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Green Synthesis of Copper Nanoparticles Using *Coffee Arabica*: Larvicidal and Biochemical Study.

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ABSTRACT

Aedes aegypti is a major problem in transmitting dangerous diseases to humans, including dengue fever. Therefore, this study aimed to synthesize copper nanoparticles from *Coffee arabica* and use them in the control of this dangerous insect. To confirm and characterize the formation of copper nanoparticles ultraviolet spectrophotometer, Fourier transform infrared spectrometry, and X-ray diffraction methods were used. To study the toxicity of copper nanoparticles on some biochemical parameters in the body of the insect, the amount of glucose, total protein, and metabolic enzymes such as aspartate transferase (AST), alkaline phosphatase (ALP), and lactate dehydrogenase (LDH) were analyzed. The results indicated that the highest mortality rate in *Aedes aegypti* larva was 96.3 % at a concentration of 100 ppm, and LC_{50} and LC_{90} were 5.7 and 36. 5%, respectively. It also showed that the lowest rate of emergence of pupae is 3.75 % at a concentration of 100 ppm, and the lowest rate of emergence of an adult insect is 2.5 % at a concentration of 50 ppm. Biochemical analyses indicate a significant decrease in the amount of total protein, alkaline phosphatase, and lactate dehydrogenase enzyme, but a significant increase in the amount of glucose and aspartate transferase enzyme was observed. Based on the obtained results, copper nanoparticles are considered a strong and promising candidate for controlling *Aedes aegypti* mosquito larvae and inhibiting their developmental stages.

Keywords: *Aedes aegypti*; copper nanoparticles; *Coffee arabica*; larvicidal; biochemical study

INTRODUCTION

Several deadly diseases for humans and animals are transmitted by mosquitoes (Diptera: Culicidae). Mosquitoes are the main carriers of many dangerous diseases that affect humans and animals. The biggest health problem in the world is insect-borne diseases caused by Mosquitoes (1).

Mosquitoes carry pathogens, especially protozoan parasites, in their salivary glands and pass them on to humans when they feed on blood by biting. The most dangerous mosquito species are the genus Aedes, Anopheles, and Culex, which causes malaria, Rift Valley fever, yellow fever, dengue fever, Zika virus, Chikungunya, and Japanese encephalitis. The *Aedes aegypti* mosquito is the main vector of dengue and Zika virus, which has no vaccine and medication yet (2,3). The spread of dengue fever is a major problem in many countries of the world, as the number of deaths resulting from dengue fever increases annually, especially in tropical regions where mosquito breeding increases (4).

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Fighting mosquito populations is easier when eliminating mosquito larvae at the breeding site. Thus, targeting larvae can be more effective in controlling mosquitoes (5).

The chemical insecticides used in the control of mosquitoes, in all its stages, have negative effects on the environment through damage to the soil, humans, and non-target organisms such as beneficial insects, honey bees, and others. In addition, the emergence of the problem of insect resistance to pesticides and the pollution of natural resources through the food chain leads to a decrease in biodiversity (6).

In modern times, scientists have focused on discovering new effective and environmentally friendly ways to control insects, such as biological control using microorganisms and medicinal plant extracts, which have achieved effective and good results against insects, and disease vectors, and did not cause environmental damage (7).

In recent years, nanobiotechnology has emerged as a new specialty under the name of nanoscience, which emerged from biology and nanotechnology, and demonstrated an excellent, new, effective, environmentally friendly, and low-cost alternative method in controlling insect pests (8).

During the past decade, nanotechnology research has increased significantly, and most of the research has focused on the medicine and agricultural sectors, and pest and disease vector resistance (9).

The biologically synthesized metal nanoparticles showed high activity against insect disease vectors, especially their larval stages (10).

The nanoparticles of copper, silver, gold, silica, iron, titanium, and zinc were studied as toxic agents against many types of mosquitoes (11). Its toxicity is due to its ability to enter the body of insects and penetrate their exoskeleton to reach the vital systems and organs, then the tissues and cells of the insect, and interact with biochemical compounds such as proteins and nucleic acids (12).

The information and research published on the chemical and pharmacological properties of the *Coffee arabica* plant in pest control encouraged us to use it in the synthesis of copper nanoparticles and to increase and enhance the effectiveness of the famous copper mineral in pest control (13).

