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Effects of ingestional exposure of 1,3,5-triazine derivatives on *Drosophila melanogaster*

Monika Yadav[1], Sunidhi Kashyap[1], Santosh Kumar Charan[2], Naresh Kumar Nirmal[3*]
[1] Research Scholar, Department of Zoology, Toxicology and Pharmacology Laboratory, University of Rajasthan, Jaipur, Rajasthan, India
[2] Assitant Professor, Department of Zoology, Bee Biodiversity Laboratory, University of Rajasthan, Jaipur, Rajasthan, India
[3] Assistant Professor, Department of Zoology, Toxicology and Pharmacology Laboratory, University of Rajasthan, Jaipur, Rajasthan, India

Abstract
Triazines, specifically 1,3,5-triazine derivatives, have garnered significant interest due to their diverse biological properties and potential therapeutic applications, including anti-tuberculosis, antiviral, and neuroprotective activities. This study investigates the effects of six 1,3,5-triazine derivatives on multiple life stages of *Drosophila melanogaster* (Oregon-R strain) to assess their *in vivo* biological impact. Using a high concentration of 1000 mg/L to capture acute effects, parameters such as eclosion efficiency, gut integrity, oxidative stress, mortality, morphological anomalies, and body weight were evaluated. Results indicated that derivatives with halogen substitutions—2-fluoro (T2), 3-fluoro (T3), and 4-chloro (T5)—caused significant developmental toxicity, marked by pupal morphological abnormalities, increased oxidative stress, reduced eclosion rates, and elevated mortality. Conversely, derivatives with unsubstituted phenyl (T1), 2-trifluoromethyl (T4), and 4-bromo (T6) groups showed minimal adverse effects. This study suggests that the type and position of substituents on triazine derivatives play a crucial role in determining their biological effects. Findings highlight the necessity for further research to elucidate structure-activity relationships and optimize the safe application of triazine-based compounds.

Keywords: 1,3,5-Triazine derivatives, drosophila melanogaster, acute toxicity, oxidative stress, structure-activity relationship

Introduction

Triazines, specifically 1,3,5-triazine compounds, represent a significant class of six-membered heterocyclic compounds containing three nitrogen atoms. These structures have gained renewed scientific attention due to their versatile biological properties and potential therapeutic applications. Recent studies have highlighted their potential in various biomedical fields, showcasing their anti-tuberculosis, antiviral, and anti-neurodegenerative activities. This research aims to evaluate the effects of six selected 1,3,5-triazine derivatives on multiple life stages of *Drosophila melanogaster* (Oregon-R strain), providing insight into their biological impact in an *in vivo* model.

The biological potential of triazines has been under investigation due to their ability to interact with various biological targets, demonstrating promising pharmacological profiles. For example, research by Singh *et al*. (2018) [9] reviewed the genotoxic and cellular effects of atrazine, a widely used triazine herbicide, noting its potential to disrupt protein synthesis and induce oxidative stress in different organisms, including *Drosophila melanogaster* (Singh *et al*., 2018) [9].

Additionally, McKoy *et al*. (2012) [6] explored the use of triazine derivatives in neurodegenerative disease models, demonstrating how specific compounds reduced amyloid-β aggregation, thereby extending the lifespan and improving motor functions in *Drosophila*. This underscores the neuroprotective capabilities of certain triazine derivatives (McKoy *et al*., 2012) [6].

Triazines have also shown potential as antiviral agents. Zeng *et al*. (2021) [12] reviewed the synergistic effects of flavonoids and triazine-based compounds in reducing viral replication, suggesting that these heterocyclic structures can be strategically employed in designing effective antiviral therapies (Zeng *et al*., 2021) [12].

Despite these promising findings, triazines' full spectrum of biological activities, including potential toxicological effects, remains under-explored. Studies like those conducted by Castañeda-Sortibrán *et al*. (2019) [1] utilized *Drosophila melanogaster* to demonstrate dose-dependent genotoxic effects of triazines through the somatic mutation and recombination test, highlighting their complex interactions at a cellular level (Castañeda-Sortibrán *et al*., 2019) [1].

Given these diverse findings, the current study aims to fill the knowledge gap by investigating the impact of six selected 1,3,5-triazine derivatives on the life stages of *Drosophila melanogaster*, focusing on parameters such as eclosion efficiency, gut damage assay, oxidative stress, mortality assay, morphology and average body weight. This model organism is a well-established system for *in vivo* assessments due to its conserved metabolic pathways and ease of genetic manipulation.

Materials and Methods

1. 1,3,5-triazine derivatives

The study employed six distinct 1,3,5-triazine derivatives, designated as T1 to T6, with their chemical structures.

