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The Effect of Environmentally Safe Nanosynthesis with Copper Particles by using *Citrus aurantium* Fruit Extract Against Harmful Mosquitoes

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Abstract: The global agricultural sector suffers from the damage due to insect pests on plants all over the world, forcing farmers to indiscriminately use mineral fertilisers and chemical pesticides that are toxic and affecting the environment, plants and animals, as well as causing serious health problems to farmers and consumers. Therefore, scientists and researchers were forced to investigate and discover new methods until they explored the field of nanoscience, which shows great potential for applications in diverse fields, including agriculture, chemicals and plant protection by controlling harmful insect pests. Therefore, a biological method was used to synthesise the laboratory-prepared copper nanoparticles that were tested on both eggs and larvae phases in mosquitoes as nanocides using acidic Citrus Nargin broth (10%). A total of 1 mM aqueous $CuSO_4$ of the plant extract was reduced and synthesised into stable copper nanoparticles with an average size of less than 450 nm. Then, the composition, size and percentage of the synthesised nanoparticles were determined using ultraviolet spectroscopy, X-ray diffraction, FTIR, SEM and AFM techniques. The results of this study showed high mortality and a significant difference ($P \leq 0.01$) for mosquito eggs and larvae when exposed to the nanocide, than those found in the extract of bitter leaves and the regular copper sulfate insecticide used in agriculture against insects. The results also showed a high relative efficiency of 100% with a highly significant difference ($P \leq 0.01$) after exposure of insects and their different stages to the nanocide after 72 hours. We conclude from this study the possibility of synthesising nanoparticles using mineral compounds with natural, safe and environmentally friendly bio-plant extracts as insecticides for various agricultural pests.

Key words: CuNPs, mosquito insect, mortality, relative efficiency.

Introduction

Insect pests have a large impact on the destruction of various agricultural crops leading to excessive use of chemical fertilisers and toxic pesticides, which show negative effects on farmers, plants, animals, consumers, and crop production and the environment in total (Lade et al., 2017). The concept green chemistry focusses on utilising green natural plants as a starting source for the synthesis of nanoparticles, covered with pest-resistant secondary bio-metabolites, as a potential solution to eradicate insect pests in farms. This methodology resolves the issue of chemical residues, left by the usage of chemical fertilisers and toxic pesticides, which pollutes the environment and shows safe use by minimising the toxicity for both

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animals and plants (Mohd et al*.,* 2016). The extensive experiments and studies have proven the effectiveness of the strong effect of nanoscale synthesis by using different combinations of minerals and their salts. The availability of sophisticated and high-scale nanoscale devices to identify and measure the nano-atom sizes and their applications as the size of solid nanoparticles ranging from 1 to 100 nm (Malathi et al., 2014).

Copper nanoparticles (CuNPs) have been used in various applications in medicine, industrial engineering, environmental and various technological fields (Saranyaadevi et al., 2014). Researches have confirmed the influence of some simple factors related to plant economics (Bhupinder, 2014). Methods for bio-synthesis have wide and encouraging applications because they are inexpensive, safe, fast and environmentally friendly (Asim et al., 2014). Most studies have focussed on the use of nanoscience in agriculture for several factors including enhancing nutrient uptake, disease detection, and improving the delivery of nano composed insecticides to target sites of insect pests in affected plants, which in turn enhances biological understanding of agricultural crops (Umer et al., 2012). The mosquito insect (*Diptera: Culicidae*) is greatly harmful; it acts as a vector for parasites, and causes many diseases. Small mosquitoes are usually eliminated with the usage of organophosphates, insect growth regulators and microbial control agents by applying topical spraying as insecticides, however, such chemicals show highly negative effects on human health, animals and the environment (Giovanni, 2016).