Based on the above-mentioned facts, this study aimed to synthesize copper nanoparticles biologically using the *Coffee arabica* plant and characterize these nanoparticles by common methods such as ultraviolet spectroscopy, infrared spectroscopy, and x-ray diffraction. In addition, the effectiveness of copper nanoparticles in killing the larva of *Aedes aegypti* and its ability to inhibit the emergence of its instars was tested. Also, some biochemical analyses were conducted to clarify the effect of copper nanoparticles on the physiology of the *Aedes aegypti* mosquito and to know its toxicity on the activity of some important metabolic enzymes.

MATERIALS AND METHODS

The kit reagents used in this experiment were purchased from the official agents of (Linear, Spain) and (Meril, Balgium), Indonesia branch. The fruits of dry *Coffee arabica* were brought from farms in Taiz Governorate, Yemen, which is world-famous for its quality and distinctive flavor. copper sulfate pentahydrate ($CuSO_4H_2O$) obtained from the Physical Chemistry Laboratory, College of Mathematics and Natural Sciences, Hasanuddin University, Indonesia, Merk Company, Germany.

Preparation of *Coffee arabica* extract

The fruits of the *Coffee arabica* were dried under the sun's rays and then ground by an automatic mill to obtain a fine powder. 20 grams of ground *Coffee arabica* powder was weighed and mixed with 200 ml of boiled distilled water at a temperature of 80 °C for half an hour. After that, the extract was cooled to room temperature and then filtered through Whatman filter paper No.11 with a vacuum pump. The final filtrate was kept in a refrigerator at 4°C for subsequent experiments.

Synthesis and characterization of copper nanoparticles

About 100 ml of the *Coffee arabica* extract was mixed with 100 ml of freshly prepared 40 mM $CuSO_45H_2O$ solution. A greenwash color indicated the formation of Coffee-fabricated CuNPs and left to stand for 24 hours at room temperature. Using a centrifuge (HERMLE Z 366 K1) at 12,000 rpm for 15 minutes at 4 ^{o}C, CuNPs were harvested and rinsed triple times with distilled water. To produce powdered copper nanoparticles, washed copper nanocrystals were roasted in the oven at 80°C for 18 hours **(14)**. The synthesized green-based CuNPs were further characterized by Uv-Vis (UV-2600, Shimadzu Europa GmbH), FTIR (IR Prestige-21, Shimadzu Europa GmbH) and Xray diffraction (XRD) (Shimadzu XRD-7000, 40 kV, 30 mA with CuKα radiation).

Culture of mosquito larvae

The larvae of the *Aedes aegypti* mosquito were collected from a colony stock in the entomology laboratory, Faculty of Medicine, Hasanuddin University, Indonesia. The larvae were grown until they reached the fourth instar, then divided into 20 larvae each in a plastic container containing 100 ml of distilled water and kept at a temperature between 25 and 27 °C and a photoperiod of 14 hours of light and 10 hours of darkness and it was used to carry out a larvicide experiment later. The larvae were fed daily with fresh food consisting of a mixture of biscuits and dry yeast.

Larvicide assay

Copper nanoparticles synthesized from *Coffee arabica* were prepared at different concentrations of 2, 12, 25, 50, and 100 ppm, and then applied against the fourth generation of *Aedes aegypti* mosquito larva according to the standards of the World Health Organization WHO, 2019 (15).

Mosquito metamorphosis studies

Aedes aegypti larvae treated with copper nanoparticles were observed daily to see any developmental changes in their instars. The death rate of larvae was recorded after 24 hours of treatment and the LC_{50} and LC_{90} were calculated using Probit analysis (SPSS, 22). The percentage of emergence of pupae and the adult insect was recorded until the end of the experiment. The experiment was repeated four times and the mortality rate was calculated using Abbott's formula **(16).**

Estimation of biochemistry parameters

The larvae were taken out of the experiment solution after 24 hours of treatment and then washed with cold saline. A homogenized tissue of the larvae was prepared in 0.5 ml of PBS solution by an electronic homogenizer for 0.5 minutes. The larval tissue homogenate was centrifuged at 10,000 rpm for 10 minutes at a temperature of 4 °C. Then the supernatant solution was collected and kept in the freezer at a temperature of -20 °C, and then used later to estimate the value of total protein, carbohydrates, alkaline phosphatase ALP, lactate dehydrogenase LDH, and aspartate transaminase AST Using a kit of reagents manufactured by the company of Linear, Spain and Meril, Balgium according to the methods recommended by the manufacturer. Biochemical examinations were achieved with a UV-Vis spectrophotometer (UV-2600, Shimadzu Europa GmbH), and repeated three times to ensure the validity of the calculations and statistical analyses.

