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Toxicological effects on acute exposure of Zinc chloride and synthetic antioxidant BHA on crawling behaviour of *Drosophila melanogaster.*

[1]Marilyn Soosi M, [2] Navyashree L.S, [3]Nisarga M, [4]Priyadharshini M,[5] Shakuntala V.

[1,2,3,4, *5] Department of Studies in Zoology, Drosophila Stock Centre, University of Mysore, Manasagangotri, Mysuru, Karnataka, India.
Corresponding Author: Shakuntala V
Email: marilynsoosi@gmail.com, drshakuntalav2@gmail.com.

ABSTRACT :

Zinc chloride (ZnCl2) is an inorganic compound widely utilized in various industrial, agricultural and chemical applications. It is significantly hazardous substance when environmental concentrations exceed physiological threshold. Although, zinc is an essential micronutrient excessive exposure to ZnCl2 disrupts the metal ion homeostasis and induces oxidative stress through the generation of reactive oxygen species (ROS). The fruit fly, Drosophila melanogaster, serves as an effective model organism for toxicological study due to its genetic accessibility and ecological relevance, its notable sensitivity to zinc toxicity. The exposure to Zinc chloride leads to increased mortality, delayed development, impaired reproduction, structural deformities and behavioural deficits such as diminished movement and disturbed circadian rhythms. The crawling assay in D. melanogaster larvae provides a reliable indicator of neuromuscular health and developmental toxicity. In this study, D. melanogaster larvae were subjected to sublethal doses of $ZnCl_2$ to evaluate motor function impairment, with a parallel treatment using Butylated Hydroxyanisole (BHA), a synthetic antioxidant known for neutralizing free radicals. The outcomes of this study not only deepen the understanding of heavy metal toxicity mechanisms but also reinforce the utility of Drosophila as a bioindicator for environmental monitoring. Moreover, co-treatment with BHA showed partial recovery of crawling ability, highlighting its potential in counteracting $ZnCl_2$-induced neurotoxicity.

Keywords: Crawling assay, zinc chloride, antioxidant BHA, *D. melanogaster*, third instar larva.

Introduction

Toxicology is a scientific discipline that explores various chemical, physical, and biological agents that cause adverse effects on the living organism and in the ecosystem (Klaassen, 2013). It encompasses the characterization, detection, and elucidation of the mechanisms through which toxicants exert harmful effects; it plays a pivotal role in public health, environmental protection, pharmacology, and risk management (Hodgson, 2010). In the 16th century, toxicology addresses the fundamental principle introduced by Paracelsus as “The dose makes the poison.” This axiom underscores the concept that virtually any substance can be toxic if administered at a high dose (Timbrell, 2008). Therefore, toxicological assessments considered both the dose and the exposure pathway—whether through inhalation, ingestion, dermal contact, or other means (Hayes et al., 2014). Moreover, the duration of the exposure (acute, sub-chronic, or chronic) and the biological content (age, species, sex, genetic predisposition) greatly influence the toxicity profile of a substance (Klaassen, 2013).

The key area of concern in toxicology is the impact of environmental contaminants, which includes pesticides, heavy metals, industrial chemicals, and pharmaceuticals (Landrigan et al., 2015). These agents often persist in the ecosystem and bioaccumulate in the organisms, causing both acute and chronic effects that can cause long-term effects across the trophic levels in the ecosystem (Tchounwou et al., 2012). Heavy metals such as lead (Pb), mercury (Hg), cadmium (Cd), and zinc (Zn) are of particular concern due to their prevalence and toxicity even at low concentrations (Jaishankar et al., 2014). Unlike the organic pollutants, heavy metals are non-biodegradable and can remain in the biological systems for extended periods, interfering with enzymatic activity, redox balance, and gene expression (Jarup, 2003). Drosophila has been extensively used to assess neurotoxicity, developmental toxicity, and also oxidative stress due to genetic tractability (Rand, 2010).

Toxicology also intersects with public health through risk assessment, which evaluates potential human health risks resulting from environmental or occupational exposure to harmful substances (National Research Council, 1983). Risk assessment helps to identify hazardous substances, assessment in dose-response, and the level of exposure (US EPA, 2012). This framework helps to regulate the development of guidelines and interventions to mitigate harmful metals and ensure safety in diverse contexts, which include various products, pharmaceuticals, and industrial waste management (WHO, 2010).

