Larvicidal activity of phyto-synthesized silver nanoparticle bio-reduced by *Morinda citrifolia* (noni) leaf extract against *Aedes aegypti* Linn larvae

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Abstract - *Nanotechnology has exhibited tremendous range of application within the biomedical field, one of which is the larvicidal activity on different species. The use of plants to synthesize metal nanoparticles have gained acknowledgement for it has biocompatibility, lesser toxicity, greener and ecological procedures. This study utilized Morinda citrifolia, commercially known as Noni plant, for the bio-reduction of silver nanoparticles, and was tested for its larvicidal activity on Aedes aegypti, the potent dengue viruscarrier. The synthesized nanoparticles were subjected to UV-Visible spectrophotometer, Fourier Transform Infrared Spectroscopy and Field Emission Scanning Electron Microscopefor further characterization. Its larvicidal activity against the varied concentrations was also determined using third instar of Aedes aegyptilarvae. The synthesized silver nanoparticles were characterized by its surface plasmon resonance (SPR), with an absorbance peak of 451 nm in the UV–Vis spectrum. FTIR analysis showed the existence of proteins accountable for creation of nanoparticles that plays a significant role in the development and stabilization of the produced silver nanoparticles. FESEM result images shows the synthesized nanoparticles produced have mostly spherical in its morphology and had an average size of 92 ± 5 nm. Silver nanoparticles produced from M. citrifolia leaf extract demonstrated larvicidal activity on the dengue vector, A. aegypti. Observed lethal concentrations (LC50 and LC90) towards the third instar larvae were 1.629 and 15.663 mg/L of nanoparticles respectively, after the 48 hour exposure period. Results suggest that the Morinda citrifolia leaf extract used in the biosynthesis of silver nanoparticles has larvicidal properties against Aedes aegypti.*

Keywords: *biosynthesis, larvicidal, Aedes aegypti, Morinda citrifolia, silver nanoparticles*

INTRODUCTION

Mosquitos' occurrence and distribution has been a growing source of concern among many public health communities and health systems in most tropical and subtropical countries, including the Philippines. They are vectors known to carry microorganisms which are fatal to man [1], [2]. Dengue virus (genus Flavivirus), one of the most significant arthropod-borne human viral pathogens transmitted by mosquitoes, is the causative agent of dengue and dengue hemorrhagic fever. It is shown that dengue is predominantly a pediatric infection, however, hyper-endemic locations that shows high course of infection displays an increased mean age of infected individuals [3]. With the recent detection and discovery of the fifth strain of dengue virus, additional problems may impede the control of the spread of the disease [4]. Studies have shown that the economic burden brought by dengue in the Philippines is substantial and needs to be resolved [5].

Nanotechnology has entered essentially many areas of everyday living. It's main concern comprises of the production and creation of nanoparticles of varied sizes, shapes, controlled dispersity and biochemical composition and the probable benefits it brings to man [6]. Nanoparticles are shown to be the smallest objects, typically 1-100 nanometers, that can still behave as a whole unit with respect to its size, distribution and morphology [7]. They act as conduits between bulk materials with relation to their molecular and anatomical structures. They can also display size-related properties noticeably different from fine particles or the counterpart bulk materials [8]. Numerous ways to synthesize and produce metal nanoparticles are available. The usual downside of these production methods are generally the cost, labor, and probable anger it may bring to the

environment and other living organisms [9].

Various biologic systems have the property to alter inorganic metal to become nanoparticles. This is primarily due to the reductive capacities of proteins and metabolites existing within these organisms. Biosynthesis approaches have greater compensation than other classical synthetic ways due to its availability, low-cost and environmental friendly procedures [10]. Plant-mediated nanoparticles synthesis is an effective approach that has immense application in the fields of modern medicines. Furthermore, phyto-synthesis by means of plant extracts is superior to other methods because it does not require additional intricate processes [11]. Plants during glycolysis create great quantity of H^+ ions along with nicotinamide adenine dinucleotide (NAD). The NAD acts as a tough reducing agent which is primarily the cause for development of nanoparticles. Water-soluble anti-oxidative agents are also responsible for reduction of nanoparticles [12].

