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Effects of Zinc Oxide Nanoparticles (ZnO NPs) Synthesized from River Oak *Casuarina cunninghamiana* Miq.,1848 Leaf Extracts on Three Insects Species

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Abstract

Despite the widespread use of zinc oxide nanoparticles (ZnO NPs) in various fields, there have been a growing number of reports regarding their toxicity. However, there is insufficient research available regarding the insecticidal effects of ZnO nanoparticles. Using leaves of the river oak tree (*Casuarina cunninghamiana* Miq., 1848), this study aimed to produce ZnO NPs utilizing a simple and eco-friendly biological process to evaluate the toxic effects of zinc oxide nanoparticles on the mealworm (*Tenebrio molitor* Fabricius, 1792), the superworm (*Zophobas atratus* Fabricius, 1775) and the red flour beetle (*Tribolium castaneum* Herbst, 1797). The produced zinc oxide nanoparticles were characterized using X-ray diffraction (XRD), transmission electron microscopy (TEM) and UV-visible spectroscopy .The laboratory study was conducted by feeding larvae with leaves immersed in a solution of different concentrations of zinc oxide nanoparticles. The lethal effects of the nanoparticles on the three studied insects were recorded at different time intervals. The outcomes of the statistical analysis displayed important differences in the mean mortality rate according to the composition of the zinc particles and the insects. The highest overall average larval mortality rate was 31.6% among the studied insects with 51.40% being the overall average adult emergence success rate of the studied insect larvae. The LC50 values of the extracted zinc oxide nanoparticles from the used plant, on the other hand, showed varying effects on the mealworm larvae, with a value of 396.27 parts per million (ppm). While the values for the super worm larvae were 6760.83 ppm and that for the red flour beetle larvae the values were 977.23 ppm. These results suggested that the synthesized zinc oxide nanoparticles from river oak tree leaves possess lethal properties against larvae and adults, making them an alternative to synthetic insecticides in insect control stages and environment friendly. Therefore, biologically derived zinc oxide nanoparticles can be used as a potential agent for insect control.

Keywords: Zinc oxide nanoparticle, River oak, Meal worm, Super worm, Red flour beetle

تأثير جزيئات أوكسيد الزنك النانوية (ZnO NPs) المُصنَّعة من مستخلص أوراق شجرة البلوط النـهرية(*Casuarina cunninghamiana* Miq., 1848) على ثلاثة أنواع حشريه

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الخلاصة

على الرغم من الاستخدام الشائع لجسيمات أكسيد الزنك النانوية(ZnO NPs) في مجالات مختلفة، إلا

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أن هناك عددًا متزايدًا من التقارير حول سميتها. ومع ذلك لا تتوفر أبحاث كافية حول تأثيراتها السمية على حشرات. هدفت هذه الدراسة إلى تخليق جسيمات الزنك باستخدام عملية بيولوجية بسيطة وصديقة للبيئة وذلك باستخدام أوراق شجرة البلوط النهرية (*Casuarina cunninghamiana* Miq.,1848) لتقييم التأثيرات السمية لجسيمات أكسيد الزنك النانوية على دودة الجريش الصفراء *Tenebrio molitor* (Fabricius ,1792)، والدود الخارق *Zophobas atratus*(Fabricius,1775) وخنفساء الطحين الحمراء *Tribolium castaneum* (Herbst.1797) هذا و تم توصيف جسيمات أكسيد الزنك النانوية المنتجة باستخدام تقنية التفريق بالأشعة السينية(XRD) والمجهر الإلكتروني النافذ (TEM) والأشعة فوق البنفسجية – المرئية(UV–visible). تم إجراء الدراسة المختبرية عن طريق إطعام الأوراق المغمورة في محلول لجسيمات أكسيد الزنك النانوية بتراكيز مختلفة وتم تسجيل التأثير القاتل لجسيمات أكسيد الزنك على الحشرات الثلاث المدروسة في فترات زمنية مختلفة. أظهرت نتائج التحليل الإحصائي وجود فروق معنوية في متوسط معدل الوفيات وفقًا لتكوين جسيمات الزنك والحشرات، حيث كان أعلى متوسط عام لموت اليرقات (31.6%) في الحشرات المدروسة في حين وصل نسبة نجاح ظهور البالغات من يرقات الحشرات المدروسة المعامله الى (51.40%). هذا و أظهرت قيمLC50 لجسيمات أكسيد الزنك المستخلصة من النبات المستخدم تأثيرًا متفاوتًا على يرقات الدود القنابي ، حيث كانت (396.27 جزء في المليون)، بينما كانت القيم ليرقات الدود الخارق (6760.83 جزء في المليون)، وليرقات خنفساء الطحين الحمراء (977.23 جزء في المليون). استنتجت هذه الدراسة أن جسيمات أكسيد الزنك النانوية المصنعه من أوراق شجرة البلوط النهرية تتمتع بخصائص قاتلة لليرقات والبالغين وتعتبر بديلًا للمبيدات الحشرية الاصطناعية في مراحل التحكم في الحشرات وصديقًا للبيئة. وبالتالي يمكن استخدام جسيمات أكسيد الزنك النانوية البيولوجية كأداة لمكافحة الحشرات.

