STUDIES OF COMBINATION OF *Azolla microphylla* WITH SILVER NITRATE AND ITS EFFECT ON *Aedes aegpypti*

CHE NORHIDAYAH BINTI ABD. HALIM

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by

CHE NORHIDAYAH BINTI ABD. HALIM

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DECLARATION

I declare that this thesis entitled "Studies of Combination of *Azolla microphylla* with Silver Nitrate and its Effect on *Aedes aegypti*" is the result of my own research except as cited in the reference. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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Date :_____

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Studies of Combination of Azolla microphylla with Silver Nitrate and Effect on Aedes aegpypti

ABSTRACT

Plant extract potential in synthesis the nanoparticles currently drawn attention in the field on nanotechnology. The development in nanotechnologies that contribute in many field had led to the demand of the nanoparticle production. Current nanotechnology for nanoparticle synthesis clarified toxic to the environment and costly. This impulse to find procedure that more environmentally friendly in synthesis the nanoparticles. In the view of the recently increased interest in biosynthesis of nanoparticles, this research studies about the combination of *Azolla microphylla* with silver nitrate with the purpose of synthesizing silver nanoparticles from *Azolla microphylla* extracts. This research also studying the optimal conditions in synthesis the nanoparticle and characterized their properties. In the present study, the synthesis of nanoparticle were performed by mixing the *Azolla microphylla* extract with 1mM of silver nitrate solution. The colour changes into dark brown can be observed which indicate reduction process take place. The optimal conditions for synthesis silver nanoparticle were identified by testing volume ratio, time interval and pH parameter. Synthesized silver nanoparticles were then being characterized using UV-vis spectroscopy and FTIR. UV-vis spectrophotometer showed peak at 423nm due to plasmon surface resonance excited vibrations. FTIR analysis reveals the functional group presence in the silver nanoparticles. The research further with determine the effect of the combination of *Azolla microphylla* with silver nitrate on *Aedes aegypti*. The ability of other A*zolla* species in mosquitoes control in previous studies impulse the test on *Aedes aegypti* through 24-hour bioassay. The combination does not showed effective killing effect however repellent effect were proved. As the result the biological synthesis of nanoparticle were determined through the combination of the *Azolla microphylla* with silver nitrate in mosquitoes population might be give different effect due to different species.

Kajian Kombinasi Azolla microphylla dengan Silver Nitrat dan Kesan pada Aedes aegpypti

ABSTRAK

Potensi ekstrak tumbuhan dalam sintesis nanopartikel kini menarik perhatian dalam bidang pada teknologi nano. Pembangunan di bidang teknologi nano yang telah menyumbang dalam banyak bidang telah membawa kepada permintaan pengeluaran nanopartikel itu. nanoteknologi semasa untuk sintesis nanopartikel menjelaskan toksik kepada alam sekitar dan mahal. gerak hati ini untuk mencari prosedur yang lebih mesra alam dalam sintesis nanopartikel. Dalam paparan menarik yang baru-baru ini meningkat dalam biosintesis nanopartikel, ini kajian penyelidikan mengenai kombinasi Azolla microphylla dengan nitrat perak dengan tujuan untuk mensintesis nanopartikel perak daripada ekstrak microphylla Azolla. Kajian ini juga mengkaji keadaan yang optimum dalam sintesis nanopartikel dan mempunyai ciri-ciri sifat-sifat mereka. Dalam kajian ini, sintesis nanopartikel telah dijalankan dengan mencampurkan ekstrak Azolla microphylla dengan 1mM larutan perak nitrat. Perubahan warna menjadi coklat gelap boleh diperhatikan yang menunjukkan proses pengurangan berlaku. Keadaan yang optimum untuk nano zarah sintesis perak telah dikenal pasti melalui ujian keatas parameter nisbah jumlah, selang masa dan pH. Nanopartikel perak yang disintesis kemudiannya dicirikan menggunakan spektroskopi UV-vis dan FTIR. UV-vis spektrofotometer menunjukkan puncak pada 423nm kerana plasmon permukaan resonans getaran teruja. Analisis FTIR mendedahkan kehadiran kumpulan berfungsi dalam nanozarah perak. Penyelidikan diteruskan lagi dengan menentukan kesan gabungan Azolla microphylla dengan nitrat perak pada Aedes aegypti. Keupayaan spesies Azolla lain dalam kajian sebelum ini dalam mengawal nyamuk telah mendorong bagi melakukan ujian kesan kombinasi tersebut terhadap Aedes aegypti melalui 24 jam bioesei. Kombinasi tersebut tidak menunjukkan kesan pembunuhan yang berkesan bagaimanapun kesan penghalau telah dibuktikan. Hasilnya sintesis biologi nanozarah ditentukan melalui gabungan Azolla microphylla dengan nitrat perak, dalam populasi nyamuk mungkin memberi kesan yang berbeza kerana spesies yang berbeza.

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LIST OF ABBREVIATIONS

AgNP	Silver Nanoparticles
UV-Vis	Ultraviolet Visible Spectrophotometer
FTIR	Fourier Transform Infrared Spectroscopy
hrs	Hours
Ag	Silver
NP	Nanoparticles
kg	kilogram
g	gram
nm	Nanometer
mM	millimolar
mL	millilitre

LIST OF SYMBOLS

±	Plus or minus
°C	Degree Celsius
%	Percentage

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Nanotechnology is currently been recognized to have the potential in contributing to various fields such as development of drugs, water treatment, information and communication technologies and innovative production of materials. The direction of this technologies in creating and manipulating the materials through scaling up the single groups of atoms or bulk materials refining or reducing (Benelmekki, 2015). Nanotechnology also has led to the development in the nanoparticles production which had result a new materials and functional facilities. The properties of nanoparticles that having high surface area to volume ratio made the nanoparticles ability to react efficiently and very quickly. The size of nanoparticles that been created are 1-100 nm in dimension (Abou El-Nour, Eftaiha, Al-Warthan, & Ammar, 2010) which equal to the size of human proteins. Due to this uniqueness of properties and structural characteristics nanoparticles compared to its bulk material gives advantages to the combination to be an important process that can improve many sectors such as in production of anti-reflective optical coat, agriculture and medicine.

In order to synthesize the nanoparticles, two methods that commonly used are physical and chemical methods which clarify toxic to the environment and costly (Johnson, n.d). The lack of the methods impulse the researcher to develop the biological method that synthesize nanoparticle more eco-friendly and economically cheap. Plants extract act as biological methods in the field of nanotechnology for the

synthesis of silver nanoparticles. According to Braun et al., (2007) the plants at various constitutional level rich with the source of bioactive compounds which is non-toxic product that safe for the environment. The biomolecules in the plants extract also play an important role in reduction of the metal ion for biosynthesis. In certain applications like pharmaceutical, medical and biomedical, the usage of these nontoxic materials and green methods are important for the production of metal and metal oxide nanoparticles (Korbekandi et al., 2014). Among the various nanoparticles, silver nanoparticles showed numerous application based on its characteristic in medical field such as antibacterial, antifungal, larvicidal, and anti-parasitic (Braun et al., 2007). Silver are also found have the ability in exhibit the wound healing activities according to Vittal, R. R,.& Aswathanarayan, J.B. (2011) not only as the effective tool in preventing and retarding bacteria infectious.

The combination of *Azolla pinnata* with silver nitrate in previous literature by Korbekandi et al. (2014) reveals the potential of the free-floating aquatic ferns *Azolla pinnata* in reducing silver ions into silver nanoparticles. The study had proved the ability of *Azolla pinnata* in becoming one of the good reducing agent for the silver nanoparticles biosynthesis. So far, there is no report on the synthesis of silver nanoparticles using the *Azolla microphylla* extract. In this paper, the research focus on the combination of *Azolla microphylla* with silver nitrate in forming the silver nanoparticles. In addition, the silver nanoparticles produced by the act *Azolla microphylla* extract may have the potential to be developed as a source for vector control program insecticide. Because of this situation had provide the opportunity for this research to identify the effect of the combination of *Azolla microphylla* with silver nitrate on *Aedes aegypti*. The research was conducted by synthesis silver nanoparticles with Azolla and characterize the synthesized nanoparticles by using the ultraviolet

visible spectrophotometer (UV-Vis) and Fourier transform infrared spectroscopy (FTIR). Optimization were performed in order to study the optimal conditions for formation of silver nanoparticles using *Azolla microphylla* extract. The 24-hour bioassay test were performed using World Health Organization standard method for different concentration Azolla with aid of silver nanoparticles.

