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RESEARCH ARTICLE
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EFFECTS OF ETHYL PYRUVATE IN LIPOPOLYSACCHARIDE MODEL OF PARKINSON'S DISEASE

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Abstract:
Objective: This study aims to evaluate the therapeutic effects of Ethyl Pyruvate (EP) in lipopolysaccharide (LPS)-induced mouse model of Parkinson's disease (PD).

Methods: Total 24 male Balb-c mice were used for the study. 4 day repeat injection model of LPS (1mg/kg/day) was chosen. EP was administered half hour before the LPS in co-treatment group (40mg/kg/day). ELISA test was performed for serum analysis of TNF-alpha levels. Immunohistochemistry was done to analyze the Tyrosine Hydroxylase (TH) positive cell count in Substantia Nigra (SN) of mice brain. Pole behavioral test was done by recording T_{total} in seconds.

Results: Our study showed that TH+ cell count was significantly reduced in LPS treated group as compared to control group. This count was improved after the administration of EP in co-treatment group. These effects of EP were supported by the results of ELISA, which showed significant reduction in TNF-alpha levels of co-treatment group (LPS + EP) in contrast to LPS treated group. Motor deficits were also improved as assessed by pole behavior test showing a reduction in T_{total} of (LPS + EP) group as compared to T_{total} of LPS treated group.

Conclusion: In this study we found significant improvement in dopaminergic neuron count, and motor deficits of PD model. Level of Inflammatory marker was also reduced significantly confirming the ant-inflammatory potential of EP. Together these data supports that progressive neurodegeneration of PD is slowed down after the use of EP.

Keywords: Parkinson's disease, Lipopolysaccharide, Ethyl pyruvate, neurodegeneration, glial cells, inflammation, Substantia Nigra, motor disorder

ABBREVATIONS:
TNF: Tumor Necrosis Factor,

LPS: Lipopolysaccharide,
SNPC: Substantia Nigra Pars Compacta,
TH: Tyrosine Hydroxylase,

1. Introduction:

In the list of neurodegenerative diseases, Parkinson's disease (PD) is ranked second most prevalent condition worldwide, 1st being the Alzheimer's disease (1). However in the spectrum of movement disorders, PD stood first (2). Parkinson's disease affects about 0.3 percent of individuals all over the world (3, 4). Both genetic and sporadic cases of disease are reported but frequency of sporadic cases of PD is much higher, nearly 90% of all cases (5).

PD is a combination of motor and non-motor symptoms. Motor symptoms include tremors at rest, bradykinesia, rigidity and postural instability (6). Non-motor symptoms include cognitive defects and gastrointestinal disturbances like constipation and these can be present years before the onset of motor symptoms (7, 8).

One of the most important pathological findings in PD is progressive loss of dopaminergic (DA) neurons in substantia nigra (SN) and striatum (9). Substantial neuronal loss occurs in the caudal and ventrolateral parts of SN (10, 11). It is interesting that the initial clinical features manifest in humans many years after the start of the neurodegenerative process when at least 50 percent of DA neuronal bodies in SN and around 70 to 80 percent of axonal endings in striatum has been lost (12).

Another characteristic pathology is Lewy body (LB) formation which are cytoplasmic inclusions made up of different proteins of which protein alpha-synuclein has major contribution (8).

Patients with PD exhibit severe gliosis and elevated proinflammatory markers and receptors in both CSF and neural tissue, which raises the possibility that inflammation may be a major contributing component in the causation of PD (13). Microglial cells on activation produces pro-inflammatory cytokines including interleukin-1 beta (IL-1β), interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) leading to neuronal injury and destruction (14). DA neurons are extremely susceptible to the death-promoting features of cytokines including (TNF-α) and (IL-1β) which can in turn be due to the presence of these neurons in those areas of brain where microglial cells are most abundant (15).

To further understand the pathogenesis and treatment of PD, a suitable animal model that represents the clinical and pathological aspects of the disease is required. Lipopolysaccharide (LPS) is increasingly used as an inflammatory model of PD in recent years. LPS exerts its neuroinflammatory effects by interacting with toll like receptors 4 (TLR4) and CD-14 receptor complex present on astrocytes and glial cells (16).

