THE EFFICACY AND SCREENING OF CRUDE AND HYDROLYZED METHANOLIC EXTRACTS OF *Tridax procumbens* (L.) AGAINST *Aedes albopictus* (Skuse) AND *Aedes aegypti* (L.).

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by

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS

Ae.	*Aedes*
An.	*Anopheles*
Cx.	*Culex*
DDT	Dichlorodiphenyltrichloroethane
DENV	Dengue virus
df	Degree of freedom
g	gram
GC	Gut Content
GC-MS	Gas Chromatography-Mass Spectrometry
HCl	Hydrochloric acid
HPLC	High-Performance Liquid Chromatography
IGR	Insect Growth Regulators
LC	Lethal Concentration
M	Molar mass
MANOVA	Multivariate Analysis of variance
mg	milligram
min	minute
ml	milliliter

NIST	National Institute of Standards and Technology
PC	Paper Chromatography
ppm	parts per million
RH	Relative Humidity
RNA	Ribonucleic acid
rpm	rotation per minute
SE	Standard Error
SIT	Sterile Insect Technique
SPSS	Statistical Package for the Social Sciences
T.	*Tridax*
TLC	Thin Layer Chromatography
UPLC	Ultra-Performance Liquid Chromatography
VCRU	Vector Control Research Unit
WHO	World Health Organization

KEBERKESANAN DAN PENYARINGAN EKSTRAK METANOLIK KASAR DAN DIHIDROLISIS *Tridax procumbens* (L.) KE ATAS *Aedes albopictus* (Skuse) DAN *Aedes aegypti* (L.).

ABSTRAK

Wabak denggi dan Zika yang ditular oleh nyamuk *Aedes* telah menjadi isu global dan pelbagai langkah pencegahan sedang digunakan. Disebabkan kesedaran terhadap alam sekitar, ramai memilih untuk menggunakan bioinsektisid. Objektif kajian ini adalah untuk mengkaji keberkesanan ekstrak kasar dan ekstrak dihidrolisis tumbuhan *Tridax procumbens* terhadap larva *Aedes aegypti* and *Aedes albopictus* dan untuk menilai kompaun larvisid yang ada di dalam *T. procumbens*. Menggunakan bioesei larva WHO, larva instar ketiga *Ae. aegypti* dan *Ae. albopictus* telah didedahkan kepada beberapa pelbagai kepekatan di antara 250 ppm - 2500 ppm kepada enam bahagian ekstrak metanolik *T. procumbens* (bunga, daun, batang akar, bahagian aerial yang mengandungi bunga, daun dan batang, dan seluruh tumbuhan) yang diekstrak menggunakan pengekstrakan Soxhlet. Ketoksikan tertinggi bagi *Ae. aegypti* telah direkodkan oleh ekstrak kasar batang *T. procumbens* dengan LC_{50} sebanyak 799.78 ppm dan LC_{95} sebanyak 1506.66 ppm. Manakala, untuk *Ae. albopictus*, ia dicatat oleh ekstrak kasar bunga *Tridax* dengan LC_{50} sebanyak 583.63 ppm dan LC_{95} sebanyak 2329.41 ppm. Ekstrak *T. procumbens* yang terpilih iaitu batang, bunga dan daun telah dihidrolisis dengan mendidih 2M HCl dan diekstrak semula dengan etil asetat untuk membuang glikosida dari kompaun bioaktif di dalam *T.* procumbens. Ekstrak yang dihidrolisis telah diuji semula untuk bioesei larva. Keputusan mendapati bahawa ekstrak dihidrolisis batang memberikan LC_{50} yang

terendah sebanyak 518.42 ppm dan LC_{95} sebanyak 1300.11 ppm untuk larva *Ae. aegypti,* manakala ekstrak dihidrolisis bunga *T. procumbens* telah memberikan LC_{50} yang terendah sebanyak 506.98 ppm dan LC_{95} sebanyak 1581.32 ppm untuk larva *Ae. albopictus.* Perbandingan berpasangan ujian-t telah menunjukkan kepekatan membunuh untuk ekstrak dihidrolisis *T. procumbens* didapati lebih rendah secara bererti untuk semua bahagian tumbuhan bagi kedua spesies kecuali ekstrak bunga dihidrolisis apabila diuji ke atas larva *Ae. albopictus* berbanding ekstrak bunga mentah (t=-0.933, df=29, P>0.05). Analisa kromatografi gas-spektrometri jisim (GC-MS) menunjukkan kemungkinan kewujudan kompaun bioaktif di dalam tumbuhan *Tridax* dengan keupayaan larvisid seperti metil ester, asid heksadekenoik dan n-asid heksadekenoik dan beberapa kompaun bioaktif dengan akitiviti antibakteria dan antimikrobial yang secara tidak langsung menyebabkan kematian *Aedes*. Analisa GC-MS juga telah menunjukkan hasil kompaun bioaktif di dalam ekstrak dihidrolisis adalah 1.5 hingga 9 kali lebih tinggi daripada ekstrak kasar *T. procumbens* di bawah kepekatan yang sama. Ini menunjukkan ekstrak kasar *T. procumbens* berkesan dalam membunuh kedua larva *Ae. aegypti* dan *Ae. albopictus* dan proses hidrolisis asid telah meningkatkan keberkesanan *T. procumbens* ke atas kematian larva *Aedes.* Oleh itu, ekstrak tumbuhan *T. procumbens* mempunyai potensi untuk dijadikan biolarvisid bagi mengawal larva *Aedes* jika dikaji lebih jauh dan pada skala yang lebih besar.

THE EFFICACY AND SCREENING OF CRUDE AND HYDROLYZED METHANOLIC EXTRACTS OF *Tridax procumbens* (L.) AGAINST *Aedes albopictus* (Skuse) AND *Aedes aegypti* (L.).

