PHYTOCHEMICAL STUDY, ANTI-TOXOPLASMOSIS AND ANTIMALARIAL ACTIVITIES OF TINOSPORA CRISPA MIERS METHANOL EXTRACT AND ITS BIOACTIVE FRACTIONS

LEE WEI CAI

UNIVERSITI SAINS MALAYSIA

2015

PHYTOCHEMICAL STUDY, ANTI-TOXOPLASMOSIS AND ANTIMALARIAL ACTIVITIES OF TINOSPORA CRISPA MIERS METHANOL EXTRACT AND ITS BIOACTIVE FRACTIONS

By

LEE WEI CAI

Thesis submitted in fulfilment of the requirement for the Degree of Master of Science

February 2015

This thesis is dedicated to.....

My parent

ACKNOWLEDGEMENT

First and foremost, I would like to express my sincere gratitude to my loving parents who have constantly been giving support and encouragement the entire time. I would like to convey my deep appreciation to my supervisor, Associate Prof. Dr. Roziahanim Mahmud, for her continuous support, guidance, knowledge and invaluable encouragement from the beginning to the final stage of my study.

My sincere thanks to my co-supervisor, Associate Prof. Dr. Sabariah Ismail and field supervisor, Associate Prof. Dr. Rusliza Basir, for their advice and assistance throughout my study and to Professor Rahmah Noordin for her kind guidance and advice for my *in vitro* studies.

I would like to express my gratitude to the Dean of School of Pharmaceutical Sciences, Universiti Sains Malaysia for giving me an opportunity and support to further my MSc study as a full time research student.

I also would like to thank my dear colleagues Ms. Shanmugapriya Perumal, Mr. Suthagar Pillai Piaru, Miss Lee Yuan-E for their invaluable help, support and encouragement which made the work possible. I would also like to extend my gratitude to scientific officers and lab assistants of School of Pharmaceutical Sciences for their invaluable help in instrumentation and phytochemical analysis.

The financial support of the FRGS research grant (No: 203/PFARMASI/6711189) from MOHE and USM Short-Term Research Grant (304/PFARMASI/6311013) are gratefully acknowledged. I would like to acknowledge MOSTI's MyBrain15 (MyMaster) scholarship for providing financial assistance to complete my studies.

Finally, I would like to thank all my family members and friends for their endless support and understanding throughout my study.

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

µg/mL	microgram per millilitre
ACN	Acetonitrile
AIDS	Acquired immunodeficiency syndrome
$AlCI_3.H_2O$	Aluminium chloride
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ATCC	American Type & Culture Collection
ATR	Attenuated Total Reflectance
BHT	tert-butylated hydroxytoulene
CAT	Catechin equivalents
CDC	Centers for Disease Control and Prevention
CGM	Complete Growth Medium
CNS	Central nervous system
CO_2	Carbon dioxide
DAD	Photodiode array detector
dd H_2O	Doubly-distilled water
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-diphenyl-1-picrylhydrazyl
EC_{50}	Median effective concentration
ESI	Electrospray ionization
FBS	Foetal bovine serum
$FeCl_3$	Ferric chloride
FTIR	Fourier Transform Infra-Red
g	Gram
GAE	Gallic acid equivalent
GCMS	Gas chromatography-mass spectrometry
GSH	Glutathione
GSH-Px	Glutathione peroxidase
h	Hour
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
IC_{50}	Inhibitory concentration of 50 %
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
i.p.	Intraperitoneal
$K_3Fe(CN)_6$	Potassium ferricyanide
LCMS	Liquid chromatography-mass spectrometry
LDH	Lactate dehydrogenase
mg	Milligram
mg/mL	Milligram per millilitre
min	Minutes
mL	Millitre
MIP-1α	Macrophage inflammatory protein-1 alpha

MIP-1β	Macrophage inflammatory protein-1 beta
MS	Mass spectrometry
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MTT	3-(4,5-Dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide
NaCl	Sodium chloride
Na_2CO_3	Sodium carbonate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide
$NaNO_2$	Sodium nitrate
nm	Nanometer
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PGE_2	Prostaglandin E_2
PRBCs	Parasitized red blood cells
Prep LC	Preparative liquid chromatography
PS	Penicillin-streptomycin solution
psi	Pound per square inch
R_f	Retention factor
RBCs	Red blood cells
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RPM	Revolution per minute
SEM	Standard error of the means
SI	Selectivity index
SOD	Superoxide dismutase
TFC	Total flavonoids content
TIC	Total ion counting
TLC	Thin layer chromatography
TNF-α	Tumour necrosis factor-alpha
TPC	Total phenolic content
UV-vis	Ultra violet-visible
WHO	World Health Organization
5-HT	5-hydroxytryptamine

LIST OF APPENDICES

KAJIAN FITOKIMIA, AKTIVITI ANTI-TOKSOPLASMOSIS DAN AKTIVITI ANTIMALARIA EKSTRAK METANOL TINOSPORA CRISPA MIERS DAN FRAKSI BIOAKTIFNYA

ABSTRAK

Toksoplasmosis dan malaria merupakan dua penyakit serius yang mengakibatkan bebanan terhadap kesihatan manusia. Pada masa kini, tiada banyak kemajuan yang dicapai bagi pembangunan agen-agen kimoterapeutik untuk rawatan toksoplasmosis dan malaria. Agen-agen kimoterapeutik semasa telah digunakan selama beberapa dekad dan parasit telah mencapai rintangan terhadap ubat-ubatan semasa. Secara tradisi, *Tinospora crispa* Miers telah digunakan dengan meluas untuk merawat malaria, demam dan penyakit-penyakit parasit lain yang berkaitan. Dalam kajian ini, ekstrak mentah metanol dan fraksi-fraksi bioaktif *Tinospora crispa* Miers dikaji untuk aktiviti-aktiviti anti-parasitik terhadap *Toxoplasma gondii* dan *Plasmodium berghei*, kajian fitokimia dan aktiviti anti-oksida. Ekstrak mentah telah diekstrak daripada batang *Tinospora crispa* Miers menggunakan kaedah rendaman dan dipencilkan kepada kumpulan komponen yang kecil dengan menggunakan teknik kromatografi lajur dengan pengelusi sistem pelarut heksana-aseton. Aktiviti sitotoksik ekstrak mentah dan kesemua fraksi yang mempunyai kekutuban berbeza (F1 hingga F8 masing-masing) telah ditentukan dengan *in vitro* assai MTT ke atas sel Vero. Kajian ini mendapati bahawa ekstrak mentah dan kesemua fraksi menunjukkan tiada aktiviti sitotoksik ke atas sel Vero. Ekstrak mentah dan kesemua fraksi telah dikaji selanjutnya untuk aktiviti anti-parasitik terhadap *Toxoplasma gondii*. Ekstrak mentah menunjukkan aktiviti perencatan terhadap *Toxoplasma gondii* dengan nilai EC_{50} 7.83 ± 0.12 µg/mL. Daripada kesemua fraksi, fraksi F3 dan fraksi F4

