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Neuroprotective effect of methanolic extract of *Sargassum wightii* on rotenone-induced parkinsonism-like symptoms in Wistar albino rats

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ABSTRACT

Introduction: The pathogenesis of Parkinson's disease (PD) is multifactorial in which oxidative stress, neuroinflammation, and mitochondrial dysfunction are the leading factors. Currently, the antioxidant and anti-inflammatory agents of natural sources as neuroprotectants have raised much attention. The current study aimed to explore the neuroprotective effect of methanolic extract of *Sargassum wightii* in male Wistar albino rats against rotenone-induced PD.

Methods: The rats were administered with rotenone (10 mg/kg orally) daily for 28 days to induce PD. *S. wightii* (200 mg/kg and 400 mg/kg) and levodopa+carbidopa combination (10 mg/kg) were administered to different groups of rats one hour prior to rotenone for 28 days. Behavioral parameters (akinesia, tremor, motor coordination, and locomotor activities) and body weight were recorded on days 14th and 28th of drug treatment. On the 28th day, the animals were sacrificed for the neurobiochemical analyses of brain tissue.

Results: Rotenone treatment caused a significant reduction in behavioural parameters ($P<0.001$), neurochemical deficits ($P<0.001$), and elevation of oxidative stress markers ($P<0.001$) in the brain. Pre-treatment with *S. wightii* at 200 mg/kg and 400 mg/kg doses significantly attenuated the rotenone-induced behavioral alterations and restored the mitochondrial NADH dehydrogenase activity and dopamine level in the striatum ($P<0.001$). Moreover, 400 mg/kg of *S. wightii* restored the rotenone-induced increased oxidative stress markers like malondialdehyde (MDA), superoxide dismutase (SOD), and reduced glutathione (GSH) in the striatum ($P<0.01$).

Conclusion: *S. wightii* has provided neuroprotective effect, probably by virtue of its antioxidant and dopamine restoring potential. Hence, it may offer a promising and new therapeutic lead for the treatment of PD but needs further research.

Implication for health policy/practice/research/medical education:
Sargassum wightii has a neuroprotective effect against rotenone induced Parkinsonism like symptoms. The mechanism probably involves its promising antioxidant activity. Moreover, its dopamine and mitochondrial NADH dehydrogenase restoring effect played a pivotal role in attenuating the behavioural alterations. Hence, it might be beneficial in Parkinson's disease.

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Introduction

Parkinson's disease (PD) is the second most common neurodegenerative disease that mostly affects people 60 years and above. The cardinal features of PD are akinesia, bradykinesia, resting tremor, rigidity, postural instabilities, and is often associated with cognitive dysfunction with disease progression (1). The neuropathological hallmark of PD is the selective and progressive degeneration of dopaminergic neurons in Substantia Nigra pars compacta, resulting in dopamine deficiency in the striatum (2,3).

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Though the genetic and environmental factors pose the leading causes, other factors such as reactive oxygen species induced damage, excitotoxicity, mitochondrial dysfunctions, and inflammation-mediated cell injury have also been implicated in the etiology of PD. Over the decades, experimental evidence has focused on mitochondrial dysfunction as an important pathogenic component of PD (3,5). Dopaminergic neurons are more sensitive to oxidative stress. An increase in the metabolism of dopamine generates high levels of Reactive Oxygen free radicals that cause cellular damage (6,7). Rotenone, a natural pesticide, is a highly lipophilic and specific mitochondrial complex-I inhibitor. Therefore, it has been widely used as a mitochondrial poison model (7,8). On chronic administration, it also induces oxidative stress. Both mitochondrial inhibition and oxidative stress contribute to dopaminergic neuronal degeneration and the development of PD-like symptoms (9,10).

Levodopa has been considered as the gold standard drug therapy for PD, but its side effects on long term use are alarming. Recently, neuroprotective approaches with the use of antioxidants, antiapoptotic and anti-inflammatory compounds of natural sources have taken the upper hand in preventing disease progression in PD.

