinin on pH of the digestive vacuole was investigated by using a flow cytometry-based assay. The pH of the digestive vacuole was measured by using FITC-dextran (a ratiometric pH indicator) incorporated into resealed erythrocytes by using a hypotonic dilution technique. A pH calibration curve was generated by incubating resealed erythrocytes in buffers (at different pH) in the presence of an ionophore (CCCP) to equilibrate the pH of the parasite compartments with the pH of the buffers. Ratios of the fluorescence intensity detected at 530 nm and 585 nm wavelengths were plotted on a pH calibration curve. Next, synchronised mature stage parasites were harvested by using MACS columns and infected with FITC-dextran-containing resealed erythrocytes. The probe was ingested and finally delivered to the digestive vacuole of trophozoite stage parasites. Trophozoite stage parasites (36-hour post-invasion) determined by using Giemsa-stained thin blood smears were adjusted to 5% parasitaemia (2% haematocrit) before treatment with pyrogallol at different concentrations for 4 hours. The parasites were permeabilised

with saponin (0.035% w/v) to release FITC-dextran in the cytoplasm of resealed erythrocytes, allowing only FITC-dextran accumulated in the digestive vacuole to be measured by a flow cytometer.

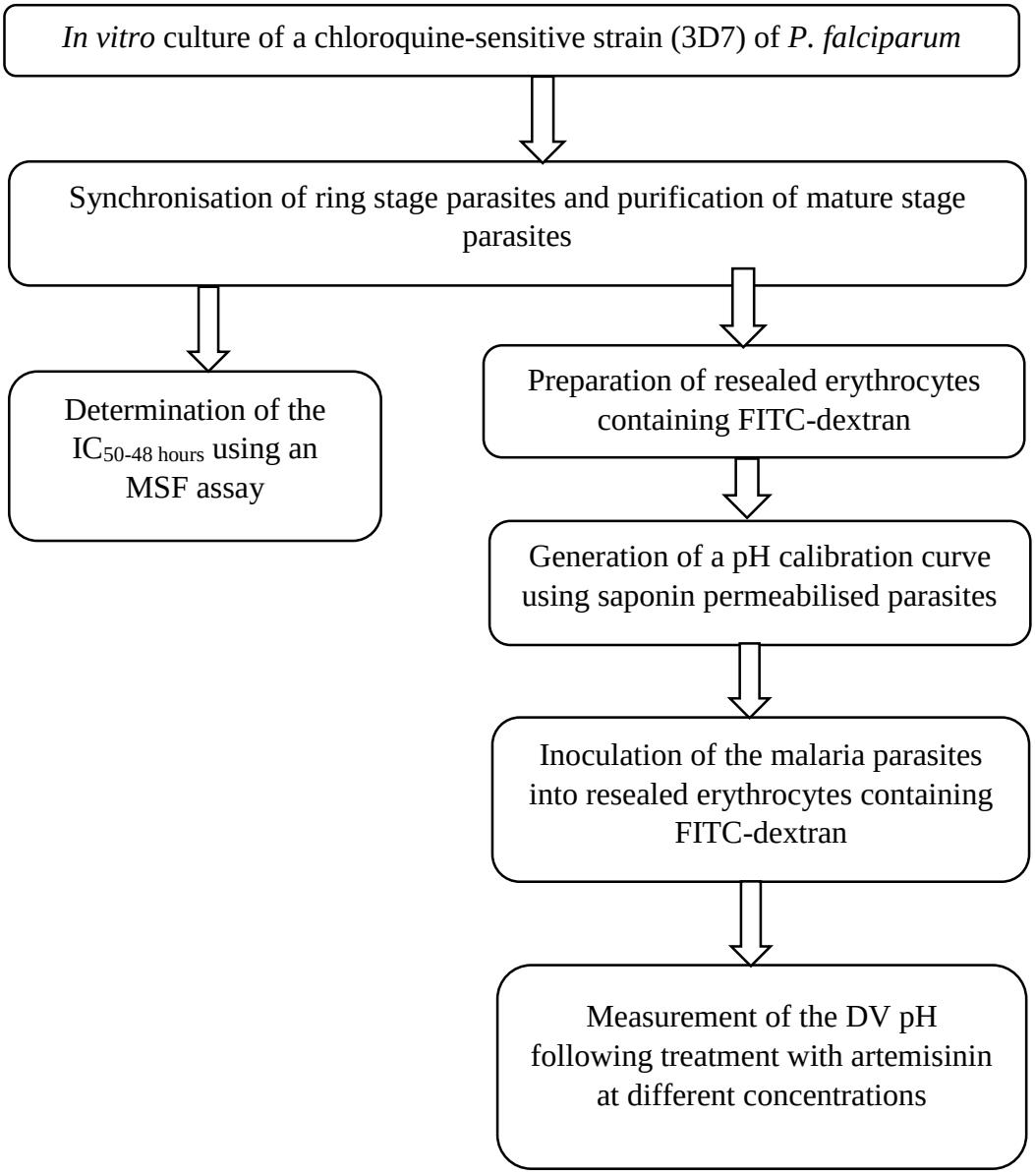

Figure 1.1: Flowchart of the experiments through all the study

CHAPTER 2

LITERATURE REVIEW

2.1 Overview of malaria

Malaria is an infectious disease caused by parasites of the genus *Plasmodium*. The disease is transmitted to a healthy human by an infected female mosquito of the genus *Anopheles*. Malaria has long been considered as one of the leading causes of morbidity and mortality around the world (Talapko et al., 2019). Malaria-like symptoms have been mentioned in historical writings of ancient nations from different parts of the world (Newfield et al., 2017). These records mentioned periodical fevers and splenomegaly (Peset et al., 2015). The prevalence of malaria in early nations was emphasised with modern diagnostic methods that detected malaria antigens in Egyptian mummies dating back to 3200 B.C. (Kenawy et al., 2015). Recently, numerous studies have been conducted and published to solve malaria-related