menunjukkan aktiviti paling bioaktif terhadap *T. gondii* dengan nilai EC_{50} 6.77 ± 0.03 µg/mL dan 6.04 ± 0.02 µg/mL, masing-masing. Siasatan lanjut untuk aktiviti antimalaria terhadap *Plasmodium berghei* dalam model tikus secara *in vivo* dijalankan dengan ekstrak mentah dan dua fraksi poten F3 dan F4 yang telah ditentukan daripada kajian *in vitro*. Tikus dijangkiti dengan malaria yang menerima dos rawatan 100 mg/kg berat haiwan ekstrak mentah, fraksi F3 dan fraksi F4 masing-masing, menunjukkan tahap perencatan parasitemia yang signifikan berbanding dengan kawalan positif klorokuin. Tahap perencatan parasitemia bagi dos 100 mg/kg berat haiwan fraksi F4 adalah lebih tinggi daripada ekstrak mentah dan fraksi F3 pada hari ke-6 selepas jangkitan, iaitu pada 54.32 ± 0.24 %, 42.85 ± 0.58 % dan 41.01 ± 0.46 % masing-masing, berbanding dengan kawalan positif klorokuin yang ditentukan pada 97.17 ± 0.03 %. Tambahan pula, tikus dijangkiti malaria yang menerima dos rawatan 100 mg/kg berat haiwan fraksi F4 menunjukkan kadar kehidupan harian yang lebih tinggi berbanding dengan tikus yang menerima rawatan dos rawatan 100 mg/kg berat haiwan ekstrak mentah dan fraksi F3. Komposisi kimia fraksi F4 yang bioaktif telah ditentukan dengan menggunakan kajian spektroskopi dan kromatografi (TLC, FT-IR, Uv-vis, HPLC, prep-LC dan MS). Komposisi kimia daripada fraksi F4 yang berpotensi telah ditentukan secara kualitatif menggunakan ESI-MS. 13(*S*)-HPODE telah didapati wujud dalam fraksi F4. Dalam aktiviti anti-oksida, ekstrak mentah menunjukkan aktiviti yang lebih tinggi berbanding dengan fraksi F3 dan fraksi F4 dalam asai DPPH, asai pelunturan beta-karotena/asid linolik dan asai kuasa penurunan. Namun, jumlah kandungan fenolik dalam ekstrak mentah adalah 1.6 kali lebih rendah berbanding dengan fraksi F4. Tambahan pula, jumlah kandungan flavanoid dalam ekstrak mentah adalah 14.8 kali lebih rendah berbanding dengan fraksi F4. Sebagai kesimpulannya, kajian ini telah mendapati ketinggian

potensi ekstrak *Tinospora crispa* Miers dalam aktiviti-aktiviti anti-toksoplasmosis dan antimalaria, di mana Fraksi F4 yang bioaktif telah menyerlahkan aktiviti farmakologi signifikan dalam assai anti-*T. gondii in vitro* dan model tikus *P. berghei* secara *in vivo*. Kajian ini membuka ruang kajian selanjutnya yang akan memberangsangkan pembangunan praklinikal agen anti-parasitik daripada sumber tumbuhan ubatan tempatan.

PHYTOCHEMICAL STUDY, ANTI-TOXOPLASMOSIS AND ANTIMALARIAL ACTIVITIES OF TINOSPORA CRISPA MIERS METHANOL EXTRACT AND ITS BIOACTIVE FRACTIONS

ABSTRACT

Toxoplasmosis and malaria are two serious illnesses resulting in huge toll on human health. To date, there is not much advancement in the chemotherapeutic agents used to treat toxoplasmosis and malaria. The current chemotherapeutic agents have been used for decades and the associated parasites have developed resistance towards the current mainstay drugs. Traditionally, *Tinospora crispa* Miers are widely used to treat malaria, fever and some other parasite-related illnesses. In this work, the crude methanol extract and bioactive fractions of *Tinospora crispa* Miers were studied for anti-parasitic activities against *Toxoplasma gondii* and *Plasmodium berghei*, phytochemical content and antioxidant activities. The crude extract was extracted from stems of *Tinospora crispa* Miers using maceration method and further fractionated into smaller group of components by elution with the hexane-acetone solvent system on column chromatographic technique. The cytotoxic activity of the crude extract and all the differing polarity fractions (F1 to F8 respectively) on Vero cell line was determined by *in vitro* MTT assay. This study found that the crude extract and all the fractions showed no cytotoxic activity on Vero cells. The crude extract and all fractions were then further investigated for anti-parasitic activity against *Toxoplasma gondii*. The crude extract showed inhibitory activity against *T. gondii* with EC_{50} value of 7.83 ± 0.12 µg/mL. Of all the fractions, fractions F3 and F4 showed the most bioactive activity against *T. gondii* with EC_{50} values of 6.77 ± 0.03 µg/mL and 6.04 ± 0.02 µg/mL, respectively. Further investigation for

antimalarial activity against *Plasmodium berghei* in the *in vivo* mice model was then carried out with crude extract and its two potent fractions F3 and F4 determined from the *in vitro* study. Malarial-infected mice receiving treatment of dose at 100 mg/kg animal weight of crude extract, fraction F3 and fraction F4 respectively, showed significant parasitaemia suppression level as compared to positive control chloroquine. The parasitaemia suppression levels of 100 mg/kg animal weight dose of Fraction F4 was found to be higher compared to the crude extract and fraction F3 on Day 6 post-inoculation, which were 54.32 ± 0.24 %, 42.85 ± 0.58 % and 41.01 ± 0.46 %, respectively in comparison with the positive control chloroquine at 97.17 ± 0.03 %. In addition, malarial-infected mice receiving 100 mg/kg animal weight dose of fraction F4 treatment showed higher daily survival rate compared to the mice receiving same doses of crude extract and fraction F3. The chemical composition of bioactive fraction F4 was determined by various spectroscopic and chromatographic studies (TLC, FT-IR, UV-vis, HPLC, prep-LC and MS). The chemical composition of bioactive fraction F4 was determined qualitatively on ESI-MS. The 13(*S*)-HPODE was found to be present in fraction F4. In the antioxidant activities, the crude extract showed better activities than fractions F3 and F4 in DPPH assay, beta carotene/linoleic acid bleaching assay and reducing power assay. However, the total phenolic content of the crude extract was 1.6 times lower compared to fraction F4, while the total flavonoids content of the crude extract was 14.8 times lower than fraction F4. In conclusion, this study have uncovered potent anti-toxoplasmosis and antimalarial activities of *Tinospora crispa* Miers extract, where its bioactive fraction F4 exhibited significant pharmacological activities in the *in vitro* anti-*T. gondii* assay and *in vivo P. berghei* mice model. This work opens the door to further promising pre-clinical development of anti-parasitic agent(s) of local medicinal plant origin.

CHAPTER 1: LITERATURE REVIEW

1.1 Apicomplexan Parasites

A parasite refers to an organism that lives on or in a host, and gets its food from or at the expense of its host (CDC, 2014). Pathogenic parasites can cause serious diseases in humans and many economically important animal species. One of the important classifications of the parasite is the apicomplexan parasites that consist of pathogenic single-cell intracellular parasites. Some important apicomplexan parasites that cause diseases in human are *Plasmodium* species that is well known for the deadly malaria, *Cryptosporidium* species for causing cryptosporidiosis and *Toxoplasma* species for causing toxoplasmosis. Two apicomplexan parasites (*Plasmodium* and *Toxoplasma*) that cause severe diseases in human population were the main highlights in the study.

