Research Article

Neuroprotective Effect of Fenugreek (*Trigonella foenum-graecum*) Seed Extract in a Rat Model of Autism Spectrum Disorder: Behavioral and Histopathological Perspectives

Arni Kusuma Dewi[1], Bambang Purwanto[2], Widjiati Widjiati[3]*, Viski Fitri Hendrawan[4]

[1]Doctoral Student, Faculty of Medicine, Universitas Airlangga, Campus A, Jl. Prof. Dr. Moestopo No.47, Pacar Kembang, Surabaya, East Java, Indonesia 60132; [2]Department of Physiology and Medical Biochemistry, Faculty of Medicine, Airlangga University, (Campus A) Prof. DR. Moestopo No.47 Street Pacar Kembang, Surabaya City, East Java, Indonesia 60132; [3]Departement of Veterinary Medicine, Faculty of Veterinary Medicine, Airlangga University, (Campus C) Dharmahusada Permai No.1 Street, Mulyorejo, Surabaya City, East Java, Indonesia 60115; [4]Department of Veterinary Reproduction, Brawijaya University, Puncak Dieng Street, Kunci-Kalisongo, Malang, East Java, Indonesia 65151.

Abstract | Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by impaired social interaction and stereotyped or repetitive behaviors. Prenatal exposure to valproic acid (VPA) is a well-established pharmacological risk factor for ASD phenotypes. This study evaluated the neuroprotective potential of fenugreek (Trigonella foenum-graecum) seed extract in a prenatal VPA-induced rat model of ASD. Pregnant Wistar rats were allocated into five groups (n = 6 per group): control (saline), VPA, VPA + fenugreek extract 100 mg/kg, VPA + fenugreek extract 200 mg/kg, and VPA + fenugreek extract 300 mg/kg. VPA (400 mg/kg) was administered as a single oral dose on gestational day (GD) 15. Fenugreek extract was administered orally once daily from GD11–GD20. Male offspring were assessed in a three-chamber social interaction test (Social Novelty Index; SNI) on postnatal day (PND) 27 and a Marble Burying Test (MBT) at PND35. Histological analysis (HandE) assessed viable neuron counts in the cerebral cortex, hippocampus CA1, and cerebellum, while myelin basic protein (MBP) immunohistochemistry quantified myelin density. The VPA group showed reduced social novelty preference and increased repetitive behavior compared with controls. Fenugreek extract at 200 mg/kg significantly improved the SNI score and reduced marble burying compared with the VPA group ($p < 0.05$). The 300 mg/kg dose showed stronger restoration of viable neuron counts, particularly in hippocampus CA1, although it did not significantly improve the SNI compared to the VPA group ($p = 0.17$). MBP intensity increased in the 200 and 300 mg/kg groups, indicating improved myelination. Fenugreek seed extract exhibits neuroprotective potential against prenatal VPA-induced ASD-like phenotypes, with 200 mg/kg showing the most consistent behavioral benefit and 300 mg/kg showing stronger histological recovery. Further studies are required to elucidate dose-dependent mechanisms and confirm bioactive compound contributions. This study supports SDG 3 (Good Health and Well-being) by exploring plant-based neuroprotective strategies for ASD. It also aligns with SDG 9 (Industry, Innovation and Infrastructure) through its potential to inspire future development of phytopharmaceuticals.

Keywords | Fenugreek seed extract, Autism spectrum disorder, Valproic acid, Neuronal viability, Good health and well-being, Industry, Innovation and infrastructure

Received | December 20, 2025; **Accepted** | January 12, 2026; **Published** | January 24, 2026
***Correspondence** | Widjiati, Widjiati, Department of Veterinary Medicine, Faculty of Veterinary Medicine, Airlangga University, (Campus C) Dharmahusada Permai No.1 street, Mulyorejo, Surabaya city, East Java, Indonesia 60115; **Email:** widjiati@fkh.unair.ac.id
Citation | Dewi AK, Purwanto B, Widjiati W, Hendrawan VF (2026). Neuroprotective effect of fenugreek (*Trigonella foenum-graecum*) seed extract in a rat model of autism spectrum disorder: Behavioral and histopathological perspectives. Adv. Anim. Vet. Sci., 14(2):293-302.
DOI | https://dx.doi.org/10.17582/journal.aavs/2026/14.2.293.302
ISSN (Online) | 2307-8316

INTRODUCTION

Autism Spectrum Disorder (ASD) is a neurodevelopmental disorder characterized by persistent deficits in social communication and interaction, accompanied by restricted and repetitive patterns of behavior. The diagnostic framework of ASD is defined in the Diagnostic and Statistical Manual of Mental Disorders (DSM-5), which integrates previously separated categories such as autistic disorder and Asperger syndrome into a single spectrum diagnosis (Hodges *et al*., 2020). Globally, ASD prevalence has continued to increase, contributing to a substantial public health and socioeconomic burden due to associated long-term medical, behavioural, and educational needs (Salari *et al*., 2022).

Given the neurodevelopmental risks of VPA and the lack of universally safe alternatives during pregnancy, there is growing interest in exploring adjunctive neuroprotective agents that could mitigate VPA-associated disruption (Cammarata, 2023). Epidemiological evidence indicates that VPA exposure during pregnancy is associated with an increased risk of neurodevelopmental disorders, including ASD, in addition to congenital malformations (Sabers *et al*., 2024). Experimental models have also demonstrated that prenatal VPA exposure induces behavioral abnormalities resembling ASD, including social deficits and repetitive behaviors, along with neurobiological changes such as neuronal loss and disrupted myelination in the hippocampus, cortex, and cerebellum (Elnahas *et al*., 2021).

Mechanistically, VPA induces neurodevelopmental toxicity via multiple pathways, including histone deacetylase (HDAC) inhibition and disruption of neurotransmitter systems, particularly gamma-aminobutyric acid (GABA) signaling (Zarate-Lopez *et al*., 2023). HDAC inhibition can alter transcriptional regulation during critical periods of neurogenesis, synaptogenesis, and oligodendrocyte maturation, potentially contributing to neuronal vulnerability and impaired myelin development. Furthermore, VPA exposure has been linked to oxidative stress and neuroinflammation, which may contribute to neuronal apoptosis and behavioural disturbances (Luhach *et al*., 2021).

Such agents could mitigate VPA-associated neurodevelopmental disruption in certain patient populations and the lack of universally applied alternatives during pregnancy, there is a growing interest in exploring adjunctive neuroprotective agents that may mitigate VPA-associated neurodevelopmental disruption. Fenugreek (*Trigonella foenum-graecum*) is a medicinal plant whose seeds contain multiple bioactive constituents, including flavonoids, steroidal saponins, and the alkaloid trigonelline, which have been reported to possess antioxidant, anti-inflammatory, and neuroprotective activity (Yang *et al*., 2022; Muhson *et al*., 2014). Experimental studies suggest fenugreek seed administration can improve cognitive function and reduce neurotoxicity in other models, suggesting a potential to influence central nervous system outcomes (Belaïd-Nouira *et al*., 2012; Riba *et al*., 2019).

