ejpmr, 2016,3(8), 441-451

SJIF Impact Factor 3.628

EUROPEAN JOURNAL OF PHARMACEUTICAL AND MEDICAL RESEARCH

www.ejpmr.com

Research Article

ISSN 2394-3211

EJPMR

MUCUNA PRURIENS SHOWS NEUROPROTECTIVE EFFECT BY INHIBITING APOPTOTIC PATHWAYS OF DOPAMINERGIC NEURONS IN THE PARAQUAT MOUSE MODEL OF PARKINSONISM

Satyndra Kumar Yadav, Sachchida Nand Rai and Surya Pratap Singh

Department of Biochemistry, Faculty of Science, Banaras Hindu University, Varanasi, Uttar Pradesh, 221005, India.

Corresponding Author: Dr. Surya Pratap Singh

Associate Professor, Department of Biochemistry, Faculty of Science, Banaras Hindu University, Varanasi, Uttar Pradesh, 221005, India.

Article Received on 30/05/2016 **Article Revised on 20/06/2016** **Article Accepted on 11/07/2016**

ABSTRACT

The neuroprotective effects of an ethanolic extract of *Mucuna pruriens* (Mp) seeds were evaluated in a Parkinsonian mice model induced by the pesticide paraquat (PQ). PQ induces classical symptoms of Parkinson's disease (PD) including motor deficits, cellular loss in the *substantia nigra* (SN) and depletion of dopamine levels. The present study investigates the mechanisms of neuroprotection elicited by Mp, a herb traditionally recognized by the Indian system of medicine, Ayurveda. Co-treatment of PQ-treated mice with Mp significantly enhanced antioxidant activity and decreased oxidant levels. Mp also improved the motor abnormalities observed in PD mice, effectively rescued the levels of dopamine and its metabolites in the SN. HPTLC analysis of the Mp seed extract identified that a large proportion of the extract consisted of L-DOPA and also identified a novel constituent, ursolic acid, present in significant quantity. Mp also facilitated neuroprotection by creating an anti-apoptotic environment indicated by reduced apoptotic (Bax and caspase-3) and increased levels the anti-apoptotic (Bcl2) protein expression, respectively. Altogether, the present study suggests that Mp treatment provides nigrostriatal dopaminergic neuroprotection against PQ induced Parkinsonism by the modulation of oxidative stress and apoptotic machinery possibly accounting for the behavioural effects.

KEYWORDS: *Mucuna pruriens,* ursolic acid, Parkinson's disease, Bax, Bcl2, caspase-3.

INTRODUCTION

Parkinson's disease (PD) is the second most common progressive neurodegenerative disease after Alzheimer's disease.[1,2] The canonical features of PD include resting tremor, postural instability, rigidity and bradykinesia. PD's principle pathology is the selective loss of dopaminergic neurons in the nigrostriatal region of mouse brain, namely the substantia nigra (SN).[2,3] The progressive neuronal loss is coupled with the depletion of dopamine levels, resulting in various motor and cognitive abnormalities.[4]

Three main factors play a crucial role in the development of PD namely age, genetic inheritance and exposure to environmental toxins.[2,5] In the environment, exposure to herbicides and fungicides used in agriculture are associated with the development of PD.[6] One commonly used herbicide called 1,1'- dimethyl-4,4'-bipyridinium (Paraquat; PQ) has been implicated in several pathological neurodegenerative disorders including PD.[7]

One of the main pathological features of PQ-induced PD is oxidative damage induced by the modulation of oxido-reductase cycling, antioxidant defence mechanisms and mitochondrial dysfunction.[8] PQ also enhances α-synuclein-induced disruption of membrane integrity and increases membrane conductance as a result of increased oxidative stress. PQ reduces the activity of complex I of the mitochondrial electron transport chain (ETC) while augmenting glial activation and free radical production through NADH oxidase. Any compromise to the ETC ultimately leads to oxidative stress, DNA damage, defective energy metabolism and cellular apoptosis.[9, 10]

There are many drugs currently used as anti-parkinsonian medications, however these treatments invoke debilitating side-effects in a large proportion of patients, such as dyskinesia. Based on the inability of current drugs to effectively relieve even the symptoms of PD, it is necessary to seek new anti-PD treatments that reduce or eliminate the onset of such neurodegenerative diseases.

In the traditional Indian system of medicine, Ayurveda, the seed powder of *Mucuna pruriens* is used to treat Parkinsonism.[11] This potent herb has been more recently classified to contain an abundance of active ingredients that could explain its powerful effects. In addition to the abundance of phytochemicals, Mp seeds contain 4-5% L-DOPA, the natural precursor to dopamine that is normally used as an allopathic treatment to PD marketed

as levodopa. Importantly, studies and medical practitioners have indicated that PD patients treated with Mp do not acquire drug-induced dyskinesia.[12] Further, studies have shown that Mp is more effective than L-DOPA at relieving the symptoms of PD.[13]

Mp is rich in alkaloids such as prurienine, prurieninine and pruriendine.[14] Triterpens and sterols such as sistosterol are also present together with proteins, amino acids and fatty acids.[15] Functionally, Mp has been shown to have a strong antioxidant effects. Mp seed extract elicits a dose dependent protection against superoxide generation, hydroxyl radical production and $FeSO_4$- induced lipid peroxidation.[16] However in the context of PD, the effect of chronic Mp administration on catecholamine levels and antioxidant properties remains elusive.

The present study aims to understand the efficacy of Mp seed extract in reversing the molecular pathology of PD. In particular, the effects of Mp on catecholamine, glutathione (GSH), lipid peroxides, superoxide dismutase (SOD) and motility deficits in a toxin-induced PD model will be investigated. In addition, the present study explores the neuroprotective mechanisms of Mp seed extract by analyzing the expression of apoptotic and antiapoptotic protein markers like Bax, caspase-3 and Bcl2, respectively.

EXPERIMENTAL PROCEDURES

Chemicals

Acetic acid, disodium hydrogen phosphate, reduced glutathione (GSH), nicotinamide adenine dinucleotide phosphate reduced form (NADPH), potassium chloride and sodium dihydrogen phosphates were procured from Sisco Research Laboratories (Mumbai, India). Folin Ciocalteau reagent, hydrogen peroxide (H_2O_2), glutathione reductase (GR) and potassium-dichromate were purchased from Merck (Darmstadt, Germany). 1-chloro 2, 4-dinitrobenzene (CDNB), dextran, histopaque 1119/1077, paraquat dihydro-chloride (PQ), sodium dodecyl sulphate (SDS), thiobarbituric acid (TBA), tri-reagent, and zinc sulphate ($ZnSO_4$) were procured from Sigma-Aldrich (St. Louis, MO, USA). cDNA synthesis kits were procured from MBI Fermentas (York, Ireland, UK). dNTPs, $MgCl_2$, Taq buffer and Taq DNA polymerase were purchased from Genei India Pvt. Ltd. (Bangalore, India). Monoclonal anti-TH antibody, biotinylated anti-mouse secondary antibodies and rabbit anti-VMAT2 antibody were procured from Sigma Aldrich. Polyclonal goat anti-DAT, monoclonal anti- β-actin, bovine anti-mouse alkaline phosphatase (AP) conjugated, rabbit anti-goat AP conjugated and bovine anti-rabbit (AP) conjugated secondary antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Streptavidin-peroxidase, normal goat serum and DAB system was procured from Genei Pvt. India Ltd. Polyvinylidene difloride (PVDF) membranes were procured from Millipore Corporation (MA, USA).

