*Section A-Research paper*

# GC-MS analysis of ethanolic extract of *Moringa oleifera leaf* and its neuroprotective effect against MPTP -induced Parkinson's mouse model.

Smriti Singh[a], Aaina Singh Rathore[c], Vijaya Nath Mishra[b], Surya Pratap Singh[c*]

[a] Interdisciplinary School of Life Sciences, Institute of Sciences, Banaras Hindu University, Varanasi

[b] Department of Neurology, Institute of Medical Sciences, Banaras Hindu University, Varanasi

[c] Department of Biochemistry, Institute of Sciences, Banaras Hindu University, Varanasi

Smriti Singh: smriti.singh11@bhu.ac.in

Aaina Singh Rathore: aainas.rathore@bhu.ac.in

Prof. Vijaya Nath Mishra: vnmishra_2000@yahoo.com

Prof. Surya Pratap Singh*: suryasinghbhu16@gmail.com

* Corresponding Author

Prof. Surya Pratap Singh

Department of Biochemistry,

Institute of Sciences,

Banaras Hindu University, Varanasi (221005)

(Uttar Pradesh), India

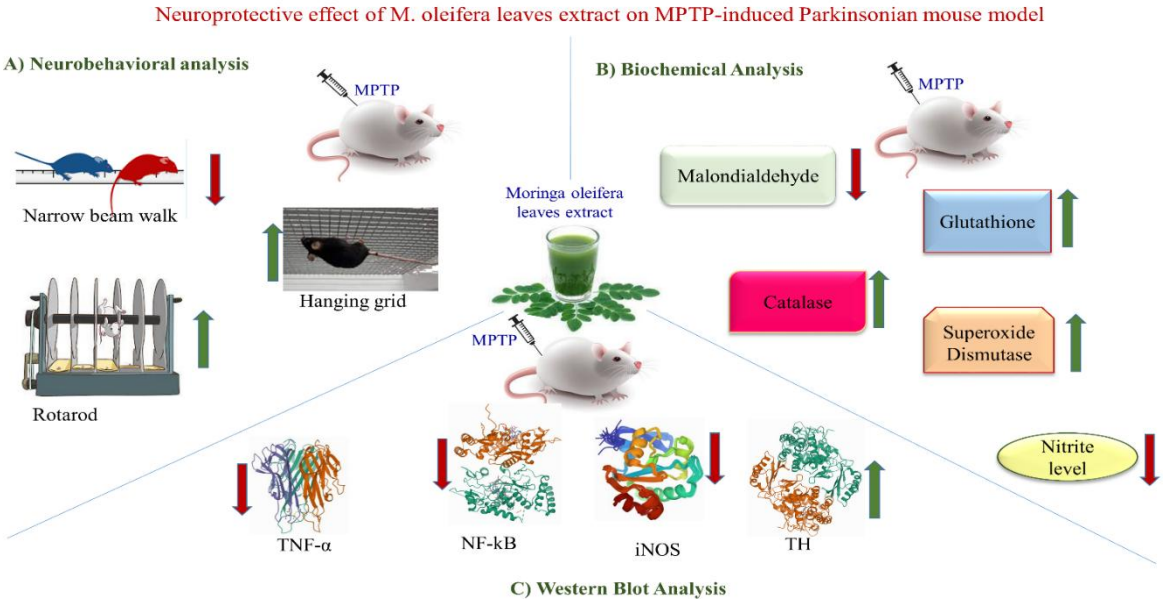
Tel.: +91-9454734930

Email address: suryasinghbhu16@gmail.com

**Abstract**

The pathological hallmark of Parkinson's disease is the progressive loss of dopaminergic neurons. The current treatment regime slows down the disease progression symptomatically but has numerous side effects. In this study, the anti-inflammatory and anti-oxidative potentials of ethanolic extract of Moringa oleifera leaf (MOE) has been assessed on MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) induced Parkinsonian mouse model. The findings of this study revealed that presence of bioactive phytochemicals in *Moringa* leaf imparts neuroprotective effects by modulating MPTP-induced neurobehavioral, biochemical deficits (enhanced endogenous antioxidative enzymes and abated oxidative stress markers) and regulating the expression of neuroinflammatory markers (TNF-α, NF-kB, and iNOS).

**Graphical Abstract**




**Keywords:** Neuroprotection · Parkinson's disease, *Moringa oleifera*, Tyrosine hydroxylase, Phytochemicals, MPTP, Neuroinflammation

**1. Introduction**

Parkinson's disease (PD) is a chronic age-related neurodegenerative disorder. It is characterized by the progressive loss of striatal dopaminergic neurons and the formation of Lewy bodies in the substantia nigra pars compacta (SNpc).[1] The motor symptoms such as bradykinesia, resting tremor, rigidity, gait difficulty, and postural instability are the major clinical features of PD. [1] Besides motor symptoms, non-motor symptoms include cognitive and memory impairment, anxiety, depression, emotional disturbances, olfactory dysfunction, constipation, pain, and sleep disturbance.[1,2] Various genetic and environmental conditions are considered the causative factors behind the disease onset but, the exact pathogenesis is still unclear.[3] Other etiopathology include oxidative/nitrosative stress, inflammation, mitochondrial dysfunction, endoplasmic reticulum stress, and excitotoxicity.[4] These multifactorial events altogether lead to the aggregation of misfolded α-synuclein[4] resulting in mitochondrial dysfunction that later induces inflammation, endoplasmic stress, oxidative stress, autophagy, and proteasomal activity.[5] The low level of glutathione and elevated calcium and iron in SNpc accelerates ROS production during dopamine synthesis.[6] The abundance of polyunsaturated fatty acids in the brain serves as a substrate during oxidative stress-induced lipid peroxidation.[7] Adverse environmental and genetic conditions lead to mitochondrial dysfunction and alleviate ROS generation, because of which the cellular antioxidant defense system gets triggered, leading to cell death.[8] Unfavorable environmental conditions and certain neurotoxins such as rotenone, lipopolysaccharides, or 1-methyl-4-phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP) trigger astrogliosis, and microgliosis, which contribute to the PD pathogenesis. [4,8,9] Activated glial cells promote PD etiology by generating pro-inflammatory markers such as nitric oxide (NO), interleukin IL-6 and IL-1β, and tumor necrosis factor- α (TNF-α), all of which contribute to PD Pathogenesis and neurodegeneration.[10,11]

With the increase in age, the prevalence of PD increases and maximizes at the age beyond 80.[12] With the increase in the aging population, the number of Parkinson's patients is estimated to be approximately 8.7 million by 2030, which is almost double the current PD cases.[13] Dopamine agonists, cholinesterase inhibitors, and monoamine oxidase inhibitors are among the current PD therapy options, however, they only provide symptomatic relief.[14] With the advancement of the disease, the efficacy of dopamine-based medications in treating the symptoms decreases. Despite the availability of various drugs and treatment strategies, the disease progression persists and is generally accompanied by various side effects.[15] As a result, it's critical to look for new treatment options with fewer adverse effects. In the past few decades, there has been a surge of interest in plant-based phytochemicals as a majority of them have been reported to have fewer adverse effects than synthetically manufactured drugs.[16]

*Moringa oleifera*, a medicinal plant, belongs to the *Moringaceae* family and is known for its anti-inflammatory and antioxidant properties.[17] The nutritious and edible pods, leaves, and flowers are exploited for medicine, food, and cosmetics.[17] The leaf of the *Moringa* plant contains abundant bioactive compounds such as vitamins, flavonoids, phenolic acids, carotenoids, alkaloids, isothiocyanates, saponins and tannins, and glucosinolates. [18] Each part of the plant is reported to have various biological effects, such as anti-inflammatory[17], anti-diabetic[19], hepatoprotective[20], and neuroprotective[21]. The anti-inflammatory and antioxidant properties of *Moringa oleifera* have been studied in several disease models, such as cholinotoxin-induced age-related dementia[22], colchicine-induced Alzheimer[23], middle cerebral artery occlusion-induced focal ischemia stroke[24], and cobalt chloride-induced brain hypoxia[25]. The neuroprotective effect of *Moringa* has also been studied on other neurodegenerative disease models such as amyotrophic lateral sclerosis[26], and learning and memory impairment.[27] Giacoppo et. al. studied the effect of glucomoringin isothiocyanate or GMG-ITC (isolated from *M. oleifera* seeds) on the MPTP-induced sub-acute Parkinsonian mouse model. The results suggest that the pretreatment with the bioactive compound

(GMG-ITC) effectively modulated inflammatory and apoptotic pathways along with modulating oxidative stress in MPTP-induced sub-acute Parkinson's mouse model.[28]

Despite several studies suggesting the anti-inflammatory and antioxidative effects of *M. oleifera*, no evidence of the antioxidative and anti-inflammatory effects of *Moringa oleifera* leaf extract on the MPTP-induced acute Parkinsonian mouse model has been reported. Thus in this study, we investigated the antioxidative and anti-inflammatory properties of ethanolic extract of *Moringa oleifera* leaf against MPTP-induced acute Parkinson's in adult male Swiss albino mice. An acute oral toxicity study was also conducted to ascertain the dose that, when administered once or over several administrations, would result in a fatality or significant toxicological consequences. The results of our study have shown that the ethanolic extract of *M. oleifera* leaf is non-toxic at the highest dose of 5000 mg/kg. In addition, the antioxidative and anti-inflammatory properties of *M. oleifera* leaf extract significantly reduced the toxic effect of MPTP by attenuating neurobehavioral and biochemical deficits and enhanced the expression of Tyrosine Hydroxylase (TH) in DA neurons within the nigrostriatal region. Moreover, the enhanced expression of pro-inflammatory cytokine (TNF-α) in MPTP-intoxicated mice was found to be suppressed by the inhibition of the NF-kB pathway after M. oleifera extract treatment, thus suggesting the neuroprotective efficacy of *M. oleifera* leaf extract.

## 2. Materials and Methods

### 2.1 Chemicals and reagents:

MPTP was procured from Sigma-Aldrich (St. Louis, MO, USA). Bradford reagent for protein estimation was bought from GeNeiTM, and potassium dichromate and hydrogen peroxide were purchased from Merck (Darmstadt, Germany). DABCO, sodium dodecyl sulfate (SDS), and Griess reagent were procured from HiMedia (Mumbai, India). Sodium Nitrite was purchased from Lobachemie, India. Acetic acid, disodium hydrogen phosphate, GSH, sodium dihydrogen phosphate, and NADPH were purchased from SRL Mumbai, India. Primary antibodies for western blot, TH (SC-25269), β-actin (SC-47778) were obtained from Santa Cruz, Biotechnology (Santa Cruz, CA, United States), and the primary antibodies for nuclear factor-κB (NF-κB; ab 16502), and tumor necrosis factor-α were procured from Abcam Life Sciences, Biogenuix Med Systems Pvt. Ltd. (New Delhi, India). Secondary antibodies for Western blotting were procured from Millipore Sigma.

