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The Effects of Zinc Oxide nanoparticles synthesized from Eucalyptus plant extracts against mealworm stages *Tenebrio molitor* L., 1758 (Tenebrionidae: Coleopetera)

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ABSTRACT

Despite the widespread utilization of Zinc Oxide nanoparticles (ZnO NPs) in different areas, there have been several studies regarding their toxicity. Thus, this study aimed to produce zinc oxide nanoparticles using a simple and eco-friendly biosynthesis process, utilizing the Eucalyptus (Eucalyptus *camaldulensis* Dehn.) as a reducing agent. It also aimed to estimate their insecticidal efficacies toward the mealworm's stages (*Tenebrio molitor* L.,1758). The produced ZnO nanoparticles were characterized by X-ray diffraction, UV–visible, and transmission electron microscopy (TEM). The laboratory experiment was carried out with one way of exposure, using the feeding method through immersing the leaf in ZnO NPs solution with different concentrations. The mortality effects on insect's stages were recorded in various periods of time. The statistical analysis results indicated the presence of noteworthy variations in the average mortality rate according to insect's stage, showing a different effect on the average of larval mortality rate. In this regard, the highest average of larval mortality rate was recorded at 5000ppm (61.25%); while the general average percentage of adult emergence was (42.53%). After three weeks, the highest general mean of adult mortality was obtained at 5000ppm (87.48%). Similarly, the LC50 value of the ZnO NPs derived from the used plant extract against the larval stage was (3630.78 ppm). This result revealed that Zinc Oxide Nanoparticles from plant extract sources have both larvicidal and adulticidal properties and they could serve as an eco-friendly alternative to synthetic insecticides for controlling insect's stages. Hence, the biogenic Zinc Oxide Nanoparticles can be used as a potential bio-insecticidal agent for the future.

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تأثير جزيئات أوكسيد الزنك النانوية (ZnO NPs) المُصنَّعة من مستخلص أوراق نبات اليوكاليبتوس على مراحل الدود القنابي *Tenebrio molitor* L.,1758 (Tenebrionidae: Coleopetera)

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الملخص

على الرغم من الاستخدام الواسع النطاق لجسيمات أوكسيد الزنك النانوية (ZnO NPs) في مختلف المجالات ، فقد كان هناك عدد متزايد من الدراسات المتعلقة بسميتها. هدفت هذه الدراسة إلى تخليق جزيئات أوكسيد الزنك النانوية باستخدام عملية تخليق حيوي بسيطة وصديقة للبيئة ، وذلك باستخدام نبات اليوكاليبتوس(*Eucalyptus camaldulensis* Dehn.) مع تقييم خصائص جسيمات أوكسيد الزنك النانوية كمبيد ضد مراحل الدود القنابي *Tenebrio molitor* L.,1758, تم تمييز الجسيمات النانوية ZnO المنتجة بواسطه حيود الأشعة السينية (XRD) والأشعة فوق البنفسجية المرئية والمجهر الإلكتروني النافذ (TEM). أجريت التجارب المختبرية بطريقة التغذية عبرغمر الأوراق في محلول ZnO NPs بتراكيز مختلفة, تم تسجيل معدل الهلاك لمراحل الحشرات في فترات زمنية مختلفة ، وأظهرت نتائج التحليل الإحصائي وجود فروق معنوية في متوسط معدل الهلاكات حسب مرحلة الحشرة ، حيث كانت أعلى نسبة لمعدل هلاك اليرقات عند تركيز ٥٠٠ppm (61.25٪) ، بينما كان المعدل العام لظهور البالغات (42.53٪) ، ومن ناحية أخرى كان أعلى معدل عام لهلاك البالغات بعد ثلاثة أسابيع عند تركيز ٥٠٠ppm (87.48٪).هذا وكانت قيم التركيز المميت النصفي لـ ZnO NPs المشتقة من المستخلص النباتي و المستخدم ضد مرحلة اليرقات (٣٦٣٠,٧٨ جزء في المليون) ، وخلصت هذه النتيجة إلى أن جزيئات أوكسيد الزنك النانوية المصنع من مصادر مستخلصات نباتية لها خصائص المبيد لليرقات والبالغات ويمكن أن تكون صديقة للبيئة وبديل للمبيدات الحشرية الاصطناعية ومن ثم يمكن استخدام الجسيمات النانوية من أوكسيد الزنك الحيوي كعامل مبيد حشري بيولوجي محتمل في المستقبل.

