EFFICACY OF NANO-SYNTHESIZED SILVER PARTICLES FROM *Azolla Pinnata* EXTRACT ON *Aedes Aegypti* LARVAE (DIPTERA: CULICIDAE)

By

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A thesis submitted in fulfilment of the requirements for the degree of Bachelor of Applied Science (Sustainable Science) with Honours

FACULTY OF EARTH SCIENCE
UNIVERSITI MALAYSIA KELANTAN

2019

DECLARATION

I declare that this thesis entitled 'Efficacy of Nano-Synthesized Silver Particles from *Azolla Pinnata* extract on *Aedes Aegypt*i Larvae (Diptera: Culicidae)' is the result of my own research except as cited in the references. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

Signature : _____

Name : _____

Date :_____

ACKNOWLEDGMENT

First and foremost, I would like to express gratitude to my research supervisor, Dr. Mohd. Faiz bin Mohamad Amin who provided insight, expertise and understanding that greatly assisted the research.

I am also grateful to Dr. Rajiv Ravi (Post-Doctoral Researcher of UMK) and Miss Nor Shaida Husna Zulkarnain (Post-Graduate student of UMK) for their co-operation, guidance and suggestions while carrying out this research. They raised many points in our discussion that have helped improved this paper.

Besides, I would also like to acknowledge Dr. Intan Haslina binti Ishak, lecturer of School of Biological Science in Universiti Sains Malaysia (USM) Penang, for her encouragement and laboratory facilities provided throughout this research. Not to forget, my gratitude also goes to Vector Control Research Unit (VCRU), USM Penang for the provision of stock solution and mosquito eggs for this project.

My heartfelt gratitude also goes to all laboratory assistants of UMK Jeli that have been helping me with all the laboratory work and facilities, especially Mr. Rohanif for the assistance and toleration.

Completing this thesis required more than academic support. Thus, I would like to thank all my colleague for giving me moral support throughout the journey, especially Maizura binti Che Mat, my Final Year Project partner for the teamwork and support for each other.

Most importantly, none of this could have happened without my family. My father, Mohd Rasli bin Awang, my mother, Wan Zakiah bt Wan Su and all my siblings who would always gave encouragement and motivation throughout the whole process. This paper stands as a testament to their unconditional love and support. Nevertheless, all praises to Allah for giving me the opportunity and strength in completing this thesis.

Efficacy of Nano-Synthesized Silver Particles from *Azolla Pinnata* Extract on *Aedes* (Diptera: Culicidae) Larvae

ABSTRACT

Dengue are a worldwide issue which is mostly found in tropical and sub-tropical climates around the world. Human being can get infected with dengue through the bite of female mosquito of *Aedes Aegypti*. The widely used synthetic insecticide in the operation of mosquito control could result in unfavorable impacts to the environment, human health and non-target organism. Considering these issues, environmental friendly insecticide from plant extract have been used as a green alternatives by recent researchers. Unfortunately, the method of using plant extract as insecticide require a large amount of raw plants to be used. In relation to this problem, the usage of nanoparticles that possesses unique characteristics including small size and potential in changing physical, chemical and biological properties of organisms were studied. Nano-synthesized silver particles from *Azolla pinnata* extract were thus investigated in this study in order to determine its efficacy as *Aedes Aegypti* larvicide. Nano-synthesized silver particles from *Azolla pinnata* extract were prepared in six different concentrations and set in plastic cups. Late third instar larvae of *Aedes aegypti* were being used in all tests. Based on the findings of the experiment, there were no mortality of larvae recorded in control groups after 24 hours of exposure. The lowest mortality recorded was at 10 ppm with only 7.5% mortality, while 95% mortality was recorded for the highest concentration which was 250 ppm. Meanwhile, the LC_{50} and LC_{95} obtained at 95% confidence interval after 24 hours of exposure were 121.570 ppm and 369.438 ppm respectively. Further studies should be done to determine the mechanisms of silver nanoparticles in aiding *Azolla pinnata* as an effective larvicide in the future.

Keberkesanan Nano-Sintesis Zarah Perak dari Ekstrak *Azolla Pinnata* pada Jejentik *Aedes* (Diptera: Culicidae)

ABSTRAK

Denggi adalah isu dunia yang kebanyakannya berlaku di iklim tropika dan sub-tropika di seluruh dunia. Manusia boleh dijangkiti dengan demam denggi melalui gigitan nyamuk betina *Aedes Aegypti*. Racun serangga sintetik yang digunakan secara meluas dalam proses pengendalian kawalan nyamuk boleh memberikan impak buruk kepada alam sekitar, kesihatan manusia dan organisma bukan sasaran. Dalam mempertimbangkan isu ini, racun serangga mesra alam dari ekstrak tumbuhan telah digunakan sebagai alternatif hijau oleh penyelidik kini. Malangnya, kaedah menggunakan ekstrak tumbuhan sebagai racun serangga memerlukan sejumlah besar tumbuhan mentah untuk digunakan. Berhubung dengan itu, penggunaan zarah nano yang mempunyai ciri-ciri unik termasuk bersaiz kecil dan berpotensi mengubah sifat fizikal, kimia dan biologi organisma telah dikaji. Oleh itu, zarah-zarah perak yang disintesis secara nano dari ekstrak *Azolla pinnata* telah dikaji untuk menentukan keberkesanannya sebagai pembunuh jentik-jentik *Aedes Aegypti*. Zarah perak yang disintesis secara nano dari ekstrak *Azolla pinnata* telah disediakan dalam enam kepekatan yang berbeza dan ditetapkan dalam cawan plastik. Jentik-jentik tahap ketiga *Aedes aegypti* digunakan dalam semua ujian yang dijalankan. Berdasarkan penemuan kajian, tidak ada kematian jentik-jentik yang direkodkan dalam kumpulan kawalan selepas 24 jam pendedahan. Kematian terendah yang direkodkan adalah pada 10 ppm dengan kematian hanya 7.5%, manakala kematian 95% dicatatkan pada kepekatan tertinggi iaitu 250 ppm. Sementara itu, LC_{50} dan LC_{95} diperoleh pada selang keyakinan 95% selepas 24 jam pendedahan di mana masing-masing berjumlah 121.570 ppm dan 369.438 ppm. Kajian lanjut perlu dilakukan untuk menentukan mekanisma zarah perak nano dalam membantu *Azolla pinnata* sebagai pembunuh jentik-jentik yang cekap pada masa hadapan.

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LIST OF ABBREVIATIONS

Ag	Silver
AgNO3	Silver nitrate
NPs	Nanoparticles
AgNPs	Silver nanoparticles
cm	Centimeter
DNA	Deoxyribonucleic acid
LC	Lethal concentration
mL	Mililitre
mm	Milimetre
um	Micrometer
nm	Nanometre
ppm	Part per million
ROS	Reactive oxygen species
Sp.	Species
UV	Ultra-violet
VCRU	Vector control research unit
WHO	World health organization

LIST OF SYMBOLS

%	Percentage
±	Plus or minus
^{o}C	Degree Celsius
>	Greater than
<	Lower than
x	Multiply
/	Divide

CHAPTER 1

INTRODUCTION

1.1 Background of Study

One of the arthropod group that plays a role as the main vector in transmitting diseases like dengue fever, malaria, and chikungunya is mosquitoes (Shanmugasundaram *et al*.,2015). Dengue are a worldwide issue which is mostly found in tropical and sub-tropical climates around the world. In Western Pacific Region, Malaysia and Philippines, the cases recorded in the year 2016 were 375 000 cases, 100 028 cases, and 176 411 cases respectively (WHO, 2017). While recently in Malaysia, 55 744 dengue cases with 131 deaths were reported from January till July 2017 (WHO, 2017).

