Spirulina Ameliorates Oxidative Damage And Inflammation In Rotenone Induced Neurotoxicity In Male Mice

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Abstract:-
Rotenone, a chemical compound produced naturally by leguminous plants, has conventionally been used as a pesticide by blocking the uptake of oxygen by body cells. Our study aimed to investigate the effect of spirulina on oxidative damage, inflammation, and neurotoxicity in male mice treated by rotenone. The experimental animals were being divided into five groups. Group (I) served as control that received dimethyl sulfoxide (DMSO); Group (II) mice treated with rotenone (1.5 mg/kg, s.c. 3 times per week); Group (III) mice received rotenone/L-dopa (25 mg/kg, P.O. daily); Group (IV) and Group (V) mice treated with rotenone/spirulina (200 and 400 mg/kg, P.O. daily) respectively for two weeks. Rotenone-treated mice showed impaired motor coordination and activity in wire hanging, wood walking, open field, and stair tests. Also, rotenone treatment caused elevation in striatal levels of MDA; NO; TNF-α; IL-1β and caspase 3 and decrement in Bcl-2; dopamine and GSH levels. Furthermore, there was severe neuronal degeneration, striatal DNA fragmentation, and an increase in striatal 8-OHdG levels and MTH1 expression in the rotenone group. On the other hand, spirulina treatment
prevented rotenone-induced motor deficits, striatal DNA fragmentation and showed good restoration of the substantial neurons with reservation of the normal dark appearance. Also, rotenone-induced biochemical changes were ameliorated by spirulina treatment as dopamine, Bcl-2, and GSH levels were increased and striatal MDA, TNF-α, IL-1β, and caspase 3 levels were decreased. Therefore, natural products as spirulina could reverse rotenone-induced neurotoxicity in male mice due to their anti-inflammatory and antioxidant properties.

**Keyword**: Spirulina, neurotoxicity, rotenone, oxidative stress, inflammation, Parkinson's disease

**Abbreviations**

MDA; Malondialdehyde; NO: Nitric oxide; TNF-α: Tumor Necrosis Factor; IL-1β: Interleukin-1 beta; GSH: Reduced glutathione; PD: Parkinson's Diseases; DA: Dopamine

1. Introduction

Parkinson's Disease (PD) is the most second progressive neurodegenerative disease after Alzheimer's that is described by bradykinesia, unbending nature, constipation, rapid-eye-movement, resting tremor, the disorder in sleep behavior, muscular rigidity, and postural instability[1,2]. The principal neurotic attribute of PD is the dynamic degeneration of dopaminergic neurons in the substantia nigra standards compacta, the loss of dopamine in the striatum, and the nearness of intracytoplasmic considerations in enduring neurons known as Lewy bodies [3].

Rotenone is an auxiliary metabolite item with bug spray angles that originated from Leguminoeae gathering plants Derris spp. and Lonchocarpus spp[4]. It is lipophilic exacerbates that just crosses organic obstruction because of its hydrophobicity, likewise allowing high ingestion and take-up in the cerebrum. As far back as its first use in the USA, as a fish toxicant has included allure among fish ranchers in numerous nations because of its adequacy and transient continuance in the lake water [5, 6]. It is a neurotoxicant, and paying little heed to be broadly dispersed in the cerebrum, rotenone can cause particular neurodegeneration in explicit locales and strong paralysis action [7, 8].

Spirulina platensis (Nordest.) Geitler or Arthrospira platensis(Nordest). The comment is a multicellular filamentous cyanobacterium of blue-green growth from the Oscillatoraceae family showing high substance of protein, alongside high measures of basic unsaturated fats and amino acids, minerals, nutrients, and other flavonoids that exhibited antioxidant and anticancer activities[9,10]. It is increasing more consideration by therapeutic researchers as a nutraceutical and as a wellspring of potential pharmaceutical.
Several studies have demonstrated spirulina to apply defensive impact in the treatment of neurodegenerative issues (Parkinsonism and Alzheimer issue). Stromberg's study additionally proposes a defensive reaction by an upgrade in the recuperation of striatal dopamine tyrosine hydroxylase (TH) positive strands and TH positive neurons in the substantia nigra standards compacta in trial rodent initiated Parkinsonism after bolstering with spirulina [11]. Spirulina applies a huge defensive impact on hippocampus neural ancestor cells against lipopolysaccharide incited intense fundamental incendiary [12].

