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Phloretin attenuates behavior deficits and neuroinflammatory response in MPTP induced Parkinson's disease in mice

Gejuan Zhang[a,1], Geqiang Yang[b,1], Jian Liu[c,*]

[a] Department of Neurology, Xi'an Jiaotong University Affiliated Xi'an Central Hospital, No.161, Xiwu Road, Xincheng District, Xi'an, Shaanxi Province 710003, China
[b] Department of Ophthalmology, Xi'an Jiaotong University Affiliated Xi'an Central Hospital, No.161, Xiwu Road, Xincheng District, Xi'an, Shaanxi Province 710003, China
[c] Department of Physiology and Pathophysiology, School of Basic Medical Sciences, Xi'an Jiaotong University Health Science Center, Key Laboratory of Environment and Genes Related to Diseases, Ministry of Education of China, No.76 Yanta West Road, Yanta District, Xi'an, Shaanxi Province 710061, China

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ABSTRACT

Neuroinflammation is one of the significant neuropathological conditions in Parkinson's disease (PD) which is due to microglial and astrocytes activation leads to progressive dopaminergic neuronal loss. To date, Current PD drugs offers only symptomatic relief with adverse effects and lack of ability to prevent the progression of neurodegeneration. Therefore, a better approach to develop a multi potent drug of natural origin would be beneficial in managing the disease. Therefore, the present study aimed to investigate the neuroprotective and anti-inflammatory effects of PHL by exploring its neuroprotective mechanism in 1-methyl-4-phenyl-1,2,3,6-tetrahydro pyridine (MPTP) induced PD in mice. MPTP intoxication in mice cause motor abnormalities, decreased dopamine (DA) levels, reduced tyrosine hydroxylase (TH) enzyme protein expression and inflammation which were effectively restored by PHL. Moreover gliotic specific inflammatory markers like glial fibrillary acidic protein (GFAP), ionized calcium-binding adaptor protein-1 (Iba-1), iNOS and COX-2 were found to be expressed more in MPTP intoxicated mice, Further the levels of proinflammatory cytokines like IL-β, IL-6, and TNF-α were significantly upregulated in MPTP intoxicated mice, these deleterious responses were diminished to extend neuroprotection by PHL treatment. Our findings strongly suggest PHL as a potent therapeutic agent in treating PD.

1. Introduction

Parkinson's disease (PD) is the second most common and progressive age related neurodegenerative disease of central nervous system (CNS) after Alzheimer's disease (AD). The hallmarks of PD pathogenesis is characterized by progressive loss of nigrostriatal neurons in the midbrain, which results in depletion of striatal DA level leads to irretrievable motor deficit such as tremor at rest, bradykinesia, rigidity and postural instability [1,2]. Since the current pharmacological therapy in PD based on supplying the neurotransmitter dopamine, which only extends a cure of reducing the clinical symptoms but cannot protect the dopaminergic neuron loss, which is a fundamental problem in PD pathogenesis [3]. Therefore, it is necessary to develop new drugs with multiple potentials in modulating overall PD pathogenesis without any side effects.

Various factors including environment, aging and genetics are associated with PD etiology. Despite extensive research studies, the etiology of dopaminergic neuronal loss in midbrain yet a thing to explore to get a clear view of PD progression [4]. In the last few decades, extensive evidences suggest that innate and adaptive immunity made a great impact in the pathogenesis of PD [5–9]. In the progression of PD, neuroinflammation mainly through the activation of resident cerebral immune cells, microglial. Microglia activation can directly damage dopaminergic neurons *via* overproduction of pro-inflammatory cytokines, including nitric oxide, TNF-α, IL-1β and IL-6. Further, the activated microglia also leads to astrocytosis as consecutively attracting an influx of peripheral immune cells and mutually making a vicious complex of inflammation. Therefore, early therapeutic intervention in attenuating the pathogenesis of PD through getting rid of inflammation by intervening the pro-inflammatory mediators could be an effective therapy to decrease the progression of neuroinflammation.

PD therapies linked with natural compounds with manifold potentials hopefully improve the quality of life with less side effects. PHL belongs to the class of flavonoids, found in apple leaves and exhibit

* Corresponding author.
E-mail address: suyuqiang_007@sina.com (J. Liu).
[1] Equal contribution.

