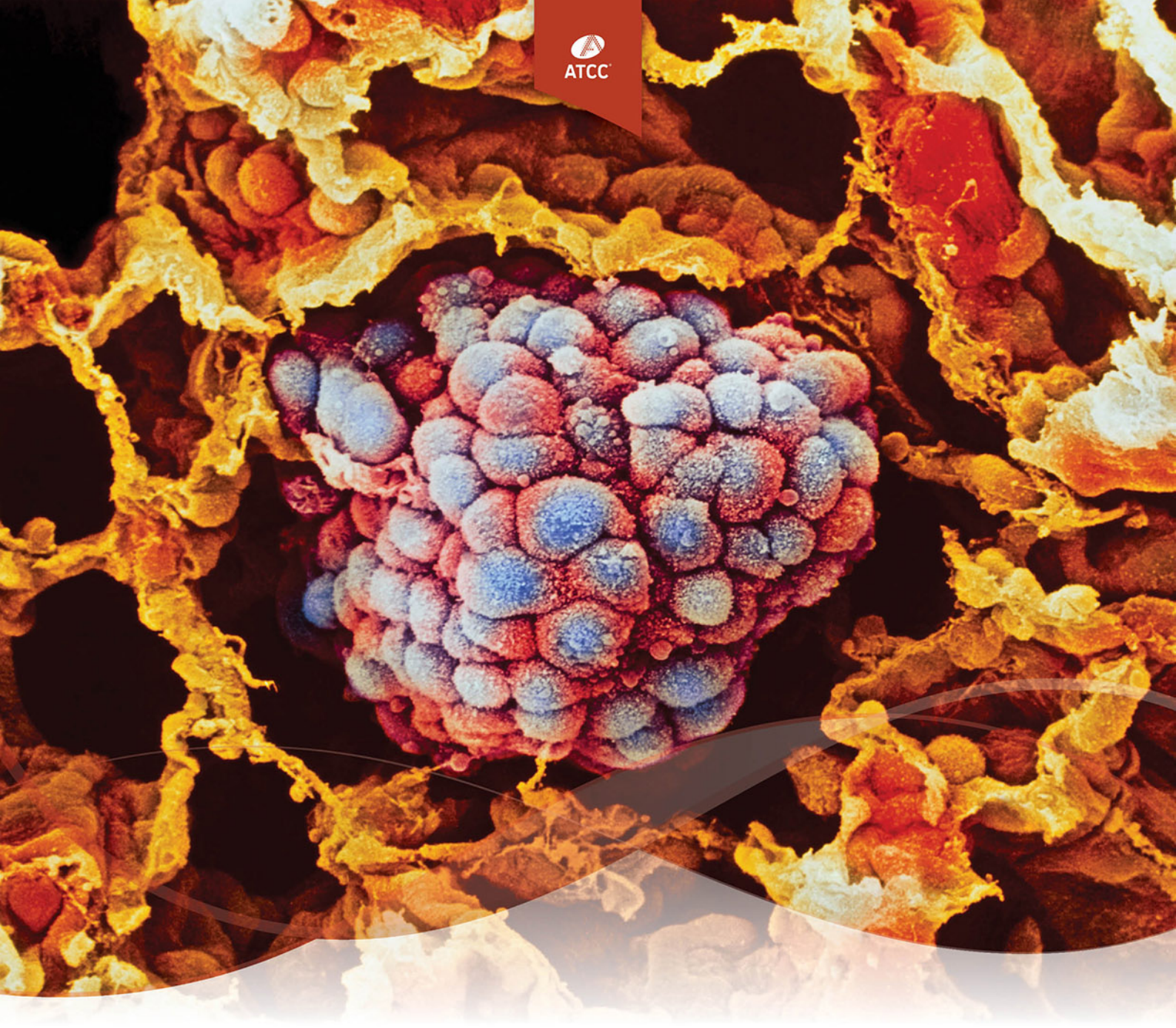

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RESEARCH ARTICLE

In vivo evaluation of the neurogenotoxic effects of exposure to validamycin A in neuroblasts of *Drosophila melanogaster* larval brain

Fatma Turna Demir[1,4] | **Eşref Demir**[2,3,4]

[1]Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts, USA

[2]F.M. Kirby Neurobiology Center, Boston Children's Hospital, Boston, Massachusetts, USA

[3]Department of Neurobiology, Harvard Medical School, Boston, Massachusetts, USA

[4]Department of Medical Services and Techniques, Medical Laboratory Techniques Programme, Vocational School of Health Services, Antalya Bilim University, Antalya, Turkey

Correspondence
Eşref Demir, F.M. Kirby Neurobiology Center, Boston Children's Hospital, 300 Longwood Avenue, Boston, MA 02115, USA.
Email: esref.demir@antalya.edu.tr

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Abstract

Agriculture commonly utilizes crop protection products to tackle infestations from fungi, parasites, insects, and weeds. Validamycin A, an inhibitor of trehalase, possesses antibiotic and antifungal attributes. Epidemiological evidence has led to concerns regarding a potential link between pesticide usage and neurodegenerative diseases. The fruit fly, *Drosophila melanogaster*, has been recognized as a reliable model for genetic research due to its significant genetic similarities with mammals. Here, we propose to use *D. melanogaster* as an effective in vivo model system to investigate the genotoxic risks associated with exposure to validamycin A. In this study, we performed a neurotoxic evaluation of validamycin A in *D. melanogaster* larvae. Several endpoints were evaluated, including toxicity, intracellular oxidative stress (reactive oxygen species), intestinal damage, larval behavior (crawling behavior, light/dark sensitivity assay, and temperature sensitivity assay), locomotor (climbing) behavior, and neurogenotoxic effects (impaired DNA via Comet assay, enhanced by Endo III and formamidopyrimidine DNA glycosylase [FPG]). The results showed that exposure to validamycin A, especially at higher doses (1 and 2.5 mM), induced DNA impairment in neuroblasts as observed by Comet assay. Both larvae and adults exhibited behavioral changes and produced reactive oxygen species. Most importantly, this research represents a pioneering effort to report neurogenotoxicity data specifically in *Drosophila* larval neuroblasts, thus underscoring the importance of this species as a testing model in exploring the biological impacts of validamycin A. The in vivo findings from the experiments are a valuable and novel addition to the existing validamycin A neurogenotoxicity database.

KEYWORDS
behavioral abnormalities, DNA damage, *Drosophila melanogaster*, gut damage, locomotor ability, neuroblast cells, neurogenotoxicity, oxidative stress, pesticide, validamycin A

1 | INTRODUCTION

The world's population is a restless tide, ever rising and falling, but over the past few decades, the tide has been slowing its pace. Even with this slowdown, the global population is still projected to swell by more than one billion people over the next 13 years, reaching 8.6 billion in 2030, which will put a strain on the agricultural food sector (United Nations, 2017). Urbanization and the subsequent decline of

arable land have resulted in the prevalence of monoculture practices that depend on agrochemicals. The global market for agrochemicals, comprising pesticides, growth hormones, and fertilizers, was anticipated to surpass a quarter of a trillion dollars with an annual growth rate of 4.5% (Wiseguyreports, 2018). Agrochemicals, however, exert detrimental effects on the environment by introducing noxious substances into ecosystems (Nuruzzaman et al., 2016). Pesticides may also result in ecosystem degradation and toxic residues in food destined for human and animal consumption due to their application to combat pest resistance (Carvalho, 2017). They entail grave health hazards, with approximately 20,000 fatalities annually ascribed to pesticide ingestion through contaminated food. These chemicals are implicated in acute and chronic health issues, including endocrine disruption, gastrointestinal disorders, and carcinoma, as well as neurodegenerative diseases such as Alzheimer's and Parkinson's (Anandhi et al., 2020).

Validamycin A, an aminoglycoside antibiotic with antifungal activity, can inhibit trehalase, an enzyme that breaks down trehalose, in plants, insects, and fungi. This antibiotic can also increase trehalose levels in genetically modified plants. Trehalose is a sugar molecule that is abundant in the blood fluid of insects and has various roles in insect physiology, such as providing energy, supporting growth, regulating metamorphosis, enhancing stress tolerance, synthesizing chitin, and enabling flight (Tang et al., 2017). The hydrolysis of trehalose is controlled by the enzyme trehalase, which holds significant importance in insect physiology by regulating metabolism and glucose production. In this regard, trehalase inhibitors have emerged as a promising candidate group of insecticides, albeit their full development is yet to be realized (Marten et al., 2020). In the field of plant pathology, validamycin A is used as a water-soluble antibiotic to inhibit *Rhizoctonia solani*, a fungal pathogen that affects various soil-borne hosts (Marten et al., 2020). Most recently, validamycin A has been shown to have larvicidal activity against *Drosophila melanogaster* (Kah et al., 2013) and to inhibit larval and pupal development in *Aedes aegypti* (Marten et al., 2020). These findings open new avenues for exploring the potential of validamycin A as an insecticidal agent in pest control and management strategies.

