TOXICITY AND SUBLETHAL EFFECTS OF *Artemisia annua* Linnaeus ON *Aedes aegypti* (Linnaeus), *Aedes albopictus* (Skuse), *Anopheles sinensis* Wiedemann AND *Culex quinquefasciatus* Say

by

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DDT	dichlorodiphenyltrichloroethane
MS	Murashige and Skoog
ml	mililitre
rpm	revolutions per minute
WHO	World Health Organizations
CDC	Centers for Disease Control and Prevention
LC	lethal concentration
ppm	part per million
SPSS	Statistical Package of Social Sciences
ANOVA	One-way analysis of variance
SE	standard error
OAI	oviposition activity index

TOKSISITI DAN KESAN SUBMAUT *Artemisia annua* Linnaues TERHADAP *Aedes aegypti* (Linnaeus), *Aedes albopictus* (Skuse), *Anopheles sinensis* Wiedemann DAN *Culex quinquefasciatus* Say

ABSTRAK

Artemisia annua adalah terkenal dengan ciri-ciri anti-malarianya tetapi kurang kajian dijalankan mengenai kesan tumbuhan ini terhadap nyamuk. Kajian ini bertumpu ke atas aktiviti larvisid dan kesan submaut ekstrak mentah *A. annua* terhadap *Aedes aegypti*, *Aedes albopictus*, *Anopheles sinensis* dan *Culex quinquefasciatus*, dan kesan ke atas aktiviti bertelur dan ovisid pada nyamuk tersebut. Sel kering *A. annua* daripada kultur terapung sel telah diekstrak dengan menggunakan hexane. *Artemisia annua* menunjukkan kesan aktiviti larvisid yang sederhana terhadap nyamuk yang diuji. *Anopheles sinensis*, *Ae. albopictus* dan *Ae. aegypti* dengan LC_{50} bernilai 244.55 ppm, 254.96 ppm dan 276.14 ppm, masing-masing mencatatkan mortaliti larva yang lebih tinggi secara signifikan berbanding dengan *Cx. quinquefasciatus* dengan LC_{50} bernilai 374.99 ppm, 24 jam selepas rawatan. Nilai LC_{50} dan LC_{95} berkurangan 48 jam selepas rawatan. Fekunditi *Aedes albopictus* dan *An. sinensis* adalah berlainan secara signifikan ($p < 0.05$) manakala penetasan telur bagi *Ae. aegypti* dan *An. sinensis* adalah berkurangan secara signifikan selepas dirawat dengan dos submaut ekstrak *A. annua* ($p < 0.05$). *Aedes aegypti* betina menunjukkan saiz yang lebih kecil secara signifikan berbanding dengan kawalan selepas dirawat dengan dos submaut ekstrak *A. annua* ($p < 0.05$). *Anopheles sinensis* betina menunjukkan jangka hayat yang lebih pendek secara signifikan dalam keadaan makmal selepas dirawat dengan dos submaut ekstrak *A. annua* ($p < 0.05$). Hanya *Cx. quinquefasciatus* tidak menunjukkan sebarang kelainan dalam ujian submaut. Semua species nyamuk tidak menunjukkan sebarang kelainan dalam generasi F1. Keputusan menunjukkan aktiviti bertelur berkolerasi dengan kepekatan ekstrak *A. annua*. Ekstrak menunjukkan kesan penghalang yang kuat ke atas aktiviti bertelur nyamuk di mana peratusan penolakkan berkesan melebihi 85 % pada 500 ppm bagi semua species nyamuk. Nilai index activiti oviposisi adalah - 0.94, - 0.84, - 0.95 dan - 0.78 bagi *Ae. aegypti*, *Ae. albopictus*, *An. sinensis* dan *Cx. quinquefasciatus*, masing-masing. Dalam kajian penetasan telur, empat species nyamuk menunjukkan peratusan penetasan telur yang lebih rendah secara signifikan berbanding dengan kawalan apabila dirawat dengan 500 ppm ekstrak *A. annua* ($p < 0.05$). Peratusan pengurangan penetasan telur adalah 45.5 %, 47.5 %, 54.7 % dan 17.6 % bagi *Ae. aegypti*, *Ae. albopictus*, *An. sinensis* dan *Cx. quinquefasciatus*, masing-masing.

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ABSTRACT

Artemisia annua is well known for its strong antimalarial properties; however, limited studies were conducted on the effects of the plant against mosquitoes. This study focuses on the larvicidal activity and sublethal effect of a crude extract of *A. annua* against *Aedes aegypti*, *Aedes albopictus*, *Anopheles sinensis* and *Culex quinquefasciatus*, and its effect on the oviposition and the ovicidal activities of the mosquitoes. Dried cells of *A. annua* from cell suspension culture were extracted using hexane. *Artemisia annua* extract showed moderate larvicidal effects against the tested mosquitoes. *Anopheles sinensis*, *Ae. albopictus* and *Ae. aegypti* with LC_{50} values of 244.55 ppm, 254.96 ppm and 276.14 ppm, respectively recorded significantly higher larval mortality compared with *Cx. quinquefasciatus* with LC_{50} value of 374.99 ppm at 24 hours post-treatment. The LC_{50} and LC_{95} values decreased at 48 hours post-treatment. The fecundity of *Ae. albopictus* and *An. sinensis* was significantly reduced in the parental generation ($p < 0.05$) while hatchability of eggs was significantly reduced for *Ae. aegypti* and *An. sinensis* after treatment with sublethal dosage of *A. annua* extract ($p < 0.05$). Females of *Ae. aegypti* showed significant reduction in size compared with the control after treatment with sublethal dosage of *A. annua* extract ($p < 0.05$). Females of *An. sinensis* had significant shorter life span under laboratory condition after treatment with sublethal dosage of *A. annua* extract ($p < 0.05$). Only *Cx. quinquefasciatus* did not show any marked differences in the sublethal effect tests. All the mosquito species did not show any marked differences in F1 generation. The