Proposed mechanisms for fenugreek include, fenugreek may reduce oxidative stress via flavonoid-mediated antioxidant effects, and modulating cyclic nucleotide signalling via phosphodiesterase (PDE) inhibition by steroidal saponins (Inoue *et al*., 1995). Although these pathways are biologically plausible in the context of VPA-induced neurotoxicity, the present study was designed primarily to evaluate behavioral and histological outcomes not to confirm specific molecular mechanisms.

This study evaluated the neuroprotective potential of fenugreek seed extract in a prenatal VPA-induced rat model of ASD by assessing social interaction (Social Novelty Index, SNI), repetitive behavior (Marble Burying Test, MBT), viable neuron counts in key brain regions, and myelin intensity via MBP immunohistochemistry. This study supports SDG 3 (Good Health and Well-being) by exploring plant-based neuroprotective strategies for ASD. It also aligns with SDG 9 (Industry, Innovation and Infrastructure) through its potential to inspire future development of phytopharmaceuticals. While mechanisms such as HDAC inhibition, caspase-3 modulation, and PDE1 pathways are biologically plausible based on previous reports, the current study was designed to primarily assess behavioral and histological outcomes. Molecular validations were beyond the scope of this initial efficacy evaluation but are strongly recommended for future studies.

MATERIALS AND METHODS

Experimental animals: Pregnant rats

Thirty healthy female Wistar rats (60 days old; mean body weight 184.29 ± 10.19 g) were obtained from the Professor Niddom Foundation, an institutional research animal breeding and housing facility in Indonesia. Animals were acclimatized for 3 days under a 12 h light/12 h dark cycle (lights on at 08:00) at 26°C with free access to food and water ad libitum. All procedures were approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, Universitas Airlangga (Approval No. 2. KEH.182.12.2023). Environmental acclimatization were carried out for 3 days with a standard life cycle of 12 hours of light and 12 hours of dark cycle (light at 8 am) and a constant room temperature of 26 degrees Celsius. Each had access to food and drink ad libitum.

Following acclimatisation, females were mated. Mating and all subsequent procedures were carried out at the Professor Niddom Foundation. Mating was performed as described previously (Umannageswari *et al*., 2020), in two stages: estrus synchronisation followed by superovulation and mating. The estrus synchronization stage were carried out by first weighing the body weight of the female and male rat which were initially kept in separate cages, then the female rats were injected with PMSG (Pregnant Mare Serum Gonadotropin) 10 IU subcutaneously at 13:00, then returned to their cages. The second stage was the female rats were injected with HCG (Human Chorionic Gonadotropin) 10 IU subcutaneously 48 hours after the PSMG injection. After the HCG injection, the females were housed with males, where 2 female rats were put in one cage with 1 male rat. After 17 hours, mating was confirmed by, pregnancy was checked by examining the presence of a vaginal plug and a vaginal smear showing the presence of sperm. The day a plug and sperm were detected was designated as gestational day (GD) 0.

Once the female is deemed pregnant, she is placed singly in a 16x35x24 cm cage until she gives birth. Pregnant rats were divided into five groups (n = 6 per group) the group that was only given saline (KS), the group that was only given valproic acid (VPA), the group that was given VPA and fenugreek seed extract 100 mg/kg (VPA+100), the group that was given valproic acid and fenugreek seed extract 200 mg/kg (VPA+200), and the group that was given valproic acid and fenugreek seed extract 300 mg/kg (VPA+300). Dams remained with their litters in the same cage until weaning on postnatal day (PND) 23 or 24.

Experimental animal pups rat

After pups were weaned, one male pup was randomly selected, so that one group consists of six rats. Figure 1 informed the schedule intervention of pups can be seen in Table 1. The detailed timeline of mating, fenugreek and VPA administration, behavioral tests, and brain collection is illustrated in Figure 1.

Table 1: Intervention timeline in rat pups.

PND	Activity
0	Birth
14–17	Eye opening
23–24	Weaning
27	Social Novelty Index (SNI) Test
35	Marble Burying Test (MBT) and Termination

Drug preparation and administration

Valproic acid: Valproic acid sodium salt (Sigma-Aldrich, Cat. No. P4543) was prepared as a stock solution (100 mg/mL) by dissolving 2800 mg in 28 mL of distilled water (Di *et al*., 2021). Pregnant rats received a single oral dose of valproic acid at 400 mg/kg on gestational day (GD) 15 to induce ASD-like phenotypes (Elnahas *et al*., 2021).

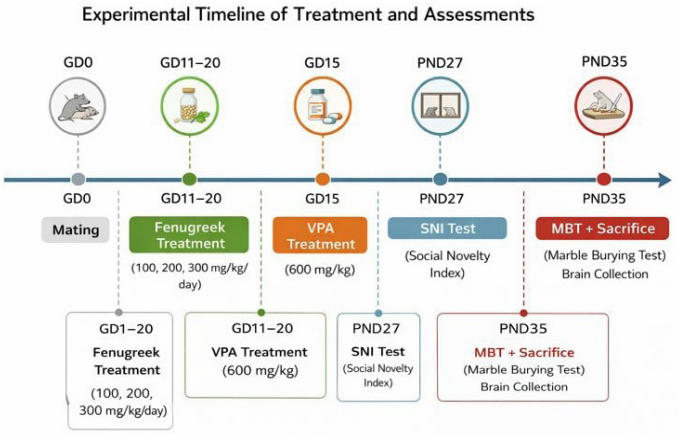

Figure 1: Experimental timeline of treatment and assessments. Mating occurred at GD0. Fenugreek extract (100, 200, 300 mg/kg/day) was administered orally on GD11–GD20. VPA (400 mg/kg) was given orally on GD15. Behavioral testing included the Social Novelty Index (SNI) on PND27 and Marble Burying Test (MBT) on PND35, followed by brain collection.

Fenugreek seed extract: Dried fenugreek seeds (*Trigonella foenum-graecum*) (100 g) were macerated in 70% ethanol for 24 h at room temperature, then centrifuged and the supernatant was collected as the crude extract (Pribac and Remenyik, 2017). The extract was evaporated to remove ethanol and reconstituted in distilled water to obtain the target dosing concentrations. The extract was administered orally once daily from GD11 to GD20 at doses of 100, 200, or 300 mg/kg.

Extract standardization: The extract used in this study was a crude preparation and was not standardized to a specific bioactive marker (e.g., total saponins, trigonelline, or total flavonoids). This limitation is acknowledged and future work should quantify key constituents to improve reproducibility and identify active compounds.

