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ORIGINAL PAPER

Tinospora cordifolia Suppresses Neuroinflammation in Parkinsonian Mouse Model

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Abstract

Parkinson's disease (PD), a neurodegenerative central nervous system disorder, is characterised by progressive loss of nigrostriatal neurons in basal ganglia. Previous studies regarding PD have suggested the role of oxidative stress along with neuroinflammation in neurodegeneration. Accordingly, our study explore the anti-inflammatory activity of *Tinospora cordifolia* aqueous extract (TCAE) in 1-methyl-4-phenyl-1,2,3,6-tetra hydropyridine (MPTP)-intoxicated Parkinsonian mouse model. MPTP-intoxicated mice showed significant behavioral and biochemical abnormalities which were effectively reversed by TCAE. It is evident that TCAE inhibits the MPTP-intoxicated Nuclear factor-κB (NF-κB) activation and its associated pro-inflammatory cytokine tumor necrosis factor-α (TNF-α) from immunohistochemistry and Western blot analysis. In MPTP-intoxicated mice, microglial and astroglial-specific inflammatory markers, ionized calcium binding adaptor molecule 1 (Iba1) and glial fibrillary acidic protein (GFAP), respectively were increased while were significantly reduced in TCAE treatment. Expression of pro-inflammatory cytokine genes, TNF-α, Interleukin-12 (IL-12) and Interleukin-1β (IL-1β) were found to be upregulated in MPTP-intoxicated mice, whereas TCAE treatment restored their levels. Additionally, anti-inflammatory factor Interleukin-10 (IL-10) gene was found to be downregulated in MPTP-intoxicated mice which were significantly restored by TCAE treatment. Tyrosine hydroxylase (TH) expression was reduced in MPTP-intoxicated mice, while its expression was significantly increased in TCAE-treated group. Our result strongly suggests that *T. cordifolia* protects dopaminergic neurons by suppressing neuroinflammation in MPTP-induced Parkinsonian mouse model.

Keywords Neuroprotection · Parkinson's disease · *Tinospora cordifolia* · Tyrosine hydroxylase · Glial cells · Neuroinflammation · MPTP

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Introduction

Parkinson's disease (PD) is a neurodegenerative disorder that mainly affects the motor activity. Its symptoms include bradykinesia, rigidity, tremor and postural instability (Kalia and Lang 2015). In PD pathogenesis, there is progressive loss of nigrostriatal neurons in the midbrain, which is an important characteristic feature of PD. Even though, the aetiology of PD is not known (Dauer and Przedborski 2003). In the pathogenesis of PD, neuroinflammation is one of the major contributors. Central nervous system (CNS) can be affected by the changes in the glial pathobiology by producing a pro-inflammatory mediators, resulting in the progression of PD (Olson and Gendelman 2016; Rai et al. 2017a).

In this regard, natural sources such as medicinal herbs could play an important role in several diseases as they contain various bioactive compounds such as alkaloid, flavonoid, etc. (Song et al. 2012). Rising evidence indicates that some medicinal herbs contain neuroprotective compounds, such as resveratrol (Zhang et al. 2010), curcumin (Ojha et al. 2012), levodopa (L-dopa) in *Mucuna pruriens* (Katzenschlager et al. 2004; Rai et al. 2017b). Thus, DA neurons can be protected using these herbs against different neurotoxin-induced neurodegeneration. In addition, these medicinal herbs have also been found to reduce the drug-induced side effects in the Parkinson's patients which has been in accordance with our previous studies. *Tinospora cordifolia* (TC), a member of Menispermaceae family of plant has been widely used for treatment in the traditional Indian system of medicine, Ayurveda (Upadhyay et al. 2010). In addition, it has been used as universal adaptogenic and prohost immunomodulator to cure various infections. Recent studies (Mishra et al. 2016) have reported the anxiolytic and immunomodulatory activity of TC as well. In addition, few studies have reported the immunosuppressive and anti-inflammatory effects along with other related pharmacological actions of the aqueous extract of TC (Pendse et al. 1977; Upadhyay et al. 2010). A recent study has shown the neuroprotective role of *T. cordifolia* ethanolic extract (Kosaraju et al. 2014), prepared from aerial parts of plant in 6-hydroxy dopamine (6-OHDA) induced rat model of PD. Current study deals with the neuroprotective and anti-inflammatory role of *T. cordifolia* aqueous extract (TCAE) in MPTP-induced Parkinsonian mice model. In this study, we have tried to show neuroprotective role of TCAE with series of experiments via NF-κB mediated inflammatory cytokines, expression of Iba1 in microglia and GFAP in astroglia in the SNpc of MPTP-induced Parkinsonian mice.

Ethical Approval

All procedures involving animals were performed in accordance with the ethical standards of the institution or practice at which the studies were conducted and in agreement with the guidelines for animal experiments established by the Institutional Animal Ethics Committees (IAECs) of the Laboratory Animal Research at Banaras Hindu University, India. The experiments were designed to minimize animal suffering and reduce the number of animals sacrificed. All mice were kept in temperature-controlled room (22 ± 2 °C) in the 12-h light and dark cycles with free access to food and water. Commercially available rodent food was provided to mice ad libitum.