All apicomplexan are parasitic and share many specific features related to parasitism (Figure 1.1). The most important feature is the presence of the apicoplast (McFadden *et al.*, 1996). Apicoplast is a non-photosynthetic organelle of algal origin (Tawk *et al.*, 2011). However, an exception to this rule is the *Cryptosporidium* parasites that loss the organelle during evolution (Zhu *et al.*, 2000). Several studies have proven that the apicoplast fulfils critical functions for parasites invasion and survival. Apicoplast is the site of several plastid-derived biochemical pathways such as biosynthesis of fatty acids, isoprenoids, haem and iron–sulfur clusters (Ralph *et al.*, 2004). Ralph *et al.* (2004) also stated that the metabolic pathways of the apicoplast contribute to lipid production and the modification of lipid-bound proteins. These findings proved that the apicoplast plays a crucial role in the successful establishment of parasite-host interaction, such as in the formation of parasitophorus vacuole during parasites

invasion. Moreover, apicoplast is needed for survival in both erythrocytic (Goodman *et al.*, 2007) and liver stages (Vaughan *et al.*, 2009; Yu *et al.*, 2008) of *P. falciparum*. In fact, the apicoplast forms close associations with mitochondria and endoplasmic reticulum (ER) during development of *Plasmodium* species (van Dooren *et al.*, 2005), while in *Toxoplasma gondii*, apicoplast is tightly associated with ER (Tomova *et al.*, 2009).

Apart from the apicoplast, the apicomplexan parasites also have a characteristically polarized cell structure, complex cytoskeletal and secretory organelle at the apical end of the cell (Dubey *et al.*, 1998). The cytoskeleton and organelles mediate the parasites' locomotion and cellular invasion (Templeton *et al.*, 2004). Apicomplexan parasites also contain the rhoptries and micronemes that are deployed during the invasion process and the delivery of parasites protein into the host cells (Anantharaman *et al.*, 2007).

Each apicomplexan lineage has evolved unique spectrum of protein that modify the host in diverse ways. The mechanisms of invasion into the hosts are largely conserved among the apicomplexan (Kim and Weiss, 2004). However, the host range, cell specificity and life cycle stages within species are quite different among the apicomplexan parasites.

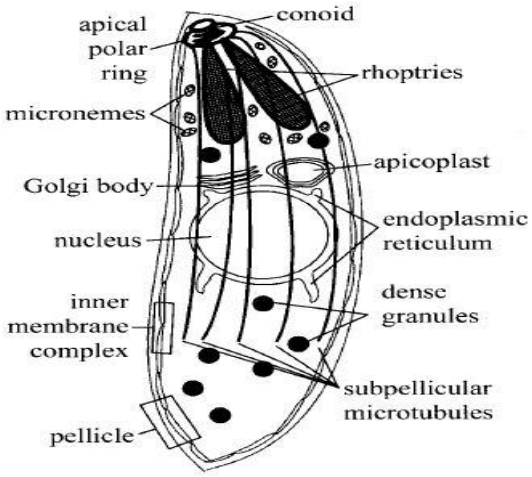


Figure 1.1: The morphology of apicomplexan parasites. Apicomplexan parasites are highly polarized cells containing a collection of organelles that are specific to the phylum. The figure was adapted from Morrissette and Sibley (2002).

1.1.1 *Toxoplasma gondii*

Toxoplasma gondii is an obligate intracellular protozoan parasite which is a significant human and veterinary pathogen (Kim and Weiss, 2004). Figure 1.2 illustrates the ultra structure of a *T. gondii* tachyzoite. *T. gondii* is capable to infect any nucleated mammalian or avian cell including humans (Dubey, 1998). This makes *T. gondii* one of the most successful parasitic organisms worldwide and can be found in most regions of the world (Innes, 2010). Felidae (cat) species are the definitive host of *T. gondii* for its reproductive cycle while the other warm-blooded organisms such as humans serve as intermediate hosts for its propagation.

The apicomplexan parasite *Toxoplasma gondii* causes a flu-like disease known as toxoplasmosis. The symptoms of toxoplasmosis include fever, headache, sore throat, cough and dizziness. Toxoplasmosis can also cause significant morbidity and mortality in the fetus and immunocompromised individuals if not treated (Montoya and Liesenfeld, 2004). According to Flegr *et al.* (2014), infection with *T. gondii* is

the most prevalent one in humans, which estimated to affect about 30-50 % of the world population. On top of that, there was a *T. gondii* seroprevalence of 55.7 % among rural Malays in a study from Malaysia (Petersen, 2007). In addition, estimation of toxoplasmosis disease burden ranks this disease at the same level as *salmonellosis* or *campylobacteriosis* in the food borne disease category (Kijlstra and Jongert, 2008).

Numerous researchers supported the use of *T. gondii* parasite as the model organism to study the apicomplexan parasites. The advantages of using *T. gondii* as model organism lays in its various biotechnology establishments that includes the well-established methodology for classic and reverse genetics of *T. gondii* (Roos *et al.*, 1999), identification of numerous *T. gondii* molecular markers and the relative ease with which the parasite can be studies using microscopic techniques (Kim and Weiss, 2004). All these factors make *T. gondii* the best model system for cell biology investigation of apicomplexan parasites. The studies of mechanisms of drug resistance, the biology of the apicoplast, and the process of host cell invasion have all been achieved in *T. gondii* (Kim and Weiss, 2004). In fact, *T. gondii* is the most experimentally tractable among the apicomplexan parasites (Roos *et al.*, 1999).

Table 1.1: Taxonomic classification of *Toxoplasma gondii* (Adapted from NCBI, 2015a).

Taxonomic Classification	
Kingdom	*Alveolata*
Phylum	*Apicomplexa*
Class	*Conoidasida*
Order	*Eucoccidiorida*
Family	*Sarcosystidae*
Genus	*Toxoplasma*
Species	*T. gondii*

