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Dr. Hardy Daniel L
Dr. Arcot Lakshmanaswamy Mudaliar Postgraduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Taramani, Chennai, India

Dr. Ravinrdan Rajan
Dr. Arcot Lakshmanaswamy Mudaliar Postgraduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Taramani, Chennai, India

Correspondence
Dr. Ravinrdan Rajan
Dr. Arcot Lakshmanaswamy Mudaliar Postgraduate Institute of Basic Medical Sciences, University of Madras, Taramani Campus, Taramani, Chennai, India

Neuro-behavioral modification of *Bacopa Monnieri* in rotenone induced hemi-Parkinson's disease model of male Wistar albino rats

Dr. Hardy Daniel L and Dr. Ravinrdan Rajan

Abstract
The dopaminergic neuronal death in Substantia nigra leads to Parkinson's disease. Though it is common in western countries, the incidence rises in developing countries. In traditional system, neurodegenerative disorders are treated by *Bacopa monnieri*. The mechanism underlying its usage in Parkinson's disease is validated in this study. Five groups of animals were studied for behavioral changes after induction of Parkinson's disease and treatment with methanolic extract of *Bacopa monnieri* for 60 days. The neuro-behavioral responses and balancing ability were studied. In T-maze test, mild cognitive impairment was observed in Parkinson's disease induced animals which improved with *Bacopa* treatment, whereas in eight arm-radial maze, the working and reference memory were enriched significantly in Parkinson's disease induced animals treated with BM. The balancing in Rota-rod and narrow beam were improved with *Bacopa* treatment in Parkinson's disease induced animals. Thus *Bacopa monnieri* improves the motor and non-motor symptoms of PD in rat models.

Keywords: Parkinson's disease, *Bacopa monnieri*, rotenone, neuro-behavior

Introduction
Parkinson's disease (PD) is a neuro-degenerative disorder caused due to the death of dopaminergic neurons in the substantia nigra region of the mid-brain. The manifestations of the disease are akinesia, rigidity and tremor along with thinking, behavioural problems and dementia in the advanced stages of the disease [1]. It is more common in the elderly age group with an estimate of 6.3 million people affected worldwide. In India the prevalence of PD is low when compared to western countries. But the incidence rises in developing countries over the past decade. The prevalence in India, which was 3.2 lakhs in the year 2005, is projected to 6.9 lakhs by the year 2030 [2].

There is interplay between genetic and environmental factors in the cause of the disease. Since, brain is exposed to low levels of the antioxidant enzymes and rich in iron, it is more prone to oxidative damage. Rotenone blocks mitochondrial complex - I leading to an upsurge in superoxide production and neuro-degeneration. Failure of synthetic drugs and its side effects have led to the assessment of herbal medicines in the treatment. *Bacopa monnieri* (BM) is used by the traditional practitioners as a neurological tonic and cognitive enhancer [3]. Since, it has significant antioxidant and neuro protective effects its efficacy in the treatment of PD is documented in this study.

The location and retrieval of food is most vital for the survival of mammals and rodents. Even though, hippocampus plays a significant role in relaying signals, other brain areas act in an intricate manner for this survival mechanism. The basal ganglia circuits are intimately involved in the learning of new skills. The behavioural discrepancies effecting from the disruption of neural structures that form the basal ganglia circuit are studied using different mazes.

In Parkinson's disease while performing a complex motor movement, inappropriate time relation between successive movements caused by the derangement of the basal ganglia structures and deficit in switching from one motor programme to another lead to disordered movements. The disrupted control process of the normal movement speed underlies the movement deficits in PD [4]. In PD, when performing a series of movements, each movement is abnormally slow, and the latency between each movement is much increased than normal [5]. The effects of unilateral striatal dopamine depletion in motor incoordination were assessed by the balancing ability of the animals over a rotating rod and a narrow beam. It was reported that, in PD failure of motor cortex stimulation lead to the performance of movement at below normal speed [6] and multiple parallel pathways within the BG when affected are associated with abnormal balance of activity [7]. Thus the present study was designed to assess the

efficacy of methanolic extract of *Bacopa monnieri* in mitigating the oxidative damage of SNpc region of midbrain by rotenone.