Behavioural test

Social test (three chamber sociability test)

This test was given to 27-day-old postnatal rats. Rats from the same group were placed in a modified cage in a chamber marked zero (one side), while rats from different groups were placed in a modified cage in a chamber marked one (one side). The data collected was the time it took the rats being tested to interact with the rats in chamber zero or chamber one. Rats were said to have interacted if there was nose-to-nose, nose-to-neck, evading-chase interaction, the rat being tested sniffing at a hole or cage in one of the chambers, or the rat being tested entering one of the cages (Elnahas *et al*., 2021). The interaction time was recorded for 10 minutes with a 5-minute adaptation period. The

results of the time recording were then calculated for Social Novelty Preference (SNI) can be seen in Figure 2. SNI is a calculation of the communication time of members of a group with members of the same group and members of different groups in a cage divided into 3 chambers. The calculation is done using the following formula:

$$\frac{S2 - S1}{S1 + S2}$$

S2= time required to communicate with animals in different groups (seconds), S1= time required to communicate with animals in the same group (seconds).

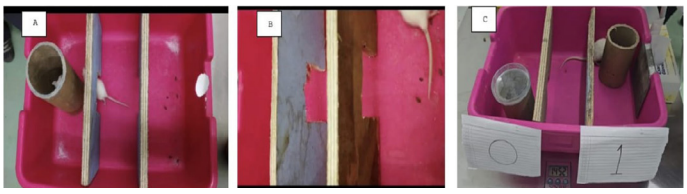

Figure 2: Three-chamber cage model for social interaction test. (A) Top view of the modified acrylic cage divided into three chambers using a wooden partition. (B) Middle partition with a central circular hole to allow pup movement between chambers. (C) Chamber 0 contains a rat from the same group; Chamber 1 contains a rat from a different group. Note: Ensure panel (A–C) are visibly labeled in the figure.

Repetitive test (marble burying test)

The test was conducted at 35 days postnatal age. The data counted was the number of marbles buried in the husk. Based on observations, marbles were buried when the rat pups repeatedly scratched for marbles as a form of repetitive behavior (Elnahas *et al*., 2021). The cage model for marble burying test can be seen in Figure 3.

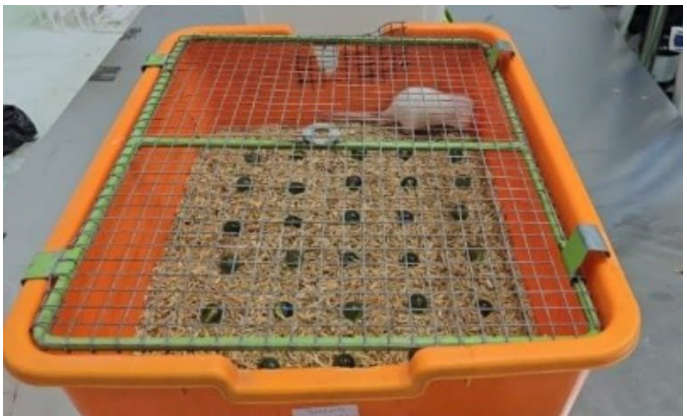

Figure 3: Histological images of the fifth layer of the cerebral cortex (Hematoxylin–Eosin staining, 400×). Arrowheads (red): Indicate degenerated neurons (pyknotic nuclei, cell shrinkage). Arrows (green): Indicate viable neurons with intact morphology. Panels: (A) Control (KS), (B) VPA, (C) VPA+100 mg/kg, (D) VPA+200 mg/kg, (E) VPA+300 mg/kg.

Histopathology and MBP quantification

MBP intensity was quantified using ImageJ from five randomly selected non-overlapping fields per section. To ensure comparability across staining batches, all slides were processed under identical staining conditions and imaging settings. Background subtraction was performed using a constant threshold value derived from negative/background regions of each section. Mean gray value (MGV) was measured and converted to reciprocal staining intensity (RSI = 255 – MGV), which is positively correlated with chromogen intensity (Nguyen and Nguyen, 2013; Cizkova *et al*., 2021).

Statistical analysis

Normality was assessed using the Shapiro–Wilk test. Data that violated normality assumptions were analyzed using non-parametric Kruskal–Wallis tests followed by Dunn's post hoc test. Normally distributed data were analyzed using one-way ANOVA followed by LSD post hoc testing. Linear regression was used to assess dose-dependent effects on viable neuron counts and MBP intensity. Statistical significance was set at $p < 0.05$.

RESULTS

Behavioral test

A total of 30 male Wistar rat pups (n = 6 per group) were used in this study. Weaning was conducted on postnatal day (PND) 21. Body weight (BW) measurements and the social novelty preference test (SNI) were performed at PND 27, followed by the marble burying test (MBT) at PND 35. Brain tissue was harvested at PND 35 for histopathological and immunohistochemical analysis. Normality tests for each group are summarized in Table 2. According to Table 2, BW data did not differ significantly across groups. However, some behavioral variables (SNI and MBT) failed the normality test, thus non-parametric Kruskal–Wallis tests were used to compare group differences. As presented in Table 3, the VPA group showed significantly lower SNI scores compared to the control and the VPA+200 groups ($p < 0.05$). Similarly, MBT scores were significantly higher in the VPA group, indicating increased repetitive behavior. Administration of fenugreek extract at 200 mg/kg improved both SNI and MBT parameters, whereas the 300 mg/kg dose showed improvement in MBT but not in SNI ($p = 0.17$).

Histopathological findings

Histological examination was performed by counting viable neurons in the cerebral cortex (layer V), hippocampus CA1, and cerebellum (Purkinje layer). Data showed normal distribution and were analyzed using one-way ANOVA followed by regression analysis. As seen in Table 4, fenugreek extract at both 200 and 300 mg/kg doses

Table 2: Body weight, SNI, and MBT distribution and normality test.

Group	BW (Mean ± SD)	Normality p	SNI (Median (IQR))	Normality p	MBT (Median (IQR))	Normality p
KS	76.83 ± 4.54	0.25	0.68 (0.60–0.75)	0.84	2.83 (2.10–3.30)	0.42
VPA	78.50 ± 4.54	0.29	-0.65 (-0.80–-0.40)	0.015*	10.0 (8.0–12.0)	0.015*
VPA+100	82.00 ± 2.76	0.49	0.54 (0.45–0.60)	0.01*	5.33 (4.70–6.10)	<0.001*
VPA+200	75.67 ± 3.61	0.82	0.50 (0.40–0.55)	0.84	1.67 (1.30–2.10)	0.001*
VPA+300	64.50 ± 6.83	0.66	0.26 (0.20–0.30)	0.33	0.83 (0.60–1.10)	0.007*

*Shapiro–Wilk normality test, *p < 0.05 indicates significant deviation from normality.