Human being can get infected with dengue through the bite of female mosquito of *Aedes Aegypti*. Within 8-12 days after female *Aedes Aegypti* mosquito acquire the virus by feeding on blood of infected person, the virus will spread to the its salivary gland (WHO, 2017).

Sustainable approach of mosquito control is important in order to improve the state of environment and public health while preventing mosquito borne disease. The widely used synthetic insecticide in the operation of mosquito control have a few adverse effects. Not only the target population, but also the non-target population

could get the effects from these synthetic insecticides, where it can give risk to the people and environment. (Ghosh *et al.*, 2012). In relation to these issues, a safe eco-friendly way like the utilization of plants that could exhibit insecticidal properties have been explored in recent past (Bagavan *et al.*, 2011). The fact that larvae and pupae breed in water ease the application of insecticide in this habitat compared to the habitat of adult stage (Bagavan *et al*., 2011).

In synthesizing metal and metal oxide nanoparticles (NPs), aquatic macrophytes are one of interesting potential research candidate that can hyper accumulate heavy metals (Korbekandi *et al.*, 2014). A plant called as *Azolla pinnata* was discovered by some researcher from past study that can prevent breeding of mosquitoes (Mwingira *et al*., 2009). In parallel to the discovery, many researchers like Ravi *et al*. (2018), and Shaida *et al*. (2018) have carried out some experiments on the plant which gave a positive feedback against *Ae. Aegypti.* This free floating aquatic pterophyte can grow rapidly where within three to five days, *A. pinnata* can cover the water surface of stagnant wetlands by doubling its biomass. *A. pinnata* can be found massively in Africa, tropical Asia, southern Japan, southern and eastern China (Swatdee, 2016).

However, smaller and efficient molecules are needed in order to increase the efficiency of the plants extract as green insecticide. These small molecules can aid in the process of absorption of the green insecticide molecules into the larvae. Nanoparticles (NPs) in the field of nanotechnology are one of the candidates that have the potential as being the smaller molecules needed. Nanotechnology is an emerging technology with variety of applications in all aspects. Nanotechnology involve the process of synthesis and the application of particles that are in the range of 1–100 nm

in terms of size which means these particles possess a high surface-to-volume ratio leading to a better contact with the organisms (Hussain *et al*., 2016). While there are several types of nanoparticles in the industries such as gold nanoparticles, the researchers have been brought to attention towards silver nanoparticles because of their antimicrobial, antiviral and suitability to be used in various fields like material sciences and medicine (H.Korbekandi *et al*., 2014).

1.2 Problem Statement

Living organisms and environment can be affected by the hazardous chemical properties of conventional insecticides. Human who are exposed to synthetic insecticide are potentially affected with problems such as kidney and liver damage, cancer, developmental and reproductive effects, and other problems.

Considering these issues, environmental friendly insecticide from plant extract have been used as a green alternatives by the recent researchers. This can be seen through one of the past research showing the efficiency of fresh and powdered *A. pinnata* against *Ae. Aegypti*. Unfortunately, the method of using plant extract as insecticide require a large amount of the plants, where the highest mortality recorded is 1853 ppm for powdered *A. pinnata* and 2,521,535 ppm for fresh *A. pinnata* (Shaida *et al*., 2018). This raised problem in the extraction process due to the vast amount of raw plants needed, making it hard to be commercialize. In addition, the size of the plant extract molecules are particularly large which decrease the rate of absorption of the extract into the larvae.

Therefore, this study uses nano-synthesis of silver particle from *A. pinnata* that act as a vector against *Ae. aegypti* larvae. Due to the small size of nanoparticles where the diameter does not exceed 100 nm, will help the nanoparticles to easily penetrate across the cell membrane, avoiding defense mechanisms (Ahbirami *et al.*, 2014). This characteristics of nanoparticles aid in the process of absorption of insecticide by the mosquitos' larvae.

1.3 Objectives

i. To determine the mortality range (between 5% and 95%) of *Aedes aegypti* larvae when tested with nano-synthesized silver particles from *Azolla Pinnata* extract.
ii. To determine the lethal concentration of 50 and 95 (LC_{50} and LC_{95}) of the nano-synthesized silver particles from *Azolla pinnata* extract in *Aedes Aegypti*.

1.4 Scope of Study

In this research, silver nanoparticle (AgNPs) solution synthesized by *A. pinnata* extract and silver nitrate ($AgNO_3$) solution was used as insecticide against late third larvae of *Ae. Aegypti*. The larvae rearing and insecticide testing was set up in Research Laboratory of School of Biological Science, of Vector Control Research Unit (VCRU) in Universiti Sains Malaysia (USM), Penang. The experiment was set up under 25 °C ± 2 °C temperature with 75 ± 5 % relative humidity.

The mortality of larvae were observed and recorded after 24 hours exposure with different concentration of reaction mixtures. The range of mortality vary according to concentration of the solution. From the mortality range recorded, lethal concentration of 50 and 95 (LC_{50} and LC_{95}) was determined by using IBM SPSS.

Silver nanoparticles was used because of its uniqueness that can potentially change biological, chemical, and physical properties. Due to their surface-to-volume ratio, these nanoparticles is assume to aid in the absorption process of insecticide into larvae. Furthermore, in some experiments of using in vitro and in vivo cultured animal tissues showed that silver nanoparticles caused an oxidative stress characterized with well reactive molecules containing free oxygen radicals (reactive oxygen species or ROS), cell apoptosissome or genotoxicity with DNA break (Kim & Ryu, 2013).

1.5 Significance of Study

This research study was proposed due to the awareness on the uses of synthetic insecticides that are harmful towards living organisms and environment.

Based on the research, it stated that *A. pinnata* was used in synthesizing silver nanoparticles that will then tested against late-third instar larvae of *Ae. aegypti*. Due to unique characteristic and mechanisms of silver nanoparticles, it has a higher potential that the insecticide is being absorbed faster by the larvae compared to synthetic insecticide or organic insecticide without silver nanoparticles. Nanoparticles can be represented as foreign elements that may interfere with the normal physiological mechanisms of the larvae resulting in a high mortality rate due to the nanoparticles physicochemical properties.

Through this research, it can justify the potential of silver nanoparticles synthesized by *A. pinnata* extract as insecticide for *Ae. aegypti* larvae. The behavior and response of each larvae could be observed along with different concentration of reaction mixtures been used. In addition, justification also can be done on the potential of silver nanoparticles that could help *A. Pinnata* extract to cut down the time and amount of *A. Pinnata* plant needed to kill the larvae of *Ae. Aegypti*. This research could contribute to new findings by further investigation to find out the mechanisms of silver nanoparticles involved in aiding *A. pinnata*, as larvicidal against *Aedes Aegypti* larvae.

CHAPTER 2

LITERATURE REVIEW

2.1 The Biology, Ecology and Morphology of *Aedes Aegypti*

Adult *Ae. Aegypti* or also known as adult yellow fever mosquito is approximately 4 to 7 millimeters, a small to medium-sized mosquito. White scales on the dorsal (top) surface of the thorax of *Ae. aegypti* adults have a shape like a violin or lyre. The appearance of stripes on *Ae. Aegypti* could be seen because of the white basal segments on each tarsal segment of the hind legs. The abdomen of *Ae. Aegypti* is generally dark brown to black, but white scales also may present (Leslie Rios, 2011).

