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Therapeutic Potency of *Camellia sinensis* Extract on Neurochemical and Oxidative Changes Correlates to Autistic Disorder in Rat Pups Model

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ABSTRACT

Background: Prenatal exposure to valproate is capable of inducing experimental autism in rat pups. This study aimed to investigate the role of *Camellia sinensis* green tea extract (GTE) in ameliorating the neurochemical changes induced by autism.

Methods: Pregnant rats were treated with a single intraperitoneal dose of sodium valproate (600 mg/kg) on the 12.5th gestational day. The treatment with GTE (300 mg/kg) orally in autistic rat pups at postnatal day 15 for 20 days.

Result: Induction of autism resulted in a significant decrease in the cerebellum and cortical 5-HT, DA, NE, GABA, glycine and taurine and a decrease in brain cholesterol and antioxidants enzymes (GSH, SOD and CAT) at P<0.05. However, it showed a significant increase in MAO, AChE, glutamate, aspartate, serine and lipid peroxidation in addition to elevation in inflammatory biomarkers in brain tissue. Treatment with GTE extract showed significant decrease in AChE and MAO in addition to reduction in the excitatory amino acid GABA in brain tissue which in accordance resulted in marked elevation in cerebellar and cortical monoamine contents. Furthermore, administration of GTE improved the antioxidant defence system in cerebellum and cerebral cortex by activating the level of GSH, SOD and CAT. These results are accompanied by marked reduction in oxidative stress biomarker (NO and MDA) and lowering in the level of TNF-α and IL-6. In conclusion, it could be stated that GTE exerts an ameliorative effect on autistic rat pups possibly due to antioxidant and anti-inflammatory effects and could be effective in the management of autism.

Key words: Autism, Brain, *Camellia sinensis*, Catecholamine, Inflammation, Valproate.

INTRODUCTION

Autism spectrum disorder (ASD) is a neurodevelopmental disorder that is thought to be caused by exposure to a wide range of toxins or a change in a constellation of genetic factors. These etiological factors can interfere with neurobehavioral development, resulting in autistic symptoms (Rossignol *et al.,* 2008). This behavioral disorder could be caused by abnormalities in several brain regions and pathways. Poor social reciprocity is one of the hallmarks of autism, inability to concentrate, hyperactivity, communication problems and repetitive behavior (Gerlai and Gerlai, 2004). People with ASD face difficulties associated with neurological disorders such as seizures (Pei-Yin *et al.,* 2021).

Pre- or postnatal environmental exposure to factors such as valproic acid (VPA), mercury, lead, viruses, air pollutants, toxins and retinoic acid can trigger oxidative stress in autism (Nicolini *et al.,* 2015). Valproate is a widely accepted antiepileptic drug utilized to manage bipolar disorder (Natasha *et al.,* 2008). Many reports suggest that VPA can produce behavioral abnormalities, difficulties in learning and multiple birth defects (Štefánik *et al.,* 2015).

It is very important to notice that, VPA resulted in an alteration in the excitation/inhibition of the cerebral cortex and cerebellum. In the cortex of VPA-exposed rats, glutamatergic activity is increased whereas GABAergic activity is decreased. The VPA rat model of autism has the same behavioral, immunological and microbiome changes as autistic persons. It is causing a reduction in the parvalbumin (PV) + Chandelier (Ch) and PV + Baskets cells (Bsk) cells, two distinct GABAergic interneuron types (Ariza *et al.,* 2018). Following intraperitoneal injection of VPA to pregnant rats on a specific day of prenatal development at a specific dose [E (embryonic day) 12.5, 400 mg/kg], the infants of these rats recorded a reduction in the number of PV+ Ch and PV+ Bsk cells in their adult cerebral cortex, similar to those in humans with autism (Liu *et al.,* 2018).

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Camellia sinensis (green tea) contains high concentrations of polyphenols and L-theanine, which are found in the tea plant's leaves and stems. The most active component of polyphenols is catechins, they act as antioxidants in the biological system and they are rapidly absorbed and distributed primarily into the mucous membranes of the small intestine and the liver; more interestingly, they can cross the blood-brain barrier. These active compounds in green tea extract (polyphenols) can neutralize free radicals and may decrease or even help to stop some of the toxicity produced by reactive oxygen species (ROS). It has been reported that, in addition to directly quenching reactive oxygen species, tea polyphenols can facilitate participation in vitamin E recycling (Truong and Jeong, 2021).

The present study aims to investigate the possible ameliorative effect of green tea extract (GTE) on the neurotoxicity in rat pups' autistic models induced by prenatal exposure to VPA.

MATERIALS AND METHODS

Chemicals

Before use, sodium valproate (VPA, Sigma Aldrich Co. PVT Ltd, USA) was dissolved in NaCl solution. The treatment of VPA was IP at a dose of (600 mg/kg) (Al-Amin *et al.,* 2015).

