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Copper Oxide Nanoparticle As a Larvicidal Agent And Its Effect On Some Physiological Parameters And Molecular Identification For *Culex pipiens* (Diptera Culicidae).

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Abstract

The present study was undertaken in order to investigate the effect of CuO nanoparticle which was prepared by green method. Also, evaluate the median concentration on total Protein and Carbohydrate content of treated larvae with *Achilea fragrantissima* extract and CuO nanoparticle comparing with control. The effects of tested materials were examined on the activities of acetylcholinesterase (AChE) and glutathione S-transferase (GST). The results revealed inhibition of all biochemical activities except in GST activity was increased in treatment by *Achilea fragrantissima* extract.

Keywords: Nano-particles, Mosquitoes control, Molecular identification, Biochemical parameters

1. Introduction

Since they create serious health problems and are thought to be the world's deadliest insect, killing over 750000 people a year, mosquitoes are well known for their significance to public health. [1]. Egypt has recognized Culex pipiens Linnaeus (Diptera: Culicidae) as a disease vector. [2], [3]. In addition to West Nile virus [4], it also spreads the Rift valley fever virus [5], Wuchereria bancrofti, which is recognised for transmitting human lymphatic filariasis [6], and Rift valley fever virus [7], [8]. Additionally, Cx. pipiens has been identified in all governorates [9], [10] and is the main filarial vector in Egypt [11], [12].
Chemical pesticides have been used to prevent diseases spread by mosquitoes, but this has led to problems with human and environmental risks as well as the development of insecticide resistance in the vectors [13], [14]. However, due to their larvicidal, adulticidal, and repellant qualities, biopesticides of plant origin, such as plant extracts and essential oils, are a desirable method for controlling vectors [15].
The introduction of particle formations ranging in size from 1 to 100 nm, using various methods of manufacture, modification, and strategy, is achieved by the burgeoning and critical field of nanotechnology. This size range changed both the individual atoms/molecules and most of the chemical, biological, and physical properties. Novel applications of nanoparticles and nanomaterials are rapidly expanding on a variety of fronts due to their wholly new or better properties related to size, distribution, and shape. [16].
The three main biosynthetic processes for nanoparticles are biomolecules, plants, and microbes. During the biogenic synthesis of nanoparticles, natural compounds found in plant and microbial extracts act as stabilizing and reducing agents. These useful elements enable the synthesis of nanoparticles from metal sources. Despite advances in approaches for creating biomolecules from microorganisms, the method for producing nanoparticles still faces various

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challenges. For example, the overall process rate is exceedingly sluggish for large-scale nanoparticle synthesis, and these procedures invariably require a range of technically and safely hazardous situations [17]. Plant extracts may be the most excellent strategy to biosynthesize nanoparticles since they can be produced in greater quantities more quickly and at lower manufacturing costs [18]. It is simple and clear to harvest plants from ecosystems indefinitely. They contain enough phytochemicals to replace expensive, very toxic, and environmentally hazardous reducing agents reagents as sodium citrate, sodium borohydride ($NaBH_4$), and ascorbate [7]. Various studies have shown that phytochemicals discovered in plant extracts, such as polysaccharides, flavonoids, phenolic acids, and quercetins, are efficient in reducing metal ions like Ag^+, Cu^{2+}, and Au^{3+} [19]–[21]. Various stabilization, capping, and chelating actions may also appear during the formation of nanoparticles. Furthermore, it is straightforward to remove key components from diverse plant sections, putting the plant in the initial stages of nanoparticle biosynthesis [22].

Several researchers have recently been noticed to be interested in using green synthesis of nano particles. This is since plants contain a wide range of physiologically active substances, including both their entire and in specific portions, which have a wide range of applications in both industry and medicine. CuO NPs have been produced successfully utilizing extracts from various plant species [23], [24]. In contrast, it was discovered that artificial CuO NPs may be utilized to clean up the environment of a variety of toxic emissions [25], rhodamine B, and methylene blue [26].

