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Original Article

# Therapeutic Potential of Trigonelline in Alleviating Valproic Acid–Induced Autism-Like Symptoms in an Animal Model: Investigating Its Antioxidative Stress Effects

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**Abstract**

**Introduction:** Oxidative stress (OS) is a contributing factor in the pathophysiology of autism spectrum disorder (ASD). Given the well-documented antioxidant and neuroprotective properties of trigonelline (TRI), this study investigated whether TRI mitigates valproic acid (VPA)-induced ASD-like behavioral abnormalities in rats via its antioxidant mechanism.

**Methods:** A single subcutaneous dose of VPA (600 mg/kg) was administered to pregnant Wistar rats (n = 14) on gestational day 12 to establish a VPA-induced ASD-like model. Then, starting from postnatal day 40, the male pups underwent a one-week treatment regimen via intraperitoneal injection of normal saline (10 mL/kg) or TRI at 10, 50, and 100 mg/kg (n = 6 per group). To evaluate neuropsychological functions, the marble burying test (MBT), elevated plus maze (EPM), shuttle box test, and three-chamber test were employed to assess repetitive behaviors, anxiety-like symptoms, memory capacity, and social interactions, respectively. Furthermore, OS markers, specifically malondialdehyde (MDA) levels and total antioxidant capacity (TAC), were quantified in the serum and hippocampus.

**Results:** The administration of VPA induced a range of behavioral abnormalities, specifically anxiety-like symptoms in the EPM ($P < 0.01$), memory impairments in the shuttle box ($P < 0.01$), repetitive patterns in the MBT ($P < 0.01$), and social dysfunction in the three-chamber test ($P < 0.001$). TRI reversed these behavioral alterations. Moreover, TRI increased hippocampal and serum TAC but reduced MDA concentrations within the hippocampal tissue and serum samples of ASD rats ($P < 0.001$).

**Conclusion:** Our findings revealed that TRI mitigated ASD-like behavioral abnormalities in rats, partially by alleviating OS markers in the hippocampus and serum.

**Keywords:** Trigonelline, Autism spectrum disorder, Oxidative stress, Valproic acid, Neuroprotection

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## Introduction

Autism spectrum disorder (ASD) is a complex neurodevelopmental condition characterized by a multifaceted clinical presentation. Its core hallmarks include profound deficits in social-communicative functioning encompassing both verbal and nonverbal communication, coupled with restricted and repetitive behavioral patterns and highly circumscribed interests (1). In addition, individuals with ASD frequently experience anxiety and depression (2).

A range of teratogenic agents, including valproic acid (VPA), ethanol, and thalidomide, have been implicated in increasing the likelihood of ASD development. VPA is widely prescribed for the management of neurological conditions such as epilepsy and seizure disorders. However, prenatal exposure to VPA can result in valproate syndrome, first described in 1984, which encompasses a spectrum of major and minor abnormalities affecting growth, neurodevelopment, and behavior (3). Additionally, it has been verified that prenatal exposure of pregnant rodents to VPA leads to the induction of ASD in offspring, indicating that VPA-induced ASD in rodents is a valid model for the study of autism (4).

The accumulation of reactive oxygen species can cause significant oxidative impairments within biological systems, which serves as a precursor to numerous chronic pathologies. Given its elevated oxygen consumption and high concentration of polyunsaturated fatty acids (PUFAs), the central nervous system exhibits a heightened vulnerability to oxidative-mediated damage. When PUFAs undergo lipid peroxidation, they generate malondialdehyde (MDA), a widely recognized indicator of oxidative stress (OS). Similar to other organs, the brain relies on antioxidant defense systems for protection. One key component of this defense is its total antioxidant capacity (TAC), which helps counteract oxidative damage. However, when OS surpasses the brain's antioxidant capacity, the imbalance leads to cellular injury and contributes to neurological dysfunction (5), suggesting that an imbalance between oxidant generation and antioxidant defenses in the brain increases its vulnerability to damage caused by OS (6-12).

In this regard, the literature has shown that agents that

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may alleviate ASD can do this by decreasing MDA and increasing TAC in the brain (12, 13). In recent decades, researchers have increasingly studied natural compounds derived from medicinal plants for various diseases, including neurological disorders (14-16). TRI is an alkaloid identified in numerous plants, particularly in the seeds of the *Fabaceae* family, including fenugreek, coffee, barley, melon, maize, onion, chickpeas, and soybeans (17).

TRI has been reported to have multiple pharmacological properties, including antidepressant, anticonvulsant, anti-inflammatory, and antioxidative stress effects (18-20). Given the role of OS in ASD development and the antioxidant properties of TRI, this study aims to examine whether TRI could alleviate VPA-induced autism-like behaviors, with particular emphasis on its potential to counteract OS.

## Materials and Methods

### *Animals*

Twenty-four Wistar rats (6 males and 18 females; weight: 200–250 g; age: 10–11 weeks) were obtained from the Royan Institute (Isfahan, Iran). The animals were housed at Shahrekord University of Medical Sciences under controlled environmental conditions, including a 12-hour light/dark cycle and a constant temperature (22 ± 122/pm 122 ± 1°C). Further, standard laboratory chow and water were provided ad libitum. It is noteworthy that all experimental protocols were conducted in strict accordance with the National Institutes of Health guidelines for the ethical treatment of laboratory animals.

### *Pregnancy and Induction of the Autism Model*

Rats were housed in cages containing one male and three females. To monitor estrous cycles, vaginal smears were collected and examined daily; the presence of spermatozoa was utilized to define gestational day (GD) 1. Following successful mating, pregnant females were randomly assigned to one of the two experimental groups. The saline control group (n = 4) received a subcutaneous injection of normal saline (10 mL/kg) on GD12, and the VPA-induced ASD group (n = 14) received a single subcutaneous dose of (VPA; 600 mg/kg) on GD12. It should be noted that the number of embryo resorptions and neonatal mortality typically varies among individual rats. On average, each dam produces around eight pups, commonly consisting of approximately four males and four females. In this study, 14 pregnant females were selected to ensure an adequate number of offspring exhibiting autism-like characteristics. Following weaning at postnatal day 21, the male pups were distributed into groups based on the predefined experimental protocols.

### *Experimental Groups*

Treatments began on postnatal day 40, corresponding to early adulthood, and continued for one week. In addition, TRI was obtained from Sigma-Aldrich (USA; CAS No. 6138-41-6), with a purity greater than 98.5% (high-performance liquid chromatography). The compound was prepared in saline containing 1% dimethyl sulfoxide (DMSO), following previously published protocols (21, 22).

