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Analyzing the Impact of Zinc Chloride and Vitamin C on Olfactory Behaviour in *Drosophila melanogaster* Larvae

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Abstract

Zinc Chloride toxicity has been a matter of concern and a major risk factor for public health in many parts of the world. Zinc is a toxic heavy metal that is widely dispersed in the environment due to various human activities such as industrial emissions and the use of zinc-based products. Inorganic zinc is highly neurotoxic due to its ability to cross the blood-brain barrier. Moreover, its exposure poses a significant threat to human health, including alterations in the central nervous system (CNS), and deficits in memory, learning, and attention. In this study, we have explored the possibility and usefulness of the vinegar fly (commonly called fruit fly) *Drosophila melanogaster* as a model organism to investigate the effects of chronic exposure to zinc chloride employing various behavioural assays. The vinegar fly offers the adaptability and toolkit needed for researchers to experimentally examine and study the behaviour and gene expression in a defined population. We have assessed the toxic effects of inorganic zinc chloride on third-instar larvae of vinegar flies for their cognitive and olfactory responses in a time and dose-dependent manner. We evaluated the olfactory response index and learning abilities of zinc-treated larvae. Our results show that chronic exposure to zinc chloride negatively affects the olfaction in the larvae of vinegar flies. Most behavioural parameters investigated were significantly rescued in the zinc-poisoned fruit flies ($p < 0.05$) when co-treated with antioxidant Vitamin C. The results suggest that Vitamin C has a protective effect against zinc chloride in *Drosophila melanogaster* and could potentially be used as a therapeutic agent for poisoning in humans.

Keywords: *Drosophila melanogaster*, zinc chloride, neurotoxicity, olfaction assay, cognitive behaviour, vitamin C, antioxidant, heavy metal toxicity, larval response, learning ability, behavioural assay, environmental toxicology.

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] [14]. 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] [18, 23].

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, and 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] [10, 24]. 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] [22]. 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] [21, 6]. 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

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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] [13, 24]. $ZnCl_2$ also causes testicular degeneration, reduces sperm quality, and alters hormonal profiles in rodents and aquatic organisms [Das *et al*., 2017; Prakash *et al*., 2015] [6, 21]. 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] [22]. 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] [20]. 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] [6]. 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] [15]. 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] [15].

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] [3]. *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* delays larval development, and impairs adult emergence [Cankaya *et al*., 2020] [5]. Co-treatment with antioxidants like vitamin C improves survival and counteracts oxidative stress markers such as ROS and malondialdehyde (MDA) [Aishwarya *et al*., 2024] [1].

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] [19]. 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 olfaction assay 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.

Larval Olfaction Assay

Background: The sense of smell is a vital sense and is important for all the organism starting from bacteria to human beings. The fruit fly, *Drosophila melanogaster* can sense and distinguish wide variety of odors with high sensitivity and varied specificity. The fruit fly has been modal choice for researchers to study olfaction, learning and memory (de Bruyne *et al*., 2001) [7] due to its automatically and genetically simple olfactory system with remarkable similarity with that of human beings. The fruit fly smells using a pair of antenna and maxillary pal which possess morphologically different types of sensilla housing olfactory sensory neurons (OSNs). Compared to the adult fly's olfactory system, the larval olfactory system is relatively simple (Gerber & Stocker, 2007) [12] since unlike the adult fly which need to navigate, the larvae reside in the medium itself. The olfactory system of adult D. melanogaster is composed of two sensory organs, the antennae and maxillary palps, which contain the olfactory receptor neurons. The larva can differentiate many different odors (Heimbeck *et al*., 1999) through three sense organs, viz., dorsal organs (DO), terminal organ (TO), and ventral organ (VO) (Gendre *et al.,* 2004) [11]. The dorsal organ (DO), also called the central dome, has 21 OSNs which innervate 21 larval antenna lobe glomeruli. (Gendre, & Stocker, 2004) [11].

There are several established olfactory behavioral paradigms for measuring the olfactory response in the fruit flies' larvae. An easy to use and highly versatile method called 'larval plate assay' this assay can be used to learn more about the attractants and repellants (Gende & Stocker, 2004) [11].

