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Neuroprotective Potential of Polydatin Against Motor Abnormalities and Dopaminergic Neuronal Loss in Rotenone Induced Parkinson Model

Potencial Neuroprotector de la Polidatina Contra las Anomalías Motoras y la Pérdida Neuronal Dopaminérgica en un Modelo de Parkinson Inducido por la Rotenona

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SUMMARY: Among the neurodegenerative disorders, Parkinson disease (PD) is ranked as second most common. The pathological hallmark is selective degeneration of the dopaminergic neurons in the nigro-striatal regions of brain with appearance of the Lewy bodies. Present study explores the neuro-protective potential of polydatin in terms of amelioration of degeneration of dopaminergic neurons in nigro-striatal regions of brain and distorted neuromotor behavior in the rotenone model of Parkinson's disease. Thirty-six male Sprague Dawley rats were divided into three groups. Group A (control), Group B (rotenone treated) and Group C (rotenone+polydatin treated). Rotenone was administrated intraperitoneally (i.p) at a dose of 3 mg/kg/body weight while polydatin was given i.p. at a dose of 50 mg/kg/body weight for four weeks. Then, animals were sacrificed; substantia nigra (SN) & striatum isolated from brain and five micron thick sections were prepared. Cresyl violet (CV), H&E and Immuno-histochemical staining using anti-TH antibody was done. Motor behavior was assessed weekly throughout the experiment using five different methods. Rotenone treated parkinsonian animals showed deterioration of motor behavior, weight loss, loss of dopaminergic neurons and diminished immune-reactivity in the sections from the nigrostriatal regions of these animals Polydatin+rotenone treatment showed contradicting effects to parkinsonism, with amelioration in weight loss, neuro-motor behavior, dopaminergic loss and immune-reactivity against dopaminergic neurons. Present study revealed a neuro-protective potential of polydatin in animal model of PD by ameliorating the neuro-motor abnormalities and degeneration of dopaminergic neurons in nigrostriatal regions.

KEY WORDS: Dopaminergic; Neurodegenration; Parkinson's disease; AntiTH antibody.

INTRODUCTION

Among neurodegenerative disease, Parkinson's disease (PD) is second most common. It is characterized by tremor at rest, rigidity postural instability and akinesia (Ma *et al*., 2017). Up to date, there is no specific treatment to halt the progression and cure of PD. Only symptomatic treatment such as levodopa is used to reduce the motor symptoms but prolonged usage may cause fluctuations of motor as well as non-motor symptoms (Dietrichs & Odin, 2017).

Polydatin, a natural phyto-polyphenol that is isolated from Polygonumcuspidum, has many pronounced pharmacological effects including anti-oxidative, anti-inflammatory, anti-tumor, hepato-protective and renal protective (Du *et al*., 2013). Neuro protective potential of polydatin has been reported in literature. Improvement in cognitive deterioration after ethanol administration (Zhang *et al*., 2015), learning & memory impairment after hypoxic–ischemic brain injury (Sun *et al*., 2014), primary

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hippocampal cells survival rate, down regulation of cyclin dependent kinase 5 activity (Zhang *et al*.) and up regulation of brain derived neurotrophic factor (Sun *et al*.) have been reported after polydatin administration. High dose of polydatin (30 mg/kg/day) has shown an up regulation of glioma associated oncogenhomolog1 (Gli1), patched-1 (Ptch1) & superoxide dismutase 1 (SOD1), down regulation of NF-kB, reduction in infarct volume, brain water contents and improvement in behavioral deficit (Ji *et al*., 2012).Some authors have suggested that neuro protection offered by polydatin is likely by preventing the mitochondrial injury of the nervous tissue (Wang *et al*., 2013) or by its anti-apoptotic property (Gao *et al*., 2016).

Present study intends to investigate the neuro protective potential of polydatin against Parkinson disease, its effects on neuro-motor behavior and dopaminergic neuronal degeneration in striatum and substantia nigra of brain in Parkinson disease using rats as mammalian model.

MATERIAL AND METHOD

Animals. Thirty six male Sprague Dawley albino rats, weighing 200-250 mg were recruited from the animal house of Baqai Medical University, Karachi and divided randomly into three groups. Group A, control, group B, rotenone only treated and Group C, polydatin+rotenone treated. All animals were kept in close observation for one week in experimental room for acclimatization.

