EVALUATION OF OVICIDAL AND LARVICIDAL EFFECTS OF PLANT EXTRACTS FROM FAMILY ANACARDIACEAE AGAINST *AEDES AEGYPTI* AND *AEDES ALBOPICTUS* (DIPTERA: CULICIDAE).

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by

YOUSAF ALI

Thesis submitted to the School of Biological Sciences, Universiti Sains Malaysia in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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PENILAIAN KESAN EKSTRAK TUMBUHAN DARI KELUARGA ANACARDIACEAE TERHADAP *AEDES AEGYPTI* DAN *AEDES ALBOPICTUS* DI PERINGKAT OVICIDAL SERTA LARVICIDAL (Diptera: CULICIDAE).

oleh

YOUSAF ALI

Tesis yang diserahkan ke Pusat Pengajian Sains Kajihayat, Universiti Sains Malaysia untuk memenuhi keperluan bagi Ijazah Falsafah Kedoktoran

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LIST OF SYMBOLS AND ABBREVIATIONS

AAPCC	American Association of Poison Control Center
Ae.	*Aedes*
An.	*Anacardium*
ANOVA	Analysis of Variance
CDC	Center for Disease Control
CNSL	Cashew Nut Shell Liquid
Cx.	*Culex*
DDT	Dichlorodiphenyltrichloroethane
DDE	Dichlorodiphenyldichloroethylene
DENV	Dengue virus
DF	Dengue Fever
d*f*	Degree of freedom
DHF	Dengue hemorrhagic fever
DNA	Deoxyribonucleic acid
GC	Gas Chromatography
Gl.	*Gluta*
HCB	Hexachlorobenzene
HPLC	High-Performance Liquid Chromatography
Hrs.	Hours
i.e.	that is
IGR	Insect Growth Regulator

JH	Juvenile Hormones
LC	Lethal Concentration
mg.	milligram
ml.	milliliter
Mn.	*Mangifera*
Ml.	*Melanochyla*
NPLC	Normal Phase Liquid Chromatography
OC	Organochlorine
OP	Organophosphate
PC	Paper Chromatography
ppm	parts per million
RH	Relative Humidity
RPLC	Reversed Phase Liquid Chromatography
®	Registered
TLC	Thin Layer Chromatography
UPLC	Ultra-Performance Liquid Chromatography
USM	Universiti Sains Malaysia
UV	Ultra Violet
VCRU	Vector Control Research Unit
WHO	World Health Organization
WHOPES	World Health Organization Pesticide Evaluation Scheme

PENILAIAN KESAN EKSTRAK TUMBUHAN DARI KELUARGA ANACARDIACEAE TERHADAP *AEDES AEGYPTI* DAN *AEDES ALBOPICTUS* DI PERINGKAT OVICIDAL SERTA LARVICIDAL (Diptera: CULICIDAE).

ABSTRACT

Fitokimia telah mendapat lebih tumpuan dalam program kawalan nyamuk sebagai pengganti kepada racun perosak sintetik pada masa ini. Objektif kajian ini adalah untuk meneroka kesan-kesan daripada *Anacardium occidentale, Gluta renghas*, *Mangifera indica* dan *Melanochyla fasciculiflora* dari keluarga Anacardiaceae terhadap vektor denggi; *Aedes aegypti* dan *Ae. Albopictus* di peringkat ovicidal serta larvicidal masing-masing dan juga perbandingan biologi progeni mereka. Vektor denggi dari strain makmal serta lapangan didedahkan dengan ekstrak metanol dari bahagian batang dan daun. Tangkai dari *Gl. renghas* menunjukan nilai LC_{95} paling rendah untuk kedua-dua strain makmal dan lapangan nyamuk *Aedes* diikuti oleh *Mn. indica, Ml. fasciculiflora* dan *An. occidentale*. *Gluta renghas* dan *Mn. indica* telah dipilih untuk penilaian lapangan sebagai perbandingan untuk Abate dan kumpulan kawalan. Ekstrak stem *Gl. renghas* adalah berkesan mengurangkan jumlah telur untuk vektor denggi dalam keadaan kering berteduh. Sebaliknya, ekstrak stem dari *Mn. indica* adalah lebih baik dalam keadaan kering dengan tidak berteduh dalam kawalan jumlah telur vektor denggi. Ekstrak daun dari *Gl. renghas* telah mempamerkan kadar hayat yang paling kurang terhadap nyamuk dewasa untuk kedua-dua musim (33.5%). Secara umumnya, kadar kewujudan *Aedes* yang telah diperhatikan akan meningkat apabila bilangan telur menjadi kurang. Begitu juga, ekstrak stem dari *Mn. indica* dapat momonjangkan bilangan hari nyamuk *Aedes* untuk mencapai dewasa secara ketara (12.42 $\pm$ 0.29 hari) berbanding dengan Abate dalam kedua-dua musim (kering: F = 8.35, d*f* = 5, P = 0.000; basah: F = 107,999, d*f* = 5, P = 0.000). Kesuburan *Aedes* generasi F1 telah dikurangkan secara ketara oleh ekstrak stem *Mn. indica* dalam kedua-dua musim (kering: F = 8.38, d*f* = 5, P = 0.000, basah: F = 4.87, d*f* = 5, P = 0.000), manakala Abate berkesan hanya dalam keadaan teduh basah (F = 4.86, d*f* = 5, P = 0.000). Pengurangan kesuburan juga menyebabkan pengurangan kadar penetasan telur oleh *Aedes*. Penetasan telur Aedes yang tertinggi adalah dari kumpulan kawalan Aedes (66.91%), manakala kadar penetasan yang terendah adalah dari kumpulan Abate (52.45%). Antara rawatan ekstrak tumbuhan yang telah diguna, ekstrak stem *Mn. indica* menunjukan peratusan telur menetas yang terendah (55.67%) yang terkurang. Dalam analisis kromatografi tumbuhan ekstrak terdapat bahawa quercetin dan apigenin adalah kumpulan utama sebatian yang mungkin bertanggungjawab menyebabkan aktiviti toksik kepada nyamuk Aedes. Hasil daripada kajian ini membuktikan bahawa *Gluta renghas* dan *Mn. indica* mempunyai aktiviti larvicidal dan sub-maut, terutamanya ekstrak stem yang mungkin boleh bersaing dengan larvicidal sintetik. Semua ini boleh dicadangkan sebagai salah satu kaedah alternatif untuk kawalan vektor denggi yang mesra alam, pensasaran spesifik serta kos fitokimia yang rendah.