When paired with specialized proteins and enzymes that are necessary for the growth and feeding of plants, copper (Cu) plays a crucial role [27]. It participates in oxidative stress responses, mitochondrial biogenesis, photosynthetic electron transport, cell wall metabolism, and hormone signaling [28]. Additionally, copper can cause changes in root systems, increased production of bioactive chemicals, and suppression of plant growth by reactive oxygen species (ROS) [29], [30]. Recently, there has been a lot of interest in the study of the production of multifunctional CuO NPs using medicinal plant-mediated methods [31]. Green CuO NP has been found to have a variety of applications in photocatalytic activity, electrocatalysis, pollutant degradation, nanomedicine, drug therapy, and catalysis [32]. CuO nanostructures have been identified in several nanoforms, include nanocrystals, nano sticks, nanotubes, nanoflowers, and nanosheets, and their adaptive and practical antifungal qualities when used as textile coatings have been proven. The third instar larvae of Cx. pipiens are influenced by the application of sublethal concentrations of plant extracts on certain biochemical parameters, including total protein, carbohydrate, and lipid contents, activity of acid and alkaline phosphatases, and inducers of AChE or GST activities [33]–[36].

Although it may be difficult to interpret these characteristics without a taxonomic background, physical characteristics were previously used to categories mosquito species. If related traits are lost, it becomes more difficult to distinguish between species [37]. A simple, precise, and user-friendly identifying solution is therefore always required [38]. Recently, DNA barcoding has shown to be reliable for classifying a variety of species, including mosquitoes [39]–[41].

The objective of the current work was to determine how green produced Copper nanoparticles affected several biochemical markers in a lab setting and to identify Cx. pipiens using PCR.

2. Materials and methods

2.1. Tested insect

Mosquito Colony: The Medical Entomology Institute provided the *Cx. pipiens* L. mosquito used in this investigation. It was raised in the Entomology laboratory, Faculty of Science, Al-Azhar University for multiple generations. Relative humidity was adjusted to be between 70% and 80%, and the amount of light and darkness was varied (16: 8 hrs.). The following research involved late third larval instars.

2.2. The plants

This study's plants were whole plants of (*Achillea fragrantissima*).

2.3. Preparation of plant extract

Two solvents were used to extract each plant (Ethanol absolute and petroleum ether 60-80). Each plant's resultant powdered components were extracted with 100 grammes each of petroleum ether and ethanol absolute. A Soxhlet device was used to carry out the extractions. Each plant's solvent extracts were evaporated and dried in a vacuum using a Labo-Rota C311 rotary evaporator set to a water bath temperature of (40 ºC) for one to two hours, yielding pet-ether and ethanol absolute. Dry crude extracts that were produced were kept in screw-capped vials and kept at 4 ºC until use.

2.4. Synthesis of CuO nanoparticles

Achillea fragrantissima plant extract was extracted with ethanol before being dropped into distilled water and forcefully agitated at 80 °C. Under magnetic stirring, 0.2 g copper acetate was added dropwise to the extract from the previous step, which had been diluted in 20 ml of distilled water. After four hours was

heating the solution mixture a few drops from NaOH were used to rich pH of the solution to an alkaline level (0.5 M). CuO nanoparticle production was occurred by the solution turning turbid then to white look.

2.5. Characterization of CuO nanoparticles
2.5.1. Transmission Electron Microscope
An electron transmission microscope was used to examine the size and shape of the produced nanoparticles (HR-TEM, JEOL-JEM-2100). Prior to analysis, the diluted CuO nanoparticle suspension was stirred continuously for 60 min in a sonication water bath. One or two drops of the suspension were applied to the testing grid, and then it was allowed to dry before being investigated.

2.5.2. Dynamic Light Scattering (DLS)
The Zeta Sizer apparatus can be employed to measure the particle size and/or zeta potential of the CuO nanoparticles generated (Nano-ZS, Malvern Instruments Ltd., ZetasizerVer, 704, UK). CuO nanoparticle suspension was first high-frequency scanned to ensure proper particle dispersion in the aqueous medium, and then the fragmented beam from the Brownian motion of the disseminated nanoparticles in solution was immediately detected using DLS.