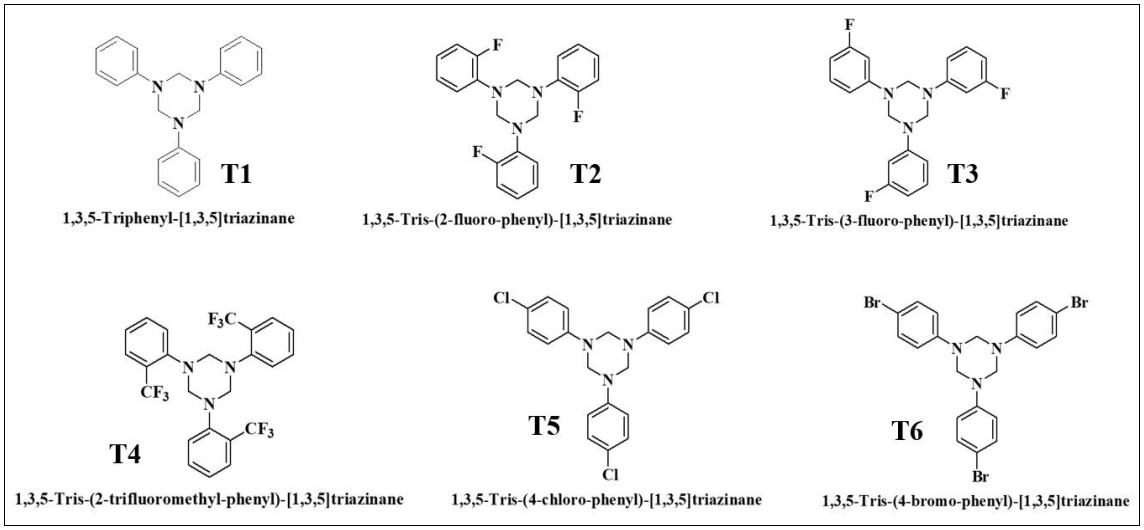

Table 1: 1,3,5-triazine derivatives, designated as T1 to T6 used in the study

Abbreviation	Full Name
T1	1,3,5-Triphenyl-[1,3,5]triazine
T2	1,3,5-Tris-(2-fluoro-phenyl)-[1,3,5] triazine
T3	1,3,5-Tris-(3-fluoro-phenyl)-[1,3,5] triazine
T4	1,3,5-Tris-(2-trifluoromethyl-phenyl)-[1,3,5] triazine
T5	1,3,5-Tris-(4-chloro-phenyl)-[1,3,5] triazine
T6	1,3,5-Tris-(4-bromo-phenyl)-[1,3,5] triazine

The detailed structural analysis was conducted to confirm their composition before use in the experimental procedures (published elsewhere).

2. Fly Maintenance

Wild-type *Drosophila melanogaster* (Oregon-R) flies were raised on a standard food medium that consisted of maize flour, yeast, sugar, agar-agar, and propionic acid. The flies were maintained in a temperature-controlled environment set at 25±1°C to provide optimal conditions for growth and development, following the protocol outlined by Ashburner and Roote (2000).

3. Preparation of Food and Exposure

Food vials were prepared by incorporating 1000 mg/L of each specific triazine derivative (T1 to T6) into the standard food medium. Triazine was properly dissolved in ethanol before adding to the food medium. Control vials contained food without any triazine derivatives. Larvae or adult flies were exposed to triazine-containing food by being transferred into these prepared food plates/vials.

4. Experimental Plan

The experimental setup involved placing 30 flies in each treatment vial, with triplicates maintained for each condition, including control and triazine treatment groups (T1 to T6). Flies were observed daily for mortality and morphological changes over a period of 30 days. The formation of pupae and the eclosion of adult flies from first instar larvae were monitored in different vials. Exposed and control third instar larvae were collected from each treatment group for gut damage and oxidative stress analysis (Fig. 1).

5. Eclosion Efficiency

Eclosion, defined as the emergence of adults from the pupal case, was used to evaluate the effects of triazine exposure on the developmental stages of *Drosophila melanogaster*, particularly from larvae to adult. The developmental progression in both treated and control groups was examined and compared to detect potential delays. Observations were conducted at regular intervals throughout the *Drosophila* life cycle. Any abnormalities or disruptions in the developmental stages, from egg to adult, provided insight into the role of stress induced by triazine exposure. To gain a clearer understanding of developmental impacts, the percentage of pupae formed and the number of adults that successfully emerged were documented.

6. Survival Assay

Survival analysis was conducted by housing freshly eclosed adult flies in vials at a density of 30 flies per vial. These flies were kept under controlled conditions and transferred to new vials with fresh food every 3 days. Mortality was recorded throughout the experiment to evaluate the impact of triazine derivatives on lifespan. Three replicates were performed to ensure statistical accuracy.