1.2 Problem Statement

Rapid growing in nanotechnology field due to its greater potential application in various fields which had led in great demand of nanoparticles. The physical and chemical method that commonly used in synthesis the nanoparticles give harm and toxic effect to the environment. Due to this situation, biological method had been recognized at recent that effectively synthesis the nanoparticle in safer way to the environment. The potential of *Azolla pinnata* in reducing silver ion to silver nanoparticles in previous studies, provide the opportunity to this research in further study on the synthesis of silver nanoparticles using different species which is *Azolla microphylla*.

Due to many benefit have been recorded of *Azolla* such as biolarvicide appeal this research on further analyse the effect of *Azolla microphylla* on the adult *Aedes aegypti*. This mosquitoes responsible in spreading the dengue and chikungunya which become a major worried for the risk of human health. However, expecting the extraction of the plant only requires the use of relatively large amount so as alternative in this research applied the combination of *Azolla microphylla* with silver nitrate to form the silver nanoparticles.

1.3 Objectives

The objectives of conducting this research are:

1. To synthesis and optimized formation silver nanoparticle using *Azolla microphylla plant* extracts.
2. To characterize the synthesized silver nanoparticle using UV-Vis and FTIR.
3. To determine the combination effect of *Azolla microphylla* with silver nitrate on *Aedes aegypti*.

1.4 Scope of Study

In order to achieve the purpose of this research, there are several main scope that the research are focusing on. The scopes are characteristics of silver nanoparticle with *Azolla* by using the ultraviolet visible spectrophotometer (UV-Vis), Fourier transform infrared spectroscopy (FTIR) and the combination effect of *Azolla microphylla* and silver nitrate on *Aedes aegypti* through 24-hours bioassay.

1.5 Significance of Study

The synthesized of silver nanoparticles with *Azolla pinnata* are importance due to the benefits that this silver nanoparticles offers to the environment, industry and health. This research provide the information that can be used in the educational purpose. The synthesis of silver nanoparticles will open up the opportunities for the other researcher to further the study using other plants and also other types of nano-materials. The ability of *Aedes aegypti* in transmit the disease made this research

important in the medical field. This research might help in control the mosquitos' population which reduces the risks for disease. The synthesis of silver nanoparticle using green plants gives a great advantages towards the environment since the process does not give any bad impact. While the other study are focusing on the potential of *Azolla pinnata* due to its biomolecules, this research provides the alternative by combining the different species which is *Azolla microphylla* with the silver nitrate. The synthesized of the silver nanoparticle properties that high in surface area to volume ratio made it more effective for application.

CHAPTER 2

LITERATURE REVIEW

2.1 *Azolla sp.*

Azolla was placed in family of *Azollaceae* that come from Kingdom of Plantae. These plant can be categorized into two sub-genera which are *Rhizosperma* and *Euazolla* where the *Azolla pinnata* and *Azolla nilotica* were listed under *Rhizosperma* while *Azolla microphylla, Azolla caroliniana, Azolla filculoides and Azolla Mexicana* were categorized under *Euazolla* (Raja, Rathaur, John & Ramteke, 2011).

The meaning of the *Azolla* name is plant that died when it dried which come from the Greek word *azo* (to dry) and *allyo* (to kill). These fern with the small, fast growing and free floating has distributed around the global. According to Raja, Rathaur, John & Ramteke (2011), these plant is an aquatic fern with dichotomous branches that naturally available on ditches, marshy ponds and moist soils which form a thick mat on surface of water and also able to cover the entire area of water.

One of the *Azolla* characteristics that completely give benefits to the environment is does not cause harm due to its non-toxic properties. A few studies in previous had proved that the *Azolla* is safe to the environments. According to Ravi et al. (2018), the exposure to the *Azolla pinnnata* plant extract did not make the aforementioned plant get wilt but somehow it become healthier. Besides, another study by Ravi et al. (2018) on Guppy fish, Poelicia reticulata toxicity test with *Azolla pinnata*

extracts. The findings of the experiment proved that *Azolla pinnata* extracts causes no harm to the fish.

2.1.1 Importance of *Azolla sp.*

Previous study on *Azolla have* provide the knowledge about the potential of this plants that give benefits to the plants and environment. There were a study that had shown the ability of *Azolla* in improving the agriculture sector that approaching to safer environment. According to Raja, Rathaur, John & Ramteke (2011), *Azolla* had been used as biofertilizer on the rice crop due to the rapid decomposing capacity and quick multiplication rates of *Azolla*. The used of the *Azolla* had increase the soil nitrogen and reduces the nitrogenous fertilizer requirement of rice crop which can reduces the chemical fertilizer usage and improve the plants and environment health.

On the other hand, another study had reported *Azolla* capabilities to remove nutrient like nitrogen and phosphorus in treating the wastewater. The utilization of the *Azolla* in waste water treatment are due to its capability in removing nitrogen and phosphorus from the water body and these plants efficacy in accumulating the heavy metals (Forni et al., 2001). In addition, the *Azolla* contribution in aquaculture sectors also been recognized since these plants widely used as fish feeds that able to promote the growth of fingerlings and adults.

2.1.2 Potential of *Azolla sp.* as source vector control program

Other than the following benefits, a few studies before had reported the finding about the potential of *Azolla sp.* plant as resources for vector biocontrol programs. This

is due to the insecticidal properties of phytochemicals in the plants as stated by Abraham & Aeri (2012) which based on their chemical nature react to larvae. Among the earliest studies about the effect of *Azolla* on mosquito breeding was done by Bao-lin (1988) from Hunan, China. The research used *Azolla filiculoids* on larval of *Culex tritaeniorhynchus* and *Anopheles sinensis*, eventhough in his observation the reduction densities of *Anopheles* was not clear but *Azolla* had reduced the density of *Culex* in average 69% by 3 months.

Another research by Pandey (2015) from Gujerat, India also proved the potential of *Azolla* as vector breeding control. This study shown the effect of *Azolla pinnata* on *Anopheles culicifacies* and *An.subpictus* mosquito oviposition in rice field. Lab study that also conducted in the same research resulted the changes of the significant behaviour and supress the egg laying properties of *Cx. quinquefasciatus* and *Cx. culicifacies* mosquitoes which effect from oviposition cause by *Azolla pinnata*.

The *Azolla* also been utilized as mosquitoes repellent. Recent study on the effectiveness of the plants with different form which is powder and fresh against the *Aedes sp*. The research found that powdered form more effective which needs lower concentration compared to fresh *Azolla* in achieving highest mortality of *Aedes sp.* (Ahmad et al., 2018). In spite of the fact many studies focus on the other *Azolla sp.* plant against with mosquitoes, there none of them have mentioned about the ability of *Azolla microphylla* as biological control agents. Hence, this study will be focused on effect of *Azolla microphylla* plants against *Aedes aegypti*.

2.2 Aedes *aegypti*

Aedes aegypti or also known as yellow fever mosquito, is a mosquito that capable in spreading some fever virus such as dengue, chikungunya, Zika, Mayaro and yellow fever and also some others disease agents. This mosquitoes have white markings on its legs and also some other marking in form of layer on its thorax upper surface which easier to be recognized. The mosquito nowadays can be found in tropical, subtropical and temperate regions throughout the world even though it actually originate from Africa. .

In life cycle of the *Aedes aegypti*, there will be four distinct stages which are eggs, larva, pupa and adult as shown in Figure 2.1. The cycle start with eggs emerge into the larvae when it was covered with water. For the larvae stage, there will be three times moulting before it transform into fourth stage which last from 6 to 8 day before forming a pupae. The pupae stage will last for about 1 to 2 days in tropic temperature but can extend until 9-12 days in cool temperature. Once the pupae develop into adult the life span can range from 2 weeks to a month depends on the environment conditions.

