Ind. J. Pharm. Edu. Res., 2026; 60(2s):s839-s852.
https://www.ijper.org

Original Article

Neuroprotective Effects of Eupatorin against Valproic Acid-Induced Autism-Like Phenotypes in Zebrafish Embryos

Abdullah Farasani[1,2,*]

[1]Department of Medical Laboratory Technology, Faculty of Nursing and Health Sciences, Jazan University, Jazan, SAUDI ARABIA.
[2]Health Research Centre, Jazan University, Jazan, SAUDI ARABIA.

ABSTRACT

Purpose: Autism Spectrum Disorder (ASD) is a neurodevelopmental condition often linked to oxidative stress and dysregulated apoptosis. Valproic Acid (VPA), a known teratogen, induces ASD-like behavioral and morphological abnormalities in zebrafish larvae. This study evaluated the neuroprotective potential of eupatorin, a naturally occurring methoxylated flavone, against VPA-induced developmental and neurobehavioral toxicity in zebrafish embryos. **Materials and Methods:** In this study, zebrafish embryos at 4 hpf were exposed to VPA (5 and 10 µg/mL) with or without eupatorin (5 nM) co-treatment until 96 hpf. Survival, heart rate, and morphology were examined microscopically; locomotor and social behaviors were assessed using automated tracking; oxidative stress biomarkers (ROS, H_2O_2, SOD) were quantified fluorometrically; and apoptotic cells were detected using acridine orange staining. **Results:** Embryos were exposed to VPA (5 and 10 µg/mL) with or without eupatorin (5 nM) co-treatment from 4 to 96 Hours Post-Fertilization (hpf). Eupatorin co-treatment significantly improved the survival rate, regularized the heart rate towards the control level, and reduced morphological defects such as pericardial edema and tail malformations. Behavioral assays revealed that eupatorin mitigated VPA-induced hyperactivity, social deficits, and stereotyped swimming behavior. Additionally, eupatorin restored redox homeostasis by lowering ROS and H_2O_2 levels while enhancing Superoxide Dismutase (SOD) activity. Acridine Orange (AO) staining confirmed reduced apoptotic cell death, particularly in the brain region. **Conclusion:** Collectively, these findings suggest that eupatorin alleviates oxidative stress-mediated neurodevelopmental impairments and may serve as a promising candidate for ASD intervention.

Keywords: Apoptosis, Behavioral phenotype, Developmental neurotoxicity, Neuroprotection, Oxidative stress, ROS.

Correspondence:
Prof. Abdullah Farasani
Department of Medical Laboratory Technology, Faculty of Nursing and Health Sciences, Jazan University, and Health Research Centre, Jazan University, Jazan-45142, SAUDI ARABIA.
Email: aofarasani@jazanu.edu.sa
ORCID: 0000000169425136

Received: 04-11-2025;
Revised: 19-12-2025;
Accepted: 24-02-2026.

INTRODUCTION

Autism Spectrum Disorder (ASD) represents a clinically heterogeneous condition of neuro-developmental disorders with persistent impairments in social communication, limited interests, and repetitive behaviors, the onset usually occurring early in childhood. The occurrence of ASD has constantly been increasing worldwide, with current estimates representing that one in 100 children is affected worldwide, stressing the need to elucidate its underlying mechanisms and efficient therapies.[1] Although the etiology of ASD remains multifactorial and heterogeneous, substantial evidence indicates that oxidative stress and increased neuronal apoptosis during critical periods of brain development contribute to the pathogenesis of the disorder.[2,3] This mechanistic link is particularly evident in environmental risk factors, with prenatal exposure to Valproic Acid (VPA), an antiepileptic medication, serving as a well-established model of ASD-like behavioral and neurochemical alterations in animal models. During embryogenesis, VPA exposure interferes with the histone deacetylase activity, which leads to abnormal oxidative stress, gene expression, mitochondrial dysfunction, and enhanced apoptosis, all of which contribute to ASD pathology.[4-7]

The zebrafish (*Danio rerio*) system has become a highly effective *in vivo* model for the investigation of neurodevelopmental disorders, such as ASD. The embryos of zebrafish exhibit broad genetic and physiological similarity to humans, and their transparent bodies and fast development render them especially suitable for real-time imaging, behavioral tests, and drug treatment.[8,9] Zebrafish larvae treated with VPA showed a range of phenotypes related to autism, including compromised social interaction, modified locomotion, enhanced anxiety-like behavior, as well as

DOI: 10.5530/ijper.20264630

brain morphology and neurotransmitter system disruptions.[10,11] As a result, this model provides an appropriate platform to determine the neuroprotective potential of natural compounds.

Eupatorin is a methoxylated flavone, naturally present in plants such as *Orthosiphon stamineus* and *Eupatorium perfoliatum*. Studies have shown that *Orthosiphon stamineus* has been found to reverse age-related deficits in short-term memory, as well as prevent and reduce the rate of neurodegeneration, while also possessing the capacity to mitigate oxidative stress in neuronal cells.[12] Eupatorin receives more attention today because it exhibits various pharmacological activities, including anti-inflammatory, antioxidant, anticancer, and neuroprotective effects.[13,14] Published findings show that eupatorin has a unique dual action that depends on the context. It acts as a pro-oxidant in cancer cells but stays an antioxidant or is not very toxic in normal cells. This selectivity is associated with fluctuations in cellular redox balance and metabolic activity. Eupatorin raises the levels of ROS inside cancer cells by a lot. This causes the mitochondrial membrane to depolarize, cytochrome c to be released, and the caspase-dependent apoptotic pathway to be activated. Significantly, antioxidant pre-treatment greatly reduces these effects, confirming ROS-mediated apoptosis as the main way it fights cancer.[15,16] On the other hand, eupatorin has much higher IC_{50} values and very little toxicity in normal cells. It has been shown to have antioxidant and anti-inflammatory effects by enhancing the body's own antioxidant systems and stopping lipid peroxidation and inflammatory mediators.[17,18] These findings confirm eupatorin as a natural antioxidant, illustrating its ability to scavenge Reactive Oxygen Species (ROS), modulate mitochondrial function, and impede apoptotic pathways in various cellular models, thereby maintaining redox homeostasis and protecting normal cells from oxidative damage. Nonetheless, studies investigating eupatorin's therapeutic efficacy in neurodevelopmental disorders remain limited. In particular, its capacity to alleviate oxidative stress and mitochondrial dysfunction in models of Valproic Acid (VPA)-induced autistic-like behavior has not been comprehensively examined, highlighting a prospective avenue for future research into its neuroprotective and antioxidant effects. The goal of this study was to find out how eupatorin affected VPA-induced ASD-like symptoms in developing zebrafish larvae, with a focus on oxidative stress and apoptotic markers. The hypothesis asserts that eupatorin, due to its antioxidant and anti-apoptotic properties, alleviates VPA-induced neurobehavioral deficits and cellular stress, thereby suggesting potential therapeutic efficacy for the treatment of ASD. This study examined the effect of eupatorin on VPA-induced ASD-like phenotypes in developing zebrafish larvae, focusing on oxidative stress and apoptotic markers.

Materials and methods

Reagents and chemicals Acridine Orange (AO), tricaine Methane Sulfonate (MS-222), eupatorin (purity ≥ 98%), and valproic acid (purity ≥ 98%) were acquired from Sigma Aldrich (St. Louis, MO, USA). The solutions of VPA and eupatorin were dissolved in DMSO. Enzyme-Linked Immunosorbent Assay (ELISA) kits (MAK528& MAK311)were purchased from Sigma-Aldrichto measure the levels of Hydrogen Peroxide (H_2O_2) and Superoxide Dismutase (SOD) activity.

Zebrafish maintenance and VPA exposure

The Institutional Ethical Committee has approved the study with approval number REC-46/11/1540. Wild-type adult zebrafish were brought from the international zebrafish main supply center and maintained in the Research Lab of Jazan University. The fish were maintained in a 14-hr/10-hr light/dark photoperiod cycle at 28±0.5ºC, and they were fed brine shrimp twice daily. Following proper fertilization, embryos were harvested at 2 hours post-fertilization (hpf), and mature embryos were selected for further research.

Approximately 50 healthy embryos in each group were exposed to VPA at concentrations of 5 and 10 µg/mL (in 0.01% DMSO) from 4 to 96 hr, where 0.01%DMSO was used as a vehicle control. The doses were selected according to a study that established the toxicity of VPA in zebrafish as a standard.[19] According to Kimmeli *et al*., (1995), zebrafish will be in the blastula stage at 4 hours post-fertilization (hpf), when the embryos are vulnerable to external stimuli.[20] Based on earlier reports that developed viable zebrafish models for autism research,[21,5] embryos were treated with VPA beginning at 4 hpf. For the Eupatorin intervention experiments, 50 embryos per group were co-exposed to VPA (5 or 10 µg/mL) with or without Eupatorin (5 nM) from 2 to 96 hpf. The Eupatorin concentration was chosen according to a previous study.[22] All experiments were independently replicated three times.

Assessment of developmental toxicity

Daily manual records of the hatching and survival rates were made. Under a stereomicroscope at 24 hpf, spontaneous movement was manually recorded by counting the number of unhatched embryos' tail flicks in 20 sec using a timer and amanual counter. Measurements of body length and heart rate were taken at 1-min intervals at 48 hpf, when the cardiovascular system is fully operational.[23] A 96-hpf value was used to calculate the malformation rate. Following MS-222 anesthesia, zebrafish larvae were examined and captured on camera using a stereomicroscope (Nikon, Japan).

Behavioral assessment

Behavioral tests were conducted in a calm environment that was kept between 27 and 28ºC. At least 2 hr in the morningwere spent acclimating the 120 hpf zebrafish larvae. Three behavioral tests were conducted utilizing a Zebra Lab Tracking System (Viewpoint Life Sciences, Lyon, France): the open-field test, the shoaling test, and the social contact test. To reducevariability among tests,

extraneous noise and vibrations were reducedthroughout the testing periods.