Over the past decade, considerable attention has been focused on researches on marine macroalgae as they offer enormous resources for novel compounds. The macroalgae (seaweeds) have been identified as the largest reservoir of structurally diverse, bioactive compounds and secondary metabolites like peptides, carotenoids, phenols, terpenoids, phlorotannins, flavonoids, complex polysaccharides, phytosterols, glycolipids, vitamins, and minerals. Hence the pharmaceutical and agri-food industries have shown the demand for seaweeds as a functional food. Moreover, scientifically seaweeds have been validated for their antibacterial, antifungal, anti-inflammatory, anticoagulant, antioxidant, hypolipidemic, anti-diabatic, hepatoprotective and neuroprotective activities (11,14). *Sargassum wightii*, brown algae that shares similar phytoconstituents such as alkaloids, phenols, flavonoids, phlorotannins, has been proved as a powerful free radical scavenger (15). In our previous study, we observed the neuroprotective effect of *S. wightii* against haloperidol induced catalepsy (16). Thus, it is hypothesized that it has potential protective effect on other neurodegenerative diseases such as PD. This study aimed to evaluate the possible neuroprotective effect of methanolic extract of *S. wightii* on rotenone induced PD model in Wistar albino rats.

Materials and Methods

Animals

Male Wistar albino rats of 150-200 g body weight were maintained in the animal house of Roland Institute of Pharmaceutical Sciences, Berhampur, under ambient conditions of temperature (24 ± 1°C), relative humidity (45-55%) and 12:12 light: dark cycle. The animals were fed with standard pellet diet and water *ad libitum* and were allowed to acclimatize to laboratory conditions for 7 days before the day of the experiment. With prior permission from the Institutional Animal Ethics Committee (IAEC), (Regd No: 926/PO/Re/S/06/ CPCSEA), the experimental procedures were conducted in accordance with the Guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) formulated by the Ministry of Fisheries, Animal Husbandry and Dairying, Government of India. All the experiments were conducted between 10.00 to 16.00 hours.

Plant material

The plant materials were collected as a gift sample from Micobiotech limited (Manufacturers of natural agricultural inputs) New Gundlav, Valsad, Gujarat, India. To prepare a methanolic extract of *Sargassum,* 40 g of powder sample was extracted with 400 mL of methanol by using Soxhlet's apparatus for 72 hours. The extract obtained was dried in the evaporator and stored at 4°C for further use. Different concentrations of drug solution were freshly prepared on the day of the experiment using 1% CMC suspension (17).

Drugs and chemicals

Rotenone, dopamine HCl, and glycyl glycine buffer were purchased from Sigma-Aldrich, USA. Nitro-blue-tetrazolium chloride, dithionitrobenzoic acid, thiobarbituric acid, and trichloro-acetic acid were purchased from HiMedia Laboratories Pvt. Ltd. Mumbai, India. All other chemicals were of analytical grade and purchased from standard manufacturers.

Phytochemical screening of plant extract

The methanolic extract of *S. wightii* was subjected to a phytochemical screening test in our earlier study and was found to contain polyphenols, terpenoids, tannins, flavonoids, and polysaccharides (16).

Acute toxicity test

The acute toxicity study was done in our previous study as per OECD Guidelines 423 [Limit test]. The extract at doses 5, 50, 1000, and 2000 mg/kg were given orally in a stepwise manner. The animals were observed for 24 hours. No mortality or behavioral changes were observed during the study period (16).

Experimental design

Thirty Wistar albino rats were randomly divided into five groups (n=6) to receive treatments as follows:

Group-I (Normal control) was administered with vehicle olive oil (1 mL/kg p.o). Group-II was treated with

Rotenone (10 mg/kg p.o.) daily. Group-III rats (standard group) received levodopa and carbidopa combination (10 mg/kg orally) and rotenone (10 mg/kg p.o) (1,6). Group-IV (*S. wightii* 200 mg/kg + rotenone 10 mg/kg) and group-V (*S. wightii* 400 mg/kg + rotenone 10 mg/kg) were considered as the test groups. All the treatments were given orally for a period of 28 days. The drugs and vehicle were given one hour before rotenone administration. Behavioral tests were performed on days 0, 14, and 28 of the study. After the last behavioral assessments on day 28, rats were sacrificed by cervical dislocation and the brains were harvested quickly for the biochemical estimations.

