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Effect of Spiromesifen on Hemocyte Level and Micronucleus Frequency in *Galleria mellonella* Larvae

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ABSTRACT

In this study, the immunotoxic and genotoxic effects of Spiromesifen on *Galleria mellonella* larvae were investigated. For this purpose, Spiromesifen was injected into *Galleria mellonella* larvae at various doses (10, 20, 30, 40, 50, 60 µg) and time intervals (24, 48, 72 hours) and its effects on hemocyte count and micronucleus frequency were investigated. As a result of the research, while the hemocyte count did not show a significant change at 48 and 72 hours at 10µg and 20µg doses compared to the control group, a serious decrease in the hemocyte count was observed at the highest dose of 60µg. In the study, it is thought that the decrease in the total number of hemocytes may be due to increased necrosis due to cellular damage in hemocytes under oxidative stress caused by high doses of spiromesifen or to the induction of apoptosis due to increased ROS under oxidative stress. With the increase in Spiromesifen concentration in the hemocytes of *Galleria mellonella*, a significant increase in the number of micronuclei was observed compared to the control group. This clearly shows that Spiromesifen induces micronucleus formation in *Galleria mellonella* hemocytes and has a genotoxic effect.

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Introduction

Chemical pesticides are essential in agricultural practices for managing insect pests, protecting crop yields, and safeguarding food security. However, their extensive application has raised significant concerns regarding potential adverse effects on ecosystem health, biodiversity, and non-target species (Meftaul et al., 2020; Rani et al., 2021). Acaricides, a class of pesticides developed to combat mites, are particularly effective in controlling phytophagous mites, which represent a considerable threat to agricultural productivity. Spiromesifen is a widely used acaricide and insecticide belonging to the ketoenol chemical group, and exhibits activity by disrupting the basic physiological processes of target organisms, inhibiting lipid biosynthesis (Yu et al., 2022). Although its efficacy against target pests is well documented, its effect on non-target species is poorly understood. There are limited studies investigating the biochemical, physiological and immunotoxic effects of spiromesifen on non-target organisms, raising concerns about potential sublethal and chronic toxicity in ecological systems (Dudwal et al., 2024).

Galleria mellonella (Greater wax moth, Pyralidae) is extensively employed as a model organism in studies of insect physiology, toxicology, and immunology, owing to its biological similarities with other insect species and its well-characterized immune responses (Ellis et al., 2013). The larval stage of *G. mellonella* is particularly valuable for toxicological studies as it has high sensitivity to environmental stressors such as chemical exposures and exhibits immune responses similar to higher invertebrates (Fernandes et al., 2017). Hemocytes, the main immune cells in insects, undertake important functions such as recognition of pathogens, phagocytosis, encapsulation and detoxification of harmful substances (Kurt & Kayis, 2015). Investigating the effects of spiromesifen on hemocyte density and subtypes is expected to yield critical insights into the immunotoxic potential of this pesticide and its broader impact on the insect immune system. Genotoxicity assessment is a crucial component of pesticide toxicity studies, as pesticides have the potential to induce DNA damage. This damage can lead to mutations, chromosomal abnormalities, and may have broader population-level effects on non-target organisms. The micronucleus test is a genetic marker commonly used to assess genotoxic damage and micronucleus formation is considered an indicator of chromosomal instability and DNA breaks (Guo et al., 2018). Assessing the frequency of micronuclei in hemocytes after spiromesifen exposure will contribute to risk assessments of environmental and ecological effects of this pesticide by revealing its potential to cause genotoxic effects.

Materials and Methods

Test Organism and Spiromesifen

Following the method outlined by Bronskill, (1961), *G. mellonella* larvae were maintained in a controlled environment with a temperature of 28°C ± 2°C and a relative humidity of 60 ± 5%. The experiments were conducted in quadruplicate, utilizing last-stage (7th instar, 200–250 mg) larvae with an average weight of 250 ± 50 mg. The larvae were cultured in glass jars and provided with semi-synthetic nutrients for optimal growth. Spiromesifen (Formula: $C_{23}H_{30}O_4$, Aarabus SC 240, Bayer) was sourced from a local market in Gaziantep, Turkey.

