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Behavioral Toxicity of Zinc Oxide Nanoparticle by Green Synthesis from Sideritis akmanii on Drosophila melanogaster

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Yeşil Sentez ile Sideritis akmanii'den Elde Edilen Çinko Oksit Nanopartikülünün Drosophila melanogaster'de Davranış Toksisitesi

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Abstract

Nanotechnology is related to nanoparticles with sizes between 1-100 nm. With the increasing technology, the usage areas of nanoparticles are rapidly spreading and today they are encountered in different areas from medicine to food industry, from textile to drug development studies. Although there are different synthesis techniques of nanoparticles, it is more preferred to obtain them through green synthesis as it is a reliable and environmentally friendly method. Within the scope of the project, the behavioral toxicity of ZnO nanoparticles, which were previously obtained and characterized by green synthesis with *Sideritis akmanii* plant, an endemic species and known as mountain tea among the public, was investigated on a model organism, *Drosophila melanogaster*. In the evaluation of behavioral toxicity; parameters such as larval weight, larval crawling, pupa formation success, pupa position test and life span were used. In all studies, nanoparticle concentrations of 0.625, 1.25, 2.5 and 5 mg/ml were applied and the obtained data were evaluated statistically. According to the experimental results, a decrease in larval weight was detected due to the increase in concentration and this decrease was significant for the concentrations of 1.25, 2.5 and 5 mg/ml when compared to the control group statistically. The changes in larval crawling, exit success and lifespan were found to be statistically significant in the 5 mg/ml application for all three experiments when compared to the control group. As a result, in the light of all these data obtained, it was determined that ZnO nanoparticles caused behavioral changes in *Drosophila melanogaster*.

Keywords: *Behavioral toxicity; Drosophila melanogaster; ZnO nanoparticle; Sideritis akmanii*

Öz

Nanoteknoloji boyutları 1-100 nm arasında olan nanopartiküller ile ilgilidir. Artan teknoloji ile birlikte nanopartiküllerin kullanım alanları hızla yayılmakta ve günümüzde tıptan gıda sanayisine, tekstilden ilaç geliştirme çalışmalarına kadar farklı alanlarda karşımıza çıkmaktadır. Nanopartiküllerin farklı sentezlenme teknikleri olmakla birlikte güvenilir ve çevre dostu bir yöntem olmasıyla yeşil sentez yoluyla elde edilmesi daha çok tercih edilmektedir. Çalışma kapsamında daha önce endemik bir tür olan ve halk arasında dağ çayı olarak bilinen *Sideritis akmanii* bitkisiyle yeşil sentezle elde edilmiş ve karakterizasyonu yapılmış olan ZnO nanopartiküllerinin bir model organizma olan *Drosophila melanogaster* üzerinde davranış toksisitesi araştırılmıştır. Davranış toksisitesinin değerlendirilmesinde; larval ağırlık, larval sürünme, pupa oluşturma başarısı, pupa pozisyon deneyi ve ömür uzunluğu gibi parametreler kullanılmıştır. Tüm çalışmalarda nanopartikülün 0.625, 1.25, 2.5 ve 5 mg/ml konsantrasyonları uygulanmış ve elde edilen veriler istatistiksel olarak değerlendirilmiştir. Deney sonuçlarına göre larval ağırlıkta konsantrasyon artışına bağlı olarak bir azalma tespit edilmiş ve bu azalma kontrol grubu ile karşılaştırıldığında 1.25, 2.5 ve 5 mg/ml konsantrasyonları için istatistiksel olarak anlamlı bulunmuştur. Larval sürünme, pupadan çıkış başarısı ve ömür uzunluğunda meydana gelen değişimler kontrol grubu ile karşılaştırıldığında her üç deney için de 5 mg/ml'lik uygulamada istatistiksel olarak anlamlı bulunmuştur. Sonuç olarak elde edilen tüm bu veriler ışığında ZnO nanopartiküllerinin *Drosophila melanogaster* üzerinde davranışsal değişikliklere sebep olduğu tespit edilmiştir.