EVALUATION OF OVICIDAL AND LARVICIDAL EFFECTS OF PLANT EXTRACTS FROM FAMILY ANACARDIACEAE AGAINST *AEDES AEGYPTI* AND *AEDES ALBOPICTUS* (DIPTERA: CULICIDAE).

ABSTRACT

Phytochemicals are getting hype in mosquito control programs as a pertinent substitute to synthetic pesticides. The objective of this study is to explore the ovicidal and larvicidal effects of *Anacardium occidentale, Gluta renghas, Mangifera indica* and *Melanochyla fasciculiflora* from family Anacardiaceae against the dengue vectors; *Aedes aegypti* and *Ae. albopictus* and biological comparisons of their progenies. Laboratory and field strains of dengue vectors were exposed to methanolic extracts of stem and leaf parts. *Gluta renghas* stem displayed the lowest LC_{95} values for both laboratory and field strains of *Aedes* mosquitoes followed by *Mn. indica, Ml. fasciculiflora* and *An. occidentale*. *Gl. renghas* and *Mn. indica* were selected for the field evaluation with comparison to Abate and control. *Gluta renghas* stem extracts effectively reduced the number of eggs for dengue vectors in dry shaded conditions. Whereas, *Mn. indica* stem extracts were better in dry unshaded conditions. *Gluta renghas* leaf extract exhibited least survival rate of adults during both seasons (33.5%). Generally, survival rate of *Aedes* was observed to increase when the number of eggs reduced. Similarly, *Mn. indica* stem extract significantly prolonged the number of days of *Aedes* mosquito to reach adulthood (12.42$\pm$0.29 days) when compared to Abate in both the seasons (dry: F=8.35, d*f*=5, P=0.000; wet: F=107.999, d*f*=5, P=0.000). Fecundity of *Aedes* F1 generation was significantly reduced by *Mn. indica* stem extract in both seasons (dry: F=8.38, d*f*=5, P=0.000, wet: F=4.87, d*f*=5, P=0.000), while Abate was effective only in wet shaded conditions (F=4.86, d*f*=5, P=0.000). Reduction in fecundity consequently resulted in less egg hatchability rate. Highest *Aedes* egg hatchability was noticed for control (66.91%), whereas the lowest hatchability rate was observed for Abate (52.45%). Among the plant extract treatments, *Mn. indica* stem extract had the least percentage of eggs hatched (55.67%). Chromatographic analysis of the plants stem extract revealed quercetin and apigenin as the major group of compounds that might be responsible for causing the toxic activities to *Aedes* mosquito. Results from this study suggested that *Gluta renghas* and *Mn. indica* have the larvicidal and sublethal activities, particularly the stem extracts that can possibly compete with synthetic larvicides. It can be proposed as one of the alternative methods for the control of dengue vector which is environment friendly, target specific and low cost phytochemicals.

CHAPTER 1

GENERAL INTRODUCTION

Family Culicidae has 112 genera containing 3549 species and subspecies of mosquitoes (Harbach, 2008). Being important vector of several diseases, they pose serious threat to human and veterinary welfare more than any other insect group (Guzman *et al.*, 2010). The common fear for mosquitoes is their role as vectors that can spread diseases such as dengue, malaria, filariasis, yellow fever and Japanese encephalitis (Ramkumar *et al.*, 2014). The most common is the dengue fever virus which is transmitted to humans by the infected females of the family "Culicidae" i.e. *Aedes aegypti* Linn. and *Ae. albopictus* Skuse, (Koenraadt *et al.*, 2006).

Mosquito borne diseases are having economic impacts in form of health and commercial losses specifically in countries within the tropical and subtropical regions (Panneerselvam & Murugan, 2013). More than 100 countries are reported to be endemic to mosquito borne diseases and around two million people die because of these diseases every year (Subramaniam *et al.*, 2011). Four countries particularly Malaysia, Philippines, Vietnam and Cambodia are confronting annual epidemics which are more than 90 % of the total reported dengue cases of the region (Chang *et al.*, 2011).

Dengue being endemic in Malaysia, spread irrespective of the urbanization level (Saifur Rahman, 2012). The transmission of dengue fever is mainly caused by two species of *Aedes* mosquito; *Ae. aegypti* and *Ae. albopictus* (Smith, 1956). *Aedes albopictus* is a semi-domestic mosquito while *Ae. aegypti* is a domestic mosquito in urban areas (Chan *et al.*, 1971). *Aedes aegypti* is anthropic in nature (Huber *et al.*, 2008) and like to rest and feed inside the houses and human dwellings (Ponlawat & Harrington, 2005). Most of these dengue vectors species rear in both natural and artificial containers such as gutters, pools, septic tanks, tree holds, leaf axils, fruit peels, discarded and unused tires, water jars, old boats and others (Aigbodion & Anyiwe, 2005; Cadena, 2013). In Southeast Asia, mostly the water reservoirs utilized for drinking and washing purposes are reported for higher densities of dengue vectors' immature stages (Thavara *et al.*, 2001; Tsuda *et al.*, 2002).