Determination of LD_{50} value

To determine the spiromesifen LD_{50} value, we selected larvae that had reached their final developmental stage. These larvae were injected with different doses of the chemical using a Hamilton-brand syringe. First, a stock solution was prepared with Aarabus SC 240 Spiromesifen. From this stock, we prepared various concentrations of solutions by carefully diluting the original mixture with distilled water. The control group was treated with only distilled water to ensure proper comparison. During the preparation, we made sure to mix everything thoroughly to avoid any residue settling at the bottom. The experiments were repeated four times, with 10 larvae tested at each dose. After 96 hours, we recorded and photographed the larvae to note how many had survived and how many had died (Finney, 1971). Using probit analysis, we determined the LD_{50} value to be approximately 13.57 µg/larva, based on an average larval weight of 250 ± 50 mg. To estimate the LD_{50}, a wide dose range (e.g., 5 µg to 80 µg per larva) was tested to obtain mortality rates spanning from 0% to 100%.

Experimental Design

Two distinct groups of *Galleria mellonella* larvae were created: the control, and spiromesifen groups. While the control group was left untreated, different doses of spiromesifen solution (10 µg-60 µg) were administered to the spiromesifen group. 10 µL of the fluid was injected into each larva's left hind leg using a Hamilton microinjection needle.

Total Hemocyte Counts

In this study, total hemocyte counts (THC) were measured at 24, 48 and 72 hours in quadruplicate, with a total of 84 larvae (21 larvae per replicate). The method described by Jones, (1962) was used to determine THC. To begin, the larvae were placed on ice to reduce movement. A lancet was then used to gently pierce the hind legs, and 5 µL of hemolymph was extracted using a pipette. The hemolymph was immediately mixed with Tauber-Yeager solution (Tauber & Yeager, 1936) in a 1:10 ratio. The mixture was homogenized by repeatedly aspirating and dispensing with a micropipette. Next, 10 µL of the homogenized mixture was loaded onto an Improved Neubauer hemocytometer (Superior, Germany), and hemocyte counts were performed under an Olympus CX21 light microscope. THC values were calculated and expressed as the number of cells per milliliter of hemolymph.

Total Micronucleus Count

Total micronucleus counts were determined following the methodology established by Venier et al. (1997). In brief, for each experimental group (including the control and each treatment dose), three larvae were randomly selected, punctured with a sterile needle, and their hemolymph was carefully spread onto separate glass slides for analysis. The smear was air-dried for 15 minutes, then fixed in methanol for 5 minutes. Following fixation, the slide was stained with a 10% Giemsa solution in Sorensen Buffer (pH 6.8) for 10 minutes. After rinsing the slide with distilled water, 1,000 hemocytes were counted per slide for each replicate. To ensure the results' reproducibility and accuracy, the procedure was repeated five times.

Statistical Analysis

The LD_{50} value of spiromesifen at 96 hours was determined using Finney's, (1971) probit analysis. The data were analyzed using the SPSS 21.0 program. For all replicates, the mean, standard error, and significance values were calculated and presented as mean ± SE. The Levene test was conducted to assess the homogeneity of the data distribution. Based on the results of the Levene test, which showed a homogeneous distribution, a one-way analysis of variance (ANOVA) was performed to identify differences between groups. To further assess the differences between group means, Student-Newman-Keuls (SNK) analysis was used. Pearson's correlation analysis was applied to evaluate relationships between continuous variables. A significance level of $p < 0.05$ was used for all statistical tests.

Results

Hemocyte Count

In the control group, hemocyte count was measured as 211.00±7.02 at 24 hours, 213.75±17.80 at 48 hours and 247.25±12.33 at 72 hours, respectively. While no significant change was observed in hemocyte count at 10-20 µg doses, a significant decrease was detected at 30-60 µg doses ($p<0.05$). At 60 µg dose, hemocyte count decreased to 127.50±19.27 at 24 hours ($p<0.05$). This decrease indicates that the immune system was negatively affected (Table 1).

