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Comparative neuroprotective effects of Moringa oleifera, Mucuna pruriens, and milk thistle extracts in a rotenone-induced Parkinson's disease model: Behavioral, biochemical, and histopathological insights

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Abstract: Parkinson's disease (PD), a complex neurodegenerative disorder, affects over six million individuals worldwide. It leads to motor and non-motor impairments. Despite persistent efforts to develop a cure for PD, researchers have not identified an effective treatment to halt disease progression or reverse neurodegeneration. This study investigates the neuroprotective efficacy of Moringa oleifera (More), milk thistle (MT), and Mucuna pruriens (Muc) aqueous extracts, both separately and in combination, in a rotenone-induced PD mouse model. PD was induced in male Balb/c mice with daily abdominal injections of rotenone (2.5 mg/kg) for 21 days. Mice received individual treatments of each plant extract (350 mg/kg) or combinations with and after PD induction. Body weight, neurological severity score (NSS), and behavioral studies were assessed to evaluate PD induction and treatment efficacy. Histological analysis and levels of superoxide dismutase (SOD), catalase (CAT), and malondialdehyde (MDA) were measured. Among the three extracts, Mor extract was more effective, even more so than levodopa, in protection and restoration. These plant extracts could be a safe and effective alternative to conventional PD treatments.

Keywords: *Behavioral assessment, Mor, Muc, MT, Oxidative stress, PD.*

1. Introduction

Parkinson's disease (PD) is considered the second most prevalent neurodegenerative ailment, after Alzheimer's disease, impacting over six million people globally [1]. The disease arises from the degeneration of dopaminergic neurons, which occurs in the substantia nigra pars compacta. Many cellular dysfunctions, including oxidative stress, apoptosis, mitochondrial dysfunction, and α-synuclein aggregation, contribute to the etiology of PD [2]. Accumulating Lewy bodies in various brain regions leads to continuous motor dysfunctions, such as muscle rigidity, tremors, and postural instability [3]. Additional symptoms encompass a range of non-motor manifestations, including sleep disturbances, cognitive deterioration, weight reduction, autonomic dysfunction, and depression [4]. The degeneration of 50%–70% of dopamine-releasing neurons is the main cause for observed symptoms, which serve as a diagnostic tool in the advanced stage of the disease [5].

Neurodegeneration often lacks a recognized remedy because of the gradual decline of neuronal function and subsequent neuronal death [6]. PD is a complex illness; almost all treatments may mitigate its symptoms, and there is considerable overlap across neurological disorders [7]. To enhance individuals' quality of life, many symptomatic treatments have been developed to ease perceptual, cognitive, sensory, and motor problems. Using herbal medications with various biological effects on the brain is essential in treating PD and slowing its advancement, as there is no confirmed treatment for PD [8, 9].

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Moringa oleifera (Mor), referred to as the "miracle tree," is a medicinal plant used to treat different maladies, including diabetes, ulcers, and cancer. There have been various attempts to use this plant to treat or protect against neurodegenerative diseases like PD, stroke, and neurotoxicity. This is because it can reduce oxidative stress and inflammatory markers in the brain, which improves motor functions [10]. Another Ayurvedic traditional medical plant that has anti-parkinsonian properties is the *Mucuna pruriens* (Muc). The seeds of this plant contain L-dopa, the precursor of dopamine [11]. L-dopa is the primary medicinal therapy for PDs, with side effects arising from long-term use. Dopamine is essential for motor tasks such as tremors and stiffness and neuropsychiatric symptoms (such as depression, psychosis, and cognitive decline) [12, 13]. *Silybum marianum*, often known as milk thistle (MT), is a therapeutic herb recognized for its efficacy in addressing numerous diseases. The main active ingredients of this plant, silymarin, have antioxidant, antiviral, and anti-inflammatory activities [14]. It treats gallbladder disorders, enhances breast milk production, prevents cancer, and safeguards the liver against alcohol-related diseases and environmental poisons. Studies suggest that MT can protect against age-related cognitive decline, reflecting the ability to protect against neurological diseases [15, 16] such as PD [17] and Alzheimer's disease [18].

This research examines the therapeutic effects of Mor, Muc, and MT extracts in a PD-induced mouse model. Histological examination, behavior assessment, and antioxidant evaluations were conducted. Research results can contribute to innovative medical approaches for PD.

2. Materials and Methods

2.1. Preparation of Aqueous Plant Extracts

Muc (krounchbeej) seeds powder was purchased from Royal Herbal Land Pvt. Ltd. (Maharashtra, India) (Pune-410401.2023). Mor (the young shoot and total aerial part were taken) and MT (seeds only) were provided and authenticated by Dr. Mohammad Tarabulsi (Ph.D. in Herbology and Homeopathy) who cultivates them under proper conditions in Nabatieh-Deir El Zahrani, Lebanon, with voucher code NABTIEH-OCT.2023-NABTIEH-MAY.2023, respectively. He identifies the plants visually and via phytochemical screening that determines the bioactive compounds.

The plants were collected, washed, and dried in a well-ventilated, clean dark environment. This phase was followed by grinding and pulverizing. Plant powders were stored in a cool, dry, dark place, maintaining appropriate humidity levels for a duration not exceeding 6 months. A 5% aqueous solution of each plant or seed was prepared by boiling 5 grams in 100 ml of distilled water. The temperature was held at 100 °C for 30 min, accompanied by continuous pH monitoring to ensure consistency across batches. After thermal treatment, the solutions underwent centrifugation at 6000 rpm for 10 min. The supernatants were filtered sequentially through sterile gauze and then through Whatman filter paper. The final filtrates were allocated into sterile containers and stored at −20 °C for future use. The solutions were prepared every week.

2.2. PD Induction and Experimental Protocol

Adult male BALB/c mice (20-30 g), approximately 6 to 10 weeks of age, were provided by the Beirut Arab University animal facility in Debbieh, Lebanon. Male mice were chosen because the incidence of PD has more predilection in males than females. The experimental protocol was approved by the Institutional Review Board (IRB) of Beirut Arab University, with the code number (IRB number: 2023-A-0052-S-M-0553), and it followed the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines as well as the Canadian Council on Animal Care (CCAC) guidelines for the usage and care of experimental animals. The mice were randomly assigned to different experimental groups and housed in appropriate cages under calibrated laboratory conditions of light (12 h light/dark cycle),

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proper room temperature, and humidity, with ad libitum feeding following a standard mouse diet and tap water. The mice were left to acclimate for one week before beginning the experiment.

A modified version of the Rocha, et al. [19] protocol, incorporating an extended study duration of 21 days, was employed to elicit more evident Parkinsonian symptoms akin to those observed in PD patients. The mice were intraperitoneally injected with the commonly used drug to induce PD, rotenone, at 2.5 mg/kg daily for 21 days successively. Rotenone was daily prepared in DMSO at a final concentration of 10 mg/ml and then diluted in sesame oil before use. Rotenone stock was sterile-filtered (0.22 μm) before dilution in sesame oil under aseptic conditions. Levodopa, the gold standard treatment for PD was taken as positive control. Mice received it at the 20 mg/kg dose, administered by gavage, daily for 21 days.

A total of 13 groups of 9 mice each were used according to Table 1. The sample size was determined by power analysis (effect size = 0.5, $\alpha = 0.05$, power = 80%) using G*Power software. The experimental treatment protocol was divided in two phases:

1. **Co**-treatment phase: For the first 21 days, mice were simultaneously administered both rotenone (2.5 mg/kg daily) and the plant extract (350 mg/kg body weight (b.w.) daily by gavage), as detailed in Table 1.
2. Post-treatment phase: Beginning on day 22, mice received only the plant extract (without rotenone) at the same dose of 350 mg/kg b.w., administered by gavage, daily for an additional 21 days.

The body weight of the mice was monitored properly every day before receiving the exact amount of treatment. At the end of the experimental study, all mice were anesthetized by intraperitoneal injection of a mixture of ketamine (50 mg/kg) and xylazine (10 mg/kg), followed by cervical dislocation. Animals were dissected 24 hours after the last treatment. After dissection, the brains were perfused with phosphate-buffered saline and then immediately collected. They were either fixed in a pre-prepared solution of 10% formaldehyde in phosphate buffer (pH = 7.4) or frozen in liquid nitrogen and stored at -80°C for later use. The study excluded any mouse exhibiting deformation or inflammatory signs that could have influenced the results. To reduce confounding factors, all procedures were done by the same researcher, and mice were returned to the same cages.

Table 1 summarizes the groups under study with the treatments provided in every experimental group. The control group consisted of healthy mice receiving no treatment, the vehicle group received sesame oil, and the positive control group was treated with Levodopa. Three additional groups—Mor, Muc, and MT—included healthy mice that received the plant extracts separately. The PD group received rotenone injections over 21 days, once daily, to induce Parkinsonian pathology. The experimental design also categorized treatment methods into co-treatment (extracts provided concurrently with rotenone) and post-treatment (extracts delivered subsequent to PD induction). This included co- and post-treatment groups for Mor, Muc, and MT extracts, establishing a systematic framework to assess both preventative and restorative benefits.

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Table 1.
Experimental groups and treatment descriptions.

Group	Description
Control	Healthy mice receiving no treatment served as the negative control group
Vehicle	Healthy mice were administered sesame oil (vehicle used to dissolve rotenone) via injection
Mor	Healthy mice were administered Mor extract orally for 21 consecutive days.
Muc	Healthy mice were administered Muc extract orally for 21 consecutive days.
MT	Healthy mice were administered MT extract orally for 21 consecutive days
PD	Mice were injected with rotenone for 21 consecutive days to induce a PD model
Positive Control	Mice were treated with Levodopa for 21 consecutive days
Co: PD+Mor	Rotenone-induced PD mice co-treated with Mor extract for 21 days.
Co: PD+Muc	Rotenone-induced PD mice co-treated with Muc extract for 21 days.
Co: PD+MT	Rotenone-induced PD mice were co-treated with MT extract for 21 days.
Post: PD+Mor	PD mice were treated with Mor extract for 21 days.
Post:PD+Muc	PD mice were treated with Muc extract for 21 days.
Post: PD+MT	PD mice were treated with MT extract for 21 days.