7. Morphology and Body Weight

Morphological observations were conducted on larvae and adult flies from both control and treated groups under a stereomicroscope. Abnormalities in structures such as eyes, bristles, wings, and abdomen were noted. The body weight of adult flies was recorded using a sensitive electronic balance, with 50 flies (25 male and 25 female) for each treatment group. Average body weights were calculated and compared across treatments.

8. Oxidative Stress Assay

Oxidative stress was evaluated using the Nitroblue Tetrazolium (NBT) assay. Haemolymph was collected from third instar larvae, and the NBT assay was performed to detect superoxide anion production. The formation of formazan, indicative of oxidative stress, was assessed spectrophotometrically at 595 nm.

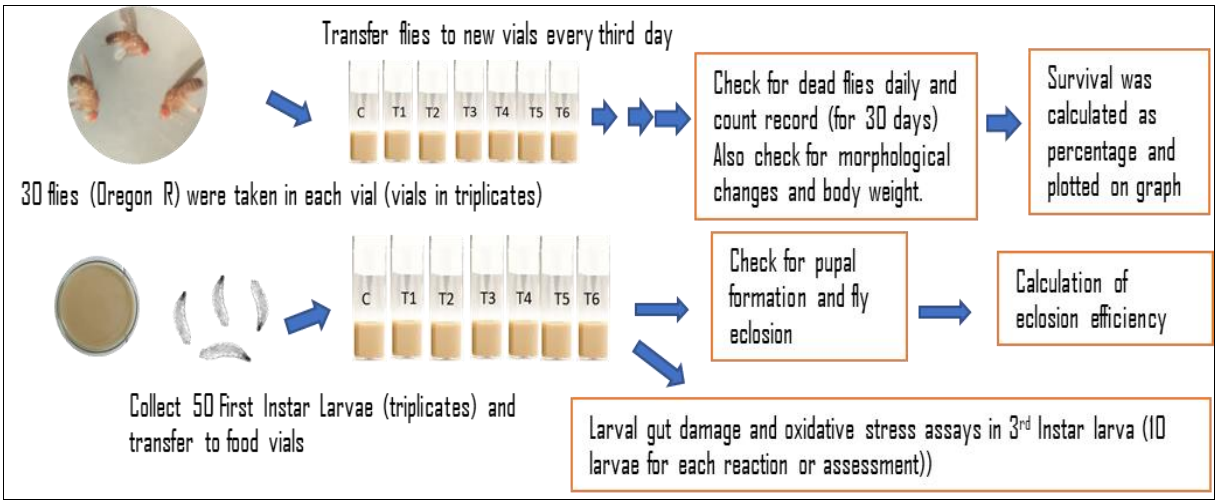

Fig. 1 Overview of the experimental plan and parameters assessed in the study. The flowchart outlines the methodology, including daily monitoring of mortality, morphological changes, and body weight over a 30-day period, followed by calculation of survival percentages. Additional evaluations include pupal formation and fly eclosion for determining eclosion efficiency, as well as gut damage and oxidative stress assays in third-instar larvae, with 10 larvae analyzed per reaction or assessment.

9. Statistical Analysis

Values were expressed as mean ± standard deviation (SD). Data were compiled and presented in graphical format. Statistical significance was determined using ANOVA, performed with GraphPad Prism (version 8.02) software. A *p* value of <0.05 was considered statistically significant.

Results

Morphological Changes

Adult flies did not show any obvious external phenotypic anomaly. Eyes were observed sunken in many cases but that may not due to the exposure as they were observed in the control groups as well (Fig. 2).

Pupal stages showed varied morphological anomalies indicated by arrows in Fig. 3. Control group showed normal pupae early, mid and late pupal stages. In T2, T3 and T5 treated pupae, incomplete eclosion, death within pupa, early pupal death, abnormal segmented pupal case, partially eclosed flies were observed (Fig. 3). There were no gross structural deformities in third instar larvae as well.

Table 2: Pupal morphological anomalies in control and triazine treated flies. A total of 90 pupae were observed in each treatment group out of which number of pupae having at least one abnormal feature are written in the table.

Pupal Stage	Anomaly Score
Control	3
T1	4
T2	12
T3	14
T4	3
T5	15
T6	2

Adult Body Weight

The analysis of adult body weight demonstrated no significant alterations due to exposure to any of the triazine derivatives tested, as shown in Fig. 5. This indicates that the derivatives did not affect the overall growth and development to the extent that would manifest in weight differences.

Eclosion Efficiency and Mortality

Significant effects on eclosion efficiency and mortality were observed with exposure to derivatives T2, T3, and T5. Fig. 6 and 7 illustrate the reduction in eclosion rates and increased mortality in groups treated with these derivatives, suggesting potential developmental toxicity associated with these compounds.

