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Promising therapeutic efficacy of kolaviron against prenatal valproate-induced autism spectrum disorder

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ABSTRACT

Objective(s): Autism spectrum disorder (ASD) is a lifelong neurodevelopmental condition marked by impairments in social communication, language, and behavior. Prenatal exposure to valproic acid (VPA) contributes to its pathogenesis. kolaviron (KV), a polyphenolic extract from Garcinia kola, has potent antioxidant and anti-inflammatory properties, offering neuroprotective potential in ASD models. Our study aimed to evaluate whether KV could improve the VPA-induced autism model through targeting serotonergic system, mitochondrial dysregulation, oxidative stress, and inflammation.

Materials and Methods: Pregnant Wistar rats received a single intraperitoneal dose of VPA (600 mg/kg) on gestational day 12.5 to induce autism-like features in offspring. Male pups were weaned on postnatal day (PND) 21 and randomly assigned to receive KV (50 or 100 mg/kg, oral), or saline until PND 49. Behavioral tests were finally conducted and brain tissue was collected for analysis of hippocampal oxidative stress, mitochondrial dysfunction, serotonin transporter (5-HTT), serotonin receptor 7 (5-HTR7), tumor necrosis factor α (TNF), and interleukin 6 (IL-6) levels. Immunohistochemical staining for glial fibrillary acidic protein (GFAP) was also performed to assess astrocytic reaction.

Results: KV-treated VPA-exposed rats showed significant improvements in social interaction and with lower repetitive behavior. Biochemically, KV decreased malondialdehyde (MDA), IL-6 and TNFα levels, improved catalase activity and mitochondrial membrane potential (MMP), and modulated serotonergic markers (5-HTT and 5-HTR7). Histologically, KV also attenuated hippocampal GFAP immunoreactivity (IRA).

Conclusion: KV showed its promising potential as a complementary therapeutic agent in ASD murine model. Further studies are still warranted to clarify its further mechanisms and clinical relevance.

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Introduction

Autism Spectrum Disorder (ASD) is a lifelong neurodevelopmental condition marked by persistent challenges in social communication, language acquisition, and the presence of restrictive or repetitive behaviors. Typically emerging in early childhood, ASD manifests through a heterogeneous array of symptoms such as delayed speech and motor complications, difficulties interpreting social cues (e.g., tone of voice, body language), hyper- or hypo-reactivity to sensory stimuli, and sometimes self-injurious behaviors (1-4). Although genetic factors contribute substantially to ASD risk, environmental influences including prenatal exposure to certain medications also play a critical role (5). To date, no curative therapy exists for ASD, underpinning the urgent need for mechanistic studies and novel interventions (6).

Valproic acid (VPA) is a branched-chain fatty acid extensively used as an antiepileptic, mood stabilizer, and migraine prophylactic (5). However, when administered during early gestation, VPA is linked to an elevated risk of ASD in the offspring (6, 7). In rodent studies, a single intraperitoneal injection of 600 mg/kg of VPA on gestational day 12.5 delays neural tube closure and triggers widespread molecular disruptions, including compromised mitochondrial function (8). This critical exposure window overlaps with peak periods of neurogenesis and serotonergic neuron migration, making the developing brain particularly susceptible to excitatory-inhibitory imbalance and oxidative damage (8). At the cellular level, VPA increases reactive oxygen species and proinflammatory mediators while diminishing inhibitory interneuron populations (5, 9). Behaviorally, VPA-exposed rodents faithfully reproduce core ASD phenotypes, social deficits in the three-chamber test, heightened repetitive actions, and atypical ultrasonic vocalizations (1, 10).

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Kolaviron (KV) is a natural mixture of bioflavonoids obtained from *Garcinia kola* seeds, traditionally used in West African medicine. Its main ingredients include Garcinia biflavonoid 1 (GB1), Garcinia biflavonoid 2 (GB2), kolaflavanone, and kolaflavones. It is recognized for its antioxidant and anti-inflammatory activities, mainly due to its polyphenolic components (11). Beyond these general properties, KV has been extensively investigated for its specific neuroprotective potential in preclinical models (12, 13). Numerous studies have demonstrated its ability to mitigate oxidative stress and inflammation in the central nervous system (12, 13). For example, KV has been shown to reverse scopolamine-induced memory impairment and prevent brain microstructural derangements and cognitive deficits in rodents exposed to various neurotoxins, including methamphetamine and cuprizone (14). In a cuprizone-induced demyelination model, KV treatment significantly restored endogenous antioxidant enzyme levels including superoxide dismutase (SOD) and glutathione peroxidase (GPx), reduced lipid peroxidation (MDA), and preserved cerebellar neuronal morphology and astrocytic health, highlighting its robust neuroprotective and anti-inflammatory actions within the brain (15). Furthermore, in a rat model of maternal deprivation, KV successfully attenuated behavioral deficits, improved memory and cognitive performance, and restored oxidative balance in hippocampal and prefrontal cortex regions by enhancing SOD levels, suggesting its capacity to disrupt excitotoxic stimuli and prevent mitochondrial dysfunctions (16). These findings from diverse *in vitro* and *in vivo* models collectively provide strong experimental evidence for KV therapeutic potential in neurological conditions characterized by oxidative stress, neuroinflammation, and neuronal damage. The relevance of KV multifaceted actions extends to the complex pathophysiology of ASD, where oxidative stress and neuroinflammation are recognized as key contributing factors (17). Given that VPA is a well-established inducer of neurodevelopmental abnormalities in animal models, partly through the generation of oxidative stress and inflammatory responses, the compelling evidence of KV ability to modulate these pathways in preclinical settings provides a strong rationale for its investigation in VPA-induced ASD model. Moreover, serotonergic system is critically dysregulated in ASD (18), which maybe a potential target for natural products like KV. In this study, KV was administered orally at 50 and 100 mg/kg after weaning in a VPA-based rat model of autism to assess its effects on behavior, oxidative and inflammatory markers, and serotonin-related targets in the hippocampus.

