THE SYNERGISTIC EFFECT OF ACETHONILIC *Ipomoea cairica* LEAF EXTRACT COMBINED WITH ENTOMOPATHOGENIC FUNGI *Metarhizium anisopliae* ON THE LARVAE OF DENGUE VECTORS

ROHAIYU BINTI MD RODZAY

UNIVERSITI SAINS MALAYSIA

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by

ROHAIYU BINTI MD RODZAY

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LIST OF SYMBOLS AND ABBREVIATIONS

AChe	Acetylcholinesterase
Ae.	*Aedes*
AMCA	American Mosquito Control Association
An.	*Anopheles*
ANOVA	Analysis of variance
ARSM	Malaysian Remote Sensing Agency
Cx.	*Culex*
DDT	Dichlorodiphenyltrichloroethane
DEN	Dengue
DF	Dengue Fever
df	Degree of freedom
DHF	Dengue Hemorrhagic Fever
DSS	Dengue Shock Syndrome
EPA	Environmental Protection Agency
Fig.	Figure
I.	*Ipomoea*
IMM	Integrated Mosquito Managements
IPM	Integrated Pest Managements
IVM	Integrated Vector Managements
LC	Lethal Concentration
M.	*Metarhizium*
Mad1	Adhesin
MANOVA	Multivariate Analysis of Variance

NDSP	National Dengue Strategic Plan
PDA	Potato Dextrose Agar
pi	Post inoculation
ppm	Parts per million
Pr1	Protease subtilisin
Pr2	Trypsin
RH	Relative Humidity
VCRU	Vector Control Research Unit
WHO	World Health Organization
WPRO	World Pacific Region Organization

KESAN SINERGISTIK EKSTRAK DAUN ASETON *Ipomoea cairica* DIGABUNGKAN DENGAN FUNGUS ENTOMOPATOGENIK *Metarhizium anisopliae* KE ATAS LARVA VEKTOR DENGGI

ABSTRAK

Tindakan yang umum dijalankan untuk menghindari wabak denggi virus selalunya bergantung kepada racun serangga kimia. Namun, berdasarkan keprihatinan orang awam dan strategi perlindungan alam sekitar, lebih banyak penyelidikan secara kawalan biologi vektor aktif dicadangkan. Penggunaan produk bio-potensi semulajadi daripada derivatif tumbuhan, iaitu *Ipomoea cairica* (pokok kembang pagi) dan kulat entomopatogenik (*Metarhizium anisopliae*) telah dinilai dalam kajian ini terhadap larva *Aedes aegypti* dan *Aedes albopictus*. Hasil daripada kajian bioasai menunjukkan bahawa rawatan ekstrak daun *I. cairica* sangat berkesan untuk membunuh *Ae. albopictus* dan *Ae. aegypti* dalam tempoh 24 dan 48 jam selepas rawatan. Hasil daripada analisis faktorial varians (ANOVA) juga menunjukkan terdapat perbezaan yang signifikan dalam aktiviti larvisidal antara spesies dan baka yang digunakan (P <0.05). Berdasarkan analisis probit, urutan keberkesanan aktiviti larvisidal asetonilik ekstrak *I. cairica* adalah dalam urutan; *Ae. albopictus* baka lapangan > *Ae. aegypti* baka makmal > *Ae. aegypti* baka lapangan > *Ae. albopictus* baka makmal. Walaupun, ekstrak *I. cairica* menunjukkan efisiensi yang tinggi terhadap *Ae. albopictus* berbanding *Ae. aegypti*, namun, adalah baik untuk kita mengenal pasti ekstrak daun *I. cairica* masih berkesan untuk rawatan terhadap larva *Aedes*. Manakala untuk *M. anisopliae*, keputusan daripada ujian bioasai menunjukkan akiviti larvisidal *M. anisopliae* META-G4 adalah tinggi

terhadap larva *Ae. aegypti* dari baka makmal dan lapangan dengan nilai LC_{50} (9.6 x 10^3, 1.3 x 10^3) dan nilai LC_{95} (1.2 x 10^6, 5.5 x 10^5). Manakala, bagi *Ae. albopictus*, nilai LC_{50} untuk baka makmal dan lapangan adalah (1.7 x 10^4, 2.7 x 10^4) dan nilai LC_{95} adalah (2.1 x 10^6, 7.0 x 10^5). Berdasarkan analisis probit, permerhatian yang dapat dibuat untuk kedua-dua spesies *Aedes* adalah baka lapangan mempunyai nilai LC_{50} dan LC_{95} yang rendah berbanding baka makmal. Hasil dari kajian ini mengenalpasti kerentangan baka lapangan nyamuk *Aedes* terhadap *M. anisopliae* adalah lebih rendah berbanding baka makmal adalah sesuatu hasil yang menarik untuk kawalan vektor. Untuk meningkatkan aktiviti larvisidal kedua-dua ekstrak *I. cairica* dan *M. anisopliae,* kami telah menunjukkan kebarangkalian menggabungkan ejen-ejen ini ke dalam formulasi sinergi yang baru. Kajian keserasian dijalankan untuk menentukan nilai kepekatan ekstrak *I. cairica* yang berpotensi untuk membunuh 100% larva *Aedes* tanpa mempengaruhi perkembangan kulat *M. anisopliae.* Kepekatan paling serasi yang telah ditentukan untuk menggabungkan agen *I. cairica* dan *M. anisopliae* pada 450 ppm dengan 6 x 10^6 konidia/ml. Kadar infektif dan persistensi dari hasil kombinasi tersebut boleh bertahan selama 15 hari dan berkurangan kepada 50% selepas 18 hari rawatan di dalam keadaan makmal. Pendekatan untuk menggunakan gabungan kulat entomopatogenik dengan ekstrak tumbuhan ini menunjukkan dapat meningkatkan persistensi produk dengan liputan kawalan jangka panjang dan mengurangkan risiko pembentukan rintangan yang akan memberi manfaat kepada kita untuk mengawal populasi nyamuk *Aedes*.