2.3. Behavioral Study

Blinded behavioral studies were conducted. The researcher administering the tests was oblivious to the group affiliation of the animal or the therapy it had received. Baseline assessments were conducted at time zero to confirm the equivalence of all mice at the start of the experiment. Behavioral assessments were conducted at 21 days (end of the cotreatment phase) and day 42 (end of the post treatment phase) to evaluate changes in each mouse's behavior, which serves as a significant indicator of both the induction of PD and the effects of treatment. Mice were allowed to acclimate to the testing environment before beginning testing. The duration and frequency of testing were minimized to reduce stress. Measures such as pre-warming before anaesthesia and systematic welfare assessments were used to reduce suffering. Following this, mice were euthanized by cervical dislocation to perform the histological and biochemical analysis.

2.3.1. Neurological Severity Score (NSS)

To investigate the neurological severity changes at sensory (proprioceptive, visual, and tactile), motor (abnormal movements and muscular status), reflex, and balance levels, a 10-measure NSS protocol was followed as provided by Zhuang, et al. [20]. The different tasks measured and their scores are summarized in Table 2. Baseline assessments were conducted at time zero to confirm the equivalence of all mice at the start of the experiment. Subsequent evaluations were performed on day 21 and day 42, and the change in neurological scores (ΔNSS) was calculated to quantify differences over time.

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Table 2.
Scores of Tasks Measured in NSS.

Task	NSS
Failure to exit a 30-cm-diameter circle 1 for 2 min	1
Presence of monoparesis or hemiparesis	1
Failure of flexion of hindlimb after raising mouse by the tail	1
Failure of flexion of forelimb after raising mouse by the tail	1
Failure of head moved >10° to vertical axis within 30 seconds after raising rodent by the tail	1
Inability to walk on a 3-cm-wide beam	1
Inability to walk on a 2-cm-wide beam	1
Inability to walk on a 1-cm-wide beam	1
Failure to Balance with steady posture on 0.5cm beam balance	1
Hugs beam and 1 limb fall from the beam (0.5cm wide)	1
Hugs beam and 2 limbs fall from the beam or spin on beam (60 seconds)	1
Attempts to balance on the beam, but falls off (> 40 seconds)	1
Attempts to balance on the beam, but falls off (> 20 seconds)	1
Falls off; no attempt to balance or hang on to beam (< 20 seconds)	1
Absence of pinna reflex	1
Absence of corneal reflex	1
Absence of startle reflex	1
Failure to have a straight walk	1
Total	18

NSS includes a structured battery of 18 neurological tasks with each one targeting a specific aspect of motor, reflex, or postural function. The failure to perform any task results in a score of 1 point, contributing to a cumulative maximum score of 18. The higher the score denotes greater neurological impairment.

2.3.2. Pole Climbs and Rotarod Tests

Motor dysfunction was evaluated by pole climb and rotarod tests as described [21]. For both tests, mice were trained before the experiment. For the pole climb test, the researcher recorded the time the mouse needed to cross half the pole length and the total length. The mice were positioned on a 70-cm vertical pole that was 1 cm in diameter. To encourage mice to drop to the cage floor, the pole was positioned in the home cage and fixed on a rectangular base stand. The experiment was repeated if the animal hesitated while descending. The pole's surface was taped to prevent it from slipping. Each animal underwent the test three times. The rotarod test was set with a starting speed of 4 rpm and hastened to reach 40 rpm gradually for 300 sec. The maximum time the mouse remained on the rod, which is proportional to the latency to fall, was recorded. Each animal underwent the test three times.

2.3.3. Forced Swim Test

A transparent tank (50 cm in length; 30 cm in width and 50 cm in-depth) filled with water was utilized to study the mice's mobility and escape-related movements within 6 min of continuously forced swimming [22]. Each animal underwent the test three times

2.3.4. Adhesive Test

A modified adhesive test was performed as described by Bouet, et al. [23]. A tape was placed on the nose of the mouse, and then the latency time of tape removal by one of the forepaws was recorded. Each trial was concluded after 1 min if the tape remained unremoved. Each animal underwent the test three times.

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2.3.5. Beam Walk Test

In this test, the mouse must walk across a narrow beam toward a safe platform, stay upright, and walk across the elevated narrow beam of 160 cm length till reaching the cage [24]. This test requires training followed by a measurement of the time each mouse takes to traverse the beam and the number of paws slips that occur during the process. Each animal underwent the test three times.

2.4. Histological Analysis

In post-behavioral analysis, mice were anesthetized and sacrificed. The organs were fixed in 10% formaldehyde. Tissue block sections of 6 µm thickness were prepared and stained by Hematoxylin and Eosin (H&E) staining. Three sections per mouse were stained and analyzed. The sections were examined under a Zeiss Primo light microscope connected to an AxioCam camera at 40x magnification.

2.5. Determination of Antioxidant Activities and Levels

2.5.1. Catalase Activity

Catalase (CAT) activity was determined according to Aebi [25] with slight modifications. Tissues were homogenized with lysis buffer (10 mM PBS containing 1 mM PMSF) at 1:9 ration w/v. The tissue homogenate (50 µl) was mixed with 1 ml reaction solution (H_2O_2+PBS) and the dynamic absorbance was read immediately at 240 nm every 15 sec for 1 min. The reference used in this experiment is the reaction solution with PBS.

2.5.2. Superoxide Dismutase Activity

Superoxide Dismutase (SOD) activity was also determined using the Bio-protocol according to Buege and Aust [26]. The tissue homogenate (50 µl) was added to 1 ml reaction mixture (100 mM PBS, 1 mM EDTA-Na_2, 130 mM Methionine, 750 µM Nitroblue tetrazolium (NBT), and 20 µM Riboflavin) and placed under light (4000 lux) for 10-15 min. Two controls are used for this assay; one in the dark and the other in light. The absorbance was then measured at 560 nm in the dark.

2.5.3. Malondialdehyde Levels

Malondialdehyde (MDA) levels were determined using the thiobarbituric acid (TBA) reactive substance assay (TBARS) [27]. The tissue homogenates (100 µl) were mixed with TBA (1ml, 0.25%) solution and placed for 15 min in a boiling water bath for chromophore development. Samples were left to cool down for 5 min in ice, and then the absorbance was measured at 532 nm and 600 nm. TBA solution with 100 µl 100 mM PBS served as a reference. MDA levels were normalized to total protein content as determined by Bradford [28] and expressed as nmol MDA per mg protein.

2.5.4. Statistical Analysis

Statistical analysis was performed using the Graph Pad Prism 10.3.0. Analysis of the mice's weight, the antioxidant tests, and all behavioral tests were analyzed by one-way repeated measure ANOVA followed by Tukey's post hoc test. All results were expressed as the mean value ± SD. A $p \leq 0.05$ was considered statistically significant.

3. Results

3.1. Plant Treatments Compensate for Body Weight Loss Due to PD

Changes in body weight of all mice, whether control, co-treated or post-treated with the extract, were systematically monitored and recorded throughout the experiment duration (Figure 1). Mice in the control group exhibited a mean body weight gain ranging between 6 to 10 g (± 1.44g), while the vehicle group demonstrated a comparable increase of 8 to 11 g (± 1.14g). In contrast the PD group

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displayed a significant reduction in body weight, with a greater than 37.5-fold decrease relative to the control group ($p< 0.0001$).

Co-treatment with Mor, Muc, MT, or their combination significantly reversed the weight loss, with 42.5-fold ($p< 0.001$), 35-fold ($p< 0.0001$), 30- fold ($p< 0.05$) and 47.5- fold ($p< 0.001$) respectively, compared to PD mice. Similarly, post-treatment with these extracts also resulted in significant weight improvements of 40-fold ($p< 0.001$) for Mor, 34-fold ($p< 0.0001$) for Muc, 30-fold ($p< 0.0001$) for MT, and 45-fold ($p< 0.0001$) for the combination treatments. Levodopa, the commonly used medication in PD treatment, yielded a modest increase in body weight of only 17.5-fold ($p< 0.05$) relative to PD group.

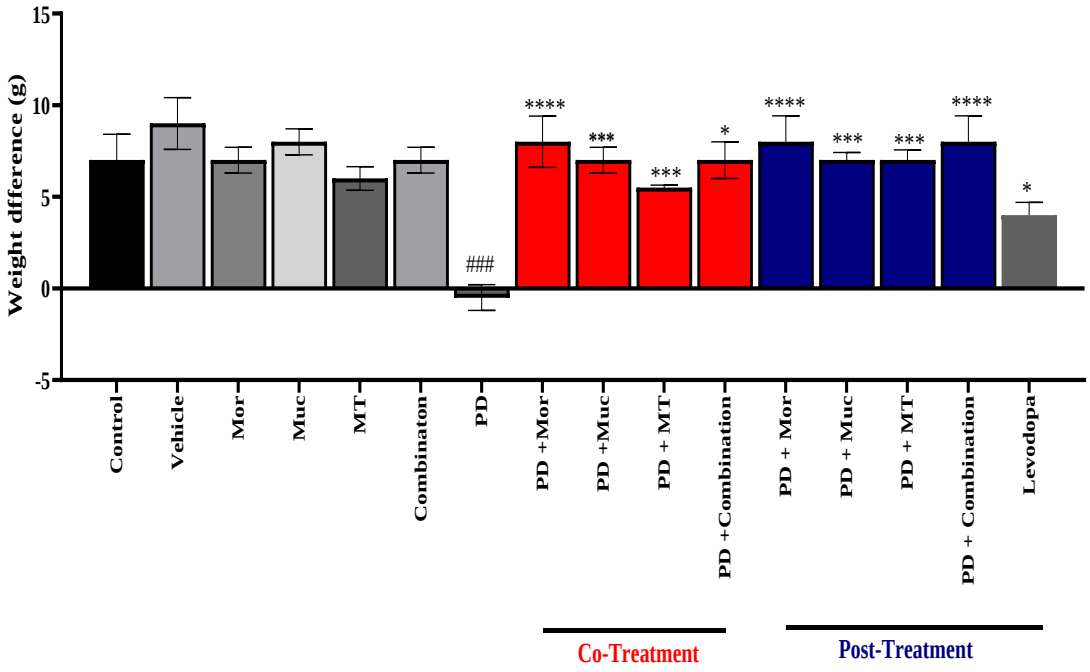

Figure 1.
The changes in body weight in all experimental groups. Weight changes for mice co-treated and post-treated with the plant extracts along PD induction.
Note: Asterisks (*) indicate significance vs. the PD group; hash mark (#) indicate significance vs. the Control group. Bars are colored red for co-treatment phase and blue for post-treatment. Data are means of 9 mice per each group ± SD. (*), (***) and (****) represent $p< 0.05$, $p< 0.001$ and $p< 0.0001$ respectively with respect to PD group.