Vector control is a very important requirement to curb diseases that lead to epidemic, and there is an urgent need to explore new and improved methods of mosquito control that are eco-friendly and safe (Naresh et al., 2012). Nargin leaves shows a composition of many compounds, such as linalool, some flavonoids such as poncirin, hesperidin, and nargin, and citric acid, including elements calcium, iron, phosphorous and vitamins A, B1, and C. The phytochemical components of Citrus Nargin have anti-toxin (Sah et al., 2011), antioxidant (Sastry et al., 2013)**,** and anti-pesticide properties (Naresh et al., 2012). The leaves can be used for important investigation to find modern methods in the manufacture of bio-nanopesticides using metals, including silver, copper, silica, etc. or metal oxides with high efficiency and wide range (Lida et al., 2016). Therefore, the present study is aimed to synthesise an environmentally friendly product, using copper nanoparticles with active bioactive components of Nargin leave extract against mosquitoes. The study also aimed to describe and identify the nanoparticles created for the citric Nargin leave extract with copper nanoparticles. In addition, the determination of the toxicity of copper nanoparticles to mosquito larvae, pupae and adults, is also targeted through this study.

Materials and Methods

Leaf Collection and Extract Preparation Nargins

Nargin leaves were collected from the house garden in the Baghdad city, washed with distilled water and dried (2 days/27±2°C) under the ceiling fan. The leave extracts were prepared from 5 gm of hand-cut leaves which were further cut into very small pieces and put in a beaker (300 ml) and 100 ml of distilled and demineralised water was poured in it. The mixture was boiled for 5 min. filtered and stored at -4°C until chemical analyses were carried out.

Mosquito Breeding

Mosquito eggs were collected from different locations (livestock tanks, temporary ponds, reservoirs, overhead tanks, trenches, etc.) in the Al-Mustansiriyah University area in Baghdad. Breeding and propagation were carried out by transportation and kept in the laboratory, placed in stainless steel trays size L × W × D was 18 × 13 × 4 cm (approximately with same number of eggs), respectively, containing 500 ml of water and incubated at 27 ± 2°C, humidity was kept 10 ± 5% and lighting period of 12 hr. The hatched larvae were fed (1 : 5) yeast and fish food, sterilised, and autoclaved at 121°C/1 atmosphere for 15 min. For pupae, 50 were placed in each container (40 × 90 × 40; door 10 × 10 cm) in 400 ml water. The hatched adult males were fed with cotton saturated with (10%) sugar solution in Petri dishes and the females were fed pigeon blood. After 2-3 days, the egg boats laid by the females were collected and transferred to the aforementioned larval rearing vessels (Zhang et al*.,* 2010). Also, glass Petri dishes lined with saturated (50 ml water) filter paper were placed inside the cages for the purpose of laying eggs by the mosquitoes**.**

Nanoparticles Toxicity

The percentage of pesticide activity was calculated by using the following equation (Capinera, 2011):

$$\text{Percentage mortality} = \frac{\text{Number of Dead larvae or pupae}}{\text{Number of larvae or pupae introduced}} \times 100$$

Preparation of Copper Nanoparticles

Nargin leaves broth (10 ml) was taken in a bowl and 1ml from 190 ml of aqueous copper sulfate ($CuSO_4$) solution was added to it gradually with constant stirring, a few drops of 1 ml of NaOH was added to the mixture for adjusting the pH to 8 in a water bath kept at 95° C, reflux (5 reflux) /10 min. was performed for 4 hrs, until the colour changed to dark black (Kerber, 2008). The copper nanoparticles was purified via centrifugation, the density was measured (UV-3600 Shimadzu spectrophotometer) with an accuracy of 1 nm (Asim et al., 2014). As shown in Figure 1, Scheme 1, CuSNPs (10 ml) were added to 90 ml of distilled water and 10% stock solution to prepare the concentrations of 100, 200 and 300 ppm, and then stored (-4°C).

Characterisation the Nanoparticles

The nanoparticles were characterised at the Nanotechnology Center, University of Technology.

