Toxicology International, *31(2) : 169-180; 2024. DOI: 10.18311/ti/2024/v31i2/35761*

Online ISSN : 0976-5131; Print ISSN : 0971-6580

Toxicology International

Contents available at: www.informaticsjournals.com/index.php/toxi



Diosgenin Mitigates Aluminum Chloride Mediated Developmental Toxicity in *Drosophila melanogaster*

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Abstract

Aluminum (Al) is a dangerous environmental neurotoxin that can cause tissue inflammation and oxidative stress. Diosgenin (DG) is a chemical that possesses therapeutic potential and is a powerful neuroprotective and antioxidant agent. Due to their unique anatomy, physiology, and diet, newborns are at a higher risk of harm from the developmental toxin Al. Researchers widely used *Drosophila melanogaster* as a popular model to study the toxicity of Al in recent years. The study investigates potential protective effects of dietary DG on *D. melanogaster*, a model of Al-toxicity. The larvae were raised on a diet containing 40 mM/kg Aluminium Chloride ($AlCl_3$) and 15 mM/kg DG. $AlCl_3$ caused oxidative stress and neurobehavioural abnormalities. However, dietary DG improved nociception and crawling deficits by restoring acetylcholinesterase levels and reducing oxidative stress. The study found that higher doses of DG reduced the negative effects of $AlCl_3$ exposure, suggesting DG's antioxidant potential could treat diseases caused by free radicals. The study reveals that Al-rich diets cause oxidative stress in *D. melanogaster*, affecting development, metabolism, and neurobehavioral function. DG, as an antioxidant, can protect against negative effects, but its benefits decrease with higher concentrations, the ideal dosage being 15 mM/kg.

Keywords: Aluminium, Developmental Neurotoxicity, Diosgenin, *Drosophila melanogaster*, Reactive Oxygen Species (ROS)

1. Introduction

Although the lithosphere is dynamic, it recycles Al, which is Earth's third most abundant element, and keeps it out of the biosphere[1]. Al, a toxic environmental and industrial by-product, is not efficiently extracted and used by the Al industry, with nearly half ending up as waste[2]. The Aluminum age began with this discovery; which made it cheap and abundant from a rare and valuable metal with an economic worth similar to gold. Most of the Al industry's growth comes from newly mined Al, which can enter and deposit in the biotic cycle, burdening biota and limiting its return to the lithospheric cycle[1].

Food processing, packing, and storage, including Al-based food additives, are significant sources of its exposure[3]. Al is a common ingredient in antacids,

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Article Received on: 01.12.2023 Revised on: 02.02.2024 Accepted on: 08.04.2024

toothpaste, and cosmetics. Until recently, the hazardous effects of consuming Al were not considered a concern due to the belief that it was non-bioavailable. Recent research suggests that consumption of high Al can increase its absorption in the gastrointestinal tract[4], accumulate in most organs, has significant bone concentrations, and also cross the blood-brain barrier[5]. Al accumulation in mammalian tissues causes toxicosis with diverse pathogenic consequences comprising circulatory, respiratory, skeletal, immune and reproductive systems[6–10]. Al deposits were linked to an increased risk of neurodegenerative disorders[11]. Therefore, it's crucial to understand its potentially harmful effects on development, especially for pregnant women[12].

The Aluminium Age has led to the presence of non-essential, neurotoxic Al enters the brain throughout all stages of human development, from infancy to old age[13]. Studies have shown that infant formula contains more Al than human milk[14]. Al is a developmental toxicant that poses a greater risk to newborns because of its unique anatomy, physiology, and nutrition[15]. Preterm newborns may experience neurotoxicity and developmental delay as well as growth impedance[16] when given intravenous solutions containing Al[17]. Even though it is neurotoxic, it is commonly used as an adjuvant in vaccines given to newborns and young children during crucial stages of brain development[18], which is associated with an increased prevalence of Autism Spectrum Disorder (ASD)[19]. Short-term use of Al hydroxide as a phosphate binder in children with chronic renal insufficiency may lead to acute encephalopathy, speech arrest, incognesia, and head tremors[20].

Researchers have used animal models from both vertebrate and invertebrate species to study the effects of Al toxicity[21]. Al-induced toxicity studies on *Drosophila melanogaster* tissues revealed developmental toxicity[22] and affected fertility, longevity, locomotion, olfactory learning, and brain health[23,24].

Various substances derived from natural sources are being investigated as therapies for Al toxicity. Bayliak *et al.*,[25] reported protective effects of dietary alpha-ketoglutarate against Al-induced toxicity at both larval and adult stages. Curcumin restored $AlCl_3$-induced developmental impairment in *Drosophila*[26]. Oboh *et al.*,[27] reported anticholinesterase activity and antioxidant properties of *Heinsia crinita* and *Pterocarpus soyauxii* in *D. melanogaster*. Ogunsuyi *et al.*,[28] investigated the modulatory capacity of *Solanum* vegetables mitigating Al-induced redox and inflammation-related neurotoxicity. Adedayo *et al.*,[29] studied the effect of *Andrographis paniculata* and *Phyllanthus amarus* leaf extracts on selected biochemical indices in the *D. melanogaster* model of neurotoxicity.

