CHARACTERISATION AND OPTIMISATION OF AZOLLA AND SILVER NITRATE MIXTURE AS AN INSECTICIDES AGAINST *Aedes Aegypti* (DIPTERA: CULICIDAE)

By

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DECLARATION

I declare that this thesis entitled 'Characterisation and Optimisation of Azolla with Silver Nitrate Mixture as Insecticides Against *Aedes Aegypti* (Dipetra: 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 degree.

Signature : _____

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Date : 6th JANUARY 2020

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Characterisation and Optimisation of Azolla with Silver Nitrate Mixture as Insecticides Against *Aedes Aegypti* (Dipetra: Culicidae)

ABSTRACT

The family of mosquitoes (Dipetra:Culicidae) contains several species which act as vectors spreading diseases to human causing a major public health. one of these species, *Aedes Aegypti*, is widely known due to its responsible in transmitting vector-borne viruses affecting humankind such as chikungunya, Zika and dengue fever. Traditionally, chemical insecticides were used to control the spreading of *Ae. aegypti*. However, the prolonged use of chemical insecticides has caused significant adverse effects on environmental and the mosquitoes has developed a resistance to insecticides, thus dramatically reducing their efficiency. Therefore, alternative and safer way was needed to combat the spreading of deadly diseases causing by *Aedes aegypti*. In that context, this research aimed to establish the efficacy of synthesized silver nanoparticles using *Azolla microphylla* extract (AZO-AgNPs) as insecticides against late third instar larvae of *Aedes aegypti*. The synthesized nanoparticles were characterized with UV-Visible spectroscopy and Fourier transform infrared spectroscopy. UV-Visible spectrophotometer showed absorbance peak in range of 410 nm to 510 nm. FT-IR analysis show peaks at 3327.91 and 1636.18 cm-1 which shows the presence reductive and capping agents in plant extract. To perform larvicidal test, third instar *Ae. aegypti* were exposed to 8 concentrations ranging from 20 to 37 ppm. The result shows the values of LC50 is 31.680 ppm while LC90 is 43.610 ppm. These result suggest that synthesized silver nanoparticles using *A. microphylla* extract (AZO-AgNPs) exhibit noteworthy larvicidal activity and should be further explored as potential source of alternative tools in the fight against insect vectors of human disease.

Pencirian dan Pengoptimuman Azolla dengan Campuran Nitrat Perak sebagai Insektida Terhadap Aedes Aegypti (Dipetra: Culicidae)

ABSTRAK

Keluarga nyamuk (Dipetra: Culicidae) mempunyai pelbagai spesies yang bertindak sebagai vektor dan berfungsi untuk menyebarkan penyakit kepada manusia justeru, menyebabkan penyakit seperti denggi. Salah satu spesies ini, adalah *Aedes Aegypti*, diketahui secara meluas kerana bertanggungjawab menjadi vektor dalam menyebarkan virus yang membawa penyakit kepada manusia seperti chikungunya, Zika dan demam denggi. Terdahulu, racun serangga kimia digunakan untuk mengawal penyebaran nyamuk *Ae. aegypti*. Kesannya, penggunaan racun serangga kimia yang berpanjangan telah menyebabkan kesan buruk yang signifikan ke atas alam sekitar. Selain itu, nyamuk telah menunjukkan ketahanan terhadap racun serangga, dengan itu mengurangkan keberkesanan racun serangga kimia. Oleh itu, cara alternatif dan selamat diperlukan untuk memerangi penyebaran penyakit maut yang disebabkan oleh *Aedes aegypti*. Kajian ini bertujuan untuk membuktikan keberkesanan nanopartikel perak yang disintesis menggunakan ekstrak *Azolla microphylla* (AZO-AgNPs) sebagai racun serangga terhadap larva instar ketiga *Aedes aegypti*. Nanopartikel yang disintesis dicirikan dengan spektroskopi UV-Visible dan FT-IR. UV-Visible menunjukkan puncak penyerapan dalam lingkungan 410 nm hingga 510 nm. Analisis FT-IR menunjukkan puncak pada 3327.91 dan 1636.18 cm-1 yang menunjukkan kehadiran ejen-ejen reduktif dan penutup dalam ekstrak tumbuhan. Untuk melakukan ujian larvicidal, instar Ae ketiga. *Ae. aegypti* terdedah kepada 8 kepekatan antara 20 hingga 37 ppm. Penemuan menunjukkan nilai LC50 adalah 31.680 ppm manakala LC90 adalah 43.610 ppm. Keputusan ini menunjukkan bahawa nanopartikel perak yang disintesis menggunakan ekstrak *A. microphylla* (AZO-AgNPs) memperlihatkan aktiviti larvikidal yang patut diberi perhatian dan harus diterokai sebagai sumber alternatif sebagai bahan alternatif dalam memerangi vektor serangga penyakit manusia.

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LIST OF ABBREVIATIONS

UV	Ultra-violet
VCRU	Vector control research unit
WHO	World Health Organization

LIST OF SYMBOLS

Ag	Silver
$AgNO_3$	Silver nitrate
NPs	Nanoparticles
AgNPs	Silver nanoparticles
AZO	*Azolla microphylla* extract
AZO-AgNPs	Biosynthesized of nanoparticles using *Azolla microphylla* extract
ppm	Part per million
Sp.	Species
%	Percentage
±	Plus or minus
^oC	Temperature (Degree Celsius)
<	Less than

CHAPTER 1

INTRODUCTION

1.1 Background of Study

The yellow fever mosquito or scientifically known as *Aedes aegypti* mosquito is one of the murderous insects in the world for humanity. The origin of *Aedes aegypti* has been traced from its ancestral home in West Africa (Powell et al., 2018) and has been spread into Asia around 1870s when the first chikungunya was reported. *Ae. aegypti* acts as the main vector in spreading viral diseases such as yellow fever, malaria, chikungunya and dengue fever to human. The ability of *Ae. aegypti* to evolve and adapt in the human environment further the viral spread and its present a great public health threat (Powell & Tabachnick, 2013).

Dengue fever is a worldwide issue. The number of cases has grown dramatically in recent decades. The disease is now endemic in more than 100 countries including Malaysia. About 506 dengue cases with two deaths have been reported in Penang in the first two weeks of January 2019. Penang Health Department state that dengue cases spike nearly 200% compared to last year which only 177 cases with no death in the corresponding period last year ("Penangites told to exercise caution as dengue cases spike nearly 200% | Free Malaysia Today," 2019). Therefore, it is important to contain the spreading of *Ae. aegypti* mosquitoes the main dengue vector.

Currently, there are no effective vaccine and no specific treatment to cure dengue fever. However, World Health Organization established a strategic approach which considered cost-effectiveness, environmental effect, and efficiency called Integrated Vector Management (Brady et al., 2012). This method includes environmental management, chemical control, and biological control. Issues arise when a few studies have reported many species of insects including *Ae. aegypti* mosquitoes had developed resistance towards insecticides (WHO., 2017) and the toxicity of insecticides bring health threat towards humans (Malik, Grohmann, & Akhtar, 2014).

