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LARVICIDAL AND ADULTICIDAL EFFECT OF GREEN SYNTHESIS COPPER OXIDE NANOPARTICLES USING *ACHILLEA FRAGRANTISSIMA* AND ITS BIOLOGICAL AND ULTRA-STRUCTURAL IMPACT ON *CULEX PIPIENS*

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ABSTRACT. Chemical pesticides are often applied indiscriminately to control vectors, which led to development and unfavorable effects on beneficial organisms. Worldwide, phytochemicals and plant extracts have a multitude of medical uses and may be employed to remove a wide range of germs and pests. Environmentally friendly nanoparticle manufacturing and nanotechnology are the newest trends in a number of sectors. Utilizing a green method, *Achillea fragrantissima* extract (AcF) was employed to prepare copper oxide nanoparticles (CuONPs@AcF). Copper oxide nanoparticle was characterized using zeta potential, DLS and transmission electron microscope (TEM). During the study of the AcF extract effect against larvicidal and adulticidal, we found that the effect of AcF extract was less than CuONPs@AcF. Also, we studied the effect of AcF and CuONPs@AcF against the female mosquito's fertility. The effect of the tested substances on the female *Culex pipiens* ovary was improved by the ultrastructure.

KEY WORDS: Nanoparticles green synthesis, Copper oxide, Mosquito, *Achillea fragrantissima* extract

INTRODUCTION

According to Mehlhorn *et al*. and the World Health Organisation (WHO) [1, 2], mosquitoes are the arthropod carriers of several illnesses, such as filariasis, dengue, and malaria, which cause significant worldwide mortality and morbidity along with rising pesticide resistance. Because the diseases vector mosquitoes transmit have a devastating impact on communities' worldwide, public health depends on their quick and effective treatment. Insecticide resistance in vectors and hazards to humans and the environment have resulted from the use of chemical pesticides to stop mosquito-borne infections [3, 4]. However, because of their larvicidal, adulticidal, and repellant qualities, plant-based bio-pesticides such as plant extracts and essential oils are the recommended method of controlling vectors [5].

The rapidly developing and crucial discipline of nanotechnology allows for the introduction of particle forms with sizes ranging from 1 to 100 nm, employing a variety of manufacturing, modification, and strategy techniques. The majority of the chemical, physical, and biological properties as well as the individual atoms and molecules have been altered by this size range. Because of their completely new or improved size, distribution, and shape features, nanomaterials

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and nanoparticles indicated special applications that are expanding quickly on several fronts [6, 7]. Nanoparticles are primarily bio-synthesized by three main sources: bacteria, plants, and biomolecules. Natural ingredients present in plant and microbial extracts serve as stabilising and reducing agents during the environmentally friendly synthesis of nanoparticles. These helpful components make it possible to create nanoparticles from metal sources. Although these methods for generating biomolecules from microbes have advanced, the process of generating nanoparticles still confronts a number of difficulties. For instance, large-scale nanoparticle synthesis processes always involve a variety of technically and safely hazardous situations in addition to, the overall process rate is incredibly slow [8]. Given their ability to be created in larger quantities faster and at a cheaper cost of manufacture; plant extracts might be the best method for biosynthesising nanoparticles [9]. It is simple and clear to harvest plants from ecosystems indefinitely. They contain enough phytochemicals to replace expensive, very toxic and environmentally hazardous reducing reagents as sodium citrate, sodium borohydride ($NaBH_4$), and ascorbate [10]. 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+} [11-14]. During the creation of nanoparticles, other stabilising, capping, and chelating functions could also manifest. Moreover, it is simple to extract essential elements from various plant parts, which places the plant in the beginning phases of nanoparticle biosynthesis [15]. Determining the toxicity and effects of green synthesised Copper Oxide nanoparticles (CuONPs@AcF) based on *A. fragrantissima* ethanolic extract as larvicidal and adulticidal agents, as well as their impact on the biological and ultrastructural parameters of *Cx. pipiens*, has been the primary goal of the current study.