Introduction

Throughout the previous several decades, nanotechnology has gained a considerable importance, particularly in enhancing the quality of life [1]. The effectiveness or performance of micro-particles is affected by various properties, such as their size, shape, and wettability [2]. Nanomaterials are typically defined as materials with dimensions ranging from 1 to 1000 nanometers in at least one dimension. However, they are commonly referred to have a diameter ranged from 1 to 100 nanometers. Metallic nanoparticles are attracting a great deal of attention because of their potential of succeeding specific processes and selectivity, especially in biological and pharmaceutical applications [3]. Zinc oxide is a material, which can have many good and unique properties [4]. Various methods have been employed to synthesize zinc oxide nanoparticles [5]. Currently, many researchers are shifting their focus towards eco-friendly approaches in order to address environmental concerns and sustain the planet [6]. Consequently, there is an increasing need for the growing of eco-friendly nanoparticle production methods that do not involve the use of harmful substances. Various methods have been employed for nanoparticle production, including chemical, physical, and biological ones. Among these methods, the usage of microorganisms, enzymes and plant extracts has been proposed as an alternative to chemical-based approaches [7]. Biological synthesis using plant species has proven to be advantageous for the large-scale production of metal or metal oxide nanoparticles, as it eliminates the need for toxic or harmful chemicals [8]. Recently, plant parts including the leaf [9], peel [10], bark [11], flower [12], seed [13], and tuber [14] extracts have mediated the biological process for the production of Zinc oxide nanoparticles. It can be investigated as potential insecticides for controlling pest populations in agriculture and other settings [15]. The utilization of ZnO NPs for insect control is currently at an early stage of research. There is an evidence indicating the potential effectiveness of ZnO NPs in controlling insect populations under certain circumstances [16]. However, it is important to note that the use of ZnO NPs for insect control raises valid concerns regarding their potential impact on non-target organisms and the environment [17]. Overall, studies reveal that ZnO can have efficacies on different insect species, including toxicological, behavioral, and physiological effects. The results reveal substantial consequences for the utilization of ZnO in agriculture and pest control [15]. Mealworms (*Tenebrio molitor* L.) Tenebrionidae are commonly used as a food source for pets, such as birds, reptiles, and small mammals. They are also considered as pests, because they feed on stored grains [18]. Consequently, the aim of this study is to biosynthesize ZnO nanoparticles derived from Eucalyptus leaf plant extract and to find out their insecticidal effects against different stages of Mealworm.

MATERIALS AND METHODS

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

Materials

The plant extract utilized for the production of Zinc Oxide nanoparticles was prepared by using *Eucalyptus camaldulensis* leaves obtained from Khabat area in Erbil province. The botanical identification of the plant was conducted by a qualified botanist. Then, distilled water, deionized water, Zinc acetate dehydrate (Zn (CH 3 CO 2) 2 ·2H 2 O) and sodium hydroxide (NaOH) were acquired from sigma-Aldrich. Mealworms were used for the purpose of application.

Collecting plant leaves and rearing of insects

Plant leaves of Eucalyptus were collected from Khabat area in Erbil province, Kurdistan region, Iraq. The plants were labelled as Education-Salahaddin University Herbarium (ESUH) as Eucalyptus (Eucalyptus *camaldulensis*) with the sample identification number 9027. While the mealworms larvae were obtained from insect farming poultries in the city center birds markets. The insect specimens were sent to the Iraqi Natural History Research Center and Museum No.455 on 9/5/2022 and identified as (*Tenebrio molitor* L.,1758). The insects were reared under laboratory conditions in plastic containers (1000 ml), fed with oat grain and lettuce deposit in incubators 25±3°C with 65 ±5% relative humidity and constant darkness [19].

Preparation of plant extract

The fresh obtained leaves were thoroughly washed with distilled water and left on dark place for a while to be dried. Then, the dried leaves were crushed and converted into powder by using ceramic grinding bowl. The powder obtained was sieved through a mesh to get its finest form. After the processes of shading, drying and grinding, (6) grams of dried leaves were mixed with 100 ml of deionized water. Then, the mixture was boiled for 1 hour at 60 °C by using Magnetic Stirrer with hot plate. Next, the mixture was put under reflux condition to be extracted. After that, aqueous plant extract of the leaves was gained by filtering the mixture using Whatmann No. 1 filter paper. Then, this aqueous plant extract was prepared to be used in further step testing [20].