Open-field test

The open-field test happened in a 5 × 5 × 1 cm arena filled with water that was almost split into 16 equal squares. The four squares in the middle were known as the center zone. We put one zebrafish larva at a time in the arena and let them swim around. A camera mounted on top recorded video for 15 min, during which time the total distance traveled and the time spent in the central zone were automatically measured. The open-field paradigm was utilized to evaluate stereotypical and repetitive swimming patterns. For this study, an additional 10-min video recording was obtained, and swimming patterns were assessed at 15-sec intervals using a double-blind scoring system. Repetitive behaviors were operationally defined as follows: walling was characterized by persistent swimming along the perimeter of the arena; big circling was defined as continuous circular swimming with a large turning radius (>50% of the arena diameter); minor circling was defined as repetitive circular movements with a small turning radius (<50% of the arena diameter); and Figure 8 behavior was identified as repeated swimming in intersecting loop patterns resembling the shape of the number eight. It was written down and looked at how many times each behavior happened.

Shoaling test

A group of zebrafish was used in the shoaling test, which measured the distance between them using the Nearest Neighbor Distance (NND) and the Inter-Individual Distance (IID).[24] Ten larvae were used per glass petri dish (10 cm in diameter) for this test. A 10-min video recording followed a 5-min adaptation phase.

Social contact test

In 6-well plates, social contact tests were conducted. The movements of ten zebrafish embryos per group were monitored for 10 min, with the average contact times (the distance between two larvae ≤ 1 body length) and time in contact beingrecorded every minute. Each group was placed in a 6-well plate with two larvae per well.

Assessment of ROS and oxidative stress levels

At 96 Hours Post-Fertilization (hpf), each experimental group of 100 larvae was homogenized on ice and then spun at 12,000 × g for 10 min at 4°C. We immediately used the supernatants for biochemical tests. We utilized a commercially available colorimetric assay kit (MAK528, Sigma-Aldrich) to quantify Superoxide Dismutase (SOD) activity. To do this, we used a microplate reader to measure how much superoxide stopped a tetrazolium salt from breaking down, following the manufacturer's instructions and what was said before.[25] We used a peroxide detection assay kit (MAK311, Sigma-Aldrich) to find out how much Hydrogen Peroxide (H_2O_2) was in the sample. This kit measures the peroxidase-mediated oxidation of a chromogenic or fluorogenic substrate that is directly related to the amount of H_2O_2 in the sample. To mitigate erroneous fluctuations in peroxide levels, homogenization and centrifugation were performed under strictly controlled cold conditions, and the data were interpreted as relative differences between experimental groups rather than absolute *in vivo* concentrations.

We also used 2′,7′-Dichlorodihydrofluorescein Diacetate (DCFH-DA) to measure Reactive Oxygen Species (ROS) inside zebrafish embryos. Embryos in the proper developmental stage were washed thrice with embryo media and subsequently placed in darkness at 28.5°C for 30 min with 10 µM DCFH-DA. After being incubated, the embryos were thoroughly washed to remove any extra dye. Then, they were immediately imaged with fluorescence. We took the pictures with a fluorescence microscope that had a FITC filter set (excitation 488 nM; emission 525 nM). All of the pictures were taken with the same microscope settings, like the objective magnification, exposure time, gain, and light intensity. To make sure the embryos stayed in the same place while being imaged, they were put on the side in low-melting agarose. We used ImageJ software to figure out how bright the fluorescence was. A standardized Region of Interest (ROI) encompassing the trunk and yolk extension was selected for each embryo, and the mean fluorescence intensity was assessed following background subtraction. We averaged the fluorescence values for each group (n>10 embryos per group) and measured them in Arbitrary Units (a.u.). We used the right comparison tests to find statistical significance, and p<0.05 was the cutoff for significance.

Assessment of apoptosis

To visualize the amount of apoptosis, 10 larvae were randomly selected from each group and stained with AO at 96 hpf, as previously described.[19] In brief, the live larvae were cleaned and then incubated for 20 min in an AO solution (5 µg/mL). They were subsequently rinsed three times with clean water. Following anesthesia, pictures of the stainedlarvae were taken using a fluorescence microscope (Nikon, Tokyo, Japan).

Statistical analysis

All statistical analyses were performed using SPSS 25.0 software (Chicago, IL, USA). Data are presented as mean±Standard Deviation (SD). Before analysis, the datasets were tested for normality using the Shapiro-Wilk test and for homogeneity of variance using Levene's test.

One-way Analysis of Variance (ANOVA) was performed to compare multiple experimental groups, followed by Tukey's multiple comparison post hoc test to control the family-wise error rate during pairwise comparisons. Tukey's test was chosen to maintain type I error control while comparing all possible group pairs with equal or near-equal sample sizes. Statistical

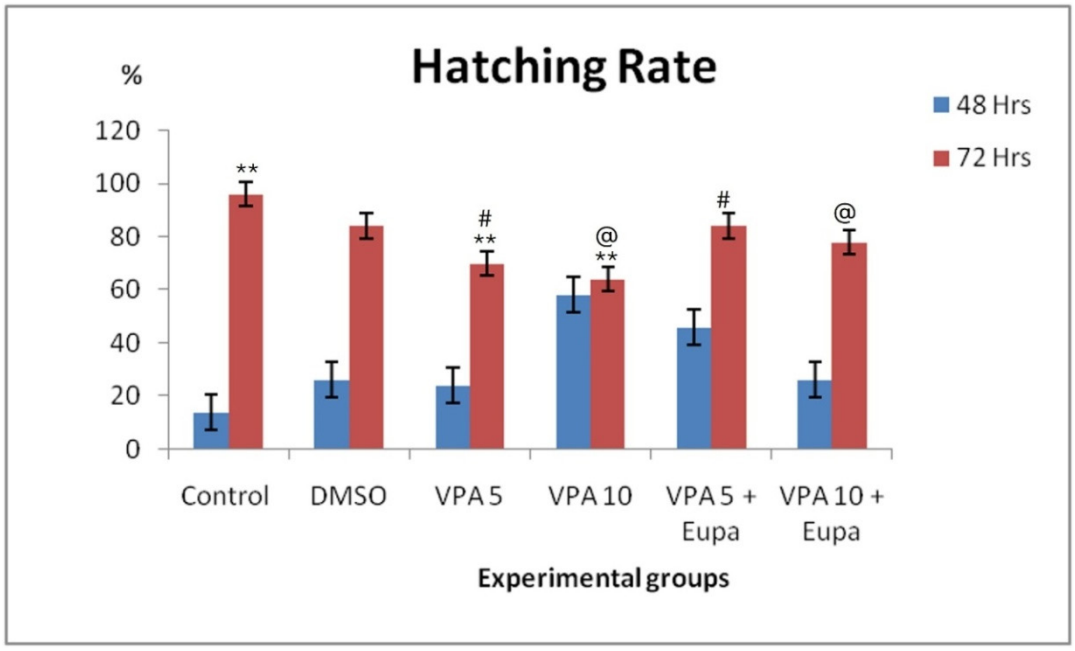

Figure 1: Hatching rate of zebrafish embryos treated with VPA and Eupatorin. (n=50 embryos per group). ** indicates p<0.05 compared to control. # indicates p<0.05 compared to VPA 5 µg/mL and @ indicates p<0.05 compared to VPA 10 µg/mL. Statistical comparisons were performed only between treatment and control groups within the same developmental stage (48 or 72 hpf), and actual p-values are now indicated in the revised figure.

comparisons were performed across all the experimental groups [Control, Vehicle control (DMSO), VPA (5 µg/mL), VPA (10 µg/mL), VPA (5 µg/mL)+Eupatorin (5 nM), and VPA (10 µg/mL)+Eupatorin (5 nM)] if not specified.

Behavioral experiments were performed with n=10 larvae per group. Three independent biological replicates from different breeding batches were used for developmental, biochemical, and molecular studies. Measurements were made in technical triplicates, as appropriate, within each biological replicate. The statistical comparisons were made using the mean of technical replicates. The threshold for statistical significance was fixed at p<0.05.

RESULTS

Hatching Rate and Early Development

The hatching dynamics of the zebrafish embryos differed in the experimental groups (Control, DMSO, VPA 5 µg/mL, VPA 10 µg/mL, VPA 5 µg/mL+Eupatorin, and VPA 10 µg/mL+Eupatorin) (Figure 1). The control group had a hatching rate that was normal for zebrafish at this stage of development, which was 48 Hours After Fertilization (hpf). Embryos that were exposed to 10 µg/mL VPA had a much higher hatching percentage than age-matched controls (p<0.05). This means that low-dose VPA exposure speeds up the start of hatching. There were no comparisons made between 48 and 72 hpf over time, so the only way to see how treatment worked was to compare it to the control groups at each time point. All groups achieved their optimal hatching percentage by 72 hr hpf, with the control group exhibiting the highest overall hatching rate. In a dose-related manner, VPA administration significantly reduced hatching rates; a 10 µg/mL dose resulted in a larger decrease than a 5 µg/mL dose. In both VPA-exposure groups, co-treatment with eupatorin significantly enhanced hatching, with a more pronounced recovery observed at the lower VPA dose.

Morphological and Developmental Outcomes

We looked at zebrafish embryos 24, 48, and 72 hr after fertilization with or without eupatorin and compared them to control and DMSO groups (Figures 2a-c). We used a set of predetermined morphological criteria to check for developmental toxicity. These included problems with developmental delay/epiboly, axial curvature, pericardial edema, craniofacial abnormalities, microphthalmia, and defects in the yolk or swim bladder. We looked at phenotypes and counted how many were present or absent in each treatment group. The embryos that were treated with DMSO and the control embryos grew normally for their stage at all times. This included complete epiboly, ordered somite formation, normal fin development, and no visible problems with the yolk morphology, body axis, or pigmentation.

At 24 hpf, exposure to VPA caused developmental problems that got worse with higher doses (Figure 2a). VPA (5 µg/mL) caused a moderate delay in epiboly and mild swelling around the heart in embryos. VPA (10 µg/mL) caused a complete stop in epiboly, severe swelling, and early craniofacial problems. Quantitative analysis revealed significantly increased anomaly ratings in both VPA groups compared to controls (p<0.05). Both the control and DMSO embryos showed normal axial elongation and initial coloring 48 hours after fertilization (Figure 2b). Embryos that

were given VPA (5 µg/mL) had craniofacial malformations, a slight bend in the spine, and swelling that didn't go away. On the other hand, VPA (10 µg/mL) embryos had very curved spines, small eyes, tails that didn't grow long enough, swim bladders that didn't inflate enough, and many other organ problems. These problems got much worse as the dose increased (p<0.01). At 72 hpf, the control and DMSO larvae had fully extended body axes, developed fins, ordered coloration, inflated swim bladders, and were swimming actively (Figure 2c). Embryos that were given VPA (5 µg/mL) still had craniofacial problems and swelling, but larvae that were given VPA (10 µg/mL) had severe axial curvature, chronic swelling, microphthalmia, poor pigmentation, and problems with multiple organs that lasted for a long time (p<0.001).