The negative effects of extensive use of chemical insecticides and limited success for Aedes control program have led to finding for a new eco-friendly insecticide. One of the many study has found that *Azolla Pinnata* can act as bioinsecticides and have a killing effect towards late third-stage larvae of *Aedes aegypti* (Rozhan et al., 2018). The potential use of *A. Pinnata* as bio insecticides may be the answer to solve problems involving chemical pollution, knockdown resistance of targeted pests and adverse effect to humans and non-target pests.

However, to increase the efficiency of plant extract as green insecticides, smaller and more effective molecules are needed. A number of scientists has invested their interest in nanotechnology for the past decades because of its physiochemical properties such as size, distribution, and morphology. Nanotechnology is the study of extremely small things in the range of 1-100 nm which known as nanoparticles (NPs)

Recently, the synthesis of nanoparticles (NPs) using plants or plants extract has attracted interest among scientist. The use of plants for synthesis of nanoparticles (NPs) is more preferred compared to other synthesis methods because it is fast, low-

cost and eco-friendly. Thus, this proposed study is to develop a novel approach for the characterization of green synthesis silver nanoparticles using *A. microphylla* extract (AZO-AgNPs). Moreover, to determine the efficacy of *A. microphylla* with silver nitrate (AZO-AgNPs) combination as Aedes insecticides.

1.2 Problem Statement

Nowadays, the extensive use of conventional insecticides in Malaysia by the Ministry of Health, the private sector and household cause the development of insecticides resistance in mosquitoes and adverse effect on the environment and non-target pest including humans. In Malaysia, evidence of resistance towards pyrethroids, DDT and bendiocarb has been recorded in *Ae. Aegypti* and *Ae. Albopictus* in Penang, Kuala Lumpur, Johor Bharu and Kota Bharu (Ishak, Jaal, Ranson, & Wondji, 2015).

As a result, a number of studies have been conducted to discover other alternatives compound and also to comply with sustainable and chemical free environment. For example, *A. Pinnata* contains bioactive molecules which shows potential and effectiveness to serve as biolarvicides for Aedes mosquito vector control (Zulkrnin et al., 2018). Unfortunately, problems arise when a vast amount of raw plants were used and only a small amount of plant extract was yield after extraction methods (Rozhan et al., 2018).

Besides, to increase the efficiency of *A. Pinnata* extract, smaller particles are needed to increase the absorption rate into mosquitos' larvae. As a result, the use of green process for the synthesis of nanoparticles is rapidly developing. There are several studies on green, biological synthesis of silver nanoparticles against mosquitoes.

Different plant of Nano synthesis possesses different efficiency in killing mosquitoes. As for that, this synthesis is intended to find discover other options of species in the family of Salviniaceae which possess higher efficacy against *Aedes aegypti sp*.

1.3 Objectives

The purpose of doing this research are:

1. To synthesise silver nanoparticles using *Azolla microphylla* plant extracts.
2. To characterize the synthesized silver nanoparticles (AgNPs) with *Azolla microphylla* using UV-Vis and FT-IR.
3. To determine the efficacy of the Nano-synthesized silver nitrate particles from *Azolla microphylla* extract (AZO-AgNPs) against third instar larvae of *Aedes Aegypti*.

1.4 Scope of Study

This study focuses on developing insecticides that comply green chemistry principle with the combination of modern nanotechnology. This study specifically used the combination of *A. microphylla* extract and silver nitrate solutions and the insecticides was used against late third larvae of *Ae. aegypti*. the susceptible lab strains eggs of Aedes aegypti was procure from Vector Control Research Unit (VCRU) at USM, Penang. The larvae rearing and insecticides testing was conducted at Research Lab, School of Biological Science in USM, Penang.

The characterization of silver nanoparticles in *A. microphylla* extract (AZO-AgNPs) will be conducted by using UV-Vis spectrometer and FTIR.

1.5 Significance of Study

The findings of this study will redound to the benefit of the society considering a new eco-friendly insecticide against *Aedes sp.* are developed. Nano synthesized silver using *A. microphylla* (AZO-AgNPs) could be the game changer in mosquitoes' vector control programme. This is due to AZO-AgNPS have higher efficacy in killing *Aedes sp.* at lower concentration and also cost effective. For the researcher, this study will help understand more about green chemistry approach that connects nanotechnology with plant.

CHAPTER 2

LITERATURE REVIEW

2.1 The Concept of Nanotechnology

The study of nanoparticles is not new. Historically, the concept of 'nanometre' was first proposed by Richard Zsigmondy. He was the first person to measure the size of gold colloids using microscopes and explained the term of nanometre, describing as characterizing of particle size (Hulla, Sahu, & Hayes, 2015). Later in the years of 1959, a physicist named Richard Feynman gave a speech titled, "There is Plenty of Room at the Bottom", in which he describe about the concept of the possibility to manipulate atoms and molecules (Fanfair, Desai, & Kelty, 2007). Richard Feynman was considered as the father of modern nanotechnology since his novel idea creates a new way of thinking and Feynman's hypotheses have since been proven correct. The word "nanotechnology" was first used by Norio Taniguchi. He defined the term "nanotechnology" as follow: "nanotechnology mainly consists of the process of separation, consolidation, and deformation of materials by one atom or one molecule".

From then on, the nanotechnology is described as a field which explores the smallest particles known as nanoscale, ranging between 1 to 100 nm. In addition, nanotechnology also discusses how to control and design the properties of nanomaterials by manipulating atoms and molecules (Alim et al., 2013). At this scale, the materials often possess change in physical, chemical and biological properties differ from their properties in bulk size (Alim et al., 2013). The unique properties

different in nanomaterials from the bulk materials, increase interest in the development of nanotechnology. Hence, producing innovative devices which the capabilities were not found in bulk materials or in nature.

2.2 The Application of Nanotechnology

The beginning of the 21st century saw an emerging field of nanotechnology. Until now, a vast number of reports and research have been published in many areas involving, medical, solar energy, food industry, human health, cosmetic dermatology and even in wastewater treatment. Some of these applications converge the knowledge of nanotechnology with other sciences including chemistry, physics, biotechnology, and engineering. As the field of nanotechnology emerges, the utilization of metal nanoparticles is the most favoured. For example, the use of carbon nanoparticles combined with protein molecules is to promote bone growth in dental and joints implants (UCLA, 2013). In the manufacturing sector, the utilization of silver nanoparticles in a fabric are to kill bacteria and make the clothes odour resistance (Anderson et al., 2016). Equally important, gold nanoparticles in manganese oxides purify the air from volatile organic pollutants (Angewandte, n.d.).