Zinc Oxide NP biosynthesis

ZnO NPs were synthesized using an eco-friendly, green synthesis method according to [20]. 30 ml of aqueous plant extract were mixed with 3 gm of Zinc acetate dehydrate and boiled over Magnetic Stirrer with hot plate at the temperature of 90 °C and stirring with 400 rpm. Then, drops of NaOH solution with 1M were added until the solution color changes from dark yellow to yellowish-white color. After the completion of reaction, 50 ml of deionized water was added to the paste and settled for 24 hours in order for NPs to be precipitated. Then, it was dried in oven at (90 °C) to get only NPs. The paste was transferred into a crucible, which was kept on furnace at

400 °C for 2 hours until the color of the precipitates changed to white color. Then, precipitates were kept in dry area for further characterizations.

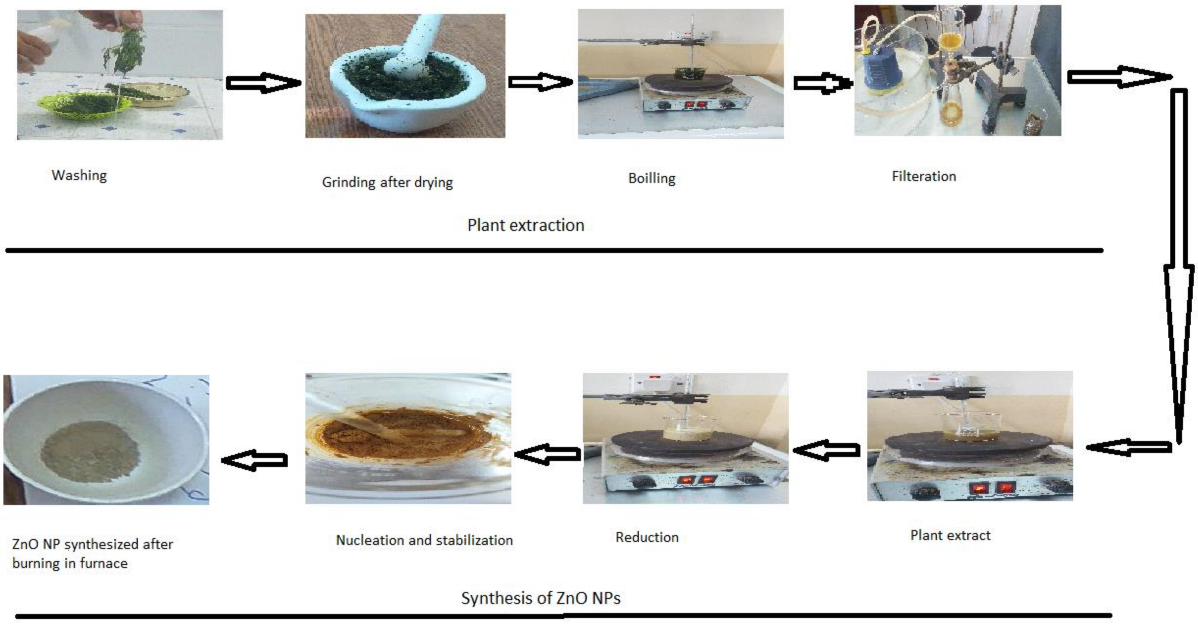

Figure خطأ! لا يوجد نص من النمط المعين في المستند.1: The process of Zinc Oxide nanoparticle synthesis

Zinc Oxide NP characterization

UV-spectrophotometer

For spectrophotometric measurements, a Shimadzu UV-Visible double beam spectrophotometer (model UV-1800, Japan) with a fixed 1nm bandwidth and a 1 cm quartz cell was used. The double beam spectrophotometer was connected to a computer for the purpose of recording the zero order spectra.

Transmission Electron Microscope

The device of transmission electron microscope (JEOL electron microscope JEM-100 CX) was used to analyze and investigate the size and morphology of synthesized ZnO NPs.

X-ray Diffraction analysis

ZnO nanoparticle's X-ray diffraction (XRD) pattern was gained by using a Rigaku X-ray Diffractometer (Rigaku, Japan) equipped with Cu Kα radiation, which has an angular resolution of 1.5418 Å (angstroms). This technique was employed to assess the sizes of the crystalline particles. For characterization purposes, a small amount of powder sample was utilized. The X-ray generators were operated at a voltage of 40 kV and a current of 30 mA, targeting the sample at room temperature [21].