Drosophila larvae are attracted to wide variety of chemical stimuli. The olfactory response to ethyl acetate, a powerful attractant, was found to be surprisingly well conserved across a variety of different wild-type strains. (Monte *et al.*,1989)

Objective:

Examine olfactory response of third instar larvae.

Material Required:

i). **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 L distilled water) with regular subculturing and maintained for all experiments at 24 °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 anesthesia. Dose administration was achieved via larval feeding for all treatment.

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

ii). Equipment Other and Items for Fly Handling

- **Stereo-binocular microscope Ringer's-Agar plate:** A 1% solution Agar.
- **Ringer's-Agar plate:** A 1% solution Agar in Ringer's solution is prepared by heating 1g Agar in 100ml Ringer's solution (the quantity of Agar and Ringer's solution can be scaled down or up depending upon how many plates are required) mixing slowly till bubbles stop coming out and agar is completely dissolved.
- **10ml of this hot Ringer's-Agar solution** is poured into each glass (or transparent plastic) Petri dish, spread evenly and allowed to cool down and solidify. This plate will be used as testing plate in the larval plate test
- **Larvae handling items:** Needles, fine brushes, forceps, etc. for larvae handling.

iii). Reagents and Solution

Odor Preparation: The required concentration (10^{-1} to 10^{-9}) of the odor compound to be tested is prepared using odorless high grade mineral oil or liquid paraffin as solvent. Ethyl acetate (EA) is one such odorant which has a sweet 'fruity' smell (similar to 'pear drops') which has been shown to be attractive for third instar larvae at lower concentrations. For testing an odorant of unknown response, one should begin with mid-range concentrations (e.g., 10^{-2} or 10^{-3} or 10^{-4}). Depending upon the response, the full concentration profile can be tested. While working with more than tone concentration at the same time, first test the lower concentration and then move to testing higher concentrations. (de Bruyn *et al*.:2007).

Experimental Details

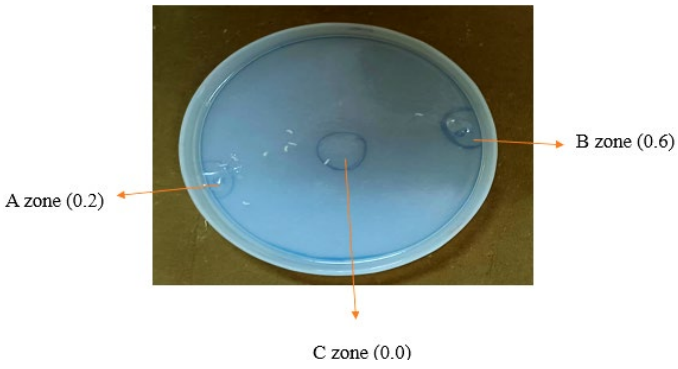

Fig 1: Larval plate assay using a Petri dish. C Zone= Control zone, A zone and B zone respectively; C Zone= area where the test larvae are initially placed.

i). Ten number of third instar larvae, required for the experiment, are obtained by keeping about 200 well-fed and 3-5 days old flies of the desired genotype/s in food bottles and allowing them to lay eggs for 12 hours, at 25 ^{0} C. After 12 hours, the flies are removed to a fresh food bottle and the bottle with eggs is kept at 25 ^{0} C for larval development till early third instar (4-5 days).

ii). Prepare the Ringer's-Agar plates on the day of testing.

iii). Collect early third instar larvae from the food bottle using soft hair brush and place in water (at 25 ^{0} C) for manually washing away the adhering food. Alternatively, after taking out the larvae from the food bottle with soft brush and washing gently with water, transfer them to a tube containing different concentration of ethyl acetate.

iv). Keep the separated larvae in ~1mL Ringer's solution in a separate Petri-dish and soon start the test.

v). the testing Ringer's Agar plate is kept on a black surface (to provide good contrast for the whitish larvae) and using fine forceps two round filter discs are placed in the A zone and B zone odorant zones each

vi). Take around 10 larvae with a soft brush, following which the larvae are quickly transferred to the center (C zone) of the testing plates.

vii). Dispense 20 µL of the test odorant (e.g., Ethyl acetate) on each of the two filter discs in A zone and B zone, quickly close the lid of the dish, and immediately switch ON timer to mark start of the odor test.