### 2.2 Plant Materials and Preparation

Fresh leaves of *Moringa oleifera* were collected from March-April 2019 from the Banaras Hindu University campus and were immediately cleaned with tap water followed by distilled water to remove dust particles. The leaves were chopped into small pieces and allowed to dry under shade to prevent the loss of volatile bioactive compounds. The dried leaves were then crushed and ground to make a fine powder. 50 g of dried *M. oleifera* leaves were extracted in 500 mL of 70% ethanol in a flat bottom flask and allowed to stand at room temperature for 72 h with occasional shaking. After 72 h, the content was filtered using the Whatman number 1 filter paper. The filtrate was allowed to stand in a hot water bath at 40$^{o}$C (7–10 h a day) to get the solvent concentrated and dried. The semisolid extract was kept at 4$^{o}$C for further study. The semisolid extract was dissolved in distilled water before usage. The *M. oleifera* leaf extract (MOE) was prepared as per the standard procedure.[54]

### 2.3 Gas chromatography/ mass spectrometry (GC/MS) analysis

GC/MS analysis of the ethanolic extract of *M. oleifera* leaf was carried out in GCMS-QP2010 SE Ultra–Shimadzu Corporation. 100 µg of concentrated extract sample was taken in a separating funnel and shaken by adding 10mL water and ethyl acetate in a ratio of 1:4 (add 2.5 mL water to 7.5 mL Ethyl Acetate). The upper layer was collected and concentrated to 1

mL in the rotary evaporator. 50 µL N,O-Bis (trimethylsilyl) trifluoroacetamide (BSTFA), and trimethylchlorosilane (TMCS) (99:1 v/v) were added and then 10 µL of pyridine was added. Samples were further heated at 60°C for 30 min. The samples were then transferred to a GC vial and dried using nitrogen gas. The samples were finally dissolved in methanol before GC-MS analysis. The temperature of the injection line was set at 260°C, ion source temperature was set at 220°C. The column flow rate was 1.21 mL/min. The oven temperature was programmed from 120°C (2 min hold) to 300°C at a rate of 4°C/min. The particle-free extract was injected into the injector (Split ratio 10:1) with a syringe. After the complete scan, mass spectra within the range of 40 amu to 650 amu were collected and the composition of the crude extract was expressed as an area percentage by peak area. Various components present in the crude extract were identified based on the retention time. The obtained mass spectra data were compared with the standards available in the mass spec libraries.

**2.4 Determination of antioxidant activity by DPPH-scavenging assay**

1,1-diphenyl-2-picrylhydrazyl (DPPH) scavenging assay was conducted to determine the free radical scavenging activity of the *M. oleifera* leaf extract using ascorbic acid as the standard.[29] The concentration of the *M. oleifera* ethanolic extract solution was 1mg/ml. In brief, a stock solution of *M. oleifera* leaf extract was prepared by adding 1 mL extract to 10 mL ethanol. The stock solution was then serially diluted in ethanol to achieve new concentrations of extract as 10, 20, 40, 80, and 160 µg/L. 75 µL of freshly prepared DPPH solution (1.3 mg/mL of ethanol) was added to each tube containing the sample and was allowed to stand in dark for 30 min. The decrease in absorbance was recorded at 516 nm using a spectrophotometer. For reference, standard Ascorbic acid was dissolved in ethanol (1 mg/mL) to make a stock solution. Serial dilution was performed in the same manner as the sample solution. A control sample was prepared by adding the same volume of DPPH solution without extract and ascorbic acid and 95% ethanol were used as the blank. The % inhibition was calculated using the equation given below:

$$\% \text{ inhibition} = [(\text{Control-sample})/(\text{Control})] \times 100$$

**2.5 Exprimental Animals**

Male Swiss albino mice (aged 8–10 weeks, weighing 25±5 g) were procured from the Animal facility of the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. Animals were ke pt in clean polypropylene cages with 24 X 7 access to water, and standard mice feed. Before experimenting, the animals were acclimatized for a week in a fully-ventilated, air-conditioned, and pathogen-free animal house with 12:12 hours alternate light and dark cycle. The temperature of the animal house was maintained at 25±2 $^0$C. Only 3 mice were kept in each cage and all the experiments were conducted in compliance with the regulation and guidelines approved by the Animal Ethics committee of Banaras Hindu University (Protocol Number: BHU/DoZ/IAEC/2021-2022/032), and every effort was made to lessen the suffering and pain of the experimental mice.

**2.6 Acute oral toxicity study (OECD 423 Guideline)**

*M. oleifera* ethanolic extract was investigated for *in vivo* acute oral toxicity as per the flow diagram of OECD (423) guidelines (Figure 1). A total of 15 male mice (3 mice per group), were randomly selected and marked for individual identification. The test group included four treatment groups with dosages of 5 mg/kg, 50 mg/kg, 300 mg/kg, 2000 mg/kg, and 5000 mg/kg body weight. Individual doses were calculated based on the body weight of the animal on the day of treatment. The test substance was orally administered in a single dose by gavage using a specially designed mouse oral intubation cannula. Animals were fasted for 3 h before dosing (only food was withheld, not water). The animals were observed manually twice daily (1 hour each during morning and evening) for mortality, appearance, and behavioral changes such as aggresiveness, anxiety, lethargy, convulsion, feed and water consumption, tremor, diarrhoea, and weakness for a period of two weeks.

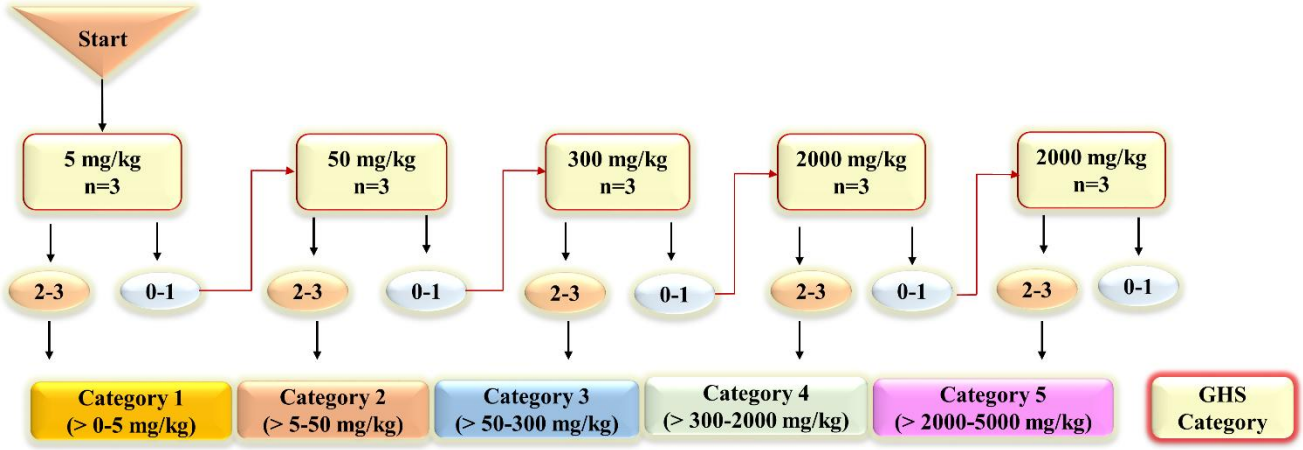



**Figure 1** The above flowchart shows the acute oral toxicity dose optimization procedure (adapted from OECD 423 guideline). The integers 0, 1, 2, and 3 represent the number of mortalities.

**2.7 Dose Standardization and Experimental Design**

After the mice were acclimatized to the laboratory conditions, they were randomly segregated into 5 groups of 6 animals each as follows: (1) Normal control group: given 0.9% saline via intraperitoneal route; (2) Disease control (DC) group: given 2 doses of MPTP (30 mg/kg body weight) within 16 h intervals via intraperitoneal injection; (3,4,5) Treatment group: animals were injected with MPTP in a similar manner as group 2: The treatment doses of 200, 400, and 600 mg/kg body weight were given orally before MPTP injection for 7 days and was continued for 14 days post MPTP injection; and (6,7,8) Positive control groups: animals were given *M.oleifera* leaf extract orally at a doses of 200, 400, and 600 mg/kg body weight . Neurobehavioral tests (rotarod, narrow beam walking test, and hanging grid test) and biochemical analysis experiments (Lipid Peroxidation, glutathione and Superoxide Dismutase, Nitrite Levels, and Catalase Levels) were conducted on all groups to analyze the optimum effective dose of *M. oleifera* leaf extract for neuroprotection.[30]

The western blotting study was done using the optimum effective dose obtained in the dose optimization study. Mice were divided into 4 groups of 6 animals each as follows: (1) Normal control group: administered 0.9% saline via the oral route; (2) Disease control (DC) group: given 2 doses of MPTP (30 mg/kg body weight) within 16 h intervals via intraperitoneal injection; (3) Treatment group: animals were injected (i.p.) with MPTP in a similar manner as group 2 and the treatment dose of 200 mg/kg body weight was given orally before MPTP injection for 7 days and was continued for 14 days post MPTP injection. (4) Positive control group: animals were given *M.oleifera* leaf extract orally (Figure 2).

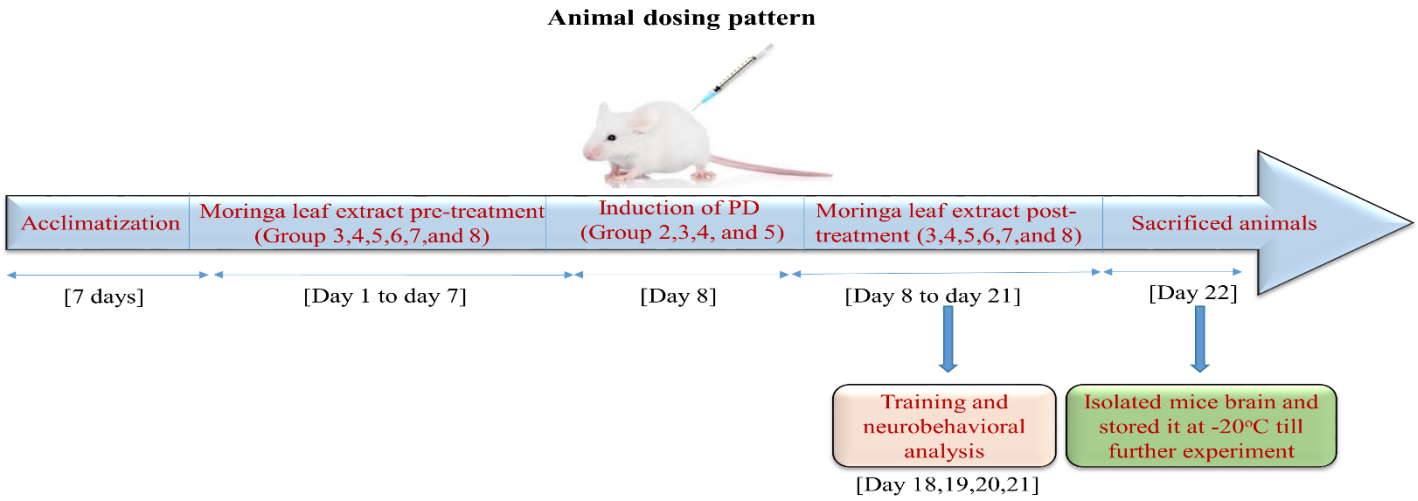



**Figure 2:** Animal dosing pattern

### 2.8 Neurobehavioural Studies

After the completion of the dosing cycle, several neurobehavioral activities were verified. Before the initialization of neurobehavioral studies, mice were subjected to training for 3 consecutive days (days 18,19, and 20) and on day 21, the final neurobehavioural studies were conducted. Several tests like the hanging test, rotarod test, and narrow beam walking test were conducted to study the motor deficits in MPTP-induced acute PD mice. The training was initiated 2 h post-treatment for each animal and was conducted thrice a day with 1 h interval between two consecutive training, and the average time was calculated for each test.