Preparation of the desired ZnO NPs concentrations

Different concentrations were obtained by diluting the initial Zinc Oxide nanoparticles with distilled water to obtain 250ppm, 1000ppm, 2500ppm and 5000ppm, plus control which contained just distilled water [22].

Test solutions and suspension preparation

A suspension of Zinc Oxide nanoparticles was prepared by adding ZnO nanopowder to deionized water with magnetic stirrer (hotplate and stirrer) of the suspension for 24 hrs. The ZnO NP suspension was gained with the final concentrations of (25, 100, 250 and 500) mg Zn NPs mL-1 corresponding to 0.25,0.1,2.5 and 5 mg Zn NPs mL-1 [23].

Bioassay Test

Different concentrations (control, 250, 1000, 2500 and 5000 ppm) of ZnO NPs solution were examined on Mealworm insect. For the bioassay tests, three replications of different stages (larva and adult) were adopted each with 10 larvae of the last instar. Insects were put in small plastic containers (25 ml) for exposure and fed (1) gm of lettuce leaflets which were immersed in ZnO NPs solution for 5 seconds. The insect's mortality percentage was adjusted using Abbot's formula. The LC50 values were calculated using the probit analysis statistical method developed by [24] depending on Abbot's formula [25] as follow:

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

As %MT is the dead larvae percentage of the treated samples, and %MC is the dead larvae percentage of the control samples.

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

Statistical analysis

SPSS version (26) was used to examine the data of bioassay tests. The data was exhibited as Mean ± SD and the means were compared by using Student's t-test at 5% significance level. Furthermore, one-way analysis of variance (ANOVA) Duncan's test was used to conduct the statistical comparisons. In all situations analyzed, P values lower than 0.05 were deemed significant.

RESULTS AND DISCUSSION

Zinc Oxide NPs characterization

UV-spectrophotometer of Eucalyptus plant

It investigated Zinc oxide nanoparticles that were produced by utilizing Eucalyptus plant extract. The confirmation of zinc oxide nanoparticles formation was achieved by utilizing a UV-visible spectrophotometer. The absorption spectrum of the synthesized zinc oxide nanoparticles was recorded within the range of 200-800 nm. The strong absorption was recorded at 271 nm, as shown in (Fig. 2) corresponding to band gap energy which was 4.75ev, by the using this calculation formula Eg=1240/λ which has a slight variance with results obtained by [26] and previously reported values [27] could be due to the disagreement in the average crystal size of NPs.

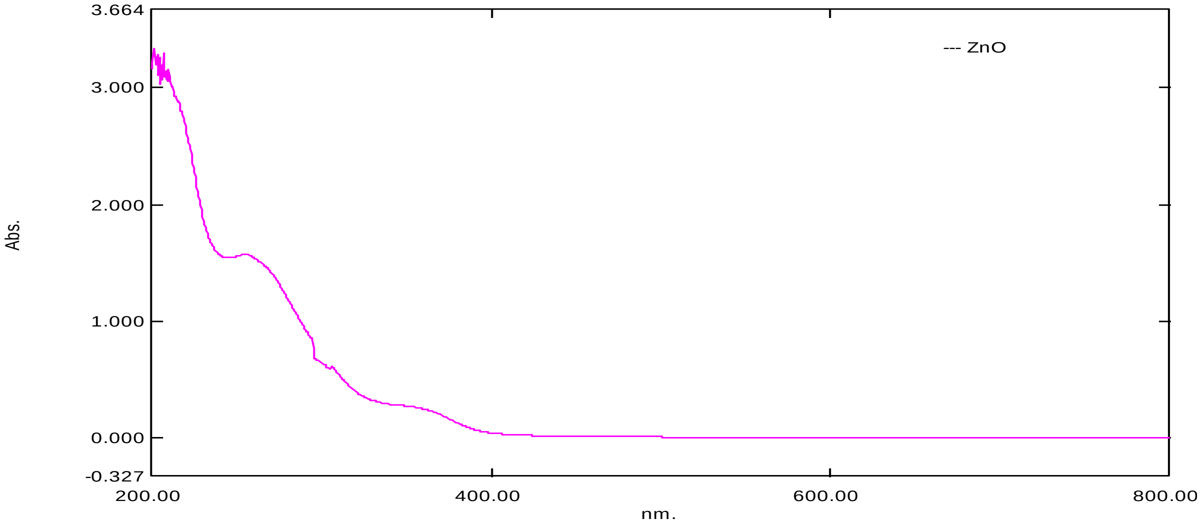

Figure 2 : UV-vis spectrum of ZnO NPs synthesized using *Eucalyptus camaldulensis* leaves aqueous extract

Transmission Electron Microscope of ZnO NPs obtained from Eucalyptus plant

Figure (3) presents the results of transmission electron microscopy that was performed to characterize the synthesized zinc oxide nanoparticles. The TEM image demonstrates that the ZnONPs exhibited a spherical shape, with an average size of approximately 18 nm at the 50 nm scale.

