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TOXICOLOGY MECHANISMS AND METHODS
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RESEARCH ARTICLE

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Effects of benzoic acid synthesized from *Cinnamomum cassia* by green chemistry on valproic acid-induced neurotoxicity in zebrafish embryos

Derya Cansız[a], Gökhan Özokan[b], Abdulkerim Bilginer[b], Semanur Işıkoğlu[c], Zülal Mızrak[c], İsmail Ünal[c], Merih Beler[c], A. Ata Alturfan[d] and Ebru Emekli-Alturfan[e]

[a]Istanbul Medipol University, Faculty of Medicine, Medical Biochemistry, Istanbul, Türkiye; [b]BioArge Laboratories, Yıldız Technical University Technocity, Istanbul, Türkiye; [c]Department of Biochemistry, Institute of Health Sciences, Faculty of Pharmacy, Marmara University, Istanbul, Türkiye; [d]Department of Biochemistry, Faculty of Medicine, Istanbul University-Cerrahpaşa, Istanbul, Türkiye; [e]Department of Basic Medical Sciences, Faculty of Dentistry, Marmara University, Istanbul, Türkiye

ABSTRACT

Benzoic acid, the most basic aromatic carboxylic acid, is produced industrially and used in cosmetic, hygiene, and pharmaceutical items as a flavoring ingredient and/or preservative. The significance of sodium benzoate, a metabolite of cinnamon, used as a food preservative and FDA-approved medication to treat urea cycle abnormalities in humans, has been shown to raise the levels of neurotrophic factors. Valproic acid (VPA), a commonly used anti-epileptic and mood-stabilizing medication, causes behavioral and intellectual problems and is a commonly used agent to induce animal model for autism. Aim of this study is to determine the effects of benzoic acid synthesized from *Cinnamomum Cassia* by green chemistry method on gene expressions related to autism development in case of VPA toxicity. Zebrafish embryos were exposed to low and high doses of benzoic acid for 72 h post-fertilization. Locomotor activities were determined. Acetylcholinesterase (AchE), lipid peroxidation, nitric oxide (NO), sialic acid (SA), glutathione (GSH)-S-transferase, catalase (CAT), and superoxide dismutase (SOD) activities were determined spectrophotometrically. *eif4b*, *adsl*, and *shank3a* expressions were determined by RT-PCR as autism-related genes. Although high-dose benzoic acid inhibited locomotor activity, benzoic acid at both doses ameliorated VPA-induced disruption in oxidant-antioxidant balance and inflammation in zebrafish embryos and was effective in improving the impaired expression of autism-related genes.

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Benozic acid; *Cinnamomum cassia*; green chemistry; valproic acid; zebrafish embryo

Introduction

Benzoic acid is the most basic aromatic carboxylic acid which has a carboxylic group that is directly linked to the benzene ring. Plants and animal tissues naturally contain it, and it can also be produced by microbiological metabolism in fermented food. It is produced industrially and used in cosmetic, hygiene, and pharmaceutical items as a flavoring ingredient and/or preservative (Zeece 2020). It is also utilized in the coolant, solvent, photochemical, plastic, textile, pesticide, paper, and dye industries as an additive, nucleating agent, intermediate, stabilizer, and/or catalyst. Benzoic acid derivatives and other similar benzenic chemicals are widely employed in a variety of industrial industries and can be obtained naturally or chemically manufactured (Del Olmo et al. 2017). Even though they are thought to be safe compounds when used in accordance with Good Manufacturing Practices (GMP) or within legal limits, adverse responses, possible toxicological effects, and public health issues have been documented and debated (Del Olmo et al. 2017) Benzoic acid has been shown to enhance the performance and nutrient digestibility, and inhibit the pathogenic microorganisms, and regulate microflora balance (Diao et al. 2016; Kluge et al. 2006). However, the information on the systematic effects of benzoic acid is very limited (Torrallardona et al. 2007).

The significance of sodium benzoate, a metabolite of cinnamon that is used as a food preservative and FDA-approved medication to treat urea cycle abnormalities in humans, has been shown to raise the levels of neurotrophic factors (Jana et al. 2013). Accordingly, these findings pointed to the possible neutrophic characteristic of cinnamon through the PKA-CREB pathway, as well as its metabolite sodium benzoate, which may be beneficial for a number of neurodegenerative diseases (Jana et al. 2013).

A severe neurological/neurodegenerative condition that causes communicative and cognitive impairments is known as autism spectrum disorder (ASD). Valproic acid (VPA), a commonly used anti-epileptic and mood-stabilizing medication, causes behavioral and intellectual problems, including autism, in human fetuses (Ornoy 2009; Feas et al. 2017). When VPA is administered to pregnant rats and mice at gestation, the offspring develops symptoms similar to autism, and this is a commonly used animal model for autism (Arndt et al. 2005). Similarly, zebrafish embryos exposed to VPA exhibit characteristics that are very similar to ASD, such as

CONTACT Derya Cansız cansizderya@yahoo.com Istanbul Medipol University, Faculty of Medicine, Medical Biochemistry, Istanbul, Türkiye
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decreased social engagement and movement (Wang et al. 2024; Li et al. 2024). Oxidative stress and apoptosis play have been suggested to play important roles in the development of VPA-induced neurotoxicity in zebrafish (Wang et al. 2024).

Green chemistry has been widely used in almost every industry, including agriculture, home products, cosmetics, and pharmacy, as a method to attain sustainability at a molecular level (Anastas and Eghbali 2010). Reducing or eliminating the production and use of chemicals that are detrimental to the environment and public health is one of the main goals of green chemistry (Özokan et al. 2024). Accordingly, the purpose of this study was to synthesize benzoic acid from Cinnamomum Cassia using green chemistry method. Then, we aimed to examine the effects of this benzoic acid on the VPA-induced autism model in zebrafish embryos focusing on oxidative stress and gene expressions related with ASD development.

Materials and methods

Chemicals used

VPA (CAS Number: 1069-66), folin reagent (F9252), 5,5′-dithiobis (2-nitrobenzoic acid) (CAS Number:69-78-3), thiobarbituric acid (CAS Number:504-17-6), vanadium (III) chloride (CAS No.:7718-98-1), H_2SO_4 (CAS Number: 7664-93-9), sodium periodate (CAS No.: 7790-28-5), H_3PO_4 (CAS No.: 7664-38-2), O-dianisidine dihydrochloride (CAS No.: 20325-40-0), Riboflavin (CAS No.:83-88-5); hydrogen peroxide (H_2O_2; CAS No.: 7722-84-1), glutathione (GSH; CAS No.:70-18-8), 1-chloro-2,4-dinitrobenzene (CDNB; CAS Number: 97-00-7) were purchased from Sigma-Aldrich, St Louis, MO. They were all analytical grade with the highest purity available.