Materials and Methods

Chemicals Required

Acetic acid, disodium hydrogen phosphate, GSH, NADPH, potassium chloride and sodium dihydrogen phosphate were procured from SRL, Mumbai, India. Streptavidin-peroxidase, normal goat serum and DAB (3,3-diaminobenzidine) were procured from Bangalore Genei Pvt. India Ltd., Bangalore, India. 1-Methyl-4-phenyl-1,2,3,6-tetra hydropyridine (MPTP) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Protein estimation kit by Bradford GeNei™, hydrogen peroxide (H_2O_2) and potassium dichromate were purchased from Merck (Darmstadt, Germany). Primary antibodies for Tyrosine hydroxylase (TH; SC-25,269), Glial Fibrillary Acidic protein (GFAP; SC-33673) and β-actin (SC-47778) were procured from Santa Cruz, Biotechnology (Santa Cruz, CA, USA) and the primary antibodies for tumor necrosis factor-α (TNF-α) (ab1793), ionized calcium-binding adaptor molecule 1 (Iba1; ab5076) and nuclear factor-κB (NF-κB; ab16502) were purchased from Abcam Life Science, Biogenuix Med systems Pvt. Ltd. (New Delhi, India), secondary antibodies for western blot were GT × Rb IgG-Biotin conjugated (62111028001A) and GT × Ms IgG Biotin-conjugated (cat#105261) and secondary antibodies for IHC, donkey anti-mouse Cy2 (AP124J) Ex max 492 nm and Em max 510 nm, procured from Millipore Sigma, Goat Anti-Mouse IgG (ab6785) & Goat Anti-Rabbit IgG Antibody, Cy3 (AP132C) Ex max 550 nm and Em max 570 nm conjugate were purchased from chemicon and DAPI(D9542-1MG) from Sigma.

Experimental Animals

Eight- to ten-week-old male Swiss albino mice (25–30 g) were procured from animal house, Institute of Medical Sciences, Banaras Hindu University, India. Before the start of experiment, mice were acclimatized to laboratory conditions for 7 days. All the animal experimental protocols were followed as approved by the Animal Ethics Committee of Banaras Hindu University, Varanasi, India.

Preparation of Plant Extract

TC was collected from wild flora adjacent to Banaras Hindu University, Varanasi, India. After drying at 37 °C for 72 h, stem of the plant was crushed into powder by maceration method. While processing, sunlight exposure was avoided to prevent the loss of the active components. 50 g of TC stem powder was dissolved in 500 ml of double-distilled water and filtered twice with Whatman no. 1 filter paper. The liquid thus extracted was kept in the water bath to evaporate excess water at 60 °C temperature for 7–10 h daily, for 2–3 days. The semisolid extract was subjected to freeze drying after keeping it overnight in the deep freezer at − 20 °C. The extract thus obtained was stored at − 20 °C to be used further in the experiment. The extract preparation was done using standardized protocol (Sengupta et al. 2011).

Dose Standardization Experiments

Dose of TC extract was optimized in different set of experiments. Animals were grouped in four different sets, of which three groups were given TC extract at doses 100, 200 and 400 mg/kg body weight, respectively, leaving one group as control (given normal saline) by oral gavage. Neurobehavioral parameters for motility were conducted including the rotarod, hanging and narrow beam walking tests, as described previously and optimum dose of the TC extract was calculated (Yadav et al. 2013).

Experimental Design

Mice were divided into four groups ($n = 6$/group). For the first group, control was treated with normal saline (i.p.), whereas second group of animals were injected with two consecutive doses of MPTP with 16-h interval (Rai et al. 2016). The third group of animals was injected with MPTP similarly as second group. TCAE was given orally to mice at a dose of 200 mg/kg body weight/day prior to MPTP treatment for 1 week and was continued for 2 weeks post MPTP treatment. Fourth group was given TCAE alone, which served as positive control.

Neurobehavioral Studies

Motor Behavioral Test

To evaluate the motor disorder in MPTP-induced Parkinsonian mice, the following behavioral tests were performed. Before starting the experiment, the mice were trained for three consecutive days and data were recorded after completion of experiment.

Rotarod Test

The animals were trained at a fixed speed of 15 rpm and the time was noted up to a maximum of 5 min after the mice fall. The average time was calculated after the experiment and repeated for four times (Manna et al. 2006). The same protocol was repeated after completing the treatment.

Hanging Test

Mice were placed on a horizontal grid and supported until they hold the grid. The grid was inverted so that the mouse hangs upside down and was allowed to stay on the grid until they lose their control and fall. The hanging time was recorded and the experiment was repeated three times (Mohanasundari et al. 2006).

Narrow Beam Walking Test

In this test, animals were trained to walk on a stationary wooden narrow flat beam (L100 cm × W1 cm) which was placed at a height of 100 cm from the floor. Time taken to walk on the beam from one end to the other was recorded and the experiment was repeated thrice (Pisa 1988).

Biochemical Studies

Sample Preparation for Biochemical Studies

Nigrostriatal tissue from the mid brain of animals from each group were collected individually and homogenized in KCl buffer (Tris–HCl 10 mM, NaCl 140 mM, KCl 300 mM, ethylenediaminetetraacetic acid 1 mM, Triton-X 100 0.5%) at pH 8.0 supplemented with protease and phosphatase inhibitors. The tissue homogenates of each sample were centrifuged at 12,000×*g* for 20 min at 4 °C, then the supernatant was collected and concentration was measured by a spectrophotometer to perform biochemical assays such as estimation of antioxidant enzymes, lipid peroxidation, etc.

Measurement LPO, GSH and SOD

Lipid peroxidation (LPO) in the nigrostriatal tissue of the mouse brain was estimated as described previously (Ohkawa et al. 1979), with slight modifications. Briefly, for measuring the concentration of malondialdehyde (MDA), an assay mixture containing 10% tissue homogenate (0.1 mL) was added to 10% SDS solution (0.1 mL) and incubated at room temperature for 5 min followed by the addition of 20% acetic acid (0.6 mL) and further incubation for 2–5 min. At last, 0.8% thio-barbituric acid (TBA; 0.6 mL) was added and the reaction mixture was incubated in a boiling water bath for 1 h. The assay mixture was cooled, centrifuged and the absorbance of the supernatant was taken at 532 nm against control. LPO levels are expressed as μM MDA/mg protein. The level of glutathione (GSH) in the brain homogenate was measured by the method described previously (Moron et al. 1979) and reported as μM GSH/mg tissue. The activity of superoxide dismutase (SOD) was measured in the nigrostriatal tissue homogenate (10%) using a standard procedure described previously (Nishikimi et al. 1972) and reported as nmol/mg protein.