Materials and Methods

Animals

Sexually mature and young male and female Wistar rats (190–240 g) were obtained from the Experimental and Comparative Research Center of Iran University of Medical Sciences (Tehran, Iran). Breeding was carried out under controlled laboratory conditions, and the male offspring were used for the subsequent experimental procedures. The animals were housed in groups of no more than four per cage, maintained on a 12-hour light/dark cycle, at a constant temperature of 22 °C and a relative humidity of 40–50%. Food and water were available to the animals at all times. All procedures were conducted in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals and approved by the Animal Ethics Committee of Iran University of Medical Sciences.

Initially, 8 virgin female Wistar rats (190–220 g) and 4 male naïve Wistar rats (200–240 g) were used with housing under standard conditions (21-23 °C, 45–50% humidity, 12/12 hr light/dark cycle) and with free access to food and water with 4 rats/cage. The day after mating was designated as gestational day 1 (GD1). On GD12.5, verified pregnant animals received a single intraperitoneal injection of either sodium valproate (600 mg/kg) (Darou Pakhsh Co., Tehran, Iran) to induce autism-like features (VPA group) or normal saline (control group). Due to the known teratogenic effects of VPA, two dams experienced fetal resorption. On postnatal day (PND) 21, pups were weaned and used in the study. For this purpose, a total of 32 male offspring (n=8 per group, 4 groups) were used. In this study, we used only male rats to reduce source of variability according to many preclinical studies, especially those focusing on neurodevelopmental disorders such as autism. ASD shows a higher prevalence in males in both clinical and experimental models. Therefore, the VPA-induced autism model is more commonly studied in male offspring, as they are more likely to display the core behavioral and neurochemical phenotypes associated with the disorder. In addition, it is well-documented that female rodents exhibit hormonal fluctuations across the estrous cycle, which can introduce variability in behavioral, neurochemical, and molecular outcomes. During our assessment period, autism-relevant behavioral traits such as reduced social interactions and repetitive/stereotyped behaviors were carefully monitored. These characteristics were assessed prior to any treatment administration to confirm the successful induction of the ASD-like condition. From PND 21 to PND 49, offspring received daily oral administration of either normal saline or KV (50 or 100 mg/kg) (Hangzhou Johoo Co., China). Doses of KV were based on its protective effect against okadaic acid-provoked cognitive impairment in the rats (13). Animals were divided into 4 groups (n = 8 each): (1) Control+Saline, (2) VPA+Saline, (3) VPA+kolaviron50, and (4) VPA+kolaviron100. Behavioral assessments were performed during PNDs 42-49. On PND 49, animals were deeply anesthetized with ketamine (120 mg/kg) and xylazine (10 mg/kg) and brains were immediately isolated. Right hemispheres were fixed in 10% formalin solution for immunohistochemical analysis of GFAP in the hippocampus and the other side was used for biochemical analyses.

KV biochemical analyses

Anti-oxidant activity of KV was measured via the DPPH radical scavenging method, calibrated with Trolox. Total flavonoid content was determined using aluminum chloride and potassium acetate, expressed as quercetin equivalents. Total phenolic content was measured by a modified Folin–Ciocalteu method, with Gallic acid as the standard. Flavonol content was evaluated after incubation with aluminum trichloride and sodium acetate, using rutin as the reference. All assays were performed in duplicate (13).

Experimental procedure

Behavioral tests

Three-chamber test

The procedure for this test has been reported before (5, 19). This test was utilized to evaluate the social interaction of rats. Initially, the rats were placed in the center of the

apparatus and given 10 min to explore their surroundings freely. Following this phase, an empty metal cage was positioned on one side of the apparatus, while a cage containing an unfamiliar rat was placed on the opposite side. The test rat was then allowed 10 min to assess its social interactions with the unfamiliar rat. The sociability index was calculated by subtracting the time spent in the non-social chamber from the time spent in the social chamber, and this result was divided by the total time spent in both chambers. Lastly, the time spent by the rats interacting with social and non-social stimuli such as cleaning, fighting, or other social behaviors was recorded. This index was utilized to analyze the social behaviors of the rats in response to various stimuli.