Materials and methods

Chemicals and Plant extract

Analytical grade of Rotenone (R8875) and Dimethyl sulfoxide (D8418) were purchased from Sigma Aldrich (USA). *Bacopa monnieri* plants were procured from a nursery in Chennai. The plants were washed and air dried in shade. The dried plants were coarsely powdered and macerated with methanol and extracted in soxhlet apparatus. The plant extract obtained was freeze dried and stored in 4ºC temperature. The extract was dissolved in 0.9% saline solution (10 mg/ml). The animals were treated orally with a dosage of 50 mg/kg body weight of this methanolic extract of BM [8].

Animals

All the experimental procedures were carried under ethical guidelines and the permission was obtained from the institutional animal ethical committee (IAEC number-01/18/2015). Five cohorts of young adult male Wistar albino rats weighing (230–280 g) were used in the present study. Each group contained six animals. The animals were housed in a temperature controlled room on a 12 h light-dark cycle with access to both food and water *ad libitum.* The first group of animals were used as control which was not lesioned and untreated. The second group were sham operated animals which were infused with 4µl of dimethyl sulfoxide (DMSO) into the left medial forebrain bundle stereotaxically. The third group included Parkinson's disease induced animals which were infused with 3µg of rotenone dissolved 4µl of DMSO into the left MFB whereas the fourth group animals were not operated but received *Bacopa monnieri* extract at a dosage of 50 mg/kg body weight for 60 days and the fifth group comprised of animals that were treated with BM for 30 days before and after surgery.

Rotenone - induced Hemi-Parkinson's disease

Stereotaxical induction of hemi-Parkinson's disease is used in the present study as there are substantial evidences to prove that this chemically induced animal model mimic human Parkinson's Disease in various aspects.

The induction of Hemi-Parkinson's disease condition was done according to the standard protocol [9]. Rotenone was dissolved in DMSO at a concentration of 0.75µg /µl. The reference for the location of medial forebrain bundle (MFB) is AP: 4.0 mm; L: 1.8 mm; DV: 7.6 mm from the bregma [10]. The animals were anaesthetised with ketamine (87 mg/kg of body weight) and xylazine (13 mg/kg of body weight) by a single dosage of intra-peritoneal injection [11] fastened on the stereotaxic frame and the head was fixed in flat skull position. A midline incision is made in the skin over the skull and a bore is made using a hand held drill for access of stereotaxic cannula (RM SBL - Braintree Scientific, U.S.A.). The cannula is inserted into the left MFB region and rotenone is infused for twenty minutes at a rate of 0.2 µl/min, using syringe pump (BS-300 - Braintree Scientific, USA.) The cannula was left in place for ten minutes and after complete diffusion of the drug it was retracted subsequently. The skin was sutured and proper post-operative care was given until the animal recovered completely. The animals were observed for spontaneous rotational behavioural abnormalities in the first three days of recovery. Following recovery from anaesthesia the animals that expressed spontaneous circling behaviour, were taken for the study.

T-maze test

The test was performed according to procedure [12]. The animals were habituated in the T-maze for 4 days. Once habituated, the animals were semi starved the previous night and training of the animals was done. Ten trials are run per day by shifting the baited goal arm and the training is continued for all the animals with ten minutes gap between each trial. The identity of the goal arm for each trial is determined by random sequences and the animals are trained to enter the baited arm. The correct arm entry, which is observed as all the four feet entering the goal arm for each animal in group, is noted. If the animal did not enter the baited arm even after 90 seconds the procedure is stopped and the animal is habituated again. The percentage of correct choices and the time spent in start arm for each animal were noted.

