

CHAPTER I

INTRODUCTION

In most developing countries, malaria stills an important public health issue. Its global burden and economic costs are still enormous, and causes about 225 million cases resulted in 781 000 deaths in 2009 (WHO, 2010).

In Malaysia, the fight against malaria, which began more than a hundred years ago, specifically in 1901, led to an impressive decrease in the disease prevalence from about 300,000 to 7000 reported cases in 2009 (WHO, 2010). However, a sudden increase in the incidence of malaria has been reported in 2008 and this may call for an urgent update to the means of eliminating the disease (Kaur, 2009). Even though indoor residual spraying of insecticides is a good method for malaria control in Malaysia (Rohani *et al.*, 2006), elimination of malaria from a community requires combining several measures for the implementation of that strategy. The 1998 Roll Back Malaria (RBM) programme, launched in Geneva by WHO, UNDP, UNICEF and the World Bank, is a people oriented plan that emphasises community involvement (WHO, 1998; Udonwa *et al.*, 2010).

The participation of the community represents one cardinal tool of malaria control programmes by the WHO as the improvement of understanding of transmission of malaria can greatly increase the realization and sustainability of malaria elimination programme (Nchinda, 1998; Govere *et al.*, 2000; Hlongwana *et al.*, 2009).

Evidence of artemisinin-resistant malaria has been reported on the Thai-Cambodian border and there is a global call to look for new anti-malarial agents from medicinal plants, which represent the main ingredients of modern anti-malarial agents (Htut, 2009). The new drugs must exhibit efficacy and safety, be inexpensive and have additional properties important for the specific disease indication (Rosenthal, 2003).

Consistent with this specification, traditional medicinal plants have several potential advantages; they are affordable, easily accessible and there is no evidence of resistance to whole-plant extracts. Moreover, traditional plants have been utilized to cure malaria for hundreds of years and provided the human with the basic components of the main malaria treatments used in the present age; artemisinin and quinine derivatives (Willcox & Bodeker, 2004; Batista *et al.*, 2009). Medicinal plants may provide anti-malarial drugs directly, as in the case for quinine from cinchona bark, or they may supply template molecules on which to base farther new structures by organic synthesis—artemisinin from *Artemisia annua*.

Although many communities have achieved successful ethnobotanical approaches in fighting malaria, very little is known about plant remedies preparation that are remain employed in the treatment of malaria in Peninsular Malaysia. The ethnobotanical approach to the search for novel anti-malarials from plant products has confirmed to be more prognostic compared to the random screening approach, where

the most important modern anti-malarial drugs are derived from the medicinal plants known to have ethnomedical standing (Saxena *et al.*, 2003).

In parallel with the implementation of Malaysia's malaria elimination programme, this study was carried out to evaluate the community's awareness regarding malaria transmission, treatment and vector control between the aboriginal and rural communities in district of Lipis, Pahang state, which still represents one of the highest prevalence of malaria cases in Peninsular Malaysia. The present study was also carried out to establish a preliminary ethnobotanical database for the plants traditionally used to treat malaria.

In vivo anti-malarial activity of four plant species, namely, *Cocos nucifera* L. *Labisia pumila* (Bl.) F.-Vill., *Languas galanga* Stuntz. and *Piper betle* L. selected based on the ethnobotanical survey and literature were evaluated against laboratory malaria model *Plasmodium berghei* to evaluate their anti-malarial activity. The acute oral toxicity (LD₅₀) was established to determine the safety of the plants extract. The phytochemical and antioxidant potentials of the crude extracts were also investigated to elucidate the possibilities of its anti-malarial effects.

1.1 OBJECTIVES OF THE STUDY

1.1.1 General Objectives:

1. To investigate the household knowledge, attitude and practices (KAP) regarding malaria in two malaria endemic communities, forest-aboriginal and rural communities, in the Lipis district of Pahang state, Malaysia.

2. To investigate the plants traditionally used in the treatment of malaria in the two malaria endemic communities of the forest-aboriginal and the rural communities, and traditional healers.
3. To evaluate the *in vivo* anti-malarial activities of selected medicinal plants traditionally used by the Malaysian people to treat malaria.

1.1.2 Specific Objectives:

1. To compare the awareness regarding malaria among the forest-aboriginal and rural communities in Lipis district, Pahang state.
2. To investigate the treatment of malaria with traditional plants remedies, including the use, preparation and administration.
3. To investigate the safety, LD₅₀ (acute oral toxicity) of the plants extracts.
4. To study the phytochemical screening of the plants extracts.
5. To evaluate the radical scavenging antioxidant capacity of the tested extracts.
6. To investigate the *in vivo* anti-malarial activity of different concentrations of the plants extract against *P. berghei* in mice.

1.2 HYPOTHESES

- 1.** Community knowledge, attitude and practices (KAP) regarding malaria in the endemic areas is inadequate.
- 2.** People in the remote and rural malaria endemic areas are using medicinal plants to fight the disease.
- 3.** Malaysian medicinal plants used by the remote and rural communities are safe and possess anti-malarial activity.
- 4.** The Malaysian folkloric medicinal application of the anti-malarial plants has a pharmacological basis.

CHAPTER II

LITERATURE REVIEW

2.1 INTRODUCTION TO MALARIA

Malaria (from the Italian *mala aria* – bad air), the oldest and cumulatively the deadliest of the human infectious diseases, seeped into our very earliest human history. Malaria has infected humans since at least 20,000 years ago (Pennisi, 2001). Hippocrates was the first to describe clearly the different types of malaria, some 2,500 years ago, depending on the periodicity of the fever—tertian and quartan fever patterns. In Hippocratic era, malaria was known as ‘the fever’, to the Romans as ‘intense burning heat’ (*febris ardens*) (Cunha & Cunha, 2008).

In the modern era, it was known to the French and English as ‘fever and chills’ and ‘seasonal fevers’, respectively. In Osler’s time, malaria was also acknowledged as the fever of summer/fall (Cunha & Cunha, 2008). Significant efforts were made to find out the causative agent of malaria, its treatment and preventive measures against it. But was not revealed, until the year 1880, when Laveran discovered disease-causing protozoa in the blood of patients with malaria in Algeria (Schulze, 2006). Seventeen years later, the anopheles mosquito was confirmed to be the vector for the disease by Roland Ross (Burfield & Reekie, 2005; Schulze, 2006). Following this the most important features of the epidemiology of malaria appeared clear, and control procedures in progress to contain this fatal threat.

Plasmodia parasites are belong to the coccidian stem of the Coccidiasina sub-class and reveal most of the representative characteristics of the Apicomplexa phylum (Table 2.1). Plasmodia demonstrate asexual development stages in the tissue and blood of the vertebrate host (intermediate host) followed by sexual development stages in the insect vector (definitive host).

Table 2.1: Scientific classification of *Plasmodium*

Sub-kingdom Protozoa
Phylum Apicomplexa
Class Sporozoasida
Sub-class Coccidiasina
Order Eucoccidiorida
Sub-order Haemospororina
Family Plasmodidae
Genus Plasmodium

Five different species of genus *Plasmodium* infects humans, *Plasmodium falciparum* (the most effective parasite in causing malaria), *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi*. *P. knowlesi*, a simian malaria parasite of the long-tailed macaque monkeys, was reported in Malaysia as a new malaria species infecting human (Singh *et al.*, 2004).

The life cycle of the *Plasmodium* genus is shown in Figure 2.1. The *Plasmodium* species, with the exclusion of *P. knowlesi* and *P. malariae* (which may also infect the higher primates) are exclusively parasites of man. Only 66 out of the 380 species of Anopheline mosquitoes can transmit malaria (Burfield & Reekie, 2005).

When a human is bitten by a female anopheline mosquito, infective sporozoites are passed into the blood stream, traveling to the liver and hepatocytes. The sporozoites undergo asexual reproduction to form exoerythrocytic schizont. The schizont matures and bursts releasing merozoites (PNAS, 2010).

Merozoites go through the bloodstream and infect red blood cells. After a few cycles of erythrocytic schizogony some merozoites develop into micro and macrogametocytes, which no longer have further activity within the human host. When vector mosquito feeds on the human blood the gametocytes are taken up and develop in its gut, where exflagellation of the microgametocytes take place to form the microgamete. The microgamete fertilized macrogamete resulting is ookinete which penetrates the epithelial lining of the midgut, and develops into an oocyst. Sporogony within the oocyst generates many sporozoites and where the oocyst ruptures, the sporozoites migrate to the salivary gland, and ready for injection into another human host (PNAS, 2010).

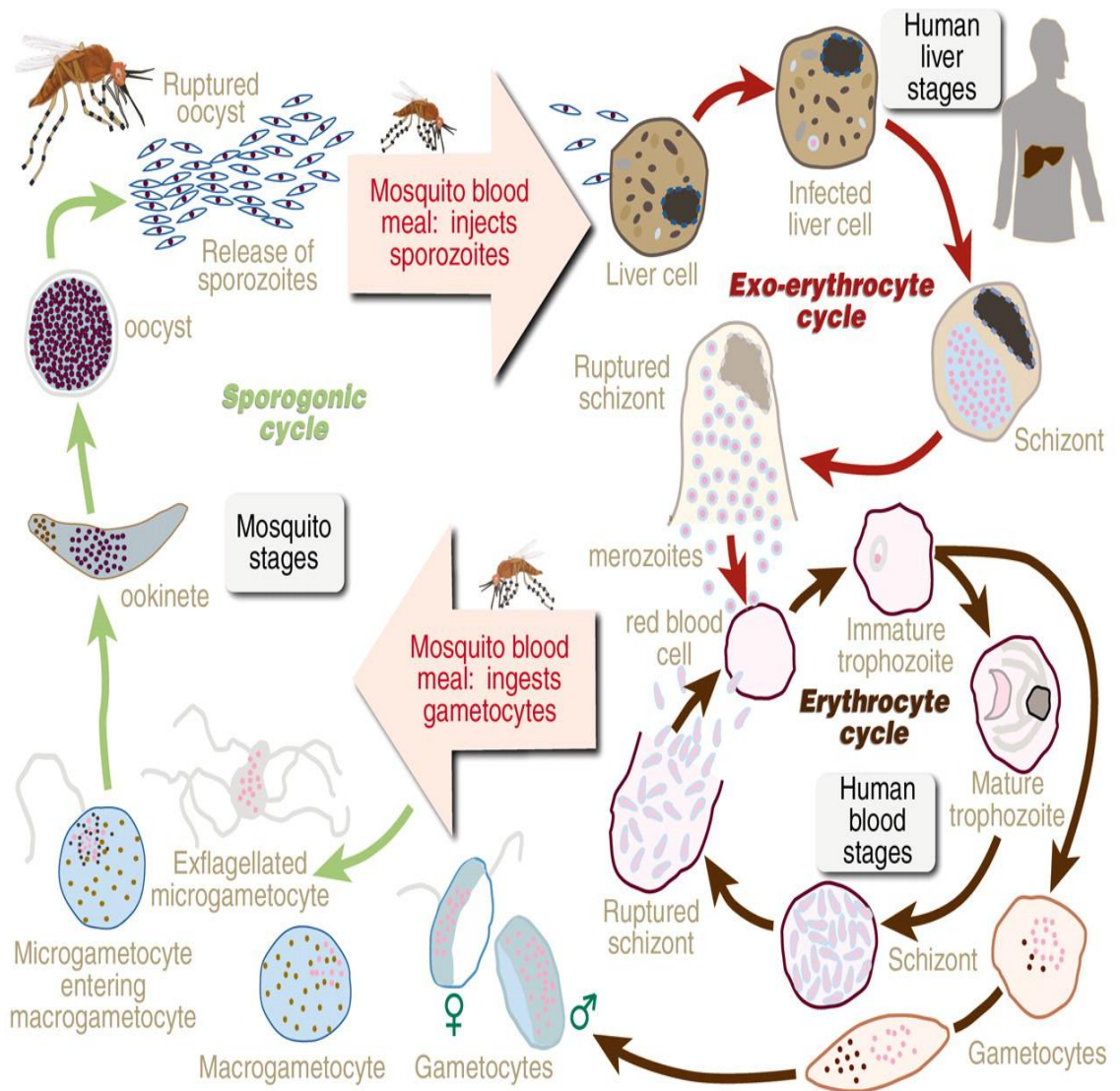


Figure 2.1: The life cycle of the *Plasmodium* genus

Source: PNAS (2010)

P. knowlesi, the fifth malaria species infecting human, occurs in most countries of South East Asia including Malaysia, Thailand, Philippine and Singapore (Jongwutiwes et al., 2004; Singh et al., 2004; Luchavez et al., 2008; Ng et al., 2008). In Malaysia, it is mostly reported in Sabah, Sarawak and Pahang (Indra et al., 2008). For diagnosis of *P. knowlesi*, molecular methods are necessary for confirmation since it is regularly misrelated as *P. malariae* because their RBCs stages are difficult to microscopically differentiated (Cox-Singh et al., 2008). In 1968, Chin et al. reported *P. knowlesi* infection as a zoonosis (animal to human) and reverse zoonosis (animal to human) in a laboratory setting. This emergence of zoonotic malaria parasite is of major public health significance especially in the rural interior region.

As highlighted in the world malaria map 2010 (Figure 2.2), three regions significantly affected by malaria are Africa, Latin America and Asia. Globally, in 2009, there were 225 million cases of malaria and almost one million deaths (WHO, 2010). More than 80% of this mortality occurs in Africa followed by about 15% in Asia and Eastern Europe. Moreover, about 66% of Africa population and 49% of Asia are at risk of infection (WHO, 2005).



Figure 2.2: Malaria world map 2010 indicating the areas; (green) in which malaria transmission occurs, (pale green) area with limited risk, and (white) no malaria

Source: WHO (2011)

2.2 MALARIA IN MALAYSIA

2.2.1 The Country Profile

Malaysia is a country of Southeast Asia; it has a maritime border with the Philippines and Indonesia from the east, west and south, and land borders with Singapore, Indonesia and Thailand from the east and south and north. Malaysia has an estimated land area of 329,758 km². Malaysia Federation consists of 13 states and three federal territories (*Wilayah Persekutuan*), and is divided into two parts: 11 states and two federal territories in Peninsular Malaysia and two states and one federal territory in East Malaysia (Figure 2.3). South China Sea separates West and East Malaysia by about 640 kilometers. Kuala Lumpur is the capital city, however, Putrajaya, is being developed as the new governmental city.

As stated by the Department of Statistics Malaysia (2010), the population of Malaysia was estimated at 28,250,000. In Malaysia, there are different ethnic groups where Malays compose 50.4%, Chinese and Indians about 30.8%. Indigenous groups, include Orang Asli (Aborigines) (Peninsular Malaysia), Kadazans (East Malaysia), Ibans (East Malaysia) and various other groups, compose 11.0% of the total population whereas 7.8% is entitled as others who have chosen to make Malaysia their home. The population density is about 85.8/km². The infant mortality rate in 2009 was six deaths per 1000 births, and life expectancy at birth in the same year was 75 years (UNICEF, 2011). The population proportion below 15 years of age was 33.3% in 2005, and the males- female proportions are 50.9%-49.1%, respectively (Al-Mekhlafi, 2008).

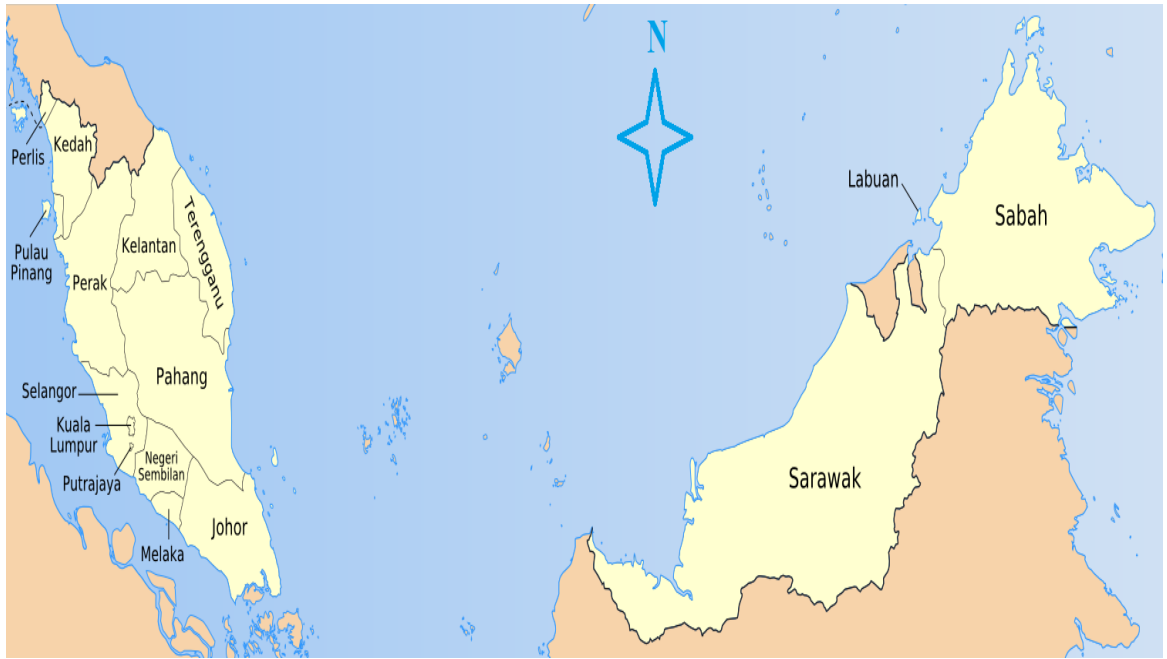


Figure 2.3: The states and federal territories of Malaysia

Source: Golbez & Mdzafrri (2009)

2.2.2 Malaria Status in Malaysia

In Malaysia, malaria stills an important public health issue in rural and aboriginal people (Orang Asli, in Malay areas). Orang Asli are the indigenous inhabitants of Peninsular Malaysia, living in remote forest areas and compose only 0.6% of the total population (Al-Mekhlafi *et al.*, 2008). About 2.2 million people are at risk of infection with the disease with incidence and mortality rates of 2.01/100,000 and 0.33 %, respectively (VBDCP, 2008). It is endemic in Sabah and Sarawak, and some areas of West Malaysia (VBDCP, 2008).

Four malaria species were reported in Malaysia including *Plasmodium falciparum*, *P.vivax*, *P.malariae* and *P. knowlesi* (Singh *et al.*, 2004; Jamaiah *et al.*, 2005; Ahmad & Mahani, 2008; WHO, 2010). A confirmed imported *P. ovale* case was detected by the Department of Parasitology, University of Malaya in the blood of a 20-year-old Nigerian male student (Yvonne *et al.*, 2010). Many malaria endemic areas have more than one mosquito vector species where each species can be either a primary or secondary vector of malaria transmission. Only ten *Anopheles* species have been reported as vectors namely *Anopheles maculatus*, *An. balabacensis*, *An. Latens*, *An. dirus*, *An. letifer* *An. campestris*, *An. donaldi*, *An. sundaicus*, *An. leucosphyrus* and *An. flavirostris* (Rahman *et al.*, 1997; VBDCP, 2008; Ahmad & Mahani, 2008).

Malaria control in Malaysia, dated back to 1901, which was developed to the malaria eradication program in 1967 and then to the vector-borne diseases control program in 1986, that resulted in a great reduction in malaria prevalence from 181,495 in 1967 to 7010 in 2009, of which 8% were imported (VBDCP, 2009; WHO, 2010) (Figure 2.4).

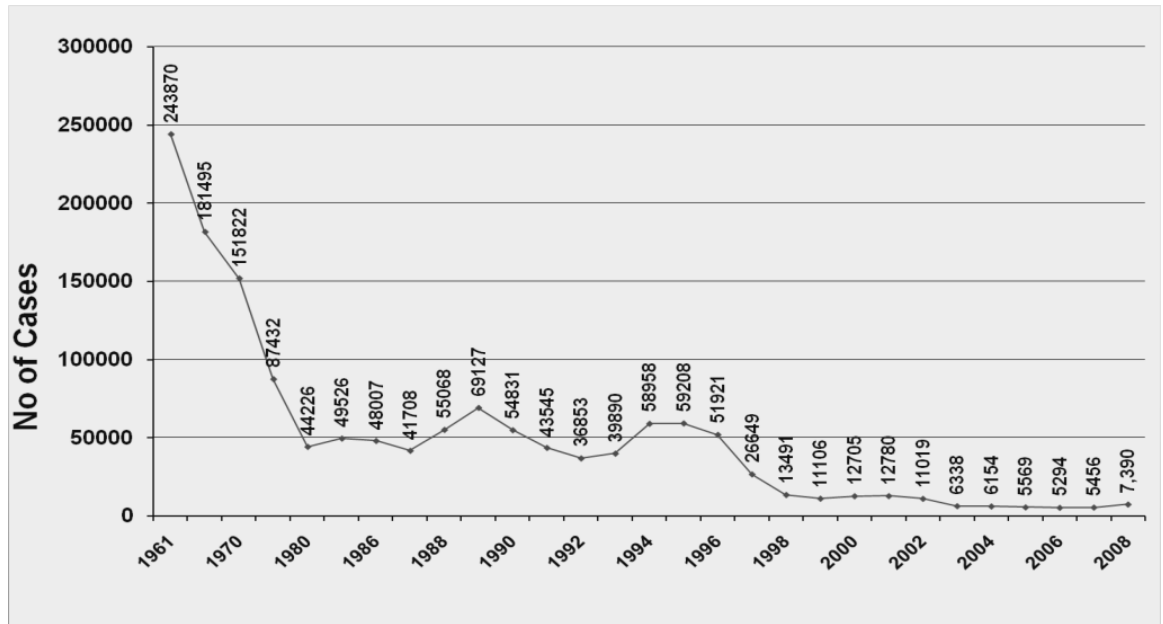


Figure 2.4: Malaria burden in Malaysia 1961-2008

Source: VBDCP, Ministry of Health, Putrajaya, 2009.

Malaysia is now aiming to eliminate the disease from the country by introducing a malaria elimination program in the 9th Malaysia Plan, in the hope to ensure there are no indigenous malaria cases by 2015. However, the data received from the VBDCP, Ministry of Health in the 2008 showed that there was an increase in incidence of malaria in some rural and remote areas (Figure 2.5). One of those areas which demonstrated an increase in the malaria infection is Lipis district located in the state of Pahang (VBDCP, 2009; WHO, 2010).

The key components of malaria control in Malaysia are indoor residual spraying (IRS), insecticide treated bed nets (ITNs) including Long-lasting insecticidal nets (LLINs) and early diagnosis and treatment supplements (WHO, 2010).

In 1963, malaria resistance to chloroquine was first reported in West Malaysia (Montgomery & Eyles, 1963), followed by several reports from East and West parts of the country (Clyde *et al.*, 1973). A widespread spread chloroquine and sulfadoxine-pyrimethamine resistant *P. falciparum* were also reported by Lokman *et al.* (1996) in West Malaysia. In 2001, existence of chloroquine and sulphadoxine-pyrimethamine combination resistance was confirmed in Terengganu and Perak states (Talisuna *et al.*, 2004).

Furthermore, the temperature in Malaysia has been anticipated to increase by 0.18 °C per decade (Chong & Mathews, 2001). Hence, tropical diseases such as malaria will rise as a result of that increase in the temperature, as mosquitoes prosper on these climatic changes. “Global warming would increase the temperature of areas where mosquitoes could not live previously and infect more people with diseases”, said, Datuk Seri Azmi Khalid (2007).

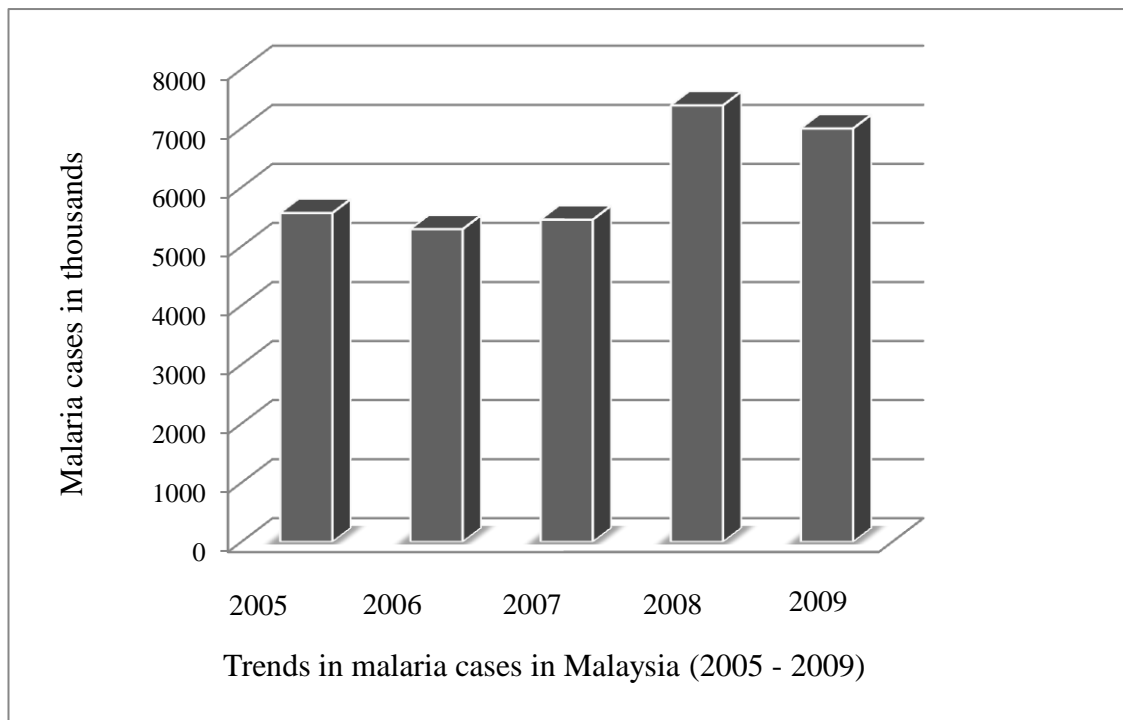


Figure 2.5: Trends in malaria cases in Malaysia, 2005–2009 (WHO, 2010)

2.3 CHALLENGES FACING MALARIA CONTROL

Despite substantial efforts to eradicate malaria in the last century, it remains serious infectious disease in most developing countries. Furthermore, this deadly disease comes back to some countries which had previously eliminated it, and transmitted to further regions such as Eastern Europe.

Control of malaria depends on a combination of different means against the insect vector and health-control interventions to treat the disease including insecticide-treated bed nets, indoor spraying, drainage, insect-growth regulators, and biological means for larval control, drug treatment and vaccination (Curtis, 1991; Takken & Scott, 2003). While these means and interventions help to decrease the prevalence of the disease by break of transmission, they do not eradicate the parasite (Takken & Scott, 2003). Moreover, it has been confirmed that it is farther complicated to eradicate an insect vector, and anywhere successful control has been arrived, this was often the result of short-term interruption of transmission to clear the human host of the parasite as has been the case for malarial disease (Takken & Scott, 2003).

