INVESTIGATION OF ANTIMALARIAL ACTIVITY OF CRUDE MALAYSIAN STINGLESS BEE PROPOLIS

BY

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LIST OF SYMBOLS AND ABBREVIATIONS

%	= percentage
°C	= degree Celsius
µg	= microgram
µg/ml	= microgram per millilitre
ACTs	= Artemisinin-based combination therapies
AIDS	= Acquired immunodeficiency disease syndrome
ARDS	= Acute Respiratory Distress Syndrome
B.elegens	= *Boerhavia elegens*
CCM	= complete culture medium
EEP	= ethanol extract propolis
FCR	= Folin-ciocalteau reagent
g	= gram
GAE	= gallic acid equivalence
GTS	= Global Technical Strategy
HEPES	= hydroxyethyl-piperazineethane-sulfonic acid buffer
HIV	= human immunodeficiency virus
IC_{50}	= 50% inhibition concentration
IL-1β	= interleukin – 1 alpha
IRS	= indoor residual spraying
ITN	= insecticide treated net
LC_{50}	= 50% lethal concentration
LPS	= lipopolysaccharide
mg	= milligram

mg/ml	= milligram per millilitre
ml	= millilitre
MOH	= The Ministry Of Health
NaHCO3	= Sodium bicarbonate
n,d	= not detected
nm	= nanometre
NO	= nitric oxide
NSPEM	= National Strategic Plan for Elimination of Malaria
P. falciparum	= *Plasmodium falciparum*
P. knowlesi	= *Plasmodium knowlesi*
P. malaria	= *Plasmodium malaria*
P. ovale	= *Plasmodium ovale*
P. vivax	= *Plasmodium vivax*
PEG	= polyethylene glycol
QBC	= quantitative buffy coat
QE	= Quercetin equivalence
RDT	= rapid diagnostic testing
RPMI 1640 medium	= Roswel Park Memorial Institute medium
SI	= selectivity index
S.surattense	= *Solanum surattense*
SP	= sulfadoxine-pyrimethamine
TFC	= total flavonoid content
TNF-α	= tumor necrosis factor alpha
TPC	= total phenolic content
w/v	= weight per volume

WEP	= water extract propolis
WHO	= World Health Organization
wt%	= weight percentage

ABSTRAK

Malaria adalah penyakit bawaan nyamuk yang disebabkan oleh spesies plasmodium dan membunuh hampir 429, 000 orang pada tahun 2015. Dengan kemunculan plasmodium kalis ubat, banyak kajian dijalankan untuk mencari ubat alternatif untuk merawat malaria. Tambahan pula, dengan kemunculan semula perubatan tradisional yang digunakan untuk merawat pelbagai penyakit pada masa kini, propolis sebagai salah satu ubat tradisional direkodkan mempunyai banyak nilai perubatan termasuk sebagai antimalaria. Propolis adalah campuran resin yang dihasilkan oleh lebah dari resin yang dikutip daripada tumbuh-tumbuhan di sekitarnya. Lebah menggunakan propolis untuk melindungi sarang daripada penceroboh luar dengan menampal propolis pada kawasan retak atau pembukaan sarang. Walau bagaimanapun kajian terhadap propolis kelulut Malaysia (*Trigona itama*) ke atas aktiviti antimalaria adalah terhad. Oleh itu, kajian ini bertujuan untuk menyiasat aktiviti antimalaria propolis kelulut Malaysia. Keputusan ujikaji *in vitro SYBR Green I-based florescence assay* terhadap Chloroquine sensitif Plasmodium falciparum (3D7) menunjukkan kedua-dua ekstrak air (WEP) dan ekstrak etanol (EEP) tidak memiliki aktiviti antimalaria. Hasil ini selari dengan tahap rendah jumlah kandungan fenolik (WEP; 16.4 ± 0.001 mg GAE / g dan EEP; 31.3 ± 0.004 mg GAE / g) dan jumlah kandungan flavonoid (WEP; 30 mg QE / g dan EEP, tiada) yang terdapat ekstrak. Disebabkan ini adalah satu kajian awal menyiasat tentang aktiviti antimalaria propolis Malaysia, oleh itu terdapat keperluan untuk melakukan kajian yang lain untuk mengesahkan keputusan tersebut.

Kata Kunci: Propolis, kelulut, antimalarial, kandungan fenolik dan flavonoid

ABSTRACT

Malaria is a mosquito-borne disease that is caused by plasmodium species and killed nearly 429, 000 people in 2015. With the emergence of plasmodium drug-resistance, many studies are conducted to find a new drug to treat malaria. Furthermore, with the resurgence of traditional medicine that is used to treat various disease nowadays, propolis as one of the traditional medicine recorded to possess many medicinal values including antimalarial property. Propolis is the resinous mixture produced by bees from the resin that collected from the surrounding plants. Bees use propolis to protect its hives from an external intruder by patching the propolis on the crack or the opening of the hive. However, there is a very limited study on the Malaysian stingless bee propolis (*Trigona itama*) on its antimalarial property. Therefore, this study was aimed to investigate the antimalarial activity of Malaysian stingless bee propolis. The results of *in vitro* SYBR Green I-based fluorescence assay against Chloroquine-sensitive *Plasmodium falciparum* (3D7) showed both water extract (WEP) and ethanol extract (EEP) do not possess antimalarial activity. The result is concordance with the very low level of total phenolic content (WEP; 16.4 ± 0.001 mg GAE/g and EEP; 31.3 ± 0.004 mg GAE/g) and total flavonoid content (WEP; 30 mg QE/g and EEP, n,d) isolated from the extracts. As this is a preliminary study on looking antimalarial property of Malaysian propolis, therefore there is a need to do another study to confirm the result.

