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Effect of Stevia Rebaudiana Bertoni extract on rat model of autism induced by prenatal exposure to valproic acid

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Keywords

- Autism
- Stevia Rebaudiana Bertoni
- Valproic acid
- Oxidative stress
- Nrf2

Abstract

Objective: Studying the antioxidantproperties of Stevia Rebaudiana Bertoni extractas a prevention therapy onvalproic acid(VPA)-induced autismrat model. **Methods:**Control group:femalerats were injected saline 0.9%IP atGD (12.5).VPA group:female rats received single IP injection of valproic acid at a dose of 500 mg/kg at GD (12.5).Stevia control group:female rats received Stevia R. extract at a dose of 200 mg/kg orally once daily for 3 weeks, through whole gestation. Stevia prevention group: Female rats received Stevia R. extract as stevia control groupand single IP injection of valproic acid at a dose of 500 mg/kg at GD (12.5). Risperidone:Female rats received single IP injection of valproic acid at a dose of 500 mg/kg at GD (12.5)and male pups received risperidone (1 mg/kg; orally) from post-natal day 23 to 43.We conducted behavioral assessments and histopathological inspection with a biochemical analysis to measure oxidative stress markers MDA and GSH levels in the hippocampal tissue. We also conducted an immunohistochemical analysis to evaluate the nuclear expression of Nrf2. **Results:** Stevia R. extractas a preventive therapy improvedanxiety and stress associated with autism. Stevia R. decreased neuronal dystrophy and MDA level compared to VPA group.Also, increased GSH levels and increased nuclear Nrf2 expression.**Conclusions:**Stevia R. extract as a preventive therapyhave a possible neuroprotective effect on VPA-induced autism rat modelthrough the Nrf2-Keap1 pathway.

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Introduction

"Autism Spectrum Disorder" (ASD) is used to describe a clinically diverse group of neurodevelopmental disorders (1). Autism Spectrum Disorder (ASD) is marked by two core symptoms: persistent deficits in social communication and interaction, alongside restricted, repetitive behaviors and unusual sensory responses(2)In the last years, the prevalence of ASD had a strong elevation (1:59 live births according to the most recent data from USA) (3), autism is perhaps the most common and handicapping neurological disorder of childhood(4).

The pathophysiology of ASD is complex, involving genetic, environmental, and neurobiological factors. ASD is strongly hereditary, with genetic causes identified in about 20–25% of cases (5). Non-genetic contributors include factors like parental age, maternal nutrition, prenatal infections, stress during pregnancy, and exposure to toxins or medications such as valproic acid. Chronic inflammation in the central nervous system (CNS) is also significant (6). Disruptions in the brain's GABAergic and glutamatergic systems, which affect excitatory and inhibitory balance, may contribute to autistic behaviors (7). Additionally, individuals with ASD often show increased oxidative stress and lower levels of antioxidant enzymes compared to typically developing children (8)&(9).

To simulate autism in rodents, various strategies have been taken over the years. Prenatal exposure to VPA is one of the best characterized rodent models of autism (3). The exact mechanism by which prenatal exposure to VPA causes autistic-like behaviors in humans and rodents remains unclear. However, recent research has shown that VPA exposure can alter gene transcription, disrupt signaling pathways, impair synaptic function, and hinder neurogenesis, all contributing to ASD development. VPA-related ASD is also associated with increased oxidative stress, neuroinflammation, and changes in the brain-gut axis. Based on these findings, researchers have suggested treatment strategies, with some drugs showing potential benefits in managing ASD symptoms (10).

There is currently no cure for autism, and its causes involve a range of heterogeneous factors (4). Recently, herbal supplements and alternative medicine havegained attention in disease prevention and treatment. Stevia rebaudiana (Stevia R.), a plant native to South America, is widely used as a natural sweetener and is known for its potential therapeutic effects, including anti-inflammatory, hypoglycemic, and antioxidant properties. Stevia contains phenolic compounds like flavonoids, which have antioxidant benefits. Some studies suggest that stevia extracts may offer neuroprotective effects in regions like the hippocampus and amygdala (11). According to (12) that Stevia R. can upregulated the expression of nuclear factor-2 erythroid-related factor-2 (Nrf2) activate antioxidant pathways, boosting enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GPx), making cells more resistant to oxidative stress (13). Does Stevia R. extract as an antioxidant acting through the Nrf2-Keap1 pathway can prevent the pathology of autism and improve the cognitive and behavioral manifestation, here is the question.

Material and method

Experimental animals

Ten pairs of male and female Sprague-Dawley rats 10-12 weeks old, with an average weight of 200-250 gm was obtained from the animal house of medical experimental research center (MERC), faculty of medicine, Mansoura university. Rats were housed in cages under controlled conditions of humidity (40-70%), in an inverted dark-light cycle (12h light/dark cycle), and temperature (20-22 °C) with food and water ad libitum. This study was approved by the IRB Committee (Code MS.22.06.2040).

Chemicals

Valproic acid was purchased from Sigma company. Stevia R. extract: Methanolic extract was prepared by pharmacognosy department – Faculty of pharmacy – Mansoura university. *Plant material*: Stevia leaves plant (S. rebaudiana) (1.5 kg), were collected from International Company from Agro-industry Product (SICAP), Cairo, Egypt.*Extraction and fractionation*: Powdered air-dried leaves of Stevia rebaudianabertoni (300 gm) were defatted with petroleum ether (60-80°C) and extracted with methanol (80%) to yield a dry extract (75 gm)(14). 3mg Apexidone (Risperidone) was purchased from multiapex pharm.

Study design

Rats were divided into five groups as the following: Control group (n=2 Females) female pregnant rats received equal quantities of intraperitoneal saline 0.9% at 12.5 (GD). Valproic acid group (n=2 Females) female pregnant rats received single intraperitoneal injection of valproic acid at a dose of 500 mg/kg body weight at 12.5 (GD)(15). Stevia control group (n=2 Females) methanolic extracts of Stevia R. was dissolved in 1.0 mL saline at a dose of 200 mg/kg and given orally via gastric gavage once daily for 3 weeks, through whole gestation(11). Valproic acid + Stevia prevention group (n=2 Females) female pregnant rats received methanolic extracts of Stevia R. that will be dissolved in 1.0 mL saline at a dose of 200 mg/kg ang given orally via gastric gavage once daily for 3 weeks, through whole gestation (11) plus single intraperitoneal injection of valproic acid at a dose of 500 mg/kg body weight at 12.5 (GD). Valproic acid + risperidone group (n=2 Females) female pregnant rats received single intraperitoneal injection of valproic acid at a dose of 500 mg/kg body weight at 12.5 day of gestation plus male pups females risperidone (1 mg/kg; orally) from post-natal day 23 to 43 (15).The average litter size is 6-8 pups from pregnant females. Three male pup per litter were selected from each group (n = 30). The weaning of the pups was done on a postnatal day (PND) 21, and further experimental procedures were carried out on the male pups while the female pups were sent for rehabilitation.