issues. Various strategies have been adopted to control and eliminate the disease including the use of antimalarial drugs, indoor residual spraying and insecticide-treated bed nets (CDC, 2020a). However, malaria still remains active and threatens public health partly due to the drug resistance crisis especially in developing countries.

2.2 Distribution of malaria

Malaria is usually distributed in tropical and subtropical countries that are characterised by several climate factors such as high temperature, humidity and rainfall

(CDC, 2020b). Up until 2018, populations of 92 countries were possible to be infected with malaria and other tropical diseases (see Figure 2.1) (WHO, 2018a). In 2017, the number of malaria cases was 231 million cases with 416 000 fatal cases. There was a slight decrease in 2018 to 228 million cases with 405 000 deaths. WHO (2019) reports that more than half of all malaria cases were found in the WHO African Region with 213 million cases (93%) in 2018. This is followed by the South-East Asia Region that had 3.4% of the cases and the Eastern Mediterranean Region with 2.1% (WHO, 2019). The slow decline in the number of malaria cases worldwide could be due to the drug resistance problem. In Malaysia, the number of malaria cases was 5 194 cases in 2010. It dropped to 85 cases in 2017. Finally, it is noticeable that there was a substantial decrease in 2018 to zero indigenous human malaria cases (Figure 2.2) (WHO, 2019). However, zoonotic *P. knowlesi* infections have been seen in number of cases in Malaysia, occurring mostly in Sabah and Sarawak (Hussin et al., 2020).

2.3 Human malaria parasites

Plasmodium species belong to the phylum Apicomplexa, which consists of a number of obligate intracellular parasites. Within the phylum Apicomplexa, *Plasmodium* species are grouped into the class Aconoidasida, the order Haemosporida and the family Plasmodiidae (Maier, 2019). There are about 156 species of *Plasmodium* that infect various species of vertebrates (CDC, 2019). Five of them can cause malaria infections in humans: *P. malariae, P. falciparum, P. ovale, P. vivax* and *P. knowlesi* (Millar et al., 2015). Out of the five human malaria parasites, *P. falciparum* is the predominant species in the world (CDC, 2019).