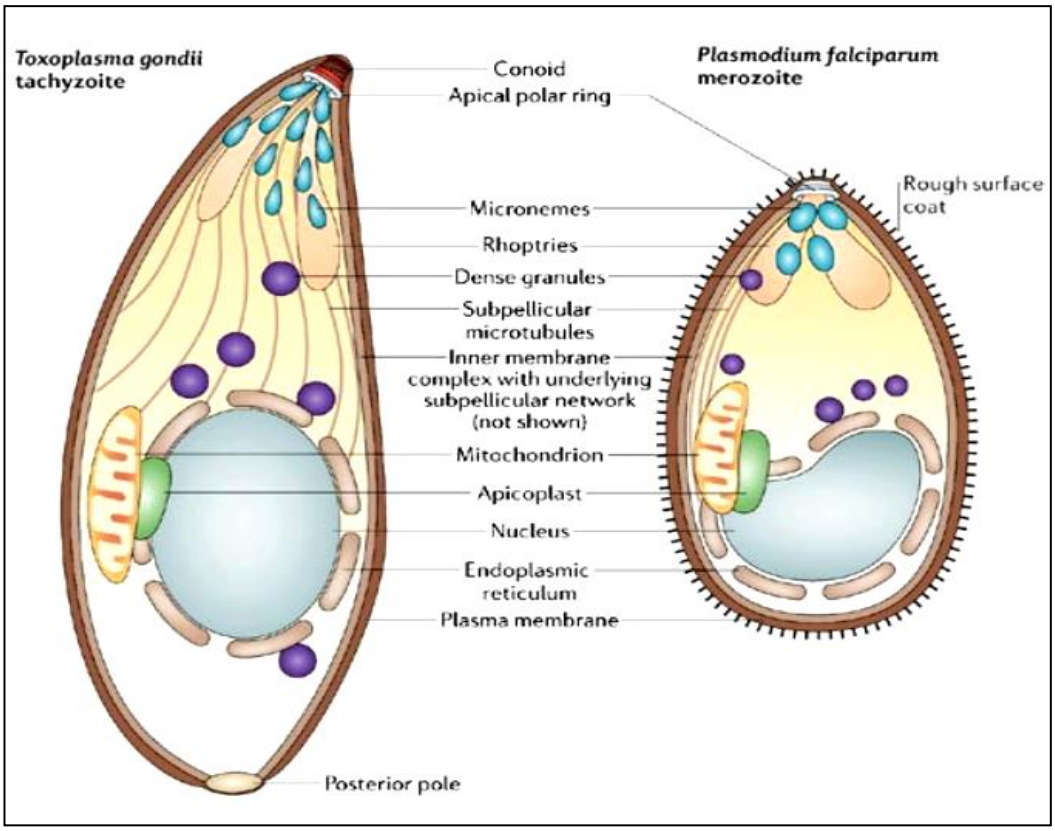


Figure 1.2: Ultra structure comparisons of a *T. gondii* tachyzoite and a *P. falciparum* merozoite (adapted from Baum *et al.*, 2006).

1.1.1.1 Life Cycle and Transmission of *Toxoplasma gondii*

The life cycle and transmission routes of *T. gondii* are illustrated in Figure 1.3. The life cycle of *T. gondii* is facultatively heteroxenous. This means that *T. gondii* has more than one host in its life cycle. There are three infectious stages of *T. gondii* during their life cycle and they are tachyzoites, bradyzoites contained in tissue cysts and sporozoites contained in sporulated oocysts. All the three stages are infectious for both intermediate and definitive hosts (Tenter *et al.*, 2000).

The sexual reproduction of *T. gondii* only occurs in the felid's intestinal tract. The cats become infected after consuming intermediate hosts contaminated with tissue

cysts or oocysts-contaminated water (Montoya and Liesenfeld, 2004). Next, the parasites invade the cat enterocytes and undergo division and differentiation into microgametocytes and macrogametocytes. The gametocytes fuse to form zygotes or oocysts that are shed into environment together with cat feces. The oocysts produce highly infectious sporozoites that are very resistant to environmental damage. The sporozoites may persist for years in warm and humid condition (Montoya and Remington, 2000). The oocysts spread and contaminate the water, soil, fruits and vegetables. Animals (intermediate hosts) will be infected following the consumption of contaminated water, soil, fruits or vegetables (Tenter *et al.*, 2000).

The asexual reproduction of *T. gondii* occurs in intermediate hosts (Montoya and Liesenfeld, 2004). Shortly after ingestion, the oocysts are rapidly transformed into tachyzoites. Tachyzoites enter all nucleated cells and replicate rapidly within the cells. Replication of the tachyzoites inside the cells eventually leading to cell death. Once the host cells are disrupted, the tachyzoites are released and disseminated via the bloodstream and rapidly invades many tissues such as central nervous system (CNS), skeletal muscle, heart, eye, placenta and fetus (Montoya and Liesenfeld, 2004). The tachyzoites cause massive tissue destruction, stimulate host immune system and cause clinical manifestations of the disease (Montoya and Remington, 2000).

Under the pressure of the immune response, tachyzoites can transform into bradyzoites to form cysts that reside and persist in tissues and muscles, especially in brain, skeletal and heart muscles for the life of the host (Montoya and Liesenfeld, 2004). The bradyzoites can be released and transformed back into active tachyzoites

that cause recrudescence infection. The cysts are the infective stages for both intermediate and definitive hosts (Montoya and Remington, 2000).

Transmission of *T. gondii* in human can occur via several routes. The main route is through oral consumption (Montoya and Liesenfeld, 2004). This happens when eating raw or undercooked cysts-contaminated meat, foods or water contaminated with cat feces or environmental samples such as fecal-contaminated soil, changing the litter box of pet cat, unwashed fruits or vegetables and unfiltered water. There was high seroprevalence of *T. gondii* in countries and regions where the humans were common to eat undercooked or raw meat (Dubey and Beattie, 1988). Second, humans can acquire toxoplasmosis during blood transfusion or organ transplantation (Montoya and Liesenfeld, 2004). However, the risk of transmission through this route is very low (Dubey and Beattie, 1988). Lastly, the fetus can get infected with *T. gondii* through transplacental infection from the pregnant mother (Montoya and Remington, 2000).