EXPERIMENTAL

Tested mosquitoes

Culex pipiens culture

Using *Culex pipiens* larvae from the Medical Entomology Institute, self-perpetuating colonies were developed and kept in the laboratory of the Entomology Department, Faculty of Science at Ain Shams University. Regulated conditions have been adopted temperature (27 ± 2 ºC), relative humidity (70% – 80%) and light–dark time (16:8 h), for raising mosquitoes. Toxicological investigations have been conducted on late third larval instars.

Preparation of plant extract

One hundred grams of the powdered plant material from *Achillea fragrantissima* were extracted using absolute ethanol. A Soxhlet device was used to carry out the extractions for 2 hours at 100 ºC (to guarantee superior outcomes, the extraction has been done three times in a row). The former step has been followed by evaporation of the absolute ethanol by using rotary evaporator (Labo-Rota C311) for two to three hours in an adjusted water bath at 40 ºC. The resulting crude extract has been weighed and stored in screw-capped vials at (-4 ºC) for later use [16].

Copper oxide nanoparticle synthesis (CuONPs@AcF)

The previously prepared *Achillea fragrantissima* plant extract has been added to 100 mL of distilled water and forcefully agitated at 80 ºC. Copper acetate 0.2 g was added dropwise to the mixture with similtanous stirring, which had been diluted in 20 mL of distilled water. A few drops of (0.5 M) NaOH were added to the solution combination after it had been heated for four hours to create an alkaline solution. The brownish black turbid solution has indicated CuO nanoparticle production.

Evaluation of copper oxide nanoparticle properties (CuONPs)

Transmission electron microscope

Transmission electron microscope used to examine the shape and size of the produced CuONPs "HR-TEM, JEOL-JEM-2100". Prior to analysis, diluted CuONPs@AcF suspension was stirred continuously for 60 min and sonicated in 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.

DLS, or dynamic light scattering, and zeta potential

The Zeta Sizer device utilised to quantify the zeta potential and/or particle size of the produced CuONPs@AcF "ZetasizerVer, Malvern Instruments Ltd., Nano-ZS, 704, UK". The suspension solution of copper oxide nanoparticles was first high-frequency scanned to ensure proper particle dispersion in the aqueous medium. Then the fragmented beam from the Brownian motion of the disseminated nanoparticles in solution was immediately detected using DLS.

Larvicidal activity

Different concentrations of CuONPs@AcF and ethanolic extract of "*Achillea fragrantissima"* were applied. The mortality data were recorded after 24 and 48 h; and analyzed by the probit analysis [17] to calculate LC_{50} and LC_{95}.

Reproductive potential of resulted females

Sub lethal concentrations of tested materials were applied on 3rd larval instar. The resulted female from these treatments and control were introduced to evaluate the following parameters.

Fecundity

Using an electric aspirator recommended by the World Health Organisation, the emerged adult females were collected, transferred and housed with normal adult males taken from the colony in wooden cages measuring 20 x 20 x 20 cm. The males and females were fed on a piece of cotton soaked in a 10% sugar solution for three days, followed by one day without sugar solution for one day. After five days, the hungry females were permitted to lay egg rafts on clean water (oviposition traps) in their cages and to have a blood meal from a pigeon. Using a binocular, the number of eggs in each egg raft was counted and the means value was then determined [18].

Hatchability of egg

El-Sheikh employed a technique to classify the eggs into two categories: hatched and non-hatched eggs [19]. Equation (1) was utilised to calculate the egg-hatchability.

$$\text{Egg-hatchability \%} = A / B \times 100 \quad (1)$$

where, A: total number of hatched eggs, B: total number of laideggs.

Index of sterility (SI)

The sterility was estimated using the formula % [18]

$$\text{Sterility percentage} = 100 - [a \times b / A \times B] \times 100 \quad (2)$$

where, a: quantity of eggs laid / amount of treated female, b: the proportion of treatment-group hatched eggs, A: quantity of eggs laid / dominating female, B: the proportion of control eggs that hatched. Data were analysed statistically using the approach that was taken from Lenner [7]. Multiple linear regressions were used to compute the LC_{50} [17, 20].