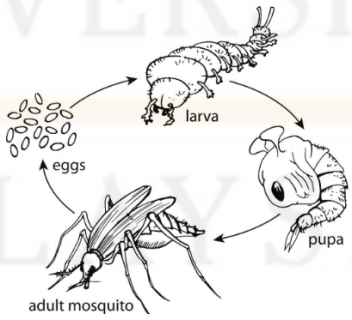

Figure 2.1: Life cycle of mosquitoes

This research aim to determine the effect of the *Azolla microphylla* plant extracts on the adult *Aedes aegypti*. To achieve the aim, the adultcidal bioassay test will be performed using WHO standard adult test method with *Azolla microphylla* extract. The acceptance of the used of plants extracts as bio-insecticide has led to increasing demand on the plants so new solution need to be discovered. Due to this situation, the synthesis of silver nanoparticles using the plant extract in field of nanotechnology recently drawn the attention.

2.3 Silver nanoparticles

2.3.1 Nanotechnology

Nanotechnology involve science, technology and engineering which conducted about 1 to 100 nanometers at nanoscales. Nanotechnology has widely used in nanomaterials production, manipulation, use and characterization. Nanotechnology mainly concern the synthesis of nanoparticles of different sizes, shapes, chemical compositions and polydispersity (Sadowski, 2012). Silver nanoparticles play an important role in nanotechnology specifically for nanomedicine compared to the others metallic nanoparticles in biomedical applications (Zhang et al., 2016). Other than that, the unique properties of silver nanoparticles include optical, high conductivity of electric, biological, electrical and thermal made them applicable to be used in several fields such as medical, foods, health care, consumer and industrial purpose.

According to Zhang et al. (2016), there are three different approaches to synthesis the nanoparticles which are physical, chemical and biological methods. The

physical methods is synthesis of silver nanoparticles that used pyrolysis and spark discharging (Pluym et al., 1993). The advantages of this methods are speed, free hazardous chemicals and used radiation as reducing agent however the lack of this method are high consumption of energy, low yield, cause solvent contamination and uneven distribution (Zhang et al., 2016). In chemical methods, used the water or organic solvent to synthesis the silver nanoparticles. The major benefits of using the methods are high yields but it has more disadvantages which are expensive methods and materials used for silver nanoparticles synthesis are toxic and hazardous (Mallick, 2004). Due to the lack of the physical and chemical methods, alternative methods which is biological methods are the better options.

2.3.2 Synthesize of Silver Nanoparticles with Green Plant

Biological methods which is green synthesis offers many benefits includes effective cost, to reducing agent it does not have any physical barrier and removes the chemical used toxic effect during the synthesis. In addition, biomolecules that contain in the plants extract such as flavonoids, phenols, alkaloids, proteins provide the ability in reducing metal ion. The chemical structure of flavonoids that available in plants extract which function as reducing agent are shown in Figure 2.2. Due to the positive impacts of the green synthesis, had attracted the attention of the researchers towards the green synthesis of nanoparticles. Numerous studies that focused on the biosynthesis of silver nanoparticles which includes various type of plants as reducing agents.

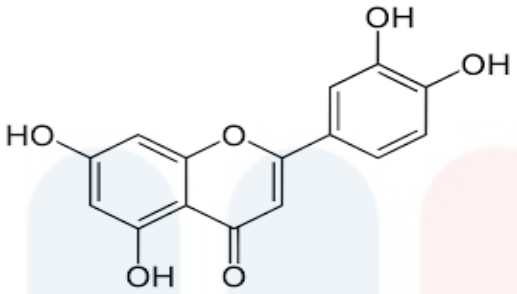

Figure 2.2: Chemical structure of flavonoids

Previous study done by Shankar et al. (2003) on *Geranium* leaf in assisting biosynthesis of silver nanoparticles. The research reported the rapid reduction of silver ion and formation of stabilized and crystalline silver nanoparticles by extracts Geranium leaf (Shankar et al., 2003). The study by Korbekandi et al. (2014) also reported the potential of *Azolla pinnata* in the biosynthesis of silver nanoparticles. The whole plant of *Azolla pinnata* were used and been extracted using hydroalcoholic with different concentration which resulted different shape and size of nanoparticles produced.

By using another plant, the research done by Krishnaraj et al. (2010) had showed effectiveness of biosynthesized silver nanoparticles in antimicrobial activity against water borne pathogens. In the research *Acalypha indica* leaf extract had been used to synthesis the nanoparticles and proved an excellent antimicrobial activity by changing the membrane permeability and respiration activity of the bacteria cell. Johnson et al. (2012) discovered the potential in cytotoxic and phytotoxic of green synthesis silver nanoparticles using *Cyathea nilgirensis Holttum*. This finding give benefits in agriculture and medical sector through the ability to control abnormal cell growth and weed plant.

2.4 Optimization of Synthesized Silver Nanoparticles

Optimal condition influenced the formation of the synthesized silver nanoparticle. The optimization showed the important role for certain activities such as catalyst, optical sensors, in data storage and antimicrobial application which this activity are size and shape dependent. Previous study by Singh et al., (2019) showed the important role of the optimum size of the silver nanoparticles for the antimicrobial activity. The optimal condition of the combination *Azolla microphylla* with silver nitrate were identified using few parameter such as volume ratio, pH and time interval.

2.5 Characterization of Synthesized Silver Nanoparticle

Characterization of the nanoparticles is needed after the synthesis process, due to the significant impact that could be have by the physicochemical properties of particles (Zhang et al., 2016). The factors that should take into account are particle size, size distribution, morphology, particle composition, surface area and solution reactivity in order to assess accurate nanoparticle toxicity (Gurunathan et al., 2009).

2.5.1 UV-Vis Spectrophotometer

For the synthesized nanoparticles primary characterization, UV-Vis spectrophotometer is a valid and applicable procedure that will also uses to monitor synthesis and stability of silver nanoparticles (Sastry et al., 1998). The unique optical properties of silver nanoparticles allow them strongly interact with the wavelength of the light specifically. The electron move freely in silver nanoparticles since the band of conduction and valence lie very close to each other. The collective oscillation of

electron of silver nanoparticles in resonance with light wave causes the free electron rise to surface plasmon resonance absorption band (Zhang et al., 2016).

Previous studies also used the UV-Vis in order to obtain the primary characterization processes on the synthesized silver nanoparticles with the *Prunus persica* plant extract. The research done by Kumar et al. (2017) shows the observation of the peak that emerged at 440 nm which cause by the excitation of the surface plasmon vibrations. Another study by Zainal Abidin Ali et al. (2016) had monitored the reduction of Ag ions which the UV-Vis spectrum that been observed were between wavelength ranging 200-700 nm.

2.5.2 Fourier Transform Infrared (FTIR)

Zhang et al. (2016) reported the ability of FTIR to detect small changes of absorbance that could differentiate the functional active residue of small absorption bands from the large background absorption of entire protein. FTIR commonly used to identify the biomolecules that might involves in synthesis of nanoparticles for academic and industrial research. FTIR is an effective technique to identify the biological molecules role during the reduction of silver ion (Zhang et al., 2016).

In order to identify the biological molecules role in the synthesis of silver nanoparticles which specifically during the reduction of silver ion process, FTIR analysis had been conducted for the determination of functional groups in the AgNPs. According to Hajra (n.d), the FTIR spectra able to clearly record the biomolecules that responsible in reducing the silver ion. The finding of her research had detected the aromatic group presence in the sample which the wavelength of 400-500 cm^{-1} had been observed. The other study conducted by Veerasamy et al. (n.d) also used FTIR

analysis to characterize the synthesized AgNPs through determining the functional group present. The study on the green synthesis of silver nanoparticles using plant mediated detected the protein group of the plants extract that involved in converting the silver nanoparticles from the silver ion.

2.6 Comparison of Result Obtained with Recent Research

Mostly, the combination of plant extract with silver nitrate in previous study showed an effective larvicidal insecticide. A research on synthesized silver nanoparticles with Cassia fistula fruit pulp against *Aedes alsopictus* and *Cylex pipiens pallens* was carried out by Fouad et al., (2017). The result obtained in this research showed higher concentration were used compared to other research on the combination between plant extract and silver nitrate. The fruit pulp aqueous solution that used were 9172 ppm and 48753 ppm which cause 95% mortality while silver nanoparticles synthesized from fruit pulp need 519.3 ppm and 2340 ppm to cause 95% mortality of mosquito larvae. This research showed the example of the ineffective of combination between plant extract and silver nitrate.

