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## Green Synthesis of Copper Oxide Nanoparticle by Using *Achillea fragrantissima* and *Nigella sativa* Extracts and Their Effects as Larvicidal, Molluscicidal and Antimicrobial Agents.

Areej, J. Al-Ghabban and Jayda, G. Eldiasty

Biology Department, Faculty of Science, "University of Tabuk", Saudi Arabia

E-mail : [a\\_alghabban@ut.edu.sa](mailto:a_alghabban@ut.edu.sa)

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### ABSTRACT

The use of plant extracts and phytochemicals can be of great significance in therapeutic treatments, and control of many pests and microorganisms globally. Nanotechnology and green synthesis of the nanoparticle is the novel trend for use in different fields. In this study, Copper Oxide nanoparticle was synthesized by a green technique by using *Achillea fragrantissima* and *Nigella sativa* extracts. The Characterization of CuO nanoparticles was carried out by TEM and DLS techniques. The effect of nanoparticles as larvicidal, and molluscicidal is more potent than using extracts themselves. The values of LC50 of Cu nanoparticle and *Achillea fragrantissima* extract against *Pirenella conica* snails were 5.65 and 279.53 ppm respectively. The larvicidal effect against *Culex pipiens* mosquitoes LC50 values were, 0.98 and 79.69 ppm for Cu nanoparticle and *Achillea fragrantissima* extract, respectively. Also, CuONPs from both ethanol and petroleum ether extracts appear high inhibitory effect against all tested organisms compared to that of crude extracts. It is observed that CuONPs demonstrated a high degree of antibacterial activity for *S. aureus* and *P. aeruginosa* and showed activity against *C. albicans* fungus.

### INTRODUCTION

*Heterophyes heterophyes* in Egypt and many areas from the Far and Middle East is an endemic disease. Human infection with *Heterophyes heterophyes* was reported in Saudi Arabia. *Pirenella conica* is the intermediate host of *Heterophyes heterophyes* (Taraschewski and Paperna, 1981).

Mosquitoes are a common vector for human and animal diseases, causing severe economic loss and many cases of death around the world. Yellow fever, dengue and dengue hemorrhagic fever, malaria, Rift Valley fever and filariasis consider important diseases which transmit by mosquitoes at epidemic and endemic levels in different countries. Vector control is a very integral part of the current global strategy for the control of mosquito-borne diseases (WHO, 2010).

The extensive uses of chemical pesticides or insecticides are responsible for the development of resistance to these insecticides which rebound vectorial capacity. Plants may be alternative sources of mosquito control agents (Attiaa, 2002; Kamel, *et al.*, 2005; Pavela, 2009; Helmy, *et al.*, 2010; Bakr, *et al.*, 2010; Kamaraj, *et al.*, 2011; El-Maghraby, *et al.*, 2012; Eldiasty, *et al.*, 2014; Alghabban, *et al.*, 2015; Kamel, *et al.*, 2015 and Rudramurthy, *et al.*, 2016).

Recently, natural products have been evaluated as sources of antimicrobial agents with efficacies against a variety of microorganisms, so alternative strategies are sought that do not use antibiotics to reduce pathogenic bacteria and fungi from foods and patients. Plants have been known for thousands of years as a source of food and treatment for some diseases. Also, some native plant extracts from Tabuk were evaluated against some snails carrying intermediate parasitic stages (Kamaraj, *et al.*, 2011; El-Maghraby, *et al.*, 2012; Eldiasty, *et al.*, 2014; Alghabban, *et al.*, 2015 and Kamel, *et al.*, 2015).

Many studies were carried out to evaluate methanolic extract of some plants were collected randomly from the Kingdom of Saudi Arabia and their fractions by different solvents, petroleum ether, chloroform, ethyl acetate and aqueous against *Plasmodium falciparum*, *Trypanosoma brucei brucei*, *T. cruzi* and *Leishmania infantum*, as well as toxicity against MRC-5 fibroblast cells (Abdel-Sattar, *et al.*, 2010; Kamel, *et al.*, 2016 and Shokeri, *et al.*, 2016). Recent studies were innovating new antibiotics, that are cheap and take a long time to produce resistance by pathogens to these new antibiotics. This has led to an increasing interest in searching for effective alternatives for the current antibiotics with a different mode of action on microbes. Hence, medicinal plants appeared to be the best alternative source for new antimicrobial drugs (Abdallah, 2011).

Nanotechnology is a new and important field for introducing particle structures size ranging from approximately 1 to 100 nm, by different ways of synthesis, manipulation and strategy. Due to this size range the chemical, biological and physical properties were changed at the level of both individual atoms/molecules and their corresponding bulk. Novel applications of nanoparticles and nanomaterials are growing rapidly on various fronts due to their completely new or enhanced

properties based on size, distribution and morphology (Elghanian, *et al.*, 1997). Biomolecules, plants and microorganisms are the three major biosynthetic paths for nanoparticles. Natural compounds are available in the microbial extracts and plant act as stabilizing and reducing agents during the biogenic preparation of nanoparticles. These functional components allow for converting metal sources into nanoparticles (Dabhane, *et al.*, 2021). The process of producing nanoparticles still faces many challenges despite the successes in methods of producing biomolecules from microorganisms. For example, these methods necessarily require a series of technically and safely serious conditions, whereas the rate of the overall process is very slow to suit large-scale nanoparticle production (Ghotekar, *et al.*, 2021). Plant extracts may be the best method for the biosynthesis of nanoparticles which can produce nanoparticles in larger amounts within a short time with high efficiency and low production cost (Srikar, *et al.*, 2016). It is easy to collect plants from ecosystems permanently. They contain a number of phytochemicals that can replace highly toxic, expensive, and environmentally harmful chemical-reducing agents such as sodium citrate, sodium borohydride (NaBH<sub>4</sub>), and ascorbate (Ahmed, *et al.*, 2016). Indeed, many studies indicated that phytochemicals such as polysaccharides, flavonoids, phenolic acids, and quercetins in plant extracts are capable of excellently reducing metal ions, e.g., Ag<sup>+</sup>, Cu<sup>2+</sup>, and Au<sup>3+</sup> (Agarwa, *et al.*, 2017; Jadoun, *et al.*, 2021 and Ong *et al.*, 2018). In addition, during the formation of nanoparticles, various stabilizing, capping and chelating functions can appear. It is also easy to extra vital compounds from different parts of plants, this makes the plant in the first stages of the biosynthesis of nanoparticles (Beyene, *et al.*, 2017).

Recently, it has been noticed that many researchers are interested in using the green synthesis of copper nanoparticles,

which is due to the presence of a large number of biologically active compounds in the plant, whether in the whole plant or its parts, in addition to their applications in medicine and industries. Synthesis of Cu NPs has been successful with extracts of various parts of plant species (Khani, *et al.*, 2018 and Ahmed, *et al.*, 2019). On the other hand, it was found that synthetic CuO NPs can be used to repair the environment from various hazardous compounds and pollutants (Patel, and Bhattacharya, 2017), rhodamine B, and methylene blue (Bordbar, *et al.*, 2017).

Copper (Cu) is a vital element when combined with specific proteins and enzymes that have essential functions in the growth and nutrition of plants (Yruela, 2005). It is involved in cell wall metabolism, mitochondrial respiration, photosynthetic electron transport, oxidative stress responses, and hormone signaling (Da Costa, and Sharma, 2016). Copper can also cause enhanced production of bioactive compounds, reactive oxygen species (ROS) plant growth inhibition, and altered root systems (Feigl, *et al.*, 2013 and Mourato, *et al.*, 2015). Copper oxide nanoparticles (CuO-N) exhibit many biological activities such as antimicrobial activity, antioxidant properties, and cytotoxic activity against tumors and cancer cells (Fouda, *et al.*, 2020) CuONPs have been fabricated either extra- or intracellularly through different biological entities, such as bacterial, fungal, actinomycetes, algae, and plants. The harnessing of fungal species as reducing, capping, and stabilizing agents to fabricate NPs is most interesting, because of various secreted metabolites, high metal accumulation, and scalability (Cuevas, *et al.*, 2015).