Giving eupatorin at the same time as VPA greatly lowered its ability to cause birth defects. At 72 hpf, embryos that were given VPA (5 µg/mL) and eupatorin had shapes that were almost normal. Their abnormality scores were much lower than those of embryos that were only given VPA (p<0.05). There were still problems in the VPA (10 µg/mL)+eupatorin group, but they were less common and less serious. The alignment of the body axis, pigmentation, and organ development was all partially restored.

Embryo Survival

We used survival analysis over the whole 96 hpf exposure period (Figure 3) to see how well eupatorin protected against developmental damage caused by VPA. Control embryos consistently demonstrated elevated survival rates (95-100%) during the observation period, whereas DMSO-treated embryos exhibited a slight, non-progressive decrease in survival, thereby confirming the safety of the vehicle. On the other hand, being exposed to VPA made survival go down a lot, and this depended on the dose and the time. The embryos given 10 µg/mL of VPA had the highest death rate. Deaths started early and rose quickly, going from almost 70% at 24 hpf to 40-50% by 96 hpf. In the VPA 5 µg/mL group, the survival rate dropped from about 80% at 24 hpf to about 60-65% at 96 hpf. This drop was not as bad, but it was still important.

Co-treatment with eupatorin significantly improved embryo survival throughout the entire developmental period. The embryos that were given VPA 5 µg/mL+eupatorin had the same survival rate as those in the DMSO and control groups, which means they were almost completely protected. Embryos that were given VPA 10 µg/mL+eupatorin showed some improvement compared to those that were given VPA 10 µg/mL alone. However, the survival rates were still lower than those in the low-dose VPA

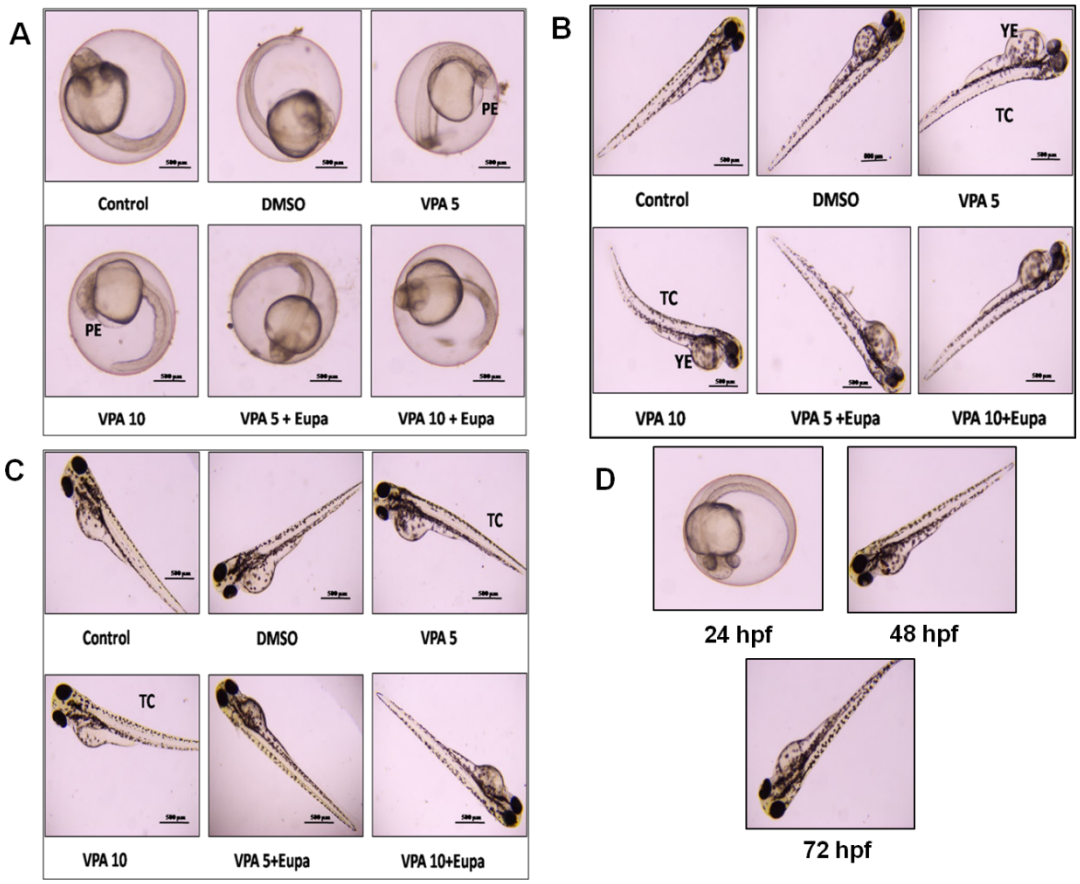

Figure 2a: VPA-induced developmental toxicity and ameliorating effect of Eupatorin at 24 hpf (A), 48 hpf (B), and 72 hpf (C) of zebrafish embryos, (D) Treated with Eupatorin alone. (n=50 embryos per group) (YE - Yolk Sac Oedema, PE - Peri Cardial Oedema, TC - Tail Curvature).

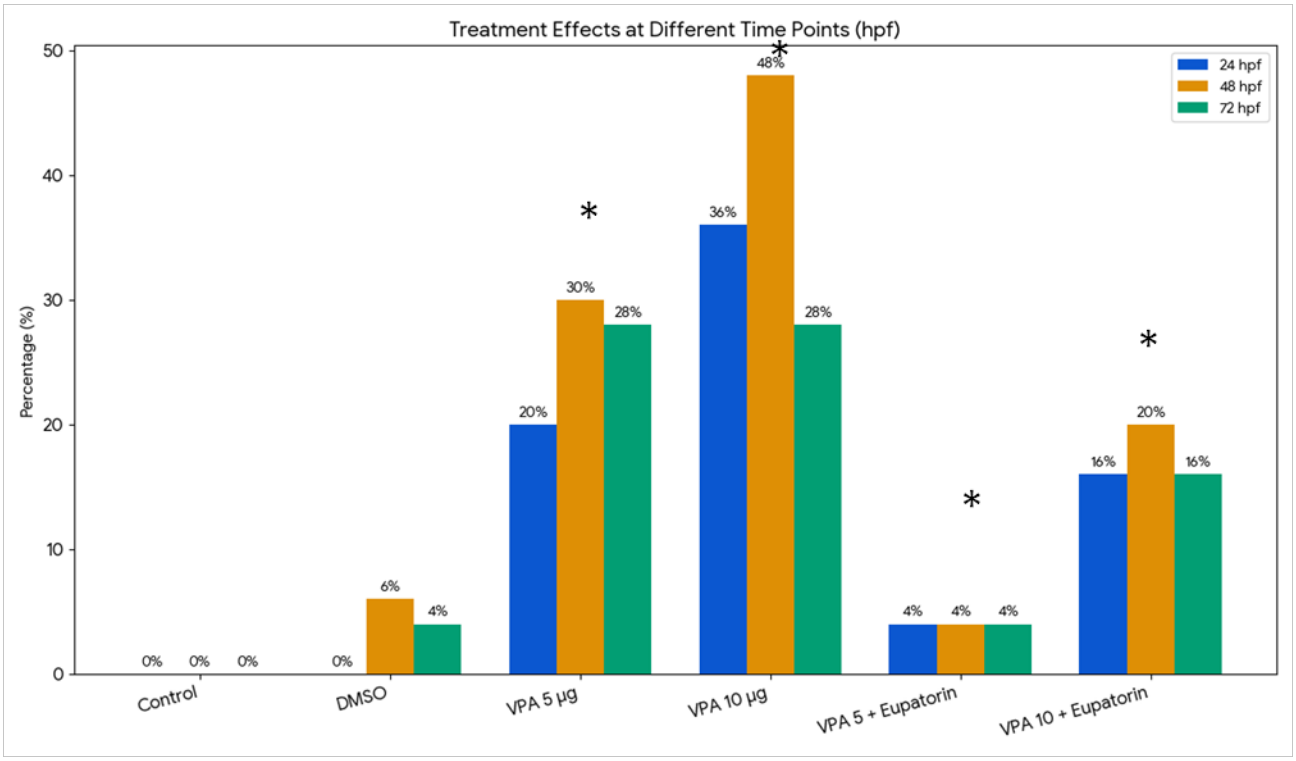

Figure 2b: VPA Induced developmental toxicity and ameliorating effect of Eupatorin at 24 hpf (A), 48 hpf (B) and 72 hpf (C) of zebrafish embryos. (*n*=50 embryos per group) * indicates $p<0.05$ compared to control.

rescue group, suggesting that eupatorin-mediated protection has a dose-dependent ceiling effect. Kaplan-Meier survival analysis demonstrated a significant divergence in survival curves between VPA-treated groups and their corresponding eupatorin co-treated counterparts, thereby validating that eupatorin effectively mitigates VPA-induced lethality throughout embryonic development rather than at a specific temporal juncture. These results show that VPA is very toxic to the development of zebrafish embryos and that the toxicity gets worse with higher doses. However, eupatorin protects the embryos very well and for a long time, especially when VPA levels are low and normal embryogenesis and survival are restored.