THE SYNERGISTIC EFFECT OF ACETHONILIC *Ipomoea cairica* LEAF EXTRACT COMBINED WITH ENTOMOPATHOGENIC FUNGI *Metarhizium anisopliae* ON THE LARVAE OF DENGUE VECTORS

ABSTRACT

The common approach to ward-off epidemics of this dengue viruses is depends on chemical insecticides. However, due to the public concerned and environmental protection strategies, more researches on biological control of the vector are actively proposed. The use of bio-potency natural products from plant derivatives, which are *Ipomoea cairica* (coastal morning glory) and entomopathogenic fungi (*Metarhizium anisopliae*) were evaluated in this study towards the larvae stage of *Aedes aegypti* and *Aedes albopictus* mosquitoes. *Ipomoea cairica* leaves plant extracts were segregated for the extraction preparation using a Soxhlet apparatus with acetone as the solvent. The results from bioassay demonstrate that *I. cairica* leaves extract treatment were highly effective to induce larvicidal mortality of *Ae. albopictus* and *Ae. aegypti* within 24 and 48 hours post-treatment. Results from factorial analysis of variance (ANOVA) also indicated there were significant differences in larvicidal activity between species and strains used ($P<0.05$). Based on probit analysis, it is interesting to notify that the sequence of effectiveness larvicidal activity of *I. cairica* acethonilic leaves extract are in the manner; *Ae. albopictus* field strain > *Ae. aegypti* laboratory strain > *Ae. aegypti* field strain > *Ae. albopictus* laboratory strain. Eventhough, *I. cairica* extracts showed high efficiency against *Ae. albopictus* compared to *Ae. aegypti*, however, it is contended for us to identify *I. cairica* leaves extract still effective towards *Aedes* larvae.

Meanwhile, for *M. anisopliae*, the results from bioassay test showed that larvicidal activity *M. anisopliae* META-G4 isolation were highly effective against larvae of *Ae. aegypti* laboratory and field strains with LC_{50} values (9.6 x 10^3, 1.3 x 10^3) and LC_{95} values (1.2 x 10^6, 5.5 x 10^5) respectively. Meanwhile, for *Ae. albopictus*, LC_{50} values for laboratory and field strains were (1.7 x 10^4, 2.7 x 10^4) and the LC_{95} values were (2.1 x 10^6, 7.0 x 10^5) respectively. Based on the probit analysis, it can be observed that the field strains have relatively lower LC_{50} and LC_{95} values compared to laboratory strains for both *Aedes* species. The findings from this study identify the susceptibility of field strains *Aedes* mosquitoes towards *M. anisopliae* was lower as compared to the laboratory strains as an attractive means for vector controls. In order to enhance the larvicidal activities of both *I. cairica* extract and *M. anisopliae*, we demonstrated the possibility of combining these agents into a new synergistic formulation. The compatibility study was conducted to determine the concentration values of *I. cairica* extract that is potential for killing 100% *Aedes* larvae without affecting the *M. anisopliae* fungi growth. The most compatible concentration for combining agents of *I. cairica* and *M. anisopliae* was at 450 ppm with 6 x 10^6 conidia/ml. The infective and persistency of this combination can last up to 15 days of 100% mortality and reduced to 50% after 18 days of post-treatment under the laboratory conditions. The current approach using a combination of entomopathogenic fungi with plant extract showed high persistence of the product with long term control coverage, and reduce the chances of resistance development which benefits us for the controlling *Aedes* mosquito population.

CHAPTER ONE

GENERAL INTRODUCTION

1.1 Introduction

Dengue is one of the most important arthropod-borne viral diseases transmitted by the two principal mosquito vectors known as *Aedes aegypti* and *Aedes albopictus*. These two main dengue vectors typically breed in both tropical and sub-tropical region since the expanding of shipping industries during 18^{th} and 19^{th} centuries, which have accelerated dengue viruses movement worldwide (Becker *et al*., 2003). The ability of these two species to adapt within different climatic condition had encouraged the establishment of dengue viruses in Southeast Asia.

Dengue virus is endemic in Malaysia since 1902 and has become major international public health concern (Becker *et al*., 2003). The transmission of classical dengue fever (DF) and dengue haemorrhagic fever (DHF) have been expanded across geographic distribution affecting tropical and sub-tropical regions around the world. The trend of dengue incidence continues to increase in Malaysia since 2001 with 72 cases recorded per 100,000 populations to 361 cases in 100,000 populations in 2014 (Mudin, 2015). Despite of continues prevention strategy implemented by Ministry of Health and Municipals, the trend of dengue incidence was still continuing to worsen. In the year of 2017, Malaysia has currently experienced 55, 744 dengue cases with 131 deaths (WHO, 2017).