Oxidative Stress

The NBT assay indicated heightened oxidative stress in the larval gut for groups treated with T2 and T5 compared to the control, as depicted in Fig. 8. This implies that these specific derivatives may induce oxidative damage in larval tissues, which could contribute to the observed impacts on developmental processes and survivorship. Although, we could not detect any significant gut damage in trypan blue exclusion assay (Fig. 4).

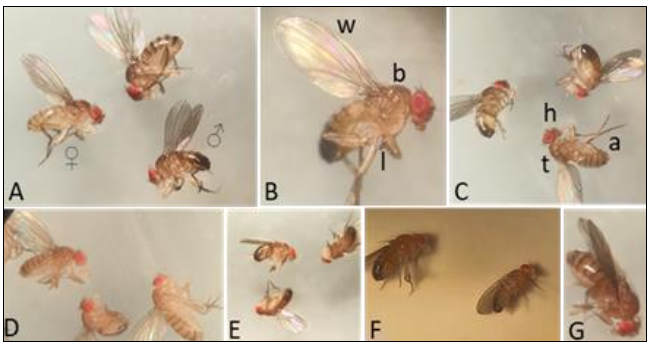

Fig 2: Morphology of adult Drosophila flies in control and treated groups. A Control, ♀: Female, ♂: Male; B T1, l: legs, b: bristles, w: wings; C T2, h: head, t: thorax, a: abdomen; D T3; E T4; F T5; G T6 exposed flies.

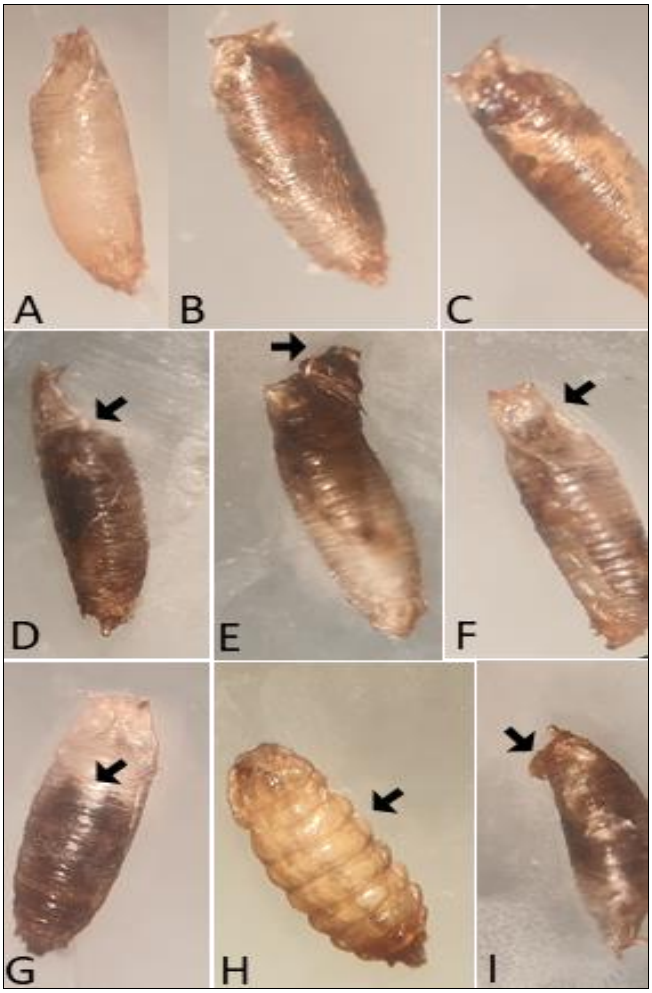

Fig 3: Pupal morphology from Control (A, B, C) and treated D to I. D-E shows incomplete and partial eclosion in larvae treated with T2. F-G display death within pupa in T3 exposed groups. H-I show abnormal segmented pupa and pupal death observed in larvae treated with T5. Images were captured using stereomicroscope.