The investigation on the synthesis of medicinal plant-mediated CuO NPs for multi-functionalized applications has received great momentum recently (Ananda Murthy, and Prakash, 2020). The green CuO NP (has been found to exhibit multifunctional applications

in photocatalysis, electrocatalysis, pollutant degradation, nanomedicine, drug therapy and catalysis (Ananda, *et al.*, 2018). As a result of finding diverse nanoforms of CuO nanostructures (nanocrystals, nano sticks, nanotubes, nanoflowers, nanosheets) versatile and useful properties as antifungals when incorporated as coatings in textiles have been demonstrated.

## MATERIALS AND METHODS

### 1. Tested Parasite (snail):

Laboratory-bred *Pirenella conica* (snails > 5 mm in length) were collected from brackish water areas around some localities of the Tabuk region (besides the Red sea shore) using a hand wire mesh scoop. They were brought to the laboratory and maintained in separate aquaria containing freshwater and aquatic plants. Snails that did not respond to gentle prodding with forceps were considered to be dead (Musee, *et al.*, 2010).

### 2. Tested Insect (mosquito):

Larvae of *Culex pipiens* were collected from different localities of Tabuk region Mosquitoes were reared under controlled conditions of temperature ( $27 \pm 2$  °C), relative humidity, R.H. (70%-80%) and light-dark period (16: 8 hrs.). Late third larval instars were used for toxicological studies.

### 3. Test Microorganisms and Culture Preparation:

Gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella sp*, *Proteus sp*, Gram-positive bacteria *Staphylococcus aureus* and the yeast *Candida albicans* were obtained from "Culture Collection of Antibiotic-Resistant Microbes (CCARM)" Military Hospital Tabuk. The bacterial strains were cultured on a nutrient agar (NA) medium at 37°C (Das, *et al.*, 2020a). The yeast strain *Candida albicans* has been maintained at 4 °C on Sabouraud Dextrose Agar (SDA) plates and sub-cultured at 25 °C in Sabouraud Dextrose Broth (SDB) before each experiment to ensure viability and purity (Bhowmick, *et al.*, 2019).

#### 4. The Plants:

The plants used in this study were the whole plant (*Achillea fragrantissima*) and the seeds of *Nigella sativa*.

##### 4.1. Preparation of Plant Extract:

Each plant was extracted with two solvents (Ethanol absolute and petroleum ether 60-80). A hundred grams of the resulting powdered materials of each plant were extracted with ethanol absolute and petroleum ether. The extractions were accomplished by means of a Soxhlet apparatus. The solvent extracts of each plant were evaporated and dried under vacuum using a rotary evaporator of a water bath adjusted at 60-70 °C. The resulting dry crude extracts were stored at 4 °C in screw-capped vials, until use.

##### 4.2. Synthesis of CuO Nanoparticles:

The plant extract (*Achillea fragrantissima*) was diluted using ethanol and dropwise added to distilled water under vigorous stirring at about 80 °C. Copper acetate (0.2 g) was dissolved in 20 ml distilled water and added dropwise to the extract solution under magnetic stirring. The solution mixture was left under heating for about 4 h. The pH of the solution was adjusted to alkaline using a few drops of NaOH (0.5 M). The solution turned turbid with the appearance of white color participated indicating the formation of CuO nanoparticles. In the case of *Nigella sativa* the solution did not turn turbid but was still clear with increasing viscosity.

##### 4.3. Characterization of CuO Nanoparticles:

###### Transmission Electron Microscope:

The particle size and shape of the prepared nanoparticles were evaluated using a transmission electron microscope (HR-TEM, JEOL-JEM-2100). The dilute CuO nanoparticles suspension was sonicated for 1 h in a sonication water bath prior to examination. The suspension was dropped onto the testing grid (one or two drops) and left for drying prior to investigation.

###### Dynamic Light Scattering (DLS):

The particle size and/or zeta

potential of the synthesized CuO nanoparticles can be estimated via the Zeta Sizer instrument (Nano-ZS, Malvern Instruments Ltd., Zetasizer Ver, 704, UK). The suspension of CuO nanoparticles was firstly sonicated to guarantee the good dispersion of these particles in aqueous media and then measured directly using DLS that measures the scattered beam resulting from the Brownian motion of dispersed nanoparticles on the solution.

#### 5. Bioassay for the Tested Materials:

##### 5.1. Molluscicidal Activity of Extracts and Nanoparticle:

A series of concentrations was prepared on the basis of volume/volume (Alghabban, *et al.*, 2015). Three replicates were used, each of ten snails (6 to 8 mm/L, for each concentration. Exposure and recovery periods were 24 h each; at  $25 \pm 1^\circ\text{C}$ . For each test, 3 replicates of control snails were maintained under the same experimental conditions in de-chlorinated water. The effectiveness of Artemether has been expressed as LC50 and LC95 (Kamel, *et al.*, 2015).

##### 5.2. Insecticidal Activity of Extracts and Nanoparticles:

Different concentrations from tested materials (plant extracts and nanoparticles) were applied. The mortality data were recorded after 48 hrs. and evaluated by a probit analysis (Finney, 1971) to calculate LC50 & LC95.

##### 5.3 Antimicrobial Assay:

The antimicrobial activity of the crude extracts was screened against the tested bacterial and fungal isolate by the agar well-diffusion method. Diluted solutions were prepared by diluting extract with DMSO for both alcohol and petroleum ether extracts. Muller-Hinton agar medium plates (prepared previously) were swabbed with 0.1 ml of bacterial and fungal suspension using sterile cotton swabs that were dipped in the suspension and streaked equally over the whole surface of the agar plate to obtain uniform inoculums. Holes were punched out from the Muller-Hinton agar using the opposite side of the sterilized

pasture pipette. 50  $\mu$ l of the crude extract was poured into respective wells with the help of a micropipette. The negative control was performed using dimethylsulfoxide (DMSO) and gentamycin, tetracycline & ampicillin were used as positive reference standards to determine the sensitivity of each microbial species tested. The treated plates were stored in a refrigerator at 4 °C for at least six hours to allow diffusion of the extracts into the agar while arresting the growth of the test microbes (Das, *et al.*, 2020a and Das, *et al.*, 2020b). The plates were then incubated for 24 hours at 37°C. Antimicrobial activity was determined by measuring the diameters of inhibition zones (DIZ) in mm. All tests were performed in triplicates and the developing inhibition zones were compared with those of the reference discs. The means and standard deviations ( $\pm$ SE) of (DIZ) were done.

## 6. Statistical Analysis of The Data:

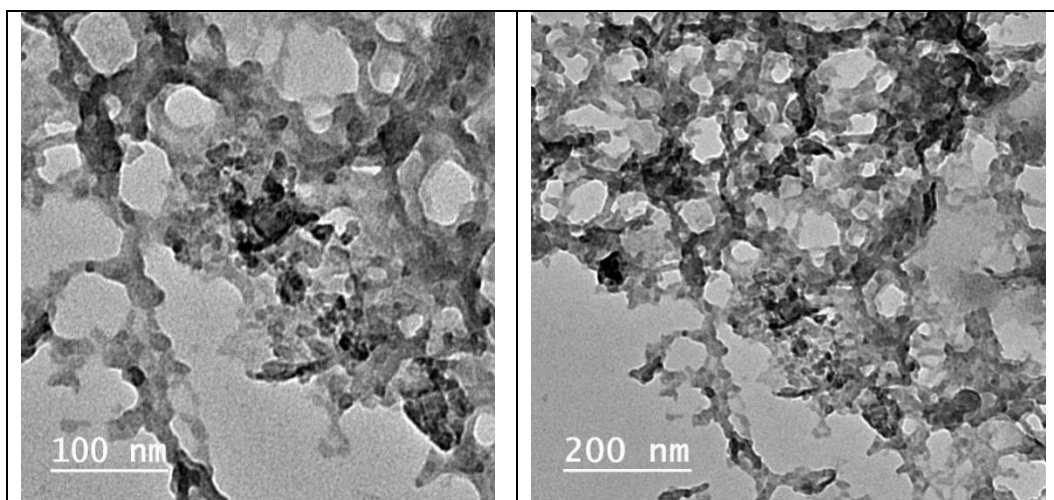
Multiple linear regressions were used to measure the LC50 (Finney, 1971). For the data obtained, the significance of the difference between means was determined using the students' *t-test*. The regression analysis was done using the least-squared method (Das, and Ghangrekar, 2019). Microsoft Excel was used for statistical purposes. Data were expressed as mean and standard error.