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various pharmacological activities such as antioxidant, anti-inflammatory, anti-cancer, anti-apoptotic and neuroprotective effects in both cellular and animal models [10–12]. Previous investigations stated that PHL able to cross the blood brain barrier and extend appreciable neuroprotective effect [13], reported that certain polar and neutral molecules like phloretin are highly lipophilic and able to cross the blood-brain barrier. Ghumatkar et al. [14] in their neuroprotective study in AD stated that high absorption of phloretin at the site of action (brain) is responsible for the potential neuroprotective effect.

Ullen et al. [15] demonstrated that intraperitoneal application and subsequent GC–MS analysis of brain lipid extracts revealed the ability of phloretin to penetrate the BBB of C57BL/6J mice. Data of their study have indicated that phloretin attenuated 2-ClHDA-mediated brain endothelial cell dysfunction by scavenging 2-ClHDA. Furthermore it was reported that, pretreatment with PHL could inhibit the lipid peroxidation and improved the antioxidant status through Nrf2 activation in rats model of cerebral injury [16]. Moreover, recently Ghumatkar et al. [14] reported that PHL treatment reinstate the impaired neuronal plasticity on Aβ (1-42)-induced hippocampal neurogenesis in rats model of AD. PHL has neuroprotective effect through suppression of reactive oxygen species and normalized mitochondrial dysfunction in rotenone exposed *in vitro* PD model [17]. The current study aimed to investigate the neuroprotective effect through the anti-inflammatory effects of PHL against MPTP induced parkinsonian mice model.

2. Materials and methods

2.1. Reagents

MPTP and PHL were purchased from sigma Aldrich (St. Louis, MO, USA). ELISA kit for Tumor necrosis factor alpha (TNF-α), Interleukins (IL-6 and IL-1β) were obtained from R&D system, China. Tyrosine hydroxylase (TH), GFAP, iNOS and COX2 were purchased from Cell signaling, USA, Iba-1 purchased from Abcam and all the other chemicals were used in this study are of analytical grade.

2.2. Animals and MPTP treatment

C57BL/6 male mice (25–30 g) were used in this study. The mice were acclimatized for 7 days. The animals were maintained at (22 ± 2 °C) on 12:12-hour light/dark cycle and they were allowed to have free access to pellet food and water. All the experimental procedures which were conducted were approved by the institutional animal ethical committee. Animals were randomly divided into four groups with six animals each as follows, control group I mice treated with normal saline (0.5 ml), mice group II of PD induction received intraperitoneal injection of MPTP (30 mg/kg bw) daily for five consecutive days [18]. Treatment group III PHL (5 mg/kg body wt.) + MPTP (30 mg/kg bw) for 14 days and MPTP was injected from the 10th day onwards. Group IV mice received oral administration of PHL alone for 14 days. At the end of the treatment schedule behavioral tests were conducted. After behavior pattern analysis animals were anaesthetized and sacrificed, immediately brain samples were dissected and kept in freezer at −80 °C for further analysis.

2.3. Rotarod test

Rotarod was used to assess the motor coordination. The assessment depends upon the period of time that mice can retain themselves on a rotating rod. Prior to the test, each animal was given 1 min trial on the moving rod. They were placed on a rotating rod with acceleration ranges from 5 to 15 rpm and were assessed for their motor coordination for 300 s. Latency of fall from rolling rod was observed. Normal mice could retain itself on the rotating rod for an indefinite duration of time. The motor performance was evaluated 3 times per day with 30 min intervals and the average retention time was calculated according to the previously studied protocol [19].

2.4. Grip test

Grip test was performed as per the previous method [20]. In brief, the test setup consists of metallic wire of 50 cm length, tied between two vertical supports of 40 cm on a flat surface. The mice were placed on the metallic wire and gently turned upside down. The animals were allowed to stay on metallic wire by holding for maximum 90 s.10 chances were given each animals with intervals of 60 s. The best longest latency to fall or release both hind limps values was recorded.

2.5. Footprint analysis

The foot printing test was performed as per the previous study with slight modification [21]. Briefly, the forepaws and hind paws of animals were dipped with nontoxic paints and the animals were immediately placed to walk across a white sheet of paper without stopping. The stride length was analyzed by measuring the distance of each step on the same side limbs.