The objective of the current study is to evaluate the antioxidant and anti-inflammatory activities of Spirulina against neurotoxicity of rotenone in adult male mice

2. Material and Methods

2.1. Rotenone was obtained from Sigma (St Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO) then given to mice of dose 1.5 mg/kg s.c., three times per week for 2 weeks [13]. Carbidopa-Levodopa ( in a dose 25mg/kg/day and (L-dopa) was given orally for 14 consecutive days [14].

2.2 Preparation of Spirulina Fusiform

2.2.1 Fifty grams of spirulina powder was soaked in 1 L of ultra-pure water and shaken continuously for 24 h at room temperature. The mixture was centrifuged and the supernatant was filtered to remove the cell debris. The sample was freeze-dried and stored at 4 °C for the experiments. The freeze-dried extract was suspended in distilled water and given via oral gavage daily, (200 and 400 mg/kg) for 14 days period of the study[15].

2.2.2 Determination of proximate composition, mineral contents, and amino acids of Spirulina: Moisture, crude fiber, ash, carbohydrate, protein, and fat of Spirulina were determined according to AOAC [16]. Amino acids were determined in the Central Service Unit, National Research Centre, Cairo, Egypt using LC3000 Amino Acid Analyzer. Individual elements (Selenium, Calcium, Phosphorus, Magnesium, and Sodium) were determined using Atomic Absorption Spectrophotometers (Perkin-Elmer 3300) according to the method of AOAC [17].

2.3 Experimental animal

Adult fifty male albino mice weighting (120 ± 10) g were kept in the laboratory under constant conditions of temperature (24 ± 2°C) at minimum one week before and through the experimental work, being maintained on a standard diet and water were ready ad-libitum. The animals were maintained by the guidelines prescribed by the faculty of Science and the study was approved by the Animal Ethics Committee of the National Research center, Egypt. The experimental mice were being divided into five groups (n=8). Group I ( DMSO) served as control; groups II to V were treated with rotenone (1.5 mg/kg,s.c.3 times per week) [13], either
alone or in combination with L-dopa (25 mg/kg, orally) [14] or spirulina (200 and 400 mg/kg, orally) [18], respectively for two weeks. Then the mice were sacrificed after behavioral studies were performed and their brains were dissected. The striatum of each brain was stored at -80 till the estimation of PD-related markers.

2.4 Behavioural tests

2.4.1 Horizontal bar (wire hanging) test:
Mice were hanged by their forelimbs into a steel rod (25 cm long, 0.2 cm in diameter), 0.25 m above the bench to evaluate their motor strength and the time for each mouse could hang suspended from the rod was recorded for three trials with a cut-off time of 180 s[19].

2.4.2 Wood walking test:
Mice were made to walk over a wooden stick (∼1 m in length, 1 cm in width) and the time each mouse spent to reach the end was recorded to assess their motor coordination[20].

2.4.3 Stair test:
To assess skilled reaching, mice were placed at the bottom of a stair (30 cm in length) placed at an angle of 55° above the bench, and the latency to climb the stair was recorded for each mouse[21].

2.5 Biochemical analysis

2.5.1 Dopamine determination assay
DA level was measured by ELISA assay (eBioscience, San Diego, CA) in striatum tissues homogenate according to Sperk [22].

2.5.2 Apoptotic and anti-apoptotic biomarkers assays
ELISA method was used for the quantitative determination of both Caspase3 and Bcl2 assays in tissue homogenate according to the method described by Saunders et al. [23] and Paxinos and Franklin [24] respectively.