DG, a steroidal saponin found in fenugreek seeds and yam tubers (*Dioscorea spp*.) has antioxidant, anti-inflammatory, anti-ageing, anti-diabetic, hypolipidemic, and neuroprotective properties[30–34] and is used to treat a range of conditions such as cancer, atherosclerosis, inflammation and leukaemia[35–37]. Based on the literature, DG has a promising pharmacological profile, with the potential to be used as a medication for various illnesses.

The aim of this study was to investigate the protective effect of DG against the developmental toxicity caused by $AlCl_3$ in *D. melanogaster*, along with its ability to revert oxidative stress and abnormal neurobehavioral responses due to Al-intoxication.

2. Materials and Methods

2.1 Chemicals and Reagents

Aluminum chloride ($AlCl_3$), Bradford reagent and Diosgenin (D1634) (Sigma-Aldrich, USA), Methyl-paraben, Nitrobluetetrazolium (NBT), Agar, Agarose, Dimethyl sulfoxide (DMSO) and other chemicals were procured from Himedia, India

2.2 *Drosophila melanogaster* Stock Culture

The Oregon-R strain of the wild-type *D. melanogaster* was obtained from the IISER, Pune, *Drosophila* research laboratory and maintained at the Department of Zoology, SPPU. Flies were cultured on a standard diet containing cornmeal with 5 % sucrose and 5 % w/v brewer's yeast at ± 22°C, 70 % humidity and a 12:12h dark and light cycle. No ethical permission is required for *Drosophila* studies.

2.3 Experimental Setup

2.3.1 Treatment

Larvae were reared on food containing 5% sucrose, 5% dried yeast, 1% agar and 0.18% methyl paraben were used as control group. Larvae were cultivated on the same food and supplemented with 40 mM/kg $AlCl_3$ for treatment group, concentration of which was based on previous studies on Al toxicity in *D. melanogaster*[24,29].

2.3.2 Standardization of Diosgenin (DG)

Identification and purity of DG was checked using HRMS technique. First instar larvae were transferred to a standard medium containing DG (0, 5, 10, 15, 20 and 25 mM/kg diet) and its effect on development was observed. For ameliorative study, dose optimisation of 15 mM/kg was done based on the survival data from the above concentrations of DG.

2.3.3 $AlCl_3$ and DG Co-treatment

First instar larvae were exposed to a standardised dose of 15 mM/kg diet of DG with 40 mM/kg $AlCl_3$. Thereafter, four final groups were selected for further experimentation as follows:

Group 1: Control
Group 2: 40 mM/kg $AlCl_3$
Group 3: 40 mM/kg $AlCl_3$ + 15 mM/kg DG
Group 4: 15 mM/kg DG
(n =3 vials per group; 30 larvae/vial).

2.4 Effect on Larval Development

2.4.1 Larva Body Weight

By selecting third-instar larvae (n=10) from each set, the body weight of the larvae was calculated and compared with the control group. This experiment was carried out three times.

2.4.2 Gustatory (Food Intake) Assay

Animals rely on their dietary preferences to choose a healthy diet and avoid harmful substances. This method is simple, accurate, and repeatable. To investigate the preferred feeding methods of the larvae, a gustatory test was conducted by adding a red food colouring dye, Sulforhodamine B (0.1mg/mL) as followed by the previously outlined spectrophotometric method by Aryal *et al.*,[38] for measuring the amount of food consumed at a wavelength of 550nm.

2.4.3 Pupation Percentage

Pupation was calculated in percentage on each day from the larvae to the pupal stage. Daily pupal count served to evaluate the pupation dynamics of the various treatments. The total number of pupae formed within ten days represented larvae-to-pupae viability. Each experiment was repeated at least four times.

2.4.4 Pupal Height

The distance from the food surface to each pupa was measured. The pupation height was measured in mm as described by Krittika *et al.*[39]

2.4.5 Adult Eclosion

The adult eclosion percentage was calculated as the emergence rate of flies.

2.5 Phenotypic Changes

After eclosion from pupae, the adult flies from each vial were anaesthesised and checked for abnormal fly phenotype as well as in eyes and wings. Morphological deformities were observed under a digital Stereomicroscope (Nikon SMZ1270) and photographed using MIchrome 6 camera.