ABSTRACT

The epidemic of dengue and Zika outbreak transmitted by *Aedes* mosquitoes has been a global issue and plenty of preventive measurements are being used. Due to environmental awareness, people are opting on bioinsecticides. The objective of this research is to study the effectiveness of crude and hydrolyzed extracts of *Tridax procumbens* plant on *Aedes aegypti* and *Aedes albopictus* larvae and to investigate the larvicidal compound present in *T. procumbens*. Using WHO larval bioassay, third instar larvae of *Ae. aegypti* and *Ae. albopictus* were exposed to different concentration ranging between 250-2500 ppm of six different parts of *Tridax* plant methanolic extracts (flower, stem, leaves, roots, aerial part containing flower, stem and leaves and whole plant) extracted using Soxhlet extraction. The highest toxicity for *Ae. aegypti* was recorded by *Tridax* stem crude extract with the LC_{50} of 799.78 ppm and LC_{95} of 1506.66 ppm. Meanwhile for *Ae. albopictus*, it was recorded by *Tridax* flower crude extract with the LC_{50} of 583.63 ppm and LC_{95} of 2329.41 ppm. The selected extract of *T. procumbens* which were the stem, flower and leaves were then hydrolyzed by boiling with 2M of HCl and re-extracted with ethyl acetate to remove glycosides from the bioactive compounds in *T. procumbens*. The hydrolyzed extracts were tested again for larval bioassay. The results were found that the hydrolyzed stem extract gave the lowest LC_{50} of 518.42 ppm and LC_{95} of 1300.11 ppm for *Ae. aegypti* larvae, while hydrolyzed flower extract of *T. procumbens* gave

the lowest LC_{50} of 506.98 ppm and LC_{95} of 1581.32ppm for *Ae. albopictus* larvae. Paired t-test comparison showed that the lethal concentration of the hydrolyzed extracts of *T. procumbens* was found to be significantly lower for all the plant parts for both species except on hydrolyzed flower extract when tested on *Ae. albopictus* larvae when compared with the crude flower extract ($t=-0.933$, $df=29$, $P>0.05$). Gas chromatography-mass spectrometry (GC-MS) analysis indicated the possibilities of the presence of bioactive compounds in the *Tridax* plant extracts with larvicidal properties such as hexadecenoic acid, methyl ester and n-hexadecenoic acid and several bioactive compounds with antibacterial and antimicrobial activities which indirectly causes *Aedes* larvae mortality as well. The GC-MS analysis also showed that the yield of bioactive compounds in the hydrolyzed extracts were 1.5 to 9 times higher than crude extracts of *T. procumbens* under the same concentration. This indicates that the crude *T. procumbens* extracts is effective in killing both *Ae. aegypti* and *Ae. albopictus* larvae and acid hydrolysis process enhances the efficacy of *T. procumbens* on the mortality on the *Aedes* larvae. Therefore, *T. procumbens* plant extracts has the potential to be a biolarvicide in controlling *Aedes* larvae if researched further and at a larger scale.

CHAPTER 1

GENERAL INTRODUCTION

Mosquitoes are known to be insects which are the largest cause of mortality in human and animal around the globe due to their abundance and their large distribution throughout the world (Versteirt et al., 2013). According to Crosby (2005), before the implementation of vector-borne disease control, millions of deaths were recorded by the transmission of viruses by mosquitoes, responsible for diseases like yellow fever and malaria. Around 34 genera were reported with 3,100 species of mosquitoes recorded worldwide from the family Culicidae comprising of three subfamilies which are Culicinae, Toxoryhnchitinae and Anophelinae. The most important subfamilies which are responsible for disease transmission are subfamily Culicinae and subfamily Anophelinae (Abu & Yap, 2003).

Aedes mosquitoes are found to be black in colour with white stripes found distributed along their bodies (WHO, 1997). *Aedes* mosquitoes are among the important vectors that are responsible in transmitting dengue, dengue hemorrhagic fever, Zika, Chikungunya and yellow fever. *Aedes aegypti* have been discovered to be the primary vector while *Aedes albopictus* were known to be the secondary vector in transmitting dengue. The number of dengue cases recorded in Malaysia in 2018 till June was 32435 cases with 53 deaths and there is an increasing trend in the dengue cases from January 2018 till June 2018 (WHO, 2018). The invasive behaviour of the *Aedes* mosquitoes has caused them to be able to colonize most parts of the world.

The excessive usage of synthetic chemicals has caused health problems to the people and harming the environment. The excessive usage of these chemicals have also caused insecticide resistance among mosquitoes. For example, in Selangor, it was

found that larvae of *Culex quinquefasciatus* is extremely resistant against Dichlorodiphenyltrichloroethane (DDT) and malathion (Nazni et al., 2005). Due to these causes, there is a need for the development of biopesticides that is both effective and does not leave any harmful side effects on the environment (Mazid et al., 2011). There are plenty of ways bio pesticides were made and among them is extracted from plant derivatives which can be used as insecticides which could be a possible alternative for synthetic chemicals. Different from the conventional pesticides which comprises of a single active ingredient, plant derived pesticides have a combination of a chemical compound that acts on the pests. This blend of chemical compounds makes the development of resistance by pests to be difficult (Ghosh et al., 2012).

Tridax procumbens or commonly known as coat buttons or tridax daisy is a plant which is native to Central and tropical South America (Ikewuchi, 2012). The local name for *T. procumbens* in Malaysia is pokok kancing baju. This plant is under the family of Asteracaea and the plant is known to have a daisy like appearance with yellow center and three toothed ray florets which are usually white in colour. Leaves of this plant are short, hairy and arrow-shaped (Jahangir, 2001). It was once known as an unwanted invasive weed which colonized almost all parts of the world but it is now discovered to have lots of uses. Among the use of *T. procumbens* includes having wound healing activity, antimicrobial activity and anti-diabetic activity (Navin et al., 2014). Besides that, the *Tridax* plant was known to have larvicidal property on mosquitoes like *Anopheles* and *Culex* (Devan et al., 2013). The source of such biopesticides needs to be easily accessible as to help in reducing the cost of manufacturing them.