Table 3: Intergroup differences in SNI and MBT scores (Kruskal–Wallis + Dunn's Post Hoc).

Variable	Kruskal–Wallis p	Comparison	p-value
SNI	0.001*	VPA vs KS	<0.001*
		VPA vs VPA+100	0.001*
		VPA vs VPA+200	0.004*
		VPA vs VPA+300	0.17
MBT	<0.001*	VPA vs KS	0.011*
		VPA vs VPA+100	0.51
		VPA vs VPA+200	<0.001*
		VPA vs VPA+300	<0.001*

* Kruskal–Wallis test followed by Dunn's post-hoc test. p < 0.05 is considered statistically significant.

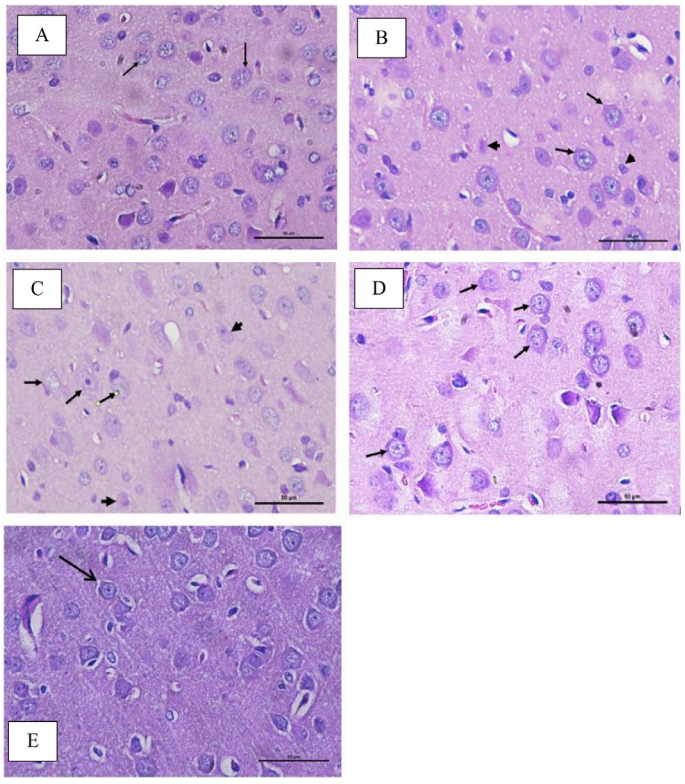

Figure 4: Histological images of the hippocampus CA1 region (H and E staining, 400×). Arrowheads (red): Degenerated pyramidal neurons. Arrows (green): Healthy pyramidal neurons with visible nuclei and cytoplasm. Panels: (A) Control (KS), (B) VPA, (C) VPA+100 mg/kg, (D) VPA+200 mg/kg, (E) VPA+300 mg/kg.

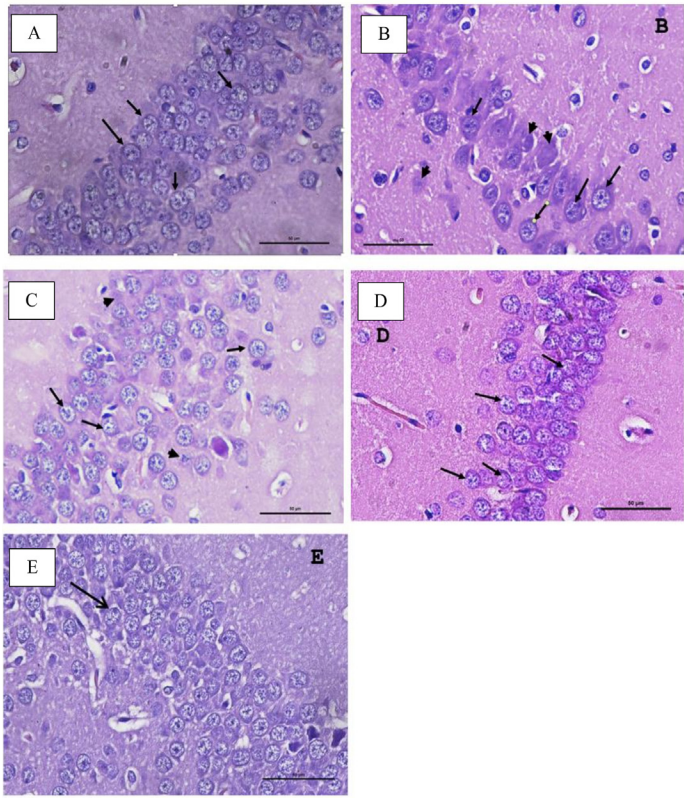

Figure 5: Histological images of the cerebellum (Purkinje layer) (H and E staining, 400×). Arrowheads (red): Degenerated Purkinje cells (dark-stained, shrunken). Arrows (green): Intact Purkinje cells with round nuclei and clear cytoplasm. Panels: (A) Control (KS), (B) VPA, (C) VPA+100 mg/kg, (D) VPA+200 mg/kg, (E) VPA+300 mg/kg.

increased viable neuron counts compared to the VPA group. The hippocampus CA1 region showed the most significant neuronal recovery. Linear regression analysis (Figure 6) demonstrated a positive dose-response relationship between fenugreek extract and viable neuron count across all brain regions. MBP immunohistochemistry results (Table 5) revealed increased MBP intensity in the cerebrum and hippocampus CA1 of rats treated with 200 and 300 mg/kg fenugreek extract, compared to the VPA group. No significant differences were observed in the cerebellum. Regression analysis (Figure 10) showed a significant positive correlation between extract dose and MBP intensity in the cerebrum and hippocampus CA1.

Table 4: Viable neuron counts in brain regions (ANOVA + LSD Post Hoc).

Brain Region	ANOVA p	Comparison	Mean ± SD	Post Hoc p	Significance
Cerebral Cortex	0.002	VPA vs KS	13.97 ± 3.67 vs 15.32 ± 3.21	0.042	NS
		VPA vs VPA+100	13.97 ± 3.67 vs 25.03 ± 1.68	0.018	*
		VPA vs VPA+200	13.97 ± 3.67 vs 19.38 ± 1.97	0.109	NS
		VPA vs VPA+300	13.97 ± 3.67 vs 23.90 ± 5.97	0.164	NS
Hippocampus CA1	0.001	VPA vs VPA+100	20.73 ± 4.97 vs 32.90 ± 1.94	0.040	*
		VPA vs VPA+200	20.73 ± 4.97 vs 29.07 ± 2.46	0.031	*
		VPA vs VPA+300	20.73 ± 4.97 vs 32.67 ± 13.19	0.038	*
Cerebellum (Purkinje)	0.004	VPA vs VPA+100	3.45 ± 0.67 vs 4.67 ± 0.74	0.053	NS
		VPA vs VPA+200	3.45 ± 0.67 vs 4.87 ± 0.46	0.026	*
		VPA vs VPA+300	3.45 ± 0.67 vs 6.17 ± 1.27	<0.001	*

* ANOVA followed by LSD post-hoc. * = p < 0.05 vs VPA group. NS = Not significant.

