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Evaluation of the protective effect of *Clitoria ternatea* L. against propionic acid induced autistic spectrum disorders in rat model

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Abstract

Background: As a result of aberrant neural development, autism spectrum disorder (ASD) is characterised by complicated behavioural and memory issues. The dearth of knowledge about the actual etiopathology of autism makes treatment extremely difficult. There is a collection of plants known as "Medhya drugs" in the Ayurvedic system of medicine because of their capacity to boost brain and neuron-related activities including learning and memory. *Clitoria ternatea* L. is a "Medhya drug" that has been shown to improve memory. In this study, the ethanolic root extract of *Clitoria ternatea* L. was evaluated for its neuroprotective effects against propionic acid induced neuroinflammation in autistic rat modells.

Method: Adult Wistar rats were segregated into 4 groups and administered the vehicle/extract for 28 days. Induction of autism was done by intra-cerebro-ventricular infusion of propionic acid between 22nd and 28th day of the study. During this infusion period, rats were subjected to various in vivo behaviour and memory evaluation methods by performing actophotometer test and Morris water maze test. On 29th day of the study, animals were sacrified to get the brain tissues. Extracted brain tissues were utilised for the estimation of various neuroinflammatory markers levels (TNF-α and IL-6), immunohistochemical analysis of neuroinflammation, and histopathological analysis.

Results: Pre-treatment of rats with extract at two dose levels (250 mg/kg and 500 mg/kg) significantly ($p < 0.0001$) reduced the neuroinflammation, memory and cognitive impairment produced by the propionic acid in a dose dependent manner.

Conclusion: Study results showed the potent neuroprotective effects of *Clitoria ternatea* L. roots against the propionc induced autistic neuroinflammation.

Keywords: Neuroinflammation, Cytokines, Memory impairment, Propionic acid, Autism spectrum disorders

Background

Autism spectrum disorder (ASD) is a set of disorders marked by social impairment, communication and language difficulties, and a limited set of interests and activities that are both unique to the individual and directed repetitively (Sala et al. 2020).

Epilepsy, depression, anxiety, and attention deficit hyperactivity disorder (ADHD) are all ordinary co-occurring maladies in people with ASD. Individuals with ASDs have a wide range of intellectual abilities, ranging from severe disability to excellent abilities (Chen et al. 2019). The global prevalence of ASD is rapidly increasing each year, yet effective therapy is lacking due to a lack of understanding of ASD pathogenesis. Many neurodegenerative illnesses, including ASD, have been documented to have neuroinflammation as a defining hallmark (Bjorklund et al. 2016). Neuroinflammation is a response

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in which neurons, microglia, and macroglia in the central nervous system (CNS) participate (Schain and Kreisl 2017). Throughout their lives, autistic people commonly had altered inflammatory responses and neuro-immune system abnormalities. Postmortem investigations have confirmed this idea, revealing significant neuroinflammation in multiple brain regions of ASD patients (Vargas et al. 2005).

PPA (propionic acid) administration via ICV (intra-cerebroventricular) routes has been shown to cause ASD in laboratory rodents (Meeking et al. 2020). Increased free acyl-carnitine (a cofactor used to transport long-chain and very-long-chain fatty acids into the mitochondria) in rats exposed to PPA is hypothesised to produce systematic mitochondrial dysfunction (MD) (Rose et al. 2018). MD and high amounts of carnitine-bound unprocessed long-chain and very-long-chain fatty acids were detected in more than 30% of ASD patients, adding to the evidence for a relationship between PPA and ASD (Meeking et al. 2020). However, it is yet unknown how MD and/or abnormal fatty acid metabolism contribute to the autistic phenotype. There have been reports of attempts to reintroduce autistic-like behaviour in mice by exposing them to PPA at various developmental phases (Rose et al. 2018; MacFabe et al. 2008). In rats, for example, intracerebroventricular administration of PPA resulted in elevated IL-6, TNF, and interferon cytokine levels, fatty acid metabolic disturbances, and significant astrogliosis and microglia over-proliferation (MacFabe et al. 2008).

Neural stem cells create neuroepithelial progenitor cells (NPCs), which then grow into neuronal or glial cells (Pardo and Meffert 2018). Glial cells, including oligodendrocytes and astrocytes, help neurons create, connect, and defend themselves. During traumatic brain damage, reactive glial cells increase and release fibrillary acidic protein (GFAP) to avoid defective axonal regrowth, resulting in gliosis. Furthermore, glial and microglial cells generate inflammatory cytokines to clean up wounded cells and toxins, resulting in neuro-inflammation (Paudel et al. 2020). Some researchers believe that gliosis is a protective mechanism because it clears away damaged cells and stops the regrowth of damaged axons. If gliosis occurs during the early stages of brain development, it is safe to expect that it will have a substantial impact on neuronal architecture and connection (Verkhratsky et al. 2019; Abdelli et al. 2019). The ASD brain showed disrupted neural connections as well as increased regional cell density in the cortical, limbic, and cerebellar sections. Glial cells, on the other hand, outnumber neurons by a large margin (Yang and Zhou 2019; Falk and Götz 2017).