2.5.4 Anti-Inflammatory Biomarkers assay
TNF-α and IL-1β levels were determined by ELISA assay using Biosource International and Camarillo, California, USA Kits. (Europe S.A. Belgium and Pierce Biotechnology, Inc.), according to the procedure reported by Brouckaert et al. [25] and Allan et al. [26] respectively.

2.6 Oxidative Stress and antioxidant Biomarkers assays
Both MDA and Nitric oxide levels were evaluated in striatum homogenate as oxidative stress biomarkers in all treated groups according to Ohkawa et al. [27] and Montgomery and Dymock [28] respectively. Reduced glutathione level was measured according to the method described by Beutler et al.[29].

2.7. DNA damage
Serum oxidative DNA damage, 8-hydroxy-2'-deoxyguanosine level, was measured in all prepared sera of treated groups as described by Crawley [30, 24].

2.7 Histopathological examination

Brain specimens of control, Parkinson's model, and different treated groups were fixed in 10% buffered neutral formalin. Formalin-fixed brain specimens were routinely dehydrated by alcohol, cleared in xylol, and finally embedded in paraffin. Paraffin blocks were serially sectioned at 4–5 µm thickness and stained with H&E stain according to Bancroft and Gamble [31].

2.8 Statistical analysis

The data were expressed as mean ± standard error. The statistical significances between means were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. p < 0.05 was considered as statistically significant. Multivariate analysis was done with a post hoc Bonferroni test with SPSS Inc 16.0 software.

3. Results

3.1 Results of behavioural tests

In the open field test, there was no significant change in the grooming of mice in group II that receive rotenone when compared to the control group. On the other hand, there was a significant decrease (P≤0.001) in raring and ambulation in the rotenone treated group compared to the control group with 62.56% and 65.3% respectively. The latency period showed a significant increase (P≤0.001) in the rotenone group when compared to the control. There was a significant amelioration of raring, ambulation, and latency period in groups III-V that were treated with L-dopa or spirulina in combination with rotenone when compared rotenone group (Figure 1).

Our study reported that rotenone-treated mice displayed a shorter hanging time when suspended from a steel rod by 68.95% than the control group. Treatment with spirulina (200 and 400 mg/kg) prolonged the hanging ability by 446% and 466.5% respectively versus the rotenone group. The time taken by mice treated with rotenone to traverse a wooden stick was significantly increased by 38% than the time taken by control mice, while treatment with L-dopa and spirulina in combination with rotenone almost reversed the rotenone effect in the wood walking test. Mice that receive rotenone (group II) exhibited an increment in time taken to ascend the stairs by 155% when compared with control on other hand treatment with L-dopa and spirulina (groups III-V) reversed the effect of rotenone (Figure 2).

3.2 Biochemical results

3.2.1. Dopamine content
In the present study, dopamine level was significantly decreased (P≤0.001) in groups II and IV by percent 59.9% & 16.6% respectively when compared with dopamine level in group I while there was a significant improvement in dopamine levels in groups III – V comparing with group II (Figure 3).

3.2.2 Apoptotic and anti-apoptotic biomarkers
Treatment with L-dopa and spirulina (groups III-V) attenuated striatal apoptosis induced by rotenone in group II, as shown in figure 4. There was a significant increase (P≤0.001) in the caspase-3 level in the rotenone group and a significant decrease in Bcl2 level in the same group with percent 476.9% and 52.8% respectively as compared with those of the control (group I).

3.2.3 Anti-Inflammatory biomarkers
From the current study, both TNF-α and IL-1β levels were significantly increased (P≤0.001) in rotenone treated group with percent 532.7% and 321.8% respectively when compared with the control group. Treatment with L-dopa and spirulina (200 & 400 mg /kg) reduced the elevated levels of these cytokines but could not restore the normal level of the control (Figure 5).

