PI3K/AKT/mTOR Signalling Inhibitor Chrysophanol Ameliorates Neurobehavioural and Neurochemical Defects in Propionic Acid-induced Experimental Model of Autism

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Research Article

Abstract

Autism spectrum disorder (ASD) is a complex neurodevelopmental disorder characterized by social-communication deficits and repetitive behaviour. Several studies have revealed that overactivation of the PI3K/AKT/mTOR signalling pathways during brain development plays an important role in the pathogenesis of autism. The PI3K/AKT/mTOR signalling pathway overexpression produces neurological abnormalities by increasing cell death, neuroinflammation, and oxidative stress. Chrysophanol, also known as chrysophanic acid, is a natural substance derived from the plant Rheum palmatum, a well-known Chinese herbal remedy with potential pharmacological applications. The purpose of this study was to look into the neuroprotective effect of CPH on neurobehavioral, molecular, neurochemical, and gross pathological changes in ICV-PPA-induced autism-like rats, with a particular emphasis on its effect on PI3K/AKT/mTOR downregulation in the brain. Furthermore, we looked at how CPH affected the levels of myelin basic protein (MBP) in rat brain homogenate, as well as apoptotic markers such caspase-3, Bax, and Bcl-2 levels in rat brain homogenate and blood plasma samples. Rats were examined for behavioural abnormalities, like neuromuscular dysfunction using actophotometer, motor coordination by beam crossing task (BCT), depressive behaviour with forced swim test (FST), cognitive deficit, and consolidation of memory using Morris water maze (MWM) task. Prolonged oral CPH administration from day 12 to day 44 of the experimental schedule reduces autistic-like symptoms in PPA-treated rats. In addition, cellular, molecular, cell death markers, neuroinflammatory cytokines, neurotransmitter levels, and oxidative stress indicators have been examined in rat brain homogenates, blood plasma, and CSF samples. The current findings suggest that CPH also restores the altered neurochemical levels and potentially prevents autism-like gross pathological changes, including demyelination volume in the rat brain.

1.0 Introduction

Autism spectrum disorder (ASD) is a severe neurodevelopmental disorder characterized by stereotyped or repetitive behaviour, memory and cognitive dysfunctions, and disturbances in sensory and motor functions(Gvozdjáková et al., 2014; Neumeyer et al., 2019). Early childhood ASD symptoms arise immediately after birth, leading to lifelong disabilities(Jin et al., 2015). Autism-like repetitive and stereotypical behaviours are caused by various neurochemical changes in specific brain regions such as the hippocampus, cerebellum, amygdala, and cerebral cortex (Rahi et al., 2021; Lee et al., 2016; Morimoto et al., 2020; Sacai et al., 2020). Previous studies revealed that a subset of children with autism has gastrointestinal symptoms and abnormal gut microflora (Kang et al., 2018; Finegold et al., 2012).

Autistic patients have higher levels of clostridium and desulfovibrio, which are known to produce short-chain fatty acids like PPA (Finegold et al., 2011). Propionic acid (PPA) is a short-chain fatty acid that easily penetrates the gut–blood barrier and can reach the CNS either through the blood-brain barrier or gradually by the monocarboxylate transporter system (Mirza et al., 2019; Shams et al., 2019). PPA accumulates inside cells, inflicting intracellular acidification, causes mitochondrial dysfunction (Mehan et al., 2020), elevates oxidative stress markers (Rahi et al., 2021), alters neurotransmitters (Tiwari et al., 2021), and impairs synaptic transmission in autistic rat brains (Meeking et al., 2020). According to Sharma et al., stereotaxic PPA injection into the cerebral cortex area causes stereotypical behaviour, and neurochemical alterations in experimental models of autism in rats (Sharma et al., 2019).

PI3K/AKT/mTOR signalling pathway is principally involved in different neuronal functions such as synaptic plasticity, neuronal development, consolidation of memory, and protein synthesis (Kassai et al., 2014; Takei et al., 2014). It also regulates various cellular and biological processes, including neuronal growth, axon guidance, proliferation, and differentiation of cells (Rai et al., 2019; Jafari et al., 2019).

In various brain regions, upregulation of the PI3K/AKT/mTOR signalling pathway has also been linked to cerebral cell proliferation, axonal disruption, and megalocephaly (Kim et al., 2015; Subramanian et al., 2015; Hutsler et al., 2010). The previous study suggests that the PI3K/AKT/mTOR pathway regulates translation in dendritic spines, and therefore its activation increases dendritic spine dysfunction, resulting in autism-like symptoms (Ganesan et al., 2019; Soltani et al., 2017; Maiti et al., 2015). The PI3K/AKT/mTOR pathway overexpression resulted in learning and memory deficits (Sharma and Mehan, 2021), social impairment (Mattioli et al., 2013), and abnormalities in synaptic plasticity (Li et al., 2010). The PI3K/AKT/mTOR signalling pathway have also been associated with the pathogenesis of various neurodevelopmental and neuropsychiatric disorders, including depression (Neis et al., 2020), cognitive development-associated brain malformation (Rivière et al., 2012), and epilepsy (Xiao et al., 2015; Brandt et al., 2018).

The PI3K/AKT/mTOR pathways are upregulated in the development of various neurodegenerative disorders, including Huntington's disease (Abd-Elrahman et al., 2019), Alzheimer's disease (Hodges et al., 2018), and brain trauma (Xu et al., 2020). Furthermore, motor neuron diseases such as Multiple Sclerosis (Giacoppo et al., 2017) and Parkinson's disease are caused by dysregulation of the PI3K/AKT/mTOR pathways (Chen et al., 2019).

Interestingly PI3K/AKT/mTOR inhibitors (Wortmannin, LY294002, Rapamycin) were employed in Male Sprague Dawley rats to restore the long-term memory and cognitive deficits (Sui et al., 2008). Rapamycin was also used to treat learning and memory deficits, social interaction deficits, hyperexcitability, and anxiety-like behaviour in autistic rats(Murakami et al., 2019). Fluoxetine is another potential candidate to treat autism (Sun et al., 2016).

The PI3K/AKT/mTOR inhibitors have also been used to treat CNS disorders, including LY294002 in Huntington's disease (Colin et al., 2005), Wortmannin in Parkinson's disease (Jiang et al., 2021), Rapamycin in infantile spasm (Yang et al., 2019) and Subarachnoid Hemorrhage (Li et al., 2019). PI3K/AKT/mTOR inhibitors also show beneficial effects in various brain diseases such as Fingolimod in Ischemic brain Injury (Li et al., 2019), Sulforaphane in Alzheimer disease (Yang et al., 2020), Propofol in Subarachnoid hemorrhage (Zhang et al., 2019) and Everolimus in Alzheimer disease (Cassano et al., 2019).

Chrysophanol (CPH) is a 1, 8-dihydroxy-3-methyl derivative of the 9, 10-anthracenedion ring first identified from rheum rhubarbarum to a herbaceous perrinary plant belonging to the Polygonaceae family (Singh et al., 2013). CPH has a wide spectrum of pharmacological effects and biological activities such as anti-depressant, anti-bacterial (Rokaya et al., 2012), and anti-cancer (Su et al., 2020). CPH also has anti-microbial, anti-inflammatory (Lu et al., 2016), antiviral properties (Lian et al., 2017), and is used in the treatment of various neurodegenerative diseases (Chae et al., 2017; Jeong et al., 2018).

Preclinical studies revealed that CPH improves cognition deficits and neuronal loss against streptozotocin-induced diabetic encephalopathy (Chu X et al., 2018). CPH has been shown to provide neuroprotection in various motor neuron illnesses, including MS (Lee et al., 2017) and PD (Chae et al., 2017). CPH is also associated with ameliorating cerebral ischemia/reperfusion injury.

To summarize the interaction of CPH with potential targets, Wang and Lvvalidated the interaction of CPH with mTOR against malignant meningioma by inhibiting mTOR signalling and activating NF2 signalling. Previous studies on colorectal cancer (Deng et al., 2019) and colon cancer (Lee et al., 2011) found that CPH decreased the PI3K/AKT/mTOR level, alleviating the conditions.

Based on the above findings, we hypothesize that CPH can downregulate the aberrant PI3K/AKT/mTOR signalling mechanisms, thereby alleviating the neuropathological abnormalities in PPA-induced autism-like rats. In addition,

CPH as a PI3K/AKT/mTOR inhibitor can be a novel therapeutic option for autism patients and other neurodevelopmental disorders.

Therefore, the current study investigated the upregulation of PI3K/AKT/mTOR involved in the pathogenesis of Autism. We examined the neuroprotective effect of CPH on behavioural, neurochemical and morphological characteristics in ICV-PPA-induced autism in rats. CPH shows neuroprotection in autistic rats by downregulation of the PI3K/AKT/mTOR signalling pathway, which was confirmed by studying neurochemical parameters in biological samples such as CSF, blood plasma and brain homogenates.

2.0 Materials And Methods

Experimental animals

A total of 36 rats were used in the current study. All experiments were conducted on six-month-old adult Wistar rats' weight 250−300 g. Each group contains six rats, either sex; they were obtained from the Central Animal House, ISF College of Pharmacy, Moga, Punjab. India. Animals were housed in an acclimatized environment with a 12-hour light-dark cycle with food and water at room temperature at 23 ± 2°C. The Institute for Animal Ethics Committee (IAEC) approved the project as 816/PO/ReBiBt/S/04/CPCSEA as IAEC/CPCSEA/Meeting No: 27/2020/Protocol No. 454, following the guidelines provided by the government of India. The rats were randomly divided into six groups based on a validated animal sampling method by Charan and& Kantharia, 2013. Animals were acclimatized to laboratory conditions before experimentation.