As in modern day medicine treatment option for ASD is limited, much interest is paying towards the development of good remedy from traditional systems of medicines, because traditionl medicine especially Ayurveda involves many prescribed formulations which has been using for many neurological issues. One such formulation is 'Medhya Rasayana.' These "Medhya medicines" referenced in Ayurvedic writings are supposed to boost mental abilities such as learning and memory enhancement, as well as having an antidepressant effect that helps to minimise mood swings. (Dash et al. 1983; Babalola et al 2021) The anti-inflammatory/antioxidant actions of the listed "Medhya medicines" are primarily responsible for these results. *Clitoria ternatea* L. (CT) is one of the Medhya drugs listed (Rai et al. 2001).

CT is an elliptic, obtuse perennial herbaceous plant with elliptic leaves. It thrives in damp, neutral soil and grows as a vine or creeper. The hue of its blooms, which are white and solitary with light golden lines, is the most remarkable element of this plant. This plant is native to equatorial Asia, including South Asia and Southeast Asia, but it has also spread to Africa, Australia, and the Americas. In Ayurveda, a concoction made up of C. *ternatea* seeds and roots, is used as a 'nerve tonic,' alternate, and laxative. As an active constituent of 'Medhya Rasayana,' it has been utilised to treat a variety of neurological problems. It is regarded a medicine by many groups of people who use it for skin ailments, eye, and throat infections, as well as urinary disorders, ulcers, and antidote action (Mukherjee et al. 2008). Many researchers were tried to give scientific validations to the reported traditional medicinal properties of CT. Shahnas and Akhila (2014) reported the memory enhancing effects of CT aqueous extract, Margret et al. (2015) reported the anti-depressant effects of the CT ethanolic extract, Kamkaen and Wilkinson (2011) reported the antioxidant effects of aqueous and ethanolic extract of CT, Ach enhancing effects and neurogenic potential of CT extracts were reported by Vyawahare et al. (2007) and Rai (2010).

The neuroprotective effects of ethanolic root extract of *Clitoria ternatea* L. (EECT) against PPA-induced neuroinflammation in an autistic rat modell were investigated in this study.

Methods

Chemicals

Propionic acid, estimation kits (TNF–ELISA Kit and IL-6 ELISA Kit), and other chemicals and reagents were obtained from Sigma Aldrich–Merck, Bengaluru, India, and delivered by Southern India Scientific Corporation, Kandanchavadi, Chennai, India. All the chemicals and reagents used were of analytical quality.

Collection and preparation of *Clitoria ternatea* L. (CT) root extract

Fresh wild CT roots were collected, and shade dried at room temperature to reduce moisture, after which they were roughly ground using an electric grinder. Using ethanol and the Soxhlet equipment, the powdered materials were subjected to hot continuous extraction (Mukherjee 2019). The extract was collected and concentrated using a rotarat vacuum evaporator after moderate heating. The concentrated extract was then weighed, the percentage yield estimated and then stored. Various preliminary phytochemical assays (Yadav and Agarwala 2011; Mukherjee 2019; Murthy et al 2021) and HR LCMS analysis were performed on the extract (Jiji and Muralidharan 2021a, b).

Experimental design

"The study employed healthy Wistar male rats that were 4 to 8-weeks-old and weighed around 150–180 g. For one week before and during the studies, animals were housed in polypropylene cages at a temperature of 25–30 °C, relative humidity of 35–45%, and light and dark cycles of 12 and 12 h, respectively. The animals were given unlimited access to a regular mouse pellet meal and water. Throughout the experiment, every precaution was taken to ensure that the animal suffered as little as possible" (Jiji and Muralidharan 2021a, b).

A total of twenty-four rats were used in the experiment. Before beginning the experiment, all the rats had a surgical procedure for cannula implantation (MacFabe et al. 2008). The rats were divided into four groups, each including six Wistar rats, after 14 days of operation. "The rats were assigned to the following groups of six rats each.

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

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

Group III Received Ethanolic extract of *Clitoria ternatea* L. (EECT) at dose of 250 mg/kg-suspended in 1% Tween-80 solution *p.o.* (Jiji and Muralidharan 2021a, b).

Group IV Received Ethanolic extract of *Clitoria ternatea* L.(EECT) at dose of 500 mg/kg-suspended in 1% Tween-80 solution *p.o.,* (Jiji and Muralidharan 2021a, b).

"For 28 days, the vehicles and extract were given orally using an intragastric tube. Each group of animals, except group I, received intracerebroventricular (ICV) infusions of propionic acid (PPA) (4.0 l of a 0.26 M solution PPA was dissolved in phosphate-buffered saline (PBS) vehicle) daily for 7 days after extract administration (between 22nd and 28th days). Between the 22nd and 28th days, Group I rat received ICV infusions in PBS (4.0 l of 0.1 M PBS)" (Jiji and Muralidharan 2021a, b; MacFabe et al. 2007, 2008; Sharma et al. 2019).

Assessment of locomotion and memory was done on each specific day (Day 22nd to Day 28th) following the infusion of PPA (MacFabe et al. 2007, 2008). Memory and retention assessments were done by using in vivo morris water maze test. Locomotor activity of the animals was evaluated by utilizing actophotometer. On 29th day of the study, animals were sacrificed by ethically approved euthanasia method-cervical dislocation, and the extracted whole brains were utilised for histopathological and immunohistochemical analysis (Sharma et al. 2019). Alternatively, other sets of extracted brain tissues were homogenised using a tissue homogeniser, and it was preserved /stored at -80˚C for further in vitro analysis.

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

Neurobehavioural examination

Locomotor activity

An actophotometer was used to assess locomotor activity (horizontal activity). A beam of light falling on the photocell is shut off as the rat moves, and a count is recorded and displayed digitally. The rats were divided into four groups, and each group was placed in the actophotometer for 10 min to acquire a basal activity score (Thirupathi et al. 2010).