Catalase

Catalase (CAT) activity was assayed by measuring the rate of decomposition of hydrogen peroxide (H_2O_2) by the spectrophotometric method as described by previous studies (Kumar et al. 2010). Briefly, 10% w/v tissue homogenate was added in phosphate buffer pH 7, distilled water, hydrogen peroxide (0.02 M) and incubated at room temperature for 1 min then potassium dichromate and acetic acid (1:3) solution was added and incubated for 15 min in boiling water bath and O.D. was taken at 570 nm. The enzymatic activity was measured in nmoles/min/mg protein.

Western Blot Analysis

Western blot analysis was performed as described previously (Gupta et al. 2010). Primary antibody dilutions included TH (1:1000), NK-κB (1:1000), TNF-α (1:1500) and β-actin (1:500). The blots were visualized using DAB and H_2O_2 as substrates. Relative band density was calculated with respect to β-actin and their expression was indicated as of fold change.

Immunofluorescence Staining of TH, Iba-1 and GFAP, TNF-α and NF-κB in SNpc

Immunohistochemical staining of TH-positive DA neurons, Iba-1-positive microglial, GFAP-positive astroglial cells and pro-inflammatory markers, TNF-α and NF-κB was performed in SNpc region of the brain using standard procedure (Gorbatyuk et al. 2008). Briefly, the perfused mice brains were post-fixed with 4% paraformaldehyde and were collected. Further, 25 m-thick coronal brain sections were cut at the SNpc level using a cryomicrotome (Leica, Wetzlar, Germany). The sections were washed twice with 0.01 M PBS at pH 7.4 and then incubated with blocking reagent (10% normal goat serum in PBS 0.3% Triton-X 100) for 1 h. The sections were then incubated with the primary polyclonal anti-mice antibody against TH at 1:1000 dilution, Iba-1 (1:1000), polyclonal anti-rabbit NF-κB p65 antibody in 1:1000 dilution, anti-mouse monoclonal TNF-α in 1:700 dilutions and monoclonal anti-mouse against GFAP 1:1000 dilution for 16 h at 4 °C. The sections were washed five times in PBST and incubated with Cy2-conjugated secondary antibodies in 1% BSA blocking solution for 1 h at room temperature. Sections were then washed three times and mounted using mounting media, fluoro shield (Sigma-Aldrich). The images with ×20 magnifications were taken under fluorescent microscope, Nikon (Nikon, Japan). Images were analyzed by Image-J-software (NIH, USA) and reported in mean percentage area value.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

Total RNA was isolated from the SNpc using the Trizol RNA isolation reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The RNA yield was quantified on Nanodrop 1000 and the RNA purity was determined based on the A260/A280 ratio. For cDNA preparation, 2 μg total RNA (kept equal for each amplification) was subjected to reverse transcription using 20U M-MLV reverse transcriptase (Fermantas, Germany), 1 × RT buffer, 20 mM dNTPs (New England Biolabs, USA), 20U RNasin (Fermentas, Germany), 0.1 M DTT with DEPC treated water, and 100 ng of random hexamers (Fermentas, Germany). Gene expression profile analysis was done on ABI7500 Fast system. PCR reaction was done according to previously reported studies with few modifications (Peinnequin et al. 2004). Briefly, 10 μl of real time mix contained 5 μl of SYBER green master mix (Applied Biosystem), 1 μl cDNA, 2 μl nuclease free water, 0.5 μl each of forward and reverse primers, 1 μl RNase inhibitor. PCR conditions were set with an initial incubation at 50 °C for 2 min, followed by denaturation at 95 °C for 10 min, and 40 cycles at 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 40 s. The abundance or declines of mRNA were normalized to the geometric average of endogenous control GAPDH for ΔCt. The fold change (ΔCt) was calculated using $2^{-\Delta\Delta Ct}$ method and reported as arbitrary unit.

Statistical Analysis

Statistical analysis of differences between means of groups was determined using one-way analysis of variance (ANOVA) followed by Student–Newman–Keuls test using Graph Pad Prism 5.0 software. Data are expressed as mean ± standard errors (SEM) for separate groups and differences are considered statistically significant when p values were $p < 0.05$.

Result

Neurobehavioral Parameters

In rotaroad test, MPTP-treated mice were found to spend less time on the rotating beam as compared to control ($p < 0.001$; Fig. 1a). While when compared to MPTP-treated mice, the MPTP + TCAE mice group spent significantly longer time on the rotating beam ($p < 0.01$). Hanging time of PD-mice was found to be significantly less ($p < 0.001$) when compared with control (Fig. 1b). However, on TCAE administration the hanging time was significantly enhanced in PD mice ($p < 0.01$). On treatment with TCAE, the animal showed significant improvement ($p < 0.01$) in walking time on narrow beam as compared to the MPTP mice (Fig. 1c). Likewise in comparison with MPTP group, we observed significantly enhanced narrow beam walking time ($p < 0.001$) in control mice.

Biochemical Parameters

TCAE Decreased the Oxidative Stress and Restored the GSH Level

We observed that TCAE reduced oxidative stress and increased antioxidant level in the SNpc of MPTP-intoxicated animals via inhibition of lipid peroxidation and restoration of the GSH level. Thus, TCAE was found to exhibit neuroprotective activity by adjusting the GSH and lipid peroxidation levels. Paralleled to MPTP group, significantly reduced MDA level ($p < 0.01$; Fig. 2a) and enhanced GSH levels ($p < 0.05$) have been shown by TCAE-administered Parkinsonian mice. Additionally, MPTP-intoxicated mice were observed to have elevated levels of MDA ($p < 0.001$; Fig. 2a) and reduced levels of GSH ($p < 0.01$; Fig. 2b) as compared to control group.