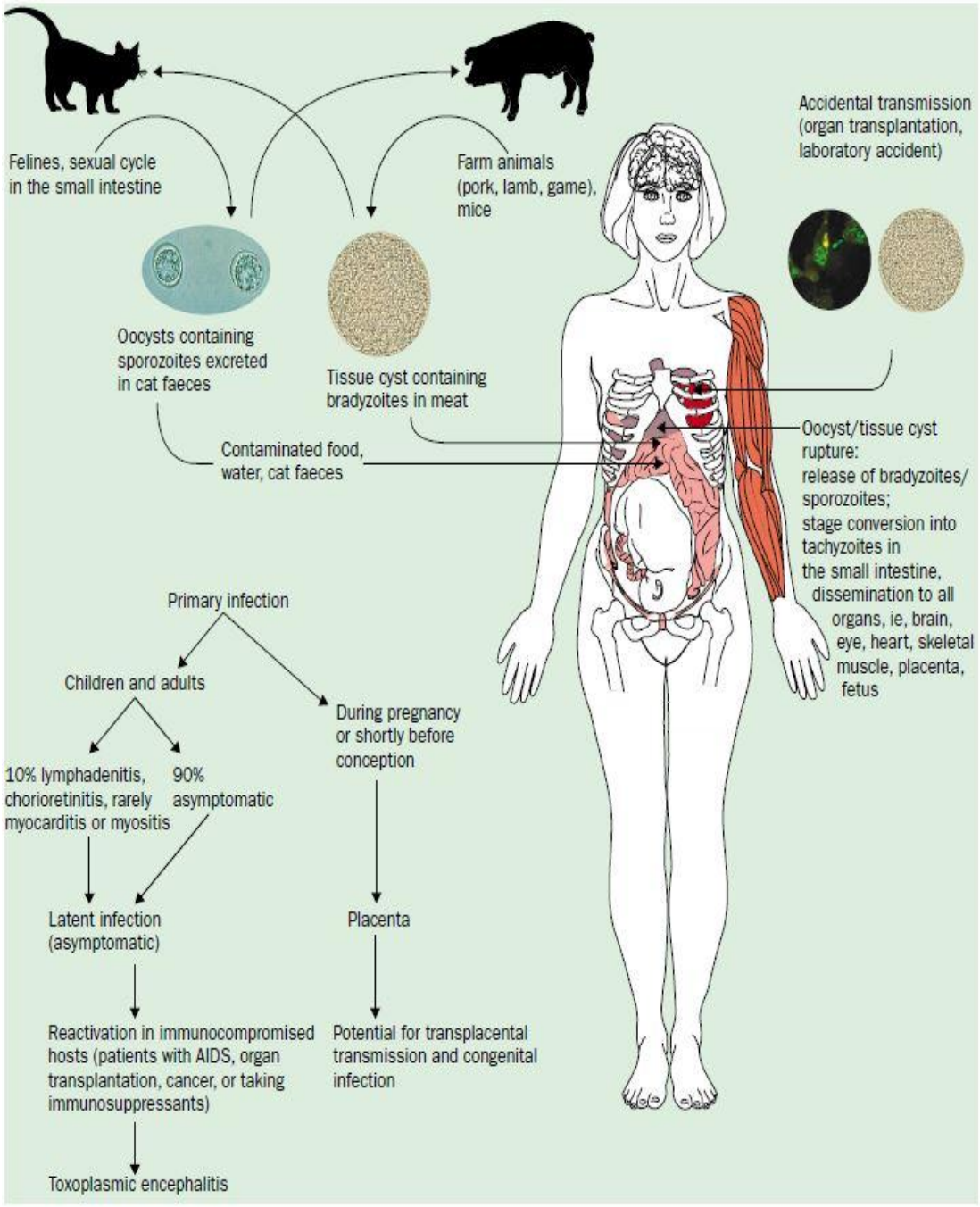


Figure 1.3: Life cycle, transmission route and clinical manifestations of *T. gondii* (Adapted and modified from Montoya and Liesenfeld, 2004).

1.1.1.2 Clinical Manifestations and Pathogenesis of Human Toxoplasmosis

The clinical manifestations and pathogenesis of toxoplasmosis in humans mainly depend on the patient's immune status and the clinical setting such as immunocompetent, immunocompromised, congenital toxoplasmosis, or ocular disease (Montoya and Liesenfeld, 2004).

(i) Immunocompetent people

The primary infection usually causes a self-limited and non-specific flu-like illness. The general symptoms of toxoplasmosis are fever, lymphadenopathy, headache, sore throat, cough, myalgia and dizziness. However, infection can remain for life and reactivation of *T. gondii* infection can occur when the immune system is compromised. Though very rarely, myocarditis, polymyositis, pneumonitis, hepatitis or encephalitis can occur as a result of *T. gondii* infection (Montoya and Liesenfeld, 2004).

(ii) Immunocompromised and immunosuppressed patients

Toxoplasmosis can be life-threatening (Liesenfeld *et al.*, 1999) and always happens as a result of reactivation of chronic infection in immunocompromised individuals (Porter and Sande, 1992). The most common clinical manifestation of toxoplasmosis in acquired immunodeficiency syndrome (AIDS) patient is toxoplasmic encephalitis. In fact, toxoplasmic encephalitis is the most common cause of death among toxoplasmosis patients with AIDS (Dubey, 2004). Other clinical manifestations include mental status changes, seizures, focal motor deficits, cranial nerve disturbances, sensory abnormalities, cerebellar signs, movement disorders, and neuropsychiatric findings (Montoya and Liesenfeld, 2004). Severe manifestations of toxoplasmosis may also include chorioretinitis, pneumonitis, or multiorgan

involvement with acute respiratory failure and haemodynamic abnormalities similar to septic shock (Liesenfeld *et al*., 1999). Severe symptoms of toxoplasmosis can also be observed in immunosuppressed patients with malignancies and after transfusions or transplant with immunosuppressive therapy (Dubey and Jones, 2008).

(iii) Congenital toxoplasmosis

Infection with *T. gondii* during pregnancy causes serious consequences. The severity of the infection depends on the stage of pregnancy at the time of infection (Montoya and Liesenfeld, 2004). The tachyzoites of *T. gondii* can cross the placenta to infect the fetus, thus causing congenital infection. Chances of congenital infection are 10-25 % in untreated women in the first trimester of pregnancy (Emelia, 2009). The incidence of fetal infection ranges between 30-54 % and 60-65 %, respectively, if infection occurs during second and third trimester of pregnancy (Lynfield and Guerina, 1997). Consequences to fetus are more severe if the infection occurs in early stages of pregnancy (Remington and Desmonts, 1990). Some of the consequences that might occur include miscarriage, intra-uterine growth retardation and premature birth. Moreover, neonatal clinical manifestations of congenital toxoplasmosis vary widely. These may include hydrocephalus, microcephaly, intracranial calcifications, chorioretinitis, strabismus, blindness, epilepsy, psychomotor or mental retardation, petechia due to thrombocytopenia, and anaemia (McAuley *et al.*, 1994; Swisher *et al.*, 1994).

(iv) Ocular disease

Toxoplasmic chorioretinitis refers to severe eye infection and it results from congenital or postnatally acquired infection of acute infection or reactivation (Montoya and Remington, 1996; Holland, 1999). The symptoms include white focal lesions in retina with overlying and intense vitreal inflammatory reaction. The retina lesion will slowly resolve and leaving a focus of retinochoroidal scarring (Koch *et al.*, 1943). Besides, mild ocular pain, blurred vision and new onset of floating spots, segmental periarteritis, retinal and vitreous haemorrhage, choroidal or retinal neovascularization are among other symptoms of active toxoplasmic choretinitis (Emelia, 2009).

1.1.1.3 Treatment of Toxoplasmosis

There is not much advancement in the search for new anti-toxoplasmosis drugs. The current mainstay anti-toxoplasmosis agents have been used for decades. The classic clinical drug combinations to treat toxoplasmosis are combination of pyrimethamine, sulfadiazine and folinic acid (Montoya and Remington, 2000).

The immunosuppressed patients such as recipients of organs transplant need to be tested for anti-*Toxoplasma* antibodies, and highly effective prophylaxis trimethoprim/sulfamethoxazole combinations are given (Montoya and Liesenfeld, 2004). Clindamycin is given to the patients with sulfonamides intolerance.

For women with suspected or confirmed acute *T. gondii* infection during pregnancy, spiramycin treatment is recommended for the first and early second trimester while pyrimethamine/sulfadiazine is recommended for late second and third trimester

(Gilbert *et al.*, 2001; Gras *et al.*, 2001). Suspected or confirmed maternal infection acquired during gestation must be confirmed by prenatal diagnosis by PCR of amniotic fluid (Rormand *et al.*, 2001). Prenatal treatment with pyrimethamine/sulfadiazine in women suspected or confirmed with fetal infection reduces the sequelae of the disease in newborn (Foulon *et al.*, 1999).

Toxoplasmic infection can cause severe eyes infection and the treatment should start when severe inflammatory responses, proximity of retinal lesions to the fovea or optic disk, or both are found (Holland, 1999). The combination of drugs used is pyrimethamine, sulfadiazine and prednisone. Besides, clindamycin or trimethoprim/sulfamethoxazole for a minimum of 3 weeks can also be used to treat the disease (Luft *et al.*, 1986). Spiramycin is not recommended to treat toxoplasmic chorioretinitis due to high recurrence rate (Ghosh *et al.*, 1965).