Morinda citrifolia (Noni plant) is a tropical plant that belongs within the Plantae family Rubiaceae. The plant has been appreciated medicinally, as it has been habitually nurtured for several of its part such as leaves, rootsand fruits. Certain part of the Noni plant has been commonly used, therapeutically, for relief of rheumatic and complementary pains. It has also been shown to have broad range of beneficial and nutritional value to man [13], [14] and studies have also also shown the plant to have anti-oxidative properties [15], [16]. Studies have also shown that synthesized silver nanoparticles from plants can induce larvicidal activity [17], [18]. So far, no report regarding the bio-synthesis of silver nanoparticles by utilizing the leaf extract of *M. citrifolia*, and testing its larvicidal activity against *Aedes aegypti*, has been tested and reported. This study aims to phyto-synthesize nanoparticles using *Morinda citrifolia* leaf extract from a silver nitrate source solution. The nanoparticles produced were subjected to larvicidal bioassay using third instar *Aedes aegypti* larvae in terms of their lethal concentration.

Figure 1. *Morinda citrifolia* (Noni) leaves

METHODS

Plant Material Collection and Identification

Matured leaves of the *Morinda citrifolia* (Noni) were collected from Simlong, Batangas City. The freshly collected leaves were authenticated at the Bureau of Plant Industry, Malate, Manila. The leaves are shown in Figure 1.

Plant Extraction

The leaves of *M. citrifolia* were washed thoroughly with distilled water to wash any dirt and debris present on the surface of the leaves. They were then dried in an oven at a temperature of 40°C for 48 hours. The fine powder from the dried leaves was obtained using an electronic blender. Twenty grams of leaf powder were mixed with 200 mL of distilled water. The mixture was kept in a water bath filled with boiling water for 10 minutes. The extracts were filtered using Whatman filter paper no. 1 [18].

Biosynthesis of Silver Nanoparticles

An aqueous solution of 1mM silver nitrate ($AgNO_3$) was used for the biosynthesis of silver nanoparticle. The reaction mixture was prepared by adding 10 mL of the Noni leaf crude extract to 90 mL of 1mM $AgNO_3$ (0.167 g of $AgNO_3$dissolved in 1 L of distilled water) solution in a sterile glass container [19] and was stirred at room temperature for about 10 minutes [18]. The primary detection of silver nanoparticles was carried out by the visual observation of development of the reddish brown coloration in the solution mixture [20].

Purification of Silver Nanoparticles

The fully reduced silver nanoparticle solution was centrifuged at 5000 rpm for 30 minutes. The supernatant was discarded while the pellet obtained was subjected for re-dispersal in distilled water. The centrifugal process was repeated three times to wash any absorbed substance present at the surface of the synthesized silver nanoparticles [18].

UV-Visible Spectroscopy

The characterization was performed to the extract after the bio-synthesis of silver nanoparticles. The sample was first filtered using a Millipore filter (0.22 microns). Small aliquots of the filtered solution were prepared for the UV-Visible spectroscopy using the double beam UV-VIS spectrophotometer BS0013 (Shimadzu). Aliquots of reaction mixture was withdrawn for analysis of the surface plasmon

resonance of synthesized silver nanoparticles using the UV-Vis spectrophotometer at the resolution of 1 nm in range of 300 to 700 nm [18].

Fourier-Transform Infrared Spectroscopy

The sample was allowed to evaporate overnight under laminar hood to sediment the particles and to obtain the powder form of the silver nanoparticles. FTIR analysis was carried out by the FTIR spectrophotometer IRAffinity-1 (Shimadzu) at the range of 400–4000 cm^{-1}. Twenty milligrams of silver nanoparticle was mixed with dried 180 mg of potassium bromide (KBr), crushed well in an agate mortar and pestle to prepare a thin pellet for the analysis. Thirty scans per sample were taken in the given range [21].

Field Emission Scanning Electron Microscope

The morphology and size of the synthesized silver nanoparticles was confirmed using the, Focus Ion Beam Field Emission Scanning Electron Microscope (FIB FE-SEM), Helios NanoLab 600i. The settled sediments obtained from the evaporation process were used for the FESEM analysis. Images were taken at 50,000 and 100,000 x magnification at an accelerating voltage of 2.00 kV and a Beam current of 86 pA.

Larvae

The third instar larvae of *Aedes aegypti* was obtained from the Medical Entomology Laboratory – Insectary, Research Institute for Tropical Medicine. The obtained larvae were reared in a plastic containers filled with 500 ml of distilled water. They were fed once a day with fish flakes. Formation of residual food artifacts that was harmful to the larvae's growth and development were carefully checked and periodically removed [22]. Conditions were sustained at 27 ± 3 °C, [23] 75 ± 5% relative humidity and photoperiod of 12 hour light and 12 hours dark [24] for optimal larvae living conditions.