Medicinal plants and preparation of extracts

Mp seeds were collected from the Institute of Medical Sciences, Banaras Hindu University, Varanasi, India. The extraction was carried out by the method of [17] using ethanol as the solvent.

Animal treatment

All experimental procedures were performed in accordance with the National Guidelines on the proper care and use of animals in laboratory research. The study was approved by the Institutional Ethics Committee for use of laboratory animals. Swiss albino mice were obtained from the animal house of the Institute of Medical Science, BHU, Varanasi, India. Male Swiss albino mice weighing 25 ± 5 g were used in the experiment and maintained under standard conditions of temperature (22 ± 5 ºC), humidity (45–55 %) and light (12/12-h light/dark cycle). The animals were fed with standard pellet diet and water ad libitum. The animals were divided into three groups each having 6 mice. One group served as vehicle treated control. In the PD group, mice were treated twice weekly (Tuesday and Saturday) with PQ (10 mg/kg body weight) intraperitoneal (i.p.) for 9 weeks. The third group was co-treated with both PQ and daily with Mp seed extract (100 mg/kg body weight) orally.

Neurobehavioral parameters

For the foot printing test, mice were trained to walk across a white sheet of paper without stopping. The forepaw of the mice were dipped in blank ink and stride length was determined by measuring 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.[18]

For the rotarod test, animals were trained for three consecutive days before the final treatment was administered. All training and testing were performed at a fixed speed (15 rpm) for a maximum of 5 minutes, and the time it took for the mouse to fall was recorded. Experimental tests were conducted 24 hours after the final treatment in all animal groups. Tests on treated animals were conducted at least 4 times and the experimental average was calculated.

Biochemical parameters

Lipid peroxidation in the nigrostriatal tissue of the mouse brain was estimated according to a method described earlier[19] 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 h. The assay mixture was cooled, centrifuged and the absorbance of the supernatant was read at 532 nm against control. LPO levels were expressed as nmoles of malondialdehyde (MDA)/mg protein. The level of glutathione reductase (GSH) in brain homogenate was measured by the method described previously[20] and reported as μM GSH/mg

tissue. The activity of superoxide dismutase (SOD) was measured in the nigrostriatal tissue homogenate (10%) using a standard procedure described previously[21] and reported as µM/mg tissue.

Measurements of dopamine, DOPAC and HVA by HPLC

Animals were sacrificed by cervical dislocation. Brains were dissected out and immediately kept in liquid nitrogen and prepared for biochemical analyses. The nigrostriatal region was isolated and the levels of dopamine, DOPAC and HVA were measured as described previously.[22]

RNA isolation and cDNA preparation

Trizol reagent was used for isolation of total RNA from mouse nigrostriatal region according to company protocols. Revert aid TM minus MuLV reverse transcriptase was used to synthesize cDNA from total RNA as previously described.[23]

RT-PCR of CYP2E1 DAT and VMAT-2 mRNA

The primers and PCR amplification conditions for CYP2E1[24], VMAT-2[25], DAT[26] and GAPDH[27] were used as described previously. PCR products were visualized by agarose gel, quantified by computerized densitometry and normalized against GAPDH.

Western blot analysis

Western blot analysis was performed as described previously[28] Primary antibody dilutions included TH (1:1000; mouse polyclonal), GFAP (1:500; rabbit polyclonal), Bcl2 (1:2000; rabbit polyclonal), Bax (1:500; rabbit polyclonal) and caspase-3 (1:1000; mice monoclonal). The blots were visualized using DAB and H_2O_2 as substrates. Relative band density was calculated with respect to β-actin and their expression was expressed as of percent of control.

Identification of L-DOPA and triterpenoids by HPTLC

The ethanolic extract of Mp seed was obtained by the use of Soxhlet apparatus. Preliminary TLC patterns of the ethanolic extract of Mp seeds were preformed to identify L-DOPA and triterpenoids. To quantify the level of major constituents, HPTLC fingerprints of the ethanolic extract of Mp was conducted. All protocols and methods have been previously described[29]

Statistical analysis

Results are expressed as mean ± standard error of mean (SEM) for separate groups. One-way analysis of variance (ANOVA) was used for all statistical analyses. Students Newman Keul's post hoc analyses were used for between group comparisons. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Mp rescues PD-like behavioural deficits

Both the foot printing (Fig. 1a) and rotarod tests (Fig. 1b) were conducted to measure motor coordination and balance, respectively. There was a significant interaction between PQ- and Mp+PQ-treatments in both the foot-printing $F(17,2) = 171.96$, $p < 0.001$) and rotarod $F(17,2) = 480.01$, $p < 0.0001$) tests due a significant rescue of behavioural deficits in the Mp group ($p < 0.001$).

Mp rescues deficits in dopamine metabolism

Dopamine is the key neurotransmitter down regulated in PD and the key marker of PD progression. There was a significant interaction in the level of dopamine expression in the nigrostriatal region between Mp and PQ+Mp treated animals as determined by HPLC $F(17, 2) = 420.2$, $p < 0.001$**.** PQ-treatment significantly attenuated dopamine levels as compared to controls ($p < 0.001$) whereas Mp co-treatment elicited a significant increase in dopamine content compared to PQ treated animals ($p < 0.05$; Fig. 2a). Similarily for DOPAC, the main metabolite of dopamine, there was a significant interaction of DOPAC levels between Mp and PQ+Mp treated groups $F(17,2) = 223.33$, $p < 0.001$) further indicated by a decrease in the PQ-treated animals as compared to controls ($p < 0.001$; Fig. 2b) and the rescuing of expression with Mp co-treatment ($p < 0.001$). A similar interaction $F(17,2) = 118.94$, $p < 0.0001$) and significance was noted for HVA values, another metabolite of dopamine, in PQ ($p < 0.001$) and PQ+Mp ($p < 0.001$) treated animals (Fig. 2c).

Tyrosine hydroxylase (TH) is the rate-limiting enzyme of dopamine production. TH expression in the nigrostraital region was assessed by western blotting. A significant interaction was found between the PQ and PQ+Mp treated groups $F(17,2) = 82.731$, $p < 0.001$) indicated by a decreased expression in the PQ-treated group compared to controls ($p < 0.001$) with a significant rescue by Mp treatment ($p < 0.001$; Fig. 2d).