Table 5: MBP intensity in brain regions (ANOVA + LSD Post Hoc).

Brain Region	ANOVA p	Comparison	Mean ± SD	Post Hoc p	Significance
Cerebral Cortex	0.02	VPA vs KS	45.50 ± 9.77 vs 83.86 ± 40.20	0.002	*
		VPA vs VPA+100	45.50 ± 9.77 vs 52.83 ± 9.06	0.106	NS
		VPA vs VPA+200	45.50 ± 9.77 vs 70.67 ± 6.19	0.011	*
		VPA vs VPA+300	45.50 ± 9.77 vs 67.83 ± 5.19	0.019	*
Hippocampus CA1	0.03	VPA vs KS	59.13 ± 9.18 vs 83.29 ± 8.57	0.020	*
		VPA vs VPA+100	59.13 ± 9.18 vs 71.33 ± 6.19	0.110	NS
		VPA vs VPA+200	59.13 ± 9.18 vs 79.67 ± 6.91	0.003	*
		VPA vs VPA+300	59.13 ± 9.18 vs 76.83 ± 8.64	0.010	*
Cerebellum	0.01	VPA vs KS	61.79 ± 12.46 vs 92.14 ± 15.77	0.003	*
		VPA vs VPA+100	61.79 ± 12.46 vs 73.33 ± 8.69	0.021	*
		VPA vs VPA+200	61.79 ± 12.46 vs 81.29 ± 8.89	0.009	*
		VPA vs VPA+300	61.79 ± 12.46 vs 77.67 ± 7.62	0.018	*

* ANOVA followed by LSD post-hoc. * = p < 0.05 vs VPA group. NS= Not significant.

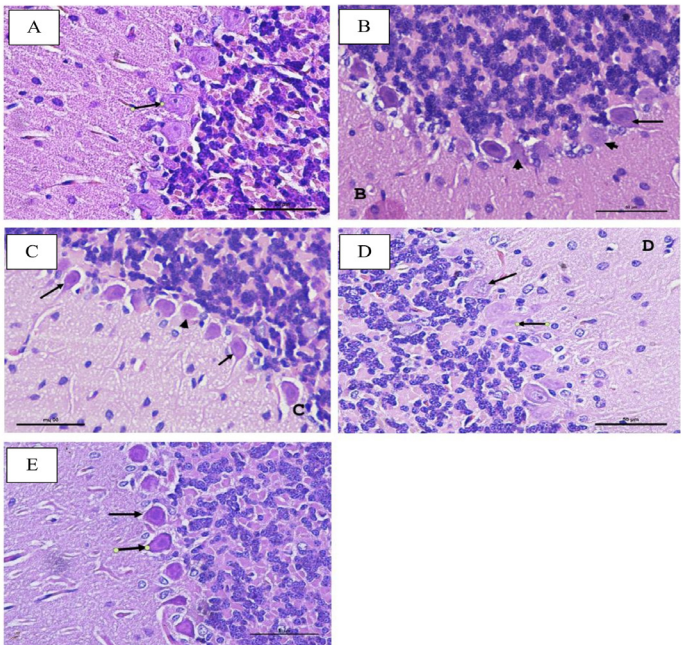

Figure 6: Dose-dependent effect of fenugreek seed extract on viable neuron count. Bar graph comparing the number of viable neurons in the cerebrum, hippocampus CA1, and cerebellum. Shows dose-dependent increase in neuron count with fenugreek extract. Significance indicated by asterisks (p < 0.05).

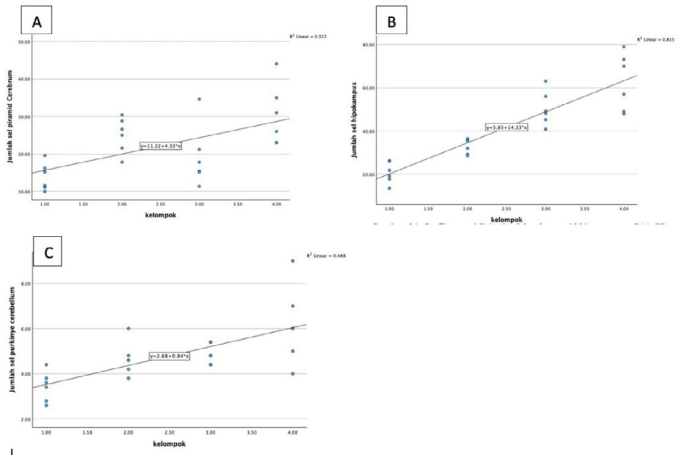

Figure 7: Linear regression analysis of viable neuron numbers. Regression plots showing the relationship between fenugreek dose and viable neuron counts in: (A) Cerebral cortex, (B) Hippocampus CA1, (C) Cerebellar Purkinje layer. X-axis: Treatment groups (1 = VPA, 2 = VPA+100 mg/kg, 3 = VPA+200 mg/kg, 4 = VPA+300 mg/kg). Y-axis: Number of viable neurons (mean ± SD).

DISCUSSION

This study evaluated the neuroprotective effects of

fenugreek seed extract in a rat model of ASD induced by prenatal exposure to valproic acid. The behavioral data demonstrated that the 200 mg/kg dose of fenugreek extract significantly improved social interaction and reduced repetitive behavior. Interestingly, while the 300 mg/kg dose improved histological parameters such as viable neuron count and MBP expression, it did not yield significant improvements in SNI behavior, indicating a possible dissociation between behavioral and structural recovery. The observation that 200 mg/kg improved social behavior, while 300 mg/kg yielded superior histological recovery, may reflect a differential sensitivity of behavioral circuits versus structural neuronal repair. Social behaviors involve complex cortical and limbic networks not fully captured by MBP or neuron count alone.