Keywords: Propolis, stingless bees, antimalarial, total phenolic and flavonoid content.

CHAPTER 1

INTRODUCTION

1.1 Research background

Malaria is one of the deadliest mosquito-borne disease that killed many people around the world which recorded about 212 million new cases globally in 2015. Malaria has devastating disease manifestation especially in severe malaria, the patient may experience severe anaemia, kidney failure, Acute Respiratory Distress Syndrome (ARDS), and others.

Malaria is a detrimental disease, ranging from absent or very mild symptoms presented to more severe disease and could even cause death if not treated appropriately. The incubation period usually took about 10 – 15 days after the infected mosquito bite. The early symptoms demonstrate resemble flu-like symptoms which include high fever, headache, sweats, chills, and vomiting. The infected pregnant mother may pass the malaria to her fetus. As a result, the fetus may experience congenital malaria.

The establishment of Global Technical Strategy (GTS) for Malaria 2016-2030 aims to reduce malaria incidence and mortality rates globally by at least 90% compared to 2015 by the year 2030 (WHO, 2015). In addition, GTS also targets to eliminate malaria from at least 35 countries in which malaria was transmitted in 2015 and to prevent the re-establishment of malaria in all countries that are malaria free.

Malaria is caused by the parasite of the genus Plasmodium. There are five species of plasmodium that specifically infect human such as *P. falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi* (Ministry of Health Malaysia, 2013). The medium of

transportation of parasite is through the infected Anopheles mosquito that transfer the sporozoites into human body during the blood meal. The highest prevalent of malaria-related death that occurs in Africa continent is caused by *P. falciparum* while in the countries outside sub-Saharan Africa, the malaria-related death is dominated by *P. vivax* (WHO, 2016a).

The Africa Region that is under surveillance of World Health Organization, WHO accounted for most global cases of malaria (90%) followed by the South-East Asia Region (7%) and the Eastern Mediterranean Region (2%) (WHO, 2016a). The most susceptible group are children below five years old that accounted for about 303, 000 of death in 2015.

According to the Management Guidelines Of Malaria in Malaysia (2013), malaria has been reported in Malaysia even before 1900s in which at the beginning there were about 50, 500 cases. However the number of cases decrease significantly until 2006 where the mortality rate recorded was less than 0.001 per 1,000 population.

In Malaysia, although there are significantly decline in malaria cases, however the frequent and affordable travel especially to endemic countries with the addition of influx of workers into Malaysia from the endemic countries have kept the number of imported malaria cases on a constant high. This become a real threat of re-introducing malaria into areas which have successfully eliminated the disease (Ministry of Health Malaysia, 2013). The Ministry of Health, MOH has established the National Strategic Plan for Elimination of Malaria (NSPEM) 2011 – 2020 with the objective of eliminating locally acquired human-only malaria by 2020 (Ministry of Health Malaysia, 2013). Several strategies are outlined including conducting relevant research to find the treatment for malaria including the plasmodium-drug resistance strain.

Propolis is a substance that produced by the bees in order to protect their hives against an external intruder. Propolis has strongly adhesive properties that are formed from resinous substance collected, transformed and is used by bees as protection inside the honeycombs (Burdock, 1998). Besides that, the propolis is used to seal and patch the area of crack or opening on the hives and also help to smoothen out the internal wall of hives (Burdock, 1998). Thus, its help with the reconstruction of the bees hive and also protection against the external hazard such as weathering threat like wind and rain (Fokt, Pereira, Ferreira, Cunha, & Aguiar, 2010).

Generally the bee collects resin from some part of the plant such as plant exudate and mixed with the bee's enzyme to produce the propolis. Although the composition of propolis is highly affected by the geographical origin, generally, propolis is composed of resin, vegetables balsam, wax, essential and aromatic oils, pollen and other substances (Burdock, 1998). Propolis has various medical attribution due to its highly content of chemical compound such as polyphenol (flavonoids, phenolic acids and their esters), trepenoids and aminoacids (Pujirahayu, Ritonga, & Uslinawaty, 2014). Because of the propolis is rich in phenolic and flavonoid content, thus propolis has been widely used as a health supplement and cosmetic ingredient. Propolis has high medical values as its exhibit pharmacological properties such as antibacterial, antifungal, antiviral and many others properties (Fokt et al., 2010).

As the propolis recorded a very excellent medical properties, it was also reported that propolis had a prophylactic efficiency against malaria in endemic areas in Brazil (Gama, 1993). Therefore, throughout this study we are able to find out the efficacy of Malaysian stingless bee propolis as antimalarial agent and to evaluate the potential of Malaysian propolis as a new drug to malaria.

1.2 Research objective

General objectives:

- To investigate the Malaysian stingless bee propolis and its properties as antimalarial agent.

Specific objectives:

- To compare the yield between water extraction Malaysian stingless bee propolis (WEP) and ethanol extraction Malaysia stingless bee propolis (EEP).
- To quantitate the phenolic and flavonoid contents between WEP and EEP.
- To evaluate the preliminary toxicity of Malaysian stingless bee propolis extracts.
- To investigate the effect of Malaysian stingless bee propolis extracts on *Plasmodium falciparum* strain 3D7 culture.