Behavioral assessments

Anti-Parkinson's activity

On chronic administration of rotenone, the animals developed Parkinson's-like symptoms such as tremor and akinesia. The severity of tremor was measured by giving different scores like no tremors (score 0), occasional tremors (score 1), moderate tremors (score 2), and continuous tremors (score 3). To measure akinesia, the tail of the animal was held in hand and the animal was allowed to move forward on his forelimbs. The number of steps taken by the fore-limbs of the animal was counted for three minutes (18, 19).

Motor coordination

The motor coordination was evaluated by using the rotarod apparatus (Inco, Ambala, India). The performance was measured by the duration of animal stay on the rod in relation to the rod speed. The rats were allowed to adjust their postures and balance them on the rotating rods at different speeds. On the day of the experiment, the rats were placed individually on the rotating rod with a speed of 25 rpm, the fall-off time was recorded and the cut-off time was fixed at 180 seconds (20).

Spontaneous locomotor activity

The locomotor activity (horizontal activity) was measured using a digital photoactometer (Inco, Ambala, India). The animals were placed individually in the activity cage and acclimatized for 5 minutes. Interruption in the photo beams inside the cage by the movement of the animals represents activity count, which was recorded for five minutes (20). The chamber was swabbed with 10% ethanol each time to avoid animal odor.

Biochemical estimations

Preparation of solubilised brain mitochondrial sample

To obtain a solubilized mitochondrial sample, the method of Berman et al, was followed (21). The rats were decapitated, and the whole brains were removed quickly, washed, minced, and homogenized at 4°C in 10 mL of isolation medium (225 mM mannitol, 75 mM sucrose, 5 mM HEPES, 1 mM EGTA, 1 mg/mL bovine serum albumin, pH 7.4), then centrifuged at 2000 g for 5 minutes. The pellets were resuspended with 10 mL isolation buffer and recentrifuged as above. The resulting supernatants were collected in separate tubes and centrifuged at 12 000 *g* for 8 minutes. The pellets, including the fluffy synaptosomal layer, were resuspended with an isolation medium and centrifuged at 12 000 *g* for 10 minutes to get brown-colored pellets. These pellets containing mitochondria without the synaptosomal layer were resuspended in 10 mL of isolated medium and recentrifuged as above. Finally, the mitochondrial pellets were resuspended with 50 µL of medium and used as the sample.

Mitochondrial nicotinamide adenine dinucleotide dehydrogenase (NADH) (Complex-I) enzyme activity

The method of King and Howard was followed for the estimation of NADH dehydrogenase enzyme activity. It involves catalytic oxidation (releasing H^+) of NADH to NAD^+ with subsequent reduction of cytochrome C. The reaction mixture contained 0.2 M glycylglycine buffer (pH 8.5), 6 mM NADH in 2 mM glycylglycine buffer, and 10.5 mM cytochrome C. The reaction was initiated after the addition of the prepared solubilised mitochondrial sample and the absorbance was measured spectrophotometrically at 550 nm for 2 minutes band. The values were expressed as nM/mg protein (22, 23).

Dopamine assay

Determination of dopamine was done using high-performance liquid chromatography (reverse phase method), and the total brain dopamine content was expressed in micrograms per gram of tissue by using formula (24).

Monoamine (µg/g tissue) =AT/AS× CS× dilution factor

AT= Area under the curve for the sample, AS= Area under the curve for the standard, CS= concentration of the standard µg/mL and dilution factor=10.

Lipid peroxidation assay

The quantitative analysis of lipid peroxidation was assayed by measuring the level of malondialdehyde (MDA) in the brain tissue following the method of Okhawa. The MDA activity was determined by measuring the absorbance of thiobarbituric acid reactive species spectrophotometrically (Shimadzu UV 1800) at 532 nm (25).

Superoxide dismutase assay

The superoxide dismutase (SOD) activity in brain tissue homogenates was assayed by measuring the inhibition of reduction of nitro-blue-tetrazolium following the method of Marklund et al and was expressed in terms of units/mg tissue (26).

Reduced glutathione assay

The reduced glutathione (GSH) of brain tissue homogenates was determined by the method of Sedlak et al. The principle was based on the reduction of Ellman's reagent by –SH groups of GSH to form 2-nitro-s-mercaptobenzoic acid. The absorbance of yellow colour imparted by nitromercaptobenzoic acid anion was recorded spectrophotometrically at 412 nm (Shimadzu UV-1800). The amount of GSH in tissue was expressed as µg/mg tissue (27).