Plant extract

From the Egyptian Herbal Market in Cairo, Egypt, we purchased green tea. The procedure outlined by Dulloo *et al.* (1999), was used to get the watery extract. According to Banji *et al.* (2011), the animals received daily oral treatment at a dose of (300 mg/kg).

Three adult male and six adult female rats weighing 100-150 g were obtained from the company for biological products and vaccines (VACSERA), Cairo, Egypt and acclimated for one week in a very specific pathogen-free (SPF) barrier area where the temperature (25±1) and humidity (55%). Rats were kept under continual control at the Laboratory of Physiology, Faculty of Science, Helwan University, Cairo, Egypt, with a 12-hour light/dark cycle. The animals were fed a typical laboratory meal and given unlimited access to water. Females were placed with males (2 females/male) overnight. On the following morning, females with sperms in their vagina were thought to be pregnant which day was designated as gestational day 0 (GD0). Three pregnant females received a single i.p injection of VPA 600 mg/kg at (GD12.5) and therefore the control females received physiological saline at the identical time as previously described. Females were housed individually until the time of birth. The newly born rats are left with their mothers for 15 days and therefore the sexes are separated.

Only male pups were taken to continue the experiments and divided into four groups each of 8 pups. Group (1) control group. Group (2) of normal pups received daily oral administration of GTE (300 mg/kg). Group (3) of autistic pups model received daily oral administration with saline. Group (4) of autistic pups model received daily oral administration of GTE (300 mg/kg). All animals start treatment at postnatal day 15 for 20 days. By the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals, 8th edition, all protocols and animal handling at the Department of Zoology, Faculty of Science, Helwan University were approved by the Committee on Research Ethics for Laboratory Animal Care (approval number: HU/Z/010-19).

At the end of the experimental period, rats were weighed and suddenly decapitated. Brains were rapidly excised from skulls, blotted and chilled. The brain tissue was rapidly wiped dry with filter paper. Dissection was performed on an ice-cooled glass plate into the cortex and cerebellum from the primary half for assay of monoamines and free amino acids and stored at (-70°C). Also, the other half of the brain was stored at (-70°C) for the determination of other biochemical parameters.

Monoamines assay

Weighing and homogenizing the tissue in 1/10 weight/volume of 75% aqueous HPLC grade methanol was the first stage in the HPLC procedure for determining the monoamines in the brain (cerebellum and cerebral cortex). The homogenate was centrifuged and subjected to solid phase extraction using a CHROMABOND column in the NH2 phase, Cat. No. 730031. After that, the sample was directly injected onto an AQUA column 150 54.6 mm 5C18, which was obtained from Phenomenex in the USA, with the following operating parameters: mobile phase 97/3 20 Mm potassium phosphate, pH 3.0/methanol, flow rate 1.5 ml/min, UV 270 nm. After 12 minutes, the monoamines were separated. Finally, the content of each monoamine was determined as g/gram of brain tissue. The resulting chromatogram revealed each monoamine location and concentration from the sample in comparison to that of the standard (Pagel *et al.,* 2000).

Free amino acids assay

High-performance liquid chromatography (HPLC) was used to identify the free amino acid neurotransmitters GABA, Glycine, Taurine, Glutamate, Aspartate and Serine in the cerebellum and cerebral cortex using the precolumn PITC derivatization method developed by Heinrikson and Meredith (1984).

Monoamine oxidase and acetylcholinesterase assay

The colorimetric technique established by Ellman *et al.* (1961) was used to evaluate the acetylcholinesterase (AChE) activity in the brain. Following the method described by Dar *et al.,* (2005), the MAO activity was evaluated fluorometrically at 550 nm (excitation wavelength) and 404 nm (emission wavelength) using 5-hydroxytryptamine (500 mM) as a substrate.

Oxidative stress biomarkers assay

Malondialdehyde (MDA), a lipid peroxidation (LPO) biomarker, was quantified in the brain tissue using the method described by Ohkawa *et al.* (1979). Nitric oxide (NO) level was determined using the Griess reagent, according to Green *et al.* (1982).

Antioxidant biomarkers assay

Glutathione (GSH) was detected by reducing Elman's reagent (5,5′ dithiobis (2-nitrobenzoic acid; DTNB) with GSH to produce a yellow molecule. The reduced chromogen's absorbance at 405 nm is directly inversely proportional to its GSH content. Catalase (CAT) activity was estimated by Aebi (1984). The superoxide dismutase (SOD) activity was assessed using the Nishikimi *et al.* (1972) technique.

