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MUCUNA SANJAPPAE SHOWS PROMISING ANTI-PARKINSON'S ACTIVITY BY REDUCING OXIDATIVE STRESS IN MPTP INDUCED MOUSE MODEL

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ABSTRACT

To evaluate anti-Parkinson's activity of ethanolic extract of *Mucuna sanjappae* in MPTP induced mouse model. In this study, our efforts on the evaluation of neuroprotective efficacy of newly described *Mucuna* species, *M. sanjappae* in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) induced mouse model of Parkinson's disease (PD). Twenty Swiss albino mice were randomly divided into five groups: Control, MPTP and three different doses of MS seed ethanolic extract in MPTP induced mice group (50, 100 and 150 mg/kg body weight (wt)). After 21 days, the behavioral studies, neurobiochemical studies and immunohistochemical studies were performed in different groups of animal. There was improvement in behavioral parameters of the MS treated mice with optimum dose of 100 mg/kg body wt. Our results demonstrate generation of oxidative stress in the nigrostriatal region of MPTP exposed mice. There was a marked increase in lipid peroxidation, nitrite level and catalase activity and decrease in glutathione level in Parkinsonian mice whereas; significant restoration was observed due to the MS treatment of 100 mg/kg body wt. The protection of dopaminergic neurons was confirmed by TH immunostaining. MS reduces behavioral impairments, oxidative stress generated due to MPTP and also prevents dopaminergic neurodegeneration thereby showing its efficacy as a future anti-Parkinsonian agent.

KEYWORDS: Parkinson's disease, *Mucuna sanjappae*, MPTP, Tyrosinase hydroxylase, Dopamine, Oxidative.

1. INTRODUCTION

Parkinson's disease (PD) also known as "Shaking Palsy" is a chronic progressive neurodegenerative disorder associated with aggregation of protein α-synuclein and degeneration of nigrostriatal dopaminergic neurons.[1,2] It is characterized by a slowness of movement, muscular stiffness, rigidity, tremor, poor posture, imbalance and sensory-motor integration deficits.[3, 4] A combination of genetic susceptibilities and environmental factors seems to play a critical role in etiology of PD.[5] Numerous biochemical and molecular mechanisms have been identified as mediators of neuronal cell death in PD.[6, 7] One of the most common etiologies for PD is oxidative stress induced by reactive oxygen species (ROS) in the brain and nervous system.[8] Till date the complete cure of this disease is doubtful because, most of the treatments available can only slow down its progression and alleviate symptoms.[9] L-DOPA is the drug of choice for PD treatment since; dopamine cannot pass through blood brain barrier but L-DOPA, a precursor of dopamine, passes through it and gives symptomatic relief from the disease. Unfortunately, L-DOPA therapy develops some secondary complications like fluctuations in motor performance, end-of-dose failure and troublesome involuntary movements known as Levodopa-induced dyskinesias (LIDs).[10]

In ancient medical systems of Asian countries such as India, China, Japan and Korea, herbal medicines are used to treat PD, which was created on experience-based theories.[11] The plants like green tea,[12] *Ginkgo biloba*,[13] *Nardostachys jatamansi*,[14] *Withania somnifera*,[15, 16, 17] *Acanthopanax senticosus,*[18] *Bacopa monnieri,*[1] *Curcuma longa,*[19] *Mucuna pruriens,*[20, 21] etc. have been proved effective for PD management. *M. pruriens* has been profitably exploited for the PD treatment since long time.[21] It has been evidenced that, *M. pruriens* is more effective than L-DOPA, might be not only due to L-DOPA but some other properties like antioxidants present in it.[22, 23, 24] *M. pruriens* is one of the species of genus *Mucuna*; the genus which comprises about 100 species distributed throughout tropical and subtropical regions of the world. But unfortunately, most of the *Mucuna* species remains uninvestigated for their potential in disease treatment including PD. However, as the demand is constantly increasing, it is a need of time to exploration and exploitation of another species of *Mucuna* for the treatment of Parkinson patient.[25] Hence,

we have focused our efforts to evaluate the efficiency of MS in the prevention of neurodegeneration in PD.

M. sanjappae is a newly described, neglected species of *Mucuna* from north- Western Ghats, India comprising 7.3% of L-DOPA.[25] In the present study, we examined the effect of MS seed ethanolic extract (MSEE) on MPTP induced Parkinson mouse model. MPTP induces dopaminergic toxicity[26] and can be used as a robust model of PD in animals.[27] Our study covers the study of behavioral pattern, neurobiochemicals parameters related to oxidative stress and immunohistochemistry of dopaminergic neuronal cells to study the expression of tyrosine hydroxylase enzyme in the SNpc of brain.

2. MATERIALS AND METHODS
2.1. Plant material and preparation of extracts
The MS seeds were collected from the north- Western Ghats, Maharashtra, India and also deposited in Department of Botany, Shivaji University, Kolhapur. The seeds were grinded in mechanical grinder to make fine powder, which was used for further experiments. The extraction was carried out by Soxhlet apparatus using ethanol as a solvent. The extracts were evaporated to dryness under reduced pressure and temperature below 50^0C in rotary vacuum evaporator. The plant extract was expressed in terms of dry weight.