Spontaneous behavior assessment in the open field

Plastic box (50 cm × 50 cm × 40 cm) was separated into 16 equal-sized blocks and 4 center blocks, called the grid. The animals were put in the center block, and the movement of the animals was recorded for 5 minutes using a video camera. The total distance, immobility time, duration spent in the center or periphery of the arena, number of rearing and grooming and the number of urination and fecal boli were recorded (15).

Anxiety-like behavior assessment by elevated plus-maze test

The test will be executed by introducing the test rat in the center of the apparatus and allowing it to habituate for 5 min. The animal behavior will be video tracked, and the cumulative duration in the open arm and closed arm will be reported. Also, the number of entries in either arm will be documented ((15).

Animal sacrifice and sample collection

One day after completing the behavioral test recordings, the rats were deeply anesthetized and euthanized with a high dose of sodium thiopental (120 mg/kg) administered via intraperitoneal injection. Intracardiac perfusion was then performed with 100 ml of heparinized saline (9). The brain was carefully dissected, and the hemispheres were separated. One hemisphere was fixed in 10% formaldehyde at room temperature for 24 hours to prepare for histopathological and immunohistochemical assessments. The other hemisphere was dissected to separate the hippocampus which was immediately weighed, frozen, and stored at -80°C for biochemical analysis of oxidative stress markers (16).

Histopathological examination

The fixed cerebral hemisphere tissue was processed using a standard paraffin embedding procedure, and serial sections were cut at a thickness of 20µm. The sections were stained with hematoxylin for 15 minutes, treated with HCl alcohol solution for 35 seconds, then immersed in eosin for 10 minutes and 90% ethanol for 40 seconds (11). At least two coronal sections from different hippocampal levels were examined for each animal. Neuronal dystrophy was identified in neurons exhibiting a shrunken appearance with eosinophilic cytoplasm and dark pyknotic nuclei, while neurons without such changes were classified as normal. The percentage of neuronal change was calculated by subtracting the number of normal neurons from the total number of neurons in all hippocampal regions (CA1, CA2, CA3, CA4) per coronal section and dividing by the total cell count for neuronal dystrophy. The results were graded as follows: 0 (no changes), 1 (mild changes: 1–25% neuronal degeneration), 2 (moderate changes: 26–75% neuronal degeneration), or 3 (severe changes: 76–100% neuronal degeneration) (11).

Biochemical study

To prepare the hippocampal homogenate, we added 10 ml of ice-cold phosphate-buffered saline for each gram of hippocampal tissue and homogenized it using a Teflon-glass homogenizer. The homogenate was then centrifuged at 5000 rpm and 4°C for 15 minutes (11). The supernatant was collected for the measurement of oxidative stress markers, including reduced glutathione (GSH) and malondialdehyde (MDA), using a colorimetric assay according to the manufacturer's instructions. The assay kits were purchased from Bio Diagnostic, Giza, Egypt, with catalog numbers GR 25 11 for GSH and MD 25 29 for MDA.

Immunohistochemical examination

Deparaffinized tissue sections were rehydrated, cleaned, immersed in 3% hydrogen peroxide, and treated with pepsin to retrieve antigens. After blocking non-specific binding with serum, the sections were incubated overnight at 4°C with an anti-Nrf2 polyclonal antibody (catalog # sc-13032 X; Santa Cruz Biotechnology, Santa Cruz, CA, USA). A brown signal was developed using a diaminobenzidine/peroxidase substrate. The sections were then washed, mounted, dehydrated, and counterstained. Phosphate-buffered saline (PBS) replaced the primary antibody in adjacent sections to serve as a negative control (6). The

mean integral optical density (IOD) values of positive cells in the CA1, CA2, CA3, and DG subregions of the hippocampus were analyzed using an Optika light microscope (Optika B-10, OPTIKA Microscopes, Italy) at 40× magnification. Initially, five sections with the greatest differences in staining intensity were chosen, and the most appropriate analysis parameters were selected to maximize the number of hippocampal pyramidal cells while minimizing background cell interference. These parameters were then used to reanalyze all the stained sections. Finally, GraphPad Prism 5.0 software was used to analyze differences between the groups.Nrf2 expression was evaluated by semi-quantitative analysis that was performed using Fiji Image J software version 2.9.0, measuring Nrf2 expression as mean gray value percentage(17).

Statistical analysis

Data analysis was conducted using IBM SPSS Statistics software (Version 26). For parametric data, group comparisons were made using a one-way ANOVA test followed by post hoc Bonferroni analysis. Non-parametric data were analysed using the Kruskal-Wallis test, and bivariable comparisons were performed using repeated Mann-Whitney U tests. Data were reported as Mean ± SD, median with interquartile range (IQR), or frequency, depending on the data type. Statistical significance was set at a p-value of less than 0.05. Correlations between different parameters were assessed using Pearson's or Spearman's correlation tests. Graphical representations were created with GraphPad Prism version 8.0.2 for Windows (GraphPad Software, Inc., San Diego, CA).

Results

Developmental analysis

100% of the male pups exposed to valproic acid only during pregnancy had kinked tail **(Fig 1).** Also, in the risperidone treated group, about 85% of rats show that sign. While concomitant administration of Stevia R. extract with valproic acid decreases this percentage to about 20%. Male pups not exposed to valproic acid during pregnancy as in control and Stevia R. control group had normal tail.