1.3 The scope of study

This study was conducted to investigate the Malaysian stingless bee propolis and its properties as antimalarial agent. The propolis was extracted using water and 70% ethanol through maceration method with frequent agitation for few days. The percentage yield of each extract was determined and compared. The total phenolic and flavonoid content of propolis extracts that responsible for the biological activity were determined and compared between the extracts. In addition, the preliminary study on the toxicity for both propolis extracts was determined using simple method of brine shrimp toxicity assay. From the test, the LC_{50} of water extraction propolis and ethanol extraction propolis were determined. Moreover, the effect of both propolis extract on *Plasmodium falciparum* strain 3D7 was evaluated using in vitro SYBR Green I- based fluorescence assay, a spectrophotometric method. The effect of inhibition of extracts against *P. falciparum*

strain 3D7 was plotted and the median inhibitory concentration, IC_{50} of extracts was determined from the graph. Finally, the selectivity index (SI) was calculated to evaluate the suitability of extracts as antimalarial agent.

1.4 The significant of study

This study may become the pioneer of discovering the properties and therapeutic effect of Malaysian stingless bee propolis since there is still lack of studies on the Malaysian propolis. In addition, the Malaysia National Budget 2016 has gladly announced about the rearing the stingless bee production as a first priority under Modernizing Agriculture Sector because nowadays stingless bees become the key-potential in Malaysia economic growth (Malaysia National Budget, 2016). This includes the production of propolis as one of the bee's product and moreover with the advantages of stingless bee as it able to pollinate the small flower and produce more propolis. Hence, with the provided budget, many opportunities are served to discover more about the properties of propolis. This study may discover the potential of Malaysia propolis as a new medicine for antimalarial as a replacement for Chloroquine and as the preparation to combat the emergence of plasmodium drug-resistance.

CHAPTER 2

LITERATURE REVIEW

2.1 Malaria epidemiology

In 2015, *Plasmodium falciparum* responsible about 99% of total death whereas the *Plasmodium vivax* responsible about 3100 of death. Therefore, *P.falciparum* is the most dangerous and recorded as the highest prevalence of plasmodium species among all others plasmodium species (WHO, 2015, 2016a)

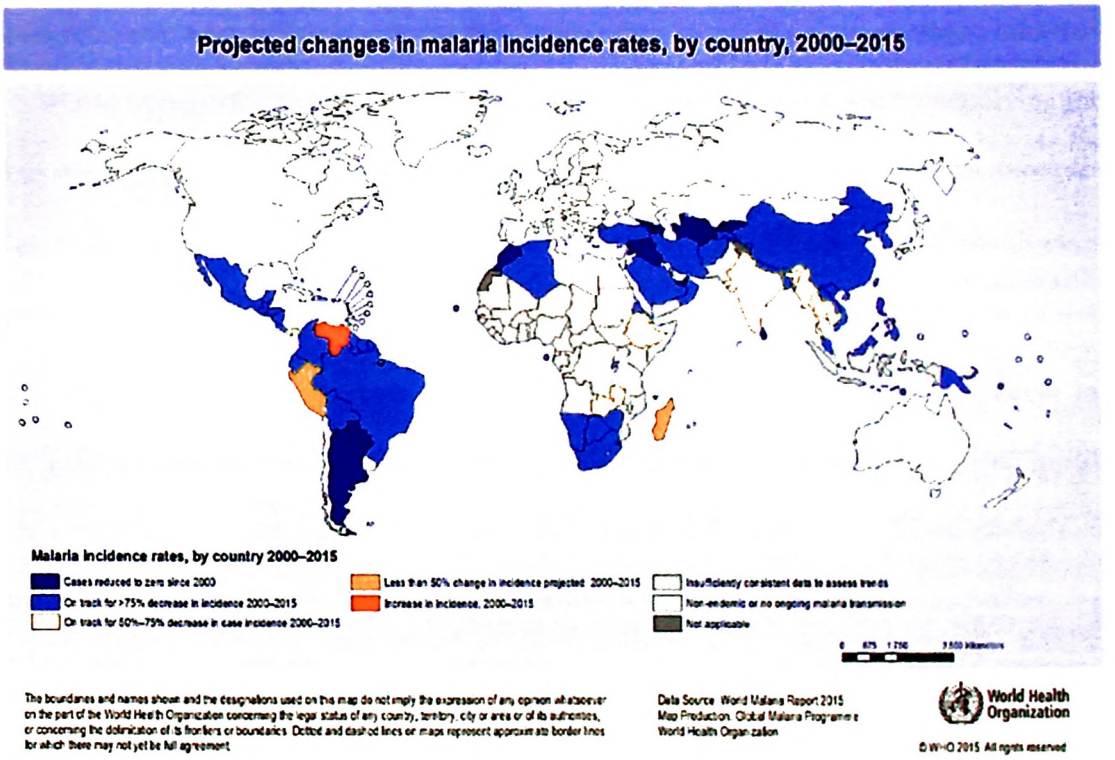


Figure 2.1: Projected changes in malaria incidence rates, by country, 2000 – 2015.

Adapted from World Health Organization (2015)

Based on Figure 2.1, shows that the African Region is the highest prevalence of malaria that represent about 90% of malaria cases and 92% of malaria death. It is estimated that about 3.2 billion of people globally from 95 countries and territories are at risk of getting malaria infection. In addition, it is also reported that about 1.2 billion of people are at high risk for malaria which is about more than one person per 1000 of the population having a chance of getting malaria in a year. Besides that, the Figure 2.1 shows that, about 91 countries and areas had the ongoing malaria transmission (WHO | Malaria, 2017).

Although one of the highest malaria burdens is carried by sub-Saharan Africa, in 2015 the number of infected people is dropping from 131 million in 2010 to 114 million. The mortality rate of malaria is estimated about 429, 000 of death globally in 2015. Nowadays, the Africa region remains the highest mortality rates among other countries. The World Malaria Report (2016) revealed that the malaria intervention by supplying the medical care in the Sub-Saharan region has improved tremendously. This provides opportunity for them to gain the medical access and early treatment for malaria. As a result, the mortality rate and new cases of malaria drop significantly in 2016.

