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Original Article

# Quinoxaline, a Bioactive Compound Abundant in Marine Mangroves, Attenuates Acrylamide Induced Neurotoxicity in Zebrafish via Antioxidant, Anti-Inflammatory and Anti-Apoptotic Mechanisms

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## ABSTRACT

**Background:** Acrylamide, a widespread dietary and industrial contaminant, is a known neurotoxicant that promotes oxidative stress, inflammation, and neurodegeneration. Identifying effective neuroprotective agents is critical for counteracting its harmful effects. **Objectives:** This study aimed to investigate the neuroprotective potential of quinoxaline, a heterocyclic compound with antioxidant properties, against acrylamide-induced neurotoxicity in adult zebrafish (*Danio rerio*). **Materials and Methods:** Adult zebrafish were exposed to acrylamide to induce neurotoxicity, followed by quinoxaline treatment. Behavioral assays were performed to evaluate locomotor and cognitive function. Biochemical analyses quantified oxidative stress and inflammatory markers, including Superoxide Dismutase (SOD), Catalase (CAT), Lactate Dehydrogenase (LDH), Myeloperoxidase (MPO), Nitric Oxide (NO) and Glutathione (GSH). Histopathological examinations assessed neuronal integrity, while qRT-PCR evaluated the expression of stress-related genes (MPO and HSP-70). **Results:** Acrylamide exposure caused significant motor and cognitive impairment, decreased SOD and CAT activity and increased LDH, MPO, NO and GSH levels, reflecting oxidative stress, nitrosative stress and inflammation. Quinoxaline treatment restored antioxidant enzyme activity, reduced pro-inflammatory markers and ameliorated behavioral deficits. Histological analysis showed preserved neuronal structures in quinoxaline-treated groups. Additionally, quinoxaline downregulated MPO and HSP-70 expression, confirming its protective role. **Conclusion:** Quinoxaline demonstrated significant neuroprotective efficacy against acrylamide-induced toxicity by restoring redox balance, reducing inflammation and maintaining neuronal integrity. These findings highlight quinoxaline as a promising therapeutic candidate for managing acrylamide-induced neurotoxicity and related neurodegenerative disorders.

**Keywords:** Acrylamide, *Danio rerio*, Neuroprotective, Quinoxaline, Zebrafish.

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## INTRODUCTION

The human brain during its developmental stages is particularly sensitive to damage from toxic substances (Bondy and Campbell, 2005). Various environmental contaminants, such as organic solvents, pesticides, heavy metals and persistent organic pollutants are known to interfere with neurodevelopmental processes (Bal Price and Fritsche 2018). These disruptions have been implicated in the increasing prevalence of neurodevelopment disorders among children, such as Autism Spectrum Disorder (ASD), Attention Deficit Hyperactivity Disorder (ADHD) and learning disabilities (Bennett *et al.*, 2016; Grandjean *et al.*, 2017). It is estimated that the number of patients with neurodegenerative diseases will increase due to the aging of the global population and these conditions are typically marked by the buildup of misfolded proteins, reduced synaptic connectivity, functional neuronal impairments, and eventual cell death (Memon *et al.*, 2020). While genetic predisposition plays a role, exposure to neurotoxic agents has emerged as a contributing factor. These toxins can induce selective neuronal loss and are frequently used in experimental models of disorders like Parkinson's Disease (PD), Amyotrophic Lateral Sclerosis (ALS) and Alzheimer's

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Disease (AD). The endogenously derived neurotoxins such as metabolic dysfunction, oxidative stress, or genetic mutations often resulting from irregular metabolic processes, can impact the Central Nervous System (CNS) and sometimes penetrate the blood-brain barrier, which is responsible for supplying vital nutrients and oxygen to brain tissue (Wu *et al*., 2021). These toxins impair neurotransmission by interfering with ion channels potassium, calcium, sodium, disrupting neurotransmitter receptor functions e.g., acetylcholine receptors, and inhibiting essential enzymes like Tyrosine Hydroxylase (TH) (Tipton *et al*., 1994; Soddu *et al*., 2015). Additionally, these compounds contribute to neurodegeneration by suppressing mitochondrial function, elevating oxidative stress, initiating neuroinflammation, and promoting apoptotic pathways (Cao *et al*., 2021).

Acrylamide is a recognized neurotoxic compound that has been widely utilized for decades in various industrial processes. It is predominantly used in the synthesis of high-molecular-weight polymers like polyacrylamide, with applications in industries such as paper production, textile manufacturing, wastewater treatment, and medical technology (*Smith et al*., 1991). Additionally, acrylamide is generated during high-temperature cooking methods such as frying, roasting, and baking making it a common contaminant in a range of food products including cereals, baked goods, crackers, and snack items (Tareke *et al*., 2002; Sirot *et al*., 2012). It is also detected in personal care products and numerous industrial materials like dyes and refined sugar. In its monomeric form, acrylamide poses substantial health risks, demonstrating carcinogenic, teratogenic, and neurotoxic properties (Parng *et al*., 2007). Notably, its neurotoxicity has been confirmed through both animal experiments and human epidemiological studies, also indicating potential developmental and genotoxic consequences (Zhao *et al*., 2022). The acrylamide induced neurotoxicity is believed to involve the covalent binding of acrylamide to cysteine residues in presynaptic neuronal proteins. This interaction hampers neurotransmitter function and synaptic transmission by deactivating critical neuronal proteins (LoPachin *et al*., 2011). Moreover, oxidative stress is a key mediator in ACR-induced neurotoxic outcomes, serving as both a biochemical signal and a physiological contributor to neuronal injury (Zhao *et al*., 2017; Zamani *et al*., 2018).

Zebrafish (*Danio rerio*) offer numerous advantages as an *in vivo* model for studying developmental neurotoxicity. Their small size allows for convenient handling and housing, while rapid neural development occur within three days post-fertilization makes them particularly suitable for early-stage neurotoxicological study (-Romeu *et al*., 2016; Kalueff *et al*., 2016; Tagkalidou *et al*., 2025). Zebrafish exhibit significant genetic, morphological, and physiological similarities to humans, particularly with respect to the CNS (Howe *et al*., 2013; Kalueff *et al*., 2016). The mechanisms and stages of CNS development in zebrafish are highly conserved across vertebrate species, including humans (Budroni *et al*., 2011). Notably, the formation and function of the Blood-Brain Barrier (BBB) in zebrafish closely similar to those found in mammals, a critical factor in neurotoxicity studies, given the BBB's role in safeguarding neural tissue from harmful substances. Additionally, the structural and functional mapping of the zebrafish brain has proven highly effective for translational research, as many behavioral and neuropharmacological studies have demonstrated strong parallels with human brain functions (Eliceiri *et al*., 2012; d'Amora *et al*., 2018). Both humans and zebrafish exhibit similar physiological responses upon acrylamide exposure, including neurotoxicity symptoms such as lethargy, body weight reduction, gait disturbances, muscle weakness, polyneuropathy and numbness in extremities (Parng *et al*., 2002). These shared outcomes further support the relevance of zebrafish as a reliable model for studying acrylamide induced neurotoxic effects.

Quinoxaline ($C_8H_6N_2$), also referred to as 1,4-diazanaphthalene or benzopyrazine, is a nitrogen containing heterocyclic compound of a benzene ring combined with a pyrazine ring, as illustrated in Figure 1. This structural configuration contributes to its broad spectrum of biological activities, which include anti-bacterial (Vieira *et al*., 2014), anti-fungal (Newahie *et al*., 2019). Anti-diabetes (Shintre *et al*., 2017), anti-cancer, anti-viral and anti-protozoal effects (Montana *et al*., 2020). Recent studies using LC-MS analysis have identified quinoxaline as a major phytochemical constituent in *Rhizophora apiculata* (mangrove) extracts, indicating both its abundance and therapeutic potential, particularly its anticancer properties (Vinod Prabhu *et al*., 2014; Vinod Prabhu *et al*., 2018; Vinod Prabhu *et al*., 2021; Cecileya Jasmin *et al*., 2025). Quinoxaline derivatives are also known to be rich in polyphenolic compounds, which are often associated with antioxidant and health-promoting effects. Given its pharmacological relevance, the present study focuses on investigating the neuroprotective role of quinoxaline against acrylamide-induced neurotoxicity using a zebrafish (*Danio rerio*) animal model system.