Note: Since the larval response to an odor can be affected by its concentration, the odorant can be applied neat (undiluted) or diluted to varying concentration using odorless liquid paraffin.

viii). After two min, quickly count the numbers of larvae in the zones A zone, B zone and C (control zone). Repeat the test with a given odorant three times with fresh larvae and fresh test dishes (Gender & Stocker, 2004) [11].

Statistical Analysis

The data obtained were analysed using IBM SPSS version 29.0. Mean, standard error, one way ANOVA, and Tukey's Post-Hoc test were carried out for the data obtained for Olfaction Assay. A graph of concentration Groups v/s different concentration of ethyl acetate (A, B, & C Zone) for 3rd instar larvae of *D. melanogaster.*

Result

Fig 1. The olfactory response in larvae to ethyl acetate (EA) odour decreased gradually with increasing concentration of Zinc Chloride treatment. The average response index (RI) of untreated larvae was measured as 53.3 percent. When larvae were exposed to different concentration of Zinc chloride of ZT1, ZT2 at 5 mM and 7mM respectively and ZT3 (7 mM +0.05g of vitamin c) concentrations, the average olfactory response index measured was decreased to 45.33 percent in ZT1 and 13.33 percent respectively in ZT2. When larvae were exposed to antioxidant vitamin C, the average olfactory response index measured was decreased to 30 percent inZT3. The olfactory response was not significantly affected at 5mM ZnCl2 treatment as compared to the untreated larvae (Control). There was a difference of 53.3 percent between the response of control larvae and 5 mM $ZnCl_2$-treated larvae for EA. But there was a drastic change in response of 7 mM $ZnCl_2$ larvae when compared to the control group. The effect of arsenic on the olfactory system of the *Drosophila* larvae was observed in their response to odour EA. Fig 2: Reveals the effect of Zinc Chloride and Vitamin C on the olfactory assay of *D. melanogaster* raised in control diet and treated media. According to the data obtained the larval plate assay