Statistical analysis

A statistical ANOVA test was used to determine the differences between the different groups in the experiment measuring the larval mortality rate, as well as to clarify the significant differences between the groups of the experiment of biochemical parameters and the effectiveness of measuring metabolic enzymes. A probit test was used to determine the LC_{50} and LC_{90} after 24 hours. All statistical analyses were carried out using the SPSS (version 22, IBM). A probability level of 0.05 was used to determine the significant difference between the treated groups.

RESULTS AND DISCUSSION

In the present study, we used *C. arabica* extract to synthesize copper nanoparticles, which was achieved due to the presence of various secondary metabolites in the plant extract. These compounds are the core reason for the reduction, formation, and coverage of nanoparticles. The formation of copper nanoparticles was confirmed by the characteristic green color, due to surface plasmon excitation of the copper at the nanoscale level, confirmed by UV-vis spectrophotometer (**Figure 1a**) which displayed the highest peak at a wavelength (λmax) of 264 nm and an absorbance of 1.240, also seen peaks at 537 and 564 nm. A similar peak was seen in the UV-Vis spectrum pattern generated by another plant extract (17,18). Additionally, the next 30 days after synthesis, a spectrum at 265 nm was found and absent another peak. This confirms copper nanoparticles' stability, purity, and small size (**Figure,1b**).

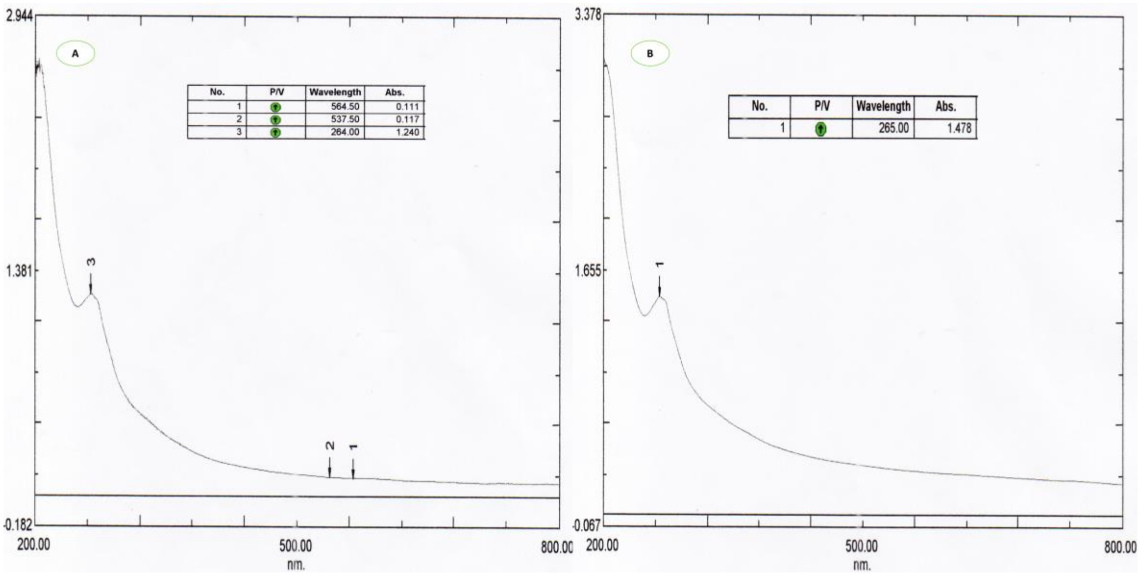

Figure 1 UV-vis spectrophotometer analysis **a**) CuNPs 3 days post-synthesis and **b**) CuNPs 30 days post-synthesis