results indicated that *A. annua* extract showed concentration dependent oviposition deterrent activity. At 500 ppm, the percentage of effective repellency was more than 85 % compared with the control for all species with oviposition activity index values of - 0.94, - 0.84, - 0.95 and - 0.78 for *Ae. aegypti*, *Ae. albopictus*, *An. sinensis* and *Cx. quinquefasciatus*, respectively. In ovicidal assay, the percentage hatchability of eggs after treatment with 500 ppm of *A. annua* extract was significantly lower than the control ($p < 0.05$) with a percentage reduction of 45.5 %, 47.5 %, 54.7 % and 17.6 % for *Ae. aegypti*, *Ae. albopictus*, *An. sinensis* and *Cx. quinquefasciatus*, respectively.

CHAPTER 1

INTRODUCTION

Mosquitoes are found all over the world and have long been known for their importance as vectors of diseases (Cheng *et al*., 2009; Wang *et al*., 2011). In addition to being a global problem, most of the mosquito-borne diseases cause human mortality and economic loss. The genera *Aedes*, *Anopheles* and *Culex* contain important vectors of mosquito-borne diseases worldwide. *Aedes aegypti* (Linnaeus) is the primary carrier for dengue fever whereas *Aedes albopictus* (Skuse) is the secondary carrier for this disease. Dengue fever is one of the main mosquito-borne diseases found in the urban areas of the tropical regions (Adanan *et al*., 2005). Besides, cases of dengue fever and dengue hemorrhagic fever have increased every year and resulted in high number of deaths in Malaysia (Lee and Zairi, 2005). *Culex quinquefasciatus* Say is the main vector of filariasis (Pavela, 2008; Govindarajan *et al*., 2011). However, in Malaysia, it is a major nuisance mosquito. *Anopheles sinensis* Wiedemann is widely distributed in the Asia region and is the important vector of malaria in China and Korea (Rueda *et al*., 2005; Ma *et al*., 2011). Malaria remains one of the most important mosquito-borne disease worldwide and one of the world's biggest killers, with 350-500 million cases occurring annually (Hemingway and Bates, 2003). Recent report from World Health Organizations (WHO) showed that about 207 million cases of malaria were reported in 2012 (WHO, 2013). In Malaysia, malaria cases are normally found in the rural areas and forests with abundant agricultural and forests activities, respectively. Most of the cases were found in Sabah and mostly involved young children and pregnant women. Chloroquine is the main drug used in malaria treatment. However, most of the malaria parasites are found to be resistant to this drug (Mak, 2000).

In those case where there is no effective cure such as dengue fever, vector control remains the main method to prevent and control the disease (Webster *et al*., 2009). Several approaches could be used to control the mosquito populations including chemical control, biological control, physical control, environmental management, personal protection and integrated pest management (Yap *et al.*, 2003a). Chemical control by larviciding and adulticiding are among the effective methods in mosquito control programmes. Adulticide is used to control adult mosquitoes that are flying or resting on surfaces while larvicide is used to control the immature stages of mosquitoes. Due to the limited area of movement of mosquito larvae compared to free flying adult mosquitoes, the control of larvae is more effective than the control of the adult (Lee and Zairi, 2005; Amer and Mehlhorn, 2006a; Rajkumar and Jebanesan, 2009; Waliwitiya *et al*., 2009). Vector control programmes using chemical and synthetic insecticides have long been utilized to prevent the transmission of mosquito-borne diseases. However, the use of these chemicals resulted in numerous problems such as insecticide resistance, environmental pollution and causing adverse effects on humans and other non-target organisms (Georghiou and Taylor, 1977; Hemingway and Ranson, 2000; Nauen, 2007; Ghosh *et al*., 2012). For example, dichlorodiphenyltrichloroethane (DDT) was first used in 1946 but case of resistance occurred a year after that (Hemingway and Ranson, 2000).

In order to resolve these problems, research on plant-based products has been carried out over the last few years (Karmegam *et al*., 1997; Isman, 2006; Jbilou *et al*., 2008). Botanical products such as plant essential oils and plant secondary metabolites are used in controlling pest because they are readily available, safe to non-target organisms, biodegradable and environmentally friendly (Isman, 2006; Jbilou *et al*., 2008; Pavela, 2008; Shekari *et al*., 2008; Cheng *et al*., 2009). Shaalan *et al*. (2005) reported that there was no resistance recorded in botanical compounds due to the limited use of the compounds in vector control programmes.

