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Assessment of Crawling Activity in Drosophila melanogaster Larvae Exposed to Zinc Chloride and Vitamin C

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ABSTRACT:

Micronutrients such as metals and vitamins are essential for physiological processes, but imbalances can result in toxic effects. Zinc plays a crucial role in enzymatic function, growth, and neural signalling, while vitamin C serves as a potent antioxidant mitigating oxidative stress. This study evaluated the impact of zinc chloride ($ZnCl_2$) and vitamin C on the locomotor behaviour of Drosophila melanogaster larvae using a crawling assay.

Third-instar larvae were subjected to $ZnCl_2$, alone and in combination with vitamin C, to monitor neuromuscular effects. Their movement patterns were recorded on agar plates, and deviations in crawling behaviour were analysed. Results showed that $ZnCl_2$ impaired larval movement, indicating neurotoxicity. However, co-treatment with vitamin C partially restored normal locomotion. These findings underscore the dual role of zinc as both essential and toxic depending on concentration, and they highlight the protective role of vitamin C in ameliorating heavy metal-induced stress.

Keywords: Drosophila melanogaster, zinc chloride, Vitamin C, crawling assay, locomotion, oxidative stress, neurotoxicity, antioxidants, behavioural response, micronutrient imbalance.

INTRODUCTION:

Toxicology is the scientific study of harmful effects caused by chemical substances on living organisms. It includes various branches such as environmental, medical, molecular, and developmental toxicology. Among environmental pollutants, heavy metals are of particular concern due to their persistence, bioaccumulation, and toxicity. Heavy metals like lead (Pb), cadmium (Cd), mercury (Hg), arsenic (As), and zinc (Zn) are often released into the environment via industrial emissions, mining, waste disposal, and agricultural runoff [Jaishankar et al., 2014]. Although some metals like zinc are essential micronutrients, they become toxic when present at high concentrations. These elements enter biological systems through contaminated food, water, or air and can disrupt physiological processes, leading to developmental delays, organ dysfunction, and oxidative stress-related diseases [Koyama et al., 2024; Tchounwou et al., 2012].

The toxic effects of heavy metals are primarily mediated through the generation of reactive oxygen species (ROS), which causes oxidative stress. This leads to damage of cellular components such as lipids, proteins,DNA. Mechanistically, heavy metals impair mitochondrial function, cause membrane lipid peroxidation, and trigger apoptosis via activation of the caspase cascade [Flora et al., 2008; Valko et al., 2005]. They also interfere with essential metal ions such as calcium and magnesium, affecting cell signalling and enzyme function. In plants, metals inhibit chlorophyll biosynthesis and root elongation, disrupt photosynthesis, and affect water balance [Rai et al., 2021]. In animals, heavy metal toxicity leads to neurotoxicity, hepatotoxicity, nephrotoxicity, and reproductive impairment. Chronic zinc exposure in animals has been linked to inflammation, oxidative stress in brain tissues, testicular damage, and reduced sperm motility [Prakash et al., 2015; Das et al., 2017]. In fish, excess zinc affects gill function and oxygen uptake, while in mammals, it impairs learning, memory, and liver function. Zinc is an essential element required for growth, immune function, wound healing, and enzymatic activity. It acts as a cofactor in antioxidant enzymes like superoxide dismutase (SOD). However, when zinc levels exceed physiological limits—particularly in the form of zinc chloride ($ZnCl_2$)—it becomes cytotoxic. At low concentrations, $ZnCl_2$ supports normal growth and metabolism, but high levels induce ROS generation, oxidative damage, and apoptosis. Zinc disrupts mitochondrial membrane potential, promotes DNA fragmentation, and impairs reproductive function in animals [Ho, 2004; Valko et al., 2005]. $ZnCl_2$ also causes testicular degeneration, reduces sperm quality, and alters hormonal profiles in rodents and aquatic organisms [Das et al., 2017; Prakash et al., 2015]. In plants, excess $ZnCl_2$ reduces seed germination, impairs nutrient uptake, and causes visible toxicity symptoms such as chlorosis and necrosis [Rai et al., 2021]. These findings underscore the dual nature of zinc—essential in trace amounts but harmful when unregulated. Vitamin C, a water-soluble antioxidant, plays a pivotal role in protecting cells from oxidative stress by directly scavenging free radicals and regenerating other antioxidants like vitamin E and glutathione. It is also involved in collagen synthesis, immune function, and iron absorption [Padayatty et al., 2003]. In the context of heavy metal toxicity, vitamin C has been shown to protect animal tissues from oxidative damage. For instance, in rats exposed to zinc and cadmium, vitamin C supplementation restored antioxidant enzyme activity, reduced lipid peroxidation (MDA levels), and improved liver and kidney histology [Das et al., 2017]. It also modulated apoptotic pathways by reducing the expression of pro-apoptotic markers (e.g., Bax, caspase-3) and increasing anti-apoptotic proteins like Bcl-2. In