Cholesterol assay

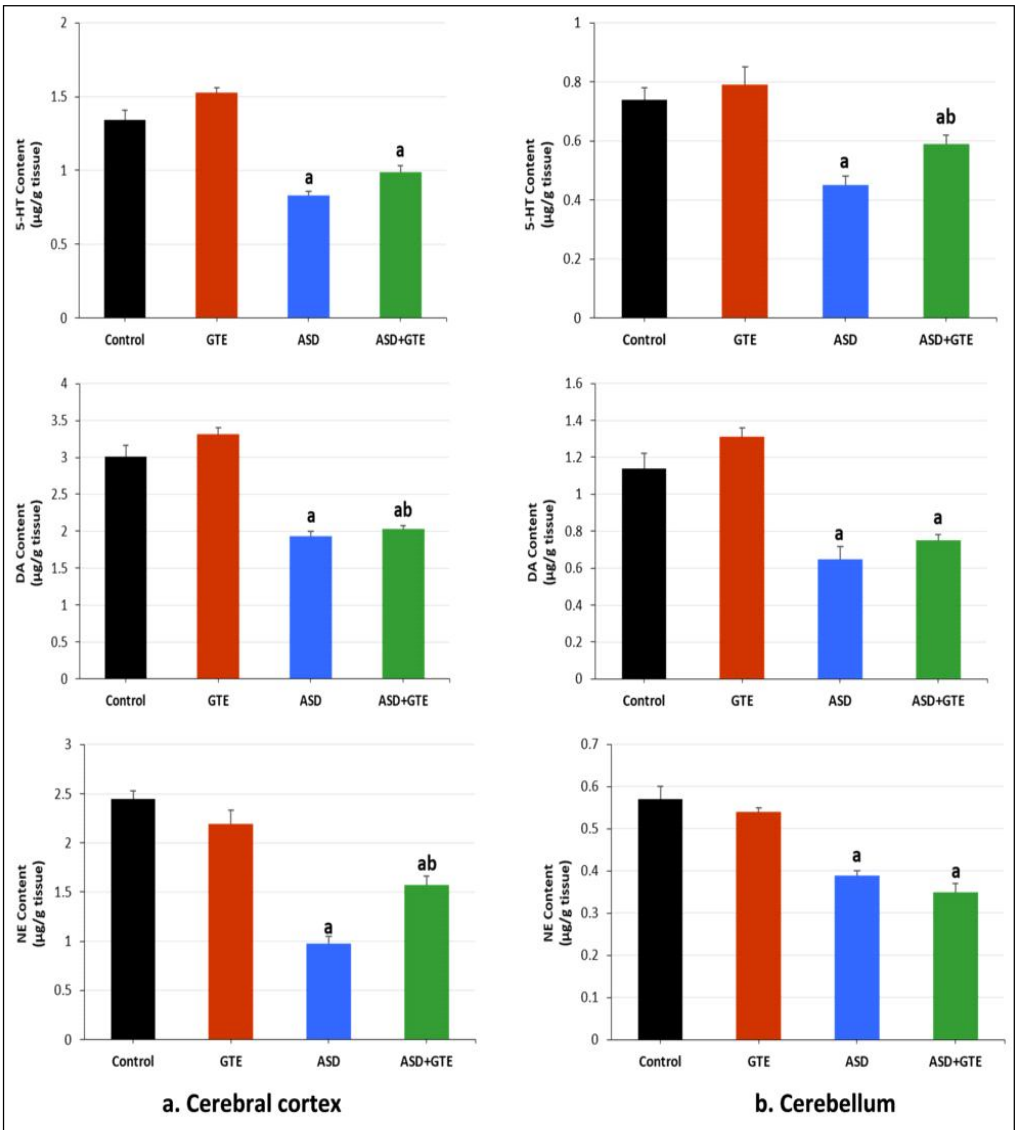
Analysis According to Abulrob *et al.* (2005), the fluorometric enzymatic approach with the Cholesterol Assay kit (Molecular Probe/Invitrogen, Eugene, OR) was used to determine the amount of cholesterol in the brain tissue samples.

Inflammation marker assay

Using commercial ELISA kits (RandD System, Minneapolis, MN, USA), TNF (tumour necrosis factor) and interleukin-6 (IL-6) concentrations were assessed as per the manufacturer's guidelines.

Statistical analysis

The design of the experiment was purely random. Using the Statistical Package for the Social Sciences, data were presented as means S.E. for the given number of



Data are expressed as means±SE (8 animals/group). a: Significance change at P<0.05 in comparison with the control group. b. Significance change at P<0.05 in comparison with the autism syndrome disease model (ASD) group.

Fig 1: The effect of oral administration of green tea extract (GTE) (300 mg/kg) for 20 days on the content of serotonin (5-HT), Dopamine (DA) and Norepinephrine (NE) in the cerebral cortex and cerebellum of autistic rat models.

independently performed experiments (SPSS 17.0 for Windows). The statistical significances within parameters were evaluated by one-way and multiple analysis of variation (ANOVA), where significant differences at P<0.05.