1 Introduction

In recent decades, nanotechnology has gained significant importance, particularly in enhancing the quality of life. The well activity of micro-particles depends on the particles' properties such as size, shape and wettability [1]. Nanomaterials (NMs) are described as materials with length of 1–1000 nm in at least one dimension. They are, however, commonly defined to be of diameter within 1 to 100 nm range. ZnO NPs have been synthesized by different chemical methods due to environmental concerns. Most of the researchers in recent times focus on their opinion of environment friendly nature to preserve the world [2]. As a result, there is an increasing demand to develop environment friendly methods for nanoparticle production that do not rely on harmful ingredients. Various techniques have been utilized to prepare nanoparticles, encompassing chemical, physical, and biological methods. Microorganisms, enzymes and plant extracts have been proposed as substitute to chemicals in the various methods used for nanoparticle preparation. The utilization of biological synthesis by plant species is advantageous in achieving large-scale production of metal or metal oxide nanoparticles without needing lethal chemicals [3]. Recently, plant parts: leaf [4], peel [5], bark [6], flower [7], tuber [8] extracts have mediated biological processes for the synthesis of zinc oxide nanoparticles. These nanoparticles are effective on many microorganisms and protozoan parasites [9, 10] rather than vertebrate [11] and invertebrate, particularly insects that have been investigated as a potential insecticides for controlling pest populations in agriculture and other settings [12]. While the use of ZnO NPs for insect control is still in the early stages of research, there is evidence suggesting that they could be effective in controlling insect populations in certain situations [13]. Overall, studies demonstrate that ZnO NPs can have a range of effects on different insect species, including toxicological, behavioral and physiological effects. These findings have important implications for the use of ZnO NPs in agriculture and pest control. *Tribolium castaneum* (Herbst.1797) is a common pest of stored grain products, found all over the world. The beetle can cause significant damage to stored food supplies [14]. Mealworms *Tenebrio molitor* (Fabricius ,1792) are commonly used as a food source for pets such as birds, reptiles and small mammals. They are also consumed by humans in some parts of the world and are considered as pests as they feed on stored grains in others. Super worms *Zophobas atratus* (Fabricius, 1775), though similar in appearance to mealworms, are larger, and commonly used as a food source for pets such as reptiles, birds and small mammals. They are also sometimes used as fishing bait. As super worms are also used in scientific research as a model organism [15], this study objective was to biosynthesize ZnO NPs derived from leaves of river oak to accomplisg the aim of determinung their efficacy towards different insect stages of mealworm, superworm and red flour beetle under laboratory conditions.

Materials and Methods

Experiments were conducted in both material laboratory (Physics department) and advanced insect laboratory (Biology department) in the College of Education, Salahaddin University, Erbil, Iraqi Kurdistan region.

1.1 Materials

The plant extract used for synthesis of zinc oxide nanoparticles was from river oak leaves purchased from Erbil city, were identified by botanist. Distilled water, deionized water, zinc acetate dehydrate (Zn (CH 3 CO 2) 2 ·2H 2 O) and sodium hydroxide (NaOH) were purchased from sigma-Aldrich. Mealworms, superworms and red flour beetles were used for the purpose of bioassay tests.

1.2 Collecting Plant Leaves and Rearing Insects

Leaves of river oak were purchased from Erbil province, Kurdistan-region, Iraq. The plants were identified within the herbarium of Biology Department, College of Education as *Casuarina cunninghamiana* Miq., 1848. While the mealworms and the superworms were obtained from insect farming poultries and the red flour beetles were collected from previously infested home flour. The insect specimens were sent to Iraqi Natural History Research Center and Museum No.455 on 9/5/2022 and were identified as *Tenebrio molitor* (Fabricius, 1792) ; *Zophobas atratus* (Fabricius, 1775) *Tribolium castaneum* (Herbst. 1797).

The mealworms and superworms were reared under laboratory conditions in containers comprising oat grain and lettuce, and wheat flour for red flour beetle, and were deposited in incubators at 25±3°C with 65 ±5% relative humidity and constant darkness for mealworms [16][17] and superworms [18]. Whereas the red flour beetles were reared in incubator under 30±3°C with 65 ± 5 % relative humidity and 8 hours darkness [19].