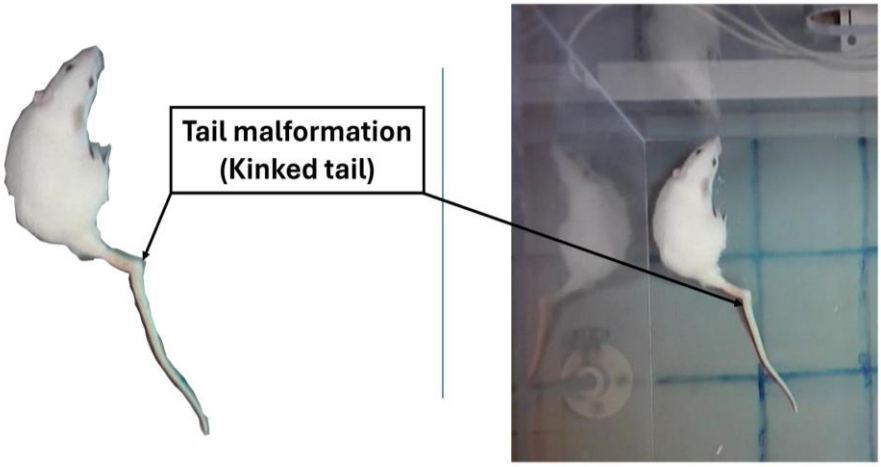

Figure 1: Tail malformation (Kinked tail).

Spontaneous behavior assessment in the open field

There were significant differences in distance (cm) travelled by rats among different groups. Assessment showed a significant increase in distance travelled by rats in VPA, stevia control and stevia prevention group compared to control or risperidone treated group**.** While there were no statistically significant differences in immobility time among different groups. Regarding rearing frequency, there were statistically significant differences among groups with the highest frequency recorded by rats in stevia control group. Grooming, as a stereotyped behavior, showed a significant higher frequency in VPA group compared to control, risperidone, stevia control and stevia prevention groups (P value <0.05)**.** The number offecal boli was assessed in each group with a significant higher number in VPA group compared to other groups**.** Whileurination frequency showed no statistically significant difference among the different groups. Duration (seconds) spent rats in open field arena may be in the center squares or the periphery of the arena. VPA group rats recorded the longest time spent in the periphery of the arena and the shortest time in the center significantly compared to other groups. Concomitant administration of stevia with VPA during pregnancy showed a significant change in that time with significant more time spent in the center and so decrease in time spent in the periphery compared to VPA group (P <0.05)**(Table 1).**

Anxiety-like behavior assessment by elevated plus-maze test

The elevated plus maze can be used to assess anxiety like behavior in rats through many parameters. Duration (seconds) spent by rats in closed arms showed a statistically significant difference among studied groups. VPA rats spent about 100% of time in the closed arms while control group rats spent the least time compared to VPA group (P <0.001)**.** However, duration spent by rats in open arms was statistically significant higher in control and stevia control group about 40 compared to VPA group. Regarding the number of entries to each open or closed arms, there were no significant differences among different groups. The number of fecal boli was also assessed with no significant difference between groups. But the number of urinations showed a statistically significant difference among studied groups with highest frequency represented in VPA group**(Table 2).**

Table 1 *Parameters of spontaneous behavior in the open field among study groups.*

	Control	VPA	Resp.	Stevia cont.	Stevia prev.	P value
Distance (cm)	586.7 ±252.38 ‡‡‡	1048.9 ±609.1§	405.4 ±243.2	1549 ±258.2§§§	1166.9 ±209.64§§	<0.0001 F= 10.4
Immobility time (sec)	212.5 (176.13- 223)	216.5 (127.2- 352)	320.7 (189.53- 342.27)	218 (210- 266)	191.7 (183.3- 237.47)	0.22 KW= 5.7
Rearing frequency	13.3 ±8.14	9.5 ±6.83	7.5 ±4.51	20.3 ±4.55§	16 ±6.57	0.012 F=4
Grooming frequency	1.5 (0-2)*	2.5 (2-3)	1.5 (1-2)*	1 (1-2)*	2 (1-2)*	0.02 k=11.4
Number of Fecal boli	2.3± 1.86**	5.7 ±1.37	1.7± 1.03***	1.5± 0.55***	3± 1.55*	<0.0001 F= 9.4
Urination frequency	3/6	5/6	3/6	2/6	3/6	0.59
Duration in center	92.3 ±34.9	57.7 ±8.02	109.3 ±24.22*	106.2 ±25.12**	105.3 ±19.27*	0.005 F=4.8
Duration in periphery	267.7 ±34.9	302.3 ±8.02	250.7 ±24.22**	253.8 ±25.12*	254.7 ±19.27*	0.005 F=4.8

The distance (in centimeters) travelled by rats in the open field, number of rearing and fecal boli and duration (in seconds) spent by rats in the center or the periphery of the open filed are represented by mean ±SD, the one-way ANOVA test with post hoc Bonferroni test was used. The immobility time (in seconds) and grooming of rats in the open field are expressed as median, min. to max, Kruskal Wallis test was used to test significance among studied groups. The presence or absence of urination is represented as frequency: number/total, Fischer exact x^2 test was used. *Significant compared to VPA group with P value <0.05. ** Significant compared to VPA group with P value <0.01.*** Significant compared to VPA group with P value <0.001. ‡ Significant compared to Stevia control group with P value <0.05. ‡‡ Significant compared to Stevia control group with P value <0.01. ‡‡‡ Significant compared to Stevia control group with P value <0.001. §Significant compared to Risperidone treated group with P value <0.05. §§Significant compared to Risperidone treated group with P value <0.01. §§§Significant compared to Risperidone treated group with P value <0.001. n= 6 in each group.

Table 2 *Parameters assess anxiety like behavior in elevated plus maze among studied groups.*

	Control	VPA	Resp.	Stevia cont.	Stevia prev.	P value
No. entries to closed arms	1 (1-2)	2 (1-8)	1 (1-3)	1.5 (1-2)	1 (1-2)	0.36 KW= 4.3
No. entries to open arms	1 (0-2)	0 (0-1)	0.5 (0-1)	1 (0-1)	0.5 (0-1)	0.38 KW=4.2
Duration in closed arms (sec)	284.8 ±3.6***	299.7 ±0.82	293.2 ±0.98***	287.3 ±3.72***	292.7 ±1.75***	0.0001 F=31.5
Duration in open arms (sec)	15.2 ±3.6***	0.3 ±0.82	6.8 ±0.98***	12.7 ±3.72***	7.3 ±1.75***	0.0001 F=31.5
Fecal boli	2.5 (0-4)	4 (3-6)	0.5 (0-10)	3 (0-4)	0.5 (0-4)	0.1 KW=7.6
Urination	0/6	6/6	0/6	1/6	1/6	0.0001

The duration (in seconds) spent by rats in closed or open arms of elevated plus maze is represented by mean ±SD, the one-way ANOVA test with post hoc Bonferroni test was used. The number of entries of rats to either closed or open arms of elevated plus maze and number of fecal boli are expressed as median, min. to max with no significance among studied groups. The presence or absence of urination is represented as frequency: number/total, Fischer exact x^2 test was used.** Significant compared to VPA group with P value <0.01.*** Significant compared to VPA group with P value <0.001.n= 6 in each group.