1.1.1.4 Current Challenges in the Treatment of Toxoplasmosis

The current standard therapies for toxoplasmosis involve the combinations of pyrimethamine with sulfadiazine, clindamycin, azithromycin, or atavaquone (Djurkovic-Djakovic *et al.*, 1999; Chirgwin *et al.*, 2002). However, these drugs do not completely eliminate the intracellular parasite and are often associated with severe side effects (Montoya and Liesenfeld, 2004). The combination of pyrimethamine and sulfadiazine is not recommended for the treatment of acute toxoplasmosis in pregnancy and AIDS patients, as they can cause bone marrow suppression, liver toxicity, nausea, vomit, seizures and other symptoms (Haverkos, 1987). Besides, clindamycin can cause adverse effects such as skin rashes and gastrointestinal problems such as diarrhoea, stomach upset and bleeding (Djurkovic-

Djakovic *et al.*, 1999). However, continuous drug therapy is essential for patients to ensure prevention of severe complications.

An effective vaccine against human *T. gondii* infection is a very desirable measure to control the disease. However, the development of effective vaccine is still in early stage of research. Alternative drugs with lesser side effects are needed to combat the disease. The knowledge of the parasite's lifecycle, transmission routes, risk groups and host immune responses has helped in the development of strategies to control the disease, reduce transmission of the parasite and limit environmental contamination.

1.1.2 *Plasmodium* parasites

The apicomplexan *Plasmodium* parasites are the causative agents of the well-known malaria disease in humans. Malaria is the most important parasitic disease in human. According to World Malaria Report 2013 by World Health Organization (WHO), malaria is endemic in 104 countries throughout the world and 3.4 billion people are at risk of malaria. An estimated 207 million people suffered from malaria in 2012 and caused about 627 000 death cases. A total of about 90 % of cases and deaths occurred in Sub-Saharan Africa and about 77 % of death cases were in children under age of 5. Malaria causes a huge toll on human health and imposes a heavy social and economic burden in the developing countries, especially in Sub-Saharan Africa and South Asia. In Malaysia, cases of malaria are restricted in distribution. Between 2000 and 2012, Malaysia successfully achieved more than 75 % decrease in microscopically confirmed malaria cases and more than 50 % decrease in reported malaria mortality rate (WHO, 2013).

The malaria-causing *Plasmodium* parasites are transmitted by *Anopheles* mosquitoes. About 30 species out of 400 different species of the *Anopheles* mosquitoes are the important vectors in transmitting the parasites (White *et al.*, 2014). The ultra structure of a *P. falciparum* merozoite is illustrated in Figure 1.2 under Section 1.1.1. There are five species of the *Plasmodium* parasites that cause malarial infections in human. Majority of the cases are caused by *Plasmodium falciparum* or *Plasmodium vivax*, but infection with *Plasmodium ovale* and *Plasmodium malariae* can lead to malaria as well. Recently, there were cases of malaria caused by monkey malaria *Plasmodium knowlesi* being reported in parts of Southeast Asia (Kantele and Jokiranta, 2011). *P. falciparum* causes the most serious cases of malaria and is responsible for almost all malaria death cases. *P. falciparum* predominates in Africa while *P. vivax* has a wider distribution due to its ability to develop in *Anopheles* mosquito at lower temperatures, and to survive at higher altitudes and in cooler climates. Besides, *P. vivax* also has a long dormant liver stage that enables the parasites to persist in human (White *et al.*, 2014).

Malaria remains one of the most important diseases in humans in terms of both mortality and morbidity, with *Plasmodium falciparum* being the most important infecting agent (White *et al.*, 2014). The increasing global spread of drug resistance to most of the available and affordable antimalarial drugs is a major concern and requires innovative strategies to combat (Nguyen-Pouplin *et al.*, 2007).

Table 1.2: Taxonomic classification of *Plasmodium* parasites (Adapted from NCBI, 2015b)

Taxonomic Classification	
Kingdom	*Alveolata*
Phylum	*Apicomplexa*
Class	*Aconoidasida*
Order	*Haemosporida*
Family	*Plasmodiidae*
Genus	*Plasmodium*
Species	*P. falciparum, P. ovale, P. vivax. P. malariae, P. knowlesi*

1.1.2.1 Life Cycle and Transmission of *Plasmodium* Parasites

The life cycle of the *Plasmodium* parasites involve three distinct stages (Figure 1.4). The liver and blood stages occur in host, whereas the sexual stage occurs in the mosquito gut (White *et al.*, 2014).

The malaria-infected female anopheline mosquito inoculates motile sporozoites into the human host during a blood meal. The sporozoites then rapidly infect and invade into hepatocytes. Inside liver cells, the sporozoites mature into schizonts and multiply to become merozoites. A successful sporozoite invasion can produce about 10000 to 30000 merozoites in 5.5-8 days inside a single hepatocyte. In *P. vivax* and *P. ovale*, infection of sporozoites into liver cells can produce hypnozoites in the dormant stage that can persist in the liver, which will then causing relapses weeks or years later. Next, the merozoites are released to invade the red blood cells. The parasites undergo asexual multiplication in the erythrocytes after the initial liver replication. An asexual cycle in the blood takes roughly 48 h for *P. falciparum*, *P. vivax*, and *P. ovale*, 72 h for *P. malariae*, and 24 h only for *P. knowlesi*. Inside the erythrocytes, the merozoites develop into ring stage trophozoite (Figure 1.5) and then mature into daughter merozoites. The erythrocytes rupture and release daughter

merozoites which rapidly invade new erythrocytes and repeat the cycle (White *et al.*, 2014).

The malaria disease is caused by the effects of erythrocytes parasitization and destruction, as well as the host immune reaction to fight against the parasites. Inside the red blood cells, the merozoites consume the cell content, altering the cell membrane to facilitate nutrients transportation and metabolise the toxic haem into harmless haemozoin (malaria pigment). Besides, some merozoites differentiate into gametocytes, which are then ingested by *Anopheles* mosquito during blood meal and initiate the sexual reproduction cycle of the parasites (Fujioka and Aikawa, 2002).

The sexual reproduction of the *Plasmodium* parasites occurs only in mosquitoes. Upon ingestion of the gametocytes, the microgametocytes fuse with macrogametocytes inside the mosquito's stomach to produce zygotes. The zygotes are then developed into ookinetes that invade the midgut wall of mosquitoes. Ookinetes develop into oocysts that rupture and release the sporozoites. The sporozoites migrate to the mosquito's salivary gland and inoculate into new host when the mosquito taken a blood meal. Inoculation of sporozoites into new human host initiates the new malaria life cycle (Fujioka and Aikawa, 2002).