3.2.4 Oxidative Stress and antioxidant biomarkers
Our data showed no significant change (P=0.421) in NO level in all treated groups when compared to control; while there was a significant increase (P≤0.001) in MDA level in group II with percent 44.5% comparing to control and a significant improvement of its level in groups III-V when compared with group II. There was a significant decrease (P≤0.001) in the level of reduced glutathione by 38.5% in group II that received rotenone when compared to control with an amendment in its level in group III-V comparing to group II (Figure 6).

3.3 DNA damage
There was a striatal DNA fragmentation and increase in striatal 8-OHdG levels and MTH1 expression in rotenone treated group. Similarly, oxidative DNA damage in the form of oxidized guanine is retained in the mitochondrial and nuclear DNA of dopamine-producing neurons of the SN in PD (Figure 7).

3.4 Chemical composition of the Spirulina powder:
The current study reported that Spirulina powder contains a high quantity of protein (60.73%), fat (5.92%), and carbohydrate (15.81%) which make it supplying high calories (359.44 Kcal/100 g). Results of minerals content showed that the Spirulina powder contained high concentrations of Calcium (153.80 mg/100 g), Phosphorus (101.10mg/100 g), and Sodium (762.70mg/100 g). Results of amino acids profile showed that the Spirulina powder contained
high amounts of Tyrosine (89.67 mg/g protein), Methionine (71.81 mg/g protein), Leucine (66.72 mg/g protein), and Isoleucine (60.24 mg/g protein) (Table 1).

3.5 Histopathological examination results

Microscopic examination of different sections of the substantia nigra of control mice showed normal darkly stained substantial neurons (Figure 8A). The striatum of those control mice showed a normal histological appearance of striatal neurons (Figure 9a). While examination of brain sections of rotenone administrated mice revealed marked histological alterations in both the substantia nigra as well as the striatal areas. Regarding the substantia nigra of rotenone administrated mice, the examination of which showed marked neuronal degeneration with an obvious loss of the melanin dark pigmentation of those neurons and necrosis of the other neurons, some of them appeared ghost-like without any nuclear structures (Figure 8B). Most mice showed a marked loss of the substantial neurons with the appearance of remnants of necrotic neurons with prominent vacuolar degeneration of other neurons (Figure 8C). While the striata of those mice showed widespread neuronal degeneration and shrinkage with marked pericellular and perivascular edema as well as necrosis and apoptosis (Figure 9b).

Concerning the treated groups, the administration of L-Dopa could restore the observed alterations, on the other hand, the administration of the drug showed a dose-related restoration of the observed histological alterations. In regards to the substantia nigra of rotenone administrated mice and treated with L-Dopa showed restoration of most of the substantial neurons with mild vacuolar degeneration and scattered necrosis of some neurons. Mild loss of the melanin pigment from some neurons was also noticed (Figure 8D). The striatum of rotenone administrated mice and treated with L-Dopa showed mild perineuronal edema with mild degeneration of some neurons and scattered neuronophagia (Figure 9c).

The substantia nigra of rotenone administrated mice and treated with 200mg of the drug showed moderate restoration of the substantial neurons with the restoration of their content of melanin pigment and few scattered necrotic cells (Figure 8E). The striata of those mice showed a moderate degree of degenerative changes of the striatal neurons, some of which appeared shrunken with scattered necrotic neurons and few neuronophagia (Figure 9d).

The substantia nigra of rotenone administrated mice and treated with 400mg of the drug showed good restoration of the substantial neurons with reservation of the normal dark appearance and only mild vacuolation of a few of them (Figure 8F). The striatal areas of rotenone administrated mice and treated with 400mg of the drug showed a moderate degree of restoration of the striatal neurons, only a few appearing shrunken with perineuronal edema and few apoptotic cells (Figure 9e).
4. Discussion

Rotenone administration has been appeared to emulate key parts of parkinsonism issue (PD) pathology, for example, dopaminergic neurodegeneration in the substantia nigra (SN). These outcomes in the exhaustion of associated neurotransmitters ultimately result in motor dysfunction [32, 33], oxidative pressure, neuroinflammation, and α - synuclein conglomeration[34].