Male mosquitoes of *Ae. Aegypti* are smaller than female. They have a few distinguishing properties which include a bushier antennae of male mosquitoes than female mosquitoes. Male mosquitoes fed on pollen and nectar from flowers while females consume pollen only for energy and they need a blood meal instead as a protein supply in producing eggs. (Clements & A.N, 2000).

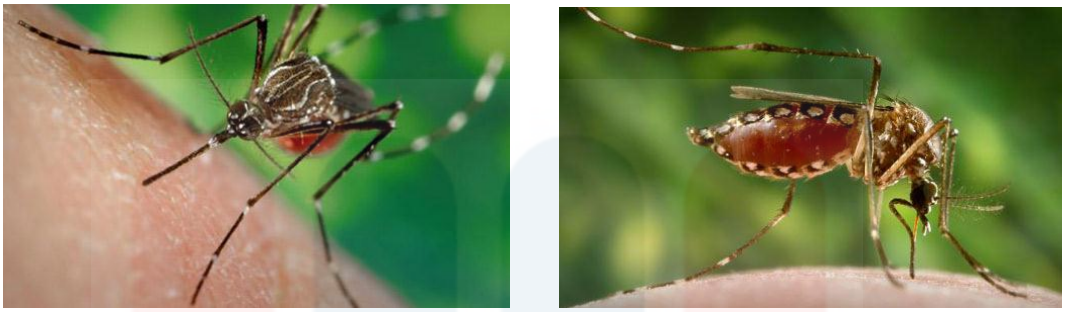

Figure 2.1: Adult *Aedes Aegypti* (Source: Featured Creatures, 2008)

2.1.1 Life Cycle of *Aedes Aegypti*

Ae. aegypti is holometabolous insect, meaning that they have a complete cycle of metamorphosis which include egg stage, larva stage, pupa stage, and adult stage.

After consuming a complete blood meal, an average of 100 to 200 eggs per batch are produced by female mosquitoes. However, blood meal size could affect the number of eggs produced where a bigger blood meal produces more eggs and vice versa (Nelson, 1986). The eggs are approximately one millimeter long which have an ovoid shaped and smooth texture. Eggs appear white when first laid but within a few minutes will become shiny black. In cooler climates, development of the eggs can take up to a week while in warmer climates, like the tropics, eggs may develop in as little as two days (Fosterand & Walker, 2002). As stated by Nelson in his study (1986), for months, the eggs can survive desiccation and will not hatch unless submerged in water.

The action of wiggling sporadically in water when being disturbed made mosquito larvae to be called as ‘wigglers’ or ‘wrigglers’. Based on Nelson (1986), the body of larvae hang vertically in water while the siphon is held above the surface of water, allowing them to breathe oxygen. For feeding process, larvae use their labral mouth brushes by creating water current in order to properly choose particles that will be pass to their mouth (Clements & A.N, 2000).

Pupae stage will appear as soon as the fourth instar ended. Pupae is also known as ‘tumblers’, and this tumblers can respond to stimuli and very active especially when being disturb. Pupae take approximately two days to develop and they do not feed while developing into adult stage (Clements & A.N, 2000).

Once developed, pupae skin will break by bloating their abdomen by swallowing air. Adult mosquitoes emerge their head first once the skin is broken. Depending on the environmental factors, adult mosquitoes can live within two weeks, and up to a month (Maricopa, 2006). The life cycle of *Ae. aegypti* can be summarize as in Figure 2.2.

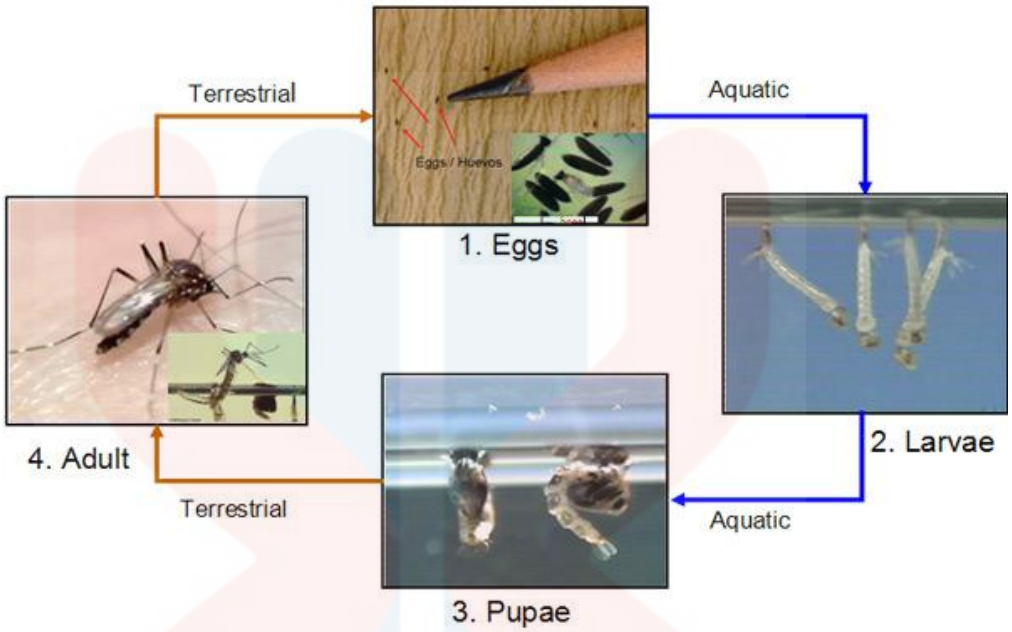

Figure 2.2: Life cycle of mosquito from eggs to adults (Source: Centers for Disease Control and Prevention, 2012)

2.2 Mosquito Vector Control in Malaysia

Controlling the mosquito vector which are both adult mosquitoes and larvae is being the only way to prevent dengue which do not have any commercial vaccine (WHO, 2013). The conventional way being used in controlling dengue vector in Malaysia is through the applications of chemical controls. However, this control program have some weakness in environmental factors such as the insecticide resistance or effectiveness, wind direction, and technical problem like treatment timing and droplet size (Lee, 2000).

2.3 Nanoparticles Aids in Mosquito Larvae Vector Control

Nanoparticles (NPs) are widely used in the process of new material production owing to their unique characteristics of having a 100nm dimension or less. Various benefits could be achieved because of the small size including a large surface area per unit mass, high internal pore volumes, high chemical reactivity, enhanced cell penetrability and surface property (Kim & Ryu, 2013).

2.3.1 Silver Nanoparticles, AgNPs

The small size of AgNPs can be seen greatly relevant to their toxicity. Silver nanoparticles can caused a higher DNA damage, oxidative stress and lethality based on several studies on silver nanoparticles. This might be due to their small size that enhanced the toxicity in mammalian cells by their potential of penetrating the cell more efficiently (Kim *et al*., 2013).

Owing to their small size, smaller silver nanoparticles have a larger surface area to volume ratio that are available for interactions with cellular organelles once they are internalized. This factor thus resulted in a more significant toxicity (Liu *et al.,* 2010). Besides, smaller silver nanoparticles also have a higher potential in releasing a higher toxic Ag+ ions from their surface.