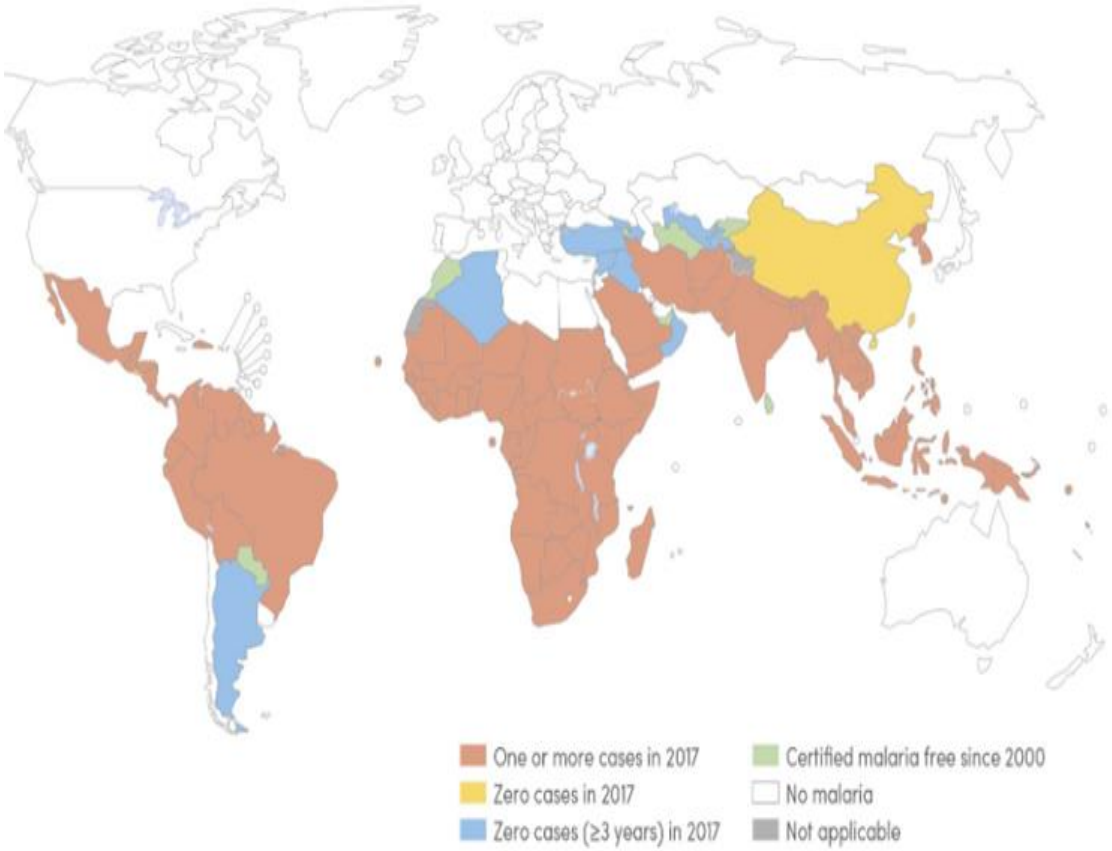

Figure 2.1: The malaria incidence rates worldwide from 2000-2017

Most of the countries in China and the European region reported zero indigenous cases in 2017. Several regions including Southeast Asia were still in active malaria transmission. Modified from World Malaria Report WHO (2018a).

Figure 2.2: The malaria incidence rates from 2000-2017 in Malaysia

Malaysian government was successful in reducing the number of malaria cases to zero indigenous human malaria cases. Modified from World Malaria Report WHO (2019).

2.4 Life cycle of the malaria parasite

The malaria parasite has a complex life cycle that is involved in two different hosts: a human host and a female *Anopheles* mosquito.

2.4.1 Sexual cycle of the malaria parasite within a mosquito

The sexual life cycle of the malaria parasite occurs once a female mosquito swallows macrogametocytes (female) and microgametocytes (male) from an infected human during a blood meal (see Figure 2.3) (Guttery et al., 2015). Eight of microgametes are produced due to exflagellation of microgametocytes that each fuses with a macrogamete to yield a zygote in the mosquito's midgut. The zygote becomes a motile ookinete throughout meiosis. Subsequently, the ookinete penetrates the midgut wall and becomes an oocyst. After ten days of maturation, the oocyst produces and releases numerous sporozoites. The sporozoites immediately migrate to the salivary gland and are ready to be delivered to a human host during another blood meal of the infected mosquito (Bennink et al., 2016; Tachibana et al., 2018).