Source reduction can be an effective way to manage the mosquito population (Dame & Fasulo, 2003). Therefore, the effective control of these possible dengue vectors at initial larval stages is utmost important (Romi *et al.*, 2003). Application of the control strategies at the larval stages helps to destroy the dengue vectors before their dispersal into the human habitats and also their inability to escape the control measures (Killeen *et al.*, 2002a). Presently, mosquito control is dependent on insecticides but its persistency has been challenged by the increasing resistance incidence in the vector populations, side effects on beneficial insects, contamination of food chain and also the enduring impacts on the environment (Barik, 2015; Rodrigues *et al.*, 2006).

Bio-pesticides are well considered as effective component of vector control programs for future as they have been reported as attractant for oviposition, insect's growth regulation and having deterrent and repellent activities against different insect pests (Amer & Mehlhorn, 2006a; Bagavan *et al.*, 2008). Therefore, focus on phytochemicals for their activities as larvicide, pupicide, adulticide, repellent, antifeedant, and ovipositional deterrent has increased (Panneerselvam & Murugan, 2013). Promsiri *et al.* (2006) declared herbal extracts as safer alternative control method, especially certain medical plant extracts containing natural toxins that are effective against mosquito larvae.

Tropical rain forests of Malaysia are rich sources of natural resources with active compounds of phytochemicals (Kamiabi, 2013). Increasing interest in the phytochemicals for the vector control programs originated the need to search for local Malaysian plants with biologically active compounds to control different immature stages, particularly larval stage. For this purpose, four plants from family Anacardiaceae were selected for ovicidal and larvicidal effects. The plants selected were *Gluta renghas*, *Mangifera indica*, *Anacardium occidentale* and *Melanochyla fasciculiflora*. Two parts of these plants were selected for the study i.e. fully developed fresh leaves and stem. The target dengue vectors selected for this study were *Ae. aegypti* and *Ae. albopictus*. This study was initiated with the following objectives:

1. To compare the larvicidal effects of methanolic extracts of plant parts (leaves and stem) of *An. occidentale*, *Gl. renghas*, *Ml. fasciculiflora* and *Mn. indica* against laboratory and field strains of *Ae. aegypti* and *Ae. albopictus* at the laboratory.
2. To evaluate the field efficacy of two best plant extracts in different seasons and conditions against the dengue vectors.
3. To determine the ovicidal effects of plant extracts on the dengue vectors and biological comparisons of their progenies.
4. To determine the chemical compositions and active ingredients of plant extracts.

CHAPTER 2

LITERATURE REVIEW

2.1 *Aedes* as Vector

Aedes aegypti and *Ae. albopictus* are reported as potential vectors of several viruses including dengue fever virus, yellow fever virus and chikungunya virus and to date have become the leading vectors in the transmission of dengue and dengue hemorrhagic fever in tropical and subtropical regions (Centers for Disease Control and Prevention, 2005). *Aedes albopictus* has a wide host range including man, domestic and wild animals, with peaks of biting times generally in the early morning and late afternoon. *Aedes* mosquito is mostly found near to the human habitats and it mostly breeds in standing water. It can be usually found in shady areas in shrubs where it rests (Koehler & Castner, 1997). Indeed, the recent outbreaks of these mosquito borne diseases is due to the higher number of breeding places in today's throw-away society and to the raising mosquitoes resistance to current commercial insecticides (Ciccia *et al.*, 2000).

2.2 Dengue and Dengue Hemorrhagic Fever

Dengue is a disease caused by a retrovirus belonging to the family of Flaviviridae, genus *Flavivirus* (Urdaneta *et al.*, 2005). It is transmitted by a mosquito vector of the genus *Aedes*. There are 4 serotypes of dengue virus (DENV-1, DENV-2, DENV-3 and DENV-4) and all are found in Malaysia (Abubakar & Shafee, 2002). Dengue virus infection is a most important health problem and signifies an enormous economic burden to the public and health services in tropical countries around the world, with a fourfold increase in the number of cases in the last thirty years (Guzman & Istúriz, 2010). Their geographical spread is increasing with only nine countries documented dengue in the 1950's but to date there are more than 100 countries around the world reporting the incidence of DF and DHF (Guha-Sapir & Schimmer, 2005).

2.3 Dengue and DHF in Malaysia

In Malaysia, the classical dengue was first reported in 1901-1902 in Penang (Skae, 1902) while the first reported DHF cases were in Penang in 1962 (Rudnick *et al.*, 1965). Major outbreaks were reported in 1974, 1978, 1982, 1990 and 1995 (Hairi *et al.*, 2003). Since then, the disease has become endemic throughout the country (Singh, 2000). In the last decade, cases of dengue have become more severe (Hairi *et al.*, 2003). The incidence rate of dengue has increased from 8.5 to 148.73 per 100, 000 respectively in 1988 to 2010 (Ministry of Health Malaysia, 2010). Since 1999, dengue cases in Malaysia have been reported on the increase. Number of cases reported from 1998-2005 are 27381, 10146, 6692, 15446, 30807, 30221, 32422 and 15862 cases respectively. The increase in mortality rate was also recorded. Number of deaths reported in 2005 were 107 cases compared to 37 cases only in 1999 (World Health Organization, 2014). Ministry of Health, Malaysia recorded 39,222 dengue cases along with 83 mortalities in 2013; in 2014, dengue cases were 103,610 with 199 deceases and 23,966 dengue cases with 62 expiries in only three months till March 2015 (Joanne *et al.*, 2015).