TCAE Modulates the Activities of Antioxidant Enzymes (CAT and SOD) in MPTP-Intoxicated Mice

Since, TCAE has shown beneficial effects on lipid peroxidation and GSH levels, we tried to estimate the activities of antioxidant enzymes, SOD (Fig. 2c) and CAT (Fig. 2d) which act as Reactive oxygen species (ROS) scavenger. In MPTP-intoxicated mice, there was significant reduction in SOD ($p < 0.001$) and CAT activities ($p < 0.001$) as compared to the control group. Further, TCAE treatment to Parkinsonian mice significantly improved the enzymatic activity of SOD ($p < 0.01$; Fig. 2c) and CAT ($p < 0.01$; Fig. 2d) as compared to the MPTP-intoxicated group.

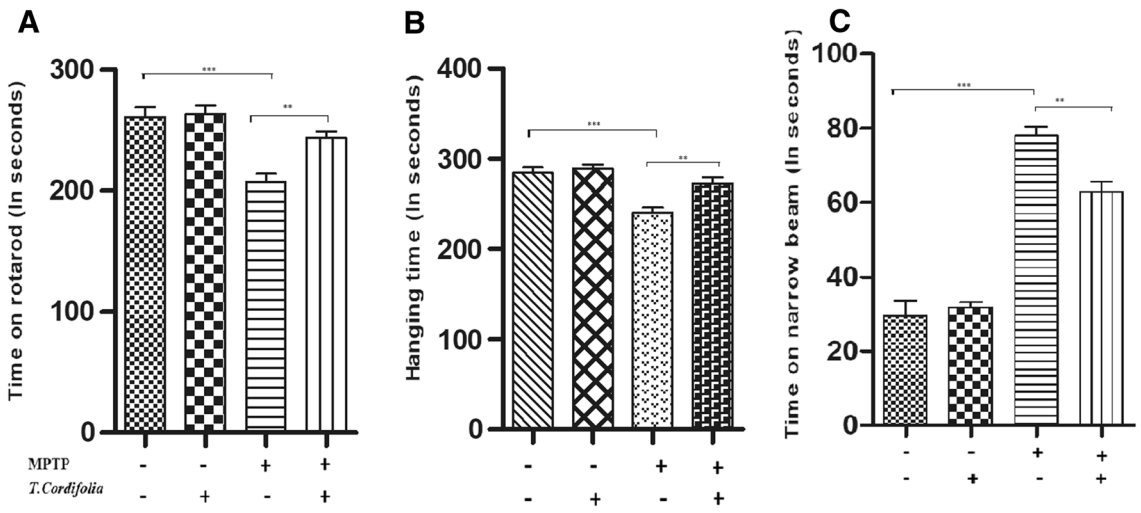

Fig. 1 Effect of TC on behavioural parameters. **a** Rotarod test, **b** hanging test, **c** narrow beam walking test. Data are expressed in terms of mean ± SEM ($n = 6$), (**$p < 0.01$, ***$p < 0.001$). *CONT* control, *SEM* standard error of mean