Artemisia annua Linnaeus from the family Asteraceae has been used to treat fever since ancient times (Tawfiq *et al*., 1989; Paniego and Giulietti, 1994; Balint, 2001). Artemisinin, a compound found in this plant is effective against *Plasmodium falciparum*, the parasite that causes malaria (Tawfiq *et al*., 1989; Paniego and Giulietti, 1994; Bhakuni *et al.*, 2001). Thus, *A. annua* is a potential antimalarial plant that is used in the treatment of malaria in malaria endemic regions, such as countries in Africa. Besides, it has the ability to treat cancer and act as antibacterial, antifeedant and anti-inflamatory agents (Bhakuni *et al.*, 2001; Baldi and Dixit, 2008). Liu *et al.* (1992) reported that extract from cell suspension culture of *A. annua* showed antimalarial properties against *P. falciparum*. *Artemisia annua* is well known to have strong antimalarial properties, however, report on this plant against mosquitoes are limited. Hence, the potential of this plant as an insecticide against mosquitoes is of great value to study. Therefore, the objectives of this study are:

1. To investigate the toxicity effects of *A. annua* extract on *Ae. aegypti*, *Ae. albopictus*, *An. sinensis* and *Cx. quinquefasciatus* at the larval stage through larval bioassays at 24 and 48 hours post-treatment

2. To evaluate the sublethal effects (fecundity, fertility, wing length and longevity) of the parental and F_1 generation larvae of *Ae. aegypti*, *Ae. albopictus*, *An. sinensis* and *Cx. quinquefasciatus* that survived after treatment with a sublethal dosage of *A. annua* extract
3. To examine the effects of *A. annua* extract on the oviposition deterrent activity and ovicidal activity of *Ae. aegypti*, *Ae. albopictus*, *An. sinensis* and *Cx. quinquefasciatus*

CHAPTER 2

LITERATURE REVIEW

2.1 Mosquito

There are more than 3000 species of mosquitoes worldwide and they are mostly found in the tropical regions (Peng *et al*., 1998; Ree, 2003). According to Goma (1966), mosquitoes will exist with the presence of human. However, some genera of mosquitoes are found only in certain regions. Mosquitoes are the most crucial household insect pests found in tropical climates (Adanan *et al*., 2005) that caused a lot of problems to public than any other group of arthropods by transmitting numerous medical important diseases (Ghosh *et al*., 2012). Although some of the species are not vector of diseases, they cause serious biting nuisance to the public (Service, 1997).

Mosquitoes are classified under the order Diptera and family Culicidae. The biting Diptera is a two-winged flying insect that sucks blood from human and animals. The body of a mosquito comprised of three parts: head, thorax and abdomen and are covered with scales. An adult mosquito has a long slender body, long legs and long needle-shaped mouthparts, also known as proboscis (Figure 2.1). An adult mosquito is 2 - 19 mm in length (Service, 1997). The family Culicidae is subdivided into three subfamilies: Anophelinae, Culicinae and Toxorhynchitinae. Among these, subfamily Anophelinae and subfamily Culicinae play an important role as vector of diseases while the subfamily Toxorhynchitinae does not have any medical importance. The genera *Anopheles*, *Aedes* and *Culex* comprise of species of mosquitoes that are important vectors in transmitting mosquito-borne diseases (Peng

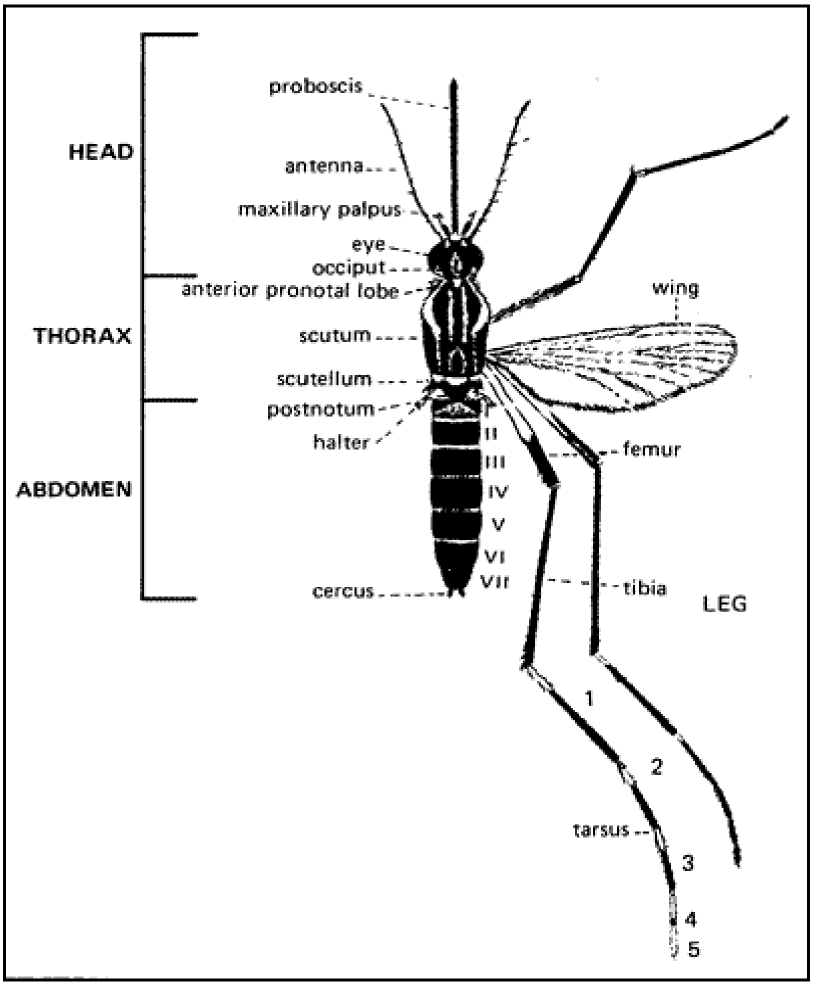

Figure 2.1 Diagram of a female adult mosquito

Adapted from http://www.cdc.gov/malaria/about/biology/mosquitoes/female_diagram.html *et al*., 1998). Furthermore, *Anopheles* and *Culex* are known as cosmopolitan genera because they can be found all over the world (Goma, 1966).