2.6 Neurobehavioral Assays

2.6.1 Chemical Nociception

The nociception experiment was conducted using 9% Hydrochloric Acid (HCl). Third-instar larvae were taken out from the food and cleaned with PBS. Larvae were placed in the centre of the petri dish. 9% HCl was dripped over using a micropipette from a height of 1cm. The continuous observation until the larvae started rolling in a "crossscrew" motion indicated larval distress. The movement was monitored using a recording device[40]. From the video recordings, the latency time of the pain response was measured. The evaluation of each larva's behaviour was carried out once. Groups of ten larvae from each diet were taken for the experiment[41].

2.6.2. Crawling Behaviour

To observe the larval behaviour of crawling, a previously described method was used[42]. Third instar larvae were placed on 2% agarose plates to examine the behaviour when subjected to various treatments. PBS-washed larvae from the treatment groups were placed at the centre of the agar plates. Graph paper was used to calculate the distance and determined the speed in centimetres per minute. The larval crawling pattern was observed, and the trail pattern was marked. This behaviour assay was performed in triplicate for each treatment using three different sets of larvae.

2.7 Biochemical Estimations

2.7.1 Homogenate Preparation

Third instar larvae from each group were weighed, homogenized, and centrifuged at 10,000 rpm for 10 min at 4°C in a 0.1 M Potassium phosphate buffer with a pH of 7.4. The recovered supernatants were utilised for biochemical tests. The proteins were normalised as 1mg/mL for each sample and then used further for biochemical estimations.

2.7.2 Metabolic Profiling

2.7.2.1 Protein Concentration

The Bradford test was used to quantify the protein concentration in the sample[43].

2.7.2.2 TAG and Glucose Levels

Levels of Triglycerides (TAG) and glucose were measured using standard kits (Tulip Diagnostics, India) following the manufacturer's instructions. The absorbance of TAG was taken at 520nm and for glucose at 530 nm (Absorbance for Glucose).

2.7.3 Oxidative Stress

2.7.3.1 Total Reactive Oxygen Species Using Nitroblue Tetrazolium

To measure the amount of ROS caused by Al toxicity in the larval hemolymph, Nitroblue Tetrazolium Reduction (NBT) test was conducted following the procedure given by Sabat *et al*[44]. Hemocytes were collected from each group then incubated with NBT for 1 hour and stopped with acetic acid following method by Pappus *et al*[45]. Treated and untreated larval cells are incubated with NBT for 1 hour, and stopped with acetic acid. The absorbance at 595nm was measured using a plate reader.

2.7.3.2 Acetylcholinesterase (AChE) Activity

In order to measure the activity of acetylcholinesterase, Ellman *et al.,*[46] method was followed. The absorbance was measured on spectrophotometer at 412nm. The AChE level was estimated as units/mg protein.

2.8 Statistical Analysis

Standard Error of the Mean (SEM) is used to express all experimental data. Analysis of Variance (ANOVA) and Tukey's post hoc test were used to evaluate the data using GrapPad Prism 5 statistical software.

3. Result

3.1 Diosgenin Identification by LC-HRMS

Results obtained from HRMS analysis confirmed the presence of DG in the sample procured.

3.2 Effect on Larval Development

In this study, 40 mM/kg $AlCl_3$ concentration was chosen based on the larval development and neurotoxicity. Al was found to decrease larval viability as reported earlier leading to decreased pupation[25].

3.2.1 Effect of $AlCl_3$ on Larval Body Weight

Body weight is a valuable indicator of toxic effects on body development. Larvae were weighed after four days of exposure, which showed its body mass was lowered in diets containing $AlCl_3$. Whereas larvae reared on the diets containing DG and co-treated with $AlCl_3$ increased in body mass. Reduced intensity of larval metabolism appears to be the most likely consequence of lower body mass of larvae on Al-supplemented diet.

3.2.2 Gustatory Assay

The larvae fed on the Al-treated diet had a significantly reduced food intake (28% less consumption) compared to those on the control diet. These findings suggest that larvae tend to avoid consuming hazardous food. However, we observed that when DG was added to media containing $AlCl_3$; the larvae ingested more food. This suggests that DG either reduces the toxicity or improves the flavour of food that contains $AlCl_3$.

3.2.3 Effect of $AlCl_3$ on Percent Pupation, Pupation Height, and Adult Eclosion

One of the factors considered while determining toxicity is pupation height. In comparison to the control diet, pupation height was seen to be reduced with $AlCl_3$, but it significantly improved when combined with DG. It was discovered that 40 mM/kg of $AlCl_3$ also reduced the percentage of pupae and eclosion. However, there was no significant difference (p-value=0.0825) observed. Our findings demonstrate that larval Al toxicity affects development.