2.6. Bioassay for the materials under consideration
2.6.1. Extracts and nanoparticles have insecticidal action.
Plant extracts and nanoparticles evaluated in various concentrations were used. After 48 hours, the mortality data was recorded. and calculated by a probit analysis [42] to determine the LC50 & LC95.

2.6.2. Biochemical Parameters of *Cx. pipiens* Larvae:
To assess the effects of the investigated plant extracts on the principal body metabolites, lethal concentration (LC50) levels were employed to determine the total protein and carbohydrate content, as well as Acetylcholinesterase (AChE) and Glutathione S-transferase activity (GST). Concentration levels with each extract examined were determined using the techniques given in detail by [36]. 25 larvae in their third instar were given treatments using various quantities of each extract, and they were kept at room temperature for 1 day. After gathering the larvae, the methods for making biochemical estimates were immediately put to use. For the spectrophotometric observations, a Shimadzu UV-VIS Recording 2401 PC was utilized (Japan).

2.6.2.1. Determination of Total Carbohydrate Content: The carbohydrate concentration of the whole-body homogenate was measured using the method described by [43].

2.6.2.2. Determination of Total Protein Content: The total amount of protein was measured using the folin phenol reagent technique [44].

2.6.2.3. Determination of Acetylcholinesterase (AChE) Activity:
Thiocholine, which is created when ChE breaks down acetylcholine, reacts with dithio-bis (nitrobenzoate) to create 2-nitro-5-mercaptobenzoate, which may be measured using bio diagnostic kits and identified spectrophotometrically at 405 nm. Three batches of the previously mentioned larvae concentration were homogenized with a homogenizer and a 10 ml solution of 0.1 M-phosphate buffer, pH 7.5 (KH2PO4-NaOH), containing 1% Triton X-100. The lysates were centrifuged for 60 minutes at 4oC and 15.000 rpm in a Heraeus Labofuge 400R, Kendro Laboratory Products GmbH, Germany. The resulting supernatant was utilized for the in vitro inhibitory experiment on the enzyme without further purification. The procedure described in [45] was used to measure the activity of acetylcholinesterase (AChE). According to the recommendations in the booklet, 10 µl aliquots of supernatant were obtained for spectrophotometric studies and AChE

2.6.2.4. Glutathione S Transferase (GST) Efficiency Determination:
The activity of glutathione S-transferase (GST) was measured spectrophotometrically using the method of [46] employing bio diagnostic procedures. The polymerization of reduced glutathione and S-2, 4-dinitrophenyl glutathione is measured using this approach (CDNB). To track the synthesis of CDNB adduct, the total increase in absorbance at 340 nm above the blank was calculated. To assess the concentration of GST, the treated larvae were homogenised in a homogenizer with 10 ml of sodium phosphate buffer (pH 8.0) containing 1% Triton X-100. The homogenate was centrifuged for 60 minutes at 4°C at 15,000 rpm using a Heraeus Labofuge 400R from Kendro laboratory products GmbH in Germany. According to the brochure's instructions, 50 µl aliquots of supernatant were collected for spectrophotometric tests, and GST activity was determined.

2.7. Extraction of DNA from mosquito larvae
The mosquito larvae were dissected and deposited in 1.5 µl Eppendorf tubes. The DNA was extracted using a Qiagen DNA tissue Mini Kit (Qiagen®, Cat. No. 56304, Helden, Germany) according to the manufacturer's instructions.

2.7.1. Polymerase chain reaction (PCR)

Specific primers amplifying the cytochrome oxidase C.O. subunit I (COI) of mosquito mitochondrial DNA LCO1490:5′-GGTCAACAAATCATAAAGATATTGG-3′ as a forward primer and HCO2198:5′-TAAACTTCAGGGTGACCAAAAAATCA-3′ as a reverse primer were employed to identify mosquito species [45]. The final reaction volume for the PCR amplification was 2X (50 µl), which contained 25 µl of 2X master mix (i-Taq, iNtRON, Seongnam, Korea), 0.2 mM of each primer, 4 µl of template DNA, 0.2 mg/ml of BSA, and 14.5 µl of nuclease-free water. The PCR was conducted under the following conditions: initial denaturation at 94°C for 10 min; 40 cycles of denaturation at 94°C for 1 min; annealing at 45°C for 1 min; and extension at 72°C for 1 min; and final extension at 72°C for 10 min. a transilluminator was used to view the PCR findings after they had been stained with ethidium bromide and run on a 1.5% agarose gel (U.V. transilluminator, Spectroline, Westbury, USA).