1.3 Plant Extract Preparation

The freshly obtained leaves were thoroughly washed with distilled water and were then left in dark place for a while to dry. The dried leaves were then crushed and converted into powder by using ceramic grinding bowl and sieved through a mesh to get finest powder form. A mixture was then prepared by combining 6 grams of dried leaves with 100 milliliters of deionized water through the process of grinding. The mixture was then boiled for 1 hour at 60^0C by using magnetic stirrer on a hot plate. Later the mixture was put under reflux condition to be extracted. The aqueous plant extract of the leaves was gained by filtering the mixture using Whatman No. 1 filter paper. Thus the aqueous plant extract was made ready to be used for further testing [20].

1.4 Biosynthesis of Zinc Oxide NPs

Zinc oxide was synthesized by using green synthesis method according to [20]. Thirty ml aqueous of plant extract was mixed with 3 gm of zinc acetate dehydrate and boiled over using magnetic stirrer on a hot plate at 90^0C and at 400 rpm. Drops of NaOH solution were then added until the solution color changed from dark yellow to yellowish-white. After completion of reaction, 50 ml of deionized water was added to the paste and settled for 24 hours in order to precipitate the NPs. The mixture was then dried in oven at 90^0C till only NPs remained. In order to obtain pure ZnO NPs, the paste was transferred into a crucible which was then calcinated at 400^0C for 2 hrs. The content was washed thoroughly and repeatedly with distilled water to remove basic solution. Finally, the result sample was calcinated at 400^0C for 2 hours and then kept in dry area for further characterization.

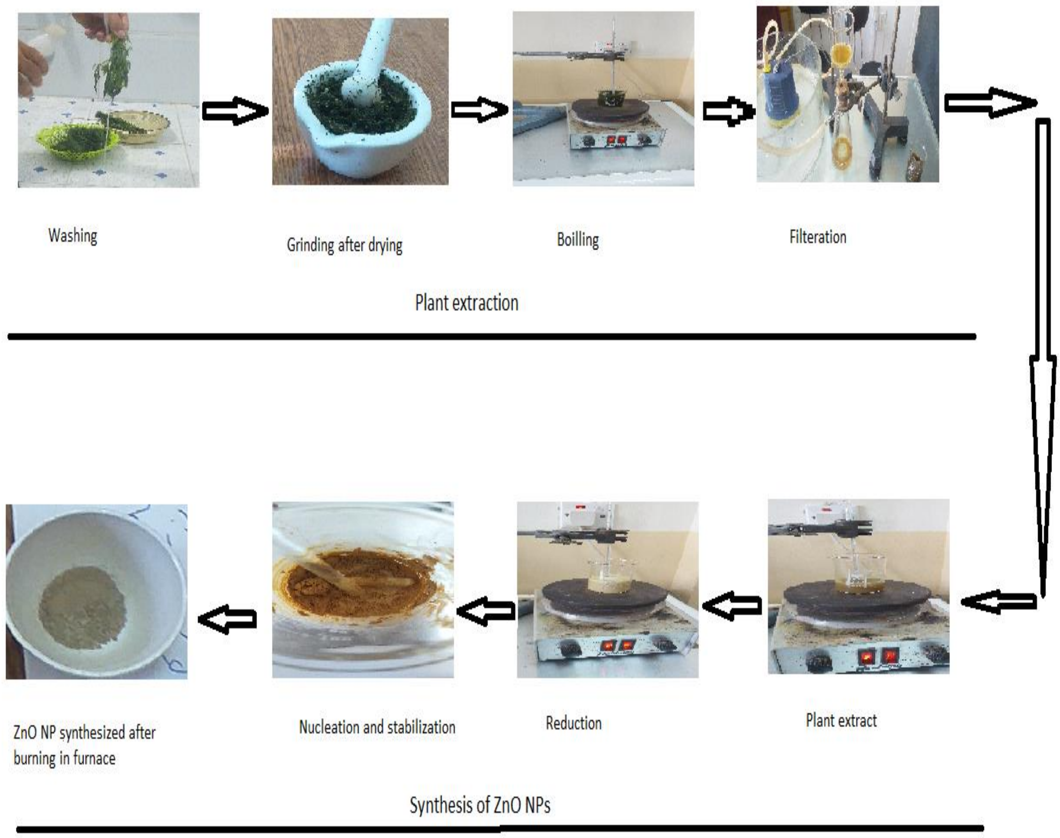

Figure 11: The process of zinc Oxide nanoparticle synthesis.

1.5 Zinc Oxide NP Characterization

1.5.1 UV-spectrophotometer:

A Shimadzu UV-visible double beam spectrophotometer from Japan, specifically the UV-1800 model, was employed for spectrophotometric analysis. The instrument had a constant bandwidth of 1nm and utilized a 1 cm quartz cell. A computer was connected to the double beam spectrophotometer to record the zero-order spectra.