Recent researchers have now focused to look for molecules which can target the basic mechanisms involved in death of DA neurons. Pyruvate is an important end product of glycolysis which is delivered to mitochondria as a fuel for ATP production. Disturbances in pyruvate metabolism can affect tissues with high energy demands such as neurons. Considered to be a potent source of pyruvate, ethyl pyruvate (EP) is a steady and lipophilic derivative of pyruvate. Ethyl pyruvate has role in reducing neuroinflammation due to its ability to block glial cell excitation and subsequent release of IL-1β and TNF-α and at the same time reducing apoptosis by reducing the caspase-3 levels (17). Based on the neuroprotective roles of EP, we postulate that supplementation of EP in a mouse model of PD can show improvement in behavioral deficits and pathological abnormalities.

2. Materials and Methods:

Ethical approval was granted by Institutional Research Ethical Board of Khyber Medical University (KMU) Peshawar, Pakistan. It was a lab based experimental study, in which a mouse model of PD was established using the toxin LPS and co-treatment was done with Ethyl pyruvate to investigate if EP has any beneficial role in treatment of PD. Study was carried out in Animal house and immune-histochemistry lab of Khyber medical university (KMU), Peshawar.

Total 24 animals were selected for the study, which were further divided into 4 groups named as Control group (n=6), LPS group (n=6), LPS+ EP group (n=6) and EP group (n=6). Male Balb-c mice, of age group 8 to 10 weeks were selected for the study. Mice were kept in animal house of IBMS,

KMU at normal room temperature (25° C) and under a light and dark cycle of 12/12 hours. Special cages were selected for the mice with a measurement of 16 X 10 X 10 inches. Food and water were provided *ad libitum.* Weight of the mice in each group was recorded using electronic weighing machine and weight chart was made for calculation of unit-dose of the drug.

Chemicals and Antibodies:
Lipopolysaccharide (Strain Salmonella enterica Abortus equi) CAT# L5886-10MG was purchased from the Sigma-Aldrich. Ethyl Pyruvate (EP) was purchased from Alfa Aesar (CAS# 617-35-6).The antibody against Tyrosine Hydroxylase (TH) was a mouse anti-TH monoclonal antibody from Santa Cruze Biotechnology.

Drug administration:
After 1 week of acclimatization, drug administration was started according to the weight of the animals. LPS was injected intraperitoneally (i.p) at a dose of 1mg/kg/day for four days, according to previously used protocol (7). Control group was injected an equal amount of Phosphate buffer Saline (PBS). EP was administered at a dose of 40mg/kg/day for 4 days and injections were made subcutaneously (18). In LPS+EP group, EP was injected half an hour before the LPS injection. 1ml insulin syringe was used for drug administration. Complete map of experimental work is shown in *Figure 2-1.*

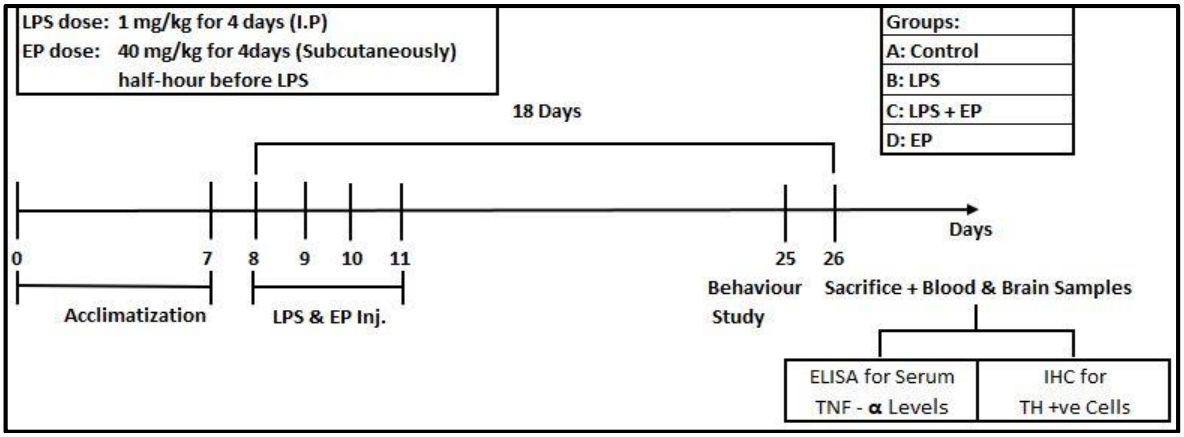


Figure # 2-1: Experimental design

Behavioral Study:
Behavioral study was carried out 1 day before the animal sacrifice, which was 18th day post-first injection. The behavioral analysis was performed on all the mice included in a group (n=6). The test was done according to guidelines present in previous studies with some changes (3). Time to return the platform of the pole was recorded in seconds (T_{total}).