2.6. Measurement of DA level

Male C57BL/6 mice were sacrificed and striatum region were collected to measure the level of DA. On the day of study, the weighed striatal tissues were homogenized in ice cold phosphate buffer saline and centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was collected and filtered through a nylon syringe 0.22 μm and striatal samples 20 μl were injected using a Rheodyne 7725 injector for analysis. The level of striatal DA was measured by High Performance Liquid Chromatography coupled to an Electrochemical Detector (HPLC-ECD, Waters, USA) with a potential set at +0.45 V [22,23]. The concentration of DA results was calculated as a μg/g wet tissue.

2.7. Determination of TNF-α, IL-1β and IL-6 cytokines

Enzyme linked immunosorbent assay (ELISA kit, R&D system, china) was used to measure the levels of IL-6, IL-1β and TNF-α. The brain samples were added into the 96 well plates and the respective antibody IL-6, IL-1β or TNF-α were added and incubated at 4 °C overnight. Followed by, 1% BSA was added to block for 1 h at room temperature. Then 50 μl of horseradish peroxidase (HRP) was added and all the wells were incubated for 45 min at room temperature. Finally, the 96 well plates were read at 450 nm and the results were expressed as pg/mg of protein.

2.8. GFAP for immunohistochemistry

Substantia nigra tissues were fixed with 10% phosphate buffered formalin for 24 h, and paraffin section were processed. The sections were then incubated with H_2O_2 (0.3%) for 10 min at room temperature in dark to exhaust endogenous peroxidase activity and the slides were incubated with blocking buffer 10% normal goat serum at 37 °C for 30 min. Then the tissue sections were incubated with primary antibody GFAP (1:250) at 4 °C overnight. Sections were washed three times in PBS, the slides were incubated with ant-rabbit HRP conjugated secondary antibody 1:5000 dilution for another 30 min at room. The sections were developed with diaminobenzidine (DAB) and the images were observed under a light microscope.

2.9. Western blot analysis

In brief, proteins were isolated using ice-cold RIPA buffer containing protease inhibitor cocktail. Protein concentration was estimated by nanodrop spectrophotometer. Accurately, 50 μg of proteins was loaded onto the 10% SDS and separated proteins were transferred into PVDF

membranes. The membranes were blocked with the 5% BSA for 1 h then the membranes were incubated with respective primary antibodies TH (1:1000), Iba-1 (1:500), COX2 (1:1000) and iNOS 1:1000 dilution in 5% BSA in Tris-buffered saline and 0.05% Tween-20 (TBST) overnight. The membrane was incubated with secondary antibody horseradish peroxide conjugate for 2 h at 37 °C. Finally, each membrane was developed using an enhanced chemiluminescence method detecting of horseradish peroxide then blots were quantified using "Image J" analysis software.

2.10. Statistical analysis

The results are presented as the mean ± standard deviation (SD) and were evaluated with one-way analysis of variance using statistical package for the social science (SPSS, software package version 12.0.) and Duncan's multiple range test was used to compare significant variation between the groups $p < 0.05$.

3. Results

3.1. PHL conspicuously mitigates MPTP induced behavioral impairments

Fig. 1A, B and C showed motor coordination in experimental animals exhibited in rotarod test. MPTP induced mice showed poor neuromuscular coordination were significantly shows reduced retention time as compared to control ($P < 0.05$). Moreover, none of the MPTP induced animals could balance themselves on the rotating rod for full cut-off time (300 s). Pretreatment with PHL distinctly enhanced balancing ability and retention time and inhibited disorientation when compared to MPTP treated mice ($P < 0.05$).

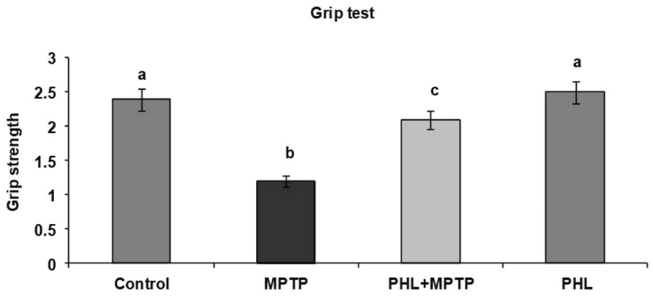

Fig. 2. Grip test performance in control and experimental mice.
The grip strength significantly reduced in MPTP induced group as compared to untreated control group. Pretreatment with PHL prior to MPTP injection has protected motor impairments as compared to MPTP alone group. Values are given as mean ± SD for six mice in each group. Statistically significant $^{b}p < 0.05$ compared to untreated control, $^{c}p < 0.05$ compared to MPTP alone group.