RESULTS AND DISCUSSION

Autism spectrum disorder (ASD) has drawn the attention of the public due to its high prevalence, substantial social costs and significant effects on families (Genovese and Butler, 2021). The results of both prospective and retrospective investigations show that maternal exposure to VPA is linked to a roughly three-fold increase in the rate of significant malformations and a potential collection of dysmorphic traits with reduced intrauterine growth (Takayama *et al*., 2022).

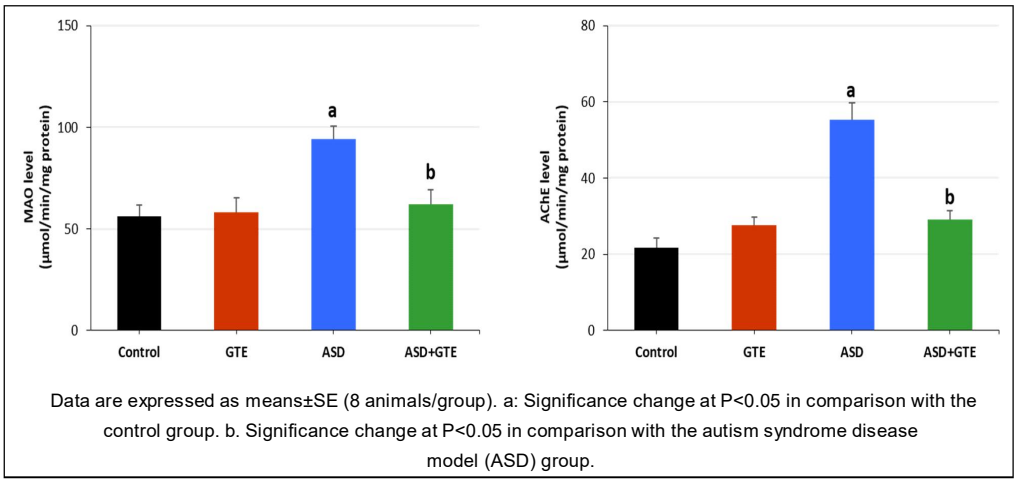
The goal of the current investigation was to assess the potential therapeutic effects of GTE (300 mg/kg) on ASD. As shown in Fig (1), the prenatal exposure to a single i.p injection of valproic acids (600 mg/kg- GD12.5) resulted in a significant decrease in 5-HT, DA and NE in both studied brain areas (cerebral cortex and cerebellum) as compared to control group (P<0.05). On the other hand, GTE administration postnatally in autistic rat pups with green tea extract resulted in a significant increase in serotonin in the cerebellum as compared to ASD group. Several studies on tryptophan retention or serotonin transporter binding in autistic patients have previously reported a similar decline in 5-HT concentration. According to Azmitia *et al.* (2011), compared to healthy controls, autistic individuals with post-mortem brain tissue from autistic donors aged 2.8 to 29 had a lower serotonin system in their brains.

The decrease in the monoamines in brain tissue in the present study suggested a disruption in monoaminergic neurotransmission and this was clear from the elevation inthe activity of MAO and AChE as compared to control. Interestingly, the levels of these parameters were considerably restored (p<0.05) by the treatment with GTE, suggesting the potent neuro-modulatory impact of GTE against autism-mediated neurotoxicity in rats (Fig 2).

The decrement recorded in DA content in the present study was also consistent with the earlier study by (Paulina and Joanna, 2022), which registered elevated dopamine hydroxylase and homovanillic acid in autistic children and reported the involvement of DA dysfunction in the production of autistic symptoms. In addition, the observed reduction in NE is consistent with the recorded decrease in DA, as vital neurotransmitters for the normal function of the brain and serve as a precursor of NE production.

The studied excitatory amino acids (Glutamate, Aspartate and Glycine) in the cerebellum and cerebral cortex significantly increased in ASD group as compared to control groups at p<0.05. The treatment with GTE (300 mg/kg) for 20 days in autistic pups resulted in a significant reduction in glutamate content in the cerebellum as compared to the ASD group (Fig 3). The data represented in Fig (4) recorded a significant decrease in inhibitory amino acids (GABA, Taurine and serene) in animals prenatal exposed to valproate as compared to the control group. GTE treatment caused a significant elevation in the inhibitory amino acids in both studied areas as compared to the ASD group.