CHAPTER 3

MATERIALS AND METHODOLOGY

3.1 Sample Collection

A total 20 kg of healthy and fresh *Azolla microphylla* shown in Figure 3.1 was collected from Pasir Mas, Kelantan. The plants were identified based on morphological phyllotaxis structure. To remove any impurities of other sources before it can be used for extraction process, the *Azolla pinnata* plants were washed by using tap water.

Figure 3.1: Fresh *Azolla microphylla*

3.2 Plant Extracts Preparation

For the preparation of *Azolla pinnata* extracts, leaves of the fresh plants were washed and then leaved for sun-dried (30°C ± 4°C room temperature) following Ravi et al. (2018) for 2 days. In order to get the plants in powder form, the dried leaves

mechanically powdered using electrical blender and then were sieved into more fine powder form.

Next, the extraction of the plants process is proceed using the Soxhlet extraction method. The total 20 g of dried *Azoll*a powder were placed into the paper thimble of the Soxhlet extraction apparatus (Favorit, Malaysia) which following the recent method by Ravi et al. (2018). Then, the sample was avoided from overflowing into another part of the apparatus by putting some cotton wool on the top of thimble. The extraction solvent which is 350 millilitre of methanol was added into a round-bottom flask with heating mantle underneath.

In order to extract the plant compound into round bottom flask, the methanol solvent were heated together with the fine powder of *Azolla* for 8 hours at the temperature that not exceeding the boiling point of solvent. The extraction process in Soxhlet apparatus (Figure 3.2) was performed until the solvent in siphon arm is observing to become clear. The methanol next was evaporated using rotary evaporator until the methanol completely separated from the extract solution and the crude extract plant left in the bottom flask weighed then store in universal bottle at -4°C for next experiment studies.

Figure 3.2: The setup of Sohxlet extraction

3.3 Silver Nitrate Preparati

Silver nitrate was weighed (42.47mg) and then slowly transferred into the beaker that contain distilled water. The solution was stirred slowly in the beaker. Next, the mixture was transferred into volumetric flask and the distilled water was added until the mark of volumetric flask (250ml).

3.4 Synthesis of Silver Nanoparticles with Plant Extracts

The mixture of 12 ml of *Azolla microphylla* leaf extract with 88 mL of 1 mM silver nitrate solution was kept for 24 hours. The changes of silver nitrate from colourless into brownish occurred after few minute of incubation. The colour turned into more dark with time which indicate the formation of silver nanoparticles.

3.4.1 Optimization of Silver Nanoparticles

For the optimization, different parameter for UV-vis analysis were used such volume ratio, time interval and pH. Different volume ratio of silver nitrate and *Azolla microphylla* extract solution were mixed together in the ratio of 88ml:12ml, 100ml:1ml and 90ml:10ml. Next, the time interval parameter where the 1Mm of silver nitrate were mixed with *Azolla microphylla* solution in the ratio of 88ml:12ml and the reaction was monitored for initial, 1 hrs, 2 hrs and 24 hrs using UV-vis spectroscopy. This absorbance measure were aim to study the stability of the SPR with time. The mixture of the *Azolla microphylla* and silver nitrate solution was adjusted the pH into 7, 8 and maintain the normal condition which is pH 5.7 and analysed for the size and position of SPR peak.

3.5 Characterization of Synthesized Silver Nanoparticles

3.5.1 UV-Vis Spectrophotometer

The bio-reduction of silver ion into silver was monitored by visual inspection in addition measuring the solution spectrum using UV-Vis. 1mL of silver nanoparticles was diluted with distilled water and then UV-Vis spectrum of the solution was observed of reaction time on HACH DR 6000 UV at resolution 1 nm between wavelength 200-800 nm following the previous study by Ali, Yahya, Sekaran, & Puteh (2016).

3.5.2 Fourier Transform Infrared (FTIR)

According to Hu et al. (2012), FTIR was used to determine the functional group in silver nanoparticles. Silver nanoparticles were analysed for the possible functional group that might presence during the reduction of silver ion (Sundaravadivelan et al., 2013). The range that was used by the FTIR for carrying the analysis is 400 - 4000 cm^{-1}.

3.6 Source of *Aedes aegypti* Egg

The *Aedes aegypti* egg were obtained from Vector Control Research Unit (VCRU) in Universiti Sains Malaysia (USM), Penang. The mosquito's life cycle were treated and maintained at the temperature of 25 - 30°C, humidity of 80 ± 10% RH and pH of 6.95 to 7.03 following Rajiv et al., (2018) methods.

3.7 Rearing *Aedes aegypti* Larvae

The method were followed the method used by the Zuharah et al., (2017) in the previous studies as shown in Figure 3.3. The eggs were hatched into a plate that contained seasoned water for 24-hours. In order to trigger the hatching process, 0.2g of larval food with ratio 2:1:1:1 of dog food, beef liver, yeast and milk powder. The changing of diet and seasoned water were done until pupae stage. Then, there is no diet or moisture needed on the cup that were placed the pupae. The pupae then were placed in a mosquito's cage ($20cm^3$) at room temperature and fed with diet mixture (Mishra, Kumar, & Malik, 2011). According to WHO, the *Aedes aegypti* take 8 to 14 days to develop in adult which were used for bioassay.

3.9 Adultcidal Bioassay

For the adultcidal bioassay, the method was performed by followed WHO method (2013). The concentration for the aqueous crude extract was tested at 5000, 10000, 15000 and 20000 ppm and AgNPs were tested at 1, 2, 3, 4, 5, 10, 50, 60,70, 80, 90, 100, 500, 700, 900, 1300 and 1500 ppm concentrations. The Whatman no. 1 filter papers (12cm x 15cm) were impregnated with 2ml of aqueous *Azolla microphylla* extract and AgNPs. After dipping, the paper were allowed to air-dried and then placed in plastics tube of WHO test kits (125mm length x 44mm diameter) which is the exposure tube. The blank paper were treated with silver nitrate and distilled water that used as control.

Twenty five female, non-blood fed and 3-5 days mosquitoes were collected and introduced into holding tube for 1 hour which allowed the mosquitoes to

acclimatize. The mosquitoes then were transferred to exposure tube with treated paper. After 1 hour exposure in the exposure tube, the mosquitoes were returned to holding tube and kept for 24-hour recovery period. A pad of cotton that soaked with 10% sucrose solution was placed on the mesh screen of the tube. Each concentration include a set of control (silver nitrate and distilled water) were replicate into five for each test.

Figure 3.3: Adulticidal bioassay set-up according to WHO (2013)

The mortality of the *Ae. Aegypti* were recorded if after the exposed of the adult *Ae. aegypti* with the concentration for 24 hours. The sign such as not able to move even after being touched was considered as dead. Figure 3.9 showed mortality calculation by Hassan et al. (2015):

$$\text{Observed mortality} = \frac{Total\ No\ of\ Dead\ flies}{Total\ sample\ size} \times 100 \qquad (3.1)$$

CHAPTER 4

RESULT AND DISCUSSION

4.1 Synthesis of Silver Nanoparticles

4.1.1 Visual Observation

Figure 4.1 (A) shows a bottle of synthesized silver nanoparticles by mixing the 1 mM of silver nitrate and *Azolla microphylla* extract solution. The solution of *Azolla microphylla* extract without $AgNO_3$ showed light green in colour while the colourless of silver nitrate solution can clearly be observed in Figure 4.1(B). The colour however slowly changed when the solution were mixed and turned into dark-brownish colour after several minutes of reaction. The appearance of the observed brownish colour point out the presence of silver nanoparticles in the reaction mixture.

According to Insciences, (2011) the colour changing of the solution is due to the excitation of surface plasmon vibration in the silver nanoparticles and also indicate the reduction from Ag^+ to Ag^0 (Yasin et al., 2013). The colour changing of the solution that caused by the bio-reduction of silver ion to silver is due to the presence of the active compounds in the plants extract such as phenols, alcohols and proteins (Abirami et al., 2016). The changes of this colour is similarly with the other studies of green synthesis using different type of plant extracts. From this, the reduction of Ag to Ag^+ by the *Azolla microphylla* aqueous extract was confirmed has taken place.