#### 2.8.1 Rotarod test

Before the initiation of the final rotarod test, the mice were trained three times a day with an interval of 1 h between two consecutive training. The training was initiated 2 h post-treatment and was conducted for three consecutive days (days 18,19, and 20) at a fixed speed of 5 rpm. The time taken by mice to fall was recorded. Mice were subjected to a maximum of 5 minutes on the rotarod. The analysis was repeated thrice for each animal, and the average time was calculated accordingly from the data obtained during the final test analysis. This method was adopted from the protocol described by Manna et al.[31]

#### 2.8.2 Narrow beam walking test

This test was conducted to assess the motor coordination of mice, which is required to verify the balance during movement on the narrow beam. Before the conduction of the final experiment, mice were trained to walk on the wooden narrow flat beam of dimension 100 cm X 1 cm (L X W), which was positioned at a height of 100 cm above the floor. Each animal was trained thrice a day (1 h interval between each training) for three consecutive days. The time taken by the animals to cross the beam from one end to another was recorded, and the experiment was repeated three times for each animal. This method was adopted from the original protocol described by Pisa.[32]

#### 2.8.3 Hanging Test

A horizontal grid was used for conducting the hanging test. Two hours' post-treatment, mice were placed on the grid and were allowed to hold the grid firmly. The grid was then inverted so that mice hang upside down till they lose their control

and fell from the grid. The hanging time was then recorded, and the same experiment was repeated thrice for each animal. Mice were trained thrice a day (1 h interval between each training) for three consecutive days before the conduction of the final hanging test. This method was adopted from the original protocol described by Mohanasundari et al.[33]

**2.9 Biochemical studies**

**2.9.1 Sample preparation**

After neurobehavioral analysis, animals were sacrificed using the cervical dislocation method, followed by decapitation with minimal pain. The brains were extracted from the mice and stored in a deep freezer for experimental studies. Nigrostriatal tissue was collected from the extracted brain and homogenized in a KCl buffer (Tris–HCl 10 mM, NaCl 140 mM, KCl 300 mM, ethylene diamine tetra acetic acid 1 mM, Triton-X 100 0.5%) at pH 8.0 supplemented with phosphatase and protease inhibitors. The tissue homogenate was centrifuged for 20 min at 12,000 g at 4°C. The supernatant obtained was collected and subjected to the measurement of concentration using a spectrophotometer for biochemical assays like lipid peroxidation, and quantification of antioxidant enzymes.

**2.9.2 Estimation of lipid peroxidation, glutathione, and superoxide dismutase**

With slight modification, lipid peroxidation (LPO) was estimated in the nigrostriatal tissue of the mouse brain in accordance with the method described by Ohkawa et. al.[34] Briefly, 10% SDS was added to 10% tissue homogenate, followed by the addition of 20% acetic acid. Later, 0.8% thiobarbituric acid (TBA; 0.6 mL) was added to this solution and the reaction mixture was incubated in boiling water for 1 h. and allowed to cool before centrifugation. After centrifugation, the supernatant was collected and absorbance was recorded at 523 nm against the control. The LPO level was measured and expressed in µM MDA/mg protein (micromoles of malondialdehyde (MDA) per milligram of protein). A method described by Moron et al.[35] was used for estimating glutathione (GSH) levels in the brain homogenate. The values of GSH were reported in terms of µM GSH/mg tissue.

The nigrostriatal tissue homogenate was used to determine the SOD levels using the method described by McCord and Fridovich.[36] NADH was used as a substrate to assay the SOD activity and the values of the SOD assay were reported in terms of nmol/mg protein. The absorbance was recorded at 560 nm for both tubes against a blank reagent. The difference between reference and experimental absorbance readings provides the value of inhibition of Nitro Blue Tetrazolium chloride (NBT) reduction by an enzyme source. Also, protein estimation was done by the enzyme source. The SOD enzyme activity unit was defined as the amount of enzyme required to inhibit the optical density at 560 nm of NBT reduction by 50% in one minute under the assay condition.

**2.9.3 Estimation of Nitrite Levels**

Nitrite content was measured in accordance with the procedure defined by Granger et al.[37] Ammonium chloride and Griess reagent was added to 10% of the tissue homogenate. The solution was left undisturbed for 30 min at 37°C and absorbance was recorded at 540 nm. Further, the reaction mixture was incubated for 30 min at 37°C and the absorbance of the supernatant was recorded at 540 nm. A standard curve for sodium nitrite (10-100 µM) was used to calculate the nitrite content in terms of micromoles per milliliter.

**2.9.4 Estimation of Catalase Levels**

The decomposition rates of hydrogen peroxide ($H_2O_2$) were used to assay the catalase (CAT) activity using spectrophotometric analysis in accordance with the procedure described by Kumar, et al.[38]. In 10% w/v tissue homogenate,

hydrogen peroxide (0.02M) was added along with distilled water and phosphate buffer at pH 7.0. After 1 min, potassium dichromate and acetic acid (1:3) were added to this solution, and the final solution was incubated in a water bath for 15 min. The OD of this solution was recorded at 570 nm and the value obtained was reported in terms of nmoles/min/mg protein.

**2.10 Western Blot Analysis:**

The mouse brain was isolated after being sacrificed via cervical dislocation and the nigrostriatal region of the mouse brain was isolated and homogenized in RIPA buffer and agitated for two hours at 4$^{o}$C. After homogenization, the homogenate was centrifuged at 12,000 rpm for 30 min at 4$^{o}$C. The supernatant was collected and the protein concentration was quantified by the Bradford assay. A total of 40 µg of protein extract was loaded into each well of the polyacrylamide gel, and the proteins were transferred onto the PVDF membrane after the completion of electrophoresis. The PVDF membrane was incubated overnight at 4$^{o}$C with primary antibodies TH (1:1000), NF-kB (1:1000), iNOS (1:1000), TNF-α (1:1500), and β-actin (1:500). After that, the blots were washed with TBST buffer (thrice for 10 min each) and were further incubated with HRP-conjugated secondary antibody for 2 h at room temperature followed by TBST washing (twice for 10 min each). The blots were developed using ECL as the substrate (luminol + $H_2O_2$) and the emission (428 nm) was captured and visualized by a Biorad gel doc imager. The band density of each blot was quantified using Quantity One software (Biorad, Windows) and the relative band density was calculated with respect to the band density of β-actin.

**2.11 Statistical Analysis:**

The statistical analysis of the data was carried forward by one-way analysis of variance (ANOVA) using the Newman-Keuls Multiple Comparison test via Graph Prism 5.0 software. The results are articulated in terms of mean ± SEM. *p* values < 0.05 are cogitated statistically significant.

**3. Results**

**3.1 Phytochemical composition of the extract by GC-MS analysis**

The ethanolic extract of *Moringa oleifera* leaf powder showed nineteen peaks in the GC-MS chromatogram (Figure 3), which were screened based on the retention time on the fused silica capillary column. Most of the compounds detected were hydrocarbons, terpenoids, esters, alcohols, and ketones. Nineteen phytochemical compounds were detected in the ethanolic extract and the major compounds detected were 1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxy phenyl)-prop-2-enoyl]-oxy-1,4,5trihydroxycyclohexane-1-carboxylic acid (23.65%) followed by 3,5,7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one (20.16%), n-Hexadecanoic acid (Palmitic Acid) (13.18%), and phytol (6.28%) (Table 1).

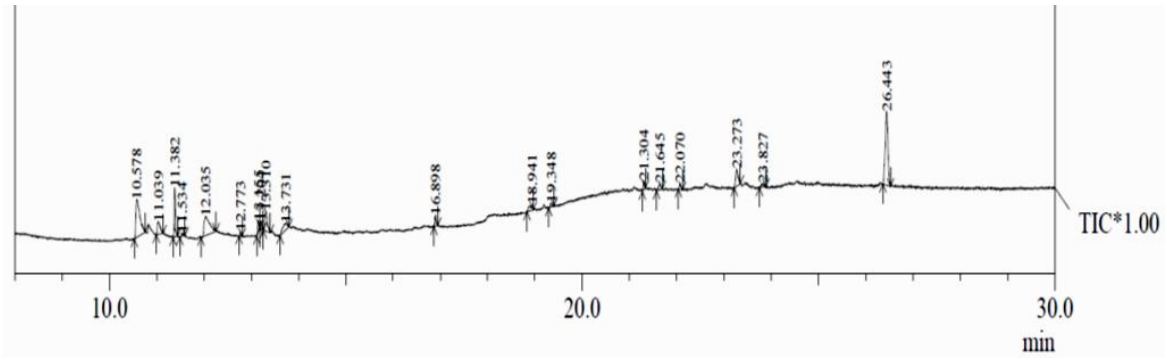
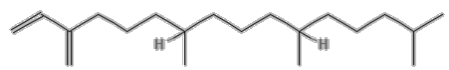
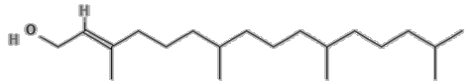
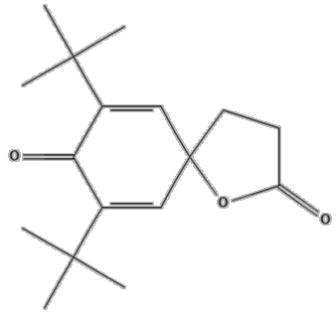
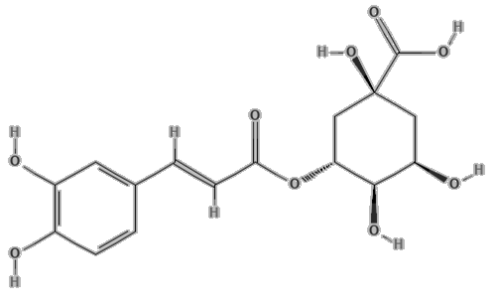
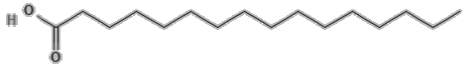
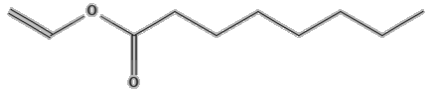
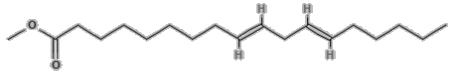
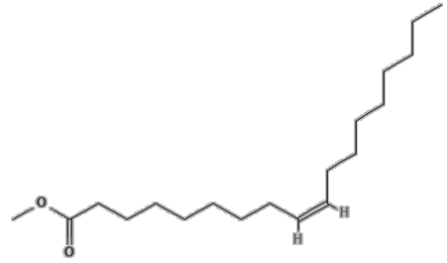
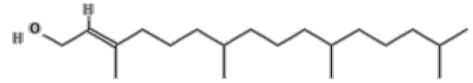
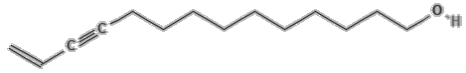
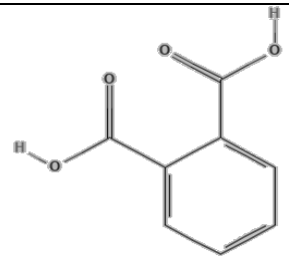
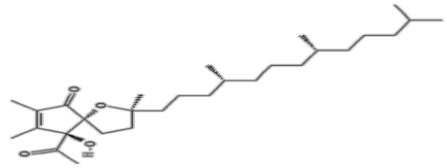