Furthermore, silver nanoparticles are of interest compared to other nanoparticles because of their unique properties which can be applied into antiviral aspects, antimicrobial aspects, cryogenic super-conducting materials, biosensor materials, electronic components and cosmetic products. Their unique characteristics are also referred to their surface plasmon resonance, physico-chemical properties, biological applications or surface-enhanced raman scattering (SERS). Biomedical and industrial research are the examples that uses biologically synthesized silver nanoparticles (Trefry, 2011).

Due to the antimicrobial properties of silver nanoparticles, various mechanisms of the properties have been proposed. The mechanisms that being proposed are mostly based on the mechanisms of silver ion toxicity. This is due to the silver nanoparticles and silver ions (Ag+) that have the same elemental composition.

The proposed mechanisms as an antiviral agents include disruption of membrane potential, depletion of intracellular ATP, reactive oxygen species (ROS) production, DNA damage, inactivation of proteins, and silver ions productions from the silver nanoparticles which then would mediate the aforementioned mechanisms (Trefry, 2011). This means, silver nanoparticles have a high potential in inducing mortality to *Ae. aegypti* larvae owing to the disturbance of silver nanoparticles against the larvae's system.

Apart from that, there are evidences from a few studies showing that silver nanoparticles act as a good insecticide in mosquito vector controls nowadays (Patil et al., 2012). One of the present study reports, the larvicidal activity of silver nanoparticles against *A. subpictus*, *C. quinquefasciatus* and *A. aegypti* synthesised

using actinobacterium which is *Streptomyces* sp. M25 from Western Ghats ecosystem, Tamil Nadu, India (Shanmugasundaram & Balagurunathan, 2015). In addition, PMA-capped silver nanoparticles synthesized by UV-irradiation was found to be effective as larvicide towards *A. aegypti* (Sap-Iam *et al.,* 2010).

2.4 Plant Derived as Mosquito Vector Control

Some plants have the ability to act as mosquito vector control due to their insecticidal properties. In 2004, Park identified that *Piper nigrum* and *Piper longum* could act as a great larvicide against larvae of mosquitoes. A few of herbal products from herbs, shrubs, trees and ornamental plants have been used against mosquito vector of different species to see the mosquito insecticidal properties (Ghosh *et al.*, 2012).

Low degree of toxicity in plants resulted in a lower hazard to human health could contribute to a lower accumulation of hazardous residues in the environment (Isman, 2006). The phytochemicals in some plants may have the potential of being involved in the mechanisms as insecticidal. Abraham in 2012 stated that the phytochemicals in plants react to larvae based on their chemical nature. The examples of phytochemicals in some plants derived secondary material including alkanes, alkenes, alkynes, terpenes, alkaloids, stereiods, flavonoids, pterocarpens and lignans (Abraham *et al.*, 2012).

2.5 Properties of *Azolla pinnata*

2.5.1 Taxonomy of *Azolla Pinnata*

Two classifications of *Azolla sp*. are Euazolla and Rhizosperma (Lumpkin *et al.*, 1980). These two sub genus have their own species as listed in the Table 2.1 below.

Table 2.1: List of species for each sub genes

Sub genus	
Eauazolla	***Rhizosperma***
Azolla caroliniana sp.	*Azolla nilotica sp.*
Azolla filiculoides sp.	*Azolla pinnata sp.*
Azolla microphylla sp.	
Azolla Mexicana sp.	
Azolla rubra sp.	

2.5.2 Distribution of *Azolla pinnata*

Azolla sp. are generally native to Asia, Africa and America and they are commonly known as water fern, mosquito fern, fairy moss, and duckweed fern. *A. pinnata* can be found naturally in marshy ponds, swamps, lakes, rivers, and paddy fields in most of Asia and the coast of tropical Africa. *A. pinnata* can cover the

entire water surface areas and form a thick mat owing to their ability to grow rapidly in around 2-3 days (Lumpkin *et al.*, 1980).

2.5.3 Morphology of *Azolla pinnata*

A. pinnata as shown in Figure 2.3 are small and fast growing free floating aquatic fern. Each plant has an average diameter size of 1-2.5 cm. *A. pinnata* has a triangular shape with its midsection is typically straight and pinnately arranged side branches that are no longer towards their base (R.C., 1921).

Figure 2.3: Pictures of *Azolla pinnata* (Source: Ravi *et al.*, 2018)

2.5.4 Utilization of *Azolla pinnata*

The usage of this plants is not limited to agriculture as green manure only, but *A.pnnata* also have a high potential in phytoremediation instead. The strong phytoremediation capacities are due to their ability and properties to grow rapidly, have a higher protein content, higher biomass production and also the ability to adapt to a wide range of environmental conditions (Mandakini *et al.,* 2016).

Apart from that, *Azolla sp*. also can contribute to paddy cultivation that act as bio fertilizer and incorporated into the soul that helps in increasing nutrient content and humus of the soil (Peters, 2014).

2.5.5 Utilization of *Azolla pinnata* as Larvicidal and Mosquito Repellent

Recent study on *A. pinnata* utilization in the form of powder and fresh *A.pinnata* have been done against late-third larvae of *Aedes sp*. Through the research they found that at 1853 ppm of powdered *A. pinnata* and 2,521,535 ppm of fresh *A. pinnata*, all the larvae tested were found dead (Shaida *et al.*, 2018).

In parallel to the research, a research of using different solvent in extraction process of bioactive compound from *A.pinnata* that will then be used against *Aedes sp.* larvae were also conducted (Ravi *et al*., 2018). From the research, methanol solvent and acetone solvent that were used showed 95% larvae mortality at 1293 ppm and 1302 ppm respectively.

This can be due to the existence of active compounds in *A. pinnata* that act as larvicidal against the larvae. This can be proven by the presence of 27 phytochemical compounds from extracted *A.pinnata* using methanol as the solvent. The mortality of the larvae tested were due to the larvicidal and insecticidal properties of the phytochemical compounds (Ravi *et al.*, 2018).

Besides, a research in Tanzania showed a surveyed that has been done on *Azolla pinnata* at different percentage of coverage. On 105 % water bodies covered with *Azolla sp.*, 80 % were covered with green *Azolla sp*. that seems like mat on water surface while 25 % covered with brown *Azolla sp.* mat. The results showed that water bodies infested with green *Azolla sp*. have less mosquito larvae productivity than those covered by brown colored *Azolla sp.* substrates (Mwingira *et al.*, 2009). It shows that although they are in different growth stage, but still both of them have the potential to disrupt mosquito larvae productivity.

2.6 WHO Protocol for Mosquito Larvicidal

In evaluating mosquito larvicidal, there are three stages involved including laboratory studies, small scale field trial and large scale field trial according to WHO protocol (WHO, 2015). This research involved laboratory study which used laboratory-strain *Aedes aegypti* late-third instar larvae.

The larvae biological activity were being evaluated by exposing the larvae with the bioassay for 24 hours at various concentrations. The percentage mortality of larvae and the lethal concentration (LC_{50} and LC_{95}) were then recorded and evaluated in order to determine the efficiency of the bioassay.

2.7 Probit Analysis for Mosquito Insecticide

Probit analysis is commonly being used in evaluating dose response experiments in various fields. The probit analysis is being used in order to determine the relative toxicity of chemicals in the larvae tested.

Determination of the toxicity is being done by observing and comparing the response of organisms when tested with different concentrations of the chemical.

The response from the experiment should always be binomial (for example death or no death) and the relationship is always sigmoid. Then, the sigmoid will then transforms into linear by probit analysis and regression was run based on the relationship (Finney, 1952).