Effect of VPA and Eupatorin on Heart Rate and Tail Flicks

Based on physiological analyses, co-treatment with eupatorin demonstrated a dose-dependent protective role against zebrafish embryos, whereas Valproic Acid (VPA) significantly decreased cardiac and locomotor activity (Figures 4 and 5). The vehicle (DMSO: 148±12 beats/min; 3 tail flicks/min) and control (158±9 beats/min; 4 tail flicks/min) group's baseline values were comparable. These features decreased in a concentration-dependent manner under the influence of VPA. VPA brought the heart rate downconsiderably to 112±10 beats/min and the frequency of tail flicks to 1 event/min at 5 µg/mL. On administration of 10 µg/mL VPA, suppression was even greater, with heart rate decreasing to 82±6 beats/min and tail flicks increasing only slightly to 2/min, both of which were significantly lower than controls ($p<0.05$). These deficits were reduced by co-administration with eupatorin. Both locomotor activity (4 tail flicks/min) and heart rate (143 beats/min) were restored to control values within the VPA (5 µg/mL)+eupatorin group. Both parameters were partially recovered at the higher dose with VPA (10 µg/mL)+eupatorin treatment; heart rate (135 beats/min) and tail flicks (3/min) were significantly improved compared to VPA alone ($p<0.05$), butnot restored to baseline levels. Taken together, these findings indicate that eupatorin strongly protects zebrafish embryos from cardiotoxicity and locomotor deficits induced by VPA, with maximal effectiveness at lower VPA exposure levels.

VPA-Induced ROS and Protective Effect of Eupatorin

Fluorescence imaging and quantitative ROS analysis indicated that VPA exposure markedly and dose-dependently elevated oxidative stress in zebrafish embryos (Figure 6). The control and DMSO-treated groups, which showed normal redox conditions, had modest amounts of ROS (2.5±0.3 and 3.1±0.4 a.u., respectively) and very little green fluorescence. In contrast, exposure to VPA led to a statistically significant increase in ROS levels, rising to 3.5±0.5 a.u. at 5 µg/mL and significantly to 10.3±0.8 a.u. at 10 µg/mL ($p<0.05$ vs. Control). Fluorescence imaging showed strong and extensive green fluorescence linked to ROS all along the embryonic body axis. Co-treatment with eupatorin dramatically reduced oxidative damage caused by VPA. In the VPA 5 µg/mL+eupatorin group, ROS levels decreased to 3.5±0.4 a.u., nearing control values, with only faint and localized fluorescence detected. Likewise, the co-treatment with

eupatorin markedly diminished ROS levels in the VPA 10 µg/mL group (5.6±0.6 a.u.), remaining marginally above baseline yet significantly lower than the VPA 10 µg/mL alone (p<0.05). These findings combined indicate that eupatorin significantly alleviates VPA-induced oxidative stress in zebrafish embryos, exhibiting enhanced protective efficacy at reduced VPA exposure levels.

Effect of VPA and Eupatorin on H_2O_2 Levels

VPA exposure dramatically raised H_2O_2 production in zebrafish embryos, which is in line with the ROS results (Figure 7). H_2O_2 levels were low in the control and DMSO groups (1.0 and 1.2 a.u.), but they were increased to 2.0 and ~2.4 a.u. by the VPA 5 µg and VPA 10 µg/mL treatments, respectively, compared to the Control/DMSO group (p<0.05). Although values were still slightly higher than baseline, co-treatment with eupatorin considerably decreased H_2O_2 accumulation: VPA 5 µg/mL+Eupatorin decreased levels to 1.6 a.u. compared to VPA 5 µg/mL (p<0.05), and VPA 10 µg+Eupatorin decreased levels to 1.7 a.u. compared to VPA 10 µg/mL (p<0.05). These results suggest that eupatorin limits the accumulation of H_2O_2, particularly at higher oxidative loads, and thus exhibits an antioxidant effect.

Effect of VPA and Eupatorin on Antioxidant Enzyme (SOD) Activity

In a dose-dependent manner, VPA dramatically reduced the activity of SOD (Figure 8). While VPA 5 µg/mL decreased SOD to 0.6 a.u., and VPA 10 µg/mL further lowered activity to 0.4 a.u. Compared to the Control/DMSO group (p<0.05), the controland DMSO groups showed robust activity (0.9 and 1.0 a.u.). SOD activity was brought back to levels close to baseline by eupatorin. SOD activity was not statistically different from Control/DMSO. It was considerably higher in the VPA 5 µg/mL+Eupatorin (0.8 a.u.) and VPA 10 µg/mL+Eupatorin (0.9 a.u.) groups than in the equivalent VPA-only groups (p<0.05). These findings suggest that eupatorin protects against enzymatic antioxidant depletion induced by VPA.

VPA-Induced Apoptosis and Ameliorating Effect of Eupatorin

We employed acridine orange staining to check for apoptosis in zebrafish embryos (n=50 per group). We took pictures of the larvae in a uniform lateral orientation so that we could compare them directly between groups (Figure 9). Embryos treated with DMSO and control embryos showed very little fluorescence, which means that there was not much basal apoptosis. VPA exposure caused apoptosis in a way that depended on the dose. Embryos subjected to VPA at 5 µg/mL had significant apoptotic fluorescence predominantly in the head and trunk regions, while VPA at 10 µg/mL induced intense and extensive fluorescence over the entire body axis. We used ImageJ to measure apoptosis as the total acridine orange fluorescence intensity per embryo. Quantitative analysis revealed a substantial increase in apoptosis in both VPA-treated groups compared to the control (p<0.05). Co-treatment with eupatorin significantly diminished apoptotic fluorescence at both VPA doses. It is important to note that apoptosis was considerably reduced in the VPA 10 µg/mL+eupatorin group than in the VPA 10 µg/mL group alone

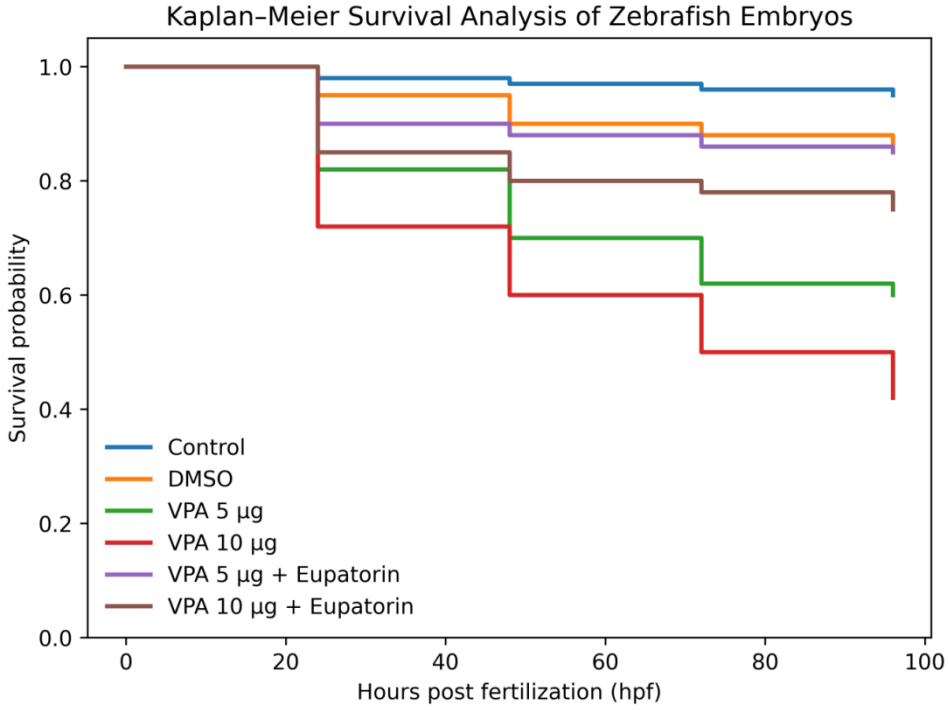

Figure 3: VPA-induced developmental toxicity and ameliorating effect of Eupatorin on the survival rate of zebrafish embryos. (n=50 embryos per group) p<0.05 vs. VPA 5 µg/mL; ## p<0.05 vs. VPA 10 µg/mL.

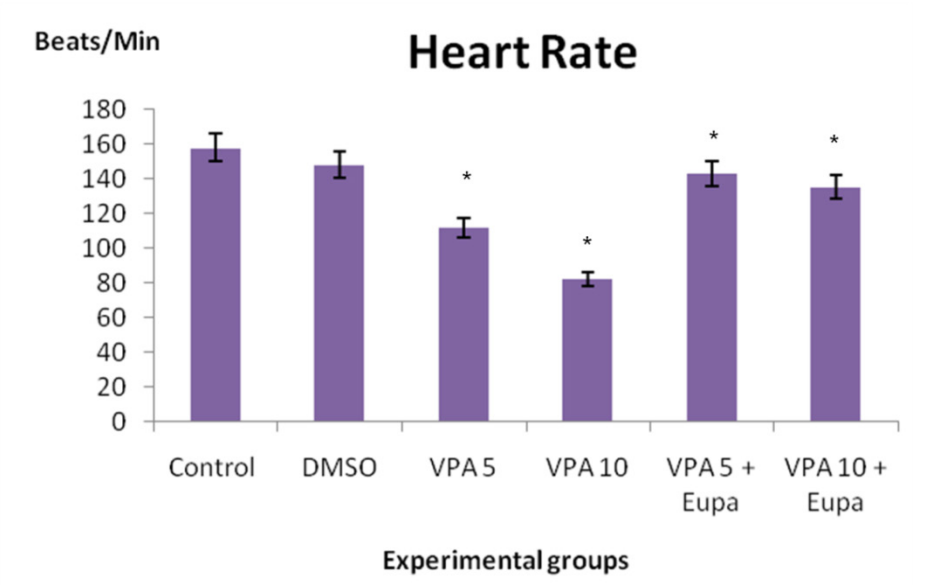

Figure 4: VPA-induced developmental toxicity and ameliorating effect of Eupatorin on the heart rate of zebrafish embryos treated with VPA and Eupatorin. (*n*=50 embryos per group) * indicates $p<0.05$ compared to control.