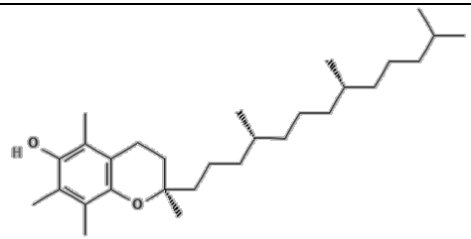
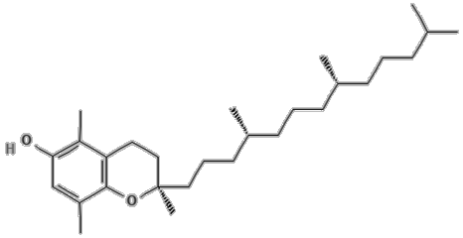
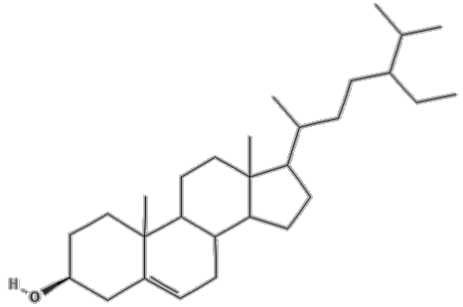

**Fig 3: GC-MS chromatogram of ethanolic extract of *Moringa oleifera* leaf**

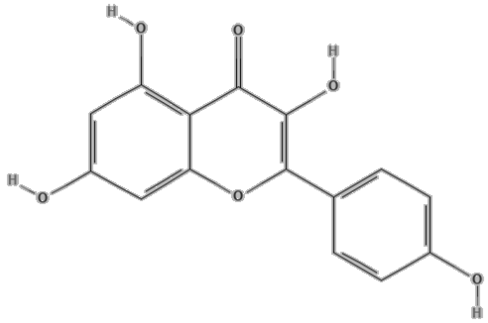
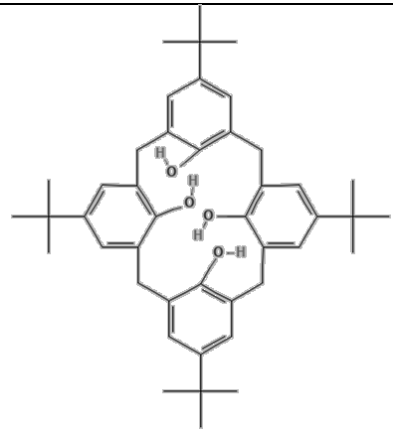
**Table 1: Phytochemical constituent of ethanolic extract of *Moringa oleifera* leaf**

| Peak | Retention Time | Area | % Area | Name | Molecular Weight (g/moL) | Molecular Formula (PubChem ID) | Molecular Structure |
|---|---|---|---|---|---|---|---|
| 1 | 10.578 | 1261628 | 5.65 | Neophytadiene | 278.5 | $C_{20}H_{38}$ (10446) |  |
| 2 | 11.039 | 305500 | 4.52 | 3,7,11,15-tetramethyl-2-hexadecen-1-ol | 296.53 | $C_{20}H_{40}O$ (5366244) |  |
| 3 | 11.382 | 499123 | 6.38 | 7,9-di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione | 276.4 | $C_{17}H_{24}O_3$ (545303) |  |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 4 | 11.534 | 48152 | 23.65 | (1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)-prop-2-enoyl]-oxy-1,4,5trihydroxycyclohexane-1-carboxylic acid (Chlorogenic acid) | 354.31 | $C_{16}H_{18}O_9$ (1794427) |  |
| 5 | 12.035 | 891652 | 13.18 | n-hexadecanoic acid | 256.42 | $C_{16}H_{32}O_2$ (985) |  |
| 6 | 12.773 | 40765 | 0.60 | Vinyl caprylate | 170.25 | $C_{10}H_{18}O_2$ (13164) |  |
| 7 | 13.155 | 112563 | 1.66 | 9,12-octadecadienoic acid, methyl ester, (e,e)- | 294.00 | $C_{19}H_{34}O_2$ (5362793) |  |
| 8 | 13.204 | 78220 | 1.16 | 9-octadecenoic acid (z)-, methyl ester | 296.50 | $C_{19}H_{36}O_2$ (5364509) |  |
| 9 | 13.310 | 424813 | 6.28 | Phytol | 296.50 | $C_{20}H_{40}O$ (5366244) |  |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 10 | 13.731 | 284656 | 4.21 | 13-tetradece-11-yn-1-ol | 208.34 | $C_{14}H_{24}O$ (543337) |  |
| 11 | 16.898 | 121870 | 1.80 | 1,2-Benzenedicarboxylic acid | 166.13 | $C_8H_6O_4$ (1017) |  |
| 12 | 18.941 | 172435 | 1.55 | Farnesol Isomer A | 222.00 | $C_{15}H_{26}O$ | Structure not available |
| 13 | 19.348 | 59152 | 0.87 | Alpha Tocospiro A | 462.70 | $C_{29}H_{50}O_4$ (21674156) |  |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 14 | 21.304 | 116781 | 1.73 | Vitamin E | 430.00 | $C_{29}H_{50}O_2$ (14985) |  |
| 15 | 21.645 | 122027 | 1.80 | Beta tocopherol | 430.00 | $C_{28}H_{48}O_2$ (6857447) |  |
| 16 | 22.070 | 78291 | 0.51 | 2,2-bis(tert-butyldimethylsiyloxy)-4,6-bis | 497.00 | $C_{16}H_{30}F_6N_3O_2Psi_2$ | Structure not available |
| 17 | 23.373 | 377700 | 3.31 | Stigmast-5-en-3-ol, (3.beta) | 414.00 | $C_{29}H_{50}O$ (6432744) |  |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 18 | 23.827 | 66054 | 20.16 | 3,5,7-trihydroxy-2-(4-hydroxyphenyl)chromen-4-one (Kaempferol) | 286.24 | $C_{15}H_{10}O_6$ (5280863) |  |
| 19 | 26.443 | 1701841 | 0.98 | 5,11,17,23-tetratert-butylpentacyclo[19.3.1.1 | 648.90 | $C_{44}H_{56}O_4$ (335377) |  |
| | | 6763223 | 100 | | | | |

### 3.2 Antioxidant activity analysis using DPPH assay

*M. oleifera* leaf extract showed a comparable level of antioxidant activity at different concentrations tested. The antioxidant activity of the test sample was compared to the antioxidant activity of ascorbic acid taken as a standard.

The results were expressed in terms of % inhibition, as shown in **figure 4**. No significant difference ($p < 0.05$) was observed in the percentage inhibition of MOE extract compared to the percentage inhibition of ascorbic acid. Thus indicating a potent antioxidant activity of MOE extract.

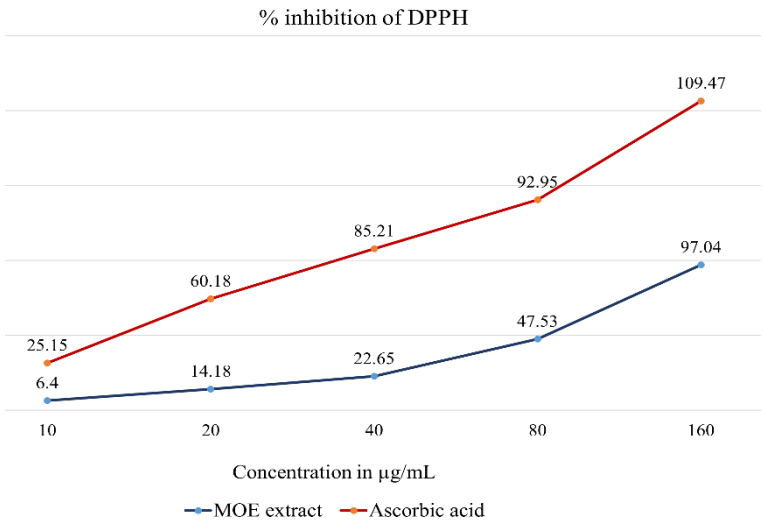



**Figure 4:** Percentage inhibition of free radicle (DPPH) by *M. oleifera* extract /ascorbic acid at 517nm.

### 3.3 Acute oral toxicity

After dosing with 5 mg/kg, 50 mg/kg, 300 mg/kg, 2000 mg/kg, and 5000 mg/kg of body weight, mice were observed for acute oral toxicity level by considering the following parameters: changes in body weight, convulsion, morbidity, mortality, sleep, lethargy, tremor, diarrhoea, skin, and fur. The above-mentioned parameters were observed during days zero, one, seven, and fourteen. All the observations were recorded individually for each animal systematically

There was no significant difference observed in the body weight of the mice starting from day zero to day fourteen, and no mortality was observed. Further, there was no significant difference observed in the duration of fourteen days in the following parameters including convulsion, morbidity, mortality, sleep, lethargy, tremor, diarrhoea, skin, and fur. The result indicates that the hydroethanolic extract of *M. oleifera* leaf extract is safe for consumption and did not show any adverse or toxic effects, even at higher doses.

### 3.4 Effect of *Moringa oleifera* leaf extract on MPTP induced Neurobehavioral deficit mice:

**Rotarod test:** The test was conducted to study the balance and coordination of the mice by placing them on a rotarod. On the 21$^{st}$ day after the dose completion, mice were taken for the final rotarod test analysis and it was observed that the time spent by the MPTP-intoxicated mice on the rotarod was significantly less ($p < 0.001$) compared with the control (**Figure 5a**).

The treatment of MPTP-intoxicated mice with different doses (400 and 600 mg/kg b.w.) of MOE significantly ($p < 0.001$) increased the retention time on the rotarod compared with the non-treated MPTP-intoxicated mice, and the results were observed to be non-significant between MPTP intoxicated and 200 mg/kg of MOE-treated mice. Among the doses of 400 mg/kg and 600 mg/kg, mice treated with the 400 mg/kg dose were observed to spend more time on the rotarod compared with 600 mg/kg. Thus, the dose of 400 mg/kg body weight was selected as the optimum dose for treatment in comparison to the doses of 200 and 600 mg/kg b.w. The retention time of the mice receiving only MOE extract at different doses (200, 400, and 600 mg/kg b.w.) was observed to be significantly higher ($p < 0.001$) compared to the MPTP-intoxicated mice and non-significant ($p < 0.01$) in comparison to the control group.

**Narrow Beam Walking Test:** The test was conducted after the final dose completion (21$^{st}$ day). The time taken by MPTP-intoxicated mice was found to be significantly ($p < 0.001$) more compared with the normal control **(Figure 5b)**. MOE-treated mice (400 and 600 mg/kg b.w.) showed a significant ($p < 0.001$) decline in the time taken to cross the narrow beam compared to non-treated MPTP-intoxicated mice. Thus, the dose of 400 mg/kg was considered the optimum treatment dose compared with 200mg/kg and 600mg/kg. The treatment group receiving 200 mg/kg b.w. of MOE did not show a significant decline in time compared with the other two (400 and 600 mg/kg b.w.) treatment groups. No significant difference ($p < 0.01$) was observed between the control group and the group receiving different doses of MOE (200, 400, and 600 mg/kg). On the other hand, a significant difference ($p < 0.001$) was observed between MPTP-intoxicated group and group administered with MOE at different doses (200, 400, and 600 mg/kg).

**Hanging Grid Test:** The motor function deficit was evaluated using a hanging grid test on the 21st day of dosing completion. The latency when an animal fell from the hanging grid was recorded. The gripping strength showed a significant decline ($p < 0.001$) in MPTP-intoxicated mice compared with control mice **(Figure 5c)**. The time latency on the hanging grid was found to be significantly increased ($p < 0.001$) after MOE treatment (400 mg/kg b.w.). Accordingly, 400 mg/kg b.w. was considered the optimum treatment dose rather than the dose of 200 mg/kg and 400 mg/kg body weight of MOE. The treatment group receiving 200 mg/kg b.w. of MOE did not show a significant increase in time latency compared with the other two (400 and 600 mg/kg b.w.) treatment groups. No significant difference ($p < 0.01$) was observed in the time latency between the control group and positive control groups (200, 400, and 600 mg/kg). However, a significant ($p < 0.001$) decline in the time latency was observed in the MPTP-intoxicated group in comparison to the positive control groups (200, 400, and 600 mg/kg).