1.5.2 Transmission Electron Microscope (TEM):

The size and morphology of the ZnO nanoparticles were examined by transmission electron microscope (JEOL electron microscope JEM-100 CX).

1.5.3 X-ray Diffraction Method (XRD) Analysis:

The distribution of ZnO nanoparticles was obtained by performing X-ray diffraction (XRD) analysis using a Rigaku X-RAY Diffractometer from Rigaku in Japan. The diffractometer utilized Cu Kα radiation with an angular resolution of 1.5418 angstroms. It was utilized to assess the sizes of the produced crystalline particles. A small amount of powder sample was utilized to perform characterization. The X-ray generators operated at a voltage of 40 kV and a current of 30 mA was applied to the target at room temperature [21].

1.6 Preparation of ZnO NPs Concentrations

Original ZnO NPs nanoparticles were diluted with deionized water to achieve various concentrations (250ppm, 1000ppm, 2500ppm and 5000ppm), plus the control which contained just distilled water [22].

1.7 Bioassay Test

Different concentrations (control, 250, 1000, 2500 and 5000 ppm) of ZnO NPs solution were examined on the tree insect species (mealworm, superworm and red flour beetle). Three replications, each with 10 larval stages, were adopted for the bioassay tests. Each insects' larvae were put in small plastic containers (25 ml) for exposure through feeding 1 gm of lettuce leaflets which were immersed in ZnO NPs solution concentrations for 5 seconds for mealworms and superworms respectively and 1 gm of flour sprayed with 0.5 ml of ZnO solution concentrations for red *T. castanium*. The insects mortality percentage was calculated accurately according to Abbot's formula, and LC_{50} values were determined by utilizing Finney's probit analysis statistical method [23].

Abbot's formula: [24]

Corrected Mortality (%) =$\left(\frac{\%\,\mathrm{MT}-\%\mathrm{MC}}{100-\mathrm{MC}} * 100\right)$

Where T = No. of dead larvae in treated replicates.

C = No. of dead larvae in control replicates.

Toxicity index for LC50=$\frac{\text{LC50 of the most effective compound}}{\text{LC 50 of the least effective compound}} \times 100$

1.8 Statistical Analysis

SPSS version 26 was used to examine data of the bioassay tests. All data was presented as mean ± SD and the mean values were compared using Student's t-test at the 5% probability level. Furthermore, Duncan's test was used to perform statistical comparisons. *P* values less than 0.05 were regarded significant in all situations analyzed.

2 Results and Discussion

2.1 Zinc oxide NPs Characterization

2.1.1 UV-spectrophotometer of River Oak Plant

The investigation focused on studying zinc oxide nanoparticles (ZnO NPs) that were synthesized using an aqueous extract of *Casuarina cunninghamiana*. The confirmation of the production of ZnO NPs was achieved through UV-vis analysis. The generated zinc oxide's UV-vs spectra were examined for the samples between 200 and 800 nm wavelengths. The spectrum revealed that the absorbance peak occurred at 305 nm (Figure 2). This peak corresponded to the characteristic band of zinc oxide nanoparticles. Our results was close to the results of [25] which was 310 nm.

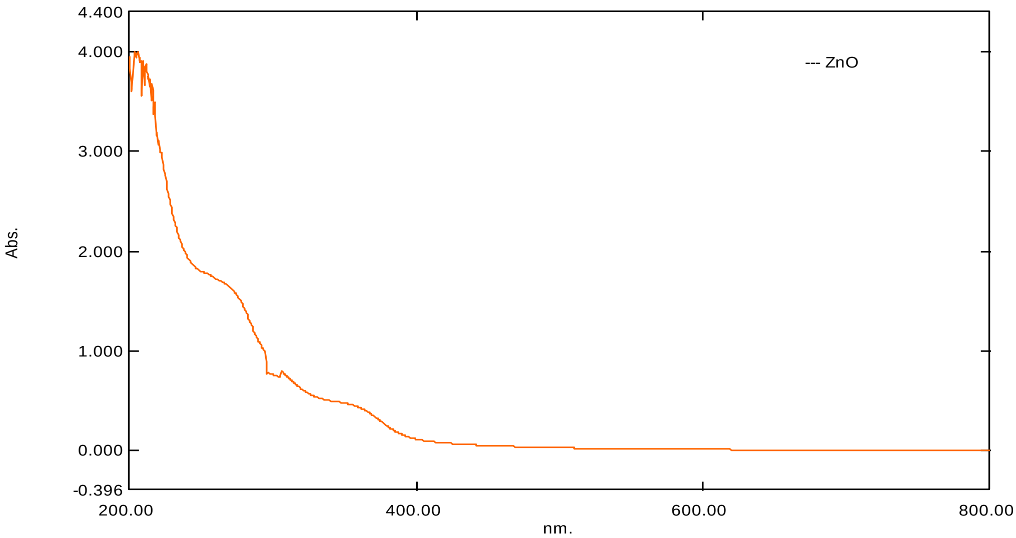

Figure 2: The UV-vis spectrum of ZnO NPs synthesized using river oak plant.