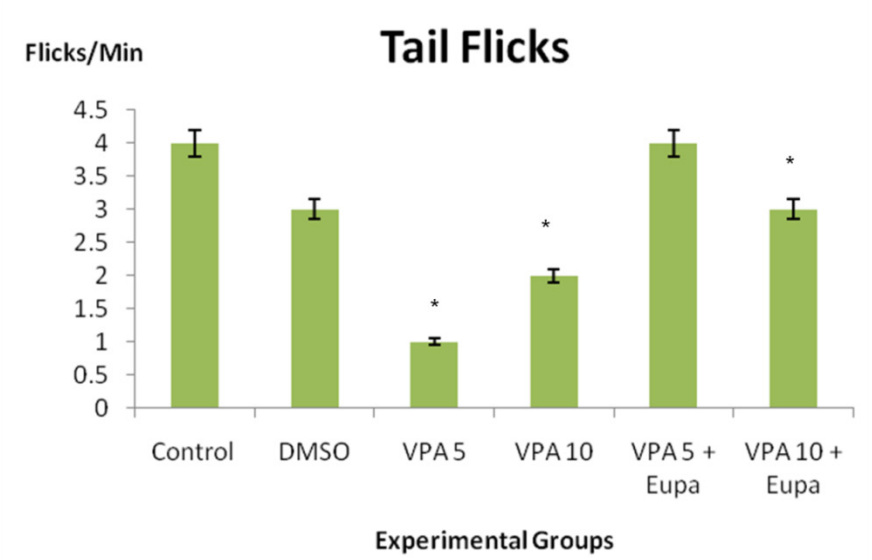

Figure 5: VPA-induced developmental toxicity and ameliorating effect of Eupatorin on tail flicks of zebrafish embryos treated with VPA and Eupatorin. (*n*=50 embryos per group) * indicates $p<0.05$ compared to control.

($p<0.05$). The VPA 5 µg/mL+eupatorin group had apoptosis levels similar to the control at the lower dose. The data are shown as Mean±SD, and a one-way ANOVA followed by Tukey's *post hoc* test was used to do the statistical analysis.

Behavior changes in zebrafish larvae

Behavioral experiments were performed to assess VPA-induced neurobehavioral changes and the possible modulatory effects of eupatorin in zebrafish larvae (Figure 10). The open field test was used to measure locomotor activity and anxiety-like behavior, and shoaling and social interaction tests were used to measure social behavior. In the open field test, larvae exposed to VPA showed a substantial rise in overall locomotor activity and center zone occupancy compared to control and DMSO-treated groups ($p<0.05$), indicating hyperactivity and diminished thigmotaxis. VPA-treated larvae spent more time in the arena's center and moved in different ways than the controls. Co-treatment with eupatorin significantly mitigated VPA-induced hyperactivity, evidenced by a reduction in total distance traveled and center zone time, with the VPA 5 µg/mL+eupatorin group exhibiting values approximating control levels ($p<0.05$ vs. VPA alone).

The control and DMSO groups showed little activity in the central zone and on the ground. The limited movement exhibited by these groups indicates typical exploratory behavior at the

assessed developmental stage and tracking threshold settings, which have been elucidated in the updated Methods section. In the social interaction assay, VPA administration significantly diminished both the frequency and length of social contact relative to controls ($p<0.05$), indicating compromised social conduct. Eupatorin co-treatment significantly enhanced social interaction metrics compared to the comparable VPA-treated groups ($p<0.05$), the results did not completely revert to baseline at the elevated VPA dosage.

Shoaling analysis revealed alterations in inter-larval distance after VPA exposure, with no clear linear trend observed across the tested doses. VPA 5 µg/mL increased shoal dispersion, although VPA 10 µg/mL only partially decreased it. These conflicting findings are now recognized as indicative of diversity in behavioral sensitivity at varying exposure levels, underscoring the necessity for careful interpretation. Furthermore, larvae subjected to VPA (5 and 10 µg/mL) exhibited a markedly increased incidence of stereotyped behaviors, such as walling, big circling, and micro-circling, in comparison to controls ($p<0.05$). Eupatorin co-treatment considerably diminished the occurrence of these stereotyped behaviors, especially at the lower VPA dosage. The current data indicate that eupatorin somewhat improves VPA-induced autism-like behaviors toward control levels; nevertheless, we recognize that the limited sample size ($n=10$ per group) constrains statistical power. More extensive cohort studies are necessary to further substantiate these behavioral consequences.

DISCUSSION

The heterogeneous neurodevelopmental disorder ASD is characterized by restricted and repetitive behaviors and impaired social interaction and communication.[26] The disorder's multifactorial aetiology includes environmental, genetic, and epigenetic factors that modify neurodevelopmental pathways. Oxidative stress, mitochondrial impairment, dysregulated apoptosis, and epigenetic dysregulation contribute significantly to the pathophysiology of ASD.[27,5]

One of the leading pathogenic mechanisms involved in the etiology of ASD and manifestation of its behaviour is oxidative stress. It is due to a mismatch between the defense antioxidant system and production of Reactive Oxygen Species (ROS), with the consequence of oxidation of proteins, lipids, and DNA that damages neurons.[27,5] Children with autism are also reliably shown to exhibit significantly reduced activities of key antioxidant enzymes, such as SOD and GPX, with heightened concentrations of markers of oxidative damage, including Malondialdehyde (MDA) and Nitric Oxide (NO), according to recent multi-cohort clinical research. These biomarkers are closely related to higher behavioral impairment of ASD.[27,28] As per Camussi *et al.*, (2024), damage impacts synaptic plasticity and connections within the brain, which are vital for sensory processing, social behavior, and communication, all of which are central domains affected in ASD.[26] Oxidative stress also initiates apoptosis via caspase-dependent mitochondrial mechanisms, resulting in abnormal neuronal population dynamics during critical developmental windows.[5] Additionally, it impinges on epigenetic regulation by modifying histone modifications and DNA methylation, thereby altering gene expression patterns involved in behaviour and neurodevelopment.[29,30] Oxidative stress-mediated neuroinflammation exacerbates neuronal injury and worsens behavioural abnormalities linked with ASD. Together, the mechanisms set up a sophisticated relationship between oxidative stress and the abnormalities in brain circuits that cause symptoms of ASD, such as repetitive behaviours and social impairments.[26]

In ASD pathophysiology, oxidative stress is tightly connected with mitochondrial dysfunction and forms a vicious circle. Both the primary source of energy and a major source of ROS are mitochondria. ATP production is reduced and ROS

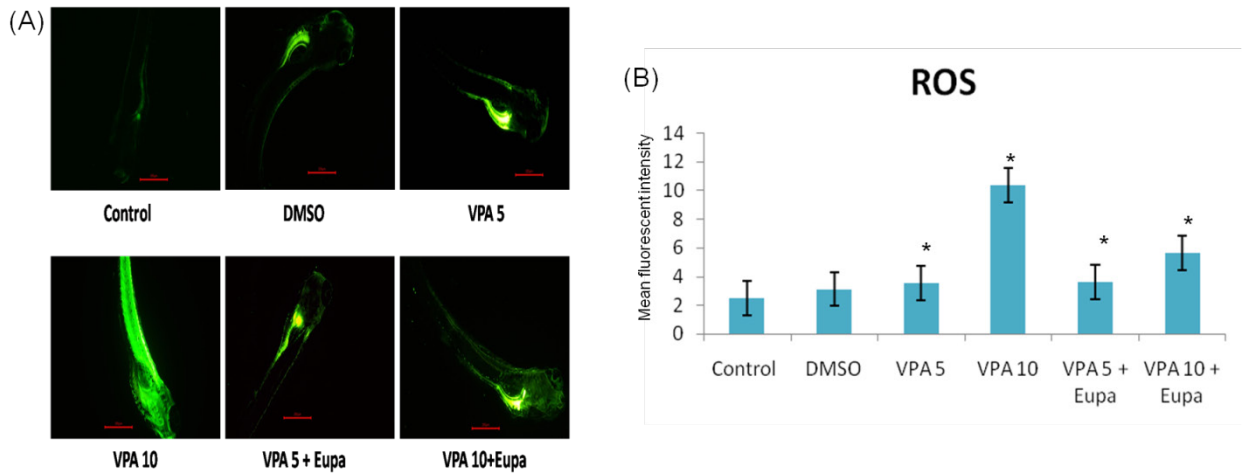

Figure 6: VPA-induced developmental toxicity and ameliorating effect of Eupatorin on ROS level of zebrafish embryos treated with VPA andEupatorin. (A) fluorescent-stained images were captured with different treatments. (B) The mean fluorescence intensity was quantified. (n=50 embryos per group) * indicates $p<0.05$ compared to control.

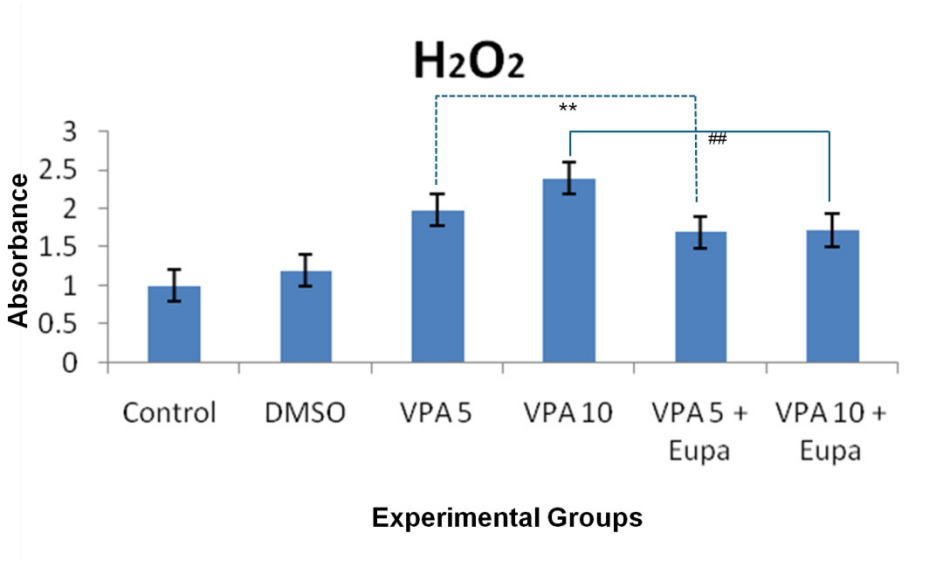

Figure 7: VPA-induced developmental toxicity and ameliorating effect of Eupatorin on H2O2 of zebrafish embryos treated with VPA and Eupatorin. (*n*=50 embryos per group) ** indicates $p<0.05$ compared to VPA 5 µg/mL and ## indicates $p<0.05$ compared to VPA 10 µg/mL.