There was a delay in larval development upon exposure to $AlCl_3$, but co-treatment with DG

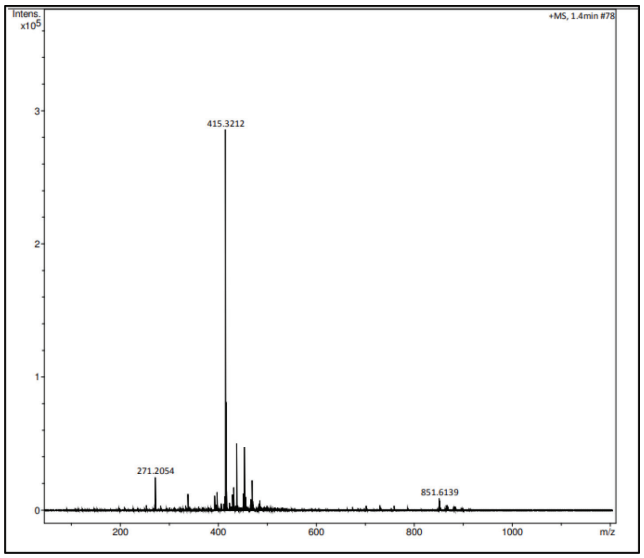


Figure 1. HRMS analysis of Diosgenin

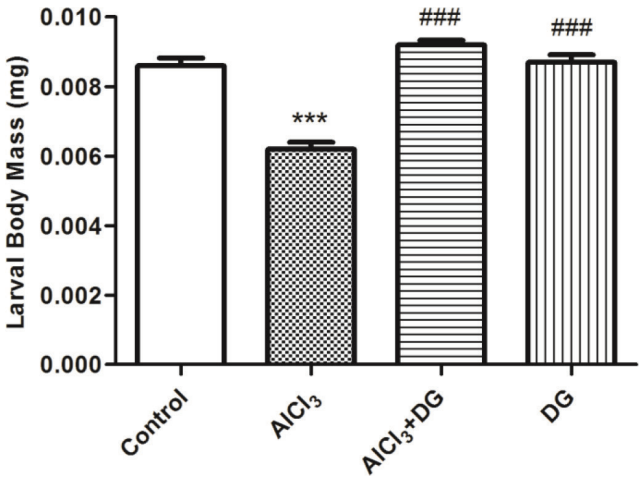


Figure 2. Effect of $AlCl_3$ on Larvae Body Weight (***indicates a significant difference at $p \leq 0.001$ compared with control group; ###indicates a significant difference at $p \leq 0.001$ compared to $AlCl_3$ group).

mitigated this effect without adverse impact on larval development.

3.3 Phenotypic Changes

The treatment with $AlCl_3$ revealed deterioration of the eye and a loss of retinal pigments in contrast to the control group, demonstrating its neurotoxic action. However, the damage was reduced when DG was given alongside the $AlCl_3$-treatment, although exposure to

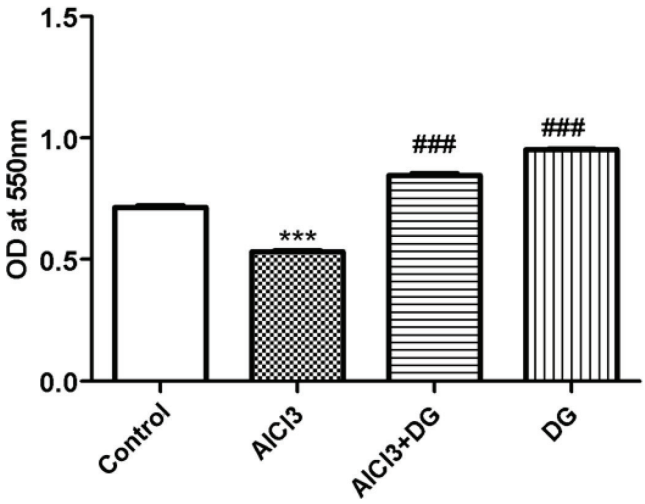


Figure 3. Effect of $AlCl_3$ on Food Intake (***indicates a significant difference at $p \leq 0.001$ from the control group; ###indicates a significant difference at $p \leq 0.001$ compared with $AlCl_3$ group)

$AlCl_3$ caused a shorter-winged phenotype, the overall shape of the wings was not significantly changed in all treatment groups.

3.4 Effect of $AlCl_3$ on Neurobehaviour of Larvae

3.4.1 Crawling Activity

Larvae can move from one place to another by squeezing their bodies together and forming a wave. These muscle contractions are directly regulated by the motor neurons found in the larval brain. Any anomalies in these neurons may thus cause an incorrect crawling pattern. The larval following paths in the all treatment groups are shown in Figure 6. Larvae treated with $AlCl_3$ displayed slower and more erratic movement in contrast to those co-treated with DG. This is the first such report of Al-toxicity on crawling behaviour, which is restored by DG due to its potent neuroprotective activity.

