ANTIMALARIAL ACTIVITY, TOXICITY AND PHYTOCHEMICAL SCREENING OF *Quercus infectoria* GALL CRUDE EXTRACTS

NIK NOR IMAM BINTI NIK MAT ZIN

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ANTIMALARIAL ACTIVITY, TOXICITY AND PHYTOCHEMICAL SCREENING OF *Quercus infectoria* GALL CRUDE EXTRACTS

by

NIK NOR IMAM BINTI NIK MAT ZIN

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LIST OF SYMBOLS, ABBREVIATIONS AND ACRONYMS

~	approximately
%	percent
^{o}C	degree Celsius
=	equal
±	plus minus
<	less than
≤	less than or equal to
>	more than
≥	more than or equal to
× *g*	gravitational force
µM	micromolar
µg/mL	microgram
µL	microliter
cm	centimetre
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	mililiter
mM	millimolar
n	number of subjects
nm	nanometre
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
[^{3}H] hypoxanthine	tritiated hypoxanthine
[^{3}H] ethanolamine	tritiated ethanolamine
pfKelchl 3	*Plasmodium falciparum* Kelch 13 gene
pfhrp2	*Plasmodium falciparum* histidine-rich protein type 2 gene
pfhrp3	*Plasmodium falciparum* histidine-rich protein type 3 gene
$AlCl_3$	aluminium chloride
AAS	atomic absorption spectroscopy
ACTs	artemisinin-based combination therapies
ADP	adenosine diphosphate
ANOVA	analysis of variance
ATP	adenosine triphosphate
BCECF	5'(and 6')-carboxy-10- dimethylamino-3-hydroxy-spiro[7H-benzo[c]xanthene-7,1'(3H)-isobenzofuran]-3'-one
BSLT	brine shrimp lethality test
Ca^{2+}	calcium ion
CO_2	carbon dioxide
Cr	chromium
Cu	copper
CC_{50}	cytotoxic concentration that reduces cell viability by 50%
CCCP	carbonyl cyanide m-chlorophenylhydrazone
CPG	2-(6-benzoyl-β-d-glucopyranosyloxy)-7-(1α, 2α, 6α-trihydroxy-3-oxocyclohex-4-enoyl)-5 hydroxybenzyl alcohol
DFd	degree of freedom denominator
DFn	degree of freedom numerator
DNA	deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide

DPPH	2,2-diphenyl-1-picrylhydrazyl
E-64	calpain inhibitor N-acetyl-leucinyl-leucinyl-norleucinal
EtBr	ethidium bromide
EC_{50}	effective concentration that requires to scavenge 50% free radicals
EDTA	ethylenediaminetetraacetic acid
$FeCl_3$	ferric chloride
FACS	fluorescence-activated cell sorting
FCS	flow cytometry standard
FITC	fluorescein isothiocyanate
FSC	forward scatter
GAE	gallic acid equivalent
GC-MS	gas chromatography–mass spectrometry
H^+	proton/hydrogen ion
HCl	hydrogen chloride/ hydrochloric acid
HEPES	hydroxyethyl piperazineethanesulfonic acid
HNO_3	nitric acid
HRP-II	histidine-rich protein II
HUVEC	human umbilical vein endothelial cells
I_g	fluorescence intensity collected at green channel
I_y	fluorescence intensity collected at yellow channel
IC_{50}	inhibitory concentration at half of maximal response
INFORMM	Institute for Research in Molecular Medicine
ISO	International Standard of Organization
ITNs	insecticide-treated bed nets
IU	international unit
K_d	equilibrium dissociation constant
KC_{50}	killing concentration at half of maximal response
KCl	potassium chloride
KOH	potassium hydroxide
LC_{50}	lethal concentration that causes 50% shrimp mortality

LDL	less than the detection limit
Mg^{2+}	magnesium ion
$MgCl_2$	magnesium chloride
MACS	magnetic-activated cell sorting
MES	2-[N-morpholino] ethane sulfonic acid
MSF	malarial SYBR Green-I fluorescence-based
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N	normality
Na_2CO_3	sodium carbonate
NaCI	sodium chloride
NaF	sodium fluoride
NaH_2PO_4	sodium dihydrogen phosphate monohydrate
Na_3PO4	trisodium phosphate
$NaNO_2$	sodium nitrite
NIH/3T3	mouse embryo fibroblast cell line
NMPC	Natural Medicinal Products Centre
Pfhrp2	*Plasmodium falciparum* histidine-rich protein type 2
Pfhrp3	*Plasmodium falciparum* histidine-rich protein type 3
PfFP-2	*Plasmodium falciparum* falcipain-2
PfFP-2'	*Plasmodium falciparum* falcipain-2'
PfFP-3	*Plasmodium falciparum* falcipain-3
PfHAP	*Plasmodium falciparum* histoaspartic proteinase
PfPM1	*Plasmodium falciparum* plasmepsin 1
PfPM2	*Plasmodium falciparum* plasmepsin 2
PfPM4	*Plasmodium falciparum* plasmepsin 4
Pb	plumbum
PBS	phosphate buffered saline solution
RDT	rapid diagnostic test
RNA	ribonucleic acid
PE	phycoerythrin
PPi	pyrophosphate
R^2	correlation coefficient

R_{gy}	fluorescence ratio
RPMI	Rosewell Park Memorial Institute
SD	standard deviation
SI	selectivity index
SNARF	seminaphthorhodafluor
SSC	side scatter
TFC	total flavonoid content
TPC	total phenolic content
TRIS	tris (hydroxymethyl) aminomethane
UIAM	Universiti Islam Antarabangsa Malaysia
USM	Universiti Sains Malaysia
V-type H^+-ATPase	vacuolar-type proton-pumping ATPase
V-type H^+-pyrophosphate	vacuolar-type proton-pumping pyrophosphatase
Vero	African green monkey kidney epithelial cell line
WHO	World Health Organization
Zn	zinc

AKTIVITI ANTIMALARIA, TOKSISITI DAN SARINGAN FITOKIMIA EKSTRAK MENTAH BIJI *Quercus infectoria*

ABSTRAK

Penurunan keberkesanan ubat antimalaria akibat penularan *Plasmodium falciparum* yang rintang ubat memerlukan usaha mencari ubat antimalaria dengan sasaran yang baharu. *Quercus infectoria* telah digunakan secara tradisional sebagai ubat herba bagi rawatan pospartum dan penyakit disebabkan parasit. Walau bagaimanapun, tiada sebarang aktiviti antimalaria yang pernah dilaporkan bagi tumbuhan ini. Oleh itu, kajian ini bertujuan menilai aktiviti antimalaria ekstrak mentah biji *Q. infectoria* secara *in vitro*. Kajian ini turut direka bagi menilai profil toksisiti dan menyaring sebatian fitokimia dalam tumbuhan ini. Potensi antimalaria ekstrak aseton, metanol, etanol dan akueus terhadap strain *P. falciparum* yang sensitif klorokuina (3D7) ditentukan melalui asai malaria berasaskan pendarfluor hijau SYBR I (MSF). Hanya ekstrak aseton dan metanol menunjukkan aktiviti antimalaria yang baik dengan kepekatan perencatan 50% (IC_{50}) masing-masing iaitu 5.86 (1.64) dan 10.31 (1.90) µg/mL. Ujian sitotoksisiti ekstrak dinilai menggunakan sel selanjar fibroblas tikus (NIH/3T3), sel selanjar epitelial ginjal monyet (Vero) dan sel primer endotelial vena umbilikal manusia (HUVEC) melalui asai 3-(4, 5-dimetiltiazol-2-il)-2, 5-difeniltetrazolium bromida (MTT). Ekstrak aseton dan metanol memaparkan kepekatan sitotoksisiti 50% (CC_{50}) dalam julat antara toksik secara sederhana dan tidak toksik terhadap semua sel normal yang diuji. Penilaian sitotoksisiti menggunakan ujian kemautan udang brin (BSLT) turut menunjukkan semua ekstrak tidak toksik ke atas udang air garam berdasarkan indeks toksisiti Meyer. Selain asai hemolisis, asai

antioksidan menggunakan 2,2-difenil-1-pikrilhidrazil (DPPH) juga dilakukan terhadap ekstrak-ekstrak bagi melihat hubungkaitnya dengan hemolisis eritrosit manusia (kumpulan darah A^+, B^+, AB^+ dan O^+). Tiada kesan hemolitik berlaku terhadap eritrosit yang dirawat dengan semua ekstrak. Semua ekstrak turut mempamerkan aktiviti pemerangkapan radikal DPPH yang baik. Kandungan logam surih (plumbum, zink, kromium, tembaga dan kadmium) dalam ekstrak menunjukkan kepekatan di bawah tahap yang dibenarkan mengikut garis panduan WHO yang telah dianalisa dengan spektroskopi penyerapan atom (AAS). Pemeriksaan kandungan fitokimia menunjukkan kehadiran tanin dan flavonoid serta jumlah kandungan fenolik (TPC) dan jumlah kandungan flavonoid (TFC) yang tinggi dalam semua ekstrak. Kesan ekstrak aseton yang telah menunjukkan aktiviti antimalaria yang paling baik dengan nilai indeks selektiviti (SI) yang memuaskan terhadap pH vakuol pencernaan *P. falciparum* telah disiasat menggunakan penunjuk pendafluor bersifat ratiometrik, fluoresein isotiosianat (FITC)-dektran yang dimuatkan ke dalam parasit malaria peringkat trofozoit dan dianalisa dengan sitometri aliran. pH vakuol pencernaan yang dirawat dengan ekstrak aseton berubah secara signifikan mengikut kepekatan apabila dibandingkan dengan parasit yang tidak dirawat ($p < 0.001$). Secara keseluruhan, kajian ini memberikan pemahaman asas yang berharga mengenai keupayaan biji *Q. infectoria* sebagai calon antimalaria yang selamat dan diyakini.