Finally, The mRNA expressions of the dopamine transporter (DAT) and vesicular monoamine transporter (VMAT)-2 were also assessed. For both markers, there was a significant interaction in expression between PQ and PQ+Mp-treated groups $F(17,2) = 80.289$, $p < 0.0001$) and $F(17,2) = 52.464$, $p < 0.05$, respectively . DAT expression was significantly increased ($p < 0.001$; Fig. 3a) while VMAT expression was suppressed ($p < 0.001$; Fig. 3b) in PQ-treated mice whereas in both cases, Mp treatment significantly improved their relative expression ($p < 0.001$ and $p < 0.01$, respectively).

Mp rescues oxidative damage by boosting antioxidant mechanisms

To investigate the effect of the Mp seed extract on the level of lipid peroxidation, MDA expression was examined in the nigrostriatal region of the mouse brain. A significant interaction between duration of PQ treatment and PQ+Mp was observed ($F(17,2) = 155.93$,

$p < 0.0001$). Post-hoc analyses indicated that MDA levels were significantly increased in the PQ-treated mouse compared to controls ($p < 0.001$). Further, co-treatment of Mp in the PQ-treated mouse significantly reduced the levels of MDA compared to the PQ-treated mice ($p < 0.001$; Fig. 4a).

There was a significant interaction in the level of GSH between the PQ- and PQ+Mp-treated mice in the nigrostriatal region $F(17,2) = 24.57$, $p < 0.05$). The level of GSH was significantly lower in the PQ treated mouse ($p < 0.001$) while co-treatment with Mp significantly increased GSH activity as compared to PQ-alone treatment ($p < 0.01$; Fig. 4b).

Mp treatment elicited a significant interaction between SOD activity in the PQ- and PQ+Mp-treated mice $F(17,2) = 19.113$, $p < 0.05$). SOD activity was significantly increased in the nigrostriatum of PQ treated mice compared to the respective control ($p < 0.001$) though Mp treatment failed to reduce this activity ($p > 0.05$; Fig. 4c).

GFAP is an indicator of neuroinflammation and an indirect measure of iNOS activity, a producer of the oxidant nitric oxide. There was a significant interaction of GFAP protein expression between the PQ- and PQ+Mp-treated groups $F(17,2) = 36.532$, $p < 0.05$) due to a significant decrease in the PQ-group ($p < 0.001$) that was rescued by Mp (Fig. 5a; $p < 0.01$).

CYP2E1 acts as an antioxidant by metabolizing xenobiotics. In PQ-treated mice, there is a significant interaction in the mRNA expression of CYP2E1 between PQ- and PQ+Mp-treated mice $F(17,2) = 1027.9$, $p < 0.001$). CYP2E1 was significantly increased in the PQ-treated mice as compared to controls ($p < 0.001$) while Mp treatment significantly reduced its expression (Fig. 5b; $p < 0.001$).

Apoptotic markers are affected by PQ and rescued by Mp

The expression of Bcl2, Bax and caspase-3 proteins were analysed in the nigrostriatal tissue by western blot. In all three instances, there was a significant interaction between the PQ- and PQ+Mp- treated groups ($F(17,2) = 82.731$, $p < 0.001$, $F(17,2) = 358.66$, $p < 0.001$, $F(17,2) = 28.402$, $p < 0.05$, respectively). There was a significant decrease in the expression of the anti-apoptotic Bcl2 ($p < 0.001$; Fig. 6) and increase in the expression of the pro-apoptotic Bax ($p < 0.001$; Fig. 6b) and caspase-3 ($p < 0.001$; Fig. 6c) in the PQ-treated groups. All three factors were significantly improvement by the treatment of Mp ($p < 0.001$, $p < 0.001$ and $p < 0.05$ for Bcl2, Bax and caspase 3, respectively).

Determination of L-DOPA and triterpenoids in Mp seed extract

The presence of L-DOPA and ursolic acid was probed in the alcoholic extract of Mp seeds using HPTLC fingerprinting. All compounds were confirmed by comparing their R_f values to reference standards (Fig. 7 c,d; Table 1, 2). Preliminary TLC studies revealed well resolved spots for the marker and test samples and confirmed the presence of L-DOPA (Fig. 7a) and tri-terpenoids (Fig. 7b).

Each of the phytochemicals were further assessed and quantified by HPTLC finger printing patterns. L-DOPA (Fig. 7e) was found in large proportions in the Mp seed extract, with its identity confirmed by comparing standard R_f values (Fig. 7c). Notably, ursolic acid was also found in significant proportions in the seed extract (Fig. 7f) as confirmed with ursolic acid standards (Fig. 7d).

Figures and legends

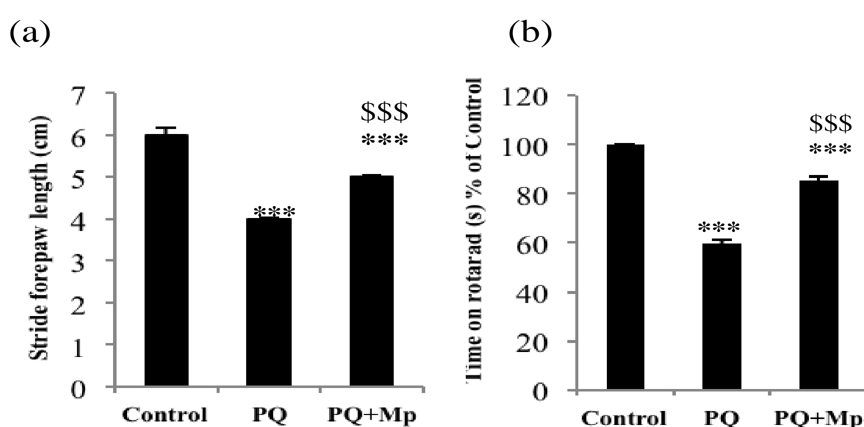

Fig.1. Behavioural characterization of PD mice. The foot-printing test (a) measured the average stride length (cm) while the rotarod test (b) measured the average time (s) animals remained on the rotating beam expressed as the percentage of controls. Each value is represented as mean ± SEM, n=6 per group. A significant difference as compared to controls is represented by *** p <0.001 and compared to PQ as $$$ p <0.001.