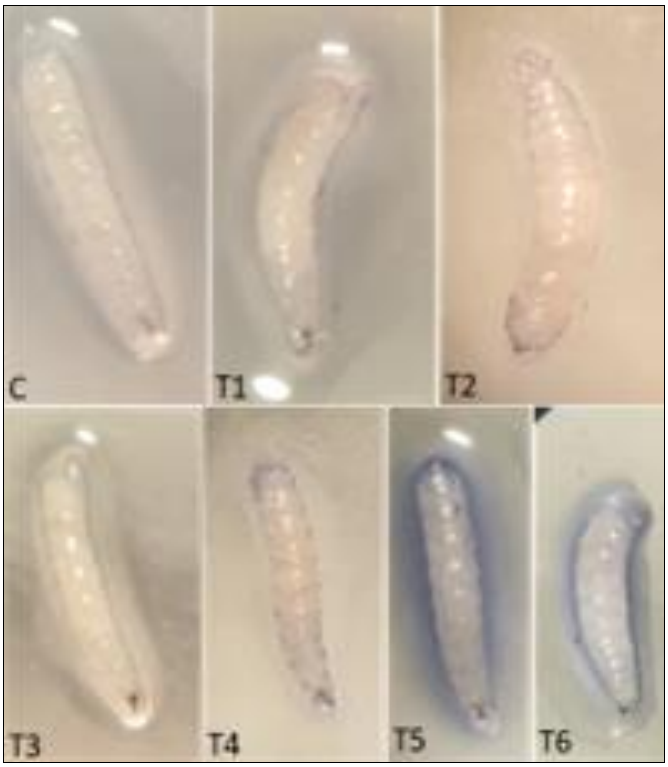

Fig 4: Gut damage assay by trypan blue exclusion exposed with different triazine derivatives (T1 to T6) and control. Blue dye uptake was not visible in any of the larvae treated.

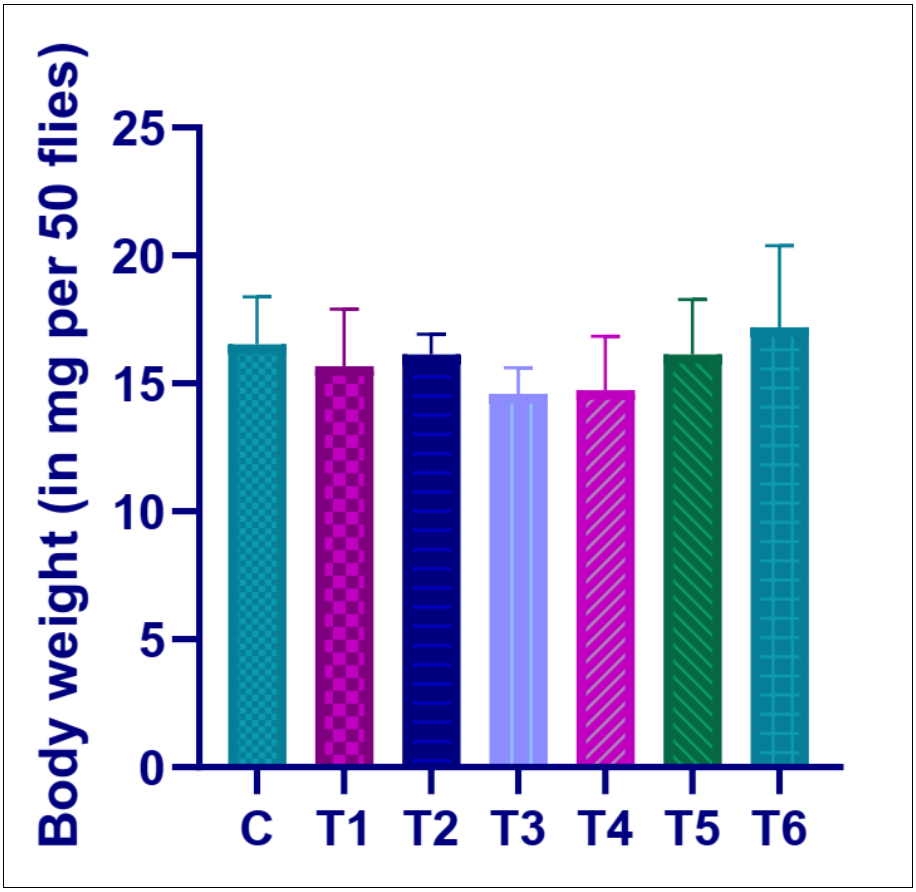

Fig 5: Comparison of body weight (in mg per 50 flies) between the control group (C) and various treatment groups (T1-T6). Each bar represents the mean body weight with standard deviation bars. The texture and color patterns differentiate the control from the treated groups, showcasing variations in body weight due to different treatment conditions.