An adult mosquito possesses a mouthpart that is developed for piercing and sucking. The mouthpart consists of several components: labellum, labium, labrum, hypopharynx, maxilla and mandible. Only female mosquitoes feed on blood meal and thus transmit diseases while male mosquitoes feed on plant nectar and honeydew for survival. The intake of blood meal is necessary for a female mosquito to obtain essential protein for egg maturation before laying the eggs. Hence, a female mosquito mouthpart is highly modified in order to assist the mosquito piercing the skin during blood feeding process (Magnarelli, 1979). Generally, male mosquito has a shorter and weaker mouthpart with reduced maxillae and mandibles (Service, 1997). However, there are some exceptions where the male and female mosquitoes in the genus *Toxorhyncites* do not feed on blood. Mosquitoes differ in their feeding and resting behavior. Species that prefer feeding indoor are known as endophagic whereas those prefer feeding outdoor are known as exophagic. Species which rest indoor are termed as endophilic while those rest outdoor are exophilic (Abu Hassan and Yap, 2003). These behaviors are important element to be considered in mosquito control programmes. For example, residual house spraying are effective against the mosquitoes that rest indoor but not to mosquitoes that rest outdoor (Pates and Curtis, 2005). Besides, there are mosquito species that prefer to feed on birds (ornithophilic), animals (zoophilic) and humans (anthropophilic) (Abu Hassan and Yap, 2003). Some species feed throughout the day while others are diurnal or nocturnal. Takken and Knols (1999) reported that olfactory plays an important role in female mosquitoes when locating a host for a blood meal. Female mosquitoes can locate their hosts by various stimuli such as carbon dioxide, host odours and body heat.

A female mosquito can lay 50 to 500 eggs a few days after taking a blood meal (Becker *et al*., 2003). The duration of digestion of the blood meal depends on the temperature; a longer time is needed for digestion at lower temperatures (Service, 1997). Gonotrophic cycle refers to the process from the beginning of blood feeding followed by egg maturation and finally oviposition. A female mosquito will repeat several gonotrophic cycles in its lifetime. There are exceptions in some species where no blood meal is needed for egg formation, and this species is known as autogenous. For example, *Culex pipiens* can develop eggs without taking a blood meal (Becker *et al*., 2003). Mosquitoes can lay their eggs directly on the water surface or on the moist rock, soil or debris above the water level. The selection of oviposition site is influenced by a variety of environmental factors such as water temperature, salinity and level of pollution (Bentley and Day, 1989).

2.1.1 Life cycle

Mosquito undergoes complete metamorphosis which also known as holometabolous where the immature stage is very different from the adult stage in terms of structure and habitat (Goma, 1966). It undergoes four distinct stages of the life cycle which comprises of egg, larva, pupa and adult. Each stage can be differentiated by their special feature. The egg, larva and pupa of a mosquito are aquatic stages while the adult mosquito is a free-flying insect.

Mosquito eggs are normally brown or blackish in color with approximately 1 mm in size. Eggs from the genus *Aedes* can withstand desiccation. In other words, these eggs can remain dry for months and are able to hatch when flooded with water. Hatching of the eggs gives rise to the first instar larvae and subsequently followed by three moltings, leading to the second, third and fourth instar larvae (Christophers, 1960). The size increases from the lower to the higher instar larvae. The larva does not possess any legs but it has a well developed head, bulbous thorax which is wider than the head and the abdomen, while the body is covered with hairs. They are called ‘wigglers’ due to the way they swim. Mosquito larvae usually feed on yeast, bacteria and small aquatic organisms while some species are carnivorous such as the subfamily Toxorhynchitinae (Service, 1997). Larva except from the subfamily Anophelinae comes to the water surface to breathe using a siphon located at the tip of the abdomen. They dive to the bottom for short periods in order to feed or escape danger (Rozendaal, 1997). According to Service (1997), larvae development time is shorter in tropical region compared to temperate region. Mosquito larvae can be found in a variety of aquatic habitats such as freshwater, swamps, rice fields, pools, drains and artificial containers.

Pupa is a resting and non-feeding aquatic stage. The mosquito pupa is comma-shaped where the head and thorax are combined to form the cephalothorax. They spend most of the time resting at the water surface to obtain oxygen through a pair of dorsal trumpets on the cephalothorax (Burgess and Cowan, 1993). When disturbed, they swim up and down in the water in a jerky fashion and because of this, they are also known as ‘trumblers’.

Adult mosquitoes emerge from the pupal case when immature development is completed. The adult will fly off from the water surface once the exoskeleton hardens and dries. The male mosquito has a pair of bushy antenna while the female mosquito has a pair of hairy antenna. This feature enables us to differentiate the sex in adult mosquitoes. In average, a female mosquito lives 2 - 3 weeks, but the male mosquito normally has a shorter lifespan (Service, 1997). In general, male mosquitoes feed on plant nectar and fluids while female mosquitoes take blood meal from vertebrate before laying eggs. Female mosquitoes will choose a suitable oviposition site in order to avoid from dangers and predators such as fish and tadpoles (Pates and Curtis, 2005). Warburg *et al*. (2011) reported that the presence of predators deter the oviposition of *An. gambiae*. It is of great importance to know the flight distance of mosquitoes to understand the transmission of vector-borne diseases (Harrington *et al*., 2005). With the information regarding the flight distance of mosquitoes, we can prevent mosquitoes from transmitting certain diseases to other places by proper control management. According to Service (1997), most mosquitoes can disperse a few hundred meters.