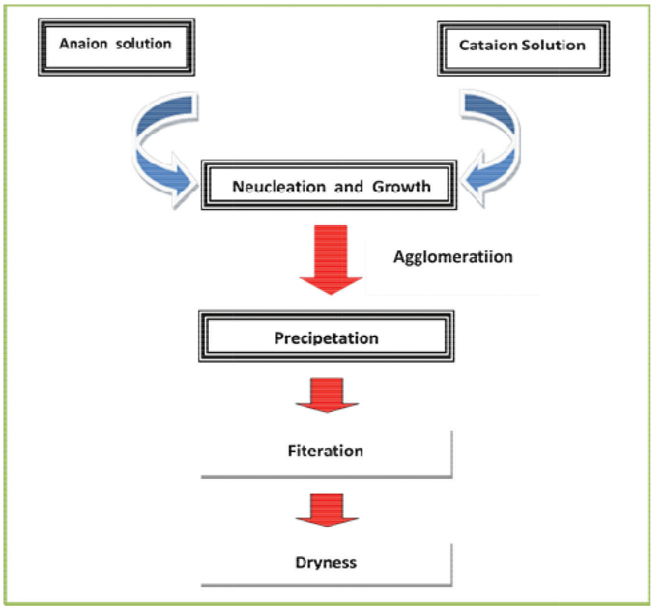
UV-Visible Spectrometry

Usually, the first visual observation of any chemical reaction is the initial detection for any colour changes; in this study a colour change from blue to reddish-brown then to dark brown was observed over time, indicating the synthesis of CuNPs. The presence and identification of copper nanoparticles were detected using UV/VIS spectroscopy in the reaction mixture with Shimadzu UV-1700, Japan, and the measuring accuracy of the 1 nm device was from 200 to 800 nm for the created sample.

X-ray Diffraction XRD Analysis

X-ray diffraction (XRD) analysis with Coca-radiation (PANanlytical X'pert Pro MPD) utilised preparing nano powder from Nargin leaves and determining the stages of the crystal structure and the size of the created copper nanoparticles using a Philips PW 1050/37 diffractometer

Figure 1: $CuSO_4$ nanoparticles.



Scheme 1: Formation of nanoparticles.

(generating 40 kV and current 30 mA, with a scan range of 100,000 to 20,000 degrees). Then, XRD patterns were recorded in the range of 0.12 °/ 0.02 L for 2 hours. A second of scanning velocity was used as a copper tube with a copper wavelength of 1.54 (Lida, 2016).

Infrared Spectrometer

The copper nanoparticles were recorded using Fourier transform infrared spectrometer (FTIR-8400S, SHIMAZW-FTIR), with a wavelength ranging from 500 to 4000 cm^{-1} (Matei et al., 2008).

Scanning Electron Microscope (SEM)

The nanoscale particles were measured using scanning electron microscope (TESCAN VEGA, USA) with an electrical voltage of 30kv under the conditions where the device is connected to a computed programme (software) to analyse the particle size.

Particle Size Distribution Analysis

Fast and accurate size distributions of iron nanoparticles (version 5.34) were analysed within a range of 6-2 nm, and dynamic light scattering at 90°, at a temperature from 5°C to 110ºC by using high power (35 mW) laser diode.

AFM Examination

A three-dimensional image was captured that showed the copper nanoparticles obtained using the deposition method.

Results and Discussion

Phytochemical Content

The results of the study represented the presence of active ingredients in abundance in the aqueous extract of Nargin leaves, as tabulated in Table 1 (Naresh et al., 2012).

Table 1: The active ingredients in the *Citrus naring* aqueous extraction

Results	*Test*
++++	Total phenol
+++	Alkaloids
++	Terpenoids
++	Tannin
+++	Protein
++	Carbohydrates
++	Steroids
++	Saponin

UV-Visible Spectroscopy

The relationship between wavelengths and absorption between 330 and 450 nm, and the peak at the wavelength 450 nm showed that the average size of the nanoparticles was 8.34 nm, also the evidence for the nanoparticles colour, which was changed from pale yellow to greenish black after chemical reaction of the copper salt, and the active component with prepared Nargin leave extract under suitable circumstance such as pH and heat, Figure 2 (Betancourt-Galindo et al., 2014; Prasad et al., 2014). Differences are observed in the absorption peak and the reason is attributed to the reaction time and the concentration of copper sulfate. The size of the particles decreased according to the high energy level, with the wavelength of 460 and 470 nm (Rai et al., 2006). On the other hand, the rate of reduction increases with the increase in the reaction temperature. Moreover, the absorption of copper nanoparticles depends on the size and shape of particles (Figure 3; Susa et al., 2003).

AFM Examination

It is a technique used to identify the three dimensions of the molecules, diagnosis and subsequent interpretation of the samples to be tested. In this study, it mostly indicates the homogeneity and agglomeration of the copper nanoparticles, as shown in Figure 4.