Morris water maze

To test spatial working memory and reference memory, the Morris water maze method is used. A round tank serves as the experimental apparatus (140 cm in diameter, 55 cm in height). 1 cm below the water's surface, an unseen platform (10 cm in diameter) was put. At a temperature of 21–23 °C, a small amount of milk was dissolved to make the water opaque. The pool was in a test room, and several cues outside the maze were visible from the pool, which the rats could use for spatial orientation. Throughout the experiment, the position of the cues was maintained. The training trials took place from the 26th to the 28th day, with one trial per day. Throughout the test period, the platform remains in the same location in the middle of one quadrant, equidistant from the pool's centre and edge. The time it took to escape to the concealed platform was recorded during each training session. The platform was removed on the 28th day, and the rats were assessed for memory, with the time spent in the target quadrant hunting for the hidden

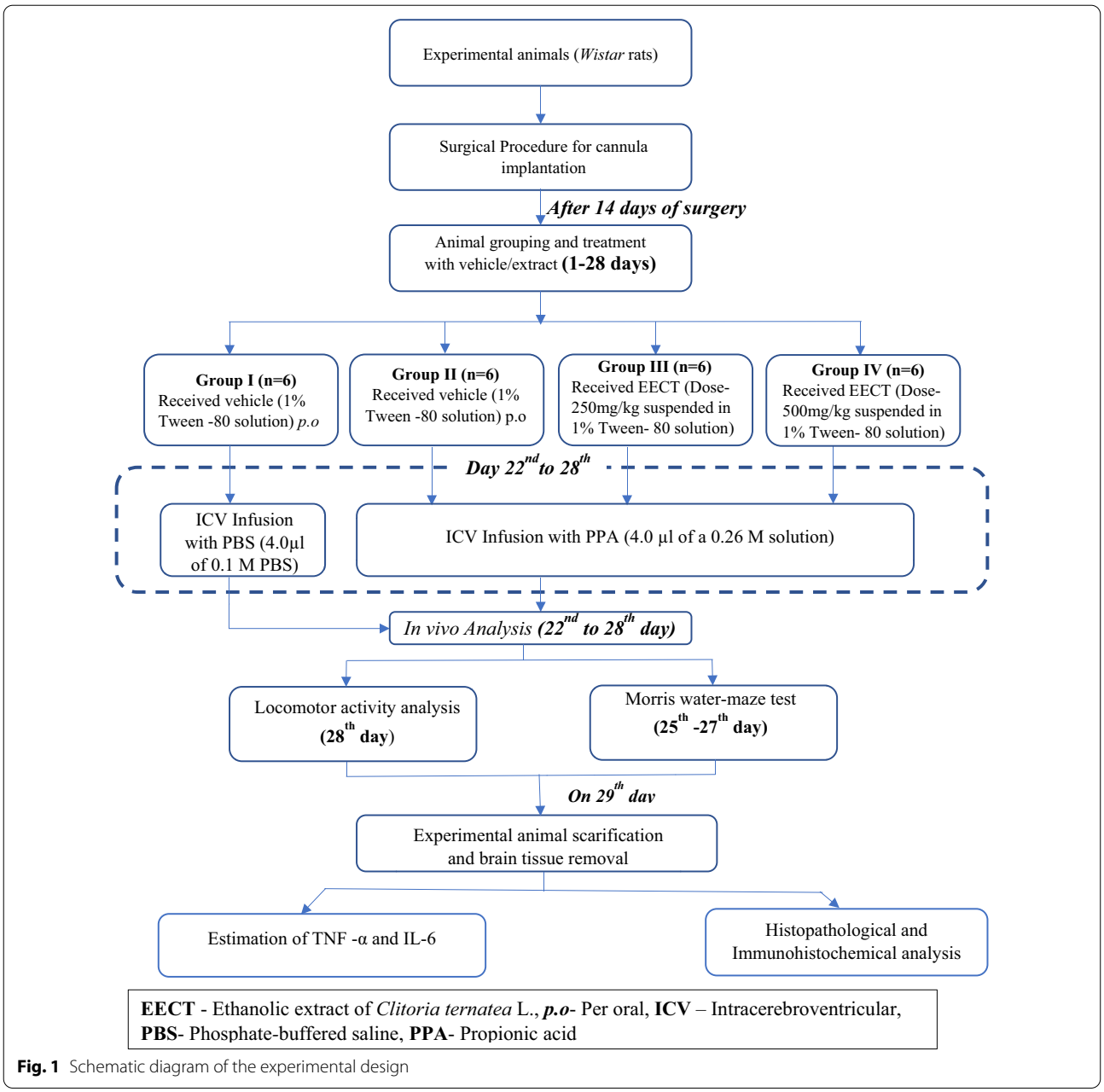

Fig. 1 Schematic diagram of the experimental design

platform recorded as an index of retrieval (Charles and Michel 2006; Morris 1984; Nunez 2008).

Biochemical examinations

To assess the levels of neuroinflammation, we evaluated the value of different cytokine-inflammatory markers (TNF-α and IL-6) in homogenised brain tissues by using TNF-α and IL-6 estimation ELISA kit. The results were obtained in picogram per milli litter (pg/ml) of wet tissues (Hensley et al. 1995).

