BIOLOGICAL SYNTHESIS AND CHARACTERIZATION OF COPPER OXIDE NANOPARTICLES PRODUCED BY PLANT GROWTH PROMOTING BACTERIA (PGPB) AND EVALUATION OF THEIR LARVICIDAL ACTIVITY AGAINST *Aedes aegypti* L. (Diptera, Culicidae)

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ABSTRACT

Copper oxide nanoparticles (CuO NPs) were synthesized by biological method using plant growth promoting bacteria (PGPB) and characterized by UV-visible spectroscopy, transmission electron microscopy (TEM) and Fourier transform infrared spectroscopy (FTIR). It was hypothesized that the synthesis of CuO NPs occurred extracellularly by reducing Cu^{2+} to Cu^{0} Based on UV spectroscopy, the absorption spectra at 205 nm can be attributed to the CuO NPs formed. TEM analysis proved that the CuO NPs were spherical and that the particle size ranged from 5-80 nm. FTIR confirmed that CuO NPs were formed through a Cu-O-C and CuO stretching from the peak at 1215.21 cm^{-1} and 1534.33 cm^{-1} respectively. The larvicidal activity of the PGPB produced copper oxide nanoparticles was tested against third instar larvae of *Aedes aegypti* and showed efficient larvicidal effect. This biologically synthesized CuO NPs exhibited LD50 and LD95 values of 644.18 ppm and 23641 ppm, respectively at 24 hours after treatment; and 156.88 ppm and 2409.9 ppm, respectively at 48 hours after treatment. Based on this preliminary study, the CuO NPs synthesized by PGPB can be a potential larvicide against *Aedes aegypti*.

***KEYWORDS*:** copper oxide nanoparticles, plant growth promoting bacteria, electron microscopy, infrared spectroscopy, *Aedes aegypti*, larvicide

INTRODUCTION

Materials at nanoscale have characteristics that made them more important than their bulk level. Currently, chemical and physical methods are employed for nanoparticle synthesis but possess hazards to non-target organism. Hence the interest is shifted towards utilizing potential of biological agents (bacteria, algae, living cells and their extracts) for nanoparticle production [1]. Among different nanomaterials, copper oxide nanoparticles are synthesized because of its low cost compared to other metal oxide nanoparticles, its antibacterial and insecticidal properties [2]. Biosynthesis of copper oxide nanoparticles using microorganisms such as bacteria, algae and yeast have been reported in the literature.

The occurrence of dengue in the Philippines led to the study for the control of mosquitoes. The *Aedes aegypti* mosquito, which will be used in the study, is the primary vector

of dengue [3]. The target is in the larval stage since at this stage, they have less mobility in breeding habitat.

METHODOLOGY

The plant growth-promoting bacteria (PGPB) used in the study was maintained at 4^{o}C in a nutrient agar. This PGPB was used as a biofactory and $CuSO_4 \cdot 5H_2O$ was used as the source of copper. After several incubation, bacterial biomass was collected by centrifugation. It was then resuspended in aqueous $CuSO_4 \cdot 5H_2O$ and the biologically synthesized CuO NPs were collected in a supernatant under sterile condition.

Different concentrations (200, 400, 600, 800 and 1000 ppm) of the CuO NPs were used for the larvicidal assay using the *Aedes aegypti.* This was done in five trials. Effectiveness of the nanoparticle was evaluated after 24 and 48 hours by calculating the percentage mortality of the larvae in each set-up. The different concentrations and their respective mortalities against *Aedes aegypti* larvae were subjected to probit analysis using PriProbit ver. 1.63.

The nanoparticles were subjected to different analysis such as UV-Vis Spectroscopy. The solutions were scanned from 200 to 800 nm to determine the λ_{max}. Fourier transform infrared spectroscopy (FTIR) was also done using Shimadzu IR Prestige-21 Fourier Transform Infrared Spectrometer equipped with Single Reflection ATR Accessory and Diffuse Reflectance Accessory. The last analysis was with the help of Transmission Electron Microscopy (TEM).