was found to be high in Zinc Chloride treated media compared to control. In between the concentration groups of control media which is significant with $p < 0.005$, $df = 2$ and $F = 30.33$. Fig 3: reveals the effect of Zinc Chloride and Vitamin C on the olfactory assay of *D. melanogaster* raised in control diet and treated media. According to the data obtained the larval plate assay was found to be high in Zinc Chloride and vitamin C treated media compared to control. In between the concentration groups of ZT1 media which is significant with $p< 0.005$, $df = 2$ and $F = 17.585$ Fig 4: reveals the effect of Zinc Chloride on the olfactory assay of *D. melanogaster* raised in control diet and treated media. According to the data obtained the larval plate assay was found to be high in Zinc Chloride treated media compared to control. In between the concentration groups of ZT2 media which is significant with $p <0.005$ $df = 2$ and $F = 35.179$. Fig 5: reveals the effect of Zinc Chloride on the olfactory assay of *D. melanogaster* raised in control diet and treated media. According to the data obtained the larval plate assay was found to be high in antioxidant treated media compared to control. In between the concentration groups of ZT3 media which is significant with $p < 0.05$, $df = 2$ and $F = 65.346$. Fig 6: reveals the effect of Zinc Chloride on olfaction assay results for 3rd instar larvae of *D. melanogaster* exposed to different concentrations of $ZnCl_2$ and Vitamin C show distinct patterns of larval movement across the zones containing ethyl acetate. In the control group, larvae were distributed relatively evenly between B zone (0.6ml ethyl acetate) and C zone (no ethyl acetate), with a lower mean in A zone (0.2ml ethyl acetate). For ZT1, the mean number of larvae in C zone increased slightly compared to control, while those in A and B zones decreased, indicating a mild preference for the C zone. At ZT2 a marked increase in the mean number of larvae was observed in the C zone, while the numbers in A and B zones remained low, suggesting a stronger avoidance of ethyl acetate at this concentration. For ZT3, with the highest mean number of larvae in the C zone, and fewer larvae in the A and B zones. Fig 7: reveals the effect of $ZnCl_2$ on olfaction assay of *D. melanogaster* raised in control diet and treated media conducted to compare the mean number of *Drosophila melanogaster* larvae in the C zone (0.0ml ethyl acetate) among the four groups: Control, ZT1, ZT2 and ZT3 the mean number of larvae in the C zone is higher in the ZT2 and ZT3 groups compared to the Control and ZT1 groups, This pattern indicates a statistically significant difference in larval distribution among the groups, with higher $ZnCl_2$ concentrations and the addition of Vitamin C leading to a greater number of larvae preferring the C zone. Fig 8: reveals the effect of $ZnCl_2$ on olfaction assay of raised in control diet and treated media *D. melanogaster* raised in control diet and treated media olfaction assay in the B zone (0.6ml ethyl acetate), the mean number of *Drosophila melanogaster* larvae in each concentration group shows a clear trend. The control group has the highest mean number of larvae, around 4, indicating that more larvae were present in the B zone when no $ZnCl_2$ or Vitamin C was added. In contrast, the ZT1 group shows a reduced mean of about 2 larvae, and the ZT2 group has the lowest mean, approximately 1 larva. The ZT3 group shows a slight increase, with a mean of about 2 larvae, similar to ZT1. The presence of $ZnCl_2$, especially at higher concentrations, significantly reduces the number of larvae in the B zone, suggesting increased avoidance of the higher ethyl acetate concentration. The addition of Vitamin C (ZT3) does not fully reverse this avoidance but results in a slight increase compared to ZT2. Fig 9: reveals the effect of $ZnCl_2$ on olfaction assay of *D. melanogaster* raised in control diet and treated media in the A zone (0.2ml ethyl acetate), the mean number of *Drosophila melanogaster* larvae in each concentration group—Control, ZT1, ZT2, and ZT3 is almost identical. All groups show a mean value of approximately 1 larva, with very little variation between them. Indicating that there is no statistically significant difference in the number of larvae present in the A zone across all tested conditions the presence of $ZnCl_2$ and Vitamin C does not influence the distribution of larvae in the A zone, as the mean number of larvae remains consistent with the control group.

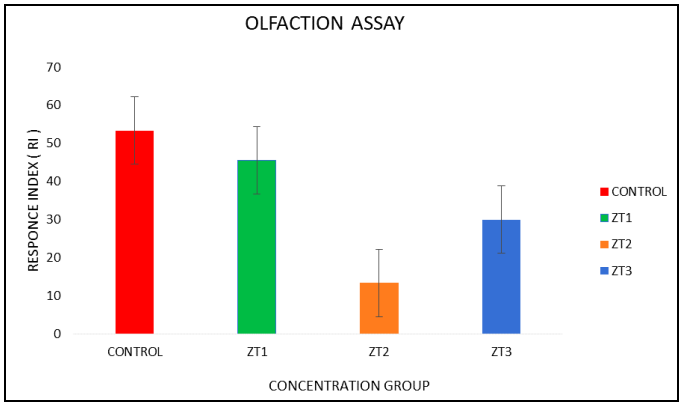

Fig 1: Effect of $ZnCl_2$ and Vitamin C showing response index on 3rd instar larvae of *D. melanogaster* in Larval olfaction assay.

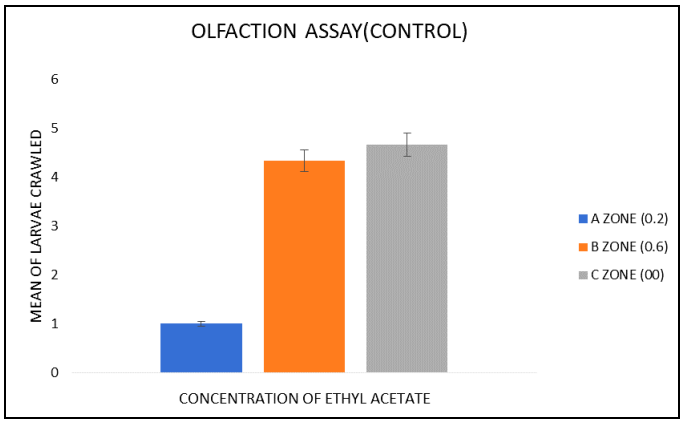

Fig 2: Effect of $ZnCl_2$ and Vitamin C in control group on 3rd instar larvae of *D. melanogaster* in larval olfaction assay.