It is pivotal to promote the antioxidants and neuroprotective activities of naturally occurring spirulina algae to improve their efficacy in experimental studies to elevate their importance to humans [35]. From the present study, it is clear that spirulina powder showed high contents of protein, fat, and carbohydrate which make it supplying high calories and high amounts of Calcium, Phosphorus, and Sodium. Also, spirulina contained some essential amino acids such as Tyrosine, Methionine, Leucine, and Isoleucine the results of the chemical composition of spirulina are in agreement with those obtained by Dolly [36] and Morsy et al. [37]

The current study reported exacerbated the mind harm brought about by rotenone in dopaminergic neurons in the striatum. Rotenone causes neuronal cell death through expanded oxidative pressure a significant pathogenetic system adding to DA-ergic neurodegeneration in human PD. Researchers accordingly demonstrated the expanded arrangement of receptive oxygen metabolite, increased lipid peroxidation items, and protein carbonyls. Social tests showed, particularly decline in dopamine consumption and diminished engine coordination with augmented bradykinesia. Our results were in agreement with data reported by Alzahranii et al. [38] and Ebenezer et al. [39] that exhibited by the hindered locomotor capacity surveyed in two well-recorded social tests, the shaft test, and open field tests.

Our results showed a significant decreased in dopamine level in rotenone treated group, with amelioration its level with L-dopa and spirulina high dose, this similar to the findings of Gu¨naydın et al.[40] which reported the biochemical examination affirmed that rotenone-treated mice reduced one-quarter of the striatal dopamine level in the vehicle-treated mice, compare with rotenone treatment with Tribulus Terrestris extract showed a significantly improved dopamine level.

From our data, there was a significant elevation in both TNF-α and IL-1β levels in the group treated with rotenone comparing with control these findings were in agreement with results obtained by Sharma et al. [41] and Wang et al. [42]. Supplementation of mice with a low and high dose of spirulina significantly reduced (p≤ 0.001) their levels in group IV and V comparing with group II. Data from the current study showed that there were a significant increase (p≤ 0.001) in MDA and NO levels with a significant decrease in GSH level in group
II that received rotenone comparing with group I, similar results were observed by Abdel-Salam et al. [43] and Zhang et al. [44]. This happened alongside stamped oxidative pressure shown by the expansion in the lipid peroxidation final result MDA and by the decline in the cell reinforcement atom GSH in tissues. Retonone infusion additionally brought about expanded articulation of the proinflammatory cytokine TNF-α and the apoptotic factor caspase-3 in tissues. While treatment mouse with spirulina improve the levels of MDA; NO and GSH these findings were in agreement with results obtained by Cho et al. [45].

Results from our study reported a significant increase (p≤ 0.001) in caspase-3 level due to nigrostriatal damage, loss of substantia nigra TH-ir, neuronal damage, focal gliosis in the cerebral cortex with a significant decrease in Bcl-2 level in group II that injected with rotenone when compared to control these similar with the findings reported by Liu et al. [46] and Jiang et al. [47], with refinement in their levels in groups treated with spirulina. Microscopic examination of brain sections of rotenone-treated mice revealed marked histological alterations in both the substantia nigra as well as the striatal areas. Regarding the substantia nigra of rotenone administrated mice, the examination of which showed marked neuronal degeneration with an obvious loss of the melanin dark pigmentation of those neurons and necrosis of the other neurons these similar findings reported by Ngala et al. [48]. Also, other research recorded control sections showed decreased staining of the substantial neurons with vacuolar degeneration, necrosis, shrinkage, and ghost appearance of most of them. [34]. Sections of the standard treatment group showed good restoration of the substantial neurons, with most of them having near to normal appearance, with only few necrotic cells with scars neuronophagia. On the other hand there were a significant improvement in both the substantia nigra as well as the striatal areas in brains of rotenone treated mice by Spirulina supplementation in groups IV and V these results with agreeing to results obtained by Wang et al. [49] and Ange et al. [50].