Dosage. Control group animals received 1 ml of dimethyl sulfoxide (DMSO) (Merck, Cat no 802912) intraperitoneally for 4 weeks. Group B animals received rotenone at a dose of 3 mg/kg/body weight intraperitoneally daily for four weeks (Tapias *et al*., 2014) and group C animals received rotenone at a dose of 3 mg/kg/body weight and polydatin at a dose of 50 mg/kg/body weight intraperitoneally daily for four weeks (Ji *et al*.).

Preparation of drugs. 300 mg of rotenone (Sigma Aldrich, Cat no R8875) was dissolved in 2 ml of DMSO (Merck Cat #802912) and then diluted with 98 ml of migloyl (Axopharma, Belgium). The final concentration of rotenone was 3 mg/ml. 500 mg of Polydatin was dissolved in 10 ml of DMSO with a final concentration of 50 mg/ml.

Neurobehavioral analysis. To record the advent of Parkinson motor symptoms and neuro protection by polydatin was done weekly between 10:00 am to 2:00 pm in the same context and place. Before the commencement of the experiment, all the animals were trained for each behavioral test for 1-2 days to refrain from fear and anxiety. **Akinesia.** Initially, each animal was acclimatized on an elevated wooden box for 5 min and akinesia was assessed by recording the time taken to move all four limbs. The test was ended if latency time exceeded 180 s (Salama *et al*., 2012).

Postural instability test. Rats were held vertically with head facing downward. Rat's nose tip was brought in zero line of ruler. One forelimb was gently restrained against rat's torso while the other forelimb permitted to plant on table. Rat allowed to move forward over single planted forelimb, up to "catch-up" step to retrieve its center of gravity. The new nose tip position indicated the body displacement required for a catch-up step (Fig. 1) (Salama *et al*., 2012; Tapias *et al*.).

Catalepsy bar test. Catalepsy bar test was used to assess the rigidity in Parkinson model (Sharma & Nehru, 2013). Fore limbs of the experimental rats were put on a steel bar, 9 cm above and parallel to the floor. Time was recorded for withdrawing both paws from the steel bar. The maximum cut off time for this test was 180 s (Fig. 2).

Adhesive removal test. Adhesive removal test was used to evaluate the motor response to sensory stimuli (Kim *et al*., 2015). A small piece of sticky tape was put on plantar surface of the forelimbs and time consumed by animals to remove the sticker was recorded. The maximum cut off time was 60 s.

Manual gait analysis. Gait analysis was done manually by measuring stride length (Glajch *et al*., 2012). Bottom of the fore limbs of rats were painted with kids paint and then they were allowed to walk on horizontal passage directed towards their cage. The passage was covered with white absorptive paper. Stride lengths were calculated by two consecutive footprints of right forelimb and average data of 4-6 steps was reported (Fig. 3).

Sacrifice, perfusion and removal of brain. At the end of experiment, all the animals were anesthetized with intraperitoneal injection of thiopental sodium at a dose of 50 mg/kg body weight (Uppalapati *et al*., 2014). Chest cavity was exposed and 100 ml of normal saline was perfused through heart to remove all the blood from the body. Immediate reperfusion with 200 ml of 10 % neutral buffered formalin was done. After decapitation, brains were removed and fixed in 10 % of neutral buffered formalin.

Isolation, sectioning and staining of substantia nigra and striatum. On hardening, brains were removed from formalin. Substantia nigra and striatum were isolated using brain slicer

(Zivic instrument, USA) (Fig. 4). Substantia nigra and striatum were sectioned by placing the knife at -2.3 to -6.04 mm and 0.70 to 1.70 mm from bregma respectively (Paxinos & Watson, 2005). Five micron thick sections were prepared and stained with cresyl violet and H&E.

Immuno-histochemical staining. Sections were deparaffinized by two washes in xylene, each for 15 min, rehydrated in descending grade of alcohol followed by washing with 1X phosphate buffered saline (PBS) for 15 min. For antigen retrieval, slides were immersed in ethylene diamine tetra acetic acid (EDTA) solution at 90 ˚C for 20 min. Sections were then permeabilized with 0.5 % Triton X100 for 10 min and then blocked in 5 % bovine serum albumin (BSA) for 2 h. Sections were then incubated in mouse monoclonal anti TH antibody overnight at 4 °C (1:25, Millipore # MAB318) and for 2 h at room temperature to obtain optimal antibody penetration. For detection of antigen signal, sections were washed thrice with PBS and incubated in avidin-biotin complex (ABC) (Millipore) solution. After washing in PBS, the sections were developed using diaminobenzidine (DAB) (Millipore, #DAB500) to visualize the brown color precipitate at the antigen sites (Cannon *et al*., 2009).