Histopathological examination

The whole brain of one animal from each group was removed and preserved in 10% formalin after washing with normal saline on day 29 when the animals were slaughtered. The tissue was cleaned, dehydrated in alcohol, clarified in xylene, and paraffin blocks were created. A rotary microtome was used to cut serial slices of 5-m-thickness. After that, the sections were deparaffinized with xylene and hydrated in descending alcohol grades. After that, the slides were immersed in haematoxylin for 10 min before being rinsed with water. These

were evaluated and then counterstained with eosin, rinsed with water, dehydrated with successive grades of alcohol, cleaned with xylene, mounted, and viewed under a light microscope for pathological symptoms to determine any architectural alterations in brain areas (Ozturk et al. 1996).

Immunohistochemical examination

Serial 4-µm-slices of the ipsilateral dorsal hippocampus were produced using a Leica microtome. The following antibodies were utilised in this study: (1) anti-glial fibrillary acidic protein (GFAP) (RRID:AB_2109645), a marker for reactive astrogliosis that has been found elevated in human autism neuropathology studies (Rosengren et al. 1994) and in cerebrospinal fluid from autism patients; (2) anti-rat CD68 antigen (RRID:AB_2291300), a marker for activated microglia that has been found elevated in human autism brain (Beach et al. 1995) and epilepsy (Kondratyev and Gale 2000); (3) anti-cleaved caspase 3 (RRID:AB_2341188), a marker for apoptotic cytotoxicity (Noyan-Ashraf et al. 2005; Shi et al. 2001).

Tissue sections were placed on glass slides and dried in a 37 °C oven overnight. For antigen recovery, sections were deparaffinized and rehydrated using normal immunohistochemistry methods (Shi et al. 2001). Endogenous peroxidase activity was inhibited for 5 min using a 3 percent hydrogen peroxide in distilled water solution. In a 1250 W microwave oven, portions were submerged in boiling 0.21 percent citric acid buffer (pH 6.0) for 30 min to recover antigens. Slides were counterstained with Gill and washed for 5 min in PBS. The primary antibodies were administered for 1 h at room temperature after a 5-min application of 10% normal horse serum in PBS solution. Following the incubation period, slices were washed in PBS and incubated for 30 min with secondary antibodies, either biotinylated anti-mouse IgG or biotinylated anti-mouse IgG. Tissues were rinsed again in PBS and stained for 30 min at room temperature with the avidin–biotin combination. After incubation, the slides were washed in PBS and treated with 3, 3-diaminobenzidine DAB chromagen for 5 min. Slides were dehydrated, cleaned, and coverslipped after final rinse (MacFabe et al. 2008). A blindfolded histopathologist viewed the slides under a light microscope.

Statistical analysis

Statistical validation of the data was done with the help of computer software GraphPad Prism version 8 (RRID:SCR_002798), statistical test utilised was ANOVA, followed by Dunnett's multiple comparison test.

Results

Extraction and phytochemical evaluation

The percentage yield obtained after the ethanolic extraction of dried root extract of *Clitoria ternatea* Linn (EECT) was found to be 10.4%w/w. Preliminary phytochemical screening showed the presence of phytochemical constituents such as carbohydrates, flavonoids, tannins, steroids, and glycosides. HR-LCMS analysis of EECT detected many compounds among the most important ones are Chelidonine, Elephantopin, Deoxysappanone B 7,3'-Dimethyl ether acetate, Pectolinarin, Levan, Maltotriose, 6,4'-Dimethoxyflavon, Mucronulatol, Isotectorigenin, and Biochanin A. (Jiji and Muralidharan 2021a, b).

Neurobehavioural examination

Effect of EECT on locomotor activity

CNS anti-depressant activity of EECT was evaluated by locomotor activity of the animal. The activity is a measure of the level of excitability of the CNS (Ozturk et al. 1996), The extract significantly increased the locomotor activity as observed in the results of the actophotometer test, represented in Table 1 and Fig. 2.

The Group II animals showed decrease in locomotory activity on comparison with Group I animals

Table 1 Effect of EECT on locomotor activity

Sl. no	Groups	Treatment given	Activity score
1	Group I	Vehicle (1% Tween-80 solution) per oral + ICV infusion of PBS	304.5 ± 3.61****
2	Group II	Vehicle (1% Tween -80 solution) per oral + ICV infusion of PPA	103.67 ± 6.9
3	Group III	EECT-dose 250 mg/kg-suspended in 1% Tween-80 solution per oral + ICV infusion of PPA	194.1667 ± 13.51****
4	Group IV	EECT-dose 500 mg/kg-suspended in 1% Tween-80 solution per oral + ICV infusion of PPA	283.17 ± 16.29****

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, non significant

****$p < 0.0001$. Upon statistical analysis, on comparison with Group II we got highly significant results with *p*-value (probability value) less than 0.0001, indicating the effectiveness of the extract