2.2. Chemicals
Acetic acid, disodium hydrogen phosphate, glutathione (GSH), potassium chloride and sodium dihydrogen phosphate were procured from Sisco Research Laboratories (SRL; Mumbai, India). Streptavidin-peroxidase, normal goat serum and the DAB (3, 3 diaminobenzidine) system were procured from Bangalore Genei Pvt. India Ltd., Bangalore, India. 1-Methyl-4-phenyl-1, 2, 3, 6-tetra hydropyridine (MPTP) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Folin Ciocalteau reagent, hydrogen peroxide (H_2O_2), potassium dichromate was purchased from Merck (Darmstadt, Germany). A primary antibody for TH was procured from Santa Cruz, Biotechnology (Santa Cruz, CA, USA).

2.3. Acute toxicity testing
Swiss albino female mice weighing 25 ± 5 gram were taken for the study. The oral toxicity testing of MS powder was done according to OECD guidelines using two groups with three animals each as toxicity test groups. The suspension of the extract was administered PO for the dose 2000 mg/kg body wt. The animals were fasted 3-4 hrs before administration of MS and 2 hrs after dosing. Control animals received 10ml/kg body wt. of distilled water PO. The animals were observed continuously for the initial 4 h and then intermittently for 14 days after dosing. The animals were observed for pre-terminal death, weight and hair loss, toxic signs and gross necropsy.

2.4. Animal treatment
The male Swiss albino mice for treatment were obtained from the animal house of the Institute of Medical Science, Banaras Hindu University, Varanasi, India. The average weight of mice was 25 ± 5 gram. Guidelines of the Institutional Ethics Committee (IEC) for use of laboratory animal were followed in this study. Animals were maintained under standard conditions of temperature (22 ± 5 ^{0}C), humidity (45–55%) and light (12/12-h light/dark cycle). The animals were fed with standard pellet diet and water *ad libitum*. For the study, animals were randomly divided into 5 experimental groups (n = 5) as follows; Group I: Mice were given intraperitoneally (ip) injections of saline (0.9%), this served as control. In Group II mice were administered ip injections of MPTP (30 mg/kg body wt.) twice within 16 hrs to induce Parkinson. Group III, IV and V mice received MPTP (30 mg/kg body wt.) twice within 16 hrs to induce PD and also treated orally with 50, 100 and 150 mg/kg body wt of MSEE respectively, for 7 days prior to MPTP treatment and 14 days after.[17] At the end of experiment, behavioral, biochemical and immunohistochemical studies were performed to understand the neuroprotective efficiency of *M. sanjappae*.

2.5. Neurobehavioral parameter
2.5.1. Rotarod Test
The muscular coordination skill of mice was measured by the rotarod test. In this test, the beam revolves around its longitudinal axis and the mice walk or run forward in synchrony. Mice were trained for 3 consecutive days before the day of final treatment at a fixed speed for 5 minutes. Mice adjust their posture in response to a moving speed of 25 rpm and the time it took for the mice to fall from the rotarod was recorded. An average of four experimental readings was calculated for each animal.[28]

2.5.2. Foot printing test
The Foot Printing test was performed as previously described[29] with slight modifications. Mice were trained to walk across a white sheet of paper. Then, the forepaws of the animal were placed in blue ink and length of forepaw steps during normal walking was measured. The distance between each step on the same side of the body; measuring from the middle toe of the first step to the heel of the second step, helped to determine the stride length. The experiment was repeated for four times and average was calculated.

2.5.3. Narrow beam walking test
The test for motor coordination requiring balance and stability was accomplished using narrow beam walking. Briefly; animals were trained to walk on a stationary wooden narrow flat beam (L100 cm × W1 cm) which was placed at a height of 100 cm from the floor. Time taken to walk the beam from one end to the other was recorded.[30] An average of four experimental readings was calculated for each animal.

2.6. Preparation of tissue samples: Mice were sacrificed by cervical dislocation after the behavior test at 21th day. Nigrostriatal tissue part was quickly dissected out from the brain over ice. It was wet weighted, placed in eppendorf tubes, homogenized in a phosphate buffer saline, sonicated and then centrifuged twice at 13,000 rpm for 15 min at 4 ∘C. The supernatant was used for further biochemical studies.[31,32]

2.7. Biochemical analysis
2.7.1. Lipid peroxidation (LPO): Malondialdehyde (MDA), which is a measure of lipid peroxidation, was spectrophotometrically quantified by means of the thiobarbituric acid assay[33] with slight modifications. In brief, 10% homogenate was mixed with 10% SDS solution followed by the addition of 20% acetic acid. Finally 0.8% TBA was added and the reaction mixture was incubated in a boiling water bath for 1 hr. The assay mixture was cooled, centrifuged and absorbance of the supernatant was read at 532 nm against control. LPO levels were expressed as nmoles of MDA/mg protein.

2.7.2. Glutathione level determination (GSH): GSH in the nigrostriatal tissue was estimated using 5, 5-dithiobis 2-nitrobenzoic acid (DTNB).[34] In brief, the homogenate (100 μl) was mixed with DTNB (2 ml in phosphate buffer, pH 8.0) and the volume was made up to 3.0 ml with phosphate buffer. Absorbance was recorded immediately at 412 nm and the GSH content was calculated in μM/mg tissue (nigrostriatal tissues) using a standard curve of GSH.