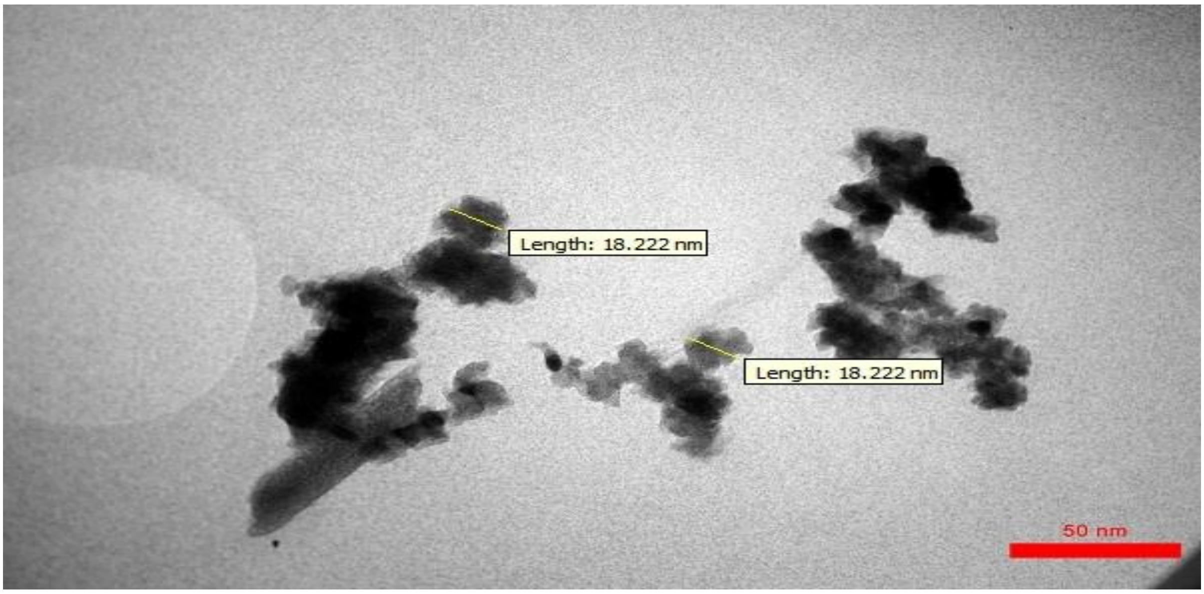

Figure 3: TEM image of green synthesized ZnO nanoparticles from Eucalyptus plant

X-ray Diffraction of Eucalyptus plant

In Fig. 4, the outcomes of the X-ray diffraction (XRD) pattern of green synthesized ZnO NPs are displayed. The synthesized ZnO nanoparticles exhibited three prominent crystalline peaks with 2θ values at 31.8°, 34.45°, and 36.3°. These peaks corresponded to the crystal planes 100, 002, and 101, respectively. Additionally, eight lower peaks were observed at 47.6°, 56.67°, 62.86°, 66.40°, 67.91°, 69.09°, 72.56°, and 76.92°, which were attributed to the crystal planes (102), (110), (103), (200), (112), (201), (004), and (202), respectively. The presence of these crystalline peaks confirmed the formation of the hexagonal wurtzite phase. The current findings are in a close agreement with the results of [28, 29] of plant-mediated ZnO NP synthesis.

