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Neuroprotective effects of *Clitoria ternatea* L. against propionic acid-induced behavior and memory impairment in autistic rat model

K. N. Jiji and P. Muralidharan*

Abstract

Background: Autism spectrum disorder is primarily characterized by complex behavioral and altered memory as a consequence of neuronal development abnormalities. The treatment of autism is highly challenging because of the lack of knowledge about its exact etiopathology. In the Ayurvedic system of medicine, there are group of plants named *'Medhya drugs'* because of their ability to improve brain- and neuron-related activities like learning and memory. *Clitoria ternatea* L. is one of the listed *'Medhya drugs'* which have been proved for its memory enhancement effects; in the present study, the ethanolic root extract of *Clitoria ternatea* L. was evaluated for its neuroprotective ability against propionic acid-induced memory and behavior impairments in an autistic rat model. The variation in behavior and memory were investigated by utilizing different procedures like rat elevated plus maze and novel object recognition test. *In vitro* assays for the estimations of glutamate and serotonin were also performed in isolated rat brain tissue homogenate.

Results: The object recognition and elevated plus maze test were showed the promising effects of *Clitoria ternatea* L. ethanolic root extract against the propionic acid-induced autism. In this study, the propionic acid infused rats (Group II) fail to recognize and explore the novel object compared to Group I (infused with phosphate-buffered saline) animals; extract treatment at two different doses (250 mg/kg and 500 mg/kg) (Groups III & IV, respectively) prevented these damage significantly ($p < 0.001$) so that extract-treated groups showed significant improvement in novel object recognition in a dose-dependent manner. Similarly, the effect of extract treatment on learning and memory of rats was investigated using transfer latency as a parameter for acquisition and retention of memory process on elevated plus maze; this further proved the memory enhancement ability of *Clitoria ternatea* L. Extract treatment also significantly reduced the concentration of different neurotransmitters like serotonin and glutamate in rat brain homogenate (Groups III &IV) in a dose-dependent manner as compared with the Group II.

Conclusion: The ethanolic root extract of *Clitoria ternatea* L. proved to be effective against propionic acid-induced memory and behavior impairments in an autistic rat model.

Keywords: Clitoria ternatea, ASD, Neurotransmitters

Background

Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by complex behavioral impairments that can be devastating for affected individuals, their families, and society. The individuals affected with ASD are manifested with symptoms like deviations in social relations, narrow or monotonous interests, diminishing in verbal and non-verbal communication, and

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purposeless motor behavior [1]. ASD prevalence estimation by Center for Disease Control and Prevention (CDC) in 2018 reported that there is a 15 percent increase in the ASD prevalence in USA to 1 in 59 children, from 1 in 68 in 2016 [2]. The exact pathophysiology of autism is still a mystery but research evidence pointing out the factors such as genetics, neural, environmental, and immunology have prominent role on ASD pathology [3, 4].

Neurodevelopmental defects harm various brain functions. Neurotransmitters play an important role in the normal development of the brain and also adequate levels each neurotransmitter are mandatory for maintaining functions such as learning and memory, motor–behavior activity in normal ways [5, 6].

Autism is not completely curable, preventable not even fully manageable with the help of the present treatment system because of poor available knowledge on its cause, pathophysiology, and due to its heterogeneous symptoms. Current synthetic drug therapy tries to improve memory, learning ability, and also trying to calm the patients, but all the drugs used for this purpose are having their profile of serious adverse effects. At this juncture, research has to be done incorporating various systems of medicine, especially the Ayurvedic system to identify the most relevant pathogenesis also to obtain a good cure or preventive medicine for autism. In the Ayurvedic system of medicine *'Medhya drugs'*—a group of herbal medicines—are known for their actions on the nervous system [7]. These *'Medhya drugs'* mentioned in Ayurvedic texts are said to improve mental abilities like learning and memory enhancement, antidepressant effect, also effective in preventing mood fluctuating disorders [8]. So these drugs can be screened against autism animal models to evaluate their efficacy and safety. *Clitoria ternatea* L. is one of the listed *'Medhya drugs'* [9]; in our present study, we have evaluated the neuroprotective effects of ethanolic extract of *Clitoria ternatea* L against propionic acid (PPA) induced memory and behavioral impairments.