Nowadays, noble-metal nanoparticle is widely used because of its incredible properties can be applied in a diverse sector. The commonly utilize of noble-metal nanoparticles in the industry are palladium, platinum, cobalt, copper, zinc oxide, manganese oxide, silver and gold. Although, there are various metals, silver nanoparticles are the most popular due to their properties to act as antimicrobial without causing toxicity to animal cell (Elechiguerra et al., 2005), as a catalyst in oxidation-reduction (Arvizo et al., 2012) and as a biocide agent (Küünal et al., 2016). Other than that, silver nanoparticles possess unique physiochemical properties, which

include a high electrical and thermal conductivity, surface-enhanced Raman scattering, catalytic activity and chemical stability and non-linear behaviour (Krutyakov, Kudrinskiy, Olenin, & Lisichkin, 2008).

2.3 The Synthesis of Nanoparticles

There are several methods that have been carried out to synthesized nanoparticles, such as physical and chemical procedures. Chemical procedures include electrochemical, photochemical (Elnashaie, Danafar, & Rafsanjani, 2015) and chemical reduction (Wang, Qiao, Chen, & Ding, 2005) produce toxic by-products which give adverse effect to the environment. Besides, chemical methods are not suitable for medical use due to hazardous chemicals attached to their surface (Pirtarighat, Ghannadnia, & Baghshahi, 2018). Furthermore, physical methods, such as physical vapour condensation (Carmona, Benito, Plaza, & Recio-Sánchez, 2017) are inconvenient because expensive and require high energy and space (Wei et al., 2012). Thus, a new approach called green/biosynthesis has been introduced. This approach has been proposed as inexpensive, safe, easy, environmental friendly and reliable. The biological methods of silver nanoparticle synthesis using various biological component, for example, yeast, fungi, bacteria and plant (Ponarulselvam et al., 2012). The use of microbe mediated synthesis of nanoparticle is less convenient due to requiring high maintenance in aseptic conditions. Unlike microbe, plant-mediated synthesis of nanoparticle gives more advantages since, it offers greater stability, free from toxic chemical and contain natural capping agents (Singhal, Bhavesh, Kasariya, Sharma, & Singh, 2011).

The novel method known as green synthesis have been recently explored by a variety of plant extract and show a great success for efficiency and rapid extracellular synthesis of silver nanoparticles. *Salvia spinosa* (Pirtarighat et al., 2018), *Aloe vera, Budleja globose* (Carmona et al., 2017), *Azadirachta indica* (Saifullah, Ikram, Swami, Ahmad, & Ahmed, 2015), *Acalypha indica* (Selvakumar et al., 2009), *Catharanthus roseus* (Ponarulselvam et al., 2012), and *Coriandrum sativum* (Khan, Tareq, Hossen, & Roki, 2018) proved its potential in reducing silver nitrate to silver nanoparticles.

A lot of literature proves, the natural biomolecule in plants extract such as proteins, amino acids, flavonoid, carbohydrates, vitamins, alkaloid, phenolic and carboxylic acid act as reducing and capping agents which provide stability to silver nanoparticles (Rashid, Bhuiyan, & Quayum, 2013). According to Abraham & Aeri, (2012), *A. microphylla* plant extract show the presence of phytochemical compounds which include carbohydrates, phenols, flavonoids, carboxylic acid, alkaloids, terpenoids, steroids, saponis and proteins. Therefore, it is almost certain that *A. microphylla* could show success in synthesizing of silver nanoparticles.

2.4 *Azolla microphylla* sp.

As shown in Figure 2.1, *Azolla microphylla is* a species of fern and also known as mosquito fern, fairy moss, duckweed fern and water fern. *A. microphylla* is from the family of Salviniaceae and belong to genus *Azolla*. *A. microphylla* is an interesting plant which flourish in the subtropical and tropical climate and widely found in Africa, Asia (Brunei Darussalam, Malaysia, China, India, Japan, Korea and the Philippines) and parts of Australia.

Figure 2.1: *Azolla microphylla* plant (Source: Ken Fern, 2012)

Azolla are grouped into two subgenera containing six species of *Azolla* as listed in Table 2.1. The four species under *Euazolla* are known to be originated from temperate sub-tropical and tropical regions of North and South America while the species under *Rhizosperma* is a native of East (Wagner, 1997).

Table 2.1: Lists of sub genes

Euazolla	***Rhizosperma***
Azolla caroliniana .	*Azolla nilotica*
Azolla filiculoides	*Azolla pinnata*
Azolla microphylla	
Azolla Mexicana	
Azolla rubra	

A. microphylla heavily grow in wetlands, lakes and paddy field. *Azolla sp*. possess phytoremediation process especially in the remediation of heavy metals (Akinbile, Ogunrinde, Che bt Man, & Aziz, 2016). Other than that, application of *Azolla sp.* is implemented in domestic wastewater for its efficiencies in removal heavy metal (Akinbile et al., 2016), act as a potential adsorbent for removal of methyl violet (Kooh, Lim, Dahri, Lim, & Sarath Bandara, 2015) and as pesticides.

2.5 *Aedes Aegypti*

Ae. aegypti is the major urban vector of dengue viruses' worldwide and the number of cases has increased significantly every year. The adaptation and close association of *Ae. aegypti* towards the urban environment is the main factor facilitating the viral spread of dengue viruses. Controlling and preventing dengue outbreak include physical and chemical methods. The physical methods such as bed nets and wearing bright coloured clothes are known to be ineffective and impractical. Chemical methods are widely used by the Ministry of Health and private sector as the effective mitigation to contain dengue outbreak.

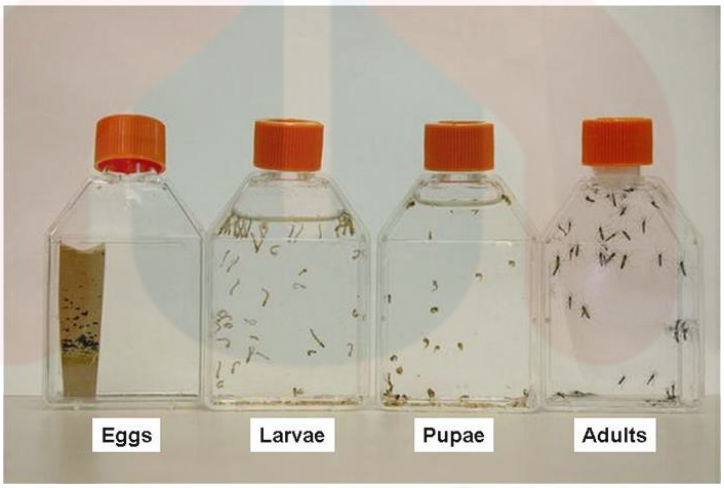

Figure 2.2: Life cycle of mosquitoes from eggs to adults (Source: Centrers for Disease Control and Prevention, 2019)

2.6 *Azolla microphylla* as Bioinsecticides

Globally, people are showing concern about the usage of chemical methods to the environment. The majority are concern about the loss of biodiversity, air quality and public health issues. Many researchers are looking forward to improve some existing insecticides to more sustainable and a chemical-free environment. For instance, the properties of *A. microphylla* extract to develop as bio insecticides for mosquito control methods.