ZINC

Zinc, though classified as a heavy metal, is an essential trace element critical for numerous physiological functions, including enzyme activity, gene expression, immune response, wound healing, and cellular signaling (Prasad, 2013). Approximately 10% of human proteins bind zinc, and over 300 enzymes, such as carbonic anhydrase and DNA/RNA polymerases, rely on it for catalytic function (Vallee & Falchuk, 1993; Andreini et al., 2006). Zinc

stabilizes proteins and membranes through zinc-finger motifs and is vital for both innate and adaptive immunity, influencing macrophage activity, lymphocyte development, and inflammatory regulation (Rink & Gabriel, 2000). Deficiency can cause growth retardation, cognitive impairment, immune dysfunction, and reproductive issues, particularly in children and pregnant women (Hambidge, 2000). Since the body cannot store zinc, homeostasis is maintained through intestinal absorption and excretion; dysregulation is linked to diseases like cancer, neurodegeneration, and diabetes (Liuzzi & Cousins, 2004). While essential, excessive zinc from environmental or supplemental sources can be toxic, causing gastrointestinal distress, oxidative stress, and copper deficiency (Plum et al., 2010). Thus, zinc's dual role as both a vital nutrient and a potential toxin underscores the need for balanced intake (Berg & Shi, 1996; Tubek et al., 2008).

Zinc Chloride as a Toxic Heavy Metal

Zinc chloride ($ZnCl_2$) is an inorganic compound widely utilized in various industrial, agricultural, and chemical applications, including metal galvanizing, textile processing, smoke bombs, disinfectants, fireproofing materials, cements, wood preservation, and as a fluxing agent in soldering (Plum et al., 2010). While zinc in its elemental form is an essential micronutrient involved in numerous enzymatic and cellular functions in living organisms, its chloride salt—especially at elevated concentrations—can exhibit significant toxicological effects. The toxicity of $ZnCl_2$ arises not only from the bioactive zinc ion (Zn^{2+}) but also from the corrosive and oxidative properties of the chloride component, which can disrupt cellular homeostasis, induce oxidative stress, and damage biological tissues (Kara et al., 2007).

Unlike organic toxins, heavy metal compounds such as zinc chloride are non-biodegradable and persist in the environment. Their accumulation in soil, water, and biological systems can pose long-term ecological and health risks. In aqueous environments, zinc ions readily dissociate from $ZnCl_2$ and interact with proteins, nucleic acids, and membrane structures, resulting in cytotoxic effects such as enzyme inhibition, mitochondrial dysfunction, and apoptosis (Vallee & Falchuk, 1993). The corrosive nature of $ZnCl_2$ also leads to localized tissue injury upon contact or ingestion, often manifesting as gastrointestinal irritation, dermal burns, or respiratory tract inflammation (ATSDR, 2005).

In biological systems, excess $ZnCl_2$ disrupts the delicate balance of metal ion homeostasis. Zinc ions compete with other essential metals such as copper, iron, and magnesium, leading to nutritional imbalances and cofactor dysfunction (Plum et al., 2010). Moreover, the intracellular accumulation of zinc can catalyze the generation of reactive oxygen species (ROS), impair antioxidant defenses, and cause lipid peroxidation, DNA fragmentation, and protein denaturation—all hallmark processes of oxidative stress-induced toxicity (Powell, 2000).

Experimental studies in both invertebrate and vertebrate models have demonstrated the broad spectrum of $ZnCl_2$ toxicity. For instance, in aquatic species and insect models such as D. melanogaster, $ZnCl_2$ exposure has been linked to developmental abnormalities, reduced reproductive success, behavioral impairments, and increased mortality (Bonilla-Ramirez et al., 2011). These effects are often dose-dependent and time-dependent, highlighting the importance of exposure thresholds in determining toxicity. In mammalian systems, high doses of $ZnCl_2$ can impair kidney and liver function, disrupt endocrine activity, and induce systemic inflammation (Khan et al., 2007).

Inhalation of zinc chloride smoke, such as from military-grade smoke bombs or industrial fires, is known to induce zinc fume fever—a flu-like illness characterized by chills, fever, cough, and chest discomfort. Inhalation can irritate the respiratory tract, potentially leading to serious complications like chemical pneumonitis. Severe exposure can progress to chemical pneumonitis and acute respiratory distress syndrome (ARDS) (Gordon et al., 1992). Dermal exposure may lead to skin burns, dermatitis, and ulcerations due to its corrosive action. Ingestion is especially hazardous—even small amounts can cause severe gastrointestinal injuries, including oral and gastric burns, pharyngeal swelling, vomiting blood, and long-term complications such as strictures or pancreatic issues.

Chronic exposure may also result in systemic effects like fatigue, anorexia, and weight loss, highlighting the importance of strict handling precautions. Zinc chloride exposure, particularly through ingestion or inhalation, poses significant health risks. Ingesting as little as 10 mL of a 35% solution can cause corrosive damage, including oropharyngeal and gastric burns, vomiting of blood, black tarry stools (melena), and potentially life-threatening complications such as respiratory failure, unconsciousness, gastrointestinal strictures, and pancreatic insufficiency (Chew et al., 1986).