2.7.2. Sequence analysis

Macrogen reagent was used to purify PCR products (Seoul, Korea). After performing single-strand DNA sequencing, the nucleotide sequences of mosquito larvae COI were aligned.

2.7.3. Bioinformatics

Using the same PCR primers used for PCR, one strand of DNA was sequenced. Chromas Pro 1.5 beta was used to put the obtained sequences together (Technelysium Pty. Ltd., Tewantin, QLD, Australia). The obtained sequences were then compared with those found in the GenBank database maintained by the National Center for Biotechnology Information using the Basic Local Alignment Search Tool (BLAST) analysis (NCBI). MEGA 11.0 software was used to align the sequences using W-cluster alignment. Sequence divergences are calculated using Kimura's two parameters (K2P) [46]. To illustrate the patterns of species divergence, N.J. trees using Tamura-Nei method [47] for *C. pipiens*. Bootstrapping was performed in MEGA 11.0 [48] with 1000 replications.

2.8. Statistical analysis of the data

Multiple linear regressions were used to measure the LC50 [42]. The data is shown as Mean SD. Minitab 17 software was used to do a one-way analysis of variance ANOVA on the data to determine the significance of the differences between means.

3. Results and Discussion

3.1. Characterization of CuO nanoparticles

3.1.1. Transmission Electron Microscope

To determine the morphological shape and size of the produced CuO NPs, TEM examination was carried out. According to Figure 1, the particles were semi-spherical and ranged in size from 15 to 40 nm. This finding suggests that the produced CuONPs were mono dispersed and crystalline.

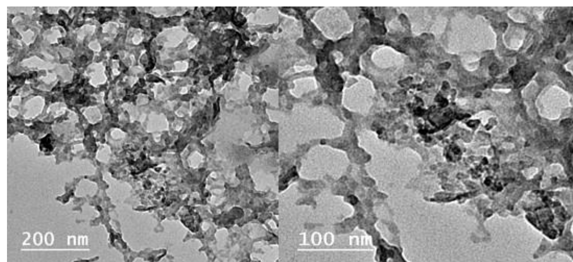

Figure (1):TEM at different magnification to CuO nanoparticles

3.1.2. Dynamic Light Scattering (DLS)

Dynamic light scattering (DLS) measurements were used to calculate the particle size of the produced CuO nanoparticles, as shown in figure (2). The average particle size shown in the figure is 219 nm. The larger particle size acquired from DLS measurement compared to that obtained by TEM measurement is because DLS provides an overall image of the nanoparticles and their aggregations while TEM only provides an image for a specific area for measurement.

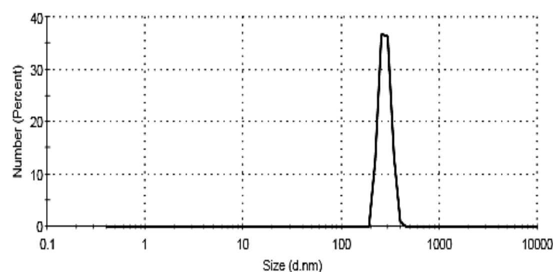

Figure (2) Particle size for CuO nanoparticles from DLS measurements

Plant-mediated nano-fabrication is a new area of nanotechnology that is favored over traditional methods due to its advantages in terms of safety, affordability, environmental friendliness, and biocompatibility. In the current investigation, plant extract was used to create the nanoparticles. The plant extract is one of many natural surfactants that are used in the production of green synthesis materials [49] and it also contains many chemicals that have reactive functional groups like hydroxyl, amino, carboxyl, and thiol groups. These substances helped reduce copper salts to CuO nanoparticles and stabilized the particles so they could float freely in the solution. However, some particles clumped together to form clusters made

up of dozens or even hundreds of distinct nanoparticles. The size of nanoparticles differs between TEM and DLS.