2.7.3. Nitrite estimation: 10% tissue homogenate was incubated with ammonium chloride and mixed with Griess reagent. The reaction mixture was then incubated at 37 ^{0}C for 30 min, and the absorbance of the supernatant was recorded at 540 nm. The nitrite content was calculated using a standard curve for sodium nitrite (10–100 μM) in terms of μmoles/ml.[35]

2.7.4. Catalase activity: Catalase activity was examined spectrophotometrically by measuring the rate of decomposition of hydrogen peroxide.[31,36] Briefly, tissue homogenate was incubated with potassium dichromate and acetic acid (1:3) for 10 minutes in boiling water and absorbance was taken at 570 nm. The enzymatic activity was measured in μmoles/min/mg protein.

2.8. TH-immunoreactivity
Immunohistochemical (IHC) staining of tyrosine hydroxylase (TH)-positive dopaminergic (DAergic) neurons was performed in SN region of mice brain sections.[37] The perfused mice brains were post-fixed with paraformaldehyde and thin sections (20 μm thick) were cut, using a cryostat. Sections were incubated with 0.5% H2O2, followed by incubation in 2% normal goat serum and washed. Then, monoclonal anti-TH antibody was added and incubated at 4 ^{0}C for 48 h and washed again. Finally, sections were incubated with biotinylated secondary antibody and streptavidin peroxidase. The

color was developed with 3, 3 diaminobenzidine and the sections were mounted with DPX after dehydrating them in graded ethanol and mounted.[38] Images were captured using a bright field microscope (Nikon eclipse P-200) at 10X magnification. Counting of TH-positive cells was done using a standard procedure reported earlier.[39] Counting of TH positive cells was done by selecting three slides from each mouse brain, of all the treated groups. The counting for every slide was done in triplicate to exclude any experimental error.

2.9. Phytochemical analysis of MSEE
2.9.1. Analysis of phenolics, flavonoids and phytic acid: The phenolic content of ethanolic extract of *M. sanjappae* seeds (MSEE) was determined spectrophotometrically according to the method described by Singleton and Rossi.[40] Total phenolics value is expressed in terms of mg of gallic acid equivalent per gram (mg GAE g $^{-1}$) of dry mass. The flavonoids content was determined according to the method by Chang *et al.*[41] Flavonoids was compared to the standard curve of quercetin concentrations and expressed as milligram of quercetin equivalents per gram (mg QUE g^{-1}) of dry weight. The phytic acid content was determined according to the method described by Gao *et al.*[42]

2.9.2. RP-HPLC-DAD analysis of L-DOPA and phenolics: The reversed phase HPLC photo diode array (RP HPLC DAD) (Shimadzu HPLC, hypersil C18 reversed-phase column) for the analysis of L-DOPA was performed. The MSEE extract was obtained by homogenizing 10 g of MS cotyledon powder in 50 ml ethanol. Extraction was carried out on an orbital shaker for 24 h at room temperature. The mixture was filtered through 0.45 μm filter and used for L-DOPA determination. The mobile phase was methanol with flow rate of 1 ml min−1 and 20 μl injection volume. L-DOPA was identified at 280 nm by comparing the retention time of sample chromatographic peaks with those of authentic standards.

For the phenolics determination, sample was prepared using continuous shaking extraction method (110±2 rpm) at controlled temperature (25±2^{0}C) for overnight. 1 gm of MSSP was extracted using 100 ml of ethanol as a solvent. The extract was filtered through 0.45 mm nylon filter (Axiva filters), final volume was adjusted with ethanol and stored in amber vial at 4^{0}C until HPLC analysis. Mobile phase consisting of Water: Acetonitrile: Glacial Acetic Acid (90:5:5) was used for separation with an injection volume of 20 μl. The flow rate was 0.9 ml/min with run time of 60 min and detection was done at 280nm.

2.10. Statistical analysis: Statistical analysis of the data was performed using two-way analysis of variance (ANOVA) followed by Student-Newman–Keuls test using Statistical Package for the Social Sciences (SPSS). Data were expressed as mean ± standard errors (SE) for

separate groups and differences were considered statistically significant, when p values were less than 0.05 ($p < 0.05$).

3. RESULTS

3.1. Oral acute study: The MS seed powder caused no mortality, hair or weight loss. Any type of toxicity signs at the dose of 2000mg/kg body wt was not observed. All the animals appeared normal and showed no clinical signs of intoxication after dosing till the end of the study (14 days). Based on this result, we selected different safe doses for our further experiment.

3.2. Neurobehavioral study

3.2.1. Rotarod test: The rotarod test was performed to examine balance, grip strength and motor coordination of mice. We observed significant ($p < 0.001$) decrease in retention time on rotarod for group II (MPTP-treated group) mice in comparison with group I, which indicates due to the action of MPTP, loss of motor coordination and grip strength was developed in mouse. The treatment with MS significantly increased retention time on rotarod in group III, IV and V mice ($F_{(3, 29)} = 167.52$, $p < 0.001$). The optimum dose found was 100 mg/kg body wt i. e. group IV. A significant difference was also observed within treatment groups when we compared group III ($p < 0.001$) and group V ($p < 0.01$) with group IV (Fig.1a).