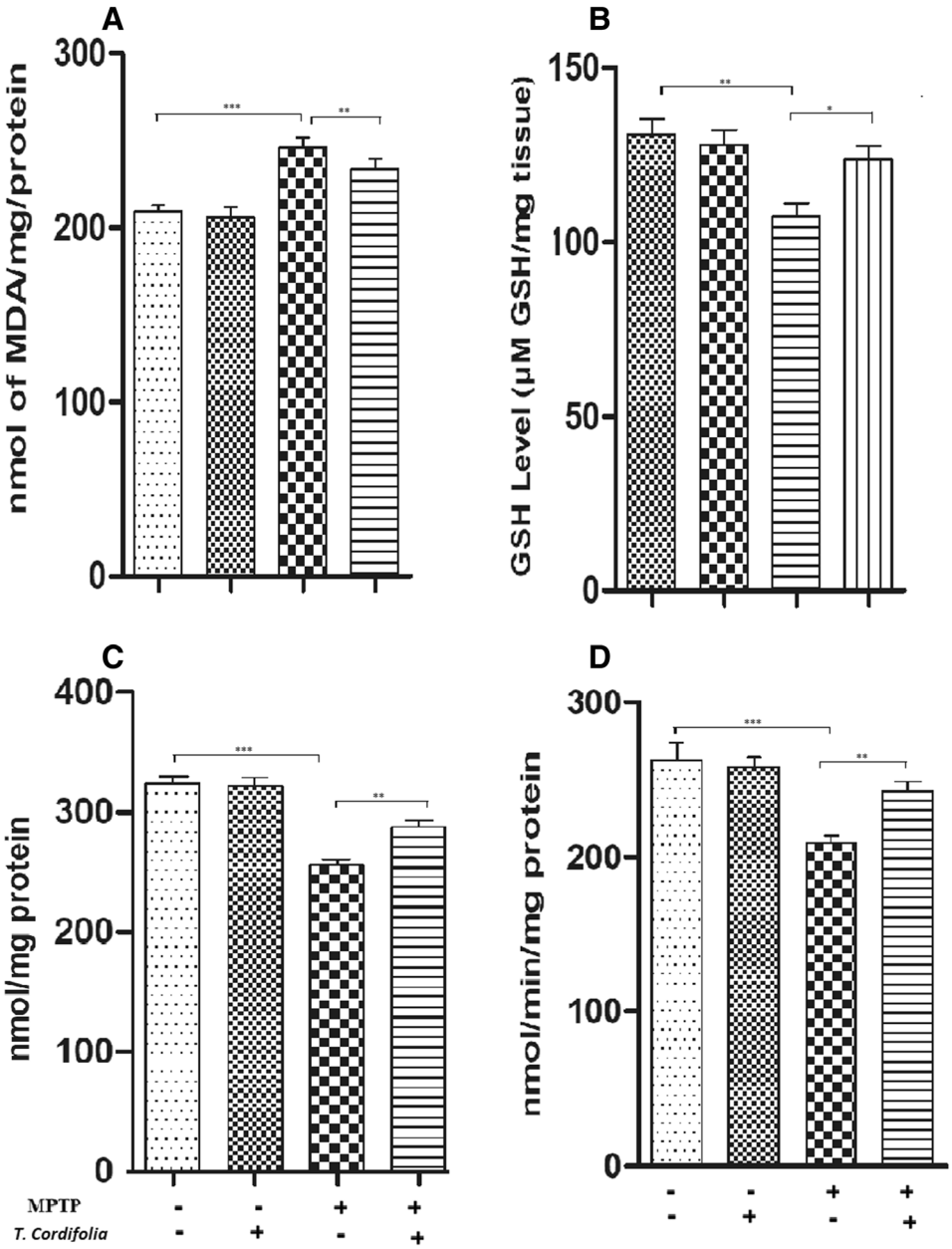

Fig. 2 Biochemical estimation of **a** MDA, **b** GSH, **c** SOD, **d** CAT in the SNpc region of the mid brain. Values are expressed as mean ± SEM ($n = 5$) (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$). *MDA* malondialdehyde, *GSH* glutathione, *SOD* superoxide dismutase, *CAT* catalase

Western Blot Analysis of TH, NF-κB and TNF-α

After assessing the behavioral and biochemical parameters, the expression levels of TH (60 kDa), NF-κB (64 kDa) and TNF-α (17.4 kDa) (Fig. 3) were estimated using Western blots in tissue lysates isolated from SNpc region. A reduced ($p < 0.01$) TH and enhanced NF-κB ($p < 0.01$) and TNF-α ($p < 0.001$) expression levels were observed in MPTP-intoxicated mice as compared to the control group. On TCAE treatment to Parkinsonian mice, increased TH level ($p < 0.01$) was observed when compared to MPTP-intoxicated mice. Conversely, reduction in NF-κB ($p < 0.01$) and TNF-α ($p < 0.001$) expression were observed on TCAE administration.

Effect of TCAE on Expression of TH, Iba1, GFAP, TNF-α and NF-κB in SNpc

MPTP administration resulted in significant loss of DA ($p < 0.001$) neurons in SNpc as compared to normal saline treated control mice (Fig. 4c). The treatment of TCAE to MPTP group, showed significant neuroprotection ($p < 0.01$) of DA neurons in comparison with MPTP injected mice. In MPTP group, expression of GFAP