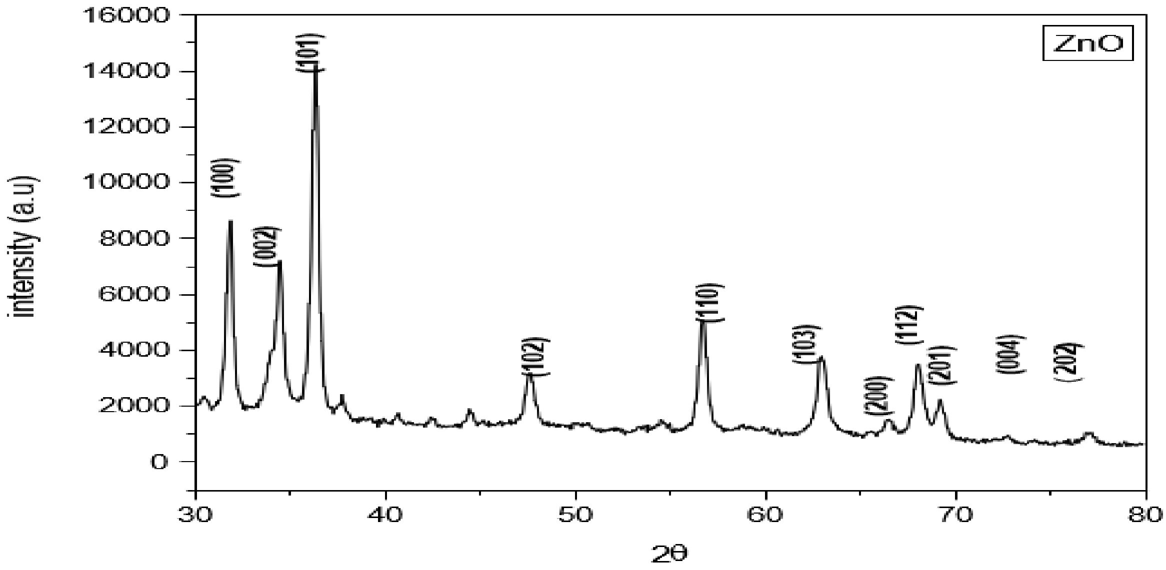

Figure 4: XRD pattern of ZnO NPs synthesized using Eucalypts plant

Bioassay of ZnO NPs synthesized from Eucalyptus plant sources against larvae and adults of mealworm species with feeding method

Larval stage

It is obvious in table (1) that ZnO NPs formed from Eucalyptus showed a different effect in the average of larval mortality rate. Hence, the highest average of the larval mortality rate was recorded at 5000ppm (61.25%), while its lowest average was at the concentration 250ppm after 24 hrs and 1 week (0%). The statistical analysis results indicated significant differences in the average mortality rate among the different studied concentrations. The ZnO NPs treatment affected the adult emerge ratio (as shown in table 2), where the general average percentage was (42.53%), while the mean of adult emerge in control treatment was (53.30%). The statistical analysis showed a significant difference between them for adult emerge percentage. Numerous studies have been conducted to explore the effects of ZnO nanoparticles (ZnO NPs) on mortality and growth of different insect larvae depending on their concentrations and species, reaching the same conclusions. For instance, a study by [30] explored the impact of ZnO NPs on the larvae of *Spodoptera litura* (Fabricius, 1775) , 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, in which the mortality percentage increased after the treatment. Moreover, [15] presented the adult emergence of *S. litura* after treating it with various concentrations of ZnO-thiamethoxam and thiamethoxam. They observed that both treatments led to an increase in adult emergence in a dose-dependent manner. This suggests that the efficacies of thiamethoxam alone and the combination of ZnO-thiamethoxam varied depending on the concentration used, declining the emergence of adults in all tested concentrations of ZnO−thiamethoxam. Moreover, [31] demonstrated that the increased concentration of Aluminum oxide (Al2O3) nanoparticles was found to reduce the number of adult emergence of *Mythimna separata* a moth of the family Noctuidae. The study showed that the reduction percentages of adult emergence increases with the increase in the concentration of nanoparticles.

Table 1: The effects of ZnO NPs produced by Eucalyptus plant with different concentrations on Mealworm larvae

Different letters indicate significant differences.