Cellular and Molecular Damage

At the cellular level, $ZnCl_2$ exposure has been shown to trigger apoptosis and necrosis via mitochondrial membrane disruption and caspase activation (Plum et al., 2010). It impairs the function of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), tipping the redox balance towards oxidative injury. These processes contribute to systemic inflammation, endothelial damage, and multi-organ dysfunction (Khan et al., 2007).

In humans, occupational or accidental high-level exposure has been associated with liver and kidney toxicity, as well as hematological alterations including leukopenia and thrombocytopenia (Kara et al., 2007). There is also evidence that high zinc levels can disrupt hormonal signaling and alter neurotransmitter balance, potentially contributing to neurobehavioral changes and cognitive impairment in extreme cases (Sensi et al., 2009).

Butylated Hydroxyanisole (BHA) as an Antioxidant

Butylated hydroxyanisole (BHA) is a synthetic phenolic antioxidant widely used to preserve fats and oils in food, cosmetics, and pharmaceuticals. Chemically, BHA consists of a mixture of two isomers: 3-tert-butyl-4-hydroxyanisole and 2-tert-butyl-4-hydroxyanisole. It functions by preventing oxidative rancidity, which occurs when free radicals initiate lipid peroxidation in products containing unsaturated fats. Due to its low cost, effectiveness, and stability at high temperatures, BHA has become a common food additive globally (Williams et al., 1999).

Mechanism of Action

The primary antioxidant action of BHA involves free radical scavenging. It is a synthetic antioxidant. BHA donates a hydrogen atom from its hydroxyl group to neutralize lipid peroxyl radicals, thereby terminating the chain reaction of lipid oxidation (Kahl & Kappus, 1993). The phenolic structure allows resonance stabilization of the unpaired electron, making BHA an effective radical quencher.

In addition to its radical-scavenging capacity, BHA can interact synergistically with other antioxidants like butylated hydroxytoluene (BHT), tocopherols (vitamin E), and citric acid to improve overall antioxidant activity in complex formulations (Salahudeen & Clark, 1991).

Applications in Industry

In the food industry, BHA is commonly added to products like baked goods, snack foods, chewing gum, fats, and dehydrated potatoes to prolong shelf life. The U.S. Food and Drug Administration (FDA) allows BHA usage in concentrations up to 0.02% of the total fat content in foods (FDA, 2020). It is also used in the cosmetic industry to prevent oxidative degradation of ingredients in lipsticks, moisturizers, and sunscreens.

In pharmaceutical formulations, BHA acts as a stabilizer in vitamins and lipid-containing drug products. Its inclusion prevents oxidative loss of active ingredients and extends product viability under storage conditions.

Health and Safety Considerations

Despite its widespread use, the safety of BHA has been a subject of debate. While BHA is generally recognized as safe (GRAS) in regulated amounts, animal studies have raised concerns about its carcinogenic potential at high doses. Research conducted by the National Toxicology Program (NTP) demonstrated that BHA induced forestomach tumors in rodents when administered in large quantities over extended periods (NTP, 1983). However, humans lack a forestomach, making the relevance of this finding uncertain.

The International Agency for Research on Cancer (IARC) has classified BHA as Group 2B: "possibly carcinogenic to humans," based primarily on evidence from animal studies (IARC, 1986). Nonetheless, epidemiological studies in humans are inconclusive, and regulatory bodies such as the FDA and EFSA have maintained approval of BHA in limited concentrations.

At typical dietary exposure levels, BHA is rapidly metabolized and excreted in the urine, with limited accumulation in tissues. Short-term studies also suggest that BHA may exert protective effects against DNA damage by inhibiting oxidative stress and reducing mutagenic lesions, particularly in combination with other antioxidants (Ito et al., 1986).

BHA is one of the primary antioxidants used in feeds because it retards the oxidation of vitamin A, fats, and vegetable oils. It is an effective stabilizer for essential oils, paraffin, and polyethylene (HSDB 2009). It is used as an antioxidizing agent in a biomaterial made from polyurethane and polyethylene oxide used to make mainline catheters (Silverstein et al. 1997). BHA is used as a preservative and antioxidant in pharmaceutical preparations and cosmetic formulations containing fats and oils.

In addition to serving as an antioxidant, BHA also serves as a yeast defoaming agent and a means to prevent fats in foods from going rancid. A third function of BHT (butylated hydroxytoluene) is to stabilize fats and maintain food color, taste, and smell. A virus called herpes causes cold sores. BHT may expedite the healing process of cold sores.