2.1.2 Transmission Electron Microscopy of ZnO NPs Obtained from River Oak Plant

This application produced zinc oxide nanoparticles, and transmission electron microscopy at 100 nm scale has been demonstrated in Figure 3. The ZnO NPs were found to be spherical in shape and of average size ranging between 10-13 nm with some bulks reaching 119 nm in size.

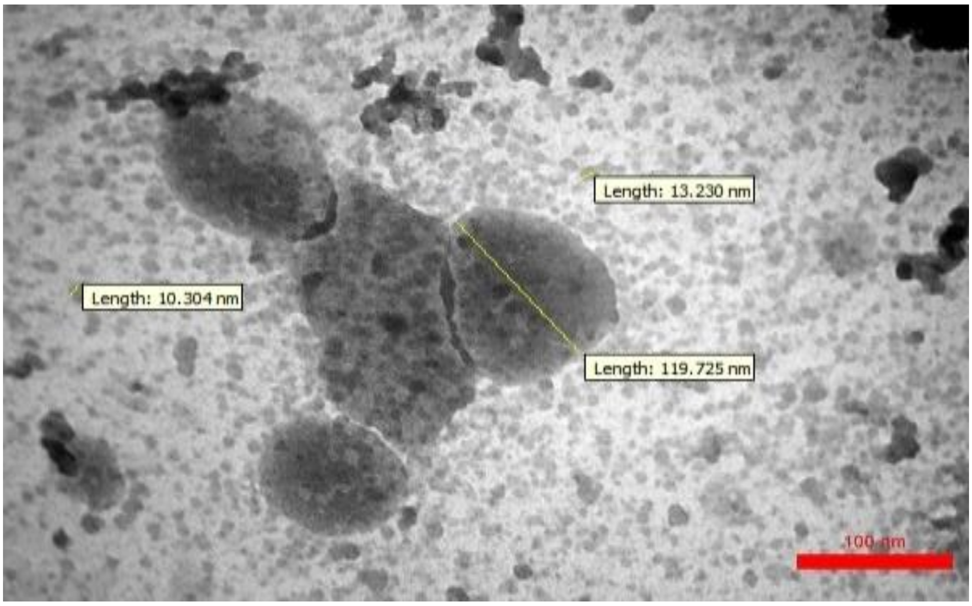

Figure 3: TEM image of green synthesized ZnO nanoparticles from leave extracts of river oak plant.

2.1.3 X-ray Diffraction Method (XRD) Analysis of ZnO NPs Obtained from River Oak

Figure 4 shows the X-ray diffraction pattern of green zinc oxide nanoparticles that were produced. Three major crystalline peaks with 2θ values at 31.74, 34.2 and 36.17 in the produced ZnO NPs patterns were indexed to the planes 100, 002 and 101 respectively. The 102, 110, 103, 200, 112, 201, 004 and 202 planes were responsible for the eight low peaks at 47.55, 56.67, 62.86, 66.40, 67.91, 69.09, 72.56 and 76.92 respectfully. These crystallographic peaks demonstrated that the hexagonal wurtzite phase had formed. This result was consistent with the outcomes of Vijayakumar *et al.* and Umavathi*et al.* [26, 27] of plant-mediated ZnO NP synthesis.