Self-grooming test

On the test day, each animal was individually placed in a Plexiglas cage measuring 30 × 30 × 45 cm. To familiarize them with the environment, the rats were acclimated to the empty cage for 10 min on the previous day. Following this, their total grooming time across all body areas was recorded over a 10-minute period (20).

Marble burying test

An apparatus measuring 40 cm × 40 cm × 40 cm was prepared, filled with nesting material and set up with 20 marbles arranged in 5 rows. Rats were then placed inside for 30 min, during which they could explore freely. The number of marbles they buried was recorded as an indicator of repetitive digging behavior (5, 19).

Social interaction test

To measure social interactions, an open field apparatus (60 × 60 × 40 cm) was set up. In this test, rats were separately adapted to the environment one day before the main session for 10 min. Then, after one day, testing rats were separately encountered with an unfamiliar rat to assess social interaction factors including sniffing, licking, crawling or mounting, approaching, or following the other rat (20).

ELISA assay

The concentrations of TNFα (Cat # sc-52746, Santa Cruz Biotechnology, Inc., USA), IL-6 (Cat # sc-57315, Santa Cruz Biotechnology, Inc., USA), 5-HTT (Cat # MBS2533556, MyBioSource, Inc., USA), and 5-HT7R (Cat # MBS282566), MyBioSource, Inc., USA) in the hippocampal tissues were determined using enzyme-linked immunosorbent assay (ELISA) kits, following the manufacturer protocols.

Hippocampal biochemical assessments

Assessment of hippocampal malondialdehyde (MDA)

The amount of malondialdehyde (Cat # KMDA96, Kiazist, Iran), a marker of lipid peroxidation, was evaluated using thiobarbituric acid dissolved in acetic acid solution. The experiment was conducted in a thermoblock set at 95 °C for 30 min. After this period, the samples were allowed to cool and then centrifuged for 5 min with a speed of 1500 rpm. Finally, the optical absorbance was measured at a wavelength of 535 nanometers with tetraethoxypropane as the standard (21).

Assessment of hippocampal catalase activity (CAT)

Catalase activity was assessed by its specific kit (Cat # KCAT96, Kiazist, Iran). In this method, the enzyme activity was assessed by first mixing 20 µl of supernatant with 100 µl of buffer and 20 µl of diluted substrate. This mixture was allowed to react for 20 min, facilitating the enzymatic reaction. After this period, the reaction was halted by adding 30 µl of diluted KOH. Next, 10 µl of KIO4 are introduced to the solution, and after waiting for 5 min, the absorbance was measured at 540 nm using a spectrophotometer. The amount of breakdown of H2O2 with catalase (CAT) was the basis of this assessment (13).

Assessment of hippocampal nitrite levels (NIT)

The nitrite content (Cat # KNIT96, Kiazist, Iran) in the tissue was measured using Griess reagent, which consisted of 2.5% phosphoric acid, 1% sulfanilamide, and 0.1% naphthyl ethylenediamine dihydrochloride. After a 10-minute incubation, the optical density was measured at 540 nm, with sodium nitrite used as the calibration standard (22, 23).

Assessment of mitochondrial membrane potential (MMP)

The assessment of mitochondrial membrane potential (MMP), which serves as a key diagnostic marker for mitochondrial integrity and health, was performed using Rhodamine 123 (Cat # R8004, Sigma-Aldrich, USA). In this procedure, samples were first subjected to centrifugation at 10,000 rpm. Subsequently, 20 µl of Rhodamine 123 was combined with 180 µl of phosphate-buffered saline (PBS) at a pH of 7.4 and after a 30-min incubation, the emission was taken at 525 nm after excitation at 488 nm (24, 25).

Immunohistochemical evaluation of hippocampal GFAP IRA

Paraffin embedding and standard histological processing were performed on the hippocampal tissue blocks, after which serial sections (a thickness of 5 µm) were obtained. Tissue sections were initially deparaffinized, rehydrated through descending concentrations of ethanol, and rinsed thoroughly in phosphate-buffered saline (PBS). To block endogenous peroxidase, a diluted 0.5% H2O2 solution was used for 10 min. To permeabilize the tissue, the slides were incubated with Triton X-100 in PBS for 15 min. Non-specific binding was blocked by exposing the sections to 10% normal goat serum (NGS) in PBS at room temperature for 1 hour. Subsequently, the sections were incubated overnight (12 hr at 4 °C) with a primary monoclonal mouse anti-GFAP antibody (1:55 dilution, Cat. # sc-33673, Santa Cruz Biotechnology, USA). The next day, samples underwent a 4-hr incubation at room temperature with an HRP-conjugated secondary antibody (1:75 dilution, Cat. # sc-516102, Santa Cruz Biotechnology, USA). To visualize antibody binding, 3,3′-diaminobenzidine tetrahydrochloride (DAB; Cat. # D5905, Sigma-Aldrich, USA) was used as the chromogenic substrate in the presence of H_2O_2, producing a brown staining pattern indicative of GFAP immunoreactivity (IRA). Sections were lightly counterstained with hematoxylin for 15 sec to enhance tissue contrast. Finally, GFAP immunoreactivity was quantified and expressed as positive loci per mm^2. All images for GFAP IRA were analyzed using ImageJ software (Version 1.53, National Institutes of Health, USA). To ensure objectivity and minimize bias, all histological assessments were conducted using coded slides and analyses were performed by a single blinded investigator.