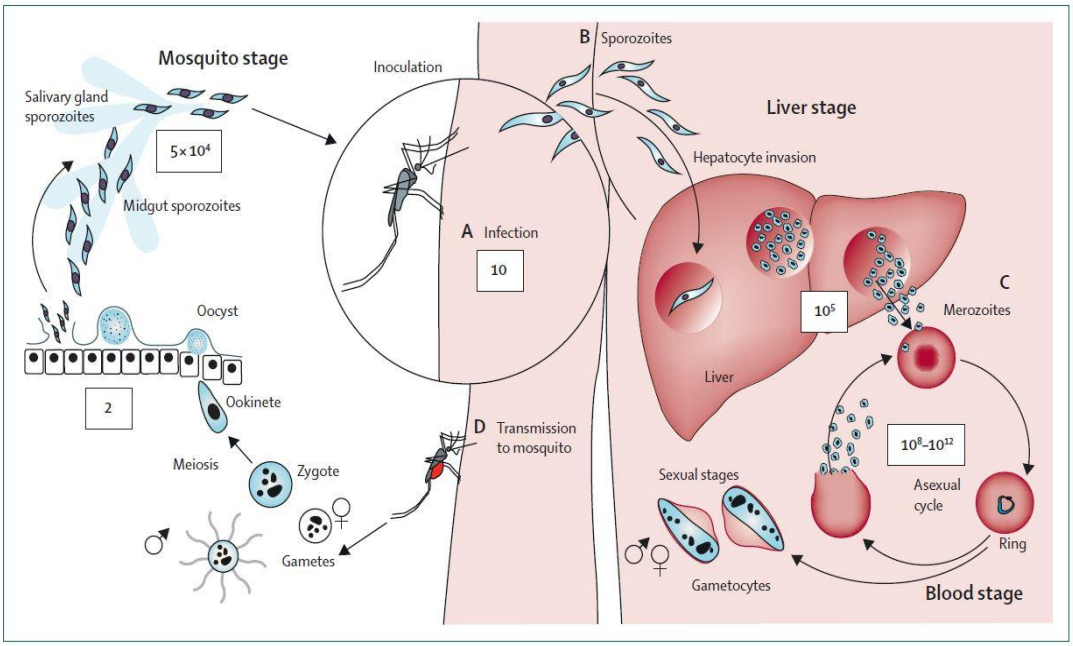


Figure 1.4: Life cycle of *Plasmodium* parasites in the human body and the anopheline mosquito (adapted from White *et al.*, 2014).

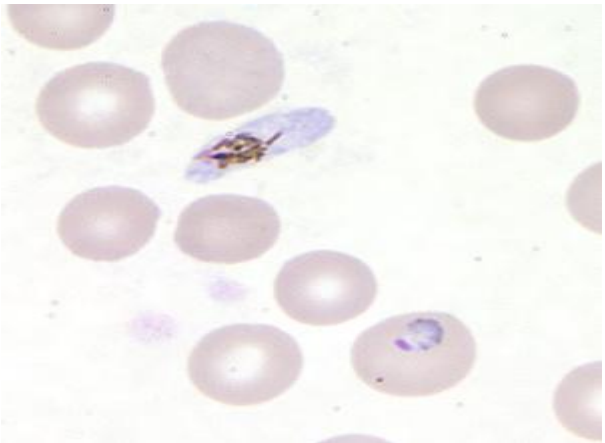


Figure1.5: Gametocyte and trophozoite (ring form) of *P. falciparum* in a thin blood smear [Adapted from Centers for Disease Control and Prevention (CDC) USA, 2014].

1.1.2.2 Clinical Manifestations and Pathogenesis of Human Malaria

Malaria causes severe clinical manifestations in human. The initial symptoms of malaria are nonspecific and they include absence of wellbeing, headache, fatigue, anaemia, muscle aches, abdominal discomfort and followed by irregular fever. Other symptoms such as vomiting, nausea and hypotension also occur frequently. Malaria caused by falciparum parasites is also associated with generalised seizures and might be followed by coma that is caused by cerebral malaria. For uncomplicated malaria infections, most patients have fewer physical symptoms. Since malaria parasites infect the liver, hepatomegaly may often be found in young children while mild jaundice is more likely to occur in adults. Recurrent malaria infection causes chronic anaemia and splenomegaly in young children (White *et al.*, 2014).

Infection with *P. falciparum* can cause the severe malaria type. In falciparum malaria, the infected erythrocytes tend to adhere to vessel walls and also to each other. The erythrocytes adherence causes sequestration of red blood cells containing mature parasites into vital organs particularly the brain (White *et al.*, 2014). Inside the brain, the sequestered parasites will interfere with microcirculatory flow and the metabolism and normal functioning of vascular endothelium (Pongponratn *et al.*, 2003). The erythrocytes in malaria caused by other parasites species are not sequestered substantially. Severe falciparum malaria is mainly attributed by extensive parasitised erythrocytes sequestration and consequently dysfunction of vital organs. However, age is the main factor determining the manifestation of severe falciparum malaria (Dondorp *et al.*, 2008). In children, severe anaemia and hypoglycaemia occur more frequently whereas acute pulmonary oedema, acute kidney injury, and jaundice are more common in adults. Besides, acidosis and

cerebral malaria following by subsequent coma can occur in all age. The mortality rate increases as the proportion of infected red blood cells exceeds 2 %. However, uncomplicated falciparum malaria has a low mortality rate of about 0.1 % when properly treated with effective antimalarial drugs (Dondorp *et al.*, 2008).

Severe malaria can also cause other clinical complications such as invasive bacterial infections by enteric bacteria in children (Bronzan *et al.*, 2007). Malaria can accelerate the progression and transmission of HIV infection (Kublin *et al.*, 2005). The mortality rate of clinical and severe malaria is increased in adults with HIV infection and compromised immune status (Whitworth *et al.*, 2000; Chalwe *et al.*, 2009). Moreover, the concomitant HIV infection increases the severity and mortality of severe falciparum malaria in children (Berkley *et al.*, 2000; Hendriksen *et al.*, 2012).

Falciparum malaria can cause serious complications in pregnant women. The risk of low birth weight (< 2.5 kg) increases when the mother get infected. The lower birth weight is associated with increased infant mortality (Desai *et al.*, 2007). Other malaria complications such as high parasitaemia, severe anaemia, hypoglycaemia and acute pulmonary oedema are more frequent in pregnant women compared to non-pregnant women (McGready *et al.*, 2012). Fetal distress, premature labour and stillbirth often occur in pregnant women with severe malaria (Desai *et al.*, 2007). There is a higher risk of infant death if maternal malaria occurs during late pregnancy (Bardaji *et al.*, 2011). Besides, maternal death caused by haemorrhage during childbirth is correlated with the malaria-induced anaemia (Rijken *et al.*, 2012).

1.1.2.3 Treatment of Malaria

Most of the antimalarial drugs target the asexual erythrocytic stage of the parasite. During parasitic growth, the parasites metabolize haemoglobin. The haemoglobin degradation produces free haem that is able to react with oxygen, thus producing reactive oxygen species as toxic by-products. A major pathway of haem detoxification is through polymerization to malaria pigment haemozoin (Pagola *et al.*, 2000). Most of the antimalarial drugs act to disturb the polymerization of haem, thus killing the parasites with its own metabolic waste (Egan and Marques, 1999). The main chemical classes of antimalarial drugs are as listed in Table 1.3. Some antibiotics are also used to treat malaria in combination with other drugs.