Thus, the result obtained from the above-mentioned neurobehavioral tests suggests that the ethanolic extract of *M. oleifera* leaf extract (400 mg/kg) imparts its neuroprotective potential by improving MPTP-induced motor deficits in PD mice.

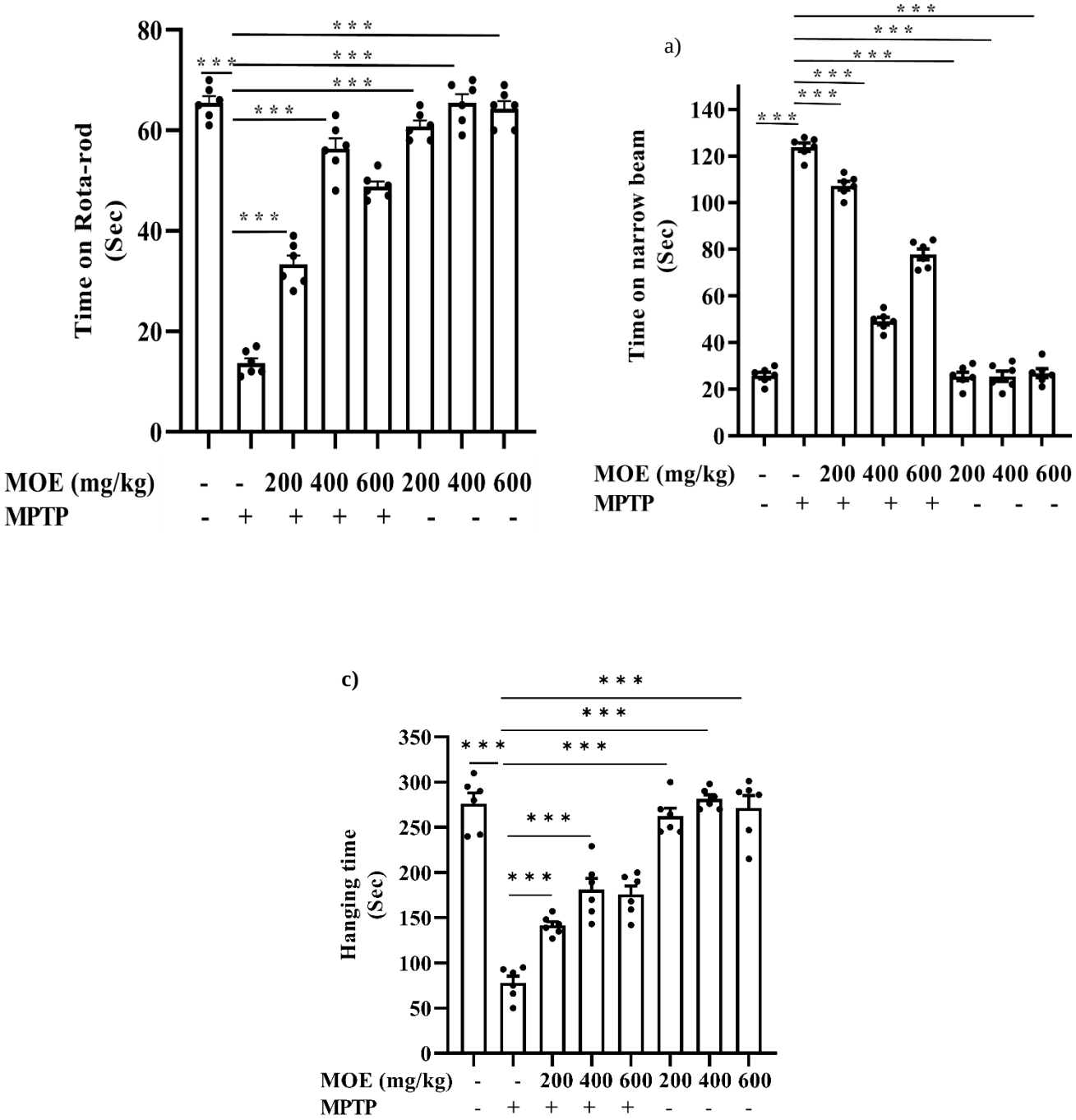



**Figure 5: Neurobehavioral analysis of mouse: MOE ameliorates MPTP-induced neurobehavioral deficit. (a)** Time latency on the rotarod, **(b)** Time spent on a narrow beam, and **(c)** Time on a hanging grid. The values are expressed in terms of mean ± SEM (n=5). *$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$. The results were analyzed via one-way ANOVA followed by the Newman-Keuls test using Graph Pad Prism software.

**3.5 Effect of *Moringa oleifera* leaf extract on MPTP-induced biochemical deficits in mice:**

After the completion of the behavioral studies, biochemical tests were conducted to study the antioxidant level properties of MOE**.**

**3.5.1 MOE regulates nitrite level and inhibits Lipid peroxidation in MPTP-intoxicated mice:**

MPTP intoxication significantly ($p < 0.001$) elevated the MDA levels compared with the control group (Figure 6a). However, pre-treatment with MOE extract at 200, 400, and 600 mg/kg b.w. significantly ($p < 0.001$) reduced the elevated MDA level.

The level of nitrite was estimated in the SNpc regions of the brain in different groups. The level of nitrite was found to be significantly ($p < 0.001$) elevated after MPTP intoxication compared with the control group (Figure 6b). However, in the MOE pre-treated groups (400, and 600 mg/kg b.w.) the level of NO was observed to be significantly ($p < 0.001$) reduced compared with the MPTP-intoxicated group. The MOE at a lower dose of 200 mg/kg was found to be comparable to that of the diseased group.

A significant difference ($p < 0.0001$) was observed in MDA and Nitrite levels between MPTP-intoxicated group and positive control groups (200, 400, and 600 mg/kg b.w.). On the other hand, a comparable level ($p < 0.001$) of MDA and nitrite was observed in control group and positive control groups (200, 400, and 600 mg/kg b.w.).

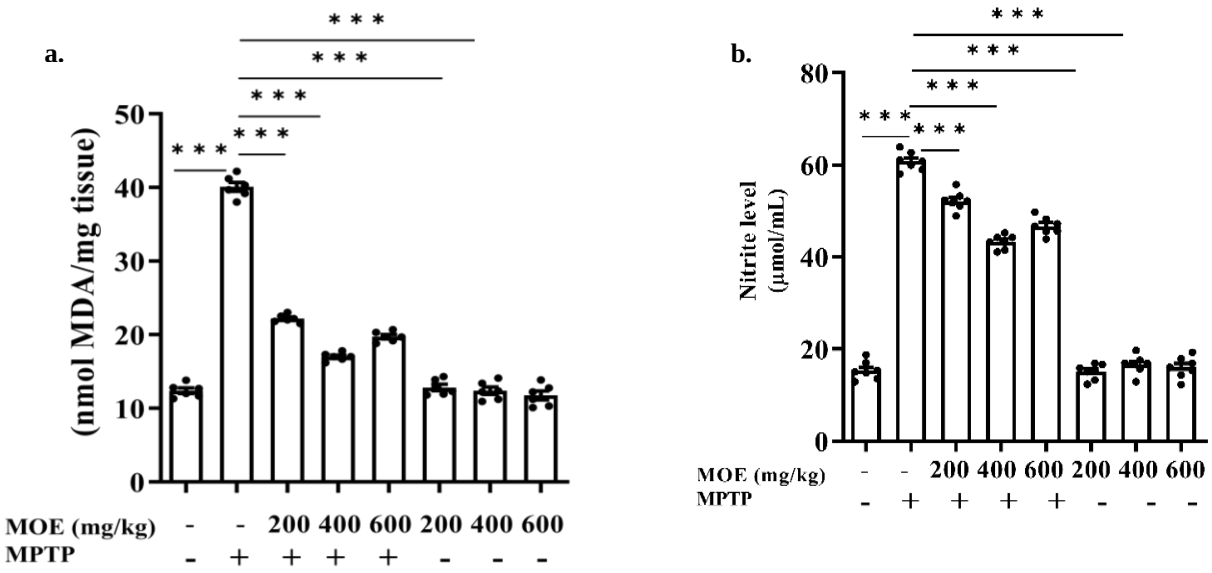



**Figure 6**: **MOE extract modulates the level of oxidative stress markers in the SNpc region of the midbrain:** MOE treatment reduced the alleviated **(a)** MDA level, and **(b)** Nitrite levels in treatment groups as compared to the diseased group. The values are expressed in terms of mean ± SEM (n=5). The results were analyzed via one-way ANOVA followed by the Newman-Keuls test using GraphPad Prism software. In this Figure, each point (•) represents the data of each mouse, the bar represents the mean, and the lines (T) the SEM.

**3.5.2 MOE modulates the antioxidant enzyme activity (SOD, CAT, and GSH) in MPTP-intoxicated mice:**

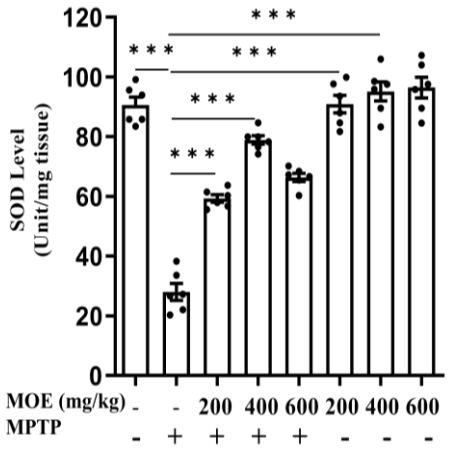
The radical scavenging activity of SOD and CAT was evaluated in the SNpc region of the brain. MPTP-intoxication significantly ($p < 0.001$) reduced the level of SOD compared with the control group **(Figure 7a)**. Pre-treatment with MOE extract at 400 and 600 mg/kg b.w. dose significantly improved the reduced SOD level in the treatment group. However, the treatment group receiving 200 mg/kg b.w. of MOE extract did not show a significant enhancement in the level of SOD enzyme. A significant decline ($p < 0.001$) in the levels of SOD was observed in MPTP-intoxicated group in comparison to positive control groups (200, 400, and 600 mg/kg b.w.). However, there was no significant difference ($p < 0.01$) observed in the levels of SOD between the control and positive control groups (200, 400, and 600 mg/kg b.w.) hence showing no adverse effect of MOE alone on mice.

The level of CAT was found to be significantly ($p < 0.001$) reduced after MPTP intoxication compared with the control group **(Figure 7b)**. In comparison to the non-treated group, treatment groups receiving MOE extract at 400 and 600 mg/kg b.w. doses showed a significant ($p < 0.01$) reduction in CAT level. However, the treatment group receiving 200 mg/kg b.w. of MOE extract did not show a significant increase in CAT level compared to the non-treated MPTP-intoxicated group. The levels of Catalase were found to be comparable ($p < 0.01$) between the control and positive control groups (200, 400, and 600 mg/kg b.w.). However, a significant difference ($p < 0.001$) was observed between the MPTP-intoxicated group and positive control groups (200, 400, and 600 mg/kg b.w.) were the positive control group doesn't show any adverse effect as shown in MPTP-intoxicated group.