Group 1 (n = 6, control animals) received saline with 1% DMSO (10 mL/kg, intraperitoneally). Further, VPA-exposed rats in group 2 (n = 6) were administered the same saline/DMSO vehicle (10 mL/kg, i.p.). In group 3 (n = 6), animals exposed to VPA received a 100 mg/kg dose of TRI via intraperitoneal (i.p.) injection. Furthermore, VPA-exposed rats in group 4 (n = 6) received 50 mg/kg of TRI via intraperitoneal (i.p.) injection. Finally, in group 5 (n = 6), animals exposed to VPA were intraperitoneally injected with a 10 mg/kg dose of TRI.

The dosing regimen and treatment duration were selected based on findings from our pilot experiments and prior literature (23). According to some previous studies, TRI has a biological half-life of approximately 5 hours (24-27) and exhibits notable neuroprotective properties. Likewise, TRI is capable of crossing the brain-blood barrier due to its high lipid solubility, allowing it to reach the brain and exert protective effects even after a single administration (28). Similarly, this alkaloid has a wide distribution and can rapidly enter all tissues (18, 20).

### *Behavioral Tests Schedule*

Behavioral evaluations were conducted following a seven-day treatment period, covering postnatal days 47 through 50. To maintain consistency across all subjects, each rat completed the entire set of behavioral tasks in a predetermined sequence. The tests were performed in this regard included three-chamber sociability test, marble burying test, elevated plus maze (EPM), and finally the shuttle box test, respectively. This organized progression enabled systematic assessment of social interaction, repetitive behaviors, locomotor and anxiety-related responses, as well as passive avoidance learning in a controlled and standardized manner.

### *Three-Chamber Sociability Test*

Social interaction was evaluated using a three-chamber Plexiglas apparatus (120 × 40 × 50 cm), comprising a middle compartment flanked by two equivalent side chambers. Following a 5-minute habituation period in the center zone, sociability was assessed by introducing a novel, gender-matched and age-matched conspecific (social stimulus) into one side chamber, while the opposite chamber contained an empty wire enclosure (non-social stimulus). The subject was allowed 10 minutes of exploration, during which time spent in each zone and interaction durations were recorded. Furthermore, the sociability index (SI) was computed as: (social stimulus 1 non-social stimulus)/(social stimulus 1 + non-social stimulus). To evaluate social preference, the previously vacant chamber was replaced with a second, unfamiliar, age-matched and gender-matched rat (social stimulus 2). After another 10-minute exploration period, the social preference index (SPI) was calculated: SPI = (time with social stimulus 2 –time with social stimulus 1) / (time with social stimulus 2 + time with

social stimulus 1). It should be noted that SI and SPI served as the primary parameters to characterize social behavioral profiles (29-31).

### *Elevated Plus Maze*

Anxiety-like tendencies were assessed using an EPM assembly, comprising a plus-shaped configuration of two open and two closed arms, positioned 50 cm above the ground. During each 5-minute exploration period, rats were positioned on the central platform with their orientation toward a closed arm. Moreover, behavioral metrics, including the frequency of arm entries and time intervals spent in both open and closed arms, were manually documented. An entry was strictly defined as the moment the animal's four paws crossed into an arm, and higher durations within the closed arms were indicative of increased anxiety. To ensure consistency and mitigate olfactory cues, the maze was sanitized with 70% ethanol between trials. Additionally, all procedures were performed in a controlled, low-noise, and dimly lit environment (32).

### *Marble Burying Test*

Animals were subjected to the marble-burying task, a well-established paradigm for quantifying compulsive-like behavior. Twenty marbles were placed on the bedding of a Plexiglas cage in a consistent grid pattern. Each rat was allowed 20 minutes to freely explore the cage. A marble was considered buried when at least two-thirds of its surface was obscured by the bedding; subsequently, the total count of buried marbles was documented (33).

### *Shuttle Box Test*

The animals' passive avoidance memory was measured in a standard shuttle box, a two-compartment device (light and dark sides) joined by a gate and built with electrified grid floors. The experimental protocol spanned four days. The acquisition phase commenced on the third day following an initial 48-hour habituation period, during which rats explored the device freely for five minutes per session. In this phase, rats were introduced into the illuminated compartment; upon crossing the door into the dark zone, they received a transient 1-second electrical stimulus (1 mA). Then, the latency to traverse into the dark chamber was measured. During the retention test, the time taken for the rats to enter the dark compartment (capped at 60 seconds) was recorded on the fourth day. To prevent olfactory or sensory interference, the apparatus was sanitized between each trial (34).

### *Euthanasia and Sample Collection*

Once the behavioral testing battery was completed, deep anesthesia was induced in the rats by administering ketamine (60 mg/kg) in combination with xylazine (15 mg/kg) (35). Once an adequate depth of anesthesia was confirmed, blood was obtained through cardiac puncture. In addition, the brains were excised following blood collection, and the hippocampal tissue was carefully isolated. The samples were promptly frozen and maintained at −70°C until further analysis. Likewise, blood specimens were centrifuged at 3,200 rpm for 5 minutes. The resulting plasma fraction was then transferred into designated storage tubes and preserved at −70°C for subsequent biochemical assays.

### *Hippocampal Isolation*

After opening the skull using appropriate surgical instruments, the brain was gently removed and transferred to a chilled Petri dish placed on ice. Next, the brain was separated into two hemispheres, and the hippocampi were precisely isolated from their anatomical regions. Each hippocampus was homogenized with a mechanical homogenizer in 0.03 M phosphate-buffered saline buffer (pH: 7.4) in order to prepare the tissue for subsequent biochemical analyses (36, 37).

### *Determination of Total Antioxidant Capacity*

TAC was determined in serum and hippocampal tissue homogenates employing the ferric reducing ability of plasma method. Briefly, the assay exploits the ability of antioxidants to reduce $Fe^{3+}$-TPTZ complexes to $Fe^{2+}$ under specified conditions (37°C, pH: 3.6), producing a blue chromophore whose intensity is proportional to antioxidant content. Absorbance measurements were taken at 593 nm on an automated plate reader (LQ-300 + II-Epson, USA), and concentrations were derived from a standard curve and reported as µg/mL (38).