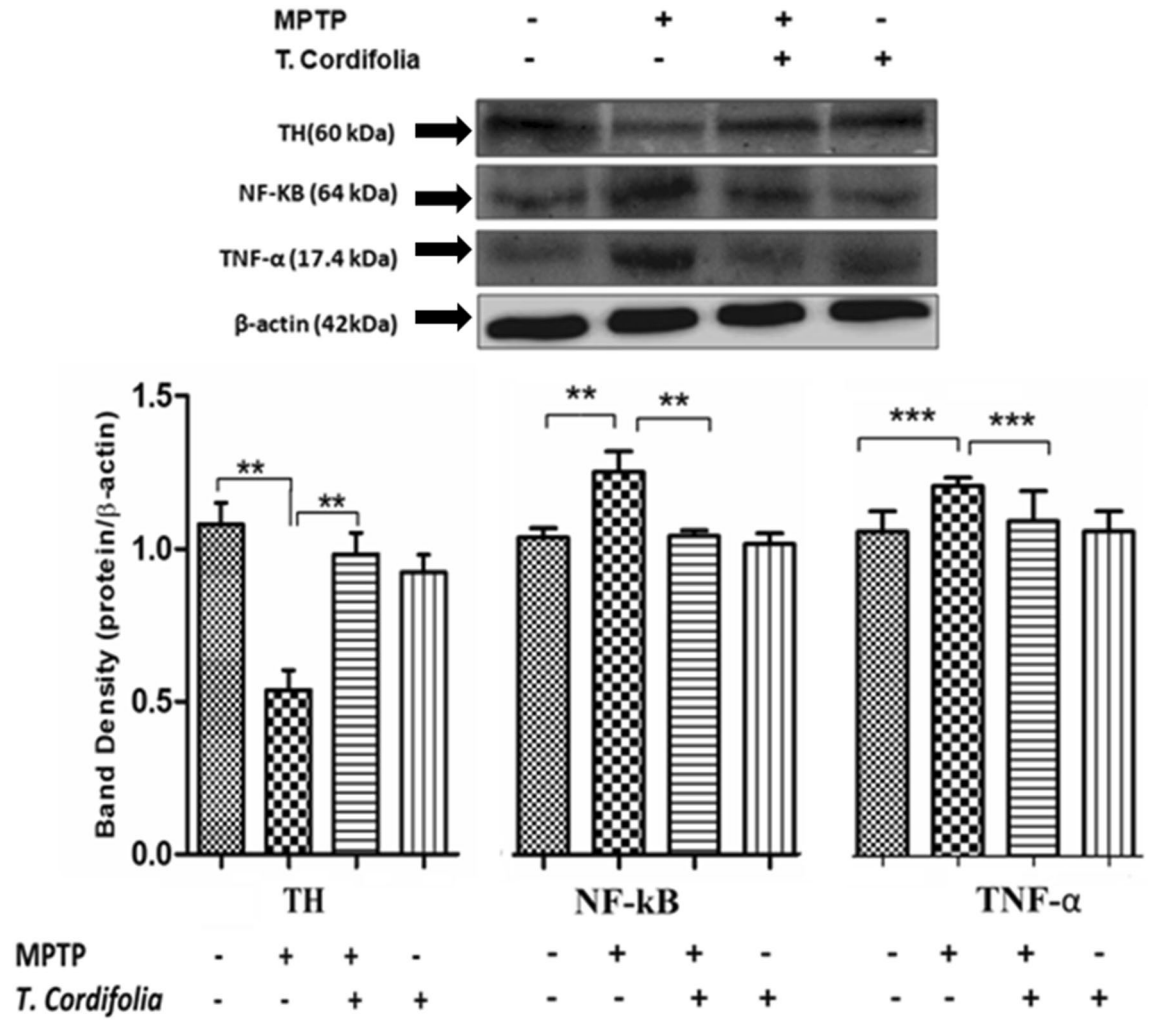

Fig. 3 Western blot analysis to detect the expression level of tyrosine hydroxylase (TH), TNF-α and NF-κB in the SNpc tissue. Values are expressed as mean ± SEM ($n = 5$) (**$p < 0.01$, ***$p < 0.001$). Expression levels of TH (60 kDa), NF-κB (64 kDa) and TNF-α (17.4 kDa) in the SNpc region of mice brain. TNF-α and NF-κB expression was increased in the MPTP group as compared to the CONT group. TC treatment followed by MPTP injection downregulated the expression of TNF-α and NF-kB as compared to MPTP group. Similarly, TH expression was declined in MPTP group as compared to CONT group. TC treatment increased TH expression as compared to MPTP group

($p < 0.01$; Fig. 4a) and Iba-1 ($p < 0.01$; Fig. 4b) was significantly increased. On treatment with TCAE, GFAP ($p < 0.01$) and Iba-1 ($p < 0.01$) expression was found to be decreased. Besides different cell types, we have also measured the pro-inflammatory markers; NF-κB and TNF-α in the SNpc of PD mice. An increase in NF-κB ($p < 0.001$; Fig. 5b) and TNF-α ($p < 0.001$; Fig. 5a) expression was found in MPTP-intoxicated mice as compared to control. However, on TCAE administration there was significant reduction in the expression levels of NF-κB ($p < 0.01$) and TNF-α ($p < 0.01$) in TCAE-treated Parkinsonian group as compared to MPTP group.

The Quantitative RT-PCR Further Confirmed the Anti-inflammatory Role of TCAE by Downregulating Inflammatory Cytokines

The qRT-PCR was done (Fig. 6) with GAPDH, taken as endogenous mRNA control. qRT-PCR validated the potential role of TCAE in regulating neuroinflammation in Parkinsonian mice model. The qRT-PCR revealed upregulation of TNF-α (5.1 fold), IL-1β (4.2 folds) and IL-12 (3.6 fold) in MPTP-intoxicated mice with respect to normal healthy control, while Interleukin-10 (IL-10) (0.8 fold) was found to be downregulated. After treatment

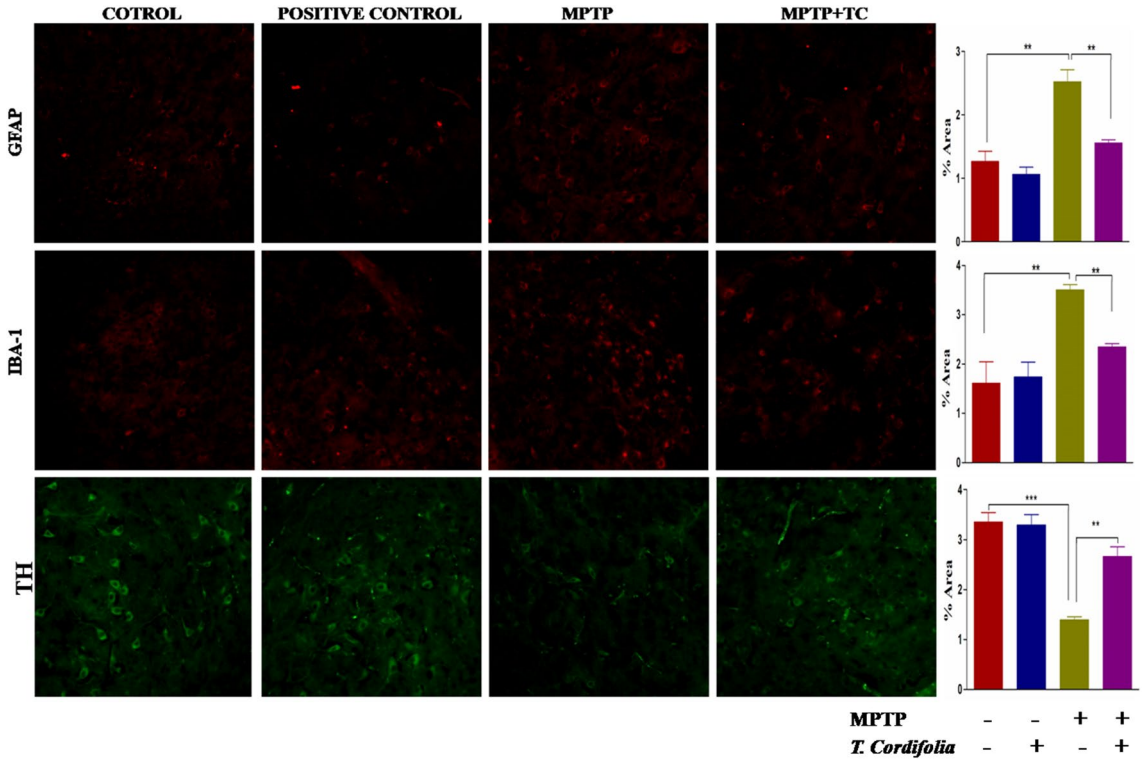

Fig. 4 Immunohistochemical staining of GFAP, Iba-1 and TH in the SNpc region of mice brain. With ×20 magnification image staining. Expression of **a** GFAP, **b** Iba-1, **c** TH. Value expressed as mean ± SEM ($n = 5$) (**$p < 0.01$, ***$p < 0.001$)