Larvicidal Bioassay

The larvicidal activity was assessed following WHO protocols (2005) with slight modifications. A stock solution was made by adding 100 mg of biosynthesized silver nanoparticles to 1 L of distilled water. From the stock solution, the nanoparticle solutions were diluted using distilled water as a solvent according to the desired concentrations of the synthesized silver nanoparticles (4, 2, 1, 0.5 and 0.25 mg/L).

For the bioassay proper, third instar larvae of *Aedes aegypti* were taken in three batches of 20 in 200 mL of water with the desired concentration of silver nanoparticles. Positive and negative control was set up with sodium hypochlorite (NaOCl) [25] and distilled water respectively. The control was treated the same as other test experiments. Each test was performed by placing 20 *A. aegypti* mosquito larvae into 200 mL of distilled water with nanoparticles into a container. Symptoms of treated larvae were observed and recorded immediately at timed intervals. The number of dead larvae was counted after 0.5, 1, 4, 20, 24 and 48 hours of exposure, and the percentage of mortality was reported from the average of three replicates. Larvae were considered dead when they fail to move following a gentle tap using an applicator sticks for a minimum of five times. Moribund larvae were excluded from the mortality record data [26].

Statistical methods

The average larval mortality data was subjected to statistical analysis (ANOVA) for calculating LC50 and LC90. Other analysis such as 95% fiducial limit of upper confidence limit, lower confidence limit values was calculated by using the software SPSS.

RESULTS AND DISCUSSION

Biosynthesis of silver nanoparticles

The mixture of *Morinda citrifolia* leaf extract-silver nitrate solution color was initially yellowish green (a), but color changed to reddish brown (b) after an hour (Figure 2). The first color change noted (yellowish green) signifies that nanoparticles were starting to be formed [27].

The final reddish brown coloration indicates that the *Morinda citrifolia* leaf extract have successfully reduced the solution to turn into silver nanoparticles.The color change can primarily be attributed to the surface plasmon resonance of the synthesized silver nanoparticles. This was also noted on several studies, such as Roopan [18], Borase [21] and Sathishkumar [19]. However, the color reactions noted on those studies were more rapid in comparison to this study, with the fastest being at 10 minutes. The reaction may be chiefly due to the plants individual anti-oxidative and reduction capabilities. The study [28] explains the advantage of a plant along with a basic ph of the solution in the synthesis of nanoparticles. The observed visual color change of reddish brown in the study is a primary indicator that nanoparticles were being successfully produced and synthesized [20].

Figure 2. Biosynthesized silver nanoparticle visual observation a.) initial color reaction b) final color reaction

UV-visible spectroscopy

The UV-Vis spectroscopy was done to determine the unique property of nanoparticles termed as the surface plasmon resonance (SPR). This observable characteristic they possess mainly arises due to the strong interaction between the silver nanoparticles from the conduction of electrons towards the metal surface that experience combined oscillation upon excitation of light. This results in remarkably elevated absorption coefficients and scattering properties visibly seen on the UV-visible spectrum wavelength range. These properties allow silver nanoparticles to have developed sensitivity in optical detection approaches [29].

A schematic depiction of the UV-Visible spectrum can be easily gleaned on Figure 3. The phyto-synthesize nanoparticles gave an absorption peak at 451 nm. Each noble metal nanoparticle have specific absorption peaks that determines its presence in a solution [30].

SPR for different nanoparticles have been reported by numerous studies. For gold it is 540 nm, for zinc, it is usually seen around 315 nm and for silver nanoparticles, peaks usually arises around at 450 nm [21], [31]. Formation of SPR provides proof for successful formation and the bio reduction of the silver nanoparticles from the silver nitrate solution source, by utilizing the leaf extract of *Morinda citrifolia*.

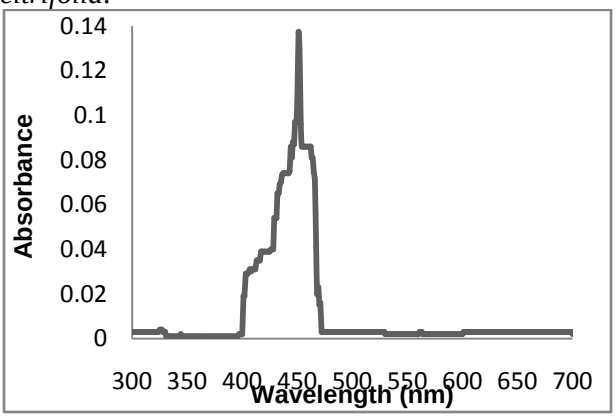

Figure 3. UV-visible spectra of silver nanoparticle

Fourier-transform infrared spectroscopy

The Fourier-transform infrared spectroscopy (FTIR) analysis of the synthesized silver nanoparticles was performed to detect potential interaction among the proteins present and silver nanoparticles produced. The obtained peak values in the infrared absorption spectrum are shown in Figure 4. The FTIR results showed absorbance peaks at 3378, 1655, 1378, and 1077 cm^{-1}.