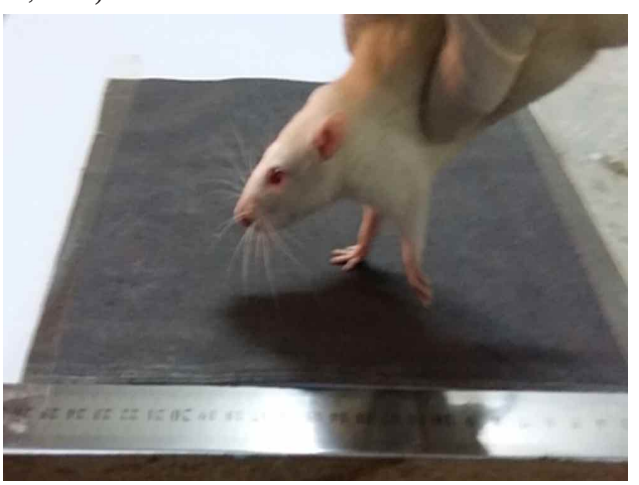

Fig. 1. Postural instability test.

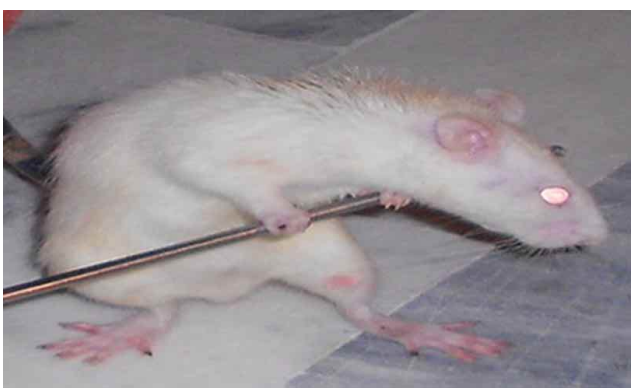

Fig. 2. Catalepsy bar test.

Fig. 3. Manual gait analysis.

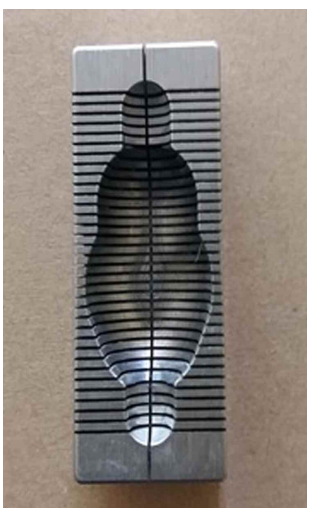

Fig. 4. Brain slicer.

RESULTS

Body weight. In week zero and one, no significant difference (P>0.05) was recorded in mean body weights between treated and control animals. In second (P<0.05), third and fourth weeks (P<0.001), mean body weights of the polydatin+ rotenone treated animals were significantly higher (P<0.001) than the rotenone treated parkinsonian animals. When compared to control animals, they were insignificant in second (P>0.05) while significantly (P<0.05) lower in third and fourth weeks (Fig. 5).

Akinesia. Mean akinesia scores of the control and treated animals were insignificantly different (P>0.05) in week zero. In first, second, third and fourth weeks, these scores of the polydatin+rotenone treated animals were significantly lower (P<0.001), than the rotenone treated parkinsonian animals. When compared to control animals, these scores were insignificant in first while significantly (P<0.05) lower in second, third and fourth weeks (Table I).

Postural instability test. There was no significant difference (P>0.05) in mean postural instability scores in week zero among the groups. Mean scores of polydatin+rotenone treated animals were insignificant (P>0.05) in week one (P>0.05) but significantly lower in second, third & fourth week (P<0.001) than rotenone treated parkinsonian animals When compared to control animals, these scores were insignificant in first (P>0.05) while significantly (P<0.05) higher in second, third and fourth weeks (P<0.001) (Table I).

Catalepsy bar test. No significant difference (P>0.05) was recorded in mean scores of catalepsy bar test between control and experimental animals in week zero. Mean scores of

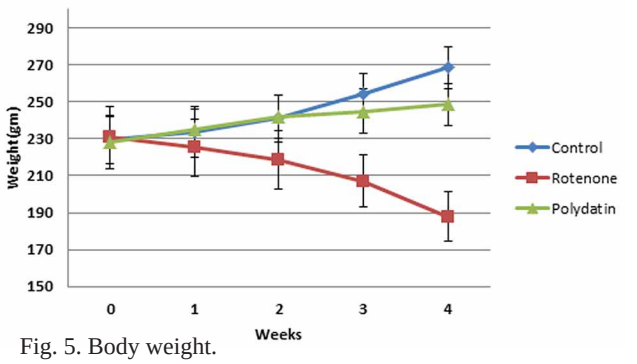

Fig. 5. Body weight.