Pesticides are essential for agriculture, as they mitigate or prevent the damage inflicted by harmful organisms such as insects, weeds, fungi, and bacteria on crops. By applying pesticides, farmers can ensure that the food they produce is safe for human consumption and devoid of contaminants. Pesticides also enable farmers to reduce the cost of production and enhance the yield and quality of crops. In the absence of pesticides, more than half of the global agricultural output would be compromised by diseases and pests that would diminish the quantity and quality of food available (OECD-FAO Agricultural Outlook, 2012). Traditional pesticides, once hailed as a savior of the agricultural industry, are now being increasingly scrutinized for their considerable environmental drawbacks. These chemicals are often indiscriminately sprayed, meaning that they can adversely affect beneficial organisms, such as bees. For example, a study by Carriger et al. (2006) found that only 0.1% of aerially sprayed pesticides actually reach their intended targets, while the remaining 99.9% pollute the surrounding ecosystem, posing risks to both human and animal health. Besides their environmental impact, pesticides can also contaminate soil and water. This can lead to acute and chronic poisoning, which has been linked to around 100,000 human fatalities worldwide. Vulnerable populations, such as children and women in developing countries, are especially at risk (WHO Global Health Statistics, 2015).

In light of these concerns, it is essential that we develop more efficient risk assessment methods to evaluate the potential environmental impact of new pesticides, such as trehalase inhibitors like validamycin A. This is especially important for nanopesticides, which are a relatively new class of pesticides that have not been extensively studied. Therefore, we urgently require efficient risk assessment methods to swiftly investigate the neurotoxicity, ecotoxicity, and genotoxicity of pesticides and nanopesticides. We have been well aware that pesticides and engineered nanopesticides can be released into the environment, causing contamination and exposing nontarget organisms during the process of production, transformation, and utilization (Demir, 2020; Demir et al., 2022). Despite their extensive use, we still have limited information about the potential neurotoxic and genotoxic effects of validamycin A on nontarget organisms like the fruit fly, *D. melanogaster*. *Drosophila* has been a reliable model organism for assessing the neurotoxicity and genotoxicity of various pesticides, including nanopesticides (Demir, 2020).

It is generally known that pesticides and engineered pesticides (nanopesticides) have the potential to be discharged into the environment and cause pollution, exposing humans to nontarget species during the processes of manufacture, transformation, and utilization. Despite being widely used and applied, little is known regarding the potential neurotoxicity and genotoxicity of validamycin A on nontarget organisms like *D. melanogaster*. We conducted a comprehensive evaluation of the biological impact of validamycin A on *Drosophila* at different doses using a combined approach. This is a pioneering study to explore the neurotoxic, genotoxic, and reactive oxygen species (ROS) production effects of validamycin A in *D. melanogaster*. We assessed a variety of parameters, including DNA damage in neuroblasts using the Comet assay (genotoxicity), egg-to-adult survival (toxicity), intestinal damage, light/dark sensitivity assay, measurement of crawling behavior, thermal sensitivity assay, locomotor behavior, and intracellular ROS production in neuroblasts.

2 | MATERIALS AND METHODS

2.1 | Reagents and substances used

The chemical compound validamycin A ($C_{20}H_{35}NO_{13}$, 73.7%, CAS No. 37248-47-8) was acquired from Merck (Darmstadt, Germany) for use in the study. Various reagents and substances, along with their respective CAS numbers and purities, were obtained from Sigma Chemical Co. (St. Louis, MO, USA). These include ethyl methanesulfonate (EMS; CAS No. 62-50-0), giemsa (CAS No. 51811-82-6), methanol (≥99.9% purity, CAS No. 67-56-1), ethanol (99.5% purity; CAS No. 64-17-5), HEPES sodium salt (≥99% purity, CAS No. 75277-39-3),

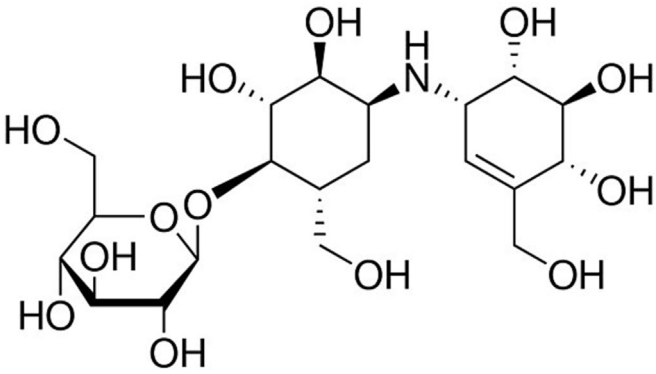

FIGURE 1 The structural formula of validamycin A, a macrolide antibiotic used to treat bacterial infections in animals. The molecule consists of a large lactone ring with several attached sugar groups. The molecular formula of validamycin A is $C_{20}H_{33}NO_{13}$.

potassium chloride (CAS No. 7447-40-7), ethylenediaminetetraacetic acid (EDTA; CAS No. 60-00-4), bovine serum albumin (≥98% purity, CAS No. 9048-46-8), endonuclease III from *Escherichia coli* (recombinant, ≥90% purity, E0526), Fpg protein from *E. coli* (≥90% purity, F3174, CAS No. 78783-53-6), and hydrogen peroxide (H_2O_2; CAS No. 7722-84-1).

Prior to its utilization in the experiments, validamycin A was dissolved in sterile distilled water, which was used as a solvent control (Demir et al., 2022). For the Comet assay, 4 mM EMS was utilized as the positive control (Demir, 2022; Turna Demir & Demir, 2022a, 2022b, 2023), while 0.5 mM H_2O_2 was employed to assess ROS (Demir, 2022; Turna Demir & Demir, 2022a, 2022b, 2023). Figure 1 shows the structural formula of validamycin A (https://www.sigmaaldrich.com/US/en/product/sial/32347).

2.2 | Toxicity (viability) of validamycin A

A wild strain of *D. melanogaster* (Canton-S) was used to investigate the toxic potential of validamycin A. The toxic doses had been determined before other studies (genotoxicity and intracellular ROS tests) were conducted. *Drosophila* eggs were exposed to varying doses, and the viability rates were determined. For each dose (0.01, 0.1, 1, 2.5, 5, 7.5, and 10 mM), 50 *Drosophila* eggs were transferred to plastic vials containing 4 g of instant medium. The number of adult flies that emerged from the eggs was then counted.

The food culture was previously saturated with various doses (10 mL) of validamycin A (0, 0.01, 0.1, 1, 2.5, 5, 7.5, and 10 mM). These concentrations corresponded to nominal doses of 4.975, 49.75, 497.5, 1243.75, 2487.5, 3731.25, and 4975 μg/mL, which, when dispersed in the food culture, represented 0.012, 0.124, 1.244, 3.109, 6.219, 9.328, and 12.438 mg/g food, respectively. The doses of validamycin A were determined based on data from prior genotoxicity and toxicity experiments (Demir et al., 2022; Marten et al., 2020). The greatest dose of validamycin A was 10 mM, and five replicates were used for each dose. The number of surviving adult flies was recorded after exposure to the test materials, and the survival rate was calculated relative to the control group. The validamycin A doses are expressed in millimolar throughout the paper to avoid confusion and to maintain uniformity.

2.3 | Quantification of ROS production in larval neuroblasts

The harmful effects of physical and chemical substances are regulated by oxidative stress. In order to investigate the effects of validamycin A on intracellular levels of ROS, 96 h old *Drosophila* larval neuroblasts were exposed to the compound for 24 h in third instar larvae. The generation of intracellular ROS levels was detected using a fluorescent probe called DCFH2-DA. DCFH2-DA is converted into the green fluorescent product 2′,7′-dichlorofluorescein (DCF) inside cells that have ROS. Following our previous protocols, neuroblasts were harvested and incubated with 5 μM DCFH2-DA for 30 min at 24°C (Demir, 2022; Turna Demir & Demir, 2022a, 2022b, 2023). After the incubation period, ROS production levels were detected using fluorescence microscopy with a green filter (485 nm excitation and 528 nm emission) at 20× magnification. Distilled water was used as a negative control and 0.5 mM H_2O_2 as a positive control for chemical assays. Using the ImageJ software package, fluorescence microscopy image data were analyzed (Schneider et al., 2012).

2.4 | Trypan blue cell viability assessment

In this study, trypan blue staining was employed to assess gut damage in *Drosophila* larvae. Trypan blue is a cell membrane-impermeable stain that is widely used to test cell survival (Strober, 2001). The protocol for trypan blue staining was modified slightly from previous studies (Turna Demir & Demir, 2023; Turna Demir et al., 2022; Zhang et al., 2020). Four replicates (20 larvae per replicate) were performed for each group. The larvae were all placed on 0.8% agarose plates with 5% sucrose and 10% trypan blue stain for 30 min after being cleaned with phosphate-buffered saline (PBS) solution. After 30 min, the larvae were washed with PBS for 15 min and then analyzed using stereomicroscopy to calculate the ratio of blue-stained larvae (SLX-2 STEREOZOOM) and then assess the severity of gut damage in the exposed larvae.