3.4.2 Nociception Activity

Drosophila larvae exhibit diverse nociceptive responses, depending on the type of stimulation received. Nociception is an essential sensory ability in all animal species, which aids in the identification and avoidance of potentially harmful stimuli. $AlCl_3$ exposure resulted in a decrease in the latency of nociception. This is the

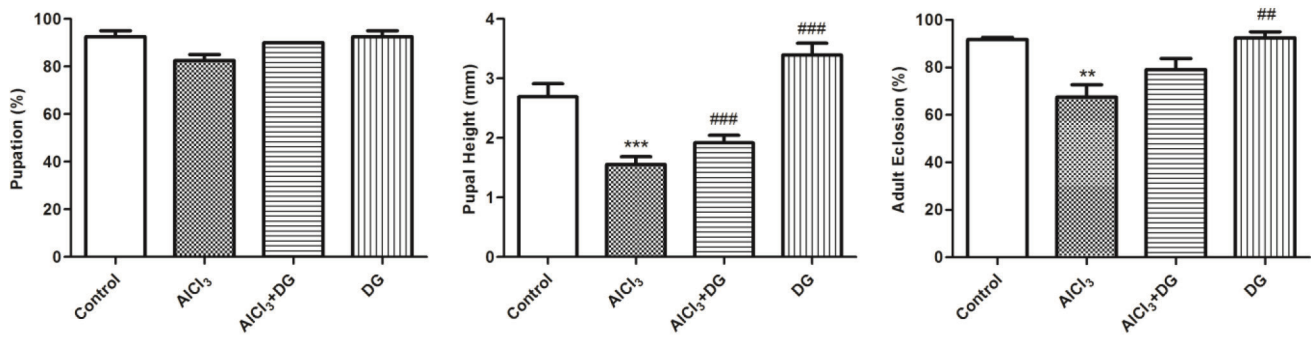


Figure 4. Effect of $AlCl_3$ on the, Percent Pupation, Pupal Height and Adult Eclosion (**, ***indicates a significant difference at $p \leq 0.01$, $p \leq 0.001$ compared with the control group; $^{\#\#}$,$^{\#\#\#}$ indicate significant difference $p \leq 0.01$, $p \leq 0.001$ compared with $AlCl_3$ group.)

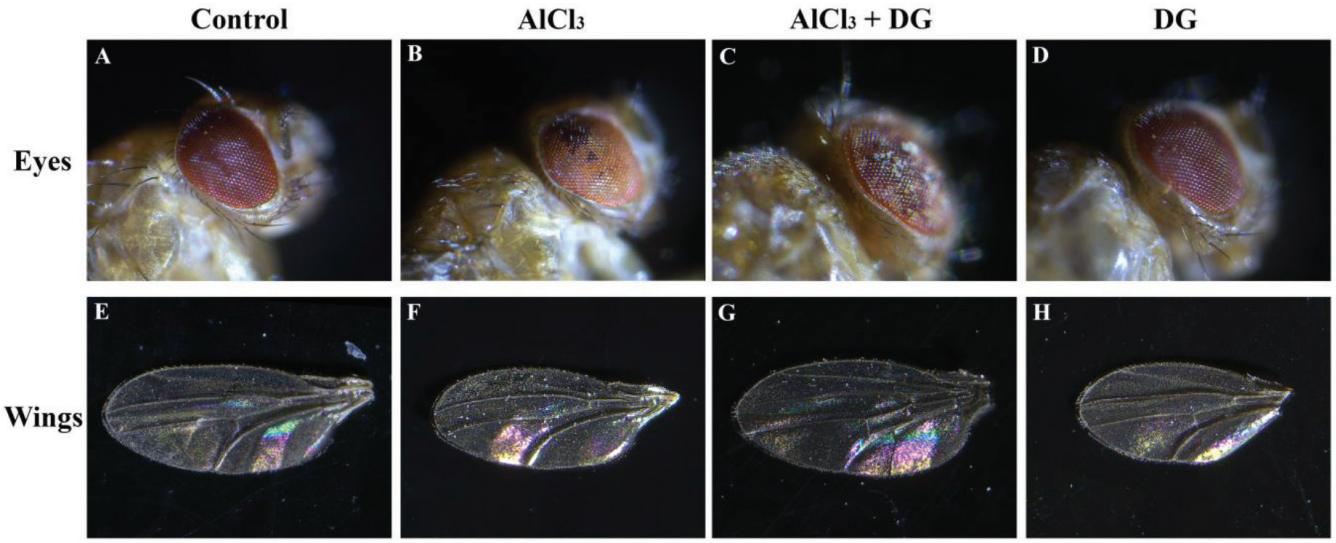


Figure 5. Effect of $AlCl_3$ and Co-treatment with DG on flies (A,B,C and D are eyes of flies and E,F,G and H are wings of flies indicating Control, $AlCl_3$, $AlCl_3$+DG and DG respectively).

first such report of Al-toxicity on nociception behaviour, whereas the dietary DG led to its restoration.