Despite outstanding progress, across the Africa Region particularly, the people are still lack of access to the health care and with the funding shortage and unstable health care systems, they are unable to obtain the appropriate treatment. Since malaria is mosquito-borne disease, the malaria transmission is remaining to spread.

Another factor that possessed the real threat to malaria is the emergence of insecticide and the drug resistance. Out of 70 countries that WHO monitored for indoor residual spraying, IRS and insecticide treated net, ITN 63 of its have reported that mosquito resistance to at least one class of insecticide. Meanwhile, the parasite resistance toward one of the best antimalarial available, Artemisinin has been reported in five countries of the Greater Mekong Subregion (WHO, 2016)

2.2 Symptoms of malaria

The initial symptom of malaria is fever before it gradually becomes worst and can cause death if not treated early. There are variable of clinical features of illness such as fever, chills, headaches, muscular aching and weakness, vomiting, cough, diarrhoea and abdominal pain (WHO | Malaria, 2011). As the illness progress, the organ starts to dysfunction. The patient may experience acute renal failure, pulmonary oedema, generalised convulsion, or circulatory collapse, then followed by coma and death (Ministry of Health Malaysia, 2013). The incubation period is about 7 days or longer. After 7 days, the onset of acute febrile illness begin and the treatment should be given as early as 24 hours after the onset, if not, the delayed treatment could be fatal.

2.3 High-risk group of malaria

According to WHO (2015), high risk group malaria includes pregnant women, infants, children under five years old, HIV/AIDS patient, non-immune migrant, mobile population and traveller. These groups of people are susceptible to malaria due to some circumstances such as the migrant and refugees that lack of immunity to malaria, limited access to preventive, diagnostic testing and treatment aids. The children under five years old including infants are vulnerable to infection especially in the endemic area due to low immunity which killed about 303 000 children. However, the mortality rates significantly decline by 35% between 2010 and 2015. Nevertheless, this was not enough as it responsible for the major killer of children under five that claimed to kill about 1 child every 2 minutes. In HIV or AIDS patient, malaria usually exist as co-infection that may also worsen the patient condition. Infected pregnant women may experience maternal and fetal anaemia, stillbirth, spontaneous, abortion, low birth weight and neonatal death. In 2012, WHO recommended the intermittent preventive treatment of pregnant women for

malaria with Sulfadoxine-Pyrimethamine (SP) to be implanted in high risk countries such as Africa (WHO, 2005). The *P. falciparum* parasites that are able to carry quintuple mutation is linked to SP resistance which therefore causing therapeutic failure of SP.

2.4 Diagnostics

The primary diagnosis of malaria is based on the symptoms demonstrated by the patient. However some symptoms overlap with another disease which make it harder to decide based on physical examination solely (Tangpukdee, Duangdee, Wilairatana, & Krudsood, 2009). Therefore, the blood test is conducted. Because of the plasmodium species are the blood parasite, these parasites are determined microscopically. The laboratory diagnosis consists of the thick and thin peripheral blood smear or the concentration techniques such as quantitative buffy coat (QBC). The blood smear is stained with 10% Giemsa stain, the thick smear is used to screen the presence of malaria parasite whereas thin blood smear is used to define the species and to calculate the parasitemia. Each species of plasmodium has a different characteristic in every stage of parasites. Meanwhile, the QBC method is used to enhance the detection of parasite microscopically.

Besides that, the Rapid diagnostic testing (RDT) was introduced for more rapid detection of parasites. It is widely over the past decade to differentiate between malaria fever and non-malaria fever before any treatment is conducted. In 2015, WHO reports stated that about 51% of children with fever was able to receive malaria diagnostic test. This is a good sign that enable early treatment can be given which can reduce the mortality rates.

2.5 Treatment

Chloroquine and Artemisinin are the drugs used to treat malaria. In the population that are not Chloroquine or Artemision resistance malaria, these drugs are still used for the treatment. The antimalarial drug resistance occur when parasite gain ability to survive despite the administration or the absorption of drug that are given in doses at equal or higher than usually recommended but within the tolerance of subject (Bloland, 2001). The mutation cause the drug resistance of parasite. The drug resistance may require single mutation from the parasite or sometime could be multiple mutation that needed to become the drug resistance parasite. It is detrimental to have antimalarial drug resistance parasite because it could lead to the treatment failure since the drug available has no effect on the parasites.

Artemisinin-based combination therapies (ACTs) has been used as a primary treatment against *P. falciparum*. It is highly effective for the treatment of complicated and uncomplicated malaria. The Artemisinin are used in combination with another drug as to provide an adequate cure and to delay any drug resistance. ACTs is a drugs therapy that used against drug resistance *P. falciparum*, by combining more than one drug that comes from different class with the fast-acting Artemisinin-based compounds. The companion drug are such as lumefantrine, mefloquine, sulfadoxine/pyrimethamine and others. By combining two types of drugs, it will form a co-formulated anti-malarial drug into one tablet. However, the limitation of ACTs is the availability and affordability of companion drugs. ACTs have been widely used in sub-Saharan Africa and in areas with multi-drug resistance in Southeast Asia. Therefore to maintain the effectiveness of ACTs, it is critical to ensure the dose, delivery, access and cost are taken appropriately. (Artemisinin-based Combination Therapy, 2017)

2.6 Malaria interventions

The primary intervention to encounter malaria from spreading is by vector control. There are two ways of vector control that are effectively used which are insecticide-treated mosquito nets (ITNs) and indoor residual spraying (IRS). ITNs are being implemented particularly in Sub Saharan Region for over five years. As a result, in 2015 it is estimated about 53% of risk population slept under treated net. Whereas IRS is largely used by national malaria programmes in the targeted areas which able to protect 106 million of population at risk (World Malaria Report, 2016)