Total Micronucleus Count

While the number of micronuclei was observed at very low levels in the control group, a significant increase in the number of micronuclei was observed with the increase in Spiromesifen dose ($p<0.05$). At 60 µg dose, the micronucleus number was measured as 2.00±0.81 at 48 hours and a significant increase was recorded compared to the control group ($p<0.05$). These findings suggest that Spiromesifen may have genotoxic effects (Figure 1, Table 2)

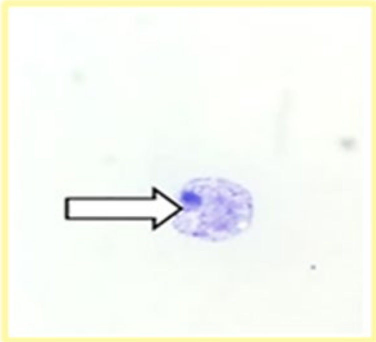

Figure 1. Light microscope image of micronucleated cells

Table 1. Effect of spiromesifen on total hemocyte count in G. mellonella larvae

		24 Hours (mean±SE)	48 Hours (mean±SE)	72 Hours (mean±SE)
Hemocyte	Control	211.00±7.02a,b,c,d,e	213.75±17.80a,b,c,d,e.f	247.25±12.33a,b,c,d
	10µg	195.50±36.09f,g,h,i,j	199.25±6.65a,g,h,i	227.50±26.98e,f,g,h
	20µg	166.25±7.36a,f,k,l	177.25±10.21b,j	219.25±19.25i,j,k,l
	30µg	161.00±13.08b,g,m	181.25±11.67c,k,l	173.25±26.14a,e,i,m
	40µg	148.75±11.95c,h	166.50±21.12d,g	138.75±22.72b,f,j,m
	50µg	138.00±14.07d,i,k	158.50±9.74e,h,k	149.50±25.52c,g,k
	60µg	127.50±19.27e,j,l,m	152.00±11.16f,i,j,l	145.75±11.75d,h,l

Data were expressed as mean ± Std., Same letters (a, b, c, d, and e) in the same column indicate statistical differences between groups at the P<0.05 level.

Table 2. Effect of spiromesifen on total micronucleus count in G. mellonella larvae

		24 Hours (mean±SE)	48 Hours (mean±SE)	72 Hours (mean±SE)
Micronucleus	Control	0.25±0.50a, b	0.00±0.00a	0.00±0.00a, b
	10µg	0.25±0.50c, d	0.50±0.57b	0.25±0.50c
	20µg	0.50±0.57e	0.25±0.50c	0.25±0.50d
	30µg	1.25±0.95	0.75±0.95d	0.25±0.50e
	40µg	0.75±0.50	0.25±0.50e	0.50±0.57f
	50µg	1.50±0.129a, c	0.75±0.50f	1.00±0.81a
	60µg	1.75±0.95b, d,e	2.00±0.81a, b,c,d,e.f	2.00±0.81b, c,d,e,f

Data were expressed as mean ± Std., Same letters (a, b, c, d, and e) in the same column indicate statistical differences between groups at the P<0.05 level.