Statistical methods

Statistical analyses were performed using GraphPad Prism 9. Normality of data distribution was assessed via the

Kolmogorov-Smirnov test, and outliers were identified and excluded using Grubbs' test. Inter-group differences were found using one-way ANOVA followed by Tukey's *post hoc* test. Results are presented as mean ± SEM, and statistical significance was defined as $P<0.05$.

Results

Table 1 shows the obtained findings on total phenols, flavonoids, and flavonols in the KV product.

Table 1. Total phenols, flavonoids, and flavonols in the kolaviron

Total antioxidant activity (equivalent to Trolox/gram)	2.25±1.6
Total phenols (mg equivalent of gallic acid/gram)	18.7±1.9
Total flavonoids (mg equivalent of quercetin/gram)	19.3±1.7
Total flavonols (mg equivalent of rutin/gram)	17.2±1.5

Behavioral findings

Self-grooming test

The results of the self-grooming test which reflects stereotyped and repetitive behaviors in animals are presented in Figure 1a. One-way ANOVA analysis revealed significant differences among the experimental groups [F (3, 26)=39.4, $P<0.001$]. Further evaluation using Tukey's multiple range test indicated that VPA group has significantly higher repetitive behaviors as compared to the control group ($P<0.001$). Moreover, treatment with KV in the VPA model groups at both doses of 50 and 100 mg/kg led to a marked decrease of such behaviors as compared to the VPA-damaged group. This reduction was significant for both doses ($P<0.001$; 33.7% and 44.1% for 50 and 100 mg/kg doses, respectively). These findings highlight the potential of KV in mitigating VPA-induced stereotyped behaviors.

Three-chamber test

Figure 1b presents the results of the three-chamber test, which is used to assess social interaction and social avoidance behaviors. The statistical analysis using one-way ANOVA showed a significant difference between the groups [F (3, 27)=6.1, $P<0.01$]. Further analysis revealed that the VPA model group exhibited a significant reduction in social interaction compared to the control group ($P<0.001$). In the KV-treated groups, the 100 mg/kg dose caused a significant improvement in social interaction compared to the VPA group ($P<0.05$; this increase was equivalent to 341.9%). These findings suggest that KV treatment, especially at the 100 mg/kg dose, can enhance social interaction in VPA model of social deficits.

Open field test

Figure 1c represents the time in the social zone, which assesses social behavior. One-way ANOVA statistical analysis indicates a significant difference among the studied groups [F (3, 24)=23.9, $P<0.001$]. Furthermore, our additional analyses revealed that the diseased group (VPA group) exhibited a significantly lower social behavior time compared to the control group ($P<0.001$), with a reduction of 71.2%. In the VPA groups treated with the KV, it was observed that the VPA+KV 50 mg/kg group has a significant increase ($P<0.01$) in social behavior time, with an improvement of 132.12%. Similarly, the VPA+KV 100 mg/kg group exhibited a comparable pattern, significantly having a higher index as compared to the VPA group ($P<0.01$), with an improvement of 118.01%.

Marble burying test

Figure 1d shows the results of the marble burying test, which is used to assess anxiety and repetitive behaviors in rats. The one-way ANOVA analysis revealed a significant difference among the groups [F (3, 26)=7.3, $P<0.01$]. Further