Transmission electron microscope studies (TEM) on adult mosquito

The ovary of adult *Cx. pipiens* previously treated with CuONPs@AcF, were observed using transmission electron microscopy (TEM) after 48 h of treatment. The adult, both treated and untreated, were prepared following the methodology of Disbrey and Rack [21]. Ten adults were selected for each treatment and were placed in a 1.5% (v/v) glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.2) for 48 h at 4 °C. The samples were dehydrated using ethanol solution and embedded in Araldite. The abdominal integument was examined using a light microscope after staining with toluidine blue, while ultrathin sections (0.1 µm) were stained with uranyl acetate and lead citrate before being analyzed microscopically using TEM (JEOL 1000, Japan) at the Electron Microscope Unit, Central Lab., National Research Center Giza, Egypt.

RESULTS DNA DISCUSSION

Synthesis of Achillea fragrantissima (AcF) extracts in-situ copper oxide nanoparticles (CuONPs@ AcF)

Achillea fragrantissima extract's phytochemical analysis reveals the presence of several distinct components. In particular, phenolic substances such as rhamnose, simple sugar, isothiocyanates, and glucosinolates are abundant in this type of plant [22, 23]. It has been discovered that while CuONPs@AcF are being prepared, *Achillea fragrantissima* extract possesses the right reducing and stabilising properties. While the glucosinolates have been oxidised to gluconic acid, the sugar has reduced the cation into CuO by acting as an aldehyde to produce CuONPs@AcF and stabilise [24]. Certain organic compounds with reductive groups like -OH, -SH, -NH, etc. can reduce the copper cation. Examples of specific organic compounds that may be isolated from *Achillea fragrantissima* are displayed in Figure 1 (pseduosolasodine diacetate **1**, quercetin **2**, methylinosine **3**, tanaphillin **4**, cosmosiin **5**, apressin **6**, and *p*-hydroxy-phenethylamine IV **7**) [23, 25]. The reaction's colour shift has been monitored during the production of CuONPs@AcF from Cu^{+} using *Achillea fragrantissima* extract.

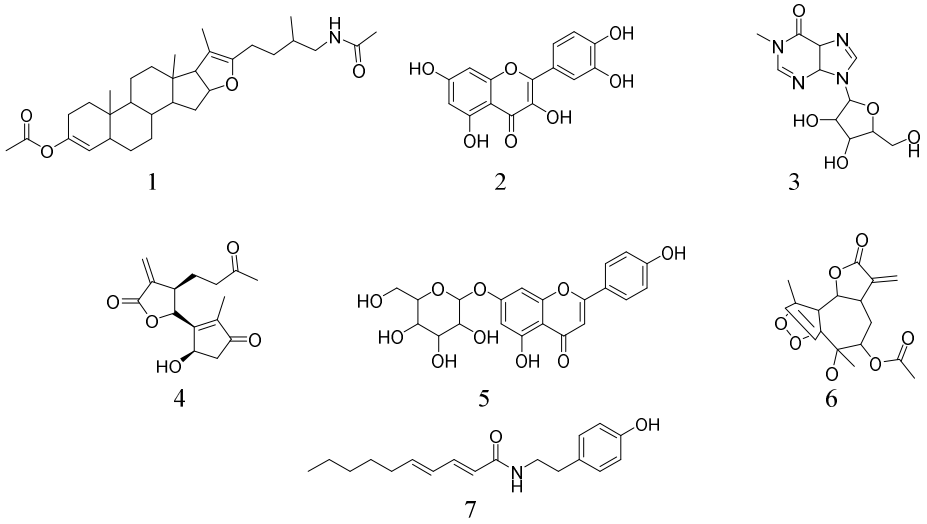

Figure 1. Structures of selected phytochemicals from *Achillea fragrantissima.*

Evaluation of CuO nanoparticle properties

Transmission electron microscope

Without exhibiting any indications of aggregation, the produced nanoparticles have dispersed throughout the aqueous solution, that may be because the plant extract's functional organic ingredient has stabilised them. The characterization of CuONPs@AcF colloidal nanoparticles was validated by transmission electron microscopy (TEM) distributed in an aqueous solution without the development of aggregations. Figure 2 indicated that copper oxide nanoparticles CuONPs@AcF obtained using these green methods have spherical shape morphology with average size from 4.61 to 6.97 nm. This finding suggests that the produced CuONPs@AcF were monodispersed and crystalline.