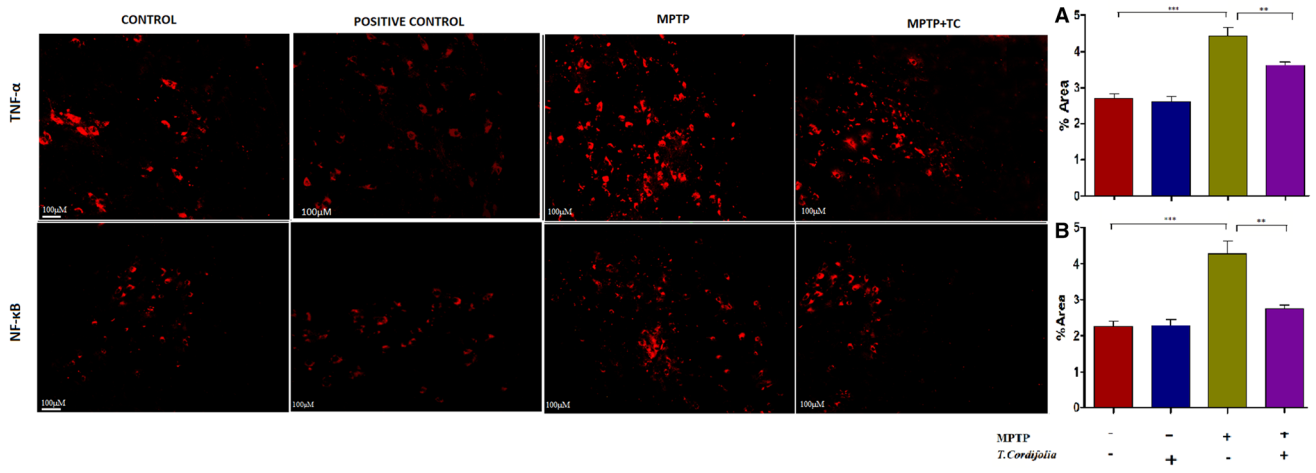

Fig. 5 Immunohistochemical staining of TNF-α and NF-κB. With ×20 magnifications after staining. The level of pro-inflammatory molecule TNF-α and NF-κB were significantly increased MPTP-injected mice as compared to CONT group. While TC treatment significantly decreased the level of both molecule as compared to MPTP mice. Positive control group did not find any alteration. Value expressed as mean ± SEM ($n = 5$) (**$p < 0.01$, ***$p < 0.001$)

with TCAE, there was a significant decrease in TNF-α (1.2), IL-1β (2.0) and IL-12 (1.4) and an increment was found in the level of IL-10 (1.4) as shown in Fig. 6, displaying the fact that TCAE has got significant potential to control neuroinflammation by regulating the pro- and anti-inflammatory cytokines.

Discussion

Various studies to explore the medicinal properties of TC such as, anti-oxidative (Subramaniam and Chesselet 2013), anti-cancerous (Mishra and Kaur 2013) and

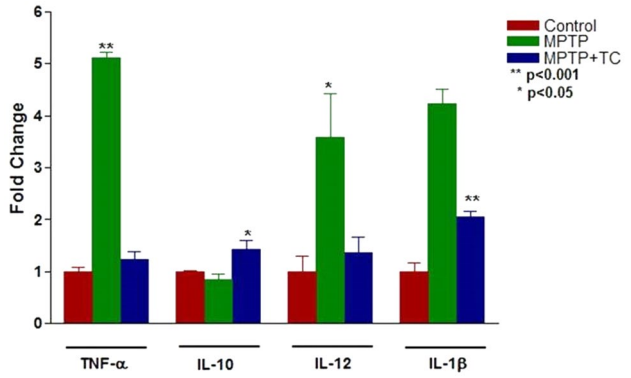

Fig. 6 Fold change in gene expression of pro and anti-inflammatory cytokines in MPTP-intoxicated and Co-treated mice, in comparison to healthy control. Expression of TNF-α, IL-12 and IL-1β was found significantly upregulated in MPTP mice, whereas it was found to be attenuated in TC-treated mice. Expression of IL-10 was downregulated in MPTP mice and upregulated in TC-treated mice. Data were normalized with endogenous control (GAPDH) and value expressed as mean ± SEM ($n = 5$) (**$p < 0.01$, ***$p < 0.001$)

immunomodulatory activities along with cognitive function improvement (Mishra et al. 2016) have been performed in ageing brain of acute sleep-deprived rat. These studies have displayed the ability of TC to efficiently cross the blood–brain–barrier and encouraged us to study the effect of TCAE in PD.

PD is associated with behavioral changes along with motor impairment as observed in MPTP-intoxicated Parkinsonian mice, thus suitably used for such studies (Blum et al. 2001). MPTP mice model show behavioral (Singh et al. 2009) and pathophysiological characteristics of neurodegenerative diseases, such as oxidative stress, DA neuronal loss and neuroinflammation-associated glial cell activation (Blesa et al. 2015).

Rotarod test has been widely used to check the brain and motor co-ordination in animals, as they try to move and make balance simultaneously on a rotating beam (Duan and Mattson 1999). Motility and muscle strength of the mice were assessed using hanging test, while coordination of movement to see balance and stability in mice was measured using narrow beam walking test (Rai et al. 2016; Singh et al. 2018). These behavioural deficits were found to be improved by TCAE treatment. Thus, TCAE treatment has significantly improved gait impairment and helped in rescuing the motor deficits caused by MPTP intoxication.