2.5 | Larval behavior

2.5.1 | Observation of crawling behavior

In this study, we employed a larval crawling assay to detect early signs of neuronal dysfunction. The crawling pattern of wild-type larvae typically involves moving in a straight line toward the edges of a petri dish. However, exposed larvae displayed altered crawling patterns, characterized by multiple turns and stops. The assay procedure involved washing third instar larvae (72 + 4 h old) with PBS to

eliminate excess particles of food medium. Subsequently, the larvae were placed at the center of a 2% agar plate and allowed to crawl freely for 1 min. The distance covered by the larvae was then marked on the agar plate, and the results were graphed. This assay has been previously utilized in similar studies to evaluate the impact of different compounds on neuronal activity in *Drosophila* larvae (Nichols et al., 2012; Priyadarsini et al., 2019; Sabat et al., 2016).

2.5.2 | Assessment of light and dark sensitivity

To assess the light-sensing ability of third instar larvae, a light/dark sensitivity assay was conducted. In this experiment, petri plates and their lids were divided into four quadrants, with 50% of the plate painted black, while the other half contained 2% agar. Third instar larvae, both from the control group and those exposed to various doses of validamycin A, were placed in the dark condition for 6 h before the assay. During the assay, the larvae were positioned at the center of the plate, and their attraction toward light and dark regions was observed and counted every 5 min. This process was repeated at least six times to ensure reliable results. In wild-type third instar larvae, the preference is often to stay in the light region. By comparing the preference of the control larvae with the validamycin A-treated larvae, conclusions regarding the impact of the compound on the light sensitivity of the larvae were drawn (Mishra & Barik, 2018; Priyadarsini et al., 2019).

2.5.3 | Evaluation of thermal sensitivity

The crawling behavior of *Drosophila* larvae was used to assess their ability to sense temperature when exposed to hot and cold plates. The observed effect on their crawling behavior served as an indicator of temperature sensitivity. This assay was specifically designed to evaluate the functionality of transient receptor potential channels, which are responsible for detecting temperature changes in larvae. Larvae from both the control group and those exposed to validamycin A were forced to crawl on plates with a wide range of temperatures. For this temperature sensitivity assay, temperatures of 4°C (cold) and 75°C (hot) were chosen based on previous research by Priyadarsini et al. (2019). These specific temperatures were chosen because *Drosophila* larvae cannot survive at extremely low or high temperatures, resulting in increased lethality when exposed to such conditions (Priyadarsini et al., 2019). Throughout the test, seven larvae from each validamycin A dose were allowed to crawl on agar plates maintained in both hot and cold environments. By comparing the crawling behavior of control and exposed larvae at different temperatures, the functionality of the channel proteins involved in temperature sensing was examined.

2.6 | Assessment of larval motility

Following the methods established in previous literature (Anand et al., 2017; Demir, 2021, 2022; Demir et al., 2022; Priyadarsini et al., 2019), the locomotor behavior of the flies was evaluated using a climbing assay. In this experiment, 10 flies from both the control and study groups were placed in separate vials and allowed to acclimate for 15 min at room temperature. The vials were then gently tapped to prompt the flies to move down to the bottom, and their climbing ability was assessed by recording the number of flies that successfully climbed above the 7 cm mark within 10 s for each group. The climbing assay was repeated 10 times for each group after each treatment, ensuring reliable and consistent results in assessing the flies' locomotor behavior.

2.7 | Neuroblast collection

To detect translocation of validamycin A throughout *Drosophila* third instar larvae brain, neuroblasts were collected (Sierra et al., 2014). The developed protocol included the use of third instar larvae treated in the food during 24 ± 2 h. For neuroblasts, chilled 96 ± 2 h old larvae were removed from food media, washed in water, and dried. The larvae brain from about 100 larvae 96 ± 2 h old was disrupted with two fine forceps in 500 µL PBS (1X) on a silicone dissecting plate, after carefully clearness and dryness.

2.8 | Detection of genotoxicity in larval neuroblasts by Comet assay

The Comet assay is a valuable tool for conducting genotoxicity and DNA repair tests. In this study, third instar (72 + 4 h old) *Drosophila* larvae of the wild Canton-S strain were subjected to different treatments: exposure to validamycin A, negative control (distilled water), and positive control (4 mM EMS). For the oral exposure to validamycin A, instant *Drosophila* culture media (4 g) from Carolina Biological Supply Co, Burlington, NC, USA, was mixed with varying doses (0.01, 0.1, 1, and 2.5 mM) in a volume of 10 mL. The larvae were then fed with this mixture for a duration of 24 ± 2 h. Following the oral exposure, neuroblast cells were collected from the flies using the method proposed by Sierra et al. (2014). This approach allowed the researchers to conduct the Comet assay on the collected neuroblast cells, enabling the assessment of genotoxicity and DNA repair responses to the different treatments. After isolating the neuroblasts, the Comet assay was conducted following the method described by Singh et al. (1988), with some minor modifications. To ensure the viability of the isolated neuroblasts, the trypan blue exclusion assay was employed, as outlined by Ghosh et al. (2012). The neuroblasts (20 µL) were mixed with low melting agarose (75%, 120 µL) and spread on normal melting point (1%) agarose-coated slides. Coverslips were mounted on the slides, and they were stored on ice for 5 min to embed the neuroblasts into the slides. Subsequently, the coverslips were removed, and a second low melting agarose (80 µL) spreading was applied, followed by another 5 min of storage on ice (Singh et al., 1988; Tice et al., 1990). Next, the opened slides were immersed in fresh chill lysing solution (10 mM Trizma base, 100 mM EDTA,

2.5 M NaCl, 1% Triton X-100, and 1% N lauroyl sarcosinate at pH 10) for 2 h at 4°C in a dark chamber. This lysing step was essential for breaking down the cell membranes and allowing the DNA to migrate during the subsequent steps of the Comet assay. To prepare the lysing solution for the Comet assay, dimethyl sulfoxide was intentionally excluded due to its potential to cause unnecessary damage to the tissues of *Drosophila*, as reported by Mukhopadhyay et al. (2004). The entire process, including the lysing step, was conducted under dim light conditions to minimize any additional DNA damage that strong light exposure might cause. After the lysing step, the slides were transferred into an electrophoresis buffer (consisting of 1 mM EDTA and 300 mM NaOH, with a pH of approximately 12.8) for 20 min. Electrophoresis was then carried out for 20 min at 300 mA and 1 V/cm. Subsequently, the slides were washed three times for 5 min each in a neutralization buffer (Tris buffer, pH 7.5) to neutralize the alkaline conditions from the previous step. To fix the DNA, the slides were treated with cold 70% ethanol for 5 min and then dried before staining with ethidium bromide (EtBr) (20 mg/mL). The staining process involved immersing the slides in EtBr solution for 20 min to facilitate the visualization of DNA fragments under fluorescence microscopy (using a filter with a wavelength range of 515–560 nm). The Comet assay analyses were performed using CaspLab software (version 1.2.3b2), following the procedure indicated in the previous study conducted by Turna Demir and Yavuz (2020). This approach ensured consistency and comparability with the previous research in the analysis of the Comet assay results. CaspLab is a dependable and widely used open-source software for quantifying DNA damage in Comet assay images (Końca et al., 2003; Turna Demir & Yavuz, 2020). In this study, the analysis focused on 100 randomly selected cells, performed in triplicate for each treatment, resulting in a total of 300 cells analyzed. The percentage of DNA in the tail (% DNA tail) served as the measure of DNA damage. Mean and standard error values were calculated in terms of % DNA tail to accurately represent the level of DNA damage observed in the different treatments. This rigorous analysis provided reliable and statistically sound results, enabling the researchers to draw conclusions based on the measured DNA damage levels.

2.9 | Detection of induced oxidative DNA damage

The evaluation of oxidized bases induction by validamycin A was conducted following the procedures outlined in a 2022 study by Demir. Enzyme treatments were incorporated to modify the standard Comet assay. After the lysis step, the slides were washed three times for 5 min each at 4°C using an enzyme buffer solution composed of 40 mM HEPES, 0.1 M potassium chloride, 0.5 mM EDTA, and 0.2 mg/mL bovine serum albumin, with a pH of 8.0. Following the washes, 100 µL aliquots of buffer containing bacterial enzymes Endo III (Sigma, >10,000 units/mg protein) or FPG (Sigma, >20,000 units/mg protein), or no enzyme (control), were placed onto the agarose-coated slides. The slides were then sealed with a cover glass and incubated for 30 min at 37°C. The enzyme concentration used was 1/1000 relative to the commercial dose. The standard alkaline Comet test protocol was used to process the cell samples after the enzyme treatments in order to assess the induction of oxidized bases. This modified assay allowed the researchers to specifically target and quantify the oxidized bases in the DNA, providing valuable insights into the impact of validamycin A on DNA damage caused by oxidized bases.

2.10 | Statistical analysis

Statistical analyses were performed to evaluate the data obtained from various experiments. The normality of variance was assessed using the Kolmogorov–Smirnov and Shapiro–Wilk tests, while the homogeneity of variance was examined using Levene's test. For data with a normal distribution and equal variance, Student's *t*-test was applied for the Comet assay, crawling behavior, and light/dark sensitivity assay. On the other hand, one-way analysis of variance (ANOVA) was used for the climbing assay. These analyses were conducted using SigmaPlot version 11.0 (SPSS, Chicago, IL). Data with unequal variance or skewed distribution, such as viability/toxicity, gut damage, and ROS production, were analyzed using the nonparametric Mann–Whitney U test. The presented data are the means of two independent experiments, each with duplicates, unless mentioned otherwise. The values are reported as arithmetic means ± standard error. Statistical significance was considered when $P \leq 0.05$, indicating that the results were statistically significant.