There are several aspects which contribute to these challenges including the lack of efficient vaccine, the drug resistance which is an increasing problem in Asia, Africa and South America, ineffective control of malaria mosquito vector. Furthermore, failure to diagnose malaria in the early stage of infection often leads to mortality that could have been avoided by timely therapy (Fischer & Bilek, 2002). Poverty and malnutrition increases the susceptibility of individual in endemic areas to malaria and increases the mortality rate (Sharma, 2009).

Pesticides such as dichlorodiphenyltrichloroethane (DDT) are still applied in indoor residual spraying to control the mosquito vector in many countries. Even though it has to some extent useful in reducing the incidence of malaria, it has also caused various long-term negative affects and a resistance of mosquito vectors to DDT were reported (Talisuna *et al.*, 2004).

Global warming represents a new challenge facing malaria control. Conjectures on the prospective effect of climate change on human healthiness commonly meet on mosquito-borne diseases. Relevant studies proposed that higher climate temperature will increase the transmission rates of mosquito-borne diseases (Reiter, 2001). In the coming decades, forecasts indicate a massive increase in malaria cases that would happen in endemic areas, and the transmission would reach the higher altitudes (Reiter, 2008).

Population development, urbanization, deforestation, new agricultural and irrigation projects and immigration have all created new breeding sites for the disease vector, resulted in increasing of malaria transmission and farther epidemics (Hardwicke, 2002; Wangchuk, 2004).

All these factors represent serious problems facing malaria control programmes in many parts of the world and leading too much higher rates of disease and deaths.

2.4 MALARIA DRUG RESISTANCE

Malaria drug resistance is a real obstacle facing malaria control efforts. The appearance of drug resistance led to increasing the disease mortality rates and drugs cost. Resistance of *P. falciparum* against a variety of drugs is prevalent and showing fast decline in drug sensitivity for the period of the past decade. To a slight level, resistance of the others *Plasmodium* species has been reported in some countries such as Indonesia, Myanmar and India. Drug resistance is responsible for the extent and recurrence of malaria to new regions that had previously eradicated the disease and also taken part in the occurrence and seriousness of epidemics (Ridley, 1997).

The main mechanism responsible for the emergence of resistance to anti-malarial agents is biologically acquired genetic mutations in *Plasmodium* species with the aim to give a survival advantage for the malaria parasite. These mutations are the responsible for the decline that exhibited in drug sensitivity (Nagelschmitz *et al.*, 2008). Medication of a high biomass infection by giving sub-therapeutic dosage or sub-standard drug is unable to kill mutant parasites leading to a selective pressure for resistance. These mutant parasites with acquired resistance are subsequently transmitted to other hosts by vectors. Additionally, it is more likely that drug with long half-time to be selected in treating resistant parasites rather than low drug concentrations which only be able to cure sensitive species (Falade *et al.*, 2008).

2.4.1 Drug Resistant *P. falciparum*

Southeast Asia has showed a significant responsibility as a center for the growth of drug resistance of *P. falciparum*. *P. falciparum* resistant to chloroquine first reported almost at the same time in Thai-Cambodian border, Thailand and Colombia, Latin America in late 1950s (Spencer, 1985; Wernsdorfer & Payne, 1991). In the early sixties, the emergency of chloroquine resistance resulted in a considerable rise in death rate (Thimasarn, 1999). In the early seventies, chloroquine resistant *falciparum* strains had reached all endemic areas of Southeast Asia, South America and India (Sehgal *et al.*, 1973; Peters, 1987). At the end of the eighties, *P. falciparum* resistant to chloroquine was prevalent in almost all Asia, sub-Saharan Africa and Oceania (WHO, 1997).

The widespread of the resistance of *P. falciparum* strains to chloroquine led to the use of other anti-malarial drugs like quinine, sulphadoxine-pyrimethamine and Mefloquine in the seventies. Resistance to sulphadoxine-pyrimethamine and quinine was early observed in 1960s from Thai-Cambodian border (Bjorkman & Phillips-Howard, 1990; Pickard & Wernsdorfer, 2002). Since then, sulphadoxine-pyrimethamine and quinine resistance have been observed in several places of Southeast Asia, Western Oceania, South of China, South America and Africa (Aramburu *et al.*, 1999; WHO, 2001a; Jelinek *et al.*, 2001; Zalis *et al.*, 1998). In the late eighties, the sulphadoxine-pyrimethamine resistance it was no less than 90% in Brazil (Souza, 1992), sulphadoxine-pyrimethamine resistance was detected in India (Choudhury *et al.*, 1987) and mefloquine resistant *P. falciparum* strains was first reported from the Thai-Cambodian border (Wongsrichanalai *et al.*, 2001).

Cross-resistance among halofantrine and mefloquine is proposed by reduced response to halofantrine when used to treat mefloquine failures (Kuile, 1993). Multidrug resistant is typically referring to resistance to both chloroquine and sulphadoxine-pyrimethamine, but may also include resistance to other compounds as well (Bloland, 2001).

Artemisinin (known as qinghaosu) and its derivatives are the most recent anti-malarial drugs. They possess the most rapid action of all current drugs against *P. falciparum*. However, evidence of artemisinin-resistant *falciparum* malaria has been reported on the Thai-Cambodian border (Pickard *et al.*, 2003; Dondorp *et al.*, 2009).

2.4.2 Drug Resistant *P. vivax* and *P. malariae*

Chloroquine resistance in *P. vivax* was unidentified until 1989, when Australians sent home from Papua New Guinea failed regular treatment (Rieckman *et al.*, 1989). Following reports confirmed that finding, where chloroquine resistant *P. vivax* strains were reported from Indonesia, Myanmar and India (Schwartz *et al.*, 1991; Marlar-Than *et al.*, 1995; Dua *et al.*, 1996). Surveys in Indonesia exposed a higher risk in the east of the country (Baird *et al.*, 1996; Sumawinata *et al.*, 2002). Resistance of *P. malariae* to chloroquine was observed in Indonesia (Ridley & Fletcher, 2008). Drug resistant *P. ovale* as well as *P. knowlesi* parasites have not yet been documented.

2.5 URGENT STRATEGIES TO CONTROL MALARIA

2.5.1 Combination Therapies

The growth of malaria resistance holds back the anti-malarial control tactics. Anti-malarial drugs combination to control multidrug resistant malaria is an urgent need. The benefits of each candidate treatment of drugs combination must suspiciously be taken into account for particular malaria endemic areas, where the disadvantages of the selection may have an effect on upcoming drug policies and ability to manage mortality due to this serious disease (Farooq & Mahajan, 2004).

WHO (2001b) advocates all territories suffering resistance to regular monotherapies, for instance chloroquine and sulfadoxine/pyrimethamine, be supposed to employ artemisinin-based combination therapies (ACTs) used for *P. falciparum*.

The idea of using drug combinations to delay malaria drug resistance came from Peters (1990). The rational for experiencing this combination is properly launched in tuberculosis treatment and cancer chemotherapy (Yeung *et al.*, 2004). The curative rates through employing combinations can be sustained and this tactic keeps medicines in a mutual fashion (Olliaro & Taylor, 2004). However, the basis to success in this issue is to keep using these combinations against the parasites that are still sensitive to the used drugs (Bloland, 2001).

The chance of *Plasmodium* species developing resistant at the same time to two drugs is:

Mutation frequencies/parasite \times the total No. of parasites subjected to drugs (White, 1999; Yeung *et al.*, 2004).

The options for malaria treatment are restricted to the following drugs: chloroquine, sulphadoxine/pyrimethamine (SP), amodiaquine, mefloquine, dihydroartemisinin, artesunate, the recently registered atovaquone/proguanil, chlorproguanil/dapsone (LapDap), dihydroartemisinin-piperaquine, and artemether/lumefantrine (Olliaro & Taylor, 2004; WHO, 2006).

2.5.2 New Anti-Malarial Agents from Medicinal Plants

As showed above in the discussion of combination therapies to overcome malaria resistance, this strategy represents a delay of malaria resistance to the treatments currently available in the markets. Currently no single drug is successful for treating multi-drug resistant *Plasmodium* malaria. In addition resistances to artemisinin-derivatives (Pickard *et al.*, 2003; Dondorp *et al.*, 2009) and to drug combination therapies (Wichmann *et al.*, 2004) have already appeared. Hence, in the lack of an effective, safe and commonly available malaria vaccine, attempts to develop new anti-malarial drugs continue to being urgently needed.

Consistent with this specification for the production of new anti-malarial agents, traditional medicinal plants have several potential advantages; they are affordable, easily accessible and there is no evidence of resistance to whole-plant extracts. Moreover, traditional medicinal plants have been used to cure this fatal disease for hundreds decades and they represent the ingredients of the two major classes of modern anti-malarial drugs; artemisinin and quinine derivatives (Cordell *et al.*, 1994; Willcox & Bodeker 2000; Willcox and Bodeker, 2004; Batista *et al.*, 2009).

The first major research on anti-malarial activities from plant extracts was started in 1947 by the screening of 600 species of plants belong with 126 families but it was only in the mid 1980s that the testing of these plant extracts was completed (Phillipson, 1999). The research found several plants, particularly in two families; the Simaroubaceae and Amaryllidaceae were active against avian malaras (Phillipson, 2001).

2.6 FURTHER STRATEGIES TO CONTROL MALARIA

Different strategies to the above are at present being studied in an effort to eradicate or at least contain the malaria problem. The most important of these are discussed below.

2.6.1 Small interfering RNAs (siRNA)

David Baulcombe's group interferes the RNA interference (RNAi) pathway by the addition of double stranded RNA (dsRNA) to the pertinent cells or organism (Hamilton & Baulcombe, 1999). The double stranded RNA is then cleaved into smaller RNA fragments by RNAase III-like enzyme (Dicer) (Schulze, 2006). The mature single-

stranded siRNA are degraded and translationally inhibited by direct them to bind to untranslated regions of target mRNAs (Xue *et al.*, 2008).

Researches who take advantage of this pathway in the fight malaria are on in full swing. However, to date no RNA interference (RNAi) gene candidates have been identified in the experimental *Plasmodium* species, *P. berghei* (López-Fraga, 2008).

2.6.2 Structure-Based Drug Discovery

Structure-based drug discovery is a further tool to discover potent anti-malarial drugs; it is still in its infancy for most targets. This drug development approach involves the identification of practicable target proteins and detection of its three dimensional structure by X-ray crystallography method. Lactate dehydrogenase, triosphosphate isomerase and plasmepsin II *Plasmodium* enzymes are the most highly developed targets of this pathway of anti-malarial drug discovery to offer significant progress in better understanding the selective inhibition of these enzymes as well as mutational changes leading to drug resistance (Mehlin, 2005).

Protein expression remains a basic challenge facing this tool of anti-malarial drug development, and there is, up to now, no simple solution to this difficulty (Mehlin, 2005). Moreover, the function of about 60% of the encoded genes of *P. falciparum* genome sequence is not understood (Gardner *et al.*, 2002).

2.6.3 Genetically-Modified Mosquitoes

Genetically-Modified Mosquitoes is aimed to design genetically modified mosquitoes, which are unable to transmit malaria parasites (Catteruccia *et al.*, 2000; Schulze, 2006; Wilke *et al.*, 2009). Recently, this was realized in the mosquito *Anopheles stephensi* Liston, which was transformed in order that binding of the malaria parasite *Plasmodium berghei* to the midgut membrane of *A. stephensi* and sporozoite passage through the epithelium of the salivary glands were considerably reduced (Ito *et al.*, 2002). In view of the fact that Ito's trial was carried out using rodent malaria, further studies are required using a human model (Schulze, 2006).

Perspectives works in this field is the successful application of the RIDL system to *Drosophila melanogaster* (Thomas *et al.*, 2000) which can potentially be adopted to be used in mosquitoes and other vectors of human pathogens. The RIDL system has recently been adopted for use in *Aedes aegypti*, based on a non-female-specific construct (LA513) (Phuc *et al.*, 2007), which produces mosquitoes that die as larvae without tetracycline, but can develop normally when raised in the presence of this drug (Wilke *et al.*, 2009). In addition, new genetic constructs have been proposed that rely on the use of a promoter specifically began in immature *Aedes aegypti* females, known as *Act4* (Muñoz *et al.*, 2004). These systems have shown hopeful results in the laboratory and field tests are expected to have begun in Malaysia (Lee *et al.*, 2008).

2.6.4 Malaria Vaccine

A vaccine is hard to develop against malaria infection because of the incidence of antigenic polymorphism. Genetic polymorphism of the different species as well as stages of malaria species is a main reason behind the ability of the parasite to survive regardless of the immune responses produced by the human (Martin *et al.*, 1987). Furthermore, the slow progress of immunity in the infected hosts residing in malaria endemic areas is in agreement with the suggestion that efficient immunity merely develops following experience to several of genetically diverse *Plasmodium* species strains (Talisuna *et al.*, 2004).

The existing clinical trials to develop vaccines against malaria utilize a number of stage specific antigens. The proteins targeted by the vaccines include sporozoite surface antigen-2 (SSP-2), circumsporozoite protein (CSP), merozoite surface antigen (MSA-1), apical merozoite antigen (AMA), liver stage antigen-1 (LSA-1), serine rich antigen (SERA), a sexual stage antigen of *P. falciparum* (Pfs25) and schizont export antigen 5.1 (Schulze, 2006). Some of these trials unfortunately met with no success.

To date RTS, S/AS02A is one of the most useful vaccine efforts to develop important protection against *Plasmodium* infection. It is containing containing circumsporozoite protein (CSP) combined with a hepatitis B surface antigen. It affords limited protection against malaria in naive and hyper-immune adult volunteers (Alonso *et al.*, 2004; Dubois *et al.*, 2005; Schulze, 2006). The Walter Reed Army Institute of Research developed that vaccine to protect their troops sent into endemic areas of malaria (Bojang *et al.*, 2001; Kester *et al.*, 2001). The vaccine has a protective activity up to six months and found to be effective, safe and tolerated in clinical trials (Alonso *et al.*, 2004; Schulze, 2006; Aponte *et al.*, 2007). Many other candidate vaccines are in

progress, but the trial results of this one, published in the Lancet, is the most promising yet. This strategy is being studied further (Schulze, 2006).

2.7 AWARENESS OF COMMUNITY REGARDING MALARIA

Ignoring community's knowledge, attitudes and practices (KAP) about malaria has led to the lack of ability to attain sustainable malaria control programmes. Knowledge community's awareness of transmission, symptoms, treatment and prevention of malaria is a vital step to control the disease (Govere *et al.*, 2000; Simsek & Kurcer, 2005). The 1998 Roll Back Malaria (RBM) programme, launched in Geneva by WHO, UNDP, UNICEF and the World Bank, is a people oriented plan that emphasises community involvement (Udonwa *et al.*, 2010). The participation of the community represents one cardinal tool of malaria control programs by WHO as the improvement of understanding of transmission of malaria can greatly increase the realization and sustainability of malaria elimination program (Hlongwana *et al.*, 2009).

There have been many studies regarding the knowledge, attitudes and practices concerning malaria in different parts of the world. Various knowledge, attitudes and practices (KAP) surveys show these misconceptions related to malaria exist and practices for the control of malaria have been inadequate (Nyamongo, 2002; Swe & Pearson, 2004; Oguonua *et al.*, 2005; Xia *et al.*, 2007; Joshi & Banjara, 2008; Udonwa *et al.*, 2010). Providing efficient health education has often been showed as a potential response so those communities residing in malaria endemic areas are made aware of the transmission, the preventive measures and the seriousness of the disease.

Knowledge, attitudes and practices surveys to study awareness of a community concerning malaria are appropriate to plan and enhance malaria control programmes, put epidemiological and behavioural database and ascertain guides for supervising a programme's success (Macheso *et al.*, 1994). The outcomes of these studies can be adopted to fit the local requirements of the community based on the information resulted from such survey data (Ongore *et al.*, 1989). For instance, a community's awareness of malaria transmission, treatment-seeking behaviour and vector control can be applied to establish clearer knowledge, attitudes and practices of the population enabling the use of efficient tools for health education and improving and sustaining good practices for malaria prevention. (Miguel *et al.*, 1999; Mazigo *et al.*, 2010). Accordingly, the results of these types of studies will assist the health authorities into the decision making processes, the design of interventions with active community participation, and the implementation of educational schemes (Paulander *et al.*, 2009). Furthermore, community awareness about a particular disease prepares the ground for the work of specialized studies on the disease in that community. For example, the study of people's knowledge on anti-malarial plants requires studying the community's knowledge of the disease itself. People's awareness of the disease lays the groundwork for any study related to the disease. In this study, this methodology has been followed.

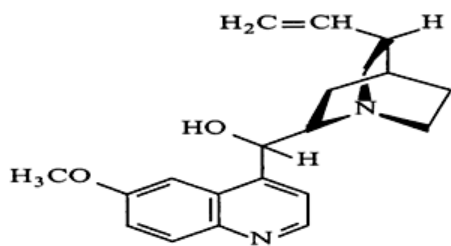
Although malaria control program in Malaysia has been going on more than a hundred years, but there is no survey on knowledge and practice of the population concerning malaria. In parallel with the implementation of Malaysia's malaria elimination programme, this study was carried out to investigate the knowledge, attitude and practices about malaria transmission, treatment and vector control between the hill/forest and the rural communities in Peninsular Malaysia.

2.8 ETHNOBOTANICAL PLANTS AS A SOURCE OF ANTI-MALARIALS

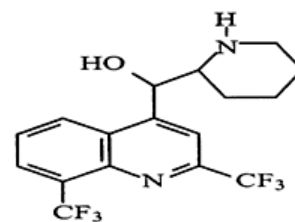
The use of plants for medicinal purposes is an ancient idea, as confirmed by the earliest documented uses uncovered in Babylon and ancient Egypt (1770 BC and 1550 BC, respectively). Ancient Egyptians believed that remedial plants were even efficient in the life after death of their Pharaohs, as revealed by the plants recovered from the Giza pyramids (Veilleux & King, 1996). In the Third World, 80% of people are estimated to rely on plant remedies (WHO, 1993). Moreover, the medicinal plants are responsible for the development of many modern synthetic anti-malarial agents (Figure 2.6).

Quinine is an aminoquinoline alkaloid that isolated from *Cinchona* species bark was the first anti-malarial agent of plant sources. It was discovered in 1820 by Caventou and Pelletier (Phillipson, 2001). The quinine molecule has been considered responsible for the production of synthetic drugs of 4- and 8-aminoquinolines, such as chloroquine and primaquine after the Second World War (Coatney, 1963). With the discovery of these drugs, malaria was eradicated from the developed countries and parts of the developing countries in South America and Asia and the seeking for novel anti-malarial drugs became less important for these countries (Wangchuk, 2004).

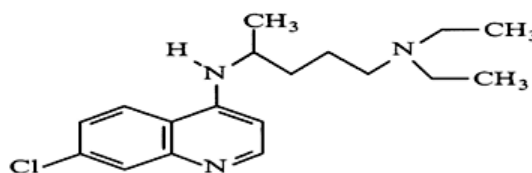
Chloroquine was used as the treatment of choice against all species of malaria parasites for many years as a result of its high curative activity, low toxicity, low cost and still used to treat malaria in some areas in the developing countries where drug resistance has not yet reported (Krettli *et al.*, 2001).



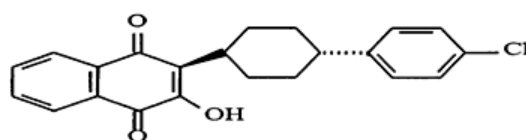
Quinine



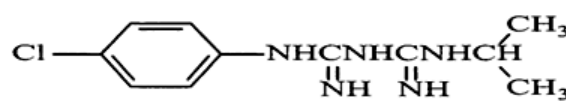
Mefloquine



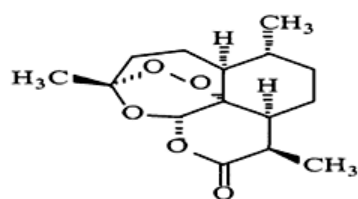
Chloroquine



Atovaquone



Proguanil



Artemisinin

Figure 2.6: Structures of important anti-malarial molecules of current use to treat malaria

Source: adapted from Krettli *et al.*, 2001 and Batista *et al.*, 2009.

From *Tabebuia* species indigenous to South America, naphthoquinones compounds were produced (Heinrich *et al.*, 2004). They have supplied a molecule template on which atovaquone was developed. A combination of Atovaquone and proguanil was approved for clinical anti-malarial use (Edstein *et al.*, 2005).

Artemisinin is another example of a novel sesquiterpene endoperoxide anti-malarial agent that developed in 1972 from a traditional medicinal plant, *Artemisia annua*. It is used for thousands of years to cure malaria in Chinese folkloric medicine (Krettli *et al.*, 2001; Saxena *et al.*, 2003). The isolation of artemisinin led to reiterating many research groups to search for new anti-malarial agents from plants that have ethnopharmacological basis and provoked the evaluation of anti-malarial activity of natural peroxides (Klayman, 1985; Krettli *et al.*, 2001).

Thus, it becomes clear that plants have proved to be an important basis of malaria treatments. Therefore, it is vital that other medicinal plants that have ethnobotanical standing are investigated, to ascertain their efficacy, and to reveal their ability as supplies of new drugs in coping with the spread of malaria parasites resistance.

2.8.1 Ethnobotanical Approach to Anti-Malarial Plant Selection

Ethnobotany is the study of the useful association between human and plant including medicinal uses (Hershberger, 1896). Usage of medicinal plants by people regularly concerns to their importance in the community, medicinally, religiously or traditionally. Medical ethnobotany has great importance in drug discovery; it saves effort and confirms the suspicion. The plants studied through the medical ethnobotany tend to be safe and active and therefore laboratory tests will be mostly positive (Gottlieb *et al.*, 2002). Moreover, medical ethnobotanical information create a prediction by the researchers regarding the biological activity of plant, for example, if the designated plant used against malaria, and laboratory tests showed negative results that this plant may be has indirect effects, it may be that the plant is very useful in reducing the body temperature. Medical ethnobotany is very important in keeping information about the plants used to treat malaria and circulation these information between generations (Koch, 2005). Many novel drugs have been discovered through the active medical heritage of medicinal plants such as quinine. Thus, the search of anti-malarial drugs through ethnobotanical survey could lead to the discovery of new therapeutic structures of anti-malarial drugs.

Ethnopharmacology and medical ethnobotany: ethnopharmacology is the study showing a relationship among ethnic groups, their health, and how it correlates to their physical habits and methodology in making and using medicines (Etkin, 1996). On the other hand, medical ethnobotany searches for incorporate ethnobotany and ethnopharmacology by exploring the field study factors affecting medicinal plant knowledge followed by laboratory analysis of the toxicity and activity of the plants used

by the people to increase the plant's therapeutic understanding values for people who depend on them and for possibly discovering new drugs (Lewis, 2003; Koch, 2005).

Currently, efforts to document the data about anti-malarial plants used in traditional plant medicine become important for the additional anti-malarial laboratory investigations and for isolating and identifying of new anti-malarial drugs (Cox, 1994), which is reflected by intensive ethnobotanical studies (Phillipson 2001; Zhang *et al.*, 2002; Weniger *et al.*, 2004; Asase *et al.*, 2005; Zirihi *et al.*, 2005; Botsaris, 2007; Nguyen-Pouplin *et al.*, 2007; Namsa *et al.*, 2010).

2.8.2 Anti-Malarial Activity of Traditional Medicinal Plants Crude Extracts

The discovery of artemisinin from *Artemisia annua* and its successful in the treatment of malaria have evoked the interest in investigating medicinal plants as sources for new anti-malarial agents (Taylor & Berridge, 2006). Currently, efforts to study the anti-malarial activity of plants have increased dramatically. This subheading presents some anti-malarial studies of crude extracts from medicinal plants that showed significant activity during the last years.

In Malaysia, Sahidan *et al.* (1994), Nik-Najib *et al.* (1999), Kit-Lam *et al.* (2004), Noor Rain *et al.* (2007) and Wan Omar *et al.* (2007) studied the anti-malarial activity of some Malaysian traditional plants crude extracts. *Eurycoma longifolia*, *Piper sarmentosum*, *Andrographis paniculata*, *Tinospora crispa*, *Jasminium sambac*, *Xylocarpus granatum*, *Goniothalamus scortechinii* and *Goniothalamus macrophyllus* showed considerable anti-malarial effects.

In Japan, *Hydrangea macrophylla* leaf water extract showed a potent *in vivo* anti-malarial activity against *Plasmodium yoelii* in mice. The treated ICR mice exhibited a reduction in *P. yoelii* parasitaemia to undetectable level following a transient recrudescence assay (Ishih *et al.*, 2001).

In Myanmar, *Zingiber cassumunar* rhizomes, *Ferula foetida* latex, *Myristica fragrans* whole fruits and *Piper nigrum* seeds (Hlaing *et al.*, 2008) displayed good effects against *P. falciparum in vitro*.

In India, *in vivo* anti-malarial dose of one g/kg of *Aegle marmelos*, *Artemisia scoparia*, *Cinnamomum tamala*, *Enicostema hyssopifolium*, *Jurinea macrocephala*, *Momordica dioica*, *Nyctanthes arbor-tristis*, and *Prunus persica* were found to possess potent schizontocidal activity (50% and above) against *Plasmodium berghei* in mice (Misra *et al.*, 1991). The following five species used to treat fever or malaria in India seems to be of special significance for further anti-malarial studies: *Casearia elliptica*, *Holarrhena pubescens*, *Pongamia pinnata*, *Plumbago zeylanica* and *Soymida febrifuga* (Simonsen *et al.*, 2001). In 2011, Samy and Kadarkari studied the *in vivo* anti-malarial activity of 81 plants crude extracts against *P. berghei* NK65 in mice, 55.5% of the plants extracts gave significant parasitaemia chemosuppression activity.

In Brazil, *in vivo* anti-plasmodial activity of traditional plants species was evaluated experimentally in mice. The crude extracts were investigated in mice at up to one g/kg for four days against *P. berghei*. *Esenbeckia febrifuga*, *Acanthospermum australe*, *Tachia guianensis* and *Lisianthus speciosus* were partially active against the rodent malaria (Carvalho *et al.*, 1991). *Potomorphe umbellate*, traditional anti-malarial plant in Brazil showed strong anti-malarial activity against *P. berghei* subjected to the

4-day suppressive test at 250 and 1250 mg/kg in mice (Amorim *et al.*, 1988). The essential oil obtained from leaves of *Viola surinamensis* caused 100% inhibition against development of the young trophozoites to schizonts stage (Lopes *et al.*, 1999).