3.2.2. Foot printing test: Fig.1b shows the mean stride length differences in the control, MPTP and MPTP plus MS treated groups. The step difference significantly ($p < 0.001$) decreased in the group II (MPTP-treated group) relative to the controls. While, treatment with MS (group III, IV and V) significantly ($F_{(3, 29)} = 16.673$, $p < 0.001$) improved walking impairment relative to the MPTP-intoxicated mice. The optimum dose for improvement was 100 mg/kg body wt i.e. group IV ($p < 0.001$ when compared with group II). The significance level between treatment groups was also determined and it was ($p < 0.001$) and ($p < 0.01$) respectively, for group III and IV in comparison with group IV.

3.2.3. Narrow beam walking: Narrow beam walking time was significantly increased ($p < 0.001$) in the Group II (MPTP exposed mice) in comparison with group I (control mice). MS treatment declines the time significantly ($F_{(3, 29)} = 83.027$, $p < 0.001$) in comparison with group II. Here also, 100 mg/kg body wt dose was an optimum dose for the treatment i. e. group IV (Fig. 1c). The significant difference between group III and IV was $p < 0.001$ whereas between group IV and V was $p < 0.01$.

3.4. Biochemical analysis

3.4.1. Lipid peroxidation (LPO): The extent of lipid peroxidation was measured in terms of MDA level and it was found to be significantly increased ($p < 0.001$) in the nigrostriatal region of group II (MPTP exposed mouse) in comparison with group I. Whereas, the treatment of MS reduces the LPO level extensively in the PD mouse

($F_{(3, 29)} = 43.270$, $p < 0.001$) when we compare group IV with group II, as group IV again proved as an optimum dose. Within the treatment groups, significance level between group III and IV was ($p < 0.001$) while group V does not showed any significant difference with group IV (Fig. 2a).

3.4.2. Glutathione Level: Fig. 2b gives comparatively the level of GSH found in control, MPTP and MS treated groups. It can be seen that, the concentration of GSH is significantly decreased ($p < 0.001$) in group II in comparison with group I. The treatment of MS again proved beneficial for the improvement of GSH level with 100mg/kg body wt (($F_{(3, 29)} = 107.04$, $p < 0.001$)) as an optimum treatment dose.

3.4.3. Nitrite estimation: The results showed that, administration of MPTP significantly increases the nitrite levels in the Parkinsonian mouse nigrostriatal region (Group II) compared to control (Group I) ($p < 0.001$) and MS seed extract possess the potential to reduce it and ultimately oxidative stress. The optimum dose found was 100 mg/kg body wt (Group IV) which significantly decreases ($F_{(3, 29)} = 172.97$, $p < 0.001$) the nitrite level in comparison with MPTP alone treated group (Group II) (Fig. 2c).

3.4.4. Catalase Activity: The level of catalase was also significantly increased ($p < 0.001$) in the MPTP treated mouse (Group II) when compared to their respective control (Group I). The treatment with MS has improved catalase activity in MPTP induced mouse. The optimum dose was 100mg/kg body wt with significance level ($F_{(3, 29)} = 50.092$, $p < 0.001$) in comparison with MPTP group (Group II). The significance level between MS treatment group was $p < 0.001$ when compared group IV (optimum dose) with group III and group V (Fig. 2d).

3.5. TH Immunoreactivity

The effect of ethanolic extract of MS seed on PD mice was estimated by IHC analysis of TH-positive DAergic neurons in SNpc part of mouse brain. A significant interaction between doses for treatment of mice with TH positive DAergic neurons was obtained after a one-way ANOVA analysis. The response for different doses of MS treatment was different with optimum dose of 100mg/kgbody wt. A significant ($F_{(3, 29)} = 124.07$, $p < 0.01$) increase in TH-positive DAergic neurons was observed at this optimum MS treated group as compared to MPTP treated group (Fig. 3a and 3b).

3.6. Analysis of phenolics, flavonoids and phytic acid

The essential secondary metabolites present in MSEE were quantified. MSEE showed 7.929 ± 0.52 mg GAE g^{-1} and 39.73 ± 0.89 mg RUE g^{-1} of phenolics and flavonoids respectively. The phytic acid content of MSEE was 114.5mg/gm.