## RESULTS AND DISCUSSION

### 1.Characterization of CuO Nanoparticles:

#### 1.1. Transmission Electron Microscope:

TEM analysis was performed to know the morphological shape and size of the synthesized Cu NPs. From Figure (1), the particles found in semi-spherical shape within the range of 15–40 nm in size. This result indicates that the synthesized Cu NPs were crystalline and monodispersed.

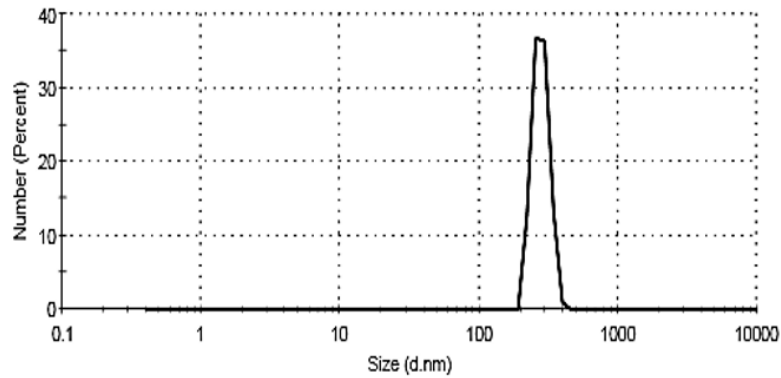


**Fig. 1:** TEM of CuO nanoparticles at different magnification.

#### 1.2. Dynamic Light Scattering (DLS)

The particle size of the synthesized CuO nanoparticles was determined by dynamic light scattering (DLS) measurements as shown in figure (2). The particle size indicated in the figure has an average of 219 nm. The larger particle size

obtained from DLS rather than that obtained by TEM measurement is that the TEM provides an image for a certain area for measurement while DLS provides an overall image for the nanoparticles and their aggregations.



**Fig. 2:** Particle size from DLS measurements for ZnO nanoparticles.

Plant-mediated nano-fabrication is an emerging field of nanotechnology which is preferred over conventional techniques because of its safety, cost-effectiveness, eco-friendliness and biocompatibility properties. In the present study, the nanoparticles were prepared by using plant extract. The plant extract is considered one of a variety of natural surfactants in the field of green synthesizing material (Shah, *et al.*, 2022) and also the extract also contains different compounds having reactive functional groups such as hydroxyl, amino, carboxyl and thiol groups (El-Sayed, *et al.*, 2020). These compounds participated in the reduction of copper salts into CuO nanoparticles and stabilized the particles to be suspended in the solution. However, some particles agglomerated into clusters that consist of tens, or hundreds of individual nanoparticles. The difference in the size of nanoparticles between TEM and DLS because the DLS gives a hydrodynamic radius of nanoparticles (hydrated particles) or coated particles in an aqueous solution, whereas, TEM provides the dried diameter of nanoparticles (Fan, *et al.*, 2012).

## 2. Molluscicidal Activity of Extracts and Nanoparticle:

The Molluscicidal activity for 4 extracts from *Achillea fragrantissima* and seeds of *Nigella sativa* was carried out against *Pirenella conica* snails. In general, the nanoparticle showed high potency than the effect of extract alone and no big difference in nanoparticles activity prepared

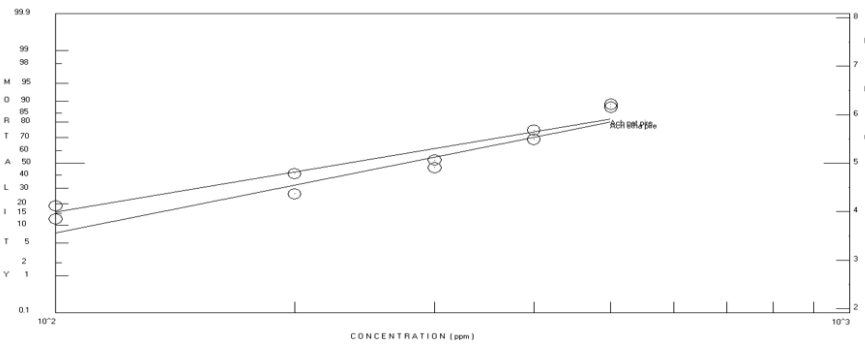
from both extracts as illustrated in Table (1). The values of LC50 of Cu nanoparticles from both extracts were 5.65 and 5.62 for pet-ether and ethanol, respectively. The pet-ether extract of *Achillea fragrantissima* showed low potency than the ethanolic extract as stated in Table (1). The coefficient limits and LC50, and LC95 after 48hr were recorded as shown in Table (1). The lowest value (i.e., high potency) of LC50 and LC95 were 233.32 and 921.40 ppm for pet-ether extracts of *Achillea fragrantissima*, respectively. On the other hand, the highest value (i.e. low potency) of LC50 and LC95 were 606.27 and 2191.62 ppm for ethanolic extracts of *Nigella sativa*, respectively. Both extracts of tested plants and Copper nanoparticles of *Achillea fragrantissima* showed the highest slope from all tested extracts as stated in Table (1) and Figures (3-5) which showed the regression lines of susceptibility for *Pirenella conica* snails to selected extracts of the present study. The present data showed that survival rates of adult *Pirenella conica* snails were markedly reduced which may arise from metabolic disorders as a result of saponine compounds present in the plant extracts or the type of solvent used. These results agree with (Saad, *et al.*, 2019 and Ibrahim & Ghoname, 2018). The improvement of the molluscicidal activity of *Anagalis arvensis* ethanolic extract was made using CuO NPs by forming plant nanocomposite (ACuO NC) against *Biomphalaria alexandrina* snails. In most recent studies, the toxicity of CuO NPs

increased when the rate of Cu<sup>++</sup> ions increased and bioavailability increased in solution (Aruoja, *et al.*, 2009 and Mortimer, 2010). The nanoparticle showed high potency than the effect of extract alone, the same conclusion was revealed by (Azzam, *et al.*, 2019) who stated that lupine extracts nanoparticles have more effect than copper sulphate nanoparticles on both aquatic or terrestrial snail mortality. Also, (Zayed, *et*

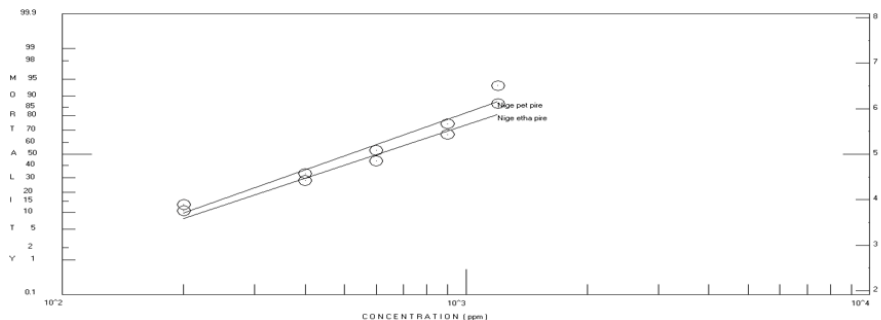
*al.*, 2021) demonstrate the molluscicidal effects of Ag-NPs against the three species of snails, including two species of intermediate snail host of *Schistosoma mansoni* (*Biomphalaria alexandrina* and *Biomphalaria glabrata*) and one species of intermediate snail host of *S. japonicum* (*Oncomelania hupensis*), and the cercaricidal effects of Ag-NPs against *Schistosoma mansoni* cercariae.