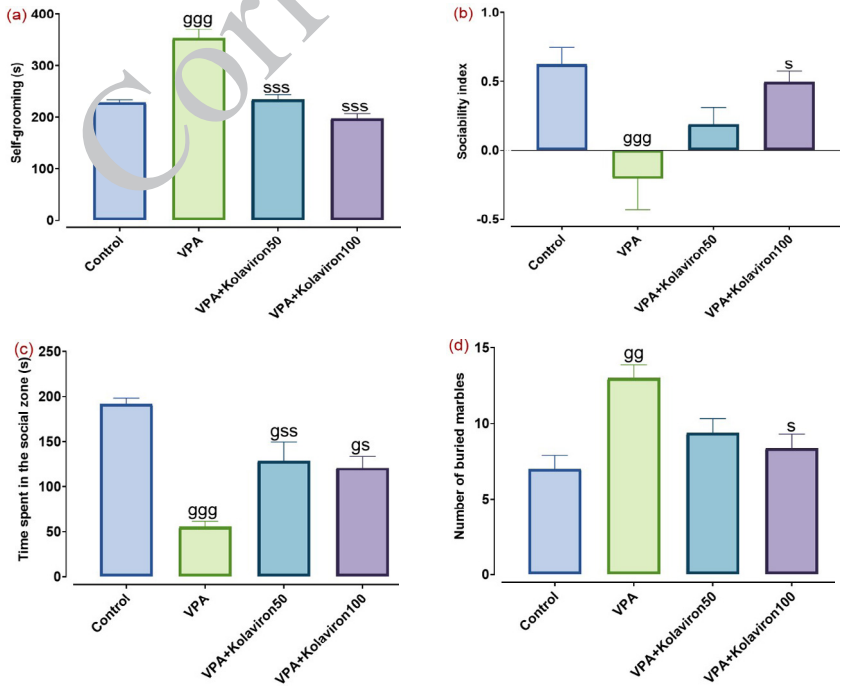

Figure 1. Behavioral assessments of control and prenatal VPA-induced ASD rats in different tasks on post-natal days 42-49 (a) Self-grooming test, (b) three-chamber social test, (c) open field test, and (d) Marble burying test

s denotes a significant difference compared to the disease model group ($P<0.05$), ss ($P<0.01$), and sss ($P<0.001$). g represents significance compared to the control group ($P<0.05$), gg ($P<0.01$), and ggg ($P<0.001$). Data are presented as mean±SEM.

evaluation with Tukey's *post hoc* test indicated that the VPA group has significantly higher anxiety and repetitive behaviors as compared to the control group (P<0.01). In the KV-treated groups, the 50 mg/kg dose did not produce a significant change in anxiety or repetitive behaviors. However, the 100 mg/kg dose of KV significantly reduced these behaviors compared to the VPA group (P<0.05). This decrease was equivalent to 35.6%. These findings suggest that KV at the 100 mg/kg dose can effectively reduce anxiety and repetitive behaviors induced by the VPA.

Biochemical assessments

In this study, we also assessed parameters related to the oxidative stress, mitochondrial dysregulation, inflammation, and two factors associated with the serotonergic system.

Oxidative stress analyses

Malondialdehyde (MDA) assessment

In these assessments, for MDA a significant increase was observed in the VPA group compared to the control group in the hippocampus (P<0.001). Additionally, in the treated disease group with 100 mg/kg of KV, a significant reduction was observed compared to the disease group (P<0.05, a reduction by 31.2%) (Figure 2a).

Catalase assessment

A significant decrease in the Cat activity was observed in the VPA group compared to the control group (P<0.05). Furthermore, the disease group treated with KV at 100 mg/kg showed a significant increase compared to the disease group (P<0.05, an increase by 73.4%) (Figure 2b).

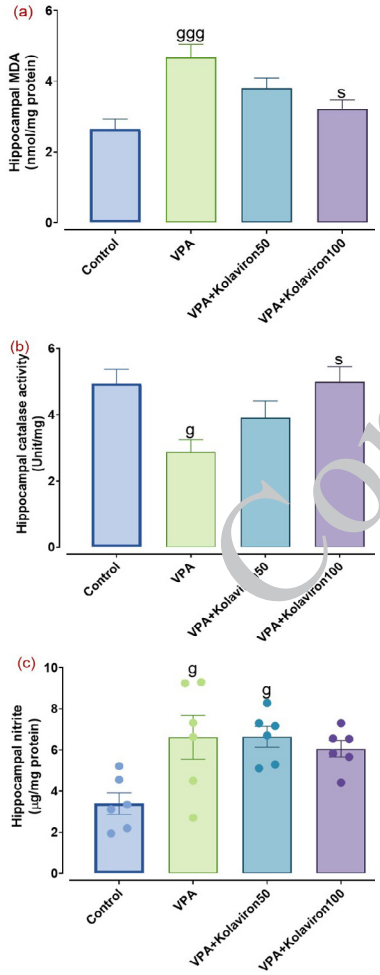

Figure 2. Assessment of hippocampal oxidative stress in control and prenatal VPA-induced ASD rats (a) malondialdehyde level, (b) catalase activity, and (c) nitrite level across different experimental groups
s denotes a significant difference compared to the disease model group (P<0.05). g represents significance compared to the control group (P<0.05) and ggg (P<0.001). Data are presented as mean±SEM.

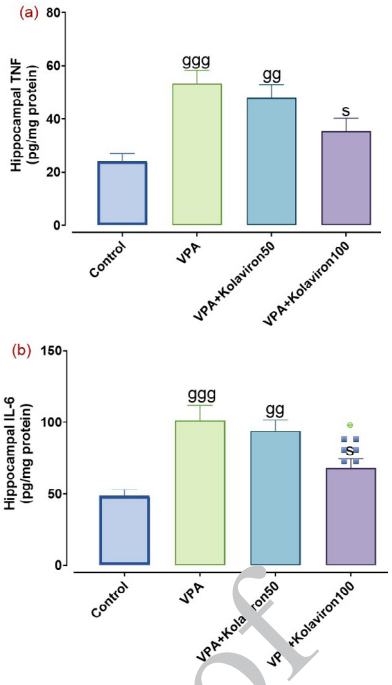

Figure 3. Assessment of hippocampal inflammation-associated factors in control and prenatal VPA-induced ASD rats (a) tumor necrosis factor α (TNFα) and (b) interleukin 6 (IL-6) levels across different experimental groups
s denotes a significant difference compared to the disease model group (P<0.05). gg represents significance compared to the control group (P<0.01), ggg (P<0.001). Data are presented as mean±SEM.