Drugs and chemicals

PPA was purchased from Sigma−Aldrich (USA). CPH was provided as an ex-gratia sample from BAPEX, India. All other chemicals utilized in the experiments are of analytical grade. Before using the drugs and chemicals, fresh solutions were prepared. CPH was given orally by dissolving in an aqueous solution of 2 % ethanol (Chu et al., 2018). The dosing of chrysophanol was determined based on previous research findings in various brain diseases, including ischemic brain injury (Zhao et al., 2016), learning and memory deficits (Dong et al., 2010), cognition deficits and neuronal loss (Chu et al., 2018), and Cerebral Ischemia (Zhang et al., 2014).

Experimental grouping of animals

The total duration of the experimentation was of 44 days. Propionic acid (PPA) was injected from day 1st to day 11th into the intracerebroventricular (ICV) region of the rat brain to induce autism. CPHwas administered orally from day 12th to day 44th. Animals were randomly assigned into six groups. Group 1- vehicle control; Group 2- sham control; Group 3- Chrysophanol perse (20 mg/kg., p.o.);Group 4- PPA (10μl/0.26M, i.c.v.); Group 5- PPA (10μl/0.26M, i.c.v.) + Chrysophanol (10 mg/kg., p.o.);Group 6- PPA (10μl/0.26M, i.c.v.) + Chrysophanol (20 mg/kg., p.o.). The present study was unblinded, and the experimenter was known regarding the care of animals. All behavioral parameters were conducted from day 1 to day 44th. On day45th, after completing the protocol schedule, the blood plasma, CSF was collected from adult Wistar rats. Besides, Sodium pentobarbital 270 mg/ml, i.p., was used to anaesthetize the animals deeply. After anesthetization, the fresh brain was removed and preserved with ice-cold PBS (0.1 M) PBS for further neurochemical analysis. The experimental protocol is summarized in (Fig. 1).

Experimental animal model of ICV-PPA induced autism in rats

The PPA-induced experimental model of Autism in rats was established and validated by Tiwari et al., 2021. Experimental rats were treated with a PPA-ICV injection of 10μl/0.26M for consecutive 11th days. The study by Rahi et al., 2021 suggests that PPA causes behavioral and neurochemical alteration similar to an experimental animal model of autism and is regarded as a validated experimental model for researching the pathophysiological changes identical to those seen in Autistic patients.

Rats were allowed to be habituated in a laboratory environment. Eventually, rats were anesthetized by intraperitoneal injection of 75 mg/kg ketamine. Then, the rats were placed on the stereotaxic instrument (Stoelting Co., Wood Dale, IL, USA) in a skull-flat position. The positioning of the head was adjusted prior to the surgery to ensure that the bregma and lambda coordinates were similar and at the same level. The rats' heads were shaved, the scalp had been cleaned with 70% ethanol and incised with a blade (mid-sagittal), the skin was removed, and the skull was exposed to spot bregma and lambda that was indicated to assist in defining ICV injection coordinates. Wet cotton swabs were put on rat eyes to prevent dehydration, and cotton buds were used to stop bleeding. A hole was drilled in the skull (Stereotaxic coordinates: AP= -1.3mm; ML= -1.8mm; DV= -3.0mm), a cannula inserted in the burr hole, and the cannula was closed using a plastic ear-pin. The dental cement was filled in the hole and then sutured with an absorbable surgical suture attached to the sterile surgical needle (Gonzalez-Fraguela et al., 2013).

For post-operative care, rats were kept independently in a polyacrylic cage that contains warm cloth. Special care was needed until they restored spontaneous movement, which occurred approximately 2−3 hours after anesthesia. The room temperature was set at 25 ± 3°C. For 2−3 days, milk and glucose water are provided inside the cages to avoid physical trauma after surgery. To prevent sepsis, gentamycin (35 mg/kg) was given intraperitoneally for three days, and lignocaine gel was applied to the sutured area to relieve the pain. Neosporin powder was sprinkled on them to prevent bacterial infection of the skin. After surgery, the body's overall health and clinical symptoms such as dehydration, body weight, infection, and other physical changes were closely monitored.

2.1 Parameters

Measurement of weight variations

Assessment of body weight

The body weight was measured on the 1st, 13th, 23rd, 33th, and 43rd days of the experiment protocol schedule (Sharma et al., 2019).

Measurement of relative brain-body weight ratio

The relative brain-body weight ratio was calculated on the 45th day of the experimental protocol schedule (Gopi et al., 2019).

Behaviour parameters

Morris water maze task (MWM)

The Morris water maze test was conducted to evaluate memory and cognitive impairment (Morris, 1984). Escape latency time (ELT) was measured using MWM on the 40th, 41st, 42nd, and 43rd, days of the protocol schedule. Time (seconds) taken by rats to reach the target platform was considered as escape latency. On day 44th, rats were exposed to swim in the pool containing a hidden platform; for 120 seconds, and time spent in the target quadrant (TSTQ) was recorded. The TSTQ represents the degree of memory consolidation, which occurred after learning (Duggal et al., 2020).

Locomotor activity

The locomotor activity was performed on the 1st, 13th, 23rd, and 43rd days of the experimental protocol schedule using an actophotometer (INCO Group of Companies Dubai, United Arab Emirates). The behaviour parameter was evaluated using the method described by Mehan et al., 2018. The animal was placed in a digital actophotometer equipped with infrared photocells. They are then observed for five minutes in a square, the closed arena. The value of a digital actophotometer begins as counts per 5 minutes (Mehan et al., 2018).

Beam crossing task (BCT)

The beam crossing task was conducted on days 1st, 13th, 23rd, and 43rd of the experimental protocol schedule to evaluate motor coordination ability. During each trial, the number of foot slips was recorded, and additionally, the direction of an animal's fall was observed against the cut-off time of five minutes (Sharma et al., 2019).

Forced swim test (FST)

The forced swim test was used to measure the depressive-like behaviour of rats on the 1st, 13th, 23rd, and 43rd days. The first exposure of rats in the tank during the training phase is for 15 minutes, and the second is after 24 hours later for 5 minutes. A single six-minute exposure is used during the testing session. The first two minutes serve as a habituation period, with the final four minutes serving as the test itself, which determines the length of immobility (Minj et al., 2021).

Neurochemical parameters

Collection and preparation of biological samples

Blood plasma collection and separation

On day 45th of the protocol schedule, anesthetized the rats with the chloroform before sample collection. Immediately after anesthetization, a capillary tube is placed at the medial canthus of the eye, and then the sinus is ruptured. Instantly 1–2 ml of blood was collected from the rats through retro-bulbar puncture (Kumar et al., 2017). The obtained blood samples were then cold centrifuged at 10,000×g for 15 minutes to separate the plasma. Then separated plasma was carefully stored at -80° C deep freezer for biochemical analysis.

CSF collection

Rats were deeply anaesthetized with 270 mg/ml sodium pentobarbital through i.p. injection. The rats' head was fixed using a holder to reveal the Arachnoid membrane and a skin incision was made, and a translucent dura mater was exposed. A maximum volume of 100μL CSF was obtained by direct inserting a 30-gauge needle at a 30° angle into the cisterna magna. Within 20 minutes after collection, the sample was centrifuged at 2000 g for 10 minutes at 4°C. After centrifugation, the supernatant was stored at -80°C until further analysis(Kozler et al., 2015).

Brain homogenate preparation

Rats were sacrificed by decapitation on the 45th day of the treatment schedule. The whole fresh brain was removed, washed with ice-cold isotonic saline solution, and homogenized with 0.1M (w/v) of chilled phosphate buffer saline (pH = 7.4). The rat brain homogenate was then centrifuged at 10,000×g for 15 minutes, the supernatant was separated, and the aliquots were preserved. The samples were stored in a deep-freezer at -80°C to be used as and when the need for various neurochemical estimations (Rahi et al., 2021).

Assessment of cellular and molecular markers

Estimation of PI3K, AKT and mTOR level

PI3K protein level was measured in rat brain homogenate (David et al., 2015) and CSF (Tong et al., 2019) using an ELISA kit. According to the instruction given by ELISA assay kits (E-EL-H1019/PI3K; Elabsciences, Wuhan, Hubei, China). The AKT level was estimated in rat brain homogenate (Schipper et al., 2000) and CSF (Dong et al., 2018) (E-AB-21082/AKT; Elabsciences, Wuhan, Hubei, China), mTOR level was measured in rat brain homogenate (Wang et al., 2017) and CSF (Li et al., 2015) samples (E-EL-H1655/mTOR; Elabsciences, Wuhan, Hubei, China).

Estimation of MBP level

The MBP levels were assessed in rat brain homogenate using an ELISA kit (E-EL-R0642/MBP; Elabsciences, Wuhan, Hubei, China). The values were expressed in μg/mg protein (Rahi et al.,2021).

Assessment of Apoptotic Markers

Caspase-3 concentrations were assessed in brain homogenate (Rahi et al., 2021) and blood plasma (Guo et al., 2018) using an ELISA kit. Bax protein level was determined in brain homogenate (Tiwari et al., 2021) and blood plasma (Wang et al., 2019). The anti-apoptotic protein such as Bcl-2 levels was estimated in brain homogenate (Sharma et al., 2019) and blood plasma (Wang et al., 2018) using ELISA commercial kits (E-EL-R0160/Caspase-3; E-EL-R0098/Bax/Bcl2Elabsciences, Wuhan, Hubei, China). The values are expressed in ng/gm protein in brain homogenate and ng/ml in blood plasma.