2.4 Differentiation of *Aedes aegypti* and *Aedes albopictus*

Adults of *Ae. albopictus* and *Ae. aegypti* can easily be recognized by the distinct black and white shiny scales on their dorsal side of thorax (Hawley, 1988). *Aedes albopictus* has a single line of white scales positioned in the middle of thorax (Goddard, 1993) while *Ae. aegypti* has two straight lines surrounded by the curved lines on the side (Ahmad & Yap, 1997). The larvae of these two *Aedes* species can easily be differentiated by the pectin teeth on the siphon and shape of comb scales on eight segment of the abdomen. In *Ae. aegypti* larvae, the pectin teeth have less distinct denticles but the comb teeth have well-defined lateral denticles whereas *Ae. albopictus* larvae have three well distinct pointed denticles on the pectin teeth but comb teeth are without lateral denticles (Cheong *et al.*, 1986).

2.5 Control of *Aedes* Mosquito

Yap and Zairi (2003) reported the mosquito control subdivided into four types, namely; (i) Source reduction and environmental management, (ii) physical barriers and personal protection, (iii) chemical control and (iv) biological control.

2.5.1 Source Reduction & Environmental Management

The first most important and most economical, effective and environment friendly control measures for the mosquito control programs is source reduction which can be achieved by eradicating their breeding habitats. Normally, standing water regions of the river channels are considered to have higher density of mosquitoes because of their preferences for standing water, however, the healthy biodiversity of river channels having vast range of natural enemies like predators and parasites, have very less or no mosquito population (Barik, 2015).

Planning and organizing the environmental factors for their employment in controlling the vector population and their interaction with the human beings is termed as environmental management by World Health Organisation (1982). This may include the long lasting changes such as destruction or removal of vegetation, land water and filling of the potential sites for breeding with soil to preclude the breeding sites for mosquitoes. In short, it implicates changing of the breeding sites of the mosquitoes into unattractive and unfavorable for their breeding (Al-Akel & Suliman, 2013; Bond *et al.*, 2007). This environmental management is considered as long term strategy for the control of mosquito vectors having no or less detrimental ecological effects. Cases have been reported for efficient control of population density of mosquitoes in countries like Brazil (Killeen *et al.*, 2002b; Utzinger *et al.*, 2001).

2.5.2 Physical barriers and personal protection

Second step is to use the physical barriers in order to minimize the human and disease vectors' interaction. Personal protective measures (PPM) is a single approach that can be used for broad spectrum of vector pests and can avoid arthropod bites (Maia & Moore, 2011). Use of screens in the accommodation and work areas on the windows and doors, eliminating the holes in roofs, walls and other gaps and closing the eaves around the building may reduce the entrance of the disease vectors to houses, resulting in lesser risk of vector borne diseases (Kirby *et al.*, 2009; Njie *et al.*, 2009). Treated and untreated bed nets are also considered as an effective barrier for several insect pests, reducing the vector bites risk (Chappuis *et al.*, 2007), whereas, proper covering of the body with proper clothes may also reduce the human–vector interaction (Joy, 1999; Schoepke *et al.*, 1998).

2.5.3 Chemical Control

Among different pest control approaches, the use of insecticide in chemical control appears to be good for both house hold insect pest control and also public health (Zaim & Jambulingam, 2007). With the introduction of organic insecticides in 1940s, chemical control became the most popular approach for controlling mosquito (Yap *et al.*, 2003). Beside many short and medium term benefits in controlling vector borne diseases, number of evidences has shown that there is a great threat to both human and environment health due to continuous pesticide use (Lu & Kacew, 2002; Schulz, 2004). Prolonged and continued low dose exposure towards pesticides are thought to be one of the causes of chronic health problems in humans including respiratory, reproductive and carcinogenic effects (Mansour, 2004). Pyrethroids, Carbamates, organochlorines and organophosphates are the general insecticides used (World Health Organisation, 2006).

2.5.4 Biological Control

Biological control involves the use of natural products and natural enemies of the mosquito vectors like fishes, nematodes, predators and pathogens which have different mode of actions, mechanisms and sites to control the population density (Al-Akel & Suliman, 2013; Ramirez *et al.*, 2009).

2.5.4.1 Plant Extracts

Biological products like plant extracts offer boundless possibilities for new phytochemical discoveries due to the higher availability of vast range of chemicals (Cos *et al.*, 2006). Extraction is the basic step to get these chemical compounds out of these plants (Fabricant & Farnsworth, 2001). Different solvents are used for extraction of botanical compounds and these solvents affect the compounds extracted i.e. different solvents extract different botanical compounds (Cos *et al.*, 2006). Around 80% of the world population, for their basic health needs depends on the traditional medicines (Sasidharan *et al.*, 2011). Wide range of substances are found in these plants which are used for traditional medicines to control infectious diseases (Duraipandiyan *et al.*, 2006). Thousands of biochemicals in more than 20,000 medicinal plants are confirmed for their safety and effectiveness with fewer side effects. These compounds have antimicrobial, anticancer, antidiarrheal, antioxidant, analgesic and larvicidal activities (Sasidharan *et al.*, 2011).

Family Anacardiaceae has around 76 genera and about 600 species worldwide, mostly found in tropical regions (Hartley, 1998) and most of which are reported for their medicinal properties (Eloff, 2001). Family Anacardiaceae was considered for this study because of their larvicidal activities of its species reported by several researchers (Mukhopadhyay *et al.*, 2010; Oliveira *et al.*, 2011).

a. *Anacardium occidentale*

Anacardium occidentale is a perennial tall tree grown mostly in tropical and subtropical regions (Mukhopadhyay *et al.*, 2010), with a height of up to 12 meters, with simple and alternate leaves, numerous flowers and having pear shaped, red or yellowish soft fruit called Cashew apple. *Anacardium occidentale* has been used as herbal medicine for the treatment of warts, corns, ulcers, dysentery, fever, piles, tumor, fungicidal and insect repellent (Orwa *et al.*, 2009). Phytochemical analysis shows a range of metabolites like flavonoids, alkaloids, terpenoids, triterpenoids, phenols, steroids, tannins, glycosides and carbohydrates in different parts of *An. occidentale* (Doss & Thangavel, 2011).