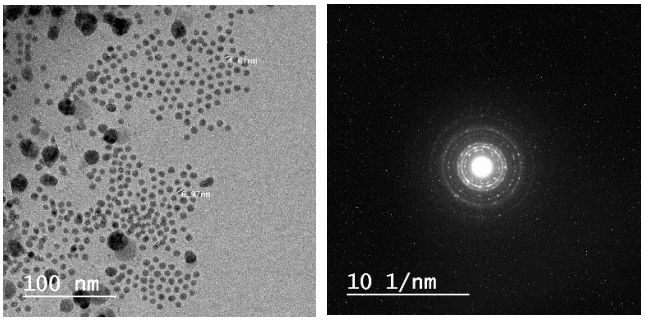

Figure 2. TEM of CuONPs@AcF.

Zeta potential and dynamic light scattering (DLS)

Dynamic light scattering (DLS) measurements were used to find the particle size of the produced CuONPs@AcF, as shown in Figure 3. According to the figure, the average particle size is 80.34 nm. Since TEM only introduces a picture for a limited field of view, DLS provides an overall image of the nanoparticles and their aggregations, accounting for the higher particle size obtained from DLS measurement compared to that received by TEM measurement. Zeta potential measures to be -29.9 mV suggesting that there is higher degree of stability in the electro-kinetic effect and surface charge present on the particle as in Table 1.

Table 1. Particle size and Zeta potential of CuONPs@AcF.

Compounds	MPS (nm)	PDI	SD	Zeta potential
CuONPs@AcF	80.34	0.58	±6.324	-29.9

Main particle size (MPS), polydispersity index (PDI).

Plant-mediated nano-fabrication is a new area of nanotechnology that is favoured over traditional methods due to its advantages in terms of safety, affordability, environmental friendliness, and biocompatibility. Plant extract was utilised in the current study to make the nanoparticles. One of the numerous natural surfactants utilised in the creation of green synthesis materials is plant extract [26] and it also includes a large number of substances with reactive functional groups, such as thiol, carboxyl, amino, and hydroxyl groups. These compounds stabilised the copper salts so they could float freely in the solution and assisted in reducing them to CuO nanoparticles. However, some particles clumped together to form clusters made up of dozens or even hundreds of distinct nanoparticles.

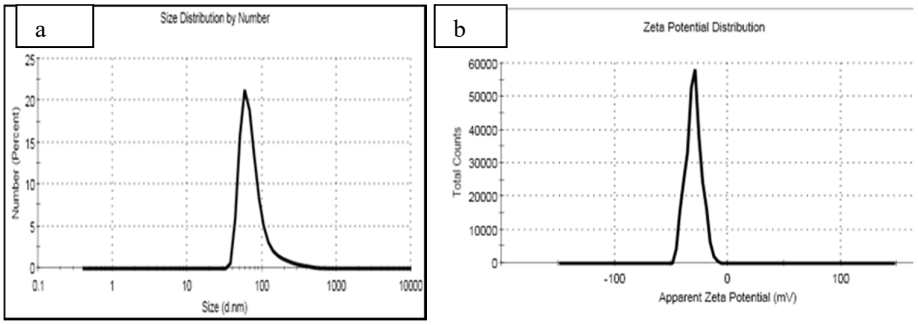

Figure 3. a) Particle size for CuONPs@AcF, b) Zeta potential for CuONPs@AcF.