2.1.2 Medical importance

Mosquitoes probably have a greater harmful effect on human compared with any other insects. They have long been known for their importance as pests and vectors of diseases (Goma, 1966). Despite their small size, mosquitoes are of various economic and medical importances. Mosquito-borne diseases cause economic loss and are commonly found in the tropics than in temperate regions (Adanan *et al*., 2005). However, no part of the world is free from vector-borne diseases (Fradin and Day, 2002). Female mosquitoes acquire pathogens or viruses from infected host during blood feeding, pathogens will incubate in the mosquito's body and finally transmitted to other hosts during the next blood feeding. This bloodsucking creature transmits several life-threatening diseases. For example, *Aedes* is the major vector for dengue and dengue hemorrhagic fever, *Culex* is the vector for filariasis and Japanese encephalitis while *Anopheles* is the sole vector for malaria (Hemingway and Ranson, 2000). Most of the mosquito-borne diseases are of global problem and caused mortality and economic loss. For example, dengue fever had found predominantly in Africa and South America; lymphatic filariasis in India and Africa while malaria cause severe death in Africa (Cheah *et al*., 2013).

2.2 Biology of mosquito

2.2.1 *Aedes aegypti*

Aedes aegypti can be recognized by the black and white markings on the legs and a lyre-shaped marking on the thorax. Study has shown that this species was originated from West Africa and was then transported to the New World such as the Americas (Christophers, 1960). This species is commonly found in the tropical and subtropical regions of the world. The adult prefers to rest indoor and feeds on humans. There are two periods when this mosquito is active in biting: early morning after daybreak and in the evening before dark (Gubler, 1998). The eggs are black, ovoid in shape and laid singly on damp substrates above the water surface (Service, 1997). Naturally, the eggs can be found in tree holes, rock pools and leaves on pond edges (Abu Hassan and Yap, 2003). Besides, the eggs can withstand desiccation for a long period of time. This is an important adaptation that makes the mosquito control very challenging because repopulation will occur when the eggs in the containers are flooded and the dried eggs come in contact with water again. This characteristic allows the eggs to be easily spread to other location. Unfortunately, there is still no effective way to control the eggs (CDC, 2012b).

Aedes aegypti is the primary carrier of the dengue virus that causes dengue fever and dengue hemorrhagic fever. Dengue is prevalent in more than 100 countries and affected more than 2.5 billion peoples in the world (Mahesh Kumar *et al*., 2012). There are four dengue virus serotypes, named DEN-1, DEN-2, DEN-3 and DEN-4 that belong to the family Flaviviridae and genus *Flavivirus* (Gubler, 1998; Günther *et al*., 2007). *Aedes aegypti* also transmits Chikungunya and yellow fever.

2.2.2 *Aedes albopictus*

Aedes albopictus was first described as 'the banded mosquito of Bengal' by Skuse (1894). This species is also known as the Asian tiger mosquito and is the secondary carrier of dengue virus (Gubler, 1998). Adult *Ae. albopictus* has a white stripe in the middle of the scutum (Service, 1997; Abu Hassan and Yap, 2003). The adult is dark in color with a white dorsal stripe and banded legs. This species is dominant in the tropical and subtropical regions such as countries in Asia, but they can adapt themselves to cooler regions. *Aedes albopictus* was originally indigenous to Southeast Asia and the islands of the western Pacific and spread to Africa, Europe and America in the recent decades. It is believed that the spread was due to the shipment and the importation of tires (Gratz, 2004). *Aedes albopictus* eggs can withstand desiccation and remain viable in tires, and when flooded with water, it will hatch and subsequently infest the area. Service (1997) and Gubler (1998) had reported that *Ae. albopictus* was introduced into America in the early 1980s. Export of the tires had facilitated the infestation of this species to other countries.

This species was found to breed in natural and artificial containers such as tree holes, water pots, tin cans and vehicle tires. Because dengue fever can be transmitted transovarially (Günther *et al*., 2007), removing man-made containers could help in reducing the dengue cases. Female mosquitoes lay eggs singly at the edge of water surface which normally found in the vegetation around the house (CDC, 2012a). Besides, they prefer to bite during the day especially at dusk and dawn (Abu Hassan and Yap, 2003; CDC, 2012a). Besides dengue virus, it also transmits several other diseases such as yellow fever, Chikungunya, Eastern equine encephalitis, West Nile virus and Japanese encephalitis (Gratz, 2004; CDC, 2012a).

2.2.3 *Anopheles sinensis*

Anopheles sinensis can be recognized by the alternate pale and dark marks on its wings. The resting position of adult mosquitoes are different from culicine mosquitoes where *Anopheles* sp. rest at a 45° angle to their resting surface (Abu Hassan and Yap, 2003). *Anopheles sinensis* is an important malaria vector in China and Korea (Rueda *et al*., 2005; Ma *et al*., 2011). It has a wide distribution in Asia particularly in China, Japan, Korea, Taiwan and Malaysia. Rueda *et al*. (2005) reported that *An. sinensis* is the most common anopheline species found in Japan. Although malaria has been eradicated in Japan, this species remain abundant in the country. Female mosquitoes deposit eggs singly at night. The eggs are approximately 1 mm in size and have floats on its side (Abu Hassan and Yap, 2003). Larvae are normally found in clear and clean water with vegetation and exposed to sunlight. The larvae lie parallel to the water surface and they are filter feeders. The females feed at night and will rest outdoor after feeding.