3.2. Treatments With the Extracts to Restore Normal Brain Morphology

Histological examination of the brains using H&E staining is shown in Figure 2. The control, Veh, Mor, Muc, MT, and Com groups (Figure 2 A-F respectively) demonstrated normal neuronal architecture, characterized by intact cells and an absence of degeneration signs showing normal color of the neural cells in the brain substantia nigra.

The PD group (Figure 2 G) exhibited significant neuronal degeneration, extensive vacuolation, and marked gliosis.

Co-treatment with Mor (Figure 2 H) significantly enhanced brain architecture, decreased vacuolation, and demonstrated substantial neuroprotection while Post-treatment with Mor (Figure 2 I) indicated signs of recovery, accompanied by mild residual alterations.

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Co-treatment (Figure 2 J) or post-treatment (Figure 2 K) with Muc restored normal brain architecture revealing neuroprotective plant potential.

Both co-treatment (Figure 2 L) and post-treatment (Figure 2 M) with MT demonstrated sustained degeneration and limited recovery.

The combined group (Figure 2 N) exhibited enhanced neuronal density and decreased vacuolation, indicating a synergistic neuroprotective effect. The post-combined treatment (Figure 2 O) significantly restored near-normal structure with minimal edema, indicating the most effective recovery among all groups.

Post-levodopa-treated animals (Figure 2 P) exhibited moderate recovery, accompanied by residual vacuolation, indicating partial neuroprotection.

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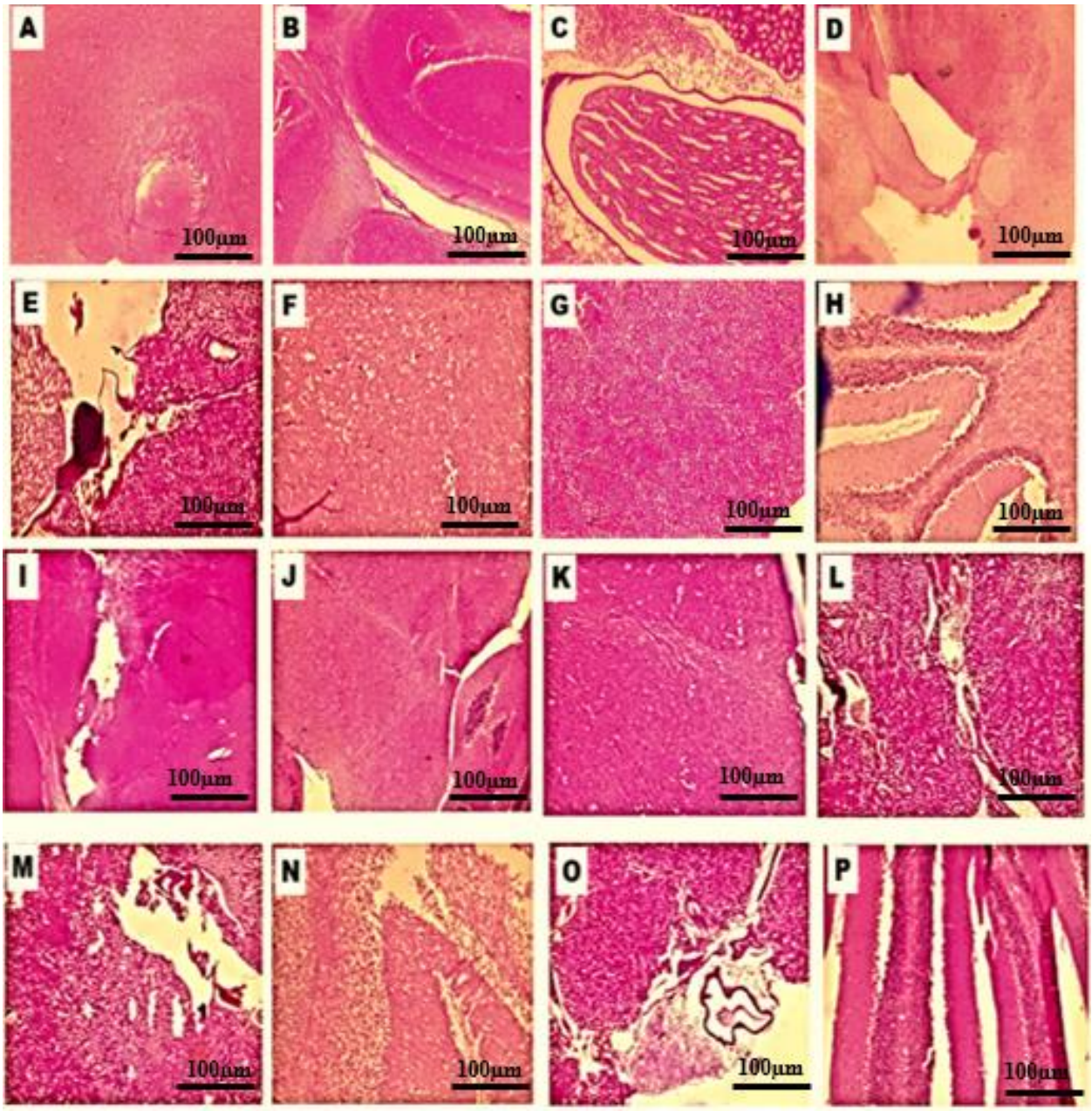

Figure 2.
Representative H&E-stained brain sections (40× magnification) for all groups.

(A) control group, (B) vehicle group, (C) Mor treated group, (D) Muc treated group, (E) MT treated group, (F) combination-treated group, (G) PD group, (H) cotreated-Mor group, (J) post-treated Mor group, (J) co-treated Muc group, (K) post-treated Muc group, (L) co-treated MT group, (M) post-treated MT group, (N) combined group, (O) post-combined group, (P) post-levodopa treatment.

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3.3. Treatment With the Extracts Enhanced PD Behavior

3.3.1. Evaluation of Neurological Severity Score (NSS)

Neurological function was assessed by the NSS score for all experimental groups (Figure 3). Control, vehicle, Mor, Muc, MT, and combination groups maintained a score of zero in different experimental time points (at the beginning of the experiment, after 14 days and at the end of the experiment) indicating normal neurological behaviour. However, the PD group exhibited a significantly elevated NSS score of 2.67± 0.58 ($p< 0.0001$).

Following co-treatment with each plant extract or their combination, the mice exhibited a complete reversal of NSS where this score declined back to 0, in comparison to control groups.

Similarly, post-treating PD mice with these extracts caused a significant decline (NSS=0) in post-Mor treatment, post-Muc treatment, post-MT treatment, and post-combination treatment, while treating with levodopa yielded an NSS of 1 (0.63-fold reduction), indicating partial improvement in neurological function.

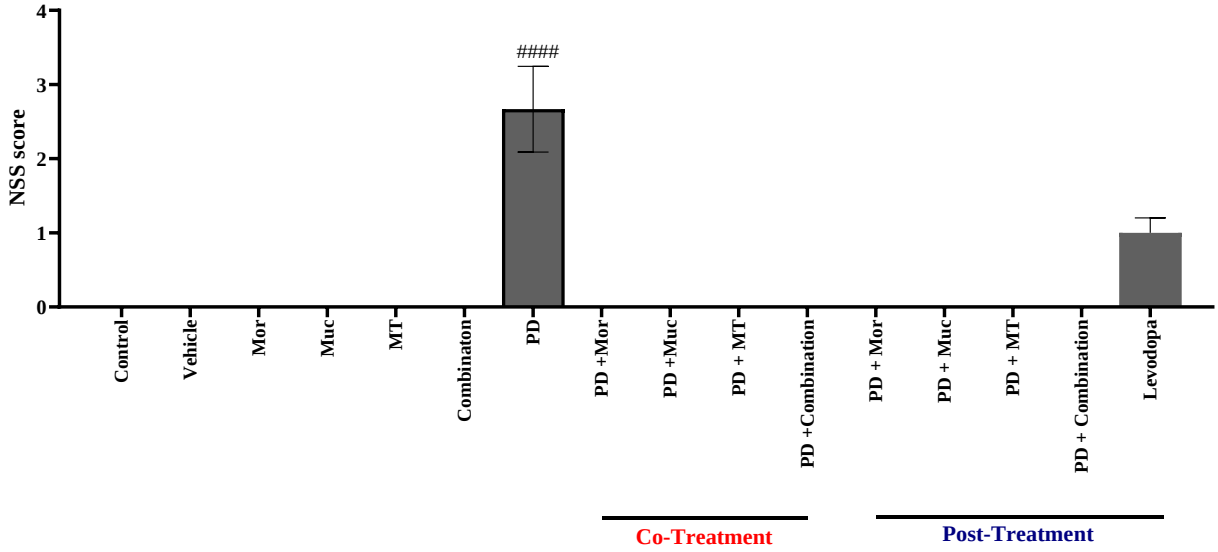

Figure 3.