GABA is formed from glutamate by the enzyme glutamic acid decarboxylase (GAD), which is the rate-limiting step of the synthesis of GABA (Hussman, 2001 and Dade *et al.,* 2020). Most theories regarding these amino acids neurotransmitters in autism suggest that the GABAergic system is suppressed resulting in an elevation in the glutamate system. The overactivity of glutamate could result in excitotoxicity which could cause aberrant neuronal development (Bittigau and Ikonomidou, 1997). Excessive glutamatergic stimulation is also associated with seizures,



Data are expressed as means±SE (8 animals/group). a: Significance change at P<0.05 in comparison with the control group. b. Significance change at P<0.05 in comparison with the autism syndrome disease model (ASD) group.

Fig 2: The effect of oral administration of green tea extract (GTE) (300 mg/kg) for 20 days on the level of monoaminoxidase (MAO) and acetylcholinesterase (AChE) in brain tissue of autistic rat models.

which are common among individuals with autism (Hussman, 2001).

Green tea's main free amino acid, L-theanine, has been shown to exhibit neuronal protection and tumor inhibition (Yang *et al.,* 2013). L-Theanine is absorbed in the small intestine after oral injection into the bloodstream and easily crosses the blood-brain barrier (Vuong *et al.,* 2011). Theanine interacts with glutamate receptors because it has a structural similarity. Regarding the possible mechanisms, theanine showed an antagonistic effect on AMPA/kinate type glutamate receptors. In addition, it inhibits the glutamate/ glutamine cycle and thus blocks the reuptake of glutamate (Jens *et al.,* 2021). On the other hand, some neurochemical studies reported that L-theanine caused increased brain DA, 5-HT and GABA levels (Liu *et al.,* 2009).

Fig 5 and 6 represented a significant raise in MDA and NO suggesting alternation in the brain oxidative state, after autism induction. On the other hand, the data recorded a significant reduction in GSH, SOD and CAT in brain tissue homogenate of autistic animal models as compared to the control group ($P<0.05$). The treatment with GTE postnatally for 20 days considerably reduces the oxidative stress and increase the content of antioxidant enzymes significantly as compared to the ASD group.