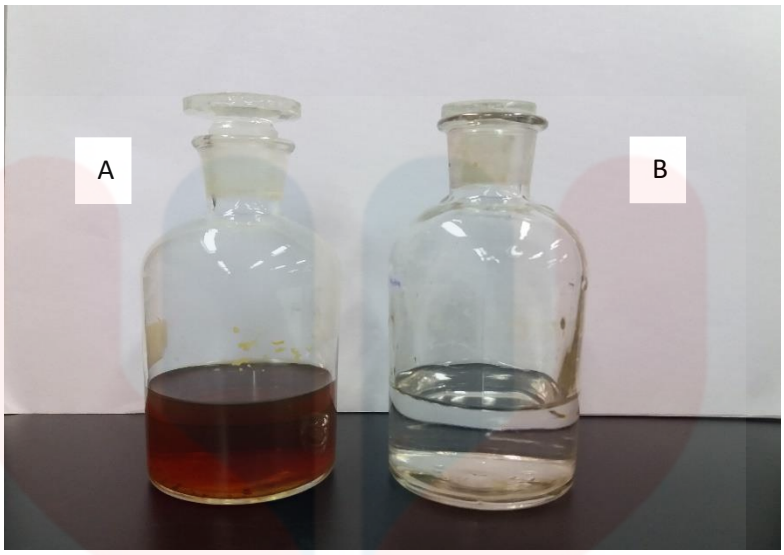

Figure 4.1: Synthesized AgNPs (A) and aqueous solution of 1mM AgNO3 (B)

4.2 Optimization of Silver Nanoparticles Synthesis

4.2.1 Volume ratio

The optimization of volume ratio ranging 88ml:12ml, 100ml:1ml , 90ml:10ml of 1 Mm silver nitrate and *Azolla microphylla* aqueous extract were used. Different volume ratio that been tested were determined based on the volume ratio that used in synthesized of silver nanoparticles from the other reported studies. The studies by the Muthukumaran et al. (2016) used the 88ml:12ml in the synthesis of silver nanoparticles while in some other reports, volume ratio of 100ml:1ml was used. Previous research from Mondal et al., (2014) also synthesized the silver nanoparticles using *Azolla pinnata* extract in 90ml:10ml of volume ratio. According to Dada et al., (2018) different literature showed the biological method or green synthesis route where for better formation of the silver nanoparticles more silver ion is needed.

Among the volume ratio tested, maximum silver nanoparticles occur in volume ratio 88ml:12ml which was further confirmed by the formation of sharper and highest

peak in spectroscopy as shown in figure 4.2. Sharper peak indicate formation of smaller sized silver nanoparticles (Mondal et al., 2014) while higher absorbance value indicate higher formation of silver nanoparticles. Thus, this ratio obtained were considered as optimum condition and next parameter was performed based on this ratio.

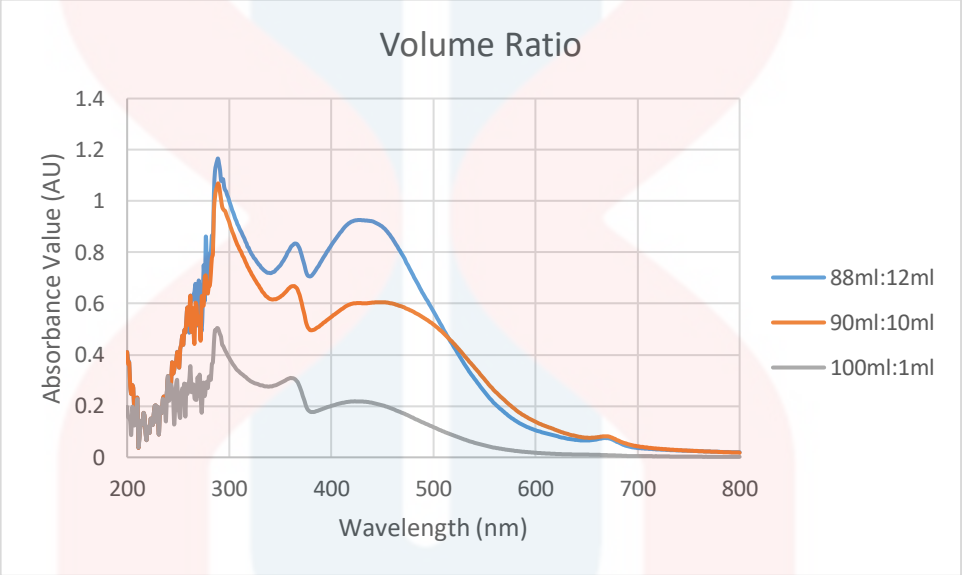

Figure 4.2: UV-vis spectroscopy results showing different volume ratio of silver nitrate and *Azolla* aqueous extract

4.2.2 Time Interval

The effect of the reaction time was showed in the Figure 4 where the UV-vis spectroscopy was measured at different time interval for the AgNPs formation. The effect of the reaction time was investigated by monitoring the formation of AgNPs for initial, 1, 2 and 24 hours at room temperature. From figure 4.3, the intensity of the absorbance obtained were increased along with the time increased which indicated the amount of AgNPs produced from the mixture. The best surface plasmon resonance peak was observed from the UV-vis spectroscopy results is within 458 nm at 24 hours.

Increasing the contact time enhances excellent plasmon band formation because large amount of Ag+ has been converted to Ag0. However, further increase in the contact time leads to noticeable decrease in the absorption intensity and wavelength which is an indication of some aggregation of silver nanoparticles leading to increase in particle size (Dada et al., 2018). The other parameter was carried out at 24 hours for further investigation since it is the optimum time obtained.

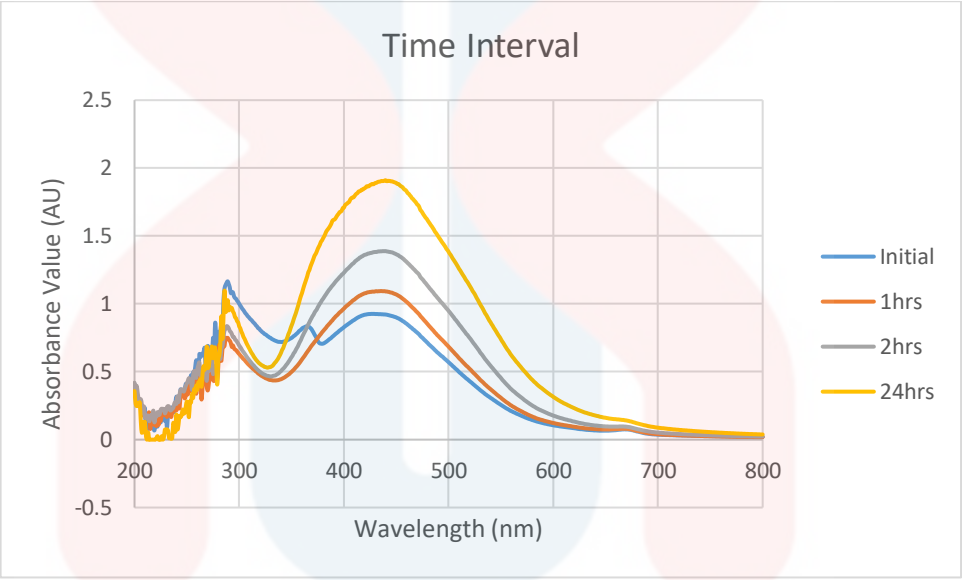

Figure 4.3: UV-vis spectroscopy result showing AgNPs measured at different time intervals

4.2.3 pH

The pH play an importance role in the synthesis of silver nanoparticle. The mixture solution was adjusted into various pH values to determine the UV-Visible spectra in order to investigate the influence of pH on the synthesis of AgNPs. The absorbance value of silver nanoparticles with different pH such as 7, 8 and normal condition which is 5.4 (weak acid) are shown in Figure 4.4.