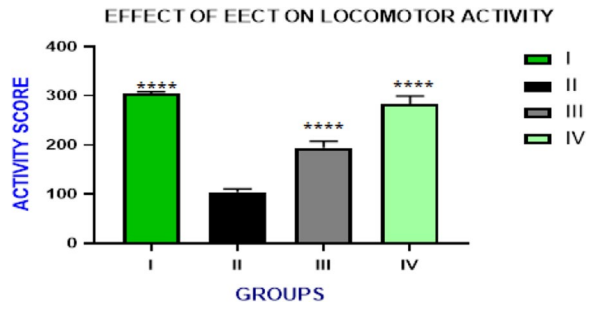

Fig. 2 Effect of EECT on Locomotor Activity

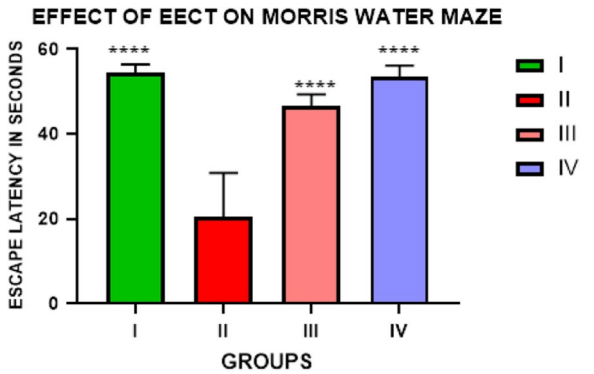

Fig. 3 Effect of EECT on Morris water maze test

($p < 0.0001$). Treatment with EECT (250 mg/kg and 500 mg/kg) increased the locomotory activity siginificantly ($p < 0.0001$ and $p < 0.0001$ for Group III and Group IV, respectively).

Effect of EECT on Morris water maze task

Morris water maze is a behavioural modell for assessing spatial and related forms of learning and memory. The Group II animals showed increase in escape latency period on comparison with Group I animals ($p < 0.0001$). Treatment with EECT (250 and 500 mg/kg) decreased the latency time significantly ($p < 0.0001$ and for $p < 0.0001$ Group III and Group IV, respectively) on comparison with Group II. Results are shown in Table 2 and Fig. 3.

Biochemical examinations

Effect of EECT on TNFα levels

TNF-α is a proinflammatory cytokine formed by monocytes in the periphery and microglia, neurons, and astrocytes in the CNS. As a kingpin in inflammation, TNF-α serves as a main regulator of acute phase inflammation, introducing inflammatory cytokine signaling cascades. The brain TNFα level of Group II animals was increased significantly ($p < 0.0001$) on comparison with Group I animals. All treatment groups treated with EECT 250, and 500 mg/kg (Group III and IV, respectively) exhibited significant ($p < 0.0001$ for both the groups) decrease in the level of TNFα in comparison with Group II animals. Results are represented in Fig. 4.

Effect of EECT on IL-6 Levels

Interleukin -6 (IL-6) is a major cytokine in CNS and its levels get upregulated whenever neuroinflammation is expected. The brain IL-6 levels of Group II animals were increased significantly ($p < 0.0001$) on comparison with Group I animals. All treatment groups treated with EECT 250, and 500 mg/kg (Group III and IV, respectively) exhibited significant ($p < 0.0001$ for both the groups) decrease in the level of IL-6 in comparison with Group II animals. Results are represented in Fig. 5.

Immunohistochemical examination

These studies have reliably found that rats given ICV injections of PPA display a neuroinflammatory response, characterised by increased activated microglia and reactive astrogliosis which was emphasised by the exaggeration of GFAP and CD 68 expression in brain area of hippocampus of group II animals as compared with

Table 2 Effect of EECT on Morris water maze

Sl. no	Groups	Treatment given	Escape latency (SEC)
1	Group I	Vehicle (1% Tween-80 solution) per oral + ICV infusion of PBS	54.33 ± 2.092****
2	Group II	Vehicle (1% Tween-80 solution) per oral + ICV infusion of PPA	20.50 ± 10.26
3	Group III	EECT-dose 250 mg/kg -suspended in 1% Tween-80 solution per oral + ICV infusion of PPA	46.50 ± 2.84****
4	Group IV	EECT-dose 500 mg/kg-suspended in 1% Tween-80 solution per oral + ICV infusion of PPA	53.50 ± 2.62****

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, non significant

****$p < 0.0001$. Upon statistical analysis, on comparison with Group II we got highly significant results with *p*-value (probability value) less than 0.0001, indicating the effectiveness of the extract

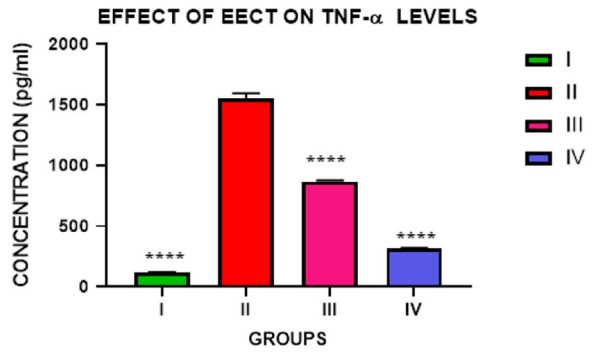

Fig. 4 Effect of EECT on TNFα Levels

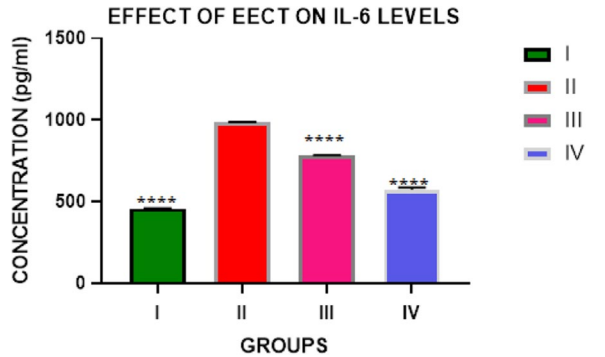

Fig. 5 Effect of EECT on IL-6 Levels

group I (represented as arrows in the figures). Plant extract treatment reduced this increased expression in dose dependent manner implicating its protective effects against neuroinflammatory response/Neuroinflammation. Neuroinflammatory process are occurs in the absence of apoptotic neuronal loss indicated by the neagativity shown in all groups with respect to caspase 3 which is a marker of apoptosis. Figure 6 representing the GFAP expression (marked with arrows), Fig. 7 representing the CD 68 expression (marked with arrows), and Fig. 8 representing the Caspase 3 expression of various groups.