## MATERIALS AND METHODS

Acrylamide (extra pure, 99%) was procured from Sisco Research Laboratories Pvt. Ltd., (Product code: 22794). Quinoxaline, with a stated purity of 98%, was obtained from Sigma Aldrich Chemicals Pvt. Ltd., USA. All additional reagents and chemicals required for biochemical and enzymatic assays were of analytical grade and were procured from HiMedia Laboratories Pvt. Ltd., India. Zebrafish (*Danio rerio*) were procured from Tarun Fish Farm, Ornamental and Research Zebrafish Farms Breeder, located in Manimangalam, Chennai, Tamil Nadu, India. The breeder sourced wild zebra fish from various river ecosystems in the southern part of the Western Ghats (Kerala and Karnataka, India) and cultured them under controlled farm conditions. All animal experiments were performed according to the rules and regulations of the Institutional Animal Ethics Committee of the

Government of India, (Project Proposal No:SU/CLATR/IAEC/XXIV/58/2024).

## Experimental setup

Adult male and female zebra fishes were selected for the investigation to evaluate the neuroprotective potential of quinoxaline. Prior to the experiment, the fish were acclimatized for a period of seven days in a well-aerated tank, which was routinely cleaned. During this time, the fish were fed twice daily using standard commercial feed. To induce neurotoxicity, acrylamide was used at a concentration of 0.75 mM, prepared in 200 mL of water within both the induction and treatment tanks. A stock solution of quinoxaline was prepared by diluting the compound in distilled water, achieving a final dose of 150 µg/kg body weight. This dosage was confirmed to be non-toxic and non-lethal for the zebrafish. The neuroprotective efficacy of quinoxaline was examined over a 72-hr period. For treatment, 100 µL of the prepared stock solution containing the determined dose of quinoxaline was administered through feeding.

## Experimental groupings

For this investigation, adult zebrafish were randomly assigned into four groups, each comprising ten specimens ($n$=10). Group I: The first group served as the control - Normal and was maintained on a standard diet provided *ad libitum*. Group II: The second group, referred to as the induction group, was exposed to 0.75 mM acrylamide in the water and fed with the same standard diet. Group III: The third group received the same acrylamide exposure (0.75 mM) but was given a diet supplemented with quinoxaline at a concentration of 150 µg/kg of body weight, mixed thoroughly with finely powdered fish feed. Group IV: A fourth group was designated as the positive control, in which the fish were exposed to acrylamide (0.75 mM) and received a feed supplemented with donepezil HCl at concentration of 10 µg/kg of body weight, a reference neuroprotective compound, administered at a safe and effective dosage.

Over the course of 72 hr, behavioral changes were assessed using a T-maze tank setup designed to evaluate locomotor activity and cognitive response. At the end of the exposure period, the fish are euthanized ethically and brain tissues were dissected for downstream analyses. These included histological evaluation, biochemical assays, and gene expression analysis of Heat Shock Protein-70 (HSP-70) and Myeloperoxidase (MPO), to determine the potential of quinoxaline in mitigating acrylamide-induced neurotoxic effects.

## Assessment of behavior using the T-Maze setup

Behavioral analysis was conducted using a T-maze to observe cognitive and locomotor responses in zebrafish from all experimental groups. The maze consisted of a main arm, measuring 20 cm, which led to two branching arms, each 38 cm in length. The starting point of the straight arm served as the entry area (compartment 1). The left arm included fish feed to act as a mild attractant, while the right arm served as the goal zone for the maze trial.

Fish from the control group were introduced into the maze first, followed by those in the acrylamide-exposed and treatment groups. Behavioral testing was performed at 72 hr. During the trials, several behavioral indicators were recorded, including the time taken to complete the maze, signs of disoriented or erratic swimming, periods of inactivity, and evidence of memory recall. All fish movements were documented using video recordings, which were later analyzed to evaluate the influence of acrylamide toxicity and the potential protective effects of quinoxaline.

## Histological examination of brain tissue

Brain samples collected from all the zebrafish groups and were carefully dissected and rinsed with 0.9% physiological saline to eliminate residual blood and debris. The tissues were then preserved in 10% buffered formalin for 24 hr to ensure proper fixation. After fixation, the samples were transferred to a 70% ethanol solution for dehydration. Standard histological procedures were followed to process the tissues, which were subsequently embedded, sectioned, and stained using hematoxylin and eosin. The prepared slides were then examined microscopically to assess histopathological changes in neuronal structures.

## Superoxide Dismutase activity assay

The enzymatic activity of Superoxide Dismutase (SOD) was measured using a modified protocol based on the method described by Misra and Fridovich (1977). For the assay, 100 µL of tissue homogenate obtained from both control and treated groups was used. The reaction mixture also contained 0.25 mL of 0.6 mM EDTA and 0.25 mL of 0.3 M carbonate buffer, adjusted to pH 10.2. Following the addition of 0.4 mL of epinephrine as the substrate, the change in absorbance was recorded at 480 nm using a UV-visible spectrophotometer. SOD activity was calculated and expressed as International Units (IU) per milligram of protein content.

## Myeloperoxidase activity assay

The myeloperoxidase activities were evaluated using tissue lysates prepared from both control and treated groups. A total of 100 µL of each sample was added to a reaction mixture composed of 50 mM potassium phosphate buffer (pH 6.0), O-dianisidinedihydrochloride as the chromogenic substrate, and 0.0005% hydrogen peroxide to initiate the reaction. The enzymatic activity was monitored by measuring the change in absorbance at 460 nm using a UV-visible spectrophotometer. MPO activity was quantified for all four experimental groups to determine variations associated with acrylamide exposure and quinoxaline treatment.

## Catalase activity assay

The catalase activity was assessed following the method outlined by (Aebi, 1984). For each sample, 100 µL of protein sample was mixed with 0.01 mL of ethanol and kept on ice for 30 min to stabilize the enzyme. After incubation, 10% Triton X was added to each sample to aid in enzyme activation. The reaction mixture consisted of 2 mL of 50 mM phosphate buffer at pH 7.0 and 1 mL of Hydrogen Peroxide ($H_2O_2$) as the substrate. The breakdown of hydrogen peroxide was monitored by measuring the decrease in absorbance at 240 nm, with readings taken every 15 sec for a total of 3 min. Catalase activity was expressed in terms of micromoles of $H_2O_2$ decomposed per minute per milligram of protein expressed as U/mL.

## Assessment of antioxidant markers: Assay of Glutathione Peroxidase levels

The Glutathione Peroxidase (GSH) activity was determined based on the method originally described by Rotruck *et al.*, (1973). A 100 µL aliquot of tissue lysate was combined with 0.4 mL of 0.4 M sodium phosphate buffer (pH 7.0) with 0.1 mL of 10 mM sodium azide. To this mixture, 0.2 mL of 4 mM reduced glutathione with 0.1 mL of 2.5 mM hydrogen peroxide was added. The total volume was adjusted to 2 mL with distilled water. The reaction mixture was incubated at room temperature for 3 min. To terminate the enzymatic reaction, 0.5 mL of 10% Trichloroacetic Acid (TCA) was added, and the mixture was centrifuged to separate the precipitate. The supernatant was removed and the pellet was treated with 4 mL of 0.3 M disodium hydrogen phosphate with 1 mL of DTNB reagent. The absorbance of the resulting solution was measured at 412 nm using a UV-visible spectrophotometer. Enzyme activity was expressed as the amount of glutathione oxidized per minute, normalized to milligrams of protein.

## Estimation of nitric oxide levels

The Nitric Oxide (NO) concentration in the tissue samples was quantified using a method adapted from (Sun *et al.*, 2003). A 100 µL portion of the protein extract was mixed with 0.1 g of activated charcoal and vortexed thoroughly to remove any color impurities that could interfere with spectrophotometric readings. The mixture was then centrifuged at 15,000 rpm for 15 min at 4°C. The resulting supernatant was treated with 1 mL of an extraction buffer consisting of 50 mM cold acetic acid (pH 3.6) and 4% zinc acetate. Subsequently, 1 mL of Griess reagent was added to initiate the colorimetric reaction. The samples were incubated at room temperature for 30 min, after which absorbance was measured at 540 nm using a UV-vis spectrophotometer. A standard curve was established using sodium nitrate solutions in the range of 1-100 µM to calculate the nitric oxide concentration in the samples.