ANTIMALARIAL ACTIVITY, TOXICITY AND PHYTOCHEMICAL SCREENING OF *Quercus infectoria* GALL CRUDE EXTRACTS

ABSTRACT

The reduced efficacy of the mainstay antimalarial drugs due to widespread of drug-resistant *Plasmodium falciparum* has necessitated efforts to discover new antimalarial drugs with new targets. *Quercus infectoria* galls have been used traditionally as a herbal remedy for post-partum medication and treatment of parasitic diseases. However, the antimalarial activity of the galls has not been reported. Thus, this study was aimed at evaluating the *in vitro* antimalarial activity of *Q. infectoria* gall crude extracts. This study was also designed to evaluate the toxicity profiles and screen the phytochemical constituents. The antimalarial potential of acetone, methanol, ethanol and aqueous extracts against the chloroquine-sensitive strain (3D7) of *P. falciparum* was assessed via malarial SYBR Green-I fluorescence-based (MSF) assay. Only acetone and methanol extracts showed a promising antimalarial activity with 50% inhibitory concentration (IC_{50}) of 5.86 (1.64) and 10.31 (1.90) µg/mL, respectively. The cytotoxicity of the extracts was evaluated against mouse fibroblast cell (NIH/3T3), monkey kidney epithelial cell (Vero) and primary human umbilical vein endothelial cell (HUVEC) via 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The acetone and methanol extracts showed 50% cytotoxicity concentration (CC_{50}) ranged from moderate toxic to non-toxic against all tested normal cells. The cytotoxicity evaluation using a brine shrimp lethality test (BSLT) showed that all extracts were non-toxic according to Meyer's toxicity index. In addition to the haemolytic assay, a 2,2-diphenyl-1-picrylhydrazyl

(DPPH)-based antioxidant assay of the extracts was performed to observe its connection with haemolysis of human erythrocytes (A^+, B^+, AB^+ and O^+ blood groups). No haemolytic effect was observed on the erythrocytes treated with all extracts. All extracts exhibited excellent DPPH radical scavenging activities. The concentration of heavy metals (lead, zinc, chromium, copper and cadmium) analysed with atomic absorption spectroscopy (AAS) in all extracts was below the permissible level according to WHO guidelines. The phytochemical screening revealed the presence of tannins and flavonoids, and high amount of total phenolic content (TPC) and total flavonoid content (TFC) in all extracts. The effect of acetone extract which previously exhibited the most promising antimalarial activity and have satisfactory selectivity index (SI) values on the pH of the parasite's digestive vacuole was examined using a ratiometric fluorescent probe, fluorescein isothiocyanate (FITC)-dextran incorporated into mid trophozoite stage-infected erythrocytes and analysed by flow cytometry. The pH of the digestive vacuole of acetone extract-treated parasites was significantly altered in a concentration-dependent manner compared to the untreated parasites ($p < 0.001$). Overall, this study provides valuable insights of *Q. infectoria* gall capability as a safer and promising antimalarial candidate.

CHAPTER 1

INTRODUCTION

1.1 Background of the study

Malaria overwhelms humans throughout the centuries. In 2018, the World Health Organization (WHO) reported 228 million cases of malaria with 405 000 deaths globally (WHO, 2019). The highest number of malaria cases and deaths was recorded in the African region, followed by the Southeast Asian, Eastern Mediterranean and Western Pacific regions. Although Malaysia was ranked among the ten malaria-affected countries in the Western Pacific region that achieved zero indigenous cases of human malaria in 2018 (WHO, 2019), malaria-related deaths have still failed to reduce since 2010. Thus, malaria continues to pose a significant threat to the health system and economic development, requiring a massive effort for a malaria-free world.

Five malaria parasites, namely *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae*, *Plasmodium knowlesi* and *Plasmodium falciparum* are transmitted to humans by female *Anopheles* mosquitoes (Cowman *et al.*, 2016). The last species accounts for the most malaria-associated deaths (WHO, 2019) and poses a great risk of severe clinical presentations after jeopardising the host erythrocytes (Talapko *et al.*, 2019). During the intraerythrocytic stage, haemozoin and other toxic factors produced by the parasite stimulate macrophages and other cells to produce cytokines and other soluble factors that trigger fever and rigours and influence other severe pathophysiological functions (Cowman *et al.*, 2016; Yusuf *et al.*, 2017).

The elimination of *Anopheles* mosquito breeding sites with insecticides, the prevention of mosquito-human contacts with insecticide-treated bed nets and the use of malaria rapid diagnostic tests significantly reduce the number of malaria cases (Gachelin *et al.*, 2018; Dhiman, 2019; Lechthaler *et al.*, 2019). In the absence of effective vaccines, the treatment measure is strengthened by the use of antimalarial drug therapies (Achan *et al.*, 2011; Guo, 2016; WHO, 2019). The decreased sensitivity of *P. falciparum* towards artemisinin-based combination therapies (ACTs) as the current front-line treatment has been reported in multiple locations of the Greater Mekong subregion (Noedl *et al.*, 2010; Tun *et al.*, 2016; He *et al.*, 2019; Tse *et al.*, 2019). This is thought to be associated with mutations in the region of the *pfKelch13* gene (He *et al.*, 2019). Therefore, finding new antimalarial agents, particularly with new mechanisms of action is urgently needed.

Medicinal plants have been studied for many years as a source of new antimalarial agents (Katiyar *et al.*, 2012; Rakotoarivelo *et al.*, 2015). Several phytochemicals of the alkaloids, terpenes and phenolic compounds groups with numerous varieties such as phenolic acids, flavonoids and tannins exhibit antimalarial properties (Muñoz *et al.*, 2000; Wink, 2012; Upadhyay *et al.*, 2013). Artemisinin, a sesquiterpene lactone isolated from the leaves of *Artemisia annua* is the example of the bioactive compound that has been commercially used as the antimalarial drug (Ashley & Phyo, 2018). Phenolic glycosides isolated from *Flacourtia indica* and polyphenolic flavonoid silymarin obtained from *Silybum marianum* have also been reported to possess antimalarial activity by inhibiting haem polymerisation activity to form haemozoin in the digestive vacuole of malaria parasite (Singh *et al.*, 2017; Mina *et al.*, 2020). Thus, the effect of phenolic-rich medicinal plants such as *Quercus*

infectoria against the malaria parasite can provide an insight into the discovery of new antimalarial drugs with novel mechanisms of action.

Q. infectoria is a plant with a long history of traditional uses for various ailments including fever treatment (Everest & Ozturk, 2005; Jamal *et al.*, 2011). The galls that also known as *biji manjakani* among Malaysians contain a high number of phenolics belonging to the compounds such as tannins, gallic acid, ellagic acid, pyrogallol, rutin and quercetin (Kheirandish *et al.*, 2016; Abdullah *et al.*, 2018; Tayel *et al.*, 2018), which might contribute to the antiparasitic activities (Sawangjaroen *et al.*, 2004; Sawangjaroen & Sawangjaroen, 2005; Ozbilgin *et al.*, 2013; Kheirandish *et al.*, 2016). In view of that, the present study was designed to determine the *in vitro* antimalarial activity and toxicity, to screen the phytochemical constituents of *Q. infectoria* gall crude extracts and to measure the pH of the digestive vacuole treated with the selected extract.