Transmission electron microscope (TEM) studies for adult female mosquito

The cross-sectioned ovaries of the adult *Cx. pipiens* without treatment were photographed using a TEM microscope Figure 4, revealing a well-organised ovarian tissue structure. The ovary is made up of several follicles at various stages of development, each of having an oocyte encircled by follicular cells. The entire single layer of closely spaced follicular cells are encircled the oocyte. The apical surface of the cells has been enlarged microvilli, increasing the surface area available for interaction between the oocyte and its surroundings. The cells displayed a well-developed basal lamina on their basal surface. The presence of mitochondria, endoplasmic reticulum, and ribosomes in the cytoplasm of the cells suggested that metabolic activity was occurring. The cell nuclei were orientated towards the basal side and had an oval shape. The outer layer of yolk granules encircled the oocyte that displayed a distinct nucleus and nucleolus. Yolk granules were also present in the follicular cells and were transferred to the oocyte for development and expansion (Figure 4). In contrast to the untreated control, the ovarian structure of the group treated with CuONPs@AcF has shown notable changes. The borders of the follicular cells were uneven, and their microvilli were shortened and distorted, resulting in a smaller surface area available for exchange with the oocyte. The cytoplasm displayed notable alterations in the number and location of organelles, and the basal lamina was damaged. There were fewer mitochondria as an evidence of stress and swelling in the endoplasmic reticulum. Nuclear damage was evident from the follicular cells' asymmetrically formed and positioned nuclei. The oocytes displayed aberrant yolk granule buildup and a fractured nucleus, both of which were indicative of degeneration. When compared to the untreated, the yolk granules were significantly fewer in number and were not evenly distributed (Figure 5). In a similar vein, Farag and Kamel reported that the NPs utilised in both experiments had an impact on the cell content (ovary and midgut). This may be related to the NPs' minuscule size, which allows them to readily permeate the cell membrane and cause deformation inside it [36].

The ovary's follicular cells showed abnormal boundary patterns, shorter and malformed microvilli, a disturbed basal lamina, and notable alterations in the quantity and distribution of organelles. The oocytes displayed aberrant yolk granule buildup and a fractured nucleus, as an indication of degeneration. According to the findings, CuONPs@AcF may deform the ovarian tissues of *Cx. pipiens* larvae, perhaps leading to structural damage and disturbance of reproductive processes.

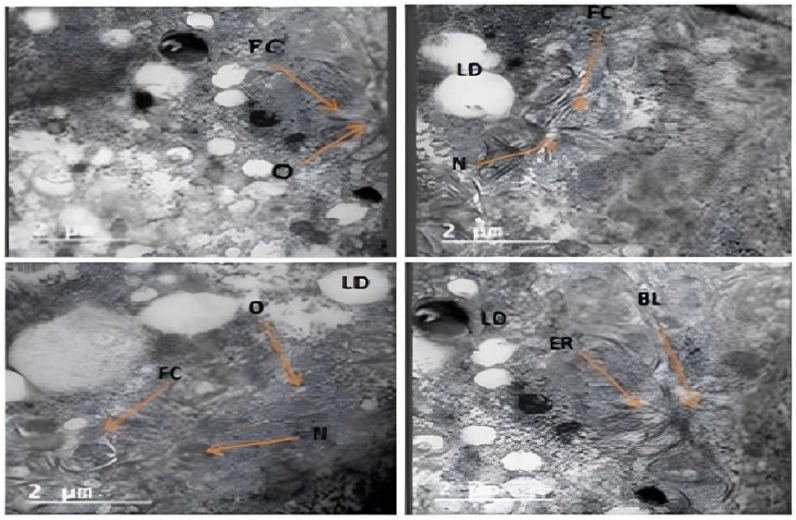

Figure 4. Transmission electron microscopy (TEM) image of the ovary of untreated *Cx. pipiens* adults showing ovary composed of several follicles at different developmental stages, each containing an oocyte (O) surrounded by follicular cells (FC) envolved by basal lamina (BL). The follicular cells were tightly packed together, the cytoplasm of the cells contained lipid droplets (LD) and endoplasmic reticulum (ER). The oocyte showed a distinct nucleus (N) surrounded by a layer of yolk granules.