Nitrite assessment

A significant increase in Nit was observed in the VPA disease group compared to the control (P<0.05). However, no significant changes were observed in the treated groups compared to the disease group (Figure 2c).

Inflammation assessment

Tumor necrosis factor (TNF) assessment

For TNF, the VPA group showed a significant increase compared to the control group in the hippocampus (P<0.001). The disease group treated with 100 mg/kg of KV showed a significant reduction (P<0.05, by 33.4%) (Figure 3a).

Interleukin-6 (IL-6) assessment

The VPA group showed a significant increase compared to the control group (P<0.001). For this parameter, the disease group treated with KV at 100 mg/kg showed a significant reduction (P<0.05, by 32.8%) (Figure 3b).

Serotonergic system assessment

In the assessment of serotonin-related parameters, we measured two parameter, including 5HTT and 5HTR7.

Serotonin transporter (5HTT) assessment

For 5HTT, the VPA group showed a significant increase compared to the control group in the hippocampus (an elevation of 120.7%). The disease group treated with 100 mg/kg of KV showed a significant reduction compared to the disease group (P<0.05, a reduction of 40.38%) (Figure 4a).

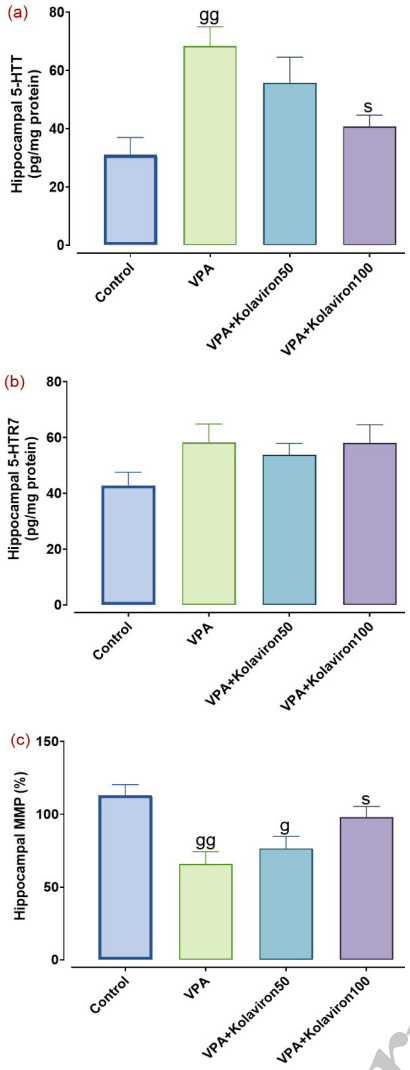

Figure 4. Assessment of hippocampal serotonergic system associated factors and mitochondrial health in control and prenatal VPA-induced ASD rats (a) serotonin transporter (5HTT) (b), serotonin receptor (5-HTR7), and (c) mitochondrial membrane potential (MMP) across different experimental groups
s denotes a significant difference compared to the disease model group (P<0.05). g represents significance compared to the control group (P<0.05), gg (P<0.01). Data are presented as mean ± SEM.

Serotonin receptor 7 (5HTR7) assessment

For 5HTR7, no significant changes were observed in any of the groups. KV administration led to a non-significant reduction in 5-HTR7 expression in VPA-exposed animals treated with KV. This reduction was more prominent at the 50 mg/kg dose (Figure 4b).

Mitochondrial membrane potential (MMP) assessment

In this study, we also assessed MMP, which reflects mitochondrial dysregulation, a key factor contributing to cellular metabolic imbalance. A significant decrease was observed in the VPA group compared to the control group in the hippocampus (P<0.01). However, among the treatment groups, only the disease group treated with 100 mg/kg of KV showed a significant increase compared to the disease group (P<0.05, by 48.9%) (Figure 4c).

Histological assessment of GFAP IRA

A significant increase in GFAP immunoreaction was observed in the VPA group compared to the control group (P<0.001). Additionally, the disease group treated with 100

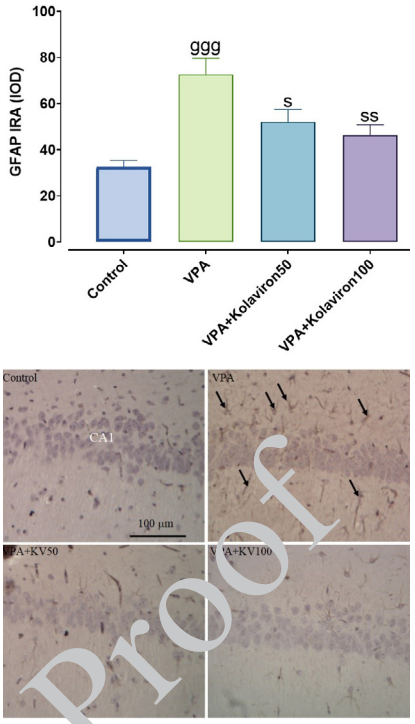

Figure 5. GFAP IRA across different experimental groups
s denotes a significant difference compared to the disease model group (P<0.05) and ss (P<0.01). ggg represents significant difference compared to the control group (P<0.001). Data are presented as mean ± SEM. Black arrows show hyperreactive astrocytes.

mg/kg of KV showed a significant decrease compared to the disease group (P<0.01, a decrease by 36.4%) (Figure 5).