1.2 The rationale of the study

The crisis of antimalarial resistance not only complicates the management of malaria but also challenges the global elimination efforts to be achieved. Although the quest for new treatment regimens and development of the vaccine continues, none of them is readily available and licensed to be used to combat single drug- and multidrug-resistant malaria. Malaria is becoming harder to treat; therefore, the usage of an affordable medicinal plant with antimalarial properties is most welcome.

The discovery of potent antimalarial drugs coming from medicinal plants such as *Q. infectoria* galls is seen as a major approach to tackle the crisis of antimalarial resistance. Therefore, this is the first study that investigates the antimalarial activity of the medicinal plant, *Q. infectoria* galls. In an attempt to bridge the knowledge gap on the antimalarial study of the galls, toxicity and phytochemical screening have also been conducted as they play a significant role in determining the overall potential of the galls, providing an insightful view of the galls as a safer and selective antimalarial candidate. Additionally, as no investigations were conducted to explore the antimalarial effect of the galls, the effect of the galls on pH of the malaria parasite's digestive vacuole has also investigated. The inhibitory activities of the haemoglobin degradation and haem detoxification by many phenolic compounds from several plants (Mamede *et al.*, 2020; Tajuddeen & Van Heerden, 2019) could affect one of the physiological states of the digestive vacuole such as pH (Spiller *et al.*, 2002; Wunderlich *et al.*, 2012). The galls likely impaired proton pumps function that responsible for pH maintenance in the digestive vacuole, thereby altering the pH of the digestive vacuole. The outcomes could provide possible explanations on the antimalarial effect of the galls which can be used as the guideline for future investigation on the molecular mechanism underlying antimalarial action and further reflects the importance of the in-depth antimalarial investigation.

The overall goal of these efforts is to provide a basic understanding of the antimalarial effect of the galls. As pH regulation of the malaria digestive vacuole is an important indicator of the physiological state of the parasite and critically crucial for haemoglobin digestion and subsequent haem detoxification in the host's erythrocytes, this novel mechanism could be a possible approach in discovering new antimalarial

candidates. It could also further broaden the research on antimalarial drug discovery as well as add valuable insights to the existing knowledge on the mechanism of antimalarial action. Comprehensive understanding of the patterns and mechanisms of a potential plant on the malaria parasite will allow specific strategies to be tailored for the improvement of mode of antimalarial drugs action, as well as isolation and biosynthesis of valuable bioactive compounds.

1.3 Objectives of the study

1.3.1 General objective

The study was aimed to determine the *in vitro* antimalarial activity and toxicity of different extracts of *Q. infectoria* galls. The study also was aimed to determine the pH changes of the digestive vacuole following treatment with the selected gall crude extract.

1.3.2 Specific objectives

1. To determine the antimalarial activity of different gall crude extracts; acetone, methanol, ethanol and aqueous against the chloroquine-sensitive (3D7) strain of *P. falciparum*.
2. To evaluate the toxicity of the gall extracts on normal mouse fibroblast cell line (NIH/3T3), normal African green monkey kidney epithelial cell line (Vero), normal human umbilical vein endothelial cell (HUVEC), brine shrimps and human erythrocytes (A^+, B^+, AB^+ and O^+ blood groups).

3. To determine the content of heavy metals of lead (Pb), zinc (Zn), chromium (Cr), copper (Cu) and cadmium (Cd) in the gall extracts.
4. To screen the phytochemical constituents of the gall extracts.
5. To measure the pH of the digestive vacuole following treatment with the selected gall extract.

1.4 Hypothesis of the study

1. *Q. infectoria* gall crude extracts have promising antimalarial activity against the malaria parasite.
2. *Q. infectoria* gall crude extracts have non-toxic effect on the normal cells, brine shrimps and normal erythrocytes and low concentration of heavy metals based on the permissible limit.
3. *Q. infectoria* gall crude extracts are rich with phenolic compounds, which could be associated with antimalarial effect.
4. Selected *Q. infectoria* gall crude extract alters the pH of the parasite's digestive vacuole, leading to the parasite death.

1.5 Experimental design

The overall flow of the study, starting with the authentication of *Q. infectoria* galls at the Natural Medicinal Products Centre (NMPC), Universiti Islam Antarabangsa Malaysia (UIAM) was summarised in Figure 1.1. The galls were macerated using solvents with different polarity (acetone, methanol, ethanol and aqueous) to produce four different crude extracts.

The antimalarial activity of the gall crude extracts was determined using a malarial SYBR Green-I fluorescence-based (MSF) assay. Parasite cultures predominantly at the ring stage were synchronised with sorbitol before treatment with the gall extracts at different concentrations for 48 hours. The SYBR Green-I solution (2× final concentration from 10 000× stock concentration) was added to the parasite suspensions after 48 hours of treatment and the fluorescence signal was measured (excitation and emission wavelengths at 490 nm and 530 nm, respectively) and analysed to determine the concentration of the gall crude extracts that inhibits parasite population at half of maximum response (IC_{50}). Giemsa-stained thin blood smears were also prepared at 24- and 48-hour post-treatment to observe the morphology of treated parasites.

The cytotoxicity of the gall crude extracts was evaluated using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Normal mouse embryo fibroblast cell line (NIH/3T3), normal African green monkey kidney epithelial cell line (Vero) and normal human umbilical vein endothelial cell (HUVEC) were treated with the gall extracts at different concentrations for 72 hours before addition of MTT tetrazolium salt solution (0.4 mg/mL final concentration). The absorbance was measured at 570 nm and analysed to determine the concentration of the gall extracts that causes the reduction of cell viability by 50% (CC_{50}). The selectivity index (SI) was calculated using a ratio of the CC_{50} to the IC_{50} obtained from the antimalarial assay.

A brine shrimp lethality test (BSLT) was conducted as another cytotoxicity testing of the gall extracts. The gall extracts at different concentrations were tested for

their toxicity on mature brine shrimps for 24 hours. The percentage of mortalised shrimps was calculated to determine the lethality concentration that kills 50% of the shrimp population (LC_{50}).

The cytotoxicity of the gall crude extracts was further investigated on normal human erythrocytes using a haemolytic assay. Washed erythrocytes (2% haematocrit) from different blood groups (A^+, B^+, AB^+ and O^+) were treated with the gall extracts at different concentrations for 45 minutes. The absorbance of haemoglobin in the supernatants was measured at 450 nm and the results were recorded as the percentage of haemolysis (%). In addition to the haemolytic assay, the antioxidant assay was performed to determine whether the antioxidant activity of the gall extracts was associated with their haemolytic effect. The gall extracts at different concentrations were exposed with a free radical 2,2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) solution and the absorbance was measured at 517 nm to determine the effective concentration requires to reduce 50% of free radical DPPH (EC_{50}).

The presence of heavy metals in the gall crude extracts was determined using atomic absorption spectroscopy (AAS). Lead (Pb), zinc (Zn), chromium (Cr), copper (Cu) and cadmium (Cd) were identified in the powdered form of the gall extracts and raw galls. The concentration of the heavy metals was compared with the permissible level commenced by WHO guidelines.

Screening of different classes of phytochemicals such as phenolics, flavonoids, tannins, alkaloids and saponin in the gall crude extracts was carried out. The total phenolic content (TPC) and total flavonoid content (TFC) of the gall crude

extracts were also determined using Folin-Ciocalteu and aluminium chloride colourimetric methods, respectively.

The effect of the crude extract, which exhibited a promising antimalarial activity and acceptable toxicity, on the digestive vacuole pH was investigated using a flow cytometry-based assay. The pH of the digestive vacuole was measured by the use of ratiometric pH indicator, the fluorescein isothiocyanate (FITC)-dextran incorporated into resealed erythrocytes via hypotonic dilution technique. A pH calibration curve was generated by incubating resealed erythrocytes in buffers at different pH in the presence of an ionophore, carbonyl cyanide-m-chlorophenylhydrazone (CCCP) to equilibrate the pH of the erythrocyte compartments with the pH of the buffers. Ratios of the fluorescence intensity detected at two different wavelengths (530 and 585 nm) were plotted on a pH calibration curve.

Synchronised mature stage parasites were harvested using MACS columns and inoculated with resealed erythrocytes preloaded with FITC-dextran to initiate infection. As the parasite matures, the probe along with haemoglobin was endocytosed and eventually accumulated in the digestive vacuole of the trophozoite stage parasites. Mid trophozoite stage parasites (~34-hour post-invasion) examined using Giemsa-stained thin blood smears were adjusted to 5% parasitaemia (2% haematocrit) before treatment with the crude extract at different concentrations for 4 hours. The parasites were permeabilised with saponin (0.035% w/v) to release FITC-dextran in the host cell cytoplasm, allowing only FITC-dextran entrapped in the digestive vacuole to be measured by flow cytometry.