Methods

Chemicals

Propionic acid and neurotransmitters estimation kits (Glutamate Assay Colorimetric kit, Serotonin ELISA kit) were purchased from Sigma-Aldrich–Merck, Bengaluru, India, and supplied by Southern India Scientific Corporation, Kandanchavadi, Chennai, India, whereas all other chemicals and reagent were of analytical grade.

Experimental animals

Healthy *Wistar* male rats between 4 and 8 weeks of age and weighing about 150–180 gm were used for the study. Animals were purchased from the animal house of Tamil Nadu Veterinary and Animal Sciences University, Chennai. They were housed in polypropylene cages at a temperature of 25–30°C and relative humidity 35–45%, light and dark cycles of 12 and 12 h, respectively, for one week before and during the experiments. Animals were provided with free access to a standard rodent pellet diet and water ad libitum. All animal studies were performed after getting the approval from the 'Institutional animal ethical committee with an approved reference number'; also possible measures were taken to minimize the suffering of the animals.

Collection and Preparation of *Clitoria ternatea* L. root extract

Fresh roots of *Clitoria ternatea* L were collected, authentified, shade-dried at room temperature to remove moisture, and then coarsely powdered by using an electric grinder. The powdered materials were stored in an airtight container and used for further extraction.

Extraction procedure

Extraction of roots of *Clitoria ternatea* L. was carried out using ethanol by hot continuous extraction method with Soxhlet apparatus. The extract obtained was collected and concentrated by gentle heating followed by using rotarat vacuum evaporator. The concentrated extract was then weighed, calculated the percentage yield, and stored. The extract was subjected to various preliminary phytochemicals tests [10].

Experimental design

A total of twenty-four rats were utilized for the study. Before starting the study procedure, all the rats were undergone a surgical procedure for cannula implantation. After 14 days of the surgery, the rats were stratified in to four separate groups containing six *Wistar* rats each.

Group I: Received vehicle alone per oral (*p.o)* (1% Tween-80 solution).

Group II: Received vehicle alone *p.o* (1% Tween-80 solution).

Group III: Received ethanolic extract of *Clitoria ternatea* L.(EECT) [11] at dose of **250 mg/kg** -suspended in 1% Tween-80 solution *p.o.,*

Group IV: Received ethanolic extract of *Clitoria ternatea* L.(EECT) [11] at dose of **500 mg/kg**-suspended in 1% Tween-80 solution *p.o.,*