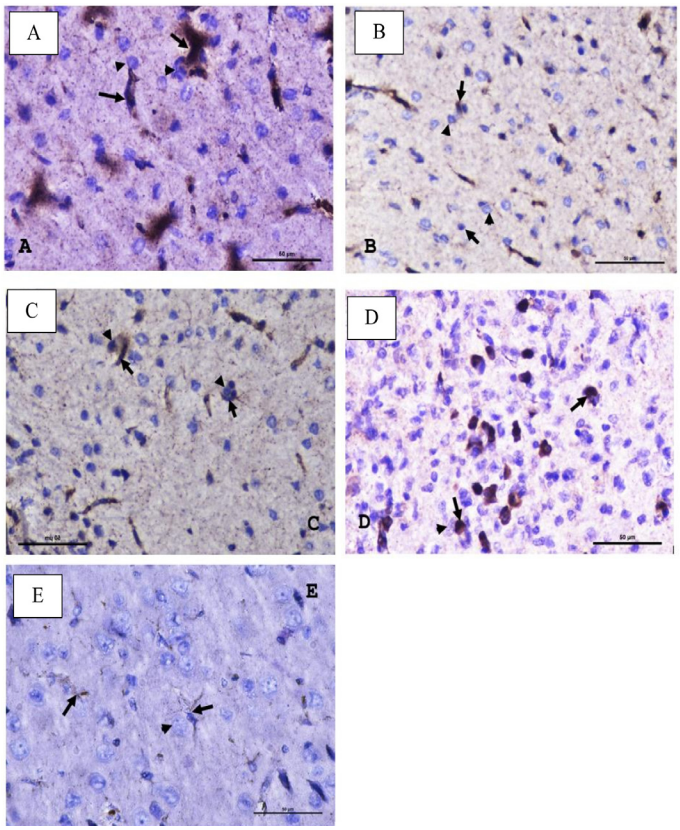

Figure 8: Immunohistochemical staining of Myelin Basic Protein (MBP) in the cerebral cortex (400×). Arrowheads (blue): Neurons. Arrows (brown): MBP-positive oligodendrocytes and myelinated fibers. Panels: (A) Control (KS), (B) VPA, (C) VPA+100 mg/kg, (D) VPA+200 mg/kg, (E) VPA+300 mg/kg.

Fenugreek extract contains flavonoids, steroidal saponins, and trigonelline, which may exert neuroprotective effects via antioxidant activity, phosphodiesterase-1 (PDE1) inhibition, and histone deacetylase (HDAC) modulation. These mechanisms may restore neuronal integrity and myelination disrupted by VPA-induced neurotoxicity. However, these pathways were not directly measured in this study and remain speculative.

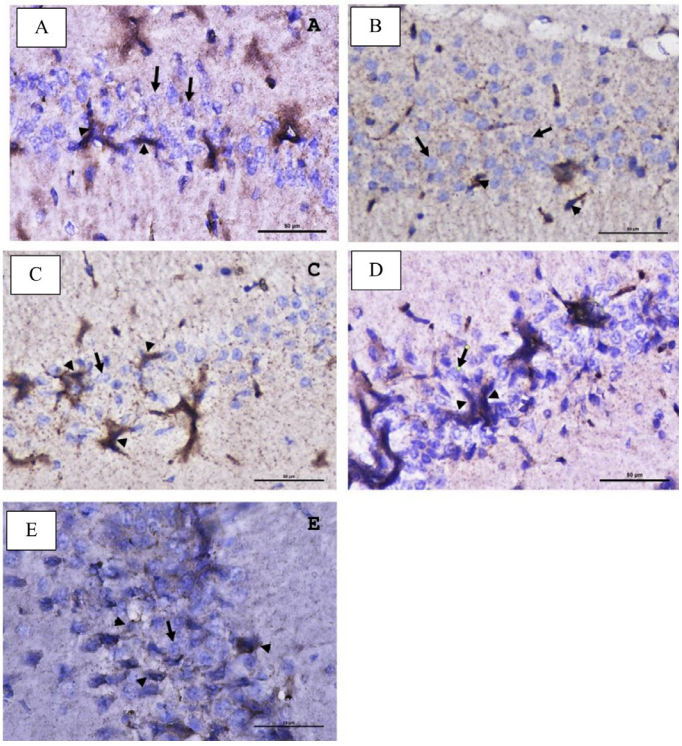

Figure 9: Immunohistochemical staining of MBP in the hippocampus CA1 region (400×). Arrowheads (blue): Neurons. Arrows (brown): MBP-positive oligodendrocytes. Panels: (A) Control (KS), (B) VPA, (C) VPA+100 mg/kg, (D) VPA+200 mg/kg, (E) VPA+300 mg/kg.

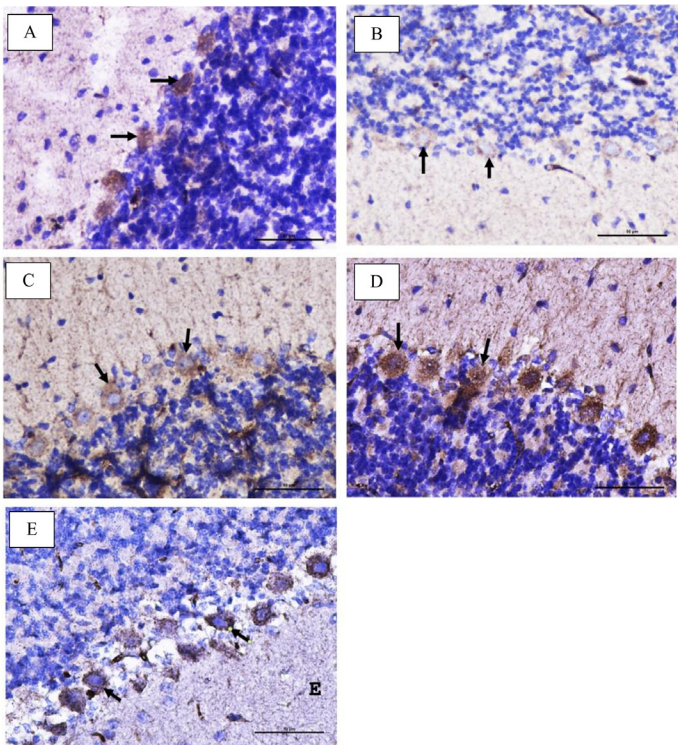

Figure 10: Immunohistochemical staining of MBP in the cerebellum (Purkinje layer, 400×). Arrowheads (blue): Neurons. Arrows (brown): MBP-positive oligodendrocytes in the white matter beneath the Purkinje layer. Panels: (A) Control (KS), (B) VPA, (C) VPA+100 mg/kg, (D) VPA+200 mg/kg, (E) VPA+300 mg/kg.