## Assessment of biomolecular modifications:

### *Estimation of protein carbonyl content levels*

The level of protein carbonyl groups, indicative of oxidative protein damage, was assessed using the method outlined by (Levine *et al.*, 1990). A 100 µL aliquot of the protein sample was mixed with 0.5 mL of 10 mM 2,4-dinitrophenylhydrazine (DNPH) prepared in 2 M Hydrochloric Acid (HCl). This reaction mixture was incubated at 37°C for 90 min to allow the formation of hydrazone derivatives. Following incubation, the tubes were cooled on ice, and 1 mL of 28% Trichloroacetic Acid (TCA) was added to precipitate the proteins. The precipitated proteins were collected by centrifugation at 4°C for 10 min, and the supernatant was discarded. The pellets were washed twice with a 1:1 mixture of ethanol and ethyl acetate to remove excess DNPH, and then air-dried. The final pellet was dissolved in 1 mL of 6 M guanidine hydrochloride in 2 M HCl and incubated at room temperature in the dark for 1 hr to facilitate color development. After a brief centrifugation at 4°C for 5 min, the absorbance of the supernatant was measured at 360 nm using a UV-visible spectrophotometer. Protein carbonyl concentration was determined using an extinction coefficient of 21.000 $M^{-1}$ $cm^{-1}$ and expressed in nmol per mg of protein.

## Assessment of HSP70 stress marker using reverse transcriptase polymerase chain reaction (RT-PCR)

The expression of the HSP70 gene was assessed using Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) following total RNA extraction from zebrafish brain tissue. The extraction procedure was based on the method described by (Lehning *et al.*, 2003). The concentration and purity of the isolated RNA were evaluated using a biophotometer (Eppendrof, Germany). Complementary DNA (cDNA) was synthesized using a commercially available cDNA synthesis kit (Pratosh Lab, Chennai, India). Approximately 0.5-1.0 µg of total RNA was mixed with 1.5 µL of random hexamer primers and incubated for 72°C for 10 min, followed by rapid cooling to ensure primer annealing. The reaction mixture was then supplemented with 5.0 µL of a premix containing 10 mM Deoxynucleotide Triphosphates (dNTPs),

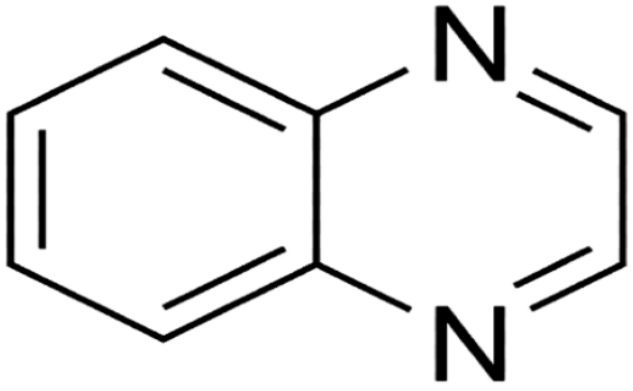


**Figure 1:** The structure of quinoxaline (C8H6N2).

3.0 µL of 10× MMLV reverse transcriptase buffer, and 1.0 µL of M-MLV reverse transcriptase enzyme. The final quantity was adjusted to 50 µL using RNase- and DNase-free water.

Reverse transcription was carried out by incubating the mixture at 42°C for 1 hr and 15 min, and the reaction was terminated by heating at 72°C for 15 min. The synthesized cDNA was stored at -20°C for subsequent amplification. PCR was conducted to amplify HSP70, Myeloperoxidase (MPO), and Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) genes using gene specific primers. The amplified products are resolved using 1% agarose gel electrophoresis stained with ethidium bromide and electrophoresed at 50 V for 2-3 hr at room temperature. The resulting DNA bands were visualized under Ultraviolet (UV) illumination, and band intensities were quantified using ImageJ software (NCBI, USA). Genes and their primers with product size are shown in Table 1.

### Statistical Analysis

All experimental data were expressed as mean values accompanied by their corresponding Standard Deviations (mean±SD), with each group comprising ten samples ($n$=10). To evaluate statistical differences between groups, Student's t-test was employed. The analyses were performed using SPSS software (version 16.0; SPSS Inc., Chicago, IL, USA). A $p$-value of less than 0.05 was measured statistically significant.

## RESULTS

### Effect of quinoxaline on acrylamide induced behavioral impairment

This study evaluated the behavioral alterations in zebrafish following acrylamide exposure, using the T-maze test as a tool to assess learning and memory function. Behavioral indicators such as time to complete the maze, instances of erratic swimming, periods of immobility, and cognitive responsiveness were recorded throughout the experiment. Zebrafish exposed to acrylamide displayed clear signs of cognitive decline and behavioral disturbance. These fish took longer to complete the maze, demonstrated irregular and disoriented swimming, and experienced longer durations of immobility suggestive of compromised neural coordination and impaired memory. As exposure continued, these deficits appeared to intensify, as shown by the progressive decline in performance across multiple sessions. The increased incidence of uncoordinated movements further indicated acrylamide-induced neurotoxicity. Zebrafish treated with quinoxaline showed significant improvements in all behavioral parameters. Their movement was more stable and organized, immobility time was significantly reduced, and abnormal swimming patterns decreased. Importantly, the fish retained their ability to remember the correct path in the T-maze, therefore the study demonstrating that quinoxaline had a protective effect against the neurobehavioral damage caused by acrylamide exposure. The results of the various parameters of the T-maze experiment are presented in Table 2. The pattern of locomotion of the zebrafish in control and the experimental groups in a T-maze tank was represented in diagrams as shown in Figure 2.

### Effect of quinoxaline on acrylamideinduced histological changes in the brain

Histological analysis of the zebrafish brain, with a particular highlighting on the Purkinje cell layer, demonstrated significant changes following acrylamide exposure, as illustrated in Figure 3. Figure 3(B) Induction: Brain sections from the acrylamide induced group exhibited significant neurodegeneration, including widespread neuronal damage, disruption of normal tissue architecture, and loss of cellular integrity. The Purkinje cells were particularly affected, with decreased cell density and enlarged intercellular spaces, suggesting cytotoxic injury induced by acrylamide. Additional alterations such as disruption of the granular layer, depletion of Purkinje cells, and an overall breakdown in structural organization further confirmed the neurotoxic impact of acrylamide. In comparison, brain samples from the control normal Figure 3(A) maintained normal histological features, with clearly defined Purkinje cells and intact tissue structure, showing no evidence of damage or disorganization. Figure 3(C) Treatment with quinoxaline appeared to offer significant neuroprotection. Brain tissues from this group showed preserved cellular structure, with minimal signs of degeneration and a more organized appearance of the Purkinje layer. These observations suggest that quinoxaline can counteract the histological damage caused by acrylamide and reinforcing its potential as a protective agent against neurotoxicity.