Histopathological analysis

Using a score assessing the neuronal dystrophy in hippocampal cells, there were statistically significant differences among studied groups un different hippocampal regions. The control group as well as stevia treated groups showed a highly significant difference compared to VPA group especially in CA1 and CA3 **(Fig 2.A & Fig 3.A).** The Risperidone treated group also showed a significant difference compared to VPA group in CA2, CA3 and CA4 hippocampal regions **(Fig 2.B & Fig 3.A&B).** In CA2, there was a significant difference in the stevia prevention group compared to stevia control group. Also, the risperidone group showed a significant difference compared to the control group **(Figure 2.B).** In CA3, the stevia prevention group and risperidone group showed a statistically significant difference compared to the control and the stevia control groups **(Fig 3.A).** In CA4, there was a significant difference in the stevia prevention group compared to the control group **(Fig 3.B).** Hippocampal histopathological changes for different groups are shown in the **(Fig. 4)**

Biochemical analysis

Rats exposed to VPA prenatally showed a significant decrease in GSH levels compared to the control group (P <0.001). Highest levels of GSH showed by stevia control and stevia prevention

groups with significant differences compared to other groups **(Fig5.A)**.Rats exposed to VPA prenatally exhibited a significant increase in MDA levels compared to the other groups. Use of stevia along with VPA during pregnancy reduced MDA level in hippocampal tissue more than that occurred in risperidone treated group. All these values are significant compared to control and stevia control group (P <0.001) **(Fig 5.B).**

Immunohistochemical analysis of nuclear Nrf2 expression

Regarding CA1, Nrf2 expression showed a significant increase in the stevia treated groups compared to the VPA group. Also, The VPA group showed a significant increase in Nrf2 expression compared to the control group **(Fig 6.A).** Also, the same findings were presented in the CA2 in addition to significant increase in stevia prevention and stevia control groups compared to risperidone **(Fig 6.B).** RegardingCA3, also stevia control and stevia prevention groups showed a significant difference in Nrf2 expression compared to VPA group. Stevia prevention showed a significant increase in Nrf2 expression compared to the control and risperidone treated groups. Stevia control group also showed a significant increase in Nrf2 expression compared to the control group**(Fig 7.A).** The findings presented in CA3 also showed by CA4 immunohistochemistry with a significant increase in Nrf2 expression in stevia control group compared to the risperidone treated group **(Fig 7.B).** Dentate gyrus Nrf2 expression showed the same findings as CA4 with a significant increase in Nrf2 expression in risperidone treated group compared to the control group **(Fig 7.C).**Nrf2 expression in hippocampal regions for different groups are shown in the **(Fig 8).**

Correlation between study variables

Spearman's rho correlation analysis revealed a significant negative correlation between the duration spent by rats in the center of the open field arena and the degree of neuronal dystrophy either in CA1 or CA3 regions (P <0.05). But there was a significant positive correlation between duration spent by rats in the open field arena and level of GSH (P <0.05).Spearman's rho correlation analysis also revealed a significant negative correlation between No. of entries to open arm in EBM and the degree of neuronal dystrophy in CA3 (P <0.001).The degree of neuronal dystrophy either in CA1 or CA3 regions showed a significant negative correlation with GSH level and a significant positive correlation with the MDA level (P <0.001), with spearman's rho correlation analysis.Regarding mean gray value % of Nrf2 either in CA1 or in CA3 showed a significant positive correlation with the GSH level (P <0.0l)), with spearman's rho correlation analysis.Also, spearman's rho correlation analysis showed a significant negative correlation between the level of GSH and the level of the MDA in hippocampal tissue**(Table 3)**

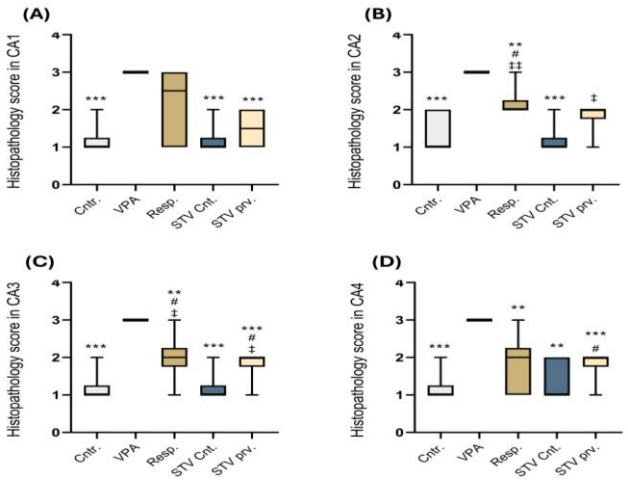

Figure 2: Effect of VPA and Stevia R. extract on hippocampal neuronal dystrophy in hippocampal regions. (A) The score of neuronal dystrophy in CA1 is represented as median, min. to max, Kruskal Wallis test was used to test significance among studied groups .*** Significant compared to VPA group with P value <0.001. **(B)** The score of neuronal dystrophy in CA2 is expressed as median, min. to max, Kruskal Wallis test was used to test significance among studied groups. ** Significant compared to VPA group with P value <0.01.*** Significant compared to VPA group with P value <0.001. ‡ Significant compared to Stevia control group with P value <0.05. ‡‡ Significant compared to Stevia control group with P value <0.01. # Significant compared to control group with P value <0.05. n= 6 in each group. **(C)** The score of neuronal dystrophy in CA3 is expressed as median, min. to max, Kruskal Wallis test was used to test significance among studied groups. **Significant compared to VPA group with P value <0.01.*** Significant compared to VPA group with P value <0.001. ‡ Significant compared to Stevia control group with P value <0.05. # Significant compared to control group with P value <0.05. **(D**) The score of neuronal dystrophy in CA3 is expressed as median, min. to max, Kruskal Wallis test was used to test significance among studied groups. ** Significant compared to VPA group with P value <0.01.*** Significant compared to VPA group with P value <0.001. # Significant compared to control group with P value <0.05. n= 6 in each group.