The vehicles and the extract were administered orally using an intragastric tube daily for 28 days. After 21 days of extract administration, each group of animals except Group I received intracerebroventricular (ICV) infusions of propionic acid (PPA) (4.0 μl of a 0.26 M solution PPA was dissolved in phosphate-buffered saline (PBS) vehicle) daily for 7 consecutive days (between 22nd and 28th
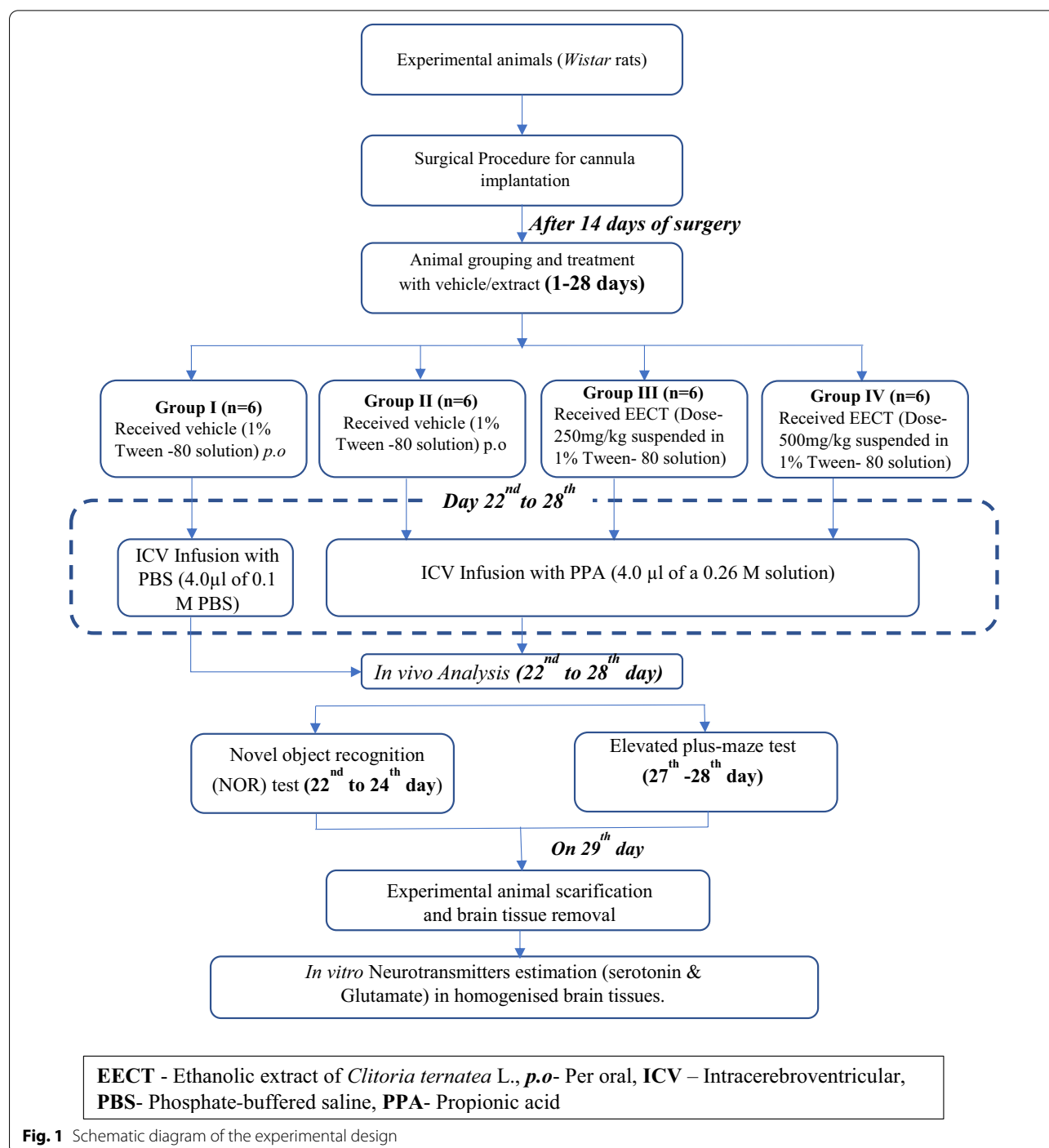
Fig. 1 Schematic diagram of the experimental design

days). Group I rats received ICV infusions PBS (4.0 µl of 0.1 M PBS) between 22nd and 28th days.

A schematic representation of the experimental design is provided in Fig. 1.

Induction of ASD

Surgical procedures for cannula implantation

Surgical procedures were completed under aseptic conditions. All rats were implanted with a 23gauge (ga) guide cannula using standard stereotaxic techniques. Before surgery, each rat was treated with atropine methyl nitrate (0.1 ml Subcutaneous injection). Before placing the stereotaxic apparatus, rats were anesthetized using inhaled isoflurane and 3% oxygen. During surgery, body temperature was maintained normal using a heating pad.

The tip of the guide cannula was placed in such a way that it will penetrate into the brain lateral ventricle just below the border of corpus callosum. For proper infusion

into lateral ventricle below the tip of the guide cannula, another 30ga injection cannula is placed so that the tip of injection cannula was further protruded 0.5 mm deepar. Each cannula was provided with an obturator closing, which can be removed before each intracerebroventricular (ICV) infusion. A Sage syringe pump with sterile PE10 tubing was attached with 30ga injection cannula for the purpose of infusion. Small screws were placed in the top of the skull, and the cannula was affixed to the skull with dental acrylic. Oral extract/vehicle administration began approximately 14 days after surgery [4, 12].