2.4.2 Asexual cycle of the malaria parasite within a human

The asexual life cycle of the malaria parasite occurs once a female mosquito transmits sporozoites to a human during a blood feeding. The sporozoites enter the bloodstream and attack the liver cells to begin the exoerythrocytic cycle (see Figure 2.4) (Cowman et al., 2016). In the liver, the malaria parasite multiplies and develops into a schizont. The schizont produces and releases numerous merozoites.

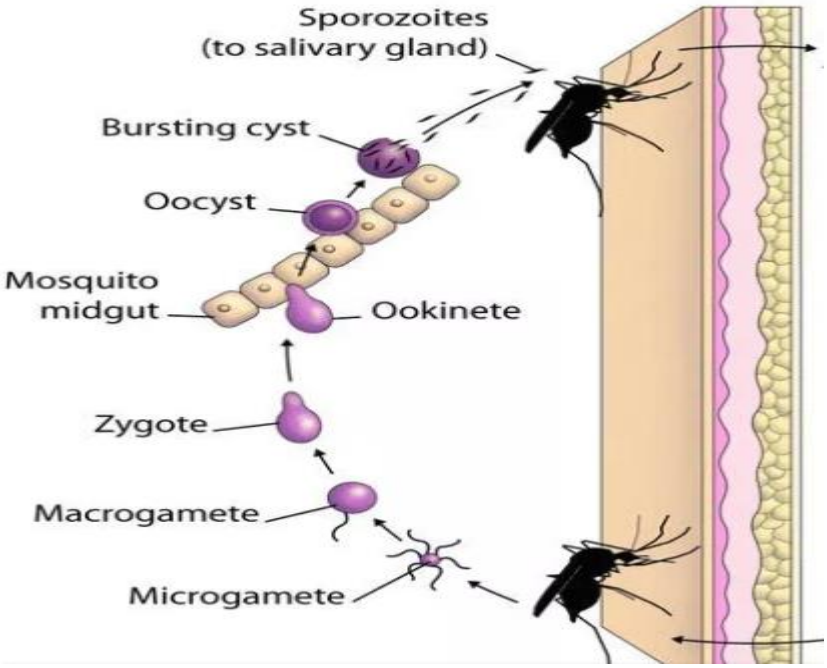

Figure 2.3: The sexual life cycle of the malaria parasite inside a mosquito

Macrogametocytes (female) and microgametocytes (male) are swallowed by a female mosquito during a blood meal and travelled to the mosquito's midgut. These gametocytes are developed into microgametes and macrogametes during maturation process. Zygotes are formed throughout fusion of the microgametes with the macrogametes and undergo meiosis to generate ookinetes. After that, the midgut wall is penetrated by the ookinetes that matures into oocysts and release numerous sporozoites when it is broken down. Then, malaria parasites are migrated to the salivary gland**.** Modified from CDC (2018).