**Table 1:** **List of genes, and their primers with their product size.**

| Name of the genes | Primers 5'-3' | Tm (°C) | Product size |
|---|---|---|---|
| GAPDH | Forward primer- ACTAATGGCACTGGACAA<br>Reverse primer-ACTGATAAGACACTGATATGGTA | 55.6 | 145 |
| MPO | Forward primer - GTGGTCGTGTCGGTTCTCTT<br>Reverse primer- GCAGATTATGCGGGCCATTG | 40. 0 | 120 |
| HSP-70 | Forward primer - CAACGTGCTGATCTTTGACC<br>Reverse primer- TCCTCTTGGCTCGTTCACAT | 42.0 | 127 |

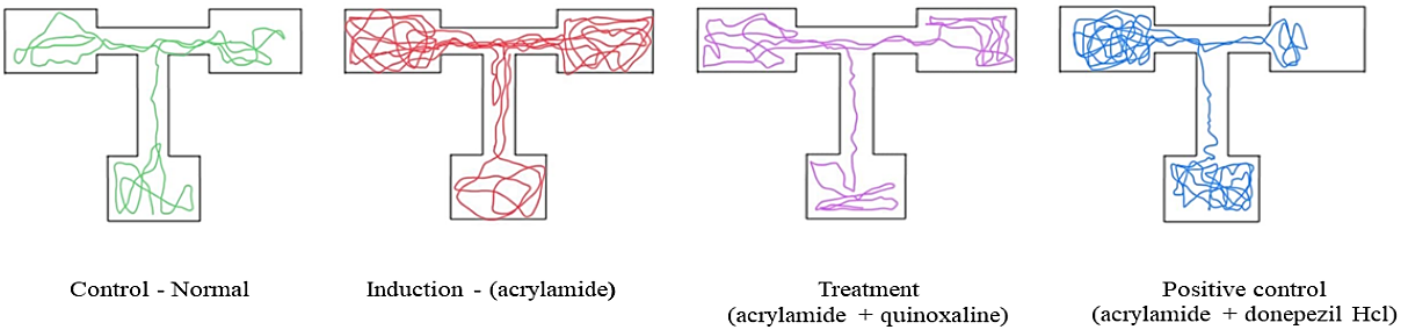


**Figure 2:** Patterns of locomotion of the control and the experimental groups in a T-maze tank.

## Effect of quinoxaline on total brain protein content in acrylamide induced neurotoxicity

The total protein content in the brain tissues of zebrafish across different groups was quantitatively assessed, and the results are illustrated in Figure 3. In the control group, the protein concentration was recorded at approximately (92.24 µg/10 mg) of brain tissue, reflecting normal metabolic function. In contrast, the acrylamide-induced group showed a reduced protein concentration of (32.8. µg/10 mg) of tissue, indicating possible protein degradation or impaired synthesis due to neurotoxic stress.

Animals that received quinoxaline treatment exhibited a noticeable recovery in protein levels (83.45µg/10 mg) when compared to the acrylamide-induced group, suggesting that quinoxaline helped in preserving protein integrity in brain tissues. The increase in protein concentration in the treatment group indicates quinoxaline could be a potential in alleviating acrylamide-induced protein loss and mitigating neurotoxicity. The protein concentration of the control and experimental groups are shown in graphical representation in Figure 4.

## Effect of quinoxaline on enzymatic markers

Administration of acrylamide led to increased levels of LDH and MPO in the brain, while the group treated with Quinoxaline showed significantly decreased levels of LDH (2135.5 (U/mL)) when compared with induction group (3197.0 (U/mL)). The MPO level of the treated groups significantly reduced to (136.7 µmoles) when compared with induction groups (437.0 µmoles). Therefore, these damages in the induction groups denote the tissue damage and inflammation in the brain of the zebrafish.

The SOD and CAT level were also significantly decreased in the Induction groups due to the damage in the production of SOD and CAT in the brain, result in oxidative stress. Comparatively, the fishes fed with quinoxaline, treated groups (11.75 u/mL) significantly restore the levels of SOD when compared with the induction group level (6.3 u/mL). The CAT level were also significantly restored to (50.0 U/mL) when compared with the induction groups (24.0 U/mL), suggesting that quinoxaline could revert back the effect of oxidative stress caused by acrylamide. The effects of acrylamide toxicity and quinoxaline treatment on the enzymatic markers such as LDH, MPO, SOD and CAT levels are show in Figure 5.

## Effect of quinoxaline on antioxidant markers

Treatment with quinoxaline significantly modulated oxidative stress biomarkers. Nitric Oxide (NO) levels were markedly reduced in the treated group (40.1 mM) compared with the induction group (75.7 mM), indicating attenuation of nitrosative stress. Conversely, Glutathione (GSH) levels increased substantially in the treated group (123.6 mM) relative to the induction group (61.1 mM). This restoration of GSH suggests that quinoxaline enhanced the antioxidant defense system, thereby contributing to its observed neuroprotective effect. The levels of antioxidant markers of NO and GSH levels of the experimental groups are presented graphically in Figure 6.

## Effect of quinoxaline on protein biomolecule modifiers

The levels of Lipid Peroxidation (LPO) and Protein Carbonyl (PC) content were assessed across the experimental groups using their respective extinction coefficients. Zebrafish exposed to acrylamide exhibited a significant increase in LPO levels (72.8 nmol), indicating elevated oxidative stress, compared to the quinoxaline treated group, which showed a reduced level of 44.4 nmol). Similarly, the protein carbonyl content was higher in the acrylamide group (8.85 nmol/mg protein) than in the group treated with quinoxaline (8.1 nmol/mg protein). The reduced levels of LPO and PC in the treatment group suggest that quinoxaline effectively mitigates acrylamide-induced oxidative damage and inflammation in the brain. These findings support the neuroprotective potential of quinoxaline in preserving cellular protein integrity under toxic stress conditions. The levels of LPO and protein carbonyl levels of the experimental groups are presented graphically in Figure 7.

## Gene expression analysis

The expression profiles of HSP-70 and MPO genes were evaluated in zebrafish subjected to acrylamide exposure. A notable upregulation in HSP-70 mRNA expression was observed in the

acrylamide-exposed group, indicating an activated cellular stress response in neuronal tissues. In contrast, the group treated with quinoxaline induces downregulation of HSP-70 expression, suggesting a reduction in cellular stress levels following treatment. The elevated expression of HSP-70 in the acrylamide group indicates the neuronal defense mechanism triggered by toxic while its downregulation upon quinoxaline administration demonstrates the compound's protective and regulatory role in alleviating acrylamide-induced neurotoxicity.

Similarly, the MPO gene expression was significantly elevated in zebrafish exposed to acrylamide, reflecting enhanced inflammatory activity. However, a considerable reduction in MPO expression was observed in the quinoxaline-treated group, further supporting its anti-inflammatory potential. The expression levels of GAPDH, HSP-70 and MPO, along with the densitometric analysis of HSP-70 and MPO fold changes, are represented in Figures 8 and 9.

## DISCUSSION

Acrylamide in its polymeric form is considered non-toxic, its monomeric form poses significant health hazards, particularly in animal models such as rats and mice (Lehning *et al*., 2003; Lopachin *et al*., 2004). The monomer is associated with multiple toxicological outcomes, including carcinogenic, teratogenic and neurotoxic effects (Favor J and Shelby 2005; Lehning *et al*., 2003; Hogervorst *et al*., 2010; Ma *et al*., 2011). Acrylamide-induced neurotoxicity is primarily linked to central and peripheral distal axonopathy, reflecting its detrimental impact on neuronal structure and function (Lehning *et al*., 2003; Lopachin *et al*., 2004). Although the developmental toxicity of acrylamide has been studied in various laboratory animals, there remains a lack of comprehensive data on its developmental neurotoxicity. This break underlines the importance of developing and validating reliable animal models to explore acrylamide-induced neurodevelopmental impairments, especially for improving the clinical assessment and management of individuals with occupational exposure to acrylamide (Lopachin *et al*., 2003; Zhu *et al*., 2021).

The zebrafish is a widely recognized vertebrate model in the field of developmental biology and is increasingly utilized in neurotoxicity research (Vesterlund *et al*., 2011). Its rapid life cycle, high reproductive capacity, and transparent embryonic structure make it highly suitable for developmental toxicity assessments (Xia *et al*., 2018). Zebrafish exhibit approximately 70% genetic similarity to humans and share several fundamental features of the nervous system, including the presence of analogous neurotransmitters (Howe *et al*., 2013; Babin *et al*., 2014; Faria *et al*., 2015, Horzmann *et al*., 2022).