Discussion

Pesticides can enter the organism through food and drinking water as a result of environmental transport to air, water, soil and food by humans or anthropogenically. This causes toxic substances to accumulate in organisms and poses a danger to living things (Wu et al., 2016). Occupational exposure to pesticides has also been linked to a wide range of conditions, including neurological diseases, cancer, reproductive disorders and nervous system disorders in the medium and long term. Exposure is usually due to pesticide use (Gangemi et al., 2016; Sharma et al., 2020). Spiromesifen is a non-systemic pesticide with contact action used in the fight against mites and whiteflies in many products such as pepper, tomato, corn and melon (Bretschneider, 2003; Nauen et al., 2003). Spiromesifen is a keto-enol insecticide/acaricide produced from tetramic acid that stops lipid biosynthesis by inhibiting acetyl-CoA carboxylase, especially damaging young stages and reproduction of adult mites (Bretschneider, 2003) and reducing total carbohydrate and glycogen content in *Drosophila melanogaster* (Kissoum & Soltani, 2016) and *Culiseta longiareolata* (Bouabida et al., 2017). In the study, the LD_{50} value was first determined to determine sublethal doses. Using probit analysis, we determined the LD_{50} value as 54.28 µg/kg. From these findings, we determined the sublethal doses by testing 10 µL concentrations at 10 µg, 20 µg, 30 µg, 40 µg, 50 µg and 60 µg for each larva. Our experimental results showed that Spiromesifen caused dose-dependent mortality in *G. mellonella*. The LD_{50} value is important in terms of evaluating the acute toxic effects of chemical substances due to short-term exposure. The blood cells known as hemocytes, which form the basis of the cellular defense system in insects, play an important role in defense against microorganisms. In addition, hemocytes play an important role in the detoxification of toxic substances and the transport of hormones and metabolites (Prakash, 2008). Therefore, it is inevitable for insects with open circulatory systems to be affected by toxic chemicals taken into the body. In the presented study, it is seen that the total hemocyte counts of *G. mellonella* larvae generally decrease under the effect of spiromesifen. In the study conducted to determine the effect of spiromefene on the hemocyte count of *G. mellonella*, in the control group, the hemocyte count remained relatively constant over the 72-hour period and reached the values of 211.00±7.02 at 24 hours, 213.75±17.80 at 48 hours and 247.25±12.33 at 72 hours. No significant change was observed in the hemocyte count at doses of 10-20 µg, indicating that these doses did not affect the immune system. However, a significant decrease in hemocyte count was observed at the higher doses (30-60 µg) ($p<0.05$), particularly at the 60-µg dose, where the hemocyte count dropped to 127.50±19.27 at 24 hours ($p<0.05$). This decrease suggests a negative impact on the immune system, potentially weakening the larvae's immune defense. Similar results were reported in the study by (Aytekin & Kayis, 2024), where a significant increase in micronucleus count in *Galleria mellonella* larvae was observed at 24, 48, 72, and 96 hours after the application of a 15-µg dose of thiacloprid. Hemocytes play an important role in the immune system of insects. The amount of hemocytes in some organisms is considered a biomarker for assessing the innate immunological effect of invertebrates (Kacsoh & Schlenke, 2012). In terms of genotoxicity, the cotrol group showed very low micronucleus count. However, a significant increase in the micronucleus count was observed as the Spiromesifen dose increased ($p<0.05$). At the 60-µg dose, the number of micronuclei was measured at 2.00±0.81 at 48 hours, representing a significant increase compared to the control group ($p<0.05$). This increase suggests that Spiromesifen may have genotoxic effects, causing DNA damage in the larvae. These findings highlight the potential risks associated with high doses of Spiromesifen, including immune suppression and genetic damage. Previous studies have demonstrated that exposure to low levels of pesticides can lead to various effects, including the formation of protein or DNA adducts, as well as the induction and inhibition of enzymes (Kocaman et al., 2014). Consistent with these findings, (Kayis et al., 2019) and (Aytekin & Kayis, 2024) reported a significant dose-dependent increase in the number of micronuclei, which aligns with the results observed in our study.

Conclusion and Suggestions

In the study, it was observed that Spiromesifen doses caused genotoxic effects, DNA damage and micronucleus formation. These findings indicate that high doses of Spiromesifen can cause serious biological effects such as oxidative stress, suppression of cellular defense mechanisms and genetic disorders in organisms. In conclusion, this study emphasizes the importance of dose regulation in pest control strategies. In addition, *G. mellonella* can be used as a bioindicator to show the effects of pollutants on non-target organisms.

Declarations

Author Contribution

Execution research project, Experimental design, Data analysis, Manuscript preparation- *H.U.A* Experimental design, Data analysis, Manuscript preparation- *H.U.A*; Materials, Supervision, Writing -review & editing. *H.U.A*; Experimental design, Data analysis, Manuscript preparation. *H.U.A*; Materials *H.U.A.*

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Ethics Committee Approval

For this type of study, formal consent is not required.

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