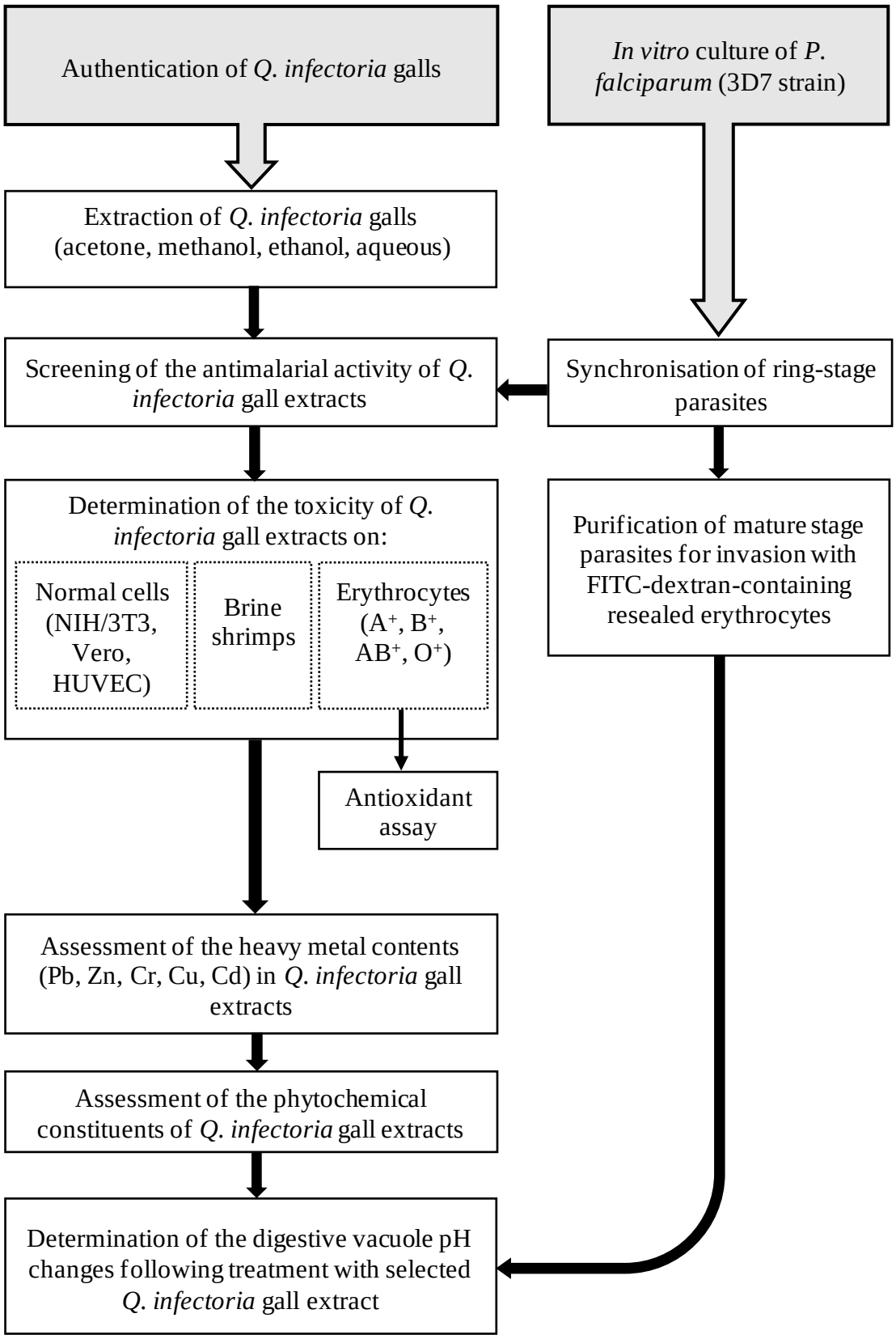


Figure 1.1: Flowchart of the experiments carried out through all the study

CHAPTER 2

LITERATURE REVIEW

2.1 History of malaria

Malaria is a parasitic disease that causes a serious burden in the world. It was initially described as a disease with high periodic fever originated from swampy air (Arifin *et al.*, 2016; Barnett, 2016; Talapko *et al.*, 2019). Others claimed that malaria was caused by a bacterium, *Bacillus malariae* until Charles Louis Alphonse Laveran (a French army physician) discovered the malaria parasite in the blood specimen in 1880 (Arifin *et al.*, 2016). Six years later, the Italian physiologist, Camilo Golgi identified several *Plasmodium* species. In 1897, Ronald Ross (a surgeon) observed that malaria was transmitted via mosquitoes and later Giovanni Battista Grassi (an Italian professor) demonstrated that female *Anopheles* mosquitoes could transmit malaria to humans. From here, many studies to control and prevent the disease have emerged.

2.2 Statistics of malaria

In 2018, the World Health Organization (WHO) reported 228 million cases of malaria with global 405 000 deaths (WHO, 2019). The highest number of malaria cases and deaths was recorded in the African region, followed by the Southeast Asia, Eastern Mediterranean, Western Pacific and American regions (Figure 2.1). Malaysia is one of the countries who has achieved zero indigenous cases of human malaria in

2018, followed by China with a second consecutive year of zero indigenous cases. In Malaysia, although the malaria cases were reduced from 5194 to 514 cases in 2010 and zero indigenous cases in 2018, the malaria-related deaths have still failed to reduce since 2010 (Figure 2.2).

Four species of human malaria parasites are found in Malaysia, i.e. *P. vivax, P, malariae, P. knowlesi* and *P. falciparum* (Cowman *et al.*, 2016; Davidson *et al.*, 2019; Talapko *et al.*, 2019). *P. falciparum* accounts for the most malaria cases and deaths, and poses a great risk of severe clinical presentations (Talapko *et al.*, 2019; WHO, 2019). *P. vivax*, formerly recognised as one of the main causes of human malaria in Malaysia has now been replaced by zoonotic *P. knowlesi*, which naturally occurs in macaques (Lim *et al.*, 2017; Davidson *et al.*, 2019). The increasing cases of *P. knowlesi* malaria have been reported among aborigines practising forestry and peasant lifestyle in Malaysian Borneo and Peninsular Malaysia (Jeffree *et al.*, 2018). Meanwhile, *P. ovale* rarely causes malaria in Malaysia, as it is widely distributed in Sub-Saharan Africa (Lim *et al.*, 2017).

2.3 Life cycle of the malaria parasite

2.3.1 Sexual cycle of the malaria parasite

Transmission of the malaria parasites from an infected human to a mosquito is mediated through sexual stage parasites called gametocytes (Figure 2.3) (Lim *et al.*, 2017; Messina *et al.*, 2018; Usui *et al.*, 2019; Venugopal *et al.*, 2020). A female *Anopheles* mosquito takes up the gametocytes during blood-feeding. The ga-