The synthesis of benzoic acid by green chemistry

Green organic synthesis techniques were applied during the synthesis of natural benzoic acid using natural benzaldehyde from cassia oil (Figure 1). Only water was used as solvent in the reaction. Purple-colored solution is prepared by dissolving 12 g of potassium permanganate in 200 g of water. A cloudy-colored solution is prepared by mixing 10 g of natural benzaldehyde in 25 g of water. Purple permanganate solution is added to this solution. Heating is applied for 45 min with slow stirring at a temperature of approximately 90 °C. When the reaction is completed, it is cooled and the brown solid (Manganese dioxide) formed is filtered through a 15-micron paper filter. Of 37% hydrochloric acid is added to the transparent colorless solution and the pH of the solution is adjusted to 1.5. The white benzoic acid crystals formed are filtered through a paper filter and washed with some cool water. Vacuuming first allows the crystals to dry slightly. White crystals are transferred to a dry beaker and dried in an oven set at 40 °C for 1 week.

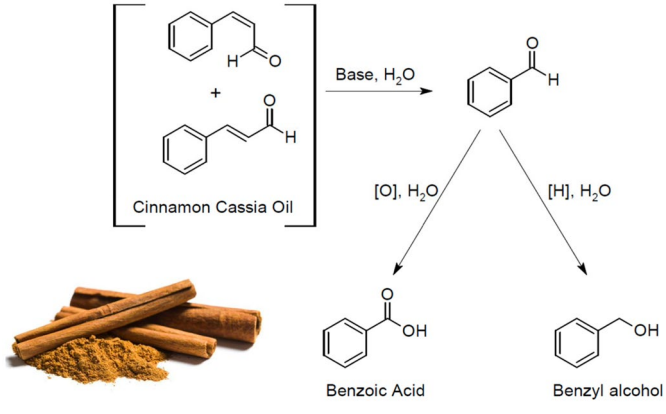

Figure 1. Synthesis of natural salicylic acid using organic synthesis and green chemistry methods.

Purity analysis using GC/MS

The Natural Benzoic Acid (Plantraction CA100) produced was purity-tested using GC-MS analysis using the following parameters for column as HP-5ms Ultra Inert, and dimensions of 30 m × 250 µm × 0.25 µm. The other programs applied were 'Beginning: 60oC, Final Temperature: 260 °C, Temperature Increase Rate: 3oC per minute, Analysis Duration: 66.6 min; Inlet Temperature 250 °C; MS Detector Temperature: 230 °C; Helium Flow Rate: 1.1 mL/min; Split Ratio: 20:1; Sample Preparation: 5 mg of Natural Benzoic Acid (Plantraction CA100) dissolved in 1.5 ml of acetone; The volume of injection was 1 µl.'

Zebrafish embryo toxicity analysis

The AB/AB strain of zebrafish was housed in an aquatic rack arrangement (Zebtec, Tecniplast, Italy) with a 14/10 h light/dark cycle and a constant temperature of 27 ± 1 °C. The fish were fed commercial flake fish meal twice a day, supplemented with live Artemia. System water has a pH between 6.9 and 7.2. Reverse osmosis water containing 0.018 mg L^{-1} of Instant OceanTM salt was used for all testing. Fertilized embryos were gathered and arranged according to their previously documented development and morphology after regular spawnings (Westerfield 1995). According to the Council of Europe (2010) and EU Directive 2010/63/EU, no ethical authorization was needed for the protocols utilized because the zebrafish embryos tested were no older than five days.

Initially, range-finding experiments were used to identify the lethal concentration of benzoic acid that causes 50% mortality (LC50) in zebrafish embryos. Concentrations below 10 mg/L was found to be the environmentally relevant quantity of benzoic acid synthesized by green chemistry that affected development. Of 10 µM VPA exposure was shown to be an effective concentration that caused ASD-like phenotype in zebrafish embryos (Joseph et al. 2022). Based on these, in well plates, zebrafish embryos were subjected to low (2.5 mg/L) and high (5 mg/L) doses of benzoic acid and 10 uM VPA for 72 h after fertilization (hpf). To prepare benzoic acid stock solution, 20 mg benzoic acid was dissolved in 20 mL E3 solution (Embryo medium:15 mM NaCl, 0.5 mM KCl, 1.0 mM $MgSO_4$, 0.15 mM KH_2PO_4, 0.05 mM Na_2HPO_4, 1.0 mM CaCl2, 0.7 mM $NaHCO_3$, pH 7.2). By diluting from the stock solution, 2.5 mg/L and 5 mg/L benzoic acid solutions were prepared. VPA stock solution was prepared at a concentration

of 25 mM in E3 solution. 10 µM VPA solution was prepared by dilution from the stock solution and used in the experiments.

The blank control was the embryo media. To maintain the exact dose-response relationships of the investigated substances, a sufficient number of healthy, recently fertilized eggs are required for each concentration level and control treatment in the toxicity tests conducted using zebrafish embryos. Embryos were randomly distributed into groups. In our study, VPA and benzoic acid exposures were performed up to 72 hpf, and at the end of 72 hpf, the embryos were sacrificed and homogenized for biochemistry and RT-PCR analyses. For each group, we prepared an appropriate RNA pool (50 embryos/pool; 3 biological replicates in every group). For the biochemical evaluations, 3 biological replicates were also prepared for each group (50 embryos/pool).

Every day, fresh exposure solutions were substituted for the old ones. Developmental parameters were monitored with the use of a stereomicroscope (Zeiss Discovery V8, Jena, Germany), and malformations were documented and photographed. The aforementioned development indicators were employed for embryo staging (Westerfield 1995). Scientists that were not aware of the experiments evaluated gene expression, biochemical data, and statistical data.

Locomotor activity

The total distance traveled (mm), average acceleration (mm/s^2), and exploration rate (%) were used to measure behavior activity. ToxTrac (Windows program designed for tracking), was used to evaluate the data, and then it was exported to Excel (Microsoft) for statistical analysis.

Biochemical analyses

To get a sufficient number of zebrafish embryos for the biochemical analyses, three biological replicates were established as pools. After homogenizing zebrafish embryos in 1 mL of PBS per pool, a fast centrifugation was performed. The supernatant was used to determine the biochemical parameters. All biochemical experiments were carried out by different researchers who computed the results without knowledge of the treatments.

Total protein analysis

The Lowry technique was used to determine the total protein level. Using this method, the proteins react with copper ions in an alkaline environment before being reduced using folin reagent. A spectrophotometer set at 500 nm is used to quantify the intensity of a blue color that forms, which is directly proportional to the protein content. The result is expressed for each protein (Lowry et al. 1951).

Acetylcholinesterase analysis

The Ellman 1961 method was utilized to assess the activity of acetylcholinesterase (AChE) present in the supernatants. Using 5,5′-dithiobis (2-nitrobenzoic acid), AChE generates thiocholine by the Ellmann method. This results in a yellow tint. The spectrophotometer was used to detect the intensity of the yellow product color at 412 nm, which is proportional to the enzyme activity in the sample (Ellman et al. 1961).