Histopathological examination

Propionic acid treatment evoked mild to moderate changes such as reactive gliosis, lymphocytic infiltrate, and passive congestion of blood vessels in the areas of corpus callosum and hippocampus, (marked with arrows in Fig. 9) of group II rats. These features are not encountered much with pre-treatment of plant extract. This reversibility of gliotic changes and lymphocytic infiltrate suggest efficacy of plant extract to prevent neuroinflammation. Efficacy of plant extract showing in dose dependent manner. Histopathological results have been picturised in Fig. 9.

Discussion

PPA is a short-chain fatty acid that occurs naturally in the human body as both a fatty acid metabolic intermediary and a fermentation end product of antibiotic-resistant enteric gut bacteria like Clostridia (Mirza and Sharma 2018). Although PPA and related short-chain fatty acids are mostly generated in the stomach, they can easily reach the brain, where they can trigger a variety of neurophysiological processes that can alter both brain function and behaviour (Bukhari et al. 2020). PPA and other enteric short-chain fatty acids may be increased in ASD, according to several lines of evidence (Witters et al. 2016; de la Bâtie et al. 2018). Intracerebroventricular (ICV) injections of PPA in rats have recently been employed as an animal modell of ASD to further investigate the link between PPA and ASD. Using this paradigm, researchers discovered that ICV treatment of PPA can cause a variety of behavioural, electrophysiological, and neuropathological abnormalities in rats that are like those seen in people with ASD (MacFabe et al. 2007, 2008; Sharma et al. 2019).

Motions and emotions are inextricably linked, ASD has been linked to changes in psychomotor behaviour (Bigelow and Agrawal 2015). When compared to the

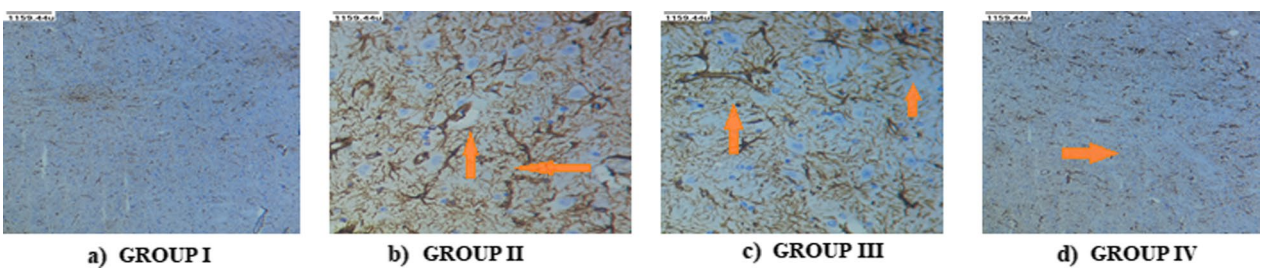

Fig. 6 Representative photomicrographs of GFAP expression on dorsal hippocampus of rats received ICV infusions of PBS or PPA for 7 days. **a** GROUP I rat brain, **b** GROUP II rat brain, c GROUP III rat brain, **d** GROUP IV rat brain

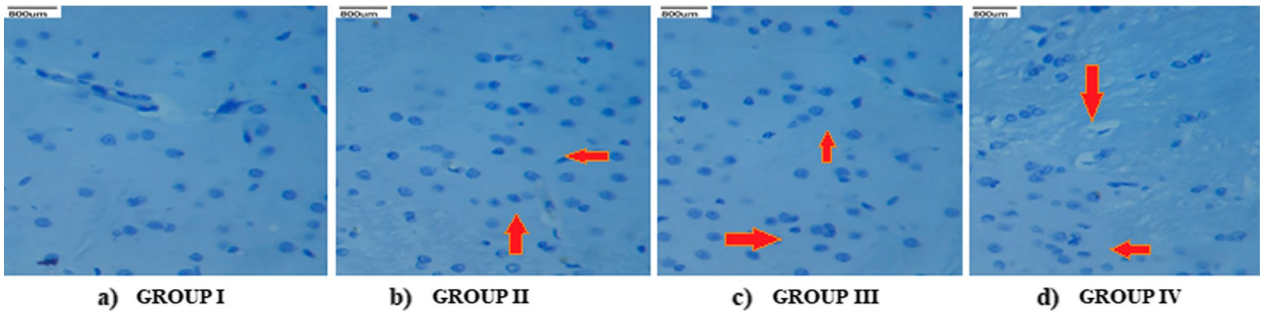

Fig. 7 Representative photomicrographs of CD-68 expression on dorsal hippocampus of rats received ICV infusions of PBS or PPA for 7 days. **a** GROUP I rat brain, **b** GROUP II rat brain, c GROUP III rat brain, **d** GROUP IV rat brain