3 | RESULTS

3.1 | Toxic potential of validamycin A

The study aimed to investigate the potential toxic effects of validamycin A at various doses (0.01, 0.1, 1, 2.5, 5, 7.5, and 10 mM) using wild-type *Drosophila* strain (Canton-S) from egg to adult stages. Results showed that lower doses (0.01, 0.1, 1, and 2.5 mM) did not exhibit significant toxic effects, but greater doses of validamycin A (5, 7.5, and 10 mM) caused lethal effects. Distilled water was used as a negative control, demonstrating 100% viability for *Drosophila* eggs. In contrast, exposure to increasing doses of validamycin A (0.01, 0.1, 1, 2.5, 5, 7.5, and 10 mM) resulted in a decrease in the percentage of exposed eggs reaching the adult phase: 93%, 88%, 77%, 63%, 42%, 26%, and 17%, respectively (Figure 2A). The absence of lethality induced by lower doses of validamycin A was confirmed by measuring larval length. Larval lengths recorded after exposure to validamycin A at 0.01, 0.1, 1, and 2.5 mM were 94%, 90%, 86%, and 83% of controls, respectively (Figure 2B). Based on this finding, nontoxic doses (0.01, 0.1, 1, and 2.5 mM) of validamycin A were chosen for subsequent experiments involving ROS assay, genotoxicity tests using the Comet assay, gut damage assessment, and climbing behavior analysis in *Drosophila* larvae. These concentrations allowed for conducting the specified experiments without causing serious toxicity in the larvae.

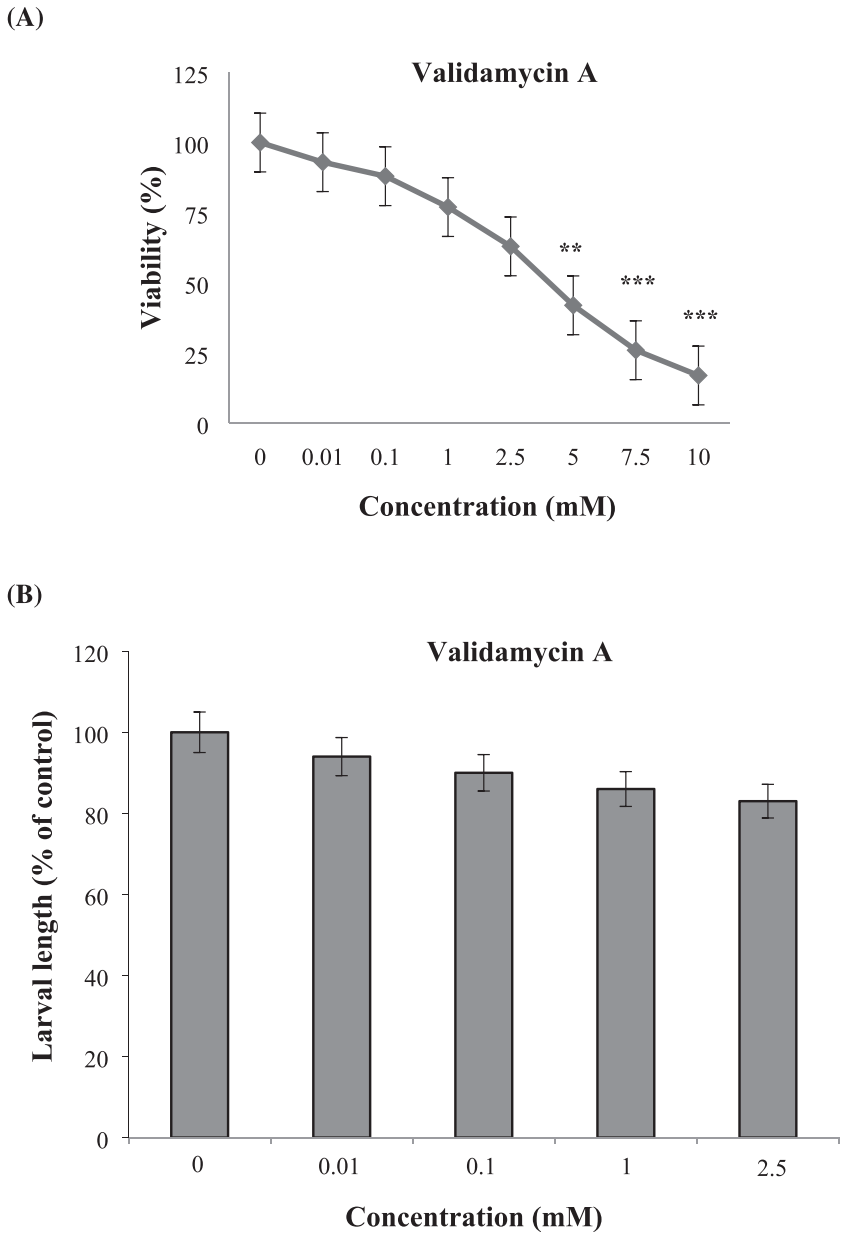

FIGURE 2 Viability (toxicity) of validamycin A in *Drosophila melanogaster* (fruit flies). (A) The loss of viability (egg to adult survival) of *D. melanogaster* exposed to various doses of validamycin A was measured relative to the control group. Ten vials per dose were used, with 50 eggs per vial. Data were analyzed by Mann–Whitney U test. (B) The lengths of *D. melanogaster* larvae exposed to validamycin A were compared with the lengths of unexposed larvae. Eighty larvae per dose were measured. Exposure lasted from the egg stage to the larval stage at 96 h. $P \leq 0.01$ and $P \leq 0.001$ indicate statistically significant differences from the control group, as determined by a Mann–Whitney U test.

3.2 | Validamycin A-induced oxidative stress in neuroblasts

Oxidative stress plays a crucial role in demonstrating the adverse effects of exposure to validamycin A. The accumulation of ROS is considered a critical indicator of harmful effects in various cells. To investigate ROS generation in third instar larval neuroblasts exposed to validamycin A, a technique using the fluorescent dye, 6-carboxy 2,7′-dichlorodihydro-fluorescein diacetate (DCFH-DA), was employed. In Figure 3A, fluorescence images depict ROS detection in the neuroblasts of *D. melanogaster* larvae. The results showed that exposure to validamycin A at doses of 1 and 2.5 mM caused statistically significant elevations in ROS levels ($P \leq 0.001$) (Figure 3). The highest oxidative stress was observed at a dose of 2.5 mM. Interestingly, the ROS values at 2.5 mM (326%) surpassed those of propionic acid at 1 mM (287%) (Figure 3B). This indicated that validamycin A induced a higher degree of ROS production in neuroblasts compared with propionic acid, and the effect was dose-dependent (Figure 3B). Overall, these findings highlight the importance of oxidative stress as a key factor in understanding the detrimental effects of validamycin A exposure, with higher doses leading to a more significant increase in ROS buildup in neuroblasts.

3.3 | Effects of validamycin A on gut integrity in *Drosophila* larvae

In the study, trypan blue staining was employed to assess intestinal cell damage in *Drosophila* exposed to propionic acid. The results indicated positive trypan blue staining, signifying intestinal damage, in