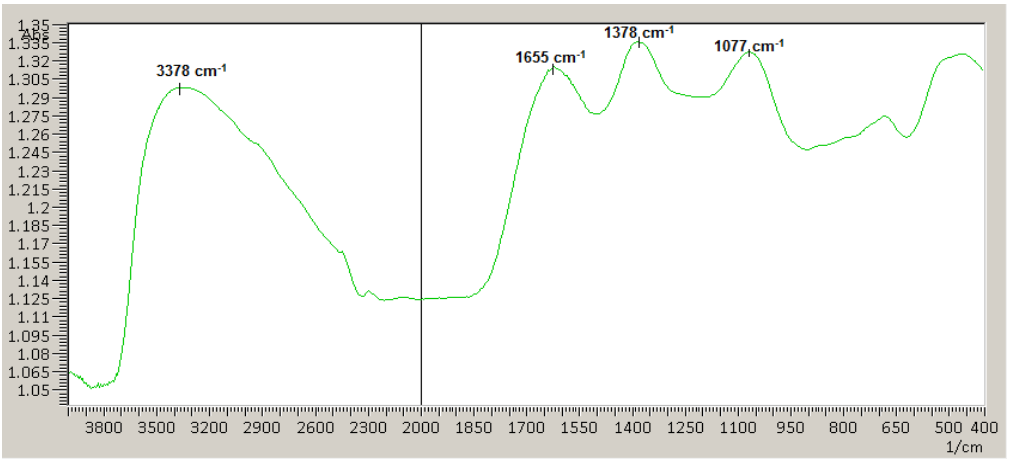

Figure 4. Fourier-transform infrared spectroscopy analysis of silver nanoparticles

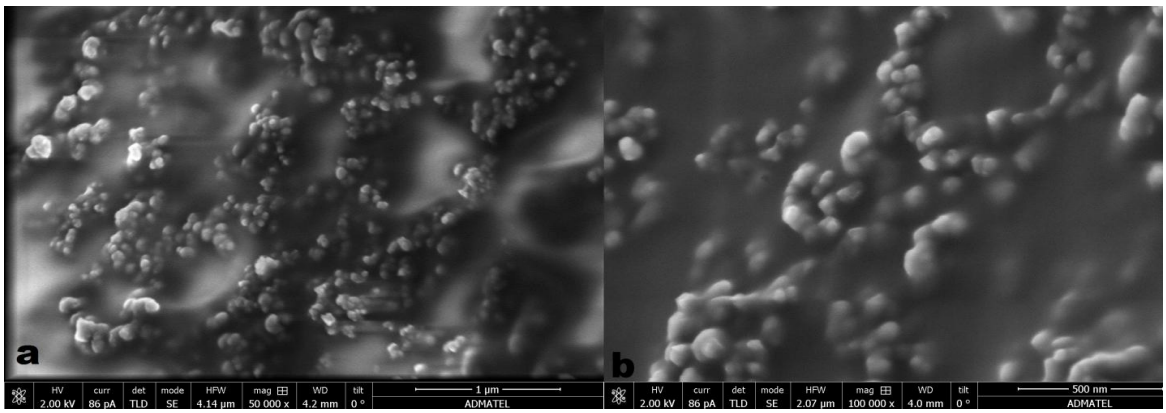

Figure 5. Field Emission Scanning Electron Microscope (FESEM) images a.) taken at 50,000x magnification b.) taken at 100,000x magnification

The peak at 1378cm^{-1} recommends the C–N stretching vibration of aromatic amines while 1077 cm^{-1} was due to the vibration on the aliphatic amines [32]. Peak present at 3378 cm^{-}correlates with the NAH stretching in amines from the proteins of plants. The strong bonds positioned at 1655 cm^{-1} may be allotted to the amide I bond of proteins ascending due to presence of carbonyl stretching of proteins [33]. The absorption peak of 1655 cm^{-1} is near to that designated for native proteins that highly propose proteins interrelating with the bio-produced silver nanoparticles. This also indicates that the secondary structure was not being change throughout the bio-reaction with silver ions, or after subsequently binding with the silver nanoparticles [34], [35].