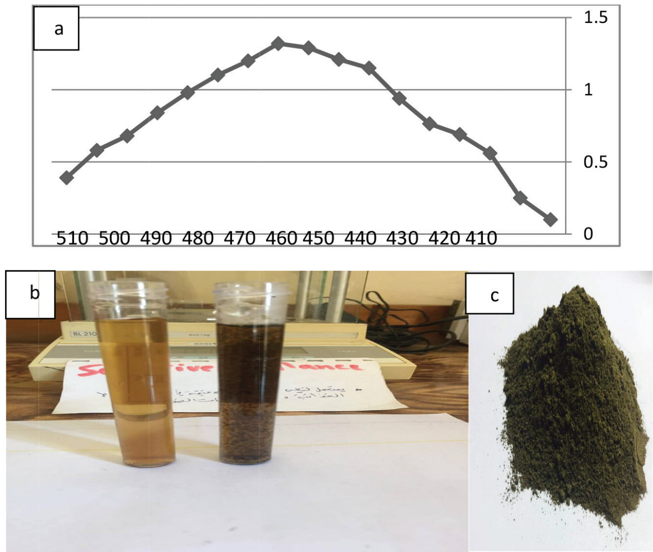


Figure 2: Ultraviolet light absorption of copper nanoparticles composed of copper sulphate and citrus Nargin leave broth: (a) examination of copper nanoparticles using UV-Visible rays and (b) the nanoparticles in the colloidal solution c-powder form the nanoparticles.

SEM examination

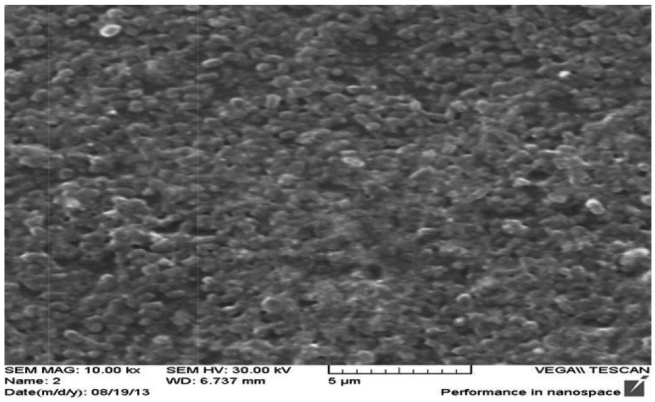


Figure 3: Scanning electron microscope (SEM) of synthesised copper nanoparticles with Nargin leaf broth.

FT-IR Characterisation

The FT-IR technique is utilised to find and characterise the bio-compounds and their functional groups present in the copper nanoparticles. The particle size decreased leading to an increase in the energy gap, and the absorption peak at a high level of energy. Naresh et al. (2012) used Nargin leaf extract in their study. The absorption of silver nanoparticles depends on the size and shape of the particles, as shown in Figure 5 (Rai et al., 2006).

The surface peak of the particles was observed at 440 nm (Raghunandan et al., 2010). Through FTIR

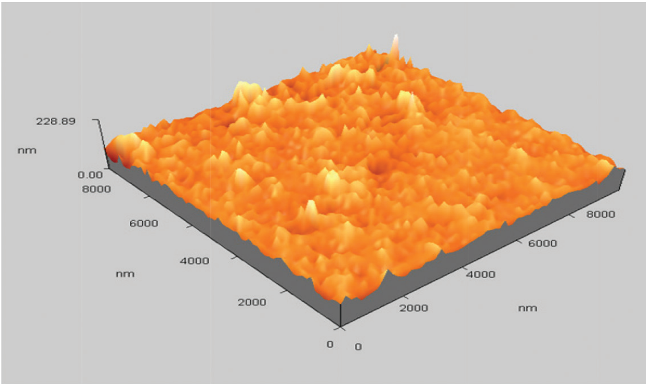


Figure 4a: AFM of synthesised copper nanoparticles with Nargin leaf broth.