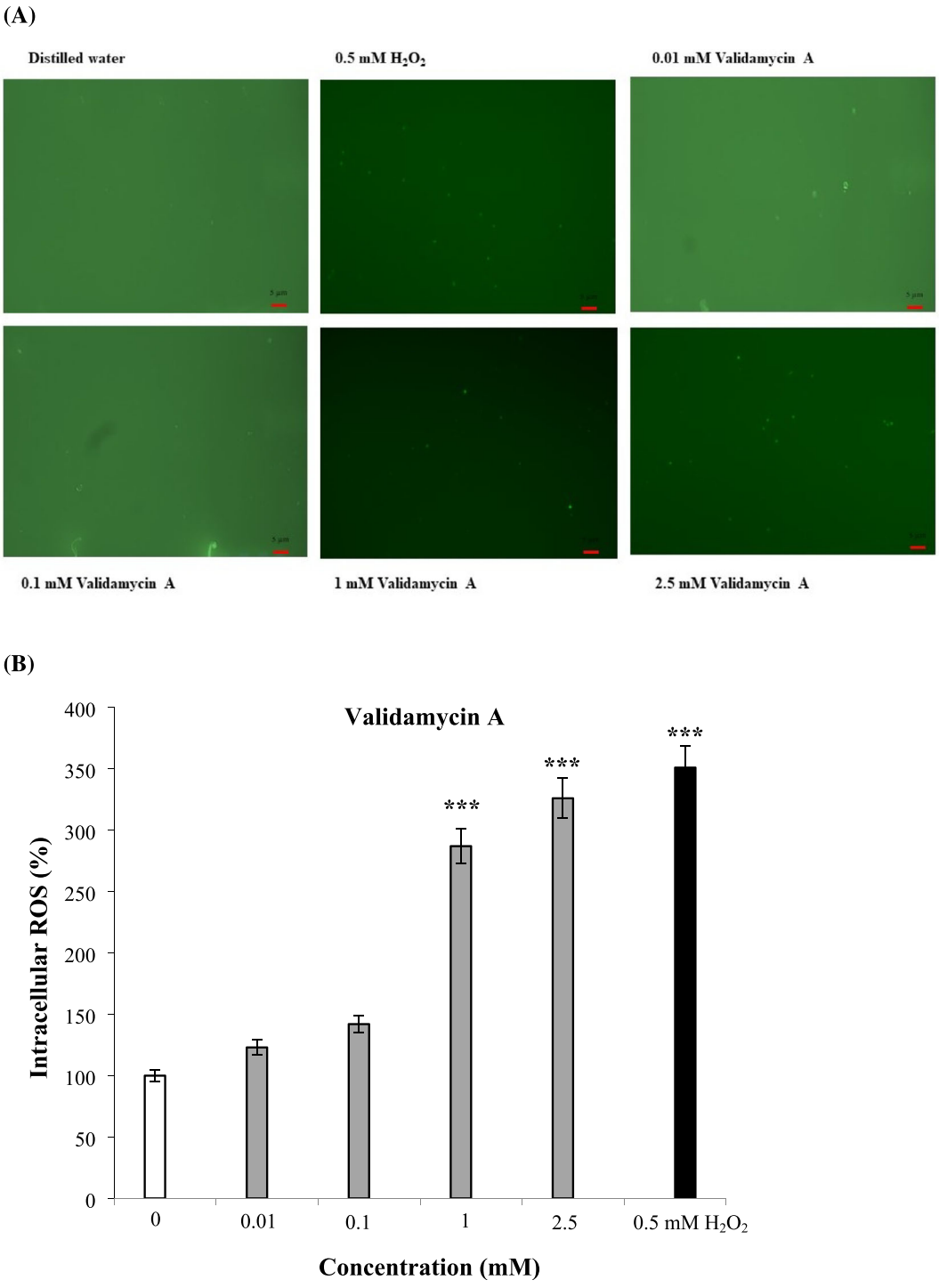

FIGURE 3 Reactive oxygen species (ROS) production in neuroblasts of third instar larvae treated with validamycin A. (A) Representative images of neuroblasts incubated with 5 μM 6-carboxy 2,7′-dichlorodihydro-fluorescein diacetate for 30 min at 24°C. The neuroblasts were treated with distilled water (0), 0.01, 0.1, 1, and 2.5 mM validamycin A or 0.5 mM H_2O_2 as a positive control. (B) Quantification of the fluorescence intensity of the neuroblasts at 20× magnification by ImageJ analysis. $P \leq 0.001$ indicates statistically significant differences from the controls, as determined by a Mann–Whitney U test. Ten representative images were used for each dose.

the flies belonging to the treatment groups (Figure 4A). Compared with the control group, exposure to validamycin A resulted in increased gut damage, particularly at the two highest doses (1 and 2.5 mM). The highest levels of gut damage caused by propionic acid were observed at the highest doses of 1 and 2.5 mM, with gut damage levels of 48% ($P \leq 0.001$) and 62% ($P \leq 0.001$), respectively (Figure 4B). The intense staining observed in the larvae suggests a substantial amount of gut damage, which may result in increased

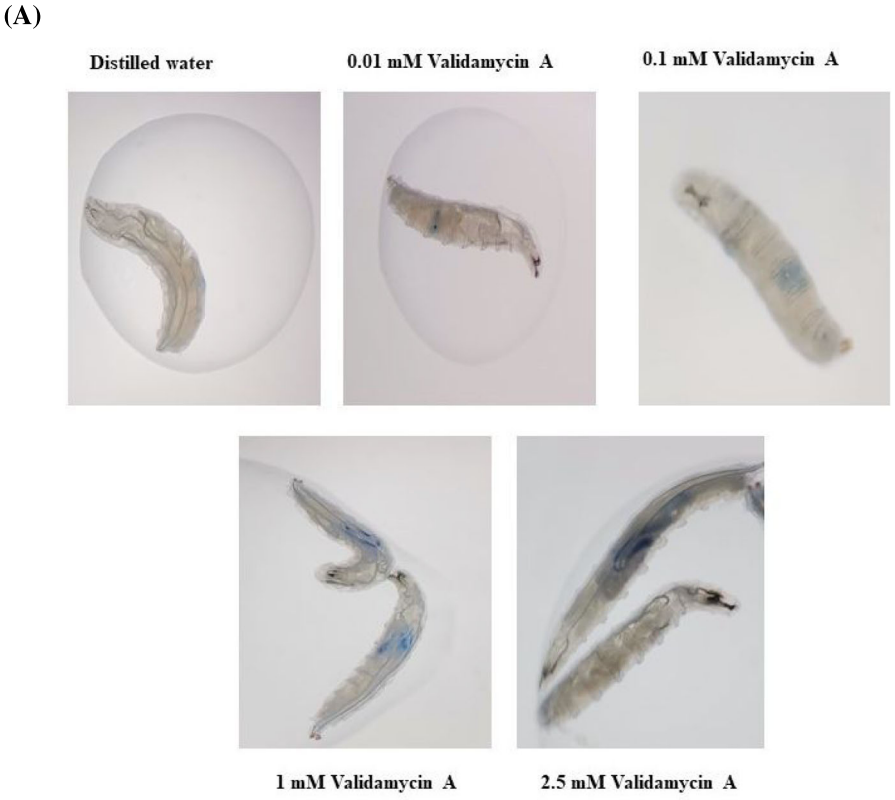

FIGURE 4 Gut damage in larvae treated with validamycin A. (A) Representative images of trypan blue-stained gut tissues from control and treated larvae. The control larvae showed no sign of tissue damage, while the treated larvae showed significant gut damage. (B) Percentage of gut damage in larvae after exposure to different doses of validamycin A. $P \leq 0.001$ indicates statistically significant differences from the controls, as determined by a Mann–Whitney U test.

toxicity beyond what was initially observed. Validamycin A could be suggested to induce a certain degree of gut damage in a dose-dependent manner, with greater doses leading to a notable increase in gut damage compared with controls, further emphasizing the potential adverse effects of the compound on the intestinal cells of *Drosophila*.

3.4 | Larval behavior

3.4.1 | Observation of crawling behavior

The crawling speed of third instar larvae was measured, and it was observed that the control group was able to cover longer distances compared with those exposed to validamycin A. In larvae treated with validamycin A, crawling speed showed an increase with the rise in the dose up to a certain extent, specifically at 0.1 mM doses of validamycin A. However, at higher doses (1 and 2.5 mM), the larvae exhibited sluggish crawling behavior. Additionally, larvae treated with doses of 1 and 2.5 mM displayed zigzag crawling behavior (Figure 5A). This observation indicates that higher doses of validamycin A negatively impacted the normal crawling behavior of the larvae, resulting in a zigzag movement pattern instead of the typical straight-line crawling seen in the control group. The results suggest that the effects on the crawling