The output obtained were used to compare the amount of chemical required to cause wanted response. The LC_{50} value represent the concentration at which 50% of the sample population responds while LC_{95} represent the concentration at which 95 % of the sample population responds (Finney, 1952).

CHAPTER 3

METHODOLOGY

3.1 Study Area and Study Design

The study area to test the experiments was in Universiti Sains Malaysia (USM), Penang Campus.

All of the experiments carried out were conducted in USM's Vector Control Research Unit by referring to WHO (2005) guidelines. The laboratory has an average room temperature of 25 °C ± 2 °C with 75 ± 5 % relative humidity that is suitable for larvae rearing environment.

3.2 Source of Nano-Synthesized Silver Particle from *Azolla pinnata*

Final concentrations of the reaction mixture containing *Azolla pinnata* extract and 1mM of silver nitrate ($AgNO_3$) solution was obtained from the experiment conducted by Maizura binti Che Mat, student of Universiti Malaysia Kelantan through her research tittled as Nano-Synthesis and Characterization of Silver Particles from *Azolla Pinnata*. Through her research, *Azolla pinnata* extract was mixed with silver nitrate ($AgNO_3$) at the ratio 1:9 respectively resulting in the production of silver nanoparticles.

Together with the nano-synthesized silver particles from *Azolla pinnata*, aqueous solution of *Azolla pinnata* was also obtained. This will be used as the control group for larvicidal testing.

3.3 Source of Mosquito Eggs

Eggs of *Aedes Aegypti* were obtained from Vector Control Research Unit (VCRU) in Universiti Sains Malaysia (USM), Penang.

To ensure they are not exposed with insecticides or repellents, the mosquito eggs were reared in enamel trays located in an insectarium. The mosquitoes cycle process were kept in room temperature of 25 °C to 30 °C and having pH of 6.95 to 7.03 in VCRU insectariums. The eggs were reared until they became as adult mosquito and hatched new eggs.

The larvae were fed daily with finely ground fish pellet until they pupated. The developed pupae were then transferred manually using a Pasteur pipette into a plastic filled with two third of seasoned water.

Then, the cup was kept in a silver frame mosquito cages (30 cm X 30 cm X 30 cm) which was enclosed with fine mesh netting cloth (20 μm mesh size) for adult mosquitoes development. Glucose meal were given to the adult mosquitoes by soaking a cotton ball with 10 % of glucose solution. The mosquitoes also were blood fed on mouse two times a week in the mosquito cage. Female mosquitoes were then let on to lay their eggs on wet filter paper located in the beakers which were half filled with water.

3.4 Rearing Mosquito Larvae

The eggs of *Aedes Aegypti* on filter paper that were obtained from VCRU, USM are shown below in Figure 3.1. With a little modifications, the eggs then were hatched according to WHO standard in VCRU USM until the larvae reached late-third instar stage. To prevent any larvae from becoming adult mosquitoes, any larvae that pupated was killed in hot water (60 ^{o}C).

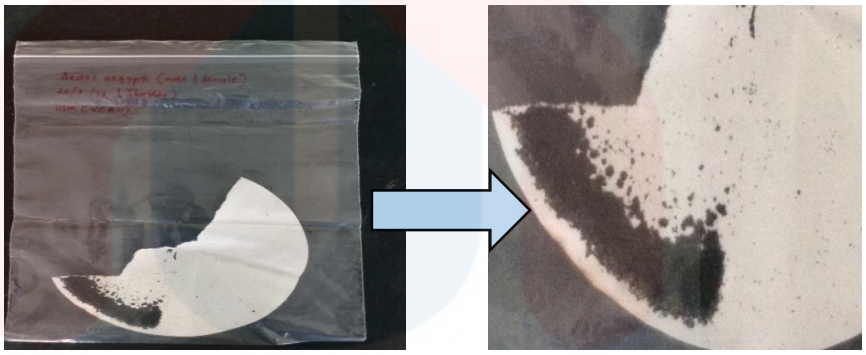

Figure 3.1: Eggs of *Aedes Aegypti* obtained from VCRU, USM

Seasoned water was changed every day and only an appropriate amount of larval food were given in order to prevent scum formation until they reached late third instar stage. Seasoned water is tap water that was left behind for at least 24 hours before been use to ensure precipitation of unwanted chlorine particle at the bottom of container. Round sized plastic container that was filled with seasoned water and covered with mesh cover was used in the process of rearing the larvae.

The larvae rearing usually took up 2-3 days after hatching for first stages, 2-4 days for second stages, 4-6 days for third stages, 6-7 days for fourth stages and 7-9 days for pupae stage. The experiment was set up as shown in Figure 3.2.

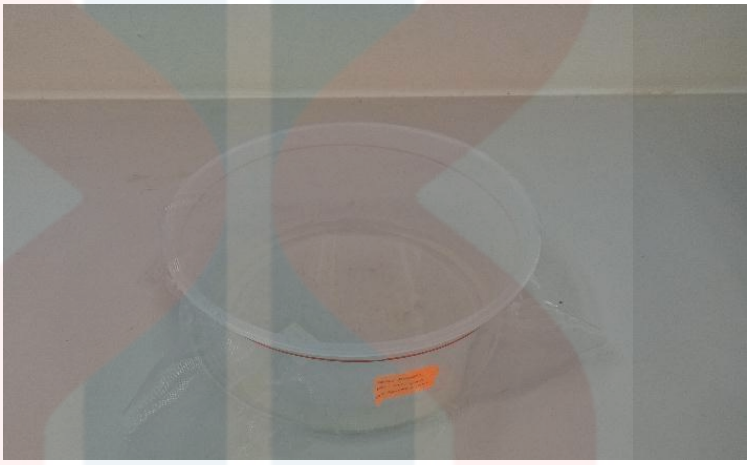

Figure 3.2: Plastic container covered with net cloth for larvae rearing

3.4.3 Larvicidal Bioassay

This experiment was being carried out as per standard World Health Organization (2005) in order to investigate the mortality of *Aedes Aegypti* larvae in different concentration of reaction mixture. Late-third instar larvae were been used as the response of newly hatched larvae (first and second stage larvae) are not completely developed. Four replicates were tested using 20 freshly hatched late-third instar larvae which in total it gave a value of 80 larvae for each concentration.

Firstly, the reaction mixtures were diluted according to the desired concentration by using the equation 3.1 below.

$$M_1V_1=M_2V_2 \quad (3.1)$$

The mosquito larvae were exposed to a wide range of concentration ranging from 5 ppm to 750 ppm. This is necessary to determine the reaction mixtures range for larvicidal activities. Then after the mortality of larvae in this vast range were determined, a narrower range of concentrations (six concentrations ranging from 10 ppm to 250 ppm) yielding between 5% and 95% mortality in 24 hours of exposure were selected as test concentrations.

Two control groups were being tested which were distilled water and the lowest concentration (10 ppm) of *Azolla pinnata* extract that have not been mixed with $AgNO_3$ solution yet. Lowest concentration of *Azolla pinnata* extract was used as the control in order to ensure that the mortality of larvae in the lowest reaction mixture was due to the aid from silver nanoparticles and not by the *Azolla pinnata* extract only. By doing this, confirmation could be done that only *Azolla pinnata* extract at 10 ppm concentration could not induce mortality towards the larvae.

Each replicate of test cup containing the larvae was given larval food before being covered with fine mesh netting cloth. After the larvae were exposed with the test concentrations for 24 hours, the mortality of larvae were observed and recorded. Unable to swim up to the surface and not being able to move even after being touched are signs that the larvae are dead. Figure 3.3 below show the set-up of the experiment in plastic cups.