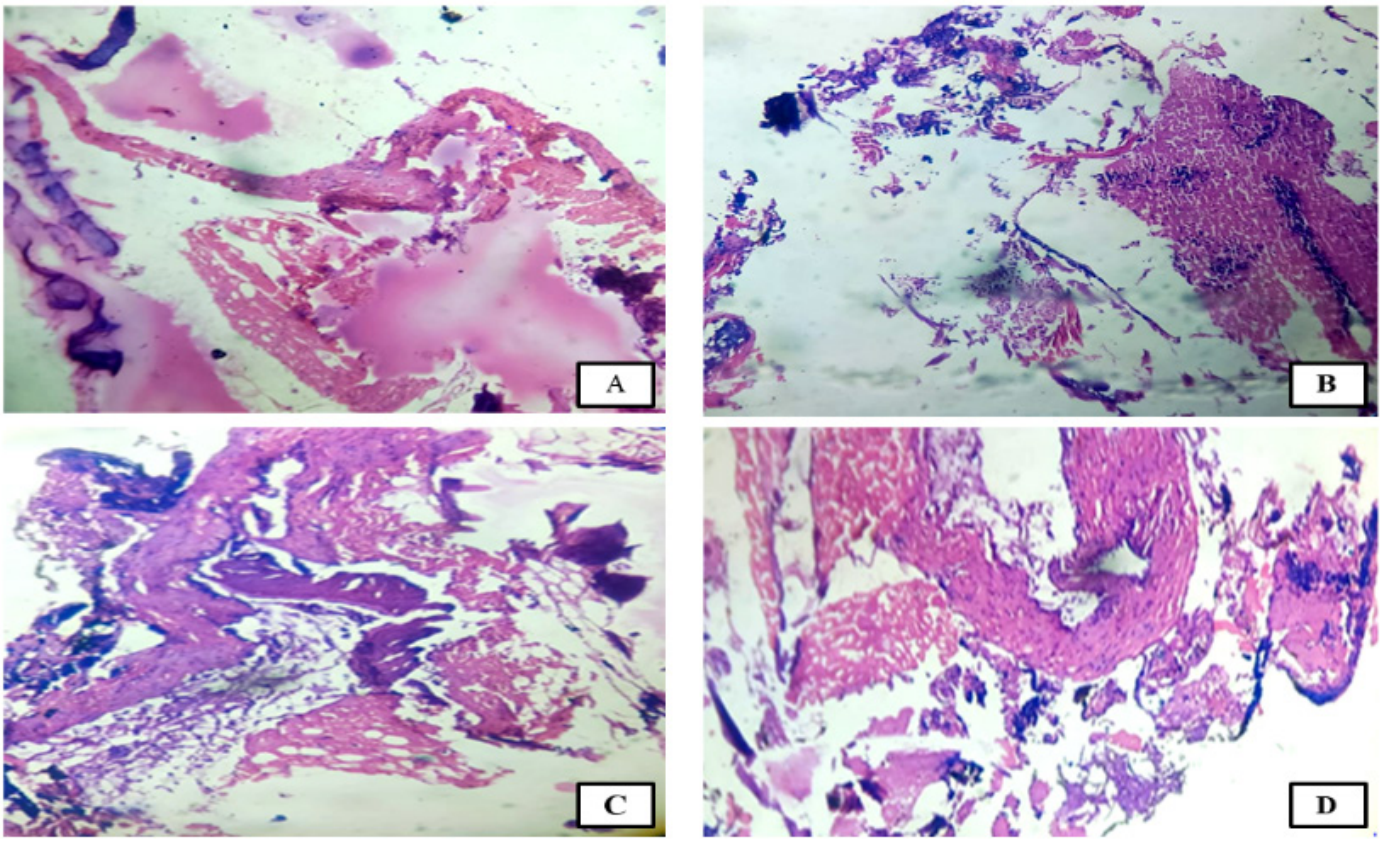


**Figure 3:** Effect of quinoxaline on acrylamide induced histological changes in the purkinje cell region of the brain in the experimental groups. (A) Control-Normal, (B) Induction (acrylamide), (C) Treatment-(acrylamide+quinoxaline) and (D) Positive control-(acrylamide+donepezil HCl).

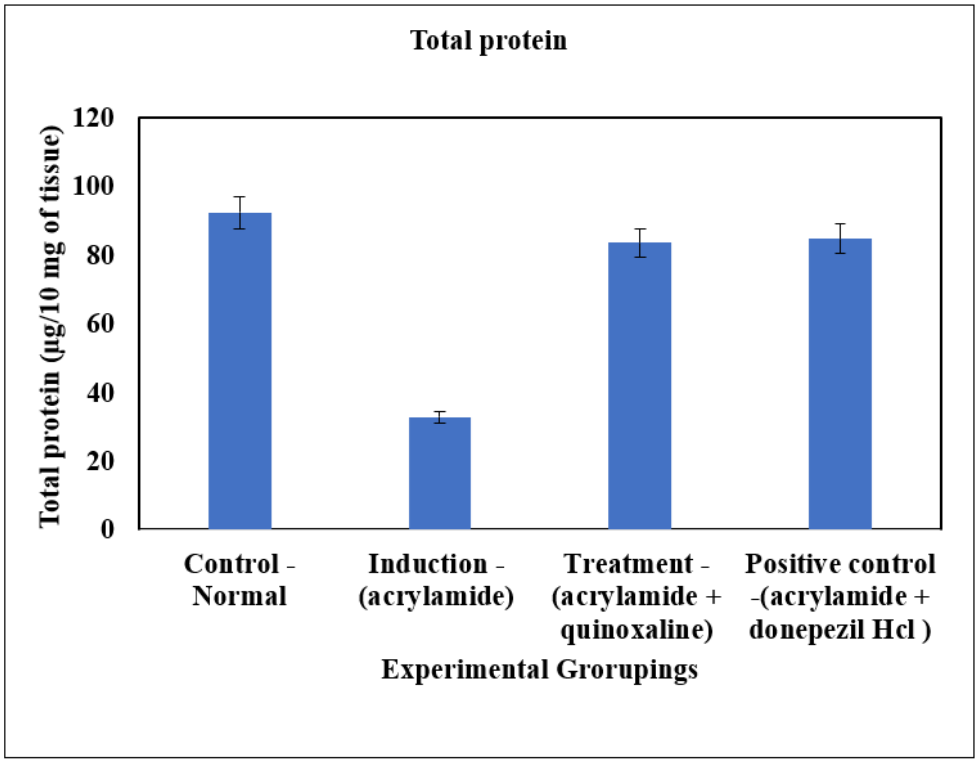


**Figure 4:** Graphical representations of the levels of the protein concentration in the experimental groups. Values are expressed as the mean±SD (*n*=3). (**p*<0.05, ***p*<0.01) are considered statistically significant from untreated group.

**Table 2: Behavioral parameters of the T-maze assay of the control and the experimental groups after 72 hr. Values are expressed as Mean±SD for three fishes in each group. Values are expressed as the Mean±SD (*n*=3). (**p*<0.05, ***p*<0.01) are considered statistically significant from untreated group.**

| Experimental Grouping | Total time taken to finish the T-maze | Total Erratic swimming time | Total immobility time |
|---|---|---|---|
| Control -Normal | 3.05±2.54 | 0.28±0.21 | 0.69±0.18 |
| Induction-(Acrylamide) | 5.35±0.32* | 4.34±0.20* | 0.19±0.12 |
| Treatment-(Acrylamide+Quinoxaline) | 2.13±0.14** | 1.17±0.23** | 0.15±0.12** |
| Positive control-(Acrylamide+Donepezil HCl) | 2.87±0.20 | 2.32±0.12 | 0.43±0.12 |

In the present study the symptoms of neurotoxicity were evident in zebrafish as early following the exposure to acrylamide. The fish displayed distinct hyperactivity and disoriented swimming behaviors in the T-maze test, which persisted for up to 72 hr. Although the intensity of hyperactivity lessened over time, disorientation and difficulties in completing the maze remained evident. This behavioral pattern indicated significant memory deficits, as the fish exhibited uncertainty in choosing the correct direction to swim. Particularly, none of the zebrafish showed immobility instead, they were consistently active, often moving in repetitive circular patterns which denote a clear indication of impaired motor coordination not observed in the control group. Exposure to 150 mg/L of acrylamide consistently induced such circular swimming, reinforcing the presence of movement disorders. But in contrast the, zebrafish treated with quinoxaline showed behavior that closely similar the control group. These Quinoxaline treated fish shown improved memory, spent less time navigating the maze, and showed a reduction in erratic and circular swimming. Additionally, the restoration of normal immobility periods suggested that quinoxaline can initiate the recovery of cognitive and motor functions.