Lipid peroxidation analysis

The Yagi technique was utilized to determine the LPO levels. Using a spectrophotometer, the absorbance of the pinkish color produced by the interaction of thiobarbituric acid and malondialdehyde (MDA), a byproduct of LPO, was determined. Using an extinction coefficient of 1.56.105 $M^{-1}cm^{-1}$, LPO was represented in MDA equivalents as nmol MDA/mg protein (Yagi 1984).

Nitric oxide analysis

Using the Miranda technique, the levels of nitric oxide (NO) were measured. Using vanadium (III) chloride, nitrate is converted to nitrite in the Miranda technique. The complex diazonium molecule was then created *via* the reaction of sulfonyl amide and N-(1-Naphthyl) ethylenediamine dihydrochloride, which was created in an acidic environment. A spectrophotometer was used to quantify the colored solution that developed at 540 nm, and the results were expressed as nmol NO/mg protein (Miranda et al. 2001).

Sialic acid analysis

The thiobarbituric acid method of Warren (1959) was used to measure the levels of sialic acid (SA). The hydrolyzate was utilized for analysis after the homogenates were incubated with 0.1 N H_2SO_4 for an hour at 80 °C. SA is oxidized with sodium periodate in concentrated phosphoric acid, and the resultant chromophore is extracted in cyclohexanone after the periodate oxidation product is mixed with thiobarbituric acid. Using a spectrophotometer set at a wavelength of 549 nm, absorbance was measured. The results were represented as mg SA/g protein (Warren 1959).

Superoxide dismutase analysis

The Mylorie method was used to measure the activity of superoxide dismutase (SOD). The effect of riboflavin-sensitized photo-oxidation of SOD, o-dianisidine, is amplified in this method. The reaction mixture including riboflavin and O-dianisidine dihydrochloride is illuminated with a fluorescent lamp to induce superoxide activity. O-dianicidin oxidation is increased by SOD and sensitized by riboflavin; the rate of increase is linear in SOD concentration. Using a spectrophotometer, absorbances were measured at 460 nm after 0 and 8 min of illumination, and net absorbances were computed. Using the standard curve that was prepared with bovine SOD (S7571, Sigma Chemical Co., St. Louis, MO) as a reference, the SOD activity of the supernatant was calculated, taking into account the dilutions made (Mylorie et al. 1986).

Catalase analysis

The modified Aebi approach, which is predicated on the catalase (CAT) enzyme's ability to convert H_2O_2 into water, was utilized to ascertain the CAT activities of the supernatants. The CAT activity of the samples was reported as U/mg protein, and this conversion was seen as a reduction in absorbance measured at 240 nm. For the CAT activity measurement, the specific extinction coefficient 0.004 $mM^{-1}mm^{-1}$ was used and results were calculated as U/mg protein and taking into account the dilutions made (Aebi 1984).

Glutathione-S-transferase analysis

The assay used to measure the activity of GSH-S-transferase involved measuring the absorbance of the product obtained from the reaction of GSH with CDNB at 340 nm using spectrophotometry (Habig and Jakoby 1981). The change in absorbance (ΔA_{340})/min is calculated. The ΔA_{340}/min for the blank reaction is subtracted from the ΔA_{340}/min for each sample. The molar extinction (ε mM) of CDNB is 0.0096 µM-1/cm. Taking into account the dilutions made, GST activity was calculated and presented as unite per mg protein.

Quantitative real-time PCR analysis

To harvest RNA from each set, three biological replicates – pools of zebrafish embryos – were made. Each replication consisted of fifty embryos. The application of the Qiacube (Qiagen, Hilden, Germany) and Rneasy Mini Kit followed the manufacturer's instructions. A single-stranded cDNA was produced from 1 µg of total RNA using RT2 Profiler PCR Arrays (Qiagen, Hilden, Germany). The DNA Master SYBR Green kit (Qiagen, Hilden, Germany) was used to perform RT-PCRs. The housekeeping gene that was used was beta-actin. Forward and reverse primers used in the study are listed in Table 1. The housekeeping gene was utilized to standardize the results of the CT method, which was then employed in a blinded fashion to determine the relative transcription levels (Livak and Schmittgen 2001).

Statistical analysis

Each analysis using zebrafish embryos was carried out with 50 embryos each trial and performed three times. To assess the normality of the data, the effects of VPA and benzoic acid on zebrafish embryos were examined using the Shapiro–Wilk test. A two-way ANOVA with the Sidak multiple comparison test was used to examine the statistical significance for the components VPA treatment and benzoic acid treatments in a blinded way. GraphPad Prism version 9 (Inc, La Jolla, CA) was used for statistical analysis. The findings were presented as mean ± standard deviation, with significance level set at $p < 0.05$.

Table 1. Forward and reverse primers used in the study.

Primers	(forward/reverse)
Gene ID: 57934- *bactin*	5′-AAGCAGGAGTACGATGAGTCTG-3′
	5′-GGTAAACGCTTCTGGAATGAC-3′
Gene ID: 334431- *adsl*	5′-CGTTTAACCGCTGCAGGCAT-3′
	5′-AGCATCGCTTATGGGGAGGC-3′
Gene ID: 336619- *eif4b*	5′-ACTCTGCTGTGCCTCATCGG-3′
	5′-TTCATAGGGACGCCCAACCG-3′
Gene ID: 557701-*shank3a*	5′-CTGTTTTACGGAGCGGACAT-3′
	5′-CCCTGAAAGGCACAACATCT-3′

Results

GC/MS analysis results

The purity analysis results of benzoic acid through GC/MS analysis revealed 100% purity (0% impurities) with a 60% (weight) yield (Figure 2).

Figure 3 displays representative images of the zebrafish embryos. In VPA-exposed embryos delayed hatching was observed at 48 hpf. At 72 hpf cardiac edema and hemorrhage were recorded. High benzoic acid caused cardiac edema at 48 hpf. Both low and high benzoic acid treatments caused delayed hatching at 48 hpf in the VPA group. Delayed hatching was also observed at 72 hpf in the high-dose benzoic acid-treated VPA group.

Results of locomotor and AChE activities

Two-way ANOVA results revealed a statistically significant interaction between the effects of VPA and benzoic acid treatments on average acceleration ($F = 10.4$, $p = 0.0002$). HBA and VPA treatments caused significant reductions in average acceleration and total distance ($p < 0.0001$; $p < 0.05$ and $p < 0.0001$; $p < 0.0001$) (Figure 4(A,B)). Moreover, both low and high doses of benzoic acid treatments decreased average acceleration in the VPA groups ($p < 0.01$ and $p < 0.0001$) (Figure 3(A,B)). On the other hand only HBA exposure caused significant reductions in total distance ($p < 0.0001$) (Figure 3(B)). When we evaluated AChE activity, which is closely related to locomotor activity, a statistically significant interaction between the effects of VPA and benzoic acid treatments was detected ($F = 8.7$, $p < 0.01$). VPA was found to decrease AChE activity compared to control group ($p < 0.001$). On the other hand, in the VPA-exposed group, HBA treatment caused a increase in AChE activity compared to VPA group ($p < 0.05$) (Figure 4(C)).

