AgNPs-*Azolla Pinnata* Extract As Larvicidal Against *Aedes Aegypti* (Diptera: Culicidae)

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$AgNP_s$ - *Azolla Pinnata* Extract as Larvicidal Against *Aedes Aegypti* (Diptera: Culicidae)

R.Ravi[1], I. H. Ishak[2], M.F. Mohd Amin[3]*, Hamzah. Z[3], M. S. M. Rasat[4], M.A.Salam[5]

[1]School of Biological Sciences, Faculty of Science and Technology, Quest International University, Ipoh, Perak, Malaysia.
[2]School of Biological Sciences, Vector Control Research Unit, Universiti Sains Malaysia, Minden, Penang, Malaysia.
[3]Faculty of Earth Science, Universiti Malaysia Kelantan, Jeli Campus, Jeli, Kelantan, Malaysia.
[4]Faculty of Bioengineering and Technology, Universiti Malaysia Kelantan, Kelantan, Malaysia.
[5]Department of Environmental Science and Disaster Management, Noakhali Science and Technology University, Noakhali, Bangladesh

E-mail: mohamadfaiz@umk.edu.my

Abstract. The widely used synthetic insecticide in the operation of mosquito control could result in unfavourable impacts to the environment, human health and non-target organism. Considering these issues, environmentally friendly insecticides from plant extract have been used as green alternatives by recent researchers. Unfortunately, the method of using plant extract as insecticide requires a large amount of raw plants. In relation to this problem, the use of nanoparticles that possesses unique characteristics including small size and potential in changing physical, chemical and biological properties of organisms were studied. Nano-synthesized silver particles (AgNPs) from *Azolla pinnata* extract were thus investigated in this study in order to determine its efficacy as *Aedes aegypti* larvicide. The present work focuses on extraction of the compounds in *Azolla pinnata* using soxhlet extraction method. The plant extract was mixed with 1 mM silver nitrate solution and the biosynthesized silver nanoparticles were then being characterized using UV-Vis spectrophotometer. AgNPs particles from *Azolla pinnata* extract were prepared in six different concentrations and set in plastic cups. Late third instar larvae of *Aedes aegypti* were being used in all tests. Based on the findings of the experiment, there was no mortality of larvae recorded in control groups after 24 hours of exposure. The lowest mortality recorded was at 10 ppm with only 7.5% mortality, while 95% mortality was recorded for the highest concentration which was 250 ppm. Meanwhile, the LC50 and LC95 obtained at 95% confidence interval after 24 hours of exposure were 121.570 ppm and 369.438 ppm respectively. Further studies should be done to determine the mechanisms of AgNPs in aiding *Azolla pinnata* as an effective larvicide in the future.

1. Introduction

The infections which caused by transmission of pathogens by certain types of arthropods are called vector-borne diseases. Ticks, lice, sand flies, black flies, mosquitoes and others related insects are the few examples of arthropods that can spread vector-borne diseases. Dengue is one of the mosquito-borne diseases mainly caused by dengue viruses [1,2]. It can be transmitted by 2 main species of *Aedes* mosquito which are *Aedes aegypti* and *Aedes albopictus* mosquitoes through blood feeding from one individual to another individual [1,2]. Currently, there are 46,713 reported dengue cases in Malaysia which accumulated from 29 December 2019 to 30 May 2020 (World Health Organization, 2020). According to World Health Organization (2020), a cumulative amount of 50,511 dengue cases in Malaysia was reported as of 13 June 2020. The resurgence or re-emergence of mosquito-borne arboviruses gives concerns to the importance of public health because it can lead to diseases outbreak which can occur globally. It also has been reported that the clinical characteristics of dengue were similar with coronavirus diseases 2019 (COVID-19) World Health Organization (2020). A green approach to mosquito control is vital in order to improve the environment and public health. The widely used chemical insecticides are reported to have a few adverse effects on the target and non-target population [3-8]. An exploration of safe and eco-friendly methods through the utilization of plants that could be promising which numerously shown by *Azolla pinnata* plant to prevent mosquito breeding [9-12]. This free-floating aquatic pterophyte can proliferate within three to five days by doubling its biomass [13].