ΔNSS of mice in all experimental groups. Hash marks (#) indicate significance vs. the Control group
Data are means of 6 mice per group ± SD. #### represents $p< 0.0001$ respectively.

3.3.2. Pole Climbs Test

To evaluate motor coordination and bradykinesia, the pole climb test was conducted by measuring the mice latency to reach the halfway (t½) and final (t_f) positions on the rod (Figure 4 A& B). The PD group exhibited a significant delay in both measures confirming the impairment of motor functions. Specifically, PD mice required 7.00 sec (± 1.00 sec; $p< 0.001$) for t½ and 12.33 sec (±1.52 sec; $p< 0.001$) for t_f, representing a 2.1-fold and 1.85-fold increase, respectively, in comparison to the control group (t½: 3.33 ± 1.53 sec; t_f: 6.67 ± 0.58 sec).

In contrast, all non-PD groups (control, vehicle, Mor, Muc, MT and combination) showed non-significant variation from the control group, with t½ and t_f values ranging within the normal limits, indicating no impact on motor coordination of these mice.

Co-treatment with individual or combined plant extracts led to marked restoration of the motor performance, where t½ was significantly reduced to 2.31 sec (± 0.56 sec; $p< 0.0001$) for Mor, 4 sec (±

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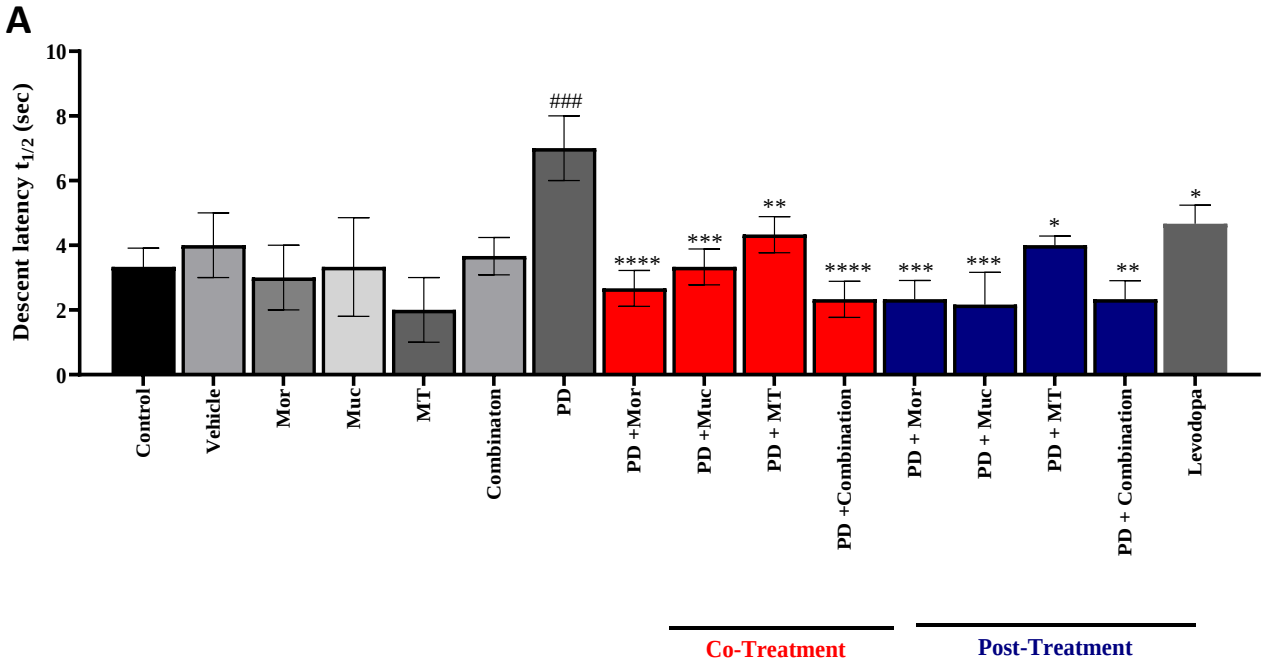
1.00 sec; $p< 0.001$) for Muc, 2.17 sec (± 0.29 sec; $p< 0.01$) for MT and 2.33 sec (± 0.58 sec; $p< 0.0001$) for combination, representing 3.03-fold, 1.75-fold, 3.23-fold and 3-fold improvement in comparison to PD mice. Similarly, t_f was reduced to 5.67 sec (± 1.15 sec; $p< 0.0001$) for Mor, 8.33 sec (0.55 sec; $p< 0.01$) for Muc, 5.33 sec (± 1.16 sec; $p< 0.001$) for MT and 5.67 sec (± 0.56 sec; $p< 0.0001$) for combination, corresponding to 2.17-fold, 1.48-fold, 2.31-fold and 2.17-fold improvement.

Post-treatment also demonstrated substantial recovery following PD induction. For t½ latency time decreased to 3.67 sec (± 0.56 sec; 1.91-fold; $p< 0.001$) for Mor, 4.33 sec (± 0.56 sec; 1.62-fold; $p< 0.001$) for Muc, 2.33 sec (± 0.54 sec; 3-fold; $p< 0.05$) for MT, 2.33 sec (± 0.58 sec; 3-fol; $p< 0.01$) for combination and 4.667 sec (± 0.57 sec; 1.5-fold; $p< 0.05$) with levodopa, all of which showed substantial reductions relative to the PD group. The t_f needed to descend completely the pole also showed significant differences across groups again. Post-treatment following PD induction showed comparable trends: PD + Mor (7.67 ± 1.16 sec;1.61-fold ; $p< 0.0001$), PD + Muc (8.33 ± 0.58 sec;1.48-fold; $p< 0.01$), PD + MT (5.67 ± 0.50 sec;2.17-fold; $p< 0.0001$), PD + combination (5.67 ± 1.50 sec; 2.17-fold; $p< 0.0001$) and PD+ levodopa (8.33 ± 1.5 sec;1.48-fold; ns) with PD + MT and PD + combination nearly normalizing the values.



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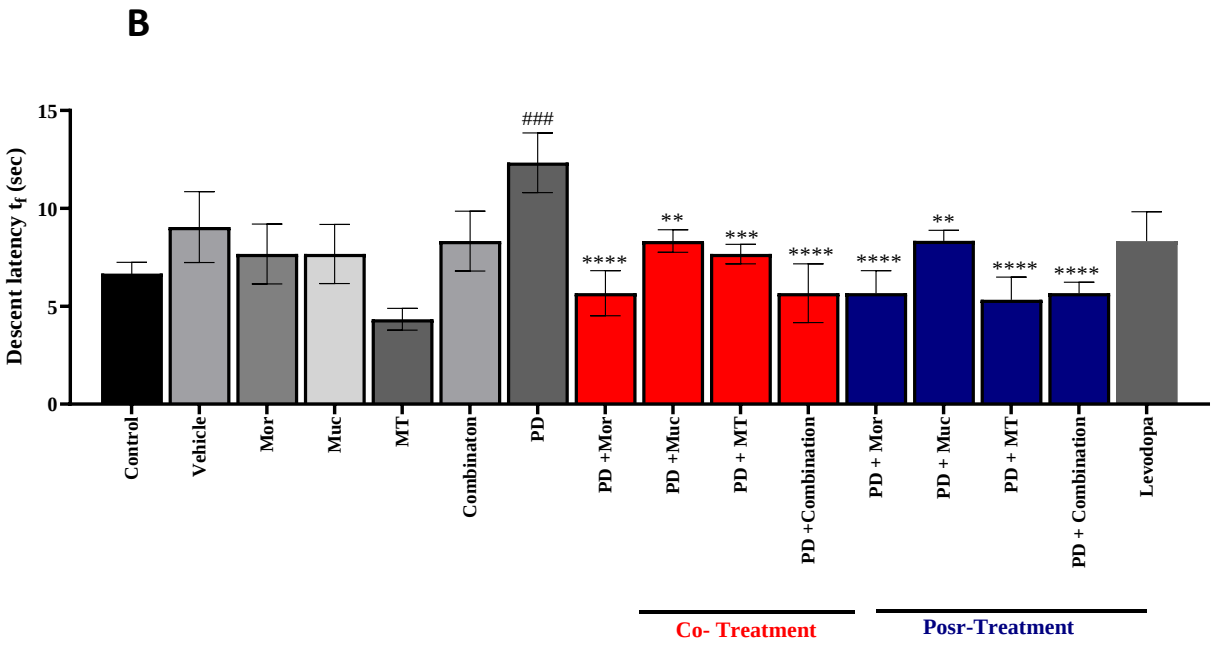

Figure 4.
Pole Climb Test. Panel A shows $t_{1/2}$ and Panel B shows t_f latency time values for both co and post-treatments for the pole climb test.
Note: Asterisks (*) indicate significance vs. the PD group; hash marks (#) indicate significance vs. the Control group. Bars are colored red for co-treatment groups and blue for post-treatment groups to distinguish treatment timing. Data are means of 6 mice per group ±SD. (*), (**), (***) and (****) represent $p< 0.05$, $p< 0.01$, $p< 0.001$ and $p< 0.0001$ respectively.

3.3.3. Rotarod Test

The Rotarod test was used to assess motor coordination and neurological deficits in mice (Figure 5). PD mice exhibited significant decline in performance (63.00 ± 32.91 sec, $p< 0.05$) compared to control groups (~210.00 sec), confirming the effectiveness of the PD model in altering baseline levels. The control, vehicle and individual plant extract groups (Mor, Muc, MT and combination) showed almost similar levels of latency time, indicating normal behavior of all mice within these groups. However, when co-treating the PD mice, these treatments significantly increased the latency to fall compared to PD alone. Notably treating PD mice with Mor, Muc, MT, or combination showed enhancement in latency time to fall with mean values comparable to or exceeding those of the control group showing 3.18-fold ($p< 0.0001$), 3.02-fold ($p< 0.001$), 2.86-fold ($p< 0.01$) and 3.65-fold ($p< 0.0001$) improvements over PD group. In the post-treatment condition, a comparable pattern was observed. The PD mice treated with Mor yielded 3.31-folds ($p< 0.001$), with 3.26-fold for Muc, ($p< 0.01$), 2.86-fold with MT ($p< 0.001$), and with combination treatment 3.26-fold ($p < 0.001$) that were even higher than Levodopa treatment (2.54-fold, $p< 0.05$).