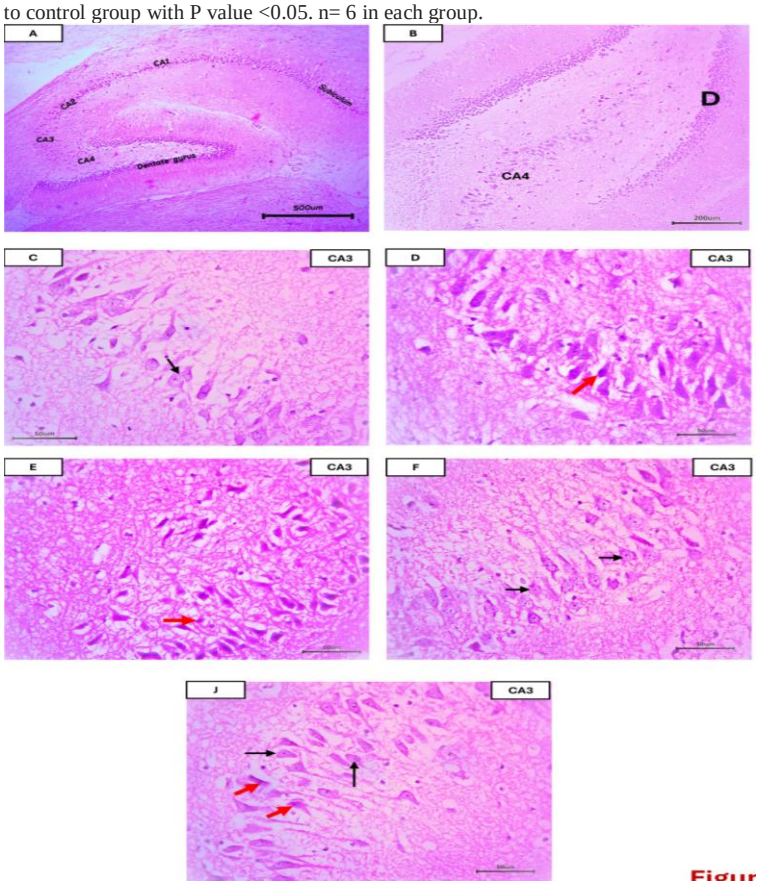

Figure 3:Morphology of different hippocampal regions in studied groups. (A) Shows normal hippocampal structure. The scale bars represent 500 µm at magnification 40x, (H&E). **(B)** Shows normal hippocampal structure mainly dentate gyrus and CA4. The scale bars represent 200µm at magnification 100x, (H&E).**(C)** Shows normal CA3 in control group. Black arrows represent the normal shape and distributions of neurons with round nuclei and prominent nucleoli.**(D)** Shows the effect of VPA on CA3 with low number of round normal cells and dense pyknotic nuclei in died neurons which represented by (red arrows).**(E)**represents the effect of VPA on CA3 showing low number of round normal cells with dense pyknotic nuclei in died neurons which represented by (red arrows) and cannot be reversed by risperidone.**(F)**represent normal CA3that are not affected by Stevia administration during pregnancy. Black arrows represent the normal shape and distributions of neurons with round nuclei and prominent nucleoli.**(J)** Represents the effect of Stevia coadministration with VPA during pregnancy on CA3 showing greater number of normal neurons with prominent nucleoli represented by (black arrows). This along with low number of died neurons with dense pyknotic nuclei represented by (red arrows).The scale bars represent 50µm at magnification 400x, (H&E).

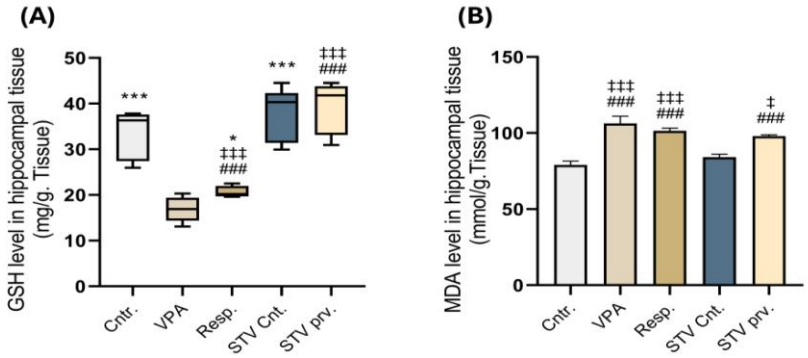

Figure 4: Effect of VPA and Stevia R. administration on (A) GSH level is expressed as median, min. to max, Kruskal Wallis test was done with a bivariable comparison using the repeated Mann-Whitney U test. *Significant compared to VPA group with P value <0.05. *** Significant compared to VPA group with P value <0.001. ‡‡‡ Significant compared to Stevia control group with P value <0.001. ### Significant compared to control group with P value <0.001. **(B)** MDA level is represented as mean ± SEM, A One-way ANOVA test with post hoc Bonferroni test was used. ‡ Significant compared to Stevia control group with P value <0.05. ‡‡‡ Significant compared to Stevia control group with P value <0.001. ### Significant compared to control group with P value <0.001. n= 6 in each group. GSH: reduced glutathione and MDA: malondialdehyde.