Acid hydrolysis is a process of chemical decomposition in which a compound is split into other compounds mediated by an acid. When it comes to plant extractions,

the hydrolysis process is used to remove sugar compound from the targeted compound to maximize yield and efficiency. The extract from *T. procumbens* has found to be having bioactive compounds like carotenoids, alkaloids, saponins, tannins and flavonoids (Jude et al., 2009). These chemical compounds are usually sugar bound in plants. Bioactive compounds in plants are produced as secondary metabolites which are used in the survivability of the plant such as causing toxic effects against pest and is not involved in the growth of the plant (Azmir et. al., 2013). It is possible that by hydrolyzing the *T. procumbens* extract, the bonds between the sugar and the chemical compound can be removed, thus increasing the efficacy of *T. procumbens* against *Aedes* larvae.

The phytochemical analysis is required to screen for the compounds responsible for the larvicidal properties in the *T. procumbens.* Among the method of phytochemical analysis is chromatography. Chromatography is a chemical analysis which is used to separate mixtures of substances into their components based on their molecular structure and molecular composition. The phytochemical analysis chosen for this study is gas chromatography-mass spectrometry (GC-MS). Gas chromatography is a process where the tested sample are vaporised and injected onto the chromatographic column head. By using the principle of adsorption and partition, gas chromatography can determine materials present in a compound even at a low concentration. Due to its simplicity and effectiveness, gas chromatography is widely used in the chemical plant, oil-industry, food industry and bio medical sciences (Sermakkani & Thangapandian, 2012).

Therefore, the purpose of this study is to determine the efficacy of *T. procumbens* on the *Ae. aegypti* and *Ae. albopictus* larvae. The result of this study is important in

understanding the effects of *T. procumbens* on *Aedes* larvae. For these reasons, the following objectives have been drawn up for this study:

- to determine the larvicidal efficacy of flower, stem, leaves, roots, aerial part and whole plant parts of *T. procumbens* against *Ae. aegypti* and *Ae. albopictus* larvae.
- to elucidate the effect of hydrolysis process on the effectiveness of *T. procumbens* plant extracts on *Ae. aegypti* and *Ae. albopictus* larvae.
- to identify the possible chemical compounds in *T. procumbens* that are responsible for the larvicidal efficacy against *Ae. aegypti* and *Ae. albopictus* larvae using gas chromatography-mass spectrometry (GC-MS).

CHAPTER 2

LITERATURE REVIEW

2.1 *Aedes* as a Vector of Dengue

Mosquitoes are notorious insect pests which invade the personal space and health of human and animals worldwide. *Aedes* mosquitoes have caused various health issues to arise as the mosquitoes can become vector which help in transferring viruses and other pathogens which causes diseases (Li et al., 2001) and one the most known disease known to be transmitted by the *Aedes* mosquitoes is the dengue fever. *Aedes* mosquitoes are responsible for transmitting the virus for dengue but *Aedes* mosquitoes are not responsible for causing dengue itself.

The virus which is known to cause dengue fever belongs under the class of Flavivirus in the family Flaviviridae. The size of the virus is small to medium with a measurement around 25-50mm. A genome of linear single-stranded RNA is also found in the spherically enveloped virus (Sarin et al., 1998). The mode of delivering the virus is during blood feeding, where the *Aedes* mosquitoes feeds on blood of an infected person. The virus then develops within the mosquito and matures to infect other people when the mosquito feeds on a different host. Four serotypes of dengue viruses were found in Malaysia which are DENV-1, DENV-2, DENV-3 and DENV-4 (Abubakar & Shafee, 2002).

There are two common *Aedes* species found in Malaysia which are the *Aedes aegypti* and *Aedes albopictus.* The difference between the two species can be found at the adult stage of the mosquitoes by viewing the dorsal side of the thorax which the white scale patterns (Figure 2.1). A curved lyre shaped lines can be seen on *Ae. aegypti*

which has a pattern of two straight lines meanwhile the middle of *Ae. albopictus'*s thorax only has a broad line (Rozendaal, 1997).

Aedes aegypti and *Ae. albopictus* can be identified and differentiated at larval stages too. The shape of the comb scale found on the eighth segment of the larvae abdomen and pecten teeth on their siphon can be differentiated between the species. Comb scales with a well-developed lateral denticles can be seen on *Ae. aegypti.* The pecten teeth of *Ae. aegypti* are known to be having less defined denticles. Meanwhile in *Ae. albopictus* no lateral denticles can be seen on its comb teeth and the pectin teeth of *Ae. albopictus* have three well-defined denticles (WHO, 1997).