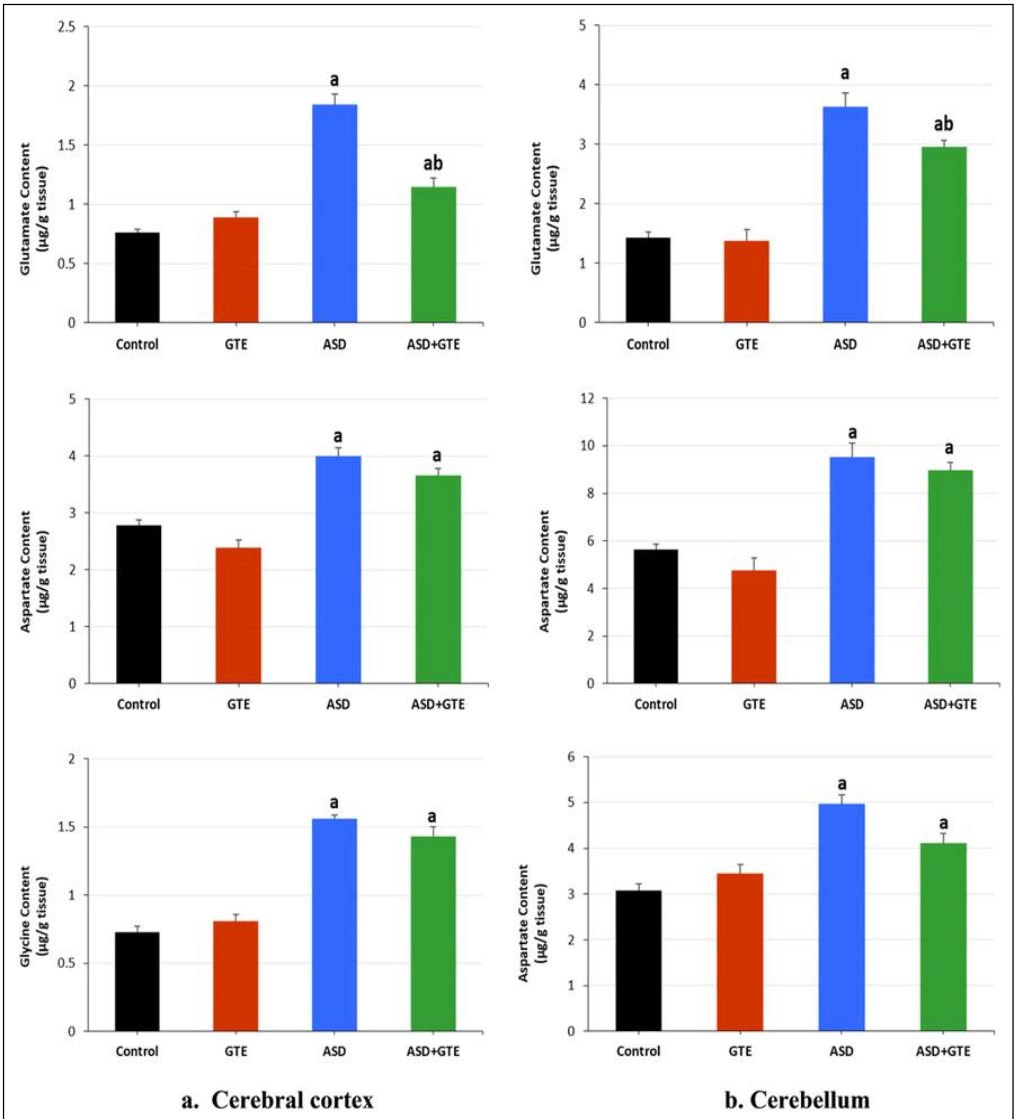


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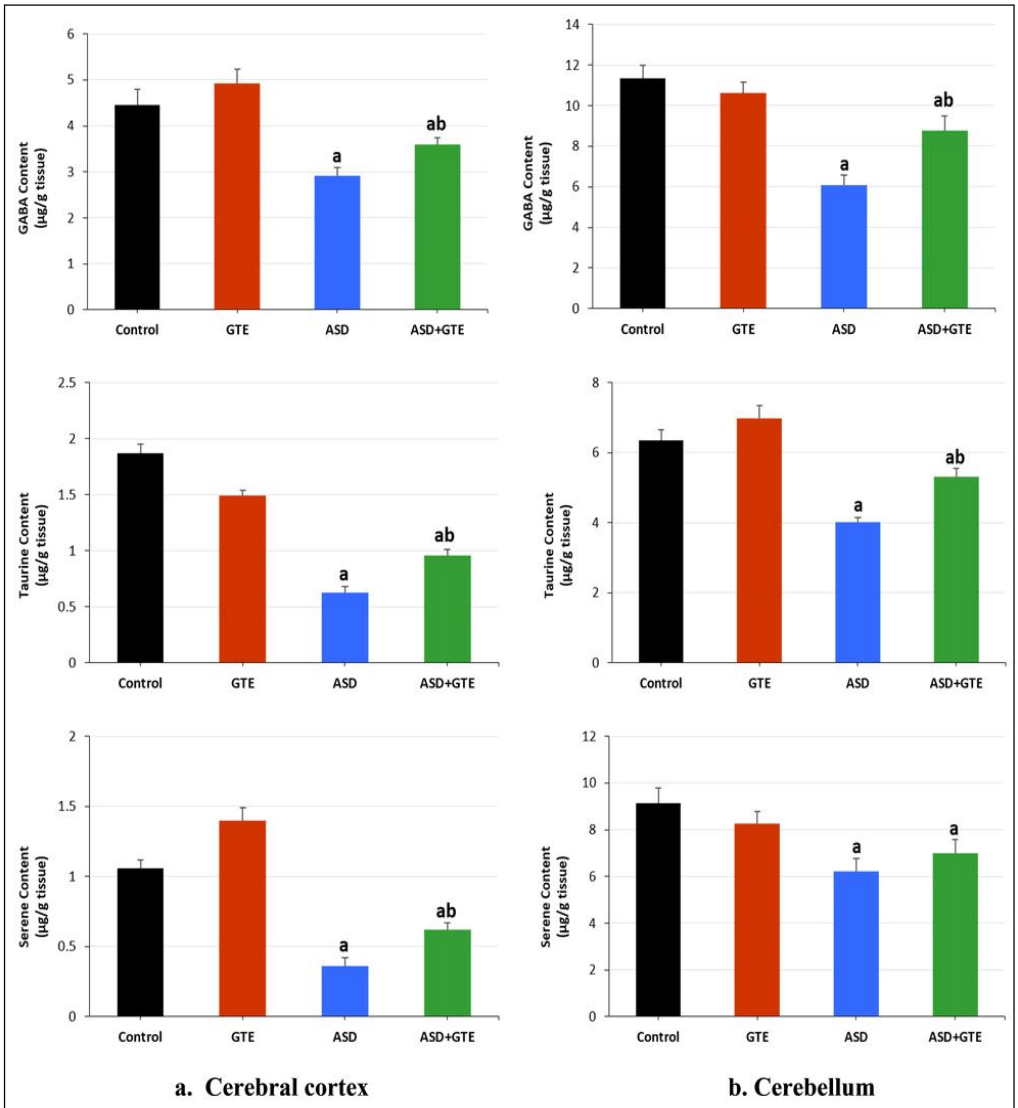
Fig 3: The effect of oral administration of green tea extract (GTE) (300 mg/kg) for 20 days on the content of free excitatory (glutamate, aspartate and glycine) amino acids in the cerebral cortex and cerebellum of autistic rat models.