The only class of drugs that is active against the gametocytes stage is of 8-aminoquinolines (Robert *et al.*, 2001) (Table 1.3). Primaquine belongs to 8-aminoquinolines class and it is widely used against the hypnozoites (liver dormants) responsible for causing relapse of *P. vivax* and *P. ovale*. Primaquine interferes with the mitochondrial function of *Plasmodium* parasites (Robert *et al.*, 2001).

The classes of sulfonamides and sulfones, biguanides, diaminopyrimidine and naphthoquinone act as nucleic acid inhibitors (Table 1.3). Sulfonamides and sulfones, biguanides and diaminopyrimidine belong to the folate antagonist class (Olliaro, 2001), though the *Plasmodium* parasites already developed resistance against this class of drugs. The inhibition of the enzymes in the folate pathway results in decreased synthesis of parasites pyrimidine, thus reduces the formation of methionine, serine and DNA necessary for parasites development. The inhibitory activity is exerted at all growing stages of the erythrocytic cycle and on young

gametocytes (Olliaro, 2001). On the other hand, atovaquone (from class naphthoquinone) inhibits the respiratory chain of malarial mitochondria at the cytochrome bc1 complex by mimicking the natural substrate, ubiquinone. This, in turn, inhibits the mitochondrial electron transfer from ubiquinol to cytochrome c (Fry & Pudney, 1992). However, the parasites develop resistance to atovaquone quickly when atovaquone is prescribed alone. Therefore, atovaquone is usually prescribed with proguanil in fixed combination to treat malaria (Robert *et al.*, 2001).

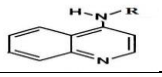
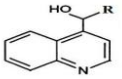
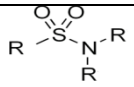
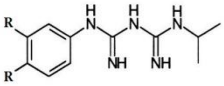
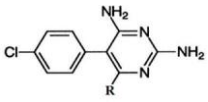
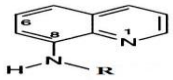
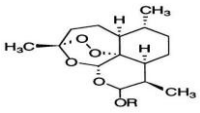
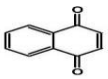
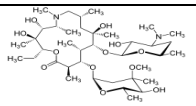
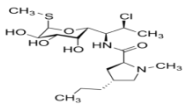
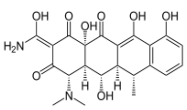
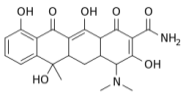
On the other hand, blood schizonticides refer to drugs that act on erythrocytic stages of parasites. The primary target is the parasite food vacuole (Olliaro, 2001). These classes of drugs include the quinoline containing drugs such as 4-Aminoquinolines and aryl-amino alcohols, as well as the artemisinin-type drugs (Table 1.3). The quinoline drugs accumulate in the acidic food vacuole within the parasites, and subsequently alter the haem detoxification processes that eventually leading to parasites death (Olliaro, 2001).

The artemisinin-type compounds include the natural extract artemisinin and its semi-synthetic derivatives. They achieve higher parasitaemia reduction rates than any other drugs (White, 1997), and are being used for the treatment of uncomplicated and severe forms of malaria. The artemisinin-type compounds mainly act through the generation of alkylating species by reaction of homolytic cleavage of endoperoxide bridge with haem (Robert *et al.*, 2001). The generated alkylating radicals then disrupt the vital biochemical processes of the parasite via alkylation of biomolecules and thus causing parasites death (Robert *et al.*, 2001).

Artemisinin-based combination therapies (ACTs) are the current first-line therapy for falciparum malaria in all endemic areas (WHO, 2013). It is also highly efficacious against the other human malarias. Artemisinin combination treatment acts rapidly and reliably effective. Artemisinin combination therapy has few adverse effects and has very high curative level (International Artemisinin Study Group, 2004). In ACTs, the artemisinin derivatives are combined with a partner drug of different mechanisms of action. The fast-acting artemisinin compound serves the purpose to reduce the main parasite load during the first three days of treatment, while the partner drug eliminates the remaining parasites (WHO, 2013). The artemisinin derivatives such as artesunate is also the first treatment of choice for all types of severe malaria (severe falciparum, vivax and knowlesi malaria) as artesunate has no important local or systemic adverse effects (Barber *et al.*, 2013). Chloroquine is still used in regions where it still remains efficacious.

Atovaquone–proguanil, doxycycline, primaquine, or mefloquine are prescribed to travellers travel to resistant *P. falciparum* endemic areas as chemoprophylaxis purpose. On the other hand, sulfadoxine–pyrimethamine was given as intermittent preventive treatment to pregnant women in Africa. A minimum of three doses of sulfadoxine–pyrimethamine are recommended to provide continuous preventive effects against malaria (White *et al.*, 2014). Besides, mefloquine is recommended for chemoprophylaxis in pregnant women travelling to areas with drug-resistant malaria. WHO (2012) recommended monthly amodiaquine and sulfadoxine–pyrimethamine (maximum four doses) to all children aged 3–59 months in Africa during the yearly malaria transmission season.

Table 1.3: Classes of chemical family for antimalarial drugs. The table was adapted and modified from WHO (2010).

Chemical Family	Common structure	Drugs
4-Aminoquinolines		Chloroquine, amodiaquine, piperaquine
Aryl-amino alcohols		Quinine, quinidine, mefloquine, halofantrine, lumefantrine
Sulfonamides and sulfones	 Sulfonamides  Sulfones	Sulfadoxine, sulfalene, dapsone
Biguanides		Proguanil, chlorproguanil
Diaminopyrimidine		Pyrimethamine, cycloguanil
8-Aminoquinoline		Primaquine
Sesquiterpene lactones		Artemisinin, arteether, artemether, artesunate, dihydroartemisinin
Naphthoquinone		Atovaquone
Antibiotics	 Azythromycin  Clindamycin  Doxycycline  Tetracycline	Azythromycin, clindamycin, doxycycline, tetracycline

1.1.2.4 Current Challenges in the Treatment of Malaria

The current antimalarial drugs such as chloroquine and artemisinin have been used for decades and there is not much advancement in the search for new effective antimalarial molecules. Even the newest antimalarial drugs, the artemisinin derivative, were introduced into market in 1990s (Faurant, 2011).

The use of chloroquine to prevent and treat falciparum malaria has led to the wide spread appearance of chloroquine-resistance strain of *Plasmodium falciparum* across the world (Rahman *et al.*, 1999). The resistance of malaria parasites has been increasingly extended to other current antimalarial drugs. This is because many of antimalarial drugs in current usage are chemically related. Thus, development of resistance to one drug can facilitate the development of resistance to others. In fact, cross-resistance towards chloroquine and amodiaquine (4-aminoquinolines) has been reported (Bloland, 2001). Besides, resistance to mefloquine may lead to resistance to halofantrine and quinine.

In such situation, World Health Organization (WHO) has recommended artemisinin-based combination therapies in all endemic countries. Moreover, WHO had also banned the use of artemisinin in monotherapy and recommended the artemisinin combination therapy instead in order to delay the development of drug resistance and to improve cure rates (WHO, 2006; Robert *et al.*, 2001). Recently, there have been signs that the efficacy of artemisinin-based combination therapy and artesunate monotherapy have declined in western Cambodia. The resistance to artemisinin has been reported around Thai-Cambodia border (Muller *et al.*, 2009) and Thailand-Myanmar border (Phyo *et al.*, 2012). The spread of artemisinin resistance parasites to