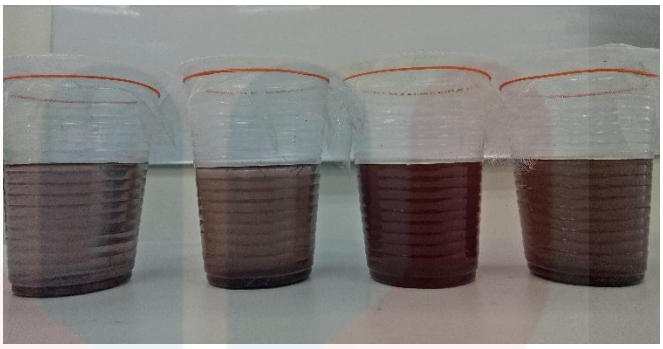

Figure 3.3: Test cup containing larvae and different concentrations of reaction mixtures

3.5 Mortality Percentage and Lethal Concentration of *Aedes Aegypti*

For all tabulation process, Microsoft Excel (2010) was used. Using equation 3.1, the average of replicate test and the percentage mortality were taken. The result of mortality will be used in probit regression analysis (Finney, 1952). To estimate the LC_{50} and LC_{95} values at 95 % confidence limits which are the concentration values for killing 50 % and 95 % of the sample population, probit analysis of the concentration-dependent mortality data was conducted and obtained from the statistical package IBM SPSS 21 software. Diagnostic dose kill 99 % of *Aedes* larvae were also calculated. Statistically significant is considered when the results shown that $P < 0.05$.

$$Observed\ Mortality = \frac{Total\ No.of\ Dead\ Larvae}{Total\ Sample\ Size} \times 100 \quad (3.2)$$

(Source: Hassan, Jaal, Ranson and Philip, 2015)

If the control mortality was above 20 %, the test should be neglected while if the control is below 5 %, the mortality could be ignored and no correction is necessary. For the control test, similar calculations were made in order to obtain a value for control mortality. If the tests were greater than 5 % but less than 20 %, the observed mortality have to be corrected using Abbott's formula as in Equation 3.3:

$$\frac{\%\ observed\ Mortality - \%\ control\ Mortality}{(100 - \%\ control\ Mortality)} \times 100\ \% \quad (3.3)$$

(Source: Hassan, Jaal, Ranson and Philip, 2015)

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Mortality Rate and Percentage of *Aedes Aegypti* larvae

The reaction mixtures of *Azolla pinnata* extract and silver nanoparticles prepared in a few concentrations ranging from 10 ppm to 250 ppm showed good results. The bioassay prepared at different concentrations were tested on larvae to observe the mortality percentage after 24 hours. The experiments were done in four replicates with each test cup containing 20 late third instar larvae which gave a total of 80 larvae. Table 4.1 show the cumulative mortality rate obtained from the experiment while Table 4.2 show the percentage mortality of *Aedes Aegypti* larvae after been exposed for 24 hours.

Table 4.1: Cumulative frequency mortality number of *Aedes Aegypti* larvae

Concentration of silver nanoparticles synthesized from *Azolla pinnata* (ppm)	**Mortality number of *Aedes Aegypti* larvae (after 24 hours)**
Control (distilled water)	0
Control (Aqueous *Azolla pinnata*)	0
10	6
50	19
100	34
150	47
200	60
250	76

From Table 4.2, higher concentrations of silver nanoparticles synthesized from *Azolla pinnata* seem to contribute to a higher percentage mortality of larvae. After 24 hours of exposure, the highest concentration (250 ppm) resulted in 95% larvae mortality while the lowest concentration (10 ppm) only show 7.5% larvae mortality. Meanwhile for control groups which are distilled water and *Azolla pinnata* (10 ppm), all larvae were active and no mortality recorded for control groups after 24 hours exposure. This means, in order for the lowest concentration (10 ppm) of *Azolla pinnata* to cause mortality to larvae, silver nanoparticles are needed to aid in the larvicidal activity.

Table 4.2: Cumulative frequency percentage mortality of *Aedes Aegypti* larvae

Concentration of silver nanoparticles synthesized from *Azolla pinnata* (ppm)	**Percentage Mortality of *Aedes Aegypti* Larvae (%)**
Control (distilled water)	0
Control (Aqueous *Azolla pinnata*)	0
10	7.5
50	23.75
100	42.5
150	58.75
200	75.0
250	95.0

4.2 Larvicidal Assay of Nano-Synthesized Silver Particles from Azolla plant on *Aedes Aegypti* larvae

Larvicidal test data were recorded in Table 4.1 and Table 4.2 which show the rate of mortality and percentage mortality of larvae at various concentration of bioassay. By using log probit regression analysis (95% confidence level) through IBM SPSS 21 software, lethal concentration 50 and 95 (LC_{50} and LC_{95}) values after 24 hours exposure were then calculated and tabulated as in Table 4.3. While LC_{99} was tabulated in Table 4.4.

Based on the LC_{50} and LC_{95} result obtained from log probit analysis, it means 121.570 ppm of nano-synthesized silver particles from *Azolla pinnata* is needed to kill 50% of larvae while 369.438 ppm of the solution is needed to kill 95% of larvae. The p-value obtained (0.025) shows significance value as it does not exceed 0.05 which is

the maximum value to be categorized as significant. This means the result obtained is statistically significance and null hypothesis is rejected. The p-value reflects the characteristics and efficiency of the test carried out on the sample population which were larvae.

Table 4.3: Lethal concentration of bioassay against *Aedes sp*. larvae after 24 hours exposure

Larvae Instar	**LC_{50} (ppm)**	**95% Confidence Limit**		**LC_{95} (ppm)**	**95% Confidence Limit**		**p-value**
		UCL (ppm)	**LCL (ppm)**		**UCL (ppm)**	**LCL (ppm)**	
Late-third	121.570	173.695	0.622	369.438	1282397 1.50	235.469	0.025

*Note: Significant p-value < 0.05; LC50- lethal concentration that kills 50% of exposed larvae; LC90- lethal concentration that kills 90% of exposed larvae; UCL- upper confidence limit; LCL- lower confidence limit

Table 4.4: Lethal concentration 99 (LC_{99}) of the bioassay

Larvae instar	**LC99 (ppm)**	**95% Confidence Limit**	
		UCL (ppm)	**LCL (ppm)**
Late third	585.518	11876469115	309.263

4.3 Comparison of Result Obtained with Recent Research

A research on *Azolla pinnata* against late-third larvae of *Aedes Aegypti* was carried out by Ravi et al. (2018). The result from the research that used only *Azolla Pinnata* extract was compared with the nano-synthesized silver particle from *Azolla pinnata* in the Table 4.5. Differences in concentration used between silver nanoparticles synthesized from *Azolla pinnata* and *Azolla pinnata* extract could be seen clearly. The highest concentration of *Azolla pinnata* extract that was used by the researchers was 1500 ppm which caused 100% mortality while the silver nanoparticle synthesized from *Azolla pinnata* only need 250 ppm to cause 95% mortality of the sample population. This proved that the existence of silver nanoparticles in the reaction mixture aids the *Azolla pinnata* to act as larvicide.