aquatic animals like fish, vitamin C has reduced gill and liver damage caused by zinc exposure. Its role extends to the central nervous system, where it protects neurons from oxidative stress, maintains mitochondrial integrity, and improves behavioural responses under toxic conditions [Kazmierczak-Baranska et al., 2020]. However, the effectiveness of vitamin C depends on the dose of the toxicant and timing of administration. At very high metal concentrations, its protective effect may be overwhelmed, and in some cases, it may even act as a pro-oxidant in the presence of redox-active metals like iron and copper [Kazmierczak-Baranska et al., 2020].

Drosophila melanogaster, the fruit fly, is a widely used model organism in developmental biology and toxicology. It offers several advantages, including short generation time, ease of culture, cost-effectiveness, and genetic similarity to humans—over 70% of human disease-related genes have Drosophila homologs [Bellen et al., 2010]. Drosophila is particularly sensitive to metal-induced stress and has been used to study the developmental effects of zinc, cadmium, and copper. Studies have shown that exposure to $ZnCl_2$ in Drosophila reduces larval viability, delays development, and impairs adult emergence [Cankaya et al., 2020]. Co-treatment with antioxidants like vitamin C improves survival and counteracts oxidative stress markers such as ROS and malondialdehyde (MDA) [Aishwarya et al., 2024].

The presence of mammalian-like antioxidant pathways in Drosophila—including SOD, catalase, and glutathione S-transferase—makes it an excellent model to evaluate the balance between toxicity and antioxidant defense [Krittika et al., 2019]. Behavioural and phenotypic changes in Drosophila, such as altered pupation height and emergence rate, serve as reliable endpoints for assessing toxicity and protective interventions. Given the toxic potential of zinc chloride at higher concentrations and the known protective properties of vitamin C, the present study investigates the effect of $ZnCl_2$ exposure on the viability of Drosophila melanogaster and the extent to which vitamin C can mitigate this toxicity. Through controlled treatments and statistical analysis, this study aims to clarify the dose-dependent effects of $ZnCl_2$ and evaluate vitamin C as a potential antioxidant therapy for metal-induced stress.

CRAWLING ASSAY:

Locomotion is a fundamental biological function essential for survival-related behaviours such as foraging, predator avoidance, and reproduction. In *Drosophila melanogaster*, locomotion involves a variety of rhythmic motor patterns, including crawling, turning, and burrowing. These behaviours are driven by energy availability, influenced by both nutrient composition and environmental factors. Excess exposure to heavy metals like zinc chloride ($ZnCl_2$) can impair locomotor functions by inducing oxidative stress, disrupting calcium homeostasis, and interfering with mitochondrial efficiency. Vitamin C (ascorbic acid), a known antioxidant, counteracts oxidative damage by neutralizing free radicals and stabilizing cellular membranes.

Every single task that an organism engages in to ensure its existence and procreation requires energy. e.g., movement, courting, and locomotion. Locating food, finding a partner, escaping from predators, defending one's territory, and reacting to stress all depend on locomotion, which is an essential activity. As a result, it is essential to the majority of animal behaviour (Jordan et al., 2007). Food is the source of energy for animals, and diets can be categorized as quantitative (food availability) or qualitative (composition). Since animals get their energy and other nutritional needs from food, the qualitative impacts are obvious. For this reason, animals' ability to survive, move, and reproduce depends on maintaining a balance between their energy intake and expenditure (Pough, 1989; Sibly, 1991). The interaction of matter intake, digestion, and the distribution of newly acquired energy among different processes, including growth, reproduction, and locomotion, determines the balance (Karasov, 1986).