**Table 1:** Lethal concentration values of tested extracts and nanoparticles against *Pirenella conica* snails under laboratory conditions.

Tested materials	Solvent for extract	Lethal concentration by ppm (Coefficient limits)		Slop
		LC50	LC95	
<i>Achillea fragrantissima</i>	Pet-ether	233.32 (208.02 - 261.68)	921.4 (692.97 - 1227.86)	2.76±0.08
	Ethanol	279.53 (253.29 - 308.48)	921.52 (713.63 - 1190.83)	3.17±0.11
<i>Nigella sativa</i>	Pet-ether	515.89 (463.55 - 574.1)	1723.2 (1362.45 - 2180.81)	3.14±0.09
	Ethanol	606.27 (542.89 - 677.04)	2191.62 (1654.2 - 2905.85)	2.9±0.09
CuO nanoparticle	Pet-ether	5.65 (5.05 - 6.32)	14.00 (11.46 - 17.13)	4.18±0.24
	Ethanol	5.62 (5.00 - 6.30)	14.5 (11.74 - 17.99)	4.00±0.22

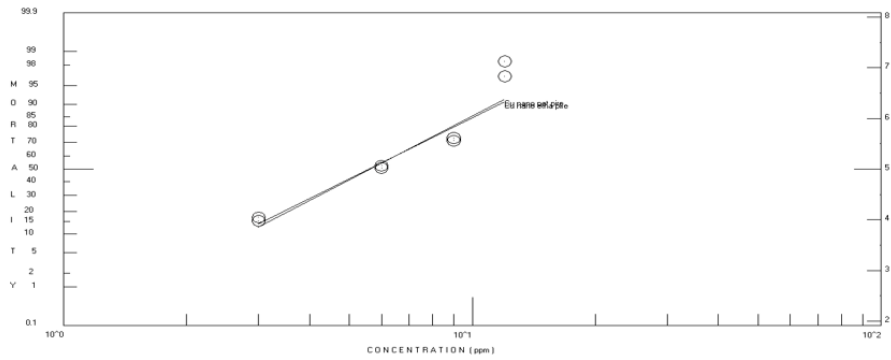


**Fig.3:** Susceptibility of *Pirenella conica* to ethanolic extract and pet ether of *Achillea fragrantissima*.



**Fig. (4):** Susceptibility of *Pirenella conica* to ethanolic extract and pet ether of *Nigella sativa*





**Fig. 5:** Susceptibility of *Pirenella conica* to CuO nanoparticle from the ethanolic extract and pet ether of *Achillea fragrantissima*.

### 3. Larvicidal Activity of Extracts and Nanoparticle:

The larvicidal activity of 4 extracts from *Achillea fragrantissima* and seeds of *Nigella sativa* was investigated against the third larval instar of the selected mosquito for this study. The results stated in Table (2) and Figures (6-8) clarify that the effect of CuO nanoparticle was high potency than the tested extracts. The LC50 of CuO nanoparticles were 0.98 and 1.04 ppm which were prepared by using ethanol and pet-ether respectively. The highest value of LC50 for the tested plant extract was 130.48 ppm for the ethanolic extract of *Nigella sativa*. The lowest value of LC50 was 58.91 ppm for the pet-ether extract of *Achillea fragrantissima*. Nanotechnology is now one of the most dynamic areas of study in many fields. Among mosquito species, *C. pipiens* has attracted much attention as it is the vector of many diseases. The management of *C. pipiens* using chemically synthesized insecticides has been unsuccessful because of resistance development, a resurgence of the vector, and environmental pollution (Aktar, *et al.*, 2009; Labbe, *et al.*, 2011 and Ozkara, *et al.*, 2016). The toxicity mechanisms of mosquito mortality on treatment with nanoparticle treatment were studied recently. So, hypothetically suggested that the mechanism of toxicity against mosquito larval is by the penetration of nanoparticles through the body. In intracellular space, nanoparticles degrade the enzymes and organelles, and it leads to the loss of cellular

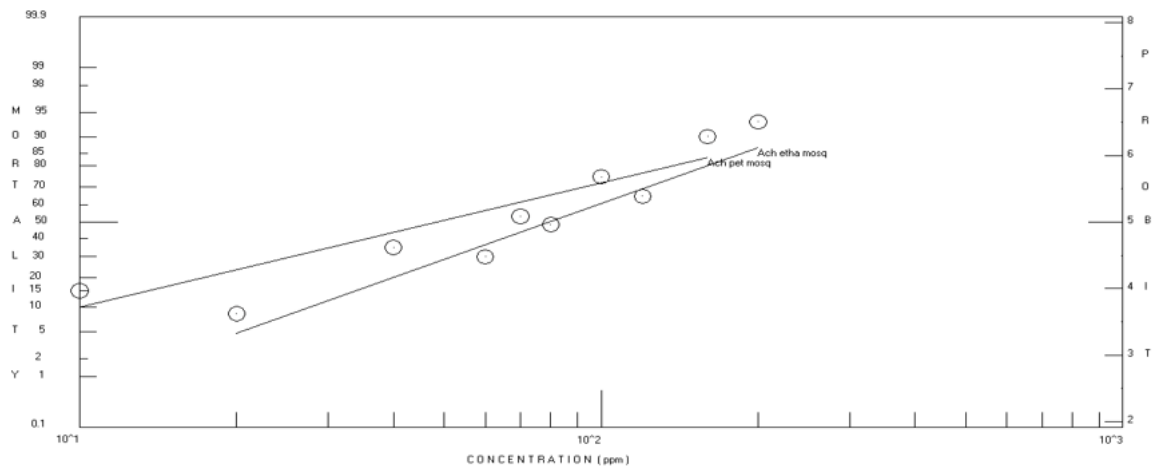
function and finally leads to cell death (Selvan, *et al.*, 2018 and Abinaya, *et al.*, 2019). The AgNPs from *Artemisia vulgaris* leaf extracts showed larvicidal activity. In the midgut of mosquito larvae, the nanoparticles will be accumulated and cause damage in the midgut, cortex region and epithelial cells. Histopathological studies of MnO<sub>2</sub>-treated larvae revealed the presence of damaged cells and tissues in the mid-gut. Similar work has been reported by (Hajra, *et al.*, 2016 and Anumathi, *et al.*, 2017) using Cadmium nanoparticles and ZnO nanoparticles respectively. Also, in seaweed-synthesized nanoparticles, many plant-synthesized nanoparticles showed considerable potency for mosquito larval control (Hajra, *et al.*, 2016 and Anumathi, *et al.*, 2017). The fruit pulp of *Cassia fistula*, *Nelumbo nucifera* and *Solanum tuberosum* synthesized AgNPs showed good larvicidal activity. Metal oxides have been scarcely explored for their larvicidal potential. The few available reports are restricted to research done in the past five years. The nanoparticles worked with include, ZnO, CuO, Bi<sub>2</sub>O<sub>3</sub>, MgO, TiO<sub>2</sub>, AgO, Fe<sub>2</sub>O<sub>3</sub>, and CeO<sub>2</sub> (Benelli, 2018). The synthesis of copper nanoparticles (CuNPs) using *Wrightia tinctoria* (Wt) R. Br extract showed also larvicidal activity against *Aedes aegypti* (Rajagopal, *et al.*, 2021). Finally, the nanoparticles prepared by using plant extracts were highly effective compared to the *Achillea fragrantissima* extract alone and also more affordable, as a smaller amount was required. These results

agree with (Alhag, *et al.*, 2021), who evaluated the effect of gold (AuNPs) and silver (AgNPs) nanoparticles synthesized