The result in Figure 4.4 showed the peak area and height of the UV-visible spectrum obtained for the reaction solution at pH 8 were highest. This formation of the

AgNPs means the pH dependent and among the various pH studied a pH of 8 was the optimal. Alkaline environment seems favored the formation of the nanoparticles. Previous studies by Prathna et al., (2011) reported that the addition of NaOH to silver nitrate solution favors the formation of silver oxide (Ag_2O) precipitate which is reduced to pure silver (Ag) in the presence of a suitable reducing agent for example reduction of Ag^+ to Ag^0 occurs on the surface of existing colloids in the system.

Reaction pH has ability to change the electrical charges of biomolecules which might affect their capping and stabilizing capacity and the subsequent growth of nanoparticles. Phenols classified as weak acids which react with and dissolve in strong bases such as sodium hydroxide (Chou et al., 2008). However, due to the intended end use of the nanoparticles in insecticidal activity studies which could be pH sensitive, a pH of 5.7 (normal condition) was used in this synthesis.

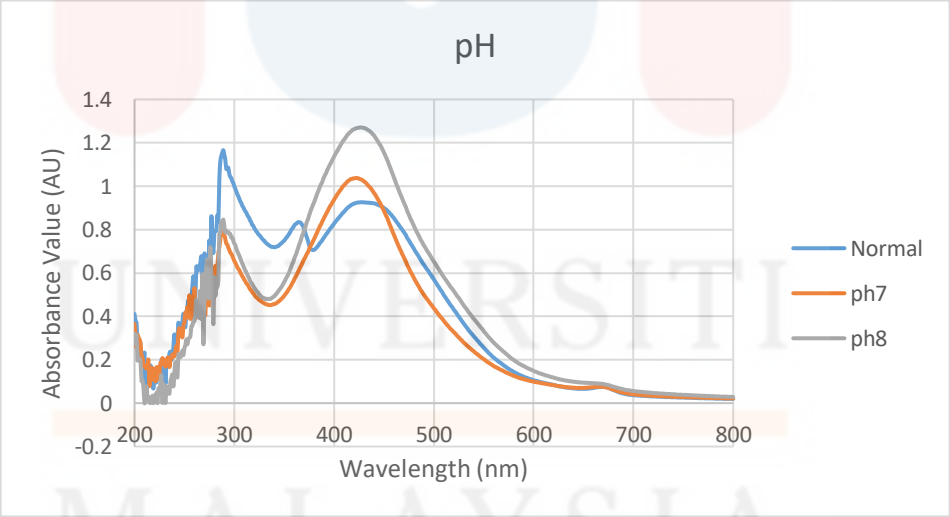

Figure 4.4: UV-vis spectroscopy showing different pH of silver nanoparticles

4.3 Characterization of Synthesized Silver Nanoparticles

4.3.1 UV-visible Spectroscopy

UV-vis spectroscopy considered as valuable tool in characterizing the structural of SNPs which become a fundamental technique in ascertaining the stable metal nanoparticles formation in aqueous medium (Braun et al., 2007). The excitation due to applied electromagnetic field of surface plasmon resonance in the silver nanoparticles were analyzed using the UV-vis spectroscopy. The collective oscillation of the electron conduction in resonance with the irradiated light wavelength had explained the appearance of the brown color. The surface plasmons resonance peaks with values of λ_{max} that typical AgNPs have are in visible range of 400-500 nm. As shown in figure 4.5, the synthesized of silver nitrate with the *Azolla microphylla* were found to have an absorbance peak at 423 nm.

The peak of surface plasmon resonance (SPR) provides a convenient spectroscopic signature for the silver nanoparticles formation through the appearance with maximum 423 nm that indicate the reduction of silver ions and formation of stable nanoparticles. (Sila et al., 2019). UV-visible absorbance of *Azolla microphylla* plant extract also showed absorbance near 315 nm indicating the phenols in the extract respectively (Figure 4.5). Absorption peak at around 315 nm shown in Figure 4.5 disappeared during the reaction which indicates the involvement and role of phenols in the reaction.

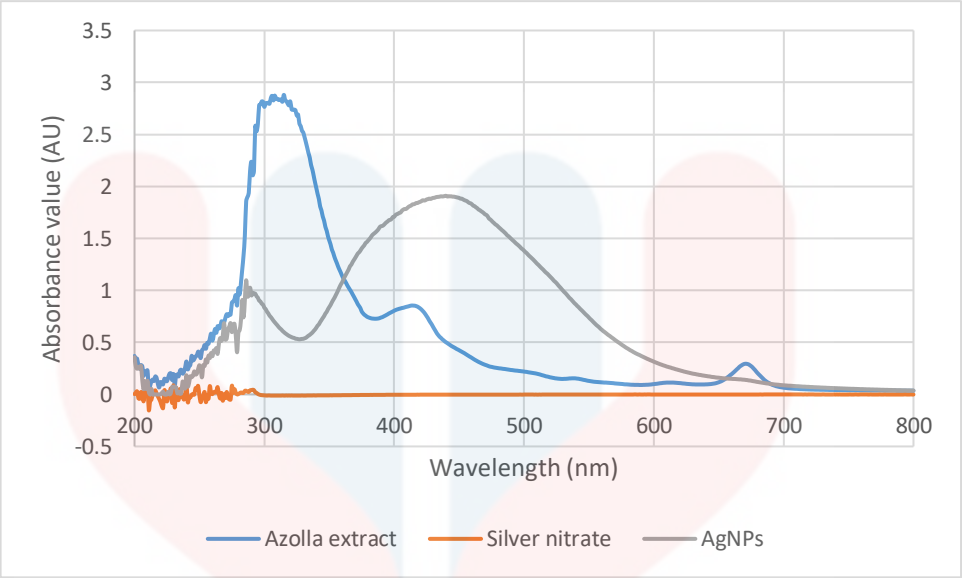

Figure 4.5: UV-vis spectra recorded for synthesis of silver nanoparticles using *Azolla microphylla*

4.3.2 Fourier Transform Infrared (FTIR)

In order to identify the interaction between the silver nanoparticles and protein, the FTIR measurement of biosynthesized silver nanoparticles was carried out. FTIR analysis of the purified nanoparticles in Figure 4.6 showed the presence of prominent absorbance band at the 3328.01 cm^{-1} and 1636.15 cm^{-1}. The absorbance band at 3328.01 cm^{-1} corresponds to O-H stretching in alcohol and phenolic compounds. The peaks at 1636.15 cm^{-1} are assigned to C-O primary amide I bond of proteins arising due to carbonyl stretch in proteins. The obtained groups responsible in stabilizing the silver nanoparticles formation and reduction of Ag to Ag^{+} (Raut et al., 2010).

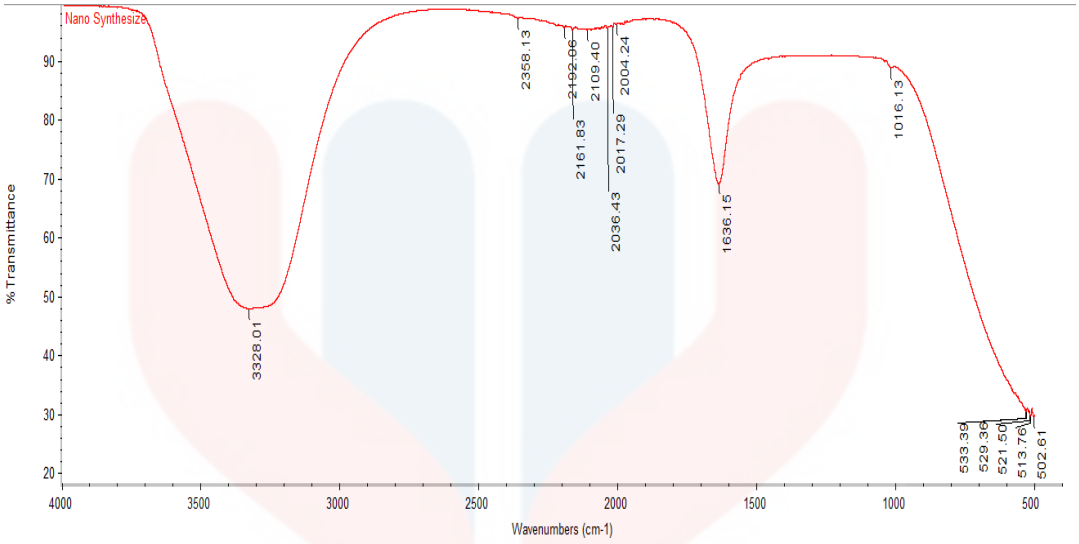

Figure 4.6: FTIR spectrum of synthesized Silver nanoparticles with *Azolla microphylla*

The absorbance band at 3328.10 cm^{-1} might also assigned by the N-H stretching and bonded O-H groups of carboxylic acid. The obtained absorbance at 1636.15 cm^{-1} also close to the reported native proteins (Jyoti, Baunthiyal, & Singh, 2015). This absorbance indicate predominant surface capping species having –C=O which involved in stabilizing the silver nanoparticles. The –C=O also relate with the formation of backbone. Protein interaction with the biosynthesized nanoparticles and unaffected secondary structure during the reaction with Ag^{+} ion or post binding with AgNPs were suggested through this evidence.