### *Measurement of Malondialdehyde*

MDA, the principal end product of PUFA peroxidation, was measured in serum and hippocampal homogenates via the thiobarbituric acid-reactive substance procedure. The reaction was performed by mixing 100 µL of sample with 900 µL of Tris–KCl buffer, followed by the addition of 500 µL of 30% trichloroacetic acid and 500 µL of 0.75% thiobarbituric acid. After incubation at 80°C for 45 minutes in a water bath and centrifugation at 3,000 rpm for 5 minutes, the absorbance of the clear supernatant was read at 562 nm on an enzyme-linked immunosorbent assay plate reader. Finally, values, normalized to protein content, were reported as nmol/mg protein (39, 40).

### *Statistical Analysis*

GraphPad Prism 8 was utilized for all statistical computations. Before conducting parametric analyses, data normality and variance homogeneity were confirmed using the Kolmogorov–Smirnov and Brown–Forsythe tests. Further, one-way analysis of variance (ANOVA), followed by Tukey's post-hoc analysis, was used to identify significant differences between groups. Values are expressed as means ± standard deviations (SD), and a $P$ value of less than 0.05 indicated statistical significance. Furthermore, G*Power software (version 3.1.7) was employed to calculate the necessary sample size for a power of 0.8 at an alpha level of 0.05

## Results

### *Trigonelline Increased the Sociability Index and Social Preference Index in Valproic Acid-Exposed Rats*

Significant variations in the SI across the experimental groups were observed via one-way ANOVA [F (4, 15) = 10.34, $P = 0.0003$]. Post hoc analysis using Tukey's test revealed a significant reduction in the SI within the VPA + saline group compared to the control + saline group ($P < 0.001$). Interestingly, administration of TRI (10 mg/kg, 50 mg/kg, and 100 mg/kg) to VPA-treated animals resulted in a substantial recovery of the SI when compared with the VPA-only group ($P < 0.05$, $P < 0.001$, and $P < 0.01$, respectively; Figure 1A). SPI followed a similar pattern, with a one-way ANOVA confirming significant between-group differences [F (4, 15) = 15.71, $P < 0.0001$]. Based on the results of Tukey's post-hoc analysis (Figure 1B), the VPA + saline group had a significantly lower SPI in comparison with the control + saline group ($P < 0.001$). Additionally, administration of TRI at 50 mg/kg and 100 mg/kg significantly elevated the SPI in VPA-treated animals relative to the untreated VPA group ($P < 0.001$).

### *Trigonelline Reduced Repetitive Behaviors in the Marble Burying Test*

One-way ANOVA revealed significant variations in repetitive behaviors among the various experimental groups [F (4, 25) = 13.71, $P < 0.0001$]. Tukey's post-hoc analysis demonstrated that the VPA-treated group exhibited a considerable elevation in repetitive behaviors compared with the saline-treated controls ($P < 0.001$). Notably, TRI administration at 50 mg/kg and 100 mg/kg significantly reduced repetitive behaviors in VPA-exposed rats ($P < 0.01$), whereas the 10 mg/kg dose yielded no statistically significant change (Figure 2).

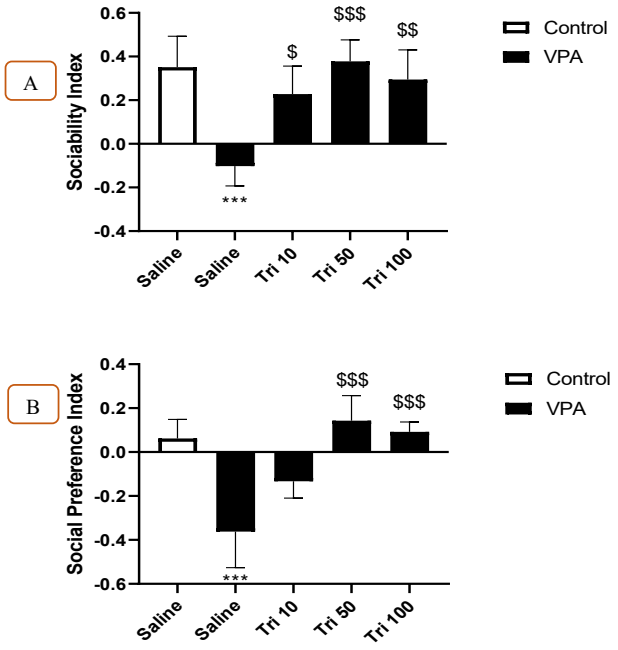


**Figure 1.** Impact of TRI on Social Behavior Across Experimental Groups: **(A)** Sociability Index and **(B)** Social Preference Index
*Note*. TRI: Trigonelline; VPA: Valproic acid; ANOVA: Analysis of variance. Values are expressed as means ± standard deviations. Statistical significance was determined by one-way ANOVA followed by Tukey's post-hoc test. $^{***}P < 0.001$ versus the saline-treated control group; $^{\$}P < 0.05$, $^{\$\$}P < 0.01$, $^{\$\$\$}P < 0.001$ versus the VPA + saline group.

### *Trigonelline Increased Latency to Enter the Dark Compartment in the Shuttle Box Test*

One-way ANOVA showed that no significant intergroup variations existed for T1 [F (4, 18) = 1.862, $P = 0.1611$; Figure 3A]. Conversely, T2 values exhibited a significant group effect [F (4, 15) = 9.708, $P = 0.0004$]. According to Tukey's post-hoc analysis, the VPA-treated saline group represented a substantially reduced secondary latency (T2) in comparison to the saline control group ($P < 0.01$). Notably, TRI administration at doses of 50 mg/kg and 100 mg/kg effectively enhanced secondary latency in VPA-exposed rats relative to the VPA-only group ($P < 0.05$ and $P < 0.01$, respectively). In contrast, the 10 mg/kg TRI dose failed to yield a significant improvement in T2 latency (Figure 3B).

### *Trigonelline Treatment Increased Open-Arm Entries and Time Spent in the Open Arms in the Elevated Plus Maze*

Remarkable variations were observed between groups in both open-arm exploration parameters. One-way ANOVA for open-arm entries revealed a significant main effect [F (4, 15) = 6.940, $P = 0.0023$), with post hoc analyses indicating that VPA-saline-treated rats had significantly fewer entries than controls ($P < 0.01$). While TRI administration at 100 mg/kg substantially rescued this behavior ($P < 0.05$), the 10 mg/kg dose failed to elicit a significant response (Figure 4A). Similarly, a significant group effect was found for the time spent in the open arms [F (4, 15) = 17.04, $P < 0.0001$]. Post-hoc analysis demonstrated that the VPA-saline group exhibited sharply reduced open-arm exploration compared to the control group ($P < 0.001$), a deficit that was markedly attenuated by TRI at doses of 50 mg/kg and 100 mg/kg ($P < 0.01$). Conversely, the 10 mg/kg TRI dose did not considerably impact this parameter (Figure 4B).