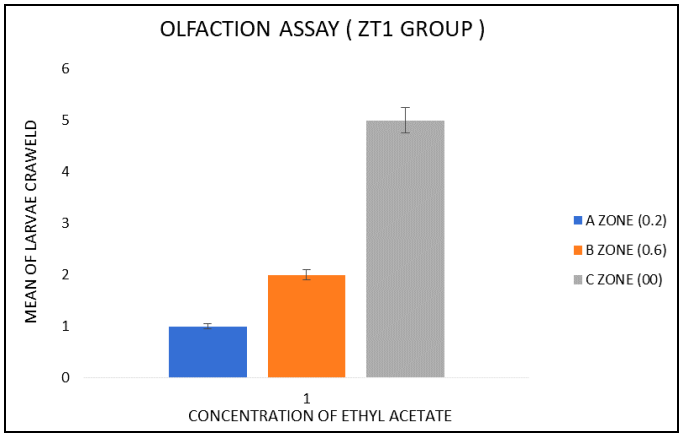

Fig 3: Effect of $ZnCl_2$ and vitamin C in ZT1 group on 3rd instar larvae of *D. melanogaster* in larval olfaction assay.

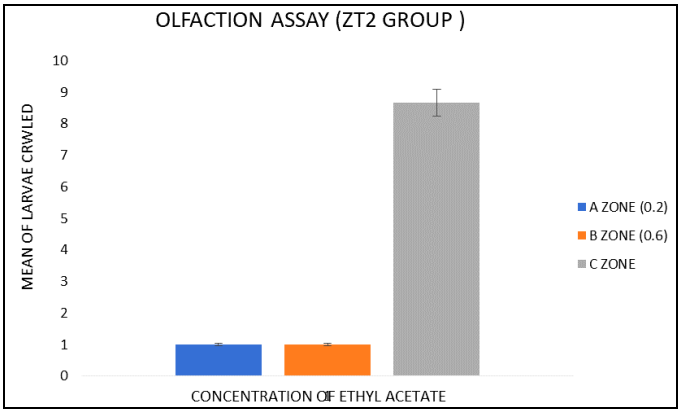

Fig 4: Effect of $ZnCl_2$ and vitamin c in ZT2 group on 3rd instar larvae of *D. melanogaster* in larval olfaction assay.

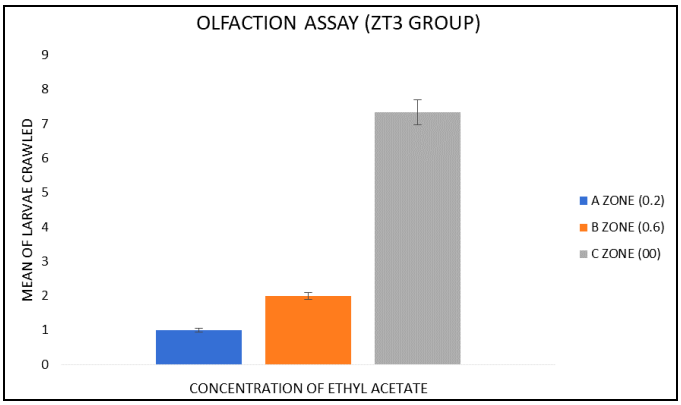

Fig 5: Effect of $ZnCl_2$ and vitamin C in ZT3 on 3rd instar larvae of *D. melanogaster* in larval olfaction assay.