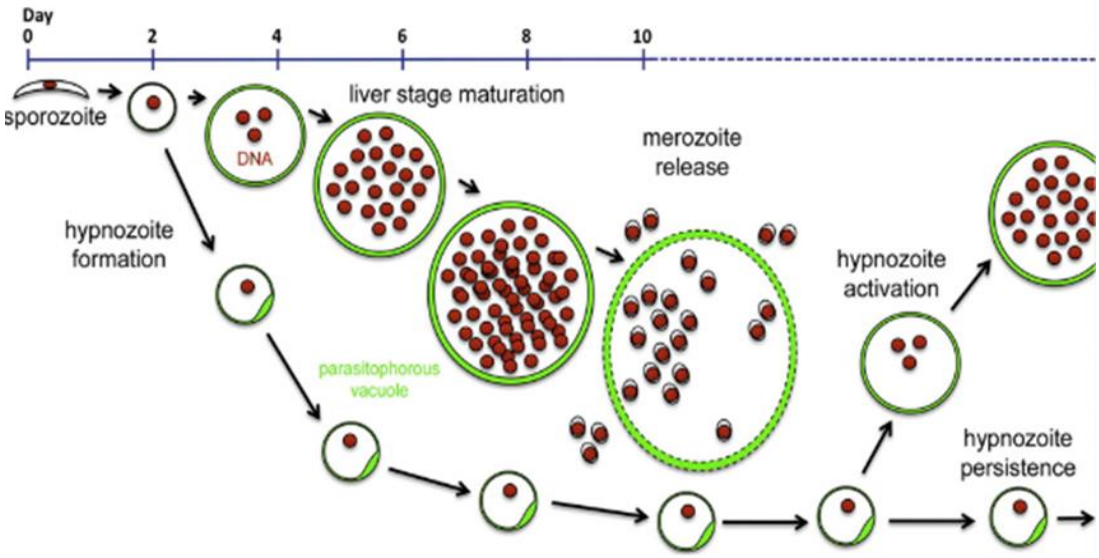

Figure 2.4: The exoerythrocytic asexual cycle of the malaria parasite within a human

The exoerythrocytic stage is occurred when sporozoites enter the blood circulation system and invade the liver cells. Modified from Mikolajczak (2015).

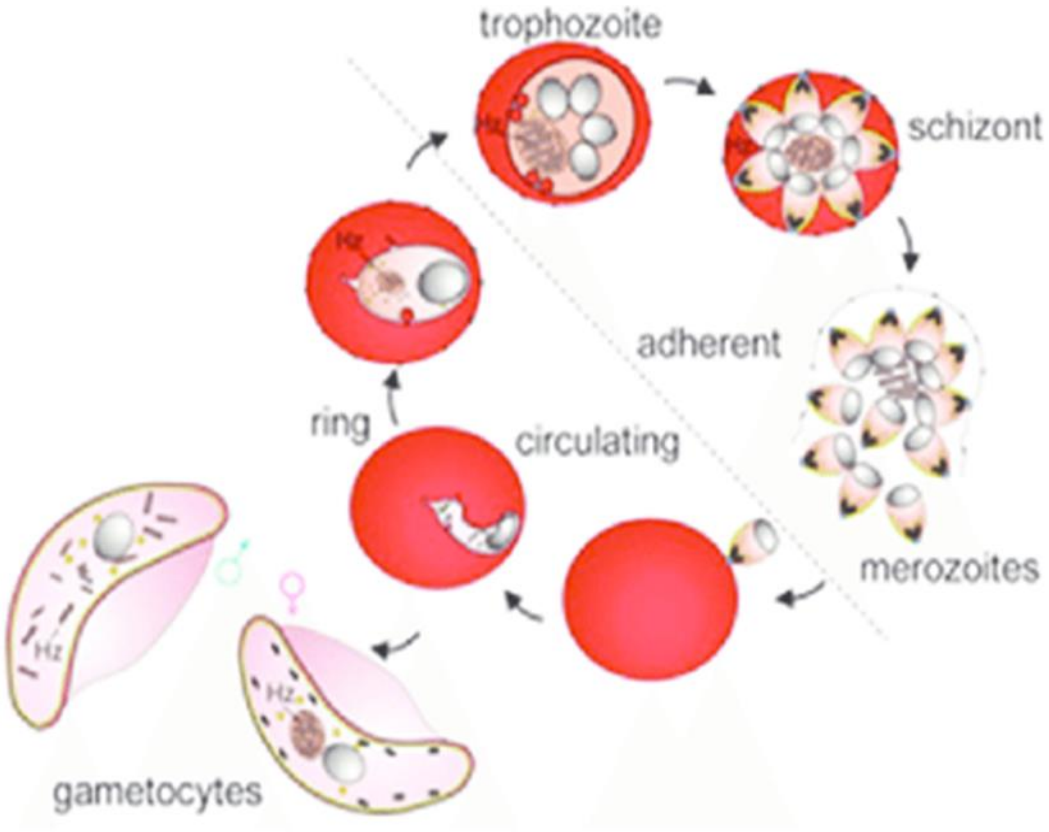

Figure 2.5: The intraerythrocytic asexual cycle of the malaria parasite within a human

Merozoites enter the erythrocytes and mature into three distinct stages; ring stage, trophozoite stage and schizont stage. Modified from Martin (2017).