RESULTS AND DISCUSSION

Synthesis of CuO from PGPB

Plant-Growth-Promoting Bacteria was used as a biofactory for the CuO NPs synthesis while $CuSO_4 \cdot 5H_2O$ was the source of Cu^{2+}. The formation of CuO NPs was initially confirmed visually, as shown in Figure 1. The change in color of the reaction mixture due to surface plasmon resonance phenomenon provided a convenient signature to indicate the formation of CuO NPs in the reaction mixture.

Figure 1. Color change during the phytoreduction of $CuSO_4$ into CuO NPs at the beginning and after resuspension in $CuSO_4 \cdot 5H_2O$.

PGPB have the ability to reduce metal to a lower redox state. Such bacterial species that can catalyze such reducing reactions are called metal-reducing bacteria which exploit metals as terminal electron acceptor in anaerobic respiration.

The reaction for the formation of CuO nanoparticles from copper (II) ion is given below:

$$Cu^{2+} + 2e^{-} \rightarrow Cu^{0} + Air\ (O_2) \rightarrow CuO$$

Components of PGPB reduced Cu^{2+} to Cu (0). Hence, PGPB, in the presence of Cu^{2+} ions, metallic copper nanoparticles can be obtained.

Larvicidal Assay in 5 Trials (Using Biologically Synthesized CuO NPs)

In the study, larvae of *Aedes aegypti* were exposed to varying concentrations in terms of volume added of synthesized CuO NPs from PGPB for 24 hours and 48 hours. The results are indicated in Figure 2.

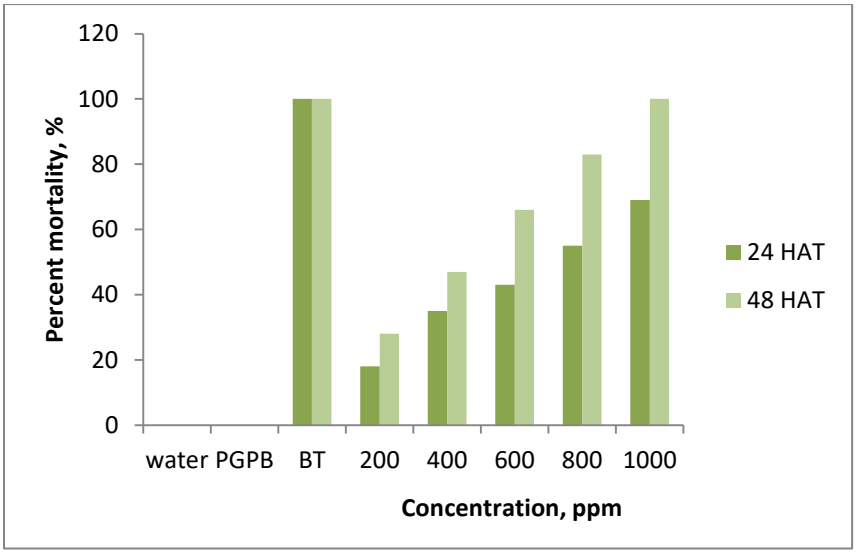

Figure 2. Percent mortalities of the Aedes aegypti larvae upon subjection to the following treatments: water, PGPB, *Bacillus thuringiensis* (BT), and CuO NPs (200, 400, 600, and 800 ppm).

The bioassay results were analyzed through the use of probit analysis by calculating the LD_{50}, LD_{95}, and fiducial limits after 24 and 48 HAT, as given by Table 1.

Fifty percent of the population of the *Aedes* larvae was killed 24 hours after treatment at a concentration of 644.18 ppm of the crude extract. This concentration corresponds to the 0.644% of the CuO NPs. This percentage implies that less than 1 % of the NP cause 50% of *Aedes aegypti* larval death.

Table 1. LD_{50} values of the CuO NPs against Aedes aegypti larvae, for both 24 and 48HAT.