Results of biochemical analyses

VPA exposure increased LPO, NO, and SA levels which were evaluated to determine the oxidant and inflammatory status of the groups. While HBA treatment ameliorated LPO ($F = 4.9$, $p < 0.05$), NO ($F = 7$, $p < 0.01$), and SA ($F = 16.79$, $p < 0.01$) levels significantly in the VPA group ($p < 0.05$, $p < 0.001$, and $p < 0.001$, respectively), LBA treatment was only effective to decrease NO and SA levels in the VPA group ($p < 0.05$ and $p < 0.01$, respectively). The decrease in LPO caused by LBA in the VPA group was not statistically significant (Figure 5(A–C)).

We analyzed CAT and GST activities to assess the antioxidant status of zebrafish embryos and observed alterations in CAT and GST activities in the groups treated with VPA and benzoic acid ($F = 6.9$, $p < 0.05$ and $F = 39.41$, $p < 0.0001$). HBA treatment increased SOD and GST activities significantly

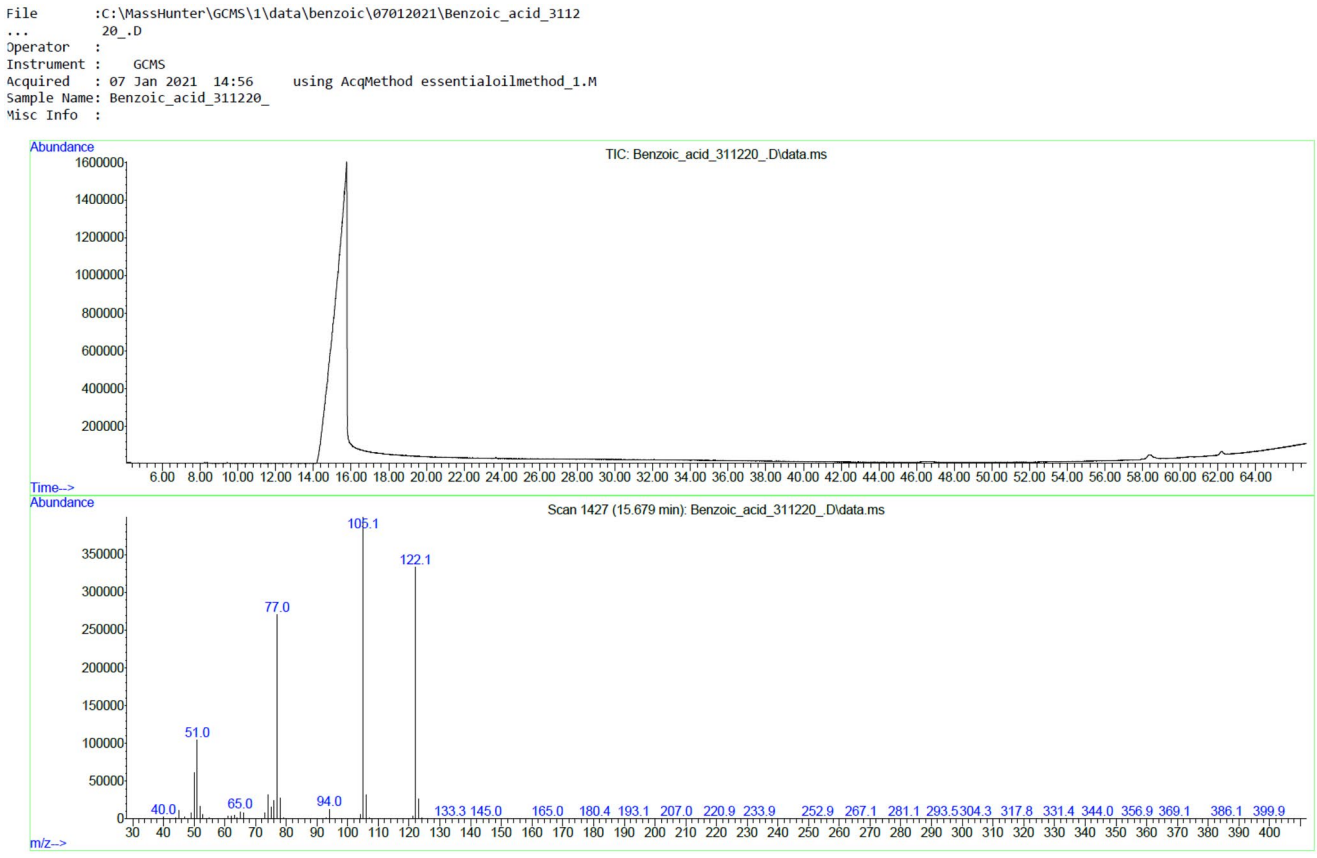

Figure 2. Chromatogram and MS spectrum of natural benzoic acid (plantraction CA100).

($p < 0.01$ and $p < 0.05$). On the hand, in the VPA group while GST activity increased ($p < 0.0001$), SOD and CAT activities decreased significantly ($p < 0.001$ and $p < 0.01$). HBA treatment in the VPA group ameliorated SOD and CAT activities ($p < 0.05$). Both LBA and HBA treatments decreased GST activities in the VPA group ($p < 0.01$ and $p < 0.0001$) (Figure 6(A–C)).

Results of gene expression analyses

Two-way ANOVA results revealed significant interactions between the effects of VPA and benzoic acid treatments on the expressions of adsl, eif4b, and shank3a, which are identified as autism-related genes ($F = 18.12$, $p < 0.001$; $F = 39.72$, $p < 0.0001$, and $F = 20.82$, $p = 0.0001$). In the VPA group, while the expressions of *adsl* and *shank3a* decreased significantly ($p < 0.001$), a significant increase was observed in the expression of *eif4b* ($p < 0.0001$). While both LBA and HBA treatments were effective in increasing *adsl* ($p < 0.05$ and $p < 0.0001$) and *shank3a* expressions ($p < 0.05$ and $p < 0.001$), the increases were more pronounced with HBA treatment. Similarly, while both LBA and HBA treatments were effective in decreasing *eif4b* ($p < 0.0001$) the decrease was more pronounced with HBA treatment (Figure 7(A–C)).

Discussion

The results of our study have shown for the first time that low and high doses of benzoic acid improve oxidant-antioxidant status, inflammation-related parameter SA, and ameliorate the expression of genes associated with ASD development in a dose-dependent manner in case of VPA toxicity in zebrafish embryos.