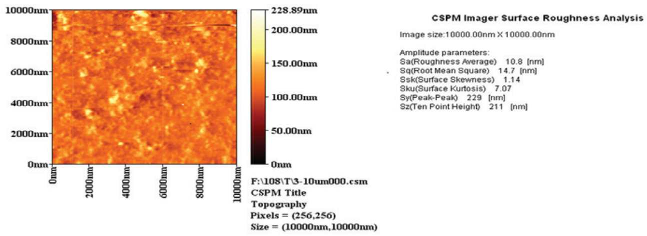


Figure 4b: AFM of synthesised copper nanoparticles with Nargin leaf extract.

analysis, copper nanoparticles with CuNPs are shown to be surrounded by a number of active organic element molecules such as polyphenols, alkaloids, and terpenoids, which are responsible for reducing copper ions to copper nanoparticles, due to their coverage and reduction of their capacity for free ions (Capinera, 2011).

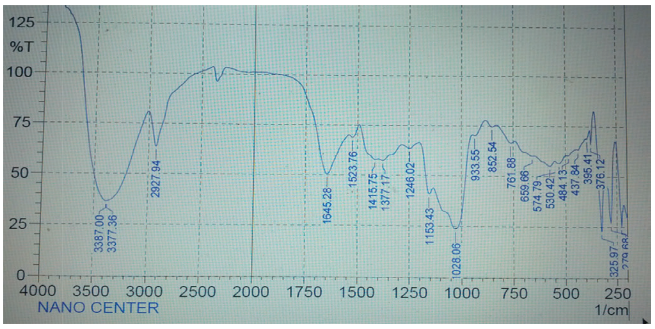


Figure 5: Infra-red light transmittance (FT-IR) of synthesised copper nanoparticles with Nargin leaf broth.

The X-ray Characterisation

The X-ray diffraction technique was performed to describe the morphological and size of the nanoparticles prepared with Nargin leave extracts, as shown in Figure 6. This analysis displays the diffraction peaks for the minutes prepared at 2θ = 38°, 50°, 65°, 78°, which refer to the (111), (020), (220), (311) planes according to Sastry et al. (2013) and Usman et al. (2012). The results of the XRD analysis suggests that there are wider peaks, which are less dense within the spectrum, and this is what represents the copper nanoparticles that were synthesised as nano crystals. Then, the peak amplitude was used to calculate the average size of the copper nano-crystals prepared with Citrus Nargin leaves using Scherer's formula following the study performed by Asim et al. (2014).

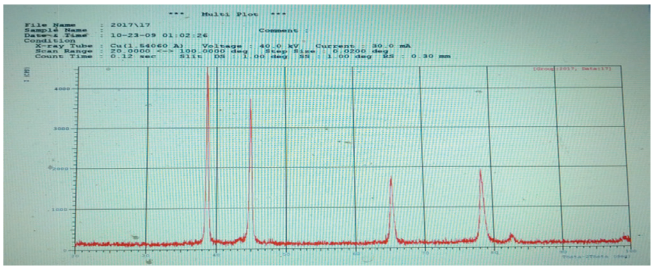


Figure 6: X-ray diffraction (XRD) pattern of synthesised copper nanoparticles with Nargin leaf broth.

Effect of Citruses Nargin Leaves Extract on the Growth Stage of Mosquito

The mortality ratio of the third, pupae and virgin stages of mosquito growth increased significantly by increasing the percentage of citruses Nargin leaves. The percentages of third phase mortality were 7.6, 18.8, and 26.8; also, the pupae phase mortality % were 3.3, 3.3, 6.4, while, the virgin phase mortality % were 3.4, 3.4, and 6.7/100, 200, 300 ppm of $CuSO_4$ respectively. These results were close to that obtained by Lade et al. (2017) (Figures 7-9).

The Fungicide Effect of Copper Sulfate on Mosquito's Growth Stages

The increase significantly prepared copper sulfate percentage. The third phase mortality % were 36.6,

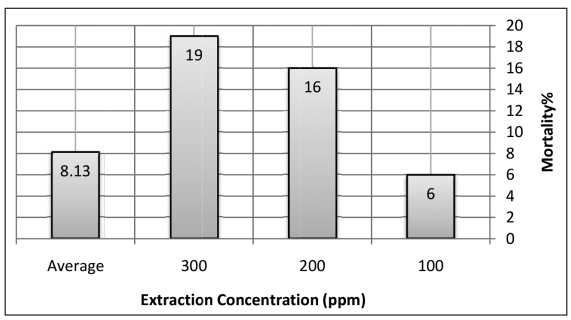


Figure 7: Effect of citruses Nargin leaves extract on the third stage of mosquitoes.