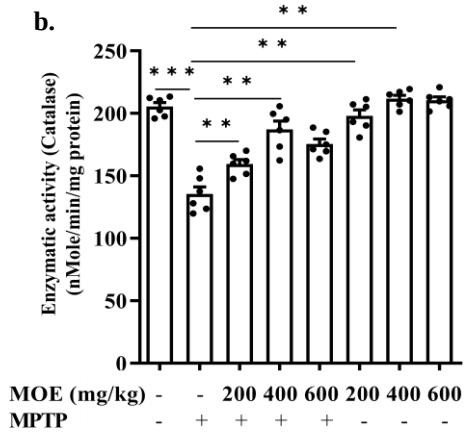
MPTP intoxication significantly ($p < 0.001$) reduced the GSH levels compared with the control group as shown in **Figure 7c**. However, pre-treatment with MOE at 200, 400, and 600 mg/kg b.w. significantly ($p < 0.001$) alleviated the reduced GSH levels. The levels of GSH was observed to be comparable ($p < 0.01$) between the control and positive control groups (200, 400, and 600 mg/kg b.w.). Whereas, a highly significant difference ($p < 0.0001$) was observed between MPTP-intoxicated group and positive control groups (200, 400, and 600 mg/kg b.w.).

Thus, the results obtained from the above-mentioned antioxidant enzyme activity assay suggest that the ethanolic extract of *M. oleifera* leaf at the dosage of 400 mg/kg is effective in ameliorating the MPTP-induced oxidative stress in PD mice.

**a.**




**b.**



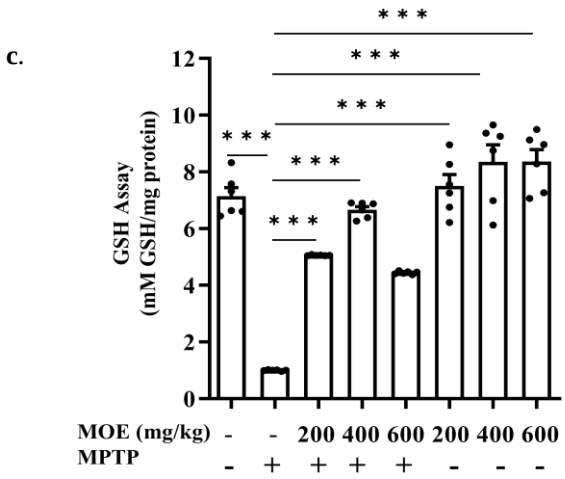
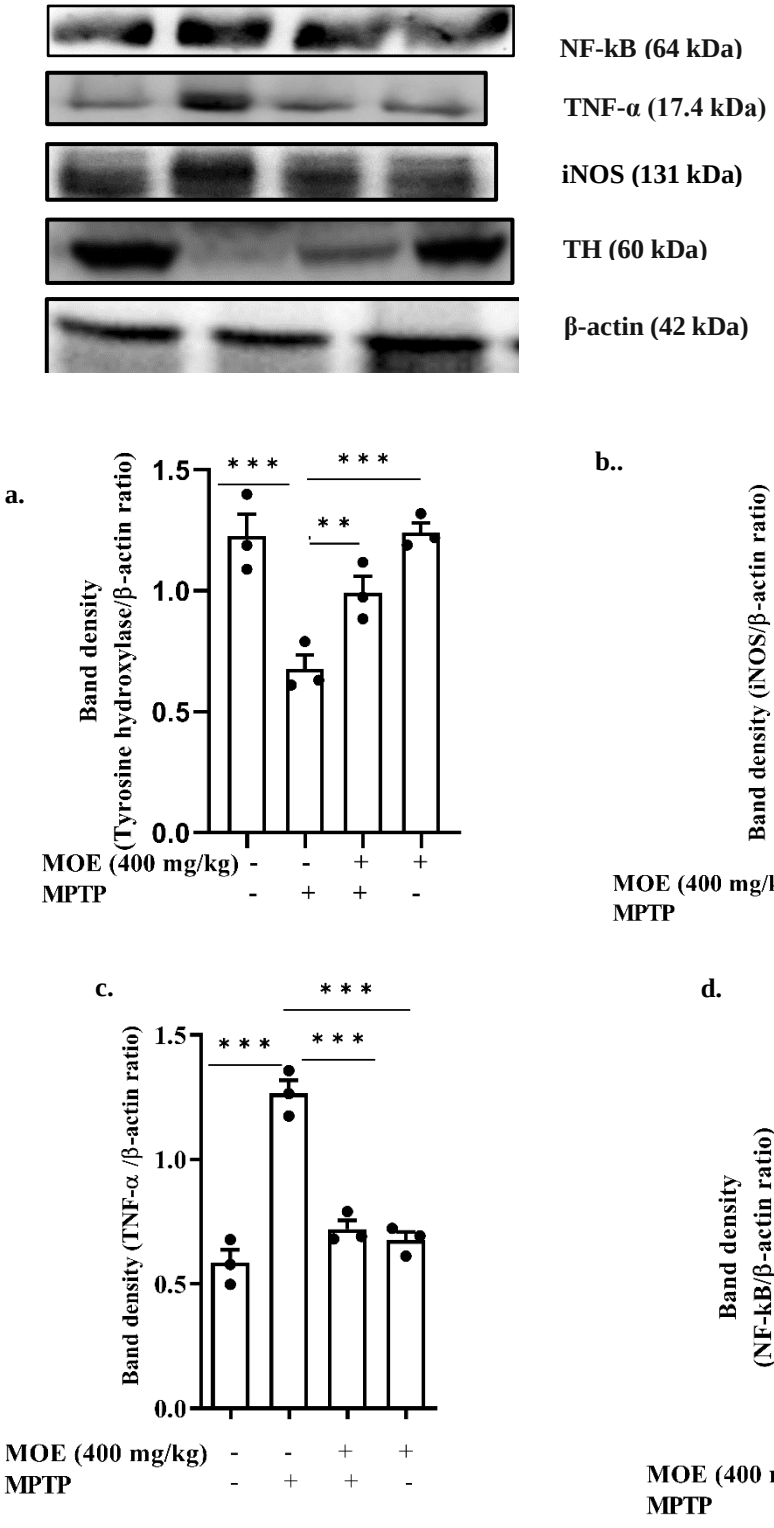



**Figure 7: MOE extract modulates the level of anti-oxidant enzyme activity (SOD, CAT, and GSH) in the SNpc region of the midbrain:** MOE treatment significantly alleviated the reduced **(a)**, **(b)** CAT, and **(c)** GSH levels in comparison to the MPTP intoxicated group. The results were analyzed via one-way ANOVA followed by the Newman-Keuls test using GraphPad Prism software. In this Figure, each point (•) represents the data of each mouse, the bar represents the mean, and the lines (T) the SEM.

### 3.6 Western Blot Analysis of Tyrosine hydroxylase (TH), Nuclear Factor kappa B (NF-kB), iNOS- inducible Nitric Oxide synthase, and Tumour Necrosis Factor-alpha (TNF-α)

The level of expression of TH (60 kDa), NF-kB (64 kDa), iNOS (131 kDa), and TNF-α was estimated in the nigrostriatal tissue lysates using Western blotting. Since the dose of 400mg/kg b.w. was observed to be an optimal dose in neurobehavioral and biochemical analysis, the same dose was used to analyze the effect of MOE extract on the level of expression of the above-mentioned proteins. The expression level of TH was observed to be significantly ($p < 0.001$) reduced in MPTP-intoxicated mice compared with the control group. Whereas, in the MOE-treated group, the expression level of TH was significantly ($p < 0.01$) improved compared with the diseased group **(Figure 8)**. The expression of iNOS, TNF-α, and NF-kB was found to be significantly ($p < 0.001$) elevated in the MPTP-intoxicated group compared with the control group. MOE treatment significantly ($p < 0.001$) reduced the enhanced expression of pro-inflammatory cytokines (iNOS, TNF-α, and NF-kB) **(Figure 8)**.

| | | | | |
|---|---|---|---|---|
| **MPTP (30 mg/kg)** | - | + | + | - |
| **MOE (400 mg/kg)** | - | - | + | + |




**4. Discussion**

**Figure 8: Evaluation of the expression level of tyrosine hydroxylase (TH), iNOS, NF-kB, and TNF-α in tissue lysate of SNpc region of midbrain:** Pretreatment with MOE extract imparts neuroprotective effect after MPTP intoxication by ameliorating the expression levels of TH, iNOS, NF-kB, and TNF-α. MOE treatment (400 mg/kg) downregulated the expression of NF-kB, iNOS, TNF-α and upregulated the expression level of TH as compared to the diseased group. The values are expressed in terms of mean ± SEM (n=5). The results were analyzed via one-way ANOVA followed by the Newman-Keuls test using Graph Pad Prism software.

Because of the high nutraceutical and medicinal properties of *M. oleifera*, it has been referred to as "the panacea," as it can cure hundreds of diseases. In India, *M. oleifera* has long been used as a traditional medicine because of the presence of bioactive phytochemicals that render the plant excellent medicinal properties.[39] Several studies have been conducted to explore the anti-oxidant, anti-inflammatory, neuroprotective, anti-cancer, hepatoprotective, anti-diabetic, and various other therapeutic properties of *M. oleifera.*[40] Most of the studies reported the neuroprotective properties of *M. oleifera* in various animal models of neurodegenerative diseases using *in vitro* and *in vivo* model.[41, 28] *M. oleifera* facilitates the scavenging of reactive oxygen and nitrogen species, induces neurogenesis, modifies neuronal stem cells and neurotransmitter activity, rendering anti-oxidative and anti-inflammatory properties and modulating mitochondrial homeostasis, thus preventing further neuronal loss.[42] The neuroprotective effect being studied hence proved the ability of *M. oleifera* to efficiently cross the blood-brain barrier and render its therapeutic effect. The neuroprotective effect of MOE on MPTP- induced Parkinson's disease has not been studied yet. In this study, we have reported the neuroprotective, anti-oxidative, and anti-inflammatory potential of *M. oleifera* ethanolic leaf extract on MPTP-induced dopaminergic toxicity for the first time. Here, we have used MPTP to induce Parkinson's because, in most of the studies, MPTP has been considered to produce the best toxin-animal model of PD by representing the behavioral and pathophysiological symptoms of PD. In general, MPTP not only encourages motor impairments in rodents but also results in DA neuronal degeneration.[61] Additionally, MPTP treatment produces behavioral, biochemical, and immunohistological traits that are strikingly comparable to those of human PD patients.[62]

We studied the acute oral toxicity dosage to determine the optimum effective dose of MOE and also focused on the mechanism behind MOE's therapeutic effectiveness in mice exposed to MPTP. The key findings of this study are (i) 1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)-prop-2-enoyl]-oxy-1,4,5trihydroxycyclohexane-1-carboxylic acid (Chlorogenic acid) followed by 3,5,7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one (Kaempferol) were detected as the major bioactive compounds in *M. oleifera* ethanolic leaf extract (ii) *M. oleifera* leaf extract is safe and tolerable even at higher doses of 2000 and 5000 mg/kg with no observed adverse effects, (iii) *M. oleifera* leaf extract is effective in improving MPTP-induced neurobehavioral deficits, (iv) *M. oleifera* leaf extract is effective in improving MPTP-induced biochemical deficit in mice, (v) *M. oleifera* leaf extract is effective in modulating the activities of antioxidant enzymes (SOD, CAT, and GSH) in MPTP-induced mice, (vi) *M. oleifera* leaf extract is effective in downregulating the expression of NF-kB, iNOS, TNF-α, and upregulating the expression level of TH in MPTP-intoxicated mice.