generation is enhanced in ASD because of a dysfunctional mitochondrial Electron Transport Chain (ETC), which overloads antioxidant enzymes and worsens oxidative damage.[31,27] Reduced activity of ETC complexes and increased oxidative damage in key brain regions involved in cognition and social behavior have been reported in individuals and models of ASD, indicating mitochondrial dysfunction.[32,33] The subsequent energy deficits disrupt synaptic transmission, ion homeostasis, and brain metabolism, all of which are required for normal neurodevelopment and behaviour.[27] Mitochondrial weaknesses are also predisposed by genetic vulnerabilities and environmental abuse associated with ASD, making this linked disease a significant therapeutic focus.[5,31]

The present study investigated Valproic Acid (VPA)-induced developmental and behavioral anomalies resembling traits associated with Autism Spectrum Disorder (ASD) using the zebrafish embryonic model. VPA, a traditional Histone Deacetylase (HDAC) inhibitor, caused morphological abnormalities that depended on the dose and time, such as yolk sac and pericardial edema, craniofacial problems, spinal curvature, delayed pigmentation, problems with swim bladder inflation, and bradycardia. These anomalies signify both neurological disruption and systemic toxicity, consistent with findings from other zebrafish and vertebrate studies on prenatal VPA exposure.[26,5] The presence of these structural anomalies highlights the translational relevance of the zebrafish model, mirroring neurodevelopmental alterations observed in human ASD pathophysiology and established animal models of VPA exposure.[26]

Notably, eupatorin alone treated group at 5 nM did not show developmental abnormalities, mortality, or physiological changes in embryos. This result was supported by previous reports of eupatorin, which indicates low cytotoxicity in normal cells at higher concentrations. As Androutsopoulos *et al*., demonstrated, eupatorin shows selective cytostatic effects in cancer cells, while displaying negligible toxicity in normal mammary epithelial cells.[16] Likewise, Hussain *et al*., stated that eupatorin exhibits antioxidant and anti-inflammatory effects on non-malignant systems without inducing cytotoxicity at low concentrations.[17] These findings support the safety of eupatorin concentration used in this study and also justify that eupatorin primarily functions as a modulatory agent against VPA-induced toxicity instead of functioning as an independent toxicant. So the eupatorin alone group was not added to further analyses, as our primary objective was to evaluate its modulatory effect on VPA induced toxicity.

Larvae exposed to VPA demonstrated hypoactivity, stereotypical swimming behaviors, impaired visuomotor responses, and social deficits, as evidenced by reduced shoaling and altered group synchronization. These characteristics align with core features of ASD, encompassing social deficits and the limitation of repetitive behaviors.[8,26] The concurrent manifestation of hypoactivity and hyperactivity in numerous experiments underscores the sensitivity of developing neural circuits to the timing and dosage of VPA exposure, highlighting the adaptability and vulnerability of neurodevelopmental processes.[8] The hatching study revealed that VPA exposure produced two effects: initially, low doses of VPA accelerated hatching, whereas subsequently, it diminished the overall success rate of hatching. Early hatching in zebrafish is often associated with stress-induced developmental acceleration or the premature activation of hatching enzymes, which may compromise embryonic viability and post-hatching survival. The subsequent decline in overall hatching success suggests that although VPA may temporarily expedite developmental

processes, extended exposure disrupts normal embryogenesis, leading to reduced developmental competence.

Such VPA-induced deficiencies were significantly reduced by co-treatment with eupatorin, a methoxylated flavone with antiapoptotic and antioxidative properties. Eupatorin enhanced the function of the swim bladder, reduced cardiac and craniofacial malformations, enhanced oedema and pigmentation, and increased survival. Global systemic protection was evidenced by normalization of physiological indicators, including heart rate.[34,26] Behaviourally, eupatorin proved capable of stabilizing neuronal networks that underlie motor control, sensory processing, and social integration through partial amelioration of locomotor activity, inhibition of stereotypical behaviour, and improvement of social cohesiveness and synchronization. Flavonoids have been reported to regulate oxidative stress and apoptotic processes in neurodevelopmental and neurodegenerative diseases,[34-36] which supports eupatorin's neuroprotective action. Recent animal experiments show that flavonoids like luteolin, rutin, and eupatorin significantly reduce neuroinflammation, oxidative stress, and repetitivebehaviors like ASD. This suggests that similar molecules have a broad range of possible medical uses.[37,38]

As evidenced by enhanced acridine orange staining in the brain and trunk, VPA mechanistically enhances ROS and hydrogen peroxide and suppresses antioxidant defences such as Superoxide Dismutase (SOD), inducing mitochondrial caspase-mediated apoptosis.[5,26] Through the partial amelioration of redox homeostasis, reduction of ROS/H_2O_2 levels, enhancement of SOD activity, and inhibition of apoptotic cell death, eupatorin offsets these effects. This is probably done through modulating pro- and anti-apoptotic proteins (Bax/Bcl-2 ratio), maintaining mitochondrial membrane potential, and blocking cytochrome c release and caspase activation.[34,35] While the immediate effects of eupatorin on HDAC activity are not yet known, its redox homeostatic activity and inhibition of apoptosis can indirectly influence gene expression and chromatin remodelling and enhance epigenetic stability in the process of neurodevelopment.[29,30]

The neuroprotective pharmacological profile of eupatorin aligns with findings in a range of pathological contexts. Earlier, we have proved that eupatorin enhances organ function in diabetic mouse models by reducing oxidative damage and hyperglycemia.[36] Eupatorin inhibits neurodegeneration and avoids amyloid-beta aggregation in Caenorhabditis elegans models of Alzheimer's disease.[39] This ability to treat redox imbalance and mitochondrial stress makes eupatorin a broad-spectrum therapeutic drug. Its exceptional capacity to restore complicated social behaviour has translational significance, highlighted by being a major obstacle in ASD drug therapy.[26]

Its restriction to early larval stages, however, is the focus of this study; impacts on juvenile and adult behaviour over longer timescales remain uninvestigated. To completely elucidate eupatorin's treatment mechanisms and effectiveness, additional studies involving multi-omics methods, direct mitochondrial functional assays, HDAC activity quantifications, and testing in mammalian ASD models are required.[26,40] Additionally, combination treatments with other flavonoids or neuroprotective agents might further improve treatment efficacy.[34,35] However, the translation of neuroprotective flavonoids from animal research studies to clinical trials faces challenges, including inefficient diffusion through the blood-brain barrier and patient response variability, which highlights a clear need to improve methods of drug delivery and patient stratification.[41] While the current assays did not include a eupatorin-only group, the normalization of VPA induced changes without supra-control effects indicates a modulatory rather than stimulatory function for eupatorin; however, more research using eupatorinalone treatment is necessary.

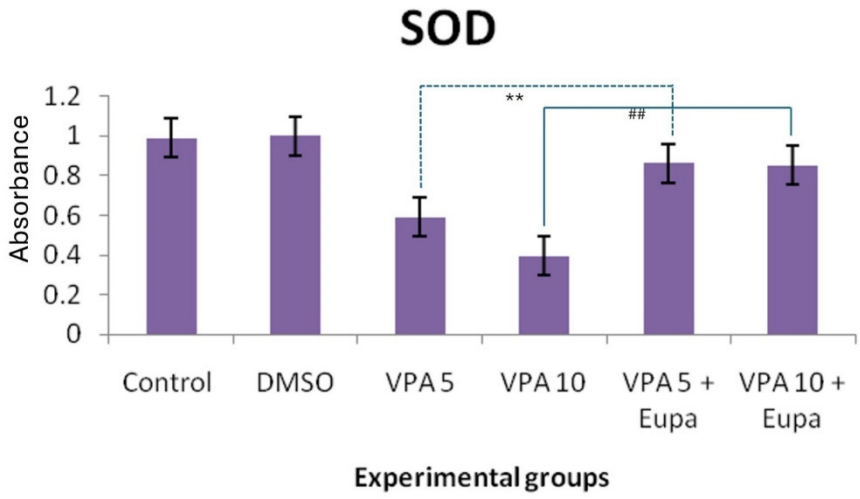

Figure 8: VPA-induced developmental toxicity and ameliorating effect of Eupatorin on SOD of zebrafish embryos treated with VPA andEupatorin. (*n*=50 embryos per group) ** indicates $p<0.05$ compared to VPA 5 and ## indicates $p<0.05$ compared to VPA 10.

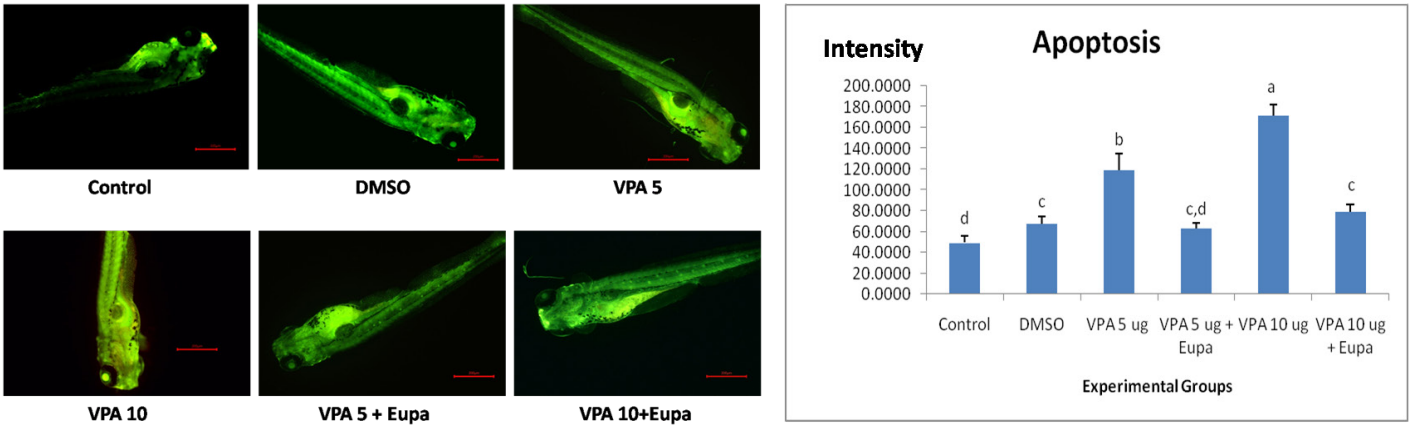

Figure 9: VPA-induced apoptosis and the ameliorative effect of Eupatorin in zebrafish embryos. (n=50 embryos per group).