Discussion

In this study, we aimed to investigate the potential effects of KV on VPA-induced model of autism. Among the various systems implicated in the pathophysiology of autism, the serotonergic system was particularly our main focus. In parallel, we also evaluated markers of oxidative stress and inflammation. By integrating behavioral assessments with histological and biochemical analyses, we sought to better understand the relationship between molecular and structural alterations and the observed behavioral changes.

In the self-grooming test, which was used for evaluation of repetitive behavior, the KV-treated groups (50 and 100 mg/kg) exhibited a significant reduction in stereotypic behaviors compared to the VPA model group. This improvement may be attributed to KV antioxidant properties and reduction in oxidative stress, potentially restoring neuronal function in brain areas responsible for repetitive behaviors, such as the hippocampus. In the three-chamber test, which specifically assesses social interaction, KV at a dose of 100 mg/kg led to significant improvement of social engagement. In the marble burying test, which is used to assess anxiety and repetitive behaviors, treatment with KV (100 mg/kg) significantly reduced both anxiety and stereotypic behaviors. These outcomes suggest that KV may be effective in alleviating anxiety symptoms, possibly through its impact on brain oxidative and inflammatory stress pathways. However, no comparable effects were observed at a dose of 50 mg/kg, indicating that this lower dose may have been insufficient to influence these specific behavioral outcomes. Finally, in the open field test, which evaluates both social

behavior and anxiety, KV significantly increased the time spent in the social zone, further supporting its potential in improving social engagement and reducing anxiety-related behaviors. This aligns with results from other behavioral assays, reinforcing KV positive impact on anxiety reduction and social behavior enhancement. These findings suggest that KV, especially at a dose of 100 mg/kg, can effectively ameliorate repetitive behaviors, anxiety, and social deficits in the VPA-induced autism model.

VPA is a short-chain fatty acid that has been widely used to model autism in rodents. When administered intraperitoneal during pregnancy, VPA can disrupt neural tube closure, leading to structural and functional abnormalities in the developing nervous system (8). This model is known to induce oxidative stress, mitochondrial dysfunction, and neuroinflammation, commonly associated with ASD. Given the high energy demands of neurons and the critical role of mitochondrial integrity in neuronal health, disruptions in these pathways can have profound effects (1). VPA also influences sodium channel activity, neurotransmitter systems, and the excitatory-inhibitory balance in the brain, contributing to a neurodevelopmental profile that mimics core features of autism, including increased reactive oxygen species (ROS) levels and altered pro-inflammatory cytokine expression (8).

KV, a bioactive compound derived from *G. kola*, is known for its potent antioxidant and anti-inflammatory properties (26). Previous studies have demonstrated that KV effectively scavenges free radicals and acts as a robust antioxidant (27, 28). By mitigating oxidative and inflammatory damage, KV may protect brain regions that are critically involved in behavioral regulation, memory, and anxiety functions and are typically impaired in autism models. Brain areas such as the hippocampus are particularly vulnerable to oxidative and inflammatory insults (29). Moreover, KV has been reported to exert neuroprotective effects against various forms of neurotoxicity and may improve social behavior and memory deficits associated with neurodevelopmental disorders such as autism (28, 30). Consistent with previous findings, our results demonstrated a significant increase in malondialdehyde (MDA) levels in the hippocampus of VPA-exposed rats, indicating elevated lipid peroxidation and oxidative damage. These findings support the hypothesis that oxidative stress plays a critical role in the neurodevelopmental abnormalities observed in the VPA model of autism. Treatment with KV at a dose of 100 mg/kg significantly reduced MDA levels in the hippocampus, suggesting a protective effect against lipid peroxidation and free radical-induced damage. Notably, such reduction may be attributed to KV's antioxidant structure and its ability to scavenge free radicals production (28). In the VPA group, a marked reduction in the activity of catalase (CAT) was also observed. This reduction indicates a compromised or impaired antioxidant defense system in the brain (2). KV treatment restored CAT levels, highlighting KV role in enhancing endogenous antioxidant defenses. Moreover, nitrite levels, used as an indirect measure of nitric oxide production and nitrosative stress, were significantly elevated in the VPA group, suggesting ongoing neuroinflammation and potential mitochondrial damage. However, KV treatment did not significantly alter nitrite levels, clearly implying that its neuroprotective effects may be more dependent on ROS-mediated pathway rather than reactive nitrogen species (RNS)-mediated cascade. To further assess the impact of oxidative stress on cellular health, MMP was also evaluated. VPA exposure led to a substantial decrease in MMP, indicative of mitochondrial dysfunction, a hallmark of oxidative stress in neurodevelopmental disorders. Interestingly, KV treatment partially restored MMP in the hippocampus, suggesting improved mitochondrial integrity and possibly partial recovery of neuronal energy balance (1, 28). The inflammatory markers TNFα and IL-6 were also assessed. In the VPA model group, both cytokines were significantly elevated in the hippocampus, indicating an activated immune response and ongoing neuroinflammation. Treatment with KV, especially at its dose of 100 mg/kg, significantly reduced these cytokines. This anti-inflammatory effect of KV may also be responsible for part of the behavioral improvements observed in the treated animals.