High performance liquid chromatography (HPLC) studies have shown the availability of phenolic acids such as chlorogenic acid, gallic acid, ellagic, and ferulic acids and the presence of various flavonoids such as quercetin, isoquercetin, astragalin, and rutin in *M. oleifera.*[43] Quercetin, myrecetin, and kaempferol are the main flavonoids found in *M. oleifera* leaf.[44] Indian *Moringa* plant varieties (PKM1 and PKM2) have had higher levels of quercetin and kaempferol.[55] In this study, we found numerous active substances, including triterpenoids, polyphenols, saponins, tannins, and flavonoids in MOE during phytochemical screening studies, which is in line with the findings of the prior study.[56] Flavonoids, including quercetin and kaempferol, and polyphenols present in MOE which are known to exhibit major antioxidant and neuroprotective effects, were detected during phytochemical screening.[57] Additionally, neophytadiene, stigmasterol, and vitamin E were detected during GC/MS analysis, which is known for their anti-inflammatory and neuroprotective properties.[58,59,60] The output phytochemical screening of MOE showed 1S,3R,4R,5R)-3-[(E)-3-(3,4-dihydroxyphenyl)-prop-2-enoyl]-oxy-1,4,5trihydroxycyclohexane-1-

carboxylic acid (Chlorogenic acid) followed by 3,5,7-trihydroxy-2-(4-hydroxyphenyl) chromen-4-one (Kaempferol) as the major bioactive compound which is believed to have major neuroprotective activity.

In this study, we performed a rotarod test, narrow beam walk test, and hanging grid test to study MOE activity in regulating behavioral changes and motor impairment. The assessment of motor coordination and balance in MPTP-induced mice has been scrutinized by the rotarod test[45] whereas, a narrow beam walking test was conducted to assess the hind limb impairment by analyzing the movement coordination[45] and a hanging grid test assesses the neuromuscular strength and motility.[46] MPTP intoxication showed balance impairment, muscle coordination impairment, and loss in muscular strength. The time latency of MPTP-intoxicated mice on the rotarod and the hanging grid was observed to be low compared to the normal control. However, in MOE-treated mice, the time latency was significantly improved. In the case of the narrow beam walk test, MPTP-intoxicated mice took a long time to walk across the beam compared with healthy controls but in the MOE-treated group, the time taken was significantly reduced.

Oxidative stress is considered one of the major causal factors behind the onset of neurodegenerative diseases followed by chronic inflammation in certain selected regions of the brain. Several reasons make the brain more susceptible to oxidative stress including mitochondrial dysfunction, neurotransmitter auto-oxidation, declined catalase level, and elevated redox-active transition metals.[47] Superoxide dismutase (SOD) and Catalase (CAT) are the first line of defense against ROS-induced oxidative stress.[48] A decline in the level of endogenous antioxidants causes the over-production of ROS, which leads to oxidative damage to biomolecules. In humans, GSH and lipid peroxidation are considered to be clinically significant biomarkers of oxidative stress.[49] Polyunsaturated fatty acids' double bonds are extremely sensitive to ROS assault and, as a result, lipid peroxidation. Malondialdehyde (MDA) is one of the most studied lipid peroxidation end products.[50] The anti-oxidative, anti-inflammatory, and neuroprotective activities of *M. oleifera* have been demonstrated earlier in the Colchicine induced animal model of Alzheimer's disease[23], Cholinotoxin induced animal model of age-related dementia[22], Scopolamine induced animal model of learning and memory impairment[27], and various other neurodegenerative disorders. In this study, we have measured the anti-oxidative properties of MOE by measuring the levels of oxidative stress markers i.e., the level of MDA and NO in the nigrostriatal region of the brain and the level of antioxidants (SOD and CAT). MPTP-intoxication significantly reduced the level of SOD and CAT. However, in the MOE pre-treated group, the antioxidant levels were significantly enhanced compared with the diseased group. The significant enhancement in oxidative stress markers (MDA and NO) by the MPTP-intoxication was reduced by MOE treatment, thus proving the anti-oxidative activity of *M. oleifera.*

NF-kB, a transcription factor, aids in the regulation of several cell-signalling pathways and plays a crucial role in the response to diverse stimuli connected to neuroinflammation. Changes in NF-kB expression could aid in illness management. [51] The most prominent proinflammatory cytokines whose production is mediated by NF-kB are iNOS, COX-2, TNF-α, and IL-1.[10] The anti-inflammatory properties of *Moringa oleifera* were studied on lipopolysaccharide stimulated macrophages and it was observed that the secretion of pro-inflammatory markers such as IL-6, IL-1β, TNF-α, and prostaglandin E2 was repressed after *Moringa* treatment.[52] Inducible NO synthase (iNOS), cyclooxygenase-2, and NF-kB enhancers have also been reported to be suppressed.[52] In a study reported by Giacoppo et al., moringin (derivative of GMG, which is extracted from *M.oleifera seeds*) was reported to reduce the expression of iNOS and TNF- α and enhance the expression of TH in MPTP-intoxicated mice[28]. Similar results were observed in this study, where the expression of iNOS and TNF-α were observed to be reduced in

the MOE pretreated group. The levels of NF-kB in the MPTP-intoxicated group were also observed to be decreased in the MOE pre-treated group. In the dopamine production pathway, tyrosine hydroxylase (TH) plays a critical role. Tyrosine hydroxylase is a marker of dopaminergic neurons in the SNpc region of the brain.[53] Our research focuses on the decline of TH-positive neurons in the SNpc of MPTP-intoxicated mice compared with the control group with intact dopaminergic neurons. TH-positive neurons were considerably recovered after MOE treatment. This indicates that MOE protects the dopaminergic neurons in MPTP-intoxicated mice, thus suggesting the neuroprotective potential of *M. oleifera* leaf extract.

**5. Conclusion**

Through the above findings, it can be concluded that the ethanolic extract of *M.oleifera* leaf can be used as a potent therapeutic agent to curb neurodegeneration and hence, the progression of Parkinson's disease. The bioactive components of *M. oleifera* confer its anti-inflammatory properties by downregulating the expression of pro-inflammatory cytokines (NF-kB, TNF α, and iNOS) and hence preventing neuroinflammation. The antioxidant activity of *M. oleifera* phytochemicals reduced the level of oxidative stress markers (MDA and NO) and enhanced the levels of endogenous antioxidative enzymes (SOD and CAT). More studies are required to establish the neuroprotective efficiency of *Moringa oleifera* leaf extract in curbing the progression of disease.

**Abbreviations**: CAT- Catalase, GSH – Reduced Glutathione; GMG- Glucomoringin; iNOS- inducible Nitric Oxide synthase; MOE- *Moringa oleifera* leaf extract; MPTP- 1-methyl-4-phenyl-1, 2, 3; NO- Nitric oxide; PD - Parkinson's disease; PVDF- Polyvinylidene fluoride; 6-tetrahydropyridine; LPO- Lipid peroxidation; MDA- Malondialdehyde; ROS- Reactive oxygen species; SNpc- Substantia Nigra Pars Compacta; SOD- Superoxide Dismutase; TH- Tyrosine Hydroxylase; TNF-α- Tumour Necrosis Factor- alpha

**Author Contributions**

SS, SPS, and VNM conceptualized this study. SPS and VNM supervised this study. SS investigated the study and wrote the original draft. SS and ASR revised the manuscript.

**Declaration of Competing Interest**

The authors declare that they have no competing interest.

**Acknowledgements**

SS and ASR are sincerely thankful to BHU-RET, for their respective fellowship. The authors are thankful to the Head, Department of Biochemistry, Institute of Sciences, BHU for providing the basic departmental facility and ISLS, BHU for their central facility.

**Funding**

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

**References**

1. L.V. Kalia, A.E. Lang, Parkinson's disease, *Lancet* , **2015**, 386, 896–912.
2. K.R. Chaudhuri, D.G. Healy, A.H. Schapira, Non-motor symptoms of Parkinson's disease: diagnosis and management, *Lancet Neurol.*, **2006**, 5, 235–245.
3. J. R. Cannon, & J. T. Greenamyre, Gene-environment interactions in Parkinson's disease: specific evidence in humans and mammalian models, *Neurobiol. Dis.*, **2013**, 57, 38–46.
4. D.J. Moore, A.B. West, V.L. Dawson, T.M. Dawson, Molecular pathophysiology of Parkinson's disease, *Annu Rev Neurosci.*, **2005**, 28, 57-87.
5. V. Ghiglieri, V. Calabrese, P. Calabresi, Alpha-synuclein: from early synaptic dysfunction to neurodegeneration, *Front. Neurol.*, **2018**, 9, 295.
6. P. Jenner, Oxidative stress in Parkinson's disease, *Ann Neurol.*, **2003**, 53, S26–S36.
7. X. Jenner, N. Yamada, W. Maruyama, T. Osawa, Formation of dopamine adducts derived from brain polyunsaturated fatty acids: Mechanism for Parkinson disease, *J Biol Chem*, **2008**, 283, 34887–34895.
8. V. Dias, E. Junn, M.M. Mouradian, The role of oxidative stress in Parkinson's disease, *J Parkinsons Dis.*, **2013**, 3 (4), 461-91.
9. R. Niranjan, C. Nath, R. Shukla, The mechanism of action of MPTP-induced neuroinflammation and its modulation by melatonin in rat astrocytoma cells, *Free Radical Res.*, **2010**, 44, 1304–1316.
10. M. L. Block, L. Zecca, J. S. Hong, Microglia-mediated neurotoxicity: uncovering the molecular mechanisms. *Nat. Rev. Neurosci.*, **2007**, 8, 57–69.
11. S. Cao, D.G. Standaert, A.S. Harms, The gamma chain subunit of Fc receptors is required for alpha-synuclein-induced pro-inflammatory signaling in microglia, *J. Neuroinflammation*, **2012**, 9, 259.
12. J.A. Driver, G. Logroscino, J.M. Gaziano, K. Tfdvas, Incidence and remaining lifetime risk of Parkinson disease in advanced age, *Neurology*, **2009**, 72, 432–438.
13. E.R. Dorsey, R. Constantinescu, J.P. Thompson, Projected number of people with Parkinson disease in the most populous nations, 2005 through 2030, *Neurology* , **2007**, 68, 384–386.
14. J.M. Ellis, M.J. Fell, Current approaches to the treatment of Parkinson's disease. *Bioorg. Med. Chem. Lett.*, **2017**, 27, 4247–4255.
15. R. Ceravolo, C. Rossi, E. Del Prete, U.A. Bonuccelli, Review of adverse events linked to dopamine agonists in the treatment of Parkinson's disease, *Expert Opin. Drug Saf.*, **2016**, 15, 181–198.

16. Z. Shahpiri, R. Bahramsoltani, M.H. Farzaei, F. Farzaei, R. Rahimi, Phytochemicals as future drugs for Parkinson's disease. A comprehensive review, *Rev Neurosci.*, **2016**, 27, 27:651–668.

17. B. Padayachee, H. Baijnath, An overview of the medicinal importance of Moringaceae, *J. Med. Plants Res.*, **2012**, 6, 5831–5839.

18. A. Leone, A. Spada, A. Battezzati, A. Schiraldi, J. Aristil, S. Bertoli, Cultivation, genetic, ethnopharmacology, phytochemistry and pharmacology of *Moringa oleifera* Leaf: An overview. *Int. J. Mol. Sci.*, **2015**, 16, 12791–12835.