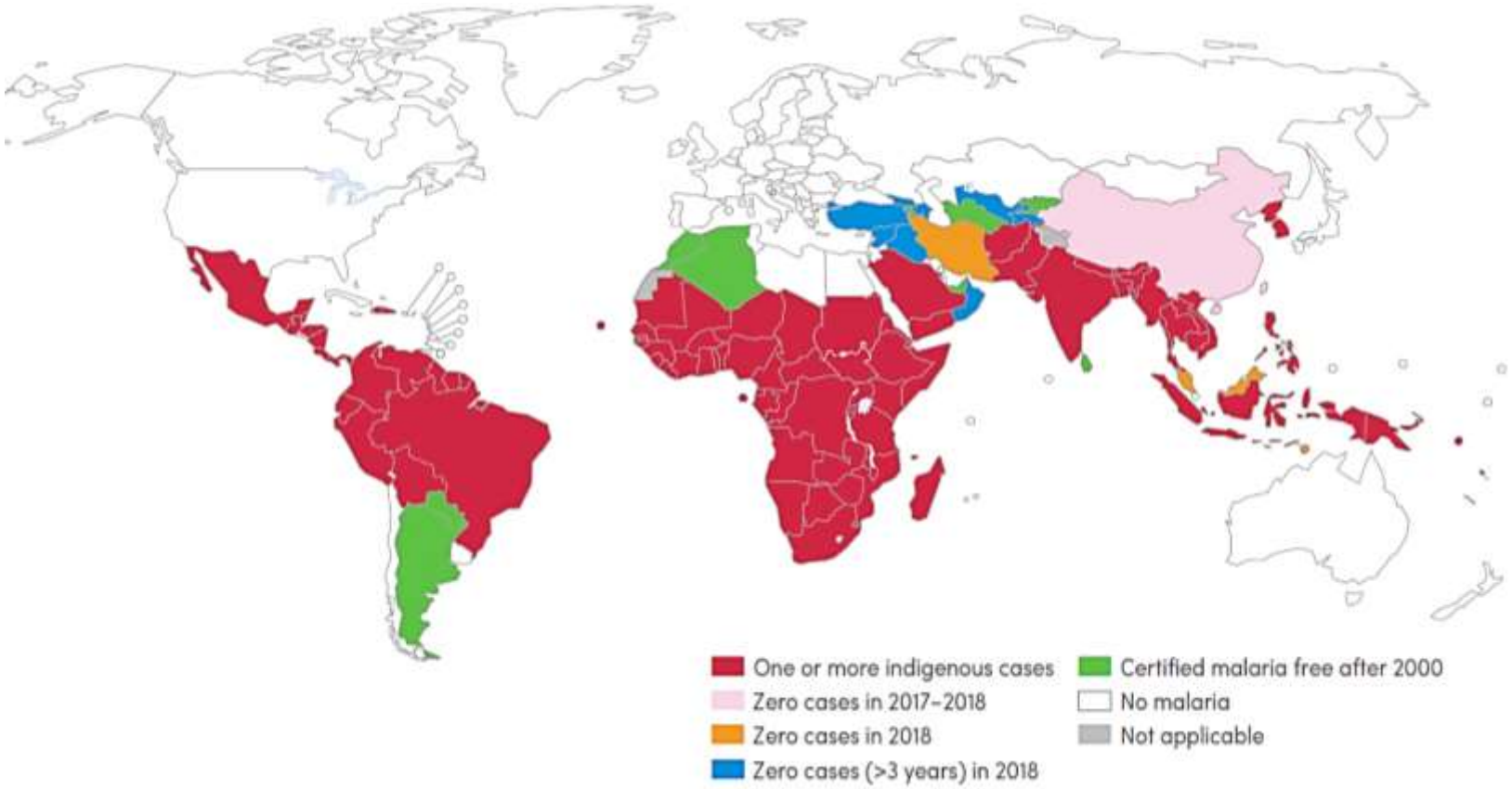
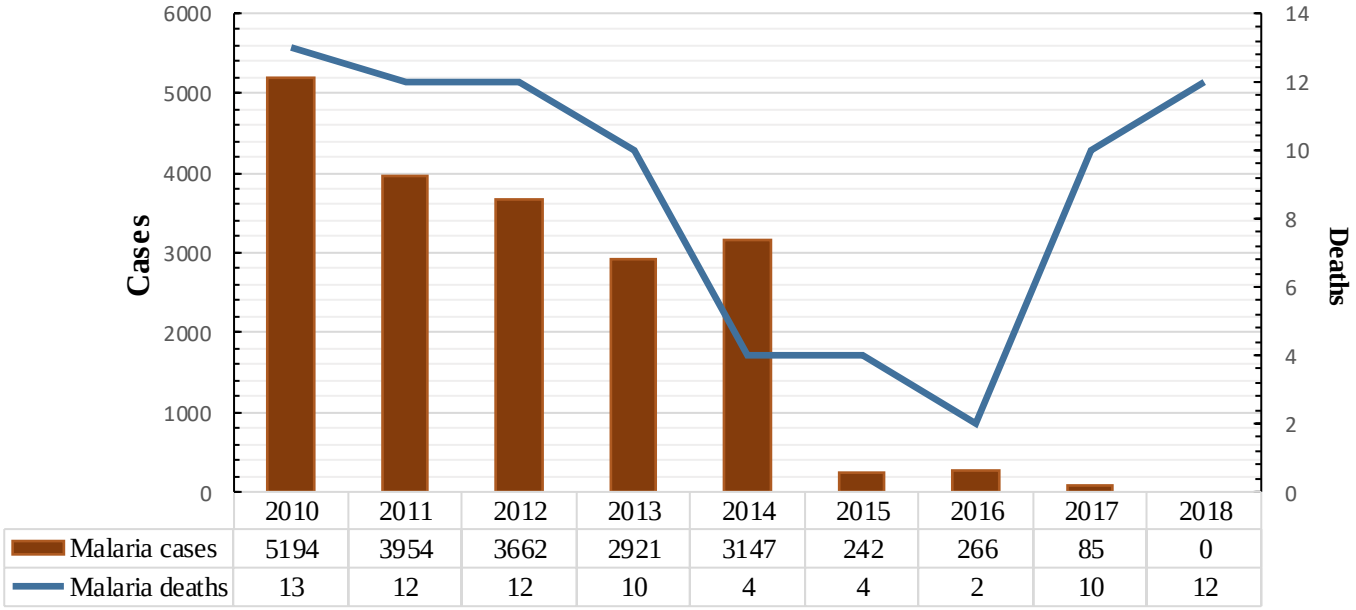


Figure 2.1: The prevalence of malaria projected by WHO in 2018

Four WHO regions that are significantly affected by malaria are African, Southeast Asia, Eastern Mediterranean and Western Pacific. European region remains to record zero indigenous malaria cases in 2018, while several countries in America and Western Pacific including Malaysia are reported to have zero indigenous cases in 2018. Modified from WHO (2019).



	2010	2011	2012	2013	2014	2015	2016	2017	2018
Malaria cases	5194	3954	3662	2921	3147	242	266	85	0
Malaria deaths	13	12	12	10	4	4	2	10	12

Figure 2.2: Malaria cases and deaths in Malaysia from 2010 - 2018

Malaysia reported decreasing cases of malaria from 5194 cases in 2010 to 242 cases in 2015 and zero indigenous cases in 2018. However, the increasing number of deaths was reported from 2017-2018. Adapted from WHO (2019).

-metocytes undergo gametogenesis within the mosquito's midgut, where the male microgametocyte divides into up to eight flagellated microgametes and the female macrogametocyte develops into a single macrogamete. The microgamete and macrogamete fuse to form a zygote. The zygote undergoes meiosis and develops into a motile ookinete. The ookinete migrates through the midgut epithelium and transforms into an oocyst (Figure 2.3A). The oocyst replicates to form thousands of sporozoites, which migrate and invade the mosquito's salivary glands before infecting a new human host during a blood meal (Figure 2.3B).

2.3.2 Asexual cycle of the malaria parasite

Sporozoites are transmitted into a human during a blood meal of an infected mosquito (Figure 2.4A) (Cowman *et al.*, 2016; Talapko *et al.*, 2019; Venugopal *et al.*, 2020). The sporozoites are taken into the liver to initiate the exoerythrocytic stage of infection (Figure 2.4B). The parasite invades the hepatocyte and subsequently produces many daughter hepatic merozoites (Figure 2.4C). The merozoites penetrate erythrocytes to start the intraerythrocytic stage of infection (Figure 2.4D). *P. falciparum* develops into distinct morphological stages around 48 hours from a young ring to a mature trophozoite and a multinucleated schizont before releasing daughter merozoites.

The ring stage parasites have a delicate cytoplasm with one or two chromatin dots observed in Giemsa-stained blood smears (Figure 2.5A) (Voulgaridi *et al.*, 2016; Mahon & Lehman, 2019). Multiple infections are commonly seen and occasional appliqué forms (a parasite appearing on the erythrocyte periphery) can be