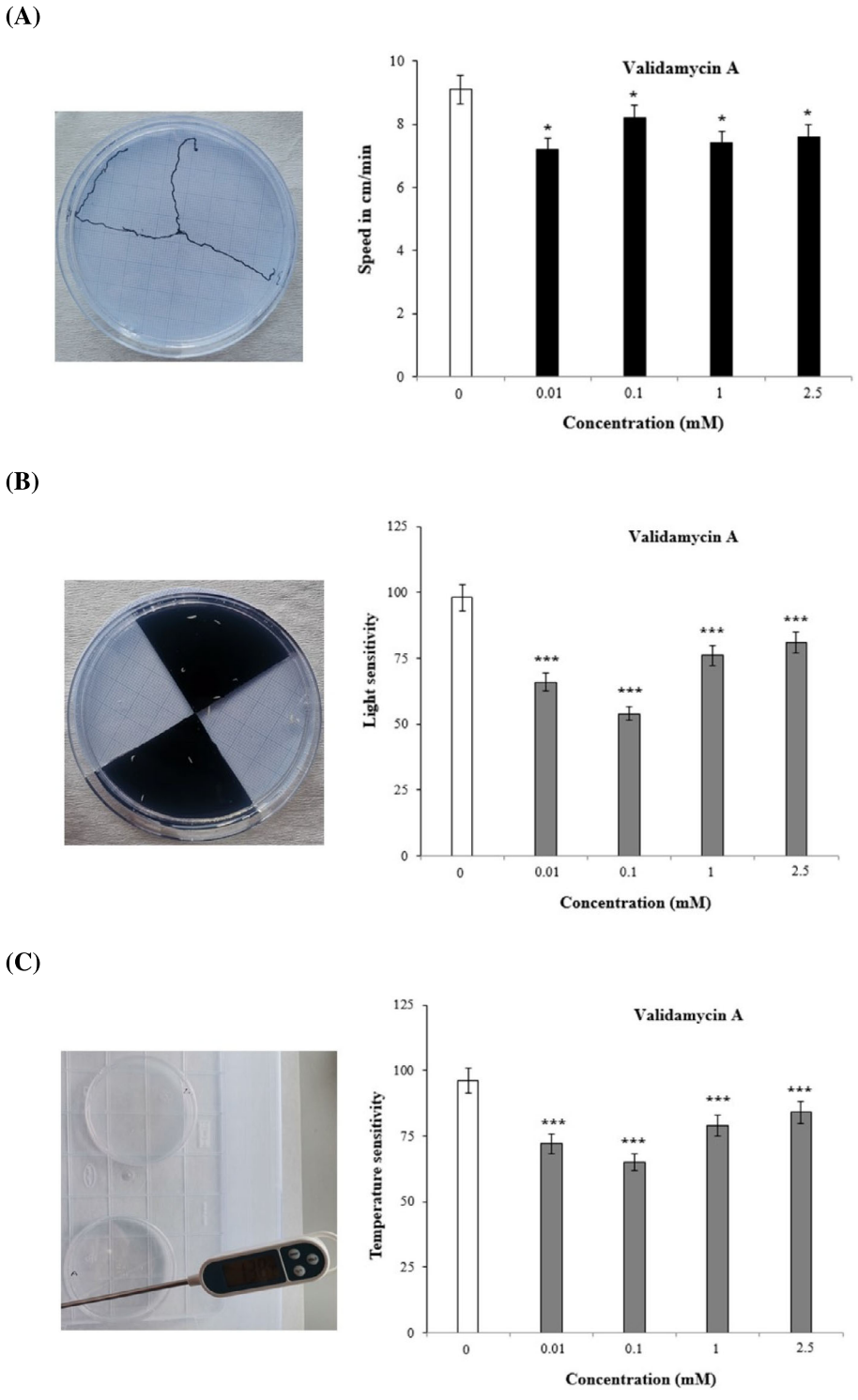

FIGURE 5 Behavioral responses of third instar *Drosophila* larvae treated with validamycin A. (A) Crawling pathways of larvae exposed to varying doses of validamycin A. The distance traveled by the larvae was significantly decreased with increasing doses of validamycin A. (B) Light/dark sensitivity assay. The plate for the light/dark experiment is shown, as well as the graph of the larvae's response to validamycin A. The larvae showed a decreased preference for the dark environment with increasing doses of validamycin A. (C) Temperature sensitivity assay. The graph shows the sensing ability of validamycin A-treated larvae toward temperature variations. The larvae showed a decreased ability to sense temperature changes with increasing doses of validamycin A. $P \leq 0.05$ and $P \leq 0.001$ indicate statistically significant differences from the controls, as determined by a Student's *t*-test.

behavior of *Drosophila* larvae are dose dependent, with low doses enhancing crawling speed and higher doses leading to sluggishness and altered crawling patterns.

3.4.2 | Assessment of light and dark sensitivity

During the assessment of light and dark sensitivity, the larvae exhibited a preference for the light region. However, upon exposure to low doses of validamycin A, the larvae displayed an increased attraction toward the dark region. As the doses of validamycin A were further increased to 1 and 2.5 mM, the larvae shifted their preference and showed a stronger attraction toward the light regions (Figure 5B).

3.4.3 | Evaluation of thermal sensitivity

The temperature sensitivity of third instar larvae was assessed by measuring their crawling speed at different temperatures. Control larvae were very active at high temperatures and became sluggish at low temperatures. The temperature sensitivity of validamycin A-treated larvae decreased with increasing doses of the compound in the body. At higher doses, they were able to move even at low temperatures. Validamycin A may have disrupted the normal temperature regulation mechanisms in *Drosophila* larvae. Lower doses may actually increase temperature sensitivity, whereas greater doses can cause the larvae to become less sensitive to temperature changes.

3.5 | Validamycin A-induced changes in climbing behavior in *Drosophila* flies

Dysfunctional locomotor behavior in flies is evidenced by their impaired climbing ability (Anand et al., 2017; Demir, 2022; Demir et al., 2022). In this study, we found that exposure to validamycin A significantly impaired the climbing ability of flies (Figure 6). The climbing efficiency of flies was measured after 7 days of exposure to varying doses of the test compound. The results showed that the climbing efficiency was significantly impaired after exposure to 0.01, 0.1, 1, and 2.5 mM. The climbing efficiency of the flies that were exposed to 0.01, 0.1, 1, and 2.5 mM was 93%, 85%, 62%, and 53%, respectively, as compared with the control group, which had a climbing efficiency of 100%. The data highlight the adverse impact of validamycin A on the locomotor behavior of the flies, as evidenced by the significant decrease in climbing efficiency at various doses, which may have resulted from the toxic effects on the nervous system that could impair the coordination and motor control necessary for climbing.

3.6 | Genotoxicity test (Comet assay)

Assessing the induction of genotoxicity is of utmost importance when evaluating the potential hazards associated with exposure to physical or chemical agents. This is because DNA damage is linked to a variety of human health problems, including cancer. The Comet assay is a well-established technique for detecting DNA damage induction, and it detects DNA breaks by measuring the migration of DNA fragments in an electric field. In this study, the Comet assay was used to assess the genotoxicity of validamycin A in neuroblasts. The results of the Comet assay showed that all tested doses caused significant increases in the percentage of DNA in the tail, a region of DNA that migrates away from the nucleus during the Comet assay. This indicates that validamycin A induced DNA breaks in neuroblasts. The largest dose (2.5 mM) caused a 39.45% increase in the DNA damage rate as compared with controls (Figure 7A). The Comet assay results also showed a significant increase in the incidence of DNA-strand breaks in neuroblasts upon exposure when compared with the controls exposed to distilled water only (Figure 7A). The findings indicate that validamycin A can be a potent toxic agent that induces genotoxicity in neuroblasts, with potential health risk to humans. Further research is needed to determine the full range of potential health impacts associated with acute and chronic exposure.

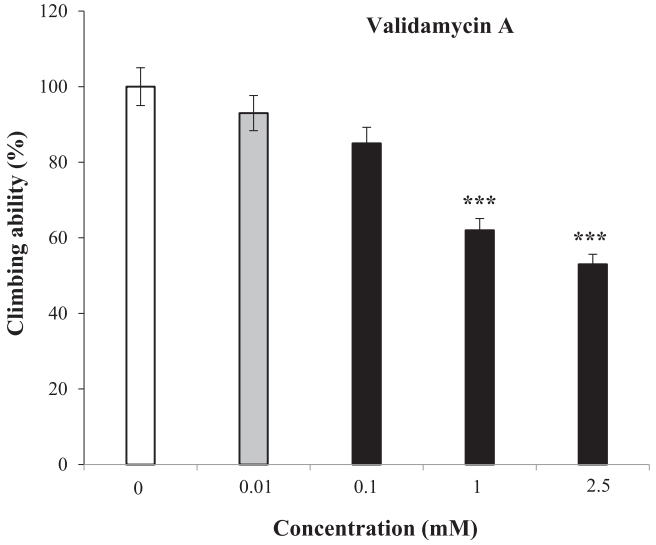

FIGURE 6 Climbing behavior of flies exposed to validamycin A, as monitored after 7 days of exposure to different doses of validamycin A. The mean number of climbs recorded after 10 s was significantly decreased in flies exposed to validamycin A, compared with the controls. $P \leq 0.001$ indicates statistically significant differences from the controls, as determined by one-way ANOVA.

The study's findings revealed that when cells were exposed to varying doses of validamycin A (0.01, 0.1, 1, and 2.5 mM), there were notable increases in oxidative DNA damage. To quantify the overall oxidative damage, the baseline level of DNA damage was subtracted from the corresponding enzyme's measured value. The results showed that the damage detected by the FPG enzymes was higher compared with the damage detected by Endo III, but this difference was not statistically significant. This suggests that oxidation at pyrimidine bases, as identified by Endo III, may not have a significant harmful effect on the DNA after being exposed to validamycin A, whose genotoxic effects were observed across all doses, as depicted in Figure 7B. The extent of such effects was assessed by measuring the percentage of DNA in the

neuroblast tail. The larval brain of *Drosophila* at the third instar stage consists of three main neurogenic compartments. Positioned on the lateral surface of the brain hemispheres is the optic lobe neurogenic center, while medially to the optic lobe is the central brain neurogenic center. On the anterior side of the brain, this central brain neurogenic center extends toward the ventral nerve cord. Within the central brain, one can find Types I and II neuroblasts (Figure 8A). In Figure 8B, the DNA damage in *Drosophila* larval neuroblasts exposed to different doses of validamycin A (0.01, 0.1, 1, and 2.5 mM) is illustrated.