Intracerebroventricular (ICV) infusion procedure

Prior to the ICV infusion of propionic acid (PPA), it was dissolved in appropriate vehicle. Phosphate-buffered saline (PBS) was used as vehicle which was buffered to pH 7.5 by means of concentrated hydrochloric acid and sodium hydroxide. All the animals except Group I received ICV infusions of PPA (4.0 μl of a 0.26 M solution) and the Group I rats received ICV infusions PBS (4.0 μl of 0.1 M PBS). The infusions were given for 7 consecutive days (between 22nd and 28th days) in twice daily manner. The infusion duration was about 60 s, but an additional 60 s was allowed for the infusion cannula to remain in the position before being removed. Assessment of habituation behavior and memory assessments were done on each specific day (day 22 to day 28) following the infusion of PPA. Habituation behavior assessment was screened by performing a 'novel object recognition test,' and memory evaluation was done using 'elevated plus maze' [4, 12]. The effects of PPA infusion and the effectiveness of the extract to prevent those effects were evaluated by performing a comparative statistical analysis with the results obtained for each group by carrying out both in vivo test—novel object recognition test and elevated plus maze test.

Novel object recognition (NOR) test

This experiment took placed in a square open enclosure of $50 \times 50 cm^2$, surrounded by four walls of $50 cm^2$

Table 1 Effect of EECT on identical object recognition

S.no	Groups	Treatment given	Time of Exploration of Object (seconds)	
			Object A1 (time in seconds)	Object A2 (time in seconds)
1	Group I	Vehicle (1% Tween -80 solution) per oral + ICV infusion of PBS	21.33 ± 7.31 ns	32 ± 5.76****
2	Group II	Vehicle (1% Tween -80 solution) per oral + ICV infusion of PPA	14.5 ± 4.51	14.67 ± 5.89
3	Group III	EECT-dose 250 mg/kg -suspended in 1% Tween- 80 solution per oral + ICV infusion of PPA	23.83 ± 3.71*	25.83 ± 6.37**
4	Group IV	EECT-dose **500 mg/kg** -suspended in 1% Tween- 80 solution per oral + ICV infusion of PPA	35.5 ± 7.97****	34.66667 ± 3.83****

Values are represented in Mean $\pm$ SD (Standard deviation), $n = 6$ (6 rats in each group)

Comparison: Group II vs Group I, Group II vs Group III, and Group II vs Group IV

ns nonsignificant

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

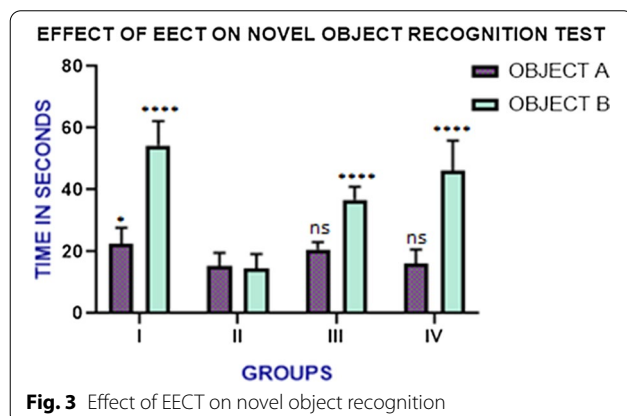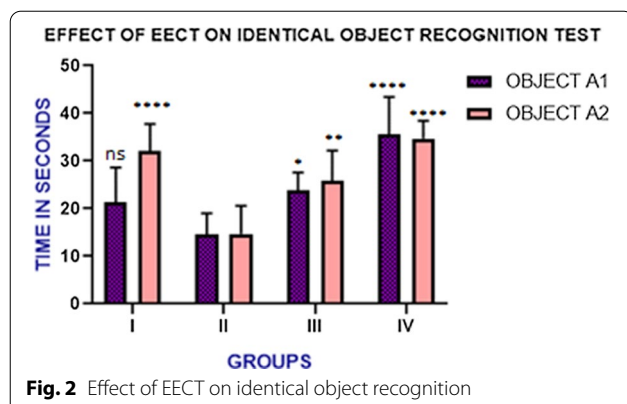**Table 2** Effect of EECT on novel object recognition