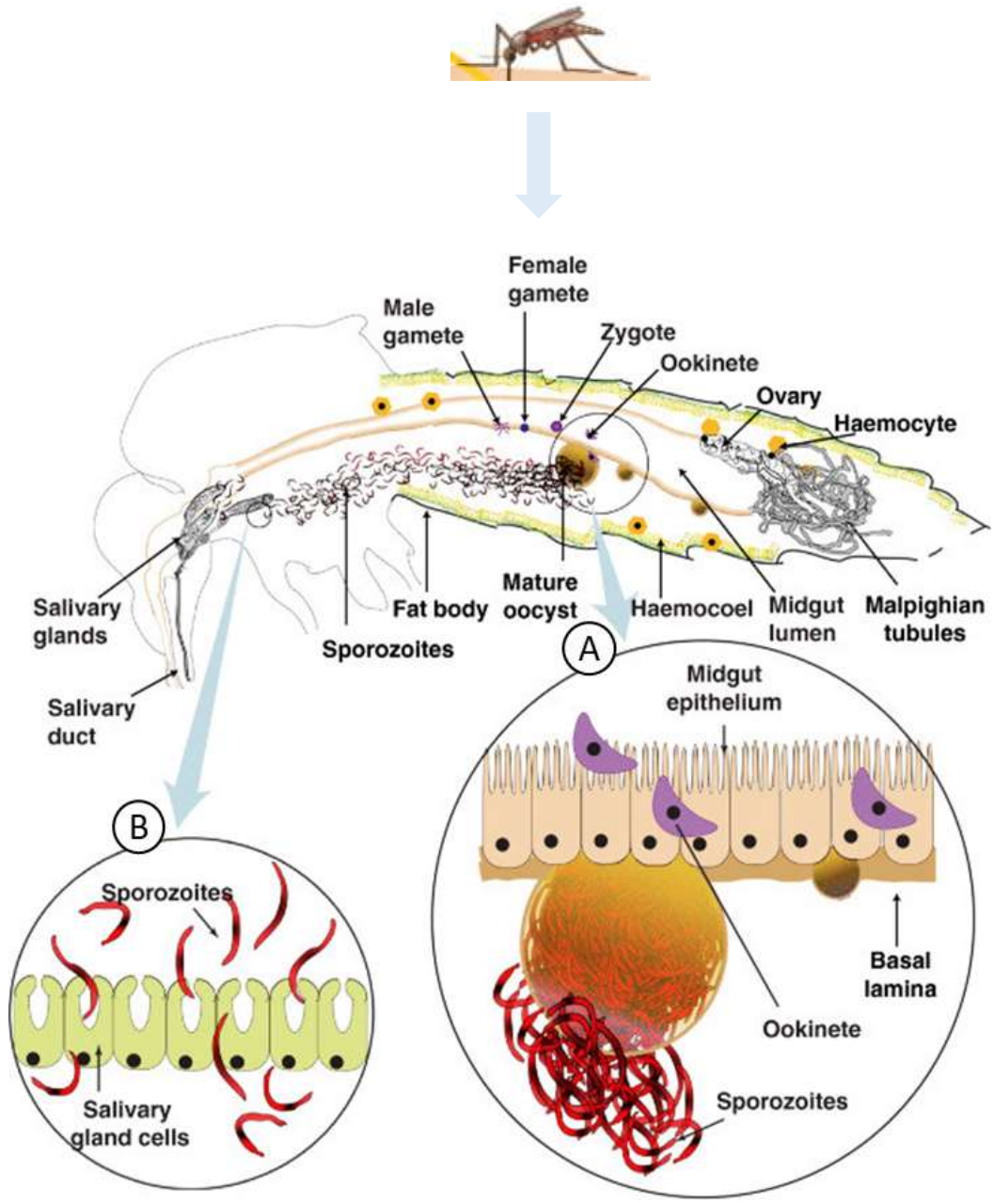


Figure 2.3: The sexual reproduction of the malaria parasite within a mosquito vector

Upon ingestion by a mosquito, the male gamete fuses with the female gamete to form a motile zygote. (A) The zygote undergoes several developmental transformations into an ookinete and then into an oocyst that matures beneath the basal lamina of the midgut's epithelium. (B) The oocyst gives rise to infective sporozoites, which travel to the mosquito's salivary glands, where they are transmitted to a new human host. Adapted from Sreenivasamurthy *et al.* (2013).

present. A dense cytoplasm of the parasite and yellow malarial pigment (haemozoin) are clearly observed in the trophozoite stage (Figure 2.5B). At this stage, haemoglobin is actively metabolised to support the parasite growth and development before progression into the multinucleated schizont (see section 2.4 for details). The schizont bursts releasing 16-32 merozoites to initiate a new cycle of infection (Figure 2.5C). Patients will develop fever, rigours and other severe pathophysiologic conditions due to the stimulation of macrophages and other cells to produce cytokines and other soluble factors by haemozoin and other toxic factors (Cowman *et al.*, 2016; Yusuf *et al.*, 2017).

2.4 Haemoglobin metabolism in the malaria parasite

P. falciparum grows within the host erythrocyte and metabolises up to 80% of the host cell haemoglobin as a source of nutrients and energy (Ginsburg, 2016; Lee *et al.*, 2018). The haemoglobin metabolism involves the breakdown of haemoglobin into haem and globin and the build-up of crystalline non-toxic haemozoin; all of which occur during the intraerythrocytic cycle of the parasite (Ginsburg, 2016; Goldberg & Sigala, 2017). Specifically, this intricate metabolism is regulated by coordinated pathways right from haemoglobin ingestion, to haemoglobin transport, haemoglobin digestion and haem or haematin detoxification.

2.4.1 Haemoglobin ingestion by the malaria parasite

Several mechanisms have been suggested for the internalisation of haemoglobin by the malaria parasite, such as cytostome-dependent endocytosis (Abu-

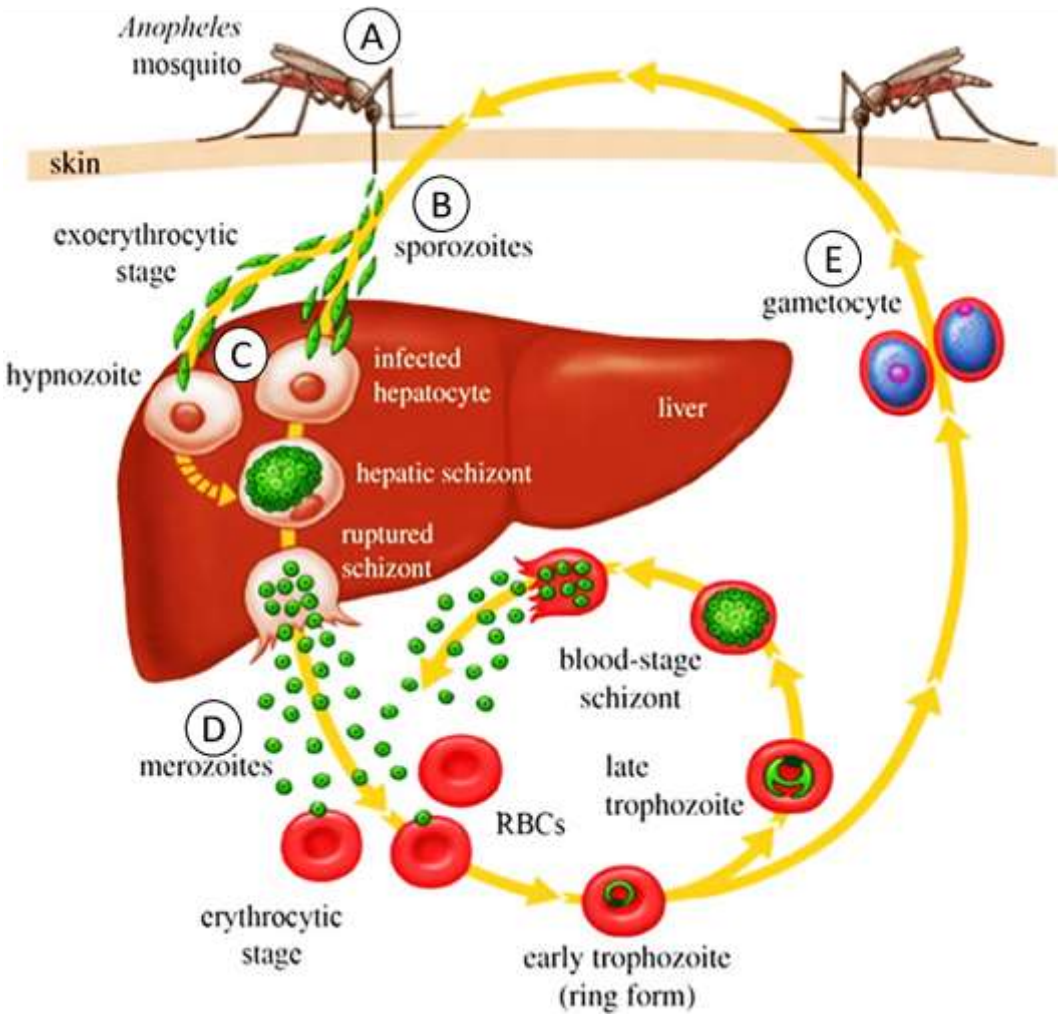


Figure 2.4: The asexual reproduction of the malaria parasite within a human host

(A) A female *Anopheles* mosquito transmits sporozoites into a human. (B-C) The sporozoites enter the liver and jeopardise the hepatocytes before releasing hepatic merozoites. (D) The merozoites infect the erythrocytes and progress into ring, trophozoite and schizont stages. Merozoites released after the schizont rupture infect other erythrocytes to repeat the intraerythrocytic cycle. (E) Some merozoites develop into gametocytes, which are taken up by a mosquito to continue the sexual reproduction, thus completing the parasite's life cycle. Modified from Hill (2011).