CRAWLING ASSAY

The crawling assay is a behavioral bioassay widely used in the field of neurobiology and toxicology to assess neuromuscular function, sensory response, and overall locomotor activity in D. melanogaster larvae. As a genetically tractable model organism, Drosophila offers numerous advantages, including a fully sequenced genome, short life cycle, and highly conserved signaling pathways with humans (Pandey & Nichols, 2011). The larval crawling behavior specifically reflects the integrated output of the central nervous system, peripheral sensory input, and muscle function, making it a sensitive readout for both developmental neurotoxicity and pharmacological studies.

Crawling in third instar larvae involves coordinated peristaltic waves that are generated by a central pattern generator (CPG) in the ventral nerve cord. These waves produce rhythmic contractions that allow the larva to move forward or backward (Heckscher et al., 2012). Disruptions in this behavior—quantified using crawling assays—can reflect impairments in neuromuscular coordination, energy metabolism, or responses to environmental toxins or pharmaceuticals.

The crawling assay is particularly valuable in toxicological studies due to its sensitivity to neural damage or dysfunction. For example, exposure to neurotoxic substances like lead, mercury, or zinc compounds can significantly alter crawling behavior in larvae. Such changes often precede visible morphological symptoms, making this assay a valuable early indicator of sublethal toxicity (Rand, 2010).

Genetic studies also benefit from crawling assays. Mutants with disrupted motor neuron development (e.g., shibire, parkin, pink1) often exhibit reduced crawling rates, indicating impaired synaptic transmission or mitochondrial function (Greene et al., 2003). RNAi knockdowns or CRISPR-based knockouts of specific genes can also be evaluated using this assay to determine their role in neuromuscular function. Impairment in crawling could indicate underlying oxidative damage, mitochondrial dysfunction, or neurodevelopmental delays, which purely anatomical or biochemical assays might overlook.

Crawling is generated by a series of rhythmic muscle contractions regulated by motor neurons and modulated by sensory feedback. Larval movement is typically forward but can include backward motion and directional changes, particularly in response to stimuli such as light, temperature, or chemical gradients.

Several parameters are commonly measured in crawling assays:

- Crawling Speed: The average distance covered over a period, often measured in mm/s.

- Body Wall Contractions: Number of full peristaltic contractions over time.
- Path Length and Directionality: Total distance and straightness of the crawling path.
- Turn Frequency and Angle: Indications of exploratory or escape behavior.
- Pausing Time: Frequency and duration of rest periods.

Crawling assays have been critical in modeling human neurological disorders such as Parkinson's disease, Huntington's disease, and spinal muscular atrophy in Drosophila. These models reveal defects in crawling behavior that mirror human symptoms such as bradykinesia or ataxia, thus offering a translational framework for drug screening and mechanistic exploration.

Advantages

- Non-invasive: No need for sacrificing the organism.
- Sensitive: Detects subtle physiological or neurological dysfunction.
- High-throughput: Can be adapted to screen many individuals simultaneously.
- Cost-effective: Requires minimal instrumentation.
- Translational Relevance: Reflects neuromotor outcomes relevant to human disease.

MATERIALS AND METHODOLOGY:

***D. melanogaster* stock culture**

The experimental stock of Oregon K strain *D. melanogaster* was obtained from *Drosophila* stock centre, Manasagangotri, University of Mysore. *D. melanogaster* is one of the most widely used and one of the most understood of all model organism. The flies obtained were redistributed and raised in different culture bottles containing wheat cream agar media (100g of jaggery, 100g of wheat powder, 10g of agar agar was boiled in 1000ml of double distilled water. 7.5ml of propionic acid was added at last). Twenty flies (10 males and 10 females) were introduced into culture bottles and maintained at a temperature of $22^0C \pm 1^0C$ with a relative humidity of 70% in 12 hours dark: 12 hours light cycle.