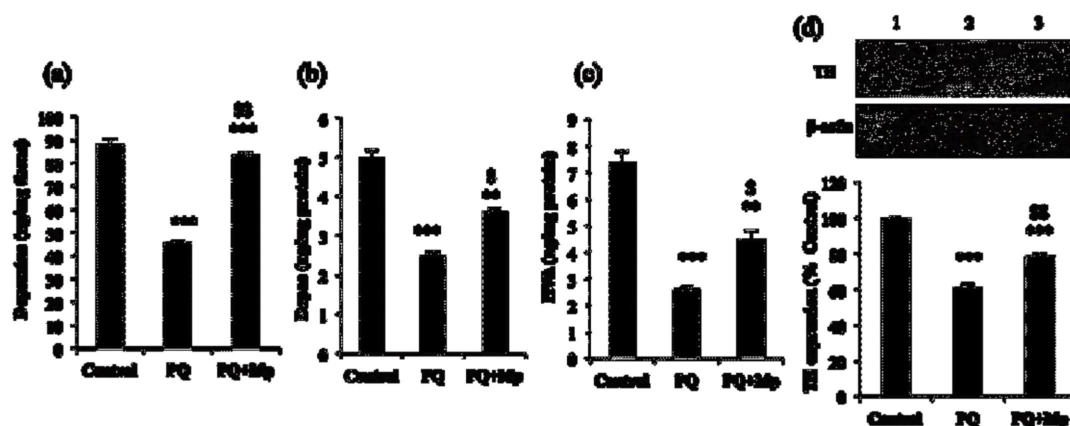

Fig. 2. Measurement of dopamine and its metabolites in the nigrostriatal region of mice. Levels of dopamine (a), DOPAC (b) and HVA (c) were measured by HPLC in both PQ- and PQ+Mp-treated mice. TH expression was determined in the nigrostriatal region by western blotting (d). Both the blot (upper) and its quantification (lower) with respect to the β-actin control are shown in (d). TH is found as a band ~60kDa whereas β-actin is represented at ~42kDa. Values are represented as mean ± SEM, n=6 for each group. A significant difference compared to controls is represented by * p < 0.05and *** p < 0.001 and as compared to PQ as $$ p < 0.01 and $$$ p < 0.001.

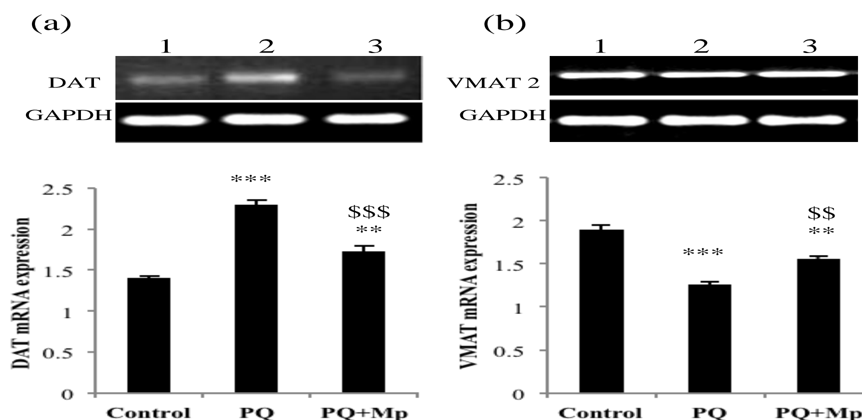

Fig. 3. mRNA expression of DAT (a) and VMAT (b) in the nigrostriatal region. Both DAT (483bp) and VMAT (507bp) expression was compared to the GAPDH (210bp) control and quantified (lower) as the band density ratio of target against control. In the blots, lanes 1, 2 and 3 represent control, PQ and MP+PQ treated animals, respectively. The data are expressed as mean ± SEM, n=6 per group. A significant difference as compared to controls is expressed as ** p < 0.01 and *** p<0.001 and as compared to PQ-treated mice as $$ p<0.01 and $$$ p < 0.001.

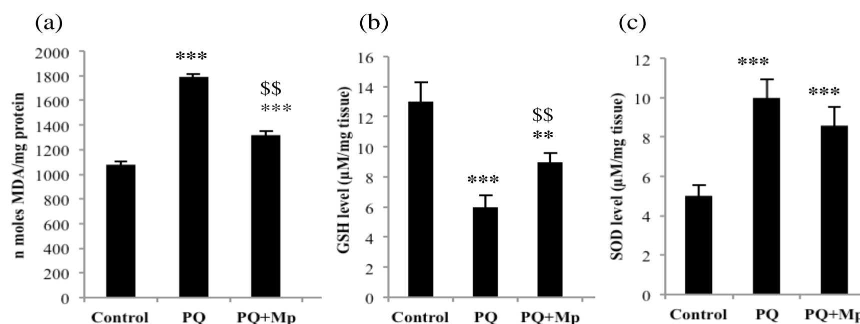

Fig. 4. Biochemical analyses of oxidative markers of PD in the nigrostriatal region. Lipid peroxidation was quantified by the level of MDA (a),where GSHlevels are quantified by reducing the amount of GSH in tissue (b) and the activity of SOD was measured (c) in control, PQ- and PQ+Mp-treated animals.Measurements are represented as mean ± SEM, n = 6 per group.A significant difference as compared to controls is represented by **p<0.01 and *** p<0.001 and as compared to PQ as $$p<0.01.

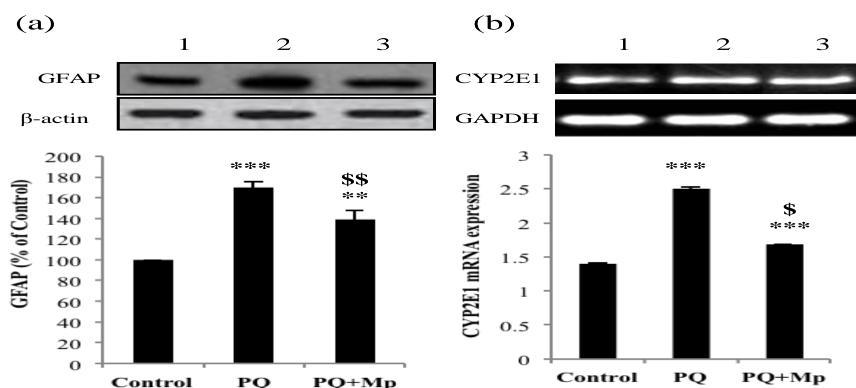

Fig. 5. Expression of the neuroinflammatory marker GFAP and the antioxidant CYP2E1 in the nigrostriatal region of mice. The blot of GFAP protein expression is shown in (a) upper at ~50kDa and compared to the β-actin control at ~42kDa. Expression is quantified in (a) lower as the ratio of GFAP to β-actin, represented as percentage of control. CYP2E1 mRNA expression is shown in (b) upper with a length of 550bp compared to the GAPDH control. Quantification of the bands as a density band ratio is presented in (b) lower. The data are expressed as mean ± SE,n=6 per group.A significant difference is expressed for the PQ group as compared to controls as ** $p < 0.01$ and *** $p < 0.001$ and as compared to the PQ-treated group as \$ $p < 0.05$ and \$\$ $p < 0.01$.