The FTIR spectrum of CuNPs and *Coffee arabica* powder are shown in (**Fig. 2 a & b**). Although there are minor differences in spectral patterns and intensity among CuNPs and *coffee arabica*, the functional groups are generally comparable. The spectrum of 3381, 2926, 2854, 1921–1517, and 1145 cm^{-1} are attributed to the functional groups OH, CH, CH 2, C=O, and COC, respectively, that all confirmed aromatic compounds and their origin including polyphenols, terpenoids, flavonoids and other organic materials in the *Coffee arabica* extract which coated CuNPs. Peaks at 979 and 927 cm^{-1} are caused by the aromatic C-H group, and

a band registered at approximately 866 cm^{-1} is attributed to the (C-H) phenolic compound on CuNPs **(19)**. Another notable peak can be seen between 500 and 800 cm^{-1}, which points to the C-Cl group of alkyl halides, aromatic alkanes, and flavonoid molecules as the stabilizing component of CuNPs. The production of CuNPs is evidenced by the sharp peaks at 617 and 1097 cm^{-1}. Similar outcomes using different plant extracts were documented in the literature (20,21,22). Results by Davarnejad *et al.*, 2022 displayed the FTIR spectrum of copper sulfate pentahydrate with strong peaks at 1153, 981, and 450-658 cm^{-1}, which is marked by the linking of sulfur to oxygen (S=O) in the $Cu_2SO_4.5H_2O$ salt, Our outcome shows a spectrum at 617 and 1097 cm^{-1} that is similar to synthesized CuNPs **(23)**.

Dried copper nanoparticles were used in XRD testing. The diffraction of X-rays traveling through the copper nanoparticles synthesized using C. arabica extract was recorded as diffraction peaks at 21.39 (100), 24.04 (75), 28.07 (88), 29.35 (54), 31.25 (79), 42.38 (46), 44.00 (50), 51.13 (50), 64.28

(50), and 77.53 (58). According to the XRD spectrum and the International Centre of Diffraction Data Card (JCPDS NO: 01-410-5040 and 01-080-1148), CuONPs and CuNPs have a monoclinic structure and are crystalline. The mean particle size of CuNPs and CuONPs was calculated using Debye-Scherer's formula, and the average crystallite size was found to be about ~16.3 nm. The crystallization size being less than 100 nm indicates the nanocrystalline nature of the green synthesized CuNPs. Similar outcomes were obtained from the synthesized CuNPs' structural XRD analysis (24,25,26). (**Figure 3**)

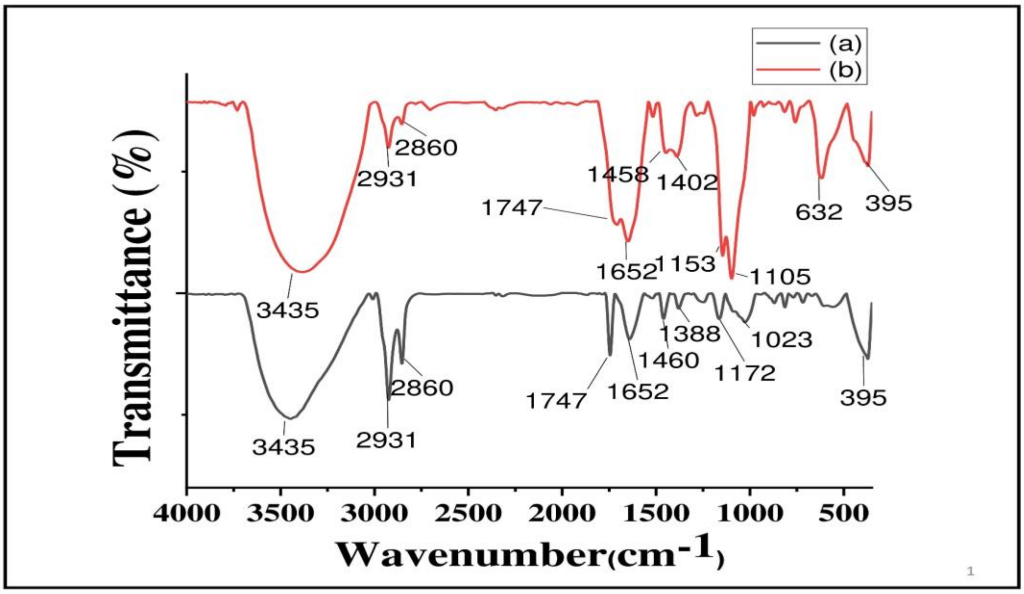

Figure 2 FTIR analysis a) *Coffee arabica* extract and b) CuNPs synthesis by *Coffee arabica*