Considering the rapid transmission of dengue virus, substantial effort to suppress mosquitoes' vector populations had been conducted. Effective control such as eliminating breeding sites, adulticide and larvicide are adopted by public health regulators (WHO, 2009). According to the World Health Organization (2009), Bi-regional Dengue Strategy (2008-2015) of WHO South- East Asia and Western Pacific regions also adopted the same control measures of dengue transmission across boundaries. In Malaysia, a structured of surveillance system were introduced since 2009 to observe the pattern of dengue virus serotypes changing in all 14 states in Malaysia (MaHTAS, 2015). In order to strengthen the management of current dengue control programmes, Ministry of Health has implemented the Integrated Management Strategy for the dengue prevention and control program since 2011 (Mudin, 2015).

One of the consented efforts focussed in this strategy is integrated vector management strategy. Following the rises of resistance profiles and mechanisms of mosquito populations towards the current insecticide used for the dengue vector control, the needed of integrated management strategy can be successful pillars to deal with insecticide resistance population. In ecological perspectives the excessive use of chemical insecticides also not only causes the mosquitoes to develop insecticide resistance, but it also threatening the life of non-target organisms (Ghosh *et al.*, 2012). Corresponding to the risk of chemical introduction, the recent researches trend focuses more on biological insecticides or the combination of both chemical and biological insecticides as an alternative to conventional chemical insecticides.

Plant extracts are rich in bioactive compound and known to be one of replacement for chemical insecticides due to its chemical properties. It has been proven to act as general toxicant, growth and reproductive inhibitor, insect repellent, larvicidal and ovicidal deterrent against mosquitos' vectors (Sukumar *et al.,* 1991; Muthukrishnan and Pushpalatha, 2001; Rajkumar and Jebanesan, 2007; Rahuman *et al*., 2008; Govindarajan *et al*., 2008, Khandagle, 2011). Studies conducted by Rahuman *et al*. (2008) found that the extractions from plant derivatives are proven as another possible alternative bio-rational insecticide safer to human being and environment. The bioactivity of plant extracts is varied significantly based on types of solvent used and mosquito species tested (Shaalan *et al*., 2005). Due to the variability of the polar solvents such as methanol and acetone together with different plant parts derivatives, the activity of the chemical compound within the plant extraction also will be different (Oliveira *et al*., 2010). As an example, *Ipomoea cairica* extracts showed excellent results with acetone (AhbiRami *et al*., 2014).

The potential of mosquito bio-rational insecticides derived from plants such as *Ipomoea cairica* (coastal morning glory) had been assessed as good potential mosquito control agents to control mosquitoes' vector populations (AhbiRami *et al.,* 2014). Besides, the high viability of this plant in the local area, *Ipomoea cairica* is considered as good potential natural plant derived products in controlling the mosquito's population. The assessment of residual from these control agents has been proven to suppress the mosquito population via their remarkable larvicidal properties with high toxicity leaded to 100% mortality of larvae (Thomas *et al*., 2004; Rajkumar and Jebanesan 2007).

Another natural product that can provide an alternate option of the used of synthetic chemicals is entomopathogenic fungi. *Metarhizium anisopliae* is one of entomopathogenic fungi commercially being assessed as biological agent for arthropod pest species. The excellency of larvicidal activity *M. anisopliae* as a killing agent for mosquito larvae has been demonstrated in various studies (Benserradj and Mihoubi, 2014; Pereira *et al*., 2009; Lee *et al*., 2015). Generally, *M. anisopliae* invades by penetrating the cuticle parts of the infected mosquito larvae (Benserradj and Mihoubi, 2014). The adhesion of fungi onto cuticles will germinate the spores to the epidermis parts of the larvae. The larval mortality can also cause by high conidia concentration within the intestine of larvae. This condition will cause blockage before germination can take place (Lage *et al*., 2001). The pathogenic feasibility of this microbial larvicide will take time to cause mortality effect against different species mosquito larvae depending on the dosage and types of fungi strain applied (Blanford *et al*., 2005; Scholte *et al*., 2003). Parallel to the variability mode of actions, *M. anisopliae* can be proposed as bio-potency agents for mosquito control.

To enhance the effectiveness of both selected bio-larvicides to be used as one of possible control methods against *Aedes* mosquitoes, we proposed to investigate the synergism effect between *I. cairica* plant extracts and *M. anisopliae* fungi. The main idea is to develop a new alternative product from the combination of natural chemical insecticides together with biological control agent for the mosquito control programme which can kill *Aedes* larvae in the short term after application by *I. cairica* component and further prolong the effectiveness of the formulation by the action of *M. anisopliae* fungi. In this study, we examined the compatibility values of *I. cairica* plant extract that is potential for killing 100% *Aedes* larvae without affecting the *M. anisopliae* fungi growth. In facts, studies have shown the possibility of combining the fungal spores with plant extracts (Borgio *et al*., 2008) and chemical insecticides (Paula *et al*., 2011) for the pest and mosquito control strategy. The dynamic combination of entomopathogenic fungi with plant extracts will increase the control strategy efficiency eventhough the concentrations of the two compounds are reduced relatively from individual concentrations (Sahayaraj *et al*., 2011; Bhan *et al*., 2015). Besides, the synergistic mixture of the compounds also will decrease the chance of pest's resistance expression due to multiple actions from two different agents. Thus, synergism are considered one of the strategy that should be applied to reduce the resistance related cases with economical costs, which are safer to human and environment (Bhan *et al*., 2015).