Anti-malarial effect of 14 traditional medicinal plant species used as anti-malarial and fever remedies in Central America were investigated against chloroquine sensitive and resistant strains of *P. falciparum in vitro*. *Xylopi* *cf. frutescens*, *S. tonduziana*, *S. pauciflora*, *Siparuna andina* and *Piper hispidum* showed significant anti-plasmodial results (Jenett-Siems *et al.*, 1999).

Panama, Nicaragua, Guatemala, Costa Rica, Colombia, Bolivia and Argentina medicinal plants; *Abuta grandifolia* leaves, *Acacia farnesiana* leaves, *Acnistus arborescens* aerial part, *Piper holtonii* aerial part, *Acnistus arborescens* leaves, *Piper cumanense* fruits, *Monochaetum myrtoideum* leaves, *Croton leptostachyus* aerial part, *Bourreria huanita* branch and *Xylopi* *aromatica* bark (Garavito *et al.*, 2006; Calderón *et al.*, 2010) displayed significant anti-plasmodial activity.

In Congo, crude extracts of *Phyllanthus niruri*, *Morinda morindoides* and *Cassia occidentalis* were investigated against *P. berghei* ANKA in mice to evaluate their *in vivo* anti-malarial activity. *M. morindoides*, *P. niruri* and *C. occidentalis* produced parasitaemia reductions of 74%, 72% and 60%, respectively (Tona *et al.*, 2001). *Albertisia villosa* is a traditional medicinal plant used in Congo against various diseases. Basic alkaloidal extracts of this plant revealed potent anti-plasmodial activity (Lohombo -Ekomba *et al.*, 2004).

Of six studies carried out in Nigeria to evaluate the anti-malarial activity of some traditional plants used to treat malaria, it was observed that crude extracts of *Myrtus communis* (essential oil), *Rosmaricus officinalis* (essential oil), *Cassia singueana* (root bark), *Artemisia maciverae*, *Xylophia aethiopica* (fruits), *Acacia nilotica* (Leaves), *Croton zambesicus* (leaves), *Cylicodiscus gabunensis* (stembark) and *Euphorbia hirta* (leaves) have significant anti-malarial activity (Milhan *et al.*, 1997; Adzu *et al.*, 2003; Okokon *et al.*, 2005; Okokon *et al.*, 2006; Ene *et al.*, 2008; Oparaocha & Okorie, 2009).

Five Cameroonian plants crude extracts (essential oils); *Antidesma laciniatum*, *Hexalobus crispiflorus*, *Pachypodanthium confine*, *Xylophia aethiopica* and *Xylophia phloiodora* were screened against *P. falciparum*. The essential oils of all five plants showed active anti-malarial effects against *P. falciparum*. The highest *in vitro* anti-plasmodial activity was shown by the essential oil of *H. crispiflorus* (Boyom *et al.*, 2003). In 2005, Tchoumboungang *et al.* studied in mice the *in vivo* anti-malarial activity of *Cymbopogon citratus* and *Ocimum gratissimum* essential oils used in Cameroon to treat malaria. *C. citratus* and *O. gratissimum* oils showed significant anti-malarial effects against *P. berghei* in the four-day suppressive assays in mice. *C. citratus* exhibited higher *in vivo* anti-malarial results than *O. gratissimum* at the same concentration with 86.6 % and 77.8 % suppressions of parasitaemia, respectively.

Kenyan plants utilized in malaria traditional treatment were investigated *in vivo* against *P. berghei* to evaluate their anti-malarial efficacy. *Vernonia lasiopus* (root bark), *Rhamnus staddo* (root bark), *Clerodendrum myricoides* (root bark), *Toddalia asiatica* (root bark), *Ficus sur* (leaves/stem bark/root bark), *Rhamnus prinoides*

(leaves/rootbark) and *Maytenus acuminata* (leaves/root bark) have significant parasitaemia suppressions against *P. berghei* NK65 in ICR mice (Muregi *et al.*, 2007).

In vivo anti-plasmodial activity against rodent malaria in mice exhibited that *Caesalpinia bonducella* leaves and *Tragia fuliaris* roots, used traditionally for treatment of malaria in Tanzania have significant anti-malarial action against *P. berghei* (Innocent *et al.*, 2009).

Hybanthus enneaspermus, *Croton lobatus*, *Nauclea latifolia*, *Fagara macrophylla*, *Funtumia elastica*, *Phyllanthus muellerianus* and *Rauvolfia vomitoria*, used to treat Malaria and fever in Benin and Ivory Coast showed significant *in vitro* activity against cultured *P. falciparum* (Weniger *et al.*, 2004; Zirihi *et al.*, 2005).

In the Middle East, *Nigella sativa*, *Acalypha fruticosa*, *Azadirachta indica*, *Dendrosicyos socotrana*, *Boerhavia elegans*, *Solanum surattense* and *Prosopis juliflora* used in the folk medicine of Yemen and Iran, showed significant anti-plasmodial activity (Abdulelah & Zainal-Abidin, 2007; Alshawsh *et al.*, 2007; Ramazani *et al.*, 2010).

Without a doubt, with all these worldwide efforts to find plants effective against this fatal disease, the goal could be achieved soon. The recently developed pharmacological techniques of evaluation, isolation and characterization of natural products have caused greater attraction in plants. Thus, the search for new additional anti-malarial drugs source from the traditional medicinal plants must keep on combating the disease.

2.9 SCREENING OF ANTI-MALARIAL PLANT EXTRACTS

As described in 2.8.2, two methods can be used to assesses the anti-plasmodial activity of plant extracts; *in vivo* using laboratory model *P. berghei* (rodents malaria parasite) or *in vitro* using cultured *P. falciparum* (human malaria parasite). However, the homeostatic mechanisms and pathways found in animals are not present *in vitro*. The active anti-malarial principles of the extract could be formed by hepatic metabolism or as a result of gut bacteria transformation (Botsaris, 2007). Additional promising mechanisms of action include immunomodulation, antioxidant activity or interference with the invasion of new red blood cells by parasites (Daubener, 1999; Anthony *et al.*, 2005; Botsaris, 2007). Hence, in this study the *in vivo* anti-malarial assays were carried out.

2.9.1 Anti-Malarial Tests in Mice

There are numbers of animal species that can be used in the laboratory to evaluate the anti-malarial activity of chemical compounds and natural products including mouse, rat, hamster, monkey and chicken (Table 2.2). Mice are simple to reproduce, obtain, maintain, control and involving little amount of plant extracts to be tested, compared to other animals (Krettli *et al.*, 2009).

Evaluation of *in vivo* anti-malarial activity of plants extract in mice represents the largest part of the *in vivo* anti-malarial tests and considered as standard procedure in this field (Peters 1965; Misra *et al.*, 1991; Carvalho, 1991; Peters & Robinson 1992; Tona *et al.*, 2001; Fidock *et al.*, 2004; Tchoumboungang *et al.*, 2005; Muregi *et al.*, 2007; Krettli *et al.*, 2009).

Table 2.2: Animal models and *Plasmodium* species used in anti-malarial tests

Animal models	<i>Plasmodium</i> species /strains or clones
Mouse, Rat, Hamster.	<i>P. berghei</i> / NK65, NY, P, KFY, ANKA, K173, RC
Mouse	<i>P. yoelii</i> (nigeriense) / MDR, 17X, ART, N67
Mouse	<i>P. vinckei</i> (petteri) / 279BY
Mouse	<i>P. chabaudi</i> /AS
Mouse	<i>P. cynomolgi</i> /Ro, M, B,
Rhesus monkey	<i>P. knowlesi</i> /W1
Rhesus monkey	<i>P. fragile</i> /Ceylon
<i>Aotus</i> and <i>Saimiri</i> monkeys, Mouse	<i>P. falciparum</i> / Uganda Palo Alto, T24, Vietnam Oak Knoll
<i>Aotus</i> and <i>Saimiri</i> monkeys	<i>P. vivax</i> / AMRU1, Palo Alto
Rhesus monkey	<i>P. coatneyi</i>
Chicken	<i>P. gallinaceum</i> / IOC, 8A

Source: Krettli *et al.* (2009).

When using anti-malarial tests in mice, some changeable need to be taken into account throughout the investigation. The course of infection and lethality vary along with the mouse strain and the rodent malaria species and subspecies (Sanni *et al.*, 2002; Fidock *et al.*, 2004).

The ICR mouse used in this study has been described by Hausckka and Mirand (1973). Many studies published in peer reviewed ISI journals have used this mouse strain to investigate the anti-malarial activity of plants extracts and new anti-malarial agents (Presber *et al.*, 1991; Coleman *et al.*, 1992; Fowler *et al.*, 1994; Ishih *et al.*, 2001; Ishih *et al.*, 2004; Muregi *et al.*, 2007; Ill-Min *et al.*, 2008; Won-Hwan *et al.*, 2008; Jong-Jin *et al.*, 2009; Ill-Min *et al.*, 2009). One of the most interested advantages of this mouse strain is its easy reproduction compared to other mice.

2.9.2 Rodent Malaria Model: *Plasmodium berghei*

Rodent malaria infects murine rodents from Central Africa (Figure 2.7). There is no evidence to the probability of human infection of these parasites and for this reason; they are recognized as valuable parasites for the study in the laboratory. They are comparable to human malaria in their life cycle including mosquito infections and match most of fundamental characteristics of structural morphology and physiology (Carter & Diggs 1977; Landau & Chabaud 1994; Krettli *et al.*, 2009).

They have been authenticated in the course of the discovery of several anti-malarial drugs including artemisinin derivatives, halofantrine, mefloquine and drug combinations, sulphonamides-pyrimethamine (Peters *et al.*, 1977; Peters, 1987; Peters *et al.*, 1987; Vennerstrom, 2000; Posner *et al.*, 2003; Fidock *et al.*, 2004). Rodent

malaria *Plasmodium* parasites also used in anti-malarial drug resistance assays, frequently via drug pressure with subcurative doses of drug (Peters, 1965; Walliker *et al.*, 1975; Peters & Robinson, 1999).

P. berghei, *P. chabaudi* and *P. yoelii* are the most important species of rodent malaria models that still a valuable tool of the anti-malarials studies including liver stage biology, vaccine development, mechanisms of drug resistance, antigenic variation, plant extracts anti-malarial assays and drug screening (Table 2.3).

Some advantages to the use of *P. berghei* in mice for *in vivo* anti-malarial tests in addition to being safe (safe handling), it is extremely virulent to mice reducing the time of experiments to about one month time, where control group of mice (non-treated) die of rodent malaria infection in one to four weeks of infections (according to the mouse and parasite strains) (Krettli *et al.*, 2009).

P. berghei is extensively used in evaluating the anti-malarial activity of plants extracts (2.8.2) and drug screening tests (Table 2.2). Genetically, *P. berghei* and *P. falciparum* complete genome sequencing demonstrate a high similarity in structure and gene content. In addition, genetic engineering technologies can manipulate *P. berghei* in the laboratory (Amino *et al.*, 2005; Janse *et al.*, 2006).

It is always necessary to take into account of certain variables during the experimental design and interpretation as rodent malaria parasite species and strains/clones can vary in the course of infection, lethality and synchronicity depending on the mouse strain, sex and age (Sanni *et al.*, 2002; Fidock *et al.*, 2004).

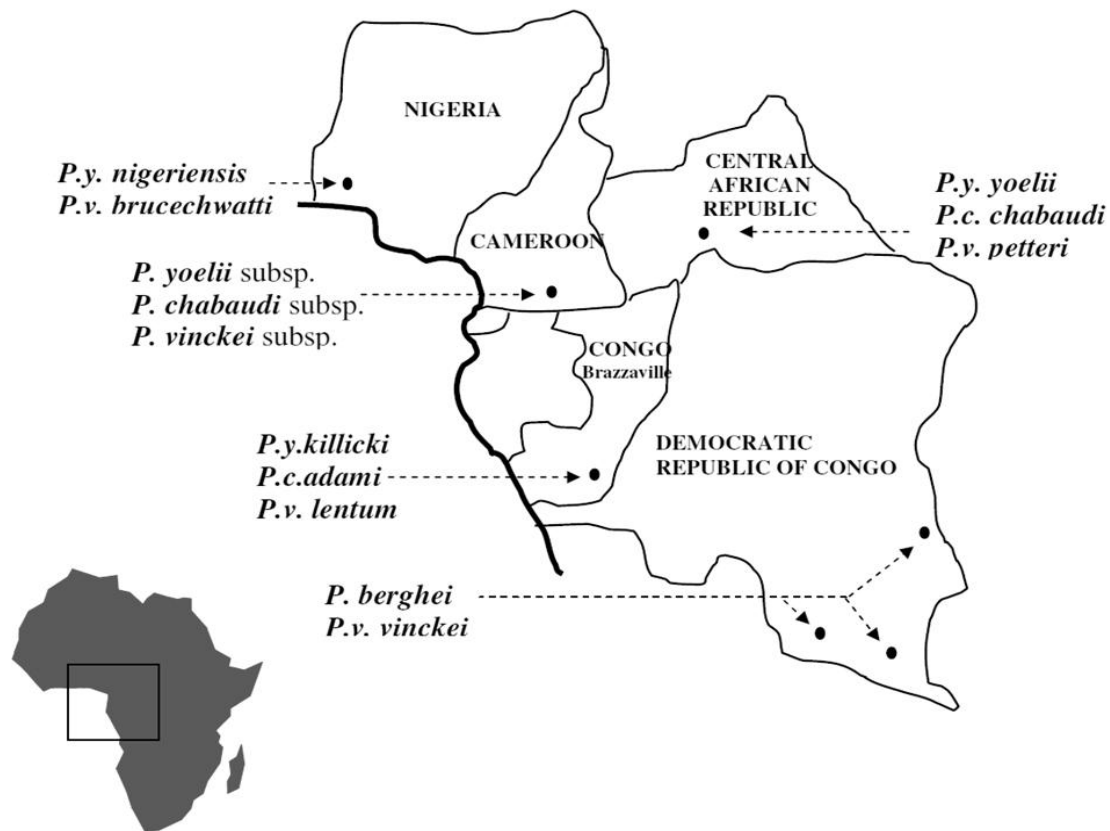


Figure 2.7: Rodent malaria species - countries of origin

Source: From Carlton *et al.* (2001).

Table 2.3: Primary uses of rodent malaria models

<i>Plasmodium</i> species	Primary uses
<i>P. berghei</i>	Plant extracts anti-malarial assays and drug screening
<i>P. yoelii</i>	Liver stage biology and vaccine studies
<i>P. chabaudi</i>	Mechanisms of drug resistance and antigenic variation

Source: adapted from Kalra *et al.* (2006).

2.9.3 *In Vivo* Anti-Malarial Screening Assays

Plant products reducing parasitaemia by 30% or more during early malaria infection are considered active (Krettli *et al.*, 2009) and a series of experiments are carried out to confirm their *in vivo* anti-malarial activities, *i.e.*, 4-Day suppressive test, curative and prophylactic methods according to Peters (1965), Ryley & Peters (1970), Peters & Robinson (1992), Peters & Robinson (1999), Abosi & Raseroka (2003), Elufioye & Agbedahunsi (2004), Bapna *et al.* (2007), Okokon & Nwafor (2009), Ill-Min *et al.* (2009), Basse *et al.* (2009), Madara *et al.* (2010) and Sathe *et al.* (2010).

In these laboratory assays, the test extracts are first evaluated in the 4-day suppressive tests in early malaria infection and further screened for their curative activity in established malaria infection and prophylactic activity in residual malaria infection according to Peters & Robinson (1992), Ryley & Peters (1970) and Peters (1965) Protocols, respectively. Most *in vivo* anti-malarial assays in mice can be performed using five animals per group (Fidock *et al.*, 2004). The flow chart of the *in vivo* anti-malarial assays used to evaluate the activity of plant extracts shown in Figure 2.8 were applied in this study.

Fundamentally, mice are infected intraperitoneally using a small needle inoculum (10^6 – 10^7 *P. berghei* parasitized erythrocytes) (Ishih *et al.*, 2004). To assess the anti-malarial activity of the test extract, thin blood films are prepared from the tail blood of the infected mice. The blood films are then stained with Giemsa's to determine *P. berghei* parasitized erythrocytes (Peters, 1965; Ryley & Peters, 1970; Peters & Robinson, 1992).

The percentage of parasitaemia was detected in random fields of the microscope by counting *P. berghei* parasitized erythrocytes out of 9,000 RBCs:

$$\% \text{ Parasitaemia} = [\text{No. of parasitized RBC} / \text{Total no. of RBC counted}] 100$$

Average percentage chemosuppression was calculated as

$$100 \left[\frac{A - B}{A} \right]$$

Where, A is the mean percentage parasitaemia in the control group (untreated group) and B is the mean percentage parasitaemia in the test group.

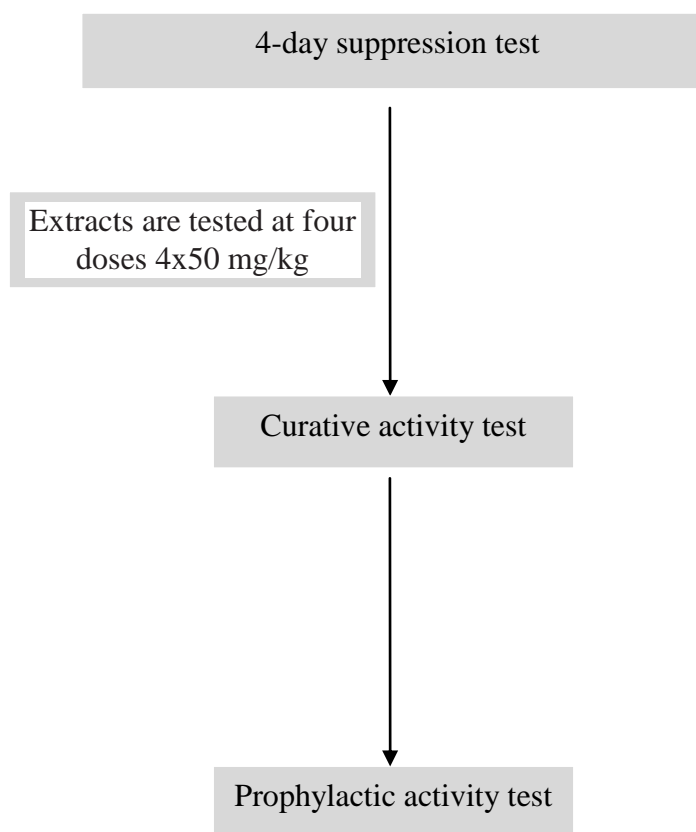


Figure 2.8: Flow chart of *in vivo* anti-malarial activity tests in rodent malaria models

Adapted from : Fidock *et al.* 2004; Elufioye & Agbedahunsi, 2004; Kalra *et al.* 2006; Krettli *et al.* 2009

2.9.4 Phytochemical screening

In this study, phytochemical screening was conducted on plants extract according to the standard procedures described by Hymete (1986), Trease & Evans (1989) and Sofowora (1993). This qualitative screening is of great importance in the prediction about the chemicals that may have caused effective suppression against malaria.

Of those chemicals are alkaloids, anthraquinones, terpenoids and flavonoids which are known as active constituents against parasites, protozoa and malaria (Jones & Luchsinger 1986; Vishwakarma, 1990; Philipson & Wright 1991; Carvalho *et al.*, 1992; Francois *et al.*, 1996; Omulokoli *et al.*, 1997; Kim *et al.*, 2004; Tasdemir *et al.*, 2006). Other chemical constituents that recently showed significant anti-malarial effects include saponin, tannin, steroids and glycosides (Nandi *et al.*, 2004; Reddy *et al.*, 2007; Libman *et al.*, 2008).

The phytochemical screening results paint a road map for future advanced tests of isolation and characterisation of active constituent if the preliminary results showed a promising activity against malaria.

2.9.5 Antioxidant Capacity

The immune system in human is stimulated by infections, including parasites, which results in the production of reactive oxygen species. On the other hand, nitric oxide (NO) is an effective mechanism to kill parasites, which is produced in macrophages in response to parasitic infection, which in turn activates the immune response against malaria (Daubener, 1999). The inhibition of nitric oxide leads to prepare good

surroundings for the development of intracellular parasite. On the other hand, these inhibition cause a decrease in an essential amino acid, tryptophan, which undergo to degradation through indolamine deoxygenase. Hence, the parasite is starved leading to its death (Daubener, 1999; Anthony *et al.*, 2005). As a result, immune stimulation and release of reactive oxygen species, the haemoglobin in red blood cells exposed to degradation (Das & Nanada, 1999; Loria *et al.*, 1999). Therefore, one of the major causes for the incidence of anaemia in malaria infection comes into view to be oxidative stress (Das & Nanada, 1999; Kremsner *et al.*, 2000; Kulkarni *et al.*, 2003).

Moreover, some recent studies have shown that the low level of antioxidants in the plasma of children with malaria may be a major cause of morbidity and mortality (Nmorsi *et al.*, 2007). Accordingly, this confirms that plants that have antioxidant activities be able to work against the oxidative damages resulting from malaria; therefore, the antioxidant defence system in the *Plasmodium* parasites may become a very hopeful drug target in the near future (Botha, 2006).

DPPH radical scavenging activity, depended on the capability of an antioxidant to provide hydrogen radical to synthetic long-lived nitrogen radical compound DPPH, is one of the oldest and most commonly applied procedures to investigate the total antioxidant capacity of food and biological extracts (Brand-Williams *et al.*, 1995; Meng *et al.*, 2009). Recently, it was observed that there is a good correlation between potential DPPH radical scavenging activity and anti-malarial activity of Argan fruit extracts (El Babili *et al.*, 2010).

2.10 MALAYSIAN ANTI-MALARIAL TRADITIONAL MEDICINAL PLANTS

In countries like Malaysia there is a popular interest in the search for therapeutic alternatives to combat the disease, both of alternative medicine based on medicinal plants, or by searching for some means known in the folklore of Malaysia. Government policies in this country supports the popular interest in alternative medicine based on medicinal plants as one of the most important resources of the country, as well as recommend widening the circle of knowledge to include the new juveniles to ensure the conservation and preservation of folk heritage information (Lin, 2006). Moreover, recent studies demonstrated the importance of cooperation with indigenous people and traditional healers in the search for the discovery of new drugs from medicinal plants (Asase *et al.*, 2005). Therefore, the assessment of the activity and safety of traditional remedies is a priority in the search for new drug for malaria, as the safe traditional remedies may be considered as an important source in the fight against the disease (Asase *et al.*, 2005).

Ministry of Health of Malaysia registered around 1300 medicinal plant products, which are available at markets (Lin, 2006). Although many communities have achieved successful specific anti-malarial ethnobotanical approaches, in Malaysia few records are accessible about the traditional medicinal plants which are still employed to treat malaria by the communities residing the malaria endemic areas.

Several plants species including *Alstonia angustiloba*, *Brucea javanica*, *Cassia siamea*, *Phyllanthus niruri*, *Eurycoma longifolia*, *Erechtites valerianaefolia*, *Eurycoma apiculata*, *Panicum palmifolium*, *Languas galanga*, *Tinospora crispa*, *Carbera odollam*, *Elaphantopus scaber* are traditionally used in East Malaysia in the treatment of malaria and fever (Kamarudin, 1997; Kulip, 1997; Fasihuddin, 2000; Fasihuddin & Holdsworth, 2003).

Very little is known about the traditional medicinal plants which are still employed to treat malaria in Peninsular Malaysia. Hence, the present study was carried out to establish a preliminary ethnobotanical database for the plants traditionally used to treat malaria among aboriginal and rural communities, and traditional healers in malaria endemic areas in Pahang, Peninsular Malaysia. *In vivo* anti-malarial activity of four plant species, namely, *Cocos nucifera* L. *Labisia pumila* (Bl.) F.-Vill., *Languas galanga* Stuntz. and *Piper betle* L. selected based on the ethnobotanical survey and literature were evaluated against laboratory malaria model *Plasmodium berghei* to evaluate their anti-malarial activity.

2.10.1 *Cocos nucifera* L.

The tree of life, *Cocos nucifera* L. (coconut) of family *Arecaceae* (palm) is grown in villages and towns in Malaysia. Coconut is native to the littoral zone of Southeast Asia (Malaysia, Indonesia, Philippines) and Melanesia (Chan & Elevitch, 2006). It is pinnate-leaved (4–6 meter long/leaf) plant and can reach 20-30 meter tall; aged leaves escape plainly, parting the trunk silky (Pradeepkumar *et al.*, 2008). *C. nucifera* fruit is the used part of this plant providing food for millions of people (Figure 2.9).

Ethnobotanically, coconut white flesh is a folk remedy for fever, flu, gingivitis, scabies, rash, venereal diseases, abscesses, bronchitis, sore throat, jaundice, dysmenorrhea, earache, erysipelas, skin care, stress relief, digestion, hair care, stomach and gastrointestinal problems, typhoid, healing of cuts, injuries, burns and swellings (Duke & Wain, 1981; Agero & Verallo-Rowell, 2004; Cano & Volpato, 2004; Peterson, 2009; Alanis *et al.*, 2005; Lans, 2006; Lans, 2007). Coconut flesh oil can also prevent cancer and heart diseases, regulate blood sugar and strengthen the immune system (Hartwell, 1984; Fife & Kabara, 2004).

The husk fibre of the coconut fruit showed several activities against helminthes (Oliveira *et al.*, 2009a), leishmania (Mendonça-Filho *et al.*, 2004) and microbes (Esquenazi *et al.*, 2002). However, studies on the phytochemical screening, acute oral toxicity of the white flesh and its activity against malaria are non-existent in the literature. Hence, this study aims to investigate and find out the phytochemical constituents, acute oral toxicity and anti-plasmodial activity of the methanol white flesh extract of *C. nucifera*.



Figure 2.9: *Cocos nucifera* L. fruit. Pos Betau, Kuala Lipis, Pahang, Malaysia. Layers of the coconut fruit: (1) Epicarp (2) Mesocarp (3) Endocarp (4) Endosperm, white flesh or meat (5) Embryo.

The figure showing the layers of the coconut fruit is from Swathivijay (2008)

2.10.2 *Labisia pumila* (Bl.) F.-Vill.

Labisia pumila or known locally as Kacip Fatimah is one of the most popular medicinal plants in Malaysia. Through ethnobotanical market survey, pharmacology and clinical trials research, one of the local herbs that have a commercial potential is Kacip Fatimah (Zainal Azman, 2007). *L. pumila* from the family of Myrsinaceae, is a small herb with a leaf of about 5-35cm long and 2-8cm wide (Figure 2.10). It is mainly found in the low land and hill forests Peninsular Malaysia at an altitude between 300 and 700 m (Zaizuhana *et al.*, 2006).