3.5 Effect of $AlCl_3$ on Metabolic Profiling

In comparison to the control larvae, the $AlCl_3$ group showed lower protein levels which were increased in the $AlCl_3$+DG group.

The data showed that $AlCl_3$ treatment significantly affects the TAG and glucose levels. After treatment with $AlCl_3$, the TAG levels in the larvae elevated significantly. Glucose levels were lowered in the DG and $AlCl_3$+DG groups compared to the control group, which may be because of hypoglycemic role of DG.

3.6 Effect of $AlCl_3$ on Oxidative Stress

3.6.1 Total ROS by Nitroblue Tetrazolium

We observed a significant difference in ROS levels between Control, $AlCl_3$, and $AlCl_3$ with DG, resulting in increased production of ROS that cause cellular damage and degrade the DNA repair system. ROS produced by innate immune cells can lead to neuronal injury and loss through neuro-inflammation.

3.6.2 Effect of $AlCl_3$ on AChE Levels

As expected, $AlCl_3$ exposure increased AChE levels compared to the control group, but no significant difference

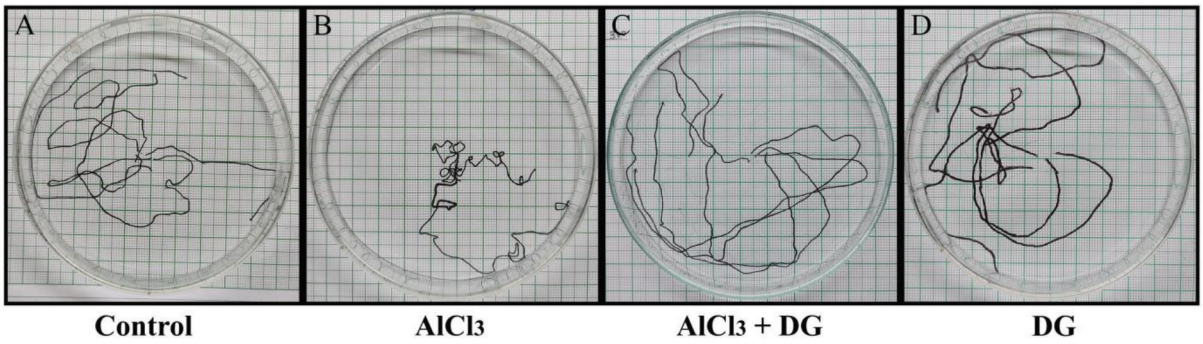


Figure 6. Crawling Activity of Larvae (A, B, C and D indicates Control, $AlCl_3$, $AlCl_3$ +DG and DG respectively).

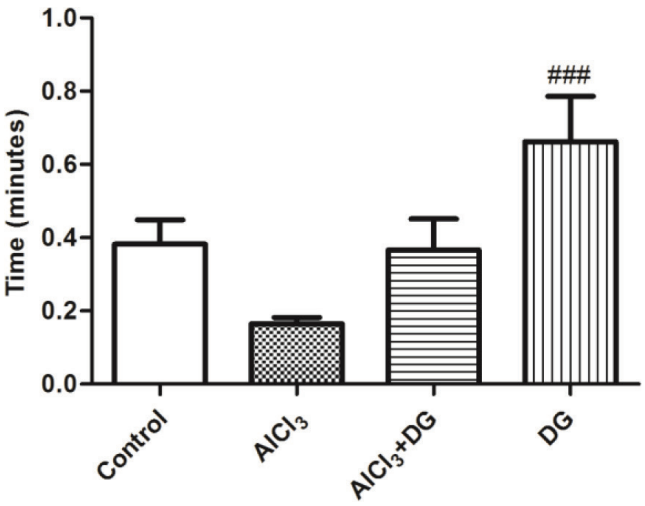


Figure 7. Nociception Activity ($^{###}$ indicates a significant difference at $p \leq 0.001$ from the $AlCl_3$ group)