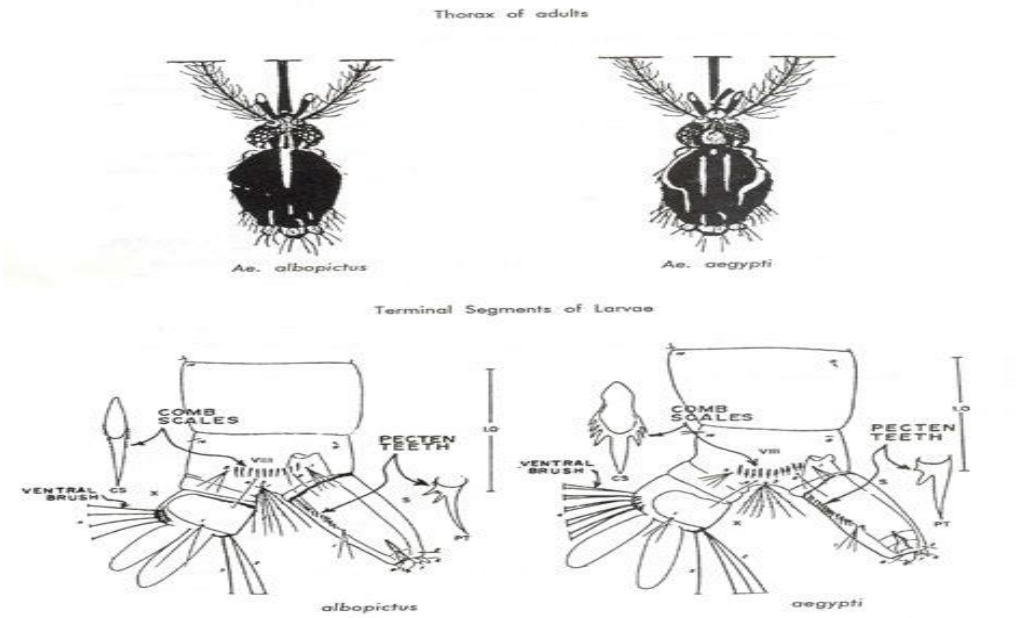

Figure 2.1: Key differences between *Aedes aegypti* and *Aedes albopictus* larvae (WHO, 1997).

2.1.1 *Aedes* Mosquitoes' Distribution in Malaysia

The *Aedes* species which can be commonly found in Malaysia are the *Ae. aegypti* and *Ae. albopictus* (Lee, 1990). The origin of *Aedes albopictus* was found to be in Southeast Asia in the tropical forest region (Smith, 1956). Africa was found to be the origin for *Ae. aegypti* which then established in Malaysia (Lo & Narimah, 1984). The first recording reported regarding the invasion *Ae. aegypti* was in 1908 by Leicester and in 1914 by Staten (Lee & Cheong, 1987) with the first recorded incidence of dengue hemorrhagic fever reported in Malaysia was in Penang in the year 1962 (Rudnick et al., 1965).

The disease has then been endemic throughout Malaysia (Singh, 2000). By 1920, *Ae. aegypti* has migrated inland and was commonly found in Kuala Lumpur. *Aedes aegypti* was then discovered to completely spread across Peninsular Malaysia (Lee & Hishamudin, 1990). The infection can be seen obviously in the urban areas where 68.1% of the total population resides and the industrialization has revealed many man-made items that aid in the breeding of *Aedes* mosquitoes (Teng & Singh, 2001).

In Penang, *Ae. albopictus* is found to be a much more dominant species than *Aedes aegypti* (Rahim et al., 2018). *Aedes aegypti* are commonly found indoor as they are highly anthropophilic and tends to be near human dwelling (Juliano & Lounibos, 2005). Meanwhile *Ae. albopictus* is commonly found outdoors as they are aggressive, exophagic and exophilic mosquitoes which usually breed outdoors (Paupy et al., 2009). However, recently it was found that both species are can now be seen in a human dwelling (Rozilawati et al., 2007).

2.1.2 Biology and Behaviour of *Aedes* Mosquitoes

Aedes mosquito undergoes has four stages in its life cycle and goes through complete metamorphosis. It's life cycle begins with an egg which then a larva emerges. The larva then transforms into a pupa before emerging as an adult mosquito to complete its life cycle (Li et al., 2001). To oviposit eggs, female *Aedes* mosquitoes require a blood meal for nourishment and the development of the eggs (Vythilingam et al., 1992). However, both male and female *Aedes* mosquitoes could rely on flower nectars for their survival. After copulation and a blood meal, it takes four to five days for the *Aedes* mosquitoes to fully develop the eggs and being ready for oviposition of these eggs. The eggs of *Aedes* are then singly laid on damp surfaces where the water line is (Vythilingam et al., 1992).

According to Rozendaal (1997), the eggs of *Aedes* can withstand desiccation up to several months and they can still hatch when coming into contact with water. The eggs are capable of hatching within 1-48 hours upon contact with water (Vythilingam et al., 1992). A water penetrating siphon can be seen at the end of the tail of the hatched *Aedes* larvae or commonly called as "wrigglers" which facilitates them breathing. The larvae then feed on decaying organic matter and microorganism found in water when growing up from the first instar stage up to the fourth instar stage (Li et al., 2001). Upon reaching the late fourth instar, the larvae then undergo a short pupae stage or "tumblers" and are usually inactive and does not generally feed at this stage. The pupae breathe through a breathing apparatus called "trumpet" (Li et al., 2001). The last stage of the *Aedes* life cycle is reached when the pupae emerge as adult mosquito stage from the pupal skin. The emerged adult mosquitoes then fly away after the wing dries which sums up the *Aedes* mosquito life cycle (Figure 2.2).