To investigate the quantity of neuronal lipid content in autistic rat model, the cholesterol was examined in brain tissue. The data recorded a significant decrease in brain cholesterol content in ASD as compared to the control group. However, daily oral administration of GTE (300 mg/kg) for 20 days in autistic rat pups resulted in a significant elevation in brain cholesterol content as compared to the ASD group at P<0.05 (Fig 7). The brain is the body's most cholesterol-rich organ and needs a lot of it to maintain the myelin sheath (Segatto *et al.,* 2019). This reduction in cholesterol level recorded in present results may be due to exceedingly concentrated amounts of 7-dehydrocholesterol (7-DHC) (Ana Sofía and Francisco, 2022). The treatment with GTE in our study resulted in amelioration in the content of brain cholesterol content. The treatment with GTE caused green tea polyphenol to increase the levels of high-density lipoprotein (HDL) cholesterol due to its free radicle scavenger's property which enhances the integral membrane enzymes (Yokozawa *et al.,* 2002 and Gao *et al.,* 2023).

Neuronal inflammation caused by prenatal exposure to VPA was diagnosed by significantly higher tissue levels of pro-inflammatory cytokines (TNF- and IL-6) than those



Data are expressed as means±SE (8 animals/group). a: Significance change at P<0.05 in comparison with the control group. b. Significance change at P<0.05 in comparison with the autism syndrome disease model (ASD) group.

Fig 4: The effect of oral administration of green tea extract (GTE) (300 mg/kg) for 20 days on the content of free inhibitory (GABA, taurine and serine) amino acids in the cerebral cortex and cerebellum of autistic rat models.

seen in the control group ($p<0.05$). GTE's anti-inflammatory efficacy in the VPA-induced autism model was noticeable in the much lower levels of these brain inflammatory responses in GTE-treated rats compared to the ASD group (Fig 8). The mechanism by which the GTE repairs the damage produced by ASD and ameliorates the studied cytokines may be due to its potent anti-inflammatory effect (Modi *et al.*, 2010). Pervin *et al.* (2018), have attributed the ability of GTE in reducing apoptosis and proinflammatory cytokines production to its catechins content which affects regulating the generation of inflammatory cytokines in the rat brain.