In addition to being a strong teratogen, VPA is a broad-spectrum antiepileptic medication. According to epidemiological research, children exposed to VPA during the first trimester of pregnancy are more likely to acquire ASD. Following VPA treatment, a number of studies on humans and animals have shown significant behavioral deficits as well as morphological alterations in the brain (Taleb et al. 2021).

In our study, delayed hatching, hemorrhage, and cardiac edema were detected in zebrafish embryos given VPA, which is consistent with its teratogenic effect (Li et al. 2024). Moreover, significant decrease in locomotor activity, measured as average acceleration and total distance, was determined in zebrafish embryos administered VPA. Consistent with our findings, Baronio et al. (2018) reported decreased locomotor activity at 5 hpf in 25 µM VPA-exposed zebrafish embryos. Zimmermann et al. (2015) showed that there was a decrease in locomotor activity at 6 hpf as a result of 48 µM VPA exposure at 48 hpf. Differences between studies may be due to the starting point of exposure, duration, exposure dose, and total duration of the experiment. In our study, we started the exposure at 0–2 hpf (the time period when the blastula stage of zebrafish embryos has not yet been completed) and performed the locomotor analyses at 72 hpf.

In the VPA-administered group, an increase in LPO and NO levels was determined, which indicate oxidative stress as

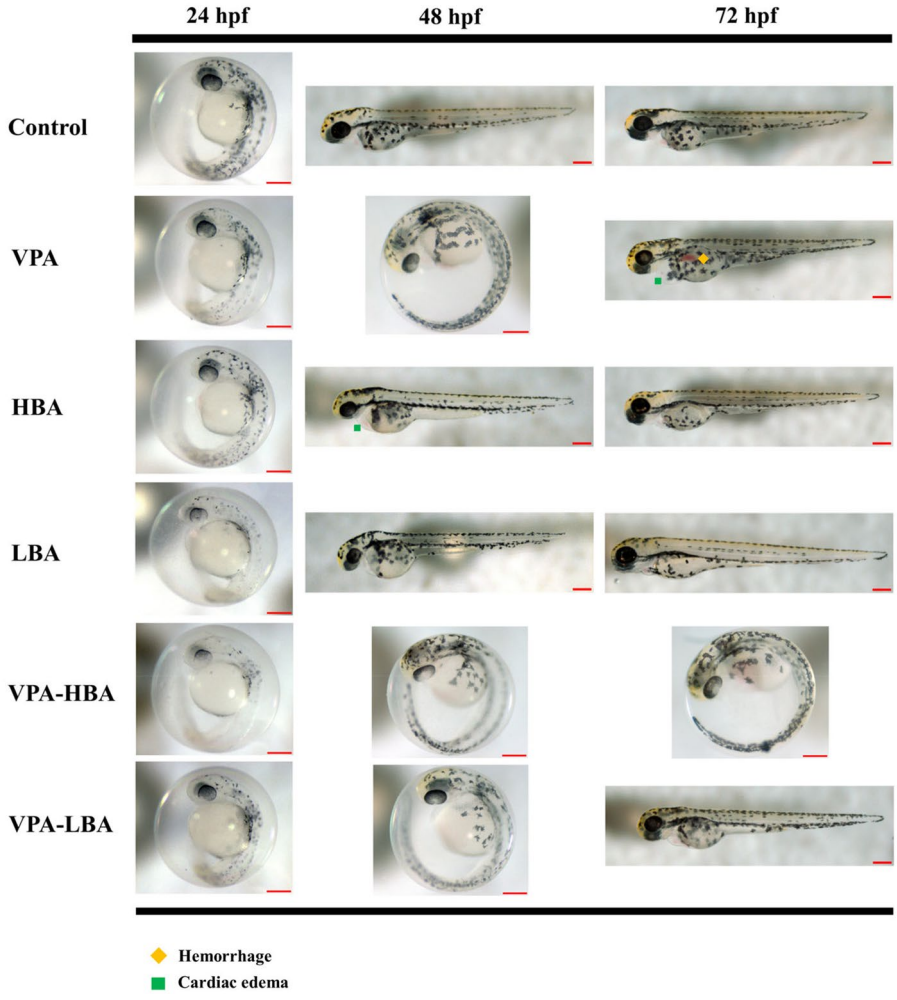

Figure 3. Representative images of the zebrafish embryos in the groups. VPA: valproic acid; LBA: low-benzoic acid; HBA: high-benzoic acid.

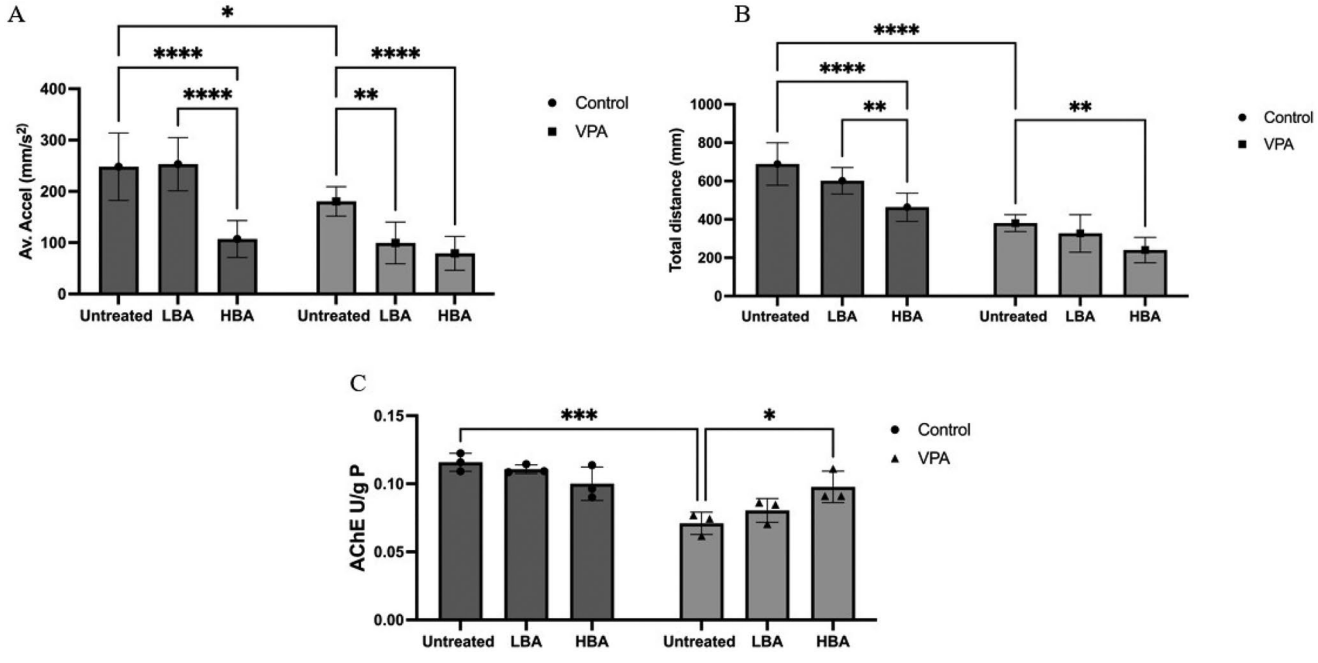

Figure 4. (A) Average acceleration ($n = 10$), (B) Total distance measurements ($n = 10$) and (C) Acetylcholinesterase (AChE) activities of the groups. For AChE activity data are expressed as mean ± SD from the three independent experiments ($n = 3$, 3 biological replicates for each group, 50 embryos/Pool). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; LBA: low-benzoic acid; HBA: high-benzoic acid; SD: standard deviation.