S. no	Groups	Treatment given	Time of exploration of object (seconds)	
			Object A (time in seconds)	Object B novel object (time in seconds)
1	Group I	Vehicle (1% Tween -80 solution) per oral + ICV infusion of PBS	22.5 ± 5.24*	54.33 ± 7.90****
2	Group II	Vehicle (1% Tween -80 solution) per oral + ICV infusion of PPA	15.33 ± 4.27	14.5 ± 4.76
3	Group III	EECT-dose 250 mg/kg -suspended in 1% Tween- 80 solution per oral + ICV infusion of PPA	20.67 ± 2.42 ns	36.67 ± 4.23****
4	Group IV	EECT-dose 500 mg/kg -suspended in 1% Tween- 80 solution per oral + ICV infusion of PPA	16 ± 4.73 ns	46.17 ± 9.79****

Values are represented in Mean $\pm$ SD (Standard deviation), $n = 6$ (6 rats in each group)

Comparison: Group II vs Group I, Group II vs Group III, and Group II vs Group IV

ns nonsignificant

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$

Fig. 2 Effect of EECT on identical object recognition

Fig. 3 Effect of EECT on novel object recognition

elevation. At the beginning of the test, each rat was allowed to liberally explore the device for 5 min in the absence of any object. Twenty-four hours later, the animals have explored two matching objects (A1 and A2) positioned at the two adjacent corners of the device at 10 cm from the walls, for 5 min; this was the 'acquisition' session. Exploration was only called when the rat had a nose directed fewer than one cm to the object while

nibbling and marking were not measured exploration. After a postponement of 24 h, the rats were again positioned in the device, but this time, one of the two identical objects was replaced by another entirely different (B). The animal is permitted to explore the two different objects for 5 min [13, 14].

The state of memory was calculated by the following equation:

$$\text{Recognition index} = \text{time spent in discovering the object B}$$

$$/\text{Sigma time spent exploring objects A and B [13].}$$

Elevated plus maze

The memory power of the rodents can be investigated efficiently by using an elevated plus maze. Rat elevated plus maze is specified with a central platform (10 cm × 10 cm) associated with two open arms (50cmx10cm) and two enclosed arms (50 cm × 40 cm × 10 cm) [15]. An elevation of 50 cm height from the floor was provided while positioning the maze. On the sixth day of propionic acid administration, the experiments were carried out to record the 'transfer latency (TL)' in seconds [15]. This was considered as a training session for each rat; before returning to its home cage rats were permitted to explore the maze for an extra 2 min. After 24 h (i.e., the seventh day of PPA infusion/28th day of the extract administration), each of the rats was examined for their retention memory [15, 16].

On the 8th day (29th day of the study), animals were killed by ethically approved euthanasia method—cervical dislocation, and brain tissues extracted and homogenized using a tissue homogenizer, and it was preserved /stored at −80°C for further analysis of neurotransmitters like serotonin [17] and glutamate [18].

Statistical analysis

Statistical validation of the data was done with the help of computer software GraphPad Prism version 8; statistical

Table 3 Effect of EECT on elevated plus maze

S. no	Groups	Treatment given	Transfer latency (time in seconds)	Retention latency (time in seconds)
1	Group I	Vehicle (1% Tween -80 solution) per oral + ICV infusion of PBS	48 ± 4**	18 ± 4.52****
2	Group II	Vehicle (1% Tween -80 solution) per oral + ICV infusion of PPA	59.5 ± 2.88	57 ± 6.48
3	Group III	EECT-dose 250 mg/kg -suspended in 1% Tween- 80 solution per oral + ICV infusion of PPA	49.67 ± 6.31**	37.83 ± 10.41***
4	Group IV	EECT-dose 500 mg/kg -suspended in 1% Tween- 80 solution per oral + ICV infusion of PPA	51 ± 6.23*	22.17 ± 8.13****

Values are represented in Mean ± SD (standard deviation), $n = 6$ (6 rats in each group)

Comparison: Group II vs Group I, Group II vs Group III, and Group II vs Group IV

ns nonsignificant

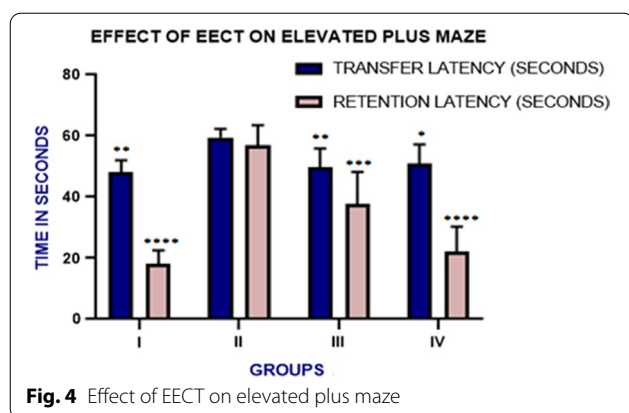* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$