For 24HAT	Value, ppm	Fiducial Limits	
		Lower Limit, LL	Upper Limit, UL
LD_{50}	644.18	284.02	5990800
LD_{95}	23641	3477	8.1880e+075
For 48HAT	Value, ppm	Fiducial Limits	
		Lower Limit, LL	Upper Limit, UL
LD_{50}	156.88	2.4157	287.11
LD_{95}	2409.9	1088.6	991160.

One-way ANOVA of the corrected percent mortalities of the three fractions was done to determine if the differences in the percentage mortalities among the fractions is significant. Comparison of the mean percentage mortalities obtained from the different concentrations reveals that there is no significant difference between the six concentrations of the CuO NPs with the controls. The result is given in Table 2.

Table 2. Percent mortalities of Aedes aegypti larvae 24 and 48 hours after treatment (HAT) with CuO NPs.

Concentration, ppm	Corrected Percent Mortality (CPM), $\boldsymbol{AVE \pm SD}$			
	24HAT	Statistical significance**	48HAT	Statistical significance**
0	0.00±0.00	G	0.00±0.00	F
200	3.60±0.89	F	5.60±0.89	E
400	7.00±1.00	E	9.40±0.55	D
600	8.60±0.55	D	13.20±1.30	C
800	11.00±1.22	C	16.60±1.14	B
1000	13.80±0.84	B	20.00±0.00	A
Bt	20.00±0.00	A	20.00±0.00	A
PGPB	0.00±0.00	G	0.00±0.00	F

**Five trials were performed containing 20 Aedes aegypti larvae. Late third instar to early fourth instar of Aedes aegypti larvae were used.*

***Means followed by a common letter are not significantly different at the 5% level by one-way ANOVA, followed by Tukey's Multiple Comparison Test.*

Characterization

Analysis by Ultraviolet-Visible Spectrophotometry

It was found out that the synthesized CuO NPs has an absorbance of 205 nm (Figure 4).

Analysis by Fourier Transform Infrared Spectroscopy

From Figure 5, the 3426 cm^{-1} shown O-H stretching due to alcoholic group; 1534.44 cm^{-1} correspond to the symmetrical and asymmetrical stretching of Cu-O and 1630.88 cm^{-1} shown N-H bending. The bands at 2960.86 cm^{-1} and 2926.14 cm^{-1} are assigned to $-CH_2$ and C-H stretching mode in alkanes. Vibration band at 1049.32 cm^{-1} was observed which corresponds to

the coordination of metal (Cu) by O-H. The metal salt (Cu-O-C) peak appeared at 1215.21 cm^{-1}. Moreover, other weak features appearing in the spectra may be due to interaction of precursor ions with the protein/surfactant/enzymes secreted by bacteria in the course of reaction.

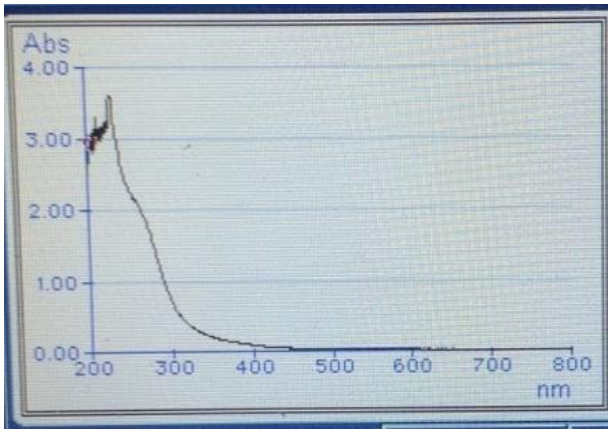

Figure 4. Spectrum of the biologically synthesized CuO NPs with absorption band detected at 205 nm.