### *Trigonelline Enhanced Hippocampal Total Antioxidant Capacity Level*

One-way ANOVA confirmed significant variations in

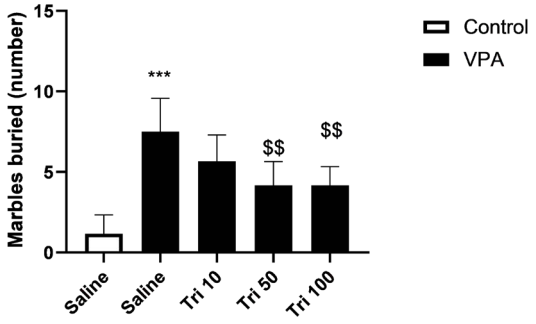


**Figure 2.** Impact of TRI Administration on Repetitive Behaviors in Experimental Groups
*Note*. TRI: Trigonelline; VPA: Valproic acid; ANOVA: Analysis of variance; SD: Standard deviation. The results are presented as means ± SD. Significant differences were identified using one-way ANOVA and Tukey's post-hoc analysis. $^{***}P < 0.001$ compared to the control-received saline group; $^{\$\$}P < 0.01$ compared to the VPA-treated group.

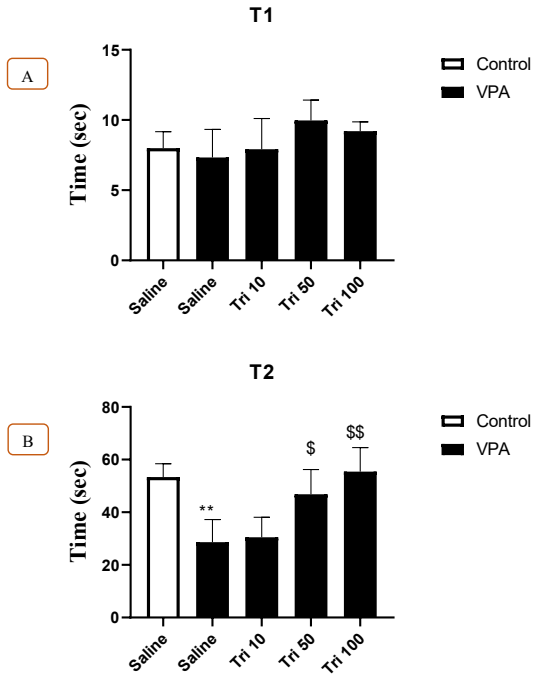


**Figure 3.** Effects of TRI on Latency Parameters in the Experimental Groups: **(A)** Initial Latency and **(B)** Step-Through (Secondary) Latency
*Note*. TRI: Trigonelline, VPA: Valproic acid; ANOVA: Analysis of variance; SD: Standard deviation. Values are presented as means ± SD. Statistical differences were determined by one-way ANOVA followed by Tukey's post-hoc test. $^{**}P<0.01$ compared to the control-received saline group; $^{\$}$ $P<0.05$ and $^{\$\$}$ $P<0.01$ in comparison to the VPA-received saline group.

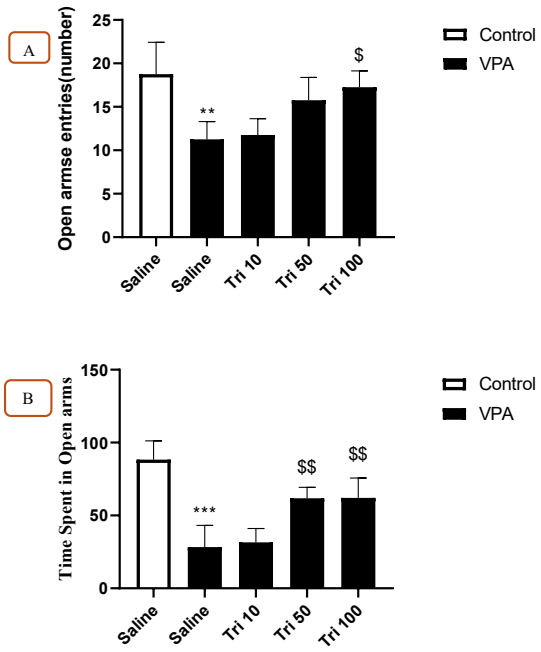


**Figure 4.** Effects of TRI on Open-Arm Exploration in the Experimental Groups: Percentage of Open-Arm Entries **(A)** and Percentage of Time Spent in Open Arms **(B)**
*Note*. TRI: Trigonelline, VPA: Valproic acid; ANOVA: Analysis of variance; SD: Standard deviation. Data, provided as means ± SD, were analyzed by one-way ANOVA followed by Tukey's post-hoc test. $^{**}P<0.01$ and $^{***}P<0.001$ indicate significant differences compared to the control saline group; $^{\$}$ $P<0.05$ and $^{\$\$}$ $P<0.01$ denote substantial differences relative to the VPA-saline group.

hippocampal TAC across the experimental cohorts [F (4, 25) = 21.01, $P<0.0001$]. Based on post-hoc comparison results using Tukey's test, VPA administration could significantly deplete hippocampal TAC compared to the saline-treated control group ($P<0.001$). Remarkably, TRI supplementation at doses of 50 mg/kg and 100 mg/kg effectively rescued this antioxidant deficit, substantially elevating TAC levels in VPA-exposed rats relative to the VPA-only group ($P<0.001$). However, no statistically significant increase in TAC was observed following the administration of 10 mg/kg TRI (Figure 5).

### *Trigonelline Enhanced Serum Total Antioxidant Capacity Level*

One-way ANOVA confirmed significant differences in serum TAC among the experimental groups [F (4, 25) = 25.93, $P<0.0001$; Figure 6]. Post-hoc analysis revealed that VPA-saline treatment could significantly diminish serum TAC levels in comparison to the control-saline group ($P<0.001$). The observed VPA-induced reduction in serum TAC was substantially attenuated following treatment with 50 mg/kg and 100 mg/kg of TRI ($P<0.01$). In contrast, TRI at 10 mg/kg produced no significant improvement in serum TAC.