Statistical analysis

The values were expressed as mean ± SEM. The data were analyzed for statistical significance applying ANOVA followed by Tukey's multiple comparison tests using GraphPad Prism 7.0. A probability values less than 0.05 were considered significance.

Results

Effect of methanolic extract of *Sargassum wightii* on rotenone induced changes on body weight

Rotenone reduced the bodyweight of rats significantly at the end of the fourth week of treatment. Treatment with levodopa + carbidopa (10 mg/kg) body weight significantly prevented the rotenone-induced loss in body weight. Further treatment with *S. wightii* at 200 mg/kg and 400 mg/kg body weight also inhibited the rotenone induced loss in body weight after 28 days of treatment ($P < 0.01$) (Figure 1).

Behavioral indices

Effect of methanolic extract of Sargassum wightii on rotenone induced akinesia and tremor

On the 14th and 28th days of rotenone administration, the rats showed a significant increase in akinesia score to 25.83 ± 3.38 and 17.83 ± 3.44, respectively compared with normal control rats ($P < 0.001$) (Figure 2A). In comparison to the rotenone control group, levodopa + carbidopa (10 mg/kg) body weight significantly prevented rotenone-induced increase in akinesia to 48.33 ± 4.40 and 60.33 ± 4.28 on the 14th and 28th days, respectively ($P < 0.001$). *S. wightii* 200 mg/kg treatment for fourteen days did not reveal any significant change in rotenone-induced akinesia, but on the 28th day, it showed a significant decrease in akinesia (38.67 ± 3.92) ($P < 0.01$). *S. wightii* with 400 mg/kg body weight significantly reduced the akinesia both on 14th (46.67 ± 3.27) ($P < 0.01$) and 28th days (50.67 ± 4.28) of treatment ($P < 0.001$) (Figure 2A). However, on the 28th day, only the higher dose of *S. wightii* (400 mg/kg) reduced the tremor score in rotenone-induced PD rats ($P < 0.05$) (Figure 2B).

Effect of methanolic extract of Sargassum wightii on motor coordination

The motor coordination of the animals was tested using rotarod apparatus. In comparison to the control rats, rotenone-treated rats showed a significant decrease in motor coordination on the 14th and 28th days of treatment (71 ± 9.93 seconds and 26.8 ± 5.17 seconds, respectively). Levodopa + carbidopa (10 mg/kg) treated rats significantly prevented rotenone-induced changes in motor coordination activity significantly on 14th day 124 ± 7.97 seconds ($P < 0.001$) and to a highly significant extent on 28th day 159.2 ± 9.60 seconds ($P < 0.001$).

Sargassum wightii 200 mg/kg and 400 mg/kg body weight after 14 days of treatment did not show any significant change in motor activity as compared to the rotenone-treated control group. Nevertheless, *S. wightii* 200 mg/kg and 400 mg/kg increased the motor coordination activity after 28 days of administration to a highly significant level 94.80 ± 11.94 seconds and 122.6 ± 8.52 seconds, respectively compared to rotenone-treated group ($P < 0.001$) (Figure 3A).

Effect of methanolic extract of Sargassum wightii on locomotor activity

The locomotor activity of the rats in rotenone-treated group was significantly reduced on the 14th and 28th

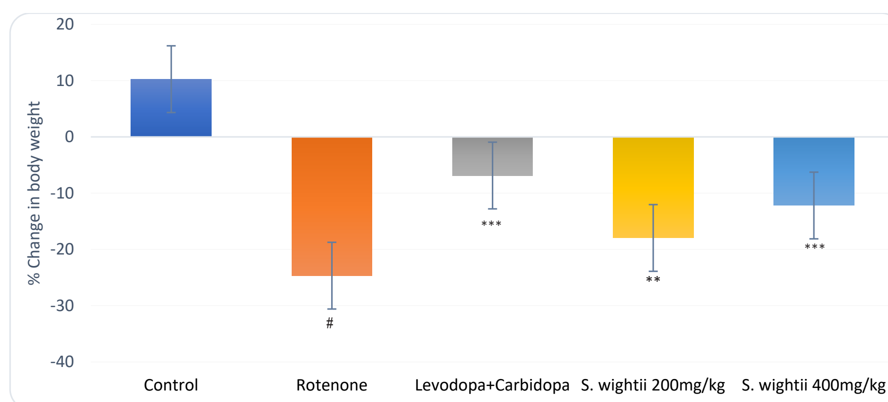

Figure 1. Effect of *Sargassum wightii* on body weight. One-way ANOVA followed by Tukey's multiple comparison tests was applied for analysis. #$P < 0.001$ compared to the vehicle control group. **$P < 0.01$, ***$P < 0.001$ compared to the rotenone control group.