3.7. HPLC Analysis of L-DOPA: The presence of L-DOPA in the ethanolic extract of MSEE was confirmed

by RP-HPLC analysis (Fig. 4.1). It is well established fact that L-DOPA is the important drug for PD management and thus, its presence signifies the potential of MSEE for the PD treatment. Phenolics determination in MSEE in comparison with standard compounds revealed that, tannic acid and gallic acid is present in the MS beans.
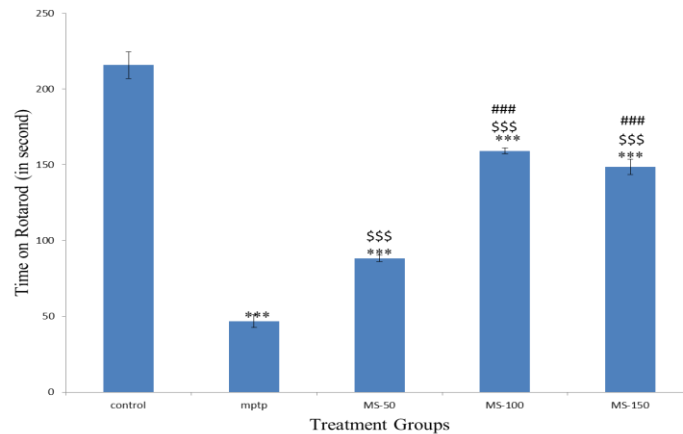
**1a. Rotarod test of mice was performed in all the different groups and a significant improvement in the time of stay on rotarod was found in MS treated mice compared to MPTP group.**
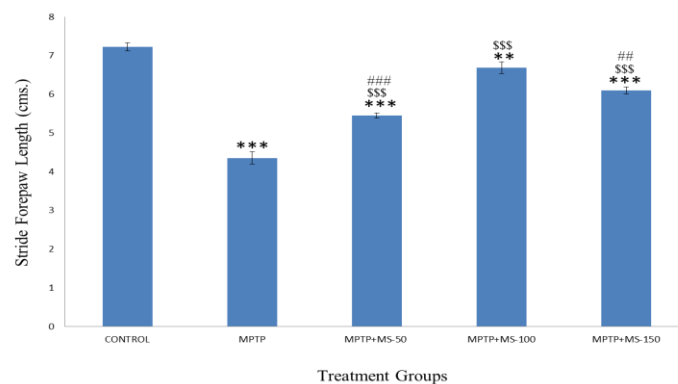
1b. Stride forepaw length was significantly reduced in the PD mouse compared to control but MS treatment restores the condition.
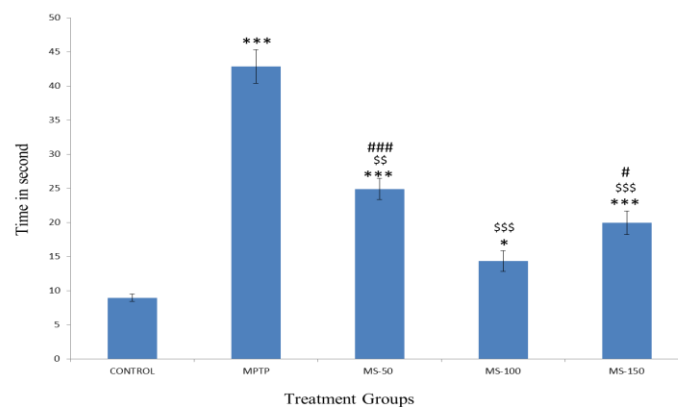

1c. Narrow beam walking time was significantly increased in the MPTP exposed mice in comparison with control group while MS treatment declines the time.
(mean ± SEM, n = 6. Significant difference ***p < 0.001, **p < 0.01, *p < 0.05 compared to control; $$$p < 0.001 and $$p < 0.01 compared to MPTP; ###p<0.001 and ##p<0.01 #p<0.05 compared to 100mg/kg body wt i.e. Group).
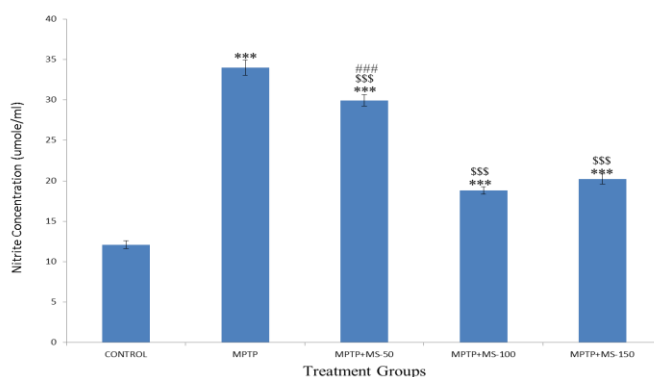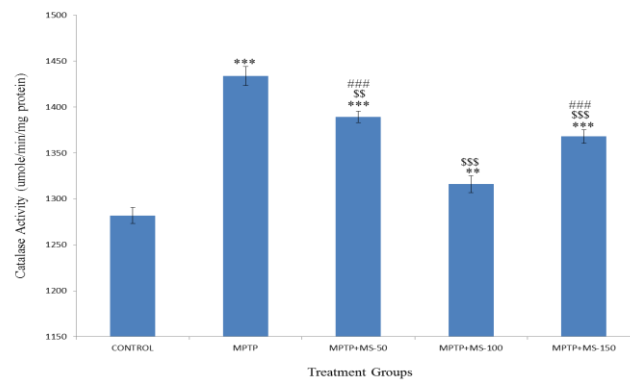**Fig.1. Effect of ethanolic extract of MS on behavioral parameters.**
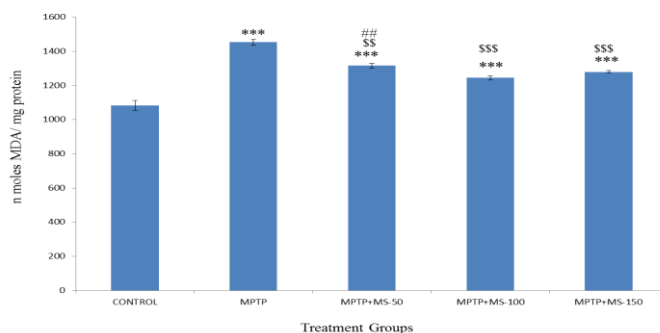
2a. Effect of ethanolic extract of MS seed on malondialdehyde (MDA) in the PD mouse brain. The PD induced mouse nigrostriatal region showed elevated levels of MDA compared to the control. The treatment of MS extract at different doses showed varying degree of decreased MDA level with optimum dose concentration as 100mg/kg body wt. (mean ± SEM, n = 6, Significant difference ***p < 0.001 compared to control; \$\$\$p < 0.001 and \$\$p < 0.01 compared to MPTP; ##p<0.01 compared to 100mg/kg body wt Group).
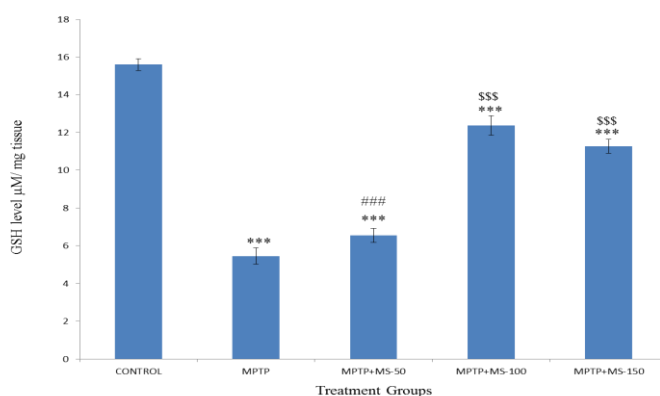
2b. Effect of ethanolic extract of MS seed on glutathione (GSH) in the PD mouse brain. The PD induced mouse nigrostriatal region showed decreased levels of GSH compared to the control. The treatment of MS extract at different doses showed varying degree of elevated level of GSH with optimum dose concentration as 100mg/kg body wt. (mean ± SEM, n = 6, Significant difference ***p < 0.001 compared to control; \$\$\$p < 0.001 compared to MPTP; ###p<0.01 compared to 100mg/kg body wt Group).