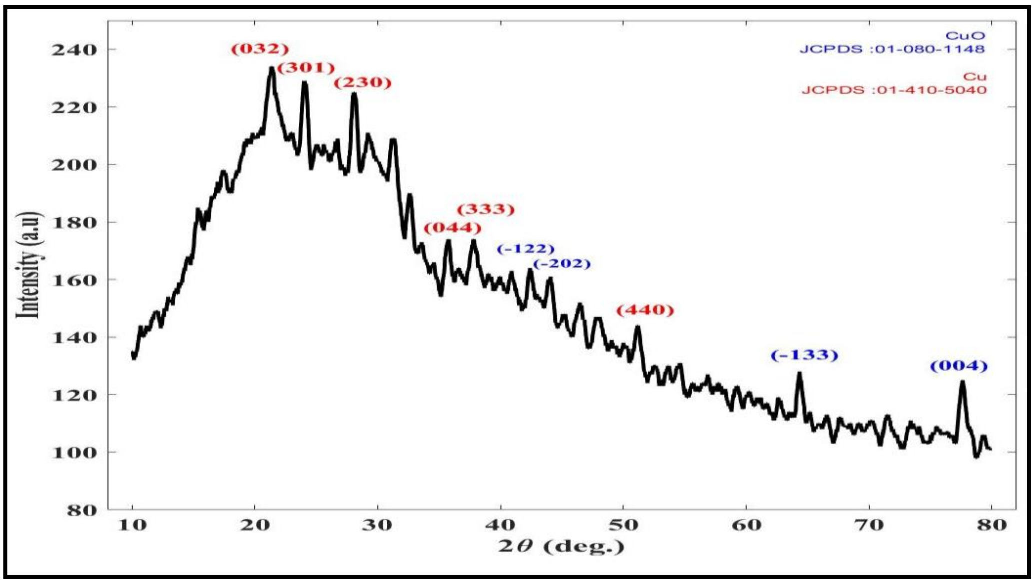

Figure 3 XRD patterns of Cu nanoparticles using *Coffee arabica* Extract

Larvicidal Bioassay of CuNPs

Copper nanoparticles synthesized from *Coffee arabica* showed good activity against *Aedes aegypti* larvae, fourth generation, at various concentrations. (**Figure 4**).

The highest mortality rate was 96.3 % at a concentration of 100 ppm of copper nanoparticles. The study recorded only 3.75 % for the emergence of nymphs from larvae treated with copper nanoparticles, while the stage of the emergence of the adult insect recorded the lowest percentage, which was 2.5 %. Statistical analysis indicated that the LC_{50} and LC_{90} doses for *Aedes aegypti* larvae treated with copper nanoparticles were 5.79, and 36.59 ppm, respectively.

Many studies have shown that biosynthetic nanoparticles have high toxicity at low concentrations against mosquito larvae (27).

The concentrations of 50 and 100 ppm of copper nanoparticles synthesized from *Coffee arabica* showed a high mortality rate, and this is due to the ability of the nanoparticles to bind and penetrate the larval section, enter the cells and tissues, and destroy the biochemical activities for the synthesis of proteins and enzymes important for the metabolism of the *Aedes aegypti* insect (28).

Similarly, a study on the synthesis of copper nanoparticles from the extract of the *Grewia asiatica* plant showed the same results in this study (29).

The extract of the *Coffee arabica* plant has effective properties against insects and its effectiveness increased when combined with copper nanoparticles. One study indicated that biologically synthesized copper nanoparticles give good results that are environmentally friendly when compared with chemical insecticides (30). These results give great hope and a promising way to control mosquitoes that transmit many diseases and preserve the environment from harmful chemical pesticides