3.2. Larvicidal activity of extracts and Nanoparticle

Two extracts from Achillea fragrantissima were tested for their larvicidal potential against a chosen mosquito's third larval instar. Table 1's results show that the effect of the CuO nanoparticles was more potent than that of the examined extracts. CuO nanoparticles produced with ethanol and pet-ether had LC50 values of 0.98 and 1.04 ppm, respectively. One of the most active areas of research right now in several fields is nanotechnology. C. pipiens, a type of mosquito, has received a lot of attention due to its role as a disease carrier. The development of resistance, the comeback of the vector, and environmental degradation has all contributed to the failure of the control of Cx. pipiens with chemically manufactured pesticides [50]–[52]. The causes of mosquito deaths following treatment with. So it was hypothetically proposed that the body's absorption of nanoparticles was the mechanism of toxicity against mosquito larvae. Nanoparticles damage the enzymes and organelles in intracellular space, which results in cellular function loss and ultimately cell death [53], [54]. The larvicidal activity of the AgNPs from Artemisia vulgaris leaf extracts was observed. The nanoparticles will develop in the midgut of mosquito larvae and harm the midgut, cortical area, and epithelial cells. The mid-gut of MnO_2-treated larvae had damaged cells and tissues, according to histopathological examinations. Similar research utilizing Cadmium nanoparticles and ZnO nanoparticles, respectively, has been published by [55], [56]. Additionally, numerous plant-based nanoparticles shown significant effectiveness for controlling mosquito larvae in addition to seaweed-based nanoparticles [55]. The larvicidal potential of metal oxides has not been widely investigated. The scant reports that are accessible are limited to studies conducted during the last five years. Nanoparticles like ZnO, CuO, Bi_2O_3, MgO, TiO_2, AgO, Fe_2O_3, and CeO_2 have all been used in research [57]. Wrightia tinctoria (Wt) R. Br extract was used in the manufacture of copper nanoparticles (CuONPs), which also shown larvicidal efficacy against Aedes agypti [58]. Finally, compared to the Achillea fragrantissima extract alone, the nanoparticles made from plant extracts were both more effective and more cost-effective because a lesser quantity was needed. These findings are in line with [59], who examined the effectiveness of gold (AuNPs) and silver (AgNPs) nanoparticles made from leaf extracts of A. calyphafruticosa in controlling the Cx. pipiens mosquito. The CuO NPs showed remarkable larvicidal activity and antifeedant activity for the larvae of Spodoptera frugiperda compared to the control [60].

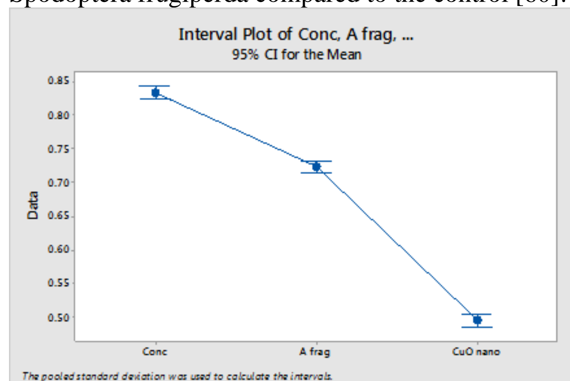

Figure 3: Interval plot of control (Conc), Achillea fragrantissima (A. frag) and CuO nanoparticle (CuO nano) for determination of total protein content.

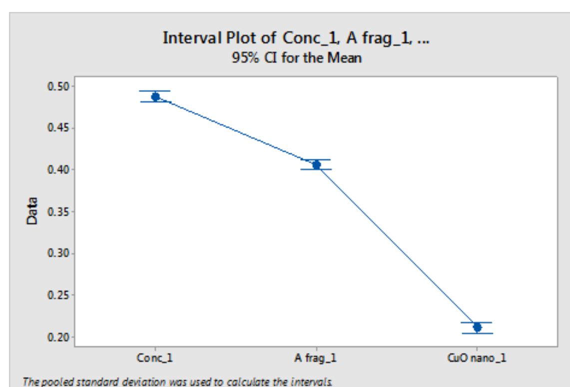

Figure 4 Interval plot of control (Conc_1), Achillea fragrantissima (A. frag_1) and CuO nanoparticle (CuO nano_1) for determination of total Carbohydrate content.