Recently, silver nanoparticles applied in the industry due to its antimicrobial, antiviral and its suitability in various fields [14]. Studies have reported that the *A. pinnata extracts* against *Ae. aegypti* require high plant concentration of 1853 ppm for crude and 2,521,535 ppm for fresh *A. pinnata* to achieve highest mortality percentage [12,13]. These are not in line with the commercialization views for the extract. In order to reduce the usage of *A. pinnata* crude extract concentrations, silver nanoparticle (AgNPs) solution was bio-synthesized with *A. pinnata* extracts, and in this study was applied as insecticides against late third larvae of *Ae. aegypti.* Thus, the objective of this study is to determine the lethal concentration of 50 and 95 (LC_{50} and LC_{95}) from the AgNPs of *A. pinnata* extract against *Aedes aegypti.*

2. Material and Methods

2.1. Study Area

All testing and experiments conducted in the Vector Control Research Unit (VCRU), USM with strict adhere to WHO 2005 [17] guidelines. The environment condition is set at an average room temperature of 25 °C ± 2 °C with 75 ± 5 % relative humidity.

2.2. Synthesis and characterization of silver nanoparticle from Azolla pinnata

The pre-extracted plant extract (10 mL) and 1mM AgNO3 (90 mL) solution were mixed at the ratio of 1:9 and kept in the dark condition for 3 hours. The colour of AgNO3 changes from colourless to brownish colour (Figure 1) after 30 minutes of incubation, and it indicates the complexation of the plant extract with the silver particles.

The complexation of Azolla extracts with the silver ions investigated through measuring the UV-Vis spectra using HACH DR 6000 UV Vis Spectrophotometer at a resolution of 1 *nm and a* wavelength ranging of 200 *nm* to 700 *nm* as the study on 'Green Synthesis of Silver Nanoparticles using Apple Extract' [14].

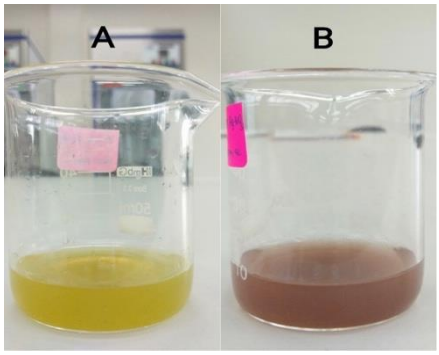

Figure 1. (A) *Azolla pinnata* extracts and (B) Synthesis of Silver Nanoparticle with *Azolla pinnata*.

2.3. Mosquito Larvae

The larvae were hatched in de-chlorinated water for 24 hours and maintained at 25℃ to 30℃ (room temperature), pH of 6.95 to 7.03, and relative humidity of 80 ± 10% and dissolved oxygen from 5.5 to 6.1 mg/L in the laboratory [11,13]. After five days, the late 3rd instar larvae used for bioassay test WHO 2005 [2].

2.4. Larvicidal Bioassay

Four replicates tested using 20 late-third instar larvae for each concentration ranging from 5 ppm to 750 ppm to find the optimum range for larvicidal activities. The second testing concentrations phase involves a lower concentration from 10 ppm to 250 ppm that yield between 5% and 95% mortality in 24 hours of exposure. Two control were created with only 10 ppm *A. pinnata* extracts. All test was carried out for 24 hours before mortality were observed and recorded. Figure 2 below shows the experiments set-up. The results were analysed with probit regression using statistical package IBM SPSS 21 software to estimate the LC50 and LC95 values.

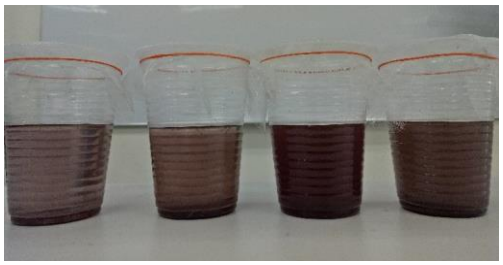

Figure 2. Larvicidal test set-up

3. Results and Discussion

3.1. UV-Vis Spectroscopy

In order to show the perfect combination of AgNPs with the azolla extract, UV-Vis analysis is applied to study the excitation of electromagnetic field of surface plasmon resonance (SPR). During the combination, the plant extract of *A. pinnata* had changed the colour of silver nitrate solution from transparent to dark brown due to the reduction of Ag ions to AgNPs within half an hour of the commencement of the reaction. These colour change arise because of the excitation of surface plasmon vibrations with the silver nanoparticles [11]. SPR peak centered near 425 nm affirmed the reduction of Ag^{+} to Ag^{0}. In particular, the absorbance range for presence of silver nanoparticles are between 420 nm to 450 nm [11]. UV- visible absorbance of reaction mixture was taken after 30 minutes of the reaction commencement which further remained constant.

The absorbance of *Azolla pinnata* plant extract showed absorbance wavelength near 240 nm and 320 nm indicating the presence of proteins and phenols in the extract respectively, Figure 3. Absorption peak at around 320 nm shown in Figure 3 disappeared during the reaction which indicates the involvement and role of phenols in the reaction.