Assessment of neurotransmitter levels

Measurement of Acetylcholine (Ach) level

Acetylcholine level was measured using a diagnostic kit (E-EL-008Ach; ELabSciences, Wuhan, Hubei, China). All samples and reagents were freshly prepared as per manual instruction provided by the kit's. The optical density of the reaction mixture was measured at 540 nm. The neurotransmitter in the supernatant was estimated and the value expressed as ng/mg protein (Rajdev et al., 2020).

Measurement of dopamine level

The dopamine levels in the striatal tissue sample were determined. Dopamine levels in the striatum are a sign of neural excitability, which leads to mood changes. The electrochemical detector was used to assess dopamine levels in the brain homogenate using the HPLC technique. The level of dopamine in brain homogenates is expressed as ng/mg protein (Sharma et al., 2021).

Estimation of glutamate level

Glutamate was assessed after derivatization with o-phthalaldehyde/-mercaptoethanol (OPA/-ME), and quantitative analysis in tissue samples was carried out according to Rahi and her coworker's method. The glutamate level in rat brain homogenate is expressed as ng/mg protein (Sharma et al., 2019).

Measurement of serotonin level

Serotonin level was measured in the brain homogenate sample by HPLC using an electrochemical detector and C18 reverse-phase column. The mobile phase consisted of sodium citrate buffer (pH 4.5) − acetonitrile (87:13, v/v). The supernatant was filtered through 0.22 mm nylon filters before being injected into the sample injector. The serotonin concentration was assessed from the standard curve generated using a standard with a concentration of 10−100 mg/ml (Sharma et al., 2019).

Measurement of inflammatory cytokines levels

The level of TNF was determined in rat brain homogenate (Mehan et al., 2018) and Blood plasma (Fu et al., 2021) by using a rat immunoassay kit. The activity of IL-1β was assessed in rat brain homogenate (Minj et al., 2021) and blood plasma (Nwangwu et al., 2017) (E-EL-R0019/TNF-α; E-EL-R0012/IL-1β; ELabSciences, Wuhan, Hubei, China) and value expressed as pg/mg protein (Yu et al., 2018).

Evaluation of oxidative stress markers

Estimation of acetylcholinesterase (AChE) level

Acetylcholinesterase concentration was estimated using spectrophotometrically. The assay mixture contained 0.05 ml of supernatant, 3 ml of 0.01M sodium phosphate buffer (pH 8), 0.10 ml of acetylthiocholine iodide, and 0.10 ml

DTNB (Ellman reagent). The change in absorbance was recorded instantly at 412nm spectrophotometrically. The enzymatic activity in the supernatant was expressed as µM/mg protein (Deshmukh et al., 2009).

Measurement of superoxide dismutase (SOD) enzymatic activity

SOD activity was measured using spectrophotometrically by auto-oxidation of epinephrine at pH 10.4. The supernatant (0.2 ml) of the brain homogenate was mixed with 0.8 ml of 50 mM glycine buffer, pH 10.4, and the reaction was initiated with the addition of 0.02 ml epinephrine. After 5 minutes, the absorbance was spectrophotometrically measured at 480nm. SOD activity was quantified as nM/mg protein (Mehan et al., 2017).

Estimation of GSH level

The level of reduced glutathione in the brain was assessed using the method described by Ellman et al. 1959. 1ml supernatant was precipitated with 1 ml of 4% sulfosalicylic acid and cold digested at 4°C for one hour. The samples were centrifuged at 1200×g for 15 min. To 1ml of the supernatant, 2.7 ml of phosphate buffer (0.1M, pH 8) and 0.2 ml of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) were added. The yellow colour appeared immediately measured with a spectrophotometer at 412 nm. The glutathione concentration in the supernatant was expressed as µM/mg protein (Bala et al., 2015).

Estimation of nitrite level

The nitrite concentration in the supernatant, indicating the formation of nitric oxide (NO) is evaluated by a colorimetric assay using a Greiss reagent (0.1% N-(1- naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid) as described by Green et al. 1982. Equal volumes of supernatant and Greiss reagent are mixed, the mixture incubated for 10 min at room temperature in the dark, and the absorbance determined spectrophotometrically at 540nm. The amount of nitrite in the supernatant is determined from a sodium nitrite standard curve and expressed as µM/mg protein (Mehan et al., 2020).

Estimation of malondialdehyde (MDA) level

The quantitative determination of malondialdehyde (MDA) was performed in brain homogenate. After its reaction with thiobarbituric acid, the concentration of MDA was measured at 532nm using a spectrophotometer and expressed as nM/mg protein (Mehan et al., 2011).

Protein estimation

The protein content was quantified by using the Coral protein estimation kit (Biuret method).

Gross pathological examination of rat brains

On the 43rd day, rats were sacrificed by decapitation; brains were removed for gross pathological analysis performed. After analysing the whole brain, coronal sections were taken (Tiwari et al., 2021). Sectioned 2-mm thick brain pieces (coronally from the anterior pole to the posterior poles of the cerebral cortex) were placed on glass slides. A digital

camera (Fujix digital camera, Fujifilm, Japan) was used to visualize all the brain regions. The demyelination region (mm) in each brain segment was measured on day 43rd after completing the procedure through MOTICAM-BA310 image plus 2.0 analysis software. The demyelination scale (mm) volume was calculated for each coronal brain segment by converting the demyelination region (mm). The demyelination size (mm^3) in each brain section was measured from the dark greyish area near the striatum by image analysis on the 43rd day. The injury's size was calculated in each coronal 2-mm-thick brain section by calculating the demyelination area (l×b×h) (Rahi et al., 2021).

3.0 Statistical Analysis

Data were analyzed using two-way ANOVA followed by Post hoc test Bonferroni and one-way ANOVA repeated measures followed by Post hoc test Tukey's multi comparison test. P < 0.001 was considered statistically significant. Data was found to be normalized, and the sample size was calculated by checking the normality distribution by the Kolmogorov Smirnov test. All statistical results were performed out by GraphPad Prism version 5.03 for Windows (GraphPad Software, San Diego, CA, USA). Statistical results were expressed as the mean ± standard deviation (SD).

4.0 Results

4.1 Effect of chrysophanol on weight variations in ICV-PPA induced autism in rats

Restoration of body weight after chronic administration with chrysophanol

Body weight was assessed on the 1st, 13th, 23rd, 33th, and 43rd days of the protocol schedule. Before initiation of treatment, there was no substantial difference in body weight between all treatment groups. Daily PPA-injection for 11 consecutive days' rats exhibited decreased body weight on the 13th day compared to a vehicle, sham, and CPH 20 perse treated groups. Prolonged oral administration with CPH 10 mg/kg and CPH 20 mg/kg showed significant restoration in body weight on the 23rd, 33th, and 43rd days as compared to PPA-treated autistic rats [two-way ANOVA: $F_{(20,120)} = 157.72$, p < 0.001]. CPH 20 mg/kg was found to be more effective than CPH 10 mg/kg in successfully recovering body weight on the 43rd day (Fig. 2a).

Restoration of relative brain-body weight ratio after chronic administration with chrysophanol

The relative brain-body weight ratio was evaluated on the last day of the protocol schedule. On the 43rd day, no significant difference in the relative brain-body weight ratio was observed among all groups. Chronic ICV injection of PPA for 11 days caused a significant reduction in relative brain-body weight ratio compared to the vehicle, sham, and CPH 20 perse treatment groups. Long-term administration of CPH 20 mg/kg and CPH 10 mg/kg resulted in an increase in relative brain-body weight ratio on the 43rd day as compared to PPA-treated autistic rats [one-way ANOVA: $F_{(5,25)} = 1.218$, p < 0.001]. While CPH 20 mg/kg remarkably restored the relative brain-body weight ratio compared to the CPH 10 mg/kg treated group (Fig. 2b).

4.2 Effect of chrysophanol in the amelioration of neurobehavioral alterations in propionic acid-induced autism in rats

Improvement in memory and cognition after chronic administration with chrysophanol

The Morris water maze test was conducted to evaluate memory and cognitive impairment. The escape latency was assessed on the 40th, 41st, 42nd, and 43rd, days of the experiment protocol schedule. PPA-treated rats show a progressive increase in escape latency time (ELT) compared to the vehicle, sham, and CPH 20 perse treatment rats. When compared to the PPA-injected group, long-term oral treatment of CPH 10 mg/kg and CPH 20 mg/kg significantly reduced the ELT in a dose-dependent manner [two-way ANOVA: $F(15,90) = 19.48$, $p < 0.001$]. Furthermore, CPH 20 mg/kg administered in rats decreased the ELT more efficiently than CPH 10 mg/kg treated animals (Fig. 2c). The time spent in the target quadrant (TSTQ) was recorded on the 44th day of the protocol schedule. Chronic PPA-infused rats have significantly lower TSTQ than vehicle, sham, and CPH 20 perse treated rats. Long term administration with CPH 10 mg/kg and 20 mg/kg increases the TSTQ in dose-dependent manner compared with PPA-treated autistic rats [one-way ANOVA: $F(5,25) = 6.594$, $p < 0.001$]. CPH 20 mg/kg-treated rats were more capable of increasing TSTQ and consolidating memory than CPH 10 mg/kg-treated rats (Fig. 2d).