In the present research, rotenone-induced a significant elevation in MDA, NO, TNF-α, IL-β and a significant decrease in dopamine, Bel-2, GSH levels compared to control with remarked change in brain tissue, these results agreed with data reported by other studies [40-43] with improved in their levels after treatment with spirulina[42-45].

In conclusion, the current study provided oxidative damage in nucleic acid is a key risk factor for experimental PD in mice induced by rotenone. The histopathological changes in brain tissues, as well as the biochemical parameters in rotenone-treated mice, were improved after
spirulina administration. This evidence a protective effect of spirulina against neurotoxicity of rotenone connects to the antioxidant and anti-inflammatory effects of spirulina.

5. References:-


Figure 1: Open field test (grooming A, rearing B, ambulation C and latency period D) in all treated groups
* Significantly difference from control group      @ Significantly difference from rotenone group

**Figure 2:** Wire hanging, wood walking and stair test results in all treated groups

* Significantly difference from control group      @ Significantly difference from rotenone group

**Figure 3:** Dopamine level in all treated groups

* Significantly difference from control group      @ Significantly difference from rotenone group

**Figure 4:** Both of caspase-3 and Bcl2 levels in all treated groups

* Significantly difference from control      @ Significantly difference from Rotenone
**Figure 5:** Anti-Inflammatory biomarkers levels in all treated groups

* Significantly difference from control group  @ Significantly difference from rotenone group

**Figure 6:** MDA, NO and GSH levels in all treated groups

* Significantly difference from control  @ Significantly difference from Rote

**Figure 7:** An agarose gel electrophoresis show DNA fragmentation

M: DNA marker with 100 bp  
Lane 1, 3, 4, & 5: DNA without streaks or laddering of groups I, III-V  
Lane 2: DNA with marked streaks and laddering (fragmented) of group II

**Table (1):** proximate composition, mineral contents and amino acids of Spirulina

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<th>Chemical composition (g/100g)</th>
<th>Amino acids (mg/g)</th>
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<td>Value</td>
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<tr>
<td>Moisture</td>
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Figure 8:

A) A photomicrograph of the Substantia nigra of control mice showing normal darkly stained substantial neurons. B) Substantia nigra of rotenone administrated mouse showing neuronal degeneration with loss of the melanin dark pigmentation and necrosis of the other neurons, some appearing ghost like. C) Substantia nigra of rotenone administrated mouse showing loss of the substantial neurons, remnants of necrotic neurons and vacuolar degeneration of the remaining. D) Substantia nigra of rotenone administrated mouse and treated with L-Dopa showing restoration of most of the substantial neurons with mild vacuolar degeneration and scattered necrosis of some neurons, notice loss of the melanin pigment from some of the neurons. E) Substantia nigra of rotenone administrated mouse and treated with 200mg of spirulina showing moderate restoration of the substantial neurons with restoration of the content of melanin pigment and few necrotic cells. F) Substantia nigra of rotenone administrated mouse and treated with 400mg of s Spirulina showing good restoration of the substantial neurons with reservation of the normal dark appearance and only mild vacuolation of few of them (H&E, X400).
Figure 9:

a) A photomicrograph of Striatum of control mouse showing normal histological appearance of striatal neurons. b) Striatum of rotenone administrated mouse showing widespread neuronal degeneration, shrinkage with pericellular and perivascular edema as well as necrosis and apoptosis. c) Striatum of rotenone administrated mouse and treated with L-Dopa showing mild perineuronal edema, mild degeneration of some neurons and scattered neuronophagia. (H&E, X400).  
d) Striatum of rotenone administrated mouse and treated with 200mg of spirulina showing moderate degree of degenerative changes of the striatal neurons, scattered necrosis and few neuronophagia. e) Striatum of rotenone administrated mouse and treated with 400mg of Spirulina showing moderate degree of restoration of the striatal neurons, only few appearing shrunken with perineuronal edema and few apoptotic cells (H&E, X200).