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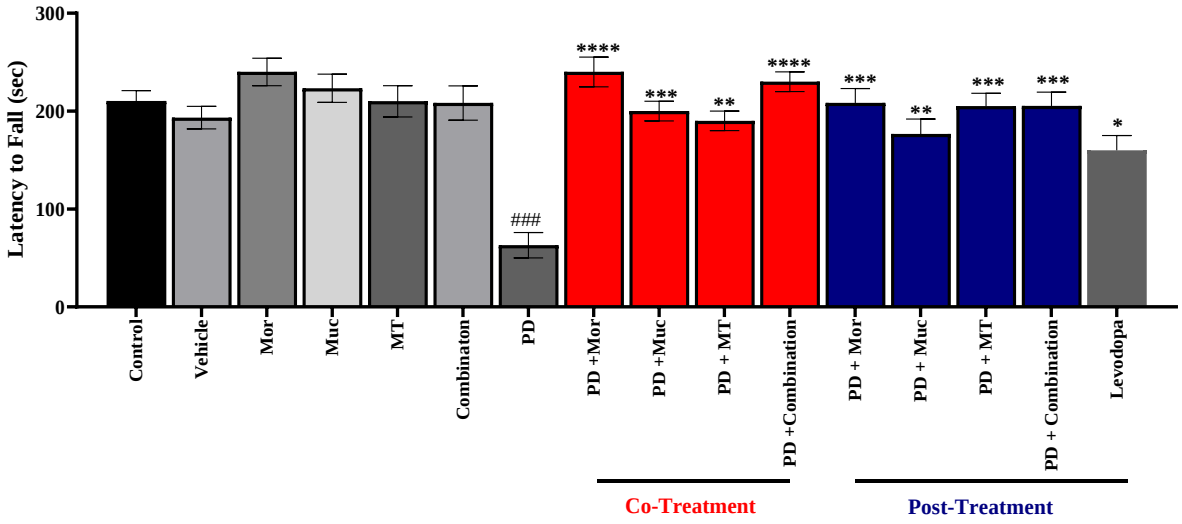

Figure 5.
Rotarod performance across experimental groups. The latency was analysed for all experimental groups for co- and post-treatment with all extracts. Asterisks (*) indicate significance vs. the PD group; hash mark (#) indicate significance vs. the Control group. Bars are colored red for co-treatment groups and blue for post-treatment groups to distinguish treatment timing.
Note: Data are means of 6 mice per group ± SD. (*), (**), (***) and (****) represent $p< 0.05$, $p< 0.01$, $p< 0.001$ and $p< 0.0001$, respectively.

3.3.4. Adhesive Test

The adhesive test was used to assess the sensorimotor deficits. The test showed that PD induction caused sensorimotor impairment with time to tape removal exceeding 15 ± 1.53 sec ($p< 0.0001$) of more than 15-fold increase in comparison to the control group, after three weeks of disease progression. However, co- and post-treatment of PD mice with Mor, Muc, MT, combination extracts, or levodopa significantly reduced removal times to below 4 sec ($p< 0.0001$), comparable to control groups as shown in Figure 6.

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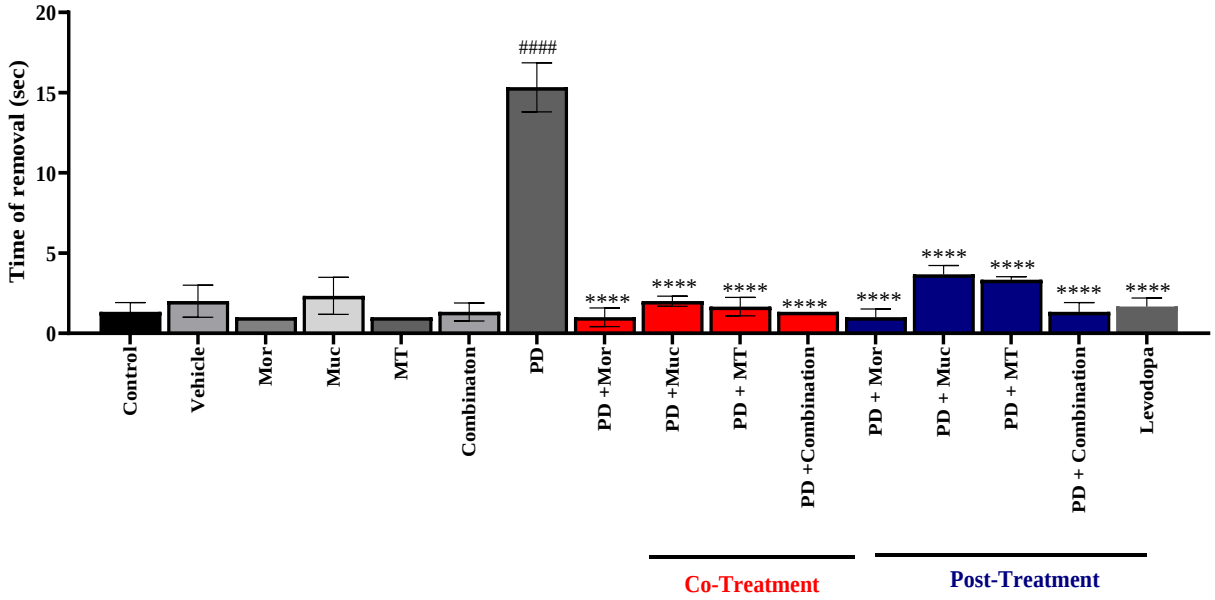

Figure 6.
Adhesive test for all experimental groups.
Note: Asterisks (*) indicate significance vs. the PD group; hash marks (#) indicate significance vs. the Control group. Bars are colored red for co-treatment groups and blue for post-treatment groups to distinguish treatment timing. Data are means of 6 mice per group ± SD. (****) represent $p < 0.0001$.

3.3.5. Beam Walk Test

The beam walk test was performed to evaluate the dynamic motor balance in mice (Figure 7). PD mice exhibited impaired dynamic motor balance, requiring 12.33 ± 1.53 sec ($p < 0.01$) to cross the beam, indicating impaired exploratory behavior or increased hesitation in comparison to control group mice (7 ± 0.58 sec). Co-treatment with Mor (3.33 ± 0.58 sec), Muc (4.67 ± 1.00 sec), MT (7 ± 1.00 sec), and combination (3.33 ± 1.53 sec) significantly reduced latency of 3.7-fold ($p < 0.0001$), 2.64-fold ($p < 0.001$), 1.76-fold ($p < 0.01$) and 3.7-fold ($p < 0.0001$) improvements compared to the PD group, with the strongest for PD + Mor and PD + Combination. In post-treatment groups, reductions in latency were also noted, particularly in PD + Mor (4.00 ± 1.00 sec; 3.08-fold; $p < 0.0001$) and PD + Muc (4.67 ± 1.53 sec; 2.64-fold; $p < 0.0001$). However, PD + MT (7.00 ± 1.00 sec; 1.76-fold; $p < 0.01$), PD + combination (6.67 ± 1.12 sec; 1.85-fold; $p < 0.01$), PD+ Levodopa (8 ± 2 sec; 1.54-fold; $p < 0.05$) were less effective, indicating treatment timing may influence efficacy.

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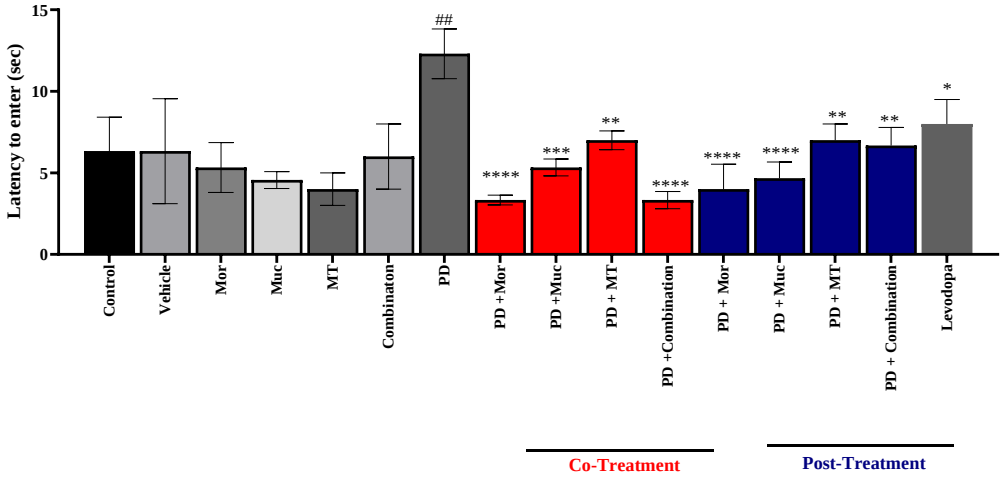

Figure 7.
Beam Walk test. The latency time to enter the cage was recorded for all experimental groups. Asterisks (*) indicate significance vs. the PD group; hash marks (#) indicate significance vs. the Control group. Bars are colored red for co-treatment groups and blue for post-treatment groups to distinguish treatment timing.
Note: Data are means of 6 mice per group ± SD. (*), (**), (***) and (****) represent $p< 0.05$, $p< 0.01$, $p< 0.001$ and $p< 0.0001$ respectively.