The FTIR spectroscopic analysis established that the carbonyl group of amino acid remains have resilient binding capacity towards metal, suggestive of the production of coating covering metal nanoparticles and acts as the capping agent providing stability [36]. The results obtain confirms the existence of probable bio-reducing and stabilizing proteins responsible for the formation of silver nanoparticles.

Field Emission-Scanning Electron Microscope (FE-SEM)

Figure 5 shows theFE-SEM images of the synthesized silver nanoparticles at 50, 000 (a) and at 100, 000 (b) x magnification. The images show that the phyto-synthesized nanoparticles had an approximately spherical morphologic shape and typically seen in clusters.A study [37] showed that agglomerate nanoparticles yield is due to the high surface energy that nanomaterials have. They tend to agglomerate to diminish this energy. The FESEM images show that the average size of the synthesized silver nanoparticles is observed to be 92± 5 nm. This is very similar to previous studies [20] that also synthesis silver nanoparticles where nanoparticles that agglomerate in powder form and have a mean size of 90 nm. The size obtained in the study is definite to that of nanoparticles.

Larvicidal Bioassay

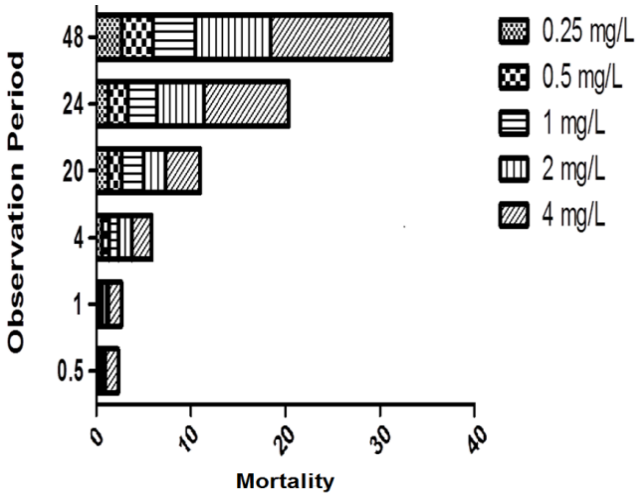

Figure 6. Mortality of *Aedes aegypti* larvae against different concentrations of silver nanoparticles

The mortality of the third instar larvae of *Aedes aegypti* larvae against the different concentration of the silver nanoparticles can be simply seen in Figure 6. It can be observed that the 4mg/L concentration of the synthesized nanoparticles imparted the most significant toxicity. The same concentration also had the most larval deaths upon the 0.5 and 1 hour observation period. This may indicate how fast the nanoparticles can induce larvicidal activity given at higher concentration. In contrast, the smaller concentrations (0.25 and 0.5 mg/L) displays almost to none mosquito larval deaths during the first few hours

of the observation period. It was also noted that there were increase in mortality seen during the 24 and 48 hour observation period.

Table 1. Mosquito Larvicidal Activity of Synthesized Silver Nanoparticles against the Third Instar Larvae of *Aedes aegypti*

Conc. (mg/L)	24hr Mortality*		48hr Mortality*	
	N	%	N	%
4	10	50	15.67	78.33
2	6.33	31.67	10	50
1	3	15	5.67	28.33
0.5	2.67	13.33	4.67	21.67
0.25	2	10	4	20
Positive control	20	100	20	100
Negative control	0	0	0	0

Control nil mortality
N: Number of deaths
%: Percent mortality
* Mean value of three replicates

Table 1 shows the larvicidal activity of synthesized silver nanoparticles towards the third instar *Aedes aegypti* larvae during the 24 and 48 hour observation period. The highest concentration (4mg/L) had an average number of deaths of 10 during the 24 hour observation and 15.67 during the end of the observation time period. It showed mortality rates of 50% and 78.33% of the larval population after 24 and 48 hours exposure period respectively.

The decreasing concentrations of silver nanoparticles resulting to mortality against the larvae also decreases with the 2, 1, 0.5, and 0.25 mg/L concentration having mortality rates of 50, 28.33, 21.67 and 20 % respectively, throughout the 48 hour observation period. The data shows the dose response relationship between the different nanoparticle concentrations and the number of deaths observed. It means that the higher the concentrations of the silver nanoparticles, the greater the mortality rates of the *Aedes aegypti* larvae. It also shows that 0.25mg/L of silver nanoparticles is sufficient for killing 20% of larvae population. These show the efficiency of the synthesized nanoparticles in the study in terms of its larvicidal activity.