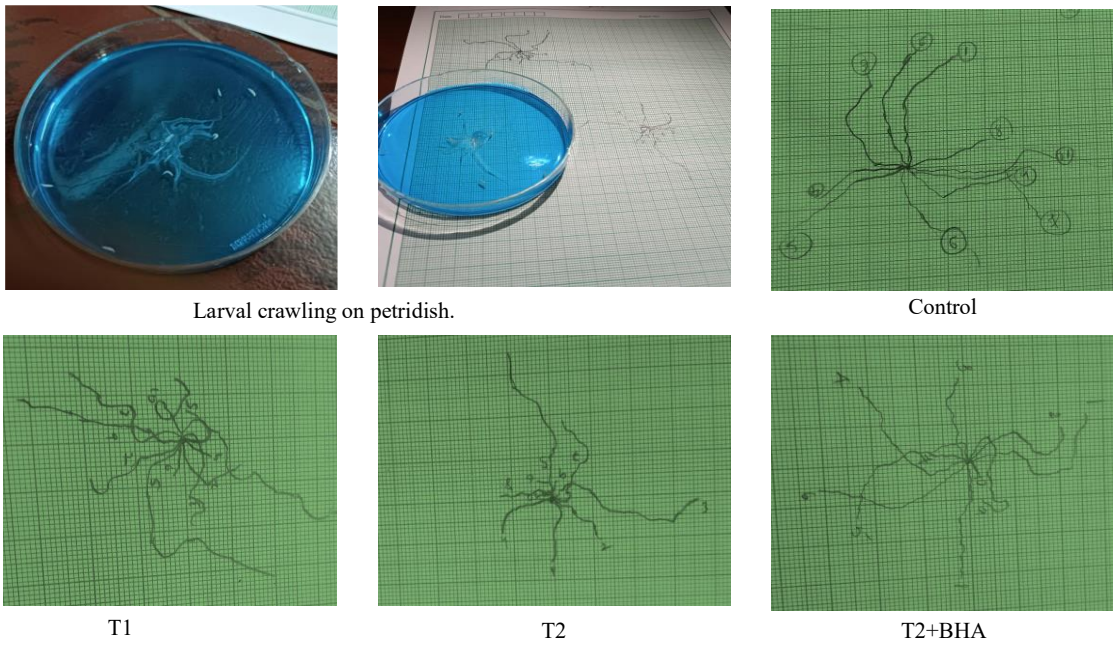
Diet preparation: Media is prepared as follows:

SI No	Ingredients	Control	Treatment 1	Treatment 2	Treatment 3
1	Wheat Soji	100g	100g	100g	100g
2	Jaggery	100g	100g	100g	100g
3	Agar	10g	10g	10g	10g
4	Propionic acid	7.5ml	7.5ml	7.5ml	7.5ml
5	Zinc Chloride	__	5mm (0.17g)	7mm (0.23g)	7mm (0.23g)
6	BHA	__	__	__	(0.045g)

PARAMETER:

CRAWLING ASSAY:

The eggs were obtained from the (Delcour procedure, 1969) media and transferred into different treatment bottles containing (zinc chloride5mm, 7mm and 7mm +BHA), maintained at a temperature of $22^0C \pm 1^0C$ with a relative humidity of 70% in 12hours dark: 12 hours light. The third instar larvae from control, T1, T2 and T2+BHA were collected. They were then transferred to a petridish containing 1% agar + 1% sucrose media [previously heated and allowed to become hard]. A thin layer of yeast paste was spread on the agar media to trace the movement of the larvae. The petridish was placed over a graph sheet and the larvae were placed on the media. The number of grid lines per cm^3 crossed per larva in 2 minutes was counted. A total of 4 replicates with 10 larvae (40 larvae total) in each group were used in each respective media.



Crawling assay of third instar larvae (control, T1, T2, T2+BHA)

Statistical analysis

The data obtained were analyzed using IBM SPSS version 30.0. Mean, standard error, one way ANOVA, and Tukey's Post - Hoc test were carried out for the data obtained for crawling assay. A graph of media v/s mean crawling assay was plotted for different concentrations of Zncl2 and antioxidant BHA.