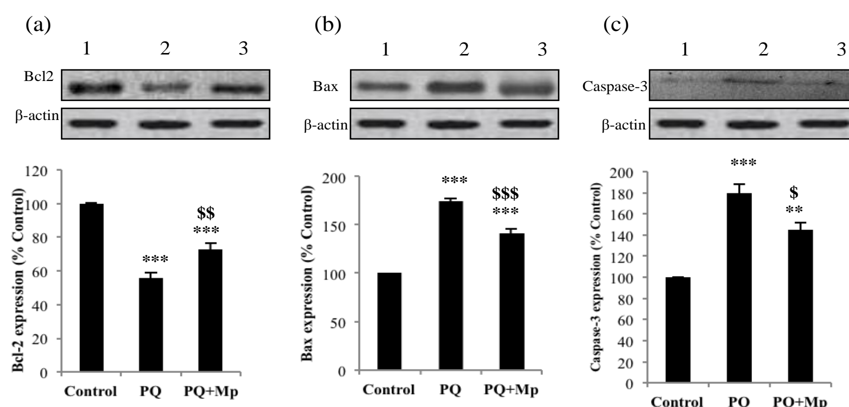

Fig. 6. Expression of apoptotic proteins in response to PQ and PQ+Mp treatment in the nigrostriatal region. (a) Bcl2 (~25kDa), (b) Bax (~23kDa) and (c) Caspase-3 (~32kDa) blots are shown with a β-actin (~42kDa) loading control in the upper portion.Quantification of the bands (lower) are represented as percentage of control. The data are expressed as mean ± SEM, n=6 per group.A significant difference as compared to controls is expressed as ** $p < 0.01$ and *** $p < 0.001$ and as compared to the PQ-treated group as \$ $p < 0.05$, \$\$ $p < 0.01$ and \$\$\$ $p < 0.001$.

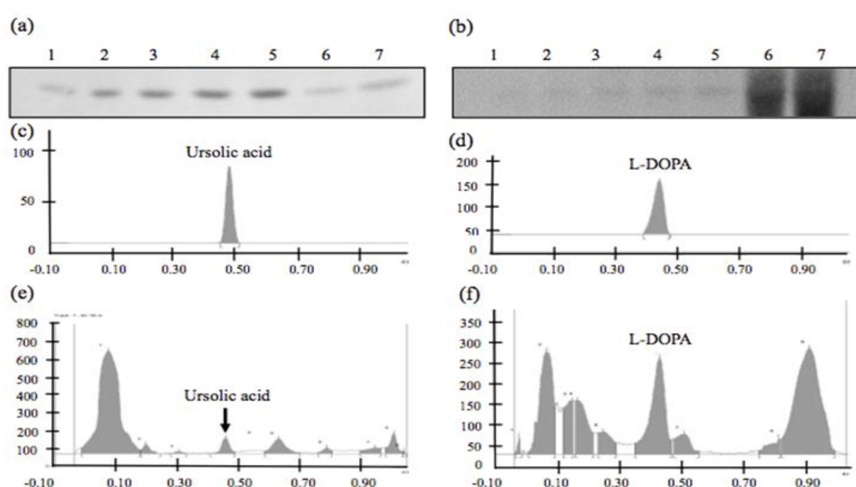

Fig. 7. Determination of the main constituents of Mp seed ethanolic extract using HPTLC. The TLC data for ursolic acid (a) and L-DOPA (b) tests is outlined. In both figures, lanes 1 through 5 represent different

concentrations of the standard, namely 1, 2, 3, 4 and 5µl of the standard. Lanes 6 and 7 are different concentrations of the Mp seed extract, namely 15 and 30µl, respectively. (c) and (d) show the HPTLC finger printing pattern for the two standard solutions, ursolic acid and L-DOPA, respectively with the corresponding R_f value. Ursolic acid was visualized at 510nm while L-DOPA at 280nm. Finally, (e) and (f) present the HPTLC finger printing plot for the Mp seed extract with the corresponding peaks associated with ursolic acid and L-DOPA, respectively. Note that in (e) the Mp plot was visualized at 510nm corresponding to the ursolic acid standard while in (f) the Mp fingerprinting plot was visualized at 280nm corresponding to the L-DOPA standard.

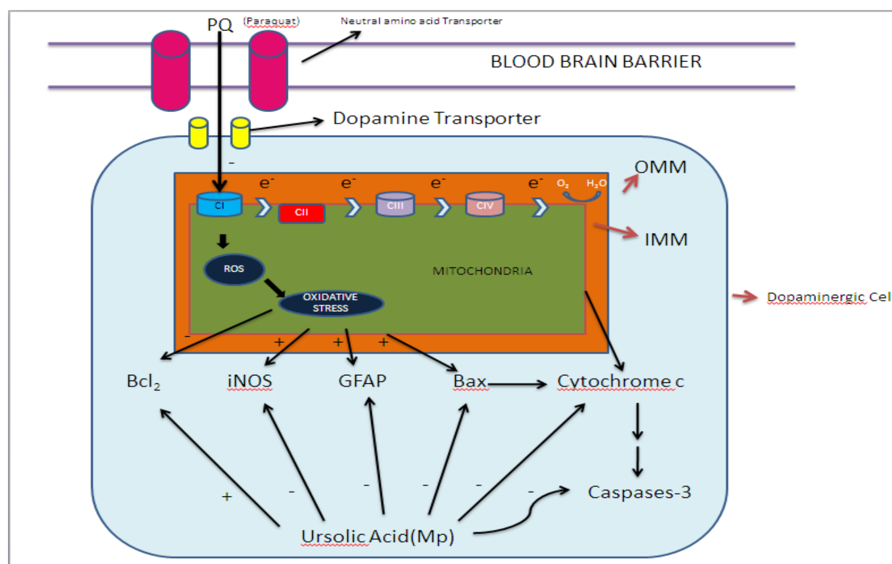

Fig. 8. The action of ursolic acid influences a variety of systems in the dopaminergic cell. Together with previous reports, we have indicated that ursolic acid potentially enhances the expression of Bcl2 while decreasing iNOS, GFAP, Bax, cytochrome c and caspase 3. These therapeutic effects rescue all of the detrimental effects elicited by PQ in the dopaminergic cell. Indirectly, we can say that ursolic acid reduces the oxidative damage induced by the action of environmental toxins in mitochondria. (OMM – outer mitochondrial membrane; IMM – inner mitochondrial membrane).

Tables

Table 1. Standard data of L-DOPA (first row) and the unknown peaks of the Mp seed extract as visualized at 510 nm. The indicated L-DOPA peak in the Mp extract is highlighted.