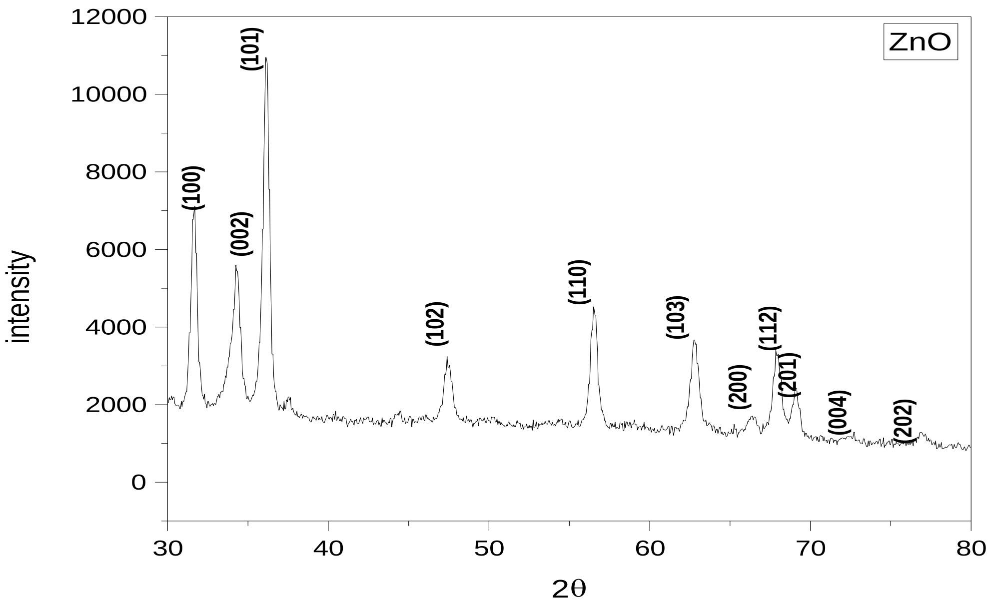

Figure 4: XRD pattern of ZnO NPs synthesized using river oak plant.

2.2 Bioassay of ZnO NPs Synthesized from River Oak Plant against Larvae of Three Insects Species using Feeding Method

The results in Table 1 show that ZnO NPs had significant influences on larval mortality where the total mean was 31.6, compared to the control where the total mean was 8.87, with significant differences between them. The results also revealed that the highest mortality rate of 61.83 was recorded in the larvae of mealworm, followed by flour beetle (22.16) and superworm (10.85) with significant differences between them. The outcome of the statistical evaluation revealed that the differences were substantial in the average mortality rate according to insect species. As for the effects of the ZnO NPs treatment on the adults, it emerged that there was a variation in ZnO NPs impacts on the three studied insects in which mealworm was more influenced through its adult emerge percentage which was 27.50%. While the superworm was more tolerant as its adult emergence percentage reached 74.18%. The statistical analysis showed a significant differences between the studied insects for their adults emergence percentage (Table 2). Several studies have investigated ZnO NPs effects on the mortality and growth of different insect larvae. For instance, a study by Elmasry [28] investigated the effects of ZnO NPs on the larvae of *Spodoptera litura*, a major pest of many crops. The study found that the ZnO NPs significantly reduced the survival rate of the larvae and caused growth inhibition, and that the mortality percentage increased after treating. Moreover, [12] presented adult emergence of *S. litura*. after exposing with various concentrations of ZnO−thiamethoxam and thiamethoxam increased in the dose-dependent manner. They observed that the treatments with different concentrations of thiamethoxam alone and ZnO−thiamethoxam declined the emergence of adults in all test concentrations of ZnO−thiamethoxam. And the results obtained by Asghar *et al.* [29] demonstrated that the mortality percentage in all tested instar *Helicoverpa armigera* larvae increased with NPs concentration. Moreover a study by Buhroo *et al.* [30] demonstrated that the increase in aluminum oxide (Al2O3) nanoparticles concentration declined adult emergence number. They also revealed that the reduction percentages of adult emergence increased with the increase of NPs concentration.

Table 1: Accumulation effects of ZnO NPs synthesized from river oak against larval stage of three insect species through feeding method after five weeks in ideal conditions:

Treatments	Insects	Mortality Mean ± SD	Total Mean	F-test	*P*-Value (Sig.)
River oak plant	Mealworm	61.83±0.94(a)	31.6(a)	17.601	0.001 (HS)
	Superworm	10.85±1.62(b)			
	Flour beetle	22.16±2.46(d)			
Control	Mealworm	13.30±0.70(b)	8.87(b)	7.370	
	Superworm	0.00±1.42(c)			
	Flour beetle	13.30±2.48(b)			

Different letters indicate significant differences.

Table 2: Efficiency of ZnO NPs synthesized from plant sources on adult emerge of the studied insect species through feeding method at ideal incubation condition:
Different Different letters indicate significant differences.

Treatments	Insects	Mean ± SD	Total Mean	F-test	*P*-Value (Sig.)
River oak plant	Mealworm	27.50±0.61(c)	51.40(b)	29.146	0.001 (HS)
	Superworm	74.18±1.90(a)			
	Flour beetle	52.51±1.52(b)			
Control	Mealworm	53.30±1.54(b)	67.77(a)	55.325	
	Superworm	90.00±0.66(a)			
	Flour beetle	60.00±2.24(b)			

2.2.1 LC_{50} Values of ZnO NPs Synthesized from River Oak Plant Sources against the Larval Stages of the Three Studied Insect Species through Feeding Method

Table 3 displays LC_{50} values of the ZnO NPs derived from the used plants that showed varying effects that differed according to the insect species. The mealworm was the most effective as its LC_{50} value was 396.27 ppm, followed by the flour beetle with 977.23 ppm LC_{50} value compared with the 6760.83 ppm LC_{50} value for superworm. The results showed that the superworm was least efficient insect among the tested species which was confirmed by the toxicity index as they gradually graded downward in their potency and reached 100, 40.55 and 5.86 for mealworm, flour beetle and superworm respectively. The toxicity curve of all the studied species showed their effectiveness compared to the control treatment is clarified (Figure 5, 6 and 7) .