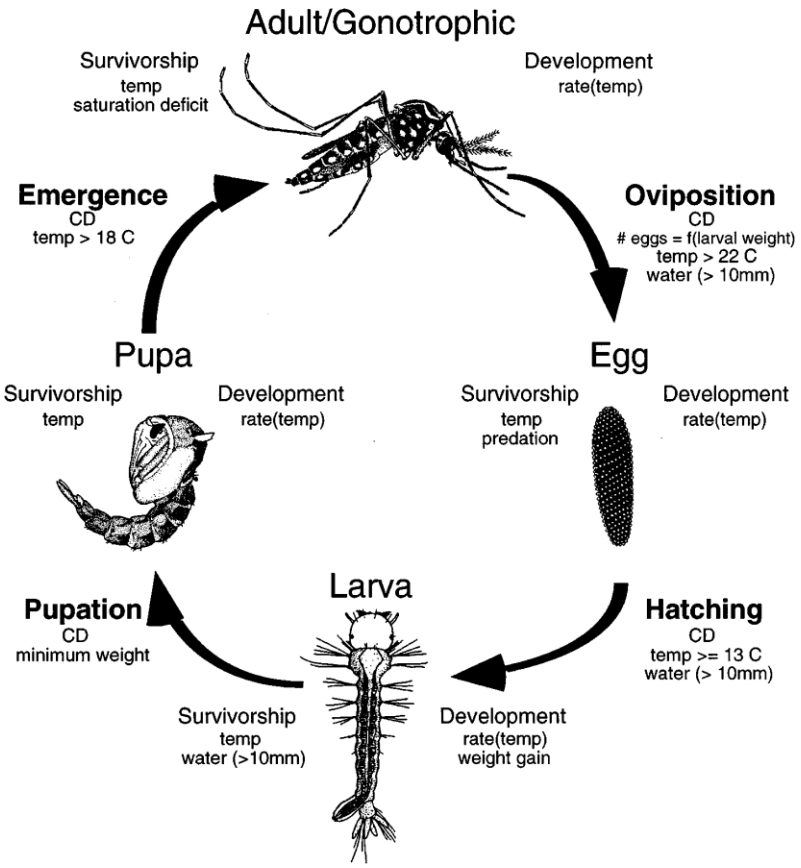

Figure 2.2: The life cycle of *Aedes aegypti* (Hopp & Foley, 2001)

2.2 Mosquitoes Control Approaches

Mosquito control has been conducted since the olden days with plenty of techniques being used regardless of the mosquitoes being disease carrier or not (Yap, 2003). Four primary approaches can be seen in controlling mosquitoes which includes environmental management, chemical control, biological control and personal protection (WHO, 2003).

Environmental management is an economical and environmental friendly measure in controlling mosquitoes. This is conducted by removing the mosquitoes breeding habitats, like containers and tires with standing water. Among these methods,

the most common method used in controlling mosquitoes is chemical control while biological control is more upcoming approach with concern to the environment. This chemical control method includes chemical larvicide, aerosol usage, thermal fogging and mosquito coils (WHO, 2003). Personal protection is an approach to minimize the interaction between human and vector. An example of such personal protection is by using screens on windows and doors and removing holes in roofs.

2.2.1 Environmental Management

Environmental management is known to be the most economical and environmentally friendly approach in the mosquito control programs as this approach revolves around source reduction. Source reduction helps in removing the breeding habitat of mosquitoes which is standing water. It was considered that standing water regions on river channels are prominent for mosquito breeding but the healthy biodiversity of the river channels contains a wide range of natural enemies to mosquito population (Barik, 2015). However, in urban areas, standing water is available in human-made containers like discarded tin cans and tyres which promotes the breeding of *Aedes* mosquitoes as there are no natural enemies to prey on the larvae in these standing waters.

It is important to consider container management in the household container uses. If the containers were found to be useless or non-essential, it is best to discard or destroy them (WHO, 2003). The destruction or elimination of unwanted artificial containers helps in reducing the population of *Aedes* mosquitoes. Method of source reduction includes cleaning up rubbish by cleaning up and packing the rubbish in disposable plastic bags and sent away to the local rubbish collection system. Discarded

tires should be disposed of in a way where they are placed away from the rain, placed under shelter and covered. The tyres can also be shredded and placed in a landfill (WHO, 2003).

2.2.2 Chemical Control

The use of chemical control as a mode of action has been in controlling house hold pest and public health (Zaim & Jambulingam, 2007). Controlling mosquito larvae by using of organic synthetic insecticides like insect growth regulators (IGRs) like methoprene are common as it inhibits the emergence of adult mosquitoes. However, this kind of chemical control may provide a long-term residual effect on larvae where at low dosages, there was no an immediate response seen. The larvicides was found only to interfere in chitin synthesis of the mosquito larvae during their moulting process (WHO, 2003). The application of larvicides at the correct timing and a proper location is essential in achieving maximum effectiveness, thus making it quite tricky to be used (WHO, 2003).

Although there are many reported short and medium duration of benefits in controlling vector that transmit diseases, several types of research have discovered that the continuous usage of such insecticides have caused a great threat to both the environment and human as well (Lu & Kacew, 2002; Schulz, 2004). Continued and prolonged low dose insecticides exposure towards human are found to be one of the cause in the rise of chronic health problems in humans. This health problems include reproductive problem, carcinogenic effects and respiratory problems (Mansour, 2004).

2.2.3 Biological Control

When it comes to biological control, natural products or natural enemies of the mosquito vectors like nematodes, fishes, pathogens and predators are often used. This control usually has different mode of actions, mechanisms and sites used in controlling the vector's population density (Ramirez et al., 2009; Al-Akel & Suliman, 2013). It was reported that *Toxorhynchites rutilus* larvae, a natural predator of mosquito larvae were found successful in controlling *Ae. aegypti* and *Culex quinquefasciatus* larvae in a field study (Focks et al., 1982). Larvicidal activity towards *Aedes*, *Culex* and *Anopheles* larvae were also reported by using *Bacillus thuringiensis,* a spore forming bacillus (Goldberg & Margalit, 1977) It was also reported that plant based extraction have proven to be effectives in controlling *Aedes* larvae and has the potential to be alternative biolarvicide (Rahuman et al., 2008).

2.2.4 Personal Protection

Personal protection can be done by using physical barriers to reduce the interaction between human and disease vectors. An example of personal protection includes the usage of screens on the windows and doors of the home and work space to hinder these mosquitoes from entering the place. Patching or eliminating holes visible from roofs, walls and other gaps helps in keeping the mosquitoes from entering the work areas or home is another example of personal protection. (Njie et al., 2009; Kirby et al., 2009). The risk of vector bites can be reduced when using an effective barrier such as treated and untreated bed nets inhibiting the invasion of several insect pests (Chappuis et al., 2007). The usage of repellents such as DEET provides

protection against *Ae. aegypti, Cx. quinquefasciatus* and *Anopheles dirus* up to six hours. (Tawatsin et al., 2001).