2.7 Life cycle of *Plasmodium falciparum*

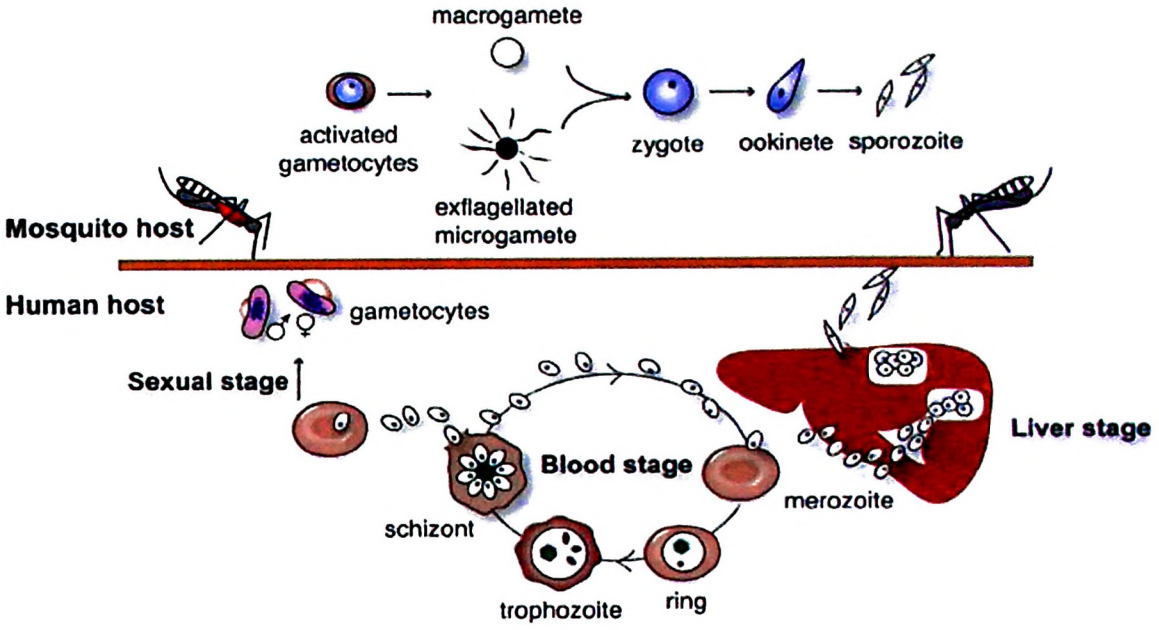


Figure 2.2: The illustration showing the life cycle of *Plasmodium falciparum,* adapted from Schmidt, Kennedy, & Tham (2015)

The life cycle of *P.falciparum* comprise of two stages; the asexual stage that occurs in mosquito and the sexual stage that occur inside the human body. The infection begins as the infected female Anopheles mosquito release the sporozoites into human body during a blood meal. Later the sporozoites will undergo exoerythrocytic stage that takes place in the liver. The parasites multiply within the liver cells and mature into schizonts which in turn begin to rupture the cells and release the merozoites that take about 5.4 days.

Later, the merozoites start to infect the human red blood cells. Another cycles production of merozoites occur in the stage known as endo-erythrocytic stage that take places inside the red blood cell when the ring stage of trophozoites mature into schizonts

and form merozoites. The red blood cell ruptures and releases merozoites within 43-48 hours. Some ring stage plasmodium differentiates into gametocyte; male gametocyte (microgametocyte) and female (macrogametocyte) during the sexual stage. These gametocytes will be taken by Anopheles mosquito during the blood meal to complete the plasmodium life cycle.

Most of malarial disease is manifested during the blood stage of parasites. The sexual stage occurs inside the mosquito stomach when the microgametocyte penetrated into the macrogametocyte and form zygotes (Eichner, 2010). The zygotes become motile and elongated which enable it to invade into midgut wall of the mosquito and become oocysts. The oocysts grow in size and then rupture to release the sporozoites. The sporozoites propagate into the mosquito's salivary gland and inoculate into human body during the blood meal. Thus the plasmodium cycle is recirculated again.

There are few natural remedies besides propolis that has been studied for the antimalarial properties. For example the ethanolic extract of *B.elegens* and *S.surattense* that reported by to have anti-plasmodial activity against *P.falciparum* of Chloroquine resistance strain (K1) and Chloroquine-susceptible strain (CY27) (Ali, Sedigheh, Soroush, Nastaran, & Navid Dinparas, 2006). Furthermore, the scientific study on *V.amigdalina* proved that *V.amigdalina* possess moderate antimalarial activity (Sha 'a, Oguche, Watila, & Ikpa, 2011).

2.8 Propolis

The bees have been exists about more than 125 million years ago and undergo successful evolutionary that enable them to become perennial species that can exploit virtually all habitats on earth (Bankova, 2005). The bees are able to produce several

products such as honey, beeswax, venom, propolis, pollen and royal jelly. The propolis has been become the subject of intense pharmacological and chemical studies for the last 30 years due to its pharmacological attributions.

The propolis which also known as the bee glue is a wax-like resin produced by honeybees from substances collected from plants, which are mixed with beeswax and other compound of bee metabolism (Fokt et al., 2010). The propolis word is derived from the Greek. *Pro* stands for 'in front of' while *polis* is for 'community' or 'city'. It describe the function of propolis as the natural defence for protecting the bee hives. The bee used the propolis for sealing and patching the cracks and the holes of the bee hives as protection against an external intruder. The waxy features of propolis and it adhesiveness is suitable for the sealing, patching, repairing and construction of the bee hives (Fokt et al., 2010).