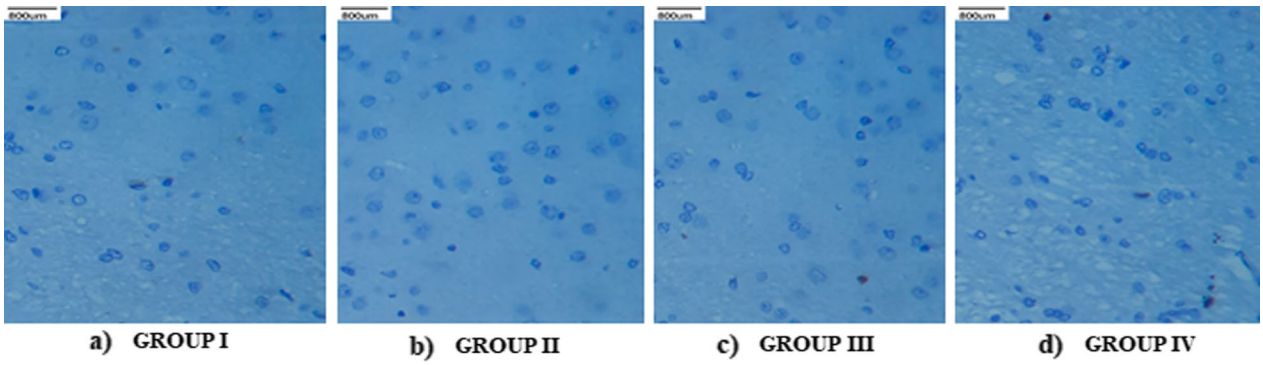

Fig. 8 Representative photomicrographs of Caspase -3 expression on dorsal hippocampus of rats received ICV infusions of PBS or PPA for 7 days. **a** GROUP I rat brain, **b** GROUP II rat brain, c GROUP III rat brain, **d** GROUP IV rat brain

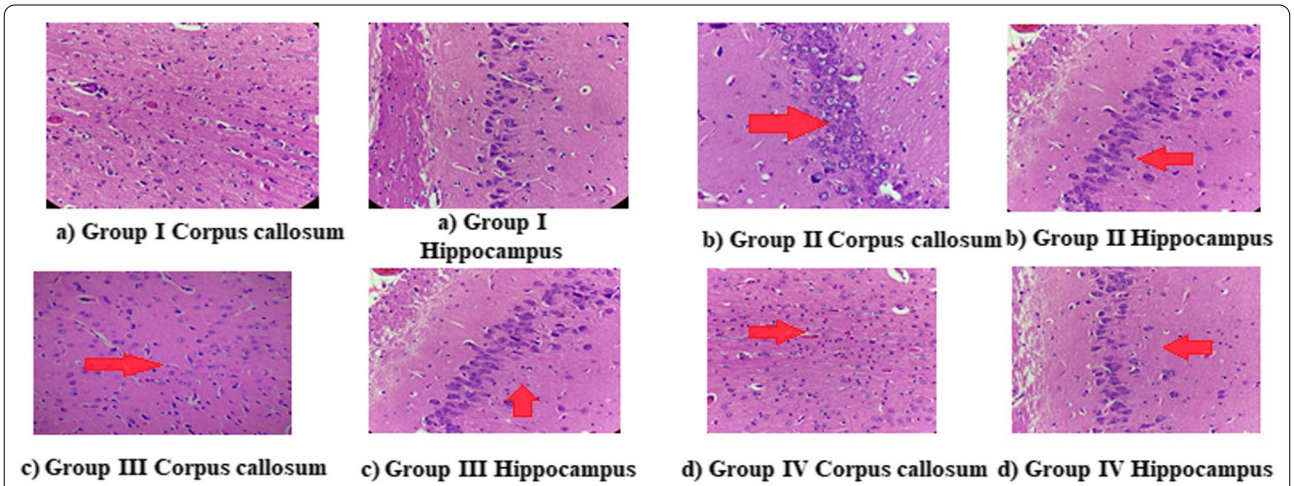

Fig. 9 Representative photomicrographs showing the protective effects of EECT against the neuropathological changes produced by the repeated ICV infusion of PPA. **a** GROUP I rat brain, **b** GROUP II rat brain, c GROUP III rat brain, **d** GROUP IV rat brain

group II rats in the present investigation, the extract significantly increased locomotor activity in the actophotometer test.

The animal must find a concealed platform to escape from swimming in a pool of water in the MWM (Morris water maze) exercise. To complete this task, the animal

uses visual stimuli from outside the testing room to create a "spatial orientation map" in the brain (Barnhart et al. 2015). The amount of time delayed before the animal climbs onto the platform to escape the water (escape latency) and the percentage of time or path length spent in the quadrant housing the platform (target quadrant) are used to evaluate learning during training (Barnhart et al. 2015). Hippocampus-dependent cognition results in spatial memory (Guan et al. 2019). In this study, the group II rats spent a less time in target quadrant, when compared with group I which indicates its inadequate spatial recognition skill and poor memory power, which could be due to neuronal degeneration in the hippocampus caused by PPA infusion. However, the EECT treated groups (III & IV) showed a dose-dependent improvement in spatial memory, which could be due to improved neuronal impulse transmission caused by the extract treatment or the extract's anti-inflammatory effects.

In the field of neurodegenerative illnesses, the significance of neuroinflammation is becoming more widely acknowledged. The word "neuroinflammation" refers to a set of activities that occur in the central nervous system (CNS) to combat potential or existing threats to the brain (Joshi et al. 2019).