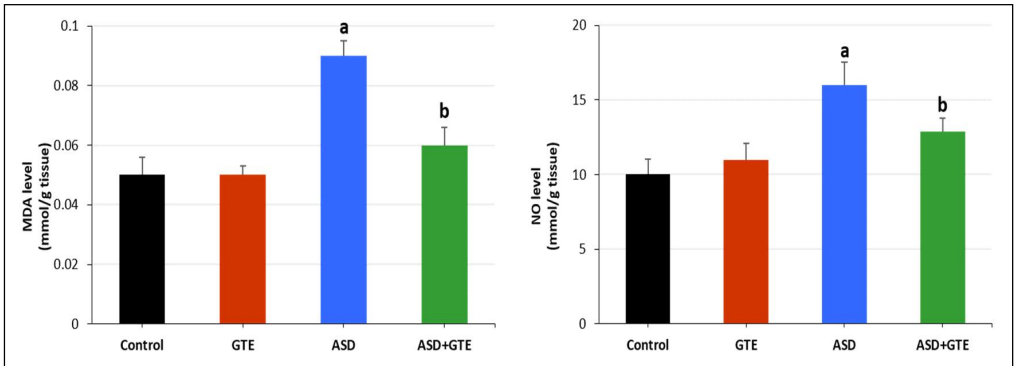


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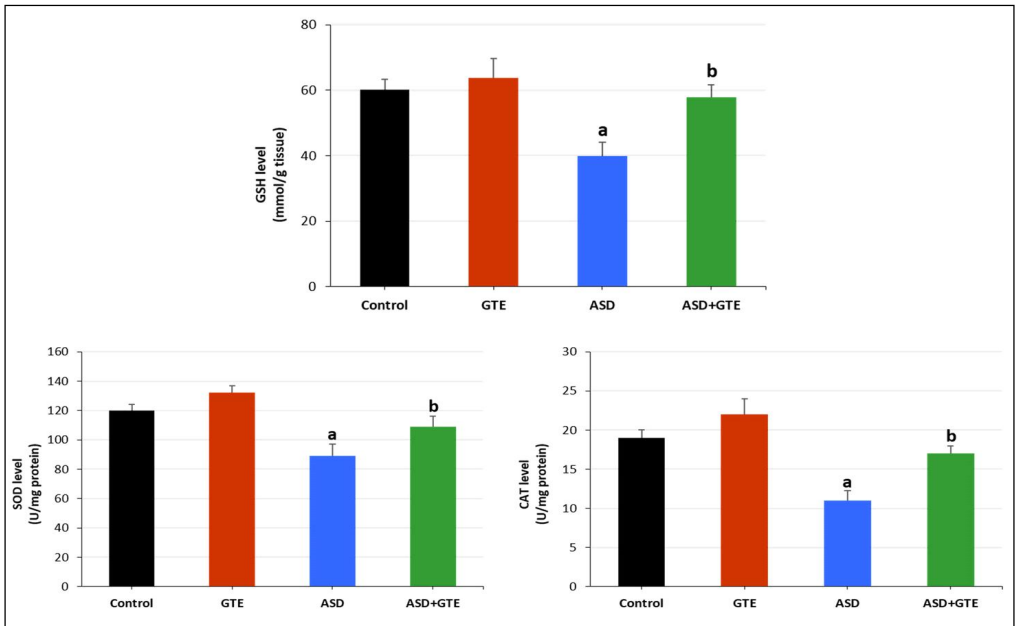
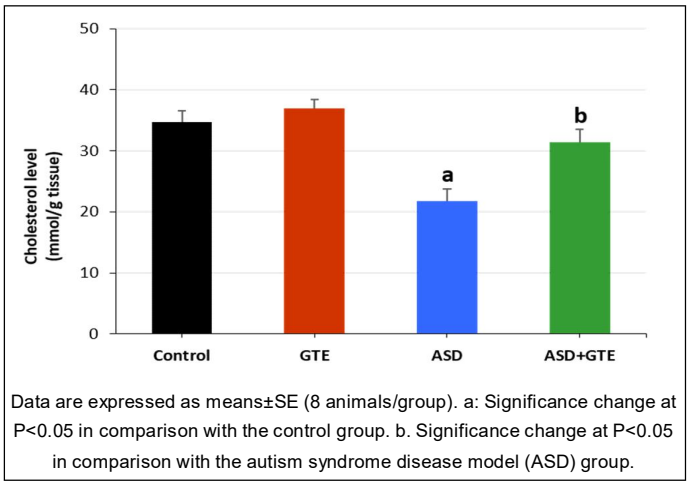
Fig 5: The effect of oral administration of green tea extract (GTE) (300 mg/kg) for 20 days on the level of oxidative stress enzymes (MDA and NO) in brain tissue of autistic rat models.



Data are expressed as means±SE (8 animals/group). a: Significance change at P<0.05 in comparison with the control group. b. Significance change at P<0.05 in comparison with the autism syndrome disease model (ASD) group.

Fig 6: The effect of oral administration of green tea extract (GTE) (300 mg/kg) for 20 days on the level of antioxidant enzymes (GSH, SOD and CAT) in brain tissue of autistic rat models.



Data are expressed as means±SE (8 animals/group). a: Significance change at P<0.05 in comparison with the control group. b. Significance change at P<0.05 in comparison with the autism syndrome disease model (ASD) group.

Fig 7: The effect of oral administration of green tea extract (GTE) (300 mg/kg) for 20 days on the cholesterol content in brain tissue of autistic rat models.

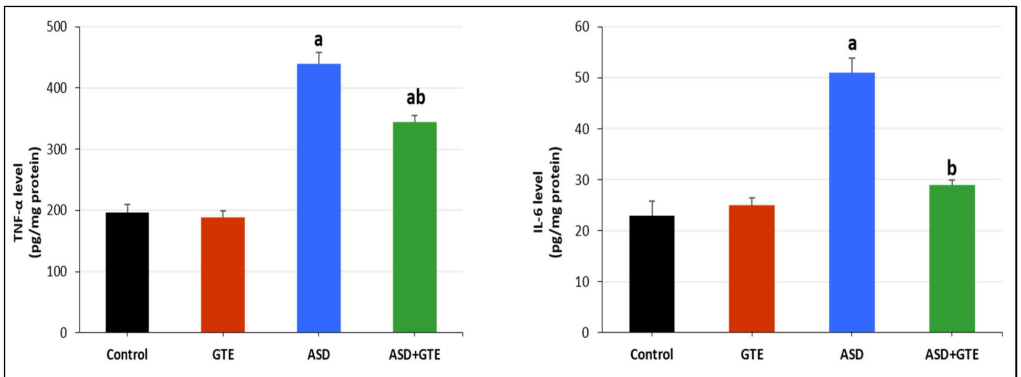


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Fig 8: The effect of oral administration of green tea extract (GTE) (300 mg/kg) for 20 days on brain neuroinflammatory markers of autistic rat models.

CONCLUSION

In conclusion, it could be stated that green tea extract was capable of attenuating VPA-induced autism. These beneficial effects of GTE could be attributed to antioxidant and anti-inflammatory action and could be effective in the management of autism. We recommend more researches on the beneficial effects of green tea extract on autistic rat pups models.

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Conflict of interest

The authors declare that there is no conflict of interest.

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