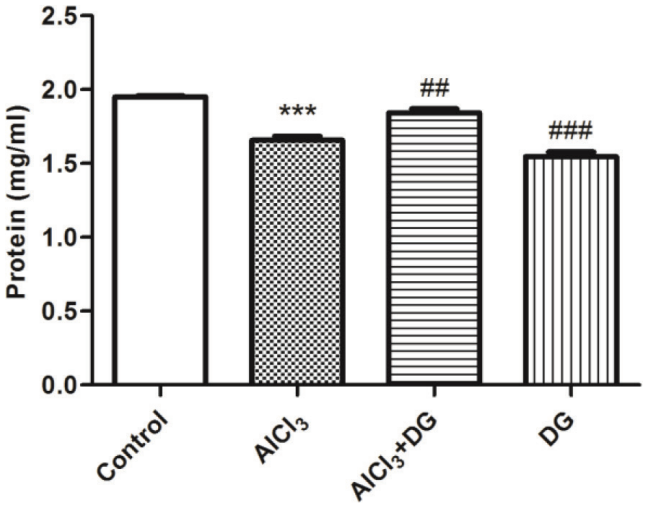
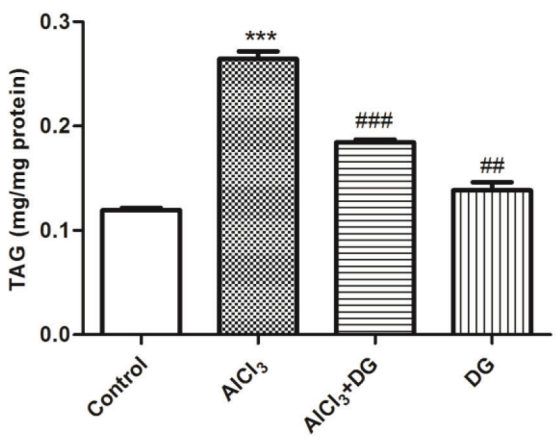


Figure 8. Effect of $AlCl_3$ on Protein Level (***indicates a significant difference at $p \leq 0.001$ from the control group; $^{##}$, $^{###}$ indicates a significant difference at $p \leq 0.01$, $p \leq 0.001$ from the DG and $AlCl_3$+DG group).



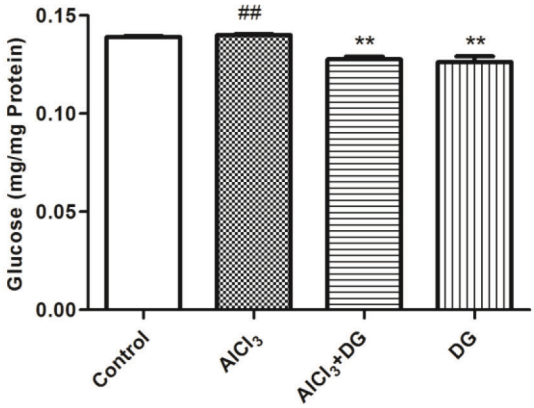


Figure 9. Effect of $AlCl_3$ on TAG and Glucose Levels in *Drosophila* Larvae (**,***indicates a significant difference at $p \leq 0.01$, $p \leq 0.001$ from the control group; $^{##}$, $^{###}$ indicates a significant difference at $p \leq 0.01$, $p \leq 0.001$, from the DG and $AlCl_3$+DG group)

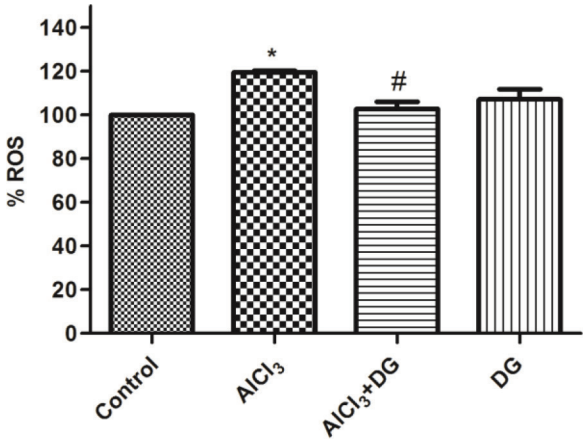


Figure 10. ROS Activity by NBT Method (*indicates a significant difference at $p \leq 0.05$ from the control group; whereas $^{\#}$indicates a significant difference at $p \leq 0.05$ respectively from the $AlCl_3$ group)

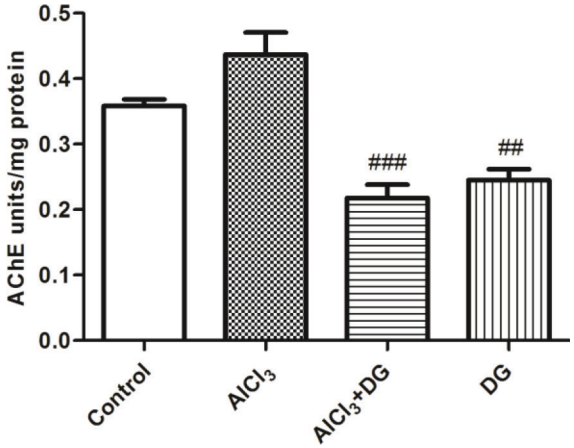


Figure 11. AChE Levels in *Drosophila* ($^{\#\#}$, $^{\#\#\#}$ indicates a significant difference at $p \leq 0.01$, $p \leq 0.001$ from the DG and $AlCl_3$+DG group)

was observed. AChE levels were significantly reduced in the DG and $AlCl_3$+DG groups compared to the $AlCl_3$ group, which may be because of the anti-AChE role of DG.