4 | DISCUSSION

Pesticides, including insecticides, are chemical substances commonly used to control pests and diseases in agriculture and public health. However, most pesticides are not highly selective, meaning they can harm not only the target pests but also various nontarget species, including humans (Costa et al., 2018). This lack of specificity poses significant risks to the environment and human health. In particular, insecticides, designed to disrupt the nervous systems of insects and pests, can also have neurotoxic effects in humans. These neurotoxic effects can occur either as a result of high acute exposure to large amounts of the pesticide or through chronic exposure to low doses over an extended period. Chronic exposure to pesticides at low doses has been the subject of numerous studies, revealing it as a significant risk factor for the development of various neurodegenerative diseases. Disturbingly, pesticide exposure has resulted in a significant death toll globally, surpassing 100,000 according to reports from the World Health Organization (WHO, 2015). Young children and female workers are particularly vulnerable to the injurious effects of pesticides, making it crucial to

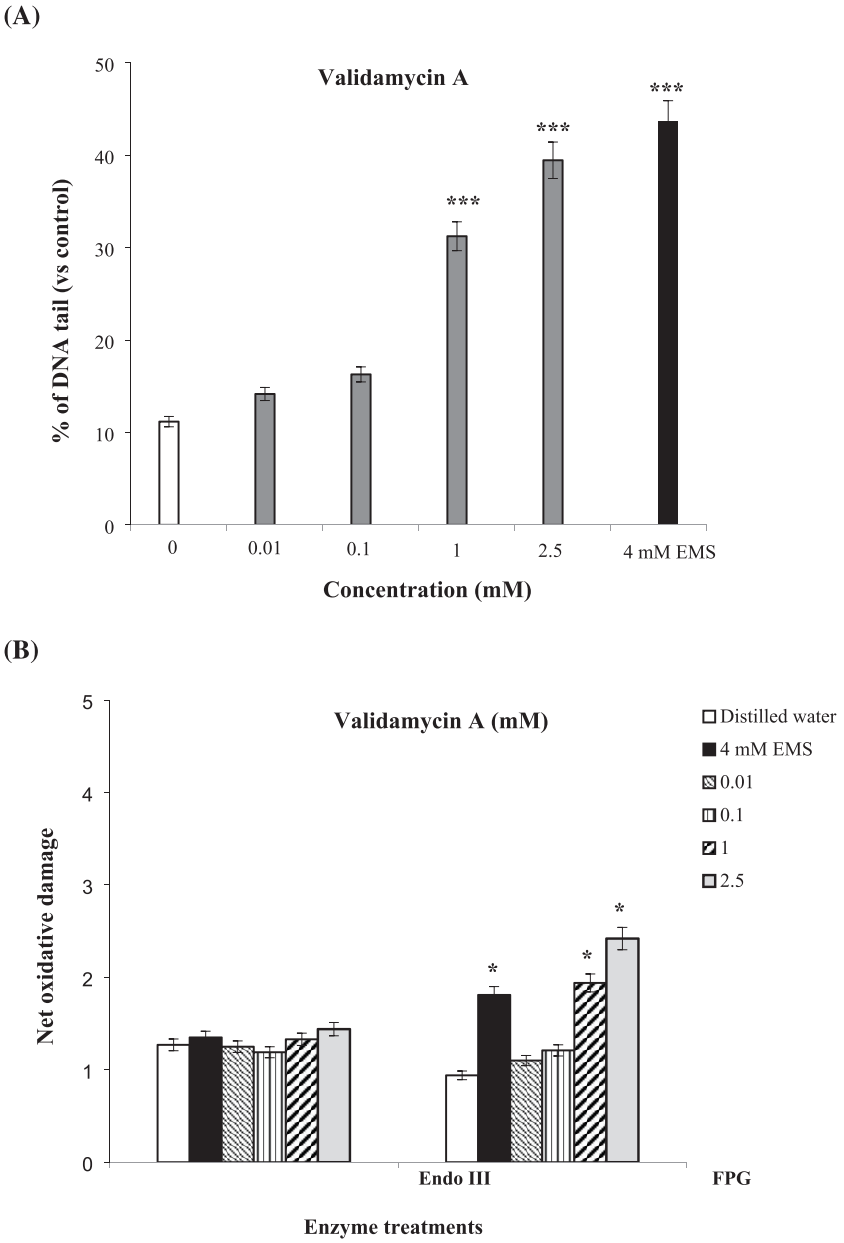

FIGURE 7 Genotoxic effects of validamycin A in the Comet assay. (A) The percentage of DNA tail induced in neuroblasts after exposure to varying doses of validamycin A for 24 h. The percentage of DNA tail was significantly increased with greater doses. (B) Net oxidative damage induction in neuroblasts after exposure at doses of 0.01, 0.1, 1, and 2.5 mM. The level of net oxidative damage showed a notable rise as the doses of validamycin A increased. $P \leq 0.05$ and $P \leq 0.001$ indicate statistically significant differences from the controls, as determined by Student's *t*-test. EMS, ethyl methanesulfonate.

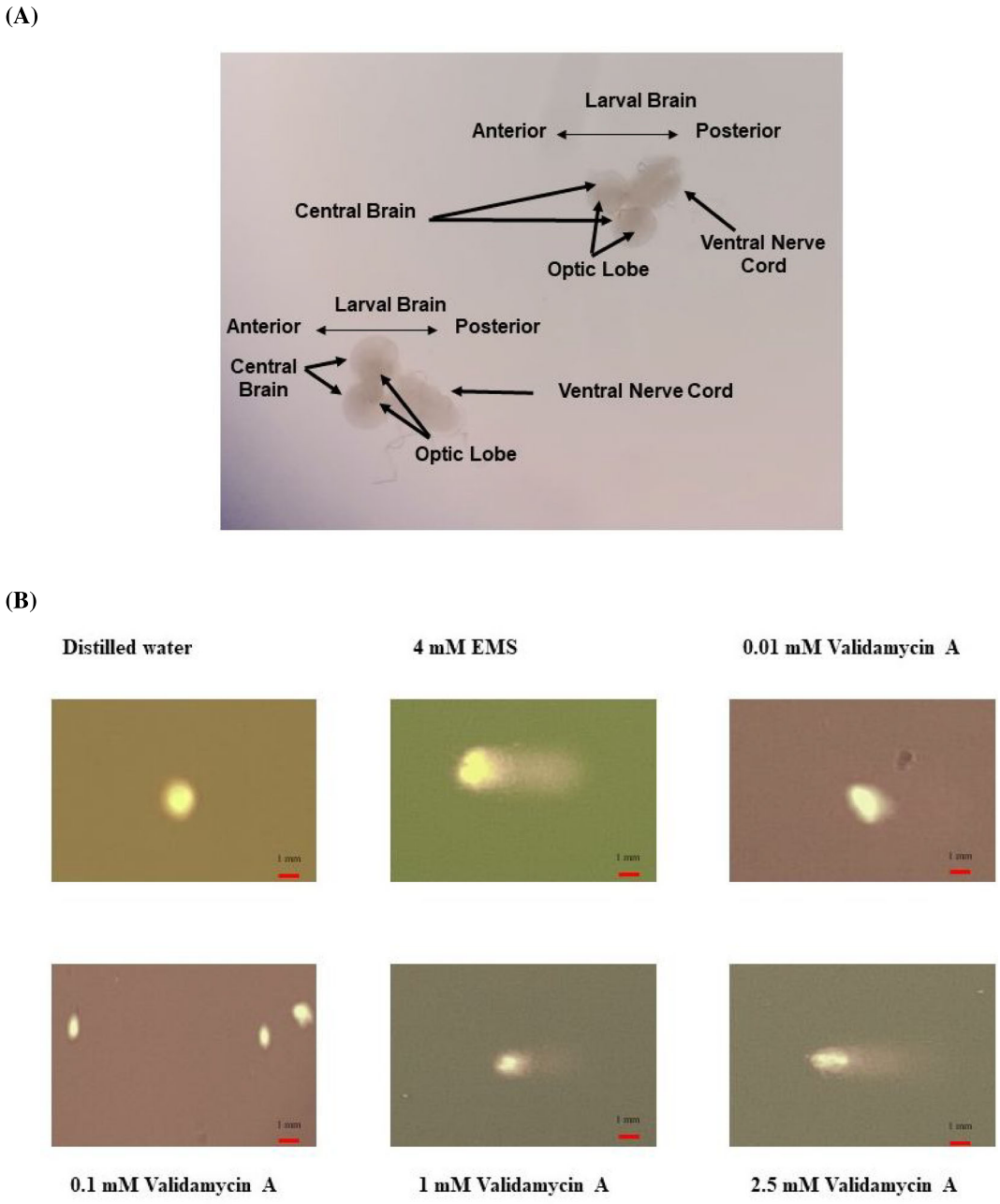

FIGURE 8 Effects of validamycin A on DNA damage in *Drosophila* larval neuroblasts. (A) Anterior/posterior view of the central nervous system of *Drosophila* third instar larvae brain. (B) Images of neuroblasts from larvae exposed to varying doses of validamycin A (0.01, 0.1, 1, and 2.5 mM). The images exhibit a dose-dependent increase in the percentage of DNA tail following validamycin A exposure. EMS, ethyl methanesulfonate.

address and mitigate these risks to protect the most susceptible populations. The adverse impacts on human health further emphasize the importance of stringent pesticide regulation, improved safety measures, and the adoption of alternative pest control practices to safeguard both human health and the environment.