Fig. 4 Effect of EECT on elevated plus maze

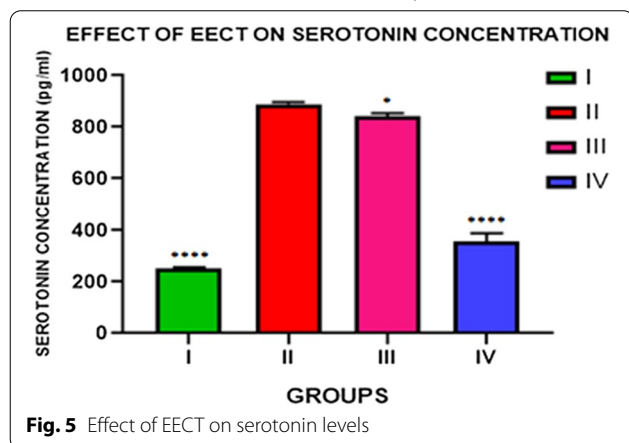test utilized was ANOVA, followed by Dunnett's multiple

Fig. 5 Effect of EECT on serotonin levels

comparison test.

All the values in the tabular column represent the Mean ± SD (Standard deviation), $n = 6$ (6 rats in each group).

Results

Assessment of habituation behavior

Effect of EECT on NOR test

NOR test is a normally used behavioral assay to investigate different aspects of learning and memory in rodents. This test was completed by 3 days: day 1-habituation day, day 2-training day, and day 3- testing day. Recognition memory of rats is its innate preference for novelty (novel object) evaluated by carrying out the NOR task. In the present study, the Group II rats showed least exploring tendency toward the identical and novel objects when compared with Groups I, III, and IV. The results obtained are given in Tables 1, 2 and represented in Figs. 2 and 3. Results pointing toward the dose-dependent effects of EECT and levels

of statistical significance are more for the Group IV rats as compared with the Group III.

Assessment of memory and retention

Effect of EECT on elevated plus maze task

Evaluation of working memory of rats was done by using this task. There was a significant reduction in retention latency in Group II animals when compared with Groups I, III, and IV implicating the declined cognitive skills of the Group II rats because of the PPA infusion. EECT treatment significantly improved the cognitive skills in a dose-dependent manner. The results obtained are given in Table 3 and represented in Fig. 4.

Effects on various neurotransmitters levels

Effect of EECT on serotonin levels

Altered 5-HT (5-Hydroxy tryptamine) system in brain is one of the key findings associated with ASD. The study results showed an increased concentration of serotonin in the brain tissue homogenates of Group II rats as compared with Group I animals. The EECT treatment at two dose levels 250 and 500 mg/kg (Groups III and IV, respectively) exhibited a significant ($p < 0.05$ and $p < 0.0001$) decrease in the level of brain serotonin in comparison with Group II animals. Results obtained are picturized in Fig. 5.

Effect of EECT on glutamate levels

Glutamate is one of the excitatory neurotransmitters well known for its prominent role in memory and cognitive skill development in humans/animals. PPA infusion in Group II rats produced an increased concentration of glutamate in brain as compared with Group I rats. The EECT treatment at two dose levels 250 and 500 mg/kg (Groups III and IV, respectively) exhibited a significant ($p < 0.0001$ for both the groups) decrease in the level of brain glutamate in comparison with Group II animals. Results obtained are picturized in Fig. 6.