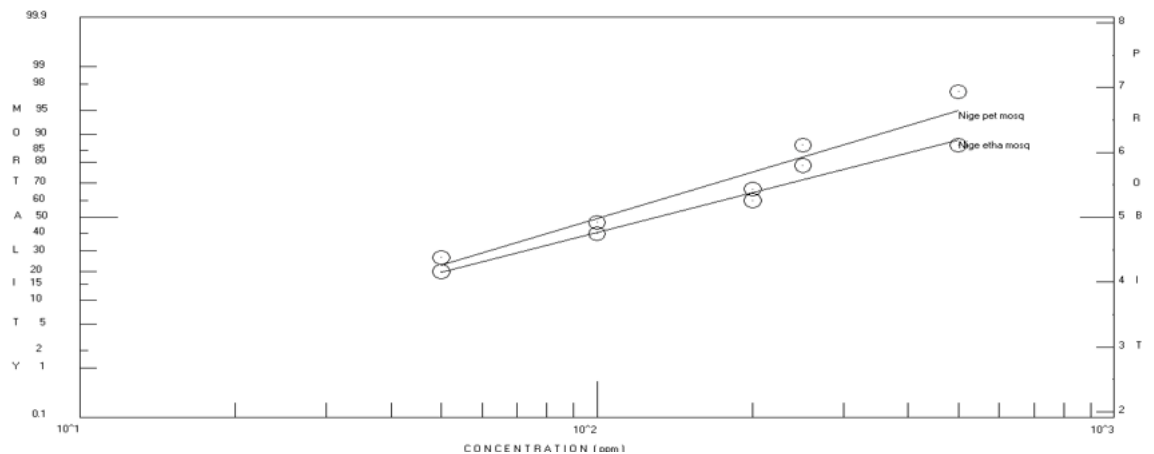
using *Acalypha fruticosa* leaf extracts to control the mosquito *Culex pipiens*.

**Table 2:** Lethal concentration values of tested extracts and nanoparticles against third larval instar of *Culex pipiens* under laboratory conditions.

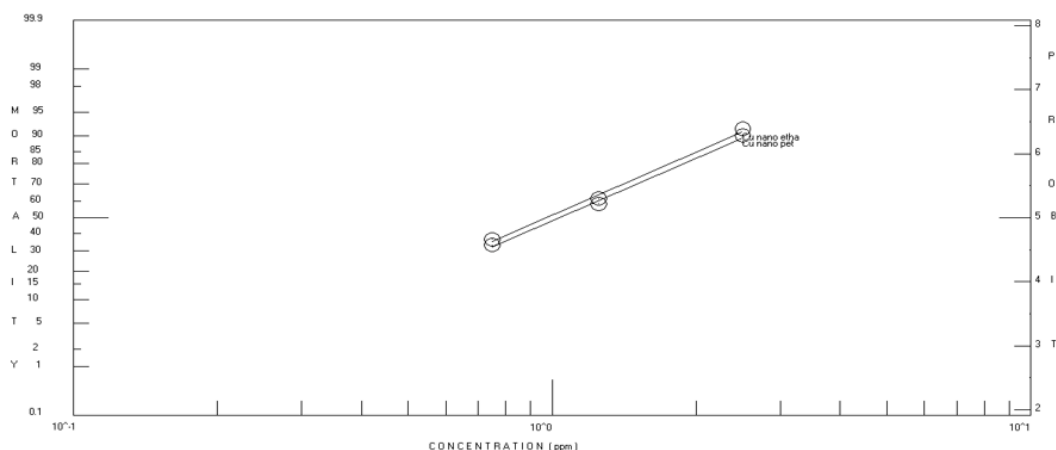
Tested materials	Solvent for extract	Lethal concentration by ppm (Coefficient limits)		Slop
		LC50	LC95	
<i>Achillea fragrantissima</i>	Pet-ether	58.91 (51.77 - 67.03)	226.18 (165.19 - 310.43)	2.82±0.11
	Ethanol	79.69 (69.71 - 91.09)	307.6 (224.53 - 422.38)	2.80±0.1
<i>Nigella sativa</i>	Pet-ether	102.24 (87.58 - 119.29)	498.74 (373.65 - 667.08)	2.39±0.05
	Ethanol	130.48 (110.94 - 153.39)	836.3 (566.39 - 1238.73)	2.04±0.04
CuO nanoparticle	Pet-ether	1.04 (0.88 - 1.20)	3.32 (2.5 - 5.33)	3.26±0.27
	Ethanol	0.98 (0.81 - 1.13)	3.11 (2.36 - 4.96)	3.27±0.29



**Fig.6:** Susceptibility of *Culex pipiens* to ethanolic extract and pet ether of *Achillea fragrantissima*.



**Fig. 7:** Susceptibility of *Culex pipiens* to ethanolic extract and pet ether of *Nigella sativa*



**Fig. 8:** Susceptibility of *Culex pipiens* to CuO nanoparticle from the ethanolic extract and petroleum ether of *Achillea fragrantissima*

### 3.4. Antimicrobial Studies:

The antimicrobial activity of the ethanolic and petroleum ether extracts of *Achillea fragrantissima* and *Nigella sativa* were determined against four tested gram-negative bacteria (*Escherichia coli*, *Klebsiella sp*, *Proteus sp* & *Pseudomonas aeruginosa*), one gram-positive bacteria (*Staphylococcus aureus*) and yeast (*Candida albicans*) using agar well diffusion method by determination of (DIZ values) at different concentrations 20, 50 and 100 mg/ml compared with the positive control, Gentamycine (10 µg), Ampicillin (10 µg), and Tetracycline (30 µ) and the results are given in Table (3). DMSO was used as negative controls not produced zones of inhibition. Depending on the diameter of the inhibition zone, the antimicrobial activity has been classified into four categories (antibacterial activity results have been expressed in mm): • ≤10 mm is considered as low activity, • >10 to 15 mm is considered as moderate activity, • >15 to 20 mm considered as strong activity and • >20 mm considered as extremely strong. Additionally, the antimicrobial activity of biosynthesized CuONPs from ethanolic and petroleum ether extracts of *Achillea fragrantissima* was assessed against tested organisms that were mentioned earlier in the same style as the extract alone.