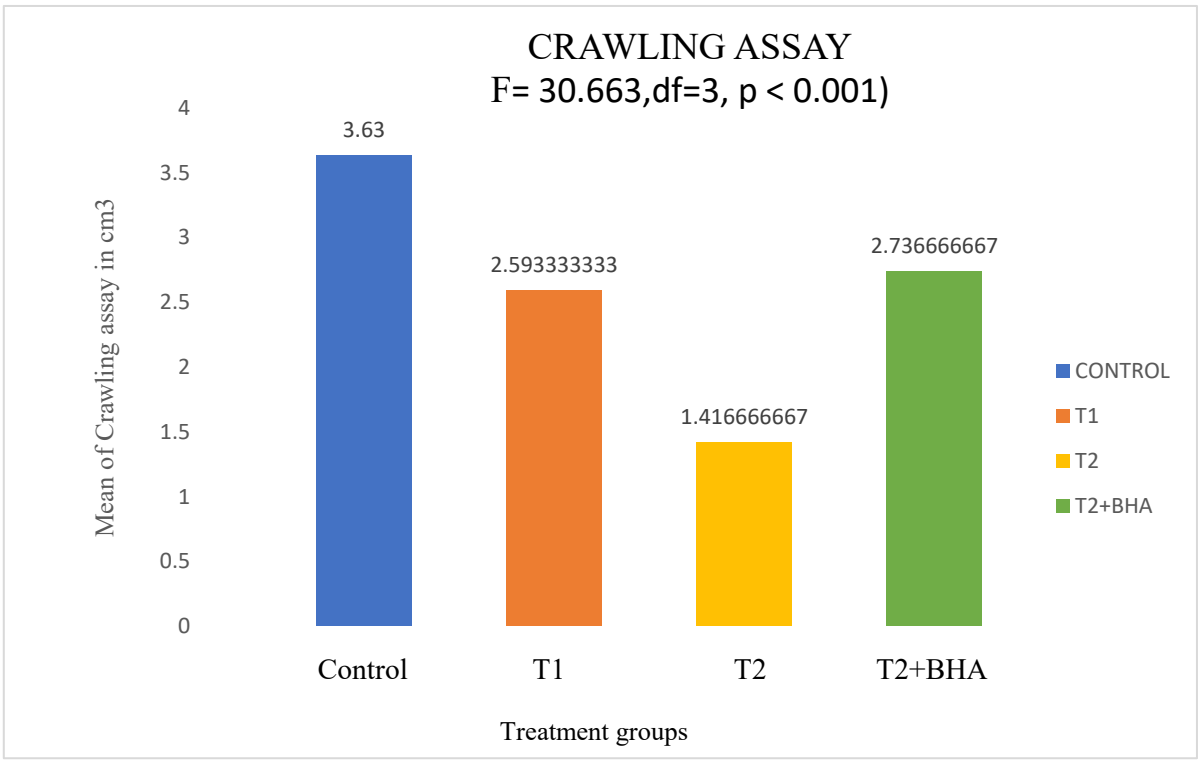
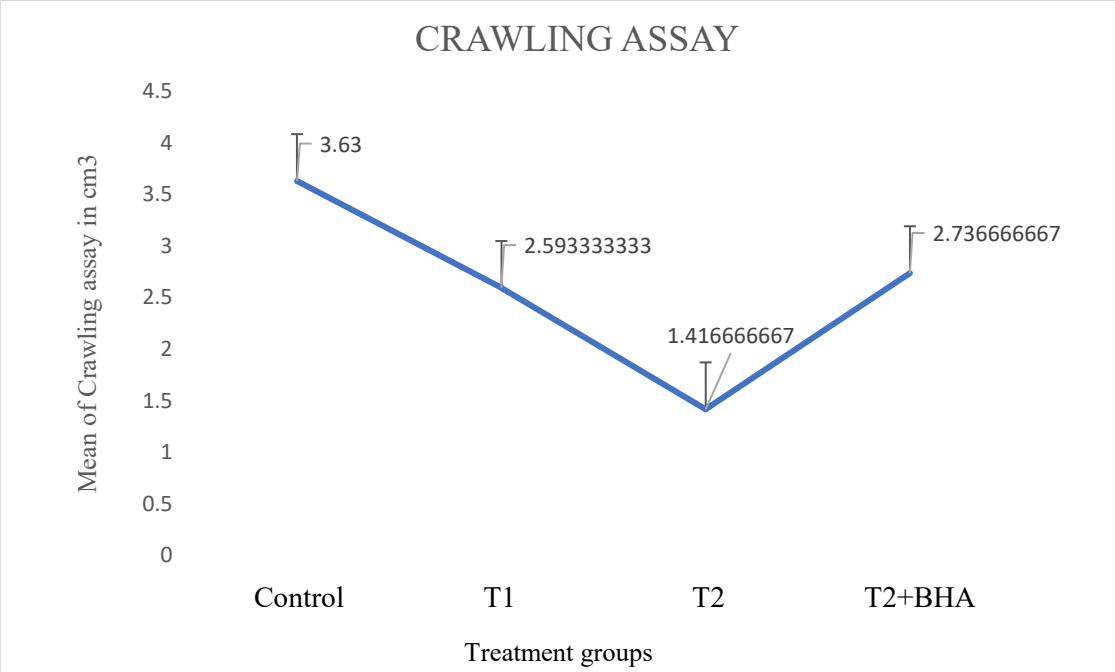


Fig1: Toxic effects of different concentrations of zinc chloride and mitigating effect of antioxidant BHA on crawling assay on third instar larvae of *D. melanogaster*.



RESULT

The graph of larval movement was drawn and the area was counted. The toxicity of zinc chloride of different concentrations and antioxidant BHA on larval crawling behaviour is provided in fig 1. It was noted that third instar larva obtained from control. The current study evaluated the toxicological impact of zinc chloride ($ZnCl_2$) at two concentrations (T1 and T2) on the locomotor behaviour of *D. melanogaster* third instar larvae. Furthermore, it examined the protective role of the antioxidant butylated hydroxyanisole (BHA) when co-administered with the higher concentration of $ZnCl_2$ (T2). The mean area crawled by larvae (in cm^2) under different treatment conditions was recorded and analyzed statistically using one-way analysis of variance ***(ANOVA).***

The bar chart (Fig. 1) visually presents the mean crawling area for each group:

Control group: 3.63 cm^2

T1 ($ZnCl_2$ low concentration): 2.59 cm^2

T2 ($ZnCl_2$ high concentration): 1.42 cm^2

T2 + BHA: 2.74 cm^2

This gradient clearly indicates a dose-dependent decline in larval crawling ability upon $ZnCl_2$ exposure, with partial restoration when BHA was co-administered.