Intervals	Concentrations (ppm)	Mean ± SD	F	P-Value	Sig.
24 h	control	0±0(a)	0.000	1.000	NS
	250	0±0(a)			
	1000	0±0(a)			
	2500	0±0(a)			
	5000	0±0(a)			
1W	control	0±0(c)	1.763	0.273	NS
	250	0±0(c)			
	1000	6.65± 0.40 (b)			
	2500	6.65±0.40 (b)			
	5000	13.35±0.40 (a)			
3W	control	10.69± 1.06 (b)	12.646	0.008	HS
	250	13.30± 1.83(b)			
	1000	24.98± 1.016 (a)			
	2500	24.98±2.20 (a)			
	5000	28.51± (a)			
5 W	control	13.35± 1.83 (c)	9.021	0.017	S
	250	19.58±1.48 (bc)			
	1000	31.11±1.85 (ab)			
	2500	38.47±1.24 (b)			
	5000	61.25±1.98 (a)			

Table 2: the efficiency of ZnO NPs produced by Eucalyptus plant sources on adult emergence of the Mealworm at optimal incubation condition

Plants	Mean ± SD	F-test	P-Value (Sig.)
Eucalyptus	42.53±1.74 (b)	77.494	0.001 (HS)
Control	53.30±1.54 (a)	55.325	

Distinct letters signify notable differences.

Adult stage

The effect of ZnO NPs synthesized from Eucalyptus with different concentrations on the Mealworm is shown in table (3). The results clearly demonstrate that there was a significant impact of ZnO NPs product on adult mortality at the studied concentrations. In this regard, the highest general mean of adult mortality was obtained after three weeks at 5000ppm (87.48%), while its lowest general mean was recorded as (0%) in the first two weeks for the studied concentration. However, the efficacy of ZnO NPs product on adult mortality was also varied between the studied concentrations after three weeks of treatment where the concentration of 5000ppm was more influenced (87.48%) as compared to the control treatment which was (33.30%). The current result shown in table (4) clarified the effects of ZnO NPs produced from Eucalyptus plant on the mealworm progeny through feeding method in which there were significant variances between the total mean of the studied concentrations (3.30%) as compared to the control treatment (4.00%). However, the effects of ZnO on insects can vary depending on such factors as the insect species, concentration and size of the nanoparticles, and methods of exposure. The results shown in table (5) and figure (5) clarified the toxicity of the synthesized ZnO NPs on the larval stage of mealworm, supporting the efficacy of the formed ZnO NPs. A study by [32] showed the mortality of *T. vaporariorum* adults within 24 hours after applying ZnO nanoparticles. The mortality rate of *T. vaporariorum* adults was found to be concentration-dependent. This means that as the concentration of ZnO nanoparticles increased, the lethality also signific,antly increased. The study reported the mortality rates at different concentrations, where 3 mg resulted in a mortality rate of 21.6%, 5 mg had a mortality rate of 35%, 10 mg had a mortality rate of 53.3%, 15 mg had a mortality rate of 73.3%, and 20 mg had the highest mortality rate of 91.6%. Moreover, mortality observed on 14th day was 36.67% for 200 ppm [33]. Additionally, [34] showed that the effects of produced AgNPs towards of the vector mosquitoes *A. stephensi*, *A. aegypti* and *C. quinquefasciatus* had the following mortality values: *A. stephensi* had 100.0% ; *A. aegypti* had 95.3%; and *C. quinquefasciatus* had 93.4% for concentration 40 μg mL−1. The study confirmed that nanoparticles can have a range of effects on the insect stages, depending on the type, concentration, and duration of exposure. The impact of silver nanoparticles on insect larvae feeding habits caused damage to the cells and tissues of the insect's midgut, resulting in cellular gaps that affected the carbohydrate and protein levels in the insect's haemolymph [34]. The use of silver nanoparticles at concentrations of 1000 and 5000 parts per million may reduce the population of these insects, thereby overcoming problems associated with the use of chemical pesticides and their harmful effects. The current study results are consistent with other studies findings, suggesting that the feeding of insects during both their larval and adult stages, whether short or long-term, has a significant impact on insect's stages.

Table 3: the effects of ZnO NPs synthesized from Eucalyptus plant source with different concentrations on the Mealworm adult stage

Intervals	Concentrations(ppm)	Mean	F	P-Value	Sig.
24 h	control	$0\pm0^{(a)}$	0.000	1.000	NS
	250	$0\pm0^{(a)}$			
	1000	$0\pm0^{(a)}$			
	2500	$0\pm0^{(a)}$			
	5000	$0\pm0^{(a)}$			
1W	control	$0\pm0^{(a)}$	0.583	0.689	NS
	250	$0\pm0^{(a)}$			
	1000	$0\pm0^{(a)}$			
	2500	$0\pm0^{(a)}$			
	5000	$0\pm0^{(a)}$			

3W	control	33.30± 0.00 (c)	11.834	0.009	HS
	250	50.07±1.60 (b)			
	1000	50.07±1.60 (b)			
	2500	74.96±1.71 (a)			
	5000	87.48±1.65(a)			

Distinct letters imply significant differences.