The average hanging time significantly decreased in MPTP induced when compared to the control mice ($P < 0.05$). Meanwhile, pretreatment with PHL in MPTP induced mice showed a significantly improved hanging time as compared to the PD induced mice (Fig. 2).

Abridged stride length is one of the chief distinctiveness of abnormal gait in PD. The result of our study demonstrated a significant decrease in forelimb and hind limb stride length in the MPTP induced mice (Fig. 3). But the stride length in the PHL and MPTP treated mice were longer than those of MPTP treated group. In addition no significant differences in stride lengths were observed between PHL alone

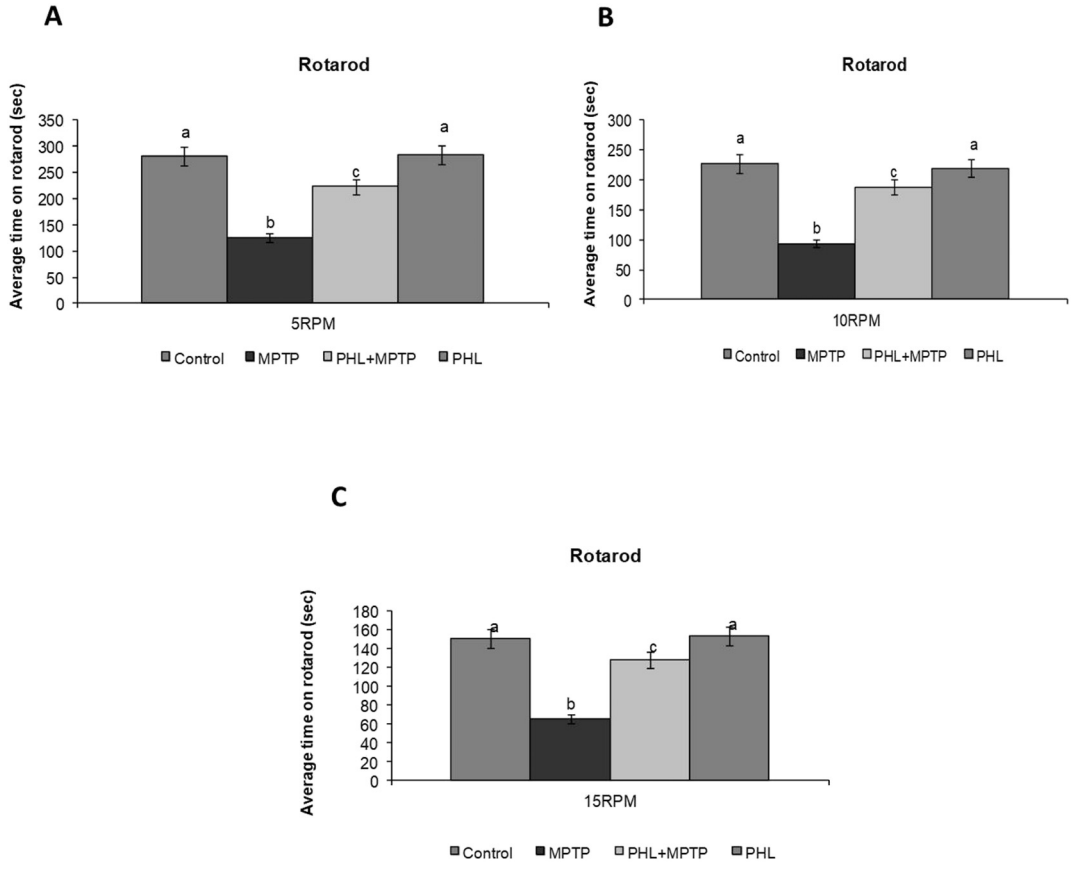

Fig. 1. A, B and C shows the rotarod performance of control and experimental animals.
MPTP induced group significantly decreased motor coordination as compared to control and PHL treatment significantly recovered in PHL + MPTP induced group as compared to MPTP alone induced animals. Data are shown as mean ± SD for six animals in each group. Statistically significant $^{b}p < 0.05$ compared to untreated control, $^{c}p < 0.05$ compared to MPTP alone group.