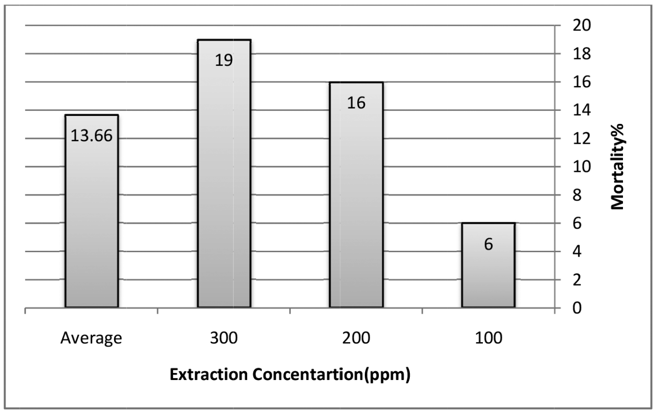


Figure 8: Effect of citruses Nargin leaves extract on the fourth stage of mosquitoes.

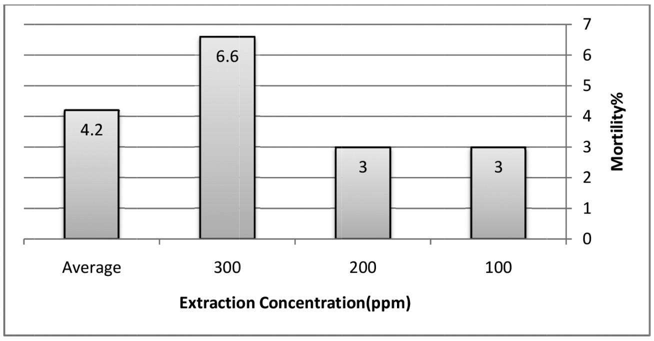


Figure 9: Effect of citruses Nargin leaves extract on virgin mosquitoes.

43.2, and 53.2. Also, the pupae phase mortality % were 49.9, 55.6 and 69.8, while the virgin phase mortality % decreased significantly to 6.6, 3.4, and 6.7 with 100, 200, and 300 ppm, respectively, that was relative to the difference in third, fourth and virginal stages of mosquitoes' membrane structures (Rao and Paria, 2013; Figures 10-12).

Effect of Prepared Nanocide (CuNPs) with Nargin Leaves Extract on Mosquito's Growth Stages

The mortality ratio of the third, pupae and virgin growth stages of mosquito increased significantly by increasing the percentage of the prepared copper salt extract. The third phase mortality % were (72.4, 78.2 and 91.3 at 100, 200 and 300 ppm concentrations of nanocide (CuNPs), respectively. Also, the pupae phase mortality % increased significantly to 100% with concentrations 100, 200 and 300 ppm, respectively, while the virgin phase mortality % declined significantly (*P*≤0.01) to 78.3, 83.4, and 90.4 at 100, 200, and 300 ppm, respectively (Mohamed et al., 2020; Vinutha et al., 2013; Figures 13-15).

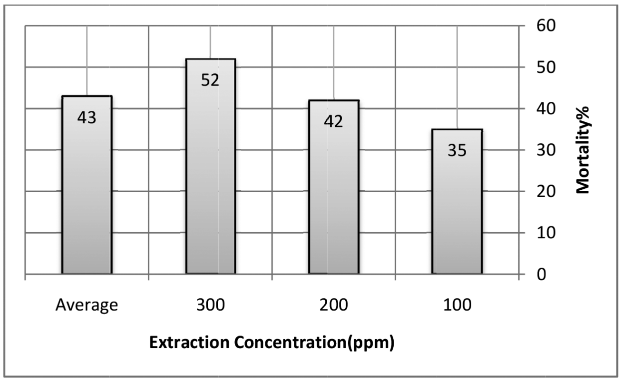


Figure 10: The results of copper sulfate on the third stage of mosquitoes.