Table 3: Estimated LC_{50} of ZnO NPs synthesized from river oak plant source against the three studied insect species larval stages through feeding method.

Insects	Intervals	Concentrations (ppm)	M	LC_{50}	Slope	R^2	Toxicity Index LC_{50}
Mealworm	Week 5	250	39.2	396.27	y=1.056x +2.25	0.94	100
		1000	53.07				
		2500	76.93				
		5000	88.33				
Flour beetle	Week 5	250	7.73	977.23	y=0.9806 x +2.064	0.78	40.55
		1000	7.73				
		2500	34.66				
		5000	38.52				
Superworm	Week 5	250	0.00	6760.83	y=3.0738 x+6.77	0.83	5.86
		1000	6.70				
		2500	10.00				
		5000	16.65				

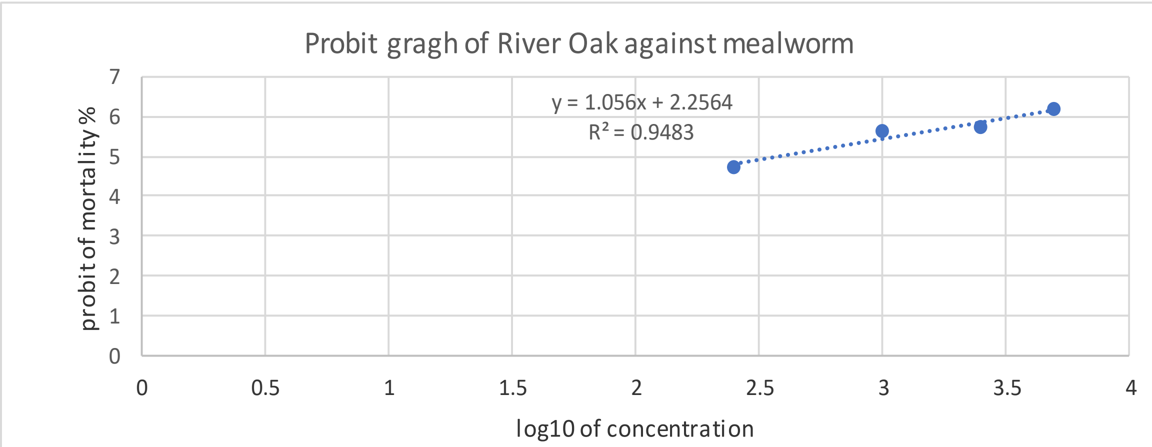

Figure 5: Toxicity curve of river oak plant against mealworm.

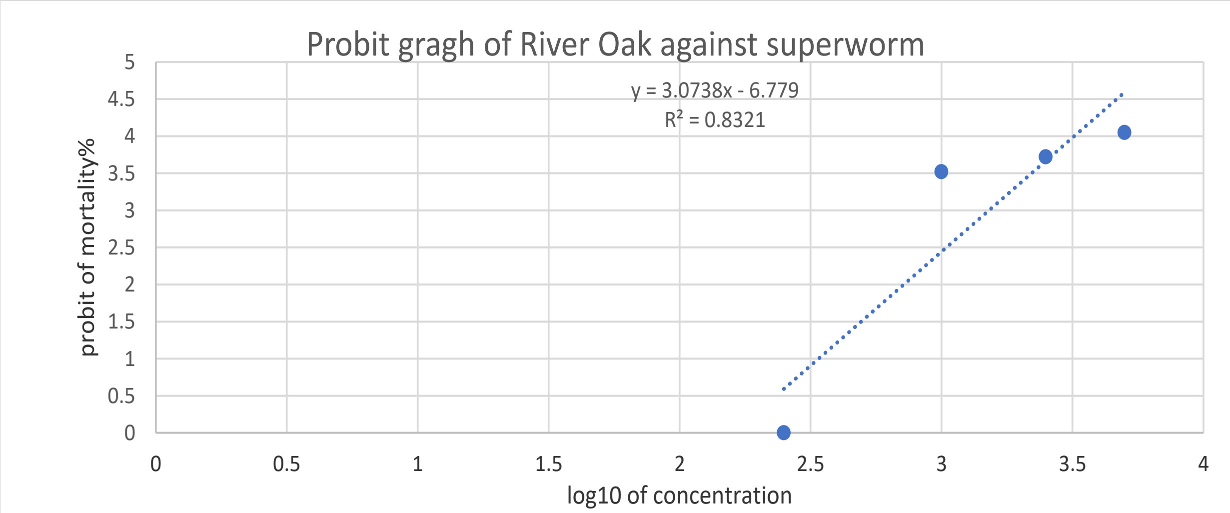

Figure 6: Toxicity curve of river oak plant against superworm.