One study by Ahmad et al., (2018) introduced *A. pinnata* as insecticides against *Ae. aegypti*. This is because the chemical composition of *A. pinnata* contain bioactive molecules such as carbohydrates, phenols, flavonoids, carboxylic acid, alkaloids and proteins. Compared to that, *A. microphylla* also contains the bioactive molecules such as carbohydrates, alkaloids, terpenoids, steroids, flavonoids, carboxylic acid and proteins (Abraham & Aeri, 2012). Due to that, *A. microphylla* plant have a high potential to act as bio insecticides.

There are several studies on the *A. pinnata* with mosquitoes, but only one of them has mentioned the process of biosynthesis of silver nanoparticle using *A. Pinnata* extract. Meanwhile, to date, no other studies have been done on the characterization of the silver nanoparticle using *A. microphylla* extract. Additionally, this study will also find the efficacy level of Nano-synthesized silver particle from *A. microphylla* extract (AZO-AgNPs) against *Ae. aegypti*. Hence, the purpose of this study is to conduct biosynthesis of silver nanoparticle using *A. microphylla* extract (AZO-AgNPS), characterize the silver nanoparticle and find the efficacy level against *Ae. Aegypti*.

CHAPTER 3

MATERIALS AND METHOD

3.1 Research Area and Design

Azolla microphylla as shown in figure 3.1 was recognized based on a morphological view of the arrangement of leaves on stem. *A* total of 20 kg fresh *A. microphylla* was collected from Pasir Mas, Kelantan, Malaysia. Following Rozhan et al., (2018) fresh *A. microphylla* was sun-dried for 2 days to remove all the water. Then, the dried *A. microphylla* were converted into powder by using electric blender and sieved to obtain the finest powder. This research was conducted according World Health Organization, WHO guidelines for mosquito larvicidal bioassay.

Figure 3.1: *Azolla microphylla* plant

3.2 Preparation of *Azolla microphylla* Extract

Soxhlet extraction methods is one of the effective method in obtaining plant extract. A paper by Ahmad et al., (2018), proves that the extraction of plant extract using Soxhlet extraction has higher yield compared to maceration method. The Soxhlet extraction was carried out using Soxhlet apparatus (Favorit, Malaysia) with a 20g of dried plant powder and place into the paper thimble. According to Rozhan et al., (2018), to avoid the sample overflow, a thin cotton wool was placed on the top part of the thimble paper. Next, a round-bottom flask was filled with 350 mL of methanol and place it on top of the heating mantle. Then, the solvent was heated at the temperature of 60^{o}C for approximately 5 hours. The extraction process was complete when the siphon arm become clear (Rozhan et al., 2018). After Soxhlet extraction complete, the remaining extract were evaporated using rotary vapour to obtain crude sample. Finally, the dry extracts were kept in universal bottle at -4^{o}C in the dark for further experimental studies.

3.3 Green Synthesis of Silver Nitrate Nanoparticles

Synthesis of silver nanoparticles was carried out by mixing 1% of *Azolla microphylla* (1 mL) with 1mM silver nitrate solution (100 mL). The mixed solution was kept in the presence of light for 24 hours and the resulting solution was observed to became red-brown in colour. The change of colour was observed which indicate the formation of silver nanoparticles.

3.4 Characterization of The Synthesized Silver Nanoparticles

3.4.1 UV-Vis Spectrophotometer

The synthesis silver nanoparticles solution with *A. microphylla* extract was monitored using UV-Vis spectrophotometer (HACH DR 6000). A small amount of sample was taken and pipetted into the cuvettes. The cuvettes were wipe tissue to avoid any disturbance. The bio reduction of Ag^{+} ions in solution was monitored at regular intervals. The de-ionized water was used as the blank. Besides, bio synthesized of AgNPs, aqueous plant extract and silver nitrate solution also monitored for further comparison. All measurement was operated at a resolution of 1nm.

3.4.2 Fourier Transform Infrared (FT-IR)

This analysis was undertaken to identify the functional groups and biomolecule existed in *A. microphylla* extract responsible in silver ion reduction. Fourier transform infrared is function to identify the presence of potential biomolecule and functional group in synthesized silver nanoparticles with *A. microphylla* extract. So the biosynthesized AgNPs was used for FT-IR spectrum analysis at wavelength range of 400 – 4000 cm^{-1} and resolution of 4 cm^{-1}.

3.5 Aedes Larvae Rearing

Susceptible lab strain eggs of *Aedes aegypti* were acquire from Vector Control Research Unit (VCRU) at University Sains Malaysia (USM), Penang, Malaysia. The eggs were reared in dechlorinated water until the eggs hatch. 0.2 g of larval food (food ratio; 2:1:1 of cat biscuit, beef liver, yeast and milk powder) was given to trigger the hatching process. The eggs and larvae were reared in a climate controlled room at room temperature (25 - 30ºC), a pH of 6.95 to 7.03 and relative humidity of 80 = -10% and dissolve oxygen from 5.5 to 6.1 mg/L in a rearing room. The eggs turned into third instar larvae around 5 to 6 days.

3.6 Larvicidal Bioassay

Larvicidal bioassays was performed in accordance to the standard WHO larval susceptibility test methods with some modification. The larvicidal activity of synthesized silver nanoparticles using *A. microphylla* was tested against *Ae. aegypti* larvaes. For bioassay test, late third instar larvae were tested in four batches containing 25 larvae in a plastic cup, with a total of 100 larvae for each concentration tested. The larvae were exposed to 8 concentration ranging between 10 to 40 ppm. The control group were included using distilled water, *A. microphylla* extract and silver nitrate solution. These procedures were conducted at room temperature of 28 ± 2ºC. The mortality of *Aedes aegypti* larvae was assessed and recorded within 24 hours. The larvae consider dead if, the larvae show no movement after being touch.

3.7 Data Analysis

All the data recorded was tabulated in Microsoft Excel (2010). The mortality results obtained from the four bioassay replicate were calculated the mean mortality per dose. With these values, dose-mortality regression was performed using log-porbit model in order to determine the LC50 and LC90 values. Calculations were done using statistical package IBM SPSS 21 software.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Visual Observation

As proven by Pirtarighat, Ghannadnia, & Baghshahi (2019), the mixing of plant extract with silver nitrate solution produced a different colour in the mixture which turn into yellowish to reddish-brown. Figure 4.1 shows, three test tubes containing *Azolla microphylla* extract (AZO), silver nitrate solution (AgNO3) and the biosynthesized silver nanoparticles using *A. microphylla* (AZO-AgNPs). The fresh and green in colour of 1% *A. microphylla* extract (1mL) was mixed with 1mM $AgNO_3$ solution (100mL), the solution colour starts to change into reddish-brown after 30 minutes of reaction. The intensity of colour gradually increases as the time of incubation passed by.