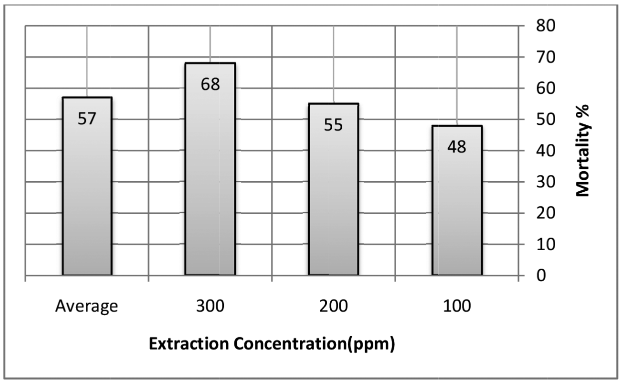


Figure 11: The results of copper sulfate on the fourth stage of mosquitoes.

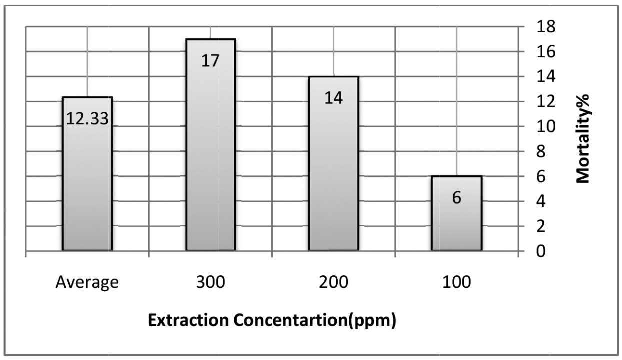


Figure 12: The results of copper sulfate on virgin mosquitoes.

The Relative Efficiency of Nanocide in Mosquito with Time Compared to Copper Salt and Nargin Leave Extract

The results of nanocide, copper salt and Nargin leave extract prepared in concentration of 100, 200, and 300 ppm were utilised, respectively, on mosquitoes mortality %, as shown in Table 2. In the field experiment, a marked increase was observed (*P*≤0.01) in mortality %, using copper nanoparticles (at concentrations of 100, 200, and 300 ppm); in pupae stage, it was

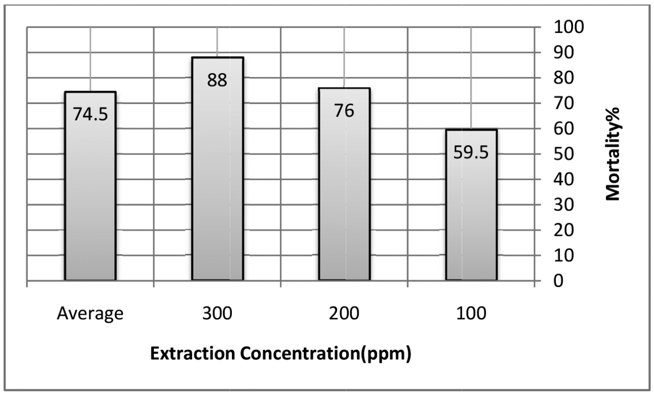


Figure 13: Effect of Nanocide (CuNPs) with Nargin leaf extract on the third phase of mosquitoes.

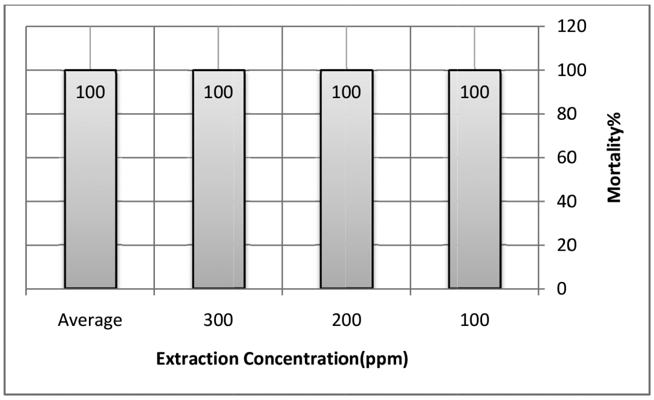


Figure 14: Effect of Nanocide (CuNPs) with Nargin leaf extract on the fourth stage of mosquitoes.