Table 4: the efficiency of ZnO NPs synthesized from Eucalyptus plant source on mealworm progeny at optimal incubation condition

Plants	Total Mean ± SD	F-test	P-Value (Sig.)
Eucalyptus	3.30±0.63(b)	38.286	0.001 (HS)
control	4.00±0.15(a)	15.142	

Different letters indicate significant differences.

Table 5: the estimated LC50 of ZnO NPs synthesized from Eucalyptus plant source against the Mealworm larval stage after 5 weeks

Plant	Intervals	Concentrations (ppm)	Corrected mean mortality	LC 50	Slope equation	R^2	Toxicity Index LC50
Eucalyptus	Week 5	250	19.58	3630.78	y=0.788x+ 2.194	0.891	10.914
		1000	31.11				
		2500	38.47				
		5000	61.25				

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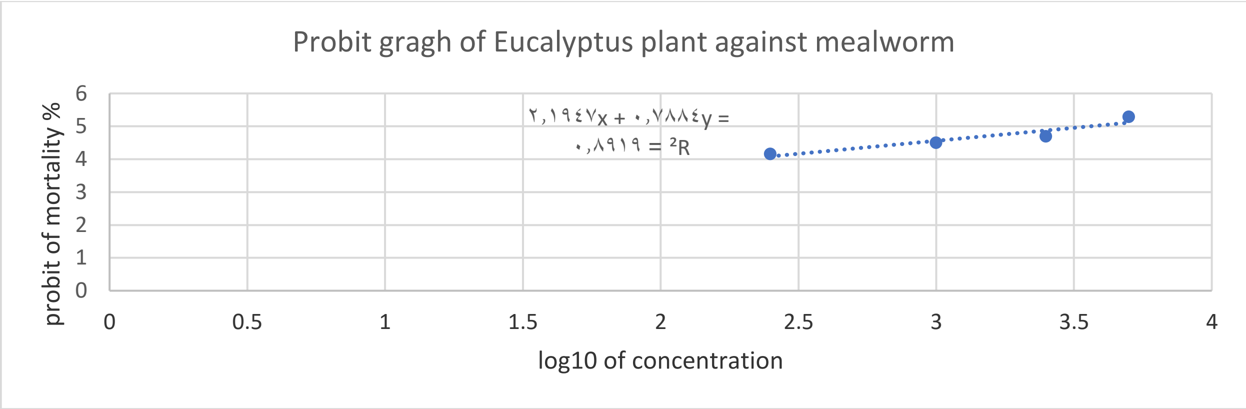

Figure 5: the toxicity curve of Eucalyptus plant against mealworm

CONCLUSIONS

The study on green method biosynthesis of zinc oxide nanoparticles involved a quick and cost-effective approach using an aqueous leaf extract of Eucalyptus. Various techniques such as UV-Vis spectroscopy, XRD, and TEM were employed to analyze and characterize the qualities of the ZnO nanoparticles synthesized through this green method. The results obtained showed that the characteristics of the ZnO nanoparticles were consistent with those produced using different methods. Additionally, the study highlighted the use of Eucalyptus plant leaf extract in the synthesis process. The study also examined the effects of ZnO nanoparticles on the mortality of Tenebrio molitor L.,1758 larvae and adults (mealworms). The highest average mortality rate of 61.25% was observed at a concentration of 500 ppm after 24 hours and 1 week, while the lowest average mortality rate of 0% was recorded at a concentration of 250 ppm during the same time period. The statistical analysis revealed significant differences in both the average mortality rate and adult emergence percentage among the studied concentrations. Furthermore, the results clearly demonstrated that after three weeks of treatment, the highest overall mean of adult mortality occurred at a concentration of 500 ppm (87.48%), whereas the lowest mean was approximately 0% for the first two weeks across all concentrations. However, after three weeks, the concentration of 500 ppm exhibited a greater impact (87.48%) compared to the control treatment (33.30%). Regarding the efficiency of ZnO nanoparticles on mealworms through the feeding method, there were significant differences in the overall mean of the studied concentrations (3.30%) compared to the control treatment (4%).

CONFLICT OF INTEREST STATEMENT

The results of the current study are part of the requirements of M.Sc. in Insects, Department of Biology, College of Education, University of Salahaddin for the first author. Also, we confirm and declare the absence of any relationship or conflict of interest with any other party.

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