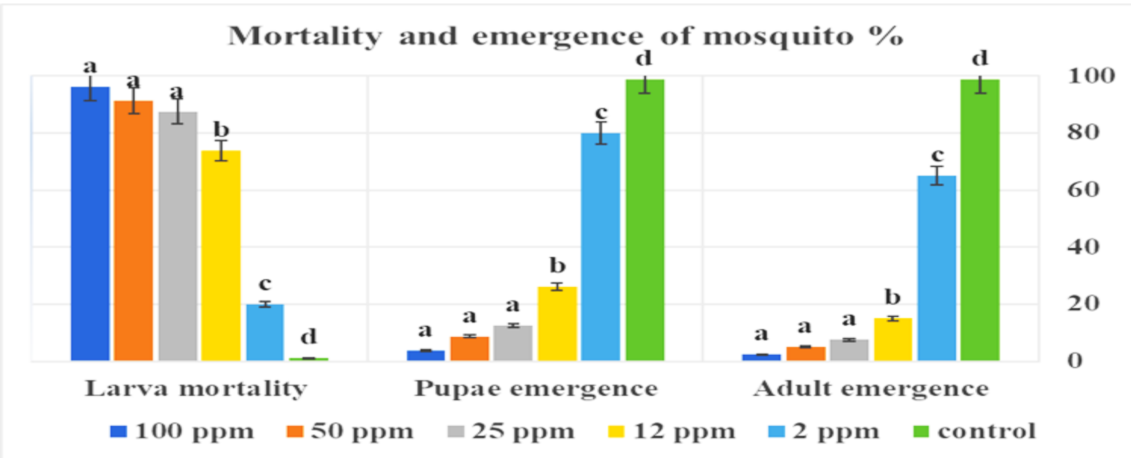

Figure 4 Larvicidal Bioassay of CuNPs

Effect of CuNPs on some biochemical parameters in *Aedes aegypti* larva

The results of the study showed that the amount of glucose increased gradually at concentrations of 12, 2, 25, 50, and 100 ppm for copper nanoparticles compared to the control group, and the difference was significant at a concentration of 15, 15, 50 ppm ($p < 0.05$), but at a concentration of 100 ppm the result of the statistical analysis was not significant ($p > 0.05$), (**figure 5a**)

The amount of total protein increased at a concentration of 2, 12, and 25 ppm and was non-significant ($p > 0.05$), while an apparent decrease with a statistically significant value ($p < 0.05$) was observed at a concentration of 50, 100 ppm for copper nanoparticles (**Figure 5b**).

Biological compounds such as carbohydrates and proteins are very important for the vital activities of all living organisms, including insects. These compounds are susceptible to being affected by oxidation factors from chemical and biological compounds (31). Environmental pollutants such as pesticides, heavy metals, and nanoparticles cause negative effects on the physiology and biochemistry of the insect body (32).

The explanation for the increased amount of protein in insects treated with insecticides is an adaptive evolution against oxidative factors caused by environmental pollutants. A study concluded that there was a significant increase in the amount of protein in the larvae of the *Galleria mellonella* insect treated with a compound oxyclozanide, as a response to the resistance of the pathogen (33). The increase in the effectiveness of AST enzymes in insects treated with nanoparticles is an indicator of cellular damage, which was reflected by increasing the amount of total protein to counteract this damage (32).

According to the hypothesis that increasing the amount of total protein is considered an adaptive activity against pathogens, our results in this study support this hypothesis. In contrast, another study on the extracts of the *Annona muricata*, *Azadira indica*, and *Pongamia glabra* reported a decrease in the level of total protein associated with the death of *Aedes aegypti* and *Aedes albopictus* larvae (34).

Most of the biological activities in insects depend on carbohydrate metabolism. The increase in the amount of glucose in this study was due to physiological stress and response to inhibit the damage caused by the copper nanoparticles, or perhaps the increase occurred as an adaptive action against the toxicity of the copper nanoparticles. Regarding the increase in glucose observed in this study, a study conducted by (35) obtained similar results on *Aedes aegypti* treated with toxic agents of fungal synthesized titanium dioxide nanoparticles revealing an increase in the amount of glucose in treated insects. The reason was attributed to the decomposition of glucose from the glycogen compound stored in the liver (fat body) under the influence of the stress that occurred (36).

In contrast, other studies reported a decrease in glucose in *Galleria mellonella* insects treated with commercial CuNPs (37). Likewise, researchers said that the larvae of the *phenacoccus solenopsis* insect treated with *pongamia glabra* and silver nanoliquids showed a significant decrease in the amount of total body glucose (38).