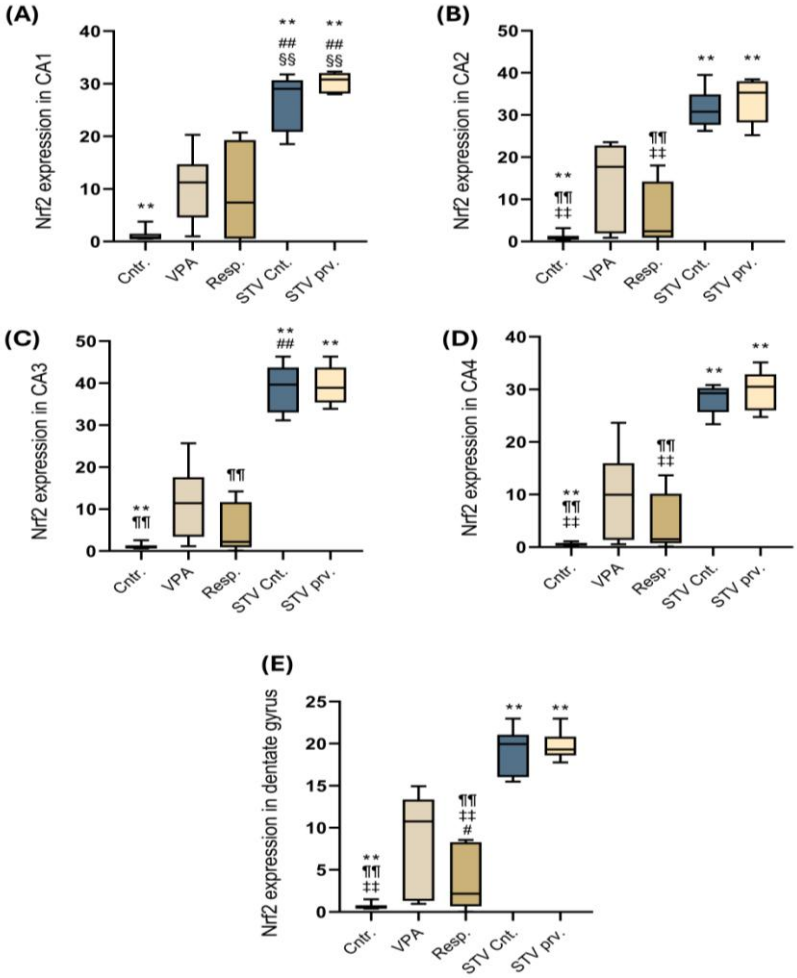

Figure 5:Effect of VPA and Stevia R. administration on Nrf2 expression in (A) CA1 of hippocampus, which is expressed as median, min. to max, Kruskal Wallis test was done with a bivariable comparison using the repeated Mann-Whitney U test. ** Significant compared to VPA group with P value <0.01. §§ Significant compared to Risperidone treated group with P value <0.01. ## Significant compared to control group with P value <0.01. **(B)** CA2 of hippocampus, which is expressed as median, min. to max, Kruskal Wallis test was done with a bivariable comparison using the repeated Mann-Whitney U test. ** Significant compared to VPA group with P value <0.01. ‡‡Significant compared to Stevia control group with P value <0.01. ¶¶ Significant compared to Stevia prevention group with P value <0.01. n= 6 in each group.**(C)** CA3 of hippocampus, which is expressed as median, min. to max, Kruskal Wallis test was done with a bivariable comparison using the repeated Mann-Whitney U test. **Significant compared to VPA group with P value <0.01. ¶¶ Significant compared to Stevia prevention group with P value <0.01. ##Significant compared to control group with P value <0.01. **(D)** CA4 of hippocampus, which is expressed as median, min. to max, Kruskal Wallis test was done with a bivariable comparison using the repeated Mann-Whitney U test. ** Significant compared to VPA group with P value <0.01. ‡‡Significant compared to Stevia control group with P value <0.01. ¶¶ Significant compared to Stevia prevention group with P value <0.01. **(E)** Dentate gyrus of hippocampus, which is represented as median, min. to max, Kruskal Wallis test was done with a bivariable comparison using the repeated Mann-Whitney U test. ** Significant compared to VPA group with P value <0.01. ‡‡Significant compared to Stevia control group with P value <0.01. ¶¶ Significant compared to Stevia prevention group with P value <0.01. #Significant compared to control group with P value <0.05. n= 6 in each group.

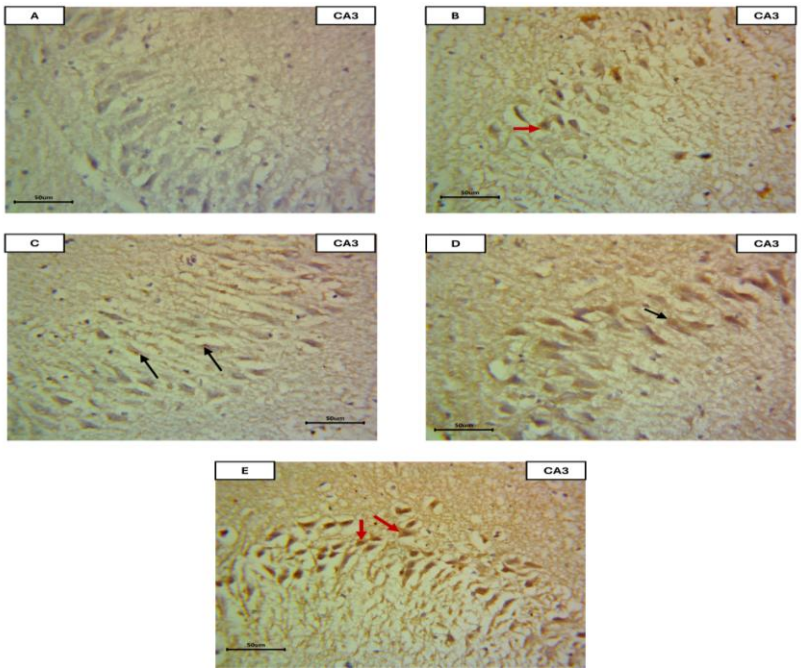

Figure 6:Representative photographs of immunohistochemical results of Nrf2 expression in different regions of rat hippocampus in studied groups. (**A**) Represents CA3 in control group showing no or minimal expression of Nrf2 is identified. Black arrows represent normal cells. (**B**) Represents the effect of VPA administration on Nrf2 expression on CA3. There is an increase in Nrf2 expression (dark brown) in normal and dystrophic neurons which are represented by red arrows.**(C)**Representsthe effect of VPA administration on Nrf2 expression on CA3 in risperidone treated group. There is an increase in Nrf2 expression (dark brown) in normal and dystrophic neurons which are represented by red arrows.**(D)** Represents the effect of Stevia R. extract administration on Nrf2 expression on CA3 in stevia control group. There is an increase in Nrf2 expression in normal neurons (dark brown) which are represented by (black arrows).**(E)**Represents the effect of Stevia R. extract administration in VPA model on Nrf2 expression on CA3 in Stevia prevention group. There is an increase in Nrf2 expression in normal neurons (dark brown) which are represented by (black arrows) and neurons showing signs of dystrophy represented by (red arrows). The scale bars represent 50µm at magnification × 400.

Table 3 *CorrelaPPearson's correlation coefficient (r) was used for parametric data, while Spearman's rho correlation coefficient (R) was used for the* Pearson's correlation coefficient (r) was used for parametric data, while Spearman's rho correlation coefficient (R) was used for the non- parametric data. * Correlation is significant at P <0.05, ** P <0.0l, *** P <0.001. n=30.