	Start Position (Rf)	Start Height (AU)	Max Position (Rf)	Max Height (AU)	Max %	End Position (Rf)	End Height (Rf)	Area (AU)	Area (%)
L-DOPA Standard									
L-DOPA	**0.38**	**2.8**	**0.44**	**120.3**	**100.00**	**0.47**	**1.5**	**3951.2**	**100.00**
Mp seed extract									
unknown 1	-0.03	2.8	-0.02	40.7	3.43	-0.02	0.0	255.7	0.48
unknown 2	0.01	4.8	0.07	249.2	20.99	0.09	14.4	9944.1	18.78
unknown 3	0.12	109.8	0.15	129.8	10.93	0.15	29.0	3184.5	6.01
unknown 4	0.16	129.4	0.17	131.2	11.05	0.22	51.7	4580.2	8.65
unknown 5	0.23	52.6	0.25	55.3	4.66	0.29	26.2	2164.8	4.09
L-DOPA	**0.35**	**26.5**	**0.44**	**233.8**	**19.69**	**0.47**	**34.8**	**9977.1**	**18.84**
unknown 7	0.48	38.3	0.51	49.5	4.17	0.56	8.7	2169.0	4.10
unknown 8	0.76	9.7	0.82	41.4	3.49	0.83	34.0	1314.4	2.48
unknown 9	0.83	37.5	0.92	256.4	21.59	1.01	9.2	19373	36.58

Table 2. Standard data of ursolic acid (first row) and the unknown peaks of the Mp seed extract as visualized at 280 nm. The indicated ursolic acid peak in the Mp extract is highlighted.

	Start Position (Rf)	Start Height (AU)	Max Position (Rf)	Max Height (AU)	Max %	End Position (Rf)	End Height (Rf)	Area (AU)	Area (%)
Ursolic acid standard									
UA standard	**0.45**	**0.4**	**0.48**	**75.5**	**100.00**	**0.51**	**0.4**	**1583.1**	**100.00**
Mp seed extract									
unknown 1	-0.01	35.9	0.07	581.1	59.40	0.18	23.0	36392.9	76.93
unknown 2	0.18	23.2	0.20	53.9	5.50	0.24	0.1	1268.7	2.68
unknown 3	0.28	2.4	0.30	13.2	1.35	0.33	3.3	326.5	0.69
UA	**0.41**	**2.5**	**0.46**	**88.4**	**9.04**	**0.49**	**9.4**	**2128.0**	**4.50**
unknown 4	0.58	14.2	0.63	83.1	8.49	0.70	3.8	3397.0	7.18
unknown 5	0.76	2.5	0.79	30.7	3.14	0.82	11.5	863.4	1.83
unknown 6	0.91	12.3	0.94	24.2	2.47	0.96	22.2	902.3	1.91
unknown 7	0.98	23.9	1.01	103.8	10.60	1.03	0.2	2027.0	4.28

DISCUSSION

There are several different pharmacological treatments for PD among which levodopa remains the most efficacious. However, long-term use of levodopa causes debilitating motor complications, particularly dyskinesia. To avoid this, many patients seek alternative treatments for PD, especially among traditional practices and medical plants. The present study was conducted to understand the effectiveness and molecular mechanisms of action of Mp in a paraquat (PQ)-induced Parkinsonian mouse model.

Mucuna pruriens (Mp) is a medicinal plant known from the Vedic era to have antiparkinsonian properties; however, the mechanism of Mp mediated neuroprotection remains elusive. Previously, an acute toxicity experiment determined the most effective dose of Mp to elicit therapeutic effects in the PQ-model.[30] Mp contains many active ingredients such as the dopamine precursor L-DOPA (4-5%) and a battery of natural antioxidant alkaloids such as prurienine, prurieninine and prurienidine.[14] Triterpenes and sterols are also found in seeds of Mp.[31] Interestingly, a previous double-blind clinical study suggested that L-DOPA alone used in long duration instigated dyskinesia whereas Mp seed powder did not while providing the same symptomatic relief to PD patients.[13] Hence, it is suggested that Mp may possess several advantages over conventional L-DOPA preparation in long term management of PD.

In this study, we investigated further the constitution of Mp seed extract by HPTLC fingerprinting analysis and while confirming the presence of L-DOPA and ursolic acid. The reported amount of L-DOPA found in Mp seeds is 4-5% though the constituents of any natural plant depend on the place and season of harvesting, the species, extraction protocol and storage conditions. We have demonstrated that the species which we use in our lab has a high percentage of L-DOPA and possibly more effective at restoring dopamine levels in the brain than other reported preparations.

Behavioural testing indicated impaired motor function in PQ treated mice, similar to PD patients. Mice demonstrated clear bradykinesia and decreased motor coordination as compared to controls. Notably, Mp reversed the behavioural deficits in all instances reflecting its potential to act as a neuroprotective agent. Such motor deterioration has been associated with the loss of dopaminergic neurons in the substantia nigra, increased dopamine turnover in the striatum.[32]

Dopamine plays a key role in movement and motor control. Reduced levels of catecholamines and oxidative stress are thought to be the main contributing factors of neurodegeneration in PD contributing to the loss of motor function observed in PD patients.[33] Notably, the autoxidation of dopamine leads to the formation of reactive oxygen species such as hydrogen peroxide, reactive quinine and reactive semi-quinine species further contributing to oxidative damage.[34] In more than one way, dopamine is a marker of PD progression.

Dopamine catabolism into DOPAC and HVA is regulated by monoamine oxidase (MAO) and catechol-o-methyl transferase (COMT), respectively.[35] In the present study, we show that dopamine, DOPAC and

HVA presence in the nigrostriatal region is significantly improved by Mp. This effect could be attributed to either the L-DOPA present in Mp seeds or the boosting action of ursolic acid on the dopaminergic neurons.

A balanced DAT and VMAT2 ratio is required for the normal functioning of the nigrostriatal dopaminergic system. In the case of neurodegeneration induced by pesticide models of PD, a higher ratio of DAT/VMAT2 is observed in the terminals of dopaminergic neurons.[36] In the present study, DAT expression was slightly increased while VMAT decreased in PQ-treated mice as compared to controls. This effect could be due to the differences in their entry into the brain directly or by transporters, metabolic fate or further transport into the nigrostriatal tissues. The decrease in VMAT2 expression directly reflects the reduced ability of dopaminergic neurons to protect against PQ-induced neurotoxicity after prolonged exposure, as VMAT2 is well known to protect dopamine from autoxidation.[37] We also showed that the expression of DAT and VMAT-2 was significantly improved by Mp seeds. As ursolic acid has been shown to protect various dopaminergic receptors (D1 and D2), it remains a strong candidate to elicit the neuroprotective action of Mp on regulating the DAT/VMAT ratio.

Oxidative stress has long been linked to neuronal cell death associated with neurodegenerative conditions such as PD.[38] In PQ-treated animals, we previously observed elevated levels of MDA and SOD and reduced levels of catalase and GSH in the nigrostriatal portion of the mouse brain.[30] Here, we further demonstrated that Mp treatment reduced oxidative damage while stimulating antioxidant defences. These observations were consistent with other studies of herbal extract mediated neuroprotection.[39] This action of Mp could be elicited by any of the antioxidant alkaloids or by the previously demonstrated antioxidant action of ursolic acid.