Improvement in locomotion after chronic administration with chrysophanol

The locomotor activity was used to observe the movement of rats. The test was performed on the 1st, 13th, 23rd, and 43rd days using the actophotometer apparatus. There were no wide variations between all treatment groups on the first day of the protocol schedule. On the 13th day, PPA-injected rats showed substantially reduced locomotion compared to the vehicle, sham, and CPH 20 perse treated groups. Persistent oral administration of CPH 10 mg/kg and CPH 20 mg/kg significantly improve the locomotion on 23rd and 43rd days, in comparison with PPA-treated autistic rats [two-way ANOVA: $F(15,90) = 644.72$, $p < 0.001$]. Compared to the CPH 10 mg/kg administered group, CPH 20 mg/kg was more effective in improving locomotor activity on the 43rd day (Fig. 2e).

Improved motor coordination after chronic administration with chrysophanol

The beam crossing task was conducted to evaluate the motor coordination ability of rats. The task was performed on the 1st, 13th, 23rd, and 43rd days. On the first day, no significant differences between treatment groups were found. Chronic PPA-treated rats had a significantly higher number of slips on the 13th day than the vehicle, sham, and CPH 20 perse treated groups. On days 23rd and 43rd, prolonged oral treatment with CPH 10 mg/kg and CPH 20 mg/kg significantly reduced the number of slips in a dose-dependent manner compared to the PPA treatment group [two-way ANOVA: $F(15,90) = 35.18$, $p < 0.001$]. On the 43rd day, CPH 20 mg/kg was significantly more effective than CPH 10 mg/kg in reducing slip count and improving beam efficiency (Fig. 2f).

Reduced depression-like behavior after chronic administration with chrysophanol

The forced swim test was used to measure the depressive-like behavior of rats. The immobility time was measured on the 1st, 13th, 23rd, and 43rd days. On the first day, there was no significant difference between any treatment groups. Compared to the vehicle, sham, and CPH 20 perse treatment groups, PPA-injected rats have longer immobility duration considerably on the 13th day of the protocol schedule. On 23th and 43rd days, long-term oral administered rats with CPH 10 mg/kg and CPH 20 mg/kg dramatically reduces the immobility time in a dose-dependent manner as compared with PPA treated rats [two-way ANOVA: $F_{(15,90)} = 910.07$, $p < 0.001$]. CPH 20 mg/kg, on the other hand, was found to be more successful in significantly reducing immobility time and recovering depressive-like behavior on the 43rd day than CPH 10 mg/kg treatment group (Fig. 2g).

4.3 Effect of chrysophanol on neurochemical alterations in ICV-PPA induced autism in rats

Decreased PI3K level after chronic administration with chrysophanol

PI3K protein level was measured in rat brain homogenate and CSF samples at the end of the experimental protocol schedule. PPA-treated rats show a considerable increase in the PI3K protein level in rat brain homogenate and CSF compared to the vehicle, sham, and CPH20 *perse* treated group. Long-term oral administration with CPH at the doses of 10 mg/kg and 20 mg/kg for 44 days consistently decreases the PI3K level compared to PPA-treated rats. In comparison with CPH 10 mg/kg treatment group, CPH 20 mg/kg treatment group was proven to be more effective in the reduction of PI3K level in rat brain homogenate [one-way ANOVA: $F_{(5,25)} = 1.136$, $p < 0.001$] and CSF samples [one-way ANOVA: $F_{(5,25)} = 0.256$, $p < 0.001$]. (Table 1a)

Table 1
Effect of chrysophanol on PI3K, AKT, mTOR and myelin basic protein level in propionic acid-induced autism in rats

S. no.	Groups	PI3K		AKT		mTOR		Myelin basic protein
		Brain homogenate (pg/g protein)	CSF (ng/ml)	Brain homogenate (pg/g protein)	CSF (ng/ml)	Brain homogenate (ng/g protein)	CSF (ng/ml)	Brain homogenate (µg/mg protein)
1.	Vehicle control	7.133 ± 0.142	2.312 ± 0.092	3.682 ± 0.103	0.470 ± 0.031	1.332 ± 0.081	7.485 ± 0.083	110.3 ± 1.972
2.	Sham control	7.123 ± 0.116	2.277 ± 0.105	3.743 ± 0.095	0.465 ± 0.029	1.308 ± 0.077	7.415 ± 0.129	110.1 ± 2.133
3.	CPH20 *perse*	7.070 ± 0.130	2.298 ± 0.097	3.822 ± 0.076	0.460 ± 0.036	1.303 ± 0.050	7.422 ± 0.084	110.7 ± 1.526
4.	PPA	28.04 ± 0.158*	4.485 ± 0.091*	19.06 ± 0.197*	1.560 ± 0.020*	7.435 ± 0.101*	11.01 ± 0.069*	50.58 ± 1.040*
5.	PPA + CPH10	21.77 ± 0.146@	3.802 ± 0.076@	14.71 ± 0.260@	1.178 ± 0.046@	5.865 ± 0.078@	9.818 ± 0.0875@	66.67 ± 1.413@
6.	PPA+ CPH20	14.16 ± 0.209@#	2.795 ± 0.094@#	9.202 ± 0.209@#	0.858 ± 0.051@#	3.532 ± 0.094@#	8.792 ± 0.129@#	76.91 ± 1.634@#

(Values expressed as mean ± SD (n = 6 rats per group). *$p < 0.001$ v/s vehicle control, sham control and CPH20Perse; @ $p < 0.001$ v/s PPA; @# $p < 0.001$ v/s PPA + CPH10 (one-way ANOVA followed by Tukey's multiple comparison test)

Decreased AKT level after chronic administration with chrysophanol

The AKT level was estimated in rat brain homogenate and CSF samples by using an ELISA kit. Chronic PPA-injected rats substantially increased AKT level in rat brain homogenate and CSF compare to the vehicle, sham, and CPH20 *perse* treatment group. Prolonged oral treatment with CPH 10 and 20 mg/kg dramatically reduced the AKT level in brain homogenate and CSF than PPA-injected groups. Moreover, CPH 20 mg/kg is found to be more effective in the reduction of AKT level in brain homogenate [one-way ANOVA: $F_{(5,25)} = 1.209$, $p < 0.001$] and CSF samples [one-way ANOVA: $F_{(5,25)} = 0.151$, $p < 0.001$] than CPH 10 mg/kg treated groups(Table 1b).

Decreased mTOR level after chronic administration with chrysophanol

The level of mTOR was measured in rat brain homogenate and CSF samples. Long-term PPA-treated rat's exhibit higher mTOR levels in rat brain homogenate and CSF samples than the vehicle, sham, and CPH20 perse treatment groups. Continuous oral administration of CPH 10 and 20 mg/kg results a significantly decreases the mTOR level in the rat brain homogenate [one-way ANOVA: $F_{(5,25)} = 1.212$, $p < 0.001$] and CSF [one-way ANOVA: $F_{(5,25)} = 0.551$, p <

0.001] in comparison to PPA treatment groups. CPH 20 mg/kg was more effective and successfully reducing mTOR levels in brain homogenate and CSF samples(Table 1c).

Restoredmyelin basic protein level after chronic administration with chrysophanol

Myelin basic protein (MBP) protein level was measure in rat brain homogenates using an ELISA kit. PPA-injected rat's show a remarkable reduction in MBP level compared with vehicle, sham, and CPH20 perse treated groups. Long-term oral administration with CPH 10 and 20 mg/kg leads to a massive rise in MBP level compared to the PPA-injected rats [one-way ANOVA: $F_{(5,25)} = 1.687$, $p < 0.001$]. CPH 20 mg/kg was more effective in restoring MBP level in rat brain homogenate than the CPH 10 mg/kg treatment group (Table 1d).

Reduction in caspase-3, Bax, and increased Bcl-2 levels after chronic administration with chrysophanol

The neuronal autophagic indicators such as Caspase-3, Bax, and Bcl-2 were estimated in rat brain homogenate and blood plasma. Chronic PPA treated rat's substantial increases in Caspase-3 and Bax protein levels in rat brain homogenate and blood plasma. Additionally, ICV-PPA treated rats show a considerable reduction in anti-apoptotic Bcl-2 levels in brain homogenate and blood plasma compared to the vehicle, sham, and CPH20 perse group.Persistent oral CPH treatment with 10 and 20 mg/kg cause a significant reduction in Caspase-3 level in brain homogenate [one-way ANOVA: $F_{(5,25)} = 0.210$, $p < 0.001$] and blood plasma[one-way ANOVA: $F_{(5,25)} = 1.052$, $p < 0.001$].

Similarly, chronic oral CPH administration at doses of 10 mg/kg and 20 mg/kg show a considerable decreased in the level of Bax in rat brain homogenate[one-way ANOVA: $F_{(5,25)} = 1.213$, $p < 0.001$] and blood plasma samples[one-way ANOVA: $F_{(5,25)} = 1.246$, $p < 0.001$]as compared to long term PPA-exposed rats, continuous oral treatment with CPH 10 and 20 mg/kg for 44 days resulted a substantially increases Bcl-2 level in rat brain homogenate [one-way ANOVA: $F_{(5,25)} = 2.193$, $p < 0.001$]and blood plasma[one-way ANOVA: $F_{(5,25)} = 3.179$, $p < 0.001$]. Compared to CPH 10 mg/kg treated rats, CPH 20 mg/kg was more efficacious in reducing autophagic markers and restoring anti-apoptotic markers in PPA-induced autistic rats (Table 2).