Antioxidant enzymes, such as SOD, CAT, MPO, and LDH, play a important role in defending against oxidative damage caused by Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS). SOD is particularly important in the body's natural defense mechanisms, as it neutralizes superoxide ions by converting them into the more stable compound Hydrogen Peroxide ($H_2O_2$). Catalase or glutathione peroxidase then further breaks down hydrogen peroxide into water, helping to maintain oxidative balance. The activities of these enzymes are finely regulated to have ROS levels at a maintained level and in low state. SOD, in particular, prevents oxidative damage in cells, including those in

tissues like the vascular endothelium that are vulnerable to ROS exposure. However, SOD levels are often reduced in neural tissues, including the brain. Due to its potent antioxidant properties, SOD protects brain cells from oxidative stress and inflammation, which can otherwise lead to cellular damage. In the present study, the induction group showed a decline in the activity of SOD and CAT enzymes in the brain, contributing to oxidative stress. On the other hand, zebrafish treated with quinoxaline exhibited a restoration of SOD and CAT levels, suggesting that quinoxaline can reduce the oxidative stress induced by acrylamide.

In the present study, the administration of acrylamide has led to an increase in the levels of LDH and MPO in zebrafish. Previous studies have reported that acrylamide-induced brain toxicity is linked to elevated MPO levels (Amirshahrokhi and Abzirakan, 2022). LDH is a cytoplasmic enzyme found in all cells and plays a central role in anaerobic metabolism. In the current study, the acrylamide exposure significantly raised both LDH and MPO levels, contributing to extensive tissue damage, inflammation, and stress in the brain. Treatment with quinoxaline significantly reduces these enzyme levels, alleviating the extent of tissue damage and inflammation.

NO contributes to tissue damage through its interaction with superoxide, leading to the formation of peroxynitrite, a potent oxidant that exacerbates inflammation. Elevated NO levels are often linked to injury and toxicity in several animal models. Additionally, nitric oxide serves as a key neurotransmitter in the central nervous system, facilitating neuromuscular junction function and blood flow regulation in the brain. It is vital for neuronal signal transduction, influencing metabolic processes and dendritic spine growth (Picón-Pagès *et al.*, 2019).

In the present study, the induction of zebrafish with acrylamide resulted in significantly increased levels of NO and GSH in the brain tissue. Decreased glutathione levels in gray matter, which is rich in neurons, make it more vulnerable to neurodegeneration due to oxidative stress. However, treatment with quinoxaline in zebrafish led to a reduction in both NO and GSH levels, mitigating the acrylamide-induced toxicity and promoting recovery from neurodegeneration.

Heat shock proteins are natural biomarkers essential for genetic adaptability and survival in organisms exposed to environmental stress. These proteins are involved in key cellular processes, such as gene transcription reprogramming, protein folding and

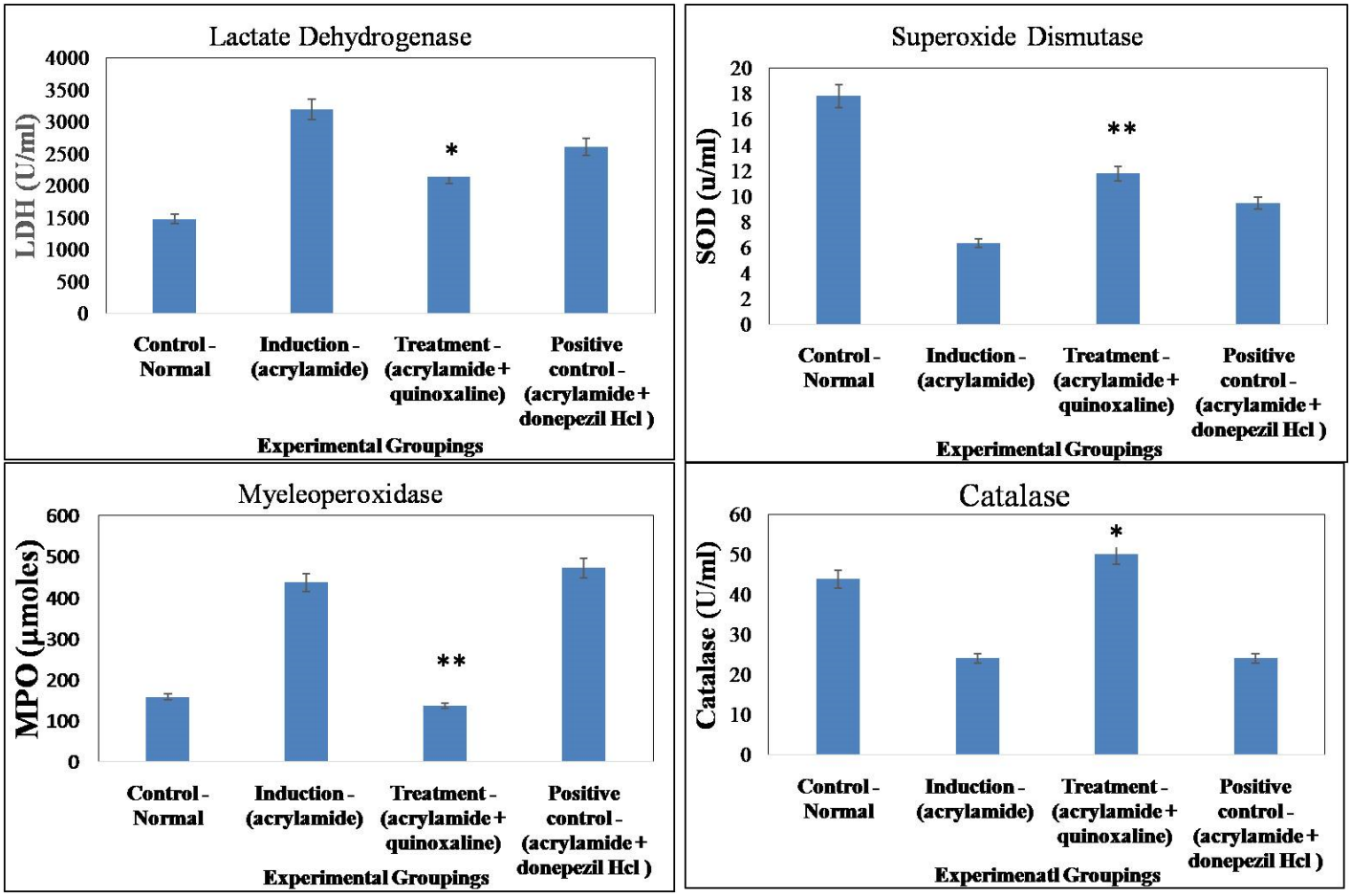


**Figure 5:** Graphical representations on the enzymatic markers levels of acrylamide toxicity and qunioxaline treatment on the enzymatic markers. A-LDH level, B-SOD level; C-MPO level; and D- CAT level. Values are expressed as the mean±SD ($n$=3). (*$p$<0.05, **$p$<0.01) are considered statistically significant from untreated group.

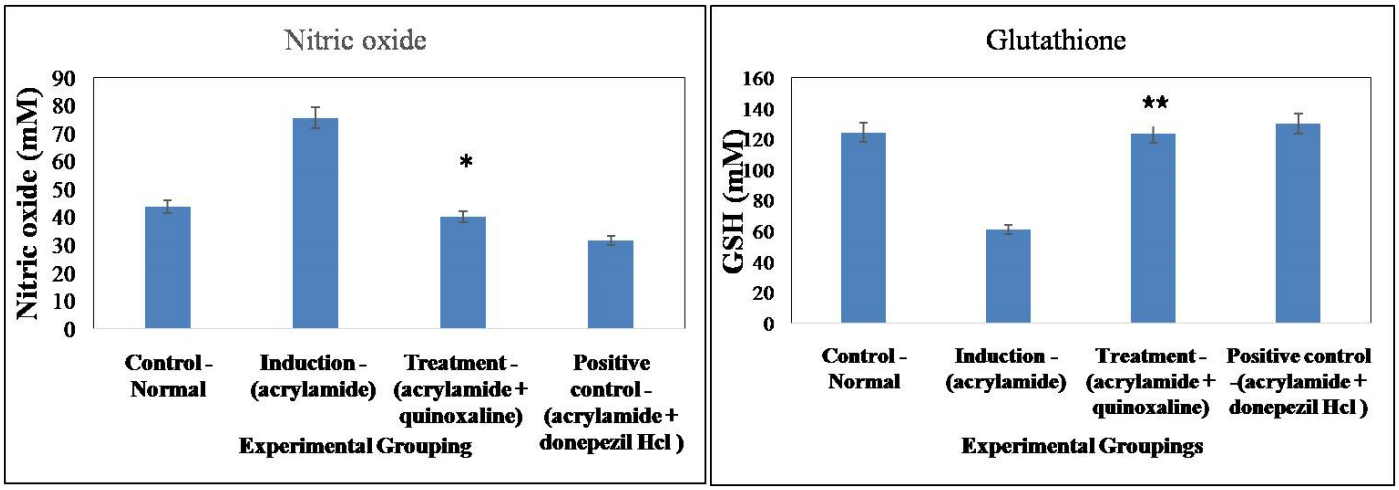


**Figure 6:** Graphical representations on the effect of acrylamide toxicity and quinoxalinw treatment on the anti-oxidant markers on nitric oxide level and glutathione levels. Values are expressed as the mean±SD (*n*=3). (*$p$<0.05, **$p$<0.01) are considered statistically significant from untreated group.