#### 3.1. Antimicrobial Activity of Ethanolic and Petroleum Ether Extracts of *Nigella sativa*:

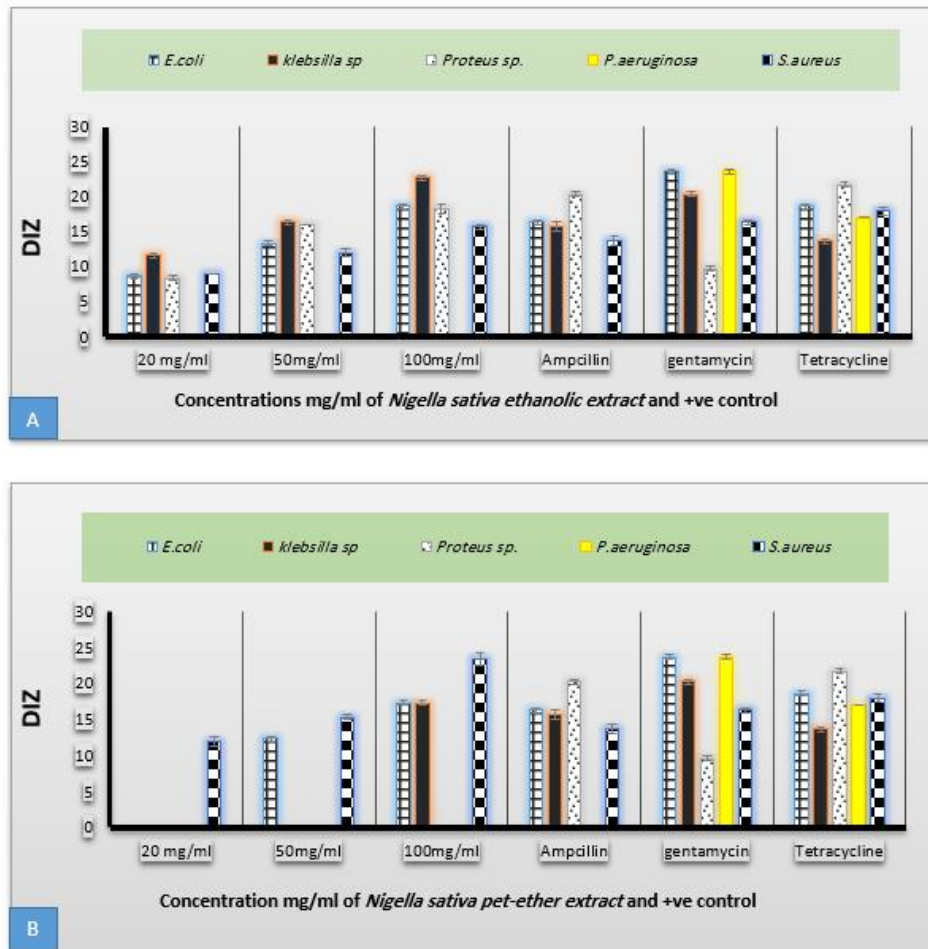
It was observed that the inhibitory effect of *Nigella sativa* on bacterial growth varied between ethanol and petroleum ether extraction as shown in Table (3) and Figure (9). It seems that ethanolic extract exhibited the highest inhibitory effect against *Klebsiella sp* with DIZ 22.6 mm at 100 mg/ml compared to positive control CN, AM & TC with DIZ 20.3, 15.6 & 13.6 mm respectively followed by the close result of inhibition of both *E. coli* and *Proteus sp* at concentrations between (20 – 100 mg/ml) with DIZ (8.6 – 18.6 mm) and (8.3 – 18.3 mm) respectively. As for *P. aeruginosa*, there is no effect. On the other side, the petroleum ether extract showed less antibacterial activity than the ethanolic extract on gram-negative bacteria where it had a moderate inhibitory effect towards *E. coli* and *Klebsiella sp* at a high concentration of 100 mg/ml with close DIZ of 17.3 mm and no antibacterial effect was detected against both *Proteus sp* & *P. aeruginosa* but regarding of *S. aureus*, the petroleum ether extract of *Nigella sativa* recorded strong activity with DIZ 23.3 mm compared to positive control AM, CN and TC with DIZ 13.6, 16.3 and 18 mm. The results regarding the antifungal effect of *C. albicans* showed a moderate effect of ethanolic extract and a weak effect of petroleum ether extract of *Nigella sativa* compared to the positive control. In this study, the antimicrobial activities of *Achillea fragrantissima* and *Nigella sativa*

crude extracts were studied in addition to the biosynthesis of copper oxide nanoparticles (CuONPs) from *Achillea fragrantissima* extracts against hospital pathogenic bacteria and fungi. The ethanol extract of *N. sativa* seeds showed better antimicrobial activities compared to that of petroleum ether extract. These results agreed with that obtained by (Kakil, 2013). In addition to similar antimicrobial activities of other plant extracts have been reported previously by (Sharmila, *et al.*,

2018; Naik, *et al.*, 2019; Sharmila, *et al.*, 2019 and Vidovix, *et al.*, 2019), and this is due to the presence of a broad spectrum of natural bioactive agents, *N. sativa* extracts. Furthermore, the antimicrobial activity of petroleum ether extract of *Nigella sativa* may be attributed to the presence of thymoquinone (Kahsai, 2002), thymohydroquinone (El-Fatraty, 1975), and thymol (Randhawa, and Al- Ghamdi, 2002) as all of which possess antimicrobial activity (Karapinar, and Aktug, 1987).

**Table 3:** Antimicrobial activity of the ethanolic and petroleum ether extracts of *Achillea fragrantissima* and *Nigella sativa*.

Microorganism		Zone of inhibition (mm $\pm$ se)							
		<i>Achillea fragrantissima</i>			<i>Nigella sativa</i>		Positive control		
		Conc. mg/ml	Ethanol	Pet-ether	Ethanol	Pet-ether	CN	AM	TC
G -ve	<i>E. coli</i>	20	0 $\pm$ 0.00	0 $\pm$ 0.00	8.6 $\pm$ 0.33	0 $\pm$ 0.00	23.6 $\pm$ 0.33	16.3 $\pm$ 0.33	18.6 $\pm$ 0.33
		50	0 $\pm$ 0.00	0 $\pm$ 0.00	13.3 $\pm$ 0.33	12.3 $\pm$ 0.33			
		100	0 $\pm$ 0.00	0 $\pm$ 0.00	18.6 $\pm$ 0.33	17.3 $\pm$ 0.33			
	<i>Klebsiella sp.</i>	20	0 $\pm$ 0.00	0 $\pm$ 0.00	11.6 $\pm$ 0.33	0 $\pm$ 0.00	20.3 $\pm$ 0.33	15.6 $\pm$ 0.66	13.6 $\pm$ 0.33
		50	0 $\pm$ 0.00	0 $\pm$ 0.00	16.3 $\pm$ 0.33	0 $\pm$ 0.00			
		100	0 $\pm$ 0.00	0 $\pm$ 0.00	22.6 $\pm$ 0.33	17.3 $\pm$ 0.33			
	<i>Proteus sp.</i>	20	0 $\pm$ 0.00	0 $\pm$ 0.00	8.3 $\pm$ 0.33	0 $\pm$ 0.00	9.6 $\pm$ 0.33	20.3 $\pm$ 0.33	21.6 $\pm$ 0.33
		50	0 $\pm$ 0.00	0 $\pm$ 0.00	16 $\pm$ 0.00	0 $\pm$ 0.00			
		100	9 $\pm$ 0.00	0 $\pm$ 0.00	18.3 $\pm$ 0.66	0 $\pm$ 0.00			
	<i>P. aeruginosa</i>	20	16.6 $\pm$ 0.33	12.3 $\pm$ 0.66	0 $\pm$ 0.00	0 $\pm$ 0.00	23.6 $\pm$ 0.33	0 $\pm$ 0.00	17 $\pm$ 0.00
		50	20.3 $\pm$ 0.33	14.6 $\pm$ 0.33	0 $\pm$ 0.00	0 $\pm$ 0.00			
		100	25 $\pm$ 0.00	19.6 $\pm$ 0.33	0 $\pm$ 0.00	0 $\pm$ 0.00			
G+ve	<i>S. aureus</i>	20	16.6 $\pm$ 0.33	12.3 $\pm$ 0.33	9 $\pm$ 0.00	12 $\pm$ 0.57	16.3 $\pm$ 0.33	13.6 $\pm$ 0.66	18 $\pm$ 0.57
		50	22 $\pm$ 0.57	15 $\pm$ 0.00	12 $\pm$ 0.57	15.3 $\pm$ 0.33			
		100	29.3 $\pm$ 0.33	16.6 $\pm$ 0.33	15.6 $\pm$ 0.33	23.3 $\pm$ 0.88			
Fungus	<i>C. albicans</i>	20	11.3 $\pm$ 0.33	0 $\pm$ 0.00	11.3 $\pm$ 0.66	6.6 $\pm$ 0.33	18 $\pm$ 0.57	15.3 $\pm$ 0.33	14 $\pm$ 0.57
		50	21 $\pm$ 0.57	0 $\pm$ 0.00	14.6 $\pm$ 0.33	9.3 $\pm$ 0.33			
		100	24 $\pm$ 0.57	15 $\pm$ 0.00	17.3 $\pm$ 0.33	11.6 $\pm$ 0.33			