Figure 4.1: Three test tube containing *A. microphylla* extract (a), $AgNO_3$ solution (b) and AZO-AgNPs (c)

The different colour is due to a phenomenon called surface plasmon resonance. Metal nanoparticles such as silver nanoparticles have unique optical properties differs from the optical properties exhibit from the same bulk materials. When the light incident on silver nanoparticles, the strong interaction occurs between light and the surface of silver nanoparticles creating surface plasmon. A group of free electron in the material start to naturally resonate with a specific frequency. When the wavelength of incident lights matches with the frequency of moving electron, the plasmon is in the resonance state where the silver nanoparticles are strongly absorbed or scattered the light, producing coloured particles (Fan, Zheng, & Singh, 2014).

In this study, the colours perceived by AZO-AgNPs complex not solely depend on silver nanoparticles but also depend on the *A. microphylla* extract medium which gives out the reddish-brown colour. This is due to, the combination of AZO-AgNPs complex possesses scattering and absorption components. The colour change to reddish-brown indicates the reduction of Ag2+ ions into Ag0 due to surface plasmon resonance of AZO-AgNPs. To prove the formation of silver nanoparticles, UV-Vis spectrophotometer was used to accurately measure the optical spectra of biosynthesis of AZO-AgNPs.

4.2 UV-Vis Spectrophotometer

Silver nanoparticles exhibit a variety of interesting optical properties that are influenced by the shape, size, concentration and agglomeration state. As mention before, silver nanoparticles exhibit a distinctive absorption peak caused by the surface plasmon excitation. The absorption band for silver nanoparticles typically between 400nm to 550nm (Zhang, Liu, Shen, & Gurunathan, 2016).

UV-Vis spectrophotometer was used to observed the absorbance of *A. microphylla* extract, $AgNO_3$ solution and Azo-AgNPs. The absorbance values were recorded shown in Figure 4.2. The 1% *A. microphylla* extract shows absorption peak intensified at 250 nm to 350 nm of wavelength, while the $AgNO_3$ solution shows low absorption peak around 300 nm to 400 nm of wavelength. After mixing 1% *A. microphylla* (1mL) into 1mM $AgNO_3$ (100mL), the absorbance spectra of the AZO-AgNPs was recorded which is shown in Figure 4.2. Various secondary metabolites from *A. microphylla* extract induced the reduction of Ag+ ions into the formation AZO-AgNPs complex. Hence, display a wide absorption peak localized between 410 nm and 510 nm which are related to the surface plasmon resonance of AZO-AgNPs complex.

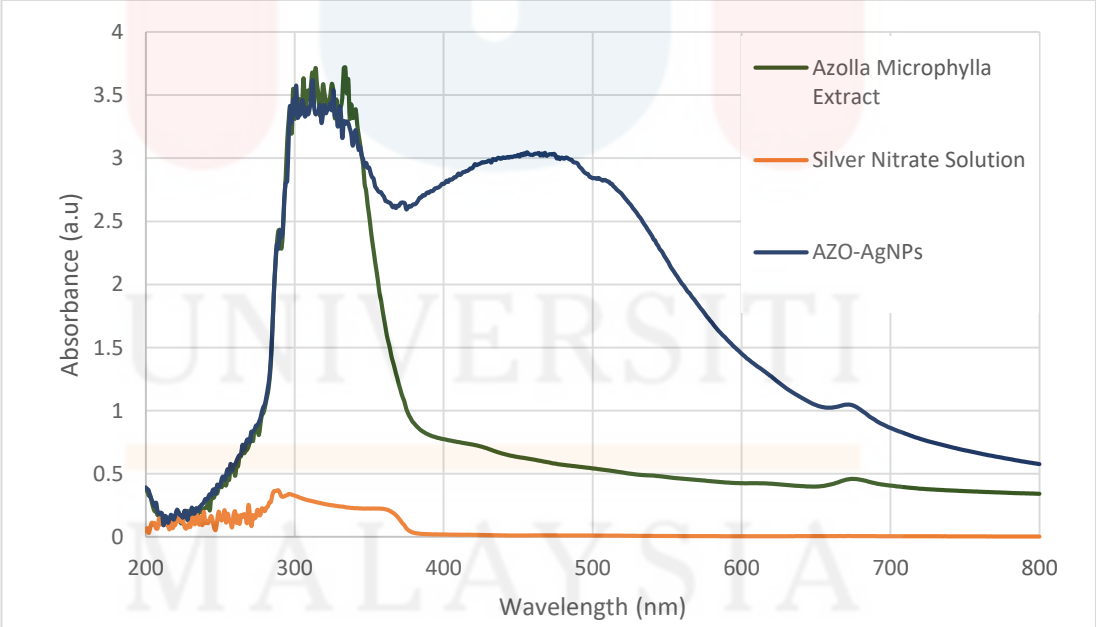

Figure 4.2: UV-Vis spectra recorded for *Azolla microphylla* extract, silver nitrate solution and biosynthesized of Azo-AgNPs.

Furthermore, as the reaction of time increase, the intensity of absorption peak will be more intensified as shown in Figure 3. This indicates the growth of biosynthesized AZO-AgNPs overreaction time (Khan, Tareq, Hossen, & Roki, 2018). As claimed by Mie's theory, the optical properties of metallic nanoparticles are influenced by localized surface plasmon resonance (LSPR) and the profile effect of LSPR bands are sensitive to the shape and size of the particles (Fan et al., 2014). Depending on their absorption spectra, single-band indicate for a spherical shape. Nevertheless, based on Figure 3, the absorption spectra are showing two plasmonic bands which can be expected for non-spherical or irregular shape (Garcia, 2011).

A popular explanation of the correlation between the wavelength and the diameter of nanoparticles is that smaller diameter nanoparticles exhibit lower wavelength refers to blue-shifting, while bigger diameter nanoparticle exhibits higher wavelength refers to red-shifting (Zhang et al., 2016). In this case, the highest absorption peak is 463 nm suggesting the size of Azo-AgNPs averaging about 20 nm to 30 nm diameters (Augustine, Kalarikkal, & Thomas, 2014). A remarkably similar results were reported in literature stating the absorbance peak at 430 nm to 480 nm indicate the formation of AgNPs by black pepper leaf (Augustine et al., 2014), *Acalypha indica* leaf (Selvakumar et al., 2009), *Eclipta prostrata* leaf (Rajakumar & Abdul Rahuman, 2011), and *Salvia spinose* (Pirtarighat, Ghannadnia, & Baghshahi, 2018).