Trehalose is a vital sugar for insects, and its breakdown is essential for their development and survival, thus inhibiting its breakdown can be lethal for them (Shukla et al., 2015). In experiments, injecting insect larvae with validamycin A and trehazolin, most common trehalase inhibitors, resulted in unsuccessful pupation and led to fatal metamorphosis, preventing them from reaching adulthood (Wegener et al., 2010). Furthermore, the inhibition of trehalose metabolism had several specific effects on insects. Tests on the migratory locust (*Locusta migratoria*) revealed that such metabolic interference could lead to hypoglycemia in the flight muscles and disrupt the normal synthesis of chitin, an essential component of their exoskeleton (Liebl et al., 2010; Wegener et al., 2010), confirming the critical role of trehalose in insect development and survival.

In this current study, the aim was to assess the possible hazards of validamycin A using *D. melanogaster*, which was not the target of

this compound. The underlying hypothesis was that trehalase inhibitors, such as validamycin A, might exhibit neurotoxic effects. To investigate this hypothesis, we performed a comprehensive comparative investigation focusing on the potential toxic and neurotoxic characteristics of validamycin A, also exploring its impact on oxidative stress, DNA damage, gut integrity, larvae behavior (crawling behavior, light/dark sensitivity assay, and temperature sensitivity assay), and locomotor abilities. Although validamycin A is widely utilized as a fungicide in agriculture, its potential toxicity to nontarget organisms remains inadequately studied. Our research contributes valuable insights into the biological properties of validamycin A and endorses the utilization of *D. melanogaster* as a dependable model organism for evaluating the compound's toxicity.

We showed that validamycin A at concentrations of 0.01–2.5 mM had concentration-dependent activity ROS and DNA damage in the larval brain, intestinal damage, alterations in crawling behavior, sensitivity to light and dark, thermal responsivity, and locomotor behavior of *D. melanogaster*. Previous research on toxicity and genotoxicity has provided evidence that exposure to pesticides can lead to lipid peroxidation and DNA damage in both humans and rodents (Kapeleka et al., 2021; Milić et al., 2018). Additionally, studies on aquatic animals have shown that pesticides can induce oxidative stress, a significant factor contributing to developmental toxicity risks (Kumar et al., 2021). These findings underscore the potential dangers of pesticide exposure across various organisms. Over the last few decades, the widespread use of pesticides, including nanopesticides, has raised considerable concerns. One major concern is that these chemicals can harm nontarget species, even those not considered pests. After conventional application methods like aerial spraying, it is estimated that only about 0.1% of the pesticides reach the intended target species, while the majority of the chemicals lead to environmental contamination (Demir, 2020). This highlights the urgent need for more environmentally friendly and targeted pest control strategies. In a previous study, we investigated the individual effects of calcium carbonate and validamycin A, finding that calcium carbonate alone did not induce any negative biological effects, such as single-stranded DNA damage, somatic mutation, recombination, lipid peroxidation, and formation of ROS or oxidative stress. However, when validamycin A and validamycin nanopesticides (with a size of 1177 ± 220 nm) were examined, they demonstrated cytotoxic and genotoxic effects. These effects were evident in various assays, including the *Drosophila* Comet assay, which detects primary DNA damage and oxidative DNA damage in hemocytes and midgut cells. Additionally, the *Drosophila* somatic mutation and recombination test revealed the potential somatic mutation and/or recombination activity in wing imaginal disc cells. We also observed increased levels of intracellular ROS in the hemocytes and midgut cells of *Drosophila* larvae after exposure to validamycin A and validamycin nanopesticides, assessing oxidative stress levels by measuring glutathione amounts and identifying lipid peroxidation product formation (malondialdehyde) as a consequence of exposure to the nanopesticides. Moreover, the study investigated the effects of the nanopesticides on the intestinal barrier, including the crossing of nanopesticides through the barrier and their effects on the intestinal lumen. Alterations in the expression of genes controlling the integrity of the intestinal barrier and general stress genes were examined, along with phenotypic changes in different generations (F0, F1, F2, and F3) and locomotor behavior (climbing and/or walking) along with the occurrence of morphological abnormalities. Notably, significant biological effects were observed after exposure to the highest doses of validamycin A and validamycin nanopesticides (1 and 2.5 mM), although no genotoxic effects were detected by the *Drosophila* somatic mutation and recombination test (Demir et al., 2022). In a similar vein, the current study's results demonstrate neurogenotoxic effects following exposure to validamycin in *Drosophila* larval neuroblasts. These findings further emphasize the importance of understanding the potential harmful effects of validamycin A and validamycin nanopesticides on various biological systems, including neurodevelopment. In our previous study, we focused on the toxic and genotoxic effects of validamycin A in hemocytes, while in this study, we have been studied brain cells known as neuroblasts.

Interestingly, trehalose serves as a stress protectant not only in *D. melanogaster* but also in mosquitoes (Thorat et al., 2012). Moreover, the use of validamycin A has been shown to hinder the flight of *A. aegypti* mosquitoes when applied during their larval stage (Marten et al., 2020). This suggests that trehalose metabolism and its inhibition by validamycin A play a vital role in the flight behavior of these insects. Moreover, the mode of action of validamycin A remained similar even when it was formulated with calcium carbonate nanoparticles. This indicates that the presence of nanoparticles does not alter the fundamental mechanism by which validamycin A exerts its effects on trehalose metabolism and insect behavior. Research into trehalose metabolism and its inhibition by validamycin A has provided valuable insights into the essential role of this sugar in insect development and survival. Understanding these mechanisms could open up potential avenues for the development of novel insecticides that target trehalose metabolism, offering more effective and targeted pest control strategies in the future. The prolonged release of the active ingredient in validamycin A when formulated with calcium carbonate nanoparticles is responsible for its long-term effectiveness (Addadi et al., 2003). This formulation has been shown to have adverse effects on the larval and pupal development of *A. aegypti* mosquitoes (Marten et al., 2020) and the larval stage of fruit flies (Kah et al., 2013).

Trehalase is expressed by arthropods, fungi, and bacteria suggesting that validamycin A may be effective against these organisms. Through encouraging dysbiosis in the mosquito microbiome, a therapy may have had a deleterious impact on mosquito development. Insect physiological processes such as chitin synthesis, stress resistance, and larval and pupal development can all be affected by trehalase inhibition (Liebl et al., 2010; Thorat et al., 2012; Wegener et al., 2010). Future studies are necessary to understand whether the validamycin A activity in *D. melanogaster* is caused by a variety of biological processes.

5 | CONCLUSION

This has been the first study to use *D. melanogaster* as an in vivo model to examine the oxidative stress and DNA damage in the larval

brain, intestinal damage, alterations in crawling behavior, sensitivity to light and dark, thermal responsivity, and locomotor behavior upon exposure to validamycin A. Our study has demonstrated the viability of *Drosophila* as a reliable in vivo model organism for detecting biological and genetic effects of validamycin A. This finding paves the way for future research that may employ *Drosophila* as a representative organism for evaluating the impacts of this compound. Moreover, the extrapolation of exposure test results in *Drosophila* to other nontarget organisms, including humans, is justified due to their developmental and physiological similarities. Nonetheless, to fully comprehend the diverse effects of these widely used substances across different biological endpoints, further investigations into their underlying mechanisms are necessary. This study not only sheds light on the potential hazards of validamycin A but also highlights the importance of adopting a comprehensive approach to assess the risks associated with various chemicals used in different fields. With more in-depth research, we can take proactive steps toward safeguarding both the environment and human health. Future toxicity testing and studies, however, are necessary to confirm the validity of various systems (in vivo and in vitro research) and model organisms to explain of the molecular mechanisms brought on by exposure to validamycin A because such studies are directly related to human and environmental health. The key takeaways from the conversation up to this point are as follows:

1. At levels lower than 5 mM, validamycin A did not cause appreciable cytotoxic effects.
2. Adult flies' ability to climb and walk was considerably hampered by validamycin A (1 and 2.5 mM).
3. Sensitivity to light and dark, thermal responsivity, and locomotor were detected in the third instar *Drosophila* larvae at treated concentrations of validamycin A exposure.
4. After exposure to concentrations of 1 and 2.5 mM of validamycin A, there were noticeable alterations in the formation of intracellular ROS, gut damage, primary DNA damage, and oxidative DNA damage.
5. Purine base oxidative damage served as a mediator for single-stranded and oxidative DNA damage.

AUTHOR CONTRIBUTIONS

Both authors made substantial contributions across all aspects of the research, demonstrating their joint effort in conceiving, designing, performing experiments, analyzing data, and creating the original manuscript. Their individual contributions were as follows:

Fatma Turna Demir: Conception; experimental design; experimental performance; data analysis; writing original manuscript. **Eşref Demir:** Conception; experimental design, experimental performance; data analysis; writing original manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest. No author has any financial or personal relationships that could inappropriately influence or bias the content of this paper.

DATA AVAILABILITY STATEMENT

All data generated or analyzed during this study are included in this published article. The raw data are not publicly available due to privacy restrictions. However, the authors will make the data available upon reasonable request.

ORCID

Fatma Turna Demir https://orcid.org/0000-0001-8045-8641
Eşref Demir https://orcid.org/0000-0002-2146-7385

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