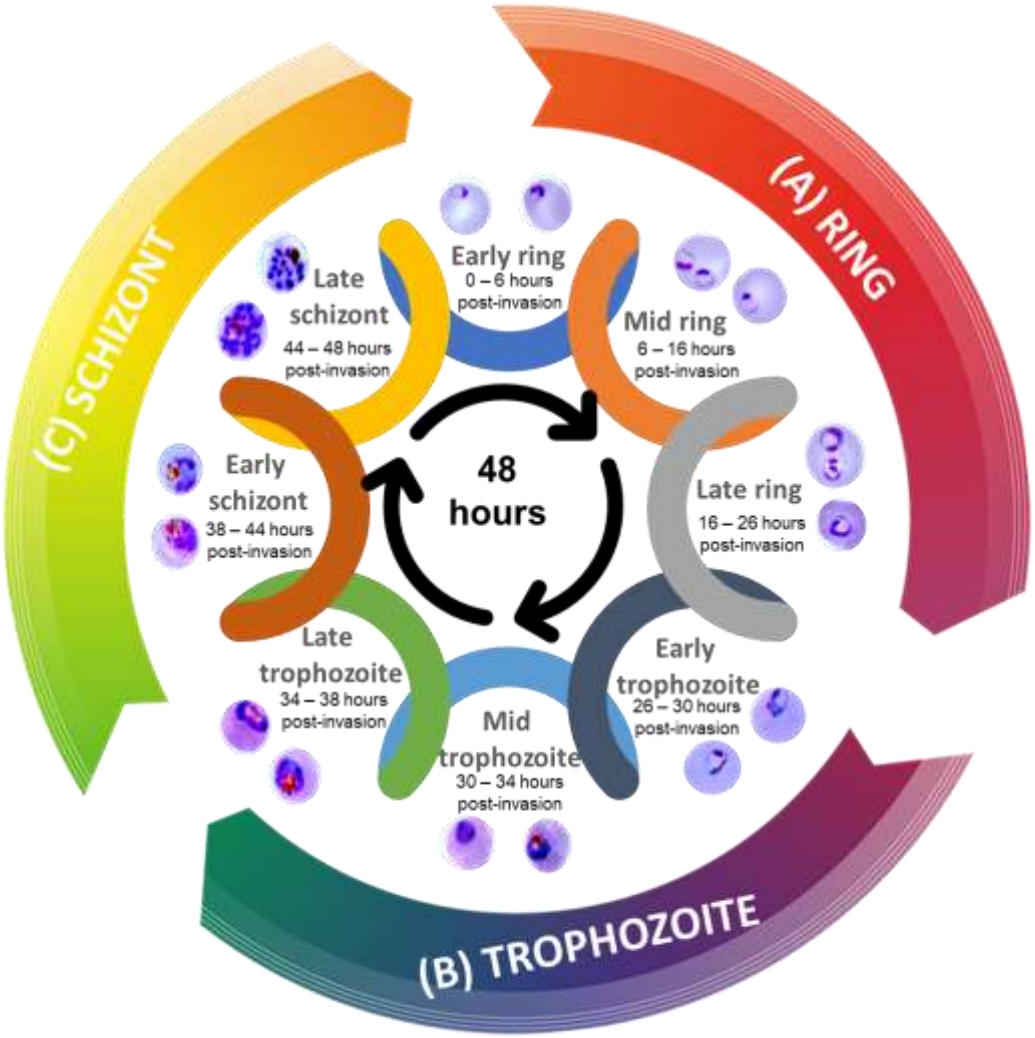


Figure 2.5: Intraerythrocytic stages of *P. falciparum*

During the intraerythrocytic cycle, the parasite differentiates into (A) a ring (B), a trophozoite and (C) a schizont that filled with merozoites. Modified from "Severe malaria" (2014).

Bakar *et al.*, 2010; Milani *et al.*, 2015). Cytostome-dependent endocytosis is a major mechanism for the uptake of haemoglobin that commences early in the parasite's intraerythrocytic development. It is also the principal pathway in the mature trophozoite stage (Figure 2.6A) (Milani *et al.*, 2015; Wunderlich *et al.*, 2012). Cytostomes are double-membrane invaginations of the parasitophorous vacuolar membrane and the parasite plasma membrane that are morphologically distinguished by the presence of electron-dense material at the interface of the parasitophorous vacuolar membrane and parasite plasma membrane neck when observed under the serial thin-section electron microscope (Wunderlich *et al.*, 2012; Goldberg & Zimmerberg, 2020; Matz *et al.*, 2020).

2.4.2 Haemoglobin transport by the malaria parasite

Pinching off at the neck of cytostomes leads to the formation of small vesicles containing haemoglobin that is surrounded by two membranes: the outer membrane derived from the parasite plasma membrane and the inner membrane derived from the parasitophorous vacuolar membrane (Figure 2.6B) (Milani *et al.*, 2015; Goldberg & Zimmerberg, 2020). The cytostome-derived haemoglobin-filled vesicles use an actin-myosin motor system to deliver haemoglobin to the acidic digestive vacuole for degradation by proteases. The outer membrane of the vesicles fuses with the plasma membrane of the digestive vacuole, resulting in the delivery of single-membrane haemoglobin-filled vesicles to the digestive vacuole (Figure 2.6C). The digestive vacuole is identifiable by light or electron microscopy due to the presence of inert haemozoin (Kapishnikov *et al.*, 2017; Pisciotta *et al.*, 2017).

2.4.3 Haemoglobin digestion by the malaria parasite

Haemoglobin is degraded primarily in the digestive vacuole of the malaria parasite by the action of proteases known as aspartic proteases (plasmepsins), cysteine proteases (falcipains) and metalloproteases (falcilysins) (Figure 2.6D) (Siklos *et al.*, 2015; Ponsuwanna *et al.*, 2016; Mishra *et al.*, 2019). Initially, plasmepsins, i.e. PfPM1, PfPM2, PfHAP and PfPM4 and falcipains, i.e. PfFP-2, PfFP-2' and PfFP-3, which function in an acidic pH of 4.0 and 5.5, respectively are responsible for cleaving haemoglobin into oligopeptides and further digested into smaller peptides by falcilysins (Bonilla *et al.*, 2007; Moura *et al.*, 2009; Xie *et al.*, 2016). Inhibition of one of the proteases by leupeptin or E-64 (calpain inhibitor *N*-acetyl-leucinyl-leucinyl-norleucinal) and disruption of the PfFP-2-encoded gene caused incomplete digestion of haemoglobin in the digestive vacuole (Wunderlich *et al.*, 2012; Siklos *et al.*, 2015). Inhibition of PfPM1 and PfPM2 appeared to affect only on ring stage parasites, indicating that each of the proteases is expressed at different stages of parasite development (Liu *et al.*, 2015).

2.4.4 The digestive vacuole of the malaria parasite

Plasmepsins and falcipains work optimally in the pH range of 4.0-5.5, which are the physiological pH of the digestive vacuole (Abu-Bakar, 2015; Liu *et al.*, 2015; Ibrahim & Abu-Bakar, 2019). The pH regulation of digestive vacuole has been demonstrated to rely on the action of proton pumps, i.e. vacuolar-type proton pump ATPase (V-type H^+-ATPase) and vacuolar-type proton pump pyrophosphatase