However, after 24 hours of incubation time, the absorbance for AZO-AgNPs showed a decrease in value. The absorption peak became border indicate that the AZO-AgNPs aggregated with time. Plant metabolites are one of the main factors in the formation of AZO-AgNPs by acting as a reductive agent and stabilizer agent. Besides plant metabolites, factors such as temperature, concentration, pH, incubation time and

electrochemical potential of metal ion can affect formations and aggregation process of nanoparticles (Mohammadlou, Maghsoudi, & Jafarizadeh-Malmiri, 2016). From the result, it is noted the aggregation of AZO-AgNPs occurred due to the high concentration of *A. microphylla* extract and also uneven concentration between *A. microphylla* extract and $AgNO_3$ solution. When all $AgNO_3$ has reacted with *A. microphylla* extract forming AZO-AgNPs, the remain *A. microphylla* will react with AZO-AgNPs, leading to the generation of larger sizes of AZO-AgNPs, resulting aggregation of AZO-AgNPs overtime. Overall these findings are under findings reported by Veerakumar, Govindarajan, & Rajeswary (2013) stated that the aggregation of AgNPs occurred due to high concentration of $AgNO_3$ since there is lack of stabilizing agents with respect to the number of nanoparticles being produced or vice versa.

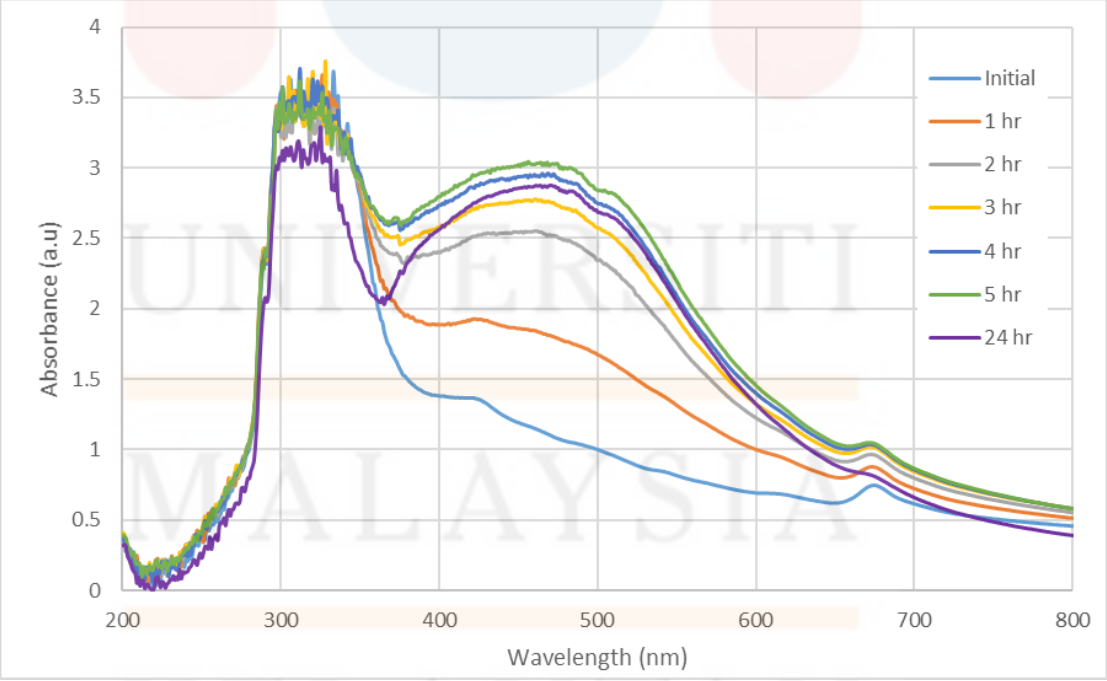

Figure 4.3: UV-Vis absorption spectra of the formation of AZO-AgNPs during reaction over time.

4.3 FT-IR

In this research, FTIR analysis was conducted with the aim to identify the potential biomolecules present in *A. microphylla* extract which is responsible for reducing and capping the bio reduced silver nanoparticles. In the green synthesis mechanism, plant extract has a dual role which acts as reducing and capping agent. As figure 4.4 indicates the FT-IR absorbance spectra of AZO-AgNPs complex which proves the presence of certain functional groups as reducing and capping agent for the AZO-AgNPs formation. Notably, the absorbance spectra show different stretches of bonds at two different peaks which were seen at 3327.91 cm-1 and 1636.18 cm-1. The spectra peak around 3327.91 assigned to OH stretching vibrations of carboxylic and phenols groups presents in the *Azolla microphylla* extract. It also noted that the peak is associated with N-H stretching vibration of NH_2 groups. Meanwhile, the sharp spectra at 1636.18 cm-1 corresponded to C=O stretching vibrations. The findings were almost the same to other AgNPs synthesized by different plants extract (Logeswari, Silambarasan, & Abraham, 2015).

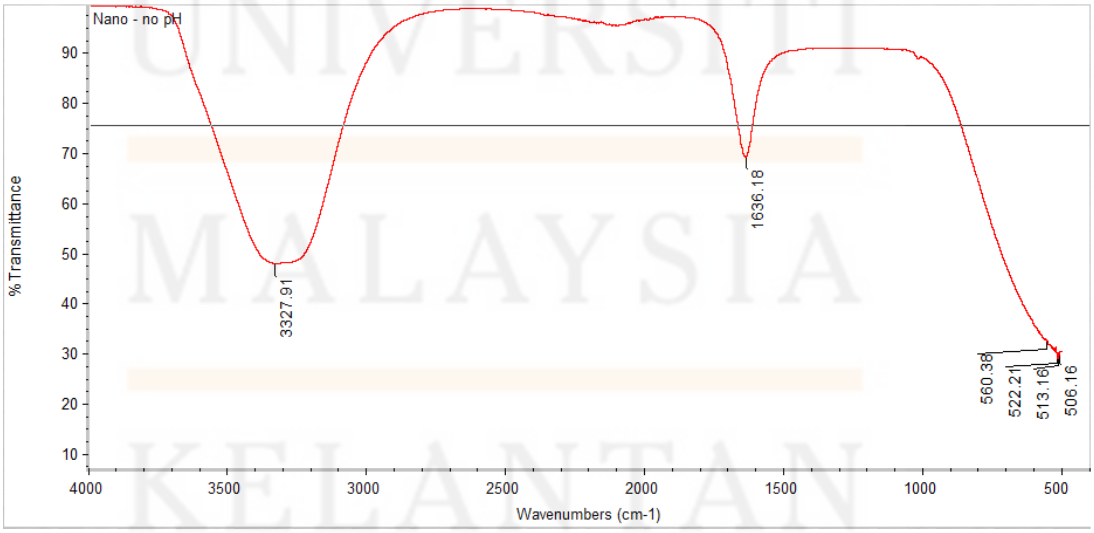

Figure 4.4: FT-IR spectra for AZO-AgNPs complex.