Efficacy of CuNPs on some metabolic enzymes in *Aedes aegypti* larvae

The value of alkaline phosphatases, lactate dehydrogenase, and acetate transaminase enzymes in the *Aedes aegypti* mosquito treated with copper nanoparticles synthesized from *Coffee arabica* was shown in (**Figure 6**).

The results showed a decrease in the enzyme alkaline phosphatase in the larvae of the *Aedes aegypti* mosquito treated with copper nanoparticles, regardless of the concentration, and the decrease was statistically significant at concentrations of 2, 12, 50, and 100 ppm, compared to the control group, and a non-significant decrease was seen at a concentration of 25 ppm. Possibly a physiological defense response against the influence of copper nanoparticles.

The results of this study agreed with another study that noted a decrease in the enzyme alkaline phosphatase in *Culex pipiens* larvae treated with silica nanoparticles and *Citrus sinensis* extract (39,40), but on the other hand, a study by El Gohary et al, 2021 conducted on *Culex pipiens* treated with *syzygium aromaticum* chitosan-nanoparticles showed an increase in the enzyme alkaline phosphatase (41). The alkaline phosphatase enzyme is a hydrolytic enzyme that works to degrade monophosphate esters in an alkaline medium. Various stresses and diseases cause a significant decrease in the activities of the enzyme alkaline phosphatase. The changes in the activity of the enzyme alkaline phosphatase may be due to the oxidative effect caused by the nanoparticles in the treated insect cells (42).

The activity of the AST enzyme and LDH enzyme is used as a biological indicator to estimate the effect of oxidative stress factors and chemical insecticide toxicants on insect cells and tissues (32).

AST enzyme transfers the amino group of amino acids to keto acids to convert between compounds of energy sources in the body of insects and mammals. LDH is a glycolytic enzyme that plays an important role in carbohydrate metabolism in living organisms and insects (37).

Figure 6 shows a significant increase in the level of AST enzyme in the tissue extract of the larva of the *Aedes aegypti* mosquito, the largest increase occurred with a concentration of 50,100 ppm of copper nanoparticles. This increase is closely related to the death rate in the previously mentioned *Aedes aegypti* larvicide experiment.

Many studies indicated that the high toxicity of insecticides and nanoparticles leads to an increase in the value of the AST enzyme in mosquito larvae.

The reason for the increased activity of metabolic enzymes in *Aedes aegypti* tissue extract may be due to cellular damage and a defect in the energy production mechanism caused by the interference of copper nanoparticles.

In another study similar to our results conducted by Halawa and colleagues 2021 on the effectiveness of chlorpyrifos and *Bacillus thuranginsis israelinsis* against *Culex pipiens*, the researchers found an increase in the value of AST enzyme in the tissue extract of the treated insects (43). Also, Sugeçti and Büyükgüzel reported that 1.5% of the oxfendazole compound caused oxidative stress in *G. mellonella* insect larvae, which was inferred by an increase in the level of AST enzyme in the treated insect's hemolymph (44).

The LDH enzyme is a glycolytic enzyme and plays an important role in the metabolism of carbohydrates in the tissues of insects its activity differs according to the level of oxidative stress and is used as an indicator of cell damage in the body (45). In this study, a decrease in the value of the LDH enzyme was observed. This decrease was associated with an increase in the concentration of copper particles and an increase in the mortality rate in the treated mosquito larva, and this decrease was statistically significant (**Figure 6**).

Results similar to this study conducted by Durairaj *et al.*, 2018 and his research team found that the value of LDH enzyme in *Culex* and *anopheles* larva treated with chitosan-based silver nanoparticles decreased according to the increase in the concentration of these particles (46). Another study found the same result regarding decreased levels of LDH in *Galleria mellonella* insects treated with titanium dioxide nanoparticles (32). On the other hand, a study found the opposite of our result regarding level LDH in *Galleria mellonella* insects treated with copper oxide nanoparticles (37).