In view of enhanced impact of these bio-rational potential products, the data from the laboratory strains mosquitoes used within this study will be the comparison to field strains from both urban and sub-urban regions in Penang. The potential of individual agents of *I. cairica* leaves extract and *M. anisopliae* will be assessed to determine the susceptibility level of *Aedes* mosquito used in the present study. Continuing our discovery for useful adjuvants, here we demonstrate for the first time the combination of *I. cairica* leaves plant extract together *M. anisopliae* for the control of *Aedes* larvae with the future aim to reduce the dengue incidence in Malaysia.

1.2 Objective

The significance of this study is to nurture our knowledge regarding a combination of two biological agents in order to suppress the population of *Aedes* mosquitoes for the long term effect. The outcome from this study will be essential for the mosquito control program that can be applied by the public health regulators. For this reason, the following objectives have been drawn up for this study:

1. To determine the effective lethal dose of *Ipomoea cairica* leaves plant extract against both *Aedes albopictus* and *Aedes aegypti* mosquitos' larvae.
2. To determine the effective lethal dose of *Metarhizium anispoliae* against both *Aedes albopictus* and *Aedes aegypti* mosquito's larvae.
3. To determine the compatibility values and the synergism effect of combining formulations of *Ipomoea cairica* plant extract and *Metarhizium anisopliae* fungi against *Aedes albopictus* and *Aedes aegypti* mosquitos' larvae.

CHAPTER TWO

LITERATURE REVIEW

2.1 Integrated vector management in vector-borne disease

Integrated approaches in controlling vector-borne diseases are one of the global public health greatest challenges in 21st century. Incorporated global strategic framework had been underlying to approach the requirement of integrated vector control (Beier *et al.*, 2008). Historically, in 19th century the greater concern has been into insect Integrated Pest Managements (IPM) in agricultural sectors. Due to broad range of other pest including pathogens and weeds, the conceptualization of IPM has been suggested to include mosquito as one of them (Axtell, 1979).

In response with the rise of vector-borne disease, integrated mosquito management has been assessed to improve future management strategies for mosquito controls (Rose, 2001). The operational framework of Integrated Mosquito Management (IMM) is based on controlling and prevention strategies using single or combination methods to manage the populations of mosquito in the environment. The implementation of IMM requires full understanding on bionomics of mosquitoes and their favourable conditions before conducting various applications including chemical methods, biological methods, source reductions, water sanitation practices and EPA-registered larvicides introduction into larval mosquito habitats (AMCA, 2009).

2.1.1 Physical control

Elimination of mosquito breeding sites or source reduction is the most practicable and encouraged control methods that can eliminate adult mosquitoes as long-term solution (Floore, 2006). The cohabitation of mosquito larvae within containers were reflected by men sanitations habits (Cheong, 1967). The reduction of breeding sites by proper disposed of plastic containers, tires or other water-holding containers around homes can prevent ovipositional opportunities of mosquitoes to breed around the residential areas (Floore, 2006). This sanitation effort is one of integral part in mosquito management methods that can decrease the inhabitations *Aedes* mosquitoes within residential areas (Chen *et al*., 2005).

2.1.2 Chemical control

2.1.2 (a) Chemical insecticides

The discovery and development of chemical compounds has led the emphasis of insecticides as an approach to insect control. From the 1930s onwards, the curiosity on chemical properties had drove the synthesis of various insecticide compounds such as DDT, organophosphates, carbamates and pyrethroids which led to profound changes in insecticides utilization (Becker *et al*., 2003). In Malaysia, vector control was mainly carried out with insecticides using temephos and pyrethroids as components for chemical controls (Manjarres-Suarez and Olivero-Vierbel, 2013). For instance, the susceptibility of *Ae.albopictus* towards temephos is still considered susceptible (Mohiddin *et al*., 2016). However, the susceptibility of *Aedes* mosquitoes towards pyrethroid demonstrated to be highly resistant recently. It was recorded that the susceptibility of primary species of *Aedes* mosquitoes; *Ae. aegypti* and *Ae. albopictus* was highly resistant towards pyrethroid with no mortality recorded after 24 hours exposure (Hasan *et al*., 2016). Understanding and monitoring the susceptibility status of *Aedes* mosquitoes towards the applied insecticides need a serious attention as such information could provide baseline data for predicting patterns of cross resistance and selection of suitable alternative insecticide that could be employed for the mosquito control efforts (Ranson *et al*., 2010).

2.1.2 (b) Botanical insecticides

In recent years, the usage of synthetic insecticides had increased insecticide resistance on mosquito. Concerning the environment sustainability, modern research prompt to look for alternative approaches developed from natural origins such as botanical insecticides. Ghosh *et al.* (2012) reviewed the efficacy of 144 plant species that exhibited mosquitocidal larvicides properties which had been proven reduced the population of target vector mosquito's species. Plant derived products have unique natural insecticidal properties comprising mixture of chemical compounds that can affect the behaviour and physiological processes of the treated sample. The effects of these insecticidal compounds vary according to plant species, geographical varieties, types of plant parts, extraction methodology, polarity of the solvents used during extraction and mosquito species exposed to the plant extracts (Sukumar *et al*., 1991). As many mixture of compound act together to form synergist between one another, there are very little chance for the treated sample to develop resistance (Ghosh *et al*., 2012).