Eight arm radial maze

The experiment was conducted according to the standard protocol [13]. The rats were habituated for 4 days in the eight arm radial maze. After habituation, the animals were trained once a day to explore pelleted food which was placed at random in 4 of the 8 arms. Novel sequences of arms were baited each day. Each rat was given 5 min to retrieve the pellets from the baited arms. The number of working memory error (entry into non baited arm) and reference memory error (re-entry into the arm that was previously visited) were noted. The training of the animals was continued until there is not more than one re-entry error per daily trial for four consecutive days. The latency period to reach the first baited arm was also noted.

Rota-rod test

The coordination of motor activities in rats was measured using a rota-rod following the procedure [14]. It comprises of a rotating rod at a fixed speed and the balancing ability of the animal in the rotating rod was observed. The rats were habituated to stay on the stationary rod for 3 minutes. After habituation the rats were allowed to balance over a rotating rod for duration of 3 minutes. The rotation was set at a relatively slow speed of 12 rotations per minute. If the animal fell down during the procedure, it was immediately replaced over the rod and allowed to balance. The apparatus was wiped with a 70% ethanol solution and dried before each trial. The number of fall and the mean latency to fall off the rod was recorded.

Narrow beam test

The competency of the animal to balance and walk over a narrow beam is tested by the standard procedures [15]. The narrow wooden beam used in the experiment was 105 cm long, 4 cm wide and 3 cm tall which is suspended at a height of 80 cm from the ground. Beneath the beam 1m wide bedding was placed to prevent injury to the animals if they fall. A line is drawn 20 cm from the start of the beam and the animals are placed before the start line and the time is noted till the animal places the first step across the start line, which is the latency period to start the task. The time is noted until the animal places all the four feet on the platform at the other end of the narrow beam. The maximum time allowed for the task was 2 minutes and a fall from the beam was also recorded as a maximum time.

Statistical analysis

All the data of the tests were analysed by one way Analysis of variance using SPSS (version 25.0) and the Tukey's honest significant difference (HSD) test for post hoc analysis by setting the P value is as 0.05.

Results

Effects of Bacopa monnieri on T-maze test

The percentage of correct choices and latency period in the start arm of T-maze was recorded after training period. The data from each of the ten trials in a single day and six animals in each group was averaged. In T-maze test percentage of correct choices as shown in (Figure 1) was insignificantly altered among test groups whereas the retention of memory (Figure 2) and the latency period in the start arm of T-maze (figure 3) were altered substantially in the PD induced group when compared to that of sham operated animals. Though, the treatment with *Bacopa monnieri* has produced a significant decrease in the latency period and improved the retention of memory in PD induced animals, it did not bring behavioural response back to normal.

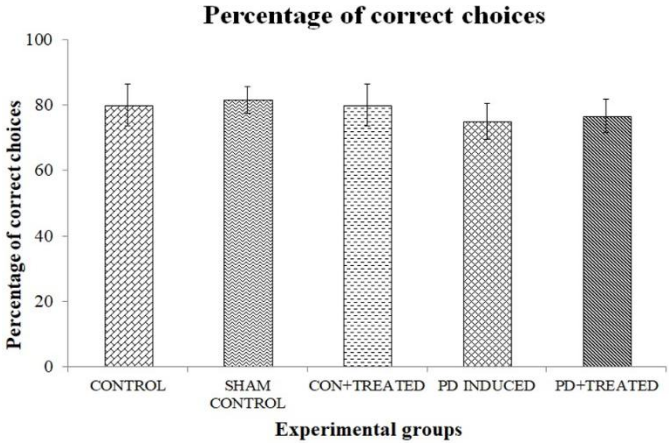

Fig 1: Showing changes in the percentage of correct choices in T-Maze test after treatment with *Bacopa monnieri.* No statistical significant change in memory was observed between the groups.