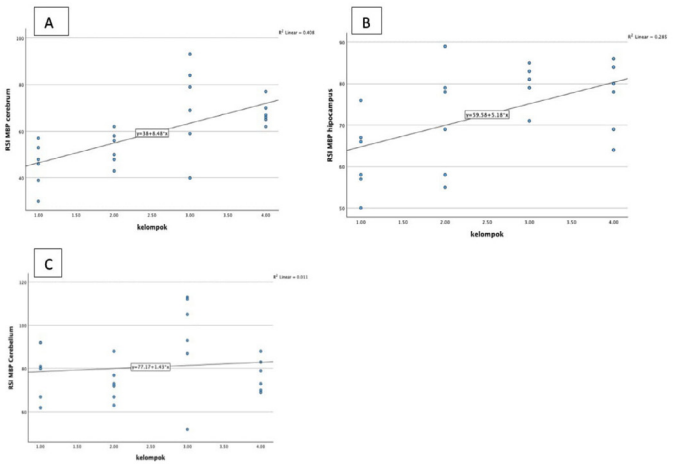

Figure 11: Linear regression analysis of MBP intensity. Graphs showing dose-dependent relationship between fenugreek extract dose and MBP intensity (RSI values) in: Cerebral cortex, Hippocampus CA1, Cerebellum, X-axis: Treatment group, Y-axis: MBP intensity (RSI = 255 – mean gray value).

The observed improvement in viable neuron counts, especially in the hippocampus CA1, aligns with embryological evidence that this region is a primary site of neurogenesis and is particularly vulnerable to VPA-induced damage. Fenugreek extract may protect or restore neuronal populations by preventing apoptosis, potentially via caspase-3 inhibition, HDAC reactivation, or modulation of mitochondrial function. However, future studies should confirm these mechanisms by evaluating markers such as TNF-α, JNK, caspase-3, and NOX2 expression.

Myelin repair was also improved with fenugreek extract, as evidenced by increased MBP intensity. This may be attributed to enhanced oligodendrocyte maturation and adenosine pathway modulation, which are essential for myelin formation and stability. The complex purinergic signaling cascade involving ATP, AMP, and adenosine is known to affect neurotransmitter release, oligodendrocyte development, and inflammation. Excess extracellular adenosine resulting from mitochondrial dysfunction, as seen in ASD models, may impair myelination via A2aR-mediated inhibition of oligodendrocyte maturation. Fenugreek extract may counteract this by reducing oxidative stress and rebalancing neurotransmitter pathways. Another limitation is the absence of a group treated with fenugreek extract alone. This restricts our ability to assess the extract's intrinsic effects on normal neurodevelopment or its potential toxicity.

Despite these promising results, the study has notable limitations. First, the exact molecular mechanisms of action were not evaluated. Second, the extract was not standardized to specific bioactive components, limiting reproducibility. Third, the absence of a fenugreek-only group precludes evaluation of its independent neurodevelopmental effects. The use of a crude, non-standardized extract limits reproducibility and mechanistic interpretation. Without quantification of key bioactives such as trigonelline or saponins, the dose equivalency across different preparations remains uncertain. Future work should employ standardized extracts and phytochemical profiling (e.g., via HPLC or LC-MS) to ensure replicability.

CONCLUSION

Fenugreek seed extract demonstrates neuroprotective potential against prenatal VPA-induced ASD-like phenotypes in rats. The 200 mg/kg dose provided the most consistent behavioral improvement, whereas the 300 mg/kg dose produced stronger restoration of viable neuron counts and myelin intensity. These findings suggest dose-dependent effects and support further mechanistic and phytochemical investigations to confirm active constituents and pathways involved.

ACKNOWLEDGMENT

The authors thank the Faculty of Medicine, Faculty of Veterinary Medicine, and the Faculty of Pharmacy Testing Service Unit, Universitas Airlangga, as well as the Professor Niddom Foundation, for supporting animal housing, facilities, and technical assistance.

NOVELTY STATEMENT

This study provides initial evidence that fenugreek seed extract may mitigate ASD-like behavioral deficits and improve neuronal viability and myelination in offspring exposed prenatally to valproic acid. The results suggest dose-dependent differences, with 200 mg/kg showing stronger behavioral effects and 300 mg/kg showing stronger histological recovery.

AUTHOR'S CONTRIBUTION

AKD and VFH carried out the histopathology staining and evaluate the result. AKD and BP participated in study design, the collection of tissue section, and statistical analysis. WW drafted the manuscript. All authors read and approved the final manuscript.

Generative AI and AI-assisted technology statement

The authors declare that no Genrative AI was used in the creation of this manuscript.

Conflict of interest

The authors have declared no conflict of interest regarding

the publication of this article.

REFERENCES

Belaïd-Nouira Y, Bakhta H, Haouas, Z., Flehi-Slim, I., Neffati, F., Najjar, M. F., Abdel-Wareth, A. A. (2012). Fenugreek seeds reduce aluminum toxicity associated with renal failure in rats. Nutr. Res. Pract., 6(6): 466–474. https://doi.org/10.4162/nrp.2013.7.6.466

Cammarata G (2023). Neurodevelopment effects of valproic acid exposure in human brain organoid.

Cao M, Wang XR, Hu WY, Yin D, Ren CZ, Chen SY, Yu ML, Wei YY, Hu TJ (2022). Regulatory effect of Panax notoginseng saponins on the oxidative stress and histone acetylation induced by porcine circovirus type 2. J. Vet. Med. Sci., 84(4): 600–609. https://doi.org/10.1292/jvms.21-0126

Circu ML, Aw TY (2010). Reactive oxygen species, cellular redox systems, and apoptosis. Free Radic. Biol. Med., 48(6): 749–762. https://doi.org/10.1016/j.freeradbiomed.2009.12.022

Cizkova K, Janousek L, Cizkova L, Tereza Foltynkova, M. Gachechiladze, Z. Tauber(2021). Comparative analysis of immunohistochemical staining intensity determined by light microscopy, ImageJ, and QuPath in placental Hofbauer cells. Acta Histochem. Cytochem., 54(1): 21–29. https://doi.org/10.1267/ahc.20-00032

Dewi AK, Purwanto B, Widjiati W, Hendrawan VF (2025). Effect of fenugreek seed extract on the improvement in folliculogenesis of female rats exposed to valproic acid. J. Vet. Sci., X(X): 1–5. https://doi.org/10.17582/journal.aavs/2025/13.10.2247.2251

Di Y, Li, Z., Li, J., Cheng, Q., Zheng, Q., Zhai, C., Kang, M., Wei, C., Lan, J., Fan, J., Ren, W., Tian, Y. (2021). Maternal folic acid supplementation prevents autistic behaviors in a rat model induced by prenatal exposure to valproic acid. Food Funct., 12(10): 4544–4555. https://doi.org/10.1039/D0FO02926B

Driel S van (2012). Cadherins in autism spectrum disorders (thesis). Utrecht: Universiteit Utrecht.