Anahtar Kelimeler: *Davranış toksisitesi; Drosophila melanogaster; ZnO nanopartikülü; Sideritis akmanii*

1. Introduction

In recent years, there are more than 200 commercial, medical, personal, and military products that use nanoparticles. There are currently hundreds of nanotechnology products on the market, including sunscreens, textiles, sports equipment, veterinary drugs, and cosmetics (Brumfiel 2006, Griffitt et al. 2007). In nanotechnology, nanoparticle synthesis can be carried out by various physical, chemical, and biological means (Baran et al. 2020). Today, scientists widely prefer green synthesis for nanoparticle synthesis (Agarwal et al. 2017). The green synthesis method involves the process of

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synthesizing nanoparticles by treating extracts containing different plant parts, such as fruits, flowers, and roots, with a metal salt. It is an environmentally friendly, rapid, and economically advantageous experimental method. Additionally, the nanoparticles obtained by this method exhibit more biologically compatible and stable structures (Karnani and Chowdhary 2013). *Sideritis* species, which are consumed as herbal tea in Mediterranean countries including Türkiye, have also been known for many years to be used as medicinal plants to relieve various diseases. The genus *Sideritis*, which has properties such as solving digestive system problems, strengthening the immune system, antimicrobial and antioxidant, anti-infection, anxiety and pain reduction, carminative, antiviral and antifungal, is distributed in our country with a total of 45 species and 15 subspecies and 60 taxa. This plant's endemism rate in Türkiye is approximately 66.6% (Gümüşçü et al. 2011). When various studies conducted on *Sideritis* species were examined, it was determined that the extracts obtained from plants belonging to this genus have anti-inflammatory, analgesic, antibacterial, antioxidant and anti-stress effects (Berber and Aksoy 2021). This plant, endemic to Afyonkarahisar, grows only in the Kumalar plateau (Aksoy et al. 2022).

In recent years, the most researched nanoparticles include gold (Au), zinc (Zn), copper (Cu), silver (Ag), titanium (Ti), iron (Fe), and palladium (Pd). The properties and sizes of nanoparticles obtained by environmentally friendly green synthesis vary depending on the plant species and plant parts studied. For this reason, researchers continue to produce various nanoparticles by green synthesis method using different plant species and plant parts and investigate their properties (Khan et al. 2021). Zinc oxide nanoparticles are one of the most widely used nanoparticles and are a group that has attracted attention in the scientific world for their unique properties. Zinc oxide nanoparticles, which are considered safe by the United States Food and Drug Administration (FDA) due to their biocompatibility, are used in various biomedical applications (Sahoo et al. 2007). Zinc oxide nanoparticles are used in important processes such as anticancer, anti-inflammatory, antidiabetic, antimicrobial, UV blocking, wound healing, bioimaging and drug delivery (Dutta and Sugumaran 2021).

Model organisms are living beings that enable studies to understand biological events and processes. Model organisms, which are easier to grow in a laboratory environment than other organisms, play a key role in explaining the biological processes of organisms that are difficult to study. *Drosophila melanogaster* (fruit fly) is one of the most frequently used model organisms and is used in many research areas such as molecular genetics, population genetics, developmental biology and neuropharmacology (Kence et al. 2010). Furthermore, approximately half of *Drosophila* proteins are similar to mammalian proteins used in human diseases, making it an advantageous model organism. More than 60% of the genes identified in human diseases are similar to *Drosophila* genes. Thus; approximately 287 genes that undergo changes due to mutation, amplification or deletion in humans are *Drosophila* orthologs (Potter et al. 2000).

There are previous studies on the toxicity assessment of different nanoparticles, but the number of studies in this area is not yet sufficient. Moreover, different nanoparticle synthesis methods may cause different effects on organisms. For all these reasons, it is important to eliminate question marks at different points in this field with this project topic. The aim of the project was to investigate whether the application of nanoparticles previously by the green synthesis method with *Sideritis akmanii* to *Drosophila melanogaster* at different rates caused behavioral changes.

2. Materials and Methods

2.1. Drosophila melanogaster

8-hour-old eggs belonging to the Oregon R+ line of *Drosophila melanogaster* were used throughout the study. When the eggs reached the 3rd larval stage (approximately 72±4 hours old), they were treated with three different doses of ZnO nanoparticles. Exposure was achieved by soaking the 72±4-hour-old larvae in medium containing approximately 4.5 grams of dry *Drosophila* Instant Medium and 9 ml of ZnO nanoparticles at different concentrations. Distilled water was used as a negative control, and data were compared with this group. All experiments were set up in triplicate.