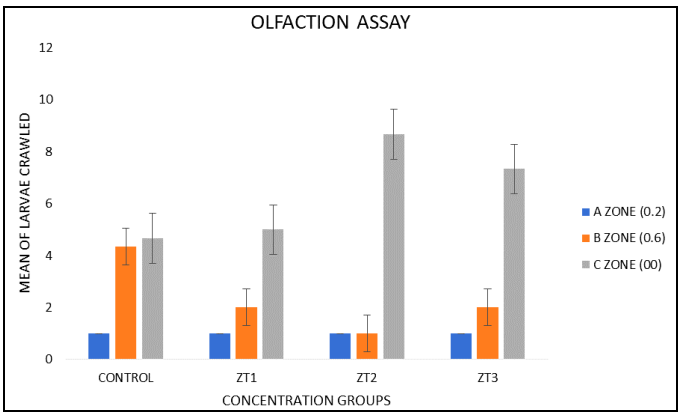

Fig 6: Effect of $ZnCl_2$ and vitamin C in different concentration groups on 3rd instar larvae of *D. melanogaster* in larval olfaction assay.

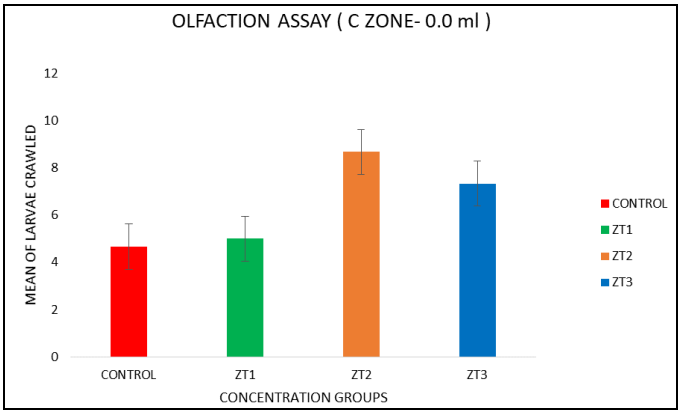

Fig 7: Effect of $ZnCl_2$ and vitamin C in C zone on 3rd instar larvae of *D. melanogaster* in larval olfaction assay

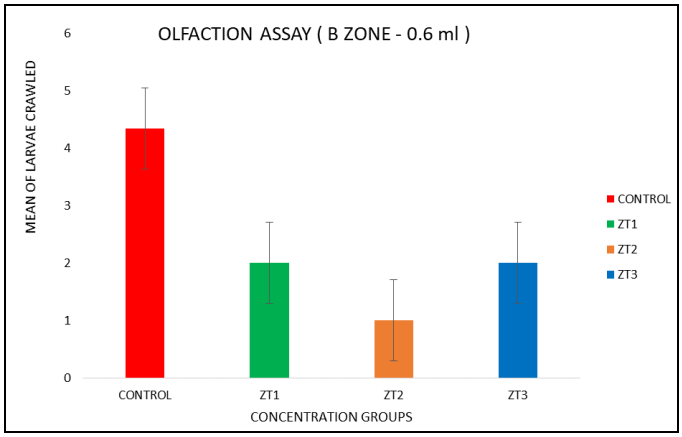

Fig 8: Effect of $ZnCl_2$ and vitamin C in B zone on 3rd instar larvae of *D. melanogaster* in Olfaction assay.

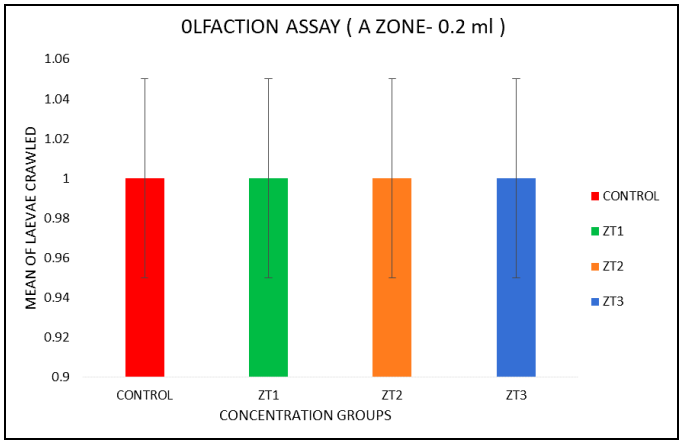

Fig 9: Effect of $ZnCl_2$ and vitamin C in A zone on 3rd instar larvae of *D. melanogaster* in Olfaction assay.