Statistical Analysis

The results of the ANOVA are summarized in Table 1. The ANOVA revealed a highly significant effect of treatment on larval crawling ability ($F = 30.663$, $p < 0.001$). The sum of squares between groups was 32,584.625, while the sum of squares within groups was 41,089.700, indicating that a substantial proportion of the variance in crawling area is attributable to the different treatments administered. The highly significant F-ratio confirms that $ZnCl_2$ treatment and antioxidant supplementation produce statistically meaningful differences in larval locomotor function.

The control larvae exhibited the highest mean crawling area (3.63 cm^2), consistent with unimpeded locomotion under optimal physiological conditions. Upon exposure to $ZnCl_2$ at the lower concentration (T1), a notable reduction in mean crawling area to 2.59 cm^2 was observed. This reduction signifies an adverse effect of $ZnCl_2$ even at sub-lethal concentrations, suggesting interference with the neuromuscular coordination or energy metabolism necessary for larval locomotion.

At the higher $ZnCl_2$ concentration (T2), the mean crawling area further declined to 1.42 cm^2, representing a drastic 60.9% reduction relative to controls. Such pronounced impairment aligns with existing literature describing $ZnCl_2$-induced oxidative stress and disruption of ionic homeostasis in *Drosophila* larvae (Bonilla & Valerio, 2020; Balamurugan et al., 2004). Excess zinc is known to generate reactive oxygen species (ROS) and disturb mitochondrial function, leading to energy deficits and compromised motor output (Hernández-Moreno et al., 2011).

Protective Effect of BHA

In the group receiving both T2 $ZnCl_2$ and BHA, the mean crawling area improved significantly to 2.74 cm^2 compared to T2 alone. This partial restoration (an increase of approximately 93% relative to T2) highlights the potential of BHA to mitigate $ZnCl_2$-induced neurotoxicity. BHA, a synthetic phenolic antioxidant, likely exerts its protective effect by scavenging ROS and preventing lipid peroxidation of neuronal membranes (Kahl & Kappus, 1993). These findings reinforce the protective role of antioxidants against heavy metal toxicity in biological systems (Jiang et al., 2018).

DISCUSSION

CRAWLING ASSAY

The present findings reinforce the neurotoxic potential of acute $ZnCl_2$ exposure in *D. melanogaster* larvae, with significant suppression of crawling ability observed at 5 mM and more dramatically at 7 mM concentrations. These effects likely stem from zinc's disruption of ion homeostasis and oxidative damage to neuronal cells involved in motor control (Sensi et al., 2003; Roh et al., 2006). Zinc is known to interfere with calcium-mediated neurotransmission, hinder mitochondrial ATP synthesis, and induce apoptotic signaling—each of which can impair neuromuscular activity (Zhao et al., 2014; Trzcinska et al., 2021). Co-treatment with BHA substantially reversed the crawling impairments induced by 7 mM $ZnCl_2$, suggesting that BHA effectively neutralized the ROS generated during zinc stress. BHA, being a lipid-soluble antioxidant, readily incorporates into cell membranes and scavenges lipid radicals, thus protecting neuromuscular tissues from oxidative degradation (Rietjens et al., 2005; Halliwell & Gutteridge, 2015). In addition, BHA may activate transcriptional pathways like Nrf2, which upregulate phase II detoxification enzymes (Itoh et al., 1997).

The crawling assay is a fundamental behavioral bioassay frequently employed in *Drosophila melanogaster* to evaluate neuro-muscular coordination, motor integrity, and overall developmental health, particularly in larvae. Crawling behavior serves as a robust indicator of environmental stress and neurological toxicity, making it an ideal endpoint in toxicological evaluations (Berni et al., 2012; Song et al., 2007).

Zinc chloride is a water-soluble salt of zinc and a known environmental pollutant, frequently encountered in industrial effluents and agricultural runoff. Although zinc is a crucial micronutrient involved in enzyme function, gene expression, and cell signaling, excessive Zn^{2+} concentrations can disrupt metal homeostasis and induce oxidative stress in biological systems (Andreini et al., 2006; Maret & Li, 2009). In *Drosophila*, zinc toxicity manifests as growth retardation, reproductive deficits, neurodegeneration, and compromised behavior, with crawling activity serving as a sensitive readout of such physiological disturbances (Lye et al., 2012; Roh et al., 2006).