The active chemical compound derived from plant extracts are secondary metabolites that protecting them from herbivores. These secondary metabolites potentially release toxic substances into target sites of the insects that feed on it (Ghosh *et al*., 2012). As an example, the disruption of receptor potential channels can result in activation or inhibition the channels pathway (George *et al*., 2014). In turns, insects will face physiological disruptions in various ways including abnormalities of nervous systems which can lead to mortality.

2.1.2 (b)(i) *Ipomoea cairica*

Ipomoea cairica is one of the species from approximately 500-600 species of genus *Ipomoea* within family Conculvulacea (Austin and Huáman, 1996). The species from this family always being notified by their twining characteristics or the existence of cells that secretes resin glycosides in their leaves parts and roots of the plants (Ralte, 2014). The origin of this plant is known to be from Mexico and now become invasive species in many tropical and subtropical regions (Samuel *et al*., 2014). In Malaysia, this showy white to lavender colour flower plant are define as weed and it growth abundantly in wild such as within grazing areas and twining around fences. As this plant has remarkable larvicidal activities towards *Aedes* mosquito (AhbiRami *et al*., 2014), it is great to know the better usage of this weed.

Laboratory bioassay tests in India also showed the excellency of *I. cairica* against four vector mosquito species, *Cx. tritaeniorhynchus* (vector of *Japanese encephalitis*), *Ae. aegypti* (vector of dengue fever), *An. stephensi* (vector of malaria) and *Cx. quinquefasciatus* (vector of lymphatic filariasis). The data obtained revealed 90% of larvae mortality could be induced by 78.3 ppm, 92.7 ppm, 109.9 ppm and

161.6 ppm against larvae of *Cx. tritaeniorhynchus, Ae. aegypti, An. stephensi* and *Cx. quinquefasciatus,* respectively (Thomas *et al*., 2004). These remarkable larvicidal efficiency supported the idea of phytochemicals properties found in *Ipomoea cairica* were responsible as larvicidal agent to destroy the mosquito larvae. The treated larvae also exhibited toxic symptoms including vibration and convulsion which occur due alteration of specific physiological process upon contact with the toxicant (Ishak *et al*., 2014; Hidayatulfathi *et al*., 2004). The persistency of *I. cairica* leaves also had been tested can be last for up to 21 days with more than 50% reduction of *Cx. quinquefasciatus* larvae (Thiagaletchumi *et al*., 2014). The results from these studies open the possibility of *I. cairica* to be used as one of larvicidal agents in mosquito control programmes.

Plate 2.1 *Ipomoea cairica*

2.1.3 Biological control

Towards the evolutionary of mosquito controls strategies which low impact to environment, the development of biological control now emerges as promising agents. Biological control aims to reduce the targets population without the usage of toxicological agents in order to preserve the ecosystems and environments (Becker *et al.*, 2003). The introduction of antagonist into mosquito populations such as naturally occurring predators, parasites and pathogens of vector insects together had been focused as bio-potential organisms for controlling mosquito.

2.1.3 (a) Entomopathogenic fungi

Entomopathogenic fungi were specifically adapted fungi developed to parasitize insects, nematodes and other invertebrates in natural environments (Deacon, 2006). The most commonly entomopathogenic fungi commonly known to attack arthropods belong to the genera *Lagenidium, Coelomomyces, Entomophora, Culicinomyces, Beuveria* and *Metarhizium* (Scholte, 2004). *Beuveria* and *Metarhizium* are considered as strong candidates for practical control of insect pests due their vast diversity in natural environments with high conidia production in laboratory culture (Deacon, 2006).

The efficiency of fungal isolations still depends on the species of treated mosquitoes, dosage applied and fungal strains (Blanford *et al*., 2005). Although the fungus isolates took times to react, but these findings identify the abilities of entomopathogenic fungi as an attractive means for vector controls (Frentiu *et al*., 2014). As fungi had relatively broad host range, the usage of their conidia should be done cautiously in order to avoid contamination into non-target organisms as low as possible (Scholte *et al*., 2004).

2.1.3 (a)(i) ***Metarhizium anisopliae***

Metarhizium anisopliae was discovered by Metscnikoff (1879) on wheat cockchafer *Anisoplia austriaca* and named it as *Entomophthora anisopliae*. Later, after the establishment of Genus *Metarhizium*, it was renamed as *Metarhizium anisopliae* by Sorokin (1883). *Metarhizium* is another commonly known entomopathogenic fungi which received interests in recent years as potential mosquito bio-control agent. *Metarhizium* spp. produced green-like conidia chains emerged from phialides termed as 'green muscardine' (Deacon, 2006). Recent taxonomic refinements based on phylogenetic DNA sequence analyses in eastern Asia identified nine species in this group (Nishi and Sato, 2017). Recently, *Metarhizium anisopliae* which is the most recognizable species for the pest management strategies had been proposed as new non-chemical approach in mosquito control (Blandford *et al*,. 2011).