3.3.6. Forced Swim Test

The forced swim test (Figure 8) assessed depressive-like behavior. Mice in control, vehicle, Mor, Muc, MT, or combined extracts showed normal swimming behavior (6 min of continuous swimming) without showing signs of immobility or exhaustion. PD mice exhibited reduced swimming times (2 ± 0.15 min) and prolonged immobility (4 ± 0.15 min) indicating the expected impairment. Upon co or post-treating the PD mice with the extracts or post treating with levodopa, an increase ($p< 0.0001$) in time of swimming (Figure 8A), and a significant decrease ($p< 0.0001$) in immobility time (Figure 8B) was observed. With co-treatment, the swimming time increased to 4.03 ± 1.15 min in the PD + Mor group, 4.97 ± 0.15 min in the PD + Muc group, 4.10 ± 0.20 min in the PD+MT group, and 5.03 ± 1.15 min in the combined group. In the same manner, post-treating PD mice with plant extract caused a significant increase in swimming time ($p< 0.0001$) to 4.00 ± 0.20 min for PD + Mor group, 3.87 ± 0.23 min for PD + Muc group, 4.07± 0.15 min for PD+ MT group, 4.97 ± 1.15 min for combined treatment, and 3.13 ±0.32 min with levodopa.

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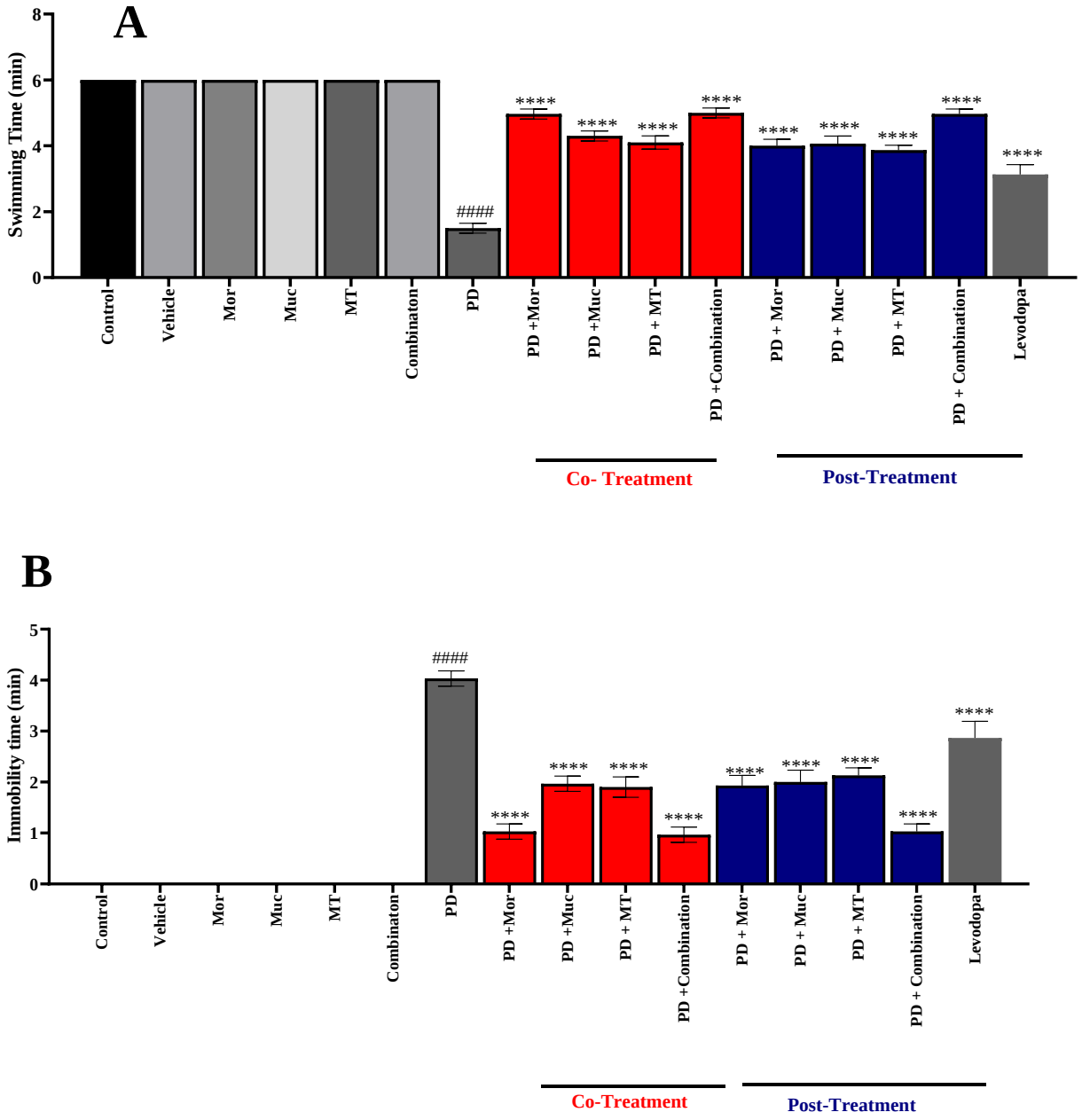

Figure 8.
Forced swim test Panel A shows swimming time recorded during the 6 min of experiment upon co-treating and post-treating PD-mice with different plant extracts. Panel B represents the immobility times recorded during the 6 min of experiment upon co-treating and post-treating PD-mice with different plant extracts. Asterisks (*) indicate significance vs. the PD group; hash marks (#) indicate significance vs. the Control group. Bars are colored red for co-treatment groups and blue for post-treatment groups to distinguish treatment timing.

Note: Data are means of 6 mice per group ± SD. (**), (***) and (****) represent $p< 0.01$, $p< 0.001$ and $p< 0.0001$ respectively.

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3.1. Effect of the Extracts on Oxidative Stress

To evaluate the effect of Mor, Muc, MT and their combinations on oxidative stress generated upon PD induction, the levels of SOD, CAT, and MDA were measured as shown in Figure 9.

The assessment of treatment effects on SOD activity revealed marked differences across groups. The PD group exhibited a significant reduction, achieving 7.6-fold ($p< 0.001$) decrease in comparison to the Control group's baseline value (0.45 ± 0.17 vs. 3.42 ± 0.03 U/mg of protein), indicating substantial impairment. Among the co-treatment groups, partial recovery was observed. The PD + Mor group restored SOD activity by 6.2-fold ($p< 0.001$) increase of that of the PD (2.80 ± 0.01 U/mg of protein), while PD + Muc (2.14 ± 0.02 U/mg of protein) showed 4.75-fold ($p< 0.01$) and PD + MT (2.64 ± 0.03 U/mg of protein) 5.87-fold ($p< 0.01$) improvement of SOD activity. The PD + combination co-treatment group showed limited recovery at 3.55-fold ($p< 0.05$) of that of the Control.

In contrast, the post-treatment groups demonstrated near-complete or full recovery. PD + Mor and PD + levodopa restored function to 8.15-fold ($p< 0.0001$) and 7.87-fold ($p< 0.001$) enhancement. PD + Muc, PD + MT and PD + combination yielded ($p< 0.001$) 7.64-fold, 6.97-fold and 7.9-fold respectively, as comparable to diseased mice.

Assessment of catalase activity in PD mice revealed a substantial drop ($p< 0.0001$) to 0.505 ± 0.045 U/mg (2.21-fold) of protein compared to the Control group (1.115 ± 0.0694 U/mg). Treatment with plant extracts significantly reduced this deficiency ($p< 0.0001$). Specifically, the PD + Mor group showed a considerable increase to 1.965 ± 0.053 U/mg protein (3.89-fold), followed by PD + MT at 1.500 ± 0.163 U/mg (2.97-fold) then PD + Muc at 1.095 ± 0.094 U/mg (2.17-fold). The combination caused an increase to 1.235 ± 0.037 U/mg (2.45-fold), showing a robust restorative impact. Post-treatment, CAT activity increased, surpassing control levels for PD + Mor (2.090 ± 0.090 U/mg of protein; 4.14-fold; $p< 0.0001$), PD + Muc (1.382 ± 0.075 U/mg; 2.74-fold; $p< 0.001$), PD + MT (2.044 ± 0.030 U/mg; 4.05-fold $p< 0.0001$), and PD + Combination (1.295 ± 0.086 U/mg; 2.56-fold; $p< 0.0001$). The largest improvements were PD + Mor and PD + MT, which exceeded the baseline. The levodopa-treated group (1.300 ± 0.081 U/mg protein; 2.57-fold; $p< 0.0001$) showed considerable improvement compared to the PD group, consistent with its therapeutic efficacy, however this effect was moderate compared to the plant extracts used in this study.

MDA, a lipid peroxidation by-product, is a direct indicator of cellular damage. Figure 25C shows the effect of the extracts on MDA levels. In PD mice, MDA levels significantly ($p< 0.0001$) increased to 13.83 ± 0.33 nmol/mg protein in comparison to the Control. However, upon treatment with the extracts, MDA levels significantly decreased ($p< 0.0001$). The Mor extract induced a significant decrease to 6.92 ± 0.30 nmol/mg protein (2-fold; $p< 0.0001$); in PD + Muc the levels decreased to 8.14 ± 0.21 nmol/mg protein (1.7-fold; $p< 0.0001$); with MT, MDA level also decreased to 8.86± 0.16 nmol/mg protein (1.56-fold; $p< 0.0001$), and in PD + combination, it decreased to 8.59 ± 0.14 nmol/mg protein (1.61-fold; $p< 0.0001$).

Post-treatment restored the parameter close to control values, highlighting the therapeutic potential of the extracts even after disease onset ($p< 0.0001$). The levels were 7.18± 0.30 nmol/mg protein (1.93-fold) for PD + Mor, 7.27 ± 0.25 nmol/mg protein (1.90-fold) for PD + Muc, 7.29 ± 0.25 nmol/mg protein (1.89-fold) for PD + MT, and 7.42 ± 0.27 nmol/mg protein (1.86-fold) for PD + combination. In the levodopa-treated group, MDA level dropped to 8.78 ± 0.025 nmol/mg protein (1.58-fold; $p< 0.0001$), also exhibiting a significant reduction compared to PD, consistent with its established efficacy, though its value remained slightly elevated relative to control.