Table 2
Effect of chrysophanol on apoptotic markers level in propionic acid-induced autism in rats

S. no.	Groups	Caspase-3		Bax		Bcl-2	
		Brain homogenate (nM/mg protein)	Blood plasma (ng/ml)	Brain homogenate (ng/mg protein)	Blood plasma (ng/ml)	Brain homogenate (ng/mg protein)	Blood plasma (ng/ml)
1.	Vehicle control	117.4 ± 0.858	1.840 ± 0.049	9.167 ± 0.269	0.833 ± 0.035	34.69 ± 0.553	9.208 ± 0.036
2.	Sham control	118.5 ± 0.622	1.830 ± 0.043	9.098 ± 0.595	0.846 ± 0.033	35.22 ± 0.437	9.143 ± 0.043
3.	CPH20 perse	117.7 ± 1.156	1.858 ± 0.046	8.690 ± 0.310	0.840 ± 0.035	34.78 ± 0.919	9.153 ± 0.054
4.	PPA	178.4 ± 1.313*	5.798 ± 0.132	15.21 ± 0.359*	5.223 ± 0.092*	23.34 ± 0.530*	1.815 ± 0.056*
5.	PPA + CPH10	161.0 ± 1.461@	3.248 ± 0.136@	13.16 ± 0.387@	4.315 ± 0.152@	25.80 ± 0.725@	4.425 ± 0.203@
6.	PPA + CPH20	147.3 ± 1.022@#	2.493 ± 0.350@#	10.92 ± 0.275@#	2.562 ± 0.301@#	29.27 ± 1.022@#	7.173 ± 0.103@#

(Values expressed as mean ± SD (n = 6 rats per group). *$p < 0.001$ v/s vehicle control, sham control and CPH20Perse; @ $p < 0.001$ v/s PPA; @# $p < 0.001$ v/s PPA + CPH10 (one-way ANOVA followed by Tukey's multiple comparison test)

Restoration of neurotransmitters level after chronic administration with chrysophanol

Neurotransmitters like serotonin, dopamine, acetylcholine, and glutamate were measured in rat brain homogenate at the end of the protocol schedule. ICV-PPA injected rats caused a massive reduction in dopamine, serotonin, acetylcholine level, and increased glutamate level observed in rat brain homogenate compared to the vehicle, sham, and CPH20 perse administered rats. Long-term oral treatment with CPH 10 and 20 mg/kg dramatically increases dopamine [one-way ANOVA: $F(5,25) = 2.546$, $p < 0.001$], serotonin[one-way ANOVA: $F(5,25) = 0.228$, $p < 0.001$], and acetylcholine[one-way ANOVA: $F(5,25) = 0.479$, $p < 0.001$] level while decreased glutamate level was estimated as compare to PPA-infused autistic rats [one-way ANOVA: $F(5,25) = 0.807$, $p < 0.001$]. Among these, CPH 20 mg/kg was more effective in restoring neurotransmitter levels in rat brain homogenate than the CPH 10 mg/kg treatment group (Table 3).

Table 3
Effect of chrysophanol on neurotransmitters level in propionic acid-induced autism in rats

S. no.	Groups	Neurotransmitters (Brain homogenate)			
		Serotonin (ng/mg protein)	Glutamate (ng/mg protein)	Dopamine (ng/mg protein)	Ach (ng/mg protein)
1.	Vehicle control	46.99 ± 0.416	119.9 ± 0.945	102.2 ± 1.867	9.047 ± 0.215
2.	Sham control	47.22 ± 0.519	120.2 ± 0.518	101.8 ± 2.186	8.955 ± 0.279
3.	CPH20 *perse*	48.09 ± 0.517	119.5 ± 0.809	102.9 ± 1.852	8.855 ± 0.189
4.	PPA	17.34 ± 0.648*	328.4 ± 1.781*	38.15 ± 1.265*	2.392 ± 0.104*
5	PPA + CPH10	24.05 ± 0.457[@]	234.4 ± 1.791[@]	54.84 ± 0.842[@]	4.278 ± 0.057[@]
6.	PPA + CPH20	31.06 ± 0.407[@#]	198.6 ± 1.690[@#]	61.91 ± 1.163[@#]	5.872 ± 0.191[@#]

(Values expressed as mean ± SD (n = 6 rats per group). *$p < 0.001$ v/s vehicle control, sham control and CPH20Perse; @ $p < 0.001$ v/s PPA; @# $p < 0.001$ v/s PPA + CPH10 (one-way ANOVA followed by Tukey's multiple comparison test)

Decreased inflammatory cytokines level after chronic administration with chrysophanol

Inflammatory cytokines such as TNF and IL-1β were measured in rat brain homogenate and blood plasma samples using an ELISA kit. Chronic ICV-PPA treated rat's show significant increases in proinflammatory mediators such as TNF and IL-1β in rat brain homogenate and blood plasma compared to the vehicle, sham, and CPH20 perse treated groups. However, persistent oral treatment with CPH 10 and 20 mg/kg results in a substantially decreases in inflammatory cytokines TNF in rat brain homogenate [one-way ANOVA: $F_{(5,25)} = 0.357$, $p < 0.001$] and blood plasma [one-way ANOVA: $F_{(5,25)} = 1.180$, $p < 0.001$].

Similarly, chronic oral dosing with CPH10 and 20 mg/kg markedly decreased the level of IL-1β in rat brain homogenate [one-way ANOVA: $F_{(5,25)} = 4.582$, $p < 0.001$] and blood plasma [one-way ANOVA: $F_{(5,25)} = 0.782$, $p < 0.001$] as compared with PPA treated rats. However, CPH 20 mg/kg treatment was found to be more efficient in reducing inflammatory cytokines compared with CPH 10 mg/kg treated rats (Table 4).

Table 4
Effect of chrysophanol on inflammatory cytokines level in propionic acid-induced autism in rats

S. no.	Groups	Inflammatory cytokines			
		TNF-α		IL-1β	
		Brain homogenate (pg/mg protein)	Blood plasma (pg/ml)	Brain homogenate (pg/mg protein)	Blood plasma (pg/ml)
1.	Vehicle control	37.84 ± 0.838	30.04 ± 0.543	18.69 ± 0.621	13.30 ± 0.664
2.	Sham control	37.05 ± 0.548	30.07 ± 0.363	18.75 ± 0.627	13.53 ± 0.336
3.	CPH20 *perse*	36.90 ± 0.267	30.22 ± 0.382	18.72 ± 0.702	13.22 ± 0.100
4.	PPA	80.45 ± 1.342*	96.78 ± 1.545*	34.89 ± 0.478*	83.91 ± 0.363*
5.	PPA + CPH10	71.40 ± 1.370[@]	80.82 ± 1.955[@]	28.84 ± 0.668[@]	69.05 ± 0.610[@]
6.	PPA + CPH20	57.61 ± 1.850[@#]	59.98 ± 0.419[@#]	23.64 ± 0.440[@#]	46.68 ± 1.080[@#]

(Values expressed as mean ± SD (n = 6 rats per group). *$p < 0.001$v/s vehicle control, sham control and CPH20Perse; @ $p < 0.001$ v/s PPA; @# $p < 0.001$ v/s PPA + CPH10 (one-way ANOVA followed by Tukey's multiple comparison test)

Amelioration of oxidative stress markers level after chronic administration with chrysophanol

The levels of oxidative stress indicators AchE, LDH, SOD, GSH, nitrite, and MDA were measured in rat brain homogenate. Chronic PPA administration rats shoed a massive rise in AchE, LDH, nitrite, and MDA levels in rat brain homogenate than the vehicle, sham, and CPH 20 perse treatment groups, ICV-PPA injected groups results in a considerable reduction in anti-oxidant enzyme levels such as SOD [one-way ANOVA: $F_{(5,25)} = 0.968$, $p < 0.001$]and GSH [one-way ANOVA: $F_{(5,25)} = 0.979$, $p < 0.001$] in rat brain homogenate as compared to PPA-exposed rats.

Chronic oral treatment with CPH 10 and 20 mg/kg for 44 days consecutively led toa significantly decreased inAchE[one-way ANOVA: $F_{(5,25)} = 1.266$, $p < 0.001$], LDH [one-way ANOVA: $F_{(5,25)} = 1.584$, $p < 0.001$], nitrite [one-way ANOVA: $F_{(5,25)} = 0.801$, $p < 0.001$], and MDA[one-way ANOVA: $F_{(5,25)} = 4.699$, $p < 0.001$] levels in brain homogenate. Similarly, continuous oral treatment with CPH 10 and 20 mg/kg results in a considerable increase in SOD and GSH levels in rat brain homogenate. Likewise, CPH 20 mg/kg is more effective in restoring anti-oxidant enzyme levels than CPH 10 mg/kg treated rats (Table 5).