### *Trigonelline Decreased Hippocampal Malondialdehyde Level*

One-way ANOVA demonstrated significant differences in hippocampal MDA concentrations among the experimental groups [F (4, 55) = 80.97, $P<0.0001$; Figure 7]. Tukey's post-hoc analysis also confirmed that VPA exposure significantly induced an elevation in hippocampal MDA levels relative to the saline-treated control group ($P<0.001$). Conversely, TRI treatment remarkably attenuated these OS markers in a dose-dependent manner, with doses of 10 mg/kg, 50 mg/kg, and 100 mg/kg yielding reductions relative to the VPA-only group ($P<0.01$ and $P<0.001$, respectively).

### *Trigonelline Reduced Serum Malondialdehyde Levels*

One-way ANOVA revealed considerable intergroup disparities in serum MDA concentrations [F (4, 25) = 30.99, $P<0.0001$; Figure 8). Although VPA exposure markedly escalated lipid peroxidation, manifested by a substantial rise in MDA levels compared to the saline control ($P<0.001$), TRI intervention demonstrated dose-dependent efficacy in mitigating this oxidative insult. Notably, TRI administration at doses of 50 mg/kg and 100 mg/kg significantly attenuated serum MDA concentrations relative to the VPA-only group ($P<0.01$ and $P<0.001$, respectively), whereas the 10 mg/kg dose failed to yield a remarkable therapeutic reduction.

## Discussion

ASD represents a prevalent neurodevelopmental condition, affecting upwards of 1% of the global population. It is fundamentally characterized by enduring deficits in social communication and the presence of restricted, repetitive patterns of behavior. Despite its relatively high prevalence, no definitive or highly effective treatment has yet been established for ASD (41). A substantial body of research points to a strong association between OS and ASD (42, 43), indicating that reducing OS may offer therapeutic

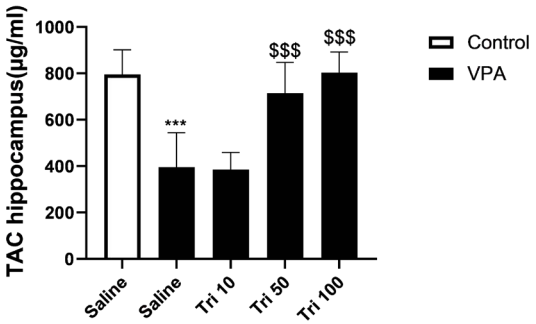


**Figure 5.** Effect of TRI on Hippocampal TAC Across Experimental Groups
*Note*. TRI: Trigonelline, VPA: Valproic acid; ANOVA: Analysis of variance; SD: Standard deviation; TAC: Total antioxidant capacity. Data are presented as means ± SD. Statistical significance was found by one-way ANOVA followed by Tukey's post-hoc test. $^{***}P<0.001$ indicates a significant difference versus the control-saline group, whereas $^{\$\$\$}P<0.001$ represents a significant difference versus the VPA-saline group.

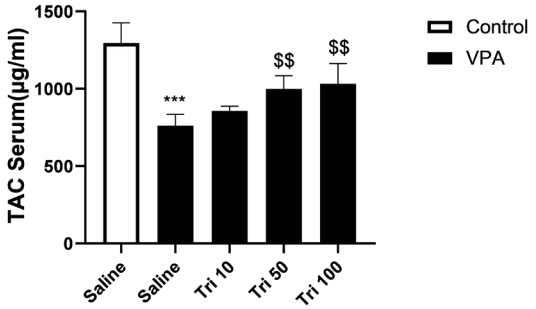


**Figure 6.** Effects of TRI on Serum TAC in Experimental Groups
*Note*. TRI: Trigonelline, VPA: Valproic acid; ANOVA: Analysis of variance; SD: Standard deviation; TAC: Total antioxidant capacity. The results are shown as means ± SD and analyzed by one-way ANOVA and Tukey's post-hoc test. $^{***}P<0.001$ compared to the control-received saline group; $^{\$\$}P<0.01$ in comparison to the VPA-received saline group.

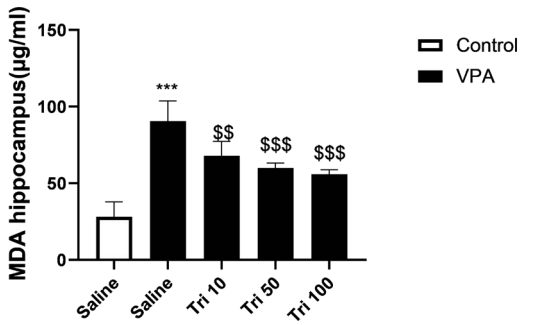


**Figure 7.** Effect of TRI on Hippocampal MDA Levels Across Experimental Groups
*Note*. TRI: Trigonelline, VPA: Valproic acid; ANOVA: Analysis of variance; SD: Standard deviation; MDA: Malondialdehyde. Data are shown as means ± SD, which were analyzed using one-way ANOVA followed by Tukey's post-hoc test. $^{***}P<0.001$ vs. the control-saline group; $^{\$\$}P<0.01$ and $^{\$\$\$}P<0.001$ vs. the VPA-saline group.

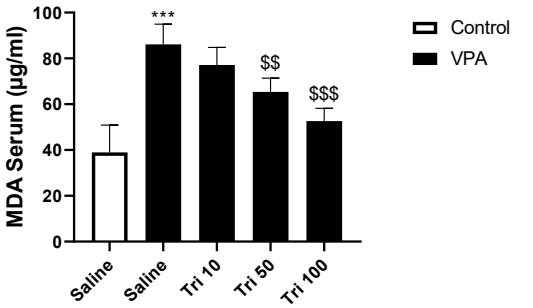


**Figure 8.** TRI-Induced Alterations in Serum MDA Levels
*Note*. TRI: Trigonelline, VPA: Valproic acid; ANOVA: Analysis of variance; SD: Standard deviation; MDA: Malondialdehyde. Data are presented as means ± SD, with statistical comparisons performed using one-way ANOVA and Tukey's post-hoc test. $^{***}P<0.001$ vs. the control-saline group; $^{\$\$}P<0.01$; $^{\$\$\$}P<0.001$ vs. the VPA-saline group.

benefits (10). Supporting this hypothesis, a growing body of evidence indicates that the reduction of oxidative damage serves to mitigate the core symptoms of ASD within various animal paradigms (44).