Tissue Collection:
On day 19 after the 1st injection day, mice were sacrificed using cervical dislocation method and brain tissue was collected (n=3 mice/group) after dissection. Dissection of midbrain section for substantia nigra pars compacta (SNpc) was done using the mouse brain atlas. Brain was placed with its dorsal surface facing upward. Cutting was performed in coronal plane. Brain was preserved in 4% paraformaldehyde after the gross cutting of midbrain section. Tissue processing was performed and formalin fixed paraffin embedded blocks (FFPE) were made. For slide formation, 5µm thin sections were cut and mounted on charged IHC slides (Leica Biosystems, Germany).

Tyrosine Hydroxylase Immunohistochemistry (IHC):
Tyrosine hydroxylase (TH) immunostaining was performed to analyze the number of TH neurons in the Substantia Nigra Pars Compacta (SNpc). After heat induced epitope retrieval (HIER), slides were

treated with Tyrosine hydroxylase (TH) antibody (Santa cruze Biotechnology) as primary antibody in a dilution of 1/100 for 1 hour at room temperature. Following this, anti-mouse secondary antibody (HRP) was applied for 40 minutes. In the final step, 3,3-diaminobenzidine (DAB) from (Leica Biosystems, Germany) was applied for 2 minutes at room temperature. Finally, the slides were dehydrated in different percentages of ethanol (70% and 95%), dipped in xylene, and covered with a thin cover glass by using DPX as mounting medium.

Microscopy and Imaging:
For analysis of TH positive cells, a Nikon multi-head microscope with display option was used. Images were taken at 4X, 10X, 20X and 40X magnifications. TH-positive cell counting was done by using Image J software.

Enzyme Linked-Immunosorbent Assay (ELISA):
For measurement of inflammatory cytokine, the 1-2 ml blood was obtained, (n=3 mice/group) via intra-cardiac puncture. Serum concentrations of Tumor Necrosis Factor-alpha (TNF-alpha) were investigated by using mouse TNF-alpha ELISA kit (CAT# E0117Mo) from Bioassay Technology Laboratory (BT LAB). The kit utilizes the principle of sandwich ELISA. An enzyme-linked immunological detector was used to determine the absorbance at 450 nm.

3. Statistical Analysis:
All the data was put into Microsoft excel sheets and then transferred to Graph Pad Prism 8.0 for statistical analysis. A one-way ANOVA (analysis of variance) followed by post hoc Tukey test were done for comparison between four groups. All the values were expressed as mean ± standard deviation. Graphs were generated. *P value* <0.05 was taken to be significant.

4. Results:
Ethyl Pyruvate Reduced the LPS-induced Motor-Dysfunction in PD-Model:
To assess the motor abnormalities of PD model, Pole behavior test was performed. We found significant increase in T_{total} to reach the platform in LPS treated group, when compared to the control group (*p value*= 0.0001).

However the time to reach the platform was significantly reduced after the administration of Ethyl Pyruvate in co-treatment group LPS + EP as compared to LPS group (*p value*= 0.002) as seen in Fig 3-1. This showed the improvement in motor deficit of LPS model after the administration of EP.

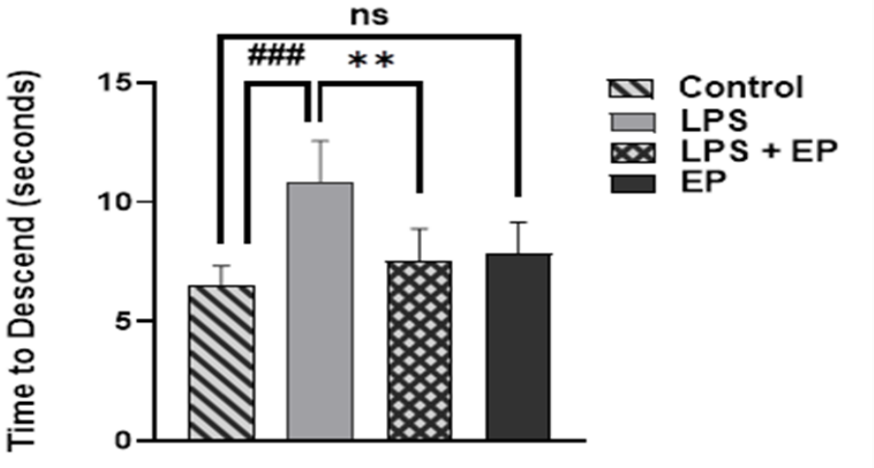


Figure # 3-1: results of Pole behavioral test. ### shows significant difference between control and LPS group (P value=0.0001). ** shows significant difference between LPS and LPS + EP group (P value=0.0020). "ns" shows no significant difference between EP and control.