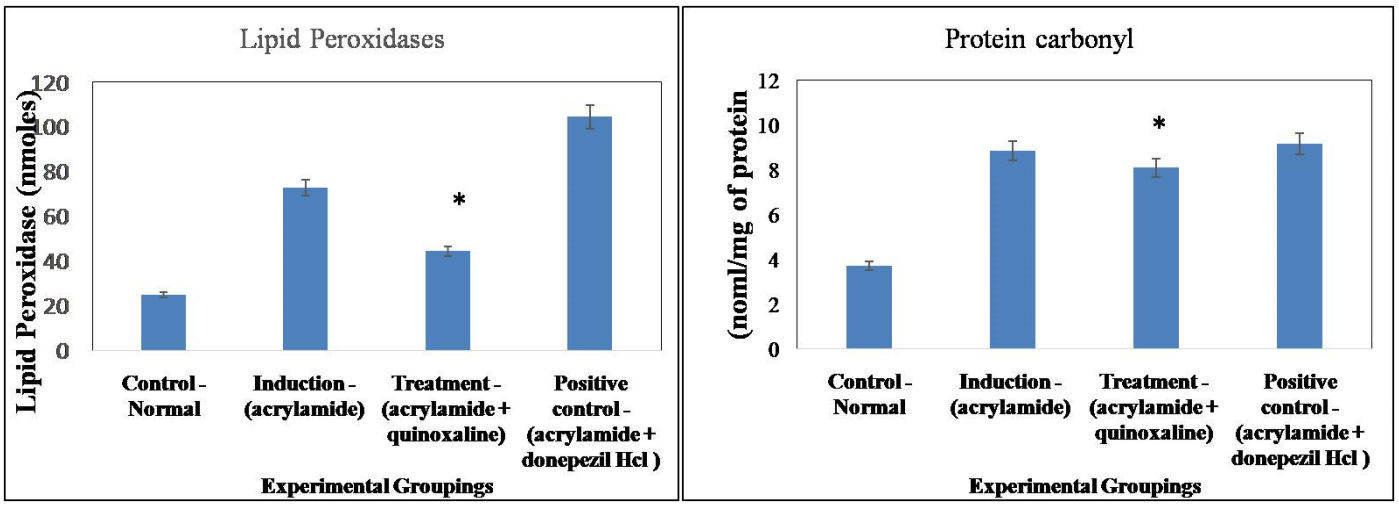


**Figure 7:** Graphical representations on the effect of acrylamide toxicity and quinoxalin treatment on LPO level and Protein carbonyl level. Values are expressed as the mean±SD (*n*=3). (*$p$<0.05, **$p$<0.01) are considered statistically significant from untreated group.

unfolding, mRNA translation regulation, and the degradation of misfolded proteins, all of which contribute to maintaining protein quality control (Mahmood *et al.*, 2014). HSPs serve as the first line of defense for cells subjected to stressors like temperature extremes, heavy metal exposure, free radicals, toxins, and viral infections. They preserve cellular integrity and ensure the proper functioning of critical cell signaling pathways necessary for regular cell operation and survival. HSP70, a major inducible protein in the HSP family, acts as a molecular chaperone, performing vital housekeeping functions within the cell (Guo *et al.*, 2017; Aiqasmi 2023; Raghunathan *et al.*, 2024). In zebrafish, exposure to toxins can lead to an upregulation of *MPO* gene expression, typically in response to oxidative stress and inflammation. *MPO*, a neutrophil-derived enzyme, plays a significant role in the generation of reactive oxygen species and is released during inflammatory and oxidative responses. Studies have shown that *MPO* levels rise in zebrafish exposed to various toxins, including fluoride, chlorpyrifos, and cigarette smoke extract (Aiqasmi 2023; Raghunathan *et al.*, 2024; Haridevamuthu *et al.*, 2025).

In this study, we investigated the HSP-70 levels in the neuronal tissues of zebrafish subjected to acrylamide induction and quinoxaline treatment since HSP-70, a heat-shock protein, is a biomarker for brain health. We have observed that acrylamide induction have led to a significant increase in HSP-70 and MPO expression, indicating the induction of oxidative stress. However, treatment with quinoxaline resulted in a reduction of HSP-70 and MPO expression levels, suggesting a protective effect against acrylamide-induced brain stress. This implies that quinoxaline could decrease brain damage and inflammation caused by acrylamide toxicity in zebrafish by retaining the cellular integrity and ensuring functioning of critical cell signaling pathways necessary for regular cell operation. These findings support the potential of quinoxaline as an effective therapeutic agent against neurotoxicity, demonstrating its ability to reduce oxidative stress and inflammation in the brain.

Microscopic analysis of brain tissues from the experimental groups revealed varying of neuronal damage induced by acrylamide exposure. In the acrylamide-induced group, significant neurodegenerative changes were observed, including granular layer depletion, prominent loss of Purkinje cells, and overall structural disorganization of brain tissue. The neurons exhibited visible intracellular vacuolation, likely resulting from acrylamide-induced neurotoxicity. The observed Purkinje cell loss correlates with the impaired motor coordination and abnormal swimming patterns seen in the behavioral tests. In contrast, the group treated with quinoxaline showed significant improvement while some neuronal damage was observed it was considerably not severe compared to the induction group. The cellular structure appeared more preserved, with reduced intracellular spacing and a lower degree of Purkinje cell loss. These histopathological observations align with the behavioral findings, indicating that quinoxaline treatment helped restore normal locomotor activity. Overall, the results support the neuroprotective role of quinoxaline in mitigating acrylamide-induced brain damage in zebrafish.