It has been established that TRI treatment facilitates the upregulation of major antioxidant enzymes, including superoxide dismutase, catalase, and glutathione peroxidase, thereby augmenting the capacity of the endogenous antioxidant system. Moreover, TRI has been reported to activate the nuclear factor erythroid 2-related factor 2–antioxidant response element signaling pathway, a central regulatory mechanism that governs cellular defense responses against OS (45). Despite its potential, the neuroprotective role of TRI in the context of neurodevelopmental disorders such as ASD remains unexplored. Consequently, this research investigated whether TRI could mitigate autism-like phenotypes induced by prenatal VPA exposure, with a particular emphasis on its antioxidant properties. Our results indicated that gestational VPA administration effectively modeled ASD-like behavioral abnormalities, characterized by heightened stereotypic and anxiety-like behaviors, along with deficits in social interaction and cognitive memory. These observations align with established literature, suggesting that prenatal VPA exposure triggers behavioral abnormalities consistent with ASD in rodent models (46, 47). Furthermore, VPA exposure in this study was associated with decreased antioxidant capacity and increased MDA levels in serum and hippocampal samples, demonstrating that TRI produced notable anti-autistic effects in rats exposed to VPA. Likewise, TRI administration showed significant therapeutic potential by mitigating repetitive behaviors during the marble burying assay and enhancing social interaction in the three-chamber sociability paradigm. Further, TRI ameliorated cognitive deficits in the shuttle box task and attenuated anxiety-like tendencies as observed in the EPM. These behavioral enhancements are in line with the findings of Amini et al, implying that VPA exposure precipitates autism-like behavioral deficits in rodent models (4).

The present investigation demonstrated that TRI administration enhances antioxidant capacity while simultaneously lowering MDA concentrations in both hippocampal tissue and serum. These observations conform to established literature regarding the neuroprotective efficacy of TRI across various neurological injury models. Notably, Pravalika et al observed a dose-dependent reduction in MDA and nitrite levels within the brain parenchyma of mice undergoing cerebral ischemia, an outcome fundamentally driven by TRI's antioxidant properties (48). Similarly, Qiu et al indicated that TRI diminishes present neural damage by modulating the

phosphoinositide 3-kinase /protein kinase B pathway and reducing OS (49). Collectively, these findings support TRI's ability to mitigate OS-induced brain injury, primarily through its strong antioxidant activity. Overall, it was revealed that TRI can significantly decrease OS at both serum and neuronal levels. The increase in TAC and the reduction in MDA levels in serum and hippocampal tissue demonstrate this compound's antioxidant ability. Given the high susceptibility of the hippocampus to OS, these antioxidant properties determine its potential neuroprotective effects. Therefore, TRI may be investigated in future studies as a natural compound with neuroprotective and antioxidative stress properties in ASD models. The lack of significant effects of TRI at a dose of 10 mg in the hippocampus may be due to the relatively short duration of treatment. Accordingly, a prolonged treatment period may be required for this dose to produce therapeutic effects.

The results of this study suggest that TRI can partially counteract the autism-like behavioral abnormalities induced by VPA, likely because it reduces MDA while increasing TAC. Nevertheless, additional investigations are required to elucidate the precise mechanisms through which TRI influences autism-related outcomes and to further define its pharmacokinetic profile. Given these observations, TRI may represent a promising natural compound for future research targeting neurodevelopmental disorders and other conditions characterized by OS. One limitation of the present work was that TRI's effects were not evaluated in female rats, leaving gender-specific behavioral responses unexplored. Future studies should therefore assess whether TRI similarly modulates autism-like behaviors in females. Another limitation was that biochemical analyses were conducted only on the whole hippocampus. Considering that distinct hippocampal subregions contribute to different cognitive and behavioral functions (50), future research should examine how TRI affects specific hippocampal areas in the VPA model of autism. Ultimately, the study did not investigate TRI's effects in non-VPA control rats, restricting conclusions regarding its broader behavioral impact.

## Conclusion

The results indicated that TRI, as a natural compound, partly mitigated the adverse effects of VPA. It was found that this compound not only alleviates behavioral symptoms associated with ASD but also exerts protective effects by enhancing antioxidant capacity while reducing hippocampal and serum MDA levels, suggesting its therapeutic potential for improving ASD-like behavioral abnormalities in this animal model.

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### Artificial Intelligence Use Disclosure

During the preparation of this manuscript, ChatGPT was used only for grammar checking to improve English. Additionally, all content generated with the assistance of this tool was precisely reviewed, validated, and revised by the authors in order to ensure accuracy, originality, and scholarly rigor. The authors assume full responsibility for the integrity and final content of the published articles.

### Authors' Contribution

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### Competing Interests

The authors declare that there is no conflict of interests.

### Ethical Approval

All experimental procedures were approved by the Animal Ethics Committee of Islamic Azad University (ethical code: IR.IAU.NAJAFABAD.REC.14031.155) and were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

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### References

1. Zeidan J, Fombonne E, Scorah J, Ibrahim A, Durkin MS, Saxena S, et al. Global prevalence of autism: a systematic review update. Autism Res 2022;15(5):778-90. doi:10.1002/aur.2696
2. Capp S, De Burca A, Aydin Ü, Agnew-Blais J, Lautarescu A, Ronald A, et al. Depression and anxiety are increased in autism and ADHD: evidence from a young adult community-based sample. JCPP Adv 2025;5(4): e70003. doi:10.1002/jcv2.70003
3. DiLiberti JH, Farndon PA, Dennis NR, Curry CJ. The fetal valproate syndrome. Am J Med Genet 1984;19(3):473-81. doi:10.1002/ajmg.1320190308
4. Amini F, Amini-Khoei H, Haratizadeh S, Setayesh M, Basiri M, Raeiszadeh M, et al. Hydroalcoholic extract of *Passiflora incarnata* improves the autistic-like behavior and neuronal damage in a valproic acid-induced rat model of autism. J Tradit Complement Med 2023;13(4):315-24. doi:10.1016/j.jtcme.2023.02.005
5. Rynkiewicz-Szczepanska E, Kosciuczuk U, Maciejczyk M. Total antioxidant status in critically ill patients with traumatic brain injury and secondary organ failure-a systematic review. Diagnostics (Basel) 2024;14(22):2561. doi:10.3390/diagnostics14222561
6. Feng J, Zheng Y, Guo M, Ares I, Martínez M, Lopez-Torres B, et al. Oxidative stress, the blood-brain barrier and neurodegenerative diseases: the critical beneficial role of dietary antioxidants. Acta Pharm Sin B 2023;13(10):3988-4024. doi:10.1016/j.apsb.2023.07.010
7. Jelinek M, Jurajda M, Duris K. Oxidative stress in the brain: basic concepts and treatment strategies in stroke. Antioxidants (Basel) 2021;10(12):1886. doi:10.3390/antiox10121886