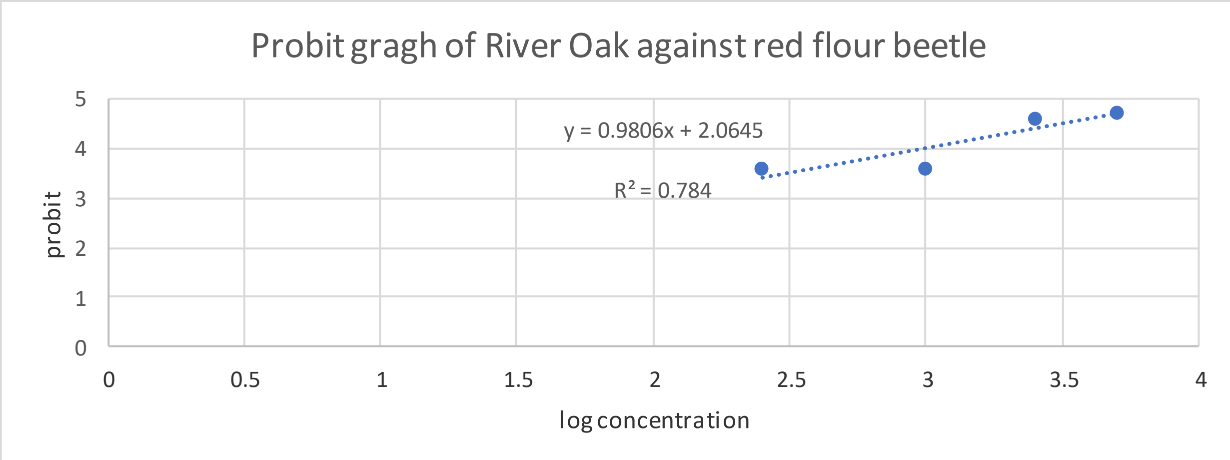

Figure 7: Toxicity curve of river oak plant against red flour beetle.

Data presented in Table 3 indicates the presence of variation in the average rates of larval mortality rate depending on the ZnO NPs derived from the used plants. The greatest proportion of mortality was seen in larvae fed with ZnO NPs derived from river oak within the period of treatment when applied at all the concentrations after five weeks of treatment. Increase in product concentration and exposure period raised mean percentage of mortality.. Highest larval mortality of 88.33 % and 76.93% was noticed in the treatment at 2500ppm and 5000ppm concentrations respectively against the larval stages of meal worm after five weeks of treatments. This mortality rate was significantly different from untreated control as shown in Table 1. Whereas, the highest larval mortality against the larval stages of super worm was obtained at 5000 ppm concentration which was 16.65%. The highest larval mortality against the larval stages of flour beetle was obtained at both 2500 and 5000 ppm concentrations reached (34.66% and 38.52% respectively. All the obtained data of our results revealed that the ZnO NPs derived from river oak were effective against the larval stage of the studied insects and that the higher the concentration, the more effective it is. The impact of silver nanoparticles on insect larvae feeding habits causes damage to the cells and tissues of the insect's midgut, resulting in cellular gaps that affect carbohydrate and protein levels in the insect's haemolymph. This was observed by Karthikeyan *et al.* [31] when fourth instar mosquito larvae were exposed to silver nanoparticles. Our study's results are consistent with his findings, suggesting that the feeding of insects during both their larval stages, whether short or long-term, had a significant impact on females' ability to lay eggs and weakened ovarian growth. The use of silver nanoparticles at 1000 and 5000 parts per million concentrations may reduce the population of these insects, thereby overcoming problems associated with the use of chemical pesticides and their harmful effects.

Conclusion

Based on our observations, river oak *Casuarina cunninghamiana* can be utilized for zinc oxide nanoparticles synthesis. The leaves of this plant are believed to contain chemical components that function as both a reducing agent and a stabilizing agent during ZnO NPs creation. According to our results, river oak has a high potential insecticidal effect on insect pests, and ,hence, can be as a good source for further studies in this field.

Acknowledgments

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Conflict of Interest Statement

The outcomes of the present study are part of the requirements of M.Sc. in Entomology of Department of Biology, College of Education, Salahaddin University, Erbil for the first author. Also, we confirm and declare no existence of any relationship or conflict of interest with any other party.

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