Ethnobotanically, it is included in many traditional medicinal practices, in particular, among Malay women. It is used to induce and expedite labour; assist contraction of the birth channel and regain body strength and act as postpartum medication to relief pain as well as it is employed to delay fertility (Zaizuhana *et al.*, 2006; Mojiol *et al.*, 2010). Some other traditional uses include treatment of bones diseases, flatulence, dysentery, dysmenorrhoea, gonorrhoea and haemorrhoids (Jamia *et al.*, 2003).

Labisia pumila showed many pharmacological effects including larvicidal activity against *Aedes aegypti* mosquito (Ibrahim *et al.*, 2003), anti-bacterial (Fasihuddin *et al.*, 1995), topical anti-inflammatory (Nik *et al.*, 2002), anti-inflammatory, anti-nociceptive and anti-pyretic (Ikujuni *et al.*, 2010). *L. pumila* also showed anti-oedema properties (Rasadah *et al.*, 2001), oestrogenicity activity (Jamia *et al.*, 2003), platelet-activating factor receptor binding inhibition (Ibrahim *et al.*, 1996).



Figure 2.10: *Labisia pumila* (Bl.) F.-Vill plant, Rimba Ilmu Botanic Garden of the University of Malaya.

2.10.3 *Languas galanga* Stuntz.

Languas galanga Syn. *Alpinia galanga* (Linn.) Stuntz. (Zingiberaceae) is native to grassland areas of Southeast Asia and Southern China (Chevallier, 1996). *L. galanga* is a robust herb and grows up to a height of 3.5 m tall. The rhizome is fragrant, copiously branched in light red or pale yellow (Figure 2.11). It is differentiated from other plants known as galangal with the common name greater galangal. In Malaysia, the rhizomes (Langkuas) of this plant are used as a spice for flavouring food.

Ethnobotanically, the rhizomes are used to treat a variety of sicknesses including coughs, headache, fever, asthma, bronchitis, inflammation, rheumatoid arthritis and colic (Burkill, 1966; Latha *et al.*, 2009).

Previous biochemical studies on *L. galanga* have shown that the rhizomes contain essential anti-microbial components (Scheffer *et al.*, 1981; de Pooter *et al.*, 1985; Mori *et al.*, 1995; Charles *et al.*, 1999; Ibrahim *et al.*, 2004). The rhizomes exhibit many pharmacological properties, including anti-tumour (Itokawa *et al.*, 1987), anti-allergic (Matsuda *et al.*, 2003), anti-ulcer (Mitsui *et al.*, 1976), anti-fungal (Janssen & Scheffer, 1985), anti-bacterial (Oonmetta-aree *et al.*, 2006) and anti-viral activities (Ye & Li, 2006). *L. galanga* rhizomes also exhibited anti-parasitic effects including anti-trypanosomal (Nahoko, 2009), anti-leishmanial (Amandeep *et al.*, 2010) and anti-helminthic (Raj, 1975).



Figure 2.11: *Languas galanga* Stuntz plant, Rimba Ilmu Botanic Garden of the University of Malaya. Apparent in the lower right is the Rhizome

2.10.4 *Piper betle* L.

Piper betle L. is a liana belonging to the Piperaceae family (Figure 2.12). It grows in most of South and Southeast Asia and prized both as a mild stimulant and medicinal plant remedy for a variety of sicknesses (Trakranrungsie *et al.*, 2006; Nalina & Rahim, 2007; Singh *et al.*, 2009).

Ethnobotanically, the leaves are used to control caries, periodontal diseases bad breath (Nalina & Rahim, 2007). It is also used for other purposes including improving appetite, tonic for brain, anti-septic for wounds and treatment for diarrhea (Pin *et al.*, 2010). The leaves are chewed alone or with other plant materials. In ayurvedic medicine, it is used to treat bronchitis, cough, asthma, pneumonia, skin diseases, fever, impotency, arthritis, anorexia, colic, flatulence, diarrhea and tonsillitis (Ayurvedic medicinal plants, 2010).

Recently, the leaves extract exhibited many significant activities against bacteria (Nalina & Rahim, 2007), leishmania parasite (Avijit *et al.*, 2008), filarial parasite (Singh *et al.*, 2009) and fungi (Trakranrungsie *et al.*, 2006) properties.



Figure 2.12: *Piper betle* L. liana, Institut Pertanian Kuala Lipis, Pahang, Malaysia

CHAPTER III

MATERIAL AND METHODS

3.1 STRATEGY OF THE STUDY

A systematic approach was used in this study in order to identify and select the plants used to treat malaria in two malaria endemic communities, forest-aboriginal and rural communities, in the Lipis district of Pahang state, Malaysia. The study began with investigation of people's knowledge, attitude and practices (KAP) regarding malaria and collection of ethnobotanical information on plants used by these communities to treat malaria. Four plants were selected to evaluate their anti-malarial activity based on the ethnobotanical information and literature reports on plant extracts. Only those plants that have not been pharmacologically investigated against malaria were chosen in this study and they are *Cocos nucifera*, *Labisia pumila*, *Languas galanga* and *Piper betle*. The flow chart of this study is shown in the Figure 3.1.

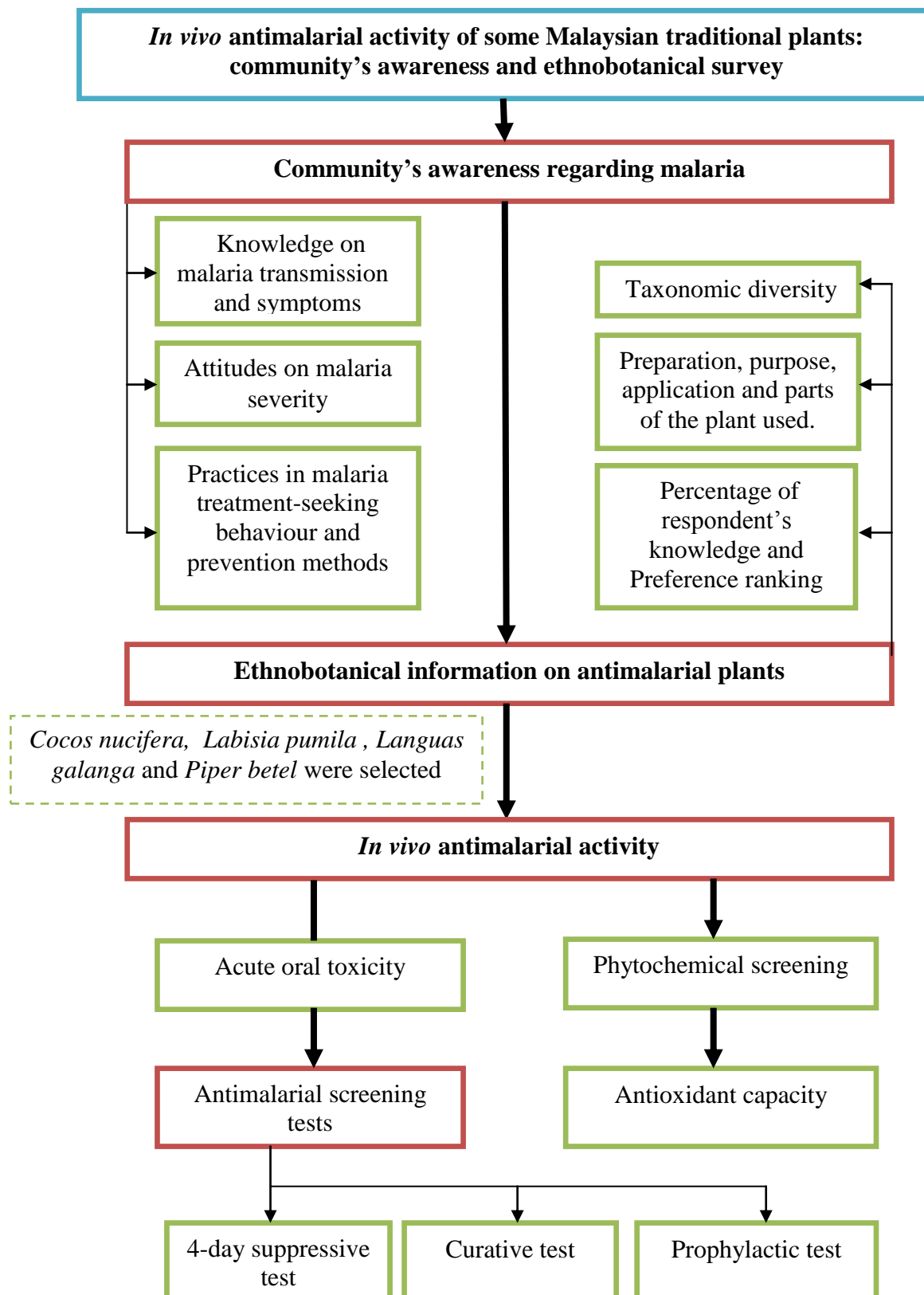


Figure 3.1: The flows charts of the study

3.2 COMMUNITY'S AWARENESS REGARDING MALARIA AND ETHNOBOTANICAL SURVEY

3.2.1 Study Area and Population

Lipis district represents the highest prevalence of malaria cases in Pahang state, Peninsular Malaysia. It is located 200 km north-east of Kuala Lumpur, Peninsular Malaysia with an area of 5,198 km². The climate is tropical with 23-32°C (an average temperature) and 1525- 3050 mm (an annual rainfall). Two rural areas inhabited by the rural population (Padang Tengku and Benta) and one forest area inhabited by the aboriginal peoples (Pos Betau) were involved in this study (Figure 3.2). The three areas are situated at a distance of about 25-50 kilometers from one another. About 59 small villages (8-40 houses in each village) existed in these areas with approximately 1,000-1,200 households. In terms of the structure of the houses, 90% of the aboriginal houses are made of bamboo and wood and have no toilets and water supply while all the houses of the two rural areas are made of cement blocks with toilets and water supply. The economy is mostly agricultural with palm and rubber plantations occupying the majority of the total land area. Malaria is endemic in the area and varies from one area to another, the highest rate of infection being in the aboriginal area (Pos Betau) followed by Padang Tengku. The foundations of malaria control performed by the Vector- Borne Diseases Control Unit in Kuala Lipis, the capital of Lipis district are impregnated bed nets, IRS, early diagnosis and treatment.



Figure 3.2: The study area of the field survey; Pos Betau, Padang Tengku and Benta, Lipis district, Pahang state, Malaysia.

3.2.2 Study Design and Data Collection

Between October and May (2009 -2010), this cross-sectional KAP and ethnobotanical survey was carried out among 223 adult households; aboriginal households (house-to-house interviews, n= 100) and rural households (interviews at outpatient clinics, n= 123) who had or had not suffered a malaria crisis. Traditional healers (n= 10) were added to the studied sample to investigate their knowledge about anti-malarial plants. The selection of the participants was according to the availability and willingness of the people. Objectives of the study and the role of community were explained during the meeting of heads of villages and some households. Participant's verbal consent was obtained. Knowledge, attitude and practices (KAP) regarding malaria and information on plant preparation, application and the parts used to treat malaria were obtained. A pre-tested semi-structured questionnaire involved open-ended questions and constructed in English and translated into the local language (*Bahasa Malayu*) was used. All participants were interviewed by local trained medical officers to fill up the questionnaire.

Semi-structured questionnaire applied in this study was flexible, allowing the extraction of as much as possible the answers to the disease during the interview. However, semi-structured questionnaire generally has a specific framework of the topics that are designed to be explored. Semi-structured questionnaire used in this study was according to the standard qualitative communication research methods (Lindlof & Taylor, 2002).

Vouchers of the plants were collected and identified by a plant taxonomist from Rimba Ilmu and deposited at the Herbarium of the University of Malaya, Kuala Lumpur, Malaysia. The floras used for identification were Malayan wild flowers and Garden Plants in Singapore (Henderson, 1954; Boo *et al.*, 2006).

3.3 PLANTS SELECTION AND EXTRACTION

3.3.1 Plants Selection Criteria

The criteria used for selecting plants for *in vivo* anti-malarial investigation were based on: (1) traditional medicinal information (ethnobotanical survey); (2) plants used orally as a remedy to treat malaria and/or fever (3) literature reports on plant extracts: only those plants that have not been pharmacologically investigated against malaria were chosen in this study. From nineteen medicinal plants, four were chosen to be investigated; *Cocos nucifera* L. (Arecaceae), *Labisia pumila* (Bl.) F.-Vill. (Myrsinaceae), *Languas galanga* Stuntz. (Zingiberaceae) and *Piper betle* L. (Piperaceae).

3.3.2 Plant Materials

Dried plant materials of *Cocos nucifera*, *Labisia pumila*, *Languas galanga* and *Piper betle* were used in this study. The plant parts were selected based on the ethnobotanical survey. The plants have been recorded as a curative and prophylactic anti-malarial remedy by the respondents. The plants were dried either at 40 °C in a hot air oven or at room temperature and then milled. The dried plants materials were provided by specialized company, Ethno Resources Sdn Bhd's, Kuala Lumpur.

3.3.3 Extraction of Dried Plant Materials

Because most of the identified anti-microorganisms aromatic and saturated organic compounds of plant components were acquired during the preliminary methanol extraction (Fransworth, 1994; Eloff, 1998; Cowan, 1999), hence, methanol solvent was used in this study to extract the crude materials of the plants. 500 grams of the plants powder materials were soaked in absolute methanol (3.5 L) for 72 hours. The extracts were then filtered with Whatman filter paper No.2 on a Büchner funnel and concentrated using a rotary evaporator to dryness at 40 °C *in vacuo*. The percentage yield (%w/w) of the *Cocos nucifera*, *Labisia pumila*, *Languas galanga* and *Piper betle* dry extracts were 4.19%, 3.76%, 3.27% and 8.69%, respectively. The freeze-dried extracts were kept at 4 °C until used.

3.3.4 Plant Extracts and Control Groups Dosages Preparation

Resulting extracts were not able to dissolve in the distilled water. Therefore, an orally non toxic dimethyl sulphoxide (DMSO) with known LD₅₀ of 16.5-24.6 g/kg was used as recommendations of OECD (2001) for vehicles other than water. The crude extracts were dissolved in dimethyl sulphoxide and distilled water to the desired acute oral toxicity and anti-malarial dosages. The concentration of DMSO in the distilled water and the plants extract was 10%. The methanol extracts in DMSO/distilled water was orally administered to mice in the test groups, whereas the control group administered distilled water containing 10% DMSO (Carrico *et al.*, 2004; Thanabhorn *et al.*, 2005; Hajimehdipoor *et al.*, 2006; Mi-Hyang *et al.*, 2006; Adeneye & Agbaje, 2008; Innocent *et al.*, 2009; Ali *et al.*, 2009; Sampaio *et al.*, 2009; Ramazani *et al.*, 2010; Azra *et al.*, 2010; Konate *et al.*, 2011).

3.4 ANTI-MALARIAL ACTIVITY OF PLANTS EXTRACTS

3.4.1 Animals

ICR mice were obtained from the Laboratory Animal Centre of Faculty of Medicine, University of Malaya. On a standard pelleted feed and water *ad libitum*, the mice were kept and maintained in standard conditions. The mice were caged of 5 animals per group and maintained in the animal laboratory in the Faculty of Medicine in conformity with the international ethics of laboratory animal use and care.

3.4.2 Acute Oral Toxicity (LD₅₀)

Different extract doses (300, 2000 and 5000 mg/kg) were inoculated orally to evaluate the acute oral toxicity according to the accepted standard described in the OECD guideline No 401 and 423; “Acute oral toxicity – acute toxic class method” (OECD, 1987; 2001). The females and males ICR mice were dosed at 5000 mg/kg. Animals extract groups that died after giving the highest dose (5000 mg/kg) were given the rest of the lower doses (2000, 300 mg/kg). A total of 10 mice (five females and five males) were tested for each extract dose (OECD, 1987). The general behaviour of the mice, clinical observations and mortality were monitored during the first 60 minutes after dosing and during the first 48 hours following dosing. The LD₅₀ was calculated by the probit analysis.

Particular consideration was focused on any observation of asthenia, piloerection, ataxia, anorexia, salivation, urination, diarrhoea, tremors, convulsions, lethargy, sleep and coma. Observations once daily were carried out for the remainder of the study. The mice were monitored for 14 days after the inoculation. On D -1, 0, 7 and 14, body weights were recorded. The LD₅₀ was calculated using probit regression analysis in the SPSS statistical package (version 13, 2004).

3.4.3 Rodent Malaria

Plasmodium berghei (NK65) was obtained from Universiti Kebangsaan Malaysia (UKM), School of Bioscience and Biotechnology and was maintained by subpassage in ICR mice. *P. berghei* strain was tolerated to 25 mg/kg total standard dose of chloroquine (not completely suppress the parasitaemia). This developed resistance may be because of the repeated passage of the parasite from the mouse to another in the context of maintaining the parasite strain in the laboratory (Adzu, *et al.*, 2003; Chawira, 1986; Okokon, *et al.*, 2005). On the other hand, when 20 mg/kg for 4 consecutive days is used to cure *P. berghei* infected mice, it suppressed parasitaemia to non-detectable levels during the 4-day suppressive test, which is also evident in this study (Ishih *et al.*, 2006; Muregi *et al.*, 2007).

3.4.4 Parasite Inoculum

A mouse having 30% parasitaemia was used as a donor to initiate the infection to the test mice. The blood was obtained through cardiac puncture or tail of the donor mouse. The blood was diluted in Alsever's solution and the parasitaemia was adjusted to the desired points. The female ICR mice, 7-8 weeks-old of about 25-28 g weight were infected intraperitoneally with about 10⁶ parasitized RBCs in 0.2 ml (Ishih *et al.*, 2004).

The inoculated animals were then randomized into five mice per group and maintained in the Animal Room, Department of Parasitology, Faculty of Medicine, University of Malaya, in accordance with the internationally accepted principles for laboratory animal's use and care.

3.4.5 Giemsa-Stained-Blood Film Preparation

The blood film was prepared from the mice tail. The mice tail end was cleansed using cotton moistened with 70 % v/v alcohol. The blood drops were obtained and thin blood smears were made on a completely clean (grease-free) and scratch-free slide. The dried blood film was stained by Giemsa's stain. The Giemsa stain was diluted with phosphate buffer saline, pH 7.2 (3% solution for 30 minutes staining). The blood film was washed with running water, placed in a draining rack and air dried.

3.4.6 Degree of Infection (Parasitaemia)

The degree of infection (parasitaemia) was estimated from the Giemsa-stained thin blood films by observing the number of parasitized RBCs in 9,000 RBCs (about 20-40 monolayer microscopic fields) using the 100x oil immersion objective. The parasitaemia (percentage) was determined by calculation the total number of infected cells per microscope field against the total number of observed red cells, using the following formula according to Peters (1965), Ryley and Peters (1970).

$$\text{The parasitaemia percentage \%} = \left[\frac{\text{No.of parasitized RBC}}{\text{Total no.of RBC counted}} \right] 100$$

3.4.7 The Percentage of Suppression (anti-malarial activity)

The effectiveness of the extracts was determined by calculating the degree of suppression. The suppression of parasitaemia (%) was determined as stated by Peters (1965), Ryley and Peters (1970), Peter and Robinson (1992) as follows:

Average percentage chemosuppression was calculated as

$$100 \left[\frac{A - B}{A} \right]$$

Where, A is the mean percentage parasitaemia in the control group and B is the mean percentage parasitaemia in the test group.

3.4.8 *In Vivo* Anti-Malarial Screening Assays

A series of *in vivo* anti-malarial assays i.e., 4-day suppressive test, curative and prophylactic methods (APPENDIX E) were carried out to assess the *in vivo* anti-malarial activities of the plant extracts at 50, 100, 200 and 400 mg/kg doses (Fidock *et al.*, 2004; Elufioye & Agbedahunsi, 2004) as compared to control groups treated with 0.2 mL distilled water (containing 10% DMSO, the solvent of the test extracts) and reference groups treated with chloroquine 20 mg/kg or pyrimethamine 1.2 mg/kg. Pyrimethamine was used during the prophylactic assay since it has a better chemoprophylactic activity than chloroquine (Bradley-Moore *et al.*, 1985). Malaria infection was established in female ICR mice by the intraperitoneal administration of donor female ICR mouse blood containing about 1×10^6 parasites.

i. 4-day suppressive activity (early malaria infection)

Suppressive activity of the extract during early malaria infection was assessed as described early by Peters and Robinson (1992). The female ICR mice were intraperitoneally infected on the first day (D 0), with 0.2 mL blood containing 1×10^6 *P. berghei* parasitized RBCs.

The infected mice were randomized into 16 extracts groups, one control and one reference drug groups of five mice each. Three hours after infection, the extracts-treated groups were treated orally with 50, 100, 200 and 400 mg/kg doses of the extracts. Chloroquine and distilled water (10% DMSO) were administered to the reference drug and control groups, respectively. The extracts and drug treatments continued daily (D0 to D3) for four days. From tail blood, thin films were prepared on D4 (the 5th day). The blood films from of each mouse were stained with Giemsa and the percentage of suppression was then calculated.

ii. Curative activity (established malaria infection)

The curative test was evaluated according to the method explained by Ryley & Peters (1970) and Saidu *et al.* (2000). The mice were selected and 0.2 mL of inoculum (1×10^6 *P. berghei*) was given to each mouse (i.p.). Seventy two hours later, the extract-tested groups were orally administered with 50, 100, 200 and 400 mg/kg doses of the extracts. Chloroquine and distilled water (10% DMSO) were administered to the reference drug and control groups, respectively. The extract and drug treatments were continued for five days. From tail blood of each mouse, thin blood films were prepared for five days. The mean differences between the test groups and control were evaluated on D6

according to Saidu *et al.* (2000). The survival time of extract-tested, reference drug and control groups were recorded over a period of 30 days, where the mean survival time differences between the test groups and control were determined.

iii. Prophylactic activity (residual malaria infection)

The prophylactic activity of the plants during residual malaria infection was assessed according to the technique described by Peters (1965). For three days (D0 to D2), each experimental animal in the studied groups was administered orally at 50, 100, 200 and 400 mg/kg of the plants crude extract, pyrimethamine and distilled water (10% DMSO) were administered to the reference group, and control group, respectively. The experimental mice were then intraperitoneally infected with 1×10^6 *P. berghei* on the fourth day. Three day after infection, the blood films from of each mouse were stained with Giemsa and the percentage of suppression was then calculated.

3.4.9 Phytochemical Screening

Standard qualitative methods for determination of chemical constituents in the plants extract were performed to expose the presence of alkaloids, anthraquinones, flavonoids, terpenoids, tannins, saponins, steroids and glycosides according to Hymete (1986) Trease & Evans (1989) and Sofowora (1993).

i. Test for alkaloids

Half a gram of each plant extract in ten mL acid alcohol were boiled for 5 minutes and filtered. Two mL of dilute ammonia followed by five mL of chloroform were added to the filtrate and mix softly. To the chloroform layer ten mL of acetic acid were added. To the mixture, Dragendorff's reagent was added. The presence of alkaloids was indicated by the appearance of a reddish brown precipitate.

ii. Test for anthraquinones

In ten mL of sulfuric acid, half a gram of each plant extract was boiled for five minutes. The hot mixture was then filtered. Five mL of chloroform were added to the filtrate and mix. In another tube, one mL of dilute ammonia was added to the chloroform layer. The presence of anthraquinones was indicated by colour changes of the mixture to brown.

iii. Test for terpenoids (Salkowski Test)

Two mL of chloroform was added to half a gram of each plant extract. Three mL of concentrated sulfuric acid was carefully added to the mixture. The presence of terpenoids was revealed by the presence of a reddish brown layer.

iv. Test for flavonoids

Half a gram of each plant extract was heated for three minutes in ten mL of ethylacetate and filtered. One mL of 1 % ammonia solution was added to four mL of the filtrate. After giving some time for the layers to separate in the mixture, a yellow colour in the ammonia layer is regarded as an indication of the presence of flavonoids.

v. Test for saponins (Froth test)

In five mL of distilled water, half a gram of each plant extract was boiled for five minutes. The hot mixture was then filtered and further four mL of distilled water was added to one mL of the filtrate. The solution was mixed strongly for 30 minutes. The presence of saponins was revealed by the persistence of a stable froth more than 30 minutes.

vi. Test for tannins

In five mL of 45 % ethanol, two 2 g of each plant extract was boiled with for five minutes. The cooled mixture was then filtered. Three drops of lead sub-acetate solution was added to one mL of the filtrate. Formation of a cream gelatinous precipitate indicated the presence of tannins.

vii. Test for steroid

To two mL of chloroform, half a gram of each plant extract was added. To the mixture concentrated sulfuric acid was added (carefully). The presence steroid was indicated by the formation of a reddish brown layer at the interface.

viii. Test for glycosides

To half a gram of each plant extract, five mL of dilute sulfuric acid was added and boiled for 15 minutes. Neutralization of the cooled mixture was performed with 20 % potassium hydroxide. A mixture (ten mL) of identical quantity of Fehling's solution A and Fehling's solution B was added. The mixture was then boiled for five minutes. The presence of glycosides was indicated by the formation of a red precipitate (more dense).

3.4.10 Antioxidant Capacity

Determination of antioxidant capacity of the plants extract was carried according to Gerhäuser *et al.* (2003). The plants extract free radical scavenging activity against DPPH (1,1-diphenyl-2-picrylhydrazyl), the stable artificial free radical, was determined photometrically with spectrophotometer. Different concentrations (1.56, 3.13, 6.25, 12.5 and 25 µg/mL) prepared with DPPH radical solution were incubated in a 96-well microplate and read at 515 nm for 3 hours at 20-min intervals. Ascorbic acid and gallic acid were used for comparison. The DPPH radical scavenging activity of the plants extract was expressed as the percentage of scavenging of the DPPH by the extract and was calculated as follows:

$$\text{DPPH radical scavenging activity (\%)} = \{[Ab-Aa]/Ab\} \times 100$$

Where Ab is the absorption of the blank sample, and Aa is the absorption of the sample.

Each test was carried out three times and the mean \pm S.E.M. was calculated. The DPPH % was presented as µg /mL of concentration.

3.5 DATA MANAGEMENT AND ANALYSIS

The data obtained were analyzed using Statistical Program for Social Scientists (SPSS 13.0.) and Microsoft Office Excel 2007 statistical analysis.

The Chi-square test was used to examine the KAP differences between aboriginal and rural participants for each of the outcome variables and $P \leq 0.05$ was considered as the level of significance. Before data entry and analysis, questionnaires were checked for coding errors, completeness and consistency.