8. Hayashi M, Miyata R, Tanuma N. Oxidative stress in developmental brain disorders. Adv Exp Med Biol 2012;724:278-90. doi:10.1007/978-1-4614-0653-2_21
9. de Oliveira CA, Iorio EL, Espíndola FS. Redox system dysfunction as a key mechanism in autism spectrum disorder pathogenesis. Int J Mol Sci 2025;26(20):9850. doi:10.3390/ijms26209850
10. Liu X, Lin J, Zhang H, Khan NU, Zhang J, Tang X, et al. Oxidative stress in autism spectrum disorder-current progress of mechanisms and biomarkers. Front Psychiatry 2022;13:813304. doi:10.3389/fpsyt.2022.813304
11. Altun H, Şahin N, Kurutaş EB, Karaaslan U, Sevgen FH, Fındıklı E. Assessment of malondialdehyde levels, superoxide dismutase, and catalase activity in children with autism spectrum disorders. Psychiatry Clin Psychopharmacol 2018;28(4):408-15. doi:10.1080/24750573.2018.1470360
12. Yui K, Imataka G, Sasaki H, Shiroki R. The role of lipid peroxidation in individuals with autism spectrum disorders. Metab Brain Dis 2020;35(7):1101-8. doi:10.1007/s11011-020-00585-4
13. Razavi SZ, Amini-Khoei H, Rahimi-Madiseh M, Bijad E, Lorigooini Z. Modulation of neuroinflammation and oxidative stress by *Echinacea purpurea* extract: therapeutic potential in maternal separation-induced autism spectrum disorder. J Psychiatr Res 2025;184:118-27. doi:10.1016/j.jpsychires.2025.02.038
14. Ratheesh G, Tian L, Venugopal JR, Ezhilarasu H, Sadiq A, Fan TP, et al. Role of medicinal plants in neurodegenerative diseases. Biomanuf Rev 2017;2(1):2. doi:10.1007/s40898-017-0004-7
15. Nikfarjam M, Heidari-Soureshjani S, Rostamian S, Kasiri K. The biochemical effects of resveratrol intake on the neurobehavioral aspects of autism spectrum disorders: a systematic review. Cent Nerv Syst Agents Med Chem 2026;26(2):167-82. doi:10.2174/0118715249387274250521054238
16. Barbalho SM, Leme Boaro B, da Silva Camarinha Oliveira J, Patočka J, Barbalho Lamas C, Tanaka M, et al. Molecular mechanisms underlying neuroinflammation intervention with medicinal plants: a critical and narrative review of the current literature. Pharmaceuticals (Basel) 2025;18(1):133. doi:10.3390/ph18010133
17. Ashihara H, Ludwig IA, Katahira R, Yokota T, Fujimura T, Crozier A. Trigonelline and related nicotinic acid metabolites: occurrence, biosynthesis, taxonomic considerations, and their roles in planta and in human health. Phytochem Rev 2015;14(5):765-98. doi:10.1007/s11101-014-9375-z
18. Nguyen V, Taine EG, Meng D, Cui T, Tan W. Pharmacological activities, therapeutic effects, and mechanistic actions of trigonelline. Int J Mol Sci 2024;25(6):3385. doi:10.3390/ijms25063385
19. Farid MM, Yang X, Kuboyama T, Tohda C. Trigonelline recovers memory function in Alzheimer's disease model mice: evidence of brain penetration and target molecule. Sci Rep 2020;10(1):16424. doi:10.1038/s41598-020-73514-1
20. Kabiri-Samani N, Amini-Khoei H, Rahimi-Madiseh M, Sureda A, Lorigooini Z. Trigonelline as an anticonvulsant agent: mechanistic insights into NMDA receptor expression and oxidative stress balance. Sci Rep 2024;14(1):14239. doi:10.1038/s41598-024-65301-z
21. Di Gioacchino M, Ricci MA, Bruni F. Science in a cup of coffee: a structural study of a trigonelline aqueous solution. J Mol Liq 2024;396:123972. doi:10.1016/j.molliq.2024.123972
22. Kothapalli P, Vasanthan M. Green RP-HPLC method for estimation of trigonelline: AQbD based development, validation, and application to nanoformulations. BMC Chem 2026;20(1):46. doi:10.1186/s13065-026-01734-z
23. Anjomshoa M, Nasiri Boroujeni S, Bagheri E, Lorigooini Z, Amini-Khoei H. Possible involvement of N-methyl-D-aspartate receptor (NMDA-R) in the antidepressant-like effect of trigonelline in male mice. Curr Pharm Des 2020;26(39):5067-71. doi:10.2174/1381612826666200610181259
24. Lang R, Dieminger N, Beusch A, Lee YM, Dunkel A, Suess B, et al. Bioappearance and pharmacokinetics of bioactives upon coffee consumption. Anal Bioanal Chem 2013;405(26):8487-503. doi:10.1007/s00216-013-7288-0
25. Wadhwa G, Krishna KV, Taliyan R, Tandon N, Yadav SS, Banerjee D, et al. Preclinical pharmacokinetics of trigonelline using ultra-performance liquid chromatography-tandem mass spectrometry and pharmacological studies targeting type 2 diabetes. Sep Sci Plus 2021;4(4):185-94. doi:10.1002/sscp.202000118
26. Ashihara H, Ludwig IA, Crozier A. Plant Nucleotide Metabolism: Biosynthesis, Degradation, and Alkaloid Formation. Hoboken, NJ: Wiley; 2020. doi:10.1002/9781119476139
27. Midttun Ø, Ulvik A, Nygård O, Ueland PM. Performance of plasma trigonelline as a marker of coffee consumption in an epidemiologic setting. Am J Clin Nutr 2018;107(6):941-7. doi:10.1093/ajcn/nqy059
28. Zhang Q, Zhang Y, Song Z, Tang Z, Wang G, Yang X, et al. Elucidating trigonelline's therapeutic mechanisms for traumatic brain injury through integrated network pharmacology and in vivo validation. CNS Neurosci Ther 2026;32(3): e70803. doi:10.1002/cns.70803
29. Mahmoudian M, Lorigooini Z, Rahimi-Madiseh M, Shabani S, Amini-Khoei H. Protective effects of rosmarinic acid against autistic-like behaviors in a mouse model of maternal separation stress: behavioral and molecular amendments. Naunyn Schmiedebergs Arch Pharmacol 2024;397(10):7819-28. doi:10.1007/s00210-024-03143-2
30. Mirzaee Khoram-Abadi K, Haratizadeh S, Basiri M, Parvan M, Pourjafari F, Aghaei I, et al. Autistic-like behaviors are attenuated by agmatine consumption during pregnancy: assessment of oxidative stress profile and histopathological changes in the prefrontal cortex and CA1 region of the hippocampus. Iran J Basic Med Sci 2024;27(3):335-42. doi:10.22038/ijbms.2023.74536.16190
31. Gouda B, Sinha SN, Chalamaiah M, Vakdevi V, Shashikala P, Veeresh B, et al. Sex differences in animal models of sodium-valproate-induced autism in postnatal BALB/c mice: whole-brain histoarchitecture and 5-HT2A receptor biomarker evidence. Biology (Basel) 2022;11(1):79. doi:10.3390/biology11010079
32. Rashova M, Akhmetova S, Tuleubaev B, Bolat Z, Mukhtarkhan U, Tazabekova K, et al. Impact of nanocellulose-based biocomposite implantation on rodent behavior in the elevated plus maze test. J Clin Med Kaz 2026;23(2):104-8. doi:10.23950/jcmk/18012
33. Karimi P, Shahgholian Ghahfarroki M, Lorigooini Z, Shahrani M, Amini-Khoei H. Umbelliprenin via increase in the MECP2 and attenuation of oxidative stress mitigates the autistic-like behaviors in mouse model of maternal separation stress. Front Pharmacol 2023;14:1300310. doi:10.3389/fphar.2023.1300310
34. Farzan M, Farzan M, Amini-Khoei H, Shahrani M, Bijad E, Anjomshoa M, et al. Protective effects of vanillic acid on autistic-like behaviors in a rat model of maternal separation stress: Behavioral, electrophysiological, molecular and histopathological alterations. Int Immunopharmacol 2023;118:110112. doi:10.1016/j.intimp.2023.110112
35. Francischi JN, Frade TI, de Almeida MP, de Queiroz BF, Bakhle YS. Ketamine-xylazine anaesthesia and orofacial administration of substance P: a lethal combination in rats. Neuropeptides 2017;62:21-6. doi:10.1016/j.npep.2017.01.003
36. Hosseinzadeh H, Parvardeh S, Nassiri Asl M, Sadeghnia HR, Ziaee T. Effect of thymoquinone and *Nigella sativa* seeds oil on lipid peroxidation level during global cerebral ischemia-reperfusion injury in rat hippocampus. Phytomedicine 2007;14(9):621-7. doi:10.1016/j.phymed.2006.12.005