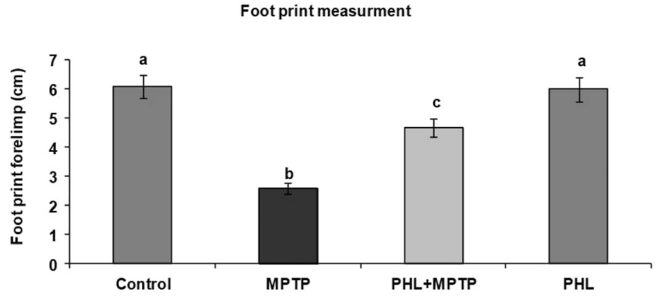

Fig. 3. Illustrate the stride length measurement in control and experimental animals. Forepaw stride length was diminished in MPTP injected group as compared to control. Treatment with PHL followed by MPTP significantly improved the forepaw distance as compared to only MPTP group. Values are given as mean ± SD for six mice in each group. $^{b}p < 0.05$ compared to untreated control, $^{c}p < 0.05$ compared to MPTP alone group.

treated and control animals.

3.2. Effect of PHL on dopamine levels

Striatal DA levels were significantly ($P < 0.05$) depleted in the MPTP treated mice compared to saline treated control mice (Fig. 4). Pretreatment with PHL significantly ($P < 0.05$) maintains DA levels compared to MPTP group. No significant difference was observed between PHL alone treated and control mice.

3.3. Effect of PH on MPTP induced TH protein expression

As shown in Fig. 5A and B TH protein expression in ST significantly diminished in MPTP treated PD induced group when compared to control group. Whereas, the expression of TH in PHL pretreated mice significantly exhibited enhancement in the TH protein expression than the MPTP induced alone group. Moreover, there is no significant differences were observed between PHL alone treated and control group.

3.4. Effect of PHL on MPTP induced TNF-alpha, IL-1 beta and IL-6 protein concentration by ELISA

MPTP induced mice showed significantly increased expression of TNF-α, IL-1β and IL-6 concentration in brain tissue when compared to untreated control group. Whereas, mice pretreated with PHL in MPTP induced mice drastically decreased the levels of TNF-α, IL-1β and IL-6 expression compared to mice treated with MPTP alone group (Fig. 6). Untreated control group and PHL alone treated group did not exhibit any significant changes.

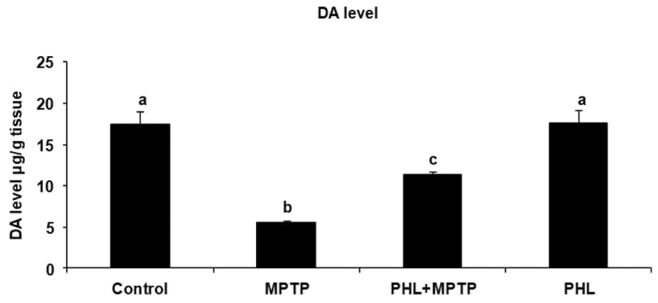

Fig. 4. Illustrate the levels of DA in control and experimental PD animals. Administration of PHL significantly protected the depletion of DA in MPTP-induced mice. Values are expressed as mean ± SD and statistically significant $^{b}p < 0.05$ compared to untreated control, $^{c}p < 0.05$ compared to MPTP alone group.