19. T.A. Oyedepo, S.O. Babarinde, T.A. Ajayeoba, Evaluation of the antihyperlipidemic effect of aqueous leaf extract of *Moringa oleifera* in alloxan induced diabetic rats, *Int. J. Biochem. Res. Rev.*, **2013**, 3, 162–170.

20. R.N. Bennett, F.A. Mellon, N. Foidl, J.H. Pratt, M.S. Dupont, L. Perkins, P.A. Kroon, Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees *Moringa oleifera* L. (horseradish tree) and *Moringa* stenopetala, *J. Agric. Food Chem.*, **2003**, 51, 3546–3553.

21. T.B. Kabirat, O. Oyetola, A.A. Victor, A.A. Ismaheel, M.A. Abayomi, A. Samuel, Protective effect of methanol leaf extract of Cnidoscolus aconitifolius against lipopolysaccharides-induced cortico-hippocampal neuroinflammation, oxidative stress and memory impairment, *Advances in Traditional Medicine,* **2021**, 23, 145-155.

22. C Sutalangka , J Wattanathorn, S Muchimapura, W Thukham-mee. Moringa oleifera mitigates memory impairment and neurodegeneration in animal model of age-related dementia. *Oxid Med Cell Longev*. **2013**; 2013, 695936. doi:10.1155/2013/695936

23. C. Roy, Role of *Moringa oleifera* on hippocampal cell morphology and senile plaque formation in colchicine induced experimental rat model of Alzheimer's disease, *International Journal of Current Pharmaceutical Research*, **2014**, 6, 2.

24. W. Kirisattayakul, J. Wattanathorn, T. Tsdvsdv, S. Muchimapura, P. Wannanon, J. Jittiwat, Cerebroprotective Effect of *Moringa oleifera* against Focal Ischemic Stroke Induced by Middle Cerebral Artery Occlusion, *Oxidative Medicine and Cellular Longevity*, **2013**, 2013.

25. A.R. Amany, M. Mohamed, K. Samah, S. Gamal, A. Haytham, Moringa oleifera extract attenuates the CoCl2 induced hypoxia of rat's brain: Expression pattern of HIF-1α, NF-kB, MAO and EPO, *Biomedicine & Pharmaco.*, **2019**, 109, 1688-1697.

26. M. Galuppo, S. Giacoppo, R. Iori, G. Rosalinda, P. Bramanti, E. Mazzon, Administration of 4-(*α*-L-Rhamnosyloxy)-benzyl Isothiocyanate Delays Disease Phenotype in SOD1G93A Rats: A Transgenic Model of Amyotrophic Lateral Sclerosis, *BioMed Research Int*, **2015**, 2015.

27. J. Zhou, W. Yang, D. Suo, Y. Li, L. Peng, L. Xu, X. Jin, *Moringa oleifera* Seed Extract Alleviates Scopolamine-Induced Learning and Memory Impairment in Mice, *Front. Pharmacol*, **2018**, 9, 389.

28. S. Giacoppo, T.S. Rajan, G.R. De Nicola, R. Iori, P. Rollin, P. Bramanti, E. Mazzon, The Isothiocyanate Isolated from *Moringa oleifera* Shows Potent Anti-Inflammatory Activity in the Treatment of Murine Subacute Parkinson's Disease, *Rejuvenation Res*, **2017**, 20, 50-63.

29. S. Anandjiwala, M.S. Bagul, M. Parabia, M. Rajani, Evaluation of free radical scavenging activity of an Ayurvedic formulation, panchvalkala, *Indian J Pharm Sci.*, **2008** , 70, 31–5.

30. K.Y. Satyndra, P. Jay, C. Shikha, P.S. Surya, Mucuna pruriens seed extract reduces oxidative stress in nigrostriatal tissue and improves neurobehavioral activity in paraquat-induced Parkinsonian mouse model, *Neurochemistry International*, **2013**, 62, 1039–1047.

31. S. Manna, D. Bhattacharyya, T. Mandal, S. Dey, Neuropharmacological effects of deltamethrin in rats, *Journal of Veterinary Science*, **2006**, 7, 133–136.

32. M. Pisa, Regional specialization of motor functions in the rat ST: implications for the treatment of Parkinsonism, *Prog. Neuropsychopharmacol. Biol. Psychiatry*, **1998**, 12, 217–224.

33. M. Mohanasundari, M.S. Srinivasan, S. Sethupathy, M. Sabesan, Enhanced neuroprotective effect by combination of bromocriptine and hypericum perforatum extract against MPTP-induced neurotoxicity in mice, *Neurol. J. Sci*, **2006**, 249, 140–144.

34. H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, *Analytical Biochemistry*, **1979**, 95, 351–358.

35. M.S. Moron, Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver, *Biochimica et Biophysica Acta (BBA)-General Subjects*, **1979**, 582, 67–78.

36. J. M. McCord, I. Fridovich, The role of superoxide anion in the autooxidation of epinephrine and simple assay for superoxide dismutase, *J. Biol. Chem.*, **1969**, 224, 6049–6055.

37. D.L. Granger, R.R.Taintor, K.S. Boockvar, J.B. Hibbs, Measurement of nitrate and nitrite in biological samples using nitrate reductase and Griess reaction, *Methods Enzymol.*, **1996**, 268, 142–151.

38. A. Kumar, I. Ahmad, S. Shukla, B.K. Singh, D.K. Patel, H.P. Pandey, Effect of zinc and PQ co-exposure on neurodegeneration: modulation of oxidative stress and expression of metallothioneins, toxicant responsive and transporter genes in rats, *Free Radic. Res.*, **2010**, 44, 1–16.

39. L. Alessandro, S. Alberto, B. Alberto, S. Alberto, Cultivation, Genetic, Ethnopharmacology, Phytochemistry and Pharmacology of *Moringa oleifera* Leaf: An Overview, *International Journal of Molecular Sciences*, **2015**, 16, 12791-12835.

40. K. Xianjuan, L. Biao, B.O. Julia, M.D. Justin, C. Ning, Nutraceutical or Pharmacological Potential of *Moringa oleifera* Lam, *Nutrients*, **2018**, 10, 343.

41. O.D. Omotoso, O.I. Olumorin, A. Sunday, A.S. Aderemi, E. Ogbonna, Neuroprotective properties of Moringa Oleifera in cadmium and herbal alcoholic beverage induced frontal cortex damage in Wistar Rats IP, *Indian J Neurosci*, **2019**, 5 ,206-213.

42. R. Letizia, D. Francesca, G. Agnese, S. Domenico, L.G. Fabrizio, L.C. Veronica, M. Emanuela, Moringin Induces Neural Differentiation in the Stem Cell of the Human Periodontal Ligament, *Scientific reports*, **2018**, 8, 9153.

43. K.S. Verma, R. Nigam, Nutritional Assessment of Different parts of *Moringa oleifera* Lam. collected from Central India, *Journal of Natural Product and Plant Resources*, **2014**, 4, 81-86.

44. B. Sultana, F. Anwar, Flavonols (kaempeferol, quercetin, myricetin) contents of selected fruits, vegetables and medicinal plants, *Food Chem*., **2008**, 3, 879–884.

45. S.P. Brooks, R.C. Trueman, S.B. Dunnett, Assessment of motor coordination and balance in mice using the rotarod, elevated bridge, and footprint tests, *Curr Protoc Mouse Biol*, **2012**, 2,37–53.

46. T.A. Wachiryah, J. Sujira, J. Suchawalee, Benefits of betanin in rotenone-induced Parkinson mice, *Metab Brain Dis*. **2021**, 36, 2567–2577.

47. J. Cobley, M.L. Fiorello, D.M. Bailey, 13 reasons why the brain is susceptible to oxidative stress, *Redox Biol.*, **2018**, 15, 490–503.

48. J.J. Sutachan, Z. Casas, L.S. Albarracin, R.S. Bernd, I. Samudio, G. Janneth, L. Morales, G.E. Barreto, Cellular and molecular mechanisms of antioxidants in Parkinson's disease, *Nutritional Neuroscience*, **2012**, 15, 120–126.

49. D. Galasko, T.J. Montine, Biomarkers of oxidative damage and inflammation in Alzheimer's disease, *Biomarkers Med.*, **2010**, 4, 27–36.

50. I. Marrocco, F. Altieri, I. Peluso, Measurement and Clinical Significance of Biomarkers of Oxidative Stress in Humans, *Oxidative Med. Cell. Longev.*, **2017**, 2017, 1–32.

51. A. Matejuk, A. Shamsuddin, IP6 in cancer therapy: Past, present and future, *Current Cancer Therapy Reviews*, **2010**, 6, 1–12.

52. T. Masoumeh, A. Palanisamy, K. Govindarajan, K.A. Siti, Bioactive Extract from *Moringa oleifera* Inhibits the Pro-inflammatory Mediators in Lipopolysaccharide Stimulated Macrophages, *Pharmacognosy Magazine*, **2015**, 11, 556–563.

53. J. Haavik, K. Toska, Tyrosine hydroxylase and Parkinson's disease, *Molecular neurobiology*, **1998**, 16, 285–309.

54. V. Boonyadist, S. Pongtip, M. Supachoke, T. Suchitra, W. Yuvadee, G. Wandee, Maximizing total phenolics, total flavonoids contents and antioxidant activity of *Moringa oleifera* leaf extract by the appropriate extraction method, *Industrial Crops and Products*, **2013**, 44, 566– 571.

55. J.P.Coppin, Y. Xu, H. Chen, M.H. Pan, C.T. Ho, R. Juliania, Q. Wu, Determination of flavonoids by LC/MS and anti-inflammatory activity in *Moringa oleifera*, *Journal of Functional Foods,* **2013**, 5, 1892–1899.

56. E.M. Fahal, A.M. Babitha Rani, M.D. Aklakur, T.I. Chanu, N. Saharan, Qualitative and Quantitative Phytochemical Analysis of Moringa oleifera (Lam) Pods, *Int J Curr Microbiol Appl Sci.,* **2018**, 7, 657–65.

57. A. Bhattacharya, P. Tiwari, P. Sahu, S. Kumar, A review of the phytochemical and pharmacological characteristics of Moringa oleifera. *J Pharm Bioallied Sci.,* **2018**, 10, 181.

58. M. Bhardwaj, V.K. Sali, S. Mani, et al., Neophytadiene from Turbinaria ornata Suppresses LPS-Induced Inflammatory Response in RAW 264.7 Macrophages and Sprague Dawley Rats, *Inflammation*, **2020**, 43, 937–950.

59. R. Pratiwi, C. Nantasenamat, W. Ruankham, W. Suwanjang, V. Prachayasittikul, S. Prachayasittikul, K. Phopin, Mechanisms and Neuroprotective Activities of Stigmasterol Against Oxidative Stress-Induced Neuronal Cell Death via Sirtuin Family. *Front Nutr.* **2021**, 8.

60. M.A. Icer, N. Arslan, M. Gezmen-Karadag, Effects of vitamin E on neurodegenerative diseases: an update. *Acta Neurobiol Exp (Wars),* **2021**, 81, 21-33.

61. D. Blum,, S. Torch, N. Lambeng, M. Nissou, A. Benabid, R. Sadoul, et al., Molecular pathways involved in the neurotoxicity of 6-OHDA, dopamine and MPTP: contribution to the apoptotic theory in Parkinson's disease, *Prog. Neurobiol.,* **2001**, 65, 135–172.

62. S. RajaSankar, T. Manivasagam, V. Sankar, S. Prakash, R. Muthusamy, A. Krishnamurti, et al., Withania somnifera root extract improves catecholamines and physiological abnormalities seen in a Parkinson's disease model mouse. *J. Ethnopharmacol,* **2009**, 125, 369–373.