In the present study we show that PQ treatment increases the expression of GFAP, a marker of neuroinflammation in glial cells. Indeed neuroinflammation has been previously implicated as a pathogenic factor in PD patients [40] and MPTP-treated mice.[41] GFAP contributes to oxidative damage as activated glial cells also have an increase in iNOS productivity and therefore production of nitric oxide. Notably, Mp treatment markedly reduced GFAP expression. We propose that Mp seeds do not only protect dopaminergic neurons in PD, but also protect the glial cells. This is important as the glial cells have many systems in place to protect and repair damaged neurons.

CYP2E1 generates free radicals owing to its oxidase activity and ability to enhance lipid peroxidation.[24] Pesticides have been functionally linked to the expression of CYP2E1[42] hence the present pesticide-model of PD was investigated for the effect of PQ on CYP2E1 expression. Indeed, PQ significantly unregulated the expression of CYP2E1. Further, Mp treatment rescued CYP2E1 expression attributing further to its antioxidant action in PD.

Ultimately, the pathology of PD is attributed to the cellular degeneration in the substantia nigra; therefore, we investigated the possible action of Mp on protein markers of apoptosis. Indeed, dopaminergic neuronal protection is partially associated with an anti-apoptotic pathway and the anti-apoptotic molecule Bcl-2 plays a major role in the maintenance of dopamine neurons in the PQ-toxic model in mice. [43] We demonstrate that PQ treatment tilts the balance of proapoptotic to antiapoptotic factors by increasing Bax and caspase 3 expressions while reducing Bcl2 expression. Further, Mp treatment was shown to restore the apoptotic balance by reinstating a health expression level of the respective factors.

To the best of our knowledge, the results of our previous[30, 44] and present study describe numerous findings which are both significant and novel. In our whole study we found that PQ treatment induces Parkinson's disease in mouse model as indicated by reduced levels of dopamine, DOPAC, HVA, Bcl-2 and increased levels of iNOS, Bax and GFAP.

Our results clearly indicate that Mp significantly reduce the neurotoxicity in Parkinson's mouse model induced by PQ as indicated by morphological and biochemical Characteristics.

CONCLUSION

Overall, this study indicates that Mp causes enhancement in level of catecholamine, improvement in motor activity, reduction in free radical generation and attenuation of activated astrocytes in a PD mouse model. In addition, Mp was shown to exhibit anti-apoptotic activity through the enhancement of Bcl2 expression and decline in level of Bax. Thus the results here elucidated the mechanism of Mp plant extract as a potent neuroprotectant. As demonstrated by the presented HPTLC data, one of the main constituents of its seed extracts namely L-Dopa and Ursolic acid. A hypothetical outline of mechanism of action of Mp on the pathology of PD has been depicted in Fig. 8.

ACKNOWLEDGEMENTS AND FUNDING

The authors sincerely thank Council of Scientific and Industrial Research (CSIR No. 37(1518/11/EMR-II)), New Delhi, India for providing research grant to SPS and SR as well as fellowships to Satyndra Kumar Yadav. Authors also thank Indian Council of Medical research (ICMR), New Delhi, India for providing research fellowship to Sachchida Nand Rai.

DISCLOSURE STATEMENT

The authors claim no conflict of interest.

REFERENCES

1. Miller RL, James Kracke M, Sun GY, Sun AY. Oxidative and inflammatory pathways in Parkinson's disease. Neurochem Res., 2009; 34: 55–65.
2. Singh MP, Patel S, Dikshit M, Gupta YK. Contribution of genomics and proteomics in understanding the role of modifying factors in Parkinson's disease. Indian J Biochem Biophys, 2006; 43: 69–81.
3. Wirdefeldt K, Adami HO, Cole P, Trichopoulos D, Mandel J. Epidemiology and etiology of Parkinson's disease: a review of the evidence. Eur J Epidemiol, 2011; 011: 9581-6.
4. Klockgether T. Parkinson's disease: clinical aspects. Cell Tissue Res., 2004; 318(1): 115-20.
5. Singh C, Ahmad I, Kumar A. Pesticides and metals induced Parkinson's disease: involvement of free radicals and oxidative stress. Cell Mol Biol., 2007; 53: 19–28.
6. Cory-Slechta DA, Thiruchelvam M, Barlow BK, Richfield EK. Developmental pesticide models of the Parkinson disease phenotype. Environmental Health Perspectives, 2005; 113: 1263-1270.
7. Mccormack AL, Thiruchelvam M, Manning-Bog AB, Thiffault C, Langston JW, Cory-Slechta DA, Di Monte DA. Environmental risk factors and Parkinson's disease: Selective degeneration of nigral dopaminergic neurons caused by the herbicide paraquat. Neuro-biology of Disease, 2002; 10: 119-127.
8. Bus JS, Aust SD, Gibson JE. Superoxide- and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat (methyl viologen) toxicity. Biochem Biophys Res Commun, 1974; 58(3): 749-55.
9. Singhal NK, Srivastava G, Agrawal S, Jain SK, Singh MP. Melatonin as a neuroprotective agent in the rodent models of Parkinson's disease: is it all set to irrefutable clinical translation?. Mol Neurobiol, 2012; 45: 186–199.
10. Uversky VN. Neurotoxicant-induced animal models of Parkinson's disease: understanding the role of rotenone, maneb and paraquat in neurodegeneration. Cell Tissue Res., 2004; 318: 225–241.
11. Manyam BV, Sánchez Ramos JR. Traditional and complementary therapies in Parkinson's disease. Adv Neurol, 1999; 80: 565-74.
12. Modi KP, Patel NM, Goyal RK. Estimation of L-dopa from Mucuna pruriens LINN and formulations containing M. pruriens by HPTLC method. Chem Pharm Bull, 2008; 56: 357-9.
13. Katzenschlager, R., Evans, A., Manson, A., Patsalos, P.N., Ratnaraj, N., Watt, H., Timmerman, L., Van der Giessen, R., Lees, A.J. Mucuna pruriens in Parkinson's disease: a double blind clinical and pharmacological study. J Neurosurg Psychiatry, 2004; 75: 1672–1677.
14. Misra L, Wagner H. Alkaloidal constituents present in Mucuna pruriens seeds. 2004; Phytochemistry, 65: 2565–2567.
15. Pant MC, Joshi LD. Identification of pharmacologically active substances in the seeds of Mucuna pruriens (Abs). Indian J Pharmacol, 1970; 2: 24.
16. Tripathi YB, Upadhyay AK. Antioxidant property of Mucuna pruriens Linn. Curr Sci., 2001; 80: 11.
17. Uhegbu AFO, Elekwa I, Ukoha C. Comparative efficacy of crude aqueous extract of Mangifera indica, carica papaya and sulphadoxine pyrimethamine on the mice infested with malaria parasite in vivo. Global J Pure Appl Sci., 2005; 11: 399– 401.
18. Tillerson, J.L., Miller, G.W., Grid performance test to measure behavioural impairment in the MPTP-treated-mouse model of parkinsonism. J. Neurosci, 2003; 123: 189–200.
19. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Anal Biochem, 1979; 95: 351-358.
20. Moron MS, Depierre JW, Mannervik B. Levels of glutathione, glutathione reductase and glutathione S-transferase activities in rat lung and liver. Biochem Biophys Acta, 1979; 582: 67–78.
21. Nishikimi M, Appaji N, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res Commun, 1972; 46: 849-854.
22. Singh S, Singh K, Gupta SP, Patel DK, Singh VK, Singh RK, Singh MP. Effect of caffeine on the expression of cytochrome P450 1A2, adenosine A2A receptor and dopamine transporter in control and 1-methyl 4-phenyl 1,2,3,6-tetrahydropyridine treated mouse striatum. Brain Res., 2009; 1283: 115-26.
23. Yadav S, Dixit A, Agrawal S, Singh A, Srivastava G, Singh AK, Srivastava PK, Prakash O, Singh MP. Rodent models and contemporary molecular techniques: Notable feats yet Incomplete explanations of Parkinson's disease pathogenesis. Mol Neurobiol, 2012; 46: 495-512.
24. Patel S, Singh V, Kumar A, Gupta YK, Singh MP. Status of antioxidant defense system and expression of toxicant responsive genes in striatum of maneb and paraquat-induced Parkinson's disease phenotype in mouse: mechanism of neurodegeneration. Brain Res., 2006; 1081: 9–18.
25. Singh S, Singh K, Patel S, Patel DK, Singh C, Nath C, Singh MP. Nicotine and caffeine-mediated modulation in the expression of toxicant responsive genes and vesicular monoamine transporter-2 in 1-methyl 4-phenyl-1,2,3,6- tetrahydropyridine-induced Parkinson's disease phenotype in mouse. Brain Res, 2008; 1207: 193-206.
26. Patel S, Singh K, Singh S, Singh MP. Gene expression profiles of mouse striatum in control and maneb? Paraquat induced Parkinson's disease phenotype: validation of differentially expressed