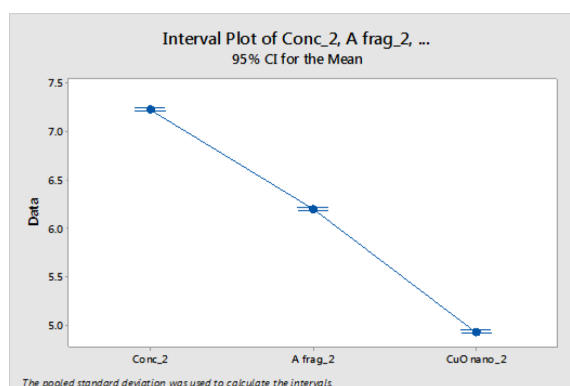

Figure 5 Interval plot of control (Conc_2), Achillea fragrantissima (A. frag_2) and CuO nanoparticle (CuO nano_2) for determination of acetylcholinesterase (AChE) activity.

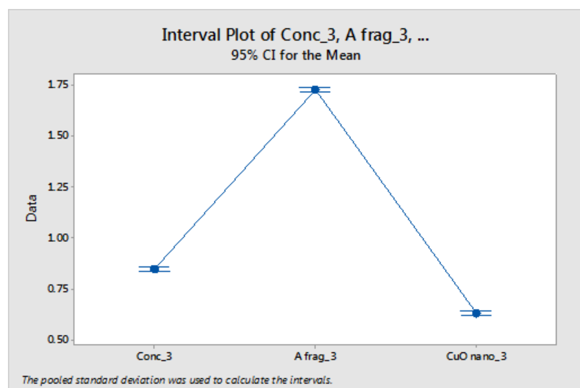

Figure 6: Interval plot of control (Conc_3), Achillea fragrantissima (A. frag_3) and CuO nanoparticle (CuO nano_3) for determination of glutathione S-transferase (GST) activity.

3.3. Biochemical Parameters of Cx. pipiens Larvae:

3.3.1. Total protein content: Total protein content was influenced by the use of plant extract and CuO nanoparticles. These were highly significant difference between two treatments and control as showed in table (2) and Fig (1). A significant difference was appearing from the F value 1460.

3.3.2. Total Carbohydrate Content: The results revealed in table (2) & Fig (2) clarifies the significant difference between the two treatments in comparing with control. The lowest value of Carbohydrate content was 0.21 mg/ml as a result of CuO nanoparticle application.

3.3.3. Acetylcholinesterase (AChE) Activity: Depending on the data from table (2) and Fig (3) the significant difference between the control and treatment compounds were high due to the F value, 14919.38 which was attribute to mean values of each application and control.