Different plant parts of *An. occidentale* are reported for its insecticidal activities. Powder and oil extracts of seeds of *An. occidentale* are confirmed for their insecticidal activities against cowpea bruchids (Ileke & Olotuah, 2012) and larvae of *Ae. aegypti* (Promsiri *et al.*, 2006), while cashew nut shell liquid (CNSL) is reported for its insecticidal activities against *Ae. aegypti* larvae in Brazil (Farias *et al.*, 2009) and France (Laurens *et al.*, 1997). Methanolic leaf extracts of *An. occidentale* have already been tested and reported for its mosquitocidal activities against the *Cx. quinquefasciatus, Ae. aegypti* and *An. stephensi* larvae (Tripathy *et al.*, 2011).

b. *Mangifera indica*

Mangifera indica is a big evergreen tree from family Anacardiaceae, found in tropic and subtropic regions (Morton, 1987). Different mango compounds (like mangiferin) and extracts have been used for their anti-inflammatory, antimicrobial, antifungal, anticancer, antidiarrheal, antioxidant, hypoglycemic, hepatoprotective and larvicidal activities (Anila & Vijayalakshmi, 2002; Aqil & Ahmad, 2003; Sairam *et al.*, 2003). Major chemical compounds found in different parts of *Mn. indica* were identified as saponins, phenolics, carotenoids, vitamins, triterpenes, hydrocarbons and fatty acids (Naef *et al.*, 2006).

Dose dependency of the *Mn. indica* leaves essential oils have shown the repellent effects against the *An. gambiae* host seeking females (Alwala *et al.*, 2010), while the acetonic leaf extracts have shown the antifeedant and toxic effects resulting in mortalities of two lepidopteran pests i.e. *Achaea janata* and *Spodoptera litura* (Devanand & Rani, 2008). Eight plants including *Mn. indica* were tested for their different parts such as leaf, flowers and stems. Methanol, chloroform, acetone, water and hot water were selected for the extraction to check the plant extracts for their larvicidal effects against the filarial vector *Cx. quinquefasciatus*, where the stem bark extracts showed the higher mortalities as compared to the leaf and flower extracts (Rahuman *et al.*, 2009).

c. *Gluta renghas*

Gluta renghas belonging to family Anacardiaceae, is naturally occurring and endemic in Malaysia and Borneo (Juperi *et al.*, 2012). This tree can grow up to 50m with a diameter of 115cm (Fern, 2014). These plants secrete noxious substances that can be very hazardous and may cause dermatitis (Meier, 2013). These hazardous compounds are present in roots, timbers, leaves and fruits (Schmidt, 2015). The presence of these detrimental substances may prove the ability of *Gl. renghas* to become a biopesticide.

d. *Melanochyla fasciculiflora*

Melanochyla fasciculiflora is a dioecious tree from family Anacardiaceae, with stilt roots, simple alternate leaves, articulate and pediculate flowers. It is a little known genus of tress and only 17 species are found in Borneo, Java, Malay Peninsula and Sumatra (Beaman, 1986). So far, no study was reported on the ovipositional deterrence and larvicidal activities.

2.6 Extraction Methods of plant materials

Extraction is a very basic and sustainable approach to isolate the biological components of the plants. New extraction techniques are very assistive in the evolution of herbal medicines. The advancement of these techniques with noteworthy advantages compare to the conventional methods will help in the accessibility of superior herbal products worldwide (Gupta *et al.*, 2012). Researchers are busy in sorting out the best extraction method to get the higher efficacy and efficiency out of the plant products. Efficacy is referred to the magnitude and potency of the bioactivity of the extract; whereas, efficiency is referred to the yield or produce of the extraction (Jadhav *et al.*, 2009).

2.6.1 Soxhlet Extraction

Soxhlet extractor was proposed for the first time by German chemist Franz Ritter Von Soxhlet in 1879 for the extraction of lipids (Soxhlet, 1879) but now it is not limited for extraction of lipid compounds, but extensively been used for extracting valuable bioactive compounds from numerous natural sources and used as a model for the comparison of new extraction alternatives (Azmir *et al.*, 2013). This process is used for both small and large scale extraction. Plant sample is placed in cellulose thimble that is positioned on the upper part of the collecting flask below the reflux condenser. A relevant solvent is introduced to the flask and then heated under reflux. Upon reaching a certain level, the condensed solvent is siphoned and recycled back to the flask beneath. The process is repeated until the solvent in the flask becomes semitransparent. The major advantage of this process is that it is a constant and continuous method (Mahdi & Altikriti, 2010; Sarker *et al.*, 2005). Soxhlet extraction is the widely used process for extracting the plant samples due to its convenience and large scale extraction, therefore, this method was selected for extraction in current study. The active compounds of the plant extracts can be detected through various techniques described below.

2.7 Separation and Detection Techniques of plant compounds

Separation and detection of the constituents of the phytochemicals are mainly done by chromatography with one or another or combination of the following chromatographic techniques: Gas Chromatography (GC), High Performance Liquid Chromatography (HPLC), Ultra-Performance Liquid Chromatography (UPLC), Thin Layer Chromatography (TLC) and Paper Chromatography (PC). Selection of the separation methods majorly depends on the volatilities and solubility of the compounds needed to separate. All these methods can be used on both large and small scales. GC is mostly used for volatile compounds, sulphur compounds, fatty acids, hydrocarbons, mono and sesquiterpenes, whereas HPLC is used for less volatile compounds. TLC is a method for the separation of lipid soluble compounds like lipids, carotenoids, steroids, chlorophylls and quinones while PC is specifically applied for the water soluble plant compounds like organic acids, phenolic compounds, amino acids and carbohydrates (Harborne, 1998).