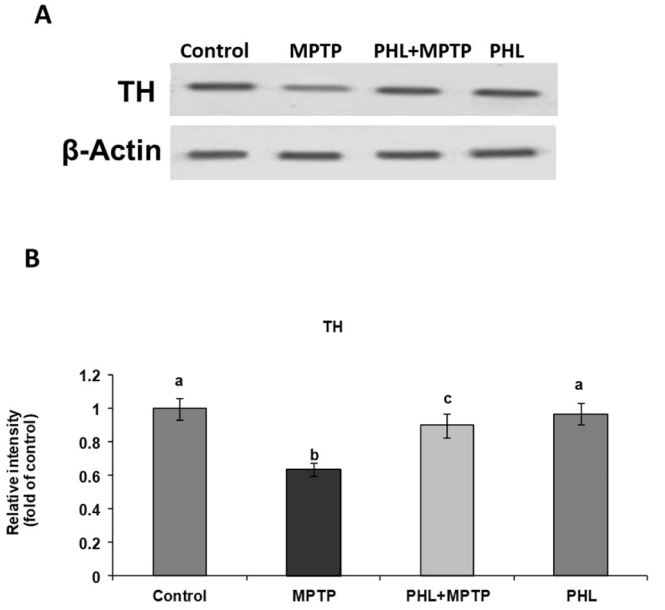

Fig. 5. Shown the effect of PHL on MPTP induced control and experimental animals. TH protein expression in control and experimental animals illustrate MPTP depletes the TH protein expression. Group I untreated control, group II MPTP, group III PHL + MPTP, group IV PHL treatment. Protein quantification was performed by densitometric analysis in image J software and the values are representing the mean ± SD of band intensity values. Statistically significant $^{b}p < 0.05$ compared to untreated control, $^{c}p < 0.05$ compared to MPTP alone group.

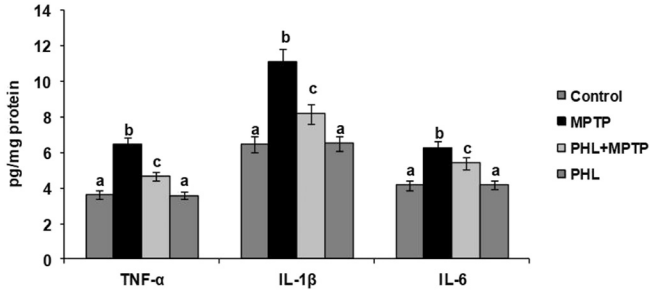

Fig. 6. Represent the amelioration in pro-inflammatory cytokine on PHL treatment. The values are representing the mean ± SD of band intensity values. Statistically significant $^{b}p < 0.05$ compared to untreated control, $^{c}p < 0.05$ compared to MPTP alone group.

3.5. Effects of PHL on MPTP induced enhanced glial markers expression

Figs. 7, 8 and 9 showed GFAP, Iba-1, iNOS and COX2 the specific markers for microglial and astrocytes. Increased expression of GFAP (Fig. 7), Iba-1 (Fig. 8), iNOS and COX2 were encountered in MPTP intoxicated mice due to increased activation of reactive astrocytes and microglial inflammatory response when compare to saline treated control mice (Fig. 9). However, PHL pretreatment dramatically lower the GFAP, Iba-1, iNOS and COX2 expression when compared with MPTP alone treated mice ($p < 0.05$). Our study results elucidated that the PHL pretreatment significantly inhibited the activation of microglia and astrocytes.

4. Discussion

PD is characterized by behavioral abnormalities, motor deficits and decline in cognition and memory due to neurodegeneration which is progressive and has no proper treatment or remedial measures to halt or reverse the disease conditions. The pathologic conditions of PD can

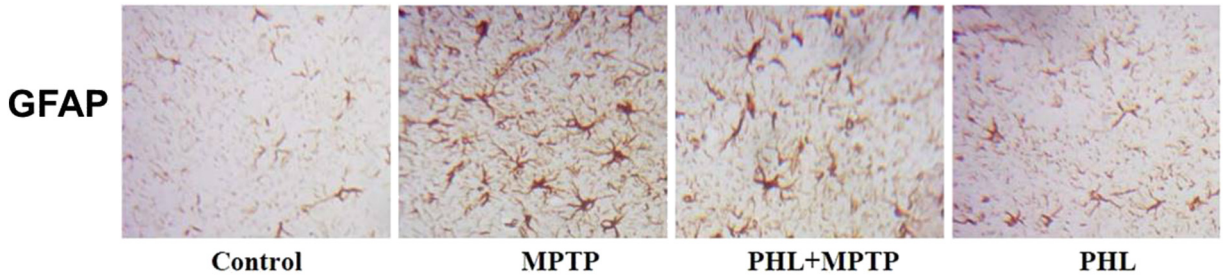

Fig. 7. Representative microphotographs of PHL on MPTP induced GFAP immunoreactivity in control and experimental animals.
GFAP immunoreactivity in the striatum of a Group I untreated control, group II MPTP, group III PHL + MPTP, group IV PHL treatment. Original magnifications at 40×.