3.3.4. Glutathione STransferase (GST) Activity: The activity of GST was affected also by increasing in case of A. fragrantissima extract as well as decreasing in case of CuO nanoparticle in comparing with control as stated in Table (2) and Fig (4) their values were 1.73, 0.63 and 0.85 U/g tissue, respectively. The overall protein content of Cx. pipiens 3rd larval instar was reduced by plant extracts tested. Similarly, [61] ascribed the decrease in protein levels to the plant extracts interfering with the hormones that regulate protein synthesis. Also, [49] found that the protein content in An. stephensi larvae treated with different phytoextracts was lowered and that this was due to the plant extracts interfering with the normal protein production pathway. Furthermore, the acquired results are consistent with those published by [50], [62], [63]. When compared to the control group, the median concentration range (LC50) of each examined extract and CuO nanoparticle considerably ($P>0.05$) lowered the total carbohydrate levels. The lack of carbohydrate content could be attributed to the use of conserved carbohydrate sources in larval tissues as a response of plant extract stress [50]. The effects of A. annua extract on carbohydrate of treated larvae is species-specific, dependent on the physiology of the larvae species; also, during treatment, the alimentary canal of Culex sp. was damaged comparably less severely and was discovered plugged with the extract [51]. The AChE enzyme is abundant in the neural tissue of insects and is regarded as a biomarker of neurotoxicity. Many publications have documented the inhibition activity of tested plant extracts on AChE activity while applying various pesticides against various insect species, such as [52] on Nilaparvata lugens and Laodelphax striatellus, wood vinegar mixed with pesticides; [53] on Musca domestica for chlorpyrifos, deltamethrin, and methomyl; [54] who discovered that Cx. pipiens larvae fed with the LC50 of Ocimum basilicum exhibited neurotoxic action as demonstrated by AchE inhibition; [50]. After 24 h of treatment with CuO NPs, the larval acetylcholinesterase enzyme levels decreased depending on dose ascending activity [60]. Cx. pipiens was treated with α-cyper, chlorpyrifos, and methomyl. GSTs, on the other hand, play a vital part in the biotransformation of foreign chemicals, drug metabolism, and the preservation of the organism against oxidative damage. The enhanced GST activity revealed a boost of the targeted larvae's detoxifying mechanism. The effect of studied plant extracts on GST activity is consistent with that reported by [55] on Helicoverpa armigera and Ostrinia furnacalis utilizing α-terthienyl; [54] Using the median lethal dose of O. basilicum against 4th instar Cx. pipiens larvae. Furthermore, it appears that lower GST activity impairs the detoxification mechanism of the treated larvae.

4.4. Molecular identification of *Cx. Pipens*:

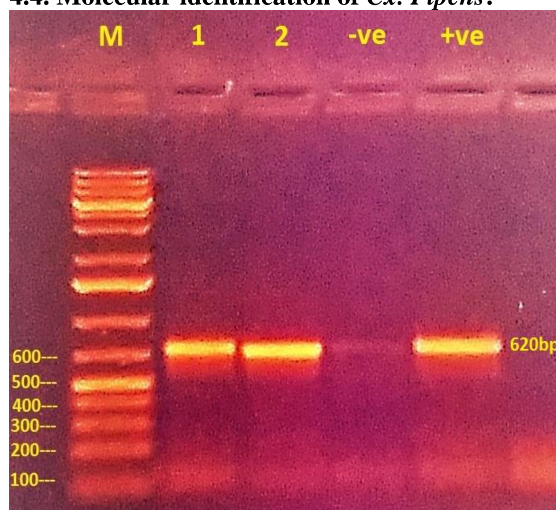

Figure 1: Agarose gel electrophoresis (1.5 % agarose): analysis of PCR amplification for detection of *Cx.*

pipiens M: 100 bp DNA marker; (1&2) *Cx. pipiens* at 620 bp; (-ve) negative control; (+ve) positive control.

PCR study of DNA from *Cx. pipiens* revealed positive amplification using primers that amplified the COI region. The amplicon was 620 pb in size (Fig. 7). The COI region (OQ248250.1) DNA sequences acquired from *Cx. pipiens* amplification exhibited a significant degree of similarity to numerous sequences of the same species which are published in GenBank. The Neighbor-Joining method was used to infer the evolutionary history [56]. The ideal tree has been displayed. The fraction of duplicate trees where the related taxa clustered together during the bootstrap test (1000 repeats) is shown below the branches [57]. The branch lengths on the tree (next to the branches) are scaled in the same units as the evolutionary distance used to estimate the phylogenetic tree. The Tamura 3-parameter approach was used to compute the evolutionary distances, which are expressed in base substitutions per site [58]. There were 19 nucleotide sequences in this investigation. Codon positions 1st+2nd+3rd+Noncoding were included. For each sequence pair, all unclear places were eliminated (pairwise deletion option). The final dataset contained 1542 locations altogether. In MEGA11, evolutionary analyses were carried out. [59].
With bootstrap values >80%, phylogenetic analysis employing COI data from species obtained in the current study indicated a tree with excellent statistical support. (Fig. 8). DNA sequences from the GenBank together with out-groups (*Anopheles arabiensis*). COI sequences recovered from *Cx. pipiens* in this investigation were determined to be 100.0% identical to *Cx. pipiens* sequences published in Kenya [64], Egypt [65], Colombia [66], and Portugal [67] in the GenBank. Biotypes pipiens are currently thought to be independent monophyletic evolutionary units that are in the early stages of ecological speciation, and as such they may be unique phylogenetic entities [68]. Only a little amount of hybridization occurs, and male-mediated introgression from this species' biotypes predominates [69]. The identical sequences could have come from the country's environment and meteorological conditions, which were 100% like those in Egypt.