Energy imbalance resulting from sedentary lifestyles, urbanization affects the organism. Heavy metals such as Zinc chloride ($ZnCl_2$) exert a dual impact on biological systems. At trace levels, Zinc is an essential micronutrient involved in enzymatic activity, gene expression, and immune regulation in both humans and *Drosophila melanogaster* (Vallee & Falchuk, 1993). However, at elevated concentrations, $ZnCl_2$ becomes toxic, disrupting cellular homeostasis by competing with essential ions like calcium and magnesium, inhibiting enzymes, and generating reactive oxygen species (ROS) (Stohs & Bagchi, 1995). In *Drosophila*, excessive zinc exposure leads to impaired locomotor behaviour, oxidative stress, mitochondrial dysfunction, and altered gene expression—effects that mirror neurodegenerative and metabolic issues observed in humans with chronic heavy metal exposure (Egli et al., 2006). Vitamin C (ascorbic acid), a potent water-soluble antioxidant, mitigates these effects by neutralizing ROS, regenerating other antioxidants, and stabilizing cellular membranes (Valko et al., 2005). Studies in *Drosophila* and mammalian models have shown that Vitamin C supplementation can reduce oxidative damage, restore behavioural performance, and support metal detoxification pathways. Thus, while zinc chloride is biologically necessary at low doses, its toxic effects at high concentrations can be significantly alleviated by antioxidant co-treatment, demonstrating conserved protective mechanisms across species such as humans and *Drosophila*. Its impact on *Drosophila's* locomotor activity and health advantages, however, is unknown. Because human diseases and *Drosophila* share many metabolic mechanisms, *Drosophila* is an excellent model organism for studying human diseases. Rhythmic movements such as respiration, digestion, circulation, and locomotion are fundamental to animal life. In *Drosophila* larvae, many behaviours have been studied such as phototaxis, learning, and navigation (Iyengar et al.,1999; Freeman et al., 2010; Luo et al., 2010). These and other behaviours are based on regulating locomotion in response to stimuli or experience. *Drosophila* larval locomotion includes a repertoire of many different types of movements, including turns, burrowing, linear crawling, and other movements (Green et al., 1983; Wang et al., 1997; Huang et al., 2007). The relative complexity of larval locomotor behaviour has precluded detailed analysis of the neuromuscular mechanism underlying any one type of movement, such as linear crawling. However, understanding the motor pattern that drives behaviour is a requisite step in understanding its cellular basis (Marder and Calabrese, 1996). Thus, the goal of the current investigation is to learn more about how different concentration of Zinc chloride and vitamin C affects *D. melanogaster's* locomotor behaviour.

MATERIALS AND METHODS:

1. **Fly culture**: Wild type *Drosophila melanogaster*- Oregon K strain (OK) was obtained from *Drosophila*Stock Centre, University of Mysore. Flies were grown and aged in culture bottles/vials on wheat cream agar media (100 Sooji, 100g jaggery, 10g agar and 7.5ml propionic acid in 1 Ldistilled water) with regular sub-culturing and maintained for all experiments at 24^{o}C with 60-70% relative humidity and ambient lighting condition with a sprinkle of live Baker's yeast. All collection of virgins, adult flies were performed under brief anaesthesia. Dose administration was achieved via larval feeding for all treatments. (D'Souza and Shakunthala, 2015)

Diet preparation	
Control	100 Sooji, 100 g jaggery, 10 g agar and 7.5 ml propionic acid in 1 L distilled water
ZT1	250 ml of control media containing 0.17 g (5 mM) of heavy metal, $ZnCl_2$
ZT2	250 ml of control media containing 0.23 g (7 mM) of heavy metal, $ZnCl_2$.
ZT3	250 ml of control media containing 0.23 g (7 mM) of heavy metal, $ZnCl_2$ and 0.05 g of Antioxidant, Vitamin C.

2. **Embryo collection:**

   A. Delcour media preparation: Add 6gm of Agar (2g of sucrose, if it is needed) to 200ml of boiling water and stir well until the agar melts into solution. Remove the beaker from heater; add 5ml of alcohol and 3ml of glacial acetic acid. Let it cool. The molten medium is poured into Petri dishes and allowed to cool and solidify. The prepared agar plates can be stored in a plastic bag at 4^{o}C for a few days.

   B. Preparation of Yeast paste: Add water to a small amount of dry Baker's yeast to bring it to a consistency of thick paste and allow it to ferment for a few hours in a closed container.

   C. Flies for embryo collection: Keep 4-6 days old flies in a fresh food bottle with additional dry yeast added for a day prior to egg collection.

   D. Note: A major fraction of the flies being transferred for embryo collection should be females. Feeding on yeast-supplemented food enhances egg-laying by females.