2c. Effect of ethanolic extract of MS seed on nitrite level in the PD mouse brain. The PD induced mouse nigrostriatal region showed elevated levels of nitrite compared to the control. The treatment of MS extract at different doses showed varying degree of decreased MDA level with optimum dose concentration as 100mg/kg body wt. (mean ± SEM, n = 6, Significant difference ***p < 0.001 compared to control; \$\$\$p < 0.001 compared to MPTP; ###p<0.001 compared to 100mg/kg body wt Group).

2d. Effect of ethanolic extract of MS seed on catalase level in the PD mouse brain. The PD induced mouse nigrostriatal region showed elevated levels of catalase compared to the control. The treatment of MS extract at different doses showed varying degree of decreased MDA level with optimum dose concentration as 100mg/kg body wt. (mean ± SEM, n = 6, Significant difference ***p < 0.001 compared to control; $$$p < 0.001 and $$p < 0.01 compared to MPTP; ###p<0.001 compared to 100mg/kg body wt Group).

**Fig.2: Effect of ethanolic extract of MS on biochemical parameters.**
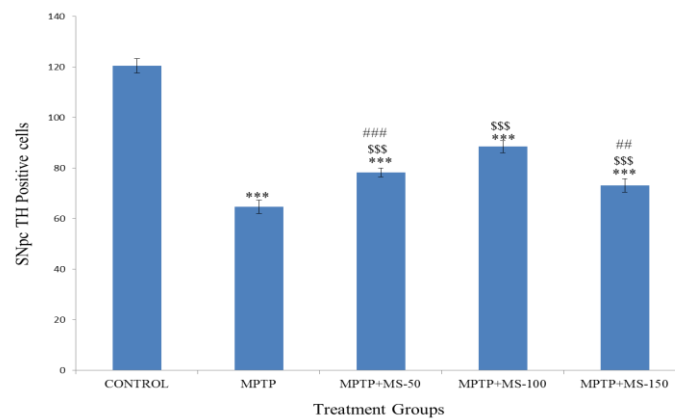
3a. Bar diagram showing number of TH positive neurons in SN region of control and treated mice. (Mean ± SEM, n = 3. Significant difference ***p < 0.001 compared to control; $$$p < 0.001 compared to MPTP; ###p< 0.001 and ##p< 0.01 compared 100mg (optimum dose) with 50mg and 150mg/kg body wt doses group.
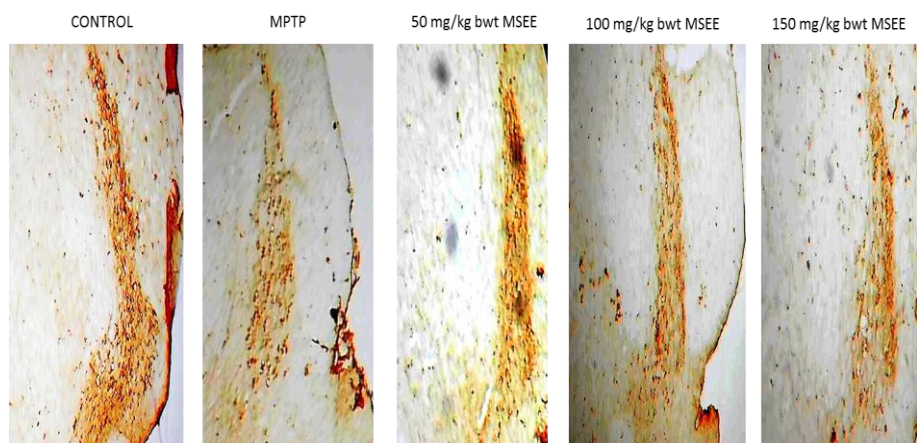
3b. TH immunoreactivity in frozen brain sections of control and treated animals.