2.2. Larval Weight

Larvae were weighed both before and after nanoparticle exposure. Larvae treated with different concentrations of nanoparticles were then collected with a fine mesh and washed with tap water to prevent contamination. Then, 10 larvae for each dose application were weighed again after 24 hours of exposure. The experiment was carried out independently with 3 replicates and the results were averaged.

2.3. Larval Crawling

Petri dishes coated with 2% agar were used to measure larval movement. Larvae were placed in the center of the petri dish and held for one minute, and the distance traveled by the larvae was measured with graph paper. The experiment was carried out independently with 3 replicates for each concentration and the average was taken.

2.4. Pupa Formation Success and Pupal Position

Larvae were fed with medium containing different concentrations of nanoparticles until the larval period began. Larvae were recorded and hatching success was calculated as a percentage with the formula; "(number of adults/50) x %100". These measurements were made according to Fauzi et al. (2020). The results were determined as a percentage of the pupa position. The measurements continued until the pupa formation ended. The 50 mL falcon tubes used in the pupa position test were divided into 4 different regions starting from the food surface and at equal intervals of 1.3 cm. These regions were named D, C, B, and A, respectively, from the food surface. After the application phase, the locations of the pupae were determined.

2.5. Lifespan

For each experimental group, 50 larvae of the 3rd instar stage were placed in a food medium prepared by wetting approximately 4.5 grams of dry *Drosophila* instant medium with 9 ml of different concentrations of ZnO nanoparticles. The experiment was performed with three repetitions for each concentration. For the sustainability of the experiment, according to Lindford et al. (2013), after the larvae became adults, they were taken to new application tubes every 2 days to ensure the continuity of the ZnO nanoparticles. Flies that died during the transfers that took place every two days were noted.

2.6. Statistical Analysis

Statistical calculations of the results obtained from the larval weight and larval movement measurements were performed with the One-way ANOVA (Dunnet) test in the SPSS 20 Package Program. Pupa formation and hatching success experiments were evaluated with the Kruskal-Wallis test; the lifespan measurement results were evaluated with the Kaplan-Mayer test; and the pupal position experiment results were evaluated with the chi-square test.

3. Results and Discussions

3.1. Larval Weight

As a result of *Drosophila melanogaster* larvae exposed to ZnO nanoparticles for 24 hours, the larval weight decreased depending on the dose increase. The decrease in larval weight at 1.25, 2.5 and 5 mg/ml application doses was significant when compared to the control group statistically. Although there was a decrease in larval weight at the 0.625 mg/ml application dose, it was not statistically significant. Larval weight changes are given in Figure 1.

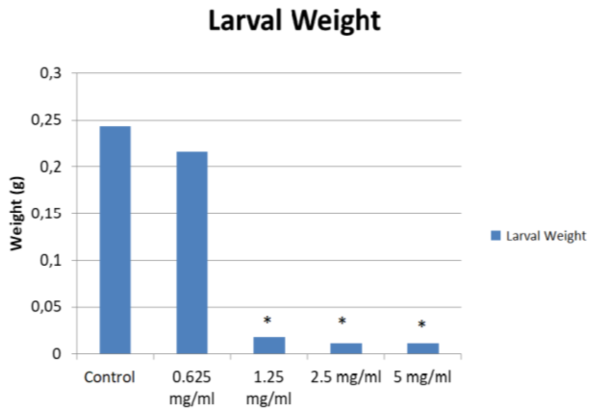

Figure 1. Larval weight changes in *Drosophila melanogaster* larvae as a result of ZnO nanoparticle exposure, *; statistically significant change ($p<0.05$).

3.2. Larval Crawling

In the larval crawling experiment, all groups that were applied with nanoparticles moved slower and covered less distance than the control group. The 0.625 mg/ml application dose showed a value close to the control group. However, as a result of the measurement of larval movement, it was determined that the application of ZnO nanoparticles at a concentration of 5 mg/ml caused a statistically significant decrease in larval movement compared to the control group. The larval crawling experiment results are given in Figure 2.