The complex composition of propolis is made up majorly by resin, vegetable balsam and a few of pollen, organic and mineral compounds. The history of propolis exists before by the discovery of honey. Honey is the main product of the bees along with the bee bread and royal jelly. Honey is well known for its high flavonoid and phenolic content and its medicinal values.

The propolis is the ancient natural medicines and has a long history of application (Castaldo & Capasso, 2002). For example the Egyptian used propolis to embalm its cadavers due to its anti-putrefactive property while the Middle Ages and Arabic physicians used propolis for antiseptic and mouth disinfectant and also as a product to heal the wound. Due to its excellence anti-bacterial activity, the propolis has been made as the official drug during the 17^{th} century by London pharmacopoeias. Later it gained more popularity between 17^{th} to 20^{th} centuries in European country (Castaldo & Capasso, 2002).

Propolis is sold in America in health store in the form of a capsule and some are as dental floss, toothpaste and mouth wash because it helps to prevent caries, treat gingivitis and stomatitis. During Second Global War, propolis was used by Soviet Clinic to treat tuberculosis infection because it was able to improve the lung problem and returning the appetites (Fokt et al., 2010).

Nowadays, with the advancement of research and technologies the propolis has been involving quite extensive studies for its biological properties. The propolis has been widely available as a food supplement for several of health benefits. It is also used to treat the cold syndrome such as upper-respiratory tract infection, common cold and flu-like infection (Fokt et al., 2010).

The propolis can improve the immune system, for example, the Brazilian red propolis is able to increase the total leucocytes, total protein and globulin concentration and also decrease the triglyceride, glutamate oxaloacetate, transaminase and glutamate pyruvate transaminase (D.S., M.S., & S.O., 2015). Besides that, propolis is also used in wound healing, treatment of burns, acnes, herpes simplex and genitalis and neurodermatitis.

The application of propolis is also extended into cosmetic, health food and beverages (Campos et al., 2015). It is commercially available in several forms such as capsule, extract, mouthwash solution, throat lozenges, powder and purified product in which the wax was removed initially.

2.9 Propolis characteristic, origin and composition

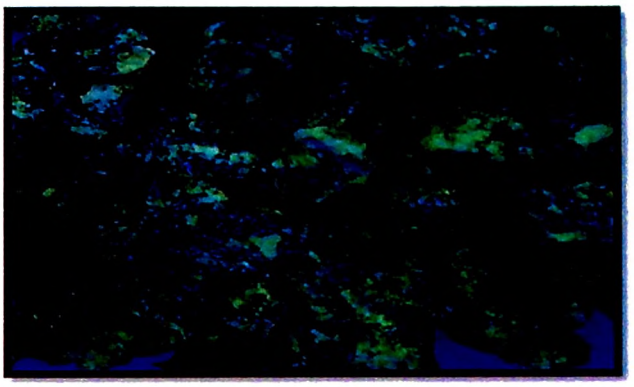


Figure 2.3: Malaysian stingless bee propolis

The raw Malaysian propolis is like chips in a small size that are soft, sticky, gummy and pliable when it is at the room temperature or in warmer condition. The colour is dark brown with yellowish substances spotting on the surface of propolis. In the frozen condition, the propolis turns into hard and brittle substances. Meanwhile, the Indian propolis becomes increasingly sticky and gummy when above 45°C (Wagh, 2013). The propolis could become liquid when it achieve its melting point at 60°C to 70°C or some other propolis could have as high as 100°C as it melting point. The Malaysian propolis produce by *Trigona itama* smell sour and their honey also have a pleasant sour taste.

The composition of propolis is highly variable and depend on it geographical origin. This is due to the resin collected by bees from different plants. Its chemical composition depends on the specificity of the local flora at the site of collection. Hence, the geographical and climate characteristic of the site greatly influence the chemical composition of propolis (Bankova, 2005). Therefore producing a large diversity of propolis composition, especially propolis originating from tropical regions. The tropical

regions serve many types of plant that could become the source of resin to the bees whereas in the temperate zone the propolis mostly originates from the resin of *Populus* species.

Besides that, the variability of the chemical composition depends on the site of collection, because of different ecosystems provide different plant exudate and secretion that could serve as a source of propolis (Bankova, Castro, & Marcucci, 2000). In addition, the knowledge about the plant source could be useful in the chemical standardisation of propolis. The study done by Kosalec, Bakmaz, & Pepeljnjak (2003) shows that the propolis sample collected various parts of Croatia do not differ markedly in contents of chrysin, pinocembrin naringenin and galangin but differ in the concentration of caffeic acid (Kosalec et al., 2003). Therefore to achieve standardisation of the propolis we must investigate their geographical origin and plant source that associate with their chemical composition.

Moreover, the chemical composition of propolis that often reflects the the biological activity does not only depends on the geographical areas but also depends on the method of extraction. For example, Blonska et al. (2004) show that ethanol extract propolis, EEP has anti-inflammatory effect in J774A.1 macrophages, while Han et al (2002) reported that the water extraction propolis, WEP showed the opposite effect on murine macrophage cell line RAW 264.7. The WEP of Korean propolis able to stimulate the macrophage to release TNF-α, IL-1β, NO production, cell morphological changes and surface molecule expression which may relate to LPS addition (Han et al., 2002).

The stingless bee propolis (*Trigona itama*) had many advantages compare to other types of bee propolis. It is able to collect flower nectar from the deepest area of the flowers. Thus, it contains very high vitamin and mineral. Moreover, the stingless bee produces sour taste honey and a lot of propolis (Kelulut Honey / Trigona Honey | Tualang Honey, n.d.)