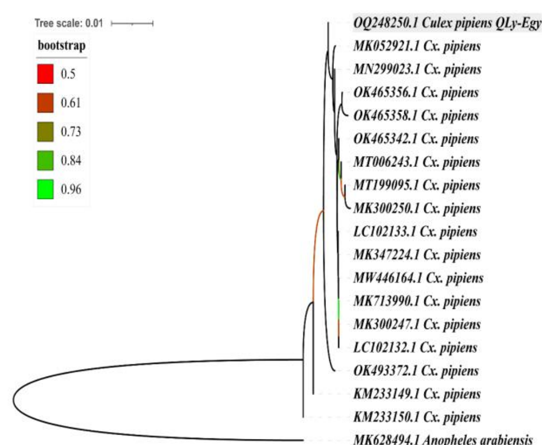

Figure 2: Neighbor-Joining phylogenetic evolutionary tree of *C. pipiens*

4.Conclusions

The author concluded that the CuONPs can be used as larvicide agent against mosquitoes depending on the results of susceptibility of larvae to tested compound. Therefore, CuO nanoparticle affect the examined biochemical parameters on this study, thus it may introduced in IPM pragrame.

Table (1): Susceptibility of third larval instar of *Cx. pipiens* to tested extracts and nanoparticles under laboratory condition.

Tested materials	Solvent used	Conc. ppm	Mortality %	Total No.	Lethal concentration by ppm (Coefficient limits)		Slop
					LC 50	LC 95	
Achillea fragrantissima	Pet-ether	10	15	9	58.91 (51.77-67.03)	226.18 (165.19-310.43)	2.82±0.11
		40	35	21			
		70	53.33	32			
		100	75	45			
		160	90	54			
	Ethanol	20	8.33	5	79.69 (69.71-91.9)	307.60 (224.53-422.38)	2.80±0.1
		60	30	18			
		80	48.33	29			
		120	65	39			
		200	93.33	56			
CuO nanoparticle	Pet-ether	0.35	13.33	8	1.04 (0.88-1.20)	3.32 (2.5-5.33)	3.26±0.27
		0.75	33.33	20			
		1.25	58.33	35			
		2.5	90	54			
	Ethanol	0.35	16.67	10	0.98 (0.81-1.13)	3.11 (2.36-4.96)	3.27±0.29
		0.75	36.67	22			
		1.25	61.67	37			
		2.5	91.67	55			

Table (2): Assessment of tested plant extract and nanoparticle on selected biochemical parameters content for third instar larvae of *C. pipiens*.

Tested parameter	Control Mean±SD	*Achillea fragrantissima* Mean±SD	CuO nanoparticle Mean±SD	*P* value	*F* value
Total Protein mg/ml	0.83±0.02a	0.72±0.02b	0.49±0.01c	0.000	1460.36
Total Carbohydrate mg/ml	0.49±0.01a	0.41±0.01b	0.21±0.01c	0.000	2305.05
acetylcholinesterase activity U/L	7.22±0.02a	6.20±0.03b	4.93±0.03c	0.000	14919.38
glutathione S-transferase activity U/g tissue	0.85±0.01a	1.73±0.02b	0.63±0.02c	0.000	14529.61

Within same rows different letters meaning significant differences (P<0.05).

5. Conflicts of interest

There are no conflicts to declare.

6. Formatting of funding sources

No funding sources for this article.

7. Acknowledgments

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