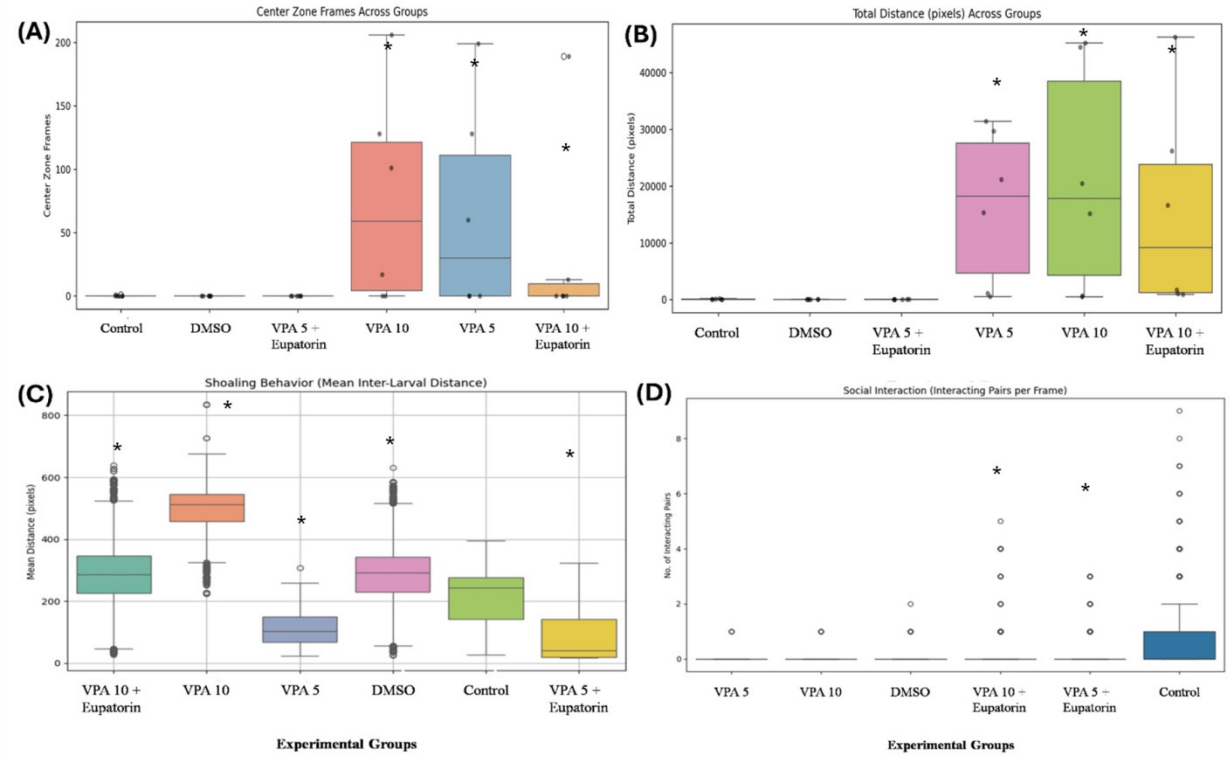

Figure 10: Valproic Acid (VPA)-induced behavioral alterations and the ameliorative effects of Eupatorin in zebrafish embryos. (A) Time spent in the center zone (B), total distance traveled (C), shoaling behavior, and social interaction were assessed to evaluate VPA-induced behavioral impairments and the neuroprotective efficacy of Eupatorin. Data represent mean values from n=10 embryos per group for (a-c) and *n*=50 embryos per group for (d). * indicates $p<0.05$ compared to control.

CONCLUSION

In conclusion, the current dataset indicates that mitochondrial dysfunction and oxidative stress are significantly correlated pathogenic characteristics in the VPA induced zebrafish model of ASD. Eupatorin treatment correlated with a mitigation of VPA induced morphological, behavioral, and biochemical anomalies, along with decreases in oxidative stress and apoptosis-related indicators. These findings suggest a possible association between eupatorin-mediated redox and cellular stress modulation and enhanced ASD-like traits; nevertheless, the existing evidence is correlational and fails to demonstrate a direct causal mechanism. Nonetheless, eupatorin presents itself as a viable multi-target candidate for further exploration in ASD and associated neurodevelopmental disorders, establishing a basis for future mechanistic and translational research.

A formal a priori power analysis was not conducted prior to the study. Although the sample sizes were selected based on commonly reported standards in zebrafish behavioral research, the modest behavioral cohort size may limit the detection of smaller effect sizes. Future studies with larger sample sizes are warranted to further validate these findings.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the funding of the Deanship of Graduate Studies and Scientific Research, Jazan University, Saudi Arabia, through Project number: (JU- 202502111 -DGSSR-RP -2025).

ABBREVIATIONS

AO: Acridine Orange; **ANOVA:** Analysis of Variance; **ASD:** Autism Spectrum Disorder; **ATP:** Adenosine Triphosphate; **a.u.:** Arbitrary Units; **BBB:** Blood-Brain Barrier; **Bax:** Bcl-2-Associated X Protein; **Bcl-2:** B-Cell Lymphoma 2; **CNS:** Central Nervous System; **DCFH-DA:** 2′,7′-Dichlorodihydrofluorescein Diacetate; **DMSO:** Dimethyl Sulfoxide; **DNA:** Deoxyribonucleic Acid; **ELISA:** Enzyme-Linked Immunosorbent Assay; **ETC:** Electron Transport Chain; **FITC:** Fluorescein Isothiocyanate; **GPX:** Glutathione Peroxidase; **H_2O_2:** Hydrogen Peroxide; **HDAC:** Histone Deacetylase; **hpf:** Hours Post-Fertilization; **IID:** Inter-Individual Distance; **IC_{50}:** Half Maximal Inhibitory Concentration; **MDA:** Malondialdehyde; **MS-222:** Tricaine Methane Sulfonate; **nM:** Nanomolar; **NND:** Nearest Neighbor Distance; **NO:** Nitric Oxide; **PE:** Pericardial Edema; **REC:** Research Ethics Committee; **ROI:** Region of Interest; **ROS:** Reactive Oxygen Species; **SD:** Standard Deviation; **SOD:** Superoxide Dismutase; **SPSS:** Statistical Package for the Social Sciences; **TC:** Tail Curvature; **VPA:** Valproic Acid; **YE:** Yolk Sac Edema.

CONFLICT OF INTEREST

The authors have no relevant financial or non-financial interests to disclose

FUNDING

The authors gratefully acknowledge the funding of the Deanship of Graduate Studies and Scientific Research, Jazan University, Saudi Arabia, through Project number: (JU- 202502111 -DGSSR-RP -2025)

ETHICS APPROVAL

This study was performed with the principles and guidelines of the Institutional Animal Ethics Committee (IAEC) and also follows the standard zebrafish welfare protocols. Approval was granted by the Ethics Committee of University REC-46/11/1540.

AUTHOR CONTRIBUTION

Abdullah Farasani solely conceived and designed the study, performed the experiments, analyzed and interpreted the data, and wrote and approved the final manuscript.

DATA STATEMENT

All data related to this research have been provided with this manuscript.

SUMMARY

Valproic Acid (VPA) exposure during embryogenesis induces Autism Spectrum Disorder (ASD)-like developmental, biochemical, and behavioral abnormalities, primarily mediated by oxidative stress and apoptosis. Using a zebrafish embryo model, this study evaluated the neuroprotective potential of eupatorin, a methoxylated flavone. VPA exposure caused dose-dependent developmental toxicity, reduced survival, impaired cardiac and locomotor function, increased reactive oxygen species and hydrogen peroxide levels, suppressed superoxide dismutase activity, and enhanced apoptotic cell death, along with autism-like behavioral alterations. Eupatorin co-treatment significantly mitigated these effects by partial amelioration of redox homeostasis, reducing apoptosis, improving survival, and ameliorating behavioral deficits, highlighting its potential as a neuroprotective candidate for ASD-related neurodevelopmental disorders.

- Eupatorin mitigates VPA-induced developmental toxicity in zebrafish embryos.
- Co-treatment restores survival, hatching, and cardiac function disrupted by VPA.
- Eupatorin reduces ROS and H_2O_2 levels while enhancing antioxidant enzyme activity.
- Apoptotic cell death is significantly decreased in the brain with eupatorin use.
- Eupatorin improves autism-like behaviors, including social interaction and activity.

REFERENCES

1. Lord C, Brugha TS, Charman T, Cusack J, Dumas G, Frazier T, et al. Autism spectrum disorder. Nat Rev Dis Primers. 2020;6(1):5. doi: 10.1038/s41572-019-0138-4, PMID 31949163.
2. Frustaci A, Neri M, Cesario A, Adams JB, Domenici E, Dalla Bernardina B, et al. Oxidative stress-related biomarkers in autism: systematic review and meta-analyses. Free Radic Biol Med. 2012;52(10):2128-41. doi: 10.1016/j.freeradbiomed.2012.03.011, PMID 22542447.
3. Chauhan A, Chauhan V. Oxidative stress in autism. Pathophysiology. 2006;13(3):171-81. doi: 10.1016/j.pathophys.2006.05.007, PMID 16766163.
4. Prince PD, Codagnone MG, Opezzo JA, Adán Areán JS, Höcht C, Arnal N, et al. Alteration in hippocampal mitochondria ultrastructure and cholesterol accumulation linked to mitochondrial dysfunction in the valproic acid rat model of autism spectrum disorders. Psychopharmacology. 2025: 1-18. doi: 10.1007/s00213-025-06899-4, PMID 40928517.
5. Wang X, Li P, Ji H, Xu Z, Xing H. Single-cell transcriptomics reveals over-activated reactive oxygen species pathway in hepatocytes in the development of hepatocellular carcinoma. Sci Rep. 2024;14(1):29809. doi: 10.1038/s41598-024-81481-0, PMID 39616235.
6. Roullet FI, Lai JK, Foster JA. In utero exposure to valproic acid and autism-a current review of clinical and animal studies. Neurotoxicol Teratol. 2013;36:47-56. doi: 10.1016/j.ntt.2013.01.004, PMID 23395807.
7. Schneider T, Przewłocki R. Behavioral alterations in rats prenatally exposed to valproic acid: animal model of autism. Neuropsychopharmacology. 2005;30(1):80-9. doi: 10.1038/sj.npp.1300518, PMID 15238991.