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	Duration spent in center of open field	No. of entries to open arm in EBM	Neuronal dystrophy CA1	Neuronal dystrophy CA3	Mean gray value % of Nrf2 in CA1	Mean gray value % of Nrf2 in CA3	GSH	MDA
Duration spent in center of open field	-							
No. of entries to open arm in EBM	R= 0.24 P= 0.2	-						
Neuronal dystrophy in CA1	R= -0.4 **P= 0.03***	R= -0.33 P=0.07	-					
Neuronal dystrophy in CA3	R= -0.4 **P= 0.03***	R= -0.57 **P= 0.001***	R= 0.76 **P= 0.0001***	-				
Mean gray value % of Nrf2 in CA1	R= 0.34 P= 0.07	R= -0.03 P=0.9	R= -0.13 P= 0.5	R= -0.08 P= 0.69	-			
Mean gray value % of Nrf2 in CA3	R= 0.3 P=0.1	R= -0.03 P= 0.88	R= -0.14 P= 0.45	R= -0.08 P= 0.68	R= 0.94 **P=0.0001***	-		
GSH	R= 0.39 **P= 0.04***	R= 0.09 P= 0.65	R= -0.59 **P= 0.001***	R= -0.53 **P= 0.003***	R= 0.45 **P= 0.01****	R= 0.45 **P= 0.014****	-	
MDA	r= -0.35 P= 0.06	R= -0.24 P= 0.2	R= 0.66 **P= 0.0001***	R= 0.63 **P= 0.0001***	R= 0.03 P=0.87	R= -0.04 P= 0.84	R= -0.61 **P= 0.0001***	-

Discussion

In this study, we found that administration of Stevia R. extract during pregnancy as a prevention therapy alleviated some of the cognitive and behavioral disturbances of autism in pups later, as well as increased the survival of pyramidal cells in the different hippocampal regions. These findings were supported by the increased level of the antioxidant marker GSH in the hippocampal tissue of the rats administered Stevia R. extract during pregnancy as a prevention therapy. Additionally, there was a decrease in the level of the oxidative marker MDA in hippocampal tissue. Also, there was a great improvement in behavioral and cognitive manifestations during behavioral assessment. This neuroprotective effect of Stevia R. extract may be mediated by Nrf2- Keap1 signaling pathway. There was an increase of the overall expression of Nrf2 in hippocampal neuronal cells. All these findings may suggest the possible role of stevia as a preventive therapy in alleviating the manifestations of autism and reversal hippocampal neuronal damage.

We prefer to use valproic acid to achieve the animal model as it was found that consumption of valproic acid during pregnancy an anticonvulsant therapy may have a potential risk of development of autism in these female children and rodents exposed to valproic acid (VPA) during prenatal development display behavioral deficits that closely mirror those observed in individuals with autism.

In the initial of our experiment, we use valproic acid dose 600mg/kg according to (15) but unfortunately this dose led to abortion. So, we used valproic acid with dose of 500 mg/kg correlatingwith literature of (4) especially Sprague-Dawley strain of rats we used in our animal lab.

Regarding Stevia R. extract as an antioxidant, a dose of 200 mg/kg daily for 3 weeks was given orally for the pregnant females form the onset of the pregnancy and after VPA injection at GD 12.5 till delivery. It is believed that this dose is sufficient to be a neuroprotective actions of stevia extract and its derivatives against the damage caused by VPA displayed in previous studies by (11)

In utero exposure to valproic acid (VPA) on embryonic day (ED) 12.5 is associated with the closure of the neural tube. So, this affects various developmental parameters as presence of tail anomaly. The Kinked tail was the most common congenital anomaly presented in all pups whose mothers treated with VPA at GD 12.5. This was observed in all rats in both VPA group and risperidone treated group compared to the control and stevia control group indicating the success of the model. Surprisingly, administration of stevia as a prevention therapy before and after VPA injection in GD 12.5 decreases the incidence of this sign up to 80% compared to VPA and risperidone treated group. This opens the door for further research on this topic to determine if stevia can prevent issues related to neural tube defects. This also reported by (18) how studied the effect of Nrf2 activation on inhibition of neural tube defects.

Regarding behavioral studies, statistical analysis showed that there is a significant difference in the distance travelled by rats in open field arena among studied groups. It was higher in the VPA group compared to control group indicating high level of anxiety compared to control group. Additionally, the distance travelled by rats was significantly decreased in risperidone treated group which can ameliorates this high level of anxiety. Similar findings were reported by the previous researchers (19). But for Stevia control and Stevia prevention groups we found a much higher distance travelled by rats in these groups which is not consistent with stevia's ability to reduce stress. There could be attributed to exploratory behavior of these rats according to (3) mentioned that there is no significant difference in distance travelled by rats between VPA group and others even it was slightly increased in VPA group treated with suramin.The number of rearings also showed another debate. It was found that there was a significant difference between the studied group and the higher frequency reported in the stevia control group compared to the risperidone treated group which had the lowest frequency. According to (15) , rearing is one of the stereotyped behavior characteristic for autism which may be alleviated by risperidone. On the other hand, according to Hirsch et al. (2020) and(20) it is a sign of exploration and so may occur frequently in normal rats. The longer the duration rats spent in the open field arena may differentiate between both types whether it is a stereotyped or exploratory behavior which decreases with time.Fecal boli frequency as an indicator of stress showed a significant difference among studied groups with the highest frequency in VPA group. This is significant difference compared to control group and other groups. Stevia prevention also showed a significant difference in the number of fecal boli compared to VPA group indicating that it can alleviate some of stress behavior accompanying autism rats. These findings were in line with a previous study by (21) assessing stress level between young and aged rats.Grooming as a stereotyped behavior, showed also a significant difference among studied groups with the highest frequency in VPA group compared to control and stevia control groups. Risperidone and stevia as preventive therapy showed a significant reduction in grooming frequency in VPA treated rats. this is consistent with what (15) reported in his study.

Time spent in the center and in the periphery of the open field could be one of the most significant indicators assessing anxiety level in the rodents. In our study, VPA treated rats spent the least time in the center of the open field compared to the other groups. Stevia as a preventive therapy showed a significant improvement in this time as the standard treatment risperidone. And consequently, VPA spent more time in the periphery compared to other groups indicating high level of anxiety and this correlates well to other previous studies as reported by (19).