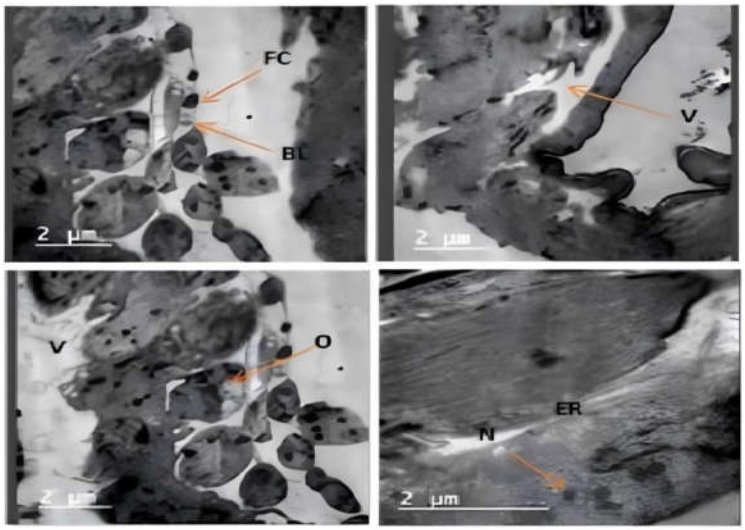

Figure 5. Transmission electron microscopy (TEM) image of the ovary of treated *Cx. pipiens* adult with CuONPs@AcF (x = 2 µm) showing follicular cells (FC) with irregular boundaries, disrupted basal lamina (BL), vacuoles (V) in the cytoplasm. Swollen endoplasmic reticulum (ER), irregular nuclei of the follicular cells (FC), degenerated oocytes (O) with a fragmented nucleus (N) and a significant reduction in the numbers and abnormal accumulation of yolk granules, compared to the untreated.

Activity of extracts and nanoparticles against larvae

Achillea fragrantissima extract and CuONPs@AcF were tested for their larvicidal potential against a chosen mosquito's third larval instar. Results in Table 2 indicated that the effect of the CuONPs@AcF was more potent than that of the examined extract. The LC_{50} and LC_{95} represented in Table 2, after 24 and 48 h after treatment by CuONPs@AcF was 1.13, 9.76 and 0.67, 3.82 and extract was 113.86, 553.18 and 76.53, 325.64 ppm, respectively. These findings suggest that CuONPs@AcF has more efficacy than the extract alone. Additionally, the investigated chemicals' efficacy varies according to the concentration and duration of application to the larvae. Because mosquitoes are susceptible to several plant ingredients, including sterols and terpenoids, plant-based insecticides are powerful substitutes for chemical insecticides [27]. Therefore, as opposed to chemical insecticides, employing plant extracts to control mosquitoes is a safer option for the environment. According to a literature search, *Achillea* species have been linked to flavonoids, terpenoids, lignans, amino acid derivatives, fatty acids, and alkamides including *p*-hydroxyphenethylamide [28-30]. Because they are inexpensive and safe for the environment, nanoparticles are being used more and more to control mosquitoes [31]. Currently, synthetic NPs made from botanical extracts are reasonably priced, and easy in application [32]. According to some research [33, 34], the primary toxicity mechanism against mosquito larvae may be caused by nanoparticles penetrating through the larval integument and affecting internal organs and organelles, enzymes, and cellular function, ultimately resulting in cell death. The outcomes of this investigation were consistent with the authors' use of additional NPs against *Culex pipiens* [35, 36].

Extract and nanoparticle's for adulticidal activities

Adulticidal activity of CuONPs@AcF and ethanolic extract of *Achillea fragrantissima* were evaluated against *Culex pipiens*. Table 3 shows the results of LC_{50} and LC_{95} for tested compounds in different intervals time. NPS has higher potency than extract, and its potency increases with concentration and exposure duration. The values of LC_{50} and LC_{95} after 24 and 48 h for CuONPs@AcF was 1.60, 11.19 and 1.00, 7.91 and for extract 134.13, 549.87 and 92.24, 452.48 ppm; respectively, as shown in Table 3. Approximately, the same result obtained from Al Thabiani (2023) starting that [39], good larvicidal mortality against *Aedes aegypti* (88.9%), *Anopheles stephensi* (84.1%), and *Culex quinquefasciatus* (81.6%) has been demonstrated by the *C. spongiosus* extract in the mosquitocidal assay. Furthermore, at 1000 ppm, the adulticidal death rates against *Cx. quinquefasciatus*, *Ae. aegypti*, and *An. stephensi* were 67.6%, 75.4%, and 78.8%, respectively. All of the earlier research concluded that the NPs based on botanical extracts had more efficacy than those containing just botanical extracts. The fact that nanoparticles are so tiny that they may more readily and swiftly infiltrate larval tissue and reach their targets with a high potency in terms of larval mortalities may help to explain this [31, 37-39].