2.7.1 High-Performance Liquid Chromatography (HPLC)

HPLC is a highly improved form of column liquid chromatography (Lundanes *et al.*, 2013). This method is used for the identification and quantification of each component of the compound. In this technique, liquid is used in the mobile phase using small packing particles with relatively high pressure. High pressure is used by a liquid (mobile phase) to push the sample in HPLC through a column packed with stationary phase, compiled by spherical shaped particles or a porous membrane.

HPLC can be subdivided into two groups, depending on the polarity of stationary and mobile phases. Methods, where stationary phase is highly polar than the mobile phase are labeled as normal phase liquid chromatography (NPLC) whereas the opposing side where stationary phase is less polar as compared to mobile phase is known as reversed phase liquid chromatography (RPLC) (Wikipedia, 2015a). The most common way to detect the substance passing through the column is ultraviolet absorption. Number of organic compounds absorb UV light where the amount of light absorbed depends on the amount of the compound. The time taken by the liquid compound to go through the column is termed as retention time which is calculated from the time of injection to the point where the maximum peak is displayed for that compound (Clark, 2007).

2.8 Biological Activities of Plant Extracts on *Aedes* Mosquito

Control measures are engaged to target the *Aedes* mosquito for the effective control of endemic diseases. Chemical and biological control are important factors in regulating the vector populations. Synthetic chemicals like organochlorines, organophosphates, carbamates and pyrethroids are proved for their activities against several mosquito spp. (Centers for Disease Control and Prevention, 2005; Chuaycharoensuk *et al.*, 2011). Besides the synthetic insecticides, phytochemicals and essential oils with poisonous activities are also point of focus for the vector control. Variety of compounds like monoterpenes, sesquiterpenes, phenols, ethers, esters, oxides, ketones, alcohols and aldehydes are found in these phytochemicals and essential oils. These compounds are reported for their biological activities like antimicrobial and antifungal (Burt, 2004), antioxidant (Cao *et al.*, 2009), cytotoxic (Sharma *et al.*, 2009), insect repellent and insecticidal (Islam *et al.*, 2009; Simionatto *et al.*, 2011).

Biological activities of the plant are based on the phytochemicals extracted present in different parts of the plant (Tuetun *et al.*, 2004). Three plants *Myrianthus arboreus, Xylopia aethiopica* and *An. occidentale* were tested for its biological activities from their bark, fruits and nuts respectively against the all the life stages of *An. gambiae* and were found effective (Akinkurolere *et al.*, 2011). Biological activities of other plants against different mosquitoes are also reported like *Lantana cramera* root and *An. occidentale* leaf against *Culex, Anopheles* and *Aedes* spp. (Tripathy *et al.*, 2011), ethanolic seed extracts of *Myracrodruon urundeuva* against *Ae. aegypti* (Souza *et al.*, 2012), essential oils of *Schinus terebinthifolia* against *Anopheles* and *Culex* spp. (Kweka *et al.*, 2011)

2.8.1 Plant Extracts as Larvicides Against Mosquitoes

Larval mortality was evidenced by *Acalypha indica* leaf extracts against the larval stages of *An. stephensi* while Pipernonaline extracted from *Piper longum* fruit also has proved the strongest larvicidal extracts towards *Ae. aegypti* larvae (Govindarajan *et al.*, 2008b). *Aedes aegypti* larvae are also found to be susceptible to the pith and bark extracts of *Rhizophora mucronata* with higher LC_{50} values of 168.3 and 157.4 ppm respectively (Kabaru & Gichia, 2009). Some of these extracts also affect the developmental growth of the insects. Sublethal doses from the neem extracts may prolong the development of *An. stephensi* larvae, pupal weight reduction, increased ovipositional deterrence and hyped mortality (Wandscheer *et al.*, 2004). Recently, essential oils of *Myrtus caryophyllus, Eucalyptus globulus* and *Cinamomum camphora* were also recognized by Pugazhvendan and Elumali (2013) for their larvicidal activities against *Cx. Quinquefasciatus, An. stephensi* and *Ae. aegypti*.

2.8.2 Biological Parameters of Mosquito Affected by Plant Extracts

2.8.2.1 Larval Developmental Time Prolongation

Insect growth regulatory activities are reported for wide range of plant extracts against different mosquito vectors. The developmental time prolongation activity was studied for the methanolic extracts of *Vitex negundo* and *Acalypha alnifolia* leaves against *Ae. aegypti* and was found prolonged up to double the time taken to complete life cycle as compared to the control treatments where all the stages including larvae, pupal and adult stages experienced prolongation (Kamalakannan *et al.*, 2015). It has also been reported by some scientists that Azadirachtin from neem and chinaberry effects and disturbs the normal larval growth (Mehdi *et al.*, 2010; Zebitz, 1987). Some of the affected larvae of *An. gambiae* treated with neem extracts, with prolonged durations are also reported for being unable to molt in to the next pupal stage (Howard *et al.*, 2009).

Sagar *et al.* (1999) affirmed the potential of ethanolic extracts of *Pongamia glabra* seeds for augmented mortality of larvae of *Cx. quinquefasciatus* and *Ae. aegypti* and their extension in their developmental period with the increase of concentration. Similar results were also found earlier for the *Mentha piperita* for its effectiveness in regulation of *Cx. quinquefasciatus* larvae (Pandian *et al.*, 1994). In India, Manimaran *et al.* (2013) testified volatile oils from eight plants; *Citrus sinensis*, *Citrus limon*, *Cymbopogon nardus*, *Myrtus caryophyllus*, *Cinnamomum veerum*, *Acorus calamus*, *Eucalyptus globulus* and *Mentha piperita* against *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* and found to be effective at higher concentrations for prolonging the larval stages, also the percent larvae developing into pupae was reduced drastically. *Melia volkensii* was observed in another study for prolonging the lifespan of larvae of *An. arabiensis* leaving the pupal stage unaffected (Mwangi & Mukiama, 1988).