2.9.1 Antimalarial

The study of a methanolic extract of Nigeria propolis against *Plasmodium berghei* in infected mice showed a significant decrease in the level of parasitemia starting from day 2 through day 5 post-treatment using concentration of 600mg/kg of body weight (Olayemi, 2014). It shows that it possesses significant anti-plasmodial activity in comparison with Chloroquine diphosphate that achieved parasite-clearance on day 5 while the methanolic propolis extract attained the parasite-clearance one or two days later. Therefore, it suggest that the propolis extract probably had anti-plasmodial activity with a different mode of action than Chloroquine diphosphate. The Chloroquine diphosphate is the blood schizonticide while propolis is a tissue schizonticide. Besides that, the Cuban propolis of red and yellow types shows inhibition against *P. falciparum* with IC_{50} of 0.2 µg/ml (Monzote et al., 2012).

Recently, the natural product has become the source of drug development because some natural product contains phenolic and flavonoid content that responsible for the biological activity. Therefore, the natural product that includes the propolis and other types of plants have been used to investigate for their anti-plasmodial activity. For example, the starfruit (*Averrhoa bilimbi L*) tropical plant contain flavonoid content of Luteolin that responsible for antimalarial activities against 3D7 and 7G8 (Prasetya, Mulia, Tantular, & Mughni, 2012). Luteolin is able to inhibit Fab I enzyme Plasmodium that related to the final reduction step of cycle extension fat Plasmodium acid chain. Within 24 hours' incubation, the IC_{50} of starfruits extract leaves is 2.805 µg/ml. Thus it shows that it is able to inhibit parasite growth at low concentration and become a potential antimalarial agent.

2.9.2 Antioxidant

Propolis also exhibits antioxidant property as mention by many researchers. The antioxidant property are depends on the geographical origin of propolis and the quantities of antioxidant compound (Kumazawa, Hamasaka, & Nakayama, 2004). The propolis collected from Anhui, China shows radical-scavenging and antioxidant activities (Yang et al., 2011). In addition, it also stated that the ethyl acetate fraction of propolis exhibit significant antioxidant and free-radical scavenging capacities.

The propolis collected from different part of Argentina possess different antioxidant activity and it is correlated with the chemical composition of propolis and the plant ecology (Moreno, Isla, Sampietro, & Vattuone, 2000). Moreover, the study by Sun, Wu, Wang, & Zhang (2015) shows that the ethanol/water solvents of propolis shows significantly high phenolic composition and antioxidant properties and suggest that 75 wt.% ethanol/water solvent might be suitable solvent to extract the phenolic content in the Propolis.

2.9.3 Antimicrobial

There is a strong association between antimicrobial activity and chemical composition. Although the chemical constitute of propolis is influenced by the geographical factor, however, the overall propolis samples from Cuban possess antimicrobial properties (Monzote et al., 2012). The Cuban propolis are divided into three; the brown propolis, red propolis and yellow propolis. It has been reported that the Cuban propolis extracts inhibit the growth of *Staphylococcus aureus* and *Trichophytonnrubrum* at low concentraction µg/ml however it is not active against *Escherichia Coli* and *Candida albicans* (Monzote et al., 2012).

Another example is the Turkish poplar type propolis, which shows a significant inhibitory activity against mutants streptococci (Arslan, Silici, Perçin, Koç, & Özgür, 2012). The reason is because the poplar type propolis contains flavanones, flavones, phenolic acids and their esters that exhibit antibacterial properties (Bankova, 2005).

2.9.4 Anti-inflammatory

The ethanol extract propolis, EEP show anti-inflammatory effect *in vitro* and *in vivo* research. The macrophages that involve in the inflammatory activity was suppressed by the flavones derivatives of EEP. Blonska et al. (2004) demonstrated that EEP is an inhibitor of LPS-induced IL-1β and nitric oxide production in J774A.1 macrophage. EEP contains caffeic acid phenethyl ester (CAPE) and galangin as the major component of propolis was reported to have anti-inflammatory in the development of inflammation induced by carrageenin in the rat paw (Borrelli et al., 2002).

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Materials

3.1.1 Disposable items

All the disposable items have been listed in the appendix A, refer to page 55.

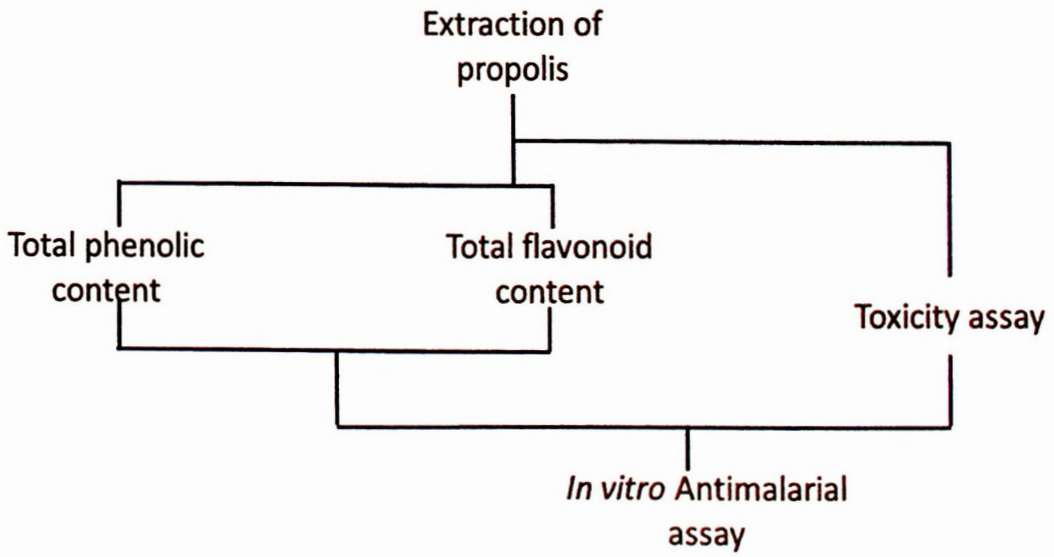
3.1.2 Laboratory Equipment

All the laboratory equipment have been listed in the appendix A, refer to page 56.