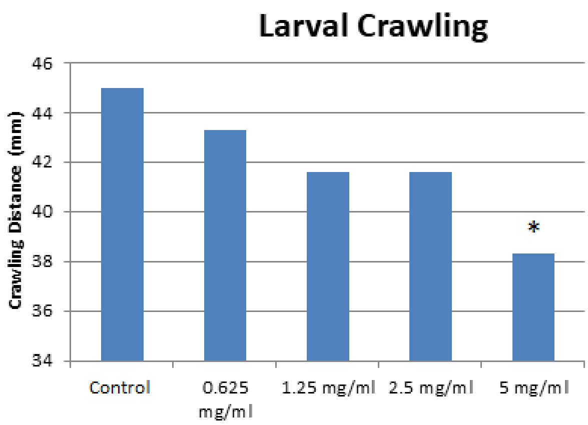

Figure 2. Changes in larval movement distance as a result of ZnO nanoparticle exposure in *Drosophila melanogaster* larvae, *; statistically significant change ($p<0.05$).

3.3. Pupa Formation Success

As a result of measuring the pupa formation success, it was reported that 86% of the larvae in the control group were able to form pupae. The application dose of 0.625 mg/ml showed a value very close to the control group with 85%. In the applications of 1.25 and 2.5 mg/ml, the success of hatching from pupae was determined as 76%

and 64%, respectively, but this change was not significant statistically. However, the highest application dose of 5 mg/ml decreased the success of hatching from pupae to 21% and this decrease was found to be statistically significant (Figure 3).

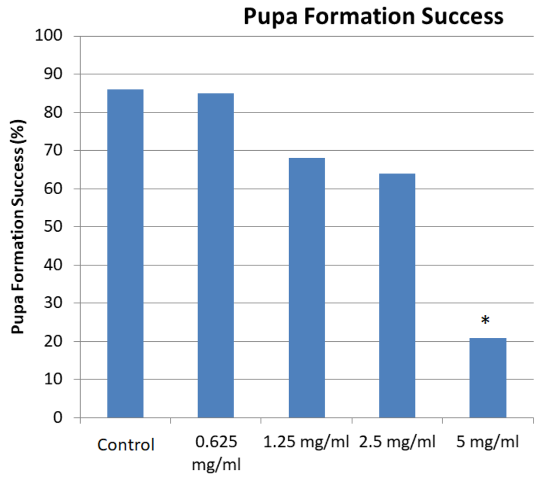

Figure 3. Pupal hatching success in *Drosophila melanogaster* larvae as a result of ZnO nanoparticle exposure, *; statistically significant change ($p<0.05$).

3.4. Pupa Position

According to the pupa position measurement results, the larvae in the control group tended to form more dense pupae in the D and C regions. Pupa positions varied in different application groups (Figure 4). However, these changes were not significant statistically. Although it was not seen as a statistically significant change, there was a move away from the D and C regions in the 5 mg/ml application group, especially preferring the A and B regions. At the 0.625 mg/ml application dose, a higher pupa formation was observed in the B region compared to the other application groups. However, all these changes did not show a statistically significant result.

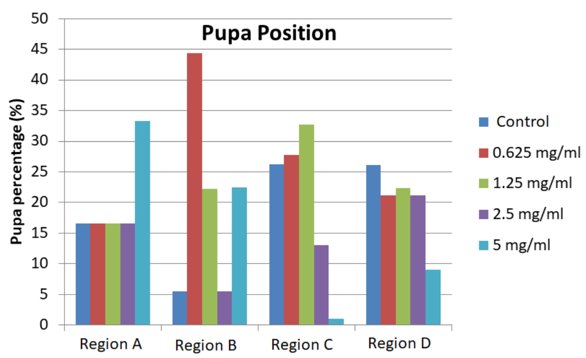

Figure 4. Pupal position placement in *Drosophila melanogaster* larvae following exposure to ZnO nanoparticles.