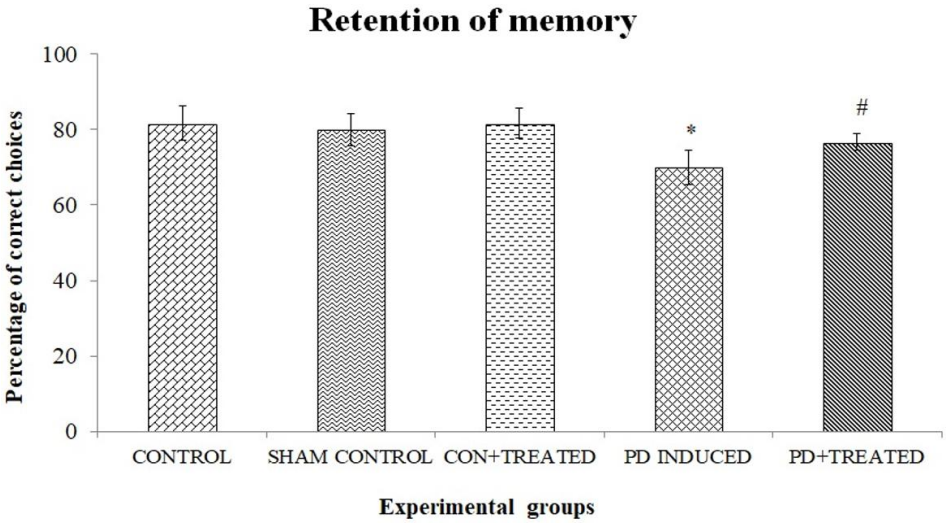

Fig 2: Showing changes in the retention of memory after *Bacopa monnieri* treatment in Parkinson's disease induced animals. A * mark shows statistical significance with Control and # shows statistical significance with Parkinson's disease induced group.

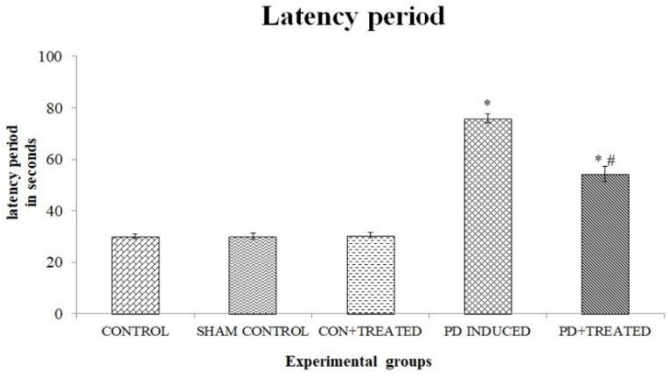

Fig 3: Showing changes in latency period in start arm of T-Maze in Parkinson's disease induced animals and *Bacopa monnieri* treatment. A * mark shows statistical significance with Control and # mark with Parkinson's disease induced group.

Effect of Bacopa monnieri on eight arm radial maze test

The changes in reference and working memory with PD induction and treatment with *Bacopa monnieri* was evaluated using an eight arm radial maze. The working and reference memory were inordinately affected in PD induced animals when compared to that of the sham operated animals as shown in figure 4 and figure 5 respectively, which improved significantly after treatment with *Bacopa monnieri* but did not bring them back to normal.

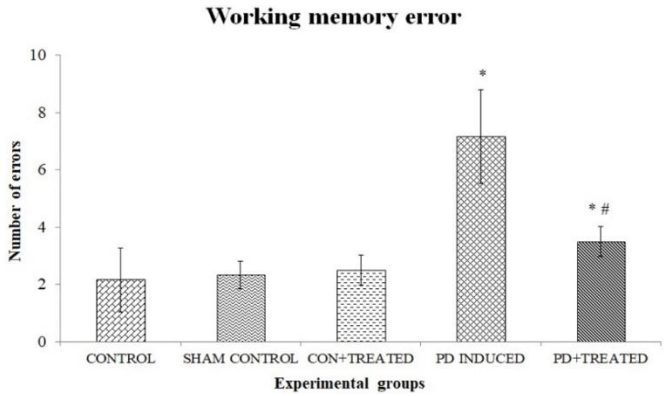

Fig 4: Showing alteration in the working memory of Parkinson's disease induced rats after *Bacopa monnieri* treatment. A * mark shows statistical significance with Control and # shows statistical significance with Parkinson's disease induced group.