Discussion

ASD is one of the leading neurocognitive disorders characterized by behavior and memory impairments. Common symptoms associated with ASD include diminished language and communication skills, repetitive or restrictive interests, stereotyped behaviors and movements, sensory disturbances, hyperactivity, and self-injury [19]. A complementary or alternative system of medicine is a reliable option in the treatment of ASD, as its cause or pathophysiology is not exactly known. Acceptability of medicinal plant origin drugs is increasing day by day because of its low cost and reduced toxicity profile. The present study indicates the neuroprotective effect of
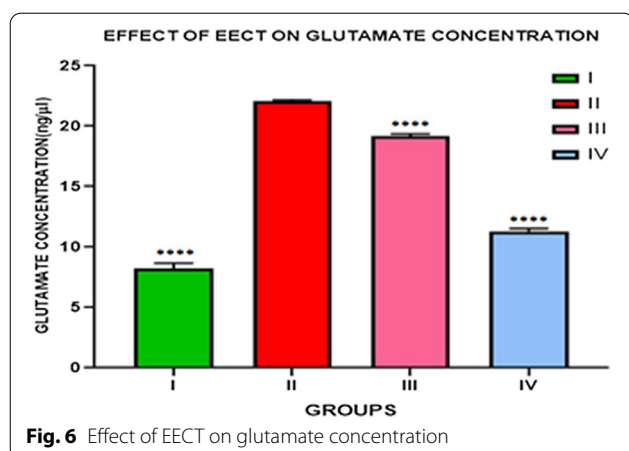
Fig. 6 Effect of EECT on glutamate concentration

ethanolic extract of *Clitoria ternatea.* L in propionic acid (PPA)-induced autism model in rats.

PPA is a metabolic intermediate of fatty acid metabolism [20] and also an end-product of enteric bacterial fermentation [21, 22]. The major application of PPA is in food industry as food preservative in wheat and dairy food products [23]. Many studies reported that repeated microinfusions of PPA into the lateral cerebral ventricles of adult rats produced behavioral, electrophysiological, and neuropathological effects comparable with ASD [24–26]. Because of this evidence, we had to choose the PPA ICV infusion as an inducing technique to produce ASD in rats.

Elevated plus maze was utilized to investigate the effects of plant extract in the rat acquisition and retention memory formation process. The evaluation was based on the transfer latency (TL); the results provided a piece of evidence for the utility of shortened TL on second-day trial in rats as a parameter (retention latency—RL) for retention or consolidation of memory [27] while the Group II animals do not show any improvement in RL, but EECT-treated groups showed significant improvement in RL in a dose-dependent manner. The results implicate the memory enhancement property of *Clitoria ternatea* L.

Habituation behavior and recognition and remembrance of rats were analyzed by carrying out the NOR task. The recognition of novelty indicates the degree of animal's cognitive skills which is primarily mediated by brain perirhinal cortex. The basic concept of the classical NOR task is when animals are allowed to explore a known and a novel object, frequency, and duration of exploring time for the unique object used to be more than the familiar one [28]. The fondness for a unique object demonstrates that the appearance of the known object subsists in the animal's memory. The utility of the NOR task can be extended from short-term memory

evaluation to long-term memory evaluation of rodents by manipulating the retention period [29]. The recognition memory formation is mainly led by perirhinal cortex and hippocampus of the brain, so any damage to these areas will lead to the compromised performance in NOR task [30]. In this study, the PPA-infused rats (Group II) failed to recognize and explore the provided novel object compared with the Group I animals; this may due to the damage of brain structures like hippocampal and cortex because of PPA-ICV infusion; EECT treatment prevented this damage so that EECT-treated groups showed significant improvement in novel object recognition in a dose-dependent manner.

Raised whole-brain serotonin is the first biomarker identified in ASD [31]. It is believed that in human body the serotonin plays a vital role in regulating mood, sleep, hunger, and sociability. It can also stimulate the intestinal muscles involved in digestion, helping for the contraction and expansion of the blood vessels, and in brain it conveys messages between neurons [32]. Serotonin functions drastically vary in infants and in adults; in infants it helps in the formation of neurons, synapse, and translocation of formed neurons to their correct locations, but in adult's brain it mainly acts as a neurotransmitter to communicate messages between neurons. The levels of brain serotonin are tightly controlled by various serotonin transporters. Both increased and decreased concentrations of brain serotonin levels show harmful effects. It is believed that in autistic patient's serotonin transporter may be altered; this could be one of the reasons for their irritability and repeated behavior [32]. In this study, ICV infusion of PPA produced an increased concentration of brain serotonin levels (in Group II rats) as compared with Group I rats. The extract treatment showed a reduction in the brain serotonin concentration.