77.0, 88.0, and 98.0% ,while the mortality % using copper nanoparticles with same concentrations in egg stage were 12.8, 20.4, and 44.5% as compared with Nargin leave extract and regular copper salts, Table 2. The death rate of the egg and pupae stage increased significantly $P \leq 0.01$ by increasing the concentration of pesticide (Lade et al., 2017; Shiny et al., 2019). The high percentage of mosquito mortality may be due to

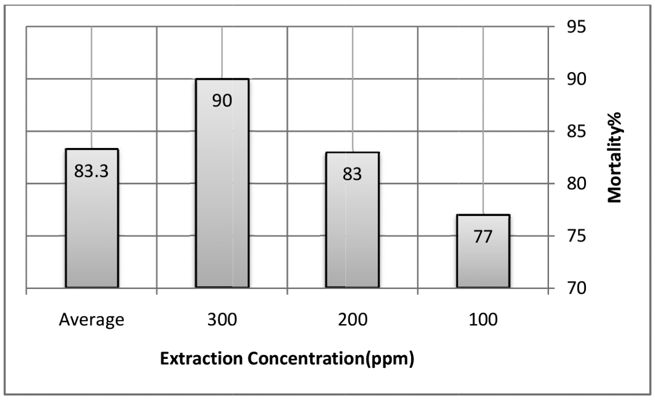


Figure 15: Effect of Nanocide (CuNPs) with Nargin leaf extract on the mosquito virgin.

the ability of the copper nanoparticles to penetrate or pass through the pores of the outer layer (Quitclaim) of mosquito skin and individual cells, where it interferes with the release of the cuticle and other physiological processes as recommended by Safaepour et al. (2009).

Relative Efficacy Control of Nanocide on Mosquitoes' Adult Stage Compared with Decis Insecticide 2.5% EC

The efficient control of Nanocide on mosquito stages compared to the Decis insecticide 2.5% EC after 24, 48, and 72 hrs is listed in Table 3, which also shows significantly high relative efficiency (P≤0.01) as compared to Nanocide extract. Nanocide results were 97.8, 98.2, and 100 % at 24, 48, and 72 hrs, respectively. While the relative efficiency of chemical Decis insecticide were 59.0, 48.5, and 40.2 at 24, 48, and 72 hrs, respectively. This is due to the differences in mosquito membrane structures. This result was similar to that obtained by Lade et al. (2017); Routray et al. (2016); Shiny et al. (2019), implying its adverse effects on human health.

Table 2: Effect of the pesticides used on the mortality % on the stages of the mosquito insect

*Phases and mortality rate of insect **	*Percentage of losses of different concentrations of pesticides*								
	Citrus Nargin leaf extract concentration			*Concentrations of regular copper salts*			*Concentrations of Nargin nanoparticle*		
	100	200	300	100	200	300	100	200	300
Egg	7.8	14.0	26.0	0.8	1.8	4.8	12.8	20.4	44.5
Death rate	15.93			2.47			25.9		
pupae	9.8	26.8	44.0	2.5	7.5	11.8	72.0	88.0	98.0
Death rate	27.0			7.3			86		

(***P***≤0.01) **;* = Ten plants for each replicate, for three treatments, as well as the comparison treatment**

Table 3: Relative efficacy of the nanocide in controlling the adult stage of mosquitoes on eggplant

Examination (hours)	*Nanocide*	*Decis insecticide 2.5% EC*
24	97.8	59.0
48	98.6	48.5
72	100	40.2
	($P \leq 0.01$)	

Conclusion

There is a possibility to use the Nano pesticide prepared by chemical reduction of copper sulfate, using Nargin leave extract. The shape of the nanoparticles, their size, and the arrangement of the active and functional groups have been distinguished using various advanced techniques (UV-visible UV spectroscopy devices, XRD, TEM and AFM). Different concentrations of copper nanoparticles with Nargin leaves were applied against mosquito activity in field experiments. The nanoparticles of the Nargin leave extract, showed a higher significant effect on the mortality rate of the mosquitoes in their third, fourth and virginal stages, in addition to its significant effect on the mortality rates of mosquitoes in the laboratory and the field. It is realised that the ready-made chemical pesticides obtained using green nanotechnology can be applied as Eco-friendly insecticide. The Nargin leave plant may be effectively utilised for the production of CuNps, which are economical in many pharmaceutical applications.

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