energy metabolizing transcripts. Mol Biotechnol, 2008; 40: 59–68.
27. Fan Q, Ding J, Zhang J. Effect of the knockdown of podocin mRNA on nephrin and a-actinin in mouse podocyte. Exp Biol Med., 2004; 229: 964–970.
28. Gupta SP, Patel S, Yadav S, Singh AK, Singh S, Singh MP. Involvement of nitric oxide in maneb- and paraquat-induced Parkinson's disease phenotype in mouse: is there any link with lipid peroxidation?. Neurochem Res., 2010; 35: 1206- 1213.
29. Pandey DK, Malik T, Banik RM. Validated HPTLC Method for Quantification of Variability in Content of Olenolic Acid in Different Variety of Lantana camara. Pharmacologia, 2013; 126.131.
30. Yadav SK, Prakash J, Chouhan S, Singh SP. Mucuna pruriens seed extract reduces oxidative stress in nigrostriatal tissue and improves neurobehavioral activity in paraquat-induced Parkinsonian mouse model. Neurochem Int, 2013; 62: 1039– 1047.
31. Siddhuraju P, Vijayakumari K, Janardhanan K. Chemical composition and 852 protein quality of the little known legume, velvet bean (Mucuna pruriens (L.) 853 DC). J Agric Food Chem, 1996; 44: 2636–2641.
32. Nasuti C, Gabbianelli R, Falcioni ML, Di Stefano A, Sozio P, Cantalamessa F. Dopaminergic system modulation, behavioral changes, and oxidative stress after neonatal administration of pyrethroids. Toxicology, 2007; 229: 194-205.
33. Mishra LC, Singh BB, Dagenais S. Scientific basis for the therapeutic use of Withania somnifera (ashwagandha): a review. Alternative Medicine Review, 2000; 5: 334–346.
34. Makes HS, Weiss C, Silides DJ, Cohen G. Coupling of dopamine oxidation (monoamine oxidase activity) to glutathione oxidation via the generation of hydrogen peroxide in rat brain homogenates. J Neurochem, 1981; 36: 589-593.
35. Jinsmaa Y, Florang VR, Rees JN, Anderson DG, Strack S, Doorn JA. Products of oxidative stress inhibit aldehyde oxidation and reduction pathways in dopamine catabolism yielding elevated levels of a reactive intermediate. Chem Res Toxicol, 2009; 22: 835-841.
36. Mun JY, Lee WY, Han SS. Effects of cypermethrin on the dopaminergic neurons in the progressive hemiparkinsonian rats. Toxicol Mech Method, 2005; 15: 399-404.
37. Fon EA, Pothos EN, Sun BC, Killeen N, Sulzer D, Edwards RH. Vesicular transport regulates monoamine storage and release but is not essential for amphetamine action. Neuron, 1997; 19: 1271-1283.
38. Jones GM, Vale JA. Mechanisms of toxicity, clinical features, and management of diquat poisoning: a review. J Toxicol Clin Toxicol, 2000; 38: 123–128.
39. Jagetia GC, Rao SK, Baliga MS, Babu KS. The evaluation of nitric oxide scavenging activity of certain herbal formulations in vitro: a preliminary study. Phytother Res, 2004; 18: 561–565.
40. Tansey MG, Goldberg MS. Neuroinflammation in Parkinson’s disease: its role in neuronal death and implications for therapeutic intervention. Neurobiol Dis., 2010; 37: 510–518.
41. Morale MC, Serra PA, L’Episcopo F, Tirolo C, Caniglia S, Testa N, Gennuso F, Giaquinta G, Rocchitta G, Desole MS, Miele E, Marchetti B. Estrogen, neuroinflammation and neuroprotection in Parkinson’s disease: glia dictates resistance versus vulnerability to neurodegeneration. Neurosci, 2006; 138: 869–878.
42. Nissbrandt H, Bergquist F, Jonason J, Engberg G. Inhibition of cytochrome P4502E1 induces an increase in extracellular dopamine in rat substantia nigra: a new metabolic pathway?. Synapse, 2001; 40: 294-301.
43. Vila M, Jackson-Lewis V, Vukosavic S, Djaldetti R, Liberatore G, Offen D, Korsmeyer S, Przedborski S. Bax ablation prevents dopaminergic neurodegeneration in the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine mouse model of Parkinson’s disease, 2001; 98: 2837–3284.
44. Yadav SK, Prakash J, Chouhan S, Westfall S, Verma M, Singh TD, Singh SP. Comparison of the neuroprotective potential of Mucuna pruriens seed extract with estrogen in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mice model. Neurochem Int, 2014; 65: 1-13.