2.3 The Development of Resistance Towards Insecticides

The over usage of insecticides like permethrin and malathion to control these pests has caused resistance towards these insecticides. The frequent usage of chemical insecticides from the same group without rotation causes the development of such resistance. The frequent usage of insecticide has caused the insect to be able to be resistant to an insecticide as the gene configuration of the insect has changed to be resistant (Serrano, 2012). For example, chemicals made to eradicate mosquitoes in the earlier days like in 1946 such as DDT has caused mosquitoes to be resistant towards DDT on the subsequent year when tested on *Aedes solicitans* and *Aedes tritaeniorhychus* due to the over usage of DDT (Brown, 1986). Over 100 mosquito species were then found to be resistant to single or several insecticides since then.

Aedes aegypti have been reported to have resistance to all four classes of insecticides which includes carbamates, organochlorines, organophosphate and pyrethroid. However, there are also reports that the insecticide resistance level in *Ae. albopictus* seems to be relatively lower compared to *Ae. aegypti*, which is probably due to a lower exposure to household insecticides as they tend to live outdoors (Ranson et al., 2010).

2.4 New Approaches in Controlling Mosquitoes

With the constant threat on the environment with persisting synthetic insecticides and the development of resistance in mosquitoes due to over usage of the same type of insecticide, people are now opting on trying new approaches in controlling these mosquito vectors. Among the reported new approaches is using *Wolbachia pipientis* in controlling *Aedes* mosquitoes. It was reported that the usage of *Wolbachia* has halved the lifespan of *Aedes* mosquitoes and it was also reported that the usage is target specific (Iturbe-Ormaetxe et al., 2011).

Another method mentioned in the new approaches is the Sterile Insect Technique ("SIT") in controlling mosquitoes. This method involves reducing the sterility in mosquitoes which indirectly reduces the population of the mosquitoes. An example of "SIT" is by using juvenile hormone in an *Aedes* breeding station which causes the mosquitoes to be unable to reach sexual maturity (Benelli, 2015). However, the success of "SIT" was highly dependent on sterile males to compete with wild males in the field and due to its cost, it cannot be done on a large scale. A safer and cheaper approach in controlling the *Aedes* mosquitoes is plant-based insecticides where plenty of researches have been conducted and proven to be effective in controlling mosquitoes.

2.4.1 Plant-Based Insecticides

There has been a rise in research studies on plant-based insecticides which are known to be successful in controlling *Aedes* larvae. These insecticides are generally complex mixtures of several secondary metabolites that might contribute in the toxicity of the extract towards these insects (Miresmialli & Isman, 2014). Secondary

metabolites are metabolites found in the plant which does not serve a purpose on the development of the plant but helps to protect the plant from attacks from insect pests. Due to this mix of secondary metabolites, the development of resistance towards plant-based insecticides is highly unlikely when compared with synthetic insecticides as synthetic insecticides are generally based on a single active ingredient (Miresmialli & Isman, 2014). The major flaw and difference of plant-based insecticide instead of synthetic chemical are in the difficulties in standardizing the active ingredients that are found within the plant compound. There can be great variability in the quality and composition of these plant extract (Miresmialli & Isman, 2014).

Few plants based insecticides have been reported to have larvicidal activities such as the morning glory, *Ipomoea cairica* extract that is capable in killing *Aedes* larvae (Rattanam et al., 2014) and neem extracts, *Azadirachta indica,* is effective in controlling *Anopheles* larvae (Okumu et al., 2007). There has been plenty of literatures reported on these plants on their effectiveness against mosquito larvae. Among the reported plants is *Tridax procumbens,* or commonly known as coat button daisies. However, there were fewer literatures made on this plant in relation to it's larvicidal effects which opens many possibilities to be researched on it.

2.3 *Tridax procumbens*

Tridax procumbens, is a plant which comes from the family Asteraceae and is once known to be a non-beneficial weed infesting many countries (Plate 2.1). However, it was later found to have many benefits. Initially it has been a part of the traditional Indian medicine as an antifungal, anticoagulant and in treating dysentery, diarrhoea and bronchial catarrh (Ali et al., 2001). It was also known to be an

indigenous medicine in treating jaundice (Saraf & Dixit, 1991) and was reported to have wound healing activities and helps in hair growth (Saraf et al., 1991). It is now a well reputed plant in Ayurvedic medicine for liver disorders (Pathak et al., 1991). Ravikumar et al., (2004) reported on the antioxidant capabilities in *T. procumbens*. Recently it was found that *T. procumbens* has effective mosquito repellence activity (Kamaraj, et al., 2011) and larvicidal properties towards *Ae. aegypti*, *Anopheles stephensi* and *Cx. quinquefasciatus* (Devan, et al., 2013).

Plate 2.1: *Tridax procumbens*, or commonly known as coat button daisies.

2.4 Plant Extraction Techniques

There are a few extraction techniques reported in regards to plant extraction. Among these extraction techniques, the known conventional extraction techniques are Soxhlet extraction, maceration and hydrodistillation (Azmir et al., 2013). Maceration was once known to be used in tonic preparation for a long time. Maceration technique involves

grinding of plant and appropriate solvent was added. The mixture is then strained and pressed to obtain mixed liquid and impurities are removed through filtration (Azmir et al., 2013). This process is generally time consuming. Meanwhile hydrodistillation is a traditional process which uses water and heat in extraction. The plant is packed in a compartment and water is added then boiled. The mixture is then cooled and the water condenses leaving the extract. The drawback is that at high temperature some volatile compounds may be lost (Azmir et al., 2013). The Soxhlet extraction is one of the more common mode of extracting plant based extracts as it was reported to be capable of extracting larvicidal components from plant extracts compared to the other extractions. (Tonks et al., 2006)