Elnahas EM, El Kerdawy AM, Mohamed DA, Abuelezz SA, Mohamad MI, Nabil MM, Abdelraouf SM, Bahaa N, Hassan GAM, Ibrahim EA, Ahmed AI, Aboul-Fotouh S (2021). Validation of prenatal versus postnatal valproic acid rat models of autism: a behavioral and neurobiological study. Prog. Neuropsychopharma. Biol. Psych., 108: 110185. https://doi.org/10.1016/j.pnpbp.2020.110185

Hodges H, Fealko C, Soares N (2020). Autism spectrum disorder: Definition, epidemiology, causes, and clinical evaluation. Trans. Pediatr., 9(8): S55–65. https://doi.org/10.21037/tp.2019.09.09

Inoue T, Nagai M, Takaishi Y, Mimaki, Y., Sashida, Y., Nikaido, T., Ohmoto, T. (1995). Steroidal saponins from the tubers of Dichelostemma multiflorum and their inhibitory activity on cyclic-AMP phosphodiesterase. Chem. Pharm. Bull., 43(5): 856–860. https://doi.org/10.1016/0031-9422(95)00117-P

Khasanah N, Liliana, A., Sandra, C. J. W. (2021). Perbandingan kecukupan ASI ibu menyusui antara konsumsi ekstrak daun kelor dengan konsumsi ekstrak biji fenugreek. J. Keperawatan. 12(2): 104–109.

Lee A, Kwon, O. W., Jung, K. R., Song, G. J., Yang, H. J. (2022). The effects of Korean red ginseng-derived components on oligodendrocyte lineage cells: distinct facilitatory roles of the non-saponin and saponin fractions, and Rb1, in proliferation, differentiation and myelination. J. Ginseng Res., 46(3): 453–465. https://doi.org/10.1016/j.jgr.2021.04.007

Luhach K, Kulkarni GT, Singh VP, Sharma B(2021). Vinpocetine amended prenatal valproic acid-induced features of ASD possibly by altering markers of neuronal function, inflammation, and oxidative stress. Autism Res., 14(11): 2270–2286. https://doi.org/10.1002/aur.2597

Muhson I, Al A (2014). Phenolic content and antioxidant activity of fenugreek seeds extract. Int. J. Pharm. Tech. Res., 6(4): 1290–1294.

Nguyen D, Nguyen D (2013). Quantifying chromogen intensity in immunohistochemistry via reciprocal intensity. Nat. Protoc. Exch., https://doi.org/10.1038/protex.2013.097

Norton WT, Poduslo SE (1973). Myelination in rat brain: Changes in myelin composition during brain maturation. J. Neurochem., 21(4): 759–773. https://doi.org/10.1111/j.1471-4159.1973.tb07520.x

Nursetiani A, Kusmiyati M, Adlina S, (2018). Potensi biji klabet (*Trigonella foenum*-graecum L) sebagai alternatif pengobatan herbal: Review jurnal. Trad. Med. J., 16(2): 475–484.

Padovan-Neto FE (2024). Beyond traditional pharmacology: Evaluating phosphodiesterase inhibitors in autism spectrum disorder. Neuropsychopharmacology, 49(4): 2023–2024. https://doi.org/10.1038/s41386-024-01860-z

Pakpahan FH, Sihite JM, Nababan L, (2022). Theory of cognitive development by Jean Piaget. J. Appl. Linguist., 2(1): 55–60. https://doi.org/10.52622/joal.v2i2.79

Petit PR, Sauvaire YD, Ponsin GR, Sauvaire YD, Hillaire-Buys DM, Leconte OM, Baissac YG, Ponsin GR, Ribes GR. (1995). Steroid saponins from fenugreek seeds: Extraction, purification, and pharmacological investigation on feeding behavior and plasma cholesterol. Steroids, 60(10): 674–680. https://doi.org/10.1016/0039-128X(95)00090-D

Pribac C, Remenyik J (2017). Analysis of phytoconstituent profile of fenugreek (*Trigonella foenum-graecum* L.) seed extracts. Studia UBB Chem., 62(2): 145–166. https://doi.org/10.24193/subbchem.2017.2.11

Riba A, Feki A, Ben Salem M (2019). Neuroprotective effect of fenugreek seed extract on aluminum chloride-induced neurotoxicity in rats. Physiol. Behav., 199: 63–70.

Sabers A, Damkier P, Lokkegaard T, Battino D, Tomson T, Bonizzoni E, Craig J, Perucca E, Sabers A, Thomas S, Alvestad S, Perucca P, Vajda F, (2024). Risk of major congenital malformations and exposure to antiseizure medication monotherapy. JAMA Neurol., Epub ahead of print.

Salari N, Rasoulpoor S, Rasoulpoor S, Rasoulpoor S, Rasoulpoor S, Shohaimi S, Jafarpour S, Abdoli N, Khaledi-Paveh B, Mohammadi M (2022). The global prevalence of autism spectrum disorder: A comprehensive systematic review and meta-analysis. Ital. J. Pediatr., 48: 112. https://doi.org/10.1186/s13052-022-01310-w

Sato A, Kotajima-Murakami H, Tanaka M, Katoh Y, Ikeda K (2022). Influence of prenatal drug exposure, maternal inflammation, and parental aging on the development of autism spectrum disorder. Front Psych., 13: 821455. https://doi.org/10.3389/fpsyt.2022.821455

Shen H, Huang N, Reemmer J, Xiao L. (2018). Adenosine actions on oligodendroglia and myelination in autism spectrum disorder. Front. Cell Neurosci., 12: 482. https://doi.org/10.3389/fncel.2018.00482

Umamageswari J, Mohan N, Bhuvaneswari M, Balasubramanian S, Krishnakumar K, Preetha SP, Vijayarani K.A (2020). A

simple and rapid staining technique to confirm mating in Wistar rats. J. Chem. Pharm. Res., 8(5): 820–823. https://doi.org/10.22271/j.ento.2020.v8.i5l.7612

Vafaee-Shahi M, Safari S, Mirzaei B, Safari S, Mirzaei B, Soheilipour F, Mohagheghi P, Riahi A, Borghei N-S, Talebi A (2022). Metabolic adverse effects of valproic acid in pediatric epileptic patients. Epilepsy Res., 183: 106941.

Yang J, Zhang Z, Wu Q, Ding X, Yin C, Yang E, Sun D, Wang W, Yang Y, Guo F. (2022). Multiple responses optimization of antioxidative components extracted from fenugreek seeds using response surface methodology to identify their chemical compositions. Food Sci. Nutr., 10(4): 3475–3484. https://doi.org/10.1002/fsn3.2949

Zarate-Lopez D, Cline BH, Bersani G, Torres-Chávez AL, Gálvez-Contreras AY, Gonzalez-Perez O (2023). Three decades of valproate: A current model for studying autism spectrum disorder. Curr. Neuropharmacol., 22(2): 260–289. https://doi.org/10.2174/1570159X22666231003121513