Previous studies have reported elevated blood serotonin levels in the VPA-induced autism-like model, suggesting a possible association between increased serotonin and the pathophysiology of autism (31). Consequently, growing attention has been directed toward serotonin due to its critical role in the nervous system and the inconsistent changes observed in different neural circuits (29). Current investigations have targeted various aspects of the serotonergic system, including serotonin itself, its transporters, receptors, and precursors. Given the known role of serotonin in mood regulation and its strong implication in ASDs, and considering the overlap between the timing of VPA injection in our model and key developmental windows for serotonergic neurons, we focused on exploring this system. In the case of the serotonin transporter (5HTT), a significant increase in its expression was observed in the VPA group in the hippocampus, suggesting altered reuptake mechanisms. Interestingly, the group treated with KV at 100 mg/kg showed a significant reduction in 5HTT levels. These findings suggest that KV treatment may modulate serotonin transporter expression and potentially restore serotonergic balance. Regarding the 5HTR7 receptor, no significant differences were observed across all groups. Although prior research suggests that 5HTR7 may play a role in behavioral disorders and neuroplasticity (31), our data did not support a direct involvement of this receptor in the observed changes within the VPA model at a significant level. This lack of significant findings could be attributed to the timing of measurement or the complex functions of this receptor in the VPA model. Collectively, these results suggest that KV may have a beneficial modulatory effect on the serotonergic system, particularly by reducing 5HTT levels, which may contribute to behavioral improvements observed in the VPA-induced model of autism. However, further studies are necessary to elucidate the precise mechanisms through which KV affects the serotonergic pathway and its potential therapeutic implications in neurodevelopmental disorders like autism.

Past studies have shown prominent astrocytic reaction, as shown by higher GFAP immunoreaction and development of astrogliosis, in prenatal valproate phenotype of ASD (32). In addition, astroglial activation in brains isolated from autistic patients and increased GFAP expression have also been reported (33, 34). Similarly, we also observed notable astrogliosis in the hippocampal region. In contrast, KV administration at 100 mg/kg was associated with lower astrocytic reaction. Consistent with such finding, attenuating effect of KV on astrocytic overreaction has been reported

following okadaic acid-provoked cognitive deficit (13).

Conclusion

In this research, KV exerted neuroprotective effects in the VPA-induced murine model of autism, primarily through its antioxidant and anti-inflammatory actions. By reducing oxidative damage, supporting endogenous defenses, and preserving mitochondrial function, KV helped restore hippocampal neuronal integrity. These biochemical improvements were associated with reduced repetitive behaviors, lower anxiety, and enhanced social interaction. The dose-dependent nature of these effects highlights the importance of optimizing therapeutic strategies. While promising, further research is still needed to fully understand KV mechanisms and its therapeutic potential in ASDs.

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Scope and Limitations

Clearly, conducting a comprehensive research study is challenging, and the limitations inherent in any research such as practical, temporal, and financial constraints are evident. In this particular study, the vastness and complexity of the changes observed in our ASD model entail that assessments be conducted across various models. Additionally, further examination of the serotonergic system in other brain regions could provide more precise and transparent insights into this field.

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Data Availability Statement

All data of this study are included in the main article. Further information can be obtained by contacting the corresponding author.

Ethics Statement

Procedures of this study were reviewed and approved by the Ethics Committee of Iran University of Medical Sciences (IR.IUMS.FMD.REC.1401.210).

Authors' Contributions

V K conducted investigation and formal analysis, prepared the original draft, and participated in reviewing and editing the manuscript. T B performed formal analysis, secured funding, supervised the project, contributed to the original draft, and reviewed and edited the manuscript. M F conducted investigation and participated in manuscript review and editing. A KK conducted investigation and took part in reviewing and editing the manuscript. S M was involved in methodology design and manuscript review and editing. M R carried out formal analysis, contributed to methodology, supervised the study, drafted the original manuscript, and engaged in review and editing.

Conflicts of Interest

The authors state that there were no commercial or financial ties that might be perceived as a potential conflicts of interest during the conduct of this research.

Declaration

We have not used any AI tools to prepare this manuscript.

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