2.4.1 Soxhlet Extraction

The Soxhlet extractor was designed in the year 1879 by a German chemist named Franz Ritter von Soxhlet for the extraction of lipids (Soxhlet, 1879) but it is now extensively used in the extraction of bioactive compounds from numerous natural sources (Azmir et al., 2013). The Soxhlet extraction follows a simple methodology where the sample (usually plant sample) is placed in a cellulose thimble with cotton placed on top. The cellulose thimble is then placed into the extractor. The extractor is then placed on the distillation flask which is placed on a heating mantle. The distillation flask is filled with the required solvent. The heating mantle is then heated up to the boiling point of the solvent, causing the solvent to evaporate and travel to the condenser. The condenser then cools down the solvent allowing the solvent to fill the extractor and causing the extract to leach out of the plant in the cellulose thimble along the way. When a designated level is reached, the condensed solvent is then flushed

back to the distillation flask at the bottom and is recycled back. The entire process will be repeated until the solvent in the flask appears semi-transparent. The setup of the Soxhlet extraction is shown in Figure 2.3.

There are some great advantages when using Soxhlet extraction. The displacement in the transfer equilibrium is facilitated by the fresh portions of the extractant when the extractant comes to contact with the sample repeatedly. As the extract is leached out through the cellulose thimble, there will be no need for filtration once the extraction is done. Moreover, only little training is required to conduct Soxhlet extraction and the extraction can extract more sample mass than other alternative extractions (Castro & Priego-Capote, 2010). However, the drawback of using Soxhlet extraction is that the duration required for the extraction is a long and large amount of solvent are wasted in the process. The reason for the Soxhlet extraction to be chosen to be used as the mode of extraction for crude *Tridax* extract is that it has been reported that Soxhlet extraction has more capability in extracting larvicidal component in a plant than several other extractions like cold extraction and reflux extraction (Tonk et al., 2006).

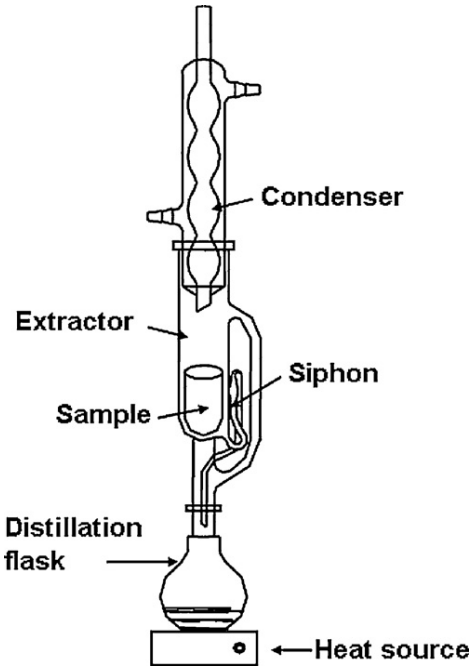

Figure 2.3: The Soxhlet apparatus setup (Castro & Priego-Capote, 2010).

2.4 Hydrolysis of Plant Extracts

It was discovered that *T. procumbens* have beneficial phytochemical compounds like saponin, tannin and carotenoid (Jude et al., 2009). However, these phytochemical compounds tend to be bound with glycosides which might hinder the efficiency in the reactivity of the compounds. It was proven that the glycosylation of compounds like flavonoids decreases the efficiency in some of the chemical activities in the plant like the antioxidant effect (Rice-Evans et al., 1996; Li et al., 2009).

A possible way to remove glycosides from the beneficial phytochemical compound is through acid-hydrolysis, where acid hydrolysis helps in breaking the bond of the glycosides leaving on the glycoside free aglycones present to be reextracted. It was also reported that a hydrolyzed fraction of a plant extract has a greater chemical activity like antioxidant effect than unhydrolyzed fractions which

contains flavonoid glycosides (Pratt, 1992). However there three different types of glycosides to be considered when approaching hydrolysis which are the easily hydrolyzed glycosides, slowly hydrolyzed glycosides and acid resistant glycosides (Bohm, 1998). Hence it best to find the right duration and temperature of hydrolysis which would help in facilitating the cleaving the glycosides bond to the bioactive compounds leaving behind the bioactive aglycones.

2.5 Phytochemical Analysis

The detection of phytochemicals in a plant extract are normally conducted through one or a combination of several chromatography techniques. These techniques include Gas Chromatography, High-Performance Liquid Chromatography (HPLC), Ultra-Performance Liquid Chromatography (UPLC), Thin Layer Chromatography (TLC) and Paper Chromatography (PC). The types of chromatography technique used in a study usually depends on the volatilities and solubility solvent used in extraction.

HPLC is normally used for compounds with low volatility while Gas Chromatography is used in volatile compounds to detect phytochemicals. TLC is a method used for separating lipid soluble compounds like chlorophylls, steroids and carotenoids, while water soluble plant compound like carbohydrates, phenolic acids, organic acids and amino acids uses PC (Harborne, 1998). Hence prior to any phytochemical analysis being conducted, it is important for us to know the type of compound that we are using to choose the suitable analysis.

2.6 Gas Chromatography-Mass Spectrometry

Gas Chromatography-Mass Spectrometry is a phytochemical analysis which comprises of two analytical methods into one. One of the methods is the gas chromatography, where this process can separate volatile and semi-volatile compounds easily but it is unable to identify them. Meanwhile, the other method which is the mass spectrometry, is a process that can provide detailed information on compounds to be identified, but could not separate them between compounds. Therefore, it was shortly after the development of the methods that the two analysis were combined in the mid 1950's. They are also known to be very compatible techniques as both techniques require the sample in the vapor phase and both technique deal with the same amount of the sample (Hites, 1997).