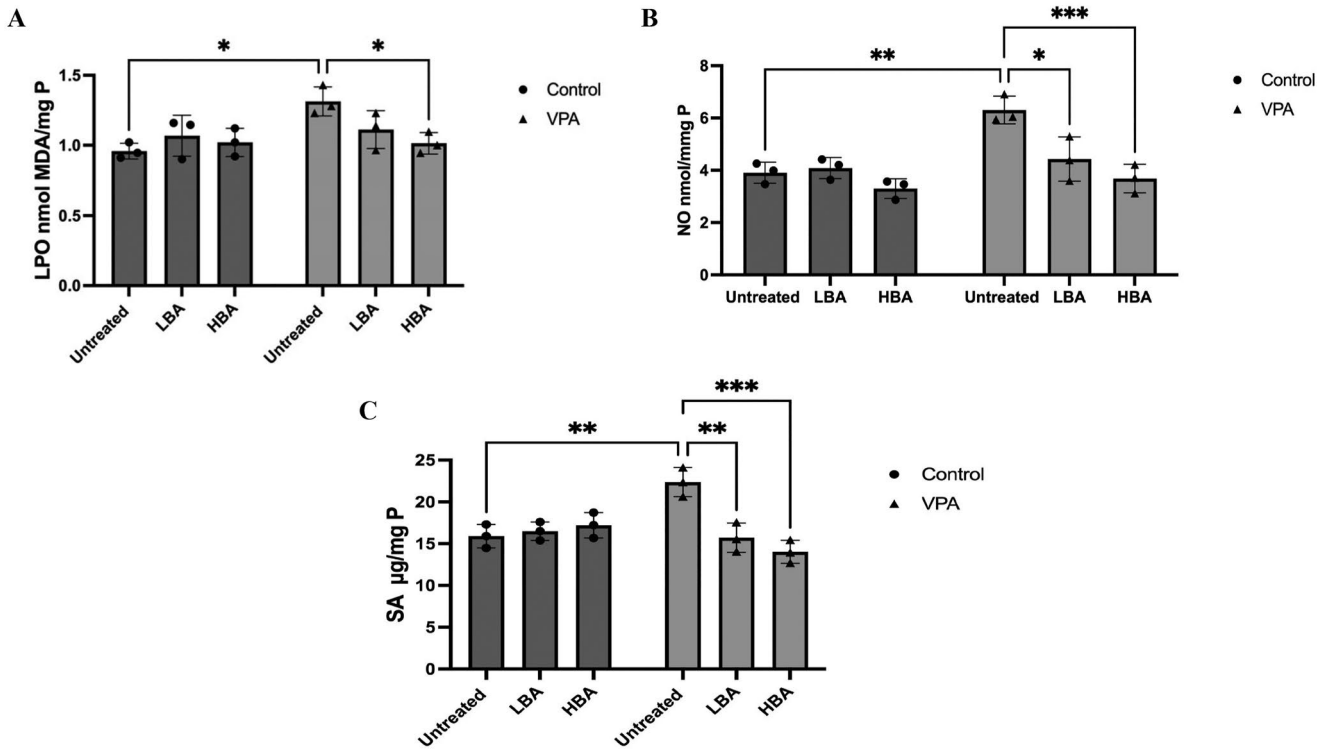

Figure 5. (A) MDA levels that serve as an index of lipid peroxidation (LPO); (B) Nitric oxide (NO) levels; (C) Sialic acid (SA) levels. Data are given as mean ± SD resulting from the three independent experiments ($n = 3$, 3 biological replicates for each group, 50 embryos/Pool). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$ SD: standard deviation.