Fig. 3: TH immunoreactivity of dopaminergic neurons in substantia nigra part of brain in control, MPTP and MS treated mice groups (n = 3 in each group).
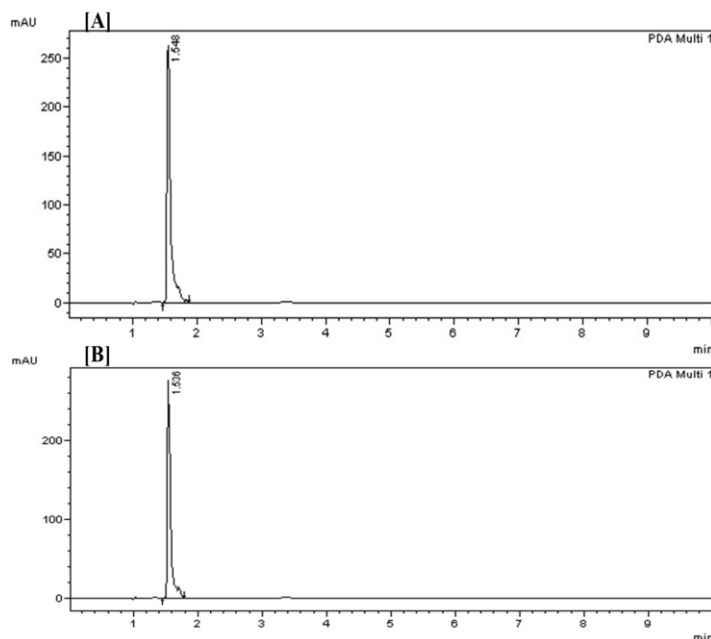
4. HPLC analysis of L-DOPA in the ethanolic extract of *M. sanjappae* (MSEE). [A] shows standard L-DOPA peak (retention time 1.548) while [B] shows L-DOPA in sample (retention time 1.536)

4. DISCUSSION

Mucuna pruriens is being used in Indian traditional medicine for the treatment of Parkinson's disease.[43] But, unfortunately, other species of *Mucuna* remains underutilized and uninvestigated till today. As demand of drug is constantly increasing, it is worthwhile to examine the neuroprotective efficacy of other *Mucuna* species also. For this purpose, we initiated the approach for future herbal drug development. We have investigated *M. sanjappae* as a representative species for its potential in neurodegenerative diseases with Parkinson disease as an example. We initiated the approach on ethanolic extract of MS to study cumulative outcome of number phytoconstituents together with L-DOPA. Initially, to know whether MS have any toxic symptoms to mice, acute toxicity experiment was conducted and results clearly showed that, it is safe to use for further research. Here, we used 2000mg/kg body wt dose for the acute toxicity study and does not found any type of toxic signs or symptoms. Also, it assisted us to specify the safe dose concentration for the experiment.

Our results was in support to earlier finding that, MPTP could cause behavioral deficits such as loss of motor co-ordination, neuromuscular strength and decrease in mean fore paw stride length similar to PD patients.[17] For this purpose, we conducted rotarod test, narrow beam walking and foot printing test to study behavioral impairments in the mouse and found that, the performance of MPTP group was significantly worse than those for the control. When Parkinsonian mice were treated with MS, they showed improvement in rotarod, narrow beam walking and significant enhancement in the stride forepaw length.

The loss of dopaminergic neurons in the ventral midbrain leading to motor abnormalities and autonomic dysfunctions is the characteristics of PD.[44, 45, 46] The SN part of ventral brain is involved in motor function and disruption of this area leads to movement disorder such as Parkinson's disease.[47] In the brain, MPTP is metabolized to its active toxin, MPP^+, by the action of monoamine oxidase (MAO) generating free radicals.[48, 49] As substantia nigra (SN) neurons are more vulnerable to oxidative stress,[50] MPTP induced oxidative stress results in increased level of malondialdehyde (MDA) and lipid hydroperoxides, a biomarker of oxidative stress damage of these neurons.[51, 52, 53] GSH in conjunction with the reductant nicotinamide adenine dinucleotide phosphate reduced (NADPH), can reduce lipid peroxidase, free radicals, and H_2O_2.[13] Thus, decrease in GSH level results in increasing free radicle load, which trigger oxidative stress induced neurodegeneration. In MPTP intoxicated mouse, we found the depletion of GSH and increased LPO level, while condition was reversed due to the MS treatment. Also, MPTP increases the level of NO production in the brain, which leads to enhanced dopaminergic neuronal damage.[54] Similarly, the formation of superoxide anions in the presence of MPP^+[15] results in increased level of catalase activity of SN.[55,56] Here, we also found the increased level of nitrite and catalase in the MPTP intoxicated mouse, which was restored by the MS treatment proving its efficiency in the PD treatment.