Table 5
Effect of chrysophanolon oxidative stress markers level in propionic acid-induced autism in rats

S.no.	Groups	Oxidative stress markers (Brain homogenate)					
		AchE (µM/mg protein)	LDH (Unit/mg protein)	SOD (µM/mg protein)	GSH (µM/mg protein)	Nitrite (µM/mg protein)	MDA (nM/mg protein)
1.	Vehicle control	23.86 ± 0.971	130.1 ± 1.206	492.3 ± 2.583	38.27 ± 0.790	6.89 ± 0.261	36.91 ± 0.315
2.	Sham control	24.24 ± 0.790	125.4 ± 0.545	491.9 ± 3.425	38.63 ± 0.886	7.11 ± 0.220	37.67 ± 0.622
3.	CPH20 *perse*	23.90 ± 0.886	126.6 ± 0.628	493.1 ± 3.004	39.10 ± 0.421	6.91 ± 0.181	37.11 ± 1.079
4.	PPA	60.25 ± 1.214*	428.7 ± 1.835*	347.8 ± 1.806	10.95 ± 0.422*	13.41 ± 0.458*	80.00 ± 0.880*
5.	PPA + CPH10	45.41 ± 0.874@	318.1 ± 1.057@	359.8 ± 0.914@	16.88 ± 0.732@	10.82 ± 0.450@	71.19 ± 0.824@
6.	PPA + CPH20	36.97 ± 1.346@#	289.8 ± 2.058@#	393.2 ± 2.183@#	24.76 ± 0.769@#	8.85 ± 0.234@#	63.37 ± 0.881@#

(Values expressed as mean ± SD (n = 6 rats per group). *$p < 0.001$v/s vehicle control, sham control and CPH20Perse; @ $p < 0.001$ v/s PPA; @# $p < 0.001$ v/s PPA + CPH10 (one-way ANOVA followed by Tukey's multiple comparison test)

4.4 Effect of chrysophanol in the restoration of gross pathological alterations in ICV-PPA induced autism in rats

Restoration of whole-brain alterations after chronic administration with chrysophanol

The normal, vehicle and CPH 20 perse treated groups had the proper brain size and morphology. The ICV-PPA treated rat brains displayed disrupted clotted outermost layer with rupture meninges compared to the vehicle, sham, and CPH 20 perse treatment groups. Prolonged oral administration of CPH at 10 mg/kg and 20 mg/kg doses repaired the morphological alterations and supported the rat brain recovery from further injuries. Similarly, rats given CPH 20 mg/kg demonstrated significant healing in the affected area of the brain and recovered brain damage compared to rats treated with CPH 10 mg/kg(Fig. 3a).

Reduction of pathological changes in brain sections after chronic administration with chrysophanol

The brain sections from the vehicle, sham, and CPH 20 mg/kg perse treatment groups' rats were structurally properly shaped and undamaged, with clearly visible basal ganglia, cortex, and hippocampus tissue. Compared to the vehicle,

sham, and CPH 20 perse, treated rats, the brain sections of the ICV-PPA treated rats showed cortical and hippocampus atrophy and atrophy in subcortical areas such as the medial thalamus, putamen, caudate nucleus, and internal medullary lamina. Prolonged oral administration of CPH 10 and 20 mg/kg restored the pathological alterations in rat brain sections (Fig. 3b).

Reduction in demyelination volume after chronic administration with chrysophanol

The normal, vehicle and CPH20 perse treated groups had no significant change in the demyelination volume. However, chronic administration of neurotoxin PPA for 11 days significantly enhanced the region of demyelination compared to normal, vehicle, and CPH20 perse treatment groups. Long-term oral treatment with CPH at doses of 10 mg/kg and 20 mg/kg significantly reduced demyelination volume compared to PPA-treated autistic-like rats.Consequently,CPH 20 mg/kg showed significant, dose-dependent effect in reducing demyelination volume when compared to CPH 10 mg/kg treated rats [one-way ANOVA: $F_{(5,25)} = 0.241$, $p < 0.001$](Fig. 4).

5.0 Discussion

In the current study, a neurotoxin PPA-induced experimental rat model was used to investigate the neurobehavioral and neurochemical alterations in autistic-like rats. Several research studies indicate that ICV-PPA injection plays a major in developing autistic-like behaviour in rats (Rahi et al., 2021; Tiwari et al., 2021; Sharma et al., 2019). PPA-exposed rats exhibit behavioural and neuropathological changes similar to those seen in ASD patients, such as hyperactivity, abnormal social interaction, stereotypic and repetitive movements, and are identified as a potential adult ASD model in rodents(Nemechek and Kathryn Moore, 2020; Chow et al., 2012).

Being a weak organic acid, PPA can passively accumulate within CNS cells, leading to a decrease in intracellular pH, with numerous physiological implications (Thomas et al., 2010). PPA has a series of physiological effects that alter brain function, reflecting the enhanced locomotion behaviour observed in PPA-infused rats (Lobzhanidze et al., 2020). It can inhibit Na+/K + ATPase, enhance intracellular calcium release, increase NMDA receptor sensitivity, and elevate nitric oxide, all of which can change neurotransmission in brain areas associated with locomotor behaviour (Meeking et al., 2020; MacFabe et al., 2007). PPA-treated rat brains exhibit microglia and astroglia activation and mitochondrial and fatty acid imbalance, elevated levels of neurotoxic cytokines and oxidative stress markers, and other abnormalities associated with the human ASD findings (MacFabe et al., 2011; Bhandariet al., 2017).

This study induces an experimental model of autism using neurotoxin PPA and investigated that CPH exerts a neuroprotective effect against PPA-mediated behavioural alterations. PPA infusion elicits ASD-like behavioural and neuroinflammatory reactions in rats. It acts as a neurotoxic agent, causing altered behavioural patterns in rats, such as learning and memory impairment, abnormal social interactions, and anxiety-like behaviour (Shultz et al., 2008; Ku et al., 2016; Wu et al., 2017). Furthermore, we evaluated various cellular and molecular markers, apoptotic markers, neurotransmitters, pro-inflammatory cytokines, and oxidative stress parameters levels in rat brain homogenate, blood plasma, and CSF samples. Insights from our behavioural and biochemical observations, long-term oral administration with CPH at two different doses exhibited a neuroprotective effect against neurobehavioral and neurochemical alterations in the PPA-induced experimental model of autism in rats.

Bodyweight evaluations on different days showed a considerable reduction in body weight after 11 days of ICV-PPA infusion in rats. Prior studies showed that PPA, a short-chain fatty acid, affects weight loss in both animal and

human individuals due to the altered fatty acid metabolism (Nankova et al., 2014) and excessive gluconeogenesis after entry into the citric acid cycle (Choi et al., 2018). In the current study, this weight loss increased in a dose-dependent manner after CPH administration. We also determined the brain-body weight ratio by dividing the fresh brain weight by the bodyweight at the end of the experimental protocol schedule. The brain-body weight ratio markedly decreased in ICV-PPA-treated autistic rats but increased dose-dependently upon CPH therapy.

The depressive-like behaviour was measured by immobility time during the forced swim test in PPA-induced autistic rats. The present study's findings demonstrated an increase in immobility time following PPA injection, which was attenuated by continuous CPH administration. The prior research report similarly showed an enhanced depressive effect following PPA treatment (Bhandari et al., 2017).

Previous research indicates that the PI3K/AKT/GSK3/mTOR/BDNF pathway is associated with the development of mood-related illnesses and has also been associated with the adaptive stress response (Lu et al., 2015). Several studies have shown that PI3K/AKT/mTOR has also been implicated in depression and anxiety (Leibrock et al., 2013; Moretti et al., 2014). CPH therapy promotes antidepressant effect by inhibiting the mTOR signalling pathway in a chronic stress model of depression in mice (Zhang et al., 2016). All of these findings supported our study's findings that CPH reduced depressive symptoms in children with autism.

In addition, the hyperactivity and repetitive behaviours that have been identified as a core symptom of autistic individuals (Kong et al., 2021). PPA and other short-chain fatty acids increase glial and intracellular neuronal acidification and calcium proportions, resulting in huge effects on neurotransmitter release, including serotonin, dopamine, glutamate, and norepinephrine, all of which play a role in the elicitation of locomotor activity (Daghestaniet al., 2017; Thomas et al., 2012).

Furthermore, PPA has been demonstrated to increase glutamatergic transmission, which leads to excitability in brain areas associated with locomotor activity. Our locomotor activity results recorded using actophotometer indicated the increased locomotor activity after ICV-PPA injection, which is similar to the findings of literature studies of Tiwari et al., 2021; Sharma et al., 2019. There was a considerable and dose-dependent improvement in hyperactive and repetitive behaviour after CPH administration at 10 mg/kg and 20 mg/kg. The beam crossing task was used to measure the balance and motor coordination by counting the number of slips during movement on a wooden beam. Our findings reveal that PPA autistic rats had an increased number of slips, indicating poor motor coordination, which was decreased in a dose-dependent manner following CPH treatment. It is commonly known that autistic children have impaired spatial memory, cognitive impairments, and intellectual disabilities (Zhang et al., 2020).

Morris water maze was used to examine long-term memory and spatial learning capabilities. Mepham et al. reported that intracerebroventricular PPA administration showed impaired spatial cognition in adult rats (Mephem et al., 2019). Our analysis revealed that increased ELT and decreased TSTQ resulted in severe memory loss in PPA-treated rats. Furthermore, CPH treatment reduces the escape latency time (ELT), although increased TSTQ in MWM indicates improved spatial memory.