Many researchers have reported that altered brain 5-HT system is there in ASD. Chugani et al. 1999 and Shoaf et al. 2000 carried out a surrogate measure to evaluate brain 5-HT synthesis, which appears to follow a changed developmental pattern in autism [33, 34]. Many researchers were tried to find out the serotonin receptor or transporter binding and its association with ASD. By conducting the platelet binding studies and neuro-imaging studies on children with autism, Goldberg et al. 2009 found a decreased 5-HT2 receptor binding in ASD [35]. A postmortem study conducted by Oblak et al. 2013 reported that there is a decrease in both 5-HT2A (5-Hydroxy tryptamine receptor 2 A) and 5-HT1A (5-Hydroxy tryptamine receptor 1 A) binding in ASD patients [36]. Serotonin is one of the major neurotransmitters which regulate the mood, emotional stability, and sleep pattern of an individual. Serotonin also got major effects on blood vessels and small intestine. High levels

of serotonin in blood and brain are considered as the first biomarker to identify ASD [37].

Glutamate is one of the chief excitatory signal conductors found in brain and spinal cord, as it got a leading role in brain development by affecting neuronal differentiation, migration, and synaptogenesis [38]. Scientific evidence also points out the glutamate involvement in general cognitive functions such as learning and memory [39]. However, excess levels of glutamate concentration act as a potent neurotoxin that leads to neuronal cell death [40]. Many research evidence showed that the ASD pathophysiology is greatly influenced by glutamatergic neurotransmission [41, 42]. The present study also justifies the above statement; here we can observe the high glutamate concentration of Group II animal brain when compared with Group I; EECT treatment reduced the glutamate concentration in a statistically significant manner.

Phytochemical evaluation of *Clitoria ternatea* L. showed the presence of tannins, alkaloids, saponins, steroids, carbohydrate, protein, triterpenoids, and various poly phenols like anthocyanin and flavonoids [43]. Researchers have reported the ability of different poylphenols to modulate the metabolism or action of various neurotransmitters [44–46]. In the present study, the EECT produced significant neuroprotection against PPA-induced autism in rats; it may be due to various phytoconstituents present in it.

Conclusions

The present study findings showed that the ethanolic root extract of *Clitoria ternatea* L. possesses neuroprotective activity with significant nootropic effects. The effects are mainly produced by manipulating the levels of serotonin and glutamate in the autistic rat brain. Preliminary phytochemical analysis and HR LCMS (High-Resolution Liquid Chromatography–Mass Spectrometer) analysis of ethanolic root extract of *Clitoria ternatea* showed the presence of various anthocyanin and flavonoids polyphenol; these may be the reason for its neuroprotective and memory enhancement effects. However, further research should be carried out in *Clitoria ternatea* to isolate the possible phytoconstituents and evaluate the same to find out their actual mechanism for neuropharmacological effects.

Abbreviations
ASD: Autism spectrum disorders; CDC: Centre for Disease Control and Prevention; EECT: Ethanolic extract of *Clitoria ternatea* L.; *P.O*: Per oral; gm: Gram; cm: Centimeter; mg/kg: Milligram/kilogram; μl: Microliter; ICV: Intracerebroventricular; PPA: Propionic acid; PBS: Phosphate-buffered saline; NOR: Novel object recognition; TL: Transfer latency; RL: Retention latency; ANOVA: Analysis of variance; SD: Standard deviation; 5-HT: 5-Hydroxy tryptamine.

Authors' contributions
PM guided JKN in planning and designing the research. PM also helped in arranging the whole facilities for the research and supervised the whole research. JKN conducted the entire laboratory works imparted in study design and interpreted the results putting efforts into statistical analysis with the guidance of PM. PM and JKN participated in the manuscript draft and have thoroughly checked and revised the manuscript for necessary changes in format, grammar, and English standard. All authors read and agreed on the final version of the manuscript. The authors read and approved the final manuscript.

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
The study approved was approved by the Institutional Animal Ethical Committee of C.L. Baid Metha College of Pharmacy, Chennai, India. Approval No: IAEC/XLIX/10/CLBMCP/2016.

Consent for publication
Not applicable.

Competing interests
Authors declared that they have no conflict of interest.

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