Table 4.5: Comparison of larvae mortality when tested against *Azolla pinnata* extract and silver nanoparticles synthesized from *azolla pinnata* extract

***Azolla pinnata* extract**		**Silver nanoparticles synthesized from *Azolla pinnata***	
Concentration	**Mortality rate (%)**	**Concentration**	**Mortality rate (%)**
500	5	10	7.5
700	25	50	23.73
800	30	100	42.5
1000	40	150	58.75
1100	50	200	75.0
1200	70	250	95.0
1300	80	-	-
1500	100	-	-

*note: data of mortality rate by using *Azolla pinnata* extract were from Ravi *et al.* (2018)

The comparison of lethal concentration for the *Azolla pinnata* extract and nano-synthesized silver nanoparticles from *Azolla pinnata* was tabulated in Table 4.6. The LC_{50} recorded by *Azolla pinnata* extract was 1093.00 ppm which is much more concentrated compared to nano-synthesized silver particles from *Azolla pinnata* which is only 121.570 ppm. While the LC_{95} of the *Azolla pinnata* extract was 1343.00 ppm and only 369.438 ppm for nano-synthesized silver particles.

This means, the presence of silver nanoparticles could aid *Azolla pinnata* in carrying out the role as larvicidal against *Aedes Aegypti* larvae. The efficacy of silver nanoparticles observed may be due to its toxic properties that resulted in larvae mortality.

Based on Trefry (2011) in his study, various mechanisms of silver nanoparticles that being proposed are mostly based on silver ion toxicity. This is due to the silver nanoparticles and silver ions (Ag+) that have the same elemental composition. The proposed mechanisms include disruption of membrane potential, depletion of intracellular ATP, reactive oxygen species (ROS) production, DNA damage, inactivation of proteins, and silver ions productions from the silver nanoparticles which then would mediate the aforementioned mechanisms

Besides, the small size of silver nanoparticles may be one of the factors that facilitate the process of absorption or ingestion of *Azolla pinnata* and silver nanoparticles into larvae. Owing to their small size, silver nanoparticles have a large surface area to volume ratio that are available for interactions with cellular organelles once they are internalized. This factor thus resulted in a more significant toxicity (Liu *et al.*, 2010).

Table 4.6: Comparison of LC_{50} and LC_{95} between *Azolla pinnata* extract and Nano-synthesized silver particles from *Azolla pinnata*

Solution	LC_{50} (ppm)	LC_{95} (ppm)
***Azolla pinnata* extract**	1093.00	1343.00
Nano-synthesized silver particles from *Azolla pinnata*	121.570	369.438

*note: LC_{50} and LC_{95} data of *Azolla pinnata* extract were from Ravi et al. (2018)

Based on the positive result obtained, the behaviors and mortality shown by the larvae may be due to the larvicidal properties of *Azolla pinnata* and silver nanoparticles. From a research by Ravi et al. (2018), *Azolla pinnata* have active ingredients which contribute to pesticidal, insecticidal, anti-parasitic and antimicrobial activities such as 3,7,11,15-tetramethyl-2-hexadecen-1-ol, neophytadiene, and methacrylic acid.

Several other researches also reported that high concentrations of extract could lead to severe morphological deformities. In a study by Ahbirami et al. (2014), they proven that there are deformities in morphological of the *Aedes Aegypti* larvae with the abdomen became blackened and twisted after treated with extract of *I. cairica* leaf.

The mixing of *Azolla pinnata* extract and silver nitrate that resulted in synthesis of silver nanoparticles has led to potentiation. This is possibly due to the small size of silver nanoparticles that allow them to pass through the wall of larvae's body into the cell where it can disturb other physiological process of larvae (Ghramh et al.,2018).

In parallel to these research, Figure 4.1 (b) and (c) showing the morphologically deformed larvae can be seen clearly with the darkening of the whole body of the larvae. This can be due to the ingestion process by larvae or absorption process of the nano-

synthesized silver particles from *Azolla pinnata* extract into the body of larvae. The differences can be seen when compared to the normal larvae in Figure 4.1(a) where a normal alive larvae has a well define abdomen. While abdomen of morphologically deform larvae could not be seen clearly and seems darker.

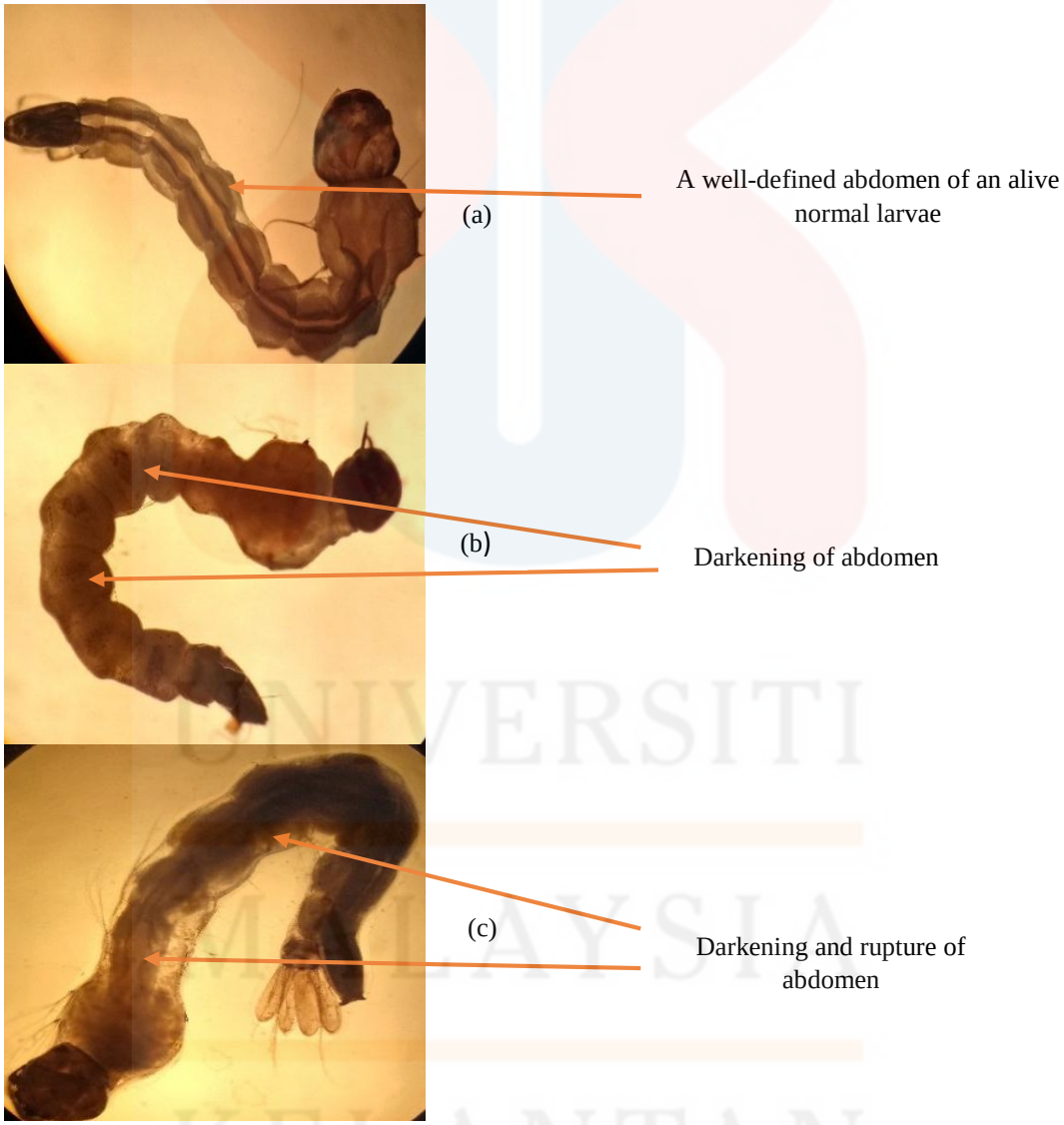

Figure 4.1: Microscope view of normal alive larvae with clear abdomen (a) and dead larvae with darkening of abdomen (b) and ruptured abdomen (c)

The larvae treated with the bioassay showed different behaviors as the time went by. Most of the larvae showed active movement such as restlessness and coiling movement in the first 7 hours after been exposed. This might be due to the adaptation process to the new aquatic environment including the new physical factors. After that, the larvae seemed to be inactive and not responding well to stimuli such as light and motion. Then, followed by settling down to the bottom of the plastic cup and died.