Ethyl Pyruvate Reduced the Dopaminergic Neuron loss in LPS-Induced PD Model:
Dopaminergic neuron loss is the key abnormality seen in Parkinson's disease. In order to evaluate the effects of Ethyl Pyruvate on LPS-induced loss of dopaminergic neurons in PD model, we analyzed the TH+ cell count of mouse brain SN region via immunostaining.
According to our results, significant reduction was seen in TH+ cell count in SN region of LPS group, when compared to control group indicating the dopaminergic neuron loss. The co- treatment group (LPS + EP) showed significant improvement in TH+ cell count as compared to LPS group showing the beneficial effects of Ethyl Pyruvate (*P value* = 0.032) as seen in Fig 3-2.

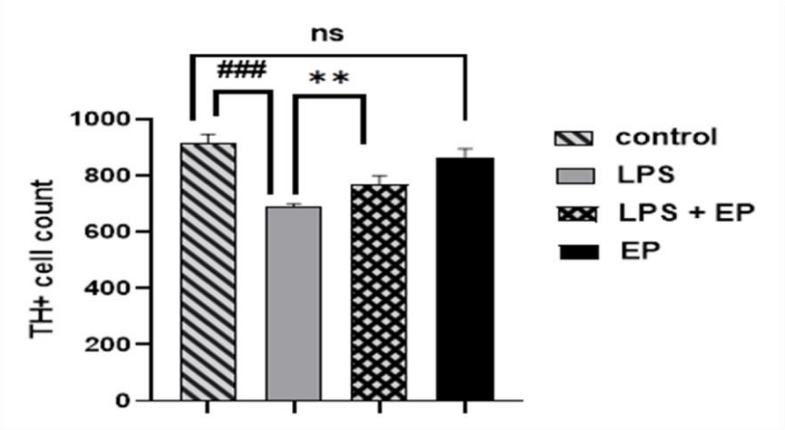


Figure # 3-2: Results of Immuno-histo chemistry for TH-+ve cell count. ### show significant difference between control group and LPS group (P value =0.0001) and ** shows significant difference between LPS and LPS+ EP group (P value= 0.0323). "ns" shows no significant difference between control and EP group.