**Fig. 9:** Antimicrobial activity of the (A) ethanolic and (B) petroleum ether extracts of *Nigella sativa*.

### 3.3.2. Antimicrobial Activity of Crude Extracts and CuONPs from *Achillea fragrantissima*:

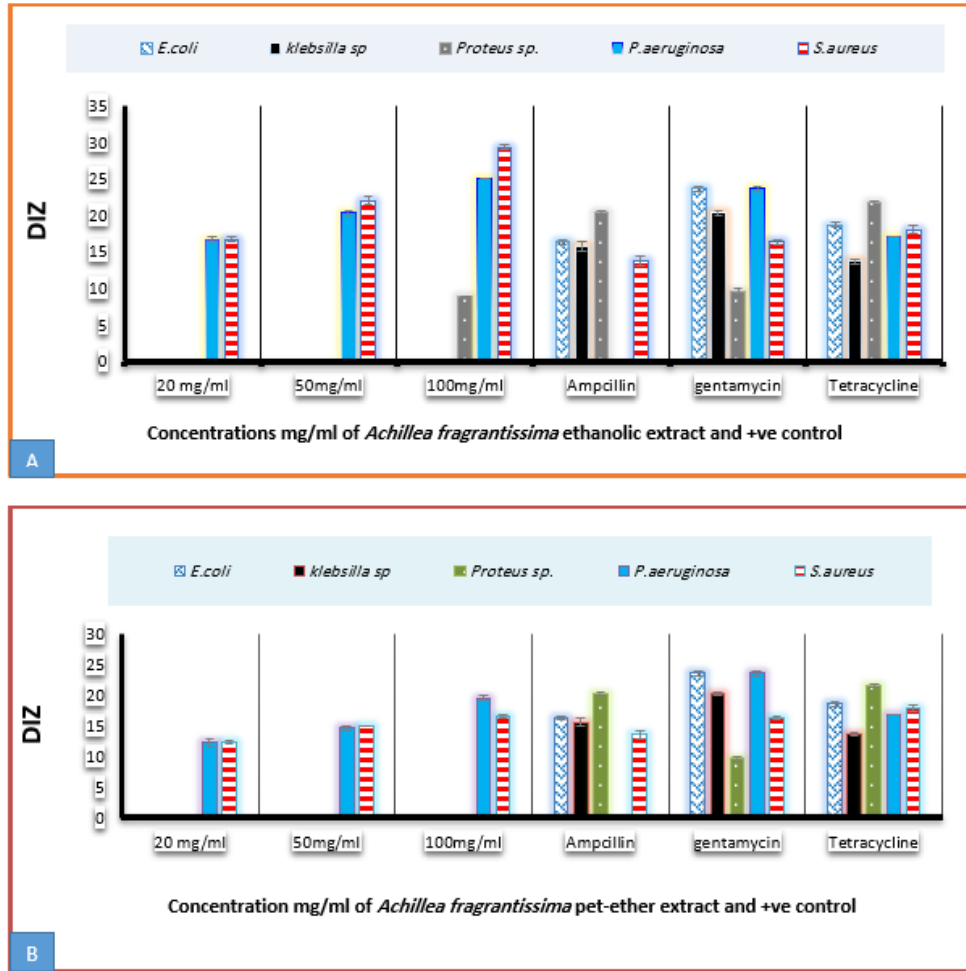
The evaluation of the antimicrobial effect *Achillea fragrantissima* crude extracts is presented in Table (3) and figure (10). It has appeared that both ethanol and petroleum ether extracts had no effect on the most of tested bacteria except *S. aureus* and *P. aeruginosa* where the efficiency of the antibacterial increased when increasing the extract concentration where the high inhibitory effect was observed using ethanolic extract with DIZ 29.3 and 25 mm followed by 16.6 and 19.6 mm at concentration 100 mg/ml of petroleum ether extract against tow bacterial strains respectively. The antifungal activity of ethanolic and petroleum ether extracts is shown in Table (3) and Figure (12). The high inhibitory effect was observed with

ethanolic extract of *Achillea fragrantissima* in concentration between (20 – 100 mg/ml) with DIZ values of (11.3 – 24 mm) whereas the antifungal activity by the petroleum ether extract was recorded at high concentration only (100 mg/ml) with DIZ 15 mm. Figures (11), (13) & Table (4) show the antimicrobial activity of different concentrations of CuONPs against five pathogenic bacteria and yeast (*Candida albicans*). It has appeared that CuONPs from both ethanol and petroleum ether extracts exhibited an obvious effect against all tested organisms compared to those crude extracts. It is observed that CuONPs demonstrated a high degree of antibacterial activity for *S. aureus* and *P. aeruginosa* with a zone of inhibitions from (17.6 to 31.6 mm) and (16 to 27.6 mm) in diameter respectively from ethanolic extract and zone of inhibitions from (14 to 24.6 mm)

and (9.6 to 17.3 mm) in diameter respectively from petroleum ether extract. Whereas, in the case of *Escherichia coli*, *Klebsiella sp* & *Proteus sp*, moderate activity is observed for CuONPs. The antifungal activity of CuONPs *Achillea fragrantissima* against the *C. albicans* was shown in Fig. (13). At all concentrations, the CuONPs from both extracts were active against *C. albicans* where the largest zone of inhibition 26.3 mm in diameter was exhibited by CuONPs ethanolic extract at 100 mg/ml in addition to good sensitivity by CuONPs petroleum ether extract with DIZ 21 mm. The obtained results from the effect of *Achillea fragrantissima* extracts appeared that ethanolic extract was found to be more effective on tested organisms than Petroleum ether extract, which is in conformity with earlier studies and also had the highest potency against gram-positive bacteria (*S. aureus*) than other gram-negative bacteria and this observation was consistent with the result of (Hazem, *et al.*, 2012) who concluded that The growth of *S. aureus* was inhibited very effectively by *A. fragrantissima* and this activity might be due to the presence of sesquiterpene lactones in their fractions and ethanol soluble fraction yields terpenoid which showed excellent activity against *S. aureus*. Additionally, our results are in agreement with (Sharma, *et al.*, 2010 and Prasad, 2014) who recorded that *A. fragrantissima* plant

extracts exerted a bactericidal effect on several gram-positive and gram-negative bacterial strains, as well as on *C. albicans*.

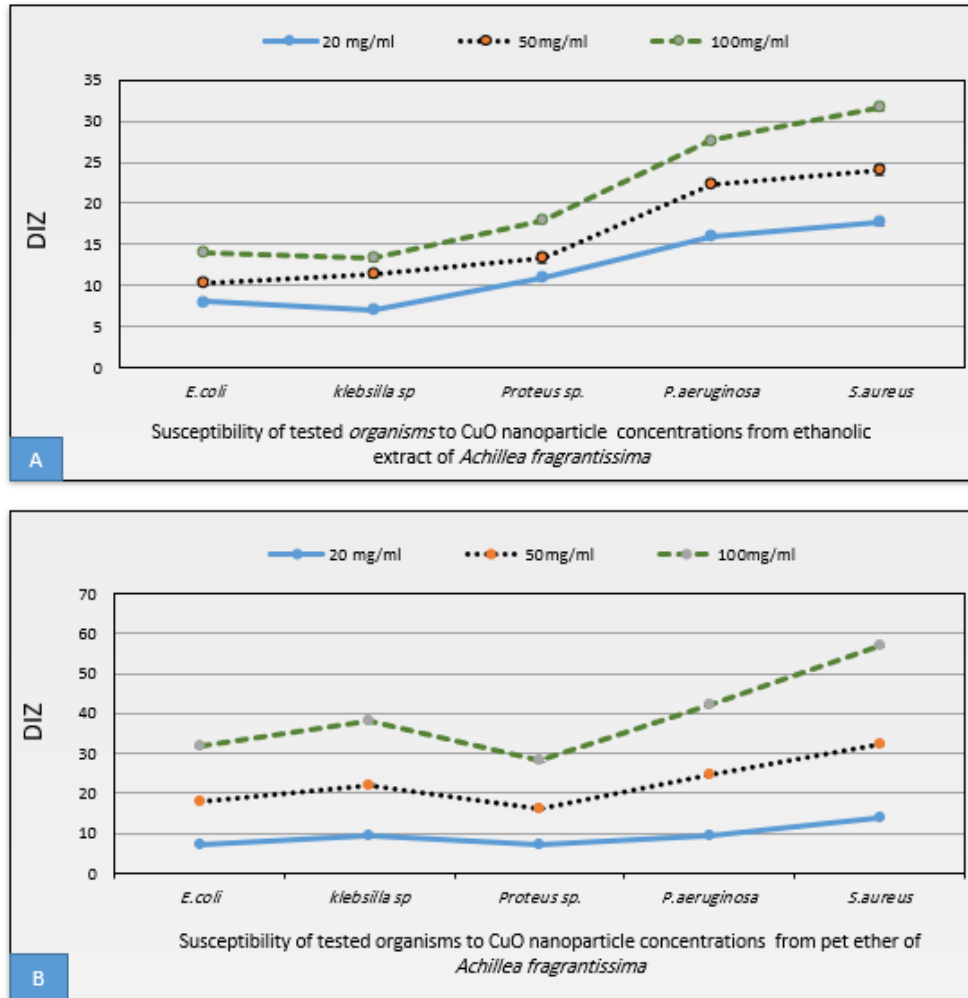
In this study, the antimicrobial activity of CuONPs recorded that all types of hospital bacterial and fungal isolates showed sensitivity to CuO nanoparticles from ethanolic and pet ether extracts of *Achillea fragrantissima*. The antimicrobial activity was also tested for different concentrations of the CuONPs and found increased activity with the increase of concentration. The antimicrobial activity of metallic nanoparticles has proven to be highly effective (Singh, *et al.*, 2020). As a result, the nanoparticles target many of the biomolecules in the bacteria, increasing the stress on the bacterial cells to fend off their resistance. Moreover, nanoparticles have outstanding properties such as small particle size, good mechanical stability, and large surface area and are suitable for clinical applications as antibacterial (Chen, *et al.*, 2020; Sharma, *et al.*, 2020 and Yin, *et al.*, 2020). It was suggested that nanoparticles through the metabolic pathway may contact cell walls and membranes of bacteria. Then it binds to components that play the active role of bacteria cells such as deoxyribonucleic acid, ribosomes and enzymes (Rajeshkumar, & Bharath, 2017 and Kumar, *et al.*, 2020).