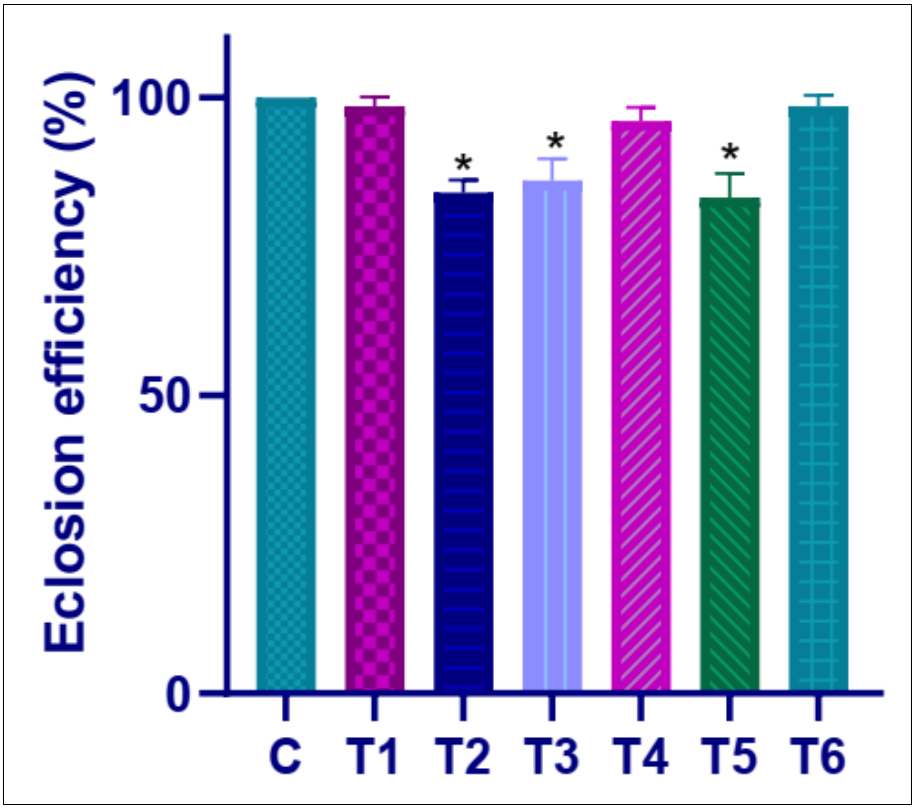

Fig 6: Eclosion efficiency (%) of the control group (C) and treatment groups (T1-T6). Each bar represents the mean eclosion efficiency with standard deviation bars. Asterisks (*) indicate statistically significant differences ($p < 0.05$) compared to the control group. The distinct texture and color patterns distinguish between the different groups, showing the impact of treatments on eclosion rates.

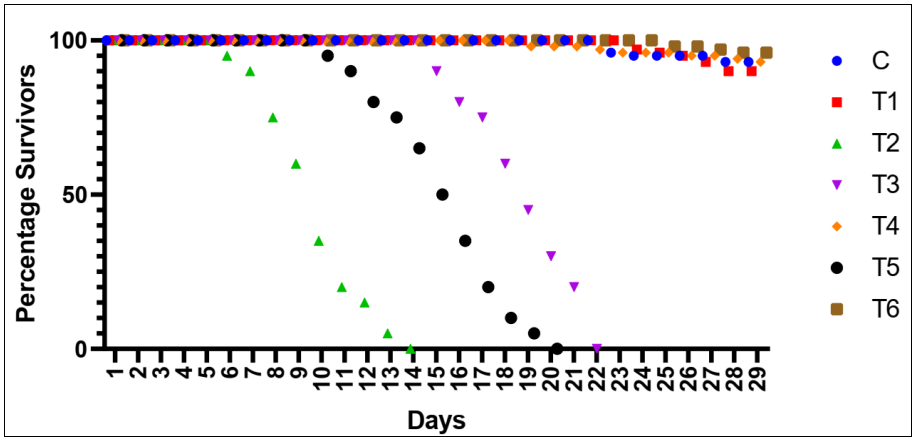

Fig 7: Survival assay over a 30-day period, illustrating the percentage of survivors in the control group (C) and treatment groups (T1-T6). The plot highlights the increased mortality rates observed in groups T2, T3, and T5, as shown by their marked decline over the duration of the assay. Each symbol represents a different group, allowing for easy comparison of survival trends across the treatment conditions.