TH is the rate-limiting enzyme responsible for converting L-DOPA into dopamine. Thus, the measurement of TH-immunoreactivity is a measure of the functionality of dopaminergic neurons present in the SN part of brain.[27] In our study, MPTP intoxicated mice showed a significant reduction in TH immunoreactivity,

indicating a loss of dopaminergic neurons due to the toxicity of MPTP. Our result is supported by earlier reports showing selective dopaminergic neuronal loss following exposure to several PD-inducing neurotoxins.[57,58,59,4] According to Blum et al,[60] the main mechanism of dopaminergic neuronal loss in these animals is oxidative stress generated in response to MPTP exposure. MS treatment produced a significant improvement in the number of dopaminergic neurons as compared to the untreated PD mice, which further support its neuroprotective efficiency. Among different doses of MS, 100mg/kg body wt was proved to be an optimum dose for improvement in behavioral pattern, restoration of neurobiochemicals and protection of dopaminergic neurons in SN. MS at the dose of 50mg/kg body wt showed poor improvement and surprisingly, we found little decrease in response at the dose of 150mg/kg body wt in comparison to 100mg/kg body wt. It indicates that, increased concentration does not follow any concentration dependent improvement pattern and may attenuate maximum effect of MS. Further research is necessary to find out the actual mechanism.

There is increasing interest in the plant secondary metabolites especially phenolics, flavonoids, alkaloids, proanthocyanidin etc. for their potential in treatment of diseases. Phenolics and flavonoids have great contribution in the antioxidative properties of plant. Flavonoids exert their effects by enhancing the activity of endogenous antioxidant enzymes, suppressing the lipid peroxidation, inhibition of inflammatory mediators, modulation of gene expression in neuronal cells.[42,61] MSEE showed considerable level of phenolics and flavonoids which might be the reason of its activity against MPTP toxicity in mice. According to Xu et al.,[62] disruption of iron metabolism and excess iron accumulation is the key pathogenesis found in PD brain. The excess iron again induces oxidative stress and further degeneration of dopaminergic cells. Phytic acid is a natural iron chelators found in various leguminous plant seeds. Number of studies has been carried out for the potential of phytic acid in PD treatment due to its antioxidant and iron chelation property.[62, 63] MSEE contain high amount of phytic acid which indicates its positive role in the effective treatment of MPTP intoxicated mice.

L-DOPA, tannic acid (TA) and gallic acid (GA) were successfully identified from MSSP under study according to retention time and spectral characteristics of their peaks compared to standard phenolic compounds peaks obtained by using HPLC coupled with UV dual detector (fig. 4.1 and 4.2). L-DOPA is the well-known drug for the PD treatment. Tannic acid is a polyphenol having antimutagenic, anticarcinogenic and antioxidant activity. Antioxidant activity of TA is essentially due to ability of iron chelation.[64] Gallic acid is another phenolics compound found in MSEE having strong antioxidant activity.[65] Study on 6-OHDA rat model

proved that GA exerts its anti-PD effect through antioxidative mechanism.[66]

Although, the exact pathway is unknown, the present study gives strong evidence for the beneficial effect of the MS on MPTP intoxicated Parkinsonian mice. Here, the neuroprotective property of MS is evident from the behavioral and biochemical recovery as well as improved TH activity in SN region of mice brain. Several natural products like polyphenols such as vitamins, flavonoids, phenolic acids, and other polyphenols have antioxidant properties and may be used for treatment of different oxidative stress related diseases including neurodegenerative diseases as promising therapeutic agents.[56,67] Recently, we have reported strong antioxidative potential of MS suggesting its ability to cure different diseases.[68] So, the bioprospecting of MS for its pharmacologically active constituents will be important task for the future drug development. Pure component as an effective drug for the specific disease usually has unavoidable side effects. On the other hand, natural drugs can cure the disease although they have mixture of several compounds.[69] In this connection, MS could be a novel therapeutic agent for the treatment of PD patient.

5. CONCLUSION
In particular, current drugs such as L-DOPA provide symptomatic relief for a limited duration and later on turns to advance secondary complications which might be associated to additional oxidative stress generated from the auto-oxidation products of L-DOPA.[61] The ability of MS to release oxidative stress and ultimately neurodegeneration indicates that it can be a good novel candidate for the PD treatment without any secondary complications.

Since ancient time, *Mucuna pruriens* is being used for the PD management by Ayurvedic practitioner and research is also going on for its therapeutic prospective. But, unfortunately, other species of *Mucuna* remains neglected and uninvestigated for their potential in disease management including PD. This is the first report showing neuroprotective properties of *Mucuna* species other than *Mucuna pruriens*. Thus, our efforts have opened the door of the number of different *Mucuna* species to assess their therapeutic significance in neurodegenerative diseases. Also, this study inspire further investigations on MS for its active constituent compounds, as possible therapeutic contribution against Parkinson's disease as well as other diseases especially related to oxidative stress.

DISCLOSURE STATEMENT
The authors claim no conflict of interest.

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