The merozoites enter the bloodstream and attack the red blood cells (RBCs) to induce the intraerythrocytic cycle (Martin et al., 2017). Inside the RBCs, the parasite matures into three stages: ring, trophozoite and schizont (Figure 2.5). Understanding the concept of sexual and asexual life cycles of the malaria parasite may facilitate control and prevent of the disease.

2.5 Malaria control and prevention challenges

The main objective of National Malaria Control Programs (NMCP) and majority of malaria activities is to decrease the morbidity and mortality rate (CDC, 2018). Therefore, several strategies have been implemented for controlling malaria such as eradicating mosquitoes, developing effective vaccines and discovering new antimalarial drugs (Benelli et al., 2017). This has led to a tremendous decrease in malaria burden in the past decade. However, achieving eradication in several countries remains a challenge (Tizifa et al., 2018). Despite attempts for developing malaria vaccines, it is demonstrated to be very problematic and there is no effective malaria vaccine yet available on the market. (CDC, 2018). Also, attempts to control *Anopheles* mosquito populations have a limited success (Wilke et al., 2015). The limitation of vector control and vaccine as well as resistance of malaria parasites to antimalarial drugs emphasises the importance of the discovery of new antimalarial agents with new mechanism of action (Burrows at al., 2017).

2.6 Treatment of malaria

Antimalarial drugs are classified into several classes based on their chemical compounds such as antifolates (Figure 2.6A) (i.e. proguanil and pyrimethamine) and quinolines (i.e. plasmoquine, quinine, mefloquine, chloroquine, primaquine and halofantrine) (Figure 2.6B) (Golden et al., 2015; NDUNG et al., 2018). Other classes of antimalarial drugs include antibiotics such as tetracyclines (i.e. doxycycline and methacycline) (Figure 2.6C) (Corral et al., 2017) and atovaquone (i.e. pyronaridine) (Figure 2.6D) (Lee et al., 2018). Currently, the most used antimalarial drugs are endoperoxides (i.e. artesunate, dihydroartemisinin, artemether, and artemisinin) that target stages of the asexual life cycle of the malaria parasite (Tilley et al., 2016). Artemisinin-based combination therapies (ACTs) are recommended by WHO for the treatment of uncomplicated and complicated malaria resulted from *P. falciparum* (WHO, 2018b).

2.6.1 Past antimalarial drugs

Quinine, an alkaloid-type compound extracted from the cinchona bark was the first effective antimalarial drug used in the 1820s (Chang et al., 2016; Gachelin et al., 2017). From the end of the 19th century, the scientists had conducted several studies on synthetic antimalarial drugs to develop quinine-based compounds and obtain more effective alternatives for quinine (Gachelin et al., 2017). This resulted in the synthesis and production of plasmoquine, the first 8 aminoquinoline in 1926 (see Figure 2.6Bii) (Van et al,.2016). However, this drug produced unwanted toxicological side effects such as induction of haemolytic toxicity in patients who are glucose-6-phosphate

dehydrogenase (G6PD) deficient (Chu et al., 2016). Because of this issue, a new class of drugs, series of side-chain modified 4-aminoquinolines known as chloroquine was synthesised in early 1940s (Figure 2.6Biii) (Bogaczewicz et al., 2017). This drug was proved invaluable in the fight against malaria, highly efficacious, inexpensive and safer as compared to quinine due to its low toxicity (Hu et al., 2020).

Chloroquine is a weak base compound, which is able to pass through the erythrocyte plasma membrane (EPM) and increases the digestive vacuole pH (Juge et al., 2015). Chloroquine becomes protonated inside the acidic digestive vacuole, thereby, the charged form of this drug cannot permeate the digestive vacuole's membrane, leading to blockade of the drug inside this organelle (Sayeed et al., 2019). It is thought that chloroquine interferes with haematin biocrystallisation in the digestive vacuole (Ibraheem et al., 2019). The aggregation of free haematin or haematin-chloroquine complex could destroy the digestive vacuole's membrane (Wang et al., 2017), resulting in the parasite death. However, in the late 1950s, the emergence of chloroquine-resistant *P. falciparum* strains occurred in Southeast Asia and in particular in Cambodia (Thu et al., 2017).