Modern exploration expanded the possibility *M. anisopliae* as pathogens for mosquito larvae (Scholte *et al*., 2004; Pereira *et al*., 2009; Bilal *et al*., 2012; Benserradj and Mihoubi, 2014). Significant larvae mortality had been remarked by these fungi when conducting larvicidal assays against *Cx. pipiens*, *Ae. aegypti* and *Ae. albopictus* with percentage of mortality reached up to more than 90% (Benserradj and Mihoubi, 2014; Pereira *et al*., 2009; Bilal *et al*., 2012). However, the virulence of these fungi depends on the strains, concentration and time exposure subjected to the studies (Scholte *et al*., 2007; Blanford *et al*., 2005; Benserradj and Mihoubi, 2014).

Generally, the route of invasions of these fungi occurs when the conidia attached to the cuticle of suitable host, forming germ tubes, developing penetration pegs and invade into the internal tissue of the host (Shrank and Vainstein, 2010). However, in aquatic conditions, the hydrophobic tensions created between the conidia and water tension resulting to failure of conidia attachment to the cuticle of mosquito larvae (Butt *et al*., 2013). Based on limited number of studies conducted to know the precise route of invasions of *M. anisopliae* on mosquito larvae, it is possibly to note that adhering of conidia in the internal tissue of the larvae might due to the ingestion of floating conidia by the larvae itself. In turns, this conidia will germinate extended into siphon tip tissue, blocked the trachea resulting in suffocation and death.

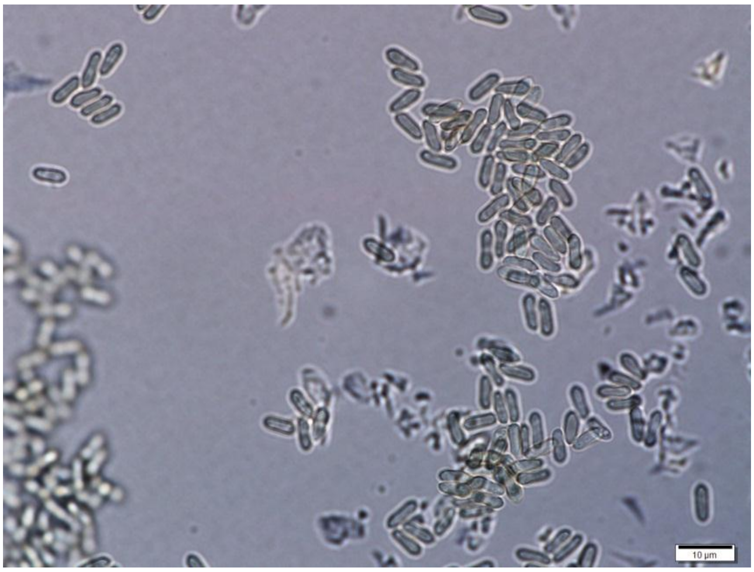

Plate 2.2 *Metarhizium anisopliae*

2.2 Synergistic action.

Many efforts have been done to increase the efficiency of the application method and products used to fight against mosquitoes. Synergism is one of applicable measures that can be used to increase the performance of individual effects of one component by the joint action with the other component in a mixture (Bhan *et al*., 2015). This synergistic mixture still providing similar control, same as the individual application or may enhance the effectiveness and has been preferred as an eco-friendly strategy; since the concentrations of the two compounds has been reduced. Concerning the rises of resistance populations, the usage of a mixture component through synergism are considerable as the probability of resistance will occur on both components in the mixture is lower compare to single component alone (Srivastava *et al*., 2011).

The selections of appropriate combinations for the application mixture are needed in order to ensure either both component acts synergistically or antagonistically with each other (Bhan *et al*., 2015). As an example, the mixture of plant extracts together with fungus might be compatible or not due to the antimicrobial and antifungal properties of botanical insecticides (Radha *et al*., 2014). By conducting detailed compatibility studies, the minimal inhibitory concentration (MIC) and optimal combination of the mixing compounds can be determined to improve the reliability and efficiency of new combined formulation (Islam *et al*., 2009).

2.3 Subfamily Culicinae

Systematics evolutionary and cladistics studies categorised mosquitoes into the family level of Culicidae with three subfamilies which are Anophelinae, Culicinae and Toxorhynchitinae. However, in 1998, the subfamilies of Culicinae were reduced into two new assignation of subfamily Toxorhynchitinae to tribal rank (Harbach and Kitching, 1998). Recent classification subdivided family Culicinae into two subfamilies and 11 tribes including most of commonly known mosquito-borne disease which are *Aedes*, *Culex* and *Mansonia* (Harbach, 2007).

Subfamily Anophelinae *(includes Genus *Anopheles*)

Subfamily Culicinae

Tribus Aedeomyiini

Tribus Aedini * (includes Genus *Aedes*)

Tribus Culicini * (includes Genus *Culex*)

Tribus Culisetini

Tribus Ficalbiini

Tribus Hodgesiini * (includes Genus *Mansonia*)

Tribus Mansoniini

Tribus Orthopodomyiini

Tribus Sabethini

Tribus Uranotaeniini

Tribus Toxohynchitini

Figure 2.1 Systematic order of the Family Culicidae (Becker *et al*., 2003)

In the cold temperate regions, female members of Culicinae known for their fiercest bite mechanism. The resting adult's positions of Culicinae are at parallel to the surface area, in contrast to Anophelinae where the abdomen parts point away from surface are to form an angle between 30° to 45°. The adult female and male mosquitoes of Culicinae can be differentiate by their palps length, where the male palp is elongated as same length as proboscis and female with shorter palps. Unlike Culicinae, Anophelinae palps of both male and females are as same length as proboscis and their sexes can be distinguish by club-like structure in palps segments of male Anophelinae (Becker *et al*., 2003).