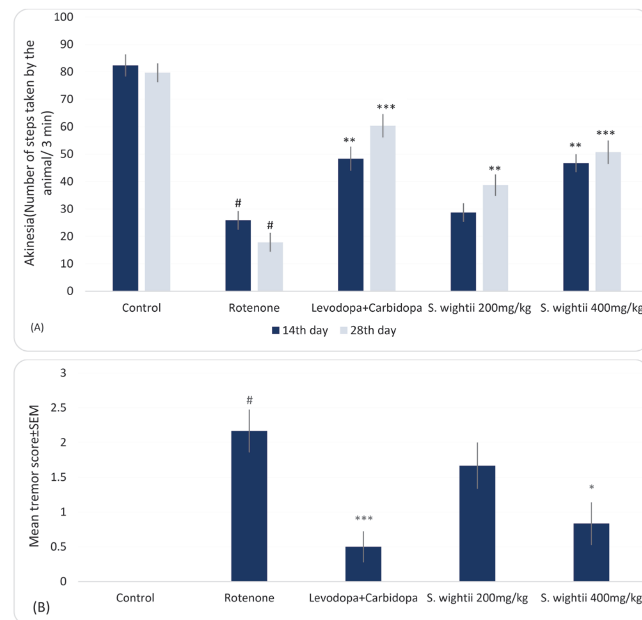

Figure 2. Effects of *Sargassum wightii* on akinesia (A) and tremor (B). Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparison tests. #$P<0.001$ compared to the vehicle control group. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared to the rotenone control group.

days of treatment by 64.5 ± 7.54 seconds and 40.5±4.05 seconds compared to the vehicle control. Administration of levodopa + carbidopa (10 mg/kg) showed a significant increase in the locomotor activity both on the 14th day (140±16.33) and 28th day (182 ± 32.57) ($P<0.001$) compared to the rotenone-treated group. *S. wightii* 200 mg/kg treatment for fourteen days did not reveal a significant change in rotenone-induced locomotor activity, but on the 28th day, it showed a significant increase in activity (114 ± 7.12) ($P<0.01$). *S. wightii* with 400 mg/kg body weight significantly increased the locomotor activity both on 14th (140.7 ± 14.9) ($P<0.01$) and 28th day (152.2 ± 12.19) ($P<0.001$) (Figure 3B).

Effect of methanolic extract of Sargassum wightii on rotenone induced biochemical changes

After 28 days of administration of rotenone, there was a significant increase in thiobarbituric acid reactive substances (4.64 ± 0.31 nM/g of brain tissue) but a significant decrease in mitochondrial NADH dehydrogenase activity (3.86 ± 0.027 nM/mg of protein), dopamine (0.26 ± 0.014 µg/g), SOD level (11.45 ± 0.93U/mg) and GSH (1.97 ± 0.19 µg/g) in comparison to that of the control group ($P<0.001$). Levodopa + carbidopa (10 mg/kg) significantly inhibited the rotenone-induced increase in TBAR to 2.25± 0.28 (nM/g). The level of dopamine, SOD, and GSH were significantly elevated to 0.50 ± 0.018 (µg/g), 23.21 ± 1.51 (U/mg) and 4.82 ± 0.35 (µg/g) following levodopa and carbidopa combination treatment ($P<0.001$). Chronic administration of *S. wightii* (400 mg/kg) for 28 days significantly inhibited the rotenone induced rise in lipid peroxidation compared to rotenone control group 2.5 ± 0.29 (nM/g) ($P<0.01$). Interestingly, *S. wightii* at 200 and 400 mg/kg significantly protected the rotenone-induced decrease in mitochondrial NADH dehydrogenase activity and dopamine level (Table 1). In comparison to the rotenone-treated group, *S. wightii* (400 mg/kg) treatment rats exhibited a significant increase in SOD activity (18.34 ± 1.48 U/mg) and GSH level (3.76 ± 0.28 µg/mg), respectively ($P<0.01$) (Table 1).