FTIR studies proved the strong ability of carbonyl group from the amino acid residues and protein to bind metal which indicate the possibility of proteins forms the nanoparticles in order to prevent the agglomeration and stabilizing the medium. Thus, suggesting the dual role of AgNPs formation and stabilization in aqueous medium by biological molecules (Sundaravadivelan et al., 2012). The result obtained confirmed

the phenols and proteins presences that function as reducing and stabilizing agents for the silver nanoparticles.

4.4 Effect on *Aedes aegypti*

4.4.1 Behavioral Response

Figure 4.7 showed behavioral effects of adult *Aedes aegypti* during the bioassay test the mosquitoes were observed assembled at the upper and bottom part of the tube. This situation occurred during the exposure period when the mosquitoes were exposed to the test paper. The responses observed due to the avoidance of the mosquitoes from in contact with the paper that treated with aqueous crude extract and synthesized silver nanoparticles with *Azolla microphylla* (Azo-AgNPs). Even though the *Azolla microphylla* extract and AgNPs did not give the killing effect on the *Aedes aegypti* but it had proved the repellency effect based on the behavioral response observed during the bioassay.

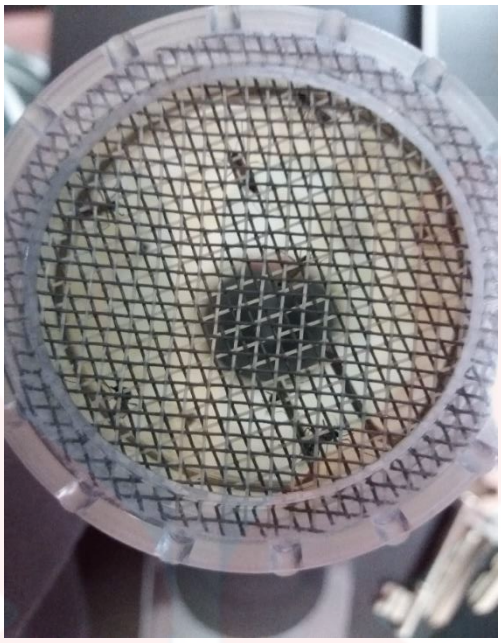

Figure 4.7: Visualization picture capture on behavioural response effects of 3-5 days adult aedes aegypti during 1-hour exposure period of bioassay test in aqueous *Azolla* extract and Azo-AgNP

4.4.2 Adulticidal Bioassay

Adulticidal activity of synthesized silver nanoparticles was tested against adult *Aedes aegypti* with concentration of 1-20,000 ppm for 24-hours. The results of the aduticidal activity of aqueous crude extract and synthesized AgNPs was noted and presented in Table 4.1 and Figure 4.8. A research on *Azolla pinnata* against adult *Aedes aegypti* was carried out by Rajiv et al., (2019). The result of adulticidal activity of *A. pinnata* were compared with the result of *A.microphylla* aqueous crude extract which showed the different of mortality effect on *Aedes aegypti* between this two species as shown in Table 4.1. At 5000 ppm, *A.pinnata* aqueous extract appeared to be effective

against *Aedes aegypti* with 88% mortality. However, the *A.microphylla* aqueous extract demonstrated nil mortality at the same concentration.

Table 4.1: Adulticidal activity of *Azolla pinnata* aqueous and *Azolla microphylla* aqueous extract against Aedes aegypti

Treatment	Dose (ppm)	Mortality (%)	Treatment	Dose (ppm)	Mortality (%)
	1000	6		5000	0
Azolla pinnata	2000	20	*Azolla microphylla*	10,000	1
	3000	72		15,000	0
	5000	88		20,000	1

The different in result showed by both species indicated that the mosquito species (*Aedes aegypti*) experienced different level of susceptibility to the plant extracts. The findings that obtained are consistent with previous findings that insecticidal effects of plants extracts vary due to several factors such as plant species, mosquito species, geographical varieties, and part used, extraction methodology adopted and six polarity of the solvents used during extraction. The factor of plant species that influences the insecticidal effect in this research was further confirmed by the comparison of chemical compound between this two species. The difference of the chemical compound between the *Azolla pinnata* and *Azolla microphylla* had been identified. Result reported by Zulkrnin et al.,(2018) showed the chemical compound of *Azolla pinnata* is highest in Diethyl Phthalate (20.449%) which this compound contribute to insecticidal activity. The other compounds of Neophytadiene that also responsible for insecticidal activity showed higher intensity in *Azolla pinnata* compared to *Azolla microphylla* as shown in Table A1 and A2 (Appendices).

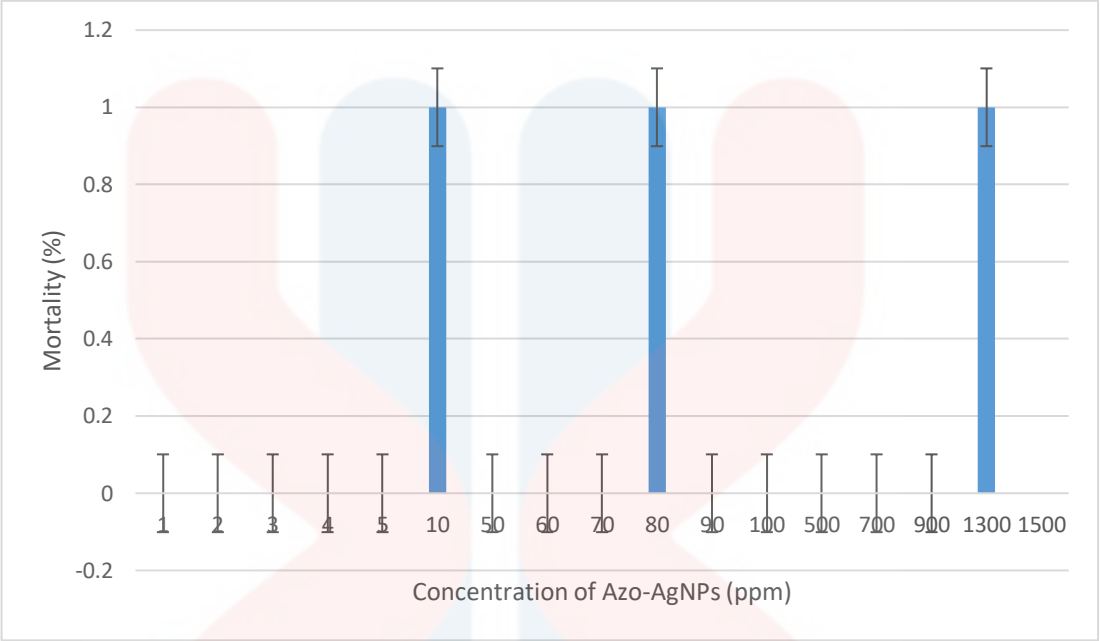

Figure 4.8: Percentage mortality of 3-5 days of *Aedes aegypti* after 24 hours to Azo-AgNPs