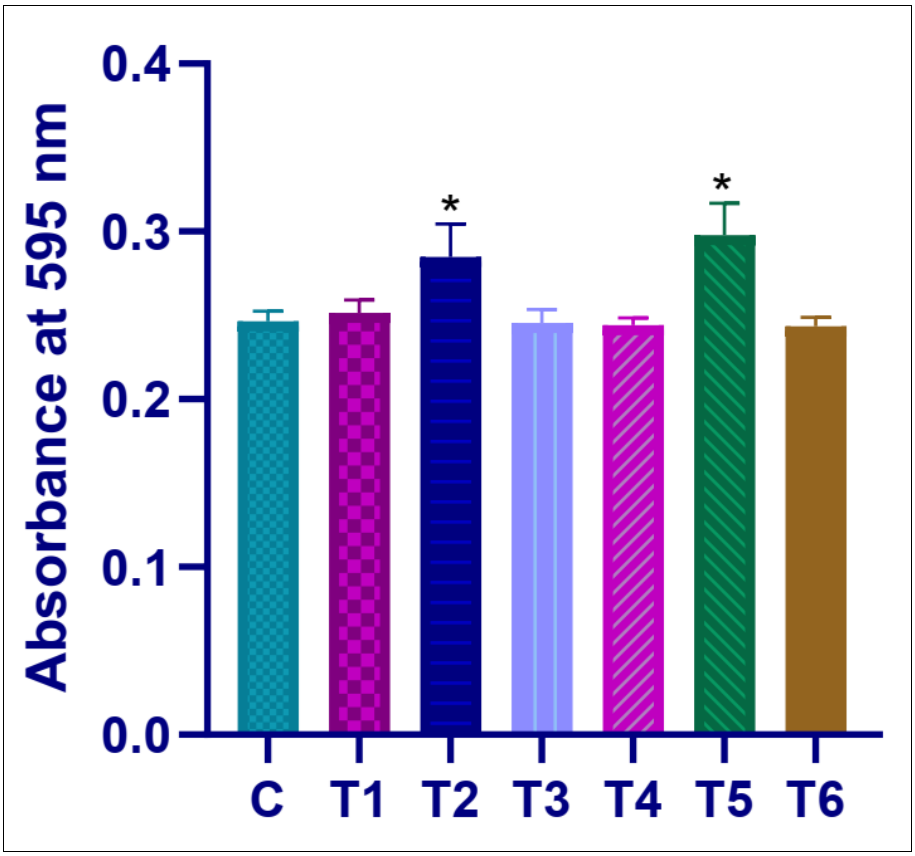

Fig 8: Oxidative stress levels in the gut as measured by the NBT assay (absorbance at 595 nm) for the control group (C) and treatment groups (T1-T6). The data indicate a significant increase in oxidative stress for groups T2 and T5, as denoted by the asterisks (*), compared to the control. Each bar represents the mean absorbance value with standard deviation bars, reflecting differences in oxidative stress induced by the treatments.

Derivatives T1, T4, and T6 did not induce significant changes in any of the parameters measured, including morphological observations, body weight, eclosion efficiency, and mortality rates.

Discussion

The results of this study, which investigated the biological effects of 1,3,5-triazine derivatives on *Drosophila melanogaster*, provide critical insights into the toxic effects of these compounds.

In the current study, pupal morphological anomalies such as pupal death, partial eclosion, abnormal pupa, were observed with 2-fluoro-phenyl, 3-fluoro-phenyl 4-chloro-phenyl triazine derivatives while triphenyl, 2-trifluoromethyl-phenyl and 4-bromo-phenyl triazines did not exhibit any effect. In contrast to our study, Smith *et al.* (2021) [10], reported that triazine concentrations of 10–100 mg/L did not significantly affect morphology over a 30-day period in a different insect model. There can be possible explanations for this deviation. Firstly, the dose level in our study was much higher and secondly, the nature of chemical nature of triazine derivative greatly influence the toxicity outcomes. The absence of significant body weight changes in the current study aligns with these findings, suggesting overall growth may not be adversely impacted even if subtle developmental disruptions occur (Smith *et al.*, 2021; Jones *et al.*, 2020) [3, 10].

The significant reduction in eclosion efficiency and increased mortality observed with T2, T3, and T5 indicate potential developmental toxicity. This should be highlighted here that in our study, 1,3,5-Triphenyl-[1,3,5]triazine, 1,3,5-Tris-(2-trifluoromethyl-phenyl)-[1,3,5] triazine and 1,3,5-Tris-(4-bromo-phenyl)-[1,3,5] triazine did not affect fly eclosion percentage from treated larvae, therefore, structure activity relationships is evident which is described below.

Studies on atrazine, a widely used herbicide, have revealed adverse impacts on different life stages in *Drosophila melanogaster*. When exposed to atrazine concentrations of 10 μM and 100 μM, *D. melanogaster* embryos and larvae experienced notable decreases in pupation and adult emergence rates, reflecting reduced developmental viability. Notably, while developmental timing and sex ratio remained unaffected, atrazine exposure induced oxidative stress, evidenced by elevated ROS levels and increased oxidative damage (Figueira *et al*., 2017) [2]. Marcus and Fiumera (2016) [5], flies were chronically exposed to atrazine concentrations ranging from 0.2 to 20 parts per million (ppm). The researchers assessed several parameters, including longevity, development time, pupation and emergence rates, body size, female mating rate, fertility, and fecundity. The results indicated that atrazine exposure decreased pupation and emergence rates, reduced adult survival, and accelerated development time. Interestingly, despite the faster development, some exposure levels led to an increase in body size. Our study is in resonance with these studies with reference to T2, T3 and T5.