2.8.2.2 Effects of Plant Extracts on Mosquitoes Adult Emergence

Prolonged larval stages due to interaction with phytochemicals may result in reduced pupal and adult emergence. Neem oil and neem seed kernel extracts have been reported for a decline in percent pupal and adult emergence for *An. stephensi* (Murugan *et al.*, 1996). Similarly, botanical extracts of *Callitris glaucophylla* dose dependently controlled *Ae. aegypti* causing death to the larval and pupal stages avoiding higher percent emergence in adults; whereas, the synthetic insecticides like fenitrothion and lambda cyhalothrin also revealed the dose dependent effects on the *Ae. aegypti* larval survival and adult emergence that was reduced by 10 folds (Shaalan *et al.*, 2005b).

Supavarn *et al.* (1974) reported 11 of 36 phytochemicals to inhibit the pupal development of *Ae. aegypti*, whereas only few of them were able to affect the larval development. Adult inhibition is severally reported by the researchers for different plant extracts against mosquito vector species. For instance, *Cx. quinquefasciatus* adults were inhibited from emergence by *Descurainia Sophia* (Mohsen *et al.*, 1990) and reduced adult emergence was observed in *An. stephensi* by *Tagetes erectes* (Sharma & Saxena, 1994). Extracts of *Calophyllum inophyllum, Samadera indica, Rhinocanthus nasutus* and *Solanum suratense* are reported to reduce the adult emergence by 50% in surviving treated larvae of *An. stephensi, Cx. quinquefasciatus* and *Ae. aegypti* (Muthukrishnan & Pushpalatha, 2001).

2.8.2.3 Effects of Plant Extracts on Mosquito Sex Ratio

Studies of Shaalan *et al.* (2005b), investigating the sublethal effects of some synthetic and botanical insecticides for determination of the sex ratio of *Ae. aegypti* highlighted the trend of favoring females, however in earlier studies the case was not the same as Robert and Olson (1989) found the *Cx. quinquefasciatus* males in higher ratio as compared to females after the exposure to resmethrin and propoxur. On the other hand, Howard *et al.* (2009) noticed no significant differences in sex ratios for the neem treated and control mosquitoes of *An. gambiae.* Similarly, various extracts of Burma rosewood, *Dalbergia oliveri* treatment didn’t detected any significant change in sex ratio when compared to the control, in the emerged adults of *Ae. aegypti* (Pluempanupat *et al.*, 2013).

2.8.2.4 Effects of Plant Extracts on Mosquito Fecundity

Effects of phytochemicals seem to continue from treated larvae through to emerging adults and then the laid eggs with the effects of decreasing viability (Shaalan *et al.*, 2005a). Phytochemicals can affect the biosynthesis of sex pheromones and fecundity of insects, causing a negative impact on the egg production (Hilker & Meiners, 2011). Neem plants are severally reported for their activities against number of insect pests. Likewise, bioactive compounds in the extracts of neem kernel are reported for their inducing male sterility, decreasing fecundity and oviposition repellency in *Cx. quinquefasciatus* and *Cx. tarsalis* (Su & Mulla, 1998). Tennakoon *et al.* (1994) specified a plant alkaloid, resperine, for the decrease in the *Cx. quinquefasciatus* fecundity. Whereas, Saxena *et al.* (1979) identified Aristolochic acid, obtained from *Aristolochia bracteata*, for inducing infertility in *Ae. aegypti*.

Extracts of *Calophyllum inophyllum, Rhinocanthus nasutus, Samadera indica* and *Solanum suratense* were found for causing the sterility in *Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus* (Muthukrishnan & Pushpalatha, 2001). Procyanidin, a type of tannin, found in the groundnuts are able to decrease the fecundity and growth rate of the aphids, *Aphis craccivora* (Grayer *et al.*, 1992) whereas, reduced fecundity was noticed in cabbage aphid, *Brevicoryne brassicae*, when exposed to Glucosinolates from *Brassica* species (Hopkins *et al.*, 1998). The diethyl ether extract of *Parthenium* *hysterophorus* leaves have been reported as a most effective oviposition repellent ensuing in most reduced fecundity of *Ae. aegypti* (Kumar *et al.*, 2011).

In India, Pushpalatha (2015) endorsed the two tropical plants; *Pogostemon quadrifolius* and *Croton hirtus* for their effects on *Cx. quinquefasciatus* adults for significantly reducing the egg production, its fecundity and hatchability. Synthetic pesticides also display sublethal effects but that can't be always anticipated. For example, pyrethroids like tetramethrin, d-allethrin and d-phenothrin synergistically decreased the egg production of *Ae. aegypti*, whereas, d-allethrin and d-phenothrin alone can only decrease the blood intake of these vector pest (Liu *et al.*, 1986).