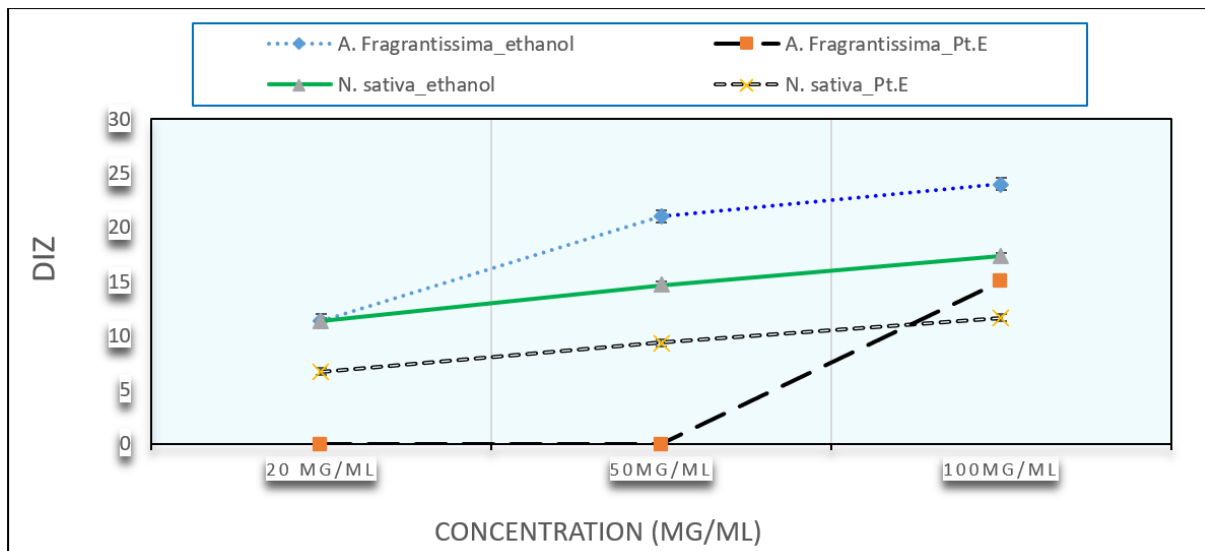
**Fig. 10:** Antimicrobial activity of the (A) ethanolic and (B) petroleum ether extracts of *Achillea fragrantissima*.

**Table 4:** Antimicrobial activity of CuONPs from ethanolic and petroleum ether extracts of *Achillea fragrantissima*.

Microorganism		Zone of inhibition (mm ±se)		
		<i>Achillea fragrantissima</i>		
		Conc. mg/ml	Ethanol	Pet-ether
G -ve	<i>E. coli</i>	20	8±0.00	7±0.00
		50	10.3±0.33	11±0.00
		100	14±0.00	13.6±0.33
	<i>Klebsiella sp.</i>	20	7±0.00	9.3±0.33
		50	11.3±0.33	12.6±0.33
		100	13.3±0.33	16±0.00
	<i>Proteus sp.</i>	20	11±0.00	7±0.00
		50	13.3±0.66	9±0.00
		100	18±0.00	12.3±0.33
<i>P. aeruginosa</i>	20	16±0.00	9.6±0.33	
	50	22.3±0.33	15±0.00	
	100	27.6±0.33	17.3±0.33	
G +ve	<i>S. aureus</i>	20	17.6±0.33	14±0.00
		50	24±0.57	18.3±0.33
		100	31.6±0.33	24.6±0.33
Fungus	<i>C. albicans</i>	20	13.3±0.33	11±0.00
		50	18.6±0.33	15.3±0.33
		100	26.3±0.33	21±0.57

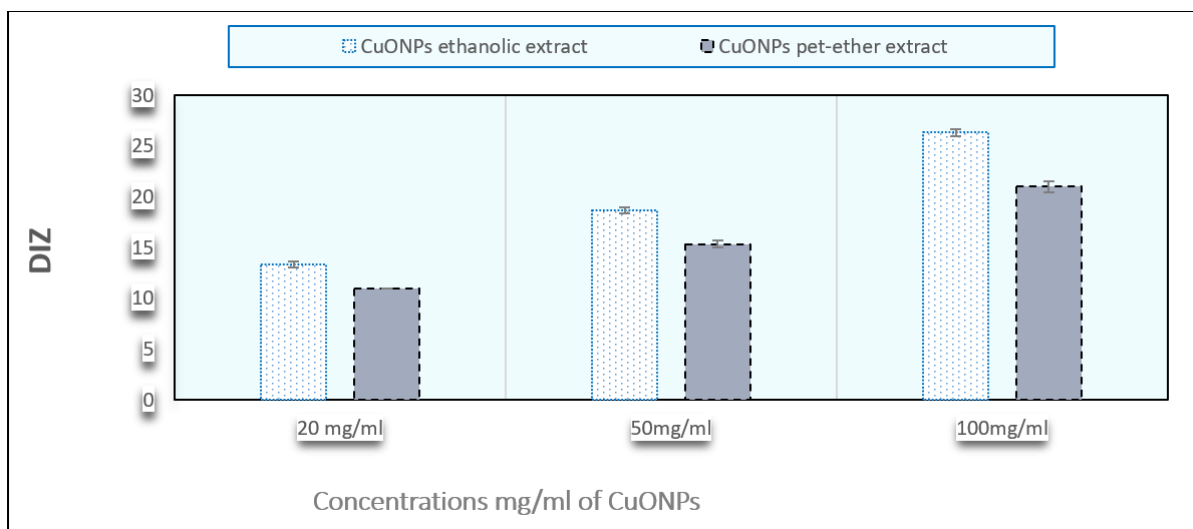


**Fig. 11:** Antimicrobial activity of CuONPs from (A) ethanolic and (B) petroleum ether extracts of *Achillea fragrantissima*.



**Fig. 12:** Antifungal activity of (A) *Nigella sativa* and (B) *Achillea fragrantissima* extracts against *C. albicans*





**Fig. (13):** Antifungal activity of CuONPs from *Achillea fragrantissima* extracts against *C. albicans*.

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