Malaria remains the most prevalent disease in the world with 200 - 450 million infections occur annually (WHO, 2010). Report showed that malaria caused serious child mortality in many countries in Africa (Black *et al*., 2003). Malaria causes by the protozoan parasite, *Plasmodium* sp. that transmitted through the bite of the *Anopheles* mosquitoes (Eede *et al*., 2009). When infected, the parasites multiply in the human liver and will infect the red blood cells. The symptoms of malaria include fever, headache and vomitting which will appear 10 - 15 days after being bitten by infected mosquitoes (White, 1996; WHO, 2013). Current drug used in malaria treatment are chloroquine, quinine and artemisinin. New alternatives are being considered due to the resistance of the *Plasmodium* sp. against the current drugs used to combat this disease (White, 1996).

2.2.4 *Culex quinquefasciatus*

Culex quinquefasciatus is a predominant house-resting mosquito found in many tropical countries. It is an important vector of filariasis and West Nile virus in some countries (Kovendan *et al*., 2012a). It is the primary vector of *Wuchereria bancrofti*, parasite of the urban form of filariasis. However, in Malaysia, it is a major nuisance mosquito. The adult abdomen is covered with brown or black scales while scutum is covered with golden and bronze narrow scales.

The eggs are long, cylindrical and brownish in color. The females lay the eggs on water surface to form a raft which normally comprises of about 300 eggs (Service, 1997). The raft remains afloat until the larvae hatch. *Culex quinquefasciatus* female prefers to breed in polluted waters with organic material such as in blocked drains, septic tanks, soak age pools and abandoned wells. The adult females are active at night time where they will bite humans and animals both indoor and outdoor throughout the night (Service, 1997). They commonly rest indoor before and after feeding; sometimes can be found in the vegetation area.

2.3 *Artemisia annua*

2.3.1 Biology and uses

Artemisia annua is known as sweet wormwood, annual wormwood and sweet annie in different regions of the world (Nallammai, 2005). It belongs to the family Asteraceae or known as Compositae, a large family of flowering plant which comprises more than 23000 species such as the daisy and sunflower. The physical characteristics of *A. annua* include an average height of about 2.0 m when mature, a single stem with alternating branches, a fern-like leaf and a bright yellow flower. It can be cross-pollinated by insects and wind. *Artemisia annua* is native to the temperate regions of Asia such as China and Vietnam (Abdin *et al*., 2003). It is commonly known as Qinghao (green herb) in China and it has been used in Chinese medicine to treat fever for more than 2000 years (Tawfiq *et al*., 1989; Paniego and Giulietti, 1994).

Artemisia annua, a plant with an important source of artemisinin is receiving worldwide attention for clinical use. Some of the derivatives such as artesunate, artemether and arteether are able to treat multidrug resistant malaria parasites (Titulaer *et al.*, 1991). In 1972, artemisinin was isolated in China from the aerial parts of the plant particularly the leaves, buds and flowers (Luo and Shen, 1987). However, the content of artemisinin was found to be very low in the plant. Different varieties of *A. annua* showed different content of artemisinin, depending on the origin of the plant and the cultivation conditions such as temperature and photoperiod (Christen and Veuthey, 2001). For example, the highest content of artemisinin was reported in the leaves of Chinese varieties of the plant (Nair *et al*., 1986). Jain *et al.* (1996) reported that more than 80 % of the artemisinin were found in the leaves and inflorescences, 5 - 15 % found in stem tissue while no artemisinin was detected in the root. Previous reports indicated that artemisinin existed in highest content before the flowering or during flowering period (Dharam *et al*., 1996).

Artemisinin found in this plant is effective against chloroquine-resistant and chloroquine-sensitive strains of *Plasmodium falciparum* (Tawfiq *et al*., 1989; Liu *et al*., 1992; Paniego and Giulietti, 1994). Therefore, *A. annua* is used in malaria treatment especially in malaria endemic countries such as Africa. *Artemisia annua* is also able to treat skin diseases and cancers such as leukemia and colon cancer (Baldi and Dixit, 2008). Besides, studies showed that several isolated compounds from this plant can act as antibacterial, antifeedant and anti-inflammatory agents (Bhakuni *et al.*, 2001).

2.3.2 Artemisinin

The chemical formula for artemisinin is $C_{15}H_{22}O_5$, with a molecular mass of 282.332 g/mol. Structural formula of artemisinin is shown in Figure 2.2. Artemisinin, also known as 'Qinghaosu' in Chinese, is an active constituent isolated from *A. annua*. However, not all plants of this species contain artemisinin and the content of artemisinin varies within the same species. Study showed that artemisinin only found in *A.annua* and minor amounts had been detected in *Artemisia apiacea* and *Artemisia lancea* (Klayman *et al*., 1984; Hsu, 2006). Artemisinin is a sesquiterpene lactone with

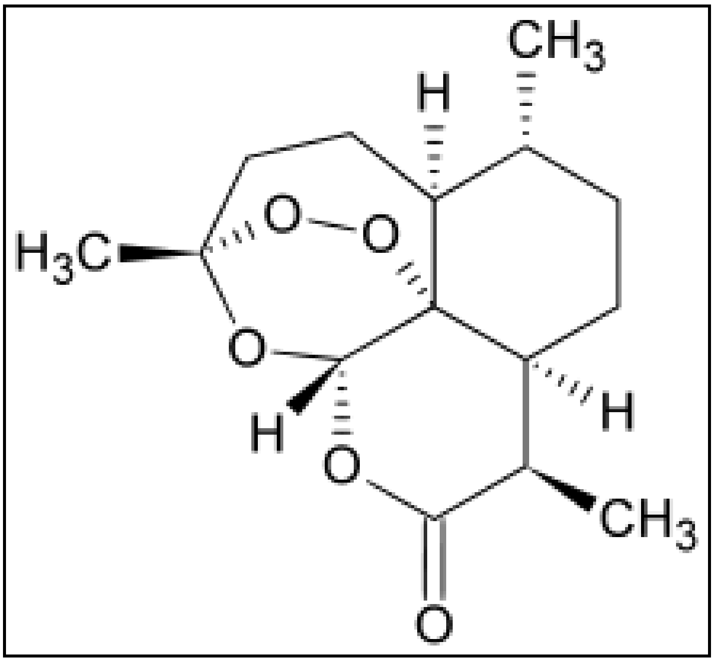

Figure 2.2 Structural formula of artemisinin

Adapted from

http://www.nature.com/srep/2013/130829/srep02513/fig_tab/srep02513_F1.html

(scientific report)