Discussion

Rotenone, a naturally derived pesticide, acts as a specific mitochondrial complex1 inhibitor and induces oxidative stress by increasing the formation of reactive oxygen species by its prominent mitochondrial complex inhibition and impaired ATP production. Evidence suggests that rotenone-induced PD is a suitable model as it simulates the behavioral and neuropathological features of disease due to selective degeneration of dopaminergic neurons (28-30). Oxidative stress may play a greater role in the demise of dopaminergic neurons indirectly by activating intracellular, cell death-related molecular pathways (30,31). During the pathogenesis of PD, the production of reactive oxygen species damages the substantia nigra through lipid peroxidation, protein oxidation, and DNA oxidation. This phenomenon seems to be induced mainly by mitochondrial dysfunction, monoamine oxidase activation, or even by changes in the antioxidant defence system. Earlier studies postulated that the neurotoxicity induced by rotenone is a consequence of dopamine oxidation that produces free radicals and damages the dopaminergic neurons, which is the key point in the neuropathology of PD (32,34). Currently seaweeds

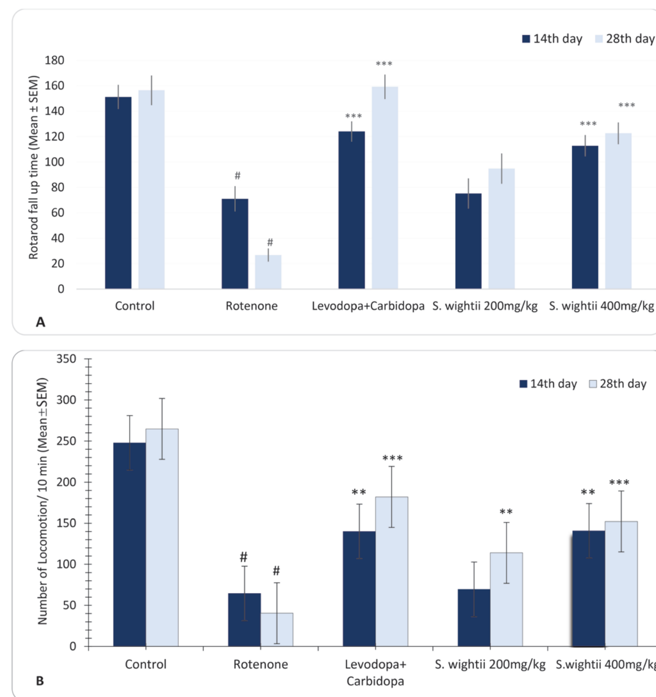

Figure 3. Effect of *Sargassum wightii* on rotarod fall up time (A) and locomotor activity (B) n=6. Statistical analysis was carried out using one-way ANOVA followed by Tukey's multiple comparison tests. # $P < 0.001$ when compared to the vehicle control group. ** $P < 0.01$, *** $P < 0.001$, test group as compared to rotenone control group.

have attracted food industries as nutraceuticals for their numerous bioactive components. *S. Wightii,* brown seaweed has been proved to possess antioxidant and anti-inflammatory activities in our earlier study. In this context, this study was an attempt to explore the potential neuroprotective effect of methanolic extract of *S. wightii* against rotenone-induced Parkinson's-like symptoms in rats. We used levodopa and carbidopa combination as the reference standard drug for comparison.

Rotenone administration (10 mg/kg) for 28 days, exhibited Parkinson's-like symptoms as evidenced from an increase in akinesia, tremor, and lack of motor coordination on rotarod test and deficit in locomotor activity in Photoactometer test. Our observations were in accordance with earlier studies (1,6). The reduced body weight of rotenone-treated group animal was seen in this study also corroborate with other reports. The reporters explained that this might be due to changes in liver enzymes levels, gastrointestinal dysfunction, and changes in neurotransmitter level (35,36). Mitochondria are a double membrane-bounded organelle residing in the cell cytoplasm involved in cellular energy metabolism, which may play a central role in survival and apoptosis. The bio-energetic dysfunction of it is an important pathogenic component in PD (37,38). In our study, rotenone depleted mitochondrial NADH dehydrogenase that explains rotenone-induced mitochondrial damage. Moreover, dopamine depletion reduced SOD and GSH levels and simultaneously enhanced lipid peroxidation in the rotenone-treated control rats. These may suggest that oxidative stress has been induced by rotenone (Table 1). In our observation, treatment with methanolic extract *S. wightii* both at 200 and 400 mg/kg reversed the rotenone-induced changes in the body weight, motor coordination,