The information obtained in the course of the ethnobotanical interviews was analyzed using the following parameters according to Asase *et al.* (2005):

1. Taxonomic diversity, preparation, application and parts of the plant used.
2. The percentage of respondent's knowledge (PRK) regarding the use of plants species to treat malaria was estimated as following: (No. of interviewed people citing species/total No. of interviewed people using plants) $\times 100$.
3. Preference ranking (PR), in this parameter the plants were ranked to three levels (one, two and three) according to their efficiency in the treatment of malaria by the respondents. A value of three (3) was given for the most effective plant.

The *iv vivo* anti-malarial activity of the test extracts, reference drugs and control groups at different doses in mice parasitized with *P. berghei* were expressed as percentage (%) for the suppression of parasitaemia. Parasitaemia were expressed as mean \pm S.E.M. (n = 5) and significance compared to controls. The Student's t-test and ANOVA were applied to examine the differences between the test and control groups. Differences between means at 5% level ($P \leq 0.05$) were considered significant (effective).

DPPH radical scavenging activity results represent means (\pm S.E.M) of triplicates of the different concentrations analyzed.

3.6 ETHICAL CONSIDERATION

The protocol of this study has been approved by the Medical Research Committee of the University of Malaya Medical Centre, and the Department of aboriginal Affairs, Ministry of Rural Development. Ethical approval was obtained from the ethical committee of the Faculty of Medicine, University of Malaya and Jabatan Hal Ehwal Orang Asli (JHEOA), Department of Aboriginal Affairs, Ministry of Rural Development, prior to study commencement.

Permission and approval for animal studies were obtained from the Faculty of Medicine, Animal Ethics committee, University of Malaya dated 05 June 2009 (Ref. No. PAR/05/6/2009/AHAA-R).

CHAPTER IV

RESULTS

4.1 COMMUNITY'S AWARENESS REGARDING MALARIA

4.1.1 General Characteristics of Participants

Out of the 260 households that participated in this survey, questionnaires were only completed for 223 households (100 forest-aboriginal and 123 rural). Table 4.1 shows the general socio-demographic characteristics of the study population. Overall, 10% of the aboriginal households had previously suffered from malaria compared to 1.6% of rural households. Almost half of the aboriginal participants had no formal education, and the proportion of men with formal education was significantly higher than that of women ($\chi^2=21.549$, $P < 0.001$). In contrast, almost all the rural participants had formal education and the proportion of women with formal education was significantly higher than that of men ($\chi^2=4.922$, $P = 0.027$).

4.1.2 Malaria Knowledge

Data about the participants' knowledge and attitudes to malaria are shown in Table 4.2. About half of the aboriginal participants believed that malaria is transmitted by mosquito bites and this was significantly associated with the educational level ($\chi^2=4.244$, $P = 0.039$). Aboriginal participants who had previously been infected with

malaria showed better knowledge of the symptoms of malaria than those with no history of infection ($\chi^2=6.810$, $P = 0.009$).

On the other hand, the majority (86.2%) of the rural participants have knowledge of malaria as a disease and 70.7% of them believed that malaria is transmitted through the bite of mosquitoes and this was found to be influenced by their level of education; participants who had better education showed better knowledge about malaria as a disease and malaria symptoms ($\chi^2=24.037$, $P < 0.001$; $\chi^2=4.416$, $P = 0.036$ respectively). Moreover, Malay participants showed a higher level of knowledge about malaria transmission than Chinese and Indian ($\chi^2=6.234$, $P = 0.013$). The findings also showed that attitudes towards the severity of malaria were significantly higher among aboriginal participants who had previously been infected with malaria ($\chi^2=4.421$, $P = 0.036$).

4.1.3 Malaria Treatment-Seeking Behaviour and Prevention

Data about treatment-seeking behaviour and prevention methods are shown in Table 4.3. The vast majority of the rural participants (95.1%) indicated that they would seek treatment from health centre and this was found to be associated significantly with the educational level and age of the participants ($\chi^2=6.236$, $p = 0.013$; $\chi^2=9.856$, $p = 0.002$ respectively). Similarly, the educational level, age and race of the rural participants were associated significantly with the practicing of effective preventive measures ($\chi^2=4.634$, $P = 0.031$; $\chi^2=5.483$, $P = 0.019$; $\chi^2=7.965$, $P = 0.019$, respectively).

Overall, the rural participants showed a higher knowledge regarding malaria and its transmission than the Aborigines ($\chi^2=6.746$, $P = 0.009$; $\chi^2=10.006$, $P = 0.002$, respectively) (Table 4.2). On the contrary, the aboriginal participants showed a higher practices in their treatment-seeking behaviour for treating febrile diseases, both in terms of the use of medicinal plants ($\chi^2=5.225$, $P = 0.022$) or belief in witchcraft and sorcery ($\chi^2=16.980$, $P < 0.001$) (Table 4.3).

Regarding the knowledge about the symptoms of malaria, the attitude towards severity of the disease as well as the use of mosquito bednets, the two communities did not show significant differences. However, the knowledge and practice of different preventive measures to combat malaria such as insecticides and the elimination of breeding areas was significantly higher among the rural population than the Aborigines ($\chi^2=23.136$, $P < 0.001$).

However, the rural population showed significant differences higher than the Aborigines in the combat against malaria in different preventive measures such as the use of insecticides and elimination of mosquito breeding areas ($\chi^2=23.136$, $P < 0.001$).

Table 4.1: Socio-demographic characteristics of the participants

Characteristics	population	
	Forest-aboriginal (n = 100)	Rural (n = 123)
	n (%)	n (%)
Age (years)		
18-40	76 (76.0)	75 (61.0)
>40	24 (24.0)	48 (39.0)
Sex		
Male	38 (38.0)	46 (37.4)
Female	62 (62.0)	77 (62.6)
Religion		
Non-religious	99 (99.0)	0 (0.0)
Muslim	1 (1.0)	74 (60.1)
Buddhist	0 (0.0)	36 (29.3)
Hindu	0 (0.0)	13 (10.6)
Education		
None	38 (38.0)	6 (4.9)
Kindergarten	5 (5.0)	2 (1.6)
Primary school	49 (49.0)	57 (46.3)
Secondary school	8 (8.0)	51 (41.5)
Tertiary	0 (0.0)	5 (4.1)
University	0 (0.0)	2 (1.6)
Occupation		
Working	31 (31.0)	38 (30.9)
Non-working	35 (35.0)	36 (29.3)
Housewife	34 (34.0)	47 (38.2)
Students	0 (0.0)	2 (1.6)
Races		
Aborigine	100 (100.0)	0 (0.0)
Malay	0 (0.0)	74 (60.1)
Chinese	0 (0.0)	36 (29.3)
Indian	0 (0.0)	13 (10.6)

Table 4.2: Participant's knowledge about malaria transmission and symptoms, and attitudes towards malaria severity

Variables	Population		χ^2 significance
	Forest-aboriginal	Rural	
	(n = 100) n (%)	(n = 123) n (%)	
Transmission of malaria			
Mosquito bites	50 (50.0)	87 (70.7)	$P < 0.01$
Use of stagnant water	46 (46.0)	6 (4.9)	$P < 0.001$
From forest	17 (17.0)	0 (0.0)	$P < 0.001$
Human-to- human	7 (7.0)	4 (3.3)	ns
From weather/sun	3 (3.0)	0 (0)	ns
No knowledge	16 (16.0)	26 (21.1)	ns
Symptoms of malaria			
Fever	76 (76.0)	95 (77.2)	ns
Chill and rigor	56 (56.0)	27 (22.0)	$P < 0.001$
Headache	30 (30.0)	24 (19.5)	ns
Vomiting	11 (11.0)	16 (13.0)	ns
Body pain/weakness	10 (10.0)	7 (5.7)	ns
Loss of appetite	9 (9.0)	9 (7.3)	ns
Red rash	8 (8.0)	18 (14.6)	ns
Abdominal discomfort	2 (2.0)	0 (0.0)	ns
No knowledge	13 (13.0)	18 (14.6)	ns
Is malaria a serious disease?			
Yes	72 (72.0)	93 (75.6)	ns
No	10 (10.0)	13 (10.6)	
No knowledge	18 (18.0)	17 (13.8)	

Table 4.3: Participant's practices in malaria treatment-seeking behaviour and prevention methods

Variables	Population		χ^2 significance
	Forest-aboriginal (n = 100) n (%)	Rural (n = 123) n (%)	
	Treatment-seeking behavior		
Go to clinic as a first line activity (within 24hrs of fever onset)	65 (65.0)	117 (95.1)	$P < 0.001$
Use plant remedies	28 (28.0)	19 (15.4)	$P < 0.05$
Believe in Witchcraft to treat malaria	13 (13.0)	0 (0.0)	$P < 0.001$
Take anti-malarial/anti-pyretic medicine	0 (0.0)	6 (4.9)	$P < 0.05$
Malaria prevention methods			
Use of mosquito bed nets	63 (63.0)	76 (61.8)	ns
Keep the house/surroundings clean	30 (30.0)	46 (37.4)	ns
Elimination of breeding sites	0 (0.0)	8 (6.5)	$P < 0.001$
Use of insecticide /spraying	3 (3.0)	33 (26.8)	$P < 0.001$
Fumigation by smoke	7 (7.0)	28 (22.8)	$P < 0.01$
Use of anti-malarials	1 (1.0)	8 (6.5)	$P < 0.05$
Use of medicinal plants	10 (10.0)	6 (4.9)	ns
No knowledge	21 (21.0)	23 (18.7)	ns

4.2 ETHNOBOTANICAL STUDY ON ANTI-MALARIAL PLANTS

The use of anti-malarial plants was significantly higher among the aboriginal community ($\chi^2=5.225$, $P=0.022$). Of the 223 interviewed respondents, 28% and 15.4% of the aboriginal and rural participants had tried self medication with anti-malarial plant remedies to cure or prevent malaria infections, respectively. Traditional healers are rarely found in the rural areas. However, some of the religious leaders such as imams of mosques and temples priests are practicing and prescribing the traditional plants to the people. For instance, *Azadirachta indica* Juss. (locally known as Margosa) has been mentioned by an Indian priests who stated that this plant has its folkloric background in the treatment of fever and malaria in the ayurvedic medicine. On the other hand, *Nigella sativa* L. (locally known as Jintan hitam), which has its religious background in the prophetic medicine, has been mentioned by a Malay imam of mosque.

As shown in Table 4.4, a total of 19 species in 17 families used to treat malaria or fever were identified. Eleven of the vouchers of the plants that were identified by a plant taxonomist, have been previously collected and deposited at the Herbarium of the University of Malaya for the purposes of different studies at the university. While eight plants namely, *Cassia siamea* L., *Cocos nucifera* L., *Languas galanga* Stuntz., *Nigella sativa* L., *Ocimum tenuiflorum* L., *Phyllanthus niruri* L., *Piper betle* L., *Hibiscus rosa-sinensis* L., were deposited for the first time during this study. The collection of plants at the Rimba Ilmu Botanic Garden of the University of Malaya, includes a large number of medicinal plants. The plants vouchers at the Herbarium of the University of Malaya are shown in the Figure 4.1.

Table 4.4: Plant species identification and voucher numbers

Species	Family	voucher numbers
1. <i>Azadirachta indica</i> Juss.	Meliaceae	KLU 33205
2. <i>Brucea javanica</i> Merr.	Simaroubaceae	KLU 34932
3. <i>Cassia siamea</i> L.	Fabaceae	KLU 46621
4. <i>Cocos nucifera</i> L.	Arecaceae	KLU 47212
5. <i>Eurycoma longifolia</i> Jack	Simaroubaceae	KLU 03593
6. <i>Labisia pumila</i> (Bl.) F.-Vill.	Myrsinaceae	KLU 09386
7. <i>Languas galanga</i> Stuntz.	Zingiberaceae	KLU 46619
8. <i>Lansium domesticum</i> Corr.	Meliaceae	KLU 44528
9. <i>Morinda citrifolia</i> L.	Rubiaceae	KLU 45405
10. <i>Nigella sativa</i> L.	Ranunculaceae	KLU 47213
11. <i>Ocimum tenuiflorum</i> L.	Lamiaceae	KLU 46618
12. <i>Phyllanthus niruri</i> L.	Phyllanthaceae	KLU 46617
13. <i>Piper betle</i> L.	Piperaceae	KLU 46620
14. <i>Hibiscus rosa-sinensis</i> L.	Malvaceae	KLU 46616
15. <i>Tinospora crispa</i> L.	Menispermaceae	KLU 45568
16. <i>Aeschynanthus</i> sp. Jack	Gesneriaceae	KLU 20482
17. <i>Alstonia angustiloba</i> Mig.	Apocynaceae	KLU 03364
18. <i>Curcuma domestica</i> L.	Zingiberaceae	KLU 41829
19. <i>Elatariospermum tapos</i> Bl.	Euphorbiaceae	KLU 42028



KLU 33205



KLU 34932



KLU 46621



KLU 47212

Figure 4.1: *Azadirachta indica* Juss. (KLU 33205), *Brucea javanica* Merr. (KLU 34932), *Cassia siamea* L. (KLU 46621), *Cocos nucifera* L. (KLU 47212).



KLU 03593



KLU 09386



KLU 46619



KLU 44528

Figure 4.1 continued: *Eurycoma longifolia* Jack (KLU 03593), *Labisia pumila* (Bl.) F. Vill. (KLU 09386), *Languas galanga* Stuntz. (KLU 46619), *Lansium domesticum* Corr. (KLU 44528).



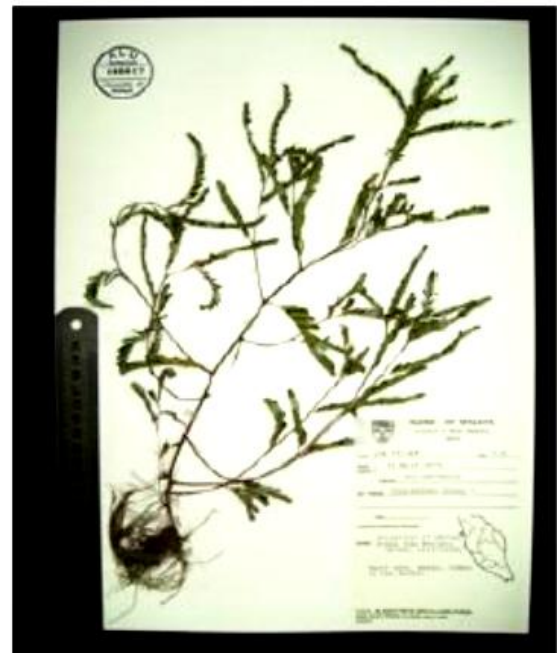
KLU 45405



KLU 47213



KLU 46618



KLU 46617

Figure 4.1 continued: *Morinda citrifolia* L. (KLU 45405), *Nigella sativa* L. (KLU 47213), *Ocimum tenuiflorum* L. (KLU 46618), *Phyllanthus niruri* L. (KLU 46617).



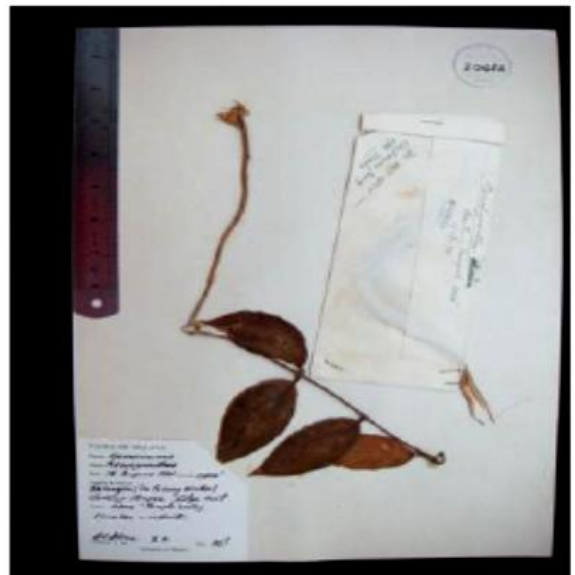
KLU 46620



KLU 46616

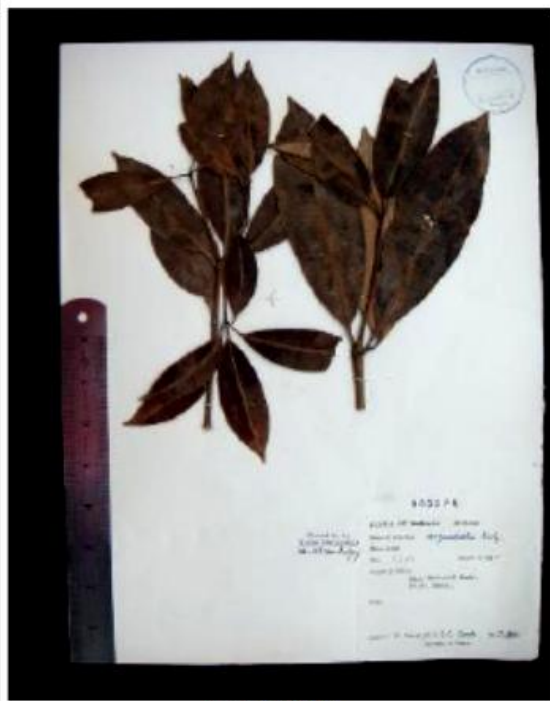


KLU 45568



KLU 20482

Figure 4.1 continued: *Piper betle* L. (KLU 46620), *Hibiscus rosa-sinensis* L. (KLU 46616), *Tinospora crispa* L. (KLU 45568), *Aeschynanthus* sp. Jack (KLU 20482).



KLU 03364



KLU 41829



KLU 42028

Figure 4.1 continued: *Alstonia angustiloba* Mig. (KLU 03364), *Curcuma domestica* L. (KLU 41829) and *Elateriospermum tapos* Bl. (KLU 42028).

The majority of the plant species (89.5%, 17/19) were identified through the interviews with the aboriginal and rural people. In spite of only 31.6% (6/19) of the plant species being identified through the interviews with the traditional healers, most of them got the highest preference ranking. Some plants were mentioned in more than one interviewed group and *Eurycoma longifolia* was the only species that was mentioned in all three interviewed groups. However, most of the identified plant species were mentioned by only one respondent (PRK 1.8). The plant species with higher PPK values were *Eurycoma longifolia* (PRK 15.8) followed by *Labisia pumila* (PRK 14.0) and *Tinospora crispa* (PRK 7.0), respectively (Table 4.5).

All of the identified plant remedies are used for curative purposes to cure malaria, and six plant species are used as curative and prophylactic remedies. Data obtained from the users of anti-malarial plants showed that 73.7% of the anti-malarial plant remedies were obtained from trees and herbs (Table 4.6).

Different parts of the plants were used in the preparation of anti-malarial plant remedies. In most of the species (63%), the remedies were obtained from the leaves and roots. Fifteen plants are used orally as decoctions or infusions, mainly three times daily until malaria is cured. Of the 15 plants used orally, the anti-malarial activity of four plants, *C. nucifera*, *L. pumila*, *L. galanga* and *P. betle* four plants are non-existent in the literature (Table 4.7).

Four plants decoctions are applied externally during bathing in combination of more than one species. In some instances, they are applied as compresses to the abdominal area accompanied by chanting some religious words.

Table 4.5 Interviewed groups, percentage and preference ranking of plants species

Species (voucher numbers)	Interviewed groups			PRK	PR
	AH	RH	TH		
<i>Azadirachta indica</i> Juss.		*	*	3.5	3
<i>Brucea javanica</i> Merr.			*	1.8	3
<i>Cassia siamea</i> L.		*		1.8	1
<i>Cocos nucifera</i> L.		*		1.8	1
<i>Eurycoma longifolia</i> Jack	*	*	*	15.8	3
<i>Labisia pumila</i> (Bl.) F.-Vill.	*		*	14.0	2
<i>Languas galanga</i> Stuntz.	*	*		3.5	2
<i>Lansium domesticum</i> Corr.		*		1.8	1
<i>Morinda citrifolia</i> L.	*			1.8	1
<i>Nigella sativa</i> L.			*	1.8	2
<i>Ocimum tenuiflorum</i> L.		*		1.8	1
<i>Phyllanthus niruri</i> L.	*			1.8	1
<i>Piper betle</i> L.	*	*		3.5	2
<i>Hibiscus rosa-sinensis</i> L.	*			1.8	1
<i>Tinospora crispa</i> L.		*	*	7.0	3
<i>Aeschynanthus sp.</i> Jack	*			1.8	1
<i>Alstonia angustiloba</i> Mig.	*			1.8	1
<i>Curcuma domestica</i> L.	*			3.5	1
<i>Elateriospermum tapos</i> Bl.	*			1.8	1

AH: Aboriginal households, RH: rural households, TH: traditional healers, PRK: percentage of respondents knowledge, PR: preference ranking.

Table 4.6: Application, local names, growth forms and purpose of species of the plants

Species/Application	Local name	Growth form	Purpose
Plants used orally			
<i>Azadirachta indica</i>	Margosa	Tree	Curative
<i>Brucea javanica</i>	Lada pahit	Tree	Curative
<i>Cassia siamea</i>	Johor	Tree	Curative
<i>Cocos nucifera</i>	Kelapa	Tree	Curative, Prophylactic
<i>Eurycoma longifolia</i>	Tongkat Ali	Shrub	Curative, Prophylactic
<i>Labisia pumila</i>	Kacip Fatimah	Herb	Curative, Prophylactic
<i>Languas galanga</i>	Lengkuas	Herb	Curative
<i>Lansium domesticum</i>	Langsat	Tree	Curative
<i>Morinda citrifolia</i>	Peremuh/ Mengkudu	Tree	Curative
<i>Nigella sativa</i>	Jintan hitam/ Habatulsawda	Herb	Curative, Prophylactic
<i>Ocimum tenuiflorum</i>	Tulsi	Herb	Curative
<i>Phyllanthus niruri</i>	Dukung anak	Herb	Curative
<i>Piper betle</i>	Sirih/Serih	Liana	Curative, Prophylactic
<i>H. rosa -sinensis</i>	Bunga Raya	Shrub	Curative
<i>Tinospora crispa</i>	Putarwali/Batang Wali	Shrub	Curative, Prophylactic
Plants used externally			
<i>Aeschynanthus sp.</i>	Sambuk	Shrub	Curative
<i>Alstonia angustiloba</i>	Pulai getah	Tree	Curative
<i>Curcuma domestica</i>	Kunyit	Herb	Curative
<i>Elateriospermum tapos</i>	Perah/parah	Tree	Curative

Table 4.7: Parts used, preparation and anti-malarial reported in the literatures

Species/Applicatio n	Part used	Preparation	Anti-malarial reported in the literatures
Plants used orally			
<i>A. indica</i>	Leaves	<i>Decoction</i>	(Obaseki and Fadunsin, 1982; Devi <i>et al.</i> , 2001; Isah <i>et al.</i> , 2003)
<i>B. javanica</i>	Fruits, leaves	Infusion	(Pavanand <i>et al.</i> , 1986)
<i>C. siamea</i>	Stem bark	<i>Decoction</i>	(Ajaiyeoba <i>et al.</i> , 2007)
<i>C. nucifera</i>	White flesh (fruit)	Infusion	Not reported in the literature
<i>E. longifolia</i>	<i>Root</i>	<i>Decoction</i>	(Hooi <i>et al.</i> , 1995; Ping-Chung <i>et al.</i> , 2003; Kit-Lam <i>et al.</i> , 2004)
<i>L. pumila</i>	<i>Leaves</i>	<i>Decoction</i>	Not reported in the literature
<i>L. galanga</i> (syn. <i>Alpinia galanga</i>)	Rhizome	Decoction	Not reported in the literature
<i>L. domesticum</i>	Peel and bark	Infusion	(Yapp & Yap, 2003; Saewan <i>et al.</i> , 2006)
<i>M. citrifolia</i>	Leaves and fruits	<i>Decoction</i>	(Ancolio <i>et al.</i> , 2002)
<i>N. sativa</i>	Seeds	Infusion	(Abdulelah & Zainal-Abidin, 2007; Wan Omar <i>et al.</i> , 2007)
<i>O. tenuiflorum</i> (syn. <i>O. sanctum</i>)	Leaves and seed	Decoction	(Devi <i>et al.</i> , 2001. Wan Omar <i>et al.</i> , 2007)
<i>P. niruri</i>	Whole plants	Decoction	(Tona <i>et al.</i> , 2001)
<i>P. betle</i>	Leaves	Chewing	Not reported in the literature
<i>H. rosa -sinensis</i>	Flowers	Infusion	(Popp <i>et al.</i> , 1967)
<i>T. crispa</i>	Stems, leaves or roots	Decoction	(Sahidan <i>et al.</i> , 1994; Nik-Najib <i>et al.</i> , 1999; Wan-Omar <i>et al.</i> , 2007)

4.3 ANTI-MALARIAL ACTIVITY OF THE PLANTS EXTRACTS

The four selected plants were investigated at the same time, conditions and controls for each test are specified below

4.3.1 *Cocos nucifera* L.

i. Phytochemical screening of *C. nucifera* white flesh

Phytochemical screening of the methanol extract of *C. nucifera* white flesh revealed the presence of terpenoids, tannins, steroids and glycosides.

ii. Radical scavenging activity of *C. nucifera* white flesh

The white flesh extract of *C. nucifera* showed a weak DPPH radical scavenging activity. At 1.56 - 25 µg/ml, the scavenging abilities of the methanol extract on DPPH radicals were 9.352%, 8.15%, 7.69%, 1.91% and 5.52%, respectively (Figure 4.2). Ascorbic acid and gallic acid showed strong scavenging abilities reaching 72.74 and 73.79% at 25 µg/ml, respectively.

iii. Acute oral toxicity of *C. nucifera* white flesh

No effects of toxicity or mortalities were recorded in any of the 10 animals post dosing and during the observation period (14 days). All animals gained body weight by D 7 and on the end of the experiment (Table 4.8), the tests and control groups did not show any significant differences. Based on the above mentioned results, the acute oral LD₅₀ in mice of the test extract was found to be in excess of 5000 mg/kg.