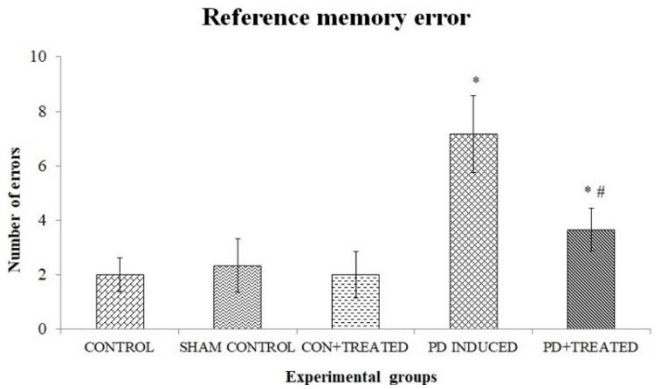

Fig 5: Showing alteration in the reference memory of Parkinson's disease induced rats after *Bacopa monnieri* treatment. A * mark shows statistical significance with Control and # shows statistical significance with Parkinson's disease induced group.

Effects on motor coordination using Rota-rod test

The number of fall from the rotating rod was distinctly increased and the latency for balancing in the rota-rod was decreased in the PD induced animals when compared to that of sham control animals. Treatment with *Bacopa monnieri* improved the coordinated motor movements in PD induced animals as depicted in figure 6 and figure 7.

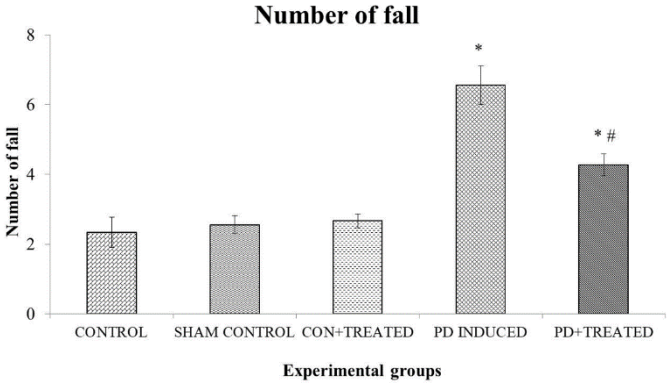

Fig 6: Showing alterations in the motor coordination in Parkinson's disease induced animals and the effects of *Bacopa monnieri* treatment over motor coordination. A * mark shows statistical significance with Control and # shows statistical significance with Parkinson's disease induced group.

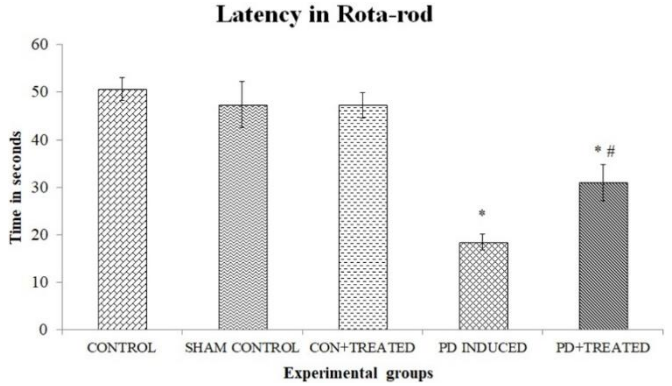

Fig 7: Showing latency in the Rota-rod of Parkinson's disease induced animals and the effects of *Bacopa monnieri* treatment over motor coordination. A * mark shows statistical significance with Control and # shows statistical significance with Parkinson's disease induced group.