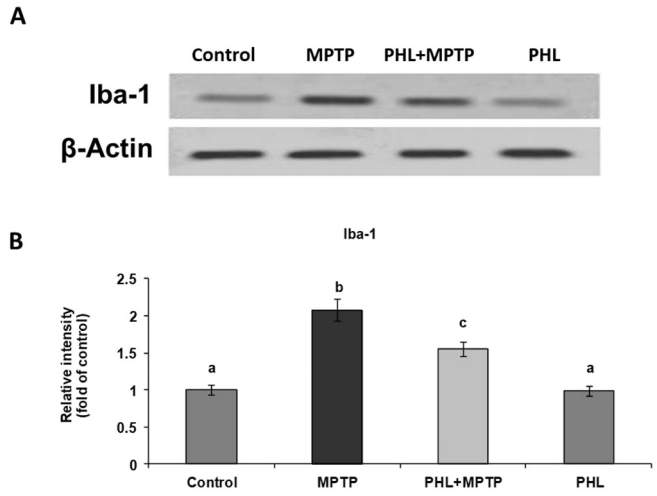

Fig. 8. PHL amelioration in Iba-1 on MPTP induced control and experimental animals. Iba-1 protein expression was determined by western blotting.
Group I untreated control, group II MPTP, group III PHL + MPTP, group IV PHL treatment. Protein quantification was performed by densitometric analysis in image J software. The values are representing the mean ± SD of band intensity values. Statistically significant $^{b}p < 0.05$ compared to untreated control, $^{c}p < 0.05$ compared to MPTP alone group.

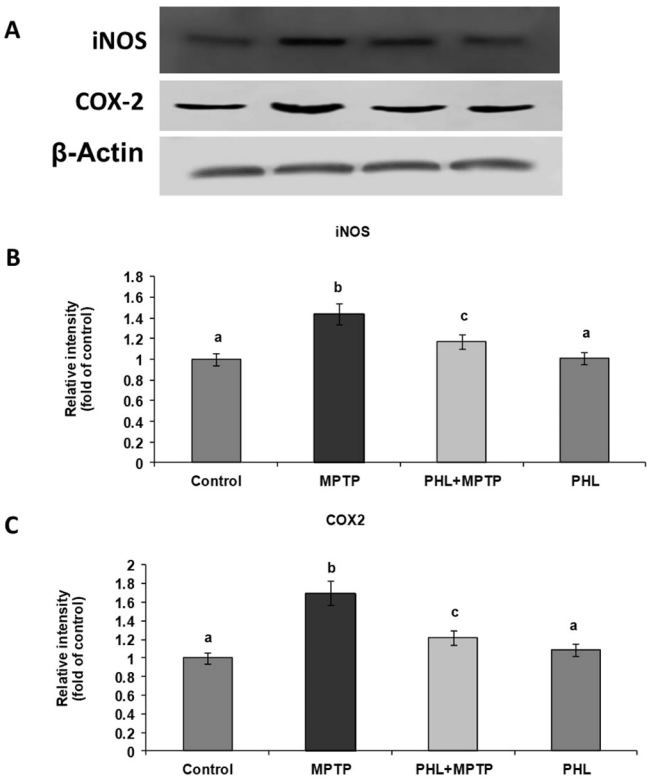

Fig. 9. Illustrate the effect of PHL against MPTP induced iNOS and COX2 protein expression in control and experimental animals.
iNOS and COX2 protein expression in the striatum of group I untreated control, group II MPTP, group III PHL + MPTP, group IV PHL treatment. Protein quantification was performed by densitometric analysis in image J software. The values are representing the mean ± SD of band intensity values. Statistically significant $^{b}p < 0.05$ compared to untreated control, $^{c}p < 0.05$ compared to MPTP alone group.

be created by MPTP administration in animals to make them as experimental PD models which exhibit odd behavioral changes and pathophysiological features of neurodegenerative disorders, including oxidative stress, dopaminergic neurodegeneration and neuroinflammation-mediated glial cell activation [24,25]. Previous studies also showed that the MPTP administration to rodents and primates induce DA neuron loss in SNpc and cause depleted a DA level which leads to severe motor dysfunction [26]. In the present study, we investigated the neuroprotective role of PHL in MPTP induced degeneration of dopaminergic neurons which is well elucidated through enhanced DA levels, improved behavioral impairments, inhibition of glial cell activation and suppression of inflammatory responses in the mice of experimental groups treated with PHL. Recent studies also showed that PHL pretreatment protect the pathological changes and enhanced subsequent behavioral performance in mice models of neurodegenerative diseases [14,17].