The reproductive potential of emerged females

Table 4 shows how female fecundity and fertility in emerging females from treated larvae are adversely affected by the sub-lethal dose of *A. fragrantissima* ethanolic extract and CuONPs@AcF. Fecundity was significantly reduced after treatment with CuONPs@AcF and *A. fragrantissima* ethanolic extract compared to the group that did not receive therapy. Benelli (2015b) observed that AgNPs are known to impair protein synthesis and gonadotrophin release, resulting in developmental and reproductive failure [40]. This has an impact on the hatchability of eggs as well.

Table 2. Larval mortality percentage and coefficient limit for *Culex pipiens* treated with CuONPs@AcF and *Achillea fragrantissima* extract at different time intervals.

Conc. ppm	Time intervals and mortality % CuONPs@AcF		Conc. ppm	Time intervals and mortality % extract	
	24 h	48 h		24 h	48 h
Control	0.00	0.00	control	0.00	0.00
0.25	12.00	22.66	20	5.33	10.67
0.75	38.67	46.67	60	25.33	37.33
1.25	53.33	69.33	100	45.33	57.33
2.00	65.33	81.33	150	61.33	74.67
2.50	73.33	97.33	200	73.33	93.33
LC_{50} (co. limit)	1.13 (1.36-0.94)	0.67 (0.55-0.78)	LC_{50} (co. limit)	113.86 (98.89-131,10)	76.53 (66.75-87.71)
LC_{95} (co. limit)	9.76 (6.23-19.68)	3.82 (2.93-5.59)	LC_{95} (co. limit)	553.18 (364.13-842.83)	325.64 (245.23-433.32)
Slope	1.76 $\pm 4.6X10^{-2}$	$2.17\pm4.8X10^{-2}$	Slope	$2.4\pm8.63X10^{-2}$	$2.62\pm7.23X10^{-2}$

Table 3. Adult mortality percentage and coefficient limit for *Culex pipiens* treated with copper oxide nano particle and *Achillea fragrantissima* extract at different time intervals.

Conc. ppm	Time intervals and mortality % CuONPs@AcF		Conc. ppm	Time intervals and mortality % extract	
	24 h	48 h		24 h	48 h
Control	0.00	0.00	control	0.00	0.00
0.25	6.67	17.33	20	2.67	9.33
0.75	25.33	37.33	60	18.67	32.00
1.25	41.33	50.67	100	38.67	50.67
2.00	53.33	68	150	52.00	64.00
2.50	69.33	85.33	200	70.67	86.67
LC_{50} (co. limit)	1.60 (1.35-1.93)	1.00 (0.18-1.18)	LC_{50} (co. limit)	134.13 (117.32-153.39)	92.24 (80.13-106.17)
LC_{95} (co. limit)	11.19 (7.04-22.66)	7.91 (5.30-14.64)	LC_{95} (co. limit)	549.87 (359.43-843.10)	452.48 (314.98-651.80)
Slope	$1.94\pm5.82X10^{-2}$	$1.83\pm4.56X10^{-2}$	Slope	2.68±0.13	$2.38\pm7.15X10^{-2}$

Table 4. Effect of sub-lethal concentration of copper oxide nanoparticles and *Achillea fragrantissima* on fecundity, fertility and sterility index of female *Culex pipiens.*

Tested compound	No. of egg laid Mean±SD	No. of hatched egg		Sterility index
		Mean±SD	%	
Control	228.3±1.77	222.8±1.4	97.59	0.00
CuONPs@AcF	110.8±2.53	23.7±1.77	21.39	89.36
A. fragrantissima	201.9±1.85	53.3±1.42	26.40	76.08

CONCLUSION

From the present study, green synthesis of CuONPs, was carried out using *Achillea fragrantissima* ethanol extract and the nanoparticle was characterized using zeta potential, DLS and TEM methods. We studied the activity of the extract and CuONPs@AcF as anti-larvicidal and anti-adulticidal. In the light of observed results, the CuONPs@AcF was more potent than the extract

itself against the larvae or adult mosquito *Culex pipiens*. Aslo, CuONPs@AcF has the promising effect on the fecundity and ovary cells of the female mosquito make distinct damage which affects the fertility and egg hatchability. Finally, the author can concluded that the CuONPs@AcF may be used effectively in the vector management program alternate to the chemical insecticides.

Disclosure statement

No potential conflict of interest was reported by the authors.

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