Discussion

The present study investigated the impact of different concentrations of zinc chloride ($ZnCl_2$) combined with vitamin C on the developmental of *Drosophila melanogaster*. The results clearly demonstrated a concentration-dependent effect, with the highest observed in the control group, followed by ZT1 and ZT3, and the lowest in ZT2. These findings are consistent with previous studies which have shown that while zinc is an essential trace element involved in several biological functions, its elevated levels can cellular toxicity and developmental impairment (Ho, 2004) [13].

Trace elements, like Zinc, found in nature play important and beneficial roles in human metabolism. Heavy metal ions can act as either cofactors or inhibitors in enzymatic pathways. It has been estimated that about half of all enzymes require a metal cofactor to be active and functional (Zhang, Y.; Zheng, J.2020; Andreini, C.2008). Over 120 million people are overexposed to zinc all over the world and 99 percent of the most serious cases are in the developing world and more than 51.3 per cent of children in Indian metros below 12 years of age have their blood levels above 10 ug/dl. Zinc poisoning (cause anorexia, vomiting and diarrhea) is a medical condition in humans and caused by increased levels of the heavy metal zinc in the body (Venkatesh *et al*, 2009).

In this study, we found that $ZnCl_2$ exposure is associated with cognitive impairment could influence cognitive performance independently. The olfactory response of early third-instar larvae was significantly affected upon getting exposed to $ZnCl_2$. Also, as the concentration of $ZnCl_2$ was increased, the olfactory response index (RI) decreased further. The findings of this study clearly indicate that the neural circuit for sensing

the odor gets affected due to $ZnCl_2$ exposure. Consequently, the response of $ZnCl_2$ and Vitamin C-treated larvae toward ethyl acetate odor decreased as compared to untreated larvae.

The sense of smell is a vital sense and is important for all the organisms starting from bacteria to human beings. The fruit fly, *Drosophila melanogaster* can sense and distinguish wide variety of odors with high sensitivity and varied specificity. The larvae on olfaction for finding food, mates and oviposition sites. The larvae have been a model of choice for researchers to study olfaction, learning and memory (de Bruyne *et al*., 2001) [7] due to its anatomically and genetically simple olfactory system with remarkable similarity with that of human beings. The fruit fly smells using a pair of antenna and maxillary palp which possess morphologically different types of sensilla housing olfactory sensory neurons (OSNs). There are around 1200 OSNs in the antenna and 120 OSNs in the maxillary palp. The OSNs express odorant receptors (ORs) and ionotropic receptors (IRs) which sense the odors. The OSNs send their axons to the antennal lobe (AL) of the brain, which is the first processing center of olfaction and contains around 43 spherical neuropils called glomeruli. The glomeruli have excitatory and inhibitory local interneurons (LINs or LNs). The output neurons from the AL, the projection neurons (PNs), innervate into the Kenyon cells (KCs) in Mushroom body (MB) and the lateral horn (LH), the higher centers of fly brain. The present study also correlated similar trends in the ability of larvae for associative learning revealing the neurotoxic effect of zinc chloride. Reported that children drinking zinc contaminated water displayed concentration, and hyperactivity, alertness. Loss of They also hypothesized that zinc acts as a xenoestrogen, which affects endocrine function and generates free radicals that decrease dopamine secretion, which in turn results in deteriorated brain growth and behavioral impairment.

Conclusion

Our results show that the olfactory in third-instar *Drosophila* larvae are dramatically decreased by chronic exposure to $ZnCl_2$ in a time- and dose-dependent manner. This is the first report on the toxic effects of $ZnCl_2$ on fruit fly larvae's cognitive abilities. To fully comprehend the molecular causes of $ZnCl_2$-induced reduced cognitive abilities in larvae.

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