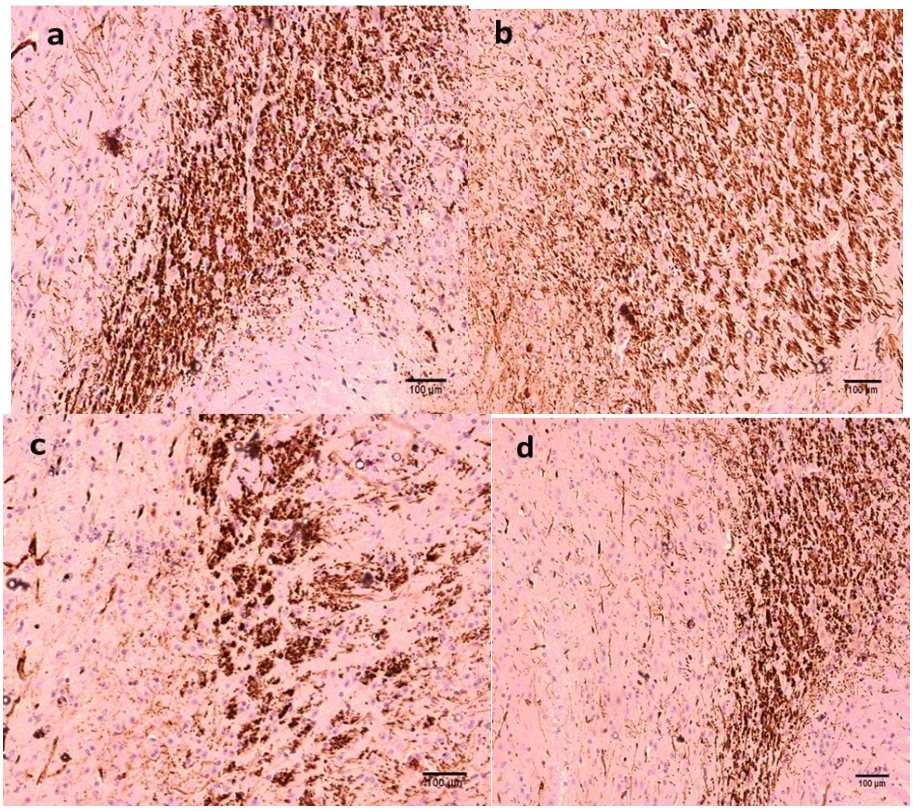
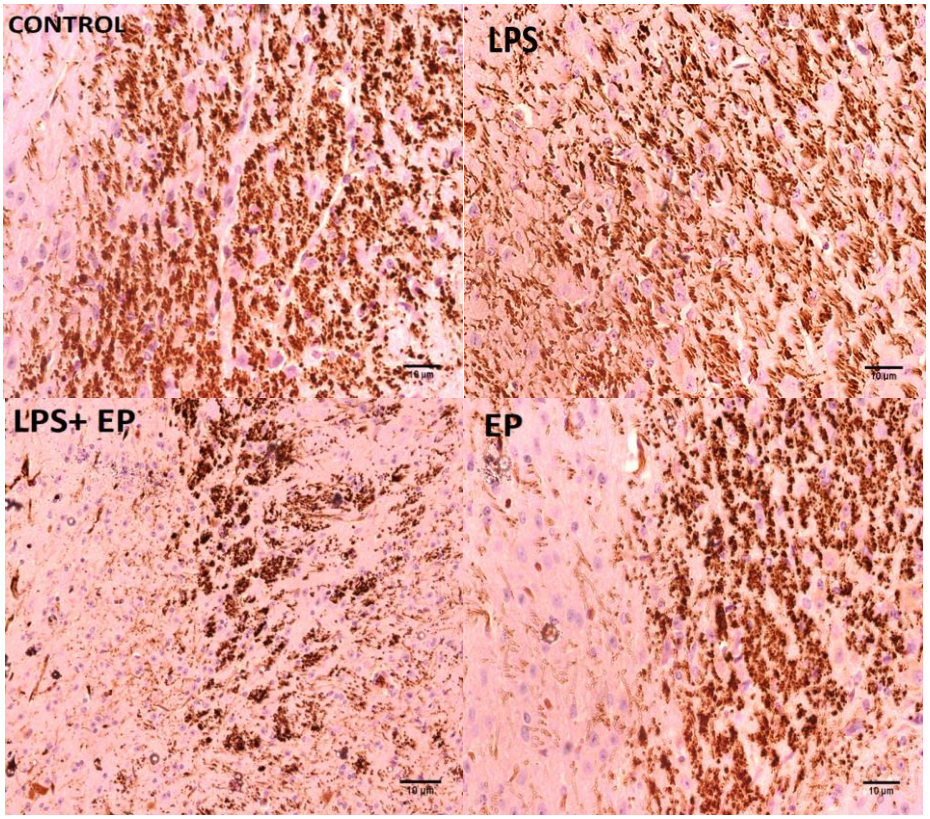


Fig # 3-3: showing the TH+ve immunohistostaining of Substantia Nigra (SN) region. Image **"a"** showing SN in control group, Image **"b"** showing SN in LPS group, Image **"c"** showing SN in co-treatment group LPS + EP, Image **"d"** showing SN in EP group. Scale bar =100µm.



Fig# 3-4: Images showing TH+ cells of four study groups at 40X magnification, scale bar=10µm

EP Protects against LPS-induced Inflammation in PD-Model:

To investigate the anti-inflammatory effects of EP, we tested the levels of inflammatory cytokine. Concentration of TNF-α was significantly higher in LPS group as compared to control group and this level was reduced after administration of EP in the co-treatment group compared to LPS group *(P value* =0.0037) as shown in the Fig 3-5