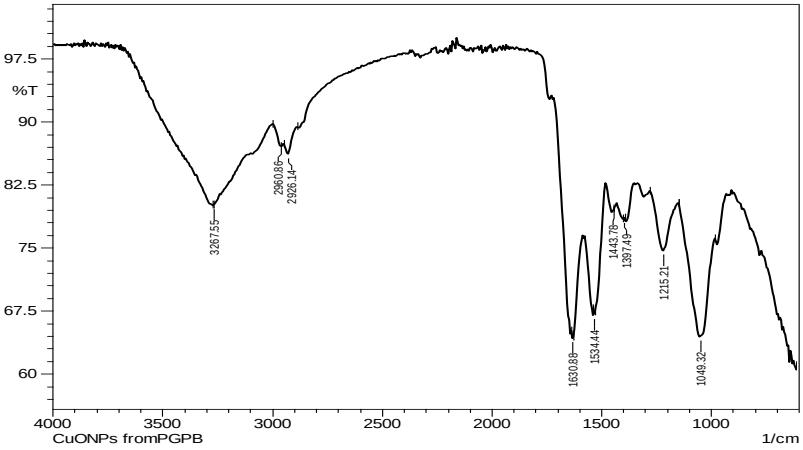

Figure 5. FTIR spectra for the CuO NPs from PGPB.

C. Analysis by Transmission Electron Microscopy (TEM)

The average particle size of the CuO NP ranges between 5 -10 nm, as seen in Figure 6.

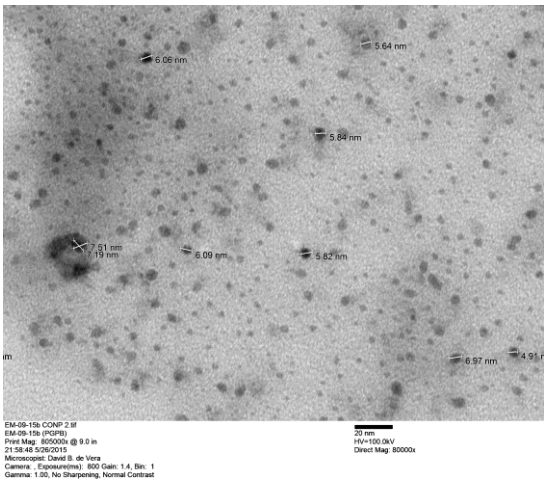

Figure 6. TEM image of CuO NPs from PGPB. (80000x Direct Magnification)

CONCLUSION

Green chemistry approach was used in the synthesis of metal oxide nanoparticles which is simple and reproducible. The method for the synthesis of Copper Oxide Nanoparticles (CuO NPs) used $CuSO_4 \cdot 5H_2O$ by reducing the Cu^{2+} ion to for Cu^0 via reduction method using Plant-Growth Promoting Bacteria (PGPB). UV-Vis spectra showed absorption at 205 nm. The FTIR for the synthesized CuO NPs showed band at 1534.44 cm^{-1} due to the symmetrical and asymmetrical stretching of Cu-O; 1049.32 cm^{-1} corresponds to the coordination of metal (Cu) by O-H; and 1215.21 cm^{-1} due to the metal salt (Cu-O-C) peak. TEM results showed that the average size of the spherical nanoparticles is from the range of 5-80 nm.

Evaluation of toxicity of the nanoparticles against *Aedes aegypti* larvae showed LD_{50} and LD_{95} values of 644.18 ppm and 23641 ppm, respectively at 24 hours after treatment, and 156.88 ppm and 2409.9 ppm, respectively at 48 hours after treatment, which suggests that NP is a potent larvicide against the *A. aegypti* larvae.

LITERATURE CITED

[1] Borase, H., Chandrashekhar, D., Salunkhem R., Narkhere, C., Salunke, B. And Patil, S. 2013. Phyto-synthesized silver nanoparticles: a potent mosquito biolarvicidal agent. Nanomedicine Biotherapeutic Discov. 3:1.

[2] Karthik, A. And Geetha, K. 2013. Synthesis of copper precursor, copper oxide and its oxide nanoparticles by green chemical reduction method and its antimicrobial activity. Journal of Applied Pharmaceutical Science. 3(5): 16-21.

[3] WHO 2014. Dengue and dengue hemorrhagic fever. Fact sheet No. 117, retrieved from http://www.who.int/mediacentre/factsheets/fs117/en on May 2014.