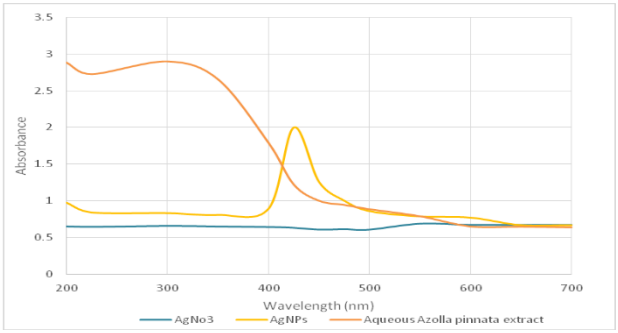

Figure 3. UV Vis spectra recorded for synthesis of silver nanoparticles using *Azolla pinnata* extract.

3.2. Larvicidal Bioassay Test

By using log probit regression analysis (95% confidence level) through IBM SPSS 21 software, lethal concentration 50 and 95 (LC50 and LC95) values after 24 hours exposure calculated as Table 1. In contrast, lethal concentration LC99 showed in Table 2.

Based on the LC50 and LC95 result from log probit analysis, it means 121.570 ppm of AgNPs from *Azolla pinnata* is needed to kill 50% of larvae while 369.438 ppm of the solution as mentioned earlier is needed to kill 95% of larvae. The p-value obtained (0.025) shows significance value as it does not exceed 0.05, which is the maximum value to be categorized as significant. The result obtained is statistical significance, and the null hypothesis is rejected. The p-value reflects the characteristics and efficiency of the test carried out on the sample populations, which were larvae.

Table 1. Lethal concentration of bioassay against *Aedes sp*. larvae after 24 hours exposure

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

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

Table 2. Lethal concentration 99 (LC99) of the bioassay

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

The larvicidal properties of *A. pinnata* and AgNPs expected to cause the larvae mortalities and behavioural changes as seen in this study. The previous study by Ravi et al. (2018) suggested that *A. pinnata* have active ingredients which contribute to pesticides, insecticidal, anti-parasitic and antimicrobial activities such as 3,7,11,15-tetramethyl-2-hexadecane-1-ol, neophytadiene, and methacrylic acid. Others also reported that high concentrations of extract would lead to severe morphological deformities. Ravi et al. [11] showed that there are deformities in morphological of the *Ae. aegypti* larvae with the abdomen became blackened and twisted after treated with extract of *I. cairica* leaf. The mixing of *A. pinnata* extract, and silver nitrate that resulted in the synthesis of silver nanoparticles has led to potentiation. This might due to the small size of silver nanoparticles that allow them to pass through the wall of larvae's body into the cell where it can disturb other physiological processes of larvae [11]

In parallel to this research, Figure 4 (a), (b) and (c) showing the morphologically deformed larvae can be seen clearly with the darkening of the whole body of the larvae. This can be due to the ingestion process by larvae or absorption process of the AgNPs from *A. pinnata* extract into the body of larvae. Meanwhile, the abdomen of morphologically deform larvae could not be seen clearly and seems darker.

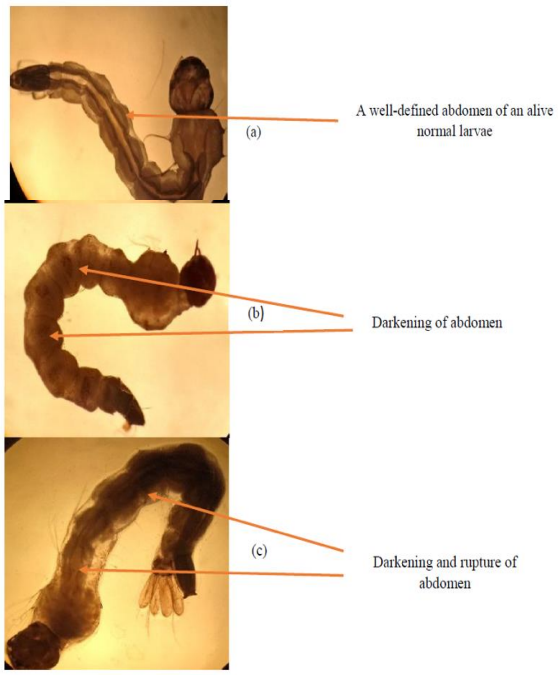

Figure 4. (a) Clear abdomen Dead (b) Larvae with darkening of abdomen (c) Ruptured abdomen

4. Conclusion

In this study, a co-synthesized complexation of AgNPs using A. pinnata extract has shown promising results in reducing the concentration of plant extract and achieving the highest larvae mortality. These are further confirmed through the morphological deformities of the dead larvae which indicated the combination potential mode of action.

Acknowledgment

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