The larvae were confirmed as dead when there is no response when being touched with Pasteur pipette. Once died, most of the larvae seemed very dark in color. This is possibly due to the silver nanoparticles that are dark in color have been absorbed or feed on by the larvae

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

5.1 Conclusion

The nano-synthesized silver particles from *Azolla pinnata* extract was found to be effective as larvicide against *Aedes Aegypti* larvae. The mortality range between 5% and 95% of *Aedes Aegypti* larvae was obtained when tested against 10 ppm to 250 ppm of nano-synthesized silver particles from *Azolla Pinnata* extract. While the lethal concentration 50 and 95 (LC_{50} & LC_{95}) could also be determined through the log probit analysis in IBM SPSS software where 121.570 ppm and 369.438 ppm of nano-synthesized silver particles from *Azolla Pinnata* extract was needed to respectively constitute LC_{50} and LC_{95} against *Aedes Aegypti* third larvae instar.

The nano-synthesized silver particles from *Azolla pinnata* extract is proven to be able to reduce the amount or concentration of *Azolla pinnata* extract that is needed to cause larvae mortality due to their unique characteristics. The effects of the silver nanoparticles and *Azolla pinnata* can be seen from the morphological deformities of the dead larvae. This result can indicate that silver nanoparticles do have larvicidal actions as well as *Azolla pinnata* extract.

5.2 Recommendations

The findings on this research can be elaborated and further up more into other study. This research only study on the mortality of *Aedes Aegypti* late-third instar larvae. Therefore, the efficacy of the *Azolla pinnata* and silver nanoparticles on other stages of *Aedes sp.* mosquito like pupae should be done in order to find out their resistance and survival.

Besides, further experiment could be done on other species of mosquitoes as each type of mosquito could have a different resistance towards the bioassay. For instances, *Aedes Albopictus* which is also a dengue vector that can cause harm to human. The findings will then can be compared on further discussion.

Next, this research did not have a living organisms as a control groups. A living organisms such as fish that live in the same habitat with larvae can be chosen sa a control group. This is necessary in order to ensure the *Azolla pinnata* extract and silver nanoparticles did not poses any threat to the other non-target organisms living in the same habitat.

Last but not least, further research on the mechanisms of silver nanoparticles that could act as larvicide against *Aedes sp*. should be done. As through this research, silver nanoparticles exhibit a high potential as an efficient larvicide against late-third larvae of *Aedes Aegypti*. Thus, a good result from the furthered research could help the application of silver nanoparticles as conventional larvicide in the future.

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APPENDICES

APPENDIX A: Generated values for Nano-Synthesized Silver Particles from *Azolla pinnata* at 24 hours exposure

		Confidence Limits					
Probability		95% Confidence Limits for Concentration			95% Confidence Limits for log(Concentration)[b]		
		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	.010	25.241	.000	66.629	1.402	-10.321	1.824
	.020	30.347	.000	73.561	1.482	-9.130	1.867
	.030	34.109	.000	78.363	1.533	-8.374	1.894
	.040	37.244	.000	82.202	1.571	-7.806	1.915
	.050	40.005	.000	85.481	1.602	-7.344	1.932
	.060	42.515	.000	88.390	1.629	-6.951	1.946
	.070	44.846	.000	91.035	1.652	-6.606	1.959
	.080	47.041	.000	93.482	1.672	-6.297	1.971
	.090	49.131	.000	95.775	1.691	-6.017	1.981
	.100	51.136	.000	97.947	1.709	-5.759	1.991
	.150	60.348	.000	107.621	1.781	-4.690	2.032
	.200	68.839	.000	116.233	1.838	-3.841	2.065
	.250	77.069	.001	124.445	1.887	-3.114	2.095
	.300	85.296	.003	132.653	1.931	-2.463	2.123
	.350	93.701	.014	141.191	1.972	-1.860	2.150
	.400	102.441	.051	150.441	2.010	-1.290	2.177
	.450	111.673	.181	160.954	2.048	-.742	2.207
	.500	121.570	.622	173.695	2.085	-.206	2.240
	.550	132.344	2.101	190.674	2.122	.322	2.280
	.600	144.270	6.985	217.059	2.159	.844	2.337
	.650	157.727	22.195	270.345	2.198	1.346	2.432
	.700	173.269	59.163	432.239	2.239	1.772	2.636
	.750	191.765	107.279	1139.450	2.283	2.031	3.057
	.800	214.692	143.530	4862.464	2.332	2.157	3.687
	.850	244.900	171.312	31038.987	2.389	2.234	4.492
	.900	289.017	198.609	344605.858	2.461	2.298	5.537
	.910	300.814	204.721	619681.130	2.478	2.311	5.792
	.920	314.176	211.271	1173936.643	2.497	2.325	6.070
	.930	329.554	218.414	2373253.641	2.518	2.339	6.375
	.940	347.621	226.369	5216205.681	2.541	2.355	6.717
	.950	369.438	235.469	12823971.497	2.568	2.372	7.108
	.960	396.826	246.265	36952778.199	2.599	2.391	7.568
	.970	433.294	259.785	135962777.011	2.637	2.415	8.133
	.980	487.013	278.331	769882023.279	2.688	2.445	8.886
	.990	585.518	309.263	11876469114.671	2.768	2.490	10.075

a. A heterogeneity factor is used.

b. Logarithm base = 10.

APPENDIX B: Rearing of *Aedes Aegypti* Larvae

Figure B1: Eggs of *Aedes aegypti*

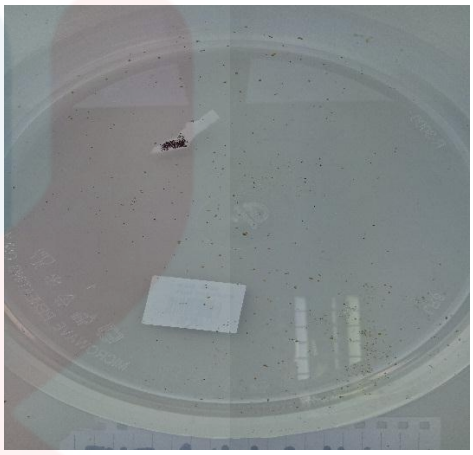

Figure B2: Eggs hatched in plastic container

Figure B3: *Aedes Aegypti* larvae rearing

Figure B4: Set-up of larvae rearing experiment

APPENDIX C: Larvicidal Testing

Figure C1: Dead Larvae

Figure C2: Bioassay with different concentrations

Figure C3: Observing dead larvae from bioassay

Figure C4: Control group (*Azolla pinnata)*