4.4 Larvicidal Bioassay

The AZO-AgNPs was used as bio-insecticides testing against late third instar larvae of *Aedes aegypti*. The larvicidal activity was conducted with concentrations of 20, 25, 30, 33, and 37 ppm for 24 hours' exposures. The mortality of *Ae. aegypti* was observed and recorded for each concentration in every replicates and the percentages mortality was tabulated in Table 4.1. From the result, it shows as the concentration increase, the percentage mortality also rises.

Table 4.1: Cumulative frequency percentage mortality of *Aedes aegypti*.

AZO-AgNPs (ppm)	**Percentage Mortality of *Aedes Aegypti* Larvae (%)**
Control (distilled water)	0
Control (Aqueous *Azolla microphylla*)	0
Control (Silver Nitrate Solution)	0
20	9.0
25	15.0
30	24.0
33	33.0
35	84.0
37	88.0

The LC50 and LC90 with a 95% confidence interval together with lower and upper confidence limit values and regression equation of AZO-AgNPs against *Ae. aegypti* was noted and tabled in Table 4.2. The mortality rates are chosen as dependent variable Y and as for independent variable X is the dose concentration. From the results, it clearly shows Y is positively relating to X indicating that mortality rates increase with the increasing dose concentration. The LC50 and LC90 values of AZO-AgNPs are 33.919 and 36.843 ppm respectively. In this study, the LC50 stipulate to

achieve 50% total kill of *Aedes aegypti*, 33.919 ppm of AZO-AgNPs was needed. Meanwhile, the values of LC90 is the concentration of AZO-AgNPs need (36.843 ppm) to kill a total of 90% of *Aedes aegypti*.

Table 4.2: Lethal AZO-AgNPs concentration of bioassay against *Aedes aegypti* larvae after 24 hours' exposure

Time	LC_{50} (ppm) with 95% confidence interval (LCL – UCL)	LC_{90} (ppm) with 95% confidence interval (LCL – UCL)	Regression Equation
24 hours	33.919 (20.513 - 35.272)	36.843 (35.425 – 63.524)	Y = 40.624 + 0.147X

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

An almost similar pattern of the result was obtained by Veerakumar, Govindarajan, & Rajeswary (2013) that the larvicidal effect of AgNPs synthesised by *Sida acuta* (Malvacea) leaf extract showed that highest mortality against the larvae of *Ae. aegypti* (LC50: 23.96 ppm, LC90: 44.05 ppm). However, other studies had reported a lower concentrations value could have high mortality of *Aedes aegypti*. As reported by Parthiban, Manivannan, Ramanibai, & Mathivanan (2019) observed the maximum efficacy in the synthesised AgNPs using Annona reticulate leaves extract shows the values of LC50 is 4.45 ppm while LC90 is 13.96 ppm against third-instar larvae of *Ae. aegypti*.

These present study proved high mortality on mosquito's larvae were achieved with a least minimum concentration of AgNPs. It may be due to the smaller size of nanoparticles can penetrate easily through the larval cellular membrane and also bind to protein and DNA. Thus, leading to denaturation of proteins and nucleic acids. As a consequence, causing destruction of cellular functions and eventually to death (J, K, Chandramohanakumar, & Balagopalan, 2012).

Over the years, it has been published that plants belonging to the genus of Azolla and family of Salviniaceae can present a raised in toxicity due the present of wide range of bioactive chemical compounds, such as flavonoids, tannins, terpenoids, and alkaloids which are a defensive chemical agent against insects (Kadimpati, Naidu, Kumar, Gunesh, & Rao, 2006). It is proven by a report stated, *Azolla pinnata* have a significant impact in body effects of larvae because of the bioactive chemical compounds present in the plant (Ahmad et al., 2018). The following study by Srivastava, Pandey, & Sharma, (1997) concluded the breeding of malaria-transmitting mosquitoes are completely vanquished in the pools, wells and ponds covered with Azolla. Similarly, the population of immature mosquitoes were significantly reduced due to the water in paddy fields were covered with mats of Azolla. A similar conclusion was also reached which revealed reduced in oviposition in India and less of adult emergence in Tanzania due to Azolla (Kadimpati et al., 2006).

Although there are others reports which investigate the synthesis of gold nanoparticles using Azolla sp. (Jha & Prasad, 2016), synthesis of Fe_3O_4 nanoparticles with the extracted pectin from the cell wall of *Azolla filicodies* (Rakhshaee, Giahi, & Pourahmad, 2011), and synthesized silver nanoparticles using *Azolla pinnata* whole plant hydroalcoholic extract (Korbekandi et al., 2014), at the moment, there are only two studies investigating the synthesized of nanoparticles using *Azolla pinnata* (Aqilah, 2019) and *Azolla microphylla* against *Aedes aegypti*.

The result demonstrated in this study notably has a lower value of LC50 and LC95 compared to the values of LC50 and LC95 by biosynthesised of silver nanoparticles by *A. pinnata* extract. The comparison of the result from biosynthesized of silver nanoparticles by *A. pinnata* extract and the AZO-AgNPs were tabulated in the Table 4.3. It shows significant difference in values of LC50 and LC95 between synthesised silver nanoparticles with *A. pinnata* and AZO-AgNPs. The LC50 and LC95 values of synthesized silver nanoparticles with *A. pinnata* are 121.570 ppm and 369.438 ppm respectively. Meanwhile, the Values of LC50 and LC90 of AZO-AgNPs are 33.919 and 37.717 ppm, noted that values were lower. Thus, showed silver nanoparticles synthesized using *A. microphylla* has higher efficacy as bioinsecticide.

Table 4.3: Comparison of larvae mortality when tested against AZO-AgNPs and Nano-synthesised silver particles from *Azolla pinnata*

Solution	**Time**	**LC_{50} (ppm) with 95% confidence interval (LCL – UCL)**	**LC_{90} (ppm) with 95% confidence interval (LCL – UCL)**
AZO-AgNPs	24 hour	33.919 (20.513 – 35.272)	37.717 (36.086 – 86.021)
Nano-synthesized silver particles from *Azolla pinnata*	24 hour	121.570 (0.622 – 173.695)	369.438 (235.469 – 12823971.50)

*Note: Result of nano-synthesized silver particles from Azolla pinnata from Aqilah (2018)

The mechanism and physiological basis of plant-synthesised AgNPs possess high efficacy in larvicidal bioassay to remain unclear and heated discussion among researchers. According to Zulkrnin et al., (2018), it was proven that the mortality of larvae is due to ingestion mechanism. After 24 hours of exposure to AZO-AgNPs, it was observed the abdomen of dead larvae turn into a dark brown colour, indicating the ingestion of AZO-AgNPs. In another context, few researchers proposed that the factor of killing by nanoparticle is the ability of nanoparticles to penetrate through the

invertebrate exoskeleton and also into insect's cells. Then, the nanoparticles bind with proteins and DNA, causing a change in on internal structure and finally their functionality. Interestingly, others reports stated that the doses of plant-synthesized AgNPs which result in high mortality to several species of mosquito larvae have a little or no effect on other non-targeted species (Ahmad et al., 2018). At the moment, the reasons for this phenomenon remain unknown.