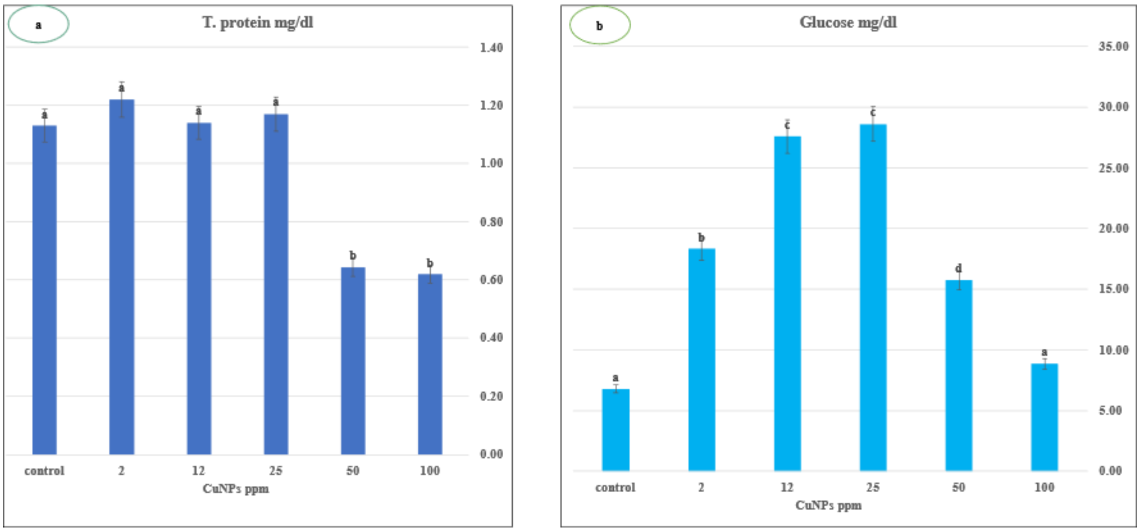

Figure 5 Effect of CuNPs on total protein and glucose in *Aedes aegypti* larvae.

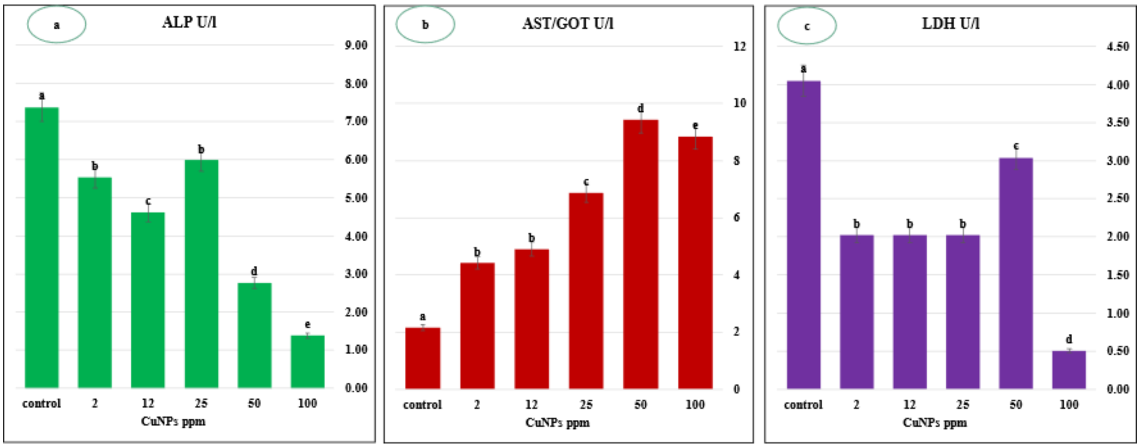

Figure 6 Effect of CuNPs on ALP, AST, and LDH enzymes in *Aedes aegypti* larvae.

CONCLUSION

The copper nanoparticles produced from the *Coffee arabica* have been used to detect the pesticide activity against the *Aedes aegypti* mosquito. The mortality average and alteration in biochemical parameters show that this nanoparticle can be used effectively to control mosquitoes. In conclusion, our results confirm that *Coffee arabica* can create copper nanoparticles that can be suggested to develop a safer and more effective larvicide alternative to chemical methods to control mosquitoes. During larval development, mosquitoes likely evolved unique physiological mechanisms involving separate biochemical systems for the detoxification of nanoparticle biocides, and these processes and systems. More broadly, research is also needed in the following areas: histopathological and genetic study to explore a mechanism larvicide, as well as CuNPs toxicity against the non-target organism.

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Conflicts of Interest:

The authors affirm that there are no conflicts of interest.

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