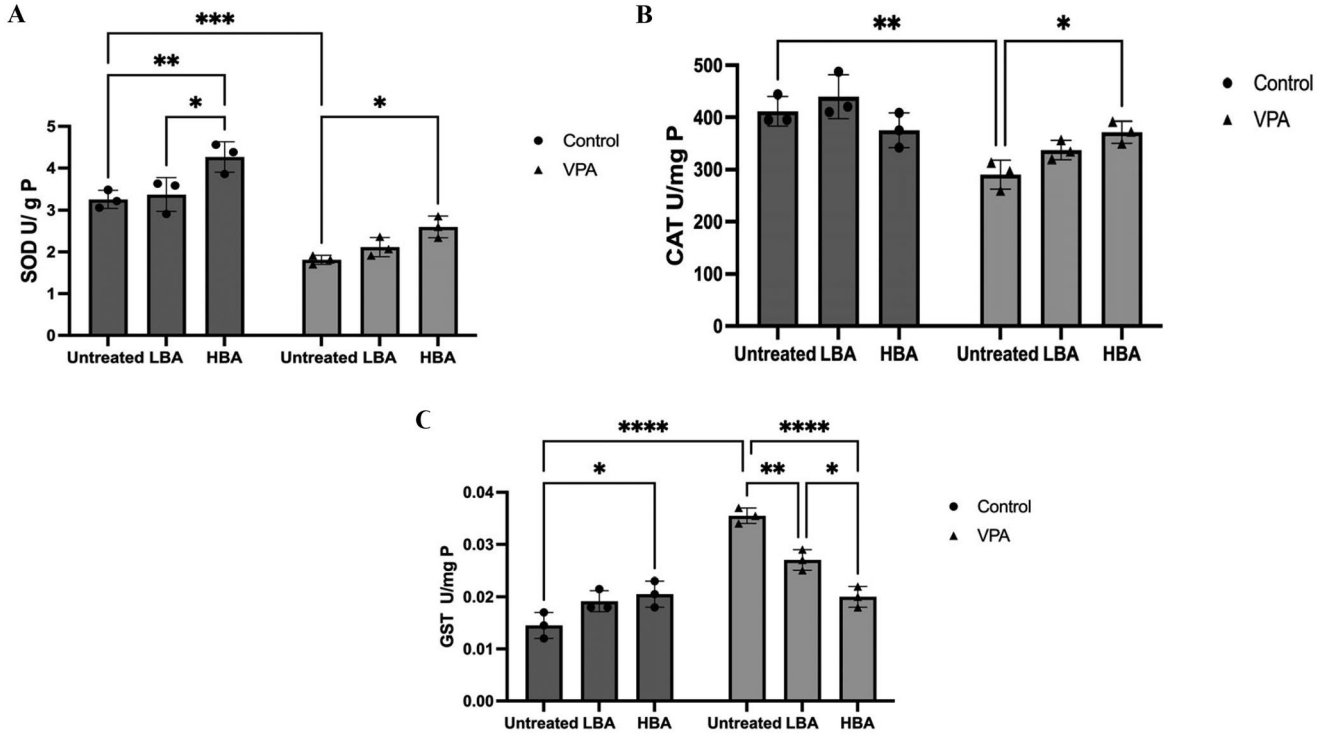

Figure 6. (A) Catalase (CAT) activities; (B) Superoxide dismutase (SOD) activities, and (C) Glutathione S-transferase activities of the groups. Data are given as mean ± SD resulting from the three independent experiments ($n = 3$, 3 biological replicates for each group, 50 embryos/Pool). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$ SD: standard deviation.

molecular markers of toxicity. LPO is an indicator of elevated free radical levels. Its increased level is a key sign that cell membranes are deteriorating, which may start with the administration of VPA (Tong et al. 2005; Cho et al. 2014). Consistent with our study, Tong et al. (2005) demonstrated that the LPO level increased in the liver tissues of rats applied

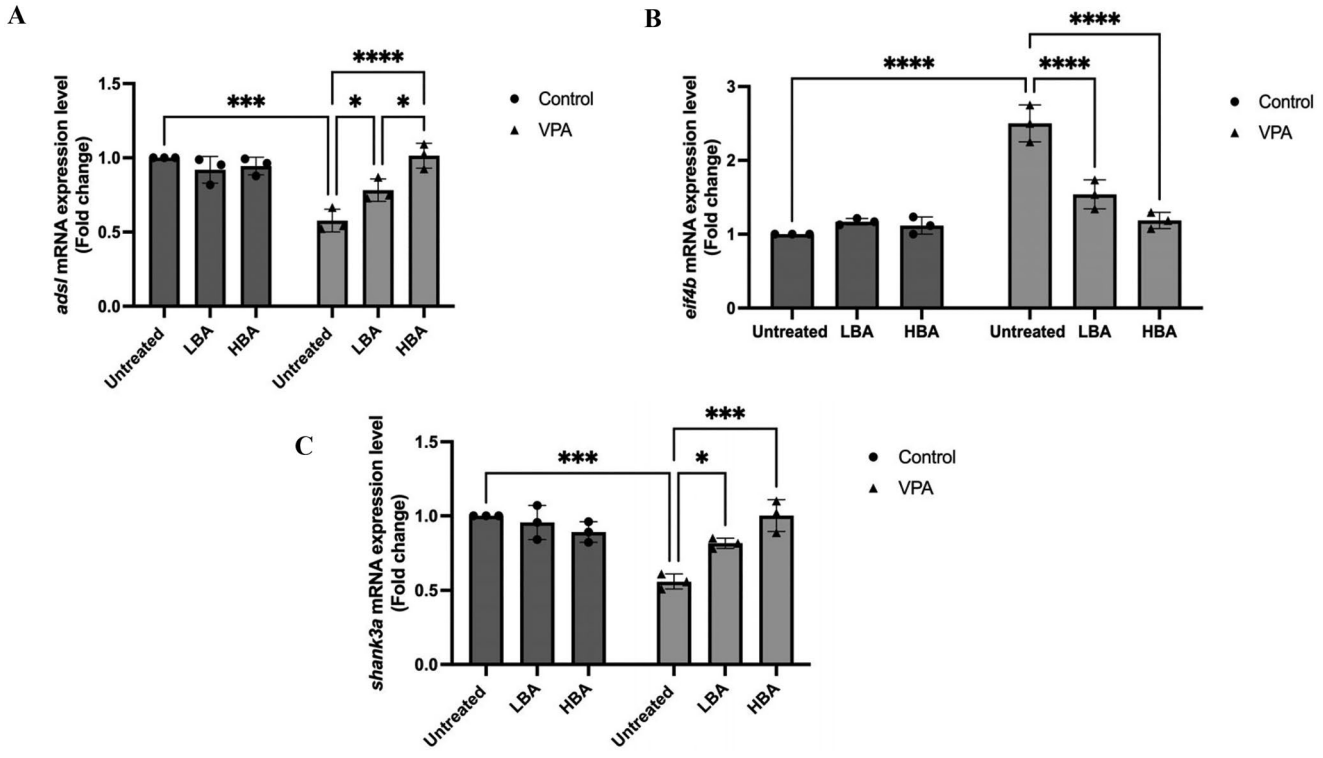

Figure 7. Bar graph presentations of the fold change of the RT-PCR-quantified transcripts of (A) *adsl* (B) *eif4b*, and (C) *shank3a*. All RT-PCR results are expressed as changes from their respective controls after being normalized to the housekeeping gene -actin. Three studies ($n = 3$, 3 biological replicates for each group, 50 embryos/Pool) were used to calculate the average values. Data presented are mean ± SD. *$p < 0.05$; ***$p < 0.001$; ****$p < 0.0001$ SD: standard deviation.

VPA. Higher ROS levels following VPA administration have also been shown by Tunali et al. (2020) in VPA-induced brain damage and by Türkyılmaz et al. (2023) in the livers of the VPA-administered rats. In accordance with the increased NO levels in our study, by blocking the CDK5-Tyr(15)-eNOS-Ser(116) phosphorylation axis, mediated by SH-PTP1, VPA has been shown to enhance the generation of NO (Cho et al. 2014).

In addition to increased oxidative stress markers, in our study, a decrease in SOD and CAT activities, which are antioxidant defense system components, was detected in embryos given VPA. Different from our findings decreased activities of SOD and CAT have been reported in liver tissues of the VPA-exposed rats (Türkyılmaz et al. 2023). This may be due to the different effects of VPA exposure in adulthood, when antioxidant defense systems are better developed, and exposure in the embryonic period. On the other hand, increased GST activity in the VPA group may be suggested to be a protective mechanism to combat increased oxidative stress which was reflected by increased MDA level and depressed SOD and CAT activities in the same group.

The brown bark of the cinnamon tree is used as a spice and flavoring for a variety of foods, including chocolate, sweets, and desserts. It also has a lengthy history of use as medicine. Cinnamon was a common ingredient in medieval medications used to treat a wide range of ailments such as sore throats, arthritis, coughing, and hoarseness. It was formerly worth so much that it was the cause of multiple wars. Apart from its manganese, dietary fiber, iron, and calcium content, cinnamon also has a significant component called cinnamaldehyde, which undergoes oxidation to produce cinnamic acid. Cinnamic acid then undergoes β-oxidation in the liver to produce benzoate, which is then present as sodium salt, sodium benzoate, or benzoyl-CoA (Jana et al. 2013; Abd El-Mawla et al. 2001). There have been reports that humans' urine also excretes a trace amount of sodium benzoate (Kubota and Ishizaki 1991).