The heightened oxidative stress in larvae exposed to T2 and T5, as measured by the NBT assay, points to ROS accumulation and potential mitochondrial disruption. This observation is also consistent with the study conducted by Figueira *et al*., 2017 [2]. In a study, Khalid *et al*. (2022) [4] investigated the impact of cyromazine (N-Cyclopropyl-1,3,5-triazine-2,4,6-triamine), a triazine based insect growth regulator, on the reproductive biology of *Drosophila melanogaster*. Their findings revealed a 58% reduction in fecundity following cyromazine treatment. Further analysis showed a significant decrease in the number of germline stem cells (GSCs) and cystoblasts (CBs) in the ovaries of treated females compared to controls.

Triazine toxicity has mixed picture in literature. There are studies which have shown negligible toxicity and even protective role of certain triazines. For instance, researchers explored the potential genoprotective and anti-inflammatory effects of newly synthesized 1,2,4-triazine derivatives against endosulfan-induced toxicity. This study builds on previous work demonstrating the neuroprotective properties of these compounds in neurotic cell lines exposed to oxidative stress and neurotoxic agents (Naderi *et al*., 2018) [8].

In another study by Velisek *et al*. (2017) [11], the authors investigated the effects of three triazine metabolites—terbuthylazine 2-hydroxy (T2H), terbuthylazine-desethyl (TD), and atrazine 2-hydroxy (A2H)—on the early life stages of marbled crayfish (*Procambarus fallax* f. *virginalis*). The research focused on chronic exposure to these metabolites, both individually and in combination, at concentrations reflecting environmental relevance. The study concluded that chronic exposure to environmentally relevant concentrations of T2H, TD, A2H, and their mixture does not adversely affect the early life stages of marbled crayfish. These findings suggest that, at the tested concentrations, these triazine metabolites pose a low risk to the development and survival of this species.

The deviations observed in this study compared to prior reports may stem from several factors: the use of a higher concentration (1000 mg/L) compared to typical 5-300 mg/L ranges in the literature, extended exposure duration up to 30 days versus shorter acute or subchronic periods, species-specific sensitivity of Drosophila melanogaster to triazine derivatives, and distinct functional groups in T2 and T5 potentially enhancing oxidative stress or interacting with developmental pathways differently from other compounds.

The observed biological effects, such as morphological anomalies, oxidative stress, increased mortality, and decreased eclosion, were noted in triazine treatments involving halogen-substituted compounds: T2 (2-fluoro), T3 (3-fluoro), and T5 (4-chloro), indicating that fluorine and chlorine substituents at various positions contribute to increased toxicity and biological stress. The specific positions of these substituents on the phenyl ring—ortho for T2, meta for T3, and para for T5—resulted in varied impacts, with T2 potentially causing higher reactivity due to its ortho position affecting steric and electronic interactions, while T5's para-positioned chlorine exerted electron-withdrawing effects that may have enhanced its interaction with biological targets. In contrast, T1 (unsubstituted phenyl), T4 (2-trifluoromethyl), and T6 (4-bromo) did not exhibit significant adverse effects. The base structure in T1 appeared non-toxic, while T4's bulky trifluoromethyl group and T6's para-bromo substituent potentially reduced interactions due to steric hindrance and lower reactivity. The electron-withdrawing properties of fluorine and chlorine in T2, T3, and T5 likely destabilized biological structures or interfered with enzymatic functions, leading to stress responses, whereas T4's CF3 group, although electron-withdrawing, may have been sterically limited in its effect. Halogens, such as chlorine and fluorine, are known to enhance hydrophobicity and binding affinity, which could explain the increased adverse impacts observed in T2, T3, and T5. Overall, the SAR analysis highlights that the type and position of halogen substituents significantly influence the biological activity of triazine derivatives, with fluoro and chloro substituents increasing toxicity, while unsubstituted or bulkier groups like trifluoromethyl and bromo may mitigate these effects.

Conclusion

This study provides critical insights into the toxicological effects of 1,3,5-triazine derivatives on *Drosophila melanogaster*, highlighting the influence of specific substituents on toxicity. Notably, derivatives with halogen substitutions—2-fluoro (T2), 3-fluoro (T3), and 4-chloro (T5)—induced significant morphological anomalies, oxidative stress, increased mortality, and reduced eclosion rates. In contrast, compounds with unsubstituted phenyl (T1), 2-trifluoromethyl (T4), and 4-bromo (T6) groups exhibited minimal adverse effects. These findings underscore the importance of substituent type and position in determining the biological activity of triazine derivatives. The observed discrepancies with previous studies may be attributed to differences in concentration, exposure duration, species-specific sensitivity, and structural variations of the compounds tested. This research emphasizes the necessity for comprehensive evaluations of triazine derivatives to inform their safe application in various fields.

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