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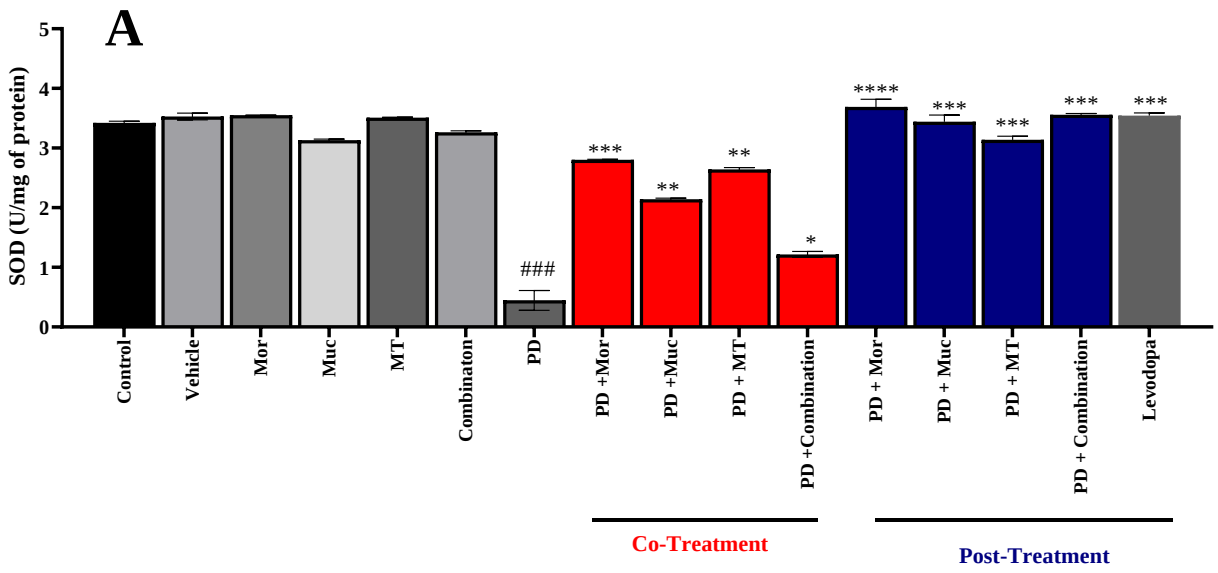

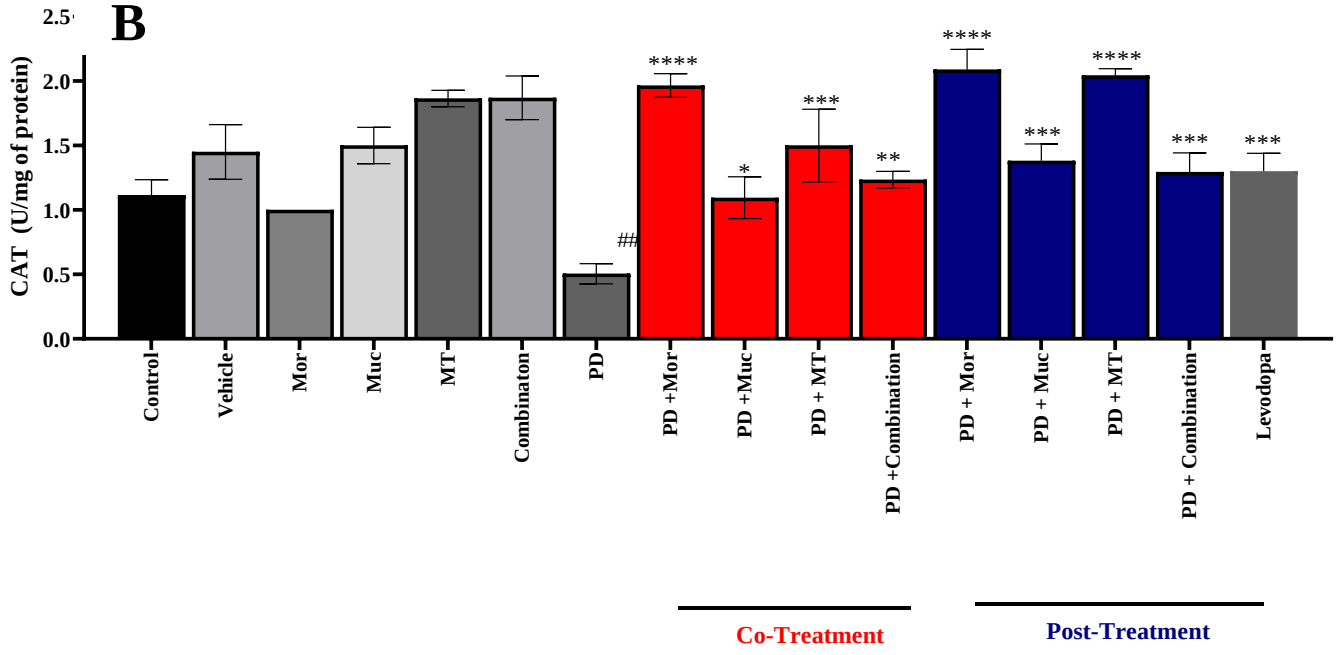

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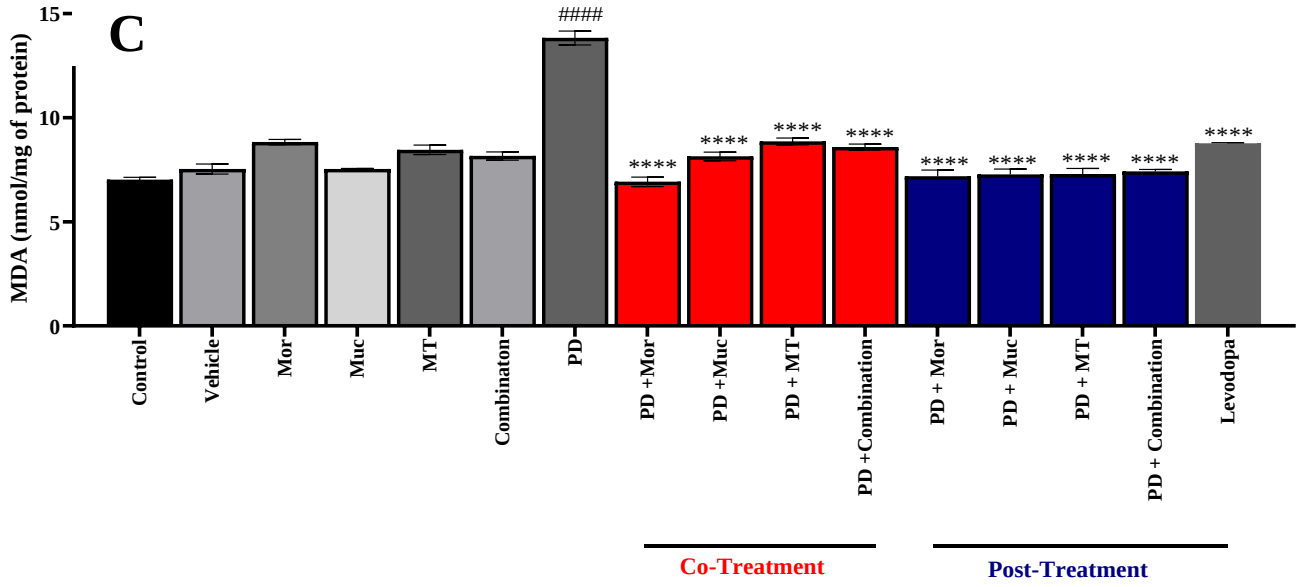

Figure 9.
Effects of Muc, Mor, MT, and the combination of the oxidative stress biomarker. SOD, CAT, and MDA) in brain tissues of all experimental groups. Panel A shows SOD activity. Panel B shows CAT activity, and Panel C shows MDA levels. Asterisks (*) indicate significance vs. the PD group; hash marks (#) indicate significance vs. the Control group. Bars are colored red for co-treatment groups and blue for post-treatment groups to distinguish treatment timing.
Note: (Data are means of 6 mice per group ± SD. (*), (**), (***) and (****) represent $p< 0.05$, $p< 0.01$, $p< 0.001$ and $p< 0.0001$ respectively.

4. Discussion

PD is a progressive neurodegenerative condition that leads to a decline in the control of both motor and non-motor functions, impacting the daily lives of patients [29].

This study employed a rotenone-induced model of PD, replicating the essential neuropathological features of human PD, including oxidative stress and behavioral impairments [30].

The research assessed the protective and therapeutic effects of Mor, Muc, and MT extracts—administered separately or in combination—against rotenone-induced Parkinson's disease in mice, comparing their efficacy to standard treatment (levodopa) and emphasizing their potential roles in both prevention and recovery from neurodegeneration associated with PD. To explore the behavioral outcomes of oxidative damage more thoroughly, we used a variety of tasks, including pole climb, rota rod, forced swim, adhesive removal, and beam walk tests.

Following 21 days of intraperitoneal rotenone administration, PD symptoms manifested, reflecting significant neurodegeneration, consistent with previous reports linking neurodegeneration to metabolic dysfunction [31, 32]. The disease presents as a notable reduction in the weight of mice, accompanied by behavioral changes following the induction of the disease. This outcome is consistent with multiple studies that explain the relationship between weight loss and progressing PD, attributing it to diminished appetite stemming from restricted brain activity in managing hunger and satiety sensations [33, 34] as well as challenges in swallowing [35]. Additional symptoms may encompass digestive issues associated with food consumption and energy expenditure because of the dysfunction of the autonomic nervous system [36]. Normal body weight returned following treating diseased mice with one of the three plant extracts or their combination, either after the onset of PD or during the induction

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of the disease (Figure 1). The weight approached values comparable to or exceeding those of the control group, showing the beneficial effects of plant extracts on weight control.