As a result, numerous antimalarial drugs have been developed and used as alternatives for chloroquine such as atovaquone, antifolates and antibiotics. Antifolates are responsible for inhibiting the formation of folate cofactors that are demanded for nucleotide synthesis (Figure 2.6A) (Estrada et al., 2016). However, the resistance of antifolate drugs occurred in *P. falciparum* due to mutations in dhfr and dhps, leading to altered structure and decreased drug affinity of dihyrofolate reductase (DHPS) and dihydropteroate synthetase (DHFR) (Verhoef et al., 2017; Joy et al., 2018).

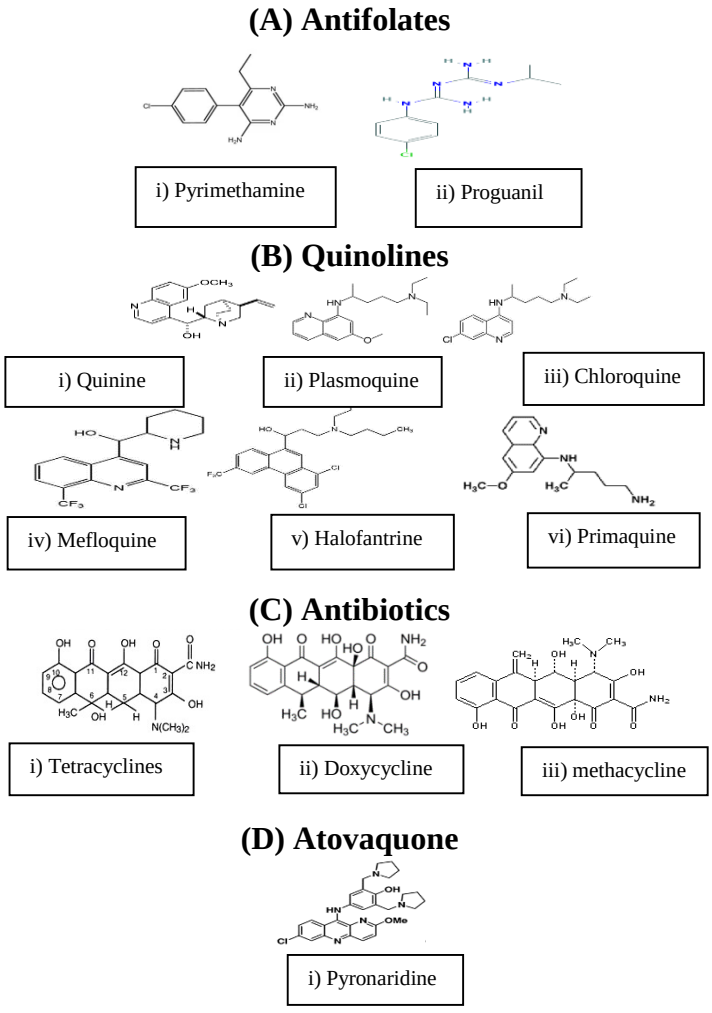

Figure 2.6: The chemical structure of the antimalarial drugs

The antimalarial drugs that have been used in the treatment of malaria infection are from the class of (A) antifolates, (B) quinolines , (C) antibiotics and (D) atovaquone.

Atovaquone, an electron transport inhibitor collapses plasmodial cells through the inhibition of parasite respiration and the depolarisation of mitochondrial membrane potential that are important for several biochemical processes of the malaria parasite (Figure 2.6D) (Gomez et al., 2018). Antibiotics inhibit protein translation inside the apicoplast of *P. falciparum* (Figure 2.6C) (Pasaje et al., 2016). Due to the resistance problem of these drugs, new strategies have been introduced as alternatives (Edwin et al., 2019).

2.6.2 Current antimalarial drugs

In order to solve the drug resistance problem, the combination of antimalarial drugs has been used for the treatment of uncomplicated malaria (Ouji et al., 2018). ACTs prove invaluable in the fight against malaria as compared to other strategies of combination therapies. However, resistance to ACTs has been existed recently (Conrad et al., 2019). This encourages scientists to conduct researches on the field of herbal medicine to produce new effective antimalarial drugs (Shen et al., 2015).