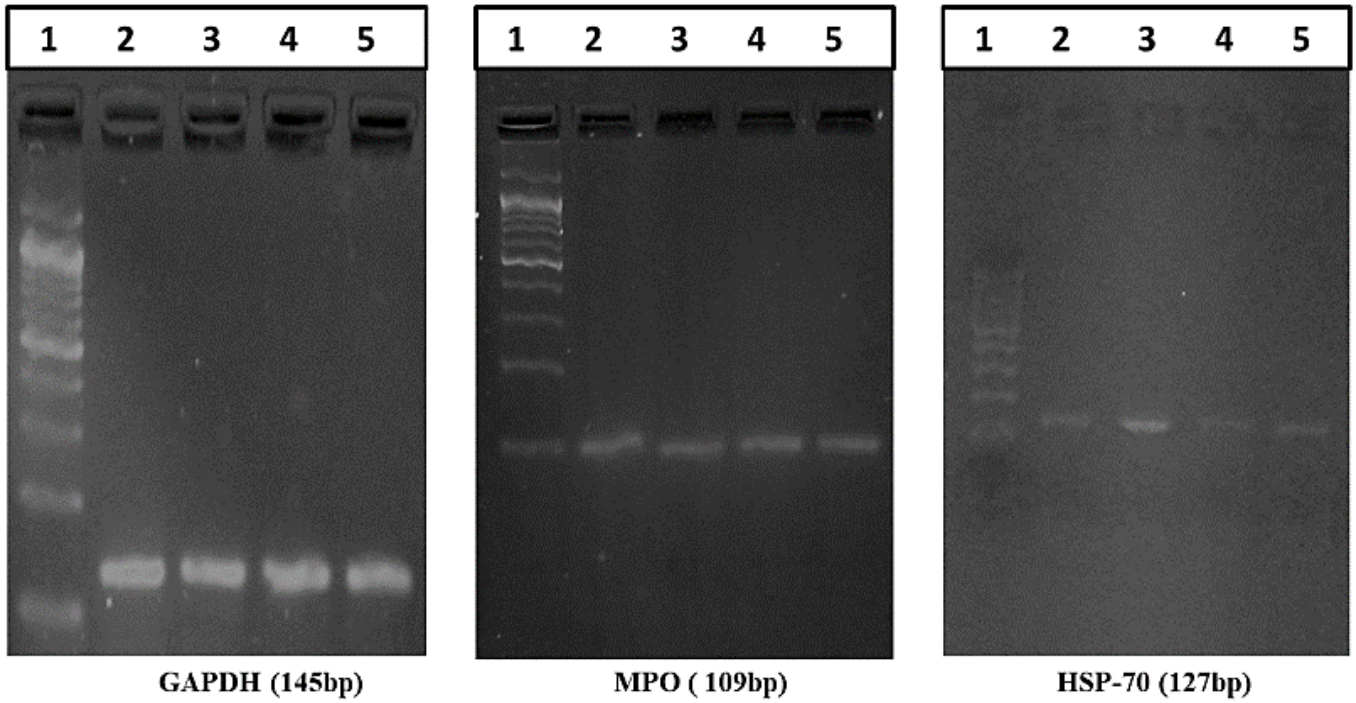


**Figure 8:** RT-PCR analysis of mRNA expression GAPDH, MPO and HSP-70, are presented as compared with Induction group. Lane 1:DNA 100 -1500bp, Lane 2: Control- Normal,Lane 3: Induction- Acrylamide, Lane 4: Treatment -Quinoxaline and Lane 5: Positive control- Donepezil HCl.

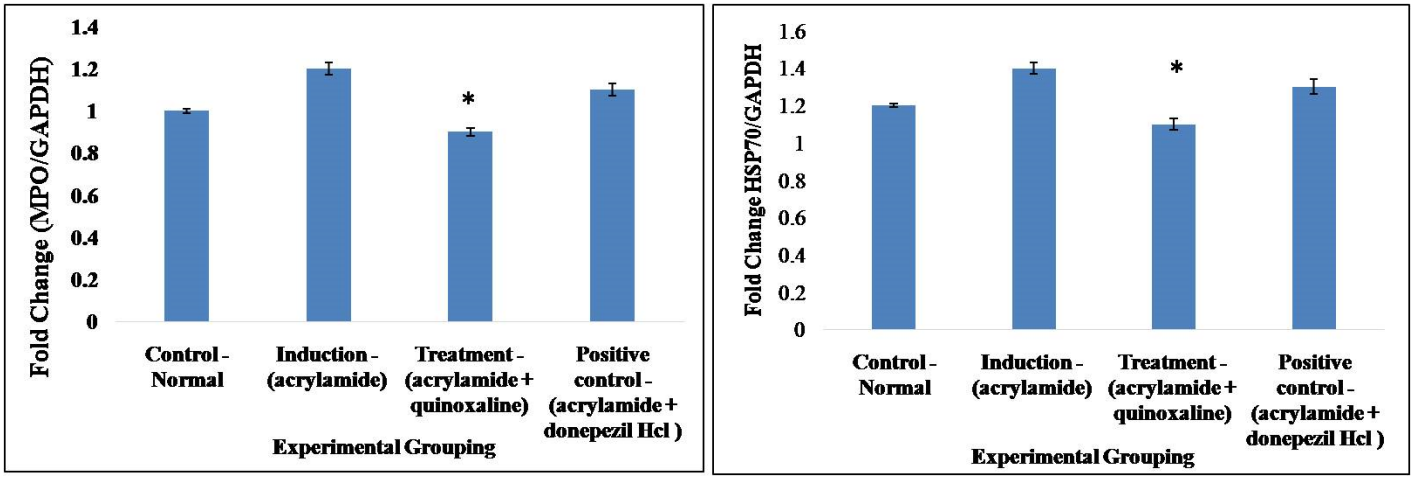


**Figure 9:** Densitometry values of mRNA expression: The densitometry values of mRNA expression of HSP-70 and MPO normalized with GAPDH are presented as compared with induction - acrylamide group. Values are expressed as the mean±SD ($n$=3). (*$p$<0.05, **$p$<0.01) are considered statistically significant from untreated group.

## CONCLUSION

This study reveals that acrylamide exposure induces significant neurotoxicity in zebrafish, affecting both behavior and brain physiology. Zebrafish exposed to acrylamide exhibited hyperactivity, disoriented swimming, and memory impairments, which corresponded with structural damage in brain tissues, including Purkinje cell loss and intracellular vacuolation. Biochemical analysis revealed elevated levels of oxidative stress markers such as LDH, MPO, NO, and GSH, along with decreased antioxidant enzymes like SOD and CAT. Additionally, increased expression of heat shock protein HSP-70 and MPO genes further confirmed the stress and inflammation induced by acrylamide.

Treatment with quinoxaline showed a significant protective effect. Zebrafish in the treatment group exhibited restored behavioral patterns, reduced oxidative stress, and improved antioxidant enzyme activity. Histopathological examination supported these observations, showing reduced neuronal damage and better-preserved brain architecture compared to the acrylamide-induced group. Moreover, the down regulation of HSP-70 and MPO gene expression in the quinoxaline treated group showed its anti-inflammatory potential. In conclusion, quinoxaline effectively reduces acrylamide-induced neurotoxicity by enhancing antioxidant defenses, reducing inflammation, and preserving neuronal integrity. These findings support the potential therapeutic role of quinoxaline in preventing acrylamide induced neurodegenerative damage in zebra fish.

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## ABBREVIATIONS

**ACR:** Acrylamide; **ASD:** Autism Spectrum Disorder; **ADHD:** Attention Deficit Hyperactivity; **PD:** Parkinson's Disease; **ALS:** Amyotrophic Lateral Sclerosis; **AD:** Alzheimer's Disease; **CNS:** Central Nervous System; **BBB:** Blood-Brain Barrier; **MPO:** Myeloperoxidase; **GAPDH:** Glyceraldehyde-3-phosphate dehydrogenase; **LPO:** Lipid peroxidation; **PC:** Protein carbonyl; **ROS:** Reactive oxygen species; **RNS:** Reactive nitrogen species; **SOD:** Superoxide dismutase; **GSH:** Glutathione peroxidase; **CAT:** Catalase; **LDH:** Lactate dehydrogenase; **NO:** Nitric oxide; **DNPH:** 2,4-dinitrophenylhydrazine; **HCl:** Hydrochloric acid; **HSP-70:** Heat shock protein-70; **RT-PCR:** Reverse Transcriptase Polymerase Chain Reaction; **qRT-PCR:** Quantitative reverse transcriptase polymerase chain reaction; **TCA:** Trichloroacetic acid; **dNTPs:** Deoxynucleotide Triphosphates.

## FUNDING

No funding has been obtained for this project.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

## AUTHOR CONTRIBUTION

CJMD and VPV: Conceptualization, Project administration, Data curation, Formal analysis, Investigation, Methodology, Writing - original draft, Writing - review and editing. MP: Data curation, Formal analysis, Investigation, Methodology. SU: Data curation, Formal analysis, Investigation, Methodology. RN: Data curation and Formal analysis.

## ETHICAL APPROVAL

All animal experiments were performed according to the rules and regulations of the Institutional Animal Ethics Committee of the Government of India, (Project Proposal No: SU/CLATR/IAEC/XXIV/58/2024).

## SUMMARY

In this study, the protective role of quinoxaline, a heterocyclic compound with strong antioxidant activity, was assessed in an adult zebrafish model. Exposure to acrylamide led to cognitive and locomotor impairments, suppressed antioxidant enzymes, and elevated oxidative and inflammatory markers. Treatment with quinoxaline reinstated superoxide dismutase and catalase activity, lowered pro-inflammatory mediators improved behavioral outcomes and also preserved neuronal structures, while qRT-PCR revealed reduced expression of stress-related genes. Overall, quinoxaline demonstrated promising neuroprotective potential against acrylamide-induced toxicity.

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