NSS evaluation further confirmed the progression of motor and sensory impairments, highlighting the efficacy of the plant extracts in restoring functionality. NSS evaluation showed that PD induction induced marked neurological dysfunction. This finding confirms the direct aspects of PD progression at sensory, motor, and reflex functions, as has been emphasized by Bieber, et al. [37]. Notably, both co-treatment and post-treatment with Mor, Muc, MT, and their combination reduced the NSS to baseline values, comparable to normal and levodopa treatment, showing their efficacy in enhancing behavioral deficits related to PD progression.

Oxidative stress has a pivotal role in the etiology of PD [38]. The brain's susceptibility is heightened by factors including an increased metabolic rate and oxygen consumption, auto-oxidation of dopamine and its metabolites, restricted antioxidant capacity, and elevated iron concentrations in the substantia nigra, which facilitate hydroxyl radical (. OH) production through Fenton reaction [39]. Our findings showed a significant reduction in antioxidant enzyme activities (SOD and CAT) alongside elevated lipid peroxidation (MDA levels) in PD mice, confirming oxidative injury. Lipid peroxidation compromises membrane integrity, enzymatic function, and membrane fluidity. MDA, the by-product of lipid peroxidation, was increased, consistent with the prior report [40] validating that oxidative membrane damage is a major pathological feature in this model. Administration of Mor, Muc, and MT extracts during disease induction decreased lipid peroxidation and enhanced antioxidant defense mechanisms demonstrating a preventive role, with Mor showing the better effects. Post-treatment of the three extracts alone enhanced the recovery of antioxidant enzymes in manner similar to levodopa, even when combined best results were obtained demonstrating curative role against oxidative damage. SOD and CAT are crucial enzymatic antioxidants that neutralize superoxide radicals and hydrogen peroxide, respectively, preventing oxidative stress progression [41]. Rotenone-induced PD models consistently show suppressed SOD and CAT activities, reflecting oxidative inactivation and enzyme depletion [42]. Co-treatment with Mor, Muc, or MT extracts led to varying degrees of partial protection comparable to levodopa treatment, with Mor achieving the most substantial recovery (82% of control). Interestingly, combination co-treatment was less effective, suggesting possible antagonistic interactions when compounds are administered together. In contrast, post-treatment approaches demonstrated superior efficacy, with nearly complete or even enhanced recovery of SOD activity. Notably, Mor post-treatment elevated SOD levels beyond baseline, hinting at a potential stimulation of endogenous antioxidant defenses. The evaluation of CAT activity demonstrated a notable increase after treatment with Mor extract, especially in the post-treatment grou. The combination therapy increased CAT activity, albeit to a lesser degree than MT alone, yet still surpassed the results of levodopa treatment, indicating that combined administration may not consistently yield synergistic effects. The pronounced antioxidant responses noted, especially with Mor and Muc extracts, underscore their potential in mitigating oxidative stress-related neurodegeneration in PD models. Restoration of these enzymatic activities by plant extracts indicates reactivation of antioxidant defenses, potentially protecting against reactive oxygen species (ROS)-mediated mitochondrial and neuronal injury. Our research supports these conclusions since mice treated with rotenone had significantly decreased activity of SOD and CAT. Decreased enzymatic activity is likely due to direct inactivation by increased H_2O_2 and the buildup of reactive oxygen species (ROS) [43]. Recent investigation highlights oxidative stress as a significant catalyst of neuroinflammation [44] mitochondrial dysfunction, endoplasmic reticulum stress, and synaptic dysfunction, which substantially contribute to the pathogenesis of PD and explain the observed behavioral outcomes in this study [45]. The excessive generation of reactive oxygen species (ROS) impairs hippocampal synaptic plasticity by diminishing long-term potentiation (LTP) and dendritic spine density, resulting in memory deficiencies and anxiety [46, 47].

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In this study, data reveals that PD mice showed significant behavioral fallback at both motor and non-motor levels. This result concurs with various studies that affirm that PD-mice behavior shows retractions at different body tasks [48]. These behavioral variations can be reflected and studied by well-organized behavioral tests [49].

Motor dysfunction, including bradykinesia, as well as balance and coordination deficits, were assessed by the pole climb and rota rod tests, which are sensitive to nigrostriatal dopaminergic deficiencies. PD mice exhibited prolonged half-time (t1/2) and total descent times (t) in the pole test, and a decreased fall latency in the rota rod test (~63 sec vs. ~190 sec in controls). Treatments using plant extracts enhanced motor function, decreasing latency periods and improving motor learning and coordination. Co-treatment with Mor and combination therapy yielded the most significant results in the pole test, while post-treatment with Mor and MT also showed substantial enhancements among plant extracts, but treating with levodopa showed better results.

Motor activities during the rotarod test improved most substantially with Mor and Muc treatments. The noticeable improvement was even better than those treated with levodopa. PD mice exhibited motor impairments, as evidenced by extended crossing durations in the beam walk test, which assesses dynamic balance and fine motor coordination. Bouet, et al. [23] used the adhesive removal test to evaluate problems in sensorimotor integration. PD mice need over 15 sec to detach the sticky tape, showing compromised cortical and striatal function. Treatment groups reduced removal times to under 5 sec, demonstrating the extracts' effectiveness in enhancing somatosensory and motor integration.

The forced swim test was used to evaluate depressive-like behaviors [50] which are becoming acknowledged as important non-motor symptoms of PD associated with oxidative stress-induced neurotransmission deficits [51]. PD mice had heightened immobility (approximately 4 min) and reduced swimming (2 min). The plants treatment increased swimming durations (about 4-5 min), even greater than those treated with levodopa, underscoring the extracts' potential for enhancing mood and reducing the depressive behavior in diseased mice.

Histopathological analysis revealed typical PD hallmarks. The disease induction led to extensive neurodegeneration distinguished by mild gliosis, degeneration, and vacuolation (Figure 3 G). Co-treating or post-treating PD models with MT extracts resulted in limited enhancement but not complete, indicating limited preventive and therapeutic efficacy in this PD model. Regarding Muc extract, co-treatment offered moderate neuroprotection by partially maintaining neuronal structure during disease induction, whereas post-treatment showed only slight histological enhancement, indicating that Muc is more effective at preventing neuronal damage than at reversing existing injury. Co-treatment with Mor during PD induction demonstrated significant neuroprotection, indicated by decreased vacuolation and maintained neuronal architecture. Post-treatment facilitated considerable recovery with only minor residual histological alterations, underscoring its dual preventive and therapeutic capabilities. Combination therapy provided synergistic neuroprotection when given during PD induction, whereas post-injury treatment resulted in nearly complete histological restoration, surpassing the outcomes of post-levodopa treatment, which only achieved partial recovery. This result aligns with other previous studies where these plants showed great potential in treating neurodegenerative disorders by reducing neuron loss in various brain regions [52-54]. These histological improvements parallel behavioral recovery, suggesting that antioxidant and neuroprotective mechanisms underlie the therapeutic benefits observed.

5. Conclusion and Limitation

Among the extracts, Mor consistently demonstrated the most substantial protective and restorative effects across behavioral, biochemical, and histological evaluations, frequently exceeding the efficacy of levodopa, the conventional treatment for PD. Significantly, whereas combination therapy had robust preventative effects during co-treatment, it did not consistently reveal synergistic advantages post-

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treatment, indicating potential antagonistic interactions. The data robustly substantiate the conclusion that Mor, Muc, and MT extracts, especially Mor, exhibit both preventative and therapeutic effects against rotenone-induced Parkinson's disease in mice. Their antioxidant, neuroprotective, and behavioral-restorative properties underscore their potential as adjunctive or alternative therapy approaches for the management of PD. Future research into the molecular processes and therapeutic application of these results is necessary. To summarize and conclude on the major findings and critical interpretations of the present study, Table 3 encapsulates the key observations, treatment effects, and scientific implications.

Table 3.
Overview of key experimental findings including the effects of botanical extract interventions, and their scientific interpretations.

Aspect Evaluated	Observation in PD Group	Effect of Plant Extract Treatment	Critical Interpretation
Weight Loss	Significant weight reduction	Partial prevention of weight loss, especially with combined extracts	Suggests metabolic preservation via neuroprotective effects.
Neurological Severity Score (NSS)	Elevated NSS indicating severe deficits	Marked improvement, notably with combination therapy	Reflects motor and sensory restoration linked to antioxidant activity.
Behavioral Tests (Pole, Wire Hanging, Traction)	Impaired motor coordination and strength	Improved performance across tests	Behavioral improvements correlate with histological protection.
Oxidative Stress Markers (MDA, SOD, Catalase, GSH)	Increased oxidative damage, reduced antioxidant defenses	Normalization of oxidative stress markers	Indicates antioxidant and free radical scavenging potential of extracts.
Histopathological Findings	Depigmentation of SNpc, neuronal loss	Reduced neurodegeneration, restored cellular architecture	Suggests direct neuroprotective action, possibly via anti-apoptotic pathways.
Neuroinflammation	Increased microglial activation (gliosis)	Decrease in gliosis post-treatment	Supports anti-inflammatory properties of plant extracts.
Limitations Identified	Single-dose regimen, short duration, lack of omics data	Treatment effects are promising but preliminary	Long-term, multi-dosage, and molecular pathway studies are necessary.

The table delineates enhancements in physiological, behavioral, biochemical, and histological characteristics, as well as the limits of the research.

Institutional Review Board Statement:

The experimental protocol was approved by the Institutional Review Board (IRB) of Beirut Arab University, with the code number (IRB number: 2023-A-0052-S-M-0553), and it followed the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines as well as the Canadian Council on Animal Care (CCAC) guidelines

Transparency:

The authors confirm that the manuscript is an honest, accurate, and transparent account of the study; that no vital features of the study have been omitted; and that any discrepancies from the study as planned have been explained. This study followed all ethical practices during writing.

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