2.7 Medicinal plants as a source of antimalarial drug candidates

Medicinal plants have been used since ancient times for the treatment of malaria (Leonti et al., 2017). For instance, many plant species have been used as antimalarial medicines in Tanzania (Nondo et al., 2015). Up until now, 80% of the world's population still depends upon traditional medicines on the treatment of malaria and other diseases (Tuo et al., 2015; Thomford et al., 2018) due to their cultural preferences, effectiveness, inexpensiveness, safety and abundant availability (National

Health Portal (NHP), 2016). Recently, many plant species have been introduced as antimalarial medicinal plants such as *Clerodendrum viscosum Vent, Duranta repens L, Lantana camara L, Nyctanthes arbor tristis L, Dracaena reflexa Lamk, Cinnamosma fragrans H, Andropogon schoenanthus/nardis L, Desmodium mauritianum D.C, Ficus megapoda Bak* and *Quercus infectoria* (Zin et al., 2019; Noronha et al., 2020).

2.8 *Quercus infectoria*

Q. infectoria is a medicinal herb that belongs to the family *Fagaceae* (see Figure 2.7) (Karimi et al., 2015). This plant is found in several countries such as Turkey, Persia, Syria, Greece and Cyprus. The parts of *Q. infectoria* that have been used for medicinal uses are mainly stem, bark, root, leaf, seed and galls (Hamad et al., 2017). In Malaysia, *Q. infectoria* galls are locally known as biji manjakani (Basri et al., 2012). The galls appear on the branches of oak trees as a result of assault by gall wasp (*Cypnis gallae-tincotoriae*) (Mohammadi et al., 2016). The wasps penetrate young branches of the tree and lay their eggs inside them (Shahid et al., 2019). The galls are green-yellow in colour, slightly odour, strongly pungent taste, globular in shape, tuberculated surface and have 0.8-2.5 cm in diameter (Figure 2.8) (Baharuddin et al., 2015). The galls have been shown to have many biological activities such as antioxidant, antibacterial and anticandidiasis activities (Baharuddin et al.,2015).

2.8.1 Phytochemistry of *Q. infectoria* galls

The phytochemical constituents in *Q. infectoria* gall extracts are 1,2,3,4,6-pentakis-ogalloyl ß-D-glucose (3.36-8.11%), 1,2,3,6-tetrakis-O-galloyl- ß-Dglucose (4.36-6.86%), digallate (1.8-2.38%), gallic acid (1.61-2.62%) and gallotannin (1-O,2-O,6-O-Digalloyl-β-D-glucose) (2.16-2.64%), respectively. Major constituents analysed resulting from polyphenolic derivatives primarily hydrolysable tannins, and gallic acid (Table 2.1) (Vaidya et al., 2013; Abdullah et al., 2017). Pyrogallol have also been found among the phytoconstituents of *Q. infectoria* (Tayel et al., 2018). Pyrogallol has been found to show antimalarial activity (Venancio et al., 2016).

2.8.1.1 Pyrogallol

Pyrogallol also known as 1,2,3-trihydroxybenzene or pyrogallic acid, an organic compound belongs to the phenol family that is naturally present in oak, hardwood plants and many fruits such as pomegranate peel and date palm (Figure 2.9) (Wang et al., 2015; Shahzad et al., 2015; El Sohaimy et al., 2015; Baharuddin et al., 2015; Panzella et al., 2017). Pyrogallol was recognised for the first time from the natural extract of *Abrus precatorius*, Linn seeds (Selvaraj et al., 2018). Nowadays, pyrogallol is commercially produced via thermal decarboxylation of gallic acid under high pressure and temperature. However, this method is limited via the strict reaction conditions, inaccessible raw material and the relatively low product yield (Wang et al., 2018). Pyrogallol possesses antimalarial, antifungal and antipsoriatic properties (Venancio et al., 2016; Han et al., 2017).

Figure 2.7: ***Quercus infectoria*** **(oak tree)**

The tree grows to 4 to 6 feet tall, crooked, with bright and smooth leaves, acorn narrow and long, downy and scaly. Modified from Yahya (2018).