Anxiety like behavior can be assessed also by elevated plus maze. Time spent in the open or closed arms and number of entries to whether open or closed arms can be used as significant parameters assessing how stressful were the experimental rats. We found that VPA group rats spent nearly the whole time of the experiment in the closed arms with very little time in the open arms. This finding is highly significant compared

to the control group and the other groups. Stevia R. extract coadministration with VPA during pregnancy can increase the time spent in the center of the open field and also increase number of entries nearly equal to the standard treatment risperidone. These findings typically presented in many previous studies as reported by (15). Furthermore, Stevia R. extract showed a significant decrease in urination frequency compared to VPA group indicating how it can reduce the stress which may be presented in autistic rats.

Regarding histopathology in our study conducted upon hippocampal tissue of the experimental rats assessing the neuronal dystrophy score, we found that the pyramidal neuronal cells of the hippocampus in different regions in VPA group showed sever neuronal dystrophy (grade 3) with shrunken appearance, eosinophilic cytoplasm and dense pyknotic nuclei. This is compared to control group or stevia control group which showed a non or a mild injury (grade 1) with preserved neuronal shape, size and nuclei were prominent. This is correlate with findings reported by (15)Surprisingly, Risperidone ,as a selective monoaminergic antagonist, significantly reduced neuronal dystrophy compared to VPA group with average score (grade 2 = moderate injury). This finding consistent with what reported by (22) that showed in his study the possible anti-inflammatory effect of risperidone and how it could decrease neuronal degeneration.For Stevia R. extract as a preventive therapy, interestingly, showed a significant reduction in neuronal hippocampal injury compared to VPA group with average score (grade 2 = moderate injury). This finding in line with that reported by (11) indicating the role of stevia in reversal the damage occurred in hippocampal tissue resulting in autism manifestations.

To further study the neuroprotective role of Stevia R. extract we investigate its antioxidant effect in hippocampal tissue. Production of reactive oxygen species (ROS) has been identified as a key factor in the development and progression of autism manifestations. So, biomarkers of oxidative stress as malondialdehyde (MDA) and reduced glutathioneGSH) were assayed. Regarding MDA, which is one of the outcomes of uncontrolled oxidative stress resulting in cell injury and neuronal death, our study showed a significant increase compared to control group and stevia control group. Also, risperidone showed a significantly higher level of MDA compared to control group. These findings are in line with that reported by (15) except for risperidone that was found to significantly decrease the level of MDA compared to VPA group. Surprisingly, Stevia prevention group showed no significant reduction in MDA level as was thought to be an antioxidant agent. This finding also reported by (23) in which stevia increased total superoxide dismutase with no significant effect on MDA or total antioxidant capacity.Regarding GSH, as an antioxidant that directly reacts with the free radicals preventing their effect, Stevia R. extract only or as a preventive therapy showed a significant increase in GSH compared to VPA group. These findings in a line with that reported by (11). Also, risperidone showed a significant increase in GSH compared to

VPA treated rats. This finding was reported by (15).

To clarify the possible underlying mechanism of neuroprotective effect of Stevia R. extract on VPA model we assess Nrf2 expression in hippocampal tissue as immunohistochemical analysis. Our data showed a significant increase in mean gray value% of Nrf2 expression in groups treated with Stevia R. extract either only or as a preventive therapy with VPA administration during pregnancy.

This compared to control and VPA group especially in CA1 and CA3 hippocampal regions. The increase in Nrf2 expression associated with stevia administration correlates with the previous study by (24) which reported that. Also, Nrf2 as a possible mechanism in alleviating the hippocampal oxidative stress in rats treated with VPA, previously reported by (25) that studied the effect of melatonin in alleviating neurogenesis disorders induced by valproic acid through antioxidant mechanism. There was also a significant increase in Nrf2 expression in VPA group compared to control group. This finding reported by (26) assumed that there is a link between increased level of ROS and increased Nrf2 expression. Controversially, (25) reported that VPA could decrease the expression of Nrf2 compared to control group. Regarding risperidone, stevia treated groups showed a significant increase in Nrf2 expression compared to the risperidone group. This may be attributed to the role of stevia as antioxidant therapy through Nrf2 expression versus risperidone with possible anti-inflammatory effect.

Conducting correlating study between different groups, we found a significant negative correlation between duration spent in the center of open field arena and neuronal dystrophy in CA1 and CA3 as well as between no. of entries to open arm in EBM and neuronal dystrophy in CA3 suggesting how hippocampal neuronal dystrophy aggravated the anxiety like behavior in autistic rats. There was also a significant positive correlation between duration spent in the center of open field arena the level of the antioxidant marker GSH as well as a significant negative correlation between degree of neuronal dystrophy in CA1 and CA3 and the level of GSH. This also beside the positive correlation between the mean gray value % of Nrf2 expression in CA1 and CA3 and the level of GSH. All these findings suggested that the underlying mechanism behind the increase in GSH is the increase oftranscription of Nrf2 as in stevia treated rats and these different antioxidant response elements could protect the hippocampal neuronal cells from damage and so reversal of cognitive and behavioral disturbances like anxiety which commonly presented in autistic rats.Furthermore, there was a significant positive correlation between the level of the MDA and the degree of neuronal dystrophy in hippocampal regions especially CA1 and CA3 and a significant correlation between the GSH level and MDA level suggesting the hypothesis that damage of hippocampal neurons related to increase level of oxidative stress markers as MDA which if is uncontrolled it may lead to neuronal dystrophy and development of autism.

Results and findings we found in our study may suggest a side plan to overcome the socioeconomic burden of autism and to prevent its development from the start. But still, we have some limitations in our study which we couldn’t afford for further

investigating the role of stevia in alleviating neural tube defect disorders. Limitations regarding behavioral studies which may be confirmed by using more devices. Also, further investigations for the underlying mechanism Nrf2-Keap1 pathway. Furthermore, study the anti-inflammatory effect of stevia and if it could be another underlying mechanism to prevent or to treat ASD. Using stevia as treatment rather than a preventive therapy may also be considered. So, we look forward to addressing these points in future studies and comparing the effects of the stevia as a preventive therapy and as treatment.

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Conflict of interest

All authors affirm the absence of any conflict of interest in this study.

Ethics approval

The Mansoura Faculty of Medicine IRB Committee approved this study (Code MS.22.04.1949).

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