an endoperoxide bridge and this structure has been proven to play an important role in the activity of this molecule (Van der Kooy *et al*., 2008). Lactone is a cyclic ester which is formed by the condensation of an alcohol group –OH and a carboxylic acid group –COOH. Sesquiterpene lactone is a class of chemical normally found in plants such as Chrysanthemum and Ragweed.

Halliwell and Gutteridge (1999) suggested that the mechanism of action for antimalarial activity involves free radicals. Artemisinin might interact with heme or iron to release free radicals. These free radicals will cause damage to the *Plasmodium* parasites and subsequently will destroy them (Meshnick, 2002). Previous study suggests that the drug is most effective against the trophozoites stage of the parasites (Geary *et al.,* 1989).

There is no resistance cases recorded in malaria parasites against artemisinin. Firstly, the drug has a short half-life and that parasites do not come in contact with it for a significant length of time. Besides, artemisinin kills the gametocytes, which is the sexual stage of the malaria parasites responsible for infection in *Anopheles* mosquitoes. This will cause parasites in patients treated with artemisinin to have a lesser chance of infecting mosquitoes and spreading it to other patients. Therefore, this reduces the risk of the development of resistance of the malaria parasites to the drug (Hommel, 2008). Artemisinin is normally used in combination with other antimalarials such as chloroquine and mefloquine in malaria treatment. This is one of the reasons why resistance is delayed in artemisinin treatment (Meshnick, 2002). Artemisinin is seldom used in monotherapy. There are no adverse effects found in patients treated with artemisinin or its derivatives. Artemisinin does not cause any effect on humans at the dosage used to treat malaria. However, in several studies, the use of artemisinin derivatives in high dose caused neurotoxicity in animals such as rats (Brewer *et al*., 1998).

2.3.3 Potential in pest control

Artemisia annua is well known as an antimalarial plant; however, the effects of this plant against animal pests are not well studied. Several studies have been performed indicating the effectiveness of *A. annua* against insect pests such as mosquitoes (Sharma *et al*., 2006a), elm leaf beetles (Shekari *et al*., 2008), stored-product beetles (Tripathi *et al*., 2000) and lesser mulberry pyralids (Roya *et al*., 2010). Liu *et al*. (1992) reported that extract from cell suspension culture of *A. annua* can exhibit antimalarial activity against the malaria parasites, *P. falciparum*. In another study, ether extract of *A. annua* with an LC_{50} value of 78.2 ppm, significantly affected the egg hatching and prolonged the larvae and pupae periods of *Cx. quinquefasciatus* (Sharma *et al*., 2006a).

2.4 Plant tissue culture technique

2.4.1 Cell suspension culture

Plant tissue culture is a technique used to maintain or grow plant cells, tissues or organs *in vitro* in a culture medium containing plant nutrient of various compositions in aseptic conditions. Using the *in vitro* culture technique, the biotic and non-biotic factors such as nutrient, temperature, humidity and light intensity can be controlled. Furthermore, this technique also provides a microbial free environment for the growth of plant cells.

Cell suspension culture is established by introducing the soft and friable callus into a liquid culture medium and agitated continuously to segregate the cell. Most of the plant cell cultures possess a typical growth pattern, a slow growth lag phase followed by exponential growth and finally the stationary phase with slow cell growth. Plant cell culture in an agitated liquid medium was first successfully carried out by Caplin and Steward (1948) followed by Muir *et al*. (1954). Liquid medium was used in the study instead of the gelled medium due to several reasons: 1) impurities in agar medium can affect the growth of tissue, 2) nutrient absorption by plant tissue in a liquid medium is more efficient because only portions of the tissues is in contact with the agar surface, 3) radioisotope tracers perform better in liquid medium than in solid medium (Narayanaswamy, 1994). In addition, cell culture will produce more cell biomass compared to callus culture due to the direct contact of cells with the nutrients in a liquid medium. Hence, cell suspension cultures are widely used in the biosynthesis of plant secondary metabolites with medicinal and commercial values. For example, cell culture techniques are used in the pharmaceutical industry to produce plant secondary metabolites such as alkaloids and terpenoids.

2.4.2 Plant secondary metabolites

Organic compounds which are not required for normal plant growth, development and reproduction are referred to as secondary metabolites. Secondary metabolites serve as a mean of defence mechanism and will be produced by plants when under stressful conditions (Ghosh *et al*., 2012). Examples of secondary metabolites are alkaloids, terpenoids, steroids and essential oil. In general, selection of elite lines is based on the production of cell biomass or the quality of the desired secondary metabolites (Ganapathi and Kargi, 1990). Torres (1989) reported that the duration of subculture cycles will affect the production of secondary metabolites. Oksman and Inze (2004) suggested that *in vitro* culture technique is highly recommended for the mass production of plant secondary metabolites. In the recent years, plant secondary metabolites are becoming economically important in producing drugs, fragrances, food additives and pesticides.