Table I. Week wise comparison of behavioral tests. a, b, c, d, e, f.

Weeks	Control	Rotenone	Polydatin+Rotenon
	Akinesia Test		
0	5.08±0.99	5.08±1.16	5.17±1.11
1	5.75±2.13	11.42±2.27[a]	6.50±1.68[b,c]
2	4.67±2.38	19.33±2.96[a]	8.50±1.98[b,d]
3	5.17±1.58	26.08±2.87[a]	9.25±2.73[a,b]
4	5.67±1.30	35.75±5.49[a]	11.83±2.17[a,b]
	Postural instability test		
0	3.52±0.88	3.57±0.88	3.46±0.78
1	3.53±0.90	4.73±1.19[d]	4.01±0.70[c,e]
2	3.54±0.64	6.34±0. 94[a]	4.98±1.03[d,f,]
3	3.64±0.65	8.13±1.18[a]	5.35±1.53[d,f,]
4	3.69±0.57	11.75±1.08[a]	6.78±0.80[a,b]
	Catalepsy bar test		
0	2.50±1.17	2.17±1.27	2.66±1.49
1	2.67±1.15	5.00±1.86[a]	4.17±1.64[b,c]
2	2.75±0.87	11.25±2.26[a]	6.91±2.71[b,d]
3	2.92±1.083	20.92±2.61[a]	7.42±2.31[a,b]
4	2.92±1.31	27.50±3.75[a]	10.00±2.69[a,b]
	Adhesive Removal Test		
0	11.92±1.24	12.17±1.53	12.33±1.55
1	12.25±1.48	15.75±1.82[a]	14.08±1.78[b,c]
2	12.58±1.51	21.75±2.14[a]	16.08±2.06[b,d]
3	12.58±1.09	25.67±4.83[a]	19.50±1.51[a,b,]
4	12.75±1.29	35.75±2.60[a]	22.00±2.05[a,b]
	Manual gait analysis		
0	4.00±0. 87	4.08±0.67	3.91±0.90
1	4.08±0. 99	5.92±1.08[a]	4.50±1.09[c,e]
2	4.08±0. 99	10.42±1.56[a]	5.75±1.06[b,d]
3	4.17±0. 83	19.17±3.10[a]	7.75±1.60[a,b]
4	4.17±0. 94	30.17±4.30[a]	10.42±2.43[a,b]

polydatin+ rotenone treated animals were significantly higher (P<0.001) than rotenone treated parkinsonian animals, from first to fourth. When compared with control, the score were insignificant in first week (P>0.05) and significantly lower in second (P<0.05), third and fourth week (P<0.001) (Table I).

a(P<0.001) when compared with control, b(P<0.001) when compared with rotenone treated animals, c(P>0.05) when compared with control, d(P<0.05) when compared with control, e(P>0.05) when compared with rotenone treated animals, f(P<0.05) when compared with rotenone treated animals

Adhesive Removal Test. There was no significant difference (P>0.05) in the mean value of adhesive removal time between treated and control animals in week zero. Animals from polydatin+rotenone treated group showed significantly less mean time (P<0.001) than rotenone treated parkinsonian animals while comparing to control, it was insignificant (P>0.05) in first but significantly lower in second third (P<0.05) and fourth week (P<0.001) (Table I).

Manual gait analysis. No significant difference (P>0.05) was recorded in the means of stride length between treated and control animals in week zero. Mean stride lengths of the polydatin+rotenone treated animals were significantly higher in first (P<0.05), second, third and fourth weeks (P<0.001) than rotenone treated parkinsonian animals. When compared to control animals, these score were insignificant in first week (P>0.05), but significantly lower in second (P<0.05), third and fourth week (P<0.001) (Table I).