   E. Setting up the embryo collection cups: Modified from Delcour (1969).

- Take a transparent plastic container whose mouth matches the size of the sucrose agar plates. Using a heated needle pierce a number of small holes (should not allow the flies to move out) on the wall of the container, to help air flow in and out of the container.
- Bring the Agar plates to room temperature (if stored in refrigerator) and place a small dab of yeast paste in the centre of the plate.
- Transfer the well-fed flies from culture bottle into the plastic container without anesthetizing them by inverting the food bottle on a funnel placed on the mouth of the container.
- Tap the container so that flies fall down to the bottom, and cover the mouth of the container with agar plate, keeping the agar side facing inward.
- Seal the plate and the rim of the collection chamber with a sticky tape.
- Keep the set up with agar plate down position, preferably in a dark place.
- Allow the flies to lay eggs on the plate for required number of hours at desired temperature depending on the experimental specifications.
- Note: In order to get embryos of different stages of development in a single collection, egg-collection may be continued for 18-20 Hr. [Delcour, J. (1969)]

CRAWLING ASSAY:

Third-instar larvae from each treatment group were gently rinsed in 1% phosphate-buffered saline (PBS) and transferred to 1% agar with 1% sucrose plates. A layer of yeast paste was applied to visualize larval movement. Plates were placed over pre-marked grid sheets, and the number of grid lines crossed by each larva within 2 minutes was recorded. Each treatment was performed in triplicate using 10 larvae per trial.

STATISTICAL ANALYSIS:

Data were analysed using IBM SPSS version 29.0. Descriptive statistics (mean, standard error), one-way ANOVA, and Tukey's Post-Hoc test were used to compare crawling performance across treatment groups. Statistical significance was set at $p < 0.05$.

RESULTS:

The larval crawling assay was conducted using 1% non-nutritive agar media (Delcour procedure, 1969)., a graph sheet and third instar larvae grown in control and treated media were used to study the effect of Zinc chloride and vitamin C on the crawling behaviour of the larva. The area of cubic squares

covered by the larvae was measured in every 2 minutes on agar media containing yeast paste. The graph of the larval movement was drawn and the area was counted. Fig. 1 shows the larval crawling assay setup with agar media. Fig.2 is a graph that depicts the movement of the larva in different directions covering different area on the graph sheet. Fig.3 is a bar graphshowing the effect of $Zncl_2$ and vitamin C on 3rd instar larvae of*Drosophila melanogaster* of crawling assay.The crawling activity of Drosophila larvae was significantly affected by $ZnCl_2$ exposure. Larvae in the ZT2group (7 mM $ZnCl_2$) showed the lowest crawling distance, indicating locomotor impairment. ZT1 larvae (5 mM$ZnCl_2$) exhibited near-normal movement. ZT3 (7 mM $ZnCl_2$ + Vitamin C) demonstrated improved crawlingcompared to ZT2, suggesting partial neuroprotection by vitamin C. ANOVA results ($F = 7.961$, $p < 0.000$)confirmed significant differences among groups.As shown in the bar graph, different treatment groups (ZT1, ZT2, ZT3) resulted in variations in crawling activity compared to the control. Tukey's Post Hoc test further confirmed that larval crawling behaviour was significantly different between specific concentrations and the control group in the Zinc chloride and Vitamin C have a significant effect on the locomotor activity of third instar *Drosophila melanogaster* larvae.