We also evaluated the effect of CPH on the PI3K/AKT/mTOR signalling pathway to examine a cellular signalling mechanism. In various studies, PI3K/AKT/mTOR upregulation was associated with the development and progression of neurodegenerative diseases (Bozdagi et al., 2013; Chen et al., 2014). This signalling pathway has recently been implicated in molecular pathways that may contribute to autism (Kwon et al., 2006). In contrast, PI3K/AKT/mTOR signalling pathway inhibition provided a neuroprotective effect used as a diagnostic marker in autistic patients (Yeung et al., 2017). Consequently, we observed increased PI3K/AKT/mTOR levels in PPA-treated

autistic rats CSF and rat brain homogenate. However, the inhibition of the PI3K/AKT/mTOR pathway by CPH treatment provides a protective role against autism by reducing cell death, inflammation, oxidative stress, and mitochondrial dysfunction in CSF and rat brain homogenate.

Using advanced techniques, researchers discovered abnormalities in the brain's white matter region associated with the pathology of autism. A prior study found that MBP levels were lower in the brains of autistic patients (Gonzalez-Gronow et al., 2015). The current study observed reduced MBP levels in rat brain homogenate of ICV-PPA rats. Furthermore, CPH therapy restored MBP levels in brain homogenate in autistic rats.

The neurochemical evaluation in our research provides a strong indication regarding CPH neuroprotective activity. Neurotransmitters, which play a role in memory, mood, and behavioural regulation, must balance normal functioning and neuronal development. Neurotransmitter dysfunction was discovered to be one of the key causes of the beginning of behavioural characteristics. Neurotransmitter imbalance is one of the significant characteristics of autism (Kuo et al., 2018). In autism, the most studied neurotransmitters include serotonin, dopamine, glutamate, and Ach. Because serotonin is linked to brain development, it is altered in autistic conditions, and its insufficiency contributes to socially impaired, repetitive, and depressed behaviour (Kane et al., 2012; Amodeo et al., 2021).

Additionally, decreased level of dopamine, Ach, and increased glutamate level was also associated with imparting autistic behaviour (Drenthen et al., 2016; Karvat et al., 2014; DiCarlo et al., 2019). Increased glutamate levels activate microglia and promote neuroinflammation, but decreased dopamine and acetylcholine levels affect neuronal excitability, resulted in mood changes (Acharjee et al., 2018). Our analysis revealed that repeated ICV-PPA injection significantly affects the number of neurotransmitters in rat brain homogenates. Dopamine, serotonin, and acetylcholine levels in ICV-PPA-treated rats decreased, but glutamate levels increased considerably, showing neuronal excitotoxicity. CPH treatment restores the level of neurotransmitters in a dose-dependent manner and ameliorates autistic-like behaviour.

TNF and IL-1β are the key contributors to oxidative damage and neuroinflammation, which are important in neurodegeneration and neurodevelopmental abnormalities (Saghazadeh et al., 2019; Mirza and Sharma, 2019). The clinical study reports on autistic children clearly showed increased inflammatory cytokine levels in CSF, leading to immune response and neuronal damage (Chez et al., 2007). Our study reveals that PPA infusion increased inflammatory cytokines such as TNF and IL-1β in blood plasma and brain homogenate. After treatment with CPH, significantly lowered inflammatory cytokine levels in blood plasma and brain homogenate result in protection against inflammation.

According to previous studies, increased oxidative stress is one of the pathological aspects of autism (Morimoto et al., 2020; Abruzzo et al., 2019; El-Ansary et al., 2012). In order to assess the severity of the condition following ICV-PPA injection and the protective effects of CPH against oxidative stress, we evaluated the levels of oxidative stress markers in brain homogenates of PPA and CPH-treated rats. Our findings clearly show an increase in AchE, LDH, nitrite, and MDA, as well as a decrease in antioxidants, specifically SOD and GSH, in PPA-induced autistic rats. PPA-induced autistic rats treated with CPH had considerably lower levels of oxidative stress markers, indicating antioxidant capabilities, in a dose-dependent manner.

Excessive cell death hampers brain maturation and is recognized as a potential risk factor in the development of autism (Eftekharian et al., 2019). After PPA exposure, the level of apoptotic markers such as Bax and Caspase 3 increased, whereas the level of anti-apoptotic marker Bcl-2 decreased. Long-term CPH therapy has been shown to protect cells from cell death by reducing the levels of Bax and Caspase-3 and increasing the levels of Bcl-2.

This research looks at the morphological structure of the brain, the whole brain sections, and the demyelination volume. Our previous studies indicate that the hippocampus area was found to be relatively vulnerable to PPA exposure. The histological and morphological finding demonstrates that the brains of PPA-treated rats differ in size and shape (Rahi et al., 2021). Chronic CPH administration improved morphological alterations such as reduced total brain mass, degraded meninges, and constricted prefrontal cortex in ICV-PPA treated autistic rats. Coronal sections of ICV-PPA-treated rats revealed deformed basal ganglia and a defragmented hippocampus area, and degraded white matter.

Moreover, the assessment of demyelination volume in rat brains revealed a substantial decrease in white matter volume following PPA injections (Rahi et al., 2021).Compared to ICV-PPA treated autistic rats, chronic administration with CPH10 mg/kg and CPH20 mg/kg recovered the abnormalities in brain sections and enabled the remyelination of degraded areas. It was found that continuous therapy with CPH reduces the severity of pathological and morphological alterations.

Our research work mainly focuses on the neuroprotective potential of CPH via downregulating the PI3K/AKT/mTOR signalling pathway and thus mitigating behavioural, neurochemical, morphological, and gross pathological abnormalities in ICV-PPA autistic rats. This study suggests that all of the neurochemical markers investigated could be employed as a useful diagnostic biomarker for the early detection of autism.

However, additional studies for cellular markers, such as Western Blot and immunohistochemistry, are expected to validate these assumptions. Concurrent studies, such as Western Blot and immunohistochemistry, are also necessary to offer molecular evidence for this idea. Despite these limitations, the neuroprotective potential of CPH in correcting or downregulating the aberrant functioning of PI3K/AKT/mTOR signalling pathways in the CNS seems promising.

6.0 Conclusion

Based on the results, we conclude that PPA injection causes significant alterations, including abnormal neural cell organization, followed by autism-like neurobehaviors and biochemical abnormalities. PPA-treated rats showed aggressive behaviour, and reduced exploratory activity. To date, no pre-clinical studies on the neuroprotective effect of CPH via downregulation of PI3K/AKT/mTOR signalling in PPA-induced autistic-like rats were reported.

Our findings show that CPH effectively improves social interaction, learning, and memory deficits in PPA-exposed rats.CPH successfully reduced oxidative stress, neuroinflammation, and neuron death by downregulating the PI3K/AKT/mTOR signalling pathway in autistic rats. Furthermore, by downregulation of the PI3K/AKT/mTOR pathway in the rat brain, CPH enhances neuronal proliferation, growth, and differentiation, suppresses cell death and elevates myelin-basic protein levels in PPA-treated autistic-like rats. Furthermore, the rapid recovery of histological and gross morphological abnormalities in the whole brain and brain sections shows CPH neuroprotective ability against PPA-induced neurological deficits.

Our remarkable findings can be used as diagnostic biomarkers in the early stages of developing a disease-modifying therapeutic compound. However, more genetic research and immuno-histological analysis are required to elucidate the underlying processes regulating such interactions. Indeed, we can consider PI3K/AKT/mTOR as a potential therapeutic target in combination with other standard pharmaceutical treatments.

Abbreviations

CNS : Central nervous system

mTOR :Mammalian target of rapamycin

PI3K :Phosphoinositide 3-kinase

CPH : Chrysophanol

AKT : Protein kinase B (PKB)

ASD : Autism spectrum disorder

AD : Alzheimer's disease

ASD : Autism spectrum disorder

BBB : Blood brain barrier

DA : Dopamine

ICV :Intracerebroventicular

PD : Parkinson's disease

PPA : Propionic acid

SOD : Superoxide dismutase

MDA : Malondialdehyde

TNF : Tumor necrosis factor

GSH : Glutathione

LDH : Lactate dehydrogenase

IL-1β : Interleukin-1β

HPLC : High performance liquid chromatography

AchE : Acetylcholinesterase

Ach : Acetylcholine

TSTQ : Time spent in target quadrant

ELT : Escape latency time

MWM : Morris water maze

BCT : Beam crossing task

FST : Forced swim test

MBP : Myelin basic protein

AD : Alzheimer's disease

Declarations

Acknowledgments

Data availability statement

All data generated or analyzed during this study are included in this article. There are no separate or additional files.

Compliance with ethical standards

Funding

Disclosure of potential conflicts of interest

The authors declare that they have no competing interests.

Ethical approval

All applicable institutional guidelines for the care and use of animals were followed.