Oxidation-inflammation theory suggests that most of the CNS diseases are strongly linked to oxidative stress followed by chronic inflammation of selected areas of the brain (Miquel 2009). Oxidative stress along with mitochondrial dysfunction plays a key role in the pathogenesis of PD (Subramaniam and Chesselet 2013). Moreover, endogenous antioxidants for example CAT and SOD potentially scavenge the free radicals produced in the PD brain (Sutachan et al. 2012). Thus, neuroprotective activity of TCAE was measured through oxidative stress parameters viz., MDA and antioxidants such as, SOD, CAT and GSH in the nigrostriatal region of brain. We found that level of MDA was significantly increased in MPTP-intoxicated mice, while TCAE potentially reduced the MDA level in the nigrostriatal region, clearly suggesting that TC has potent antioxidant properties. Antioxidant molecules such as SOD, CAT (enzymatic) and GSH (non-enzymatic) play an important role in scavenging free radicals, which otherwise can lead to oxidative damage (Sutachan et al. 2012; Celardo et al. 2014). Imbalance between antioxidant defense system and oxidative stress causes reduction in endogenous antioxidants GSH, SOD and CAT (Scapagnini et al. 2004; Pigeolet et al. 1990). We observed that level of antioxidants was significantly decreased in MPTP-intoxicated mice. Following TCAE treatment, the activities of SOD, CAT and level of GSH in the SNpc of the brain were significantly restored. This restoration signifies antioxidant and free radical scavenging activity of TC. Our study, thus additionally provides evidence about the anti-inflammatory activity of TCAE in Parkinsonian mice model.

Though, the pathogenesis of PD is not yet fully understood, but few evidences strongly suggest the role of neuroinflammation in the progressive degeneration of nigrostriatal neurons (Hirsch et al. 2012). NF-κB, a transcription factor plays an important role in response to various stimuli linked to neuroinflammation and helps in the regulation of various cell-signalling pathways. Alteration in the NF-κB expression may help in disease management. NF-κB is normally present in cytosol in the inactive form, coupled with IκB. Inflammatory mediators cause NF-κB-Iκ-B to disintegrate and produce the active form of NF-κB, which finally translocates to the nucleus and regulates the expression of the associated genes (Matejuk and Shamsuddin 2010). iNOS, COX-2, TNF-α and IL-1β are the most important proinflammatory cytokines whose production is mediated by NF-κB (Block and Hong 2007). Furthermore, NF-κB controls inflammatory responses in glial cells by promoting the expression of proinflammatory genes (Fiebich et al. 2002; Wilms et al. 2003). The DA neuronal loss is prevented by selective inhibition of NF-κB in PD (Ghosh et al. 2007). In this study, TCAE inhibits MPTP-induced activation of NF-κB and ultimately inhibits the activation of NF-κB-associated proinflammatory cytokine, TNF-α which is evident by the IHC and Western blot analysis.

TNF-α is the downstream regulator of the NF-κB responsible for the progressive degeneration of DA neurons in the nigrostriatal region. It plays the central role in neuroinflammation as it helps in activating and recruiting immune cells through its receptor TNF receptor 1 (TNFR1) (Glass et al. 2010). Additionally, TNFR1 is also capable of inducing

oxidative stress via activation of ROS and reactive nitrogen species (RNS) producing enzymes. TNF-induced oxidative stress along with inflammation promotes neurodegeneration (Frankola et al. 2011). From the IHC and Western blot data, it is clearly evident that TCAE-treated mice inhibited the TNF-α expression in nigrostriatal region as compared to MPTP-intoxicated mice.

In a study done by Arimoto et al., it was shown that inflammation induced degeneration of DA neurons in SN can be protected by IL-10 (Arimoto et al. 2007). Thus, IL-10 shows the neuroprotective activity in Parkinsonian mice. Microglia activation increases neurotoxicity, and therefore, contributes to degeneration of neurons via the release of inflammatory, and immunomodulatory cytokines, such as IL-1β, TNF-α, IL-6, IL-8, IL-12, IL-15, and IL-10. Besides microglia, astroglia also play a role in inflammatory process by releasing various cytokines (Block and Hong 2007; Teismann and Schulz 2004). Several studies suggest that astrocyte increases the expression of inflammatory cytokines such as IL-1β, TNF-α and other cytokines under pathological conditions. In addition to directly release pro-inflammatory cytokines, astrocytes can also be activated by cytokines such as TNF-α and IL-1β from microglia (Saijo et al. 2009). Thus, IL-1β, IL-12 and TNF-α are the proinflammatory cytokines, while IL-10 shows the anti-inflammatory activity in neurodegenerative diseases such as PD (Arimoto et al. 2007).

From the mRNA levels, it is clearly evident that proinflammatory cytokines IL-12, IL-1β and TNF-α are increased in MPTP-intoxicated mice, while it is significantly restored in TCAE-treated group. On the other hand, the mRNA level of anti-inflammatory cytokine IL-10 was downregulated in MPTP group while it was restored in TCAE-treated group.

TH plays a vital role in the dopamine synthesis pathway. The change seen in the pattern of TH expression is directly linked to the number of DA neurons present in SNpc (Haavik and Toska 1998). Our study deals with the reduction of TH-positive neurons in SNpc of MPTP-intoxicated mice as compared to control group in which DA neurons are seen to be intact. On TCAE treatment, the TH-positive neurons were significantly restored. Our results are in accordance with some previous studies (Yadav et al. 2017; Cheng et al. 2008). This demonstrates that TCAE exhibits neuroprotection of DA neurons in MPTP mice.

Conclusion

The study presented here delineates the neuroprotective function of TCAE against MPTP-induced DA neurodegeneration in PD mouse model. TCAE is capable of inhibiting the oxidative stress and neuroinflammation occurring in nigrostriatal tissues and simultaneously protect the TH-positive cells in SNpc of the MPTP-induced PD mouse brain. It is evident from our study that TCAE has strong neuroprotective potential which is mainly mediated by its antioxidant and anti-inflammatory activities. Taken together, TCAE appears to be a potential drug candidate in the neuroprotection of PD.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no competing interests.

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