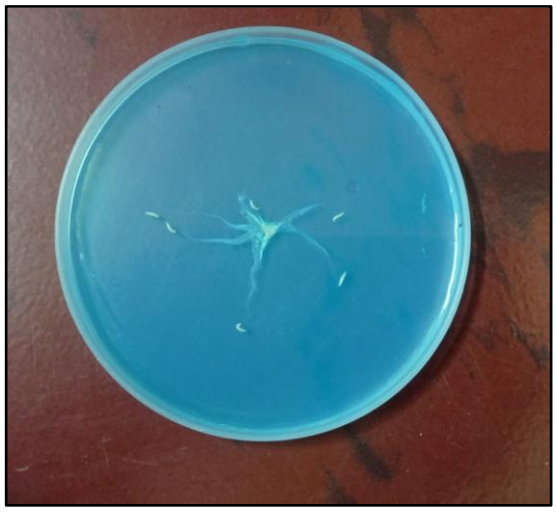

Fig. 1: Experimental setup for crawling assay

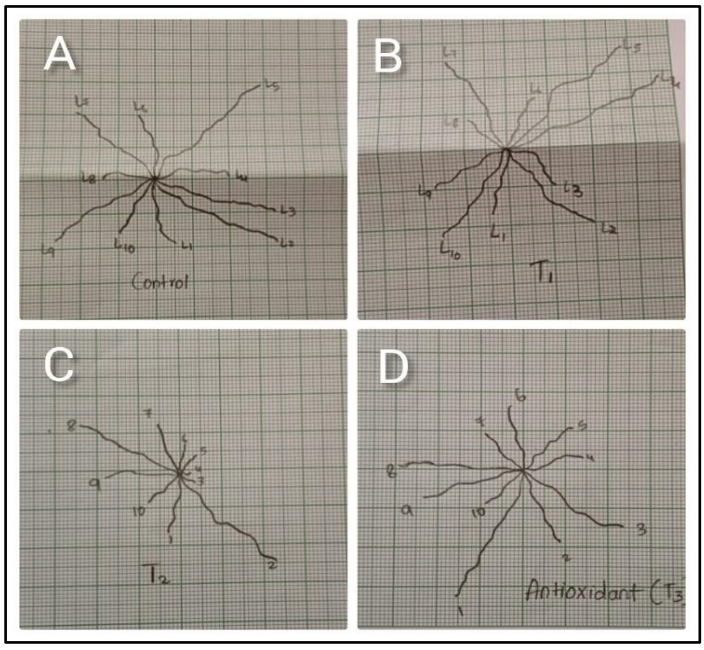

Fig. 2: Graph that depicts movement of larvae on different concentration of Zinc chloride and vitamin C. A – control group, B – 5 mM Zinc chloride, C – 7 mM Zinc chloride, D – 7 mM Zinc chloride + 0.05 g of vitamin C.

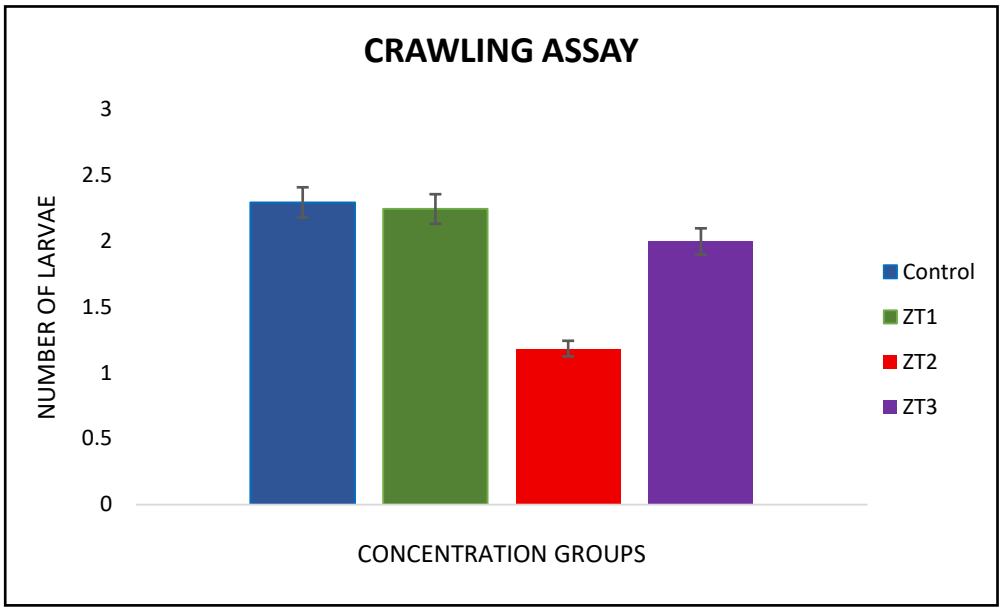

Fig.3: Effect of $ZnCl_2$ and Vitamin C on 3rd instar larvae of*Drosophila melanogaster* of crawling assay

DISCUSSION:

In the present investigation, the larval crawling assay was conducted using 1% non-nutritive agar media (Delcour procedure, 1969). A graph sheet was used to measure the area covered by third instar larvae grown in control and treated media.This graph depicts the movement of larvae in different directions, covering various areas on the graph sheet, as represented in the bar graph, reveal a clear variation in crawling behaviour of Drosophila melanogaster third instar larvae across different treatment groups—ZT1 (5 mM $ZnCl_2$), ZT2 (7 mM $ZnCl_2$), and ZT3 (7 mM $ZnCl_2$ + Vitamin C)—in comparison to the control. The control group exhibited the highest mean crawling distance, indicating normal locomotor activity in the absence of any chemical treatment. In the ZT1 group, treated with 5 mM $ZnCl_2$, the larvae displayed a crawling performance close to that of the control, with only a slight decrease. This suggests that at low concentrations, Zinc acts as a beneficial micronutrient. Zinc supports essential physiological functions without inducing toxicity. Zinc plays a crucial role in enzymatic activity, gene expression, and neural development, all of which influence motor performance (Maret, 2013). Such findings are consistent with studies where optimal dietary Zinc improved Drosophila development and behaviour without adverse effects (Sohal et al., 1993). However, in the ZT2 group, where the $ZnCl_2$ concentration was increased to 7 mM, a significant decline in crawling activity was observed. This decrease suggests that higher levels of Zinc induce toxic effects on the larvae. At elevated concentrations, Zn^{2+} ions can cause oxidative stress by interfering with cellular redox balance and by damaging proteins, lipids, and nucleic acids. This result implies that elevated Zinc levels exert neurotoxic effects, consistent with literature suggesting that excessive Zn^{2+} accumulation disrupts cellular homeostasis, induces oxidative stress, and impairs mitochondrial and neuronal function (Deshpande et al., 2005). High Zinc concentrations have been shown to inhibit motor function in Drosophila through oxidative damage and interference with calcium signalling pathways, which are critical for muscle contraction and neuron firing (Sarma et al., 2020). This group exhibited the lowest mean crawling distance among all, indicating impaired neuromuscular performance due to the toxic burden of Zinc overload. Interestingly, in the ZT3 group, which received 7 mM $ZnCl_2$ along with Vitamin C, animprovedin larval crawling behaviour was observed compared to ZT2. While the activity did not return to control levels, it was significantly improved, highlighting the protective role of Vitamin C. As a strong antioxidant, Vitamin C neutralizes free radicals and restores intracellular redox balance (Padayatty et al., 2003). It scavenges free radicals and helps to regenerate other antioxidants within the cell, thus maintaining redox balance and preventing cellular damage. This partial restoration of movement suggests that Vitamin C may alleviate some of the neurotoxic and physiological impairments caused by excessive Zinc exposure. This antioxidant-mediated recovery is supported by findings that show dietary Vitamin C improves oxidative stress tolerance and behavioural outcomes in model organisms (Kumar & Sharma, 2020).

The ANOVA table supports these observations statistically, showing a highly significant difference between the groups ($F = 7.961$, $p < 0.000$), confirming that the variations in crawling behaviour across the concentrations were not due to random chance. These findings collectively suggest that while low concentrations of $ZnCl_2$ are relatively safe or even beneficial for larval activity, higher concentrations are detrimental, and co-treatment with antioxidants like Vitamin C can effectively reduce the toxic effects and improve overall locomotor performance in Drosophila larvae.

Drosophila melanogaster is a widely used model organism in biomedical research due to its genetic tractability and short life cycle. This study explored the effects of different concentrations of Zinc chloride and vitamin C on the locomotor behaviour of Drosophila melanogaster larvae. The findings indicate that 5mM of zinc chloride that is in trace quantitypositively influences larval crawling behaviour, with larvae exposed to 7mM of Zinc chloride displaying less movement compared to those on control media and 5mM of Zinc chloride, and combined effect of 7mM Zinc chloride and 0.05g of vitamin Cnoticeable recovery in larval crawling behaviour was observed compared to ZT2. The results suggest that 7mM of Zinc chloride is more effective in reducing larval crawling behaviour than 5mM concentration of Zinc chloride.

Based on the study's findings, 7mM of Zinc chloride is more toxic than 5mM Zinc chloride in affecting the locomotory and muscular function of the organism. Therefore, it is recommended to use heavy metals such as Zinc,lead in trace quantitiesto support better locomotor behaviour in Drosophila melanogaster. This research highlights the effect of Zinc chloride and vitamin Cas a supplementin trace quantity, promoting increased physical activity and overall fitness.

CONCLUSION:

The study suggests that different concentration of Zinc chloride and combined effect of Zinc chloride with vitamin Ccan influence the crawling behaviour of Drosophila melanogaster larvae, with trends indicating increased and decreasedmovement. While individual comparisons did not yield statistically significant differences, overall analysis and post hoc tests revealed significant effects, indicating that Zinc chloride and vitamin C may stimulate larval activity. The results highlight, high concentrations of $ZnCl_2$ (7 mM) reduced larval locomotion, indicating toxicity. Co-treatment with VitaminC partially restored movement, suggesting antioxidant-mediated protection.But further research with larger sample sizes and detailed mechanistic studies are needed to confirm these findings and understand the underlying biological effects of Zinc chloride and vitamin C on larval behaviour.

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