3.5. Lifespan

The data obtained from the experiment conducted by exposing *Drosophila melanogaster* larvae to different concentrations of ZnO nanoparticles from egg onwards are given in Figure 5. As a result of the lifespan experiment, it was determined that the group that was first applied the highest concentration of 5 mg/ml ZnO nanoparticles died 8 days after they became adults. The lifespan of the other groups was close to control group. According to the statistical analysis results, a significant decrease was detected in the 5 mg/ml concentration in terms of average lifespan among the concentrations.

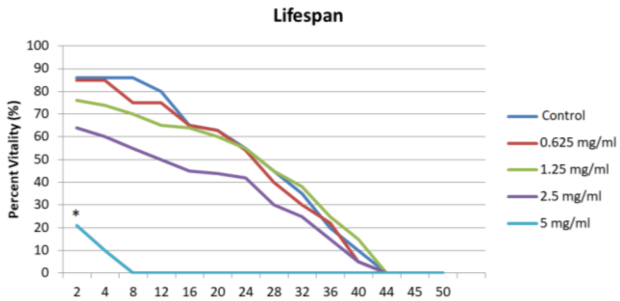

Figure 5. Lifespan in *Drosophila melanogaster* larvae as a result of ZnO nanoparticle exposure, statistically significant change ($p<0.05$).

4. Discussion

Nanoparticles are materials that are very important for humans and the environment due to their unique and extraordinary properties. The interest in the production of nanoparticles, which have many areas of use in terms of improving people's quality of life, eliminating various health problems and environmental sustainability, is increasing day by day. Various nano-scale materials designed using nanoparticles are the focus of attention in many fields, especially science and technology (Sanna and Sechi 2012). Since nanoparticles are important materials in many fields, the advantages and disadvantages of different production methods have become an important topic for scientists (Jamkhande et al. 2019). Green synthesis application, which has many advantages over physical and chemical methods, is preferred today because it is an ecologically friendly method (Shanmuganathan et al. 2019). The nanoparticles used in the study were obtained by green synthesis method and characterized by different techniques.

Nanoparticles are increasingly used in many research and technology branches such as medical diagnostic and treatment devices and consumer-related areas such as the food sector due to their application potential (Mittag et al. 2019). Their use in the preservation or improvement of foods can result in oral ingestion of nanoparticles (McClements and Xiao 2017). Due to their small size, nanoparticles can penetrate the blood and lymphatic system and as a result spread to body tissues and organs (De Matteis 2017). For all these reasons, the evaluation of the toxicity of nanoparticles that we may encounter in many places around us is a very important research topic. In this study, the behavioral toxicity of ZnO nanoparticles on *Drosophila melanogaster* was evaluated.

Metal oxide nanoparticles are known to have toxic potential (Ivask et al. 2015). The mechanism behind this toxicity is intracellular metal ion release, and the cytotoxic properties of metal oxide nanoparticles are formed in this way. The released metal ions show their harmful effects by causing inflammation and oxidative stress in the cell (Horie et al. 2018). There are also different studies showing that metal oxide nanoparticles induce genotoxicity in *Drosophila melanogaster* (Güneş et al. 2018, Ertuğrul et al. 2020). As a result of a study investigating the genotoxic effects of ZnO nanoparticles, a metal oxide nanoparticle, an increase in DNA damage and micronucleus frequency was detected in HEK293 (human embryonic kidney cell line) and NIH/3T3 (mouse embryonic fibroblast cells) cell lines exposed to this nanoparticle (Demir et al. 2014). In this study, ZnO nanoparticles were used in a similar way and when compared to the control group in the behavioral tests, statistically significant changes were detected in larval weight, larval crawling, success of hatching from pupa and lifespan experiments. Especially in the 5 mg/ml concentration application, lifespan decreased significantly compared to the control group and there were no living individuals left at the end of the 8th day. It is known that nutrient content and ambient temperature have an effect on survival and reproduction ability for *Drosophila melanogaster* (Min et al. 2021). Pompa et al. (2011) applied citrate-coated gold nanoparticles to *Drosophila melanogaster* and found that these nanoparticles reduced lifespan similar to present study. Again, Kurşun et al. (2021) found that MgO nanoparticle application had a negative effect on lifespan in *Drosophila melanogaster*.