In our study, we used benzoic acid synthesized from *Cinnamomum Cassia* by green chemistry method on gene expressions related to autism development in case of VPA toxicity. Low and high doses of benzoic acid, which were determined to be below the lethal dose, had different effects on zebrafish embryos. While high doses of benzoic acid inhibited the locomotor activities of the embryos, low doses of benzoic acid did not cause a significant change. However in the VPA given group although both low and high doses of benzoic acid decreased average acceleration rates, low dose benzoic acid did not change total distance and AChE activities.

A significant reduction of AChE activity was observed in the VPA which was also reflected in the reduced acceleration and decreased total distance. On the other hand, HBA treatment caused a significant decreased in locomotion although AChE activity did not change significantly in the same group. Moreover in the HBA-treated VPA group AChE activity increased while locomotion decreased. The balance between the dopaminergic system and cholinergic system plays an important role in maintaining locomotor activity (Myslivecek, 2021). An increase in AChE activity with benzoic acid treatment may indicate increased acetylcholine release which may be related with the alterations in dopamine levels due to benzoic acid treatment as dopamine has been shown to inhibit the release of acetylcholine

(Myslivecek, 2021; Cansız et al. 2023). The disruption of the balance between the dopaminergic and cholinergic systems can explain the decrease in locomotor activity. On the other hand, to establish the relationship between locomotor activity and AChE in the case of VPA and benzoic acid treatments, it is necessary to measure acetylcholine levels.

On the other hand, benzoic acid had an improving effect on the oxidant–antioxidant balance that was disrupted by VPA application. In the VPA group, benzoic acid was effective in normalizing the increase in oxidant parameters LPO and NO when administered at both low and high doses. Moreover, the activities of antioxidant enzymes SOD and CAT increased. GSTs are the type of enzymes that catalyze the reaction between GSH and electrotrophic substances, particularly those resulting from the biotransformation of exogenous xenobiotics (Hayes et al. 2005). While GST enzyme activity increased to ensure VPA-dependent detoxification, it decreased with benzoic acid application.

Decreased SA levels by benzoic acid treatment in the VPA-exposed embryos are another important finding of our study. The production of inflammatory cytokines including TNF-α is linked to SA (Doostkam et al. 2022). Acute-phase reactants containing SAs increase in inflammatory processes. Moreover, ROS can be scavenged by SA, which therefore inhibits the modification of biological macromolecules (Doostkam et al. 2022). In parallel with our study, Üstündağ et al. (2023) revealed that SA levels increased in zebrafish embryos exposed to 1 mM VPA.

In the VPA-exposed embryos, we also found alterations in genes linked to autism, namely *shank3*, *adsl*, and *eif4eb*. As a mediator between neural activity and protein translation, eiF4 regulates synaptic plasticity. In our study, we observed that *eif4b* expression increased with VPA exposure and both benzoic acid treatments decreased *eif4b* expressions. This result is significant because it has been demonstrated that overexpressing the translation initiation factor eiF4e in brain cells to enhance protein synthesis results in autism-like behavior in mice (Bettegazzi et al. 2017).

In our study, VPA exposed group showed reduction in *adsl2a* expressions which was ameliorated by both low and high doses of benzoic acid treatments. *shank3a* expression declines were noticeable in the VPA group. The enzyme adenylosuccinate lyase, which is required for *de novo* purine synthesis, is encoded by the *adsl2* gene (Lee et al. 2018). Different from our results, Lee et al. (2018) showed increased *adsl* expression increased and decreased shank3 expression in an experimental autism model generated by VPA in zebrafish. The difference from our results may be due to the alterations in the exposure periods as in the study of Lee et al. (2018) zebrafish were exposed to VPA for 120 h.

Both low and high doses of benzoic acid treatments ameliorated the expression of *shank3a,* coding a crucial molecule linked to the onset and progression of autism, which was decreased by VPA. Recently, Zhang et al. (2024) showed that *shank3* inhibited post-ischemia/reperfusion neuronal oxidative stress and inflammation response through *nrf2* pathway. Consistent with this report, our results showed that benzoic acid treatment had a positive effect on oxidant–antioxidant balance as well as increasing shank expression.

While VPA has a number of adverse consequences, teratogenic effects are thought to be the most serious. It is widely established that prenatal exposure to VPA causes developmental neurotoxicity in the central nervous system of children. Moreover, symptoms resembling ASD have been linked to prenatal exposure to VPA in both human and animal models (Wang et al. 2024; Li et al. 2024). In order to find new possible treatment candidates, it would be beneficial to analyze the molecular mechanism of VPA-induced ASD. Based on this point, the results of our study have shown for the first time in the literature that the treatment of benzoic acid synthesized with green chemistry in case of VPA toxicity ameliorated the VPA-induced disrupted oxidant–antioxidant system balance and inflammation in zebrafish embryos and is effective in improving the impaired expression of autism-related genes. Based on the positive effects of benzoic acid on VPA toxicity in zebrafish embryos, it can be suggested that natural sources rich in benzoic acid, such as cinnamon cassia oil, may be a suitable candidate for research on treatment strategies in the treatment of autism.

On the other hand, there is a need for more detailed research on the mechanisms of the changes caused by benzoic acid in locomotor activity and the associated changes in AChE activities. One of the limitations of our study is that the expressions of ASD development-related genes *aridib*, *chd8*, *dyrk1a*, and *syngap1*, and oxidative stress-related gene *nrf2* were not examined. We plan to validate our results by examining the expressions of these genes and also validate oxidative stress using DCF-DA in our future studies.

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Ethics approval statement

As the zebrafish embryos used were no older than 5 d old, no ethical approval was required for the protocols applied as stated by the Council of Europe (2010), EU Directive 2010/63/EU.

Consent to participate

All the authors have agreed for authorship, read and approved the manuscript, and given consent to participate.

Consent for publication

All the authors have agreed for authorship, read and approved the manuscript, and given consent for publication.

Author contributions statement

Conception/Design of study: Ebru Emekli-Alturfan; Derya Cansız Data Acquisition: Derya Cansız, Gökhan Özokan, Abdulkerim Bilginer, Semanur Işıkoğlu, Zülal Mızrak, İsmail Ünal, Merih Beler; Data Analysis/ Interpretation: Ebru Emekli-Alturfan, Derya Cansız Drafting Manuscript:: Ebru Emekli-Alturfan; Derya Cansız; Critical Revision of Manuscript: A.Ata Alturfan, Ebru Emekli-Alturfan; Supervision: Ebru Emekli-Alturfan.

Disclosure statement

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Data availability statement

Data is available on reasonable request.

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