Hematoxylin and Eosin and Cresyl violetstaining. H&E and Cresyl violet stained sections of SN & striatum from polydatin+rotenone treated animals showed a significantly (P<0.001) higher number of neurons as compared to rotenone treated parkinsonian animals and

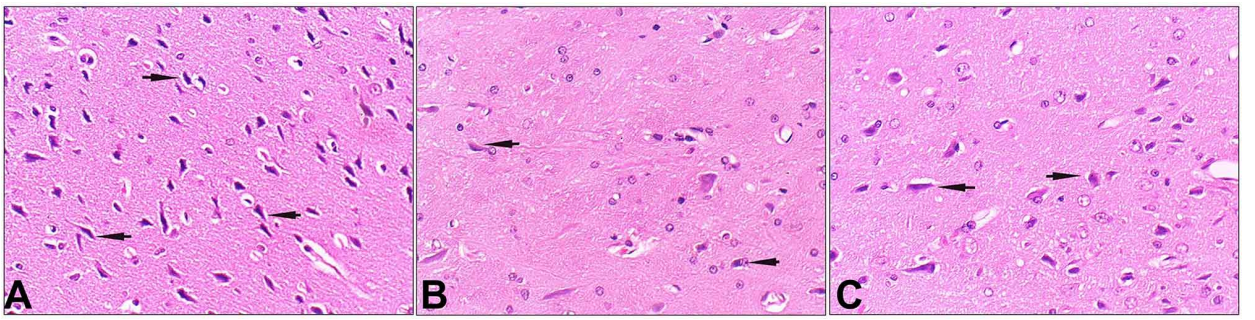

Fig. 6. Five micron thick H&E stained section of SN at 40x magnification showing neurons from control (a), rotenone treated parkinsonian animals (b) and polydatin+rotenone treated animals (c). Comparatively, a higher number of neurons is evident in section C than section B.

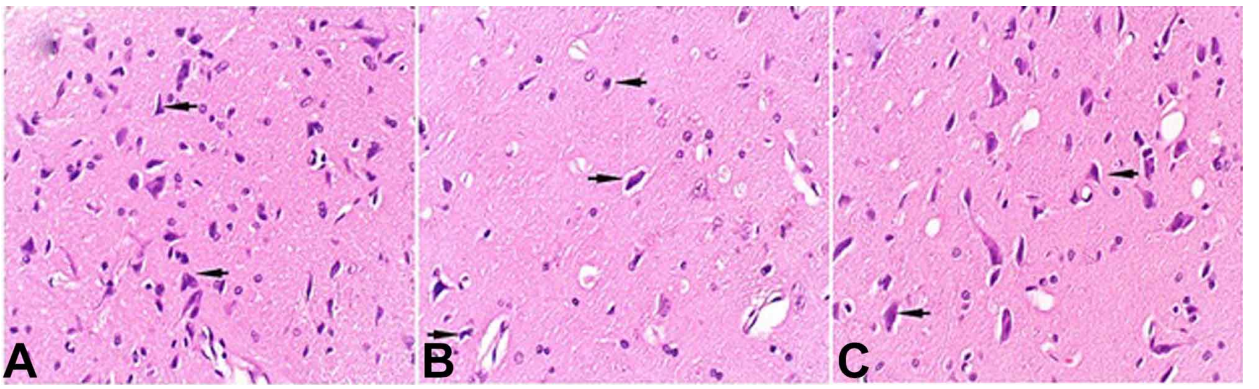

Fig. 7. Five micron thick H&E stained sections from striatum at 40x magnification showing neurons from control (a), rotenone treated parkinsonian animals (b) and polydatin+rotenone treated animals(c). Comparatively a higher number of neurons is evident in section C than section B.

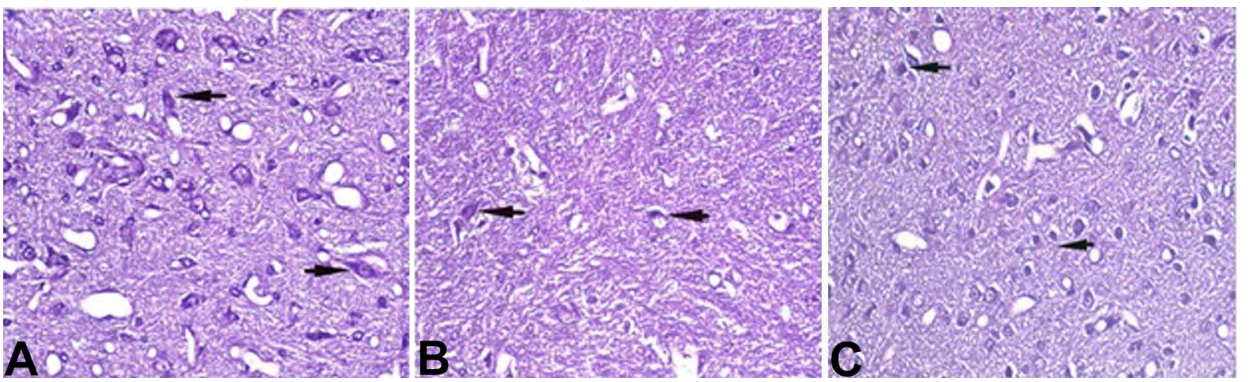

Fig. 8. Five micron thick CV, stained section of SN at 40x magnification showing dopaminergic neurons of control (a), rotenone treated parkinsonian (b) and polydatin+rotenone treated (c) animals. A higher number of dopaminergic neurons is evident in section C as compared to section B.