Because behavioral tests are the first measurable parameter of chemical exposure, they are widely used techniques in the toxicological evaluation of all kinds of chemicals and newly synthesized substances (Chen et al. 2014). Different behavioral responses can occur in living beings before structural lesions and clinical symptoms resulting from chemical exposure appear, and these changes can be determined through behavioral tests (Philbert 2010). It is possible to predict many adverse effects of the evaluated xenobiotic, nanoparticle or any chemical substance by correctly evaluating the behavioral responses.

Previous studies have determined that exposure to nanoparticles causes various behavioral disorders in the organism (Asmonaite et al. 2016, Chen et al. 2020). In their behavioral toxicity study on zebrafish, Asmonaite et al. (2016) determined that exposure to Ag^+ and Ag nanoparticles caused negative effects on development in zebrafish, prevented hatching from eggs and changed the fish's mobility, and thus caused immobility. In the current study, similarly, it was determined that there was a significant decrease in the success of hatching from pupae when compared to the control group at the last dose applications statistically. Again, in this study, larval movement decreased significantly at the last application dose of 5 mg/ml in the larval crawling experiment and similar results were obtained with the result that the mobility changed in zebrafish. In addition, similar to the inhibition of hatching from eggs seen in zebrafish, the decrease in the success of hatching from pupae in this study is parallel to the study conducted by Asmonaite et al. (2016). This situation is a good example of how study results can be similar even if the model organism of nanoparticle exposure changes.

In this study, the change in weights in 1.25, 2.5 and 5 mg/ml dose applications in the larval weight experiment was significant statistically. Similarly, Kurşun et al. (2021) detected a decrease in larval weight in *Drosophila melanogaster* with MgO nanoparticle application, but they showed that there was no statistically significant change compared to the control group, unlike the current study. Most living things have to determine their own weight throughout their lives and adjust their muscle strength accordingly in order to survive (Knight 2017). In the study conducted by Schilder and Raynor (2017) with *Drosophila melanogaster*, it is thought that they increase their muscle strength with the increase in weight and increase their mobility. In the study, the decrease in larval weight occurred in 1.25, 2.5 and 5 mg/ml dose applications, and similarly, the decrease in movement observed in the 5 mg/ml larval crawling experiment was thought to be due to a decrease in muscle mass due to weight loss.

In our study, in the pupa position measurement experiment, the application groups caused changes in the pupa positions. Although these changes did not create a statistically significant result, there was a move away from the D and C regions and a preference for the A and B regions in the 5 mg/ml application group. In fact, it is known that the position of the pupa reflects the energy of the larvae (Fauzi et al. 2020). Especially at this application dose, the preference of areas away from the food for pupa formation in our study was interpreted as a tendency to move away from the presence of nanoparticles rather than high energy.

5. Conclusion

As a result, in light of all the data obtained, it was determined that ZnO nanoparticles caused behavioral changes on *Drosophila melanogaster*. It was revealed that these changes were statistically significant in certain dose applications in larval weight, larval crawling, success of hatching from pupa and lifespan. The fact that there was an increase in these changes depending on the increase in nanoparticle dose indicates that behavioral changes may increase in higher nanoparticle applications. Nanoparticles obtained with different metal salts, which are used in many areas today, have created similar effects on various model organisms. However, more advanced studies are needed to elucidate the molecular mechanisms of behavioral disorders caused by ZnO nanoparticles on *Drosophila melanogaster*. In light of all this information, it was concluded that research on nanoparticles, which are gaining more and more place in our lives with the developing technology, should be increased.

Declaration of Ethical Standards

The authors declare that they comply with all ethical standards.

Credit Authorship Contribution Statement

Author-1: Conceptualization, methodology, Data curation
Author-2 : Conceptualization, methodology, Data curation
Author-3: Conceptualization, Resources
Author-4: Conceptualization, investigation, methodology, software, and study design
Author-5: Conceptualization, methodology and supervision and writing – review and editing
Author-6: Conceptualization, methodology and supervision

Declaration of Competing Interest

The authors declare that they have no known competing fnancial interests or personal relationships that could have appeared to infuence the work reported in this paper.

Data Availability Statement

All data generated or analyzed during this study are included in this published article

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