37. Zhang R, Zhang J, Rehman AU, Dang L, Yu X, Yang W. Isolating immune cells from mouse brain and skull. J Vis Exp 2024(209): e66861. doi:10.3791/66861
38. Wisse LE, Daugherty AM, Olsen RK, Berron D, Carr VA, Stark CE, et al. A harmonized segmentation protocol for hippocampal and parahippocampal subregions: why do we need one and what are the key goals? Hippocampus 2017;27(1):3-11. doi:10.1002/hipo.22671
39. Yadav AS. Malondialdehyde and nitric oxide levels reveal comparative toxicity of three insecticides in Wistar rats. Toxicol Mech Methods 2026;36(3):376-88. doi:10.1080/15376516.2025.2601988
40. 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
41. Hodis B, Mughal S, Saadabadi A. Autism spectrum disorder. In: StatPearls [Internet]. Treasure Island, FL: StatPearls Publishing; 2025. Available from: https://www.ncbi.nlm.nih.gov/books/NBK525976/.
42. Morimoto M, Hashimoto T, Tsuda Y, Nakatsu T, Kitaoka T, Kyotani S. Assessment of oxidative stress in autism spectrum disorder using reactive oxygen metabolites and biological antioxidant potential. PLoS One 2020;15(5): e0233550. doi:10.1371/journal.pone.0233550
43. Burke SL, Cobb J, Agarwal R, Maddux M, Cooke MS. How robust is the evidence for a role of oxidative stress in autism spectrum disorders and intellectual disabilities? J Autism Dev Disord 2021;51(5):1428-45. doi:10.1007/s10803-020-04611-3
44. 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
45. Amoushahi M, Ernst EH, Heuck A, Dueholm M, Ernst E, Lykke-Hartmann K. Trigonelline activates NRF2 and awakens dormant ovarian follicles to promote pregnancy in aging mice. iScience 2025;28(11):113717. doi:10.1016/j.isci.2025.113717
46. Mabunga DF, Gonzales EL, Kim JW, Kim KC, Shin CY. Exploring the validity of valproic acid animal model of autism. Exp Neurobiol 2015;24(4):285-300. doi:10.5607/en.2015.24.4.285
47. Vakili Shahrbabaki SS, Jonaidi H, Sheibani V, Bashiri H. Early postnatal handling alters social behavior, learning, and memory of pre- and postnatal VPA-induced rat models of autism in a context-based manner. Physiol Behav 2022;249:113739. doi:10.1016/j.physbeh.2022.113739
48. Pravalika K, Sarmah D, Kaur H, Vats K, Saraf J, Wanve M, et al. Trigonelline therapy confers neuroprotection by reduced glutathione mediated myeloperoxidase expression in animal model of ischemic stroke. Life Sci 2019;216:49-58. doi:10.1016/j.lfs.2018.11.014
49. Qiu Z, Wang K, Jiang C, Su Y, Fan X, Li J, et al. Trigonelline protects hippocampal neurons from oxygen-glucose deprivation-induced injury through activating the PI3K/Akt pathway. Chem Biol Interact 2020;317:108946. doi:10.1016/j.cbi.2020.108946
50. Fanselow MS, Dong HW. Are the dorsal and ventral hippocampus functionally distinct structures? Neuron 2010;65(1):7-19. doi:10.1016/j.neuron.2009.11.031