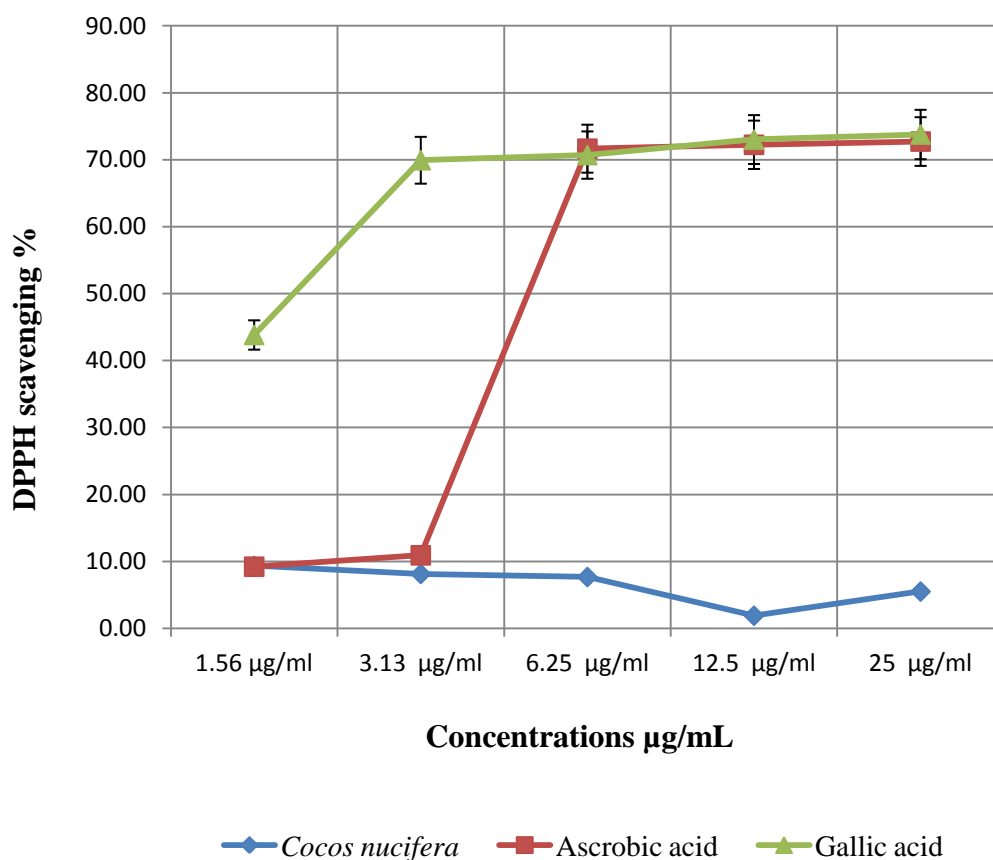


Figure 4.2: DPPH scavenging % activity of *C. nucifera* white flesh methanol extract

Table 4.8: Body weight gain of mice receiving 5000 mg/kg of *C. nucifera* white flesh methanol extract

Mice groups	Mean group weight (g) ± S.D. (g); n=5				Mean weight gain (g); n=5
	D1	D0	D7	Day 14	
Control male	28.00 ± 1.11	27.98 ± 1.28	30.52 ± 1.90	31.80 ± 1.99	3.80 ± 1.93
Test male	27.66 ± 2.42	27.98 ± 2.47	30.60 ± 2.53	31.60 ± 2.41	3.94 ± 1.94
Control female	22.44 ± 0.42	22.70 ± 0.37	24.40 ± 1.07	25.30 ± 0.72	2.86 ± 1.37
Test female	23.38 ± 0.85	23.50 ± 0.87	25.32 ± 1.00	26.04 ± 1.11	2.66 ± 1.33

iv. Suppressive anti-malarial activity of *C. nucifera* white flesh

C. nucifera white flesh methanol extract produced a dose-dependent chemotherapeutic activity in the 4-day suppressive test. Chemosuppression effects of 44.71%, 56.86%, 79.61% and 83.73% were exhibited for the 50, 100, 200 and 400 mg/kg extract doses. In comparison with the control, the 100, 200 and 400 mg/kg doses showed significant anti-malarial activity during the early malaria infection ($P < 0.05$) (Table 4.9).

v. Curative anti-malarial activity of *C. nucifera* white flesh

The methanol white flesh extract of *C. nucifera* exhibited significant ($P < 0.05$) dose dependent reductions in parasitaemia for all the extract doses in comparison with the control. A daily increases in parasitaemia reaching 9.60 % on D6 of infection was exhibited by the control group (Figure 4.3). The mean parasitaemia for the treated groups on D6 of infection were 5.90%, 4.40%, 3.20% and 3.00 for 50, 100, 200, 400 mg/kg, respectively (Table 4.10). On the other hand, the mice that received high doses of the extract during the established malaria infection showed a higher mean survival time during a period of 30 days than those received the lower concentrations. The mice treated with 20 mg/kg of the reference drug (chloroquine) exhibited a mean survival time of 27.20 days. The test mice treated with the extract at 50, 100, 200 and 400 mg/kg/day showed 13.80, 14.60, 15.20, and 15.60 mean survival time (days), respectively, the increase in the mean survival time were not significant. The control group had a mean survival time of 13.6 days (Table 4.11).

Table 4.9: Suppressive anti-malarial activity of *C. nucifera* white flesh methanol extract

Extract/drug	Dose	Parasitaemia% ± S.E.M. (n=5)	%Chemo-suppression
Control	0.2 mL	5.1 ± 0.33	
White flesh extract	50 mg/kg	2.82 ± 0.78	44.71
	100 mg/kg	2.20 ± 0.49	56.86*
	200 mg/kg	1.04 ± 0.75	79.61*
	400 mg/kg	0.83 ± 0.47	83.73*
Chloroquine	20 mg/kg	00.00	100

* $P \leq 0.05$ compared to the control group.

Table 4.10: Curative anti-malarial activity of *C. nucifera* white flesh methanol extract

Extract/drug	Dose	Parasitaemia% (D6) ± S.E.M. (n=5)	%Chemo-suppression
Control	0.2 mL	9.60 ± 0.93	
White flesh extract	50 mg/kg	5.90 ± 0.86	38.54*
	100 mg/kg	4.40 ± 0.97	54.17*
	200 mg/kg	3.20 ± 0.51	66.67*
	400 mg/kg	3.00 ± 0.51	68.57*
Chloroquine	20 mg/kg	00.00	100

* $P \leq 0.05$ compared to the control group.

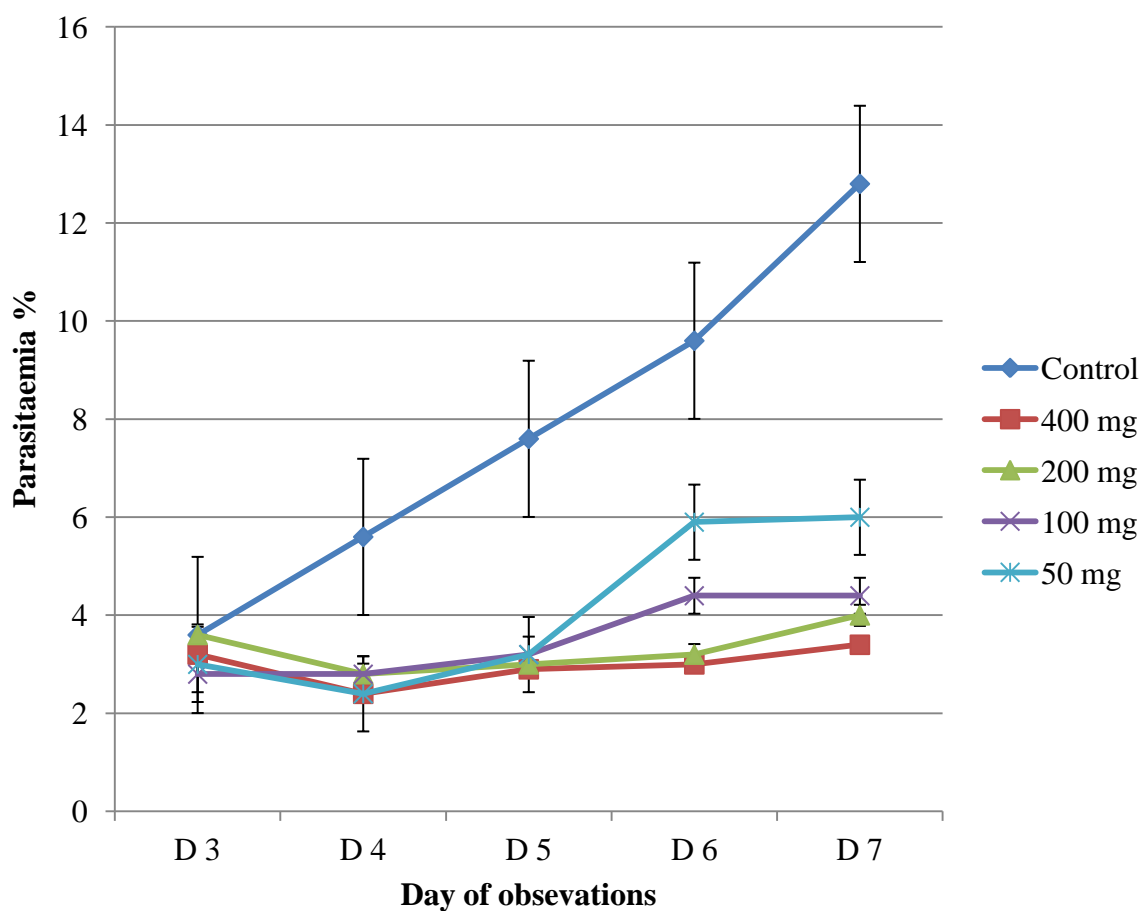


Figure 4.3: Comparison of parasitaemia chemosuppression of *C. nucifera* white flesh extract-treated groups and control from D3 until D7 after infection

Table 4.11: Mean survival time of mice treated with *C. nucifera* white flesh methanol extract

Extract/drug	Dose	Mean survival time ± S.E.M. (n=5)
Control	0.2 mL	13.60 ± 0.51
White flesh extract	50 mg/kg	13.80 ± 1.69
	100 mg/kg	14.60 ± 1.21
	200 mg/kg	15.20 ± 0.66
	400 mg/kg	15.60 ± 1.36
Chloroquine	20 mg/kg	27.20 ± 1.11*

* $P \leq 0.05$ compared to the control group.

vi. Prophylactic anti-malarial activity of *C. nucifera* white flesh

Table 4.12 presents the description of the residual infection. *C. nucifera* showed dose dependent prophylactic anti-malarial activity during. It exerted 30.43%, 43.48%, 56.52% and 73.91% suppressions, respectively. *C. nucifera* showed significant ($P < 0.05$) prophylactic anti-malarial activity during the residual malaria infection by the highest concentrations of the extracts, 200 and 400 mg/kg doses. The 50, 100 and 200 doses of the extract exhibited lower suppressions when compared with the 400 dose, which showed a prophylactic activity closed to the standard drug (Table 4.12).

Table 4.12: Prophylactic anti-malarial activity of *C. nucifera* white flesh methanol extract

Drug/extract	Dose	Parasitaemia% ± S.E.M. (n=5)	%Chemo-suppression
Control	0.2 mL	4.60 ± 0.51	
White flesh extract	50 mg/kg	3.20 ± 0.49	30.43
	100 mg/kg	2.60 ± 0.81	43.48
	200 mg/kg	2.00 ± 0.45	56.52*
	400 mg/kg	1.20 ± 0.20	73.91*
Pyrimethamine	1.2 mg/kg	1.24 ± 0.47	73.04*

* $P \leq 0.05$ compared to the control group.

4.3.2 *Labisia pumila* (Bl.) F.-Vill.

i. Phytochemical screening of *L. pumila* leaves

The presence of terpenoids, flavonoids, tannins, steroids and saponin was indicated through the phytochemical screening of *L. pumila* leaves methanol extract.

ii. DPPH radical scavenging activity of *L. pumila* leaves

L. pumila showed moderate DPPH radical scavenging activity. At 1.56 - 25 µg/ml, the scavenging abilities of the methanol extract on DPPH radicals were 6.82%, 8.80%, 17.21%, 23.03% and 40.66%, respectively (Figure 4.4). Ascorbic acid and gallic acid showed strong scavenging abilities reaching 72.74 and 73.79% at 25 µg/ml, respectively.

iii. Acute oral toxicity of *L. pumila* leaves

No effects of toxicity or mortalities were recorded in any of the 10 animals post dosing and during the observation period (14 days). All animals gained body weight by D 7 and at on the end of the experiment, the tests and control groups did not show any significant differences (Table 4.13). Based on the above mentioned results, the acute oral LD₅₀ in mice of the test extract was found to be in excess of 5000 mg/kg.

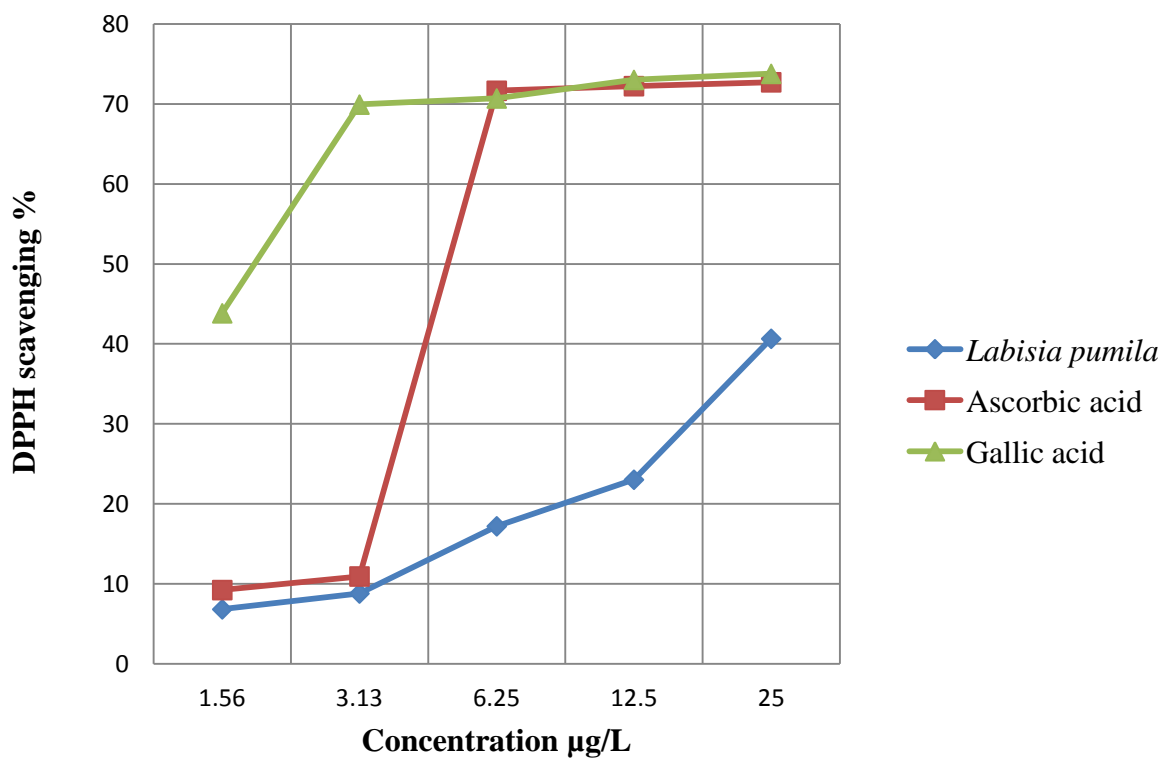


Figure 4.4: DPPH scavenging % activity of *L. pumila* leaves methanol extract

Table 4.13: Body weight gain of mice receiving 5000 mg/kg of *L. pumila* leaves methanol extract

Mice groups	Mean group weight (g) ± S.D. (g); n=5				Mean weight gain (g); n=5
	D-1	D0	D7	Day 14	
Control male	28.00 ± 1.11	27.98 ± 1.28	30.52 ± 1.90	31.80 ± 1.99	3.80 ± 1.93
Test male	28.16 ± 0.86	28.74 ± 0.98	30.12 ± 1.32	31.3 ± 1.23	3.14 ± 1.41
Control female	22.44 ± 0.42	22.70 ± 0.37	24.40 ± 1.07	25.30 ± 0.72	2.86 ± 1.37
Test female	22.08 ± 1.08	22.28 ± 1.26	23.62 ± 1.06	24.42 ± 1.01	2.34 ± 1.11

iv. Suppressive anti-malarial activity of *L. pumila* leaves

The *in vivo* anti-plasmodial activity during the early malaria infection showed that only the 400 mg/kg dose has a significant anti-malarial activity ($P < 0.05$). A dose-dependent chemosuppressive activity which ranged between 19.61% and 50.94 % in all groups of mice has been exhibited (Table 4.14).

v. Curative anti-malarial activity of *L. pumila* leaves

L. pumila leaves showed active anti-malarial activity of more than 30% reduction of parasitaemia by the 400 mg/kg dose. However, no significant activity has been revealed. Chemosuppression effects on D6 were shown for the doses of the extract (Table 4.15). Comparison of parasitaemia chemosuppression of the extract-treated groups and control from D3 until D7 after infection is shown in Figure 4.5. The mean survival did not show significant results for this plant extract (Table 4.16).

vi. Prophylactic antimalarial activity of *L. pumila* leaves

The highest percentage of parasitaemia reduction caused by the leaves extract of *L. pumila* in residual malaria infection was 36.96% for the 400 mg/kg extract dose. Again, the prophylactic test did not show any significant results. In the Table 4.17, prophylactic suppression values of 02.17%, 13.04%, 21.74% and 36.96 % was exerted for the corresponding doses of the extract, 50, 100, 200 and 400 mg/kg.

Table 4.14: Suppressive anti-malarial activity of *L. pumila* leaves methanol extract

Drug/extract	Dose	Parasitaemia% ± S.E.M. (n=5)	%Chemo suppression
Control	0.2 mL	5.10 ± 0.33	
Leaves extract	50 mg/kg	4.10 ± 0.68	19.61
	100 mg/kg	4.01 ± 1.04	21.33
	200 mg/kg	3.10 ± 0.98	39.22
	400 mg/kg	2.50 ± 0.63	50.94*
Chloroquine	20 mg/kg	00.00	100

* $P \leq 0.05$ compared to the control group.

Table 4.15: Curative anti-malarial activity of *L. pumila* leaves methanol extract

Extract/drug	Dose	Parasitaemia% (D6) ± S.E.M. (n=5)	%Chemo- suppression
Control	0.2 mL	9.60 ± 0.93	
Leaves extract	50 mg/kg	9.40 ± 2.80	02.08
	100 mg/kg	8.20 ± 0.86	14.58
	200 mg/kg	7.40 ± 1.91	22.92
	400 mg/kg	5.00 ± 0.55	47.92
Chloroquine	20 mg/kg	00.00	100

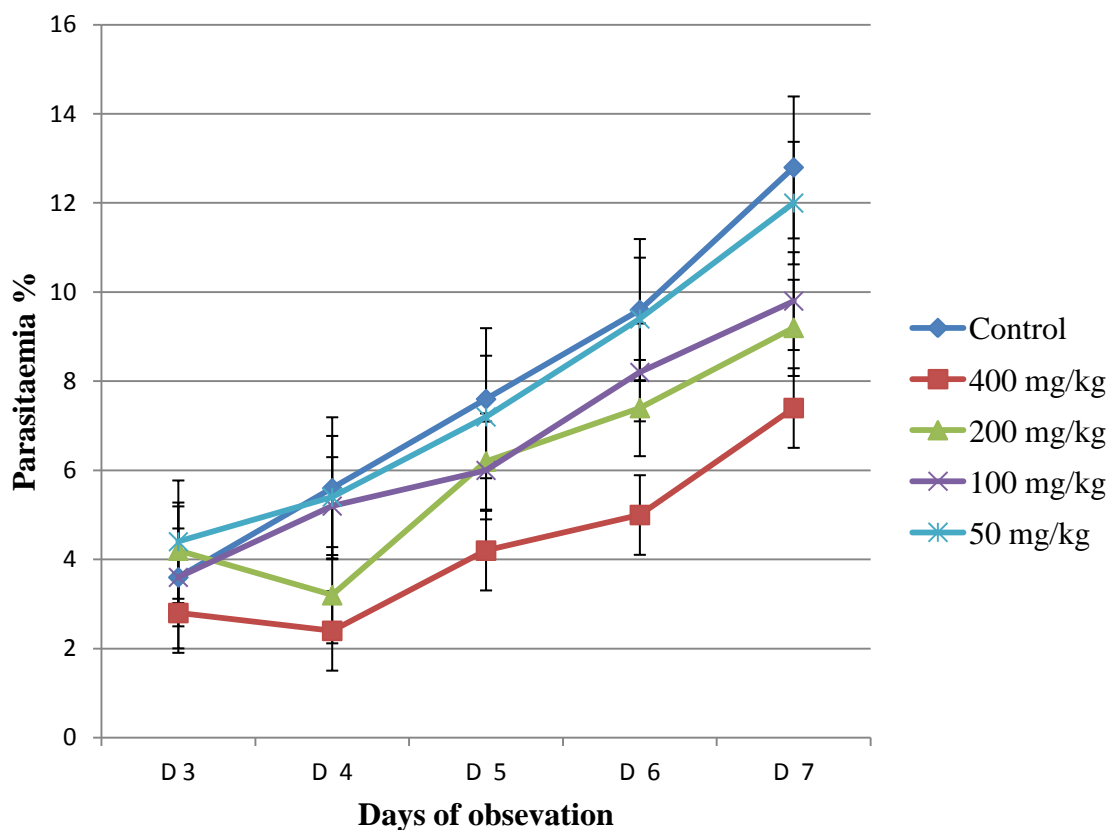


Figure 4.5: Comparison of parasitaemia chemosuppression of the *L. pumila* leaves extract-treated groups and control from D3 until D7 after infection

Table 4.16: Mean survival time of mice treated with *L. pumila* leaves methanol extract

Extract/drug	Dose	Mean survival time ± S.E.M. (n=5)
Control	0.2 mL	13.60 ± 0.51
Leaves extract	50 mg/kg	13.20 ± 0.73
	100 mg/kg	13.80 ± 1.11
	200 mg/kg	14.40 ± 0.24
	400 mg/kg	15.80 ± 1.39
Chloroquine	20 mg/kg	27.20 ± 1.11*

* $P \leq 0.05$ compared to the control group.

Table 4.17: Prophylactic anti-malarial activity of *L. pumila* leaves methanol extract

Extract/drug	Dose	Parasitaemia% ± S.E.M. (n=5)	%Chemo- suppression
Control	0.2 mL	4.60 ± 0.51	
Leave extract	50 mg/kg	4.50 ± 0.59	02.17
	100 mg/kg	4.00 ± 0.63	13.04
	200 mg/kg	3.60 ± 0.93	21.74
	400 mg/kg	2.90 ± 0.90	36.96
Pyrimethamine	1.2 mg/kg	1.24 ± 0.47	73.04*

* $P \leq 0.05$ compared to the control group.

4.3.3 *Languas galanga* Stuntz.

i. **Phytochemical screening of *L. galanga* rhizome**

The presence of terpenoids, flavonoids, tannins, saponins, steroids and glycosides was indicated through the phytochemical screening of *L. galanga* rhizomes methanol extract.

ii. **Radical scavenging activity of *L. galanga* rhizome**

The methanol rhizome extract of *L. galanga* showed moderate DPPH radical scavenging activity. At 1.56–25 µg/mL, the scavenging abilities of the methanol extract were 10.08%, 8.63%, 15.64%, 29.34% and 37.88%, respectively, as shown in Figure 4.6. At 25 µg/mL, ascorbic acid and gallic acid showed strong scavenging abilities reaching 72.74 and 73.79%, respectively.

iii. **Acute oral toxicity of *L. galanga* rhizome**

The mortality rates of orally administered *L. galanga* are shown in the Table 4.18. The most important observed behavioural indications of toxicity were asthenia, piloerection, ataxia, anorexia, urination, diarrhea, lethargy and coma. Asthenia, piloerection, anorexia, diarrhea and urination were noticed after dosing with 2,000 mg/kg and were more marked at the highest dose and continued until death, in particular among the male subjects, which showed a lethal median dose lower than that of the females. In ICR mice, the LD₅₀ of *L. galanga* rhizome methanol extract was 4,998 mg/kg.

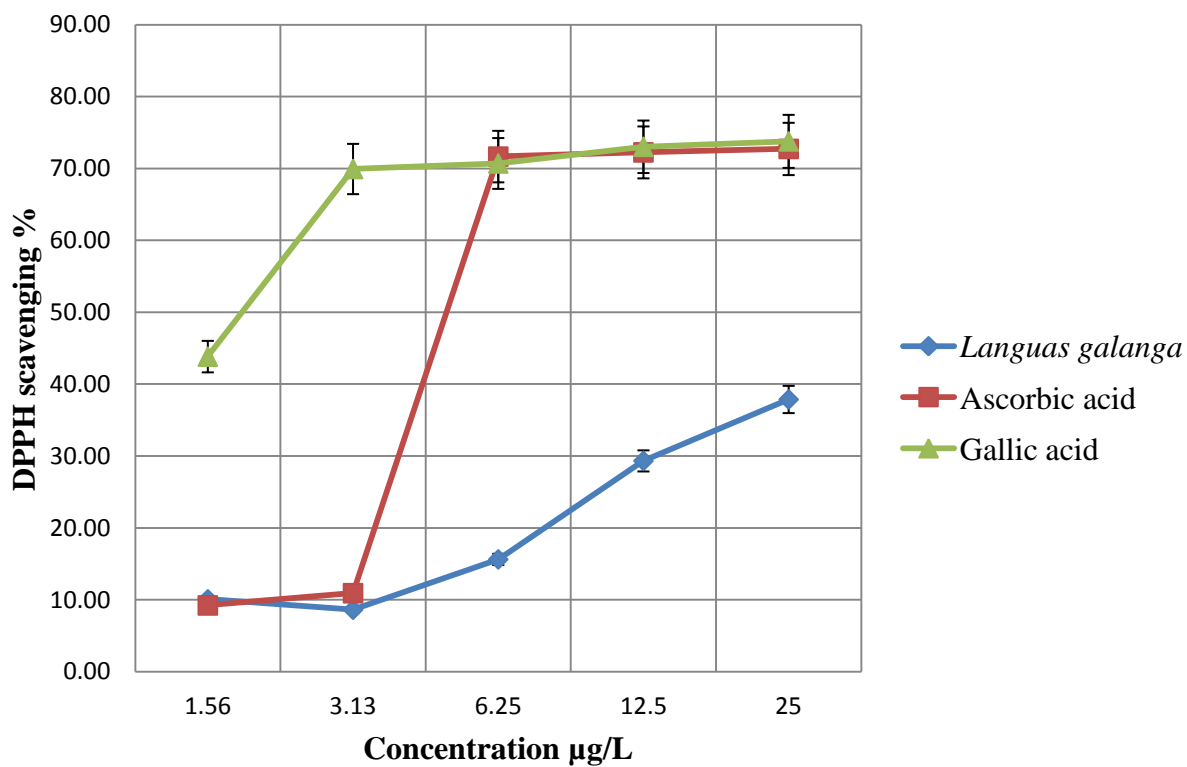


Figure 4.6: DPPH scavenging % activity of *L. galanga* rhizomes methanol extract

Table 4.18: Acute oral toxicity of *L. galanga* rhizome methanol extract in ICR mice

Dose (mg/kg)	Mortality		LD ₅₀
	Dead/tested mice	Latency (hour)	
0	0/10	-	The median lethal dose determined by the probit analysis was 4,998 mg/kg
300	0/10	-	
2,000	1/10	>36, <60	
5,000	5/10	>24, <60	

iv. Suppressive anti-malarial activity of *L. galanga* rhizome

During early malaria infection, the rhizomes extract of *L. galanga* produced a dose dependent suppressive anti-malarial activity. The 4-day suppressive effects of 29.41%, 49.02%, 62.55% and 64.51% were shown for the 50, 100, 200 and 400 mg/kg doses of the extract, respectively. After the 200 mg/kg, the anti-malarial activity of the extract showed only a marginal increase. In the exception of the 50 mg/kg dose, the extract produced significant ($P < 0.05$) anti-malarial activity (Table 4.19).

v. Curative anti-malarial activity of *L. galanga* rhizome

In comparison with the control, *L. galanga* rhizome exhibited significant ($P < 0.05$) dose dependent curative anti-malarial activity. The parasitaemia percentages for the extract-treated groups on D6 of infection were 5.80%, 3.40%, 3.40% and 3.20% for 50, 100, 200, and 400 mg/kg doses of the extract, respectively (Table 4.20). A marginal increase in the anti-malarial activity of the extract was exhibited over the 100 mg/kg dose. In Figure 4.7, parasitaemia chemosuppressions of the extracts groups and control from D3 until D7 after infection is exposed. The mean parasitaemia for the control group was 9.60. The mice group treated with the 200 and 400 mg/kg doses of the extract showed longer survival time, which was significantly different from the control group (Table 4.21).