3.1.3 Chemical and reagent

All the chemical and reagent have been listed in the appendix A, refer to page 52.

3.2 Methodology



3.2.1 Extraction of Malaysian stingless bee propolis

The raw propolis was obtained from Min House Camp, Kota Bharu, Kelantan which collected from the colony of stingless bee *Trigona itama* and was stored at -22°C before processed. The extraction was done using maceration method as described by Usman & Mohamed (2015) with some modification. Hundred (100) g of raw propolis was washed using tap water to remove the impurities and the dead bees. The liquid nitrogen was poured into the washed propolis until the propolis became hard and then was crushed using s blender. Thirty (30) g of propolis was weighed and put into two glass bottle respectively; one was mixed with 100ml of distilled water (labelled as water extract propolis, WEP), and another one was mixed with 100ml of 70% ethanol (labelled as ethanol extract propolis, EEP). The mixtures were shaken vigorously for 30 minutes. WEP and EEP were continuously shaken on a rotatory shaker (Forma Orbital Shaker, Thermo Electron Corporation) for 6 hours for 7 days at room temperature. The mixtures were filtered using filter paper Whatman size 1 and the filtrates were kept in 4°C overnight. The filtrates were filter again using filter paper Whatman size 4 in 4°C temperature. Later, EEP was allowed to evaporate for 2 days inside the fume hood and then was kept in -22°C prior to freeze dry. Both extracts were freeze dried at -50°C for three days. The freeze-dried extracts were kept at 4°C prior use.

3.2.2 Determination of total phenolic content

The total phenolic content of propolis extracts (WEP and EEP) were determined using Folin-Ciocalteau method as described by Kubiliene et al (2015) with some modification using a 96-well plate. Ten (10) µl of each extract was mixed with 150µl distilled water. Then 10µl of Folin-Ciocalteau reagent was added into the mixture. After 5 minutes, 30µl of 10% sodium carbonate was added. The microplate was incubated for 2

hours in the dark room at room temperature. The absorbance was determined at 760 nm using microplate reader SpectralMax M5, Molecular Device with SoftMax Pro 5 Software. The total phenolic content of each extract was estimated using a calibration curve of Gallic acid with a concentration of Gallic acid ranged from 10µg/ml to 100µg/ml. The total phenolic content of each extract was done in triplicate with multiple concentration ranging from 1000µg/ml to 100µg/ml. The total phenolic content was expressed in mg of Gallic acid equivalent (GAE) per g of extract.

3.2.3 Determination of total flavonoid content

The total flavonoid content of propolis extracts (WEP and EEP) was determined using Aluminium Chloride, $AlCl_3$ method as described by Wang et al. (2016) by using a 96-well plate. Hundred (100) µl of each extract in distilled water was filled into each well and mixed with 100µl of 2% $AlCl_3$. The mixture was incubated for 15 minutes in the dark room at room temperature. The absorbance of the sample was determined at 435nm using microplate reader SpectralMax M5, Molecular Device with SoftMax Pro 5 Software. The total flavonoid content was determined using calibration curve of Quercetin with a ranged of concentration of 10µg/ml to 100µg/ml. Each extract was done in triplicate with multiple concentration ranging from 100µg/ml to 1000µg/ml. The total phenolic content was expressed in mg of Quercetin equivalent (QE) per g of extract.

3.2.4 Determination of toxicity of extracts

The toxicity of extract was determined using brine shrimps toxicity test as described by Moshi et al. (2010) with slight modification. The brine shrimps were hatched prior to the test. Fifteen (15) g of brine shrimp was weighed and hatched into 150ml of

3.8% sea water. The shrimps were allowed to hatch for 48 hours under 24 hours of light exposure at room temperature. The extracts were prepared with different concentration ranging from 200µg/ml to 2000µg/ml using 3.8% salt water. Five (5) ml of each concentration was filled into the petri dish. Ten (10) brine shrimp larvae were placed into each of the triplicate vials. Five (5) ml of sea water in the petri dish with 10 brine shrimps was served as positive control while 5ml of distilled water in the petri dish serve as negative control. The petri dishes were incubated for 24 hours lighted room at room temperature. After 24 hours incubated, the nauplii were counted. The mean percentage of mortality was determined and plotted against the concentration of extracts. The lethal concentration, LC_{50} of each extract was determined from the graph.

3.2.5 *P.falciparum* (3D7) culture

Plasmodium falciparum 3D7 strain (Chloroquine sensitive strain) was cultured in 25cm tissue culture flask with blue media (RPMI 1640 supplemented with 10% human serum, HEPES and NaHCO3). Parasite culture was maintained at 2% - 3% parasitemia with 2% haematocrit and was incubated at 37°C in the gas mixture of 1% oxygen, 1% carbon dioxide and 98% nitrogen gas until 5% parasitemia reached and then further diluted for continuous culture. The antimalarial assay was performed for 0.5% parasitemia and 1% haematocrit and incubated for 48 hours in the candle jar.

3.2.6 Determination of antimalarial properties

Three thousands (3000) µg/ml of stock solution was prepared for each extracts (WEP and EEP) using complete culture media (CCM). The extracts then diluted with two fold serial dilution until concentration 23.44µg/ml was achieved. Meanwhile, the