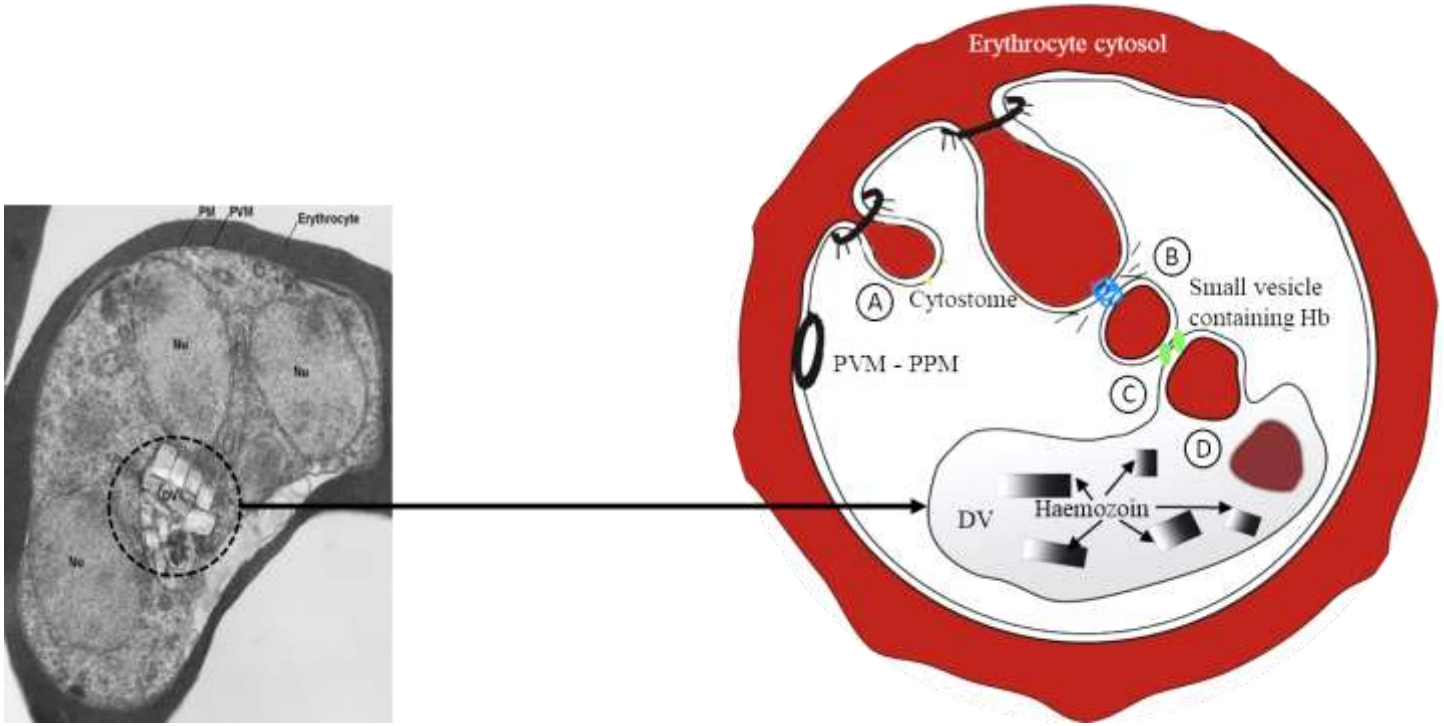


Figure 2.6: The schematic representation of the haemoglobin ingestion, transport and digestion by *P. falciparum*

(A) The malaria parasite engulfs the host erythrocyte cytoplasm by means of a cytostomal system that arises from the PVM and PPM interface. (B-C) The cytostome buds and forms a small vesicle before being transported to and fused with the digestive vacuole in which haemoglobin is digested. (D) Haemoglobin is degraded by proteases (i.e. plasmepsins and falcipains), producing globin and haem, which is then detoxified into a malarial pigment, haemozoin. Abbreviations: PVM, parasitophorous vacoular membrane; PPM, parasite plasma membrane; Hb, haemoglobin. Modified from Wunderlich *et al.* (2012) and Milani *et al.* (2015).

(V-type H^+-PPase) that are located at the vacuole's membrane (Figure 2.7) (Collins & Forgac, 2018; Dennis *et al.*, 2018; Segami *et al.*, 2018). The inhibition of V-type H^+-ATPase using the specific V-type H^+-ATPase inhibitors, i.e. concanamycin A, bafilomycin A1 and N-ethylmaleimide resulted in the alkalinisation of the digestive vacuole and the acidification of the parasite cytosol, leading to parasite death (Forgac, 2018; Tang *et al.*, 2019). The activity of V-type H^+-PPase was inhibited by the pyrophosphate analogues, i.e. aminomethylenediphosphonate, imidodiphosphate and sodium fluoride (Asaoka *et al.*, 2016; Segami *et al.*, 2018). Therefore, the digestive vacuole is essential for parasite growth and survival, and might represent a vulnerable target for future antimalarial drugs.

2.4.5 Measurement of the pH of the digestive vacuole

The pH of the malaria parasite's digestive vacuole has been extensively studied to understand the haemoglobin digestion and haem detoxification (Spiller *et al.*, 2002; Klonis *et al.*, 2007; Moura *et al.*, 2009), the mechanism of action of antimalarial drugs (Tang *et al.*, 2019; Ibrahim *et al.*, 2020) and the development of the malaria parasite resistance to antimalarial drugs (Homewood *et al.*, 1972; Kirk & Saliba, 2001; Saliba *et al.*, 2003). Given the diverse studies on the important roles of the digestive vacuole pH, methods on using pH-sensitive ratiometric fluorophores have been widely utilised for the quantification of digestive vacuole pH (Grillo-Hill *et al.*, 2014; Abu-Bakar, 2015; Chen *et al.*, 2019; Chávez *et al.*, 2020).

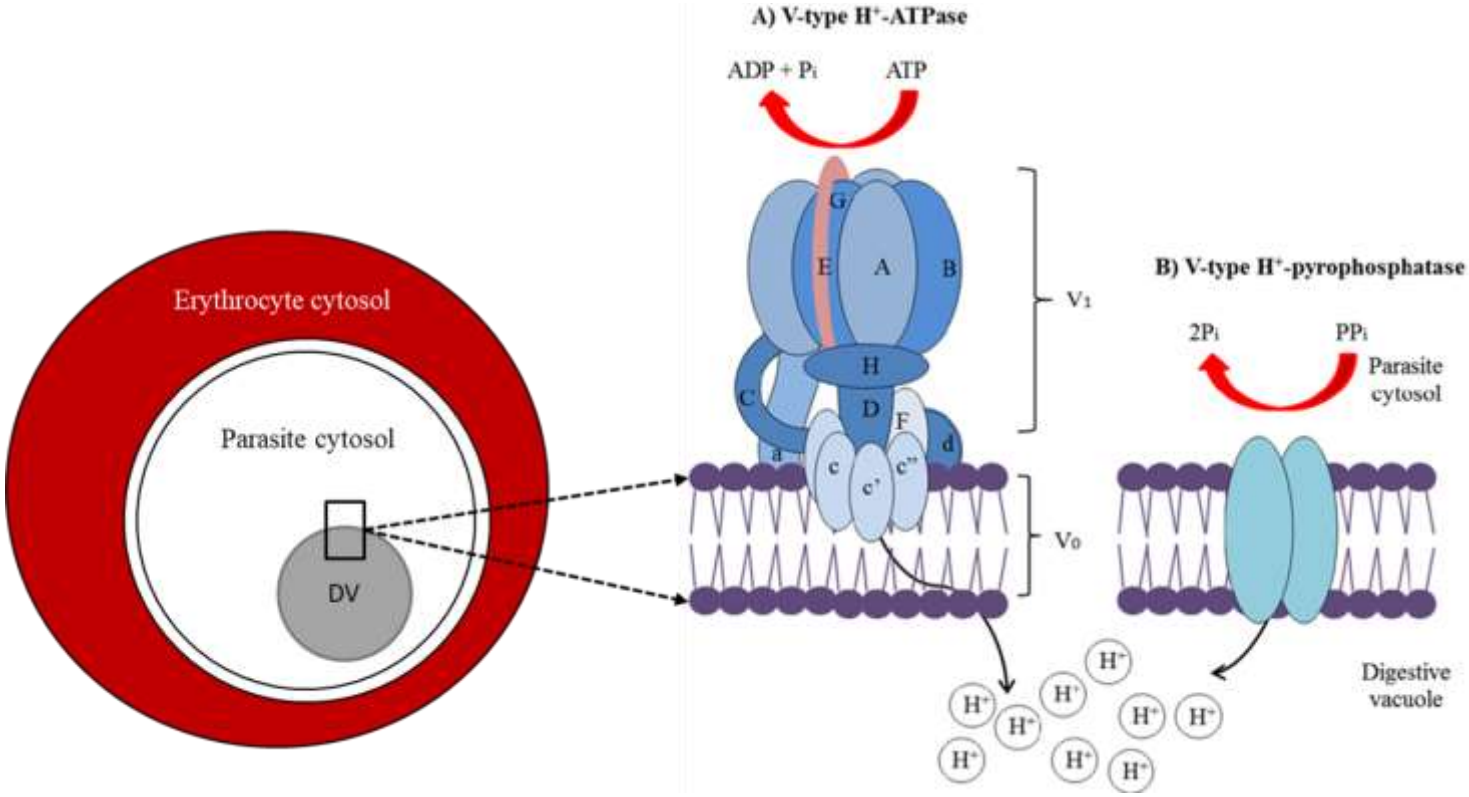


Figure 2.7: The schematic diagram of the proton pumps at the digestive vacuole's membrane

The pH regulation of the digestive vacuole is maintained by two types of proton pumps; A) V-type H^+-ATPase and B) V-type H^+-PPase. The V-type H^+-ATPase uses the energy from the hydrolysis of ATP, while the V-type H^+-PPase utilises the energy from the hydrolysis of PPi to transport H^+. ATP: adenosine triphosphate, PPi: pyrophosphate. Modified from Tresguerres (2016) and Baykov *et al.* (2013).