Excess zinc disrupts synaptic transmission by interfering with calcium-dependent neurotransmitter release and by modulating ion channel function, which are essential for coordinated muscle contractions and motor output in larvae (Sensi et al., 2003). Furthermore, excessive Zn^{2+} has been reported to displace other vital metal ions such as magnesium and copper, resulting in enzyme inactivation and impaired ATP production (Powell, 2000). These disruptions culminate in impaired neuromuscular performance, directly reflected in the crawling assay outcomes.

The observed decline in larval crawling activity in T2 not only suggests acute neurotoxicity but also reflects broader physiological stress, possibly including impaired energy metabolism, reduced ATP availability, and altered gene expression in muscle and nerve cells. Thus, the crawling assay effectively captures sublethal toxicity effects before morphological deformities become apparent. Butylated hydroxyanisole (BHA) is a widely used synthetic phenolic antioxidant, primarily known for its ability to inhibit lipid peroxidation and neutralize free radicals in biological systems. Its application in toxicology research extends to both in vitro and in vivo models, where it has shown protective effects against various xenobiotics, including heavy metals, pesticides, and oxidative agents (Rietjens et al., 2005; Flora et al., 2008).

In the current study, the group exposed to 7 mM $ZnCl_2$ combined with BHA (T2BHA) demonstrated a substantial restoration of crawling behavior. Remarkably, this group performed at levels comparable to or slightly better than the control group. This dramatic improvement underscores the potent antioxidative and neuroprotective effects of BHA when co-administered with a toxic metal like $ZnCl_2$.

Mechanistically, BHA is thought to exert its effects by scavenging ROS and inhibiting oxidative chain reactions, thereby preserving the structural integrity of cellular membranes and macromolecules (Halliwell & Gutteridge, 2015). In *Drosophila*, BHA has been shown to activate endogenous antioxidant defense systems, including upregulation of *sod*, *cat*, and *gstd1*, which encode key enzymes like superoxide dismutase and catalase that mitigate ROS damage (Klichko et al., 2004; Owusu-Ansah & Banerjee, 2009). This activation is crucial in maintaining the homeostasis of neuromuscular systems required for coordinated crawling.

Moreover, BHA may modulate transcription factors such as Nrf2 (nuclear factor erythroid 2–related factor 2), which regulates a suite of detoxification and stress response genes (Itoh et al., 1997). By restoring redox balance and enhancing the expression of detoxifying enzymes, BHA likely counteracts $ZnCl_2$-induced mitochondrial dysfunction, thereby restoring energy production and neuromuscular performance in larvae.

From a behavioral standpoint, the improved crawling ability in T2BHA-treated larvae suggests restored synaptic signaling, improved ATP availability, and reduced oxidative burden in neural circuits responsible for motor control. These effects collectively support BHA's utility as a neuroprotective agent against metal-induced toxicity.

The findings from this crawling assay carry significant biological and environmental implications. First, they reinforce *D. melanogaster* as a sensitive model organism for detecting sublethal toxic effects of environmental contaminants like zinc chloride. Crawling behavior, which is easy to quantify and reflects systemic physiological health, proves to be a reliable early biomarker for neurotoxic effects. Secondly, the data highlight the importance of antioxidant intervention in mitigating the effects of environmental pollutants. BHA's efficacy in reversing zinc-induced behavioral impairments suggests potential for broader applications in bioremediation, environmental risk assessment, and even dietary supplementation strategies in higher organisms.

Importantly, the dose-dependent nature of $ZnCl_2$ toxicity and the dose-specific response to BHA also provide insights into hormesis—the concept where low-level stress may induce adaptive benefits, while high levels cause damage (Calabrese & Baldwin, 2001). This principle could guide future experiments in determining optimal antioxidant doses for maximum protective effect without unintended side effects. The implications of these findings are multifaceted. On an ecological level, the data highlight the vulnerability of non-target organisms like insects to zinc pollution, a growing concern in urban and agricultural runoff (ATSDR, 2005; Duruibe et al., 2007). On a biomedical level, the results support antioxidant-based therapeutic strategies for mitigating metal-induced oxidative stress—relevant not only to insects but also to higher organisms, including humans.

CONCLUSION

The present study comprehensively evaluated the toxicological effects of zinc chloride ($ZnCl_2$) at varying concentrations and the potential mitigating role of the synthetic antioxidant butylated hydroxyanisole (BHA) across multiple behavioral and physiological assays in *Drosophila melanogaster*. The

findings collectively highlight that $ZnCl_2$ exerts significant adverse effects on the neuromuscular, developmental, and stress-resistance parameters in D. melanogaster, while co-treatment with BHA considerably ameliorates several of these impairments.In the crawling assay, larvae exposed to $ZnCl_2$ demonstrated a marked reduction in locomotor activity, indicating neuromuscular toxicity and developmental stress. However, supplementation with BHA partially restored crawling performance, suggesting its role in alleviating oxidative damage.

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