4. Discussion

The findings of this study demonstrated that dietary Al affects *D. melanogaster* growth, metabolic profile, behaviour, and oxidative stress. Like in mammals, exposure to Al disrupts development in the fruit fly, *D. melanogaster* as high levels of Al in insects cause metamorphosis to lengthen and pupation to delay, causing a population's development to desynchronise[22,24,26]. Additionally, research on mammals has demonstrated that Al disrupts development[47].

Reactive species can cause damage to all cellular components such as proteins, carbohydrates, lipids, and DNA. This is evident from our finding of a significant difference in ROS between the control and $AlCl_3$ group, with the latter showing a greater amount. Al exposure can disrupt nucleic acid function, enzymes, cell membrane permeability, protein production, and triglyceride metabolism, affecting their plasma levels[48,49]. Our data shows that the larvae that grew in a medium containing $AlCl_3$ had significantly less total protein and higher TAG levels compared to larvae raised on control food due to increased oxidation of glucose and activation of glycolysis in their cells, and the inhibition of the TCA cycle can be inferred from the reduced aconitase activity and increased TAG levels[25,50]. Impaired glucose can have an adverse effect on various dysfunctions, such as cerebral vascular disease with ischemia and brain injury. Changes in brain glucose metabolism have been linked to a decrease in glucose uptake at the onset of some neurodegenerative diseases[51]. DG has gained attention for treating metabolic illnesses like diabetes and hyperlipidemia[52,53]. In our study also DG co-treatment was found to reverse these conditions. Thus due to its action of controlling lipid synthesis and absorption while reducing oxidative stress and treatments can make DG beneficial in treating lipid metabolism disorders[34] including Al-toxicity.

Al-treatment caused eye degeneration and was reversed by DG co-treatment, which could be due to the neuroprotective role of DG[54]. *Drosophila* eye development requires two-thirds of critical genes, making it a valuable model for studying genetic causes of human disorders like diabetes and neurodegeneration[55,56]. Research has shown that chronic Al-toxicity can replicate the neuropathological symptoms of Alzheimer's disease[57]. Peptides and proteins linked to Alzheimer's disease cause neuron degeneration, rough texture of the eyes, and reduced mobility in both larvae and adults, which can be easily measured and analyzed[58]. Hao *et al.*,[59] reported the protective effect of DG on high glucose-induced retinal pigment epithelial cells. Similarly, we also found improved eye morphology in the DG co-treated group.

D. melanogaster is a sensitive model for studying the neurotoxicity of Al because its brain tissues absorb high levels of Al, affecting its development, life, and behaviour[25]. *Drosophila* fruit fly studies help to understand the sensory nervous system's role in nociception due to evolutionary

conservation[60]. Nociception is the process of encoding and processing noxious stimuli, enabling animals to detect and avoid harmful ones. Inflammation reduces membrane thresholds and increases sensory neuron sensitivity in the nociception pathway, leading to pain and disability as primary symptoms[61]. $AlCl_3$ induces neuro-inflammation[62] leading to neuropathic pain, which is produced by damage to the neurons in the peripheral and central nervous systems and involves sensitisation of these systems. This is the first report of altered nociception behaviour due to Al-treatment, which is restored upon DG treatment. Kiasalari *et al.*,[63] reported anti-nociceptive effect on diabetic rats by increasing mechanical and thermal nociceptive thresholds and thus lowering pain.

Abnormalities in the motor neurons of larvae affect their crawling pattern and efficiency, making the larval crawling experiments a reliable benchmark[64]. Al-treated larvae exhibited slower crawling speeds and changes in their path indicating that Al is detrimental to their neurons and DG restored these abnormalities. This could be due to altered AChE levels. The study found that administering DG inhibited the increased activity of AChE observed in $AlCl_3$-treated larvae, consistent with the results of Ogunsuyl *et al.*[65]

In *Drosophila*, Al exposure leads to oxidative stress[66]. Similarly, we also found increased oxidative stress in larvae due to Al-toxicity which is reversed by DG.

5. Conclusion

Our study found that consuming Al-rich diets leads to oxidative stress in *D. melanogaster*, which adversely affects its development, metabolism, and neurobehavioral function. These disturbances may lead to future metabolic or neurological problems. The significant protective benefits of DG may be due to its substantial antioxidant potential. By reducing oxidative stress and reversing the effects of $AlCl_3$ on abnormal development, metabolic abnormalities, and neurobehavioral disorders, DG can be used to treat diseases that are caused by Al-exposure. The most effective dosage of DG to reduce the harmful effects of Al exposure was 15mM/kg. Higher concentrations of DG reduced benefits against the developmental toxicity of Al in *D. melanogaster*.

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