It is known to be an important analysis in the identification and quantitation of volatile and semi-volatile organic compounds in complex mixtures. Through GC-MS, we can identify and determine the elemental composition and molecular weights of an unknown compound in a mixture (Hites, 1997). There are several important things to note when using GC-MS. First, the compound used for detection must be in solution form for injection. The solvent used to the mix and dilute the sample must be volatile and organic. The gas chromatographic run is usually conducted at a duration between 20 and 100 minutes and the analysis of the data will be obtained by one to 20 hours or more depending on the level of details needed.

The main component of GC-MS comprises of an ionization chamber, mass analyzer and detector which is maintained at a high vacuum (Figure 2.4). The Gas Chromatography works by first entering the mobile phase where the carrier gas, which is usually an inert gas such as helium enters the microscopic layer of polymer, inside

glass or metal tubing (column) which contains a stationary phase; a fine solid support coated with a non-volatile liquid. The sample is then moved through the column by the help of helium gas. The components in a sample are split from one another as according to the time taken for each component to pass through the column than others. When the sample exits the gas chromatography column, ionization fragments the sample, and the fragments are sorted by mass to form a fragmentation pattern. Identification of component can be made by viewing the fragmentation patterns, like retention time as they are unique (Hussain & Maqbool, 2014). Over the years, GC-MS has known to be the technological medium for profiling secondary metabolites in both plant and non-plant extract. The usage of GC-MS was also reported in several types of research to find the bioactive compounds in plants (Rukshana et al., 2017; Casuga et al., 2016) making the process to be chosen for this study.

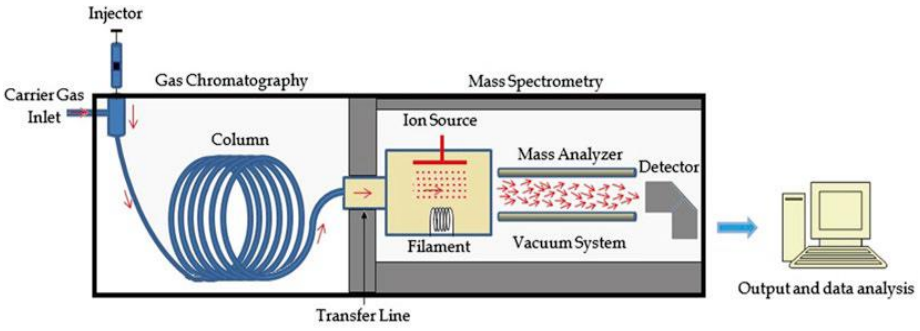

Figure 2.4: Schematic plot of the main components in GC–MS instruments (Emwas et al., 2015).

CHAPTER 3

EFFECTIVENESS OF *Tridax procumbens* PLANT EXTRACTS AGAINST DENGUE VECTOR, *Aedes albopictus* AND *Aedes aegypti* (DIPTERA: CULICIDAE).

3.1 INTRODUCTION

A huge infectious disease burden has been caused by vector-borne diseases from mosquitoes such as dengue fever, Zika, yellow fever, Chikungunya and malaria and nearly 3.5 billion people worldwide are at risk of being infected by at least one type of the vector-borne pathogen (WHO, 2004; Stanley et al., 2008). The epidemic of the dengue outbreak transmitted by *Aedes aegypti* as the primary vectors together with *Aedes albopictus* as secondary vectors has grown concern to the public. Due to the alarming rate of the number of dengue cases in Malaysia, preventive measures are being researched to halt the outbreak.

Chemical control is one of the major types of control measure being used in controlling the increasing number of *Aedes* mosquitoes (WHO, 2003). The heavy dependence on such insecticides has caused the development of resistance to insecticides (Mallet, 1989). Insect resistance is known when there is a reduction in the insect susceptibility towards an insecticide enabling the insect to withstand the insecticide upon the exposure of standard dose of the insecticide through an evolutionary process (WHO, 2012). The heavy usage of such synthetic chemical imposes a threat to human health and the environment as such chemicals tends to persist in the environment for a long time. People are now opting on using an environmental friendly approach in controlling these mosquitoes such as the usage of biopesticides and biocontrol agents.

Recently, extractions from plants derivatives have been proven to be possible alternative biolarvicides (Rahuman et al., 2008). Among the plants that can be used is *Tridax procumbens,* a plant which comes from the family Asteraceae. *Tridax procumbens* has been reported to have effective repellent activity against mosquitoes (Kamaraj, et. al., 2011), with larvicidal properties towards *Ae. aegypti*, *Anopheles stephensi* and *Culex quinquefasciatus* (Devan, et. al., 2013). It was then found that *T. procumbens* extracted with methanol was the most effective in killing *Ae. aegypti* and *Cx. quinquefasciatus* larvae at 53 ppm. Whereas, the study by Kamaraj et al. (2011) revealed that the leaf acetone extract of *T. procumbens* is toxic to *Anopheles subpictus* at 51.57 mg/ml and the leaf ethyl acetate extract on *Culex tritaeniorhynchus* at 69.16 mg/ml. It is also known to be used to treat malaria (Jude et al., 2009).

The part of the *Tridax* plant responsible for having the high larvicidal properties is still unknown. Therefore, this study is conducted to investigate the larvicidal properties of crude extract from the different parts of *T. procumbens* plant against the laboratory strain of *Ae. aegypti* and *Ae. albopictus*.

3.2 MATERIALS AND METHOD

3.2.1 Plant Collection and Extraction

Tridax procumbens were collected around Universiti Sains Malaysia (5° 21' 20.52" N, 100° 18' 4.32" E). The collected plants were separated into six parts which are the flowers, stem, leaves, roots, aerial part of the plant (consisting of flower, stem and leaves) and the whole plant (Plate 3.1). The separated parts were dried under room temperature (28±3 °C) for 10-14 days to allow all the moisture to be evaporated from the plant. The dried plant parts were then blended into a fine powder using commercial