CHAPTER 3

LABORATORY EVALUATION OF THE PLANT EXTRACTS FROM FAMILY ANACARDIACEAE AGAINST DENGUE VECTORS

3.1 INTRODUCTION

Mosquitoes are very important insect due to their vital role as a vector in the diseases transmission (Service, 1993). They can spread diseases such as dengue, malaria, filariasis, yellow fever, and Japanese encephalitis but the most hazardous are the dengue viruses which are transmitted by the infected females of *Ae. aegypti* and *Ae. albopictus* and has become a great distress for the international public health in recent years (Koenraadt *et al.*, 2006; Macoris *et al.*, 2003). Gibbons (2010) and Reiter (2001) also declared *Ae. aegypti* as the main vector for the arboviral infections of dengue viruses in tropical and sub-tropical regions. Worldwide, about 50-100 million people are infected yearly and almost 2.5 % of those infected people die (Gubler, 1998; World Health Organisation, 2014). Mosquitoes exist all over the world except for the places which are permanently frozen (Reiter, 2001). Among the 3500 species of mosquito, about 75% are native to the tropic and subtropic regions of the world (Reiter, 2001).

Control strategies need more attention these days as the resistance towards the synthetic insecticides among mosquito populations has increased (Shelton *et al.*, 2007). Mosquitoes develop resistance to every pesticide used for their control (Whalon et al., 2015) and resistance levels in both males and females are found same (Ishak *et al.*, 2015). Pyrethroid resistance in *Ae. aegypti* and *Ae. albopictus* is a global issue, however, adults were noticed for lower resistance levels in Asia, USA and Africa except Thailand where resistance is high (Wan-Norafikah *et al.,* 2010). In Americas and Caribbean region, resistance to organophosphates and in Asian region resistance to pyrethroids have already been documented for *Aedes* since 1992 (Macoris *et al.*, 2003; World Health Organization, 2000).

The harmful impacts of insecticides on health and environment has stressed on the quest of alternative environment friendly pesticide. To minimize the threats offered by the synthetic insecticides, the concern in biological control of mosquitoes grew from the early 20^{th} century (Shaalan & Canyon, 2009). The global flora encompasses massive number of phytochemicals that may now replace the synthetic pesticides (Rajkumar & Jebanesan, 2004). Number of plant species has been tested for their activities against different vectors and found to be target specific, readily degradable and environmentally safe (Govindarajan *et al.*, 2008a). A few examples on the successful effects of phytochemical from plant includes leaves of *Cassia fistula* which displayed ovicidal and larvicidal activities against *Anopheles stephensi* and *Culex quinquefasciatus* (Govindarajan *et al.*, 2008a).

Cashew nut shell liquid (CNSL) obtained from the plants of Anacardium has demonstrated significant mortality activity against termites at very low concentrations (Asogwa *et al.*, 2007). CNSL was analyzed and anacardic acid, cardanol, cardol, carbachol, orcinol, BHT and quercetin were found as main constituents. Among these constituents, cardol, cardanol and anacardic acid were affirmed to have larvicidal activities against *Ae. aegypti* (Oliveira *et al.*, 2011). *Mangifera indica* leaves essential oils are reported to have hydrocarbons, triterpenes, phenolics, carotenoids, saponins, vitamins and fatty acid as their major constituents (Naef *et al.*, 2006) and these chemicals are held responsible for repellent effect on female African malarial vector, *An. gambiae* (Alwala *et al.*, 2010).

The growing trend and positive response of the community towards the phytochemicals and its environment friendly behavior, creates an open ground for the research and innovation of the plant based insecticides. Keeping in view the toxic activities of family Anacardiaceae which were demonstrated against other mosquito species, this study was designed to test the lethal effect of plants from family Anacardiaceae against dengue vectors; *Ae. aegypti* and *Ae. albopictus*. The selected plants were *Anacardium occidentale, Mn. indica, Melanochyla fasciculiflora and Gluta renghas.* These plants were selected due to their poisonous resins and their easy availability in the urban and suburban areas of Malaysia.

This study was initiated to investigate the larvicidal effects of extracts from different part of plant (stem and leaves) derived from family Anacardiaceae against the dengue vectors; *Ae. aegypti* and *Ae. albopictus* laboratory and field strains under the laboratory conditions.

3.2 MATERIALS AND METHODS

3.2.1 Mosquito cultures

Two species from two strains were used in this experiment; *Ae. aegypti* and *Ae. albopictus* of laboratory and field strains. Laboratory strains were obtained from the insectarium of Vector Control Research Unit (VCRU), Universiti Sains Malaysia, where the mosquitoes have been cultured under laboratory conditions for more than 500 generations. The eggs collected on Whatman No. 1 filter paper were immersed in a plastic tray containing 500 ml of seasoned water. The eggs hatched after soaking in seasoned water.

The field strain of *Ae. aegypti* and *Ae. albopictus* were obtained from two locations which located at Flat Hamna (5°20'53.9" N 100°18'02.8" E) and Bukit Jambul (5°20'06.7" N 100°17'26.0" E) residential apartments using ovitrap method. Locations were selected due to high population of *Aedes* which associated with high number of dengue cases in Penang. Ovitraps were made of tin cans, painted in black and filled with 300 ml of seasoned water with wooden hardboard paddles. The function of hardboard paddle is for the attachment of eggs during oviposition. A total of ten ovitraps were placed at both locations to obtain wild field strain of *Aedes* eggs. Wooden paddles were collected weekly and replaced with new ones. This collection was carried for a month to have enough number of field strains eggs of *Ae. aegypti* and *Ae. albopictus*. The paddles collected from the field were kept in laboratory, let to dry for 48 hours, and eggs on the paddles were counted under microscope. Paddles were then soaked in seasoned water to let the eggs hatched. The eggs took about 24-48 hours to hatch. Mosquito culture was maintained at a temperature of 28 ± 3^0C, relative humidity (RH) of 70 ± 10% and a photoperiod of 12h light and 12h dark. The larvae were fed fine powdered food, a mixture of dog biscuit, yeast, beef lever and powdered milk at a ratio of 2:1:1:1 by weight. The emerged larvae were reared under laboratory conditions till adult stage. During adult stage the mosquitoes were separated according to the species. *Aedes*