Our findings revealed that PHL could effectively improve the motor coordination and gait induced by MPTP. Motor coordination ability of experimental animals was evaluated by performing rotarod test. MPTP induced mice shows poor neuro-muscular coordination and also exhibited significant reduction in retention time. PHL pretreatment distinctly enhanced balancing ability and retention time and inhibited disorientation. The average hanging time significantly decreased in MPTP induced when compared to the control, pretreatment with PHL in MPTP induced mice showed a notable elongation in, In foot print analysis, shortened stride length is one of the principal characteristics of abnormal gait in PD. The result of this study demonstrates a significant decrease in forelimb and hind limb stride length in the MPTP induced mice. Meanwhile, the stride length measurements in the PHL pre-treated mice were longer than those of MPTP animals. There is no significant changes were observed in PHL and control alone treated mice. Our results in behavioral pattern analysis were go along with the previous investigations [27].

In this study, TH plays an important role in the synthesis of dopamine. DA neurons loss in the SN halts the synthesis of dopamine and decreased the concentration of DA levels in ST due to the unavailability of TH which is well elucidated through decreased expression of TH proteins [28]. Our findings are in accordance with the findings of previous studies ([29,45]).

Quite a lot of research of evidences suggests that neuroinflammatory processes could be significant in the development of PD. T lymphocytes and activated microglial cells have been detected and reported in the substantia nigra of patients with increased expression of pro-inflammatory mediators. Moreover different findings assured that not as a primary reason but inflammatory processes are instrumental in neuronal cell death in investigations conducted in various PD models. Neuroinflammatory processes in PD are fairly involved in self-induced deleterious actions that lead to extended neuronal degeneration [30]. Inflammatory response leads to degeneration of dopaminergic neurons and cognitive dysfunction coupled with dementia [31]. Several researchers have been strongly suggested that microglia activation to be involved in neuroinflammation might play a key role in the pathogenesis of PD [32,33]. Various studies have reported that activation of microglial and subsequent neuroinflammatory induced neurodegeneration caused by MPTP lesioning [34–36].

Similar to these reports we also observed that there is significant increase in the protein expression of the microglial activation markers like GFAP and Iba-1 in MPTP induced inflammatory responses and alterations in the density of GFAP positive cells indicated the severe loss of dopaminergic neurons in the SN. Our results are well agreed with earlier findings of different studies [37–39]. Additionally, microglia activation generates numerous inflammatory mediators such as iNOS and COX2 that accordingly promotes the release of cytokines and inflammatory response leads to degeneration of dopaminergic neurons [40]. Furthermore, microglial activation leads to NF-kB nuclear translocation that upregulated the release of pro-inflammatory enzyme COX-2, iNOS, TNF-α, IL-1β in PD has also been reported [41]. In the present study up regulated expression of iNOX and COX2 associated with enhanced the levels of cytokines such as IL-1β and IL-6 were observed. PHL treatment dramatically decreased GFAP and Iba-1 expression that indicates decreased microglial activation which leads to down regulated expression of iNOS and COX-2 might be the reason for decreased levels of cytokines such as IL-1β and IL-6. Previous studies strongly suggest that inhibition of neuroinflammation as a prospective strategy in protection of motor deficit and cognitive dysfunction in PD [42–44], our findings also well agreed with these previous reports since PHL treatment dramatically reduced the entire inflammatory induction and progression.

Our results revealed that the PHL prevent degeneration of dopaminergic neuron through improved behavioral impairments, preventing dopamine depletion, restored TH expression, attenuating microglial activation and pro-inflammatory cytokine production. These findings suggest that PHL endorse neuroprotection and acting against inflammatory cascades can be a potential and effective drug candidate to treat PD. Further research and preclinical studies are warranted to extrapolate the PHL for PD management.

Declaration of Competing Interest

The authors declare that they have no competing interests.

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