Table 4.19: Suppressive anti-malarial activity of *L. galanga* rhizomes methanol extract

Extract/drug	Dose	Parasitaemia% ± S.E.M. (n=5)	%Chemo-suppression
Control	0.2 mL	5.10 ± 0.33	
Rhizome extract	50 mg/kg	3.60 ± 0.68	29.41
	100 mg/kg	2.60 ± 0.37	49.02*
	200 mg/kg	1.91 ± 0.91	62.55*
	400 mg/kg	1.81 ± 0.89	64.51*
Chloroquine	20 mg/kg	00.00	100

* $P \leq 0.05$ compared to the control group.

Table 4.20: Curative anti-malarial activity of *L. galanga* rhizomes methanol extract

Extract/drug	Dose	Parasitaemia% (D6) ± S.E.M. (n=5)	%Chemo-suppression
Control	0.2 mL	9.60 ± 0.93	
Rhizome extract	50 mg/kg	5.80 ± 1.21	39.58
	100 mg/kg	3.40 ± 0.22	64.58*
	200 mg/kg	3.40 ± 0.78	65.58*
	400 mg/kg	3.20 ± 0.77	66.67*
Chloroquine	20 mg/kg	00.00	100

* $P \leq 0.05$ compared to the control group.

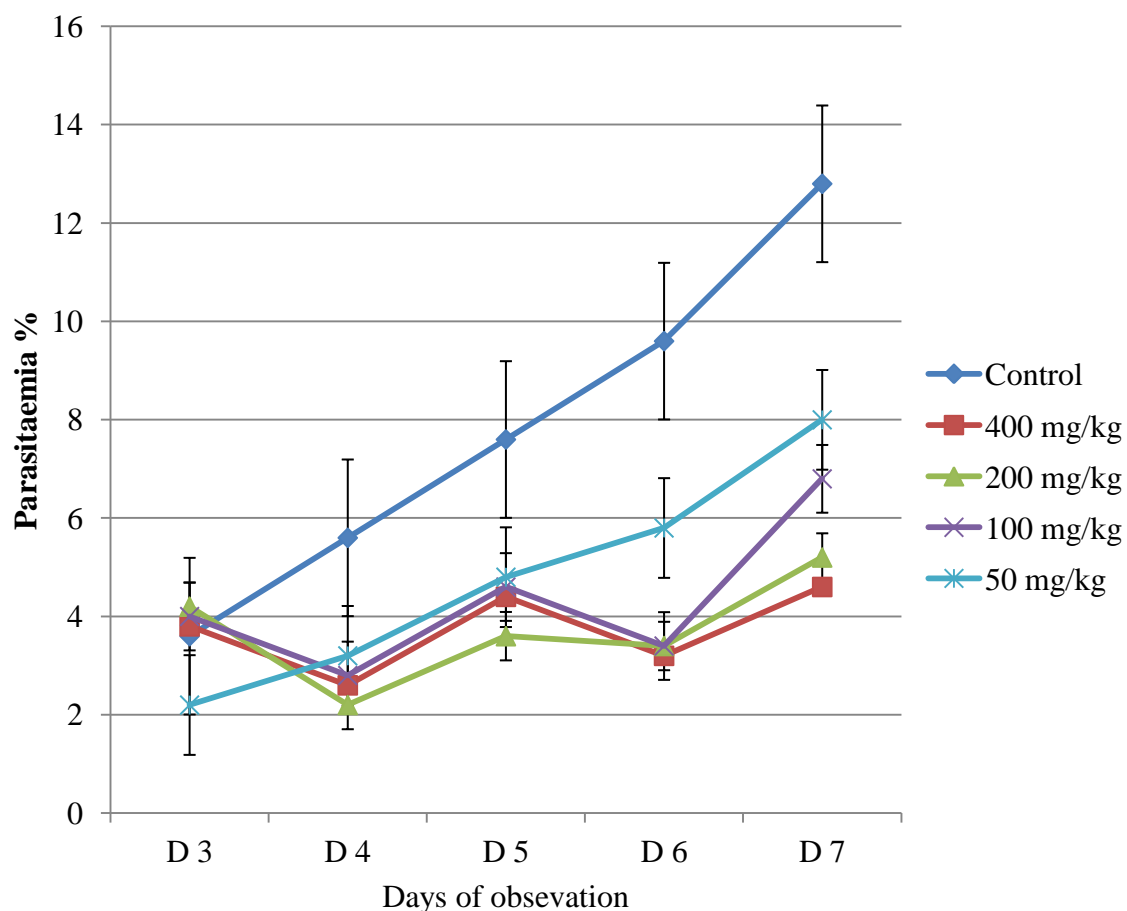


Figure 4.7: Comparison of parasitaemia chemosuppression of the *L. galanga* rhizomes extract-treated groups and control from D3 until D7 after infection

Table 4.21: Mean survival time of mice treated with *L. galanga* rhizomes methanol extract

Extract/drug	Dose	Mean survival time ± S.E.M. (n=5)
Control	0.2 mL	13.60 ± 0.51
Rhizome extract	50 mg/kg	14.40 ± 0.81
	100 mg/kg	15.60 ± 0.98
	200 mg/kg	17.60 ± 0.40*
	400 mg/kg	18.00 ± 0.55*
Chloroquine	20 mg/kg	27.20 ± 1.11*

* $P \leq 0.05$ compared to the control group.

vi. Prophylactic anti-malarial activity of *L. galanga* rhizome

As summarized in Table 4.22, the results of prophylactic activity of the rhizomes extract during the residual malaria infection exhibited dose dependent suppression at the 50-400 mg/kg doses, exerting 13.04%, 26.09%, 39.13% and 52.17% suppressions, respectively. It showed that though there were significant prophylactic activities at the doses with the highest concentrations (200 and 400 mg/kg), the chemosuppressive activity was rather low and not significant at the 100 and 50 mg/kg doses.

Table 4.22: Prophylactic anti-malarial activity of *L. galanga* rhizomes methanol extract

Extract/drug	Dose	Parasitaemia% ± S.E.M. (n=5)	%Chemo- suppression
Control	0.2 mL	4.60 ± 0.51	
Rhizome extract	50 mg/kg	4.00 ± 0.55	13.04
	100 mg/kg	3.40 ± 0.93	26.09
	200 mg/kg	2.80 ± 0.49	39.13*
	400 mg/kg	2.20 ± 0.37	52.17*
Pyrimethamine	1.2 mg/kg	1.24 ± 0.47	73.04*

* $P \leq 0.05$ compared to the control group.

4.3.4 *Piper betle* L.

i. Phytochemical screening of *P. betle* leaves

Phytochemical screening of *P. betle* leaves methanol extract exposed that the leaf extract contains alkaloids, terpenoids, anthraquinones, flavonoids, tannins, saponins and steroids.

ii. Radical scavenging activity of *P. betle* leaves

P. betle leaves exhibited a strong radical scavenging capacity comparable to the controls. At the 12.5 µg/mL concentration of the methanol extract, the scavenging activity the leaves reached 82.56 ± 1.50 %, while at the same concentration, those of the ascorbic and gallic acids controls were 72.25 ± 2.44 and 73.03 ± 2.27 %, respectively (Figure 4.8).

iii. Acute oral toxicity of *P. betle* leaves

No deaths occurred during the observation period. Some signs were observed 1 hour following administration of the extract. Ataxia and piloerection were noted in males and females; all of which resolved by 3 hours after administration. No abnormal general signs were observed in the extract treated groups. All animals gained body weight on the end of the experiment, the tests and control groups did not show any significant differences (Table 4.23).

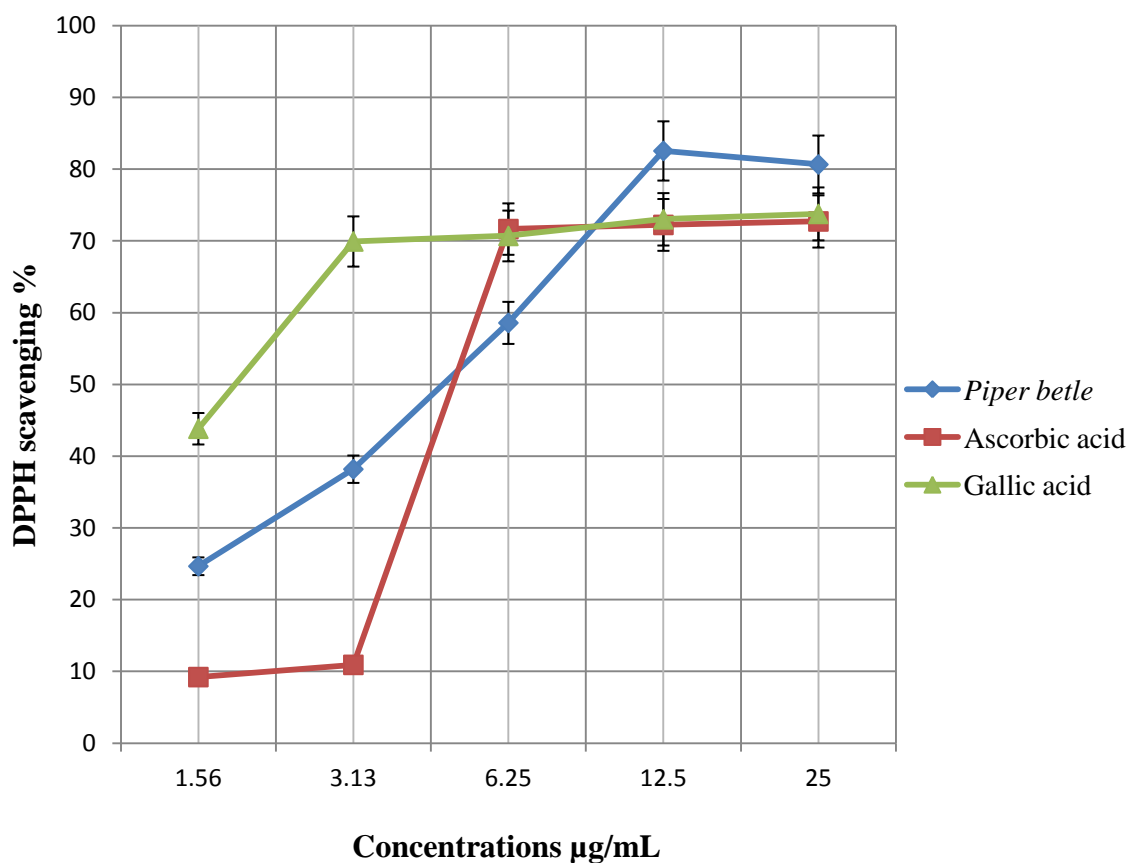


Figure 4.8: DPPH scavenging % activity of *P. betle* leaves methanol extract

Table 4.23: Body weight gain of mice receiving 5000 mg/kg of *P. betle* leaves methanol extract

Mice groups	Mean group weight (g) ± S.D. (g); n=5				Mean weight gain (g); n=5
	D-1	D0	D7	Day 14	
Control male	28.00 ± 1.11	27.98 ± 1.28	30.52 ± 1.90	31.80 ± 1.99	3.80 ± 1.93
Test male	26.26 ± 0.77	26.34 ± 0.82	27.74 ± 2.35	29.20 ± 2.72	2.94 ± 1.39
Control female	22.44 ± 0.42	22.70 ± 0.37	24.40 ± 1.07	25.30 ± 0.72	2.86 ± 1.37
Test female	22.38 ± 1.31	22.72 ± 1.21	24.60 ± 1.31	24.84 ± 3.05	2.46 ± 1.26

iv. Suppressive anti-malarial activity of *P. betle* leaves

P. betle leaves methanol extract showed a dose-dependent chemosuppressive activity which ranged between 36.47% and 82.31% (Table 4.24). A significant high degree of chemosuppression was shown by the 200 and 400 mg/kg doses of the extract that reduced the parasitaemia of the infected mice when compared to control ($P < 0.05$).

v. Curative antimalarial activity of *P. betle* leaves

The results indicated that the methanol extract of *P. betle* leaves exhibited significant ($P < 0.05$) dose dependent chemosuppression in parasitaemia for all the extract doses in comparison with the control. A daily increases in parasitaemia reaching 9.60 % on D6 of infection was exhibited by the control group (Figure 4.9). The chemosuppression effects for the treated groups on the D6 of infection were 37.50%, 45.83%, 66.46% and 70.63% for the extract doses 50 to 400 mg/kg, respectively (Table 4.25). On the other hand, the mice extract-treated groups showed longer survival times reaching 19.00 ± 1.22 days as compared to the control with 13.6 ± 0.51 days. The chloroquine-treated group had a mean survival time of 27.2 ± 2.33 days (Table 4.26).

Table 4.24: Suppressive anti-malarial activity of *P. betle* leaves methanol extract

Extract/drug	Dose	Parasitaemia% ± S.E.M. (n=5)	%Chemo-suppression
Control	0.2 ml	5.10 ± 0.33	
Leaves extract	50 mg/kg	3.24 ± 0.82	36.47
	100 mg/kg	2.40 ± 0.68	52.94*
	200 mg/kg	1.50 ± 0.63	70.51*
	400 mg/kg	0.90 ± 0.33	82.31*
Chloroquine	20 mg/kg	00.00	100

* $P \leq 0.05$ compared to the control group.

Table 4.25: Curative anti-malarial activity of *P. betle* leaves methanol extract

Extract/drug	Dose	Parasitaemia% (D6) ± S.E.M. (n=5)	%Chemo-suppression
Control	0.2 ml	9.60 ± 0.93	
Leaves extract	50 mg/kg	6.00 ± 0.84	37.50*
	100 mg/kg	5.20 ± 0.92	45.83*
	200 mg/kg	3.22 ± 0.95	66.46*
	400 mg/kg	2.82 ± 0.84	70.63*
Chloroquine	20 mg/kg	00.00	100

* $P \leq 0.05$ compared to the control group.

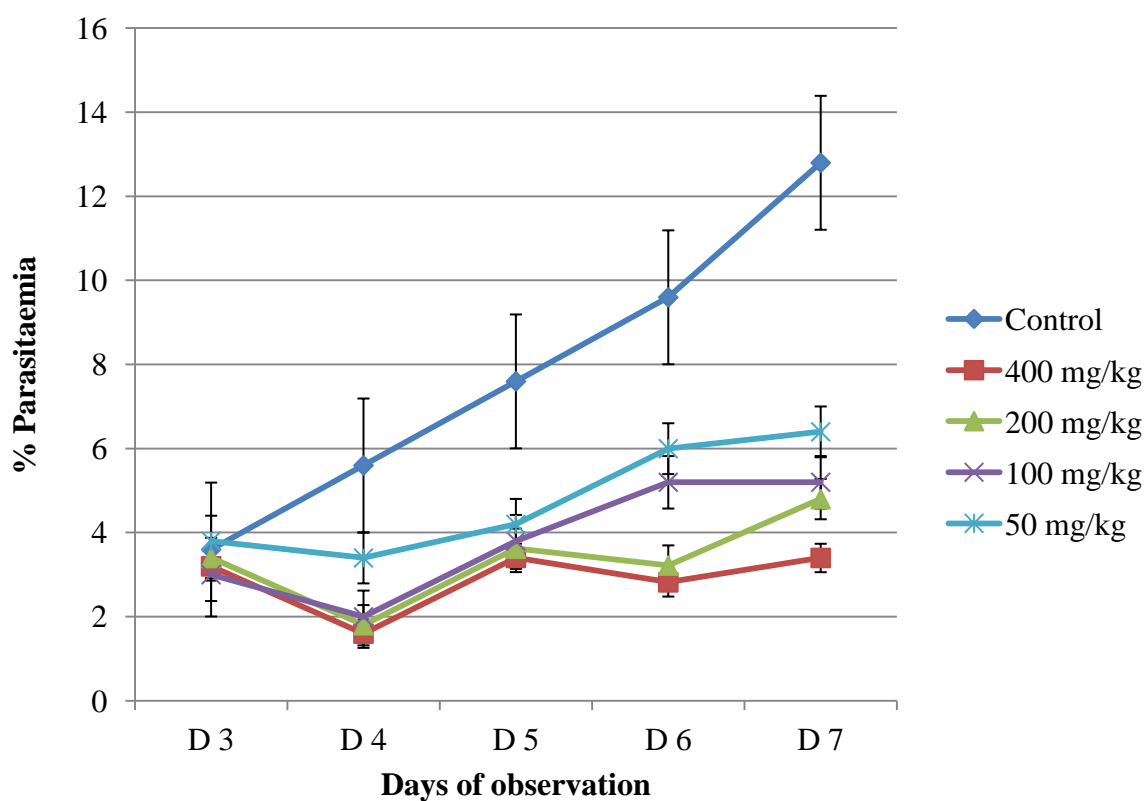


Figure 4.9: Comparison of parasitaemia chemosuppression of the *P. betle* leaves extract-treated groups and control from D3 until D7 after infection

Table 4.26: Mean survival time of mice treated with *P. betle* leaves methanol extract

Drug/extract	Dose	Mean survival time ± S.E.M. (n=5)
Control	0.2 ml	13.60 ± 0.51
Leaves extract	50 mg/kg	14.40 ± 0.93
	100 mg/kg	15.20 ± 1.16
	200 mg/kg	17.20 ± 1.56
	400 mg/kg	19.00 ± 1.22*
Chloroquine	20 mg/kg	27.20 ± 2.33*

* $P \leq 0.05$ compared to the control group.

vi. Prophylactic anti-malarial activity of *P. betle* leaves

The results of prophylactic activity of the leaves methanol extract of *P. betle* during the residual malaria infection exhibited significant ($P < 0.05$) dose dependent suppressions. The leaves extract exhibited 19.57, 34.78, 52.17 and 70.88% chemosuppressions, for the doses 50 to 400 mg/kg, respectively (Table 4.27). The chemosuppression shown by the 400 mg/kg of the extract was comparable to the standard drug.

Table 4.27: Prophylactic anti-malarial activity of *P. betle* leaves methanol extract

Drug/extract	Dose	Parasitaemia% ± S.E.M. (n=5)	%Chemo- suppression
Control	0.2 ml	4.60 ± 0.51	
Leaves extract	50 mg/kg	3.70 ± 0.30	19.57
	100 mg/kg	3.00 ± 1.34	34.78
	200 mg/kg	2.20 ± 0.37	52.17*
	400 mg/kg	1.34 ± 0.41	70.88*
Pyrimethamine	1.2 mg/kg	1.24 ± 0.47	73.04*

* $P \leq 0.05$ compared to the control group.

CHAPTER V

DISCUSSION

5.1 COMMUNITY'S AWARENESS REGARDING MALARIA

This study is the first to investigate the KAP on malaria in Peninsular Malaysia, which is directly essential to enhance community awareness of malaria. Findings of the present study indicated that there were significant differences between aboriginal and rural communities in knowledge and practices towards malaria transmission, treatment-seeking behaviour and understanding of effective preventive measures. This could be explained by the better educational level and the higher number of health facilities reported among rural population. In addition, religious practices and cultural traditions among the inhabitants of remote areas may be the reason behind the inadequate knowledge of malaria. In general, Aborigines often attribute the illness to devil, ghosts or evil spirits. In this study, these practices either through the use of plants, consultation of traditional healers or sorcerers were demonstrated in about one third of the participants.

Moreover, most of the aboriginal people of this study live in houses made up of bamboo lacking of sanitation (APPENDIX A). There is poverty, malnutrition, high prevalence of intestinal parasitic infections (Al-Mekhlafi, 2008). Insufficient quality health care and poverty that are widespread among the aboriginal people can seriously hinder the elimination of malaria (WHO, 2005; Sharma, 2009).

The present study also reported many mistaken beliefs about malaria particularly in the Aborigines where a large number of the participants demonstrated a misconception about its transmission. This emphasizes the need for effective interventions to improve the level of knowledge in this community. A noteworthy number of participants believed that malaria is transmitted by stagnant water, walking in forest, human to human via belongings and others. Although most of the participants associated malaria to mosquito bites, none of them knew that a parasite is the causative agent responsible for malaria neither how mosquitoes acquire the parasite. The role of mosquito vector in transmission of malaria was known to 50% and 70.7% of the aboriginal and rural participants, respectively. This figure was lower than that exhibited by respondents in a KAP study in Swaziland, Southern Africa (92.81%), a country earmarked for malaria elimination (Hlongwana *et al.*, 2009). Accordingly, it is clear that the lack of knowledge, among the target population, about the aetiology of the disease as well as the position of mosquitoes in causing malaria may add additional burden and costs for controlling the disease and may cause failure of malaria elimination programme.

Education plays an important role in people's perceptions and practices of treating and controlling malaria. Previous studies from Africa showed a positive correlation between the awareness of preventive measures to control malaria infection and the level of education among the community (Tarimo *et al.*, 2000; Dike *et al.*, 2006). Furthermore, the use of medicinal plants to combat malaria was lower in people who have a higher level of formal education (Dike *et al.*, 2006). In harmony with these findings, the present study showed that the better educational level of the rural community reflected a better knowledge and practices regarding malaria prevention and treatment than the aboriginal people. At the community level, the level of education

showed a significant impact on population's Knowledge, attitudes and practices on malaria.

The findings of this study showed that most people in both communities had information about symptoms of malaria and more than three quarters of the participants recognized fever, chills/rigors and headache as most common symptoms. This was in agreement with the previous studies in tropical and subtropical malaria endemic countries (Simsek & Kurcer, 2005; Swe & Pearson, 2004). It should be noted that a considerable number of participants in both communities showed confusion between the symptoms of malaria and dengue fever which is also endemic in these areas. However, this may not affect the disease control in terms of the mosquito control but, it may lead to serious complications in patients who believe that dengue fever can be cured by taking anti-pyretics and drinking fluids (Nalongsack *et al.*, 2009).

Promising results about treatment-seeking behaviour were reported; almost all the rural participants and two thirds of the aboriginal participants seek treatment at health centers within twenty-four hours of the symptoms onset. Previous studies in rural areas in Southeast Asia showed that more than half of the population opts for self-treatment without visiting a health facility (Sanjana *et al.*, 2006; Joshi & Banjara, 2008). The better behaviour reported by the present study could be due to the availability of health facilities and access to its services by all Malaysians throughout the country. Most of the aboriginal people who use medicinal plants and believe in witchcraft as a treatment for febrile diseases go to health centres for treatment, either within 24 hours exercising this practice to support the modern treatment or within 48-72 hours after waiting for the outcome of traditional recipes for a short period of time and then seek treatment from health centres. Of reasons not to seek treatment on the first day of the

onset of fever is to wait until the disease worsened to the belief that febrile diseases resolve spontaneously (Nyamongo, 2002).

Regarding the understanding of the measures for the prevention of malaria, the present study showed that most of the participants were aware that malaria can be prevented. However, many misconceptions about malaria prevention measures were reported. In both communities, more than one third of the participants do not use bed nets to prevent mosquito bites. This could be due to the reliance of these populations on the government in fighting the disease without taking enough personal precautions. The poor usage of mosquito bed nets might also be attributed to the cost and to the lack of knowledge that mosquitoes are the causative agents of malaria (Minja *et al.*, 2001; Oguonua *et al.*, 2005).

The comparison between the forest-aboriginal and rural communities has a great benefit to determine the nature of adaptations required in the plan of future interventions. Public awareness programmes to promote a better understanding on malaria transmission and active participation of the aboriginal communities in malaria control activities are deemed necessary. Such awareness and participation will bring positive changes and adaptation in their cultural beliefs and practices and this can help in reducing the incidence of malaria.

5.2 ETHNOBOTANICAL STUDY ON ANTI-MALARIAL PLANTS

The present survey has established a preliminary data base for 19 species of plant species used to combat malaria in Malaysia that are essential for further phytochemical and pharmaceutical studies. The obtained data has also revealed how different interviewed groups are able to enrich the information about the used plants in combating malaria. The majority of the species preferred by the traditional healers were considered to be active by the respondents, which suggests, in agreement with Asase *et al.* (2005), that traditional healers possessed a high knowledge of the medicinal plants.

Seven plant species namely, *Alstonia angustiloba*, *Brucea javanica*, *Cassia siamea*, *Phyllanthus niruri*, *Eurycoma longifolia*, *Languas galanga* and *Tinospora crispa* recorded in this survey are used in East Malaysia to treat malaria, Kamarudin (1997), Kulip (1997), Fasihuddin (2000) and Fasihuddin & Holdsworth (2003) while the anti-malarial use of the other 12 plant species in West Malaysia were identified and documented for the first time.

Drinking the extract decoction was the most reported preparation regarding the use of the plant remedies among the population in fight against the disease. However, the accurate quantity, standardization and quality control of the using the remedies were generally indistinguishable. The absent of quality control and standardization is seemed to be one of the major weaknesses of traditional medicine (Asase *et al.*, 2005). Moreover, the combination in the traditional remedies causes further difficulty regarding regulation, evaluation and observation of the degrees of biologically active ingredients (Asase *et al.*, 2005).

5.3 ANTI-MALARIAL ACTIVITY OF THE PLANTS EXTRACTS

Anti-malarial activity screening tests of *C.nucifera*, *L.pumila*, *L.galanga* and *P.betle* showed that each of the four plant extracts exhibited active malaria suppression activity of more than 30% against *P.berghei* infection in mice as evident from the degree of suppressions obtained based on the 4-day suppressive tests (Krettli *et al.*, 2009).