To put it another way, once the CNS is confronted with infectious agents, traumatic injuries, or other unknown components that threaten its homeostasis, it will defend itself by initiating a series of processes aimed at eliminating the pathogenic factor. Astroglia and microglia are the main players in this scenario (Gehrmann et al. 1995). These cells become involved in the generation of (and, at the same time, reaction to) inflammatory cytokines and chemokines after activation, commonly known as "gliosis," which maintains and enhances the inflammatory condition (O'Callaghan and Sriram 2005). Many research concentrating on the glial cells implicated in the brain's inflammatory responses, notably microglia and astroglia, have highlighted the dynamic and changing activity of these cells, as well as their many morphologies and activation forms, over the years. This is especially true in sick states, as glia react to any disruption of homeostasis by developing various phenotypes (DiSabato et al. 2016; Matta et al. 2019).

In this study, we discovered that ICV infusion of PPA caused a change in the neuro/glia ratio in the rat brain. Increased levels of inflammatory cytokines such as TNF – and IL-6 were responsible for these effects. The rats' cognitive abilities also deteriorated because of the neuroinflammation.

A recent neuropathological analysis of corpse material from ASD patients of various ages discovered indications of a broad innate neuro-inflammatory response with no known cause. Reactive astrogliosis, activated microglia, and cytokine abnormalities characterised this reaction (Ashwood et al. 2006). These data show that ASD patients may experience an immune-mediated response throughout their lives (Bauman and Kemper 2005). Our study results showed that increased amounts of GFAP and CD68 immunoreactivity in the hippocampus of PPA treated rats (group II) emphasizing the process of reactive astrogliosis and activated microglia. There was no neuronal death observed in the hippocampus region because caspase 3 expression was not significant when compared with group I. It can be postulated that the immune response produced by PPA may be one of the reasons for impaired behaviour and memory impairment in group II rats as compared with group I. Extract (EECT) treatment remarkably prevented this immune mediated response and provided neuroprotective effects against the neuroinflammation caused by PPA.

Histopathological examination of corpus callosum and brain hippocampus also pointing towards the protective effects of EECT against neuroinflammation.

Present study serves as the first step in new drug development for ASD by incorporating our traditional wisdom. Brain cytokines estimations, histopathological, and immunohistochemical analysis clearly demonstrated the role of neuroinflammation and excessive immune mediated response in the progression of ASD. Study gave further scientific validation for *Clitoria ternatea* L. for its promising role in neuroprotection and memory enhancement, also gave a new insight on its ability to prevent ASD progression as the extract significantly prevented the neuroinflammation (reduced TNF-α and IL-6 levels in group III and IV rat brains as compared with group II) and immune mediated response in brain (confirmed by IHC and histopathological studies). This is the first study which was conducted to evaluate the protective effects of *Clitoria ternatea* L. against the propionic acid induced behaviour and memory impairment in autistic rat modell.

EECT includes a lot of phenols and flavonoids, according to the phytochemical screening results (Jiji and Muralidharan 2021a, b). Antioxidant and anti-inflammatory effects are known to exist in these phytochemical components (Sarumathy et al. 2011). Based on our pharmacological findings, it is possible that the presence of these phytoconstituents, particularly flavanoids, is responsible for EECT's effectiveness. The current investigation has provided some preliminary insight into the neuropharmacological effects of chemicals found in *C.ternatea* root ethanolic extract.

Conclusions

Our study demonstrated the protective effects of *Clitoria ternatea* L. against intracerebroventricular infusion of PPA from the biochemical, behavioural,

histopathological, and immunohistochemical aspects. Further experiments should be carried out for isolating the possible active compounds and then to explain the actual mechanism of action of the plant extract.

Abbreviations

ASD: Autism spectrum disorders; ADHD: Attention deficit hyperactivity disorder; ANOVA: Analysis of variance; CNS: Central nervous system; cm: Centimetre; CD 68: Cluster of Differentiation 68; CT: *Clitoria ternatea* L.; EECT: Ethanolic root extract of *Clitoria ternatea* L.; GFAP: Glial fibrillary acidic protein; HR-LCMS: High Resolution Liquid Chromatograph Mass Spectrometer; IL-6: Interleukin 6; *Interferon γ*: *Interferon gamma*; *ICV*: Intracerebroventricular; MD: Mitochondrial dysfunction; NPCs: Neuroepithelial progenitor cells; PPA: Propionic acid; PBS: Phosphate-buffered saline; *p.o*: Per oral; TNF-α: Tumour Necrosis Factor alpha.

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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. Both authors read and approved the final manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Experimental animals were purchased from the animal house of 'Mass Biotech', Chennai., India. This study was approved by the Institutional Animal Ethical Committee of C.L. Baid Metha College of Pharmacy, Chennai, India. Approval no: 01/321/PO/Re/S/01/CPCSEA/dated 14-08-19. Throughout the experiment, every precaution was taken to ensure that the animal suffered as little as possible.

Consent for publication

Not applicable.

Competing interests

The authors declared that they have no conflict of interest.

Plant authentication approval

The fresh roots of wild white variety of ***Clitoria ternatea*** L. were collected from Kerala, India, and authentified by taxonomist Prof.P.Jayaraman, Ph.D., Director, Plant Anatomy & Research Centre, West Tambaram, Chennai. India. A voucher specimen (SES.CLBM.NO. 1458) is preserved at the Herbarium of Department of Pharmacognosy, C.L.Baid Metha College of Pharmacy, Chennai, India.

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