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Figures

Figure 1

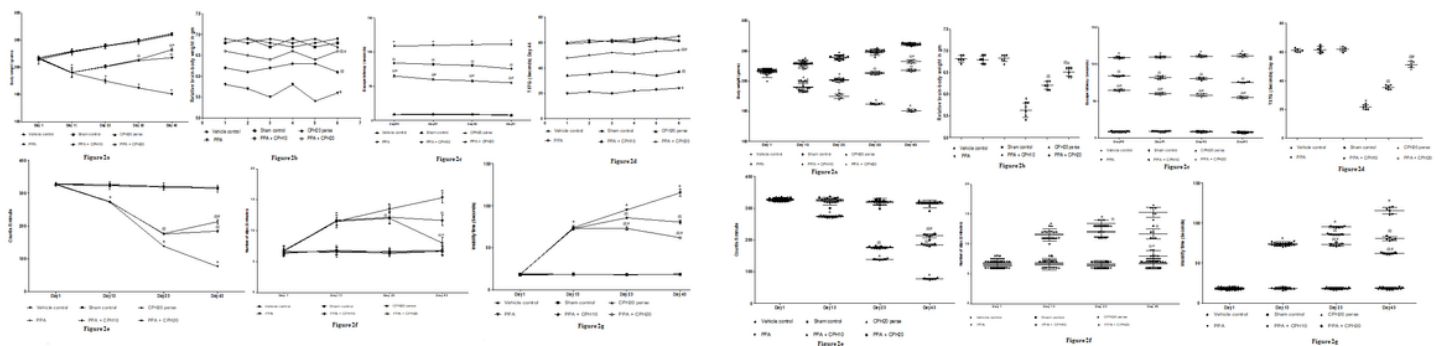Experimental protocol schedule (Behavioral and biochemical estimations)

Figure 2

(a): Effect of chrysophanol on body weight in ICV-PPA induced autism in rats. (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control, and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (two-way ANOVA followed by post hoc multiple comparison test Bonferroni) (b): Effect of chrysophanol on relative brain-body weight in propionic acid-induced autism in rats. (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (one-way ANOVA followed by Tukey's multiple comparison test) (c): Effect of chrysophanol on escape latency time in propionic acid-induced autism in rats. (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control, and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (two-way ANOVA followed by post hoc multiple comparison test Bonferroni) (d): Effect of chrysophanol on TSTQ in propionic acid-induced autism in rats. (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (one-way ANOVA followed by Tukey's multiple comparison test) (e): Effect of chrysophanol on locomotor activity in propionic acid-induced autism in rats. (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control, and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (two-way ANOVA followed by post hoc multiple comparison test Bonferroni) (f): Effect of chrysophanol on neuromuscular coordination in propionic acid-induced autism in rats. (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control, and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (two-way ANOVA 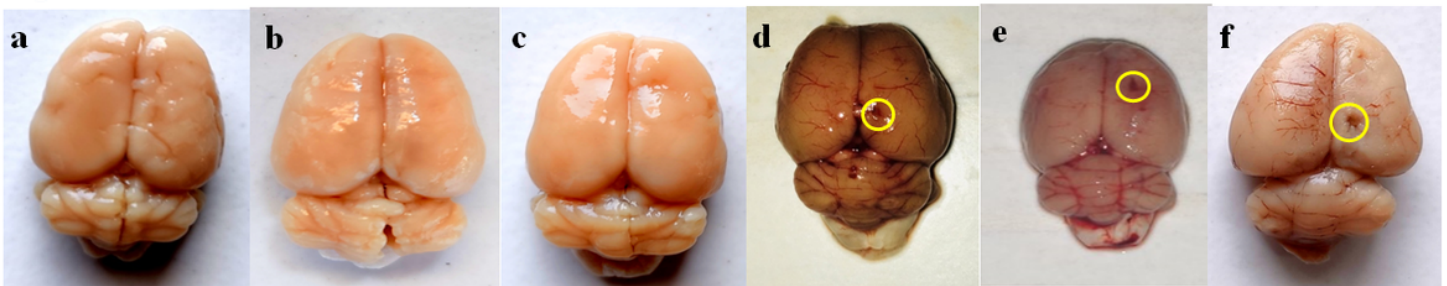followed by post hoc multiple comparison test Bonferroni) (g): Effect of chrysophanol on immobility time in propionic acid-induced autism in rats. (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control, and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (two-way ANOVA followed by post hoc multiple comparison test Bonferroni)
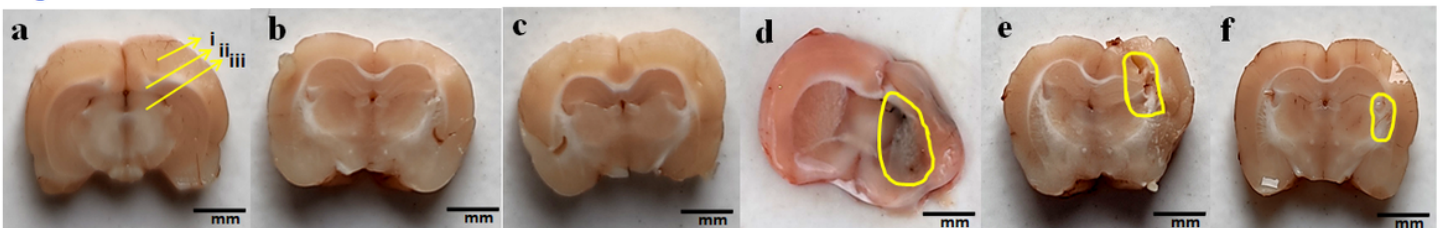

Figure 3

Effect of chrysophanol in the restoration of gross pathological alterations ofwhole rat brain and brain sections in ICV-PPA induced autism in rats (a) Whole rat brain (a) Vehicle control (b) Sham control (c) CPH20 perse (d) PPA (e) PPA + CPH10 (f) PPA + CPH20 (Scale bar = 2 mm) Note: Yellow circles are pointing to the site of the brain injury (b) Brain sections (a) Vehicle control (i) Cerebral cortex (ii) Hippocampus (iii) basal Ganglia (b) Sham control (c) CPH20 perse (d) PPA (e) PPA + CPH10 (f) PPA + CPH20 (Scale bar = 5 mm) Note: Yellow circles are pointing to the injured site.
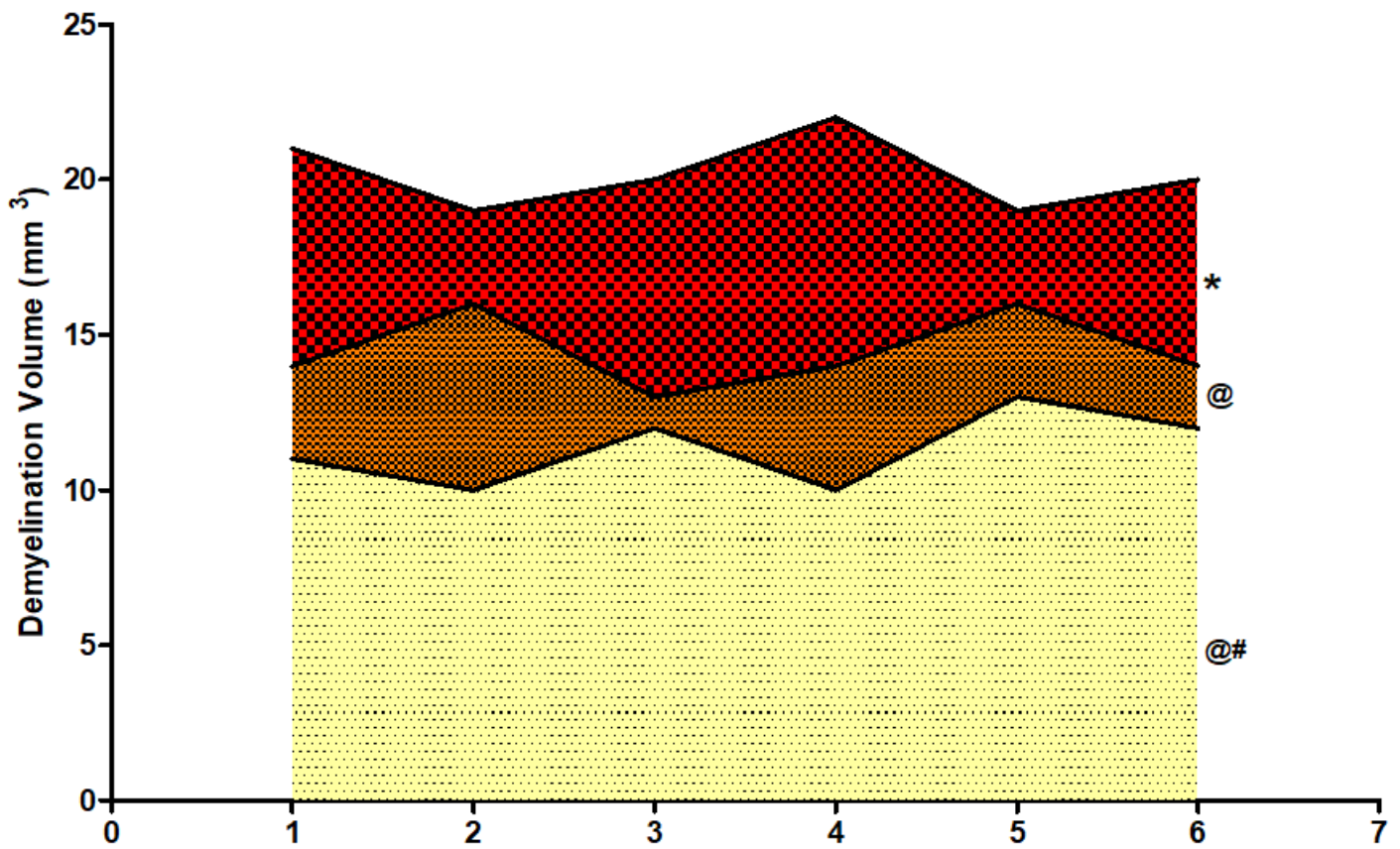
Figure 4

Effect of chrysophanol on demyelination volume in propionic acid-induced autism in rats (Values expressed as mean±SD (n=6 rats per group). *p<0.001v/s vehicle control, sham control and CPH20Perse; @ p<0.001 v/s PPA; @# p<0.001 v/s PPA+CPH10 (one-way ANOVA followed by Tukey's multiple comparison test)