2.5 Mosquito control

Mosquitoes can be controlled effectively if we have thorough knowledge of their biology. The more we know about mosquitoes' activities, the easier to control them (Goma, 1966). The main components in integrated mosquito management include surveillance, source reduction, larviciding, adultciding, biological control, as well as education to the public (Rose, 2001). According to Yap *et al*. (2003a), mosquito control approaches consist of four categories: source reduction, physical barrier or personal protection, chemical control and biological control. Control measures are generally directed against only one or a few of the most important species and the most effective methods to control them are larviciding and adulticiding. Adulticide is used to control adult mosquitoes that are flying or resting by space spraying and surface residual spray. Effective adult mosquito control with insecticides must fulfill the requirement that the small droplets can get in contact with the adults and kill them. Large droplets of insecticides that settle on the ground without getting in contact with the mosquitoes would be a waste and may cause undesirable effects to other organisms (Rose, 2001).

Due to the limited area of movement by mosquito larvae compared with that of free-flying adult mosquitoes, the control of larvae is more effective than the control of adults (Lee and Zairi, 2005; Amer and Mehlhorn, 2006a). Identification of breeding sites is an important element in controlling mosquitoes. The choice of larvicide and larvicide formulation need to be considered and applied correctly in order to achieve an optimum outcome in controlling the immature mosquitoes (Pates and Curtis, 2005). Mosquito-borne diseases became an important problem in the world due to several factors such as rapid and unorganized urbanization, increased in the immigration of people between endemic and non-endemic areas, resistance of vectors to conventional insecticides and global warming (Yap *et al*., 2003a).

2.5.1 Chemical control

Chemical control remains an important element in controlling mosquitoes since the introduction of organic insecticides in the 1940s (Yap *et al*., 2003b). Among the main groups of insecticides used are organochlorines, organophosphates, carbamates and pyrethroids. WHO (2007) reported that 547 tons of active ingredients of organochlorines, 437 tons of organophosphates, 25 tons of carbamates and 161 tons of pyrethroids were used annually during 2003 to 2005 for vector control at the global level. In year 2005, more pyrethroids were used compared with organophosphate in dengue control in Malaysia. Only pyrethroids were used in malaria control from 2001 to 2005 (WHO, 2007). Generally, organophosphates, carbamates and synthetic pyrethroids are among the insecticide group mainly used in the control of mosquitoes (Kamaraju *et al*., 2011). There are three organophosphate larvicides commonly used namely temephos which is used against clean water breeders such as *Aedes* sp. while chlorpyrifos and fenthion are used to control the polluted water breeders such as *Culex* sp. (Yap *et al*., 2003b).

However, used of these chemicals resulted in numerous problems such as insecticide resistance, environmental residues and disruption of the ecological system (Busvine, 1978; Georghiou and Taylor, 1977; Karmegam *et al*., 1997; Nauen, 2007). For example, DDT was first used in 1946 but cases of resistance occurred after a year of its introduction (Hemingway and Ranson, 2000). Insecticide resistance is defined as the ability of the species to withstand a dosage of insecticide which normally will kill them (Goma, 1966). Another class of insecticide is the insect growth regulators which comprise of two groups: juvenile hormone analogue and chitin synthesis inhibitors. Insect growth regulator is targeted against the immature stage of the insect either by interfering with the molting process or prevents the formation of chitin. It is believed that insect growth regulator is more environmental friendly and causes less harmful effect on other beneficial insects. However, they have a slower mode of action compared with synthetic insecticides. Microbial insecticide such as *Bacillus thuringiensis* and *Bacillus sphaericus* are also used in controlling mosquito larvae.

2.5.2 Botanical insecticide

Botanical insecticides which are considered as one of the biopesticides, are natural insecticides in which the toxicants are derived from plant resources. Shaalan *et al*. (2005) reported that plants were used in controlling pests before the invention of synthetic chemicals. More than 2000 plant species had been reported to produce chemicals which have medicinal and pesticidal properties (Ghosh *et al*., 2012). Asteraceae, Meliaceae, Piperaceae, Lamiaceae and Annonaceae are among the plant families which been well studied for the purpose of botanical insecticides (Isman, 2006; Pavela, 2008). In general, there are five groups of botanically derived insecticides that are commonly used: pyrethrum, rotenone, sabadilla, ryania and nicotine (Ware, 1983).

To date, no studies have shown resistance to botanical-based insecticides among vector pests due to the limited use of botanical products in vector control programmes (Shaalan *et al*., 2005). Besides, plant-based products consist of a mixture of chemicals which act on the biological and physiological properties of the pests. Therefore, plant based products are highly effective against pests by reducing the chances of the development of resistance and do not have any adverse effects on humans and wildlifes (Ghosh *et al*., 2012). Problems associated with the use of synthetic insecticide such as pollution, development of resistance and high production costs have resulted in the demand to find a new alternative in controlling pests. Kamaraj *et al*. (2010) stated that natural products of plant origin with insecticidal properties have been used in recent years for the control of a variety of insect pest and vectors, whereas Ghosh *et al*. (2012) reported that the application of plant extracts in mosquito control programmes has been impemented since ancient times. Nevertheless, phytochemicals were still not been used extensively due to the poor characterization and inefficiency in determining the active ingredients with insecticidal properties.

2.6 Sublethal effects of insecticides on mosquito

Sublethal effect is defined as the biological and behavioral changes of surviving insects after coming in contact with a sublethal dosage of insecticide that may not kill them (Lee, 2000). Knowledge of this is crucial in controlling vector pests because not all pests in the field will come into contact with the lethal dose of the