The larvae of Culicinae also can be easily differentiate according to their movement in 'S' shaped and used their siphons to get air from the water surface. However, few species such as *Mansonia* made modification on their siphons forming a hook for attachment to the aquatic plants to obtain an air. Comparing to Culicinae, the larvae of Anophelinae were usually resting in horizontal position under water surface. The characteristics of Anophelinae larvae also can be differentiate with the presents of palmate hair, tergal plate and obviously the absences of siphon.

2.3.1 Bionomics of *Aedes* mosquitoes

Due to the complement of taxonomic entities, Genus *Aedes* were enlisted under Tribus Aedini of subfamilies Culicinae (Reinert, 2001). Comprising of 41 subgenera, Genus *Aedes* globally distributed within tropical, Australian and European regions except Antarctica. Genus *Aedes* is paraphyletic due to lineages of *Aedes albopictus* and *Aedes aegypti* forming one clade generated the phylogenetic relationships of these mosquitoes belonging into subgenus *Stegomyia* whereas *Aedes triseriatus,* *Culex pipens* and *Culex tritaeniorhynchus* cluster together as sister clades (Harbach, 2007).

Subgenus *Stegomyia* were known to transmit pathogens, parasites and arboviruses including the most international public health problem which is dengue fever. In Asian countries, the competent vectors responsible for significant expansion of dengue transmission are *Ae. albopictus* and *Ae. aegypti* (Farjana *et al*., 2012). The episode of this disease outbreaks are intrinsically linked to population growth of the vectors and its relationship with the environment (Nazri *et. al*., 2013).

Their spatial distribution and abundance are associated with environmental variables such as temperature, rainfall and relative humidity (Hasnan *et. al*., 2016). Corroborates these statement, a study conducted in Guangzhou in 2009 proved that the number of dengue fever cases notified were positively associated with temperature, total rainfall and minimum relative humidity (Lu *et al*., 2009). Temperature frequently identified as important parameters to determine the constraints of dengue transmission in that regions (Brady *et al*., 2014). This is because, favourable temperature will permit the survival of adults and immature stages of *Aedes* mosquito. According to Liu *et al*. (2009), high temperature assists to shorten the extrinsic incubation period (EIP) causes the mosquito to survive long enough for the virus transmission. This statement supporting the facts that temperature does exert a strong influence to distinct the limits of vector population persistency which have cumulative effects in virus transmission.

Along with weather variables, other host factor to ensure the survival of *Aedes* mosquito is their genetic inheritance. The genetic inherits from different geographic limits, the will show a greater selection pressure for the adult survival due to their adaptation to local conditions (Brady *et al*., 2014). The increased in survival will increased the chance of vector to produce more offspring and bites resulting in a big opportunity for them to disperse the pathogen transmissions. The longevity and susceptibility of *Aedes* mosquito towards insecticide also reflecting the haplotype composition derived from the subpopulation gene pool (Grech *et al*., 2010). As example, the study conducted by Das *et al*. (2015) in Odisha, revealed the survival of *Aedes* mosquitoes in coastal plains is longer compared to northern plateau. The longer survival period of *Aedes* mosquito will enable them to trigger more arboviral transmission. Hence, it is crucial to locate the fit subpopulation which can transmit the virus more efficiently in order to employ a better vector control measures into that area.

2.3.2 *Aedes aegypti* Linnaeus

Adults *Ae. aegypti* could be easily morphologically identified by a pair of submedian longitudinal white stripes forming 'lyre-shaped' marked on the scutum (Azari-Hamidian and Harbach, 2009). The unconnected of lower and upper white patches on mesepimeron area also can be the keys for identification (Rueda, 2004). Whereas, the larvae of *Ae. aegypti* were recognized by several characteristics such as methathoracic setae parts and comb scales. The simple part to identify is at comb scales parts, where the sub apical spine forming pointed shaped like a 'triffid' appearance (Becker *et al*., 2003).

The dengue haemorrhagic fever (DHF) outbreaks during 1779-1780 in Asia, Africa and North America indicating the population distribution of *Ae. aegypti* had been globally distributed (Gubler and Clark, 1995). This species is originated from Africa and now, it is well distributed in tropical, subtropical and warm temperate regions but still limited in very dry and cold regions. During seasonal periods of northern latitudes regions, *Ae. aegypti* is appeared during summer and plausible during cold seasons (Hopp and Foley, 2001). The density of adult mosquitoes modelled by Hopp and Foley (2001) represented the establishment of *Ae. aegypti* did not occur in desert regions such as Somalia, Eritrea, Yemen, northeast Brazil and certain parts in Saudi Arabia due to less precipitation in these regions.

Aedes aegypti usually being found abundance within human settlements area due to the presence of natural and artificial containers which provides potential breeding sites for *Aedes* mosquitoes. During *Aedes* surveillance, the larvae of *Ae. aegypti* usually found breed in uncovered toilet cisterns, water tanks in toilets, vase and any potentials water recipients inside or surrounding the houses. However, higher distribution of *Aedes* populations also reported in a clean settlement area with minimal kinds of water recipients (Chen *et al*., 2005). There is a possibility of drainage system outside the residential might also provide the potential breeding sites for *Aedes* mosquitoes. This can explain that the necessity of clear water with availability of moderate contents of organic matter as food sources is needed for breeding site, but not necessity to be clean water (Jahangir *et.al*., 2003). The female *Ae. aegypti* were more preferable human blood as their host instead of domestic animals. Correlated to their host feeding preferences, this peridomestic species usually found resting indoors such as inside cupboards, behind curtains and doors (Becker *et al*., 2003).