The result in Figure 4.8, showed that combination of silver nitrate and Azolla microphylla have limited effect on adulticidal activity against Aedes aegypti mosquitoes. From 1 ppm until up to 1500 ppm maximum concentration been tested, Azo-AgNPs only achieved 20% mortality. In general, insecticide activity depends on actions and counteractions between a toxicant and insect tissues, which are simplified into three stages such as penetration of insect integument as well as membrane of the target organs, activation and detoxification of the insecticide (Chansang et al., 2018). In this research, the killing mechanism for the insecticide is through the contact with the mosquitoes. The situation was observed when the mosquitoes contact with the treated paper during the exposure period. However, the low intensity of the *Azolla microphylla* compound made this Azo-AgNPs was not strong enough to kill the *Ae. Aegypti*. In addition with the repellency effect by the insecticide make the mosquitoes even harder to contact with the treated paper since it keeps stay on the upper and

bottom part of the tube. This reported result might be the supported reason of the unaffected of combination between silver nitrate and *Azolla microphylla* against the adult *Ae. Aegypti*.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATION

5.1 Conclusion

This research is an approach to study the combination of *Azolla microphylla* and silver nitrate. The research had identified the synthesis reaction between these plant extract and silver nitrate. The reduction process occurred when the silver ion of the silver nitrate solution was exposed to the *Azolla microphylla* extract due to the capability of the biomolecules in the plant extract to reduce the metal ion. The biosynthesized of the silver nanoparticles was proved by the color changes of the plant extract because of the rise of surface plasmon resonance. The formation of silver nanoparticles was confirmed through the change of color from colorless silver nitrate to brown color.

The optimal conditions for the silver nanoparticle synthesis also were identified through this research. The volume ratio, time interval, and pH parameters were revealed to influence the formation of the synthesized silver nanoparticle. The further characterization of the synthesized silver nanoparticles was carried out by using the UV-vis spectrophotometer and FTIR. The UV-vis spectrum of the absorbance peak obtained was at 423 nm. The absorbance peak at 3328.01 cm-1 and 1636.15 cm-1 confirmed the presence of phenols and protein in the *Azolla microphylla* extract that acts as a reducing agent for synthesis AgNPs.

The result obtained showed the combination of *Azolla microphylla* with silver nitrate unable to give the killing impact against the *Aedes aegypti*. However, the observation on the behavioral response showed repellency effect on the mosquitoes. .For future further research, the *Azolla pinnata* is more preferred due to a few factors such as chemical composition and complex formation with the silver nitrate.

5.2 Recommendations

For this research, it is recommended to be repeated to validate the result obtained during this experiment. During conducting this research, some limitations were identified and the improvement should be done in the future. There is some improvement that can be done which does not expose the silver nitrate solution of the light. Before mixing the silver nitrate solution with the plant, make sure to keep the solution in dark condition for example transfer into the dark bottle reagent. This situation due to the exposure of the silver nitrate to the light might decompose its elements. The effect was afraid may give effect to the synthesized of silver nanoparticles.

Next, the process of synthesizing should be done at a short period or moment. The plant extract should be avoided to be kept for too long since it will affect the biomolecules compound of the plant. If the synthesis consumes a long-time period, it is suggested to keep on extracting the new one. The other improvement is to make sure the plant extract is keeping at -4°C. This step to ensure the possibility of the plant to degrade can be avoided.

For bioassay activity, it is recommended to always make sure to wear the glove during transferring the mosquitoes from the cage to the tube. The reason is during

transfer of the mosquitoes there might be some mosquitoes that possible bite the hand which causes the blood-fed occurrence. The presence of blood-fed increases the resistance of the mosquitoes toward the insecticide (Machani et al., 2019).

Lastly, further research could be done in the future to validate the effects of the combination of plant extract with the silver nitrate on human health and other organisms in the environment.

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APPENDICES

APPENDIX A: CHEMICAL COMPOSITON OF *A.pinnata* AND *A.microphylla*

Table A1: Chemical compound of *Azolla microphylla* (Poornima, 2016)

RT	Area (%)	Compounds Identification
16.92	0.3	1,3-di-iso-propylnaphthalene
17.87	0.52	3-ethyldibenzothiophene
19.38	0.51	Tetradecanoic acid, 12-methyl ester
20.27	1.64	Neophytadiene
21.12	0.23	2-[(Z)-9-octadecenyloxy] ethanol
21.59	1.04	9-hexadecenoic acid, methyl ester
22.02	4.8	Hexadecanoic acid, ethyl ester
22.75	1.15	3-cyano-12-isopropoxy-6,11-methanocyclodeca[g] imidazo[5,1-c](1,2,4) triazine
23.98	1.05	cis-13-octadecenoic acid, methyl ester
24.88	0.2	Hexadecanoic acid, 2,3-dihydroxypropyl ester
26.07	15.04	9-Octadecenoic acid
28.75	0.5	Hexadecanoic acid, 1-(hydroxymethyl)-1,2-ethanediyl ester
30.41	0.69	(1R)-2-(1S)-1-[2-(Methoxymethoxy) phenyl] ethyl} amino) oxy]-1-phenylethanol
31.67	0.76	3-[(tert-Butyldimethylsilyl) oxy]-1,4,4a, 9a-tetrahydro-1-phenyl-9H-xanthen-9-one
32.89	0.39	Cholest-2-en-1-ol
33.33	0.34	Oleic acid, eicosyl ester
34.94	2.04	(2R/S,4S/R,6R/S)-4-Hydroxy-2-tridecyl-1,7-dioxadispiro[5.1.5.2]pentadeca-9,12-dien-11-one
35.82	0.34	3á-(Peroxymethyl)-5-vinyl-A, B-bisnor-5á-cholestane
38.13	55.47	2,3,4,5,2',6'-Hexamethoxy-4',5'-methylenedioxychalcone
39.28	0.15	Cucurbitacin B, dihydro-
39.83	0.17	Chol-8-en-24-al, 3-hydroxy-4,4,14-trimethyl

*RT = Retention time

Table A2: Chemical compound of *Azolla pinnata* (Rajiv et al., 2018)

RT	Area (%)	Compounds Identification
25.453	20.449	Diethyl Phthalate
29.416	2.212	Sulfurous acid, cyclohexylmethyl tridecyl ester
31.003	1.279	Nonane 2,2,4,4,6,8,8-heptamethyl-
33.521	8.305	Methacrylic acid, dodecyl ester
34.484	0.257	1-Nonadecene
35.978	2.386	Neophytadiene
37.506	0.612	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
39.308	1.557	Hexadecanoic acid, methyl ester
39.497	1.010	Benzenepropanoic acid, 3,5-bis (1,1-dimethylethyl)-4
40.822	5.156	Methacrylic acid, pentadecyl ester
41.236	17.038	Sulfurous acid, cyclohexylmethyl pentadecyl ester
42.624	8.529	2,4,4,6,6,8,8-Heptamethyl-1-nonene
44.560	1.283	Behenic alcohol
47.281	2.281	Methacrylic acid, hexadecyl ester
48.891	5.244	Sulfurous acid, cyclohexylmethyl pentadecyl ester
51.529	17.960	Bis(2-ethylhexyl) methylphosphonate
53.186	4.441	Methacrylic acid, heptadecyl ester

*RT = Retention Time

APPENDIX B: MOSQUITOES CULTURE

Figure B1: Eggs emerge in the seasonal water

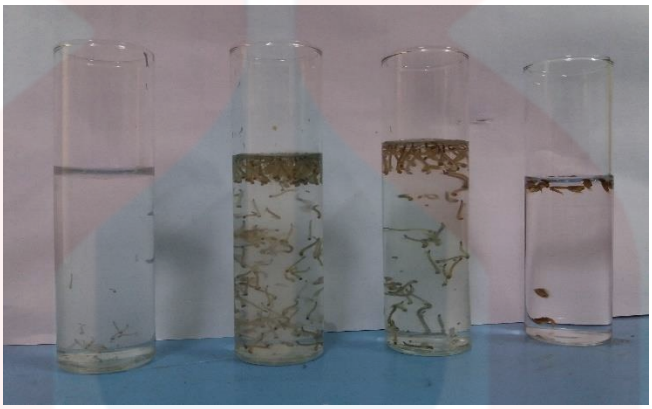

Figure B2: Larvae (stage1, 2 and 3) and Pupae

Figure B3: Mosquitoes cage for adult *Aedes aegypti*