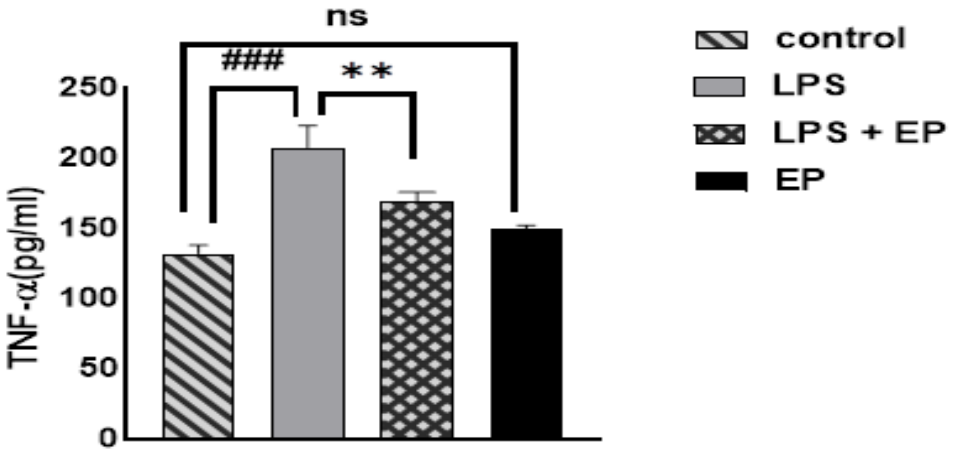


Figure # 3-5: Results of ELISA for TNF-α level. ### shows significant difference between control group and LPS group (P value=0.0001), ** shows significant difference between LPS and LPS+ EP group (P value= 0.0037). "ns" shows no significant difference between control and EP group.

5. Discussion:

The need for a drug which can halt the disease process of PD is rising day by day because of increasing life expectancy which is a cause for PD. In present study we investigated the therapeutic potential of ethyl Pyruvate (EP) against neurotoxic effects of Lipopolysaccharide-induced Parkinson's disease (PD) animal model.

Ethyl pyruvate is a stable derivative of pyruvate. The protective role of EP in CNS is proven in hypoxic-ischemic brain injury model (19). Owing to these beneficial effects of EP on neuronal cells, we evaluated the therapeutic use of EP in PD model of neurodegeneration which is consistent with its use in previous studies where EP has shown the neuro preservative effects in neurodegenerative diseases such as Alzheimer's disease, through inhibition of TLR4 receptors (20) as well as white matter injury models using rodents (17).

In this study we have specifically focused on the LPS-induced model of PD where the LPS induced neuroinflammation can lead to dopaminergic neuron loss in Substantia Nigra (SN).

Many routes can be used to administer LPS for making an LPS-induced PD model. Three of them are commonly used including intranasal, intraperitoneal and stereotaxic routes (21). Intraperitoneal route of administration of LPS was found most suitable for our study. Single as well as repeat LPS injection protocols are available to induce the PD model (22-24). Single injection requires long assessment periods (7 months) for inducing the dopaminergic neuron loss consistent with PD (25). Hence we have chosen a 4 day repeat treatment model for our study (4 day repeat LPS) which has an assessment period of 19 days (7). In current study, EP significantly reduced the motor deficit in LPS-induced mice model of PD. Our finding is in accordance to Satpute R et al; who have shown this beneficial role of EP and α-ketoglutarate by performing the rota rod test (26). The dopaminergic neuron loss induced by LPS in our study is in contrast to the results of Beier E et al who has shown almost 34% loss of TH +ve cells, as compared to 24% loss seen in our study (7). This difference in LPS induced toxicity on Dopaminergic neurons can be attributed to the difference in experimental conditions.

In our study we have demonstrated anti-inflammatory effects of EP against LPS induced neuroinflammation by measuring the mouse serum levels of TNF-alpha, an important inflammatory cytokine. These anti-inflammatory effects of EP have been demonstrated in previous studies as well (18). However, Qiu X et al has reported these anti-inflammatory effects of EP by measuring the serum levels of IL-1, in a sepsis model of LPS.

Author Contribution Statement:

1) Dr. Rabia Syed: Conceptulization, data acquisition, results analysis, writing the original draft
2) Dr. Najeeb Ullah: Conceptualization and Supervision
3) Dr. Muhammad Ikram: Data acquisition, formal analysis, chemicals arrangement and Supervision
4) Dr. Saima Mumtaz Khattak: Data acquisition, writing
5) Dr. Sarwat Jahan: Data acquisition, writing
6) Mahnoor: Chemical arrangement

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