The larval death may be inherent or innate to the mosquito larvae itself, as there were no possible ways to ensure and possibly determine the consistency of the vitality of *A. aegypti* third instar larvae. However, mortality was not observed (0%) from the negative control (distilled water) larvae units from the 24 and 48 hours observation period (Table 1). This confirmed the non-toxicity of the distilled water towards the third instar mosquito larvae, and the observed larval death where mostly due to the bio-synthesized silver nanoparticles. This may also rule out and eliminate several factors such as temperature, humidity and photoperiodicity (lighting) as possible causes of the *Aedes aegypti* mosquito larval death, since these factors was regulated among all the test sample experiment, including the control set-up.

Upon the 48 hour observation period, majority of the live larvae observed, notably that of higher concentration (4 and 2 mg/L) shows larvae to be on a moribund state. These means that upon poking with the applicator sticks, the larvae exhibited weak wriggling movement. This is a clear indicator that the larvae upon further exposure to the synthesized silver nanoparticles can lead to larval death.

Findings of the assay corresponds to previous studies [17], [18] that have shown that nanoparticles in small quantity can induce larvicidal activity against different larvae species. The study of Lallawma [17] used varying microgram per millimeter concentration of the nanoparticles, ranging from 90-500 µg/mL. On the other hand, the study of Roopan [18] study also had the same 4, 2, 1, 0.5 and 0.25 mg/L concentration of nanoparticles used in the assay. Both attained significant larval death even at lower concentration. The larvicidal property of silver nanoparticles towards the third instar larvae may be explained by its effect on the digestive tract enzymes, physical and structural deformation in DNA of the generation of reactive oxygen species [21].

Table 2. Lethal Concentration of the Silver Nanoparticles against Third Instar Larvae of *Aedes aegypti*

Larvicidal activity (mg/L)			
LC 50	**LCL-UCL**	**LC 90**	**LCL-UCL**
1.62	(0.85-3.08)	15.66	(8.26-29.69)

LC50: lethal concentration that kills 50% of the exposed larvae
LC90: lethal concentration that kills 90% of the exposed larvae
UCL: upper confidence limit
LCL: lower confidence limit

The lethal concentration (LC) of the silver

nanoparticles against third Instar larvae of *Aedes aegypti* upon 48 hour exposure to silver nanoparticles is shown in Table 2. The nanoparticles have a LC50 of 1.629 (0.859-3.088) and a LC90 of 15.663 (8.262-29.691). The lethal concentration obtain from statistical output means that 1.629 mg/L and 15.663 mg/L are needed to kill 50% and 90% of the *Aedes aegypti* larval population respectively.

With the results obtained, it can be noted that the lethal of the study is lesser in comparison to that of other earlier studies. The study of Roopan [18] had LC 50 and LC 90 of 8.72 and 23.09 mg/L respectively. Likewise, the study of Borase [21] had LC 50s ranging from 2.89 to 9.55 mg/L and LC 90s ranging from 5.17 to 15.76, depending on the plant and type of nanoparticles used in the method. Lower lethal concentration may not necessarily mean a substance is more toxic. It implies that lesser concentration is needed to kill a specific population of larvae. The data further solidifies the effectiveness of the synthesized nanoparticles bio reduced by *Morinda citifolia* in terms of larvicidal activity.

CONCLUSIONS

Morinda citrifolia (Noni) can effectively be used to bio-synthesize stable silver nanoparticles bio-reduced from the silver nitrate solution. The silver nanoparticles produced display larvicidal activity towards the third instar larvae of *Aedes aegypti*, a potent mosquito dengue carrier. This study is the first report that shows the mosquito larvicidal activity of phyto- synthesized silver nanoparticles from the *Morinda citrifolia* (noni) leaf aqueous extract solution. The use of other mosquito species is recommended to have a better understanding of the effectiveness of synthesized nanoparticles in terms of its larvicidal properties. Aside from silver, other elements can also be used for larvicidal properties. Other experiments and assay may also be used to the synthesized nanoparticles, such as its antibacterial and anti-fungal properties. Further researches using nanoparticles and nanotechnology in general can be of significant to provide additional knowledge and ideas to the science and medical field.

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