CHAPTER 5

CONCLUSION AND RECOMENDATIONS

5.1 Conclusion

The green synthesis of AgNPs using *Azolla microphylla* extract at room temperature was reported in this study. Based on the qualitative and quantitative analysis, it proves that AZO-AgNPs were successfully produced. AZO-AgNPs began to form within 30min and higher formation yield as time reaction increase as shown by the UV-vis spectrum at 463 nm. However, after 24 hours the intensity spectrum band decreased, indicating the AZO-AgNPs may be aggregated with time. The FT-IR spectrum identified the biological molecules in *A. microphylla* which perform dual functions as reducing agents for the formation of silver nanoparticles and as stabilizer agents for silver nanoparticles in aqueous medium. Overall, the results confirmed that green synthesis is an effective and eco-friendly method for producing metal nanoparticles.

Next, this study was focusing on the efficacy of AZO-AgNPs against third instar larvae of *Aedes ageypti*. The larvicidal bioassay reported that the LC50 values is 31.680 ppm while LC90 is 43.610 ppm. Based on the results, it showed the AZO-AgNPs are already efficient at low concentration. It was noted, that the effectiveness of AZO-AgNPs to *Aedes ageypti* larvae are among the highest reported for AgNPs synthesized using any plant species belonging to the Salviniaceae family.

5.2 Recommendations

The findings of this research can be elaborated and further up more into other studies. In future work, investigating how to control the formation of AZO-AgNPs might prove important. The formations of AZO-AgNPs can be controlled by changing pH, the ratio solvents, the concentration of solvents, temperature, duration of time reaction and the duration exposed to light.

Besides, due to lack of resources, this study does not explore the physical and morphological form of silver nanoparticles. Future research could be done to examine the physical formation of silver nanoparticles and might extend the explanation of the size and shape of AZO-AgNPs. This will increase in understanding the formation of AZO-AgNPs.

The findings of this research show AZO-AgNPs has high efficacy against third instar larvae of *Aedes aegypti* sp. In future research, it could be done on a different stage of *Aedes aegypti* or a completely different species of mosquitoes. Last but not least, a study should be conducted on the effect of AZO-AgNPs against non-targeted species. This is necessary to ensure AZO-AgNPs did not possess any threat to the other non-targeted species in the same habitat.

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APPENDICES

APPENDIX A: Generated values for Nano-Synthesized Silver Particles from Azolla microphylla at 24 hours' exposure.

Confidence Limits							
		concentation			log(concentation)[b]		
Probability		Estimate	Lower Bound	Upper Bound	Estimate	Lower Bound	Upper Bound
PROBIT[a]	.010	29.191	2.872	32.115	1.465	.458	1.507
	.020	29.709	3.621	32.425	1.473	.559	1.511
	.030	30.043	4.195	32.624	1.478	.623	1.514
	.040	30.296	4.685	32.776	1.481	.671	1.516
	.050	30.504	5.126	32.901	1.484	.710	1.517
	.060	30.681	5.533	33.008	1.487	.743	1.519
	.070	30.838	5.917	33.103	1.489	.772	1.520
	.080	30.979	6.283	33.188	1.491	.798	1.521
	.090	31.108	6.636	33.267	1.493	.822	1.522
	.100	31.227	6.978	33.340	1.495	.844	1.523
	.150	31.725	8.589	33.648	1.501	.934	1.527
	.200	32.126	10.130	33.903	1.507	1.006	1.530
	.250	32.475	11.666	34.132	1.512	1.067	1.533
	.300	32.791	13.241	34.348	1.516	1.122	1.536
	.350	33.086	14.884	34.560	1.520	1.173	1.539
	.400	33.369	16.624	34.778	1.523	1.221	1.541
	.450	33.645	18.490	35.011	1.527	1.267	1.544
	.500	33.919	20.513	35.272	1.530	1.312	1.547
	.550	34.195	22.725	35.586	1.534	1.356	1.551
	.600	34.478	25.150	36.003	1.538	1.401	1.556
	.650	34.773	27.775	36.641	1.541	1.444	1.564
	.700	35.087	30.434	37.822	1.545	1.483	1.578
	.750	35.428	32.630	40.290	1.549	1.514	1.605
	.800	35.812	33.988	44.849	1.554	1.531	1.652
	.850	36.265	34.804	52.043	1.559	1.542	1.716
	.900	36.843	35.425	63.524	1.566	1.549	1.803
	.910	36.984	35.546	66.714	1.568	1.551	1.824
	.920	37.138	35.670	70.378	1.570	1.552	1.847
	.930	37.308	35.799	74.656	1.572	1.554	1.873
	.940	37.499	35.936	79.758	1.574	1.556	1.902
	.950	37.717	36.086	86.021	1.577	1.557	1.935
	.960	37.976	36.254	94.032	1.580	1.559	1.973
	.970	38.296	36.454	104.936	1.583	1.562	2.021
	.980	38.726	36.709	121.450	1.588	1.565	2.084
	.990	39.413	37.098	152.983	1.596	1.569	2.185

a. A heterogeneity factor is used.

b. Logarithm base = 10.

APPENDIX B: Extraction of *Azolla microphylla* extract.

Figure B1: Soxhlet apparatus

Figure B3: Rotary evaporator

APPENDIX C: Rearing of Aedes aegypti Larvae

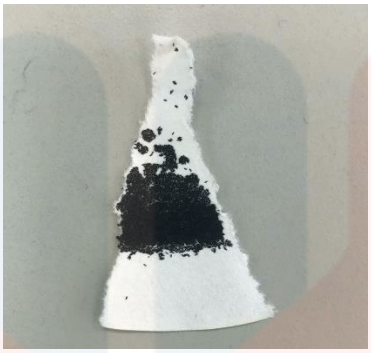

Figure C1: Eggs of *Aedes aegypti*

Figure C2: *Aedes aegypti* larvae rearing

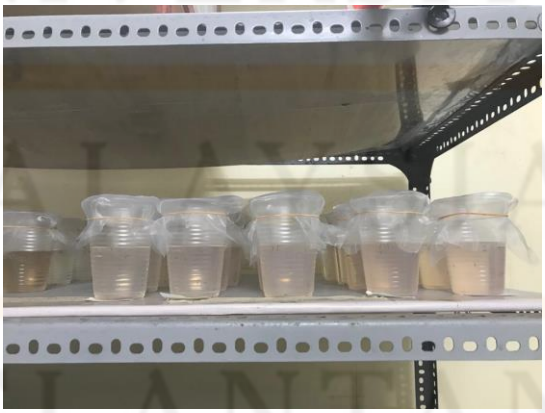

Figure C3: Set-up of larvae rearing experiment