The chemosuppressions activity showed to be dose dependent where higher concentrations of the doses caused higher degree of anti-malarial chemosuppression activity (Figure 5.1). *L.pumila* showed a clear increase proportionately among all the extract doses in all the anti-malarial tests. By increasing extract dose a clear increase in activity was observed, suggesting that more increases in the concentration showed further increases in the activity. On the other hand, marginal increases in activity of *L.galanga* over 100 mg/kg was observed by the 200 and 400 mg/kg doses in both suppressive as well as curative assays suggesting that additional increases in the concentration resulted in merely marginal increases in suppression (Elufioye & Agbedahunsi, 2004), that may represents the maximum concentration by which the anti-malarial molecules included in the extract can affect the parasite. *C.nucifera* exhibited marginal increases by the 400 mg/kg in both suppressive and curative assays. However, *P.betle* showed a slight marginal increase by the 400 mg/kg in curative assay, which may indicate that this plant can exhibit higher anti-malarial effects by increasing the dose concentrations.

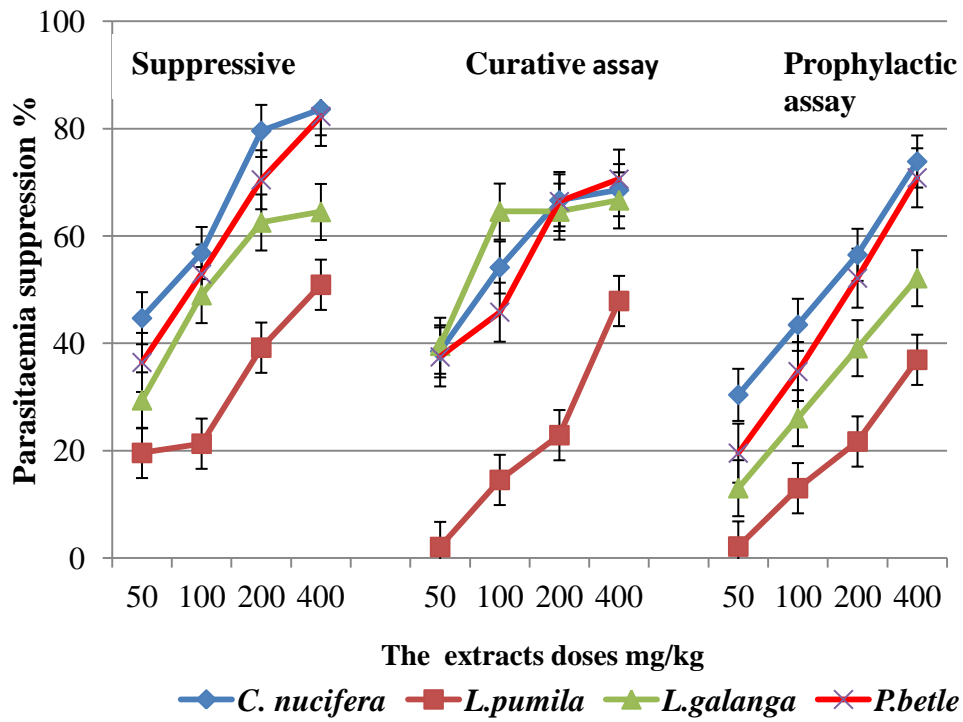


Figure 5.1: The *in vivo* anti-malarial effects of the plants extract

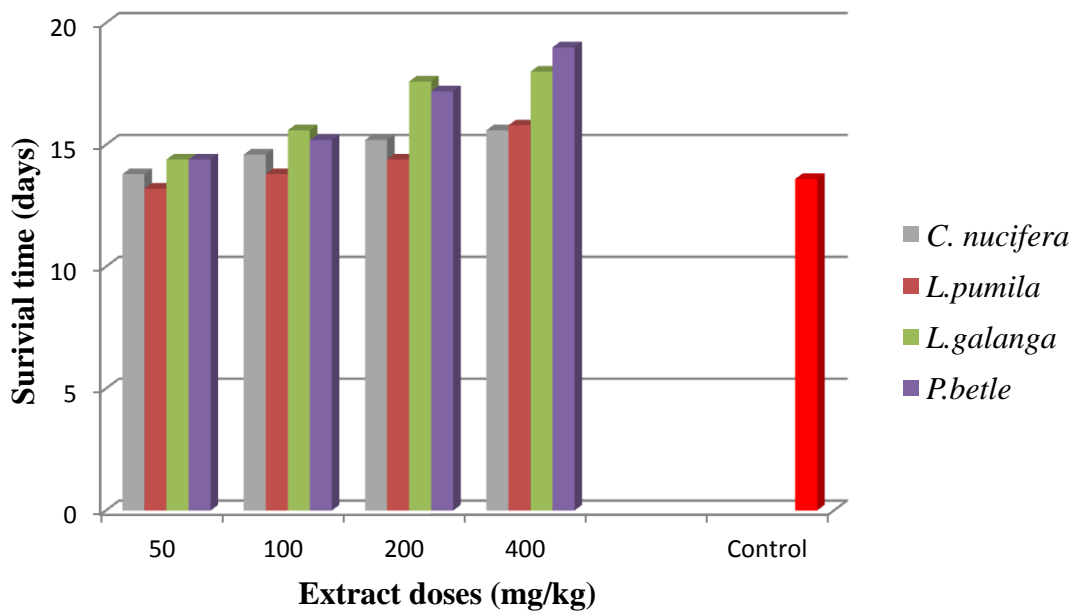


Figure 5.2: The mean survival time of the plants extract

The extracts also exhibited a curative activity with significant ($P < 0.05$) high mean survival time values during the established malaria infection particularly in the groups administered with *P.betle* and *L.galanga* (Figure 5.2). Overall results with evident of increasing the mean survival time showed that *P.betle* and *L.galanga* were the most effective followed by *C.nucifera* and *L.pumila*, respectively. Although *C.nucifera* caused high degrees of suppression comparable to *P.betle* in all the three assessment models, but the increment in the survival time of the infected mice was not significant. These activities can be interpreted as a result of individual effect by one of the chemical constituents included in *C.nucifera* and most important of which are terpenoids. This finding could be very important for further investigation of these compounds individually. The second possibility is a promising immunomodulatory effect that this plant (*C.nucifera*) may possess, which as a concern may give impression on the host-parasite interrelationship (Anthony *et al.*, 2005). Moreover, coconut oil is rich in fatty acid with more than 40% of the total fatty acids is lauric acid (Azeez, 2007). A previous study reported that injection of fatty acids during the first three days after infection protected mice infected with *P. berghei* from cerebral symptoms and increased the survival time (Moumaris *et al.*, 1995). A possible immunoregulatory role for fatty acids which enhances neutrophil-mediated killing of *P. falciparum* has been also identified (Kumaratilake *et al.*, 1997).

When standard anti-malarial drug chloroquine (20 mg/kg body weight) is used to cure mice infected with *P. berghei*, it suppressed parasitaemia to non-detectable levels (Ishih *et al.*, 2006; Muregi *et al.*, 2007), which is also evident in this study. However, it was shown that the dose that we used in curative tests did not cure all the animals. The developed resistance may be as a result of the repeated passage of the parasite in the mice in the context of maintaining the strain in the laboratory (Adzu, *et*

al., 2003; Chawira, 1986; Okokon, *et al.*, 2005). Conversely, the parasitaemia suppression pyrimethamine at 1.2 mg/kg seemed to be in agreement with previous studies conducted on the repository activity in *P. berghei* infected mice (Elufioye & Agbedahunsi, 2004; Chandel & Bagai, 2010).

The present study also showed that the test extracts are toxicologically safe by oral administration. In GHS classification: Health Effects Test Guidelines, the LD₅₀ value exceeding than 5000 mg/kg showed by *C.nucifera*, *L.pumila* and *P.betle* extracts are practically safe (Horn, 1956; GHS, 2005; Rhiouani *et al.*, 2008). On the other hand, the acute oral toxicity (LD₅₀) of *L. galanga* of 4,998 mg/kg is also safe according to Horn (1956) and Rhiouani *et al.* (2008), who stated that “plants or plant products with LD₅₀ values higher than 2,000–3,000 mg/kg are considered free of any toxicity”. This supports the logical usage of these plants in folk medicine practices.

The present study had followed a logical pathway in the extraction procedures by using the methanol solvent as an extractor. Because most of the identified anti-microorganisms aromatic and saturated organic compounds of plant components were acquired during the preliminary methanol extraction (Fransworth, 1994; Eloff ,1998; Cowan ,1999; Reuben *et al.*, 2008; Usman *et al.*, 2009; Peni *et al.*, 2010; Karthishwaran *et al.*, 2010).

The anti-plasmodial activities revealed by the plants were perhaps as a result of active chemical ingredients. Phytochemical screening of the plants methanol extracts exposed the presence of alkaloids, terpenoids, anthraquinones, flavonoids, tannins, saponins, glycosides and steroids.

Those chemical components however can differ in their potentials in causing effective malaria suppression. Those differences are depending on the active molecules involved in the reaction against the parasites. For example, the alkaloids and terpenoids isolated from *Cinchona* species and *Artemisia annua*, respectively, have different activity than those isolated from other plants. Therefore, it is very possible that each chemical constituent might has a special characteristic and different structural classes.

Alkaloids are important members of chemical constituents having anti-malarial activity. In a review article by Saxena *et al.* (2003), one hundred alkaloids from medicinal plants were accounted to reveal important anti-malarial effects in reports published between 1990 and 2003; some of these alkaloids exhibited anti-malarial activity more than chloroquine. Bisbenzylisoquinolines, Aporphine-benzylisoquinoline, Morphinan, Naphtylisoquinolines, an Indoloquinoline , Mono- and bis-indole alkaloids, Indolomonoterpenoid, Indole, Benzofenantridine, Acridone, Furoquinoline, Acridine, Tetrahydroquinoline are some of the active anti-malarial alkaloids reported of different structural classes isolated from plant sources (Oliveira *et al.*, 2009b). Meanwhile, Icajine, Vomocine, Isostrychnine, Cryptolepine, berberine , palmatine , jatrorrhizine and several berberine alkaloids derivatives showed inactive anti-malarial activity *in vivo* (Vennerstrom & Klayman, 1988; Federici *et al.*, 2000; Frederich *et al.*, 2000).

Many structural classes of terpenoids exhibited potent anti-malarial activity. Of these are keberins and friedelane triterpenoid, indanone derivative, cassane-type diterpenes, furanoterpenoids, monoterpenes geraniol and monoterpenoids; (-)-linalool, (-)-perillyl alcohol, (-)-isopulegol (-)-limonene and (±)-citronellol (Batista *et al.*, 2009). Examples of common terpenoids are farnesol and artemisinin (sesquiterpenoids). Artemisinin and related derivatives are the most vital new member of anti-malarial

structural classes, which are developed from *Artemisia annua* plant in China beginning in the 1960s (Philip & Rosenthal, 2008). Terpenoids are also have anti-bacterial effects (Ahmed *et al.*, 1993; Mendoza *et al.*, 1997; Amaral *et al.*, 1998; Singh & Singh 2003; Souza *et al.*, 2010) anti-fungal (Harrigan *et al.*, 1993; Ayafor *et al.*, 1994; Suresh *et al.*, 1997; Singh & Singh 2003) anti-viral (Fujioka & Kashiwada, 1994; Xu *et al.*, 1996) and anti-protozoal (Vishwakarma, 1990; Francois *et al.*, 1996; Ghoshal *et al.*, 1996; Delgado-Méndez *et al.*, 2008).

Flavonoids are phenolic structures synthesized by plants in response to microbial infections. Flavonoids exposed significant anti-parasitic activities against different strains of *Plasmodium* species, trypanosome and leishmania (Kim *et al.*, 2004; Monbrison *et al.*, 2006; Tasdemir *et al.*, 2006; Khaomek *et al.*, 2008; dos Santos *et al.*, 2009). The anti-malarial activities of flavonoids have not been expressed in the past, while they represent one of the most distinctive compounds in higher plants. *Artemisia annua* flavonoids were not exhibited effective anti-malarial activity (Liu *et al.*, 1992).

The results of the phytochemical screening also showed that *P. betle* leaves extract contained anthraquinones. These chemical compounds are an important member of the quinone family. Many naturally-occurring quinones are based in their structure on the anthraquinone, naphthoquinone or benzoquinone ring system. Derivatives of 9,10-anthraquinone include many important drugs including anti-malarials like rufigallol (Mahajan *et al.*, 2005). Different classes of anthraquinones showed significant anti-malarial activities in the recent concerned studies. A novel anti-malarial anthraquinone named anthrakunthone from the root bark of *Stereospermum kunthianum* was isolated (Onegi *et al.*, 2002). The bark of *Scutia myrtina* (Rhamnaceae) led to the isolation of three new anthraquinones named scutianthraquinones A, B and C. These

anthraquinones compounds exhibited significant anti-plasmodial activities (Hou *et al.*, 2009).

Saponins are a class of chemical constituents found in various plant species that can affect *Plasmodium* parasite. Philipson & Wright (1991) stated that plants having phytochemical compounds include saponins may have anti-malarial activities. In 1996, Okunji *et al.* isolated saponin spiroconazole A from *Dracaena* species that revealed pronounced anti-leishmanial, anti-malarial and molluscicidal activities. Study by Nandi *et al.* (2004) suggested that saponins can generate superoxide anions and initiate lipid peroxidation in a mechanism to kill the *Plasmodium* parasite.

A new steroidal glycoside, gongroneside A which has been isolated from the vine of *Gongronema napalense* (Wall.) Decne. (Asclepiadaceae) exhibited promising anti-malarial activity (Libman *et al.*, 2008) as well as amino steroids 8d showed potent anti-plasmodial inhibition (Sharma *et al.*, 2008). Tannin-rich fractions from *Punica granatum* L. exposed promising anti-plasmodial and anti-microbial activity (Reddy *et al.*, 2007).

It seems that the phytochemical screening showed that the plant extracts contain some vital chemical constituents which may exert the observed anti-plasmodial activity. These chemical constituents could be acting separately or in combination with one another (synergic action). *P. betle* consisted most of these chemical constituents followed by *L. galanga*, *L. pumila* and *C. nucifera*, respectively (Table 5.1).

Table 5.1: The active chemical constituents found in the plants extract

	<i>C. nucifera</i>	<i>L. pumila</i>	<i>L. galanga</i>	<i>P. betle</i>
Alkaloids	-	-	-	+
Terpenoids	+	+	+	+
Flavonoids	-	+	+	+
Anthraquinones	-	-	-	+
Tannin	+	+	+	+
Steroids	+	+	+	+
Saponins	-	+	+	+
Glycosides	+	-	+	-

The antioxidant effect of the plant extracts can represent a further system that plays a role against the malaria parasite. *P. betle* showed the highest antioxidant capacity followed by *L. pumila* and *L. galanga*. *C. nucifera* exhibited a weak DPPH scavenging % activity. The potent antioxidant capacity exhibited by the *P. betle* leaves maybe as a result of the phenolic compounds in the leaves, such as chavicol, chavibetol, chavibetol acetate and eugenol (Amonkar *et al.*, 1986; Rimando *et al.*, 1986; Nagabhushan *et al.*, 1989).

Reactive oxygen species are produced due to stimulation of immune system by malaria infection resulting in haemoglobin degradation (Das & Nanada, 1999; Loria *et al.*, 1999). Rhizomes extract of *L. galanga*, leaves extract of *P. betle* showed nitric oxide (NO) production inhibitory activities in mouse and rat peritoneal macrophages (Morikawa *et al.*, 2005; Ganguly *et al.*, 2007), respectively. Nitric oxide (NO) is an effective mechanism to kill parasites, which is produced in macrophages in response to parasitic infection, which in turn activates the immune response against malaria (Daubener, 1999). Therefore, the inhibition of NO by the extract helps in preparing good surroundings to development of intracellular parasite. On the other hand, these inhibition cause a decrease in an essential amino acid, tryptophan, which undergo to degradation through indolamine deoxygenase. Hence, the parasite is starved leading to its death (Daubener, 1999; Anthony *et al.*, 2005). Recently, it was observed that there is a good correlation between potential DPPH radical scavenging activity and anti-malarial activity of Argan fruit extracts (El Babili *et al.*, 2010).

A further anti-inflammatory effect of plants extracts may be hepatoprotective during the early stage of infection. For example, the anti-inflammatory effect of *Nigella sativa* (Al-Ghamdi, 2001) reported to be hepatoprotective in mice (Turkdogan *et al.*, 2001). It revealed a protective role against chromosomal aberrations in *Schistosoma mansoni* infection (Mahmoud *et al.*, 2002). This mechanism may afford protection in malaria infection by reducing inflammation during the early hepatic stage of infection. Anti-inflammatory effects of *C.nucifera*, *L.pumila*, *L.galanga* and *P.betle* were reported by Intahphuak *et al.* (2010), Ikujuni *et al.* (2010), Chudiwal *et al.* (2010) and Ganguly *et al.* (2007), respectively.

Anti-pyretic effects of plants may be the reason attributed to the use of the plant to control fever. Virgin coconut oil showed an anti-pyretic effect in yeast-induced hyperthermia (Intahphuak *et al.*, 2010). 100 mg/kg of *L. pumila* leaves exhibited significant anti-pyretic activity in rat (Ikujuni *et al.*, 2010). The juice of the leaves of *P. betle* is used as anti-pyretic agent (Choudhury *et al.*, 2010).

Finally, our results are in agreement with others who showed that *C. nucifera* white flesh oil, *L. pumila* leaves, *L. galanga* rhizome and *P. betle* leaves extracts possess anti-microbial activities (Table 5.2). The anti-malarial activity presented in this research may be as a result of a separate or combined action of the mechanisms discussed above. However, the responsible principles are yet to be recognized, which require advanced studies to reveal the anti-plasmodial mechanisms of their actions.

Table 5.2: Anti-microbial activities of the plants extract existing in the literature

	Anti-fungal	Anti-parasitic
<i>C. nucifera</i>	(Jolaoso <i>et al.</i> , 2010)	
<i>L. pumila</i>		Ibrahim <i>et al.</i> (2003)
<i>L. galanga</i>	(Janssen & Scheffer, 1985)	Anti-trypanosomal (Nahoko, 2009), Anti-leishmanial (Amandeep <i>et al.</i> , 2010) and anti-helminthic (Raj, 1975; Nahoko, 2009; Amandeep <i>et al.</i> , 2010).
<i>P. betle</i>	(Trakranrungsie <i>et al.</i> , 2006)	Anti-leishmanial (Avijit <i>et al.</i> , 2008), Anti-filarial (Singh <i>et al.</i> , 2009)

Table 5.2 *continued*: Anti-microbial activities of the plants extract existing in the literature

	Anti-bacterial	Anti-viral
<i>C. nucifera</i>	(Jolaoso <i>et al.</i> , 2010) and (Oyi <i>et al.</i> , 2010).	
<i>L. pumila</i>	(Fasihuddin <i>et al.</i> , 1995)	
<i>L. galanga</i>	(Oonmetta-aree <i>et al.</i> , 2006)	(Ye & Li, 2006)
<i>P. betle</i>	(Nalina & Rahim, 2007)	

CHAPTER VI

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

In spite of the permanent attempts by the government and private sectors, malaria is still a serious disease threatening millions of people around the world. Currently, the emergence of *Plasmodium* species resistant to almost all available anti-malarial drugs has become a vital challenge in malaria control efforts.

The success in combating malaria absolutely depends on combination of various methods such as IRS of insecticides and biological methods. The elimination of malaria from a community however requires several other measures including improvement on the awareness of community regarding the disease, which can greatly increase the realization and sustainability of malaria elimination programmes. In addition, considering the growing anti-malarial drug resistance problem, there is also a call for arise novel anti-malarial agents to cope with the spread of resistant malaria parasites. Traditional medicinal plants had proved to be one of the most important sources of the new anti-malarial agents.

This research was carried out to study the community's awareness regarding malaria and establish a preliminary ethnobotanical database for the plants traditionally used to treat malaria among aboriginal and rural communities, and traditional healers in malaria endemic areas in Pahang, Malaysia. Acute oral toxicity, phytochemical

screening, anti-oxidant capacity and *in vivo* anti-malarial activity of *C. nucifera* white flesh, *L. pumila* leaves, *L. galanga* rhizome and *P. betle* leaves selected based on the ethnobotanical survey were investigated.

This study revealed that the aboriginal and rural communities have shown awareness of malaria as a disease, however knowledge about transmission and prevention of malaria is inadequate, in particular, among the aboriginal population and this could be a challenging obstacle to the elimination of malaria from Malaysia. This figure was lower than that reported in another countries earmarked for malaria elimination. Hence, malaria elimination programme should consider health education and community participation will enhance prevention and instil better knowledge on malaria transmission and prevention. Accordingly, the findings of this survey would assist the health authorities to create a concept of malaria KAP of the target community, to use efficient tools for health education and to improve and sustain good practices for malaria prevention.

The ethnobotanical data reported in this research provided an ethnopharmacological database for further anti-malarial phytochemical and pharmaceutical studies. The literature search results showed that most of the plants used by indigenous Malaysians have been tested against malaria in different parts of the world and have shown significant results. These studies support the Malaysian traditional medicinal plants to be based on pharmacological basis.

The methanol extracts of *C. nucifera*, *L. galanga* and *P. betle* demonstrated significant ($P < 0.05$) anti-plasmodial activity during the suppressive, curative and prophylactic assays of the anti-malarial evaluations. The *L. pumila* extract showed significant results only in the early malaria infection at the 400 mg/kg, the highest concentration of the extract. In addition, *L. pumila* and *P. betle* exhibited clear dose-dependent chemosuppressions activities in all the three models of the anti-malarial evaluations, which may indicate that these plants can exhibit higher and more significant anti-malarial effects by increasing the dose concentrations.

The phytochemical screening showed that the plants extract contains some vital chemical constituents which may exert the observed anti-malarial activity. These chemical constituents could be acting separately or in combination with one another (synergic action). Anti-oxidant, immunomodulatory, inflammatory and anti-pyretic effects may represent the mechanisms that contribute to their anti-malarial activity. The anti-plasmodial activity presented in this research may be as a result of a separate or collective action of these effects.

Finally, it is anticipated that the results arising from this work that had been published in national and international journals (*ISI/SCOPUS Cited Publication*) would stimulate further research on these plants in the future. This study also confirms that the Malaysian folkloric medicinal application of the plants has a pharmacological basis.

The following are the findings of this study:

1. Most of the respondents in the aboriginal and rural areas have knowledge of malaria as a disease. Overall, the rural participants showed significantly higher knowledge about malaria transmission than the Aborigines and this was found to be influenced by their level of education. Fever was the dominant symptom attributed to malaria followed by chills, rigors and headache. There was no significant difference between the aboriginal and rural regarding the attitude towards seriousness of the disease.
2. Promising results about treatment-seeking behaviour were reported; almost all the rural participants and two thirds of the aboriginal participants seek treatment at health centres within twenty-four hours of the symptoms onset. Most of the aboriginal people who use medicinal plants and witchcraft as a first line activity go to health centres for treatment within two-three days. Of the 223 interviewed respondents, 28% and 15.4% of the aboriginal and rural respondents respectively had tried self medication with anti-malarial plant remedies to cure malaria or fever, respectively.
3. Elimination of mosquito breeding areas and using of insecticides as preventive measures practices to combat malaria were significantly higher in the rural community. No significant difference between the rural and aboriginal communities regarding the use of mosquito bed nets.

4. A total of 19 species in 17 families used to treat malaria or fever were identified. Twelve plant species were identified and documented for the first time. The most frequent method of treatment was oral administration of decoctions or infusions (15 of 19 plants) and 4 plants were applied externally.
5. *C. nucifera* white flesh extract contained some phytochemical constituents and is toxicologically safe by oral administration. By the 200 and 400 mg/kg doses, the extract significantly reduced the parasitaemia in the all three *in vivo* assessment assays. However, the extract did not significantly increase the survival time of the infected mice.
6. *L. pumila* leave methanol extract is toxicologically safe by oral administration and demonstrated active anti-malarial activity during the suppressive, curative and prophylactic assays of the anti-malarial evaluations. During the early malaria infection the 400 mg/kg showed significant anti-malarial suppression. However, the extract did not significantly either reduce the parasitaemia or increase the survival time of the infected mice during the established and residual infections. The extract also showed a moderate DPPH scavenging % activity and contained some phytochemical constituents.
7. *L. galanga* rhizomes methanol extract contained some phytochemical constituents and is toxicologically safe by oral administration. The extract demonstrated significant anti-plasmodial effects by the 200 and 400 mg/kg concentrations in all the three models of the anti-malarial evaluations and increased the survival time of the infected. The extract revealed the presence of

some phytochemical constituents and showed a moderate DPPH scavenging % activity.

8. *P. betle* exhibited a potent anti-oxidant ability to scavenge the free radicals, toxicologically safe by oral administration and contained some phytochemical constituents. The extract demonstrated significant anti-malarial activity during the suppressive, curative and prophylactic assays of the anti-malarial evaluations.
9. Overall results of the *in vivo* anti-malarial activity of the methanol extract of *C.nucifera*, *L.pumila*, *L.galanga* and *P.betle* during early, established and residual malaria infections with evidence of increasing mean survival time of the infected mice showed that *P.betle* and *L.galanga* were the most effective followed by *C.nucifera* and *L.pumila*, respectively.
10. The results suggest that the Malaysian folkloric medicinal application of *P.betle*, *L.galanga*, *C.nucifera* and *L.pumila* has a pharmacological basis. The extract reduced parasitaemia but it cannot be considered a cure for malaria.

6.2 LIMITATIONS

In this present study, few limitations were encountered throughout the duration of the experiments. The ethnobotanical survey took longer than expected, partly due to the process of identification of plants which need specific international standard requirements.

6.3 RECOMMENDATIONS

The following are the recommendations based on findings of this project:

1. Using efficient tools for health education and to improve and sustain good practices for malaria prevention in order to eliminate or at least to contain this serious health problem.
2. Establishment of an integrated development plan for aboriginal people which include health, social and intellectual rehabilitation.
3. Further ethnobotanical surveys in the Malaysian states endemic for malaria to enrich the documentation of plants used against malaria, and to give priority to those plants to be investigated for their activities against the parasite.
4. Regulate the alternative medicine and Witchcraft.
5. Identification and isolation of the bioactive principles present in the plants extract, in particular, *C. nucifera*, *L. galanga* and *P. betle* could results in potential anti-malarial agents.
6. Further studies on the plants extract in combination with current anti-malarial drug such as chloroquine could reveal good results.
7. Further anti-malarial studies on the plants extract using *Plasmodium* species that affect human beings are recommended.

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