Plate 2.3 Adult *Aedes aegypti* mosquito

2.3.3 *Aedes albopictus* skuse

Another highly medically important species of mosquito under the subgenus *Stegomyia* is *Ae. albopictus* (WHO, 2004). This Asian tiger mosquito was named as *Culex albopictus* before assigned into Genus *Aedes* by Edwards in 1920 (Knudsen, 1995). The adult's stage of *Ae. albopictus* can differentiate with *Ae. aegypti* by the narrow median longitudinal white stripes arises from prescutellar area to the end of the scutellum area. Whereas, the white scales patches at lower and upper parts of mesepimeron area connected forming a v-shaped (Rueda, 2004). *Ae. albopictus* larvae stage can differentiate with *Ae. aegypti* by the characteristics of comb scales without sub apical spine (Azari-Hamidian and Harbach, 2009).

During 1962, the occurrence of *Ae. albopictus* was not established within South Pacific and was mainly distributed within Oriental and Indomalayan region and thus, the title of Asian tiger mosquitoes was named by their distribution area (Belkin, 1962). Despite of limited flight range, the distribution of *Aedes albopictus* in

1994 was estimated within 537 countries including Malaysia which accidentally introduced since the industries of tyres shipment (Knudsen, 1995).

The mechanism of *Ae. albopictus* eggs that can resist desiccation up for a year or more and hatched within few hours after rehydrated enhances their distribution across continents via various transportations (Knudsen, 1995). The immature stages of *Ae. albopictus* had various potential breeding sites including natural containers and artificial container such as in tree holes, coconut shells, tin cans, polystyrenes and broken glass. Originally, the breeding habitats of *Ae. albopictus* were mainly within gardens, parks and forests, but their distributions were gradually expanding near to human settlement areas (Knudsen, 1995). *Ae. albopictus* very known of their wide varieties of feeding preferences including humans, rabbits, cows or occasionally avian hosts such as Passeriformes or Columbiformes (Niebylski *et al*., 1994). This supported that the risky potential of *Aedes albopictus* to transmit zoonotic viruses to human due to their vast host feeding preferences which includes mammals and avians (Mitchell, 1995).

Plate 2.4 Adult *Aedes albopictus* mosquito

2.4 *Aedes* mosquito and Dengue virus

At beginning of the third millennium, mankind has been plagued by emerging of various infectious disease transmitted by mosquitoes (Gubler, 2001). The advance in transportation does helping in introduction of new populations of mosquito into new geographical area. The resurgence of old and new diseases transmitted by mosquitoes had grown public health concerned.

The blood sucking behaviours of mosquitoes suggested their ability to play role in transmitting various kinds of medically important pathogens and parasites such as viruses, bacteria, protozoans and parasites (Kettle, 1995). If the host is suitable for the inhabitations of pathogens and parasites, these provide permissive conditions for them to proliferate inside the host before successfully transmitted into new hosts (Becker *et al.*, 2003). The emergence of transmission viral diseases caused by insects influenced the human social impacts including economic and political issues.

At the beginning of twenty first century, the rapid emergence of dengue fever showed intense epidemic and have been expanding its geographic distribution especially within tropics regions (Gubler, 2001). *Aedes aegypti* had been incriminated as the principal vectors responsible for the widespread of this virus which can maintain the cycles of the virus in a long period (Schule, 1928). In 1997, the transmission of dengue fever was also detected in *Ae. albopictus* as secondary vector of this virus (Kalra, 1997).

The infection of dengue fever categorised into two clinical manifestations which are severe and non-severe. The common dengue fever defined by the scale of intermediate response or no clinical complications at all, whereas dengue haemorrhagic fever/dengue shock syndrome is in severest form of infections associated with capillary leakage, liver failure which eventually leads to shock and death. The haemorrhagic term in dengue haemorrhagic fever characterised the bleeding as the acute phase of dengue infections, whereas shock in dengue shock syndrome define as the venous pooling (Halstead, 2007). Dengue fever and dengue haemorrhagic fever/dengue shock syndrome (DHF/DSS) are caused by virus from family Flaviviridae with four types antigenically distinct serological strains (DEN-1, DEN-2, DEN-3 and DEN-4). All strains presented similar epidemiology and clinical fatal effects (Raut *et al*., 2015).

2.4.1 Dengue in Malaysia

The first global pandemic of dengue occurred was in Southeast Asia during and after World War II and then expanded their demographic distribution into Pacific and American regions (Gubler, 2001). Currently, the geographic distribution of dengue fever was already endemic to more than 100 countries including within tropical and sub-tropical regions (Raut *et al*., 2015). During the year of 1995, the number of humans living in epidemic dengue areas estimated to be risk by dengue infections was approximately 2.5 billion with 5% fatality rate (Gubler and Clark, 1995).

Dengue remains as the leading arbovirus disease in Malaysia and has been declared as a national health threats to the public (Hasnan *et al*., 2016). The incidence rate of dengue infection in Malaysia is continuing to worsen with 10, 000