Table 1. Effect of methanolic extract of *Sargassum wightii* on rotenone-induced changes in brain biochemical parameters

Drug and dose (mg/kg)	Mitochondrial NADH dehydrogenase activity nM/g of protein	Dopamine (µg/g tissue)	MDA (nM/g of tissue)	SOD (U/mg of tissue)	GSH (µg/mg of tissue)
Vehicle-10 mL	14.07±0.39	0.64±0.014	1.46±0.24	25.72±1.26	5.57±0.34
Rotenone-10 mg/kg	3.31±0.20#	0.26±0.014#	4.64±0.31#	11.45±0.93#	1.96±0.19#
Levodopa + Carbidopa-10 mg/kg + Rotenone-10 mg/kg	10.50±0.36***	0.50±0.018***	2.25±0.28***	23.21±1.51***	4.82±0.35***
S. wightii 200 mg/kg + Rotenone-10 mg/kg	5.40±0.35**	0.36±0.018**	3.507±0.50	13.35±1.21	2.49±0.26
S. wightii 400 mg/kg + Rotenone-10 mg/kg	8.55±0.33***	0.41±0.014***	2.50±0.29**	18.34±1.48**	3.76±0.28**

Values are expressed as mean ± SEM, n=6. Statistical analysis was carried out using One-Way ANOVA followed by Tukey's multiple comparison tests. # $P < 0.001$ compared to the vehicle control group. ** $P < 0.01$; *** $P < 0.001$ compared to the rotenone control group.

locomotor activity, and akinesia; however, they were not comparable to levodopa and carbidopa combination. We also observed *S. wightii,* with both doses restored rotenone-induced dopamine and mitochondrial NADH dehydrogenase depletion (Table 1).

Evidence supporting the oxidative stress hypothesis in the pathogenesis of PD suggests that increased lipid peroxidation and reduced antioxidants such as SOD and GSH are associated with neuronal cell death in Substantia nigra (39). In this work, methanol extract of *S. wightii* 400 mg/kg restored the oxidative stress biomarkers like MDA, SOD, and GSH levels, revealing its antioxidant property (Table 1). Thus, we can explain that the neuroprotective effect shown by *S. wightii* is possibly due to restoration of dopamine as well as mitochondrial function. Additionally, the prevention of neurodegeneration from oxidative stress in PD could be correlated to its potent antioxidant property. However, this research is in dearth of providing the evidence correlating the neuroprotective effect of *S. wightii* with other neurochemical markers specific to PD, hence addressing further research. Also, the phytoconstituents responsible for this beneficial effect of *S. wightii* need to be isolated to provide a clue in the direction of the drug development process.

Conclusion

Results of this study conclude that *S. wightii* possesses neuroprotective activity in the animal model of PDs indicating its potential role as a novel therapeutic candidate to manage neurodegeneration and prevent PD progression. However, it needs further evaluation in different experimental and clinical models to establish its efficacy and safety in long-term use.

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Authors' contributions

BR designed and guided the study. SR conceived the research idea and performed the experiment following all experimental guidelines, provided the data and wrote the first draft of the manuscript. AK carried out the literature search, IR and PKP performed statistical analysis and helped in the interpretation of results, SKB supervised the study. All authors reviewed and approve the final manuscript.

Conflicts of interest

Authors declare that there are no conflicts of interest.

Ethical considerations

The experiment was conducted upon receiving approval from the Institutional Animal Ethics Committee (IAEC), Roland Institute of Pharmaceutical Sciences, Berhampur, Odisha, India (Ref No: 926/PO/Re/S/06/ CPCSEA). All the experimental procedures strictly adhered to the Guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) formulated by the Ministry of Fisheries, Animal Husbandry and Dairying, Government of India.

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