Effects on motor coordination using narrow beam walking test

In narrow beam walking test the time taken to cross the start line and to walk through the beam was recorded. The PD induced animals showed a marked reduction in the test performance when compared to that of the control animals, which improved with *Bacopa monnieri* treatment when compared to that of the sham operated animals as shown in figure 8 and figure 9.

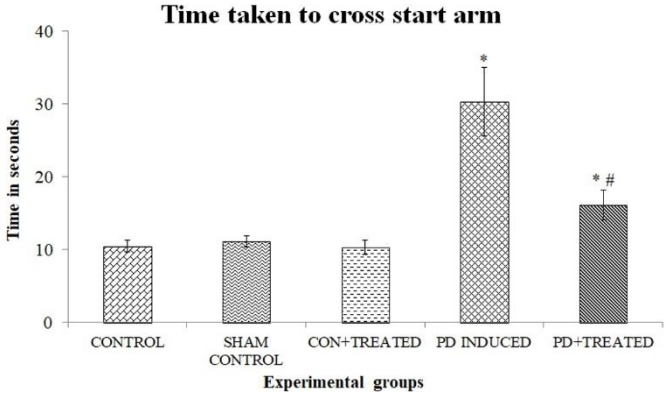

Fig 8: Showing time taken by Parkinson's disease induced animals to cross the start line of narrow beam and the effects of *Bacopa monnieri* treatment over motor performance. A * mark shows statistical significance with Control and # shows statistical significance with Parkinson's disease induced group.

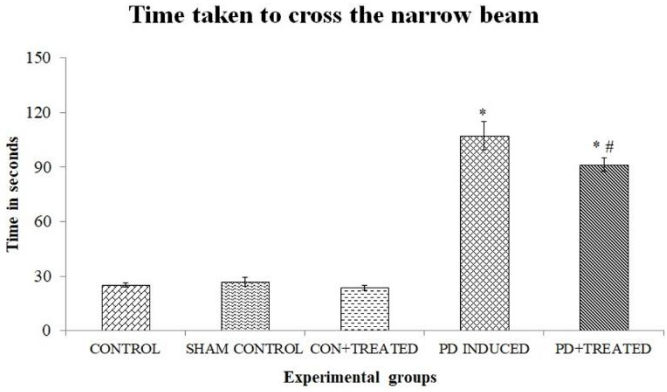

Fig 9: Showing time taken by Parkinson's disease induced animals to cross the narrow beam and the effects of *Bacopa monnieri* treatment over motor performance. A * mark shows statistical significance with Control and # shows statistical significance with Parkinson's disease induced group.

Discussion

The motor and non-motor behavioral alterations of the unilateral medial forebrain bundle (MFB)-lesioned PD model were assessed in the study. The main aim of the study was to assess the behavioural parameters for the testing of potential anti-parkinsonian effect of BM. Previous reports suggests that, infusion of rotenone into the MFB region in rats induces selective dopaminergic neuronal degeneration by oxidative damage in nigrostriatal region [16] and the lesion in MFB mimics end-stage PD [17]. The animals that exhibited spontaneous circling behaviour post-surgery were taken for the study. It was observed that, spontaneous asymmetric circling toward the hemisphere with dopamine depletion in hemi-parkinsonism [18]. The role of BM as a neuro-protective drug in rotenone induced PD is investigated in the study. It has the ability to mitigate and offer protection against rotenone induced oxidative stress [19].

In T-maze test the PD induced animals exhibited a trivial diminution in the memory pattern and the retention of memory was perceptibly impaired without side preference indicating mild cognitive impairment and the treatment with BM improved the symptoms which were not statistically significant when compared to that of the sham operated animals. This is synonymous with previous reports by that BM, a potential antioxidant, reduces oxidative stress and improves cognitive function [20]. It was also observed that the latency in the start arm of the T-maze was prominently increased in the PD induced group. The substantia nigra plays a crucial role in modulating motor movement and reward functions through the basal ganglia circuitry. The degeneration in this pathway of basal ganglia's circuitry leads to the hypokinetic state. The balance between the direct and indirect pathways of the circuitry regulates the initiation of motor output. Damage to this region decreases the cortical excitation and results in decreased movements. It is also reported that the basal ganglia is involved in the regulation of movement gain [21].

The memory pattern of the PD induced rats was declined inordinately as evidenced by increased working and reference memory errors. This is in connection with previous reports that dopamine depletion of the striatum was found to have a negative effect on cognitive performance and spatial discrimination [22]. With sub-acute treatment of BM extract, there was a substantial enhancement of memory. In hemi-Parkinson disease rotenone alters redox homeostasis and cause neuro-degeneration by oxidative stress, which ends in the Lewy body inclusion within the degenerating neurons. It was observed that, the neurons in the ventral tire of the SNpc region which projects to the dorsal region of caudate nucleus and putamen [23], is concerned mainly with functions such as flexibility, response inhibition, and working memory. The alteration in memory pattern observed in the present study may be due to the degeneration of this region. Whereas, the dorsal tier of SNpc that projects into the ventral striatum is concerned with motor sequence learning, which are affected in the advanced stages of PD which is in agreement with previous studies that, the ventral tier SNpc DA neurons less resistant than dorsal tier of dopaminergic neurons of SNpc in PD [24].

In the present study it was perceived that the coordination of motor movements was altered in the PD induced animals which was evidenced as reduced competency of the animal to balance in the rotating rod and the number of fall from the rod. Previous reports suggest that, the dopaminergic lesion produce an impaired coordination and skilled motor function in animals and also the lesion in the MFB region results in depletion of DA in the striatum [25]. This could be the probable reason for the loss of coordination in unilateral hemi-Parkinson lesion in this present study. Treatment with BM produced a substantially increased performance in rota-rod indicating its neuro-protective property.

Similarly, our results in the narrow beam walk test showed that, the time taken to cross the start line was much increased in the PD induced animals suggesting that there is a derangement in the initiation of movement. This may be due to the nuclei in basal ganglia which are involved in circuits that coordinate activation and inhibition in action selection and execution were impaired. Whereas, the treatment with BM in PD induced animals showed a significant improvement in the initiation of movements. The ability of the animal to balance in a narrow beam was also decreased in the PD induced group as compared to that of sham operated group which improved on BM treatment. It was also evidenced that in PD, the frontal areas that share basal ganglia circuits play a role in maintaining this action program, once activated. The major role of the basal ganglia is the learning and selection of the most appropriate motor or behavioral programs. Impairment of these functions of BG in unilateral lesion lead to altered movement pattern and loss of motor coordination and this may be the reason for the increase in the fall from narrow beam observed in the PD induced animals.

In the present study it was observed that BM exhibit s strong neuro-protective effect in rotenone induced neuro-degeneration. It is reported that the free radicals in the brain damages DNA, proteins, membrane lipids, and mitochondrial components [26]. In the present study it was observed that BM exert a neuro-protective effects which was attributable to the previous reports that, BM exhibit free radical scavenging [27] and increasing antioxidant activity [28] in brain. The bacosides, major constituent of BM, repair damaged neurons by enhancing kinase activity in damaged neurons, restores the synaptic activity and serves in the improvement of nerve impulse transmission [29].

Conclusion

From the present study it is clearly evident that *Bacopa monnieri* act as a neuro-protective agent in rotenone induced hemi-Parkinson's disease condition. Further the molecular mechanisms underlying its therapeutic efficacy have to be identified in such a way that it can be used as an alternate way of treatment of Parkinson's disease.

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