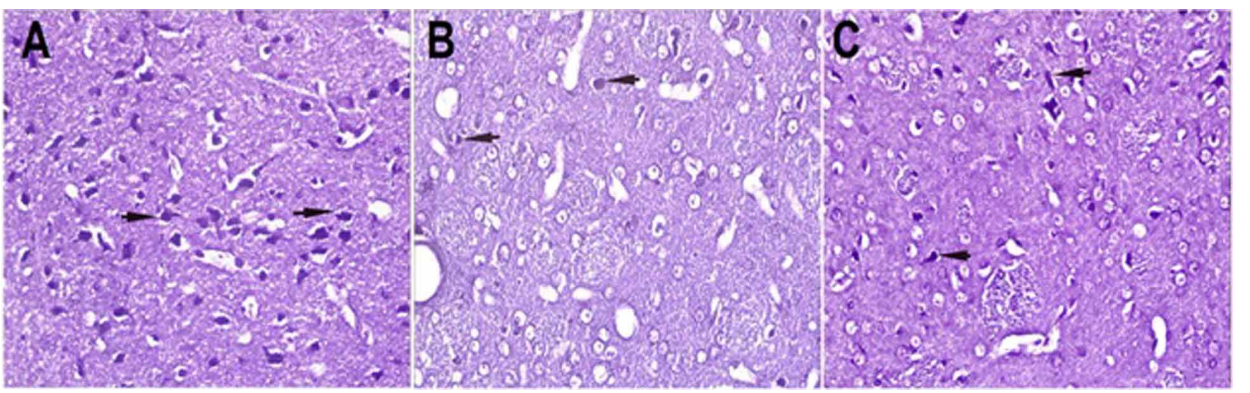

Fig. 9. Five micron thick CV stained section of striatum at 40x magnification showing dopaminergic neurons of control (a), rotenone group (b) and polydatin+rotenone group (c). A higher number of dopaminergic neurons is evident in section C as compared to section B

significantly (P<0.001) lower number of neurons as compare to control animals (Figs. 6-9) (Table II).

Anti-Tyrosine hydroxylase antibody stained sections. Anti-TH antibody stained sections of SN & striatum from polydatin+rotenone treated animals showed a significantly higher reactivity and number of neurons when compared to rotenone treated animals and a significantly (P<0.001) lower number neurons in comparison to control animals (Figs. 10 & 11) (Table II).

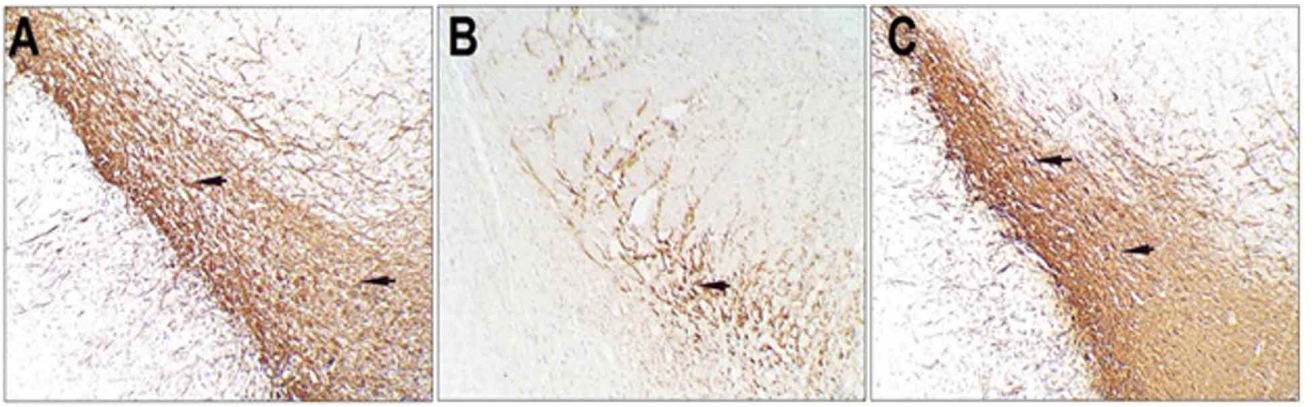

Fig. 10. Anti TH antibody immunohistochemistry of substantia nigra at 10x magnification showing brown color dopaminergic neurons and reactivity of control (A), rotenone treated parkinsonian (B) and polydatin+rotenone treated animals (C). Section B is showing decreased immunoreactivity and less number of neurons while section C is showing number of neurons and immunoreactivity comparable to section A.

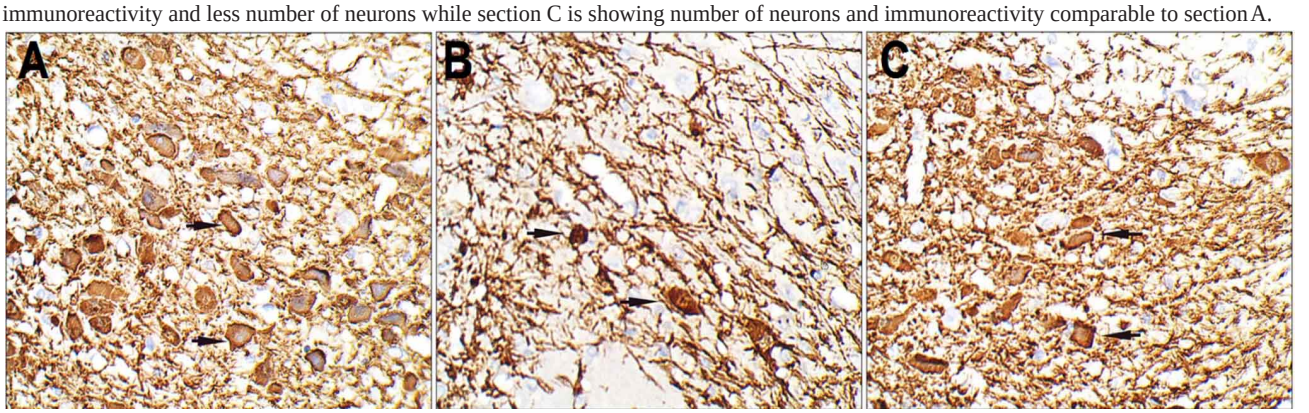

Fig. 11. Anti TH antibody immunohistochemistry of substantia nigra at 40x magnification showing brown color dopaminergic neurons and reactivity of control (A), rotenone treated parkinsonian (B) and polydatin+rotenone treated animals (C). Section B is showing diminished immunoreactivity and less number of neurons while section C is showing number of neurons and immunoreactivity comparable to section A.

Table II. Number of Neurons / field at 40x magnification. a (P<0.001) when compared with control, b (P<0.001) when compared with rotenone treated parkinsonian animals.

	Control	Rotenone	Polydatin+Rotenone
		SUBSTANTIA NIGRA	
H &E STAINING	62.83±3.95	35.83±7.54[a]	47.14±4.94[a,b]
		STRIATUM	
	60.00±3.88	28.67±2.71[a]	44.58±7.47[a,b]
		SUBSTANTIA NIGRA	
CV STAINING	61.08±5.45	31.75±6.05[a]	49.00±7.13[a,b]
		STRIATUM	
	57.50±6.21	28.17±2.91[a]	41.00±6.55[a,b]
ANTI-TH ANTIBODY		**SUBSTANTIA NIGRA**	
STAINING	58.17±5.92	28.17±3.10[a]	43.41±3.11[a,b]

DISCUSSION

Rotenone treated parkinsonian animals have shown a significant weight loss than control and polydatin+rotenone treated animals in this study. Similar results have also been reported by some earlier studies (Xiong *et al*., 2015). This weight loss could be due to the reduced food intake, disturbance in gastric motility and stool frequency (Xiong *et al*.). Contrary to this some studies have reported little or no effect of rotenone on body weight (Wang *et al*., 2015). Countering effect of polydatin on weight, recorded in this study may be due to its amelioration of GIT function secondary to neuroprotection or anti-inflammatory effect as reported earlier in mice after dextran sulphate sodium induced colitis (Yao *et al*., 2011).

In this study, rotenone treated animals developed postural instability, akinesia and rigidity which deteriorated over time (Salama *et al*., 2012). Insufficient availability of dopamine levels in substantia nigra and striatum because of degeneration of dopaminergic neurons has been accepted as the major cause in the development of these primary motor symptoms of Parkinson's disease (Salama *et al*., 2013). Histological investigations in this study have indicated a decline in the activity and loss of dopaminergic neurons in the striatum and substantia nigra. Animals treated with polydatin along with rotenone have expressed a significant fall in latency scores as compare to rotenone treated parkinsonian animals. Immune-histochemical findings of this study substantially supported this point, since the anti TH antibody immune-histochemical sections from these rats have shown a significantly higher reactivity and number of dopaminergic neurons.

Rotenone treated parkinsonian animals consumed more time to remove the sticky tape (Zhou *et al*., 2016) than the other two experimental groups during adhesive removal test in this study. This could be due to global impairment in coordinated and skilled movements of forelimb, less motivation or decrease tactile stimuli (Bentea *et al*., 2015). Decreased time to adhesive removal by polydatin+rotenone treated animals, could be due to neuro protection by boosting, tactile perception, coordination of movements and motivation secondary to amelioration in function of premotor cortex and cerebellum.

In this study, animals from rotenone group depicted a shorter stride length. This kind of gait is characteristic feature of Parkinson's disease and is a useful indicator of altered functioning of basal ganglia (Fathalla *et al*., 2016). Depleted levels of dopamine due to loss of dopaminergic neurons has been associated with altered, planning, control over movements, cortical function and cerebellar dysfunction (Georgiev *et al*., 2016; Seidel *et al*., 2017). Polydatin+rotenone treated animals displayed significantly longer stride lengths than parkinsonian rats. This could be due to neuro protection by polydatin, impeding dopaminergic degeneration, ensuring adequate levels of dopamine in nigrostriatal region and amending cerebellar function. Neuro protective potential of polydatin substantiated with immunohistochemistry is being reported first time. This study can provide a base line model for further investigation.

CONCLUSIONS

The results of this study indicate that polydatin has a neuro-protective potential against degeneration of dopaminergic neurons. Polydatin treatment has shown amelioration in the motor symptoms, enhanced reactivity and a higher number of dopaminergic neurons in the nigrostriatal regions of the brains of the Parkinsonian rats, though a full recovery was not possible.

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RESUMEN: Entre los trastornos neurodegenerativos, la enfermedad de Parkinson (EP) se clasifica como la segunda más común. El sello patológico es la degeneración selectiva de las neuronas dopaminérgicas en las regiones nigro-estriatales del cerebro, con la aparición de los cuerpos de Lewy. El presente estudio explora el potencial de protección neuronal de la polidatina en términos de la mejora de la degeneración de las neuronas dopaminérgicas en las regiones nigro-estriatales del cerebro y el comportamiento neuromotor distorsionado en el modelo de rotenona de la enfermedad de Parkinson. Treinta y seis ratas macho Sprague Dawley se dividieron en tres grupos: Grupo A (control), Grupo B (tratado con rotenona) y Grupo C (tratamiento con rotenona + polidatina). La rotenona se administró por vía intraperitoneal (i.p.) a una dosis de 3 mg/kg/peso corporal, mientras que la polidatina se administró i.p. a una dosis de 50 mg/kg/peso corporal durante cuatro semanas. Posteriormente, los animales fueron sacrificados. Se aislaron la substantia nigra (SN) y cuerpo estriado de los cerebros y se realizaron secciones de cinco micras de espesor. Se realizó una tinción de violeta de cresilo (CV), H&E y tinción inmunohistoquímica usando anticuerpo anti-TH. El comportamiento motriz se evaluó semanalmente durante todo el experimento utilizando cinco métodos diferentes. Los animales parkinsonianos tratados con rotenona mostraron deterioro del comportamiento motriz, pérdida de peso, pérdida de neuronas dopaminérgicas y disminución de la reactividad inmune en las secciones de las regiones nigroestriadas. El tratamiento con polidatina

+ rotenona mostró efectos contrarios al parkinsonismo, con mejoría en la pérdida de peso, en el comportamiento motor, en la pérdida dopaminérgica y en la reactividad inmune contra las neuronas dopaminérgicas. El presente estudio reveló un potencial de protección neuronal de la polidatina en el modelo animal de la EP al mejorar las anomalías neuro-motoras y la degeneración de las neuronas dopaminérgicas en las regiones nigroestriatales.

PALABRAS CLAVE: Dopaminérgico; Neuro-degeneración; Enfermedad de Parkinson; Anticuerpo antiTH.

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