8. Chen J, Lei L, Tian L, Hou F, Roper C, Ge X, et al. Developmental and behavioral alterations in zebrafish embryonically exposed to valproic acid (VPA): an aquatic model for autism. Neurotoxicol Teratol. 2018;66:8-16. doi: 10.1016/j.ntt.2018.01.002 , PMID 29309833.
9. Kalueff AV, Stewart AM, Gerlai R. Zebrafish as an emerging model for studying complex brain disorders. Trends Pharmacol Sci. 2014;35(2):63-75. doi: 10.1016/j.tips.2013.12.002, PMID 24412421.
10. Zimmermann FF, Gaspary KV, Leite CE, De Paula Cognato GD, Bonan CD. Embryological exposure to valproic acid induces social interaction deficits in zebrafish (*Danio rerio*): a developmental behavior analysis. Neurotoxicol Teratol. 2015; 52(A):36-41. doi: 10.1016/j.ntt.2015.10.002, PMID 26477937.
11. Liu CX, Peng XL, Hu CC, Li CY, Li Q, Xu X. Developmental profiling of ASD-related shank3 transcripts and their differential regulation by valproic acid in zebrafish. Dev Genes Evol. 2016;226(6):389-400. doi: 10.1007/s00427-016-0561-4, PMID 27562614.
12. Retinasamy T, Shaikh MF, Kumari Y, Abidin SA, Othman I. Orthosiphon stamineus standardized extract reverses streptozotocin-induced Alzheimer's disease-like condition in a rat model. Biomedicines. 2020;8(5):104. doi: 10.3390/biomedicines8050104, PMID 32365983.
13. Li Y, Ren R, Wang L, Peng K. Eupatilin alleviates airway remodeling via regulating phenotype plasticity of airway smooth muscle cells. Biosci Rep. 2020;40(1):BSR20191445. doi: 10.1042/BSR20191445, PMID 31913462.
14. Sarvestani NN, Sepehri H, Farimani MM. Anticancer effect of eupatorin via bax/bcl-2 and mitochondrial membrane potential changes through ros mediated pathway in human colon cancer. Int J Pharmacogn Phytochem Res. 2015;7:1039-46.
15. Razak NA, Abu N, Ho WY, Zamberi NR, Tan SW, Alitheen NB, et al. Cytotoxicity of eupatorin in MCF-7 and MDA-MB-231 human breast cancer cells via cell cycle arrest, anti-angiogenesis and induction of apoptosis. Sci Rep. 2019;9(1):1514. doi: 10.1038/s41598-018-37796-w, PMID 30728391.
16. Androutsopoulos V, Arroo RR, Hall JF, Surichan S, Potter GA. Antiproliferative and cytostatic effects of the natural product eupatorin on MDA-MB-468 human breast cancer cells due to CYP1-mediated metabolism. Breast Cancer Res. 2008;10(3):R39. doi: 10.1186/bcr2090, PMID 18454852.
17. Hussain Y, Mathur A, Meena A, Luqman S. Eupatorin: A comprehensive review of its pharmacological activities and underlying molecular mechanisms. Eur J Pharmacol. 2025;1008:178330. doi: 10.1016/j.ejphar.2025.178330, PMID 41197890.
18. González-Cortazar M, Salinas-Sánchez DO, Herrera-Ruiz M, Román-Ramos DC, Zamilpa A, Jiménez-Ferrer E, et al. Eupatorin and salviandulin-A, with antimicrobial and anti-inflammatory effects from Salvia lavanduloides Kunth leaves. Plants (Basel). 2022;11(13):1739. doi: 10.3390/plants11131739, PMID 35807691.
19. Zhu J, Liu C, Wang J, Liang Y, Gong X, You L, et al. Difenoconazole induces cardiovascular toxicity through oxidative stress-mediated apoptosis in early life stages of zebrafish (*Danio rerio*). Ecotoxicol Environ Saf. 2021;216:112227. doi: 10.1016/j.ecoenv.2021.112227, PMID 33848738.
20. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. Dev Dyn. 1995;203(3):253-310. doi: 10.1002/aja.1002030302, PMID 8589427.
21. Bailey J, Oliveri A, Levin ED. Zebrafish model systems for developmental neurobehavioral toxicology. Birth Defects Res C Embryo Today. 2013;99(1):14-23. doi: 10.1002/bdrc.21027, PMID 23723169.
22. Wang C, Wang P, Yang LP, Pan J, Yang X, Ma HY. Association of CYP2C9, CYP2A6, ACSM2A, and CPT1A gene polymorphisms with adverse effects of valproic acid in Chinese patients with epilepsy. Epilepsy Res. 2017;132:64-9. doi: 10.1016/j.eplepsyres.2017.02.015, PMID 28315807.
23. Thisse C, Zon LI. Organogenesis—heart and blood formation from the zebrafish point of view. Science. 2002;295(5554):457-62. doi: 10.1126/science.1063654, PMID 11799232.
24. Liu CX, Li CY, Hu CC, Wang Y, Lin J, Jiang YH, et al. CRISPR/Cas9-induced shank3b mutant zebrafish display autism-like behaviors. Mol Autism. 2018;9(1):23. doi: 10.1186/s13229-018-0204-x, PMID 29619162.
25. Zhu J, Huang M, Liu C, Wang J, Zou L, Yang F, et al. Curcumin protects against fenvalerate-induced neurotoxicity in zebrafish (Danio rerio) larvae through inhibition of oxidative stress. Ecotoxicol Environ Saf. 2023;264:115484. doi: 10.1016/j.ecoenv.2023.115484, PMID 37716069.
26. Camussi D, Naef V, Brogi L, Della Vecchia S, Marchese M, Nicoletti F, et al. Delving into the complexity of valproate-induced autism Spectrum disorder: the use of zebrafish models. Cells. 2024;13(16):1349. doi: 10.3390/cells13161349, PMID 39195239.
27. Kuźniar-Pałka A. The role of oxidative stress in autism spectrum disorder pathophysiology, diagnosis and treatment. Biomedicines. 2025;13(2):388. doi: 10.3390/biomedicines13020388, PMID 40002801.
28. Shareef AA, Kheder AH, Albarzinji N, Karim KJ, Smail SW, Mahmood AA, et al. Oxidative markers and SOD variant: predictors of autism severity and susceptibility. Future Sci OA. 2025;11(1):2483628. doi: 10.1080/20565623.2025.2483628, PMID 40160095.
29. Kazantsev AG, Thompson LM. Therapeutic application of histone deacetylase inhibitors for central nervous system disorders. Nat Rev Drug Discov. 2008;7(10):854-68. doi: 10.1038/nrd2681, PMID 18827828.
30. Nardone S, Sams DS, Reuveni E, Getselter D, Oron O, Karpuj M, et al. DNA methylation analysis of the autistic brain reveals multiple dysregulated biological pathways. Transl Psychiatry. 2014;4(9):e433. doi: 10.1038/tp.2014.70, PMID 25180572.
31. Khaliulin I, Hamoudi W, Amal H. The multifaceted role of mitochondria in autism spectrum disorder. Mol Psychiatry. 2025;30(2):629-50. doi: 10.1038/s41380-024-02725-z, PMID 39223276.
32. Rossignol DA, Frye RE. Evidence linking oxidative stress, mitochondrial dysfunction, and inflammation in the brain of individuals with autism. Front Physiol. 2014;5:150. doi: 10.3389/fphys.2014.00150, PMID 24795645.
33. Palmieri L, Papaleo V, Porcelli V, Scarcia P, Gaita L, Sacco R, et al. Altered calcium homeostasis in autism-spectrum disorders: evidence from biochemical and genetic studies of the mitochondrial aspartate/glutamate carrier AGC1. Mol Psychiatry. 2010;15(1):38-52. doi: 10.1038/mp.2008.63, PMID 18607376.
34. Muhsen M, Youngs J, Riu A, Gustafsson JÅ, Kondamadugu VS, Garyfalidis E, et al. Folic acid supplementation rescues valproic acid-induced developmental neurotoxicity and behavioral alterations in zebrafish embryos. Epilepsia. 2021;62(7):1689-700. doi: 10.1111/epi.16915, PMID 33997963.
35. Lee KM, Lee Y, Chun HJ, Kim AH, Kim JY, Lee JY, et al. Neuroprotective and anti-inflammatory effects of morin in a murine model of Parkinson's disease. J Neurosci Res. 2016;94(10):865-78. doi: 10.1002/jnr.23764, PMID 27265894.
36. Farasani A, Abdelwahab SI. Antidiabetic activity of eupatorin against streptozotocin-induced diabetes in rats: biochemical and histological studies. Indian J Pharm Educ Res. 2025;59(3):1160-9. doi: 10.5530/ijper.20251812.
37. Chunmei Z, Shuai W. Molecular mechanisms of neuroprotective effect of rutin. Front Pharmacol. 2025;16:1599167. doi: 10.3389/fphar.2025.1599167, PMID 40529506.
38. Khayyat AI, Alabdali AN, Alonazi M, Alzahrani AA, Al-Shehri E, Ben Bacha A. Luteolin mitigates oxidative stress and multi-organ impairment in a propionic acid-induced rodent model of autism. Front Nutr. 2025;12:1583119. doi: 10.3389/fnut.2025.1583119, PMID 40497022.
39. Sakellaropoulou A, Siamidi A, Vlachou M. Melatonin/cyclodextrin inclusion complexes: a review. Molecules. 2022;27(2):445. doi: 10.3390/molecules27020445, PMID 35056757.
40. Camussi D, Marchese M, Nicoletti F, Santorelli FM, Ogi A. Valproate-induced model of autism in adult zebrafish: A systematic review. Cells. 2025;14(2):109. doi: 10.3390/cells14020109, PMID 39851536.
41. Aksenov DP, Doubovikov ED. Diffusion constraints in neuroprotection: implications for clinical trials. Front Pharmacol. 2025;16:1542431. doi: 10.3389/fphar.2025.1542431, PMID 40196360.

Cite this article: Farasani A. Neuroprotective Effects of Eupatorin against Valproic Acid-Induced Autism-Like